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**THE IMPACT OF SELENIUM-RICH GREEN AND
BLACK TEA WATER EXTRACTS ON BONE HEALTH
IN VITRO, AND IN AN ANIMAL MODEL OF
OSTEOPOROSIS**

A thesis presented in partial fulfilment of the
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Abstract

The consumption of tea, as a source of dietary antioxidants, is a natural non-pharmacotherapy approach that could provide beneficial effects on bone health and represent an alternative strategy for the prevention and management of osteoporosis throughout one's life. While the benefits of tea and its bioactive chemical compounds on bone health have been increasingly investigated and reviewed, studies concerning the effects of tea with high selenium content have not yet been conducted. The purpose of the series of studies presented in this thesis was to test the hypotheses that green and black teas with high selenium content would be more effective in preventing postmenopausal bone loss than regular green and black teas, and that the positive effect of these teas on bone (if any), could be due their antioxidant and/or prebiotic-like properties. These hypotheses were investigated through a series of studies involving a variety of cellular assays, a young growing rat model, and an ovariectomy-induced bone loss rat model of postmenopausal osteoporosis. Four different teas derived from *Camellia sinensis* were assessed for their total phenolic content (TPC), antioxidant properties and prebiotic-like potential, which included a selenium-rich green tea (Se-GTE), a selenium-rich black tea (Se-BTE), a regular green tea (R-GTE) and a regular black tea (R-BTE). Aqueous tea extracts were prepared using different extraction temperatures and times to quantify the extraction efficiencies for TPC and antioxidant properties. TPC was measured using the Folin-Ciocalteu method, antioxidant activity was measured using the ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, and the prebiotic-like effect on two beneficial bacteria (*Lactobacillus acidophilus* and *L. rhamnosus*) was determined using the plate agar dilution method. Irrespective of tea selenium content, the results obtained for TPC, antioxidant properties and prebiotic-like potential of the investigated teas were highly variable dependent on the different types of tea. In addition, the optimal time and temperature of tea infusion for maximising TPC was determined to be 90 °C for 5 min (**Chapter 4**), which was then used as the standard method of preparation for aqueous tea extracts for the subsequent *in vitro* and *in vivo* work. Further, the freeze-dried aqueous tea extracts (0.001 to 10 µg/mL) were investigated for their osteogenic effects on murine pre-osteoblastic MC3T3-E1 (Subclone 4) cells, as assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), alkaline phosphatase (ALP) activity, and Alizarin Red S (ARS) staining assays. The osteoprotective effect of

the freeze-dried aqueous tea extracts against H₂O₂-induced oxidative stress during osteoblast differentiation was also evaluated. At low concentrations, all tea extracts showed an anabolic effect by enhancing matrix mineralisation in MC3T3-E1 cells. Moreover, the teas were capable of protecting and restoring the differentiated osteoblasts against the dysfunctional effects of H₂O₂-induced oxidative stress. These *in vitro* activities were irrespective of the selenium content, and were in a time- and concentration-dependent manner (**Chapter 5**). Next, their anti-osteoclastogenic effects were assessed by measuring tartrate-resistant acid phosphatase (TRAP) activity in receptor activator of nuclear factor kappa beta ligand (RANKL)-treated RAW 264.7 cells, while the numbers of TRAP-positive osteoclasts (TRAP⁺ OCLs) with five or more nuclei were quantified. All tea extracts (0.001 to 10 µg/mL), independent of selenium content, suppressed RANKL-induced osteoclastogenesis in a concentration-dependent manner, i.e. mostly significant at the higher concentrations (**Chapter 6**). In the first animal trial (**Chapter 7**), the effect consuming tea (1%, w/v) for four weeks on bone mass and strength were examined in young growing male Sprague-Dawley rats. No osteo-stimulative effects on bone parameters (i.e. serum bone resorption biomarker, bone mineral density and bone biomechanics) were observed in the rats during the rapid growth phase following tea consumption. Only Se-GTE showed prebiotic-like potential evaluated by changes in caecal parameters (i.e. decrease in caecal pH, decrease in numbers of *Clostridium* spp. (*perfringens/histolyticum* subgroup) and enhanced bacterial β-glucosidase enzyme activity). In the next animal trial (**Chapter 8**), the effects of eight-week consumption of tea (1%, w/v) on bone loss were assessed in ovariectomised mature adult female Sprague-Dawley rats. Only R-BTE significantly suppressed the serum bone resorption biomarker. Moreover, only Se-GTE and R-BTE demonstrated prebiotic-like potential in modulating intestinal microbiota composition, as seen by a marked decrease in caecal pH and enhanced activity of the bacterial β-glucosidase enzyme. Additionally, serum antioxidant capacity levels of the teas evaluated by FRAP assay in both animal trials showed mixed results. Based on the study findings, it is suggested that tea may exert stimulating effects on bone metabolism part-mediated through its prebiotic influence on gut microbiota, and not via a direct antioxidant mechanism. However, the exact mechanism underlying this effect remains unclear and needs to be investigated further. Taken together, these studies provide new insights into the potential antioxidant and prebiotic roles of teas with different levels of selenium, and their possible impact on bone health.

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If you focus on what you left behind, you will never be able to see what lies ahead.

Now go up and look around!

~ Gusteau in Ratatouille

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List of Abbreviations

α	alpha
AIN-93G	American Institute of Nutrition (growth formulation)
AIN-93M	American Institute of Nutrition (maintenance formulation)
AKT	protein kinases B
ALP	alkaline phosphatase
AMPK	5'adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
AOA	antioxidant activities
AP1	activator protein-1
ARS	Alizarin Red S
ATCC	American Type Culture Collection
ATP	adenosine triphosphatase
β	beta
β -GLU	beta-glucuronidase
β -GUS	beta-glucosidase
BA	bone area
BAP	bone-specific alkaline phosphatase
BFR/BS	bone formation rate per bone surface of proximal tibia
Bim	Bcl-2-like protein 11
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
BMP2	bone morphogenetic protein-2
BMPs	bone morphogenetic proteins
BMUs	basic multicellular units
BrdU	5-bromo-2'-deoxyuridine; bromodeoxyuridine
BSA	bovine serum albumin
BSP	bone sialoprotein
BTE	black tea extract
BV/TV	bone volume density; bone volume per total volume
BW	body weight
C	(+)-catechin; catechin
C	celsius
Ca	calcium
Ca:Cr	calcium-to-creatinine ratio
Ca-ATPase	calcium-activated adenosine triphosphatase
CAT	catalase
Cbfa1	core-binding factor alpha 1
CFUs	colony forming units
CG	(-)-catechin-3-gallate
CH-GTE	China green tea extract
c-Jun	cellular-Jun (proto-oncogene). Jun stands for "Ju-nana"; a Japanese word for 17. This gene is reputed to be a transforming gene of avian sarcoma virus 17
cm ²	square centimetre
CO ₂	carbon dioxide
CO ₂ ^{·-}	carbon dioxide radical

CO ₃ ⁻	carbonate
COL1A1	collagen type I alpha 1
COMT	catechol-O-methyltransferase
COX2	cyclooxygenase-2
CPC	cetylpyridinium chloride
CTR	calcitonin receptor
CTx-I	carboxy-terminal crosslinks telopeptides of type I collagen
CV	coefficient of variation
Cy3	cyanine 3
D	daltons
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DEXA	dual energy X-ray absorptiometry
DF	degrees of freedom
DMBA	7,12-dimethylbenz(a)anthracene
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPX	Distrene, Plasticiser, Xylene
DW	dry weight
e.g.	for example
E ₂	oestrogen; 17β-oestradiol; oestradiol
EC	(-)-epicatechin; epicatechin
ECG	(-)-epicatechin-3-gallate; epicatechin-3-gallate; epicatechin gallate
ECM	extracellular matrix
EGC	(-)-epigallocatechin; epigallocatechin
EGCG	(-)-epigallocatechin-3-gallate; epigallocatechin-3-gallate; epigallocatechin gallate
EIA	enzyme immunoassay
ERK	extracellular signal-regulated kinase
ERs	oestrogen receptors
ES/BS	eroded surface per bone surface
FCS	foetal calf serum
FDA	Food and Drug Administration
Fe ²⁺	ferrous ion
Fe ²⁺ -TPTZ	ferrous tripyridyltriazine
Fe ³⁺	ferric ion
Fe ³⁺ -TPTZ	ferric tripyridyltriazine
FeCl ₃	ferric chloride
FeSO ₄	ferrous sulphate
FeSO ₄ E	ferrous sulphate equivalent
FeSO ₄ .H ₂ O	ferrous sulphate
FISH	fluorescence <i>in situ</i> hybridization
FOXO	forkhead homeobox type O
FRAP	ferric-reducing antioxidant power
FRC	ferric-reducing capacity
g	gram
g	relative centrifugal force (RCF)
G	gauge
GAE	gallic acid equivalent

GC	(-)-galliccatechin
GCG	(-)-galliccatechin-3-gallate; galliccatechin gallate
GIT	gastrointestinal tract
GR	glutathione reductase
GSH	glutathione
GSH-Px	glutathione peroxidase
GSH-Px3	plasma glutathione peroxidase
GST	glutathione S-transferase
GTC	green tea catechins
GTP	green tea polyphenols
HAT	hydrogen atom transfer
H ₂ O	water; dihydrogen monoxide
H ₂ O ₂	hydrogen peroxide
hBMSCs	human bone-marrow-derived mesenchymal stem cells
HCl	hydrogen chloride
HFD	high-fat-diet
Hh	hedgehog
HIF1	hypoxia-inducible factor-1
HO	heme oxygenase
HO ₂ [·]	hydroperoxyl
HOBr	hypobromous acid
HOCl	hypochlorous acid
HOOCO ₂ ⁻	peroxomonocarbonate
HPLC	high performance liquid chromatography
HPr	hydroxyproline
HRT	hormone replacement therapy
HSP27	heat shock protein 27
i.e.	that is
ICAM-1	intercellular adhesion molecule 1
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectrometry
IGFs	insulin-like growth factors
IL-1β	interleukin-1 beta
IL6	interleukin-6
ILs	interleukins
J	Joule
JNK/c-Jun	c-Jun N-terminal protein kinase
KEAP1	Kelch-like ECH-associated protein 1
kg	kilogram
L	litre
LAB	lactic acid bacteria
LogP	a calculated partition coefficient value
LPS	lipopolysaccharide
LS	lumbar spine
M	molar
MAPKs	mitogen-activated protein kinases
M-CSF	macrophage-colony stimulating factor
MDA	malondialdehyde
MEDOS	Mediterranean Osteoporosis Study
MEMα	Minimum Essential Medium Alpha Modification

mg	milligram
mg E/L	milligram equivalent per litre
mg/h	milligram per hour
MIFST	Massey Institute of Food Science and Technology
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
mm ²	square millimetre
mmol/L	millimoles per litre
MMPs	matrix metalloproteinases
MQ	Milli-Q
mRNA	messenger ribonucleic acid
MRS broth	Mann-Rogosa-Sharpe broth
MS	mean square
MSCs	mesenchymal stem cells
MsrB1	methionine R-sulfoxide reductase B1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Newton
N.Oc/BS	number of osteoclast per bone surface
Na ₂ CO ₃	sodium carbonate
NAC	N-acetyl cysteine
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NASH	non-alcoholic steatohepatitis
NELL1	NEL-like protein-1
NFAT	nuclear factor of activated T cells
NFATc1	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
Nfkb2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2
NF-κB	nuclear factor kappa beta
ng/mL	nanogram per millilitre
nm	nanometre
NOXs	nicotinamide adenine dinucleotide phosphate oxidases
NRC	National Research Council
Nrf2	nuclear factor erythroid 2-related factor 2
NZD	New Zealand dollar
NZRM	New Zealand Reference Culture Collection, Medical Section
O ₂ ^{·-}	superoxide anion radical
O ₂ NOO ⁻	peroxynitrate
O ₃	ozone
OC	osteocalcin
OCLs	osteoclasts
OcS/BS	osteoclast surface per bone surface
OD	optical density
-OH	hydroxyl groups
OH [·]	hydroxyl
OH ⁻	hydroxide ions
ONOO ⁻	peroxynitrite

ONOOH	peroxynitrous acid
OPG	osteoprotegerin
OPN	osteopontin
ORAC	oxygen radical absorbance capacity
ORX	orchidectomised
OS	oxidative stress
OSCAR	osteoclast-associated receptor
OVX	ovariectomised
<i>p</i>	probability
PBS	phosphate buffered saline
pH	logarithmic measurement of hydrogen ion concentration
pK_a	the log of acidity constant
ρ -NP	para-nitrophenol
ρ -NPP	para-nitrophenyl phosphate
PPO	polyphenol oxidase
pQCT	peripheral quantitative computed tomography
PROC CORR	correlation procedure
PROC GLM	general linear model procedure
Prx	peroxiredoxin
PTH	parathyroid hormone
p44/p42 MAPK	p44/p42 mitogen activated protein kinase
QC	quality control
qPCR	quantitative real-time polymerase chain reaction
<i>R</i>	correlation coefficient
R^2	coefficient of determination
RANK	receptor activator of nuclear factor kappa beta
RANKL	receptor activator of nuclear factor kappa beta ligand
R-BTE	regular black tea
RDA	recommended dietary allowance
redox	reduction-oxidation
R-GTE	regular green tea
RO \cdot	alkoxyl
RO $_2\cdot$	peroxyl
ROOH	organic peroxides
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
Runx2	runt-related transcription factor-2
SAPK/JNK	stress-activated protein kinase/c-Jun N-terminal kinase
SAPU	Small Animal Production Unit
SAS	Statistical Analysis Software
SCFAs	short-chain fatty acids
SD	Sprague-Dawley
Se	selenium
Se-BTE	selenium-rich black tea
Se-GTE	selenium-rich green tea
SEM	standard error of mean
Sema4D	semaphorin4D
SeMet	seleno-L-methionine
Sepp1	selenoprotein P
SERMs	selective oestrogen receptor modulators

SET	single electron transfer
Sham	sham-operated
SHIME	simulator of human intestinal microbial ecosystem
SOD	superoxide dismutase
SOST	sclerostin
Tb.N	trabecular number
Tb.Sp	trabecular separation
Tb.Th	trabecular thickness
TBS	tris buffered saline
TC	Tai Chi
TFDG	theaflavin-3,3'-digallate
TFs	theaflavins
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
TPC	total phenolic content
TPTZ	tripyrindyltriazine; 2,4,6-tripyridyl- <i>s</i> -triazine
TRAP	tartrate-resistant acid phosphatase
TRAP ⁺ OCLs	TRAP-positive osteoclasts
Tris-HCl	Tris-hydrogen chloride
TRs	thearubigins
Trx	thioredoxin
TrxR	thioredoxin reductase
Trypsin-EDTA	Trypsin-ethylene diamine-tetraacetic acid
U	one unit of β-glucosidase or β-glucuronidase enzyme that releases 1 mg of para-nitrophenol or phenolphthalein per hour
μCT	micro-computed tomography
μg	microgram
UK	United Kingdom
μL	microlitre
μM	micromolar
μmol/L	micromoles per litre
US	United States
USA	United States of America
v/v	volume per volume
VCEAC	vitamin C equivalent antioxidant capacity
VDRs	vitamin D receptors
VEGF	vascular endothelial growth factor
<i>vs</i>	<i>versus</i>
w/v	weight per volume
w/w	weight per weight
Wnt	wingless-type mammary tumor virus integration site
1,25(OH) ₂ D ₃	calcitriol
¹ O ₂ Δg	singlet oxygen, electronically excited ¹ Deltag state
¹ O ₂ Σg ⁺	singlet oxygen, electronically excited ¹ Sigmag ⁺ state
8-OHdG	8-hydroxy-2-deoxyguanosine
14-kDa protein	a protein with a mass (molecular weight) of 14 kilo daltons

CHAPTER 1

INTRODUCTION

1.1 Research background

Osteoporosis is a progressive metabolic disease of bone marked by a gradual loss of bone mass and deterioration in bone microarchitecture that predisposes affected individuals to fragility fracture (National Institute of Health, 2001). Those afflicted with osteoporosis are often asymptomatic until a fracture occurs, and the condition has become a significant public health cost as fragility fractures can occur from minimal trauma injury; a consequence of diminished compression and/or torsion strength of the bone (Elliot-Gibson et al., 2004; Rachner et al., 2011). The development of osteoporosis is common in the elderly population, with the incidence of osteoporosis and osteoporosis-related fractures expected to increase significantly due to the world's increasing aged population with greater life expectancies (Cooper et al., 1992; Reginster & Burlet, 2006; Shuler et al., 2012; Åkesson et al., 2013).

Women are at a greater risk of osteoporosis than men, due to the effects of oestrogen deficiency at menopause and the accompanying accelerated loss of bone mass (Cervelatti et al., 2013). About half of postmenopausal women over the age of 50 are at higher risk of osteoporotic fractures; these are most commonly of the hip (proximal femur), wrist (distal radius) and spine (vertebra), and have become the primary cause of morbidity and mortality in elderly women (Johnell & Kanis, 2005, 2006; Khosla, 2010; Harvey et al., 2010; Ström et al., 2011; Cauley, 2013). In addition, osteoporosis leaves an enormous financial burden around the world due to expensive medications and the long-term treatment of affected individuals (Yang & Liu, 2009; Harvey et al., 2010; Ström et al., 2011; Dimai et al., 2012; Cauley, 2013; Hernlund et al., 2013; Marinho et al., 2014). The significant financial impact of osteoporosis on society is mostly due to the direct health care of osteoporotic fracture patients that involves acute treatment and rehabilitative care, as well as the indirect health care costs that are associated with decreased productivity due to premature mortality, morbidity, and disability (Office of the Surgeon General, 2004; Becker et al., 2010). The total cost of osteoporosis cases in New Zealand alone was over NZD1 billion in 2007, and is set to rise by over 30% between 2007 and 2020 (Brown et al., 2007). Thus, the principal aim of effective prevention and management of osteoporosis is to avert the risk and prevalence of fragility fractures (Qaseem et al., 2008).

Currently, a number of pharmacotherapy drugs are available to treat osteoporosis including bisphosphonates, selective oestrogen receptor modulators (SERMs) (i.e. raloxifene), strontium ranelate, parathyroid hormone (PTH), tibolone, denosumab, calcitonin, and hormone replacement therapy (HRT), as well as other emerging drugs under investigation such as cathepsin K inhibitors and new SERMs (i.e. lasofoxifene, bazedoxifene and arzoxifene) (Sandhu & Hampson, 2011; Singh et al., 2013). However, their use in therapy must be tempered with their associated risks and adverse effects that may limit their use and efficacy (Rossouw et al., 2002; Watts & Diab, 2010; Sharif et al., 2011; Reginster et al., 2013; Reginster, 2014). Furthermore, patient compliance with drug therapy can be poor especially over a long period of treatment, thus compromising the efficacy of the pharmacotherapy drugs (Weycker et al., 2006; Imaz et al., 2010; Cooper et al., 2012; Kanis et al., 2013).

Owing to the controversy surrounding the safety and efficacy of current pharmacotherapy drugs, complementary (adjunctive) and alternative non-pharmacotherapy approaches have gained a lot of interest from both researchers and consumers. At present, there is growing evidence for non-pharmacotherapy treatments including nutritional supplementations independent of calcium and vitamin D that have shown a positive impact on bone metabolism; these can be very useful for the maintenance of bone health, as well as for the prevention and management of osteoporosis (Whelan et al., 2006; Cashman, 2007; Putnam et al., 2007; Habauzit & Horcajada, 2008; Stránský & Rysavá, 2009; Horcajada & Offord, 2012; Jia et al., 2012; Nieves, 2013; Rao & Rao, 2013; Rondanelli et al., 2013; Sacco et al., 2013). Therefore, it is suggested that the natural nutritional non-pharmacotherapy approach could reduce the necessity or provide a viable alternative for current pharmacotherapy drugs, which could be used widely to prevent bone loss and fragility fractures in the large number of individuals at risk of osteoporosis.

1.2 The rational and importance of the research study

Numerous studies have suggested that the development and progression of osteoporosis is closely related to oxidative stress; a pervasive intracellular condition of increased levels of reactive oxygen species (ROS) resulting from excessive

accumulation of ROS and/or depletion of endogenous antioxidant defence systems (Shen et al., 2009a; Wauquier et al., 2009; Almeida, 2012; Rao & Rao, 2013; Sheweita et al., 2014). Oxidative stress leads to disruptions of the intracellular reduction-oxidation (redox) balance that is crucial in maintaining cellular homeostasis (Almeida et al., 2007a; Halliwell, 2007), eventually causing damaging effects on major cellular constituents such as lipids, proteins, and nucleic acids (Lobo et al., 2010; Filaire & Toumi, 2012).

It is well-known that ROS production at physiological levels is essential for regulating cell functions and mediating intracellular signals of cell death and survival (Dröge, 2002; Valko et al., 2007; Trachootham et al., 2008). Intracellular ROS production is known to increase with aging, and the accumulation accelerates with decreased oestrogen (Bokov et al., 2004; Almeida et al., 2007a; Pandey & Rizvi, 2010; Zhang et al., 2011; Bellanti et al., 2013). In bone, physiological levels of ROS are a key modulator of cell function and play a significant role in the maintenance of mineral homeostasis; where ROS contribute mostly to bone remodelling by enhancing resorption activity (Wauquier et al., 2009; Filaire & Toumi, 2012; Kanzaki et al., 2013). However, excessive intracellular ROS production results in oxidative cellular damage that leads to the initiation and progression of chronic human diseases, including in osteoporosis where they may inhibit pre-osteoblastic differentiation and/or stimulate bone resorbing activity of osteoclasts (Bai et al., 2004; Kim et al., 2006; Maiese et al., 2010; Rao & Rao, 2013).

The intake of dietary antioxidants sourced from food and beverages has been widely reported to improve endogenous antioxidant (i.e. enzymatic and non-enzymatic) defence systems (Pandey & Rizvi, 2009; Lobo et al., 2010; Poljsak, 2011; Buoayed & Bohn, 2012; Poljsak et al., 2013). Due to the significant contribution of intracellular ROS and oestrogen deficiency on oxidative stress, as well as their important roles in the increased activity of bone resorption resulting in osteoporosis, it is worth elucidating the role of dietary antioxidants in mitigating bone loss during the development of postmenopausal osteoporosis (Lean et al., 2003). There is a wide range of dietary antioxidants that have been reported for their potential as alternative strategies for prevention and management of postmenopausal osteoporosis including consumption of

tea (Scalbert et al., 2005; Mundy, 2006; Trzeciakiewicz et al., 2009; Tucker, 2009; Shirwaikar et al., 2010; Al-Anazi et al., 2011; Kalam et al., 2012).

Tea beverages made from leaves of *Camellia sinensis* are the most consumed beverage worldwide, second only to water, and they have also been used for medicinal purposes for centuries (Shi & Schlegel, 2012; Yang et al., 2014a). Tea is a dietary antioxidant that is rich in polyphenols; a group of compounds that are increasingly being investigated for their antioxidant role and health benefits in relation to various chronic human diseases (Khan & Mukhtar, 2013; Yang & Hong, 2013). Tea polyphenols act as antioxidants by scavenging reactive oxygen and nitrogen species free radicals, as well as chelating redox-active transition metal ions (Weisburger, 1999; Frei & Higdon, 2003). Consumption of tea has increasingly being reported for its potential to reduce bone loss associated with oestrogen deficiency following menopause (Trzeciakiewicz et al., 2009; Rao & Rao, 2013; Shen et al., 2013a, 2013b). Currently, there are a number of *in vitro* and *in vivo* studies that reliably indicate the positive effects of tea on bone (Shen et al., 2008a, 2008b, 2009a, 2009b, 2010a, 2010b, 2011b, 2012, 2013a; Shao et al., 2011; Oka et al., 2012; Das et al., 2004, 2005, 2009, 2013; Khan & Mukhtar, 2013; Yang & Hong, 2013), thus, suggesting beneficial impact of tea drinking in maintaining bone health.

At present, tea rich in selenium (Se) is being increasingly produced in China, and appears to be a safe, feasible and effective way to improve Se intake in low-Se populations (Hu et al., 2002; Xu et al., 2003a; Lyons et al., 2005; Li et al., 2009a). Selenium is an important trace mineral naturally available in many plant-based and non-plant based foods, and the concentration of Se in plant-based food relies solely on the total selenium content of the soil in which the plants were cultivated (Cressey et al., 2000; Rayman, 2008; Combs, 2013). The geographic distribution of Se-rich soils varies widely; from high Se levels in the United States of America (USA) to low Se levels in New Zealand (Thomson, 2004a; Letavayova et al., 2006; Sunde, 2012).

Being a crucial component of the active site of Se-dependent antioxidant enzymes known as selenoenzymes, Se plays an important role in the antioxidant defence system. Se exerts antioxidant function mainly in the form of selenocysteine incorporated into ROS-eliminating selenoenzymes, which include glutathione

peroxidases, thioredoxin reductases, methionine R-sulfoxide reductase and selenoprotein P. These selenoenzymes are involved in the antioxidant defence system through: 1) catalysing the reduction of a variety of deleterious peroxides, and/or 2) cellular redox regulation by glutathione and thioredoxin systems; eventually preventing further generation of ROS that can lead to oxidative stress (Scalbert & Williamson, 2000; Steinbrenner & Sies, 2009). Selenium has also been suggested to enhance immune activity (McKenzie et al., 1998; Rayman, 2000), and Se deficiency seriously affects the health of both humans and animals (Letavayova et al., 2006; Ebert & Jakob, 2007; Hefnawy & Tortora-Perez, 2010; Mistry et al., 2012). Furthermore, Se is found to be essential for regulation of thyroid hormones, which are necessary for bone formation (Kaprra & Krassas, 2006; Zagrodzki & Ratajczak, 2008). Above all, numerous studies have also reported links between bone health and Se intake status (Hurt et al., 1971; Tsukahara et al., 1996; Moreno-Reyes et al., 2001; Turan et al., 2003; Hejazi et al., 2009; Cao et al., 2012; Hoeg et al., 2012; Pedrera-Zamorano et al., 2012; Zeng et al., 2013a; Zhang et al., 2014).

The health benefits of Se-rich teas in providing protection against oxidative damage and preventing cancer have been increasingly investigated (Xu et al., 2007; Li et al., 2008, 2009a; Rayman, 2009; He et al., 2013; Liang et al., 2014). However, to the best of our knowledge, no information is available about the protective effect of Se-rich teas against bone loss. Meanwhile, the role and synergistic effect of Se and tea has been supported by other literature, which have shown that the high Se content in tea can significantly enhance the antioxidative effect of tea (Hu et al., 2001, 2003; Xu et al., 2003a, 2003b; Yu et al., 2007; Li et al., 2008, 2009a; Molan et al., 2009a). Existing reports of the beneficial effects of tea on postmenopausal bone loss, as well as the promising effect of Se on bone health, have led us to hypothesise that Se-rich teas may be more effective than regular teas in modulating bone loss due to ovarian hormone deficiency.

Interestingly, apart from its antioxidant properties, tea and/or its polyphenols have also been reported to exhibit a positive effect on the intestinal environment by acting as a prebiotic (Lee et al., 2006a; Molan et al., 2009a, 2010; Cardona et al., 2013). Tea may promote the growth of beneficial gut microbiota such as lactobacilli and bifidobacteria that are widely known for their health-promoting properties (Gibson &

Roberfroid, 1995; Duda-Chodak et al., 2008; Tzounis et al., 2008; López de Felipe et al., 2010; Vodnar et al., 2012; Gaudreau et al., 2013; Ruxton, 2013). Most importantly, prebiotics (non-digestible carbohydrates that promote health by selectively affecting the growth of beneficial intestinal microbiota) are suggested to be the most promising and the best investigated substances with bone health-promoting potential, compared to that similar role of probiotics (live microorganisms with health-promoting properties when administered in sufficient amounts) and synbiotics (synergism between prebiotics and probiotics present in the same food) (Scholz-Ahrens et al., 2001, 2007). It is worth noting that prebiotics affect bone health by enhancing bioavailability and uptake of bone-relevant minerals, such as calcium, magnesium, and zinc (Scholz-Ahrens et al., 2007; Slavin, 2013). Furthermore, prebiotics also indirectly influence mineral absorption by stimulating the growth of beneficial gut microbiota, which in turn, exert an effect on bone accretion independent of that of prebiotics (Scholz-Ahrens et al., 2007). This positive effect of beneficial gut microbiota on bone accretion is achieved through synthesis of bone-relevant vitamins (vitamin C, D or K), and generation of short-chain fatty acids (SCFAs) that serve as a growth substrate for the gut microbiota. These end-products result from intestinal bacterial fermentation and metabolism of complex non-digestible dietary substances that escape digestion in the small intestine (e.g. tea polyphenolic compounds) (Lopez et al., 1998; Topping & Clifton, 2001; Jacobs et al., 2009).

Although there are numerous studies that have indicated the antioxidant and prebiotic activity of tea, to the best of our knowledge, only limited information is available concerning Se-rich teas, especially for its role in bone health (Axling et al., 2012; Vodnar & Socaciu, 2012; Cardona et al., 2013; Molan, 2013; Liang et al., 2014). Recent *in vitro* studies by Molan et al. (2009a, 2010) have provided evidence that the prebiotic effect of Se-rich green tea against beneficial gut bacteria could be enhanced by its Se content. The authors also suggested that Se-rich green tea showed greater antioxidant and prebiotic-like potential than regular green tea. However, the study was limited only to Se-rich green tea, and neither *in vitro* nor *in vivo* work has been attempted to discover therapeutic potential of Se-rich teas for prevention and management of postmenopausal bone loss. The present study was undertaken mainly to determine the bone-promoting effects of green and black teas with different levels of selenium content against bone loss due to oestrogen depletion following menopause.

Furthermore, this study examined whether the tested teas exert their beneficial effect on bone properties through their antioxidative effect and/or prebiotic-like potential, both via *in vitro* and *in vivo* assays. Results obtained from this study are expected to significantly affect the lives of many individuals at risk of postmenopausal osteoporosis, by providing evidence and important insights about the role of tea drinking as an effective and safe dietary antioxidant option to prevent and overcome postmenopausal bone loss.

1.3 Research hypothesis and objectives

The hypothesis of this research work is that selenium-rich green and black teas are more effective in preventing postmenopausal bone loss than regular green and black teas, and that the positive effect of these teas on bone health (if any) could be due to its antioxidant and/or prebiotic-like properties. To verify this hypothesis, we set forth the following objectives:

1. To assess the total phenolic content and antioxidant activities of selenium-rich green and black teas in comparison with regular green and black teas, as well as the prebiotic potential of the tea water extracts to enhance the growth and viability of beneficial bacteria using pure bacterial cultures under *in vitro* conditions (**Chapter 4**).
2. To determine the osteogenic effect of selenium-rich green and black teas on *in vitro* bone formation from pre-osteoblasts in comparison with regular varieties of green and black teas, as well as the osteoprotective effect of these teas against hydrogen peroxide (H₂O₂)-induced oxidative stress in differentiated osteoblasts (**Chapter 5**).
3. To investigate the anti-osteoclastogenic effects of selenium-rich green and black teas on *in vitro* osteoclast formation from macrophage precursor cells in comparison with regular varieties of green and black teas (**Chapter 6**).

4. To investigate the potential bone-stimulating effect of selenium-rich green and black teas on bone mass in young growing rats in comparison with regular varieties of green and black teas (**Chapter 7**), by assessing any possible changes in bone density, metabolism and strength.
5. To investigate the potential bone-protective effect of selenium-rich green and black teas against bone loss in the ovariectomised mature rat model of postmenopausal osteoporosis in comparison with regular varieties of green and black teas (**Chapter 8**), by assessing any possible changes in bone density, metabolism and strength.
6. To determine whether the effects of consumption of different tea varieties (i.e. selenium-rich teas and regular teas) on bone health are related to their respective antioxidant and/or prebiotic-like properties, as assessed by using two different animal models, i.e. young growing intact rat, and ovariectomised mature adult rat model of postmenopausal osteoporosis (**Chapters 7 and 8**).

Figure 1-1 summarises the knowledge gaps identified in the existing research literature regarding the impact of tea on bone health, where the present study was conducted to bridge the gaps (Blocks I, II and III) between the three main topics of interest and bone health.

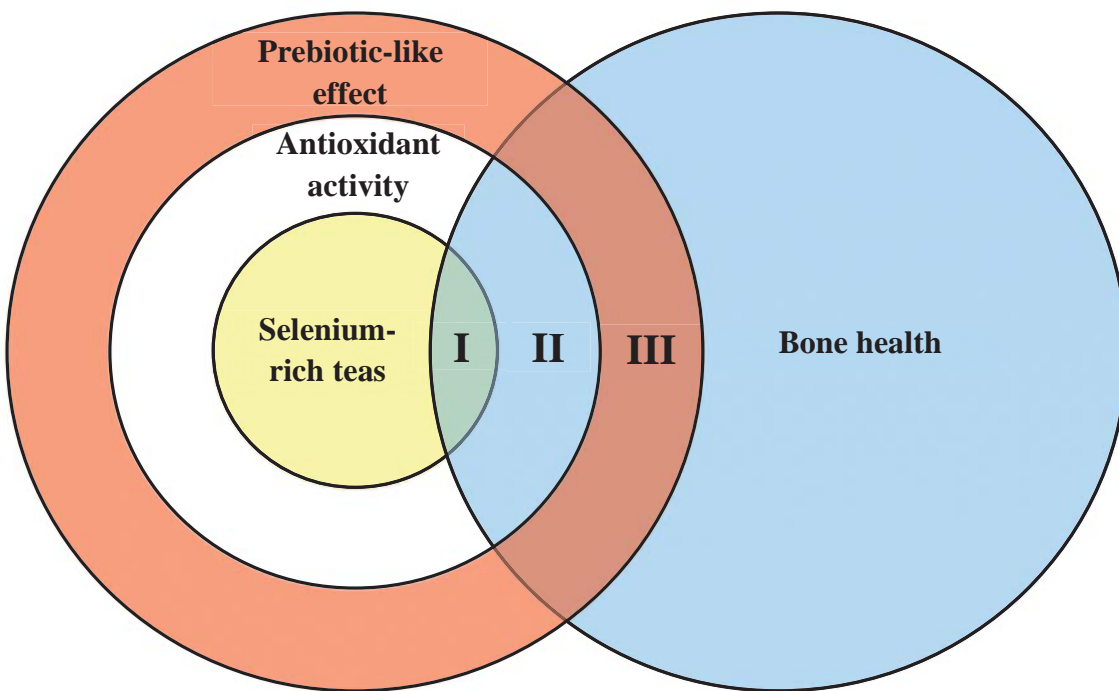
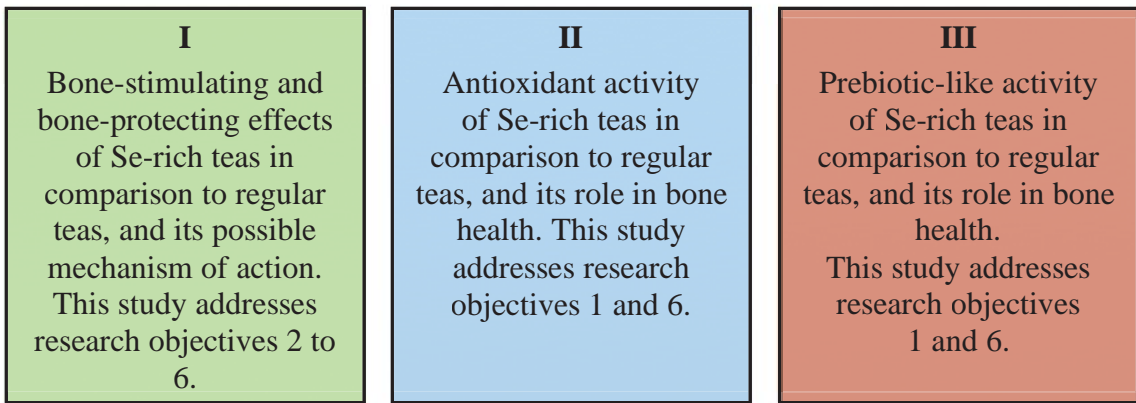


Figure 1-1. Overview of the thesis.

1.4 Thesis overview

The research presented in this thesis investigated the beneficial effects of tea on bone health using various *in vitro* and *in vivo* experimental assays and models. This thesis includes nine chapters and thirteen appendices.

Chapter 1 serves as a general introduction to the thesis; where it gives a brief overview of and postmenopausal osteoporosis, the limitations and issues with existing

pharmacological therapies for osteoporosis, and emerging evidence of the positive effect of tea as a nutritional strategy for the prevention of osteoporosis. This chapter also outlines the significance of the research study, research hypothesis and objectives of the thesis, and describes the organisation of the thesis.

Chapter 2 reviews the related literature to the current research study. This chapter is composed of three themed parts. The first part begins with laying out the physiology of bone, pathophysiology of postmenopausal osteoporosis in humans, and reviewing the link between bone metabolism and oxidative stress that may lead to postmenopausal osteoporosis. The second part provides a review of various aspects of tea properties, and critically appraises the mechanisms of antioxidative and prebiotic-like effects of tea. Finally, the third part of the chapter addressing the known role of tea in the maintenance of bone health through extensive clinical and pre-clinical research.

Chapter 3 describes the general materials and methods used throughout the experimental chapters of the thesis. This includes chemicals, equipment, sample preparation, basic techniques and procedures for cellular biology, experimental procedures for both *in vitro* and *in vivo* work, as well as analytical and statistical methods.

Chapter 4 reports the total phenolic content (TPC), antioxidant activities (AOA) and prebiotic properties of four different teas (i.e. selenium-rich green and black teas, and regular green and black teas). This chapter also evaluates the effect of different tea water extraction conditions on TPC and AOA, as well as of the correlation that may exist between TPC and AOA.

Chapter 5 is the first report to describe the osteogenic and osteoprotective effects of selenium-rich green and black teas on *in vitro* bone formation in comparison with regular teas. This chapter employs an established and widely used *in vitro* model of osteoblastogenesis, i.e. murine pre-osteoblastic MC3T3-E1 (Subclone 4) cells to investigate the effect of tea on osteogenic proliferation, differentiation and mineralisation in bone-forming cells. Furthermore, this chapter also evaluated the osteoprotective effect of various teas against hydrogen peroxide (H₂O₂)-induced oxidative stress in differentiated MC3T3-E1 cells.

Chapter 6 provided the first work that investigates the anti-osteoclastogenic effects of selenium-rich green and black teas in comparison to regular teas using an established and widely used an *in vitro* model of osteoclastogenesis, i.e. RANKL-induced osteoclast differentiation in murine macrophage RAW 264.7 cells.

Chapter 7 describes for the first time the impact of consumption of different varieties of tea, particularly selenium-rich teas in comparison with regular teas, on bone mass in young growing rats. The beneficial effect of the tested teas on bone properties in the young growing rats is assessed through determination of various bone parameters, including bone serum biomarker, biomechanical strength, and bone mineral measurements. This chapter also evaluates serum antioxidant capacity and caecal parameters in rats, in order to determine any link between the antioxidant and prebiotic potential of tea and bone mass in young growing rats.

Chapter 8 presents the first study on the influence of consumption of different varieties of tea, particularly selenium-rich teas in comparison with regular teas, on bone properties in a rat model of postmenopausal osteoporosis. This chapter presents the bone-protective effect of the tested teas on ovariectomy-induced bone loss in mature adult rats via determination of various bone parameters (i.e. bone serum biomarker, biomechanical strength, and bone mineral measurements). This chapter also evaluates serum antioxidant capacity and caecal parameters in rats, in order to determine any link between the antioxidant and prebiotic potential of tea and bone health.

Finally, **Chapter 9** summarises the main findings of the research study, provides overall discussion, identifies implications and limitations of the study, and outlines recommendations for future research work.

CHAPTER 2

LITERATURE REVIEW

This chapter has three sections; firstly the bone biology and pathophysiology of postmenopausal osteoporosis, followed by tea and its beneficial health effects, and finally the current scientific literature regarding promising effects of tea on bone health.

2.1 Bone health

2.1.1 Bone biology: An overview

Bone is a dynamic organ of mineralised connective tissues that primarily provides a structural and mechanical function for the body, i.e. as a semi-rigid body framework for load-bearing support, locomotion and protection of vital organs. Additionally, bone serves metabolic functions, **1)** as a major site of mineral homeostasis, and origin of bone matrix regulatory proteins, growth factors and cytokines; **2)** as a buffer system for acid-base homeostasis, and **3)** location of bone marrow for both haematopoietic and lipid storage systems (Office of the Surgeon General, 2004; Clarke, 2008; Kini & Nandesh, 2012). In general, bone is a natural composite material comprised of organic extracellular matrix (ECM) (30% of dry bone mass) that provides flexibility and resilience, and the predominant inorganic mineral (70% of dry bone mass) that is responsible for stiffness and strength. Aside from water and bone-associated cells, the organic ECM (also known as osteoid) is composed of osteoblast-derived proteins, i.e. collagenous fibres (primarily of collagen type I) and non-collagenous proteins (i.e. bone matrix regulatory proteins, growth factors, and cytokines); which both have a crucial role in mineral binding and regulating bone metabolism. Meanwhile, inorganic minerals are primarily of carbonated hydroxyapatite crystals (i.e. insoluble salt of calcium and phosphorus), which are found deposited into the collagen scaffolds within the ECM (Office of the Surgeon General, 2004; Clark, 2008; Bartl & Frisch, 2009; Morgan et al., 2009; Florencio-Silva et al., 2015).

2.1.1.1 Bone structure

Bone is composed of two main types, categorised largely by structure and location: cortical and trabecular bone. The cortical or compact bone is dense in appearance and forms the outer solid shell (i.e. cortex) surrounding the bone marrow cavity, which constitutes approximately 75% of the total bone mass and is mostly found in the shafts (diaphyses) of the long bones of the appendicular skeleton, and the outer shell surrounding trabecular bone both of the vertebrae and the ends of long bones. On the other hand, the trabecular or cancellous bone forms a sponge-like network of interconnecting plates and rods that line and interlace with the bone marrow cavity.

Trabecular bone is a highly porous structure that constitutes the remaining 25% of total bone mass and is mostly found in the vertebral bodies of the axial skeleton, in the expanded ends of the shaft (metaphyses), and in the ends of the long bones (epiphyses). However, it is worth mentioning that different skeletal sites may have different ratios of cortical-to-trabecular bone. The lumbar vertebra has a ratio of 25:75 of cortical:trabecular bone, calcaneus has 30:70, proximal femur has 50:50, distal radius has 75:25, and radial diaphysis has 95:5 (Office of the Surgeon General, 2004; Clark, 2008; Bartl & Frisch, 2009; Morgan et al., 2009). The cortical and trabecular bones differ mainly on the basis of porosity, i.e. the cortical bone porosity usually ranges from 5% – 50%, which is lower than the 40% – 95% porosity of trabecular bone. Accordingly, cortical bone has a lower surface area-to-volume ratio than trabecular bone: the latter has sponge-like organisation that contributes to its large bone surface area. Due to its lower surface area, cortical bone relatively has lower metabolic activity and turnover rate than trabecular bone. Hence, cortical bone is mainly capable of load-bearing support and serves mechanical functions in contrast to trabecular bone that primarily serves metabolic functions through active participation in mineral exchange, via its large bone surface and efficient blood supply (Jee & Yao., 2001; Clark, 2008; Bartl & Frisch, 2009; Morgan et al., 2009; Ma et al., 2012; Weaver & Gallant, 2013).

2.1.1.2 Bone homeostasis

As previously mentioned, bone is a reservoir for mineral homeostasis particularly of calcium, phosphorus and magnesium; which are essential minerals not only for bone, but also for other organ systems of the human body (Weaver & Gallant, 2013). These minerals are released from the bone when needed (e.g. when dietary intakes is in short supply) to maintain systemic mineral homeostasis and adequate mineral supply for cellular functions. However, this metabolic function of bone overwhelms its mechanical function as the resorption of bone mineral matrix can jeopardise the structural integrity of the skeleton, where excessive withdrawal could lead to loss of bone mass and eventually fractures. Further, bone mass and structure (both at macro- and microscopic level) are subjected to changes all through life in response to the mechanical loading environment (e.g. skeletal injury, excessive physical force) encountered during daily activity. Hence, to maintain bone structure and homeostasis in a changing environment that is influenced by internal and external forces, bone tissue continuously undergoes remodelling throughout life; a process

which enables functional adaptation to ensure both optimal skeletal integrity of the bone and adequate systemic levels of mineral homeostasis (Office of the Surgeon General, 2004; Clark, 2008; Bartl & Frisch, 2009; Morgan et al., 2009; Florencio-Silva et al., 2015).

2.1.1.3 Bone cells

Bone tissue is composed of four cell types, osteoblasts, osteocytes, bone-lining cells and osteoclasts. Among all these cell types, the first three are osteoblast lineage cells that originate from pluripotent mesenchymal stem cells (MSCs), while osteoclasts are the fusion of monocyte-macrophage lineage cells that originate from haematopoietic stem cells. Most importantly, it is the intercellular cross-talk among all these cells (including their respective progenitors) that enable their exquisitely coordinated actions in the regulation of bone remodelling (Raggat & Partridge, 2010; Florencio-Silva et al., 2015). Osteoblasts are mononucleated bone-forming cells that are usually found as a single layer along active bone-forming surfaces during osteogenesis or fracture repair. Osteoblasts increase bone mass mainly through secretion of type I collagen and other organic components (i.e. bone-matrix regulatory proteins such as osteocalcin (OC) and alkaline phosphatase (ALP)) that form the osteoid, which subsequently undergoes mineralisation (formation of bone) by the deposition of carbonated hydroxyapatite crystals. Aside from that, osteoblasts also secrete osteoclastogenic factors that play a role in stimulating osteoclastogenesis (e.g. receptor activator nuclear factor kappa beta ligand (RANKL) and macrophage-colony stimulating factor (M-CSF)) (Bellows et al., 1991; Golub & Boesze-Battaglia, 2007; Matsuo & Irie, 2008; Neve et al., 2010; Florencio-Silva et al., 2015). During osteoid mineralisation, a subpopulation of osteoblasts are progressively entrapped within the calcified osteoid and further differentiate into osteocytes that have lower collagen-producing activity than osteoblasts yet are capable of producing the bone-matrix regulatory proteins (e.g. sclerostin, bone morphogenetic proteins) implicated in mineralisation. Osteocytes are the most abundant bone cell and reside in lacunae (tiny bone chambers) that are scattered throughout the osteoid. The remaining osteoblasts either undergo apoptosis or become inactive flat-shaped bone-lining cells on the bone surface, which can be reactivated to become functional osteoblasts in response to stimuli that justify active bone formation. The osteocytes are interconnected via their dendrite-like cell processes, and their primary function is to regulate mineral homeostasis and respond to mechanical

loads, which are achieved through bone remodelling via intricate coupling mechanisms between the activities of osteoblasts and osteoclasts (Crockett et al., 2011; Schaffler & Kennedy, 2012; Sims & Martin, 2014; Florencio-Silva et al., 2015). Osteoclasts are giant multinucleated cells responsible for bone resorption, which involve both bone hydroxyapatite mineral dissolution and bone organic matrix degradation. Osteoclasts originate in the bone marrow and differentiate from hematopoietic mononuclear precursor cells of the monocyte-macrophage lineage in the presence of RANKL and M-CSF; these cytokines are secreted by both osteoblasts and osteoblast precursor cells (i.e. MSCs) (Walsh et al., 2006). Accordingly, *in vitro* osteoclast differentiation of osteoclast precursors may be sufficiently induced by RANKL and M-CSF in the absence of osteoblasts or osteoblast precursors (Theill et al., 2002; Wittrant et al., 2004).

2.1.1.4 Bone remodelling

Bone remodelling is the complex physiological process of bone synthesis and destruction that requires the asynchronous action of bone-resorbing cells (osteoclasts) and bone-forming cells (osteoblasts). The remodelling sequence and congregation of these two types of bone cells constitute the basic multicellular units (BMUs) that are either randomly distributed throughout the skeleton (stochastic act to prevent accumulation of old denser bone that is more brittle) or specifically targeted to bone affected by osteocyte death (due to immobilisation or bone macro/microdamage). The coupling activities of osteoclast and osteoblast within the BMUs is tightly-regulated by a variety of local factors (e.g. cytokines, growth factors, bone-matrix regulatory proteins) that are both secreted by bone-associated cells and released from the bone matrix during resorption in an autocrine and/or paracrine manner, and systemic factors (e.g. prostaglandins, hormones) that are brought into the BMUs by means of the vasculature. These regulatory factors are essential components of the intercellular signalling network between the bone cells (Clarke, 2008; Proff & Romer, 2009; Feng & McDonald, 2011; Sims & Martin, 2014; Florencio-Silva et al., 2015). The bone remodelling process involves four main phases: initiation/activation, resorption, reversal and formation (**Figure 2-1**); which are finely-executed under the control of multiple regulatory factors as listed, but not limited to, in **Table 2-1**. These regulatory factors are not discussed in details, but can be found in previously reviewed papers (Raisz, 1999; Yang & Karsenty, 2002; Dempster & Zhou, 2006; Hadjidakis & Androulakis, 2006;

Komori, 2006; Javed et al., 2010; Kini & Nandeesh, 2012; Long, 2012; Imai et al., 2013; Parra-Torres et al., 2013; Delaisse, 2014; Florencio-Silva et al., 2015).

Osteocytes are mainly involved in the initiation of bone remodelling as a sensor of stress (both mechanical and metabolic) signals and a coordinator of bone turnover within the BMUs. These functions of osteocytes are achieved via dynamic intercellular cross-talk facilitated by its dendrite-like processes that form the lacunocanalicular network in bone. Following stress signals transmitted by osteocytes, the activation phase begins under the stimulation of RANKL and M-CSF secreted by osteoblast lineage cells. The activation phase include origination of a new BMU, recruitment of both osteoclast and osteoblast precursors at the remodelling site, binding of osteoclast precursors to bone mineral and differentiation of osteoclast precursors into mature osteoclasts that then actively resorb bone (**Figure 2-1** and **Table 2-1**). The depth of the resorption pits created by active osteoclasts is influenced by their number, activity and life span (McNamara, 2010). Active osteoclasts then undergo apoptosis via stimulation of regulatory factors released from the resorbed bone (**Table 2-1**). Next, organic matrix remnants in the resorption pits are removed by a group of mononuclear phagocytes during the reversal phase in preparation for the deposition of new organic matrix; these cells may also release regulatory factors essential for bone formation (**Table 2-1**). Subsequent to the formation phase, osteoblast precursors differentiate into mature osteoblasts and then deposit layers of osteoid in the resorption pit that later will become mineralised. Some osteoblasts may become embedded in the bone matrix and further differentiate into stellate-shaped osteocytes. Once mineralisation is complete, osteoblasts either become inactive bone-lining cells on the surface of newly formed bone or undergo apoptosis (Clarke, 2008; Proff & Romer, 2009; McNamara, 2010; Raggat & Partridge, 2010; Crockett et al., 2011; Feng & McDonald, 2011; Sims & Martin, 2014; Florencio-Silva et al., 2015). In general, the bone remodelling process takes roughly six to nine months to complete, whereby the length of the resorption phase is shorter (two to twelve weeks) than the phase of bone formation (three to six months) (Stein & Lian, 1993; Watts, 1999; Manolagas, 2000; Eriksen, 2010).

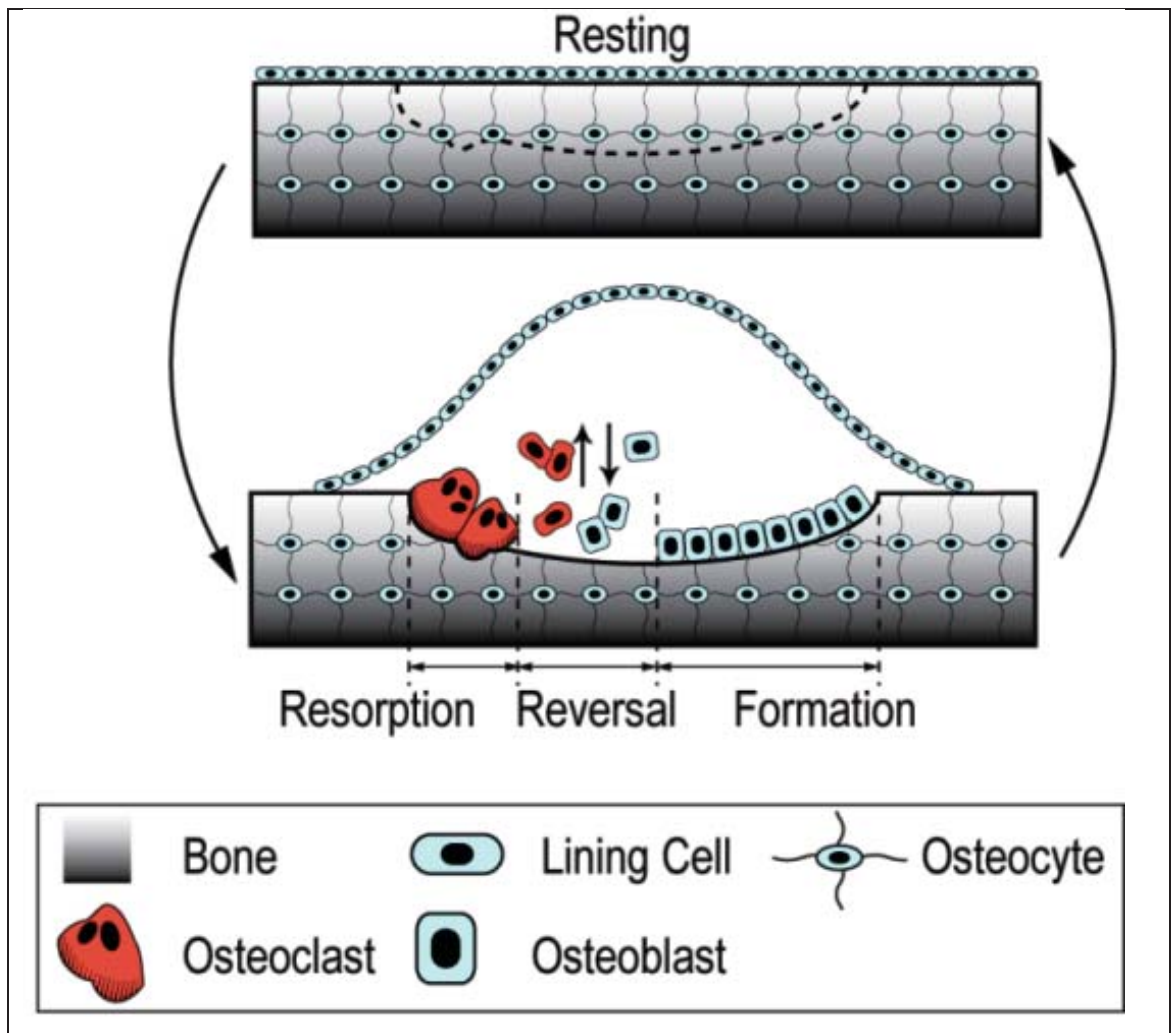


Figure 2-1. The sequence of bone remodelling within a basic multicellular unit (Reprinted with permission from the InTech Open Access Publisher. Source: © 2012 Pivonka P, Buenzli PR, Dunstan CR. Published in Cell Interaction under CC BY 3.0 license. Available from <http://dx.doi.org/10.5772/51149>).

The initiation/activation phase depends on intercellular cross-talk among osteoblast lineage cells and osteoclast precursors to initiate osteoclastogenesis. Following initiation/activation, activated osteoclasts resorb the underlying bone. During reversal phase, osteoclasts undergo apoptotic cell death, mononuclear phagocytes smooth the remaining bone matrix, and the recruited osteoblast precursors begin to differentiate. Bone formation phase is then proceeds as osteoblasts begin to fill the excavated pits with new bone. Finally, the formation of multiple layers of new bone ceases, followed by re-establishment of the resting bone surface that is maintained until the next initiation/activation of bone remodelling.

Table 2-1. Summary of local and systemic regulatory factors related to bone remodelling.

Local factor	Systemic factor	Transcription factor	References
Regulators of Initiation/Activation Phase			
<ul style="list-style-type: none"> • Interferon-γ • Tumor necrosis factor • Fibroblast growth factor • Interleukins (ILs): IL3, IL6, IL11 • Insulin-like growth factor-1 • Macrophage-colony stimulating factor • Receptor activator of nuclear factor kappa beta ligand 	<ul style="list-style-type: none"> • Androgen • Calcitonin • Oestrogen • Thyroid hormone • Parathyroid hormone 	<ul style="list-style-type: none"> • Proto-oncogene c-Fos • Nuclear factor kappa beta • Receptor activator of nuclear factor kappa beta 	<p>Raisz, 1999; Yang & Karsenty, 2002; Kini & Nandeesh, 2012; Imai et al., 2013.</p>
Regulators of Resorption Phase			
<ul style="list-style-type: none"> • Interferon-γ • Osteoprotegerin • Epidermal growth factor • Tumor necrosis factor alpha • Platelet-derived growth factor • Transforming growth factor beta • Macrophage-colony stimulating factor • Insulin-like growth factors (IGFs): IGF-I, IGF-II • Prostaglandins (PGs): PGE2, PGE1, PGG2, PGI2, PGH2 • Granulocyte/macrophage- colony stimulating factor • Interleukins (ILs): IL1, IL3, IL4, IL6, IL8, IL11, IL13, IL18 	<ul style="list-style-type: none"> • Androgen • Calcitonin • Oestrogen • Glucocorticoids • Thyroid hormone • Parathyroid hormone • Calcitriol (vitamin D) • Retinoids (vitamin A) • Parathyroid hormone-related peptide 	<ul style="list-style-type: none"> • Proto-oncogene c-Fos • Nuclear factor kappa beta • Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 • Transcription factor PU.1 	<p>Raisz, 1999; Yang & Karsenty, 2002; Hadjidakis & Androulakis, 2006; Kini & Nandeesh, 2012; Imai et al., 2013; Parra-Torres et al., 2013.</p>

Table 2-1. (*continued*).

Regulators of Reversal Phase	
<ul style="list-style-type: none"> • Interferon-γ • Interleukin-4 • Bone morphogenetic protein • Insulin-like growth factors (IGFs): IGF-I, IGF-II • Platelet-derived growth factor • Fibroblast growth factor • Osteoprotegerin • Transforming growth factor beta 	<p>Not specifically reported in any sources, but most probably of systemic hormones implicated in both resorption and formation phases.</p> <ul style="list-style-type: none"> • Core binding factor alpha1/Runt related gene 2 <p>Dempster & Zhou, 2006; Kini & Nandeesh, 2012; Delaisse, 2014.</p>
Regulators of Formation Phase	
<ul style="list-style-type: none"> • Bone morphogenetic proteins (BMPs): BMP2, BMP4, BMP6, BMP7 • Fibroblast growth factors • Insulin-like growth factors (IGFs): IGF-I, IGF-II • Interleukins (ILs): IL4, IL6, IL13 • Platelet-derived growth factors • Osteoprotegerin • Transforming growth factor beta • Vascular endothelial growth factor • Bone-matrix proteins: fibronectin, osteocalcin, osteonectin, osteopontin, bone sialoprotein, alkaline phosphatase, sclerostin 	<ul style="list-style-type: none"> • Core binding factor alpha1/Runt related gene 2 • Osterix • β-catenin • Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 • Smad transducer • Notch receptors • Activator protein 1 • Msh homeobox 2 • Signal transducer and activator of transcription 1 • Indian hedgehog • Distal-less homeobox • Activating transcription factor 4 <p>Yang & Karsenty 2002; Hadjidakis & Androulakis, 2006; Komori, 2006; Javed et al., 2010; Long, 2012; Parra-Torres et al., 2013.</p>

2.1.1.5 Systemic regulators of bone remodelling

The main systemic regulators of bone remodelling include parathyroid hormone (PTH), 1,25-dihydroxy vitamin D₃ (i.e. calcitriol (1,25(OH)₂D₃)), and oestrogen. Both calciotropic hormones (i.e. the first two) regulate serum calcium concentration through bimodal mechanisms targeting bone, the kidney and the intestine. When serum calcium decreases below a certain physiological level, the synergistic activity of PTH and calcitriol prompts the release of calcium from bone through indirect activation of osteoclast formation and function; in part, via enhancing RANKL production in osteoblast lineage cells. However, PTH also directly exerts anabolic effects on osteoblast lineage cells, which includes stimulation of Wnt signalling pro-osteoblastogenic pathways and inhibition of Wnt signalling antagonist, sclerostin (SOST). In contrast to chronically elevated serum PTH levels that favour bone resorption, low intermittent administration of PTH stimulates bone formation without significantly affecting resorption, thus suggesting its clinical importance as an anabolic agent (Mundy, 1999; Jilka et al., 2010; Crockett et al., 2011).

Calcitriol mainly enhances dietary calcium absorption in the gastrointestinal tract (GIT) and calcium reabsorption in the kidney; the former is achieved by increased formation of a calcium-binding protein in intestinal epithelial cells, i.e. calbindin D9k. While calcitriol plays a synergistic role with PTH in bone resorption, the resultant increase of serum calcium levels due to calcitriol-enhanced GIT uptake can lead to increased calcium deposition in bone. Calcitriol exerts direct anabolic effects on bone due to the presence of vitamin D receptors (VDRs) in osteoblast lineage cells, subsequently stimulating bone formation and function at different cellular stages via multiple regulatory actions. However, the effects can vary and are dependent on interactions with other regulatory factors, such as PTH. Accordingly, dietary calcium deficiency or defective GIT function may contribute to loss of bone mass (Long, 2012; Bickle, 2012; Crockett et al., 2011; Imai et al., 2013; Van Driel & Van Leeuwen, 2014).

Oestrogen is another essential hormone found in both men and women, which directly influences bone cells due to the presence of oestrogen receptors (ER α and ER β) in cells of both osteoblast and osteoclast lineages. Oestrogen primarily inhibits bone resorption by suppressing the activation of osteoclast formation and function via: inhibiting RANKL-induced osteoclastogenesis, suppressing RANKL production by

osteoblast lineage cells, increasing production of a RANKL antagonist (i.e. osteoprotegerin (OPG)), and inducing apoptotic cell death of osteoclasts. In addition, oestrogen also has a regulatory role with mechanical stimuli, and is able regulate the expression of a number of local factors and pro-inflammatory cytokines implicated in bone metabolism (i.e. interleukins and tumour necrosis factors). Further, oestrogen promotes bone formation through enhancing osteoblastogenesis and preventing apoptotic cell death in osteoblasts. Oestrogen also exerts antioxidant effects via a mechanism independent of the ERs (Behl et al., 1997; Simpkins et al., 2009; Clarke & Khosla, 2010; McNamara, 2010; Crockett et al., 2011; Manolagas et al., 2013; Florencio-Silva et al., 2015).

2.1.2 Postmenopausal osteoporosis: A bone remodelling disorder

2.1.2.1 Overview

Osteoporosis is a progressive systemic skeletal disease commonly affecting females and the elderly population. It results from the collective impact of decreased bone mass and microarchitectural impairment of bone tissue. This asymptomatic bone loss disease consequently increases skeletal fragility and susceptibility to fracture following minimal trauma injury (National Institute of Health, 2001; Rachner et al., 2011). People with osteoporosis have an increased risk for fracture (particularly of the proximal femur, distal radius, and vertebra) and this can be associated with a poor quality of life; accounting for substantial disabling pain, morbidity and mortality in the elderly population (Johnell & Kanis, 2005, 2006; Khosla, 2010). Osteoporotic fractures not only impose substantial negative psychosocial consequences due to impaired physical function, but also place an increased financial burden worldwide due to expensive direct and indirect healthcare costs associated with treatment and management (Johnell & Kanis, 2005; Yang & Liu, 2009; Harvey et al., 2010; Dempster, 2011; Ström et al., 2011; Dimai et al., 2012; Cauley, 2013; Hernlund et al., 2013; Marinho et al., 2014). Further, according to research data from populations in the United Kingdom and United States, osteoporotic fractures in women over the age of 45 years accounted for more hospitalisations as compared to other common diseases (i.e. myocardial infarction, stroke, breast cancer, chronic obstructive airways disease,

diabetes, or chronic lung disease), thus, leading to greater healthcare costs (Kanis, 1997; Singer et al., 2015).

An estimated 9 million new osteoporotic fractures worldwide were reported in 2000, with 61% of the overall osteoporotic fractures detected in women (Johnell & Kanis, 2006; World Health Organization, 2007). Odén et al. (2015) reported an estimated 158 million individuals aged 50 years or more were at high risk of osteoporotic fracture worldwide in the year 2010, and this number was expected to increase about two-fold by the year 2040. With the world's aged population increasing (Centers for Disease Control and Prevention, 2006; Population Reference Bureau, 2015), osteoporosis has become a growing health issue among the elderly and remains a significant global public health problem bringing social and economic burdens on both individuals and health service resources (Cooper et al., 1992; Harvey et al., 2010; Ström et al., 2011; Shuler et al., 2012; Åkesson et al., 2013; Cauley, 2013). Osteoporosis generally affects both women and men; however women, particularly following menopause are at increased risk of developing osteoporosis and related fractures (Johnell & Kanis, 2005, 2006; Nguyen et al., 2007; Cawthon, 2011; Cervelatti et al., 2013). The lifetime risk of developing an osteoporotic fracture at 50 years of age has been estimated within the range of 40% – 50% in women and 13% – 22% in men (Johnell & Kanis, 2005; Melton et al., 2005). The lower occurrence of osteoporosis and osteoporotic-fracture in men is due to the fact that men have greater bone mass, size, geometry and strength, have a lower life expectancy than women, and do not undergo menopause (Scane et al., 1993; Riggs et al., 2002; Nieves et al., 2005; Khosla et al., 2006).

The two main categories of osteoporosis include primary and secondary. Primary osteoporosis can be further classified into two categories, i.e. postmenopausal osteoporosis (also known as type I osteoporosis), and senile (age-related) osteoporosis (also known as type II osteoporosis) (Riggs & Melton, 1983; National Institute of Health, 2001). Postmenopausal osteoporosis is the most common form of bone loss disease, and is primarily due to ovarian hormone deficiency following menopause in aging women. Meanwhile, senile osteoporosis is mainly due to ageing and affects both women and men. On the other hand, secondary osteoporosis are bone loss disorders due to various medical conditions (nutritional deficiencies) or chronic diseases, or can also

result from certain pharmacological drugs that adversely affect bone health (National Institute of Health, 2001; Riggs et al., 2002; Office of the Surgeon General, 2004; Unnanuntana et al., 2011).

2.1.2.2 Pathogenesis of postmenopausal osteoporosis

Oestrogen has been long known to have a systemic effect or control over bone cells (Crockett et al., 2011; Florencio-Silva et al., 2015; Okman-Kilic, 2015); hence, oestrogen deficiency during the onset of menopause plays a significant role in up-regulation of bone loss in postmenopausal women (Compston, 2001; Clarke, 2008; Clarke & Khosla, 2010; McNamara, 2010; Feng & McDonald, 2011; Sims & Martin, 2014). Briefly, the decline in oestrogen concentrations after menopause accelerates the intensity of bone tissue remodelling (i.e. increased activation and number of BMUs), and exacerbates the imbalance in the regulation of bone turnover (characterised by increased resorption exceeding formation) that occurs after peak bone mass is achieved (Slemenda et al., 1996; Raisz & Seeman, 2001; Khosla et al., 2006; Lerner, 2006; Seeman, 2003, 2013).

The increase in activation and frequency of BMUs is due to an increased number of both osteoclasts and osteoblasts in response to oestrogen withdrawal. Additionally, oestrogen depletion increases the lifespan and bone-resorbing activity of osteoclasts that then cause deeper resorption pits, but concurrently decreases the lifespan of osteoblasts with impaired ability to form new bone in the resorption pits (Jilka et al., 1992; Bell et al., 1996; Manolagas, 2000; Seeman, 2003; Lerner, 2006; McNamara, 2010). Furthermore, it has been reported that about a decade after peak bone mass is achieved (around the age of 30 years), there is a low rate of bone remodelling due to an inadequate formation response of osteoblasts to resorption (Krølner & Nielsen, 1982; Nordin et al., 1990; Slemenda et al., 1996; Arlot et al., 1997; Heaney et al., 2000; Khosla et al., 2006; Almeida et al., 2007a; Manolagas, 2010). This inadequate response to resorption is age-associated, and results from the delayed initiation and completion of bone formation (osteoclast-mediated resorption in a BMU occurs in a short duration compared to the long period of osteoblast-mediated matrix formation and mineralisation that is normally meant to compensate for the bone resorbing activity) (Seeman, 2003; Raisz, 2005).

Together, these events imply a high bone turnover state at the onset of menopause that leads further to a net negative bone balance in each BMU. Subsequently, the net negative BMU balance reduces bone quantity and quality that ultimately results in increased bone fragility and thereby a higher propensity for fracture. The increase in bone fragility and susceptibility to fracture is mainly due to microstructural deterioration of both trabecular (i.e. trabecular thinning and perforation, and loss of trabecular connectivity) and cortical (i.e. cortical thinning and increased porosity) bone compartments (Arlot et al., 1990; Eriksen et al., 1990; Seeman & Delmas, 2006; Chavassieux et al., 2007; Clarke & Khosla, 2010; McNamara, 2010; Nishiyama et al., 2010; Seeman, 2003, 2008, 2013).

2.1.2.3 Osteoporosis risk factors

The mechanisms primarily underlying the osteoporotic state of bone include poor peak bone mass accrual during the years of skeletal growth, and accelerated bone turnover in adulthood attributed to an impaired formation response to increased resorption after the acquisition of peak bone mass (Hui et al., 1990; Raisz, 2005; Downey & Siegel, 2006; Sandhu & Hampson, 2011). In an attempt to prevent osteoporosis-related fractures, identifying risk factors for osteoporosis is crucial to allow appropriate therapeutic intervention strategies (both non-pharmacotherapy and pharmacotherapy approaches) for maximising peak bone mass during skeletal growth and preventing fracture during ageing. The early phase of osteoporosis is often undiagnosed in most individuals with low bone mass due to a lack of clinical signs of the disease (Gronholz, 2008).

Table 2-2 presents osteoporosis risk factors that can be categorised as non-modifiable and modifiable risk factors (Cosman et al., 2014; Cauley, 2015; Heidari et al., 2013, 2015; Morris-Naumann & Wark, 2015; Sahni & Kiel, 2015; National Institute of Health, 2015). Among the non-modifiable risk factors, heredity is the key determinant of peak bone mass, which may account for up to 90% of individual variance (Heaney et al., 2000; Recker & Deng, 2002; Khosla & Riggs, 2005; Matkovic & Visy, 2015). Meanwhile, modifiable factors also greatly influence bone health, where poor acquisition of peak bone mass is mainly the consequence of unhealthy lifestyle, i.e. nutritional insufficiency and physical inactivity (Krall, 1993; Wicks & Mahady, 2015). It is noteworthy that the most significant risk factors for postmenopausal osteoporosis

include early menopause (whereby a woman is below the age of 40 years at the onset of menopause), and menopausal duration of more than seven years (Demir et al., 2008).

Table 2-2. Non-modifiable and modifiable risk factors for osteoporosis.

Non-modifiable risk	Modifiable risk
Gender	Endocrine status
Ethnicity	Lifestyle factors:
Heredity: Small stature, low bone mass, and maternal history of osteoporosis	Excessive caffeine intake, frequent falls, low body mass index (BMI), insufficient intake of calcium and vitamin D, alcoholism, cigarette smoking, lack of weight-bearing exercise, lack of sun exposure, excessive sedentary lifestyle, low dietary protein intake
Nulliparity	
Advanced age	
History of oophorectomy/hysterectomy	
Menstrual factors: Delayed menarche, premature menopause, long menopausal duration	Gonadal hormone deficiency Medical conditions and medications predisposing to secondary osteoporosis

2.1.2.4 Reactive oxygen species (ROS) generation in biological systems

It is widely accepted that sex hormones (e.g. oestrogen) are a critical determinant in skeletal development and homeostasis throughout a person's life (Compston, 2001; Riggs et al., 2008; Khosla, 2010, 2012; Vico & Vanacker, 2010; Crockett et al., 2011; Khosla et al., 2011, 2012; Manolagas et al., 2013; Florencio-Silva et al., 2015). However, sex hormones are not the only factor that contributes to skeletal homeostasis, as there is increasing evidence indicating that bone loss following menopause is in part mediated by reactive oxygen species (Kim et al., 2006; Wauquier et al., 2009; Filaire & Toumi, 2012; Almeida & O'Brien, 2013).

Reactive oxygen species (ROS) is a term used for a range of chemically reactive oxygen-containing molecules that includes both radicals (chemical species with unpaired electrons) and non-radical derivatives (chemical species lacking free electrons that are oxidants and/or easily converted into radicals) of molecular oxygen, as presented in **Table 2-3** (Halliwell, 2006; Lü et al., 2010; Bhattacharya, 2015). Due to their unstable electronic configuration, these free radical molecules have an extremely high chemical reactivity against electron donors. In biological systems, ROS are the primary group of free radicals that are normally and constantly generated in the course of redox reactions during metabolism and functional activities by aerobic organisms (Rahman, 2007; Bhattacharya, 2015). Most ROS are formed as by-products of the mitochondrial respiratory chain involved in energy-production metabolism (i.e. oxidative phosphorylation), due to premature leakage of electrons from the electron transport systems onto molecular oxygen. The electron leakage results in partial reduction of molecular oxygen and leads to the formation of the superoxide anion radical ($O_2^{\cdot-}$), which is also the precursor for most other ROS (Cadenas et al., 2000; Turrens, 2003; West et al., 2011).

Table 2-3. Reactive oxygen species; adapted and modified from Halliwell (2006).

Radicals	Non-radical derivatives
Superoxide anion ($O_2^{\cdot-}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl (OH^{\cdot})	Hypobromous acid (HOBr)
Hydroperoxyl (HO_2^{\cdot})	Hypochlorous acid (HOCl)
Carbonate ($CO_3^{\cdot-}$)	Ozone (O_3)
Peroxyl (RO_2^{\cdot})	Singlet oxygen ($O_2^1\Delta_g$)
Alkoxy (RO^{\cdot})	Organic peroxides (ROOH)
Carbon dioxide radical ($CO_2^{\cdot-}$)	Peroxynitrite ($ONOO^-$)
Singlet oxygen ($^1O_2\Sigma_g^+$)	Peroxynitrate (O_2NOO^-)
	Peroxynitrous acid (ONOOH)
	Peroxomonocarbonate ($HOOCO_2^-$)

Additionally, ROS are also generated in other subcellular locations (i.e. endoplasmic reticulum, peroxisomes, microsomes, lysosomes, cytoplasm and membranes) as necessary intermediates of a wide variety of cytosolic enzyme- and metal-catalysed reactions (Dröge, 2002; Janssen-Heininger et al., 2008; Filaire & Toumi, 2012; Kaludercic et al., 2014; Bhattacharya, 2015; Görlach et al., 2015). Nicotinamide adenine dinucleotide phosphate oxidases (NOXs), the transmembrane proteins that catalyse the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH), are among the important enzymatic sources of ROS in most cells, which produce ROS that are required for various signalling pathways in response to a wide variety of endogenous and exogenous stimuli (Bhattacharya, 2015; Panday et al., 2015). More importantly, intracellular ROS generation can be greatly enhanced by endogenous metabolic changes (in response to growth factors, hormones and inflammatory cytokines), and various exogenous stressors including ionising radiation, environmental pollutants, ultraviolet light, chemotherapeutics, chemical oxidants, hyperoxia, xenobiotics, and transition metal ions (Janssen-Heininger et al., 2008; Bae et al., 2011; Filaire & Toumi, 2012; Poljsak & Milisav, 2013).

Moderately elevated levels and/or temporary increases of ROS are well-known for their role as essential mediators in redox regulation of cell survival (Dröge, 2002; Trachootham, et al., 2008; Halliwell, 2011; Schieber & Chandel, 2014). During normal physiological conditions, intracellular ROS are constantly scavenged by endogenous antioxidant defence systems, consisting of enzymatic (e.g. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), glutathione S-transferase (GST), heme oxygenase (HO), peroxiredoxin (Prx), thioredoxin reductase (TrxR)), and non-enzymatic antioxidants (e.g. uric acid, retinoic acid, thiol antioxidants (including thioredoxin (Trx), glutathione (GSH), and lipoic acid), ubiquinol, albumin, melatonin, bilirubin, N-acetyl cysteine (NAC), and metal-chelating proteins (i.e. ferritins, metallothioneins, transferrin)) (Bouayed & Bohn, 2010; Lennicke et al., 2015), which limit the rate of oxidation in order to maintain cellular redox homeostasis (Almeida, 2011). It has been generally accepted that redox homeostasis of cells is crucial for proper functioning of redox-dependent signalling pathways implicated in various vital cellular processes (e.g. differentiation, proliferation, senescence, and apoptosis) (Dröge, 2002; Martin & Barret, 2002; Valko et al., 2006, 2007; Bouayed & Bohn, 2010; Brigelius-Flohe & Flohe, 2011; Almeida,

2012; Ray et al., 2012; Schieber & Chandel, 2014; Holmström & Finkel, 2014; Lennicke et al., 2015).

However, any disruption in cellular redox homeostasis, i.e. depletion of endogenous antioxidant defence system, or excessive production of ROS, or both, can shift the cellular redox status towards oxidative stress (Trachootham et al., 2008; Wauquier et al., 2009; Dai & Mumper, 2010; Ray et al., 2012; Lorenz, 2013; Poljsak & Milisav, 2013; Schieber & Chandel, 2014). In general, ROS are highly reactive toward electron-rich organic molecules and have a strong affinity for lipids, carbohydrates, proteins, and nucleic acids (Augusto & Miyamoto, 2011). By affecting gene expression and enzymatic activity, oxidative stress can result in severe and permanent degradation to these cellular biomolecules (e.g. lipid peroxidation, denaturation of proteins, carbohydrate oxidation, and deoxyribonucleic acid modifications), which in turn can lead to the deterioration of physiological functions that have been ascribed to many degenerative diseases, including osteoporosis (Dalton et al., 1999; Forman et al., 2002; Djordjević et al., 2008; Johnstone & Baylin, 2010; Almeida, 2011; Cui et al., 2011; Schieber & Chandel, 2014).

2.1.2.5 The duality role of ROS in skeletal homeostasis

In accordance to the established role of ROS in the physiological control of cellular functions, a growing body of evidence also suggests bimodal physiological and pathophysiological actions of ROS in bone metabolism (Sontakke & Tare, 2002; Kim et al., 2006; Almeida et al., 2007a, 2009; Altindag et al., 2008; Filaire & Toumi, 2012). At low to moderate levels, ROS act as intracellular messenger molecules in multiple signal transduction cascades significant to the physiological process of bone remodelling, and eventually modulate gene expression of redox-dependent transcription factors that would serve to maintain skeletal homeostasis and integrity (Huh et al., 2006; Almeida et al., 2007a, 2007b, 2009; Wauquier et al., 2009; Romagnoli et al., 2013).

During physiological bone remodelling, ROS enhances osteoclast differentiation via multiple targets and through complex processes of signalling and gene expression, which primarily involves the RANKL-RANK cytokine system. It has been established that RANKL-induced ROS are essential for the activation of the RANK signalling cascades, which lead to osteoclastogenesis and stimulation of osteoclast activity. The

RANK signalling cascades include protein kinases B (AKT), nuclear factor of activated T cells (NFAT), nuclear factor kappa beta (NF- κ B), and mitogen-activated protein kinases (MAPKs; including extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK), and p38 MAPK). Further, NOX-induced ROS have been reported to be importantly involved in osteoclast-mediated bone resorption, RANKL-stimulated osteoclastogenesis, and RANKL-induced calcium signalling mechanism (calcium oscillations) during osteoclastogenesis (Armstrong et al., 2002; Theill et al., 2002; Wittrant et al., 2004; Boyce & Xing, 2008; Ha et al., 2004; Bai et al., 2005; Feng, 2005; Kim et al., 2005, 2010a; Lee et al., 2005; Pantano et al., 2006; Sasaki et al., 2009a, 2009b; Moon et al., 2011, 2012; Goettsch et al., 2013; Kanzaki et al., 2013; Mercer et al., 2014; Callaway & Jiang, 2015). Despite all these informations, the definitive molecular mechanisms of ROS stimulating osteoclast differentiation and activation of osteoclasts remain uncertain.

In a balanced state of physiological bone remodelling, the formation and activation of both osteoclasts and osteoblasts are tightly coupled and regulated through a complex network of hormonal mediators, growth factors and cytokines, which depend upon intercellular cross-talk between these cells (Matsuo & Irie, 2008; Sims & Martin, 2014). The role of ROS on osteoclastogenesis and activation of osteoclasts has been well documented in the literature (Callaway & Jiang, 2015). However, the influence of physiological ROS on osteoblast differentiation and function are not well understood (Mandal et al., 2011; Robaszkiewicz et al., 2012; Atashi et al., 2015).

It has recently been proposed that ROS-mediated antioxidant defence systems is the key factor that enhance the efficiency of osteoblastic differentiation, and stimulate activation of osteoblast function (Arai et al., 2007; Ambrogini et al., 2010; Jilka et al., 2010; Rached et al., 2010; Sequiera et al., 2011; Romagnoli et al., 2013; Sart et al., 2015). A number of studies suggest that ROS have an indirect positive contribution to osteogenesis, as achieved through ROS-mediated antioxidant defence responses (such as CAT, GSH-Px, NAC, SOD, Trx, GSH, and HO) in the maintenance of cellular redox homeostasis and in osteogenic signalling pathways (Sen, 1998; Chen et al., 2008a; Fatokun et al., 2008; Juarez et al., 2008; Barbagallo et al., 2010; Imhoff & Hansen, 2010; Mandal et al., 2011; Nojiri et al., 2011; Romagnoli et al., 2013; Yamada et al., 2013). In fact, there are various ROS-mediated and redox-dependent signal transduction

pathways that have been reported for their positive role in the regulation of osteogenesis (Chen et al., 2008a; Choe et al., 2012; Urao & Ushio-Fukai, 2013). These osteogenic molecular targets of ROS include MAPKs, NF- κ B, bone morphogenetic protein-2 (BMP2), transforming growth factor beta (TGF β), wingless-type mammary tumor virus integration site (Wnt), hedgehog (Hh), insulin-like growth factor (IGF), NEL-like protein-1 (NEL1), activator protein-1 (AP1), and hypoxia-inducible factor-1 (HIF1) (Sen, 1998; Mody et al., 2001; Feng et al., 2003; Johnson et al., 2006; Greenblatt et al., 2010; de Gorter et al., 2011; Mandal et al., 2011; Monroe et al., 2012; Atashi et al., 2015; Espinosa-Diez et al., 2015).

Most importantly, transcription factors of the forkhead homeobox type O (FOXO) family, and nuclear factor erythroid 2-related factor 2 (Nrf2) are key modulators of cellular redox status that also contribute to the regulation of skeletal homeostasis (Pervaiz et al., 2009; Manolagas, 2010; Almeida & O'Brien, 2013; Karajabille et al., 2013; Wang et al., 2013a; Atashi et al., 2015; Sun et al., 2015). It has been reported that ROS activation of FOXO (through ROS-mediated JNK kinase) and Nrf2 (through ROS-modification of the Kelch-like ECH-associated protein 1 (KEAP1) cysteine residue) are both responsible for the expression of genes implicated in antioxidant defence systems (i.e. endogenous antioxidative enzymes), which are primarily required to protect the cells from the damaging effects of ROS (Almeida et al., 2007b; Trachootham et al., 2008; Ambrogini et al., 2010; Sequeira et al., 2011; Wang et al., 2014; Atashi et al., 2015; Sun et al., 2015). Among all of the FOXO proteins (FOXO1, FOXO3, FOXO4 and FOXO6), FOXO1 is specifically identified as the main regulator of redox balance and is a positive modulator of bone mass due to its involvement in osteoblast physiology (Rached et al., 2010; Teixeira et al., 2010; Kousteni, 2011).

2.1.2.6 The implication of oxidative stress (OS) in the progression of postmenopausal osteoporosis

In contrast to the physiological role of tightly regulated ROS as signalling molecules, excess sustained accumulation of intracellular ROS (in response to various stress stimuli) and insufficient compensation by endogenous antioxidant defence systems to detoxify ROS result in OS (Valko et al., 2006; Djordjević et al., 2008; Trachootham et al., 2008; Ray et al., 2012; Poljsak & Milisav, 2013). Oxidative stress

can cause irreversible modifications to genes involved in normal bone remodelling due to dysregulation of redox-dependent signalling pathways, eventually compromising skeletal functionality. Under conditions of OS, excessive levels of ROS may elevate osteoclastogenesis and stimulate osteoclast-dependent resorption through increased activation of RANKL-RANK signalling. Simultaneously, ROS causing increased apoptosis of osteoblast progenitors, and deleterious effects on osteoblast differentiation, survival and physiological function through alterations in gene expression of transcriptional factors associated with both osteogenesis and osteoblast-mediated bone formation (Suda et al., 1993; Mody et al., 2001; Bai et al., 2004, 2005; Almeida et al., 2007a, 2007b; Sharpless & DePinho, 2007; Chen et al., 2008a; Kim et al., 2006, 2010a, 2010b; Maiese et al., 2010; Manolagas & Parfitt, 2010; Kassem & Marie, 2011; Almeida & O'Brien, 2013; Karajabille et al., 2013; Rao & Rao, 2013). Thus, the amount of bone resorbed by osteoclasts is inadequately reinstated with bone deposited by osteoblasts, resulting in accelerated bone turnover that impairs skeletal integrity and leads to progressive bone loss disease (Almeida et al., 2007a; Almeida, 2012; Manolagas, 2010; Nojiri et al., 2011). In fact, there is a considerable amount of literature that has indicated a close association between cellular redox homeostasis and bone biology, and supports the notion that oxidative stress may be among the main mechanisms that can lead to the initiation and progression of osteoporosis in humans (Shen et al., 2009a, 2011b, 2013a, 2013b; Wauquier et al., 2009; Almeida, 2012; Karajabille et al., 2013; Portal-Núñez & Esbrit, 2013; Holmström & Finkel, 2014; Philippe & Wittrant, 2014).

It is important to note that OS is commonly associated with advancing age, whereby the cells' ability to resist or adapt to the oxidative environment becomes less effective due to age-associated increased production of ROS and age-related decline in the response of endogenous antioxidant systems (Cui et al., 2011; Feng & McDonald, 2011; Poljsak et al., 2013). Thus, age-related OS is considered an important contributor to the pathogenesis of degenerative bone loss disease, i.e. osteoporosis (Manolagas, 2010; Almeida, 2012; Portal-Núñez & Esbrit, 2013). Meanwhile, evidence also suggests that oestrogen deficiency results in increased production of ROS and speeds up the effect of age-associated bone loss through depletion of endogenous antioxidant cellular defences, which in turn causes high bone turnover and bone remodelling imbalance in favour of bone resorption that subsequently results in a net decrease in bone mass and

strength. In this regard, the acceleration of age-associated bone loss by oestrogen deficiency may eventually leads to postmenopausal osteoporosis (Almeida et al., 2007a; Halliwell & Gutteridge, 2007; Wauquier et al., 2009; Pandey & Rizvi, 2010; Nojiri et al., 2011; Zhang et al., 2011; Khosla, 2012; Bellanti et al., 2013; Sims & Martin, 2014).

2.2 Tea – *Camellia sinensis* (L.) O. Kuntze (Theaceae)

2.2.1 An overview

Tea, an infusion made from the leaves of the tea plant *Camellia sinensis* is the most widely consumed non-alcoholic beverage across the world after water (Graham, 1992). Normally, young tea shoots (flush) that consist of the terminal apical buds and two or three adjacent leaves of tea plants are used in the production of various types of tea (De Costa et al., 2007). Tea is favoured not only for its pleasant taste but also for its perceived medicinal properties and health effects (Serafini et al., 2011; Hayat et al., 2015). Tea plants were originally native to Southern China, North India, Myanmar, and Cambodia (Hicks, 2001). However, the increasing growth of the global tea industry has seen cultivation of tea plants around the world, mostly in tropical and subtropical climates with plenty of rainfall, good drainage and marginally acidic soil (Hicks, 2009; Majumder et al., 2010; Chen & Chen, 2013). Indeed, tea is among the primary commodities of international trade and is a source of revenue in most of the major tea producing countries (Food and Agriculture Organization of the United Nations, 2015). Being increasingly in demand, tea is not only regarded as a part of the global beverage industry, but also has a share in the nutraceuticals and functional food market (Hicks, 2009; Sharma et al., 2010; Banerjee & Chatterjee, 2015; Blumberg et al., 2015; Food and Agriculture Organization of the United Nations, 2015).

2.2.2 Cultivated varieties

All types of teas are derived from the same plant species of *C. sinensis*, and commercial tea production mainly uses two sub-varieties that are categorised based on leaf characteristics, i.e. small-leaved *C. sinensis* var. *sinensis* (L.) and broad-leaved *C.*

sinensis var. *assamica* (Masters) Kitamura (Sealy, 1958; Wachira et al., 2013). The *C. sinensis* var. *sinensis* is reported to be originally native to and grown in China, as well as commonly cultivated in Japan and Taiwan (Chu, 1997; Harbowy & Balentine, 1997). Meanwhile, the *C. sinensis* var. *assamica* is originally derived from India and mostly cultivated in South and Southeast Asia (Chan et al., 2007; De la Paix et al., 2010). The cultivated varieties (cultivars) of tea preferred for the production of green tea is *C. sinensis* var. *sinensis*; the fresh leaves of this tea cultivar have low polyphenol content but high levels of chlorophyll and amino acids (Chu, 1997; Harbowy & Balentine, 1997; Kaur et al., 2014). *C. sinensis* var. *assamica* is not preferred for green tea production due to its high polyphenol content compared to that of var. *sinensis* (Haslam, 1989; Takeda, 1994; Harbowy & Balentine, 1997; Astill et al., 2001; Karori et al., 2007). This is due to the fact that a high concentration of unoxidised (non-fermented) catechins in the finished green tea product of var. *assamica* can cause an unpleasant, strongly astringent (metallic) taste that does not meet the requirement for high quality green tea (Kaur et al., 2014). Meanwhile, *C. sinensis* var. *assamica* is particularly suitable for high quality black tea production owing to its higher polyphenols, tannins (proanthocyanidins) and caffeine content than that of var. *sinensis*. Oxidised polymers of polyphenols (i.e. thearubigins and theaflavins), tannins and caffeine are among the compounds responsible for the colour and characteristic flavour of a finished black tea product (Nagata & Sakai, 1984; Takeda, 1994; Harbowy & Balentine, 1997; Astill et al., 2001; Anesini et al., 2008; Bancirova, 2010; Chaturvedula & Prakash, 2011). Furthermore, *C. sinensis* var. *sinensis* has the high chlorophyll content that is important in obtaining the desired shade of green of the finished green tea product, while high levels of amino acids in this tea cultivar contribute to the refreshing full-bodied (umami) taste of green tea (Kaneko et al., 2006; Kaur et al., 2014).

2.2.3 Manufacturing process

There are at least three types of tea produced around the world that are mainly distinguished by their various degrees of fermentation during the tea manufacturing process, namely green (unfermented), oolong (semi-fermented) and black (fermented) teas (Shitandi et al., 2013; Kosińska & Andlauer, 2014). In the tea manufacturing industry, fermentation of fresh tea leaves is defined as enzymatic oxidation of

monomeric catechins catalysed by the plants endogenous oxidising enzymes (primarily by polyphenol oxidase), which results in a natural browning of the tea leaves leading to the colour and aroma of the finished tea liquor (Harbowy & Balentine, 1997; Kuhnert, 2013). Additionally, these tea products vary in their chemical composition depending upon which cultivar type is used for the tea production (Cabrera et al., 2006; Wachira et al., 2013). Other infusion products of tea plant, such as white, yellow and dark (post-fermented pu-erh and brick) teas are less popular or are less consumed in particular geographical locations or communities (Saberli, 2010; Kaur et al., 2014). Green tea is produced by either pan-frying (Chinese-method of heating process) or steaming (Japanese-method of heating process) fresh tea leaves at a high temperature to inactivate polyphenol oxidase (PPO), in order to minimise enzyme-mediated fermentation and subsequently preserving catechins in the tea leaf (Kosińska & Andlauer, 2014). In line with this, it has been reported that the chemical composition of the finished green tea product is not much different from those of the unprocessed fresh leaves (Graham, 1992; Tanaka et al., 2013). On the other hand, the production of black and oolong teas involve a withering phase that aims to obtain a desirable moisture content of fresh tea leaves, in order to prepare them for a further rolling step. The withered leaves are then rolled or crushed to maximise the exposure of PPO to oxygen and to initiate catechins–PPO fermentation reactions. During the fermentation process, tea catechins are oxidised by the action of PPO into polymeric compounds that give rise to distinct characteristics (flavour and colour) of black tea, such as theaflavins and thearubigins; thus, leading to a decreased content of catechins (Cooper, 2012; Kosińska & Andlauer, 2014; Hayat et al., 2015). The final step is a drying step meant to abolish the enzyme activity by high temperature. However, a brief fermentation step during the manufacturing process of oolong tea results in partial oxidation of catechins that leaves the tea with both native and oxidised catechins. Thus, oolong tea has an intermediate range of chemical composition and characteristics between that of green and black teas (Cabrera et al., 2006; Song & Chun, 2008; Imran et al., 2012; Wachira et al., 2013). Black tea is the most popular tea drink, makes up the vast majority of tea production (78%) and is primarily consumed in Western countries and some Asian countries. Green tea accounts for about 20% of tea production and is favoured in China, Japan, Korea and Morocco. Oolong tea, makes up the remainder 2% of total tea, and is mostly favoured in China and Taiwan (Cabrera et al., 2006; Alcázar et al., 2007; Karori et al., 2007; Sharma et al., 2007; Tanui et al., 2012; Butt et al., 2014). It is worth noting that green tea consumption

has now expanded globally and gained popularity in Europe and North America for its purported beneficial health effects (Kosińska & Andlauer, 2014).

2.2.4 Chemical composition

In general, the chemical constituents of both fresh young tea shoots and finished tea are mainly composed of volatile and non-volatile compounds; these compounds affect the tea quality and determine the market value of a finished tea product (Chen & Chen, 2013; Yang et al., 2013). Volatile compounds influence the tea aroma (Ho et al., 2015), and five volatiles have been identified as the key components that can help differentiate between unfermented and fermented teas: benzaldehyde, indole, methyl-5-hepten-2-one, methyl salicylate, and trans-2-hexenal (Yang et al., 2013). Non-volatile compounds constitute the major part of the tea solids, and are responsible for the colour and flavour of a finished tea product (Chen & Chen, 2013). Non-volatiles found in fresh tea flush include complex mixtures of polyphenolic compounds (e.g. flavonoids, lignans, phenolic acids, stilbenes), purine alkaloids (methylxanthines such as caffeine), amino acids (e.g. L-theanine), enzymes (e.g. PPO), pigments (e.g. chlorophyll, carotenoids, theaflavins, thearubigins), lipids, sugars (i.e. carbohydrates such as mono- and polysaccharides), organic acids (e.g. oxalic acid), minerals (e.g. calcium), trace elements (e.g. fluoride) and vitamins (e.g. A, B complex, C, E, K) (Balentine et al., 1997; Chacko et al., 2010; Chaturvedula & Prakash, 2011; Chen & Chen, 2013; Yashin et al., 2015). Among all the tea constituents, polyphenols are the compounds of greatest interest due to their protective effect against aging- and oxidative stress-related diseases (Dai & Mumper, 2010; Queen & Tollefsbol, 2010; Hernández-Hernández et al., 2012; Vladimir-Knežević et al., 2012; Shahidi & Ambigailapan, 2015).

It is important to note that the complex chemical compositions of fresh tea flush varies widely and is greatly influenced by multiple factors including the age of tea leaf (Lin et al., 2003; Song et al., 2012; Lee et al., 2014), type of cultivars (Wachira et al., 2013; Kaur et al., 2014), climate and region of production (Shishikura & Khokhar, 2005; Owuor et al., 2008, 2010), genetic diversity among tea varieties (Owuor & Obanda, 1995; Magoma et al., 2000), geographical areas where the teas were sourced from (Bhattacharya & Sen-Mandi, 2011; Jayasekera et al., 2011), the harvesting season (Erkturk et al., 2010; Jayasekera et al., 2011, 2014; Liu et al., 2015a), fermentation

conditions of tea processing (Obanda et al., 2001), environmental conditions, and agricultural practices (Song et al., 2012; Kaur et al., 2014). Most importantly, these compounds undergo a series of biochemical changes that occur during the various phases of the manufacturing processes to produce a variety of teas (Balentine et al., 1997; Lin et al., 1998; Ahmed & Stepp, 2013; Senthil Kumar et al., 2013; Shitandi et al., 2013; Kosińska & Andlauer, 2014).

Phenolic compounds, also commonly known as polyphenols or vitamin P (Dreosti, 1996; Velderrain-Rodríguez et al., 2014), are plant secondary metabolites (i.e. chemical substances synthesised by plant cells that are essential for the plants survival) that are present as the main chemical components in tea, making up around 30% (w/w) of the dry weight of the tea leaves (Graham, 1992; Balentine et al., 1997; Astill et al., 2001; Katalinic et al., 2006; Lin et al., 2008). Polyphenols are comprised of a diverse group of compounds, which are chemically characterised by the attachment of one or more hydroxyl groups (-OH) to at least one aromatic hydrocarbon (benzene) ring. There are four major groups of polyphenols according to their carbon skeleton, i.e. flavonoids, lignans, phenolic acids and stilbenes; these are not discussed in details in this review, but can be found in previously published papers (Pandey & Rizvi, 2009; Tsao, 2010). Polyphenols range from simple molecules of low molecular weight (e.g. phenolic acids) up to complex polymerised molecules of high molecular weight (e.g. proanthocyanidins) (Crozier et al., 2009; Tsao, 2010; Sang et al., 2011). Flavonoids are a largest group of polyphenols that are widely reported to have various health benefits due to their antioxidant properties (reviewed in Kumar & Pandey (2013). Flavonoids are classified based on their chemical structures into anthocyanins, catechins, flavanones, flavones, flavonols and isoflavones (Sang et al., 2011).

Catechins, also known as flavanols (or flavan-3-ols), are the most significant subclass of flavonoids in tea (Persson, 2013; Shitandi et al., 2013). Catechins represent the most abundant polyphenols in green tea, whereby they constitute around 30% – 42% of the dry weight of the solids in green tea infusion (Balentine et al., 1997). Catechins are monomeric flavonoids mainly comprising of (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG), and (+)-catechin (C), (-)-gallocatechin (GC), (-)-catechin-3-gallate (CG), and (-)-gallocatechin-3-gallate (GCG), of which the first four are the principal catechins in

green tea. Among these four tea catechins, EGCG is the most pharmacologically active and richest compound present in green tea, accounting for approximately 65% of the total catechins (Perva-Uzunalic et al., 2006; Zaveri, 2006), thus it is frequently used in research studies (Jin et al., 2014; Ghasemi-Pirbaluti et al., 2015).

There are four major theaflavins that have been identified in black teas (representing 10% – 20% of dry weight of black tea solids): theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate. While thearubigins constitute around 60% – 70% of the dry weight of the solids in black tea infusion, its chemical structure is not well-defined and has yet to be characterised (Ho et al., 1997; Wang & Ho, 2009; Kuhnert, 2010, 2013; Bandyopadhyay et al., 2012; Da Silva Pinto, 2013). Additionally, Chen et al. (2012) recently discovered the presence of new compounds in black tea, i.e. theaflavin trigallate and tetragallate. Catechins are not common compounds in black tea, accounting for only between 3% – 10% of the solids in black tea infusion (Graham, 1992; Balentine et al., 1997). This is due to the enzymatic oxidation that occurs during the black tea manufacturing process (Cooper et al., 2005; Shitandi et al., 2013; Kosińska & Andlauer, 2014). During black tea processing, the monomeric catechins undergo enzymatic oxidation by PPO and are transformed mainly into complex polymerised molecules of higher-molecular-weight, theaflavins (dimeric form) and thearubigins (oligomeric form) that give black tea its unique chemical and liquor characteristics. The enzymatic oxidation takes place throughout the fermentation of the leaves of the tea plants (Ostadalova et al., 2011). This oxidative polymerisation eventually leads to approximately 85% less catechin content in black tea compared to green tea (Balentine et al., 1997; Astill et al., 2001; Sutherland et al., 2006; Novotny & Baer, 2013). Some catechins are still detectable in black tea as they may escape the fermentation process (Bailey et al., 1990; Rechner et al., 2002; Li et al., 2013).

2.2.5 The preparation of infusion beverages

Tea beverages are typically prepared by brewing the processed leaves of tea plants in hot water. However, there are many different tea brewing techniques, depending upon tea types, individuals, cultures and countries (Astill et al., 2001; Ahmed & Stepp, 2013; Zimmermann & Gleichenhagen, 2013). There is evidence that different

tea preparations may cause significant variation in the final biochemical composition of the brewed tea (Henning et al., 2003; Kosińska & Andlauer, 2014; Fernando & Soysa, 2015). Therefore, it is important to take into account several preparation variables that may directly affect biologically active compounds (i.e. mainly the polyphenols) present in a brewed cup of tea (Peterson et al., 2004; Komes et al., 2010), including steeping time, water temperature for tea brewing, tea-water ratio, tea particle size, brewing of loose leaves or teabags, serving additives (e.g. lemon juice, milk), and stirring (Astill et al., 2001; Khokhar & Magnusdottir, 2002; Shishikura & Khokhar, 2005; Kim et al., 2007; Molan et al., 2009b; Ryan & Petit, 2010; Jayasekera et al., 2011; Samaniego-Sánchez et al., 2011; Zimmermann & Gleichenhagen, 2011; Araújo Ramalho et al., 2013; Yang & Liu, 2013; Kosińska & Andlauer, 2014; Castiglioni et al., 2015). These preparation variables have been well-studied and may be worth manipulating to minimise degradation of polyphenolic antioxidants during tea infusion, in order to maximise the extraction of tea polyphenolic compounds and their potential health benefits. Among all these factors, the steeping conditions, i.e. time and temperature, are consistently reported as the most significant factor for efficient extraction of polyphenolic antioxidants from tea (Astill et al., 2001; Khokhar & Magnusdottir, 2002; Molan et al., 2009b; Vuong et al., 2010, 2011a, 2011b; Samaniego-Sánchez et al., 2011; Hajiaghaalipour et al., 2015; Saklar et al., 2015). While the short-time hot-water infusion method is the most common practice of tea brewing across the world, prolonged cold-water infusion (i.e. at room temperature) has recently gained popularity among tea drinkers in Taiwan and is also being adopted in North America (Yang et al., 2007; Venditti et al., 2010; Damiani et al., 2014; Lantano et al., 2015). There is a small amount of evidence that the prolonged cold-infusion method may significantly increase both total phenolic content and antioxidant activity of white tea compared to the brief hot-water infusion (Venditti et al., 2010; Damiani et al., 2014). On the other hand, the brief hot-infusion method of black and green teas results in significantly higher total phenolic content and/or antioxidant activity than that of the prolonged cold-infusion method (Venditti et al., 2010). However, reports from other studies are inconsistent (Castiglioni et al., 2015; Hajiaghaalipour et al., 2015; Lantano et al., 2015), which could be due to diverse methodological approaches (e.g. form of tea, steeping conditions).

2.2.6 Bioavailability and metabolism

In a nutritional context, bioavailability is defined as the proportion of an ingested dietary component and its metabolites that enter the systemic circulation via normal metabolic pathways (Holst & Williamson, 2008). Bioavailability is a factor that determines the biological efficacy of a pharmacologically active compound and also provides better understanding of its potential safety risks in humans (Cassidy & Kay, 2013; Parisi et al., 2014). Polyphenols are pharmacologically the most important group of tea bioactives for human health, and their health benefits are generally dependent on the actual amount consumed; however, it is the bioavailability of polyphenols that modulate the bioefficacy of polyphenols, and this is worthy of further study (Manach et al., 2005; Fairweather-Tait et al., 2010; Rein et al., 2013; Marín et al., 2015). Bioavailability of polyphenols is generally low (less than 10% of the ingested amount) in accordance to Lipinski's rule of five (Lipinski et al., 2001), which states that compounds that have either more than five hydrogen bond donors, or more than ten hydrogen bond acceptors, or a molecular weight greater than 500 daltons (D), or a calculated partition coefficient value (LogP) of more than five are poorly bioavailable. This is due to their limited absorption in the gastrointestinal tract, extensive metabolism within the enterocytes/colonocytes and hepatocytes, and rapid clearance from the body (Chow et al., 2003; Manach et al., 2005; Liu & Hu, 2007; Yang et al., 2008b; Chow & Hakim, 2011; Landete, 2012). Most importantly, bioavailability differs greatly among the individual phenolic compounds and is highly influenced by a number of internal and external variables, as reviewed by many authors (Scalbert & Williamson, 2000; Porrini & Riso, 2008; D'Archivio et al., 2010; Bohn, 2014; Valdés et al., 2015).

Polyphenols have a common phenolic feature, however, their structural diversity allows for differences in physicochemical properties (including water solubility, lipophilicity, molecular weight, stereochemistry of the glycosidic moiety, pK_a (the log of acidity constant), and degree of chemical linkages) that essentially govern their absorption mechanisms and bioavailability in the human body (Manach et al., 2009; Chow & Hakim, 2011; Sang et al., 2011; Bolca et al., 2013; Cardona et al., 2013; Carbonell-Capella et al., 2014). According to the literature, most polyphenols are natively present as glycosides (conjugated to sugar moieties), polymers (conjugated to other phenolics) and esters (conjugated to carboxylic acid). Catechins are exceptional as

they are always present in the non-glycosylated form (i.e. aglycone). While aglycones (sugar-free molecules) and simple glucosides (glucose conjugates) occur in states that are more readily absorbable, phenolic compounds in their native forms are poorly absorbed in the GIT as they are likely to escape from acidic-gastric digestion in the stomach and are less efficiently transported across the small intestinal epithelium (enterocyte) (Clifford, 2004; Manach et al., 2004; Clifford & Brown, 2006; Gao et al., 2006; D'Archivio et al., 2007; Del Rio et al., 2013, Marín et al., 2015). Polyphenol glycosides can be converted into aglycones (i.e. deglycosylation by cytosolic β -glucosidase or luminal hydrolysis by intestinal lactase-phlorhizin hydrolase) to their increase lipophilicity and enhance their uptake into the enterocytes. Meanwhile, the remaining unabsorbed polyphenols in the forms of polymers, esters and other glycosylated derivatives (e.g. rhamnose conjugates) proceed to the large intestine (colon) and undergo hydrolysis by the action of intestinal bacterial enzymes (e.g. α -rhamnosidase, β -glucosidase, β -glucuronidase) to release aglycones, which are then subjected to further degradation into simple phenolic acids of low molecular weight and ring-fission metabolites that are more readily absorbed by colonocytes (Kim et al., 1998; Li et al., 2000; Gao & Hu, 2010; Van Duynhoven et al., 2011; Cardona et al., 2013; Da Silva Pinto, 2013; Bohn et al., 2015).

Following absorption, the aglycones are rapidly and extensively metabolised by conjugation reactions (i.e. addition of polar group by transferase enzymes) that lead to the formation of conjugated metabolites. The conjugation reactions represent xenobiotic biotransformation (phase II metabolism), which is a metabolic detoxification process that restricts potential toxic effects of an ingested foreign chemical substance by increasing its hydrophilicity, and hence, facilitates rapid elimination of the substance from the body (Liu & Hu, 2007; Jančová & Šiller, 2012; Cardona et al., 2013). The phase II metabolites directly enter the liver via the hepatic portal vein for further conjugation reactions. Thereafter, the phase II metabolites formed in the liver can be either: *1*) distributed throughout the body via the systemic circulation and the unabsorbed metabolites eventually excreted in the urine via renal mechanisms, or *2*) resecreted into the small intestine with bile (via the enterohepatic circulation), undergo further alteration by gastrointestinal bacteria (i.e. hydrolysis, degradation) followed by reabsorption, and the remaining unabsorbed substances eventually excreted in the faeces (Xu et al., 2005; Crozier et al., 2009).

Due to the rapid extensive chemical modifications of the polyphenolic compounds and their relatively short-half life, both polyphenols and their metabolites do not commonly accumulate in the systemic circulation and are usually present at low nanomolar (nM) circulating plasma concentrations; therefore repeat ingestion over time is required to maintain a high plasma concentration (Nakagawa et al., 1997; Van het Hof et al., 1999; Scalbert & Williamson, 2000; Koo & Cho, 2004; Manach et al., 2004, 2005; D'Archivio et al., 2007, 2010; Chow & Hakim, 2011; Sang et al., 2011; Van Duynhoven et al., 2011; Del Rio et al., 2013). The enterohepatic circulation may allow for a longer presence of both polyphenols and their metabolites at physiological levels, however, this depends on the nature of the circulating compounds (Roberts et al., 2002; Crespy et al., 2003; Zhang et al., 2007). Of note is that, the circulating phenolic metabolites that reach the tissues are completely different from their respective parent chemicals. This means there can be difficulties in terms of identification, and determination of their biological properties (Manach et al., 2004; Pandey & Rizvi, 2009; D'Archivio et al., 2010).

2.2.7 Health benefits of tea

Consumption of tea has been associated with a wide variety of health benefits for many centuries (Shi & Schlegel, 2012; Yang et al., 2014a). Accordingly, numerous investigations performed *in vitro* and *in vivo* have provide evidence supporting the biological properties of tea, including analgesic (Mota et al., 2015), antiallergic (Tachibana et al., 2013), anticarcinogenic and antimutagenic (Singh et al., 2015), antidiabetic (Huang et al., 2015), antiinflammatory (Ramadan et al., 2015), antimicrobial (Siddiqui et al., 2016), antioxidant (Bender & Graziano, 2015), antiobesity (Xu et al., 2015) and prebiotic activities (Zhang et al., 2013). Further, there are also reports that tea may lower the risk of developing cataracts and reduce the incidence of bacterial or viral infections (Weber et al., 2003; Song et al., 2005; Gupta et al., 2009; Chan et al., 2011; Lee et al., 2012a; Shimamura et al., 2014). In fact, studies in humans have consistently shown an association between tea consumption and a reduced risk for developing health issues such as cancer, cardiovascular disease, depression, metabolic syndrome, inflammatory, neurodegenerative and skeletal diseases (Ruxton, 2013; Dong et al., 2015; Hayat et al., 2015; Li et al., 2015; Nash & Ward,

2015a; Pang et al., 2015; Saito et al., 2015; Shen et al., 2013b, 2015; Singh et al., 2015; Yang et al., 2015), indicating that low bioavailability of tea does not foreclose its biological effects. Due to their wide-ranging bioefficacies, tea and/or its bioactive compounds are commonly used in the nutraceutical and cosmetic industries, as well as in the preparation of a variety of commercial food products (Brewer et al., 2011; Jimenez-Escrig et al., 2011; Perumalla & Hettiarachchy, 2011; Vuong et al., 2011b; Wijesekera et al., 2011; Zong et al., 2012; Giavasis, 2014).

2.2.7.1 Tea as a source of dietary antioxidants

Tea beverages have engaged the attention of both the public and scientists as a dietary antioxidant that potentially delays the onset of various important diseases associated with oxidative stress (Pandey & Rizvi, 2009; Grove & Lambert, 2010; Bansal et al., 2011; Grove et al., 2011; Islam, 2011; Lambert, 2011; Yuan et al., 2011; Holzer et al., 2012; Jose & Marco, 2012; Kalam et al., 2012; Mandel et al., 2012; Oka et al., 2012; Khan & Mukhtar, 2013; Yang & Hong, 2013). This is due to the fact that the onset of oxidative stress (OS)-related disease is greatly mediated by unhealthy lifestyles choices that lead to an imbalance between oxidants and antioxidants within cells. Hence, OS-related diseases are likely preventable and irreversible by altering modifiable lifestyle risk factors, such as increasing the dietary intake of antioxidants (Hyman, 2006; Pham-Huy et al., 2008; Pandey & Rizvi, 2009; Rahman et al., 2012). In fact, exogenous antioxidants at physiological doses are crucial in the maintenance or re-establishment of cellular redox homeostasis for optimal physiological functions and general well-being (Valko et al., 2007; Bouayed & Bohn, 2010). Despite the availability of many synthetic exogenous antioxidants in the market (e.g. butylated hydroxyanisole), their restricted use due to reported adverse effects has consequently shifted attention to dietary antioxidants that are readily available in natural whole foods (Gupta & Sharma, 2006; Shahidi & Zhong, 2010; Schmidt et al., 2015).

Tea is one of the most important sources of exogenous antioxidants, and has both direct and indirect effects. Tea may not only supplement the diminished supply of endogenous antioxidants, but also modulate the gene expression of a variety of pro-oxidant/antioxidant enzymes that are critical for cellular redox homeostasis (Forester & Lambert, 2011; Serafini et al., 2011; Novotny & Baer, 2013). For example, (-)-epigallocatechin-3-gallate (EGCG), has been reported to enhance gene expression of

various endogenous antioxidant enzymes, such as glutathione peroxidase (GSH-Px) and glutathione S-transferase (GST) (Na & Surh, 2008). In fact, both direct and indirect antioxidant actions of tea have been supported by a large number of studies (*in vitro*, *in vivo* and human studies) investigating the disease-prevention benefits of different types of tea (Higdon & Frei, 2003; Meng et al., 2008; Abdul-Raheim et al., 2009; Das et al., 2009; Pandey & Rizvi, 2009; Bancirova, 2010; Lobo et al., 2010; Hu et al., 2011; Poljsak, 2011; Serafini et al., 2011; Bouayed & Bohn, 2012; Gyawali & Ibrahim, 2012; Poljsak et al., 2013).

It has been suggested that many of the beneficial health claims of tea are ascribed to its polyphenolic content, a group of bioactive compounds with wide-ranging therapeutic effects (Cartea et al., 2011; Khan & Mukhtar, 2013; Yang & Hong, 2013; Chen & Lin, 2015; Chen et al., 2015; Li et al., 2014; Hayat et al., 2015; Tenore et al., 2015). While many of the biological effects of polyphenols may aid good health, their antioxidant effect is the best-known as having the most significant impact (Graham, 1992; Frei & Higdon, 2003; Rietveld & Wiseman, 2003; Katalinic et al., 2006; Lin et al., 2008; Das et al., 2009; Molan et al., 2009a, 2009b; Perron & Brumaghim, 2009; Bouayed & Bohn, 2010, 2012; Lambert & Elias, 2010; Venditti et al., 2010; Gülçin, 2012). Depending on their chemical structure (the number, substitution, and positions of hydroxyl groups), both polyphenol compounds and polyphenol-derived metabolites exhibit direct antioxidant activity via several mechanisms: **1)** free-radical scavengers – by donating hydrogen atoms or electrons from the hydroxyl groups to free radicals, thus becoming transiently less-reactive radicals themselves, **2)** radical-chain breakers – whereby the resulting polyphenol-derived radicals are stabilised by resonance delocalisation of the unpaired electron, thus terminating the radical-chain reactions, and **3)** transition metal chelators – by chelating redox-active transition metal ions (i.e. iron and copper), thus preventing the generation of reactive hydroxyl radicals through metal redox cycling (Fenton reaction) (Graham, 1992; Weisburger, 1999; Frei & Higdon, 2003; Beecher, 2003; Chang et al., 2007; Jayathilakan et al., 2007; Das et al., 2009; Tsao, 2010; Namal Senanayake et al., 2013). Meanwhile, indirect antioxidant effects of both polyphenols and their metabolites are mostly demonstrated *in vivo* through upregulation of redox-sensitive transcription factors that are responsible for gene expression of endogenous antioxidant enzymes (i.e. FOXO and Nrf2) (Higdon & Frei, 2003; Rahman et al., 2006; Visioli et al., 2011; Chiva-Blanch & Visioli, 2012; Lorenz,

2013; Christensen & Christensen, 2014). Furthermore, some polyphenols (e.g. catechin, quercetin) are capable of enhancing synergism between ascorbic acid (vitamin C) and α -tocopherol (vitamin E) through formation of non-covalent associations with both vitamins in membranes, which potentiates regeneration of vitamin E by vitamin C (Croft, 1998; Pedulli & Lucarini, 2007; Hu et al., 2011; Fabre et al., 2015).

Paradoxically, there is emerging evidence that tea polyphenols may exert pro-oxidant effects under certain cellular environmental conditions, i.e. physiological redox status, high pH level, the presence of oxygen molecules, and high concentration of redox-active transition metal ions (Halliwell et al., 2005; Halliwell, 2008; Buoayed & Bohn, 2010; Forester & Lambert, 2011; Yordi et al., 2012; Kapetanovic, 2013); a general response seen in many antioxidants (e.g. vitamins C and E, quercetin) (Du et al., 2012; Carocho & Ferreira, 2013; Sen & Chakraborty, 2015). However, it is suggested that the limited bioavailability of tea polyphenols may restrict their hormetic properties (i.e. a biphasic dose-response) following administration at high concentrations. Therefore, tea polyphenols are unlikely to reach toxic concentrations *in vivo* (Forman et al., 2014; Ziberna et al., 2014), especially when consumed as a whole beverage (due to various interactions within complex mixtures of compounds in tea that may suppress the toxic effect of any tea bioactives) (Ulrich-Merzenich et al., 2010). Tea polyphenols are also thought to have beneficial pro-oxidant effects that produce low, subtoxic concentrations of ROS that act as second-messenger-like molecules to indirectly deliver antioxidant action against oxidative insults, i.e. through activation of redox-sensitive signalling pathways implicated in systemic endogenous antioxidant defences (Wattel et al., 2004; Liu et al., 2005; Rahman et al., 2006; Chiva-Blanch & Visioli, 2012; Halliwell, 2008; Trachootham et al., 2008; De Mejia et al., 2009; Elbling et al., 2010; Lambert & Elias, 2010; Serafini et al., 2011; Buoayed & Bohn, 2012; Lorenz, 2013; Kim et al., 2014). Hence, poor bioavailability of tea polyphenols apparently limits their pro-oxidant effect that has previously been assumed to pose a harmful risk to human health (Yordi et al., 2012).

Polysaccharides are another important constituent of tea (approximately 20% (w/w) of the dry weight of the tea leaves) that has gained much interest due to their *in vitro* and *in vivo* antioxidative potential. Accordingly, polysaccharides are considered as effective free-radical scavengers, reducing agents, and ferrous chelators in most of the

in vitro studies. However, the definitive description of their antioxidative mechanism is yet to be elucidated due to lack of functional studies on tea polysaccharides (Chen & Xie, 2001; Chen et al., 2005a, 2008b, 2009a, 2009b; Yu et al., 2007; Guoa et al., 2010; Nie & Xie, 2011; Wei, 2011; Chen, 2013; Novotny & Baer, 2013; Scoparo et al., 2013; Liu et al., 2015b; Wang et al., 2012, 2013, 2016). Interestingly, polyphenolic compounds typically form complexes with various polysaccharides, and therefore potentially contribute to the pronounced antioxidant effect of polysaccharides and *vice versa* (Scoparo et al., 2013; Wang et al., 2013b, 2016). The polyphenol-polysaccharide association leads to their low bioavailability, whereby they undergo extensive metabolism by the colonic microbiota and eventually result in various bacterial fermentation end-products (i.e. short-chain fatty acids) that could offer protection against oxidative stress (Gonthier et al., 2003; Wong et al., 2006; Hamer et al., 2008, 2009; Pandey et al., 2009; Ruijters et al., 2013; Christensen & Christensen, 2014). In addition, colonic microbiota fermentation also results in polyphenol-derived metabolites with antioxidant capacity and mechanisms differ from that of their dietary native molecules (Saura-Calixto & Bravo, 2001; Dai & Mumper, 2010; Saura-Calixto, 2010; Visioli et al., 2011; Sun et al., 2012; Yashin et al., 2012; Del Rio et al., 2013; Ross, 2014; Jakobek, 2015; Marín et al., 2015).

There are also reports of other antioxidant compounds found in lesser amounts in tea beverages, including purine alkaloids (i.e. caffeine, theobromine and theophylline), vitamins (i.e. vitamins A, B complex, C, E, and K), plant pigment (i.e. carotenoids, chlorophylls, pheophytins, lutein), trace minerals (i.e. selenium, fluoride and zinc), amino acids (i.e. L-theanine) and saponins. Each of these antioxidant compounds accounting for less than 5% (w/w) of the dry weight of the tea leaves (Mazur, 1998; Mazur et al., 1998; Higashi-Okai et al., 2001; Choi & Cho, 2009; Kim et al., 2009; Li et al., 2009b, 2009c; Cimpoiu et al., 2013; Novotny & Baer, 2013; Yashin et al., 2015). Interestingly, although vitamins A, C and E are more bioavailable than polyphenolic compounds, their antioxidant effects against oxidative stress *in vivo* are no more efficient than the polyphenols (Scalbert et al., 2002; Bouayed & Bohn, 2010, 2012).

2.2.7.2 Prebiotic-like effect of tea: Modulation of intestinal microbiota

In addition to their antioxidant properties, there is evidence that tea and/or its constituents may exert prebiotic-like effect, through which tea may offer both direct and indirect protection against development of chronic degenerative diseases. The chronic degenerative diseases are characterised by disrupted metabolic homeostasis and chronic systemic inflammation, including those of the oxidative stress-related diseases (Chiva-Blanch & Visioli, 2012; Patel & Goyal, 2012; Gomes et al., 2014; Lin et al., 2014). Prebiotics is a term that generally refers to non-digestible food substances (typically dietary fibre) that selectively modulate the composition and metabolism of beneficial intestinal microbiota known to exert beneficial influences on health, i.e. bifidobacteria and lactobacilli that have almost an exclusive saccharolytic metabolism (Gibson & Roberfroid, 1995; Crittenden & Playne, 2006; Ruxton, 2013; Slavin, 2013). Prebiotics usually consist of non-digestible carbohydrates (i.e. resistant oligosaccharides and non-starch polysaccharides) and other non-digestible substances including polyphenols (i.e. lignans, flavonoids) (Slavin, 2013; Yoder et al., 2015; Alpers & Cashman, 2016). The bacterial saccharolytic fermentation of prebiotics results in biotransformation of the parent chemicals into their active metabolites, along with production of the fermentation end-products, including gases (i.e. carbon dioxide, hydrogen, and methane), organic acids (e.g. lactate), and short-chain fatty acids (SCFAs; primarily acetate, butyrate and proprionate) (Gibson & Roberfroid, 1995; Wong et al., 2006; Hamer et al., 2008; Jacobs et al., 2009; Macfarlane & Macfarlane, 2012; Slavin, 2013).

There are important criteria shared by all prebiotics – they resist digestion and absorption processes in the upper GIT and enter the colon unmodified, undergo fermentation by the colonic microbiota, and selectively stimulate specific health-associated anaerobic bacteria mainly residing in the colon (Gibson & Roberfroid, 1995). These characteristics may be true for some tea bioactive constituents, especially polyphenolic compounds that have consistently being suggested to have prebiotic potential (Tzounis et al., 2008; Landete, 2012; Cardona et al., 2013; Exteberria et al., 2013; Ruxton, 2013; Zhang et al., 2013; Dueñas et al., 2015a, 2015b). Although tea also contains non-starch polysaccharides (cellulose, hemicellulose and pectins), which are non-digestible carbohydrates that are commonly considered as prebiotics, most are non-fermentable fibres (i.e. cellulose and some hemicellulose). The non-fermentable fibres are not broken down by intestinal microbiota and commonly associated with a faecal

bulking effect through water retention, thereby often speeding up the gastrointestinal transit rate and improving the ease of defecation. Hence, not all non-starch tea polysaccharides can be considered as prebiotics in spite of their physiological effects on gastrointestinal function (e.g. improved bowel function, aiding regular bowel movement and preventing constipation) (Hernández-Hernández et al., 2012; Eswaran et al., 2013; Slavin, 2013; Goldsmith & Sartor, 2014).

The prebiotic-like potential of tea polyphenols is primarily attributed to their polyphenol-derived metabolites that selectively modulate resident intestinal microbiota composition; a complex microbial ecosystem crucial for the development of gastrointestinal metabolism and function, and the immune system of the host (Del Rio et al., 2010, 2013; Monagas et al., 2010; Williamson & Clifford, 2010; Di Mauro et al., 2013; Purchiaroni et al., 2013; Van Duynhoven et al., 2013; Chen & Sang, 2014; Das et al., 2014). In support of this notion, a number of studies (*in vitro*, *in vivo*, and some human studies) have demonstrated bifidogenic and/or lactogenic effects of tea and/or its polyphenols (Okubo et al., 1992; Lee et al., 2006a; Duda-Chodak et al., 2008; Tzounis et al., 2008; Molan et al., 2009a, 2010; Banerjee et al., 2010; López de Felipe, 2010; Ankolekar et al., 2011; Axling et al., 2012; Jin et al., 2012; Landete, 2012; Vodnar & Socaciu, 2012; Vodnar et al., 2012; Van Duynhoven et al., 2013; Exteberria et al., 2013; Gaudreau et al., 2013; Kemperman et al., 2013; Parkar et al., 2013; Ruxton, 2013), although there is some conflicting study data (Mai et al., 2004; Lee et al., 2006a; Jaziri et al., 2009; Cueva et al., 2010; Ankolekar et al., 2011; Gaudreau et al., 2013); these discrepancies could be due to the diverse methodological approaches used in these studies, as well as well different doses and assessment tools (Lees et al., 2014; Dueñas et al., 2015a, 2015b). In fact, many authors have postulated that teas exert positive modulatory effects on gut microbiota composition through complex functional interactions between the gut microbiota and tea polyphenolic compounds (Lee et al., 2006a; Selma et al., 2009; Van Duynhoven et al., 2013; Parkar et al., 2013; Hervert-Hernández & Goñi, 2011; Duda-Chodak et al., 2015; Dueñas et al., 2015a, 2015b). Furthermore, some research has also shown the potential antibacterial effects of tea polyphenols, as shown through inhibition of the growth of pathogenic bacteria used in these studies (Lee et al., 2006a; Friedman, 2007; Michalczyk & Zawislak, 2008; Sutherland et al., 2009; Molan et al., 2009a, 2010; Ankolekar et al., 2011).

The health benefits that are gained from the prebiotic-like effects of tea may also originate from the influences of SCFAs on systemic metabolism. It has been reported that the effects of SCFAs on systemic metabolism include: **1)** enhancing the antioxidant capacity of the colonic mucosa, mainly via the effect of butyrate on increasing glutathione (GSH) concentration, **2)** as energy sources at both the colonic and systemic levels, **3)** delaying gastric emptying that helps reduce the glycaemic response to foods, **4)** inhibiting protein fermentation that can produce potentially toxic substances (e.g. ammonia and amines), **5)** decreasing the colonic pH that further increase the population size of beneficial intestinal microbiota and/or suppress the growth of pathogenic bacteria, as well as enhancing colonic absorption of minerals (e.g. calcium), **6)** decreasing the activity of undesirable bacterial enzymes (e.g. β -glucuronidase), **7)** and inhibiting formation of potential co-carcinogens from secondary bile acids (Younes et al., 1996; Coudray et al., 1997; Lopez et al., 1998; Cashman, 2003; Abrams et al., 2005; De Vuyst & Leroy, 2007; Scholz-Ahrens & Schrezenmeir, 2007; Hamer et al., 2008, 2009; Su et al., 2008; Sharma & Rao, 2009; European Food Safety Authority, 2010; Both et al., 2011; Hernández-Hernández et al., 2011; Hervert-Hernández & Goñi, 2011; Wallace et al., 2011; Legette et al., 2012; Van Dorsten et al., 2012; Slavin, 2013).

2.2.7.3 Selenium-rich tea and its associated health benefits

Selenium (Se) is a micronutrient that is essential for diverse metabolic and physiological functions. The recommended dietary allowance (RDA) of Se for adults is 55 $\mu\text{g}/\text{day}$, which is an estimated amount required for maximising plasma GSH-Px enzymatic activity (USA Institute of Medicine, 2000; Thomson, 2004b). Despite the importance of Se as a part of the antioxidant defence system, a daily Se dietary intake of greater than 400 μg per day can be toxic to the human body (based on selenosis as an adverse effect). This is due to excessive non-specific incorporation of Se in the place of sulphur in amino acids that can subsequently alter protein three-dimensional structure and impair enzymatic function (USA Institute of Medicine, 2000; Amweg et al., 2003; Winkel et al., 2012).

The physiological role of Se in the maintenance of human health is attributed primarily to its role as a co-factor of selenoenzymes. To date, there are 25 human selenoenzymes that have been identified in the human genome. Among them, several remain functionally undefined. However, some selenoenzymes are well-known for their

functions in executing oxidoreductase activity, preventing and/or reversing oxidative damage, and regulating thyroid hormone metabolism. For selenoenzymes that are functionally well-characterised, selenium in the form of selenocysteine (a cysteine analogue that contains a selenium atom in place of the sulphur atom) is located in the active site of selenoenzyme, where it serves as catalyst for redox reactions and is mostly involved in enzymatic antioxidant defence systems. The most characterised selenoenzymes with known antioxidant functions include five glutathione peroxidases (GSH-Px), the three thioredoxin reductases (TrxR), methionine R-sulfoxide reductase B1 (MsrB1) and selenoprotein P (Sepp1). These oxidoreductases are crucial for protection against oxidative damage, intracellular redox homeostasis and redox regulation of signalling pathways (Moghadaszadeh & Beggs, 2006; Steinbrenner & Sies, 2009; Yuan et al., 2012).

The GSH-Pxs (GSH-Px1, GSH-Px2, GSH-Px3, GSH-Px4 and GSH-Px6) provide protection against oxidative stress through peroxide detoxification in order to restore the cellular redox balance. In brief, the GSH-Px isoenzymes catalyse the reduction of various peroxides using glutathione (GSH) as a co-substrate. On the other hand, TrxRs (TrxR1, TrxR2 and TrxR3) are protective against oxidative stress through maintaining the reduced state of the thioredoxin system that is mainly involved in cellular redox regulation and signalling. The TrxR isoenzymes catalyse the reduction of oxidised thioredoxin using the reducing power of NADPH. In addition to thioredoxin as its main protein substrate, TrxRs directly detoxify peroxides and also catalyse reduction of a wide variety of substrates (e.g. selenium-containing compounds, α -lipoic acid, dehydroascorbic acid, ubiquinone). Further, TrxR isozymes also catalyse the regeneration of certain antioxidant compounds (e.g. selenoenzymes, lipoic acid, ascorbic acid, ubiquinol) from their oxidised metabolites. Previously known as selenoprotein R, MsrB1 is another selenoenzymes that serve as an additional antioxidant defence mechanism and is implicated in cellular redox regulation. These functions are achieved through thioredoxin-dependent reduction of methionine-R-sulfoxide (oxidised methionine) residues in protein back to methionine, sulphur-containing amino acids highly and reversibly oxidisable by ROS. The reversible methionine oxidation-reduction cycle is proposed to be an effective ROS-scavenging mechanism that is crucial for repairing and preventing oxidative damage, as well as for the maintenance of cellular redox balance. Selenoprotein P (Sepp1) is the only

selenoenzyme that contains up to 10 selenocysteines. A major selenoprotein found in plasma, Sepp1 is known for its function as a selenium transporter (from liver to peripheral tissues) and an extracellular antioxidant; the latter is demonstrated as a peroxynitrite reductase and a catalyst for reduction of membrane phospholipid hydroperoxides at the expense of GSH or Trx (Moghadaszadeh & Beggs, 2006; Luo & Levine, 2009; Steinbrenner & Sies, 2009; Steinbrenner et al., 2016).

Tea is considered a non-accumulator of Se, accumulating only moderate amounts of Se that are unlikely to pose a risk of toxicity despite being grown in seleniferous soil. It has been reported that tea plants grown on seleniferous soil can contain Se levels ranging from 1.4 to 6.5 μg per gram dry weight ($\mu\text{g}/\text{g}$ DW) of tea leaves, which is much higher than in most regular tea that contains approximately 0.1 μg Se/g DW of tea leaves (Liang et al., 2014). Accordingly, Se-rich tea is being commercially produced in certain seleniferous regions in China (e.g. the Enshi district of Hubei province in China whereby the soil Se content ranges from 20 to 60 mg/kg DW); as a part of the natural Se-biofortification practices that aim to provide for the growing demand from the general public, particularly from the low-Se status populations; (Reilly, 1998, 2008; Zhao et al., 2011; Yuan et al., 2012).

In addition, Se-enriched tea is also produced in the regions with low soil content of selenium (e.g. Ningshan district of Shaanxi province in China) (Wu et al., 2015), where a selenium enrichment procedure is performed either using selenium-containing fertiliser or spraying selenate/selenite onto the tea leaf surface (foliar spraying) (Liang et al., 2014). While both methods effectively enhance selenium content of tea, the use of selenium-containing fertiliser to produce selenium-enrichment is more favoured and able to meet the standard quality requirements for production of Se-enriched tea in China (Hu & Ding, 1998). Most importantly, the chemical compositions between natural Se-rich green tea and Se-enriched green tea have no significant difference (Hu et al., 1999).

It has been reported that the Se species found in tea is mostly the organic form, which evidently binds to different tea components including water-soluble protein, polyphenols, and polysaccharides (as reviewed by Liang et al., 2014). Plant-based Se is readily bioavailable (Litov & Combs, 1991), unlike plant-based polyphenols that have

limited bioavailability due to ingested polyphenols being recognised as xenobiotics (i.e. foreign biochemicals) by the body and therefore treated as such (Neilson & Ferruzi, 2012). Further, the bioavailability of Se is species-dependent, where organic Se are reputed to be more bioavailable and well-retained compared to the inorganic forms (Thomson, 2004b; Burk et al., 2006; Finley, 2006). Se-containing amino acid seleno-L-methionine (SeMet) is one of the primary sources of organic Se compounds present in the human body, and are mainly obtained from dietary plant intake (Ip et al., 2000; Lyons et al., 2007). SeMet is more bioavailable, beneficial and harmless than the inorganic forms of Se (e.g. selenate and selenite) (Ortman et al., 1999; Hu et al., 2001; Rayman et al., 2008; Molan et al. 2009a).

In recent years, there has been an increasing interest in high-selenium foods as potential health products, which includes Se-rich green tea (Liang et al., 2014; Gao et al., 2016; Yin, 2016). This could be attributed to the fundamental importance of selenium to human health, the beneficial role of Se-rich tea in the endogenous antioxidative defence against oxidative stress-related diseases (e.g. cancers), and also in response to extensive distribution of Se deficiency (less than 40 µg/day) in certain parts of the world, including New Zealand (Rayman, 2000, 2009, 2012; Thomson, 2004a, 2004b; Finley et al., 2005; Letavayova et al., 2006; Moghadaszadeh & Beggs, 2006; Fairweather-Tait et al., 2011; Sunde, 2012; He et al., 2013; Zeng et al., 2013a; Bermingham et al., 2014; Kong et al., 2014; Santos et al., 2014; Wu et al., 2015; Gao et al., 2016; Yin, 2016). Although a large amount of evidence shows increased antioxidant activity and prebiotic potential of green and black teas, published work on Se-rich green and black teas is limited (Molan et al., 2010; Axling et al., 2012; Patel & Goyal, 2012; Vodnar & Socaciu, 2012, 2014; Cardona et al., 2013; Molan, 2013; Liang et al., 2014).

Data from several sources have previously investigated the *in vitro* antioxidant activity and prebiotic potential of Se-rich or Se-enriched green tea, whereby Se-rich or Se-enriched green tea was shown to have greater antioxidant activity and prebiotic potential than regular green teas (Xu et al., 2003a, 2003b; Yu et al., 2007; Molan et al., 2009a, 2010). Antioxidant studies conducted by Xu et al. (2003a, 2003b) showed that Se-enriched green tea obtained by fertilisation with selenate, provided higher antioxidant activity than regular green tea, as assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging method, in both oxidised linoleic acid and

lard systems. The results of these studies were in agreement with the findings of Yu et al. (2007), who concluded that different antioxidant components of Se-enriched tea (i.e. crude tea polyphenols and crude tea polysaccharides) were greater free-radical scavengers than the antioxidant components of regular green tea. Meanwhile, Molan et al. (2009a) examined the *in vitro* antioxidant activity of water extracts of both Se-rich green tea (1.44 µg Se/g DW) and regular China green tea (0.13 µg Se/g DW), as well as their prebiotic potential against the growth of lactic acid bacteria using pure and mixed bacterial cultures under *in vitro* and *ex vivo* conditions, respectively. The results suggested that Se-rich green tea was effective as a ferric-ion reducer, DPPH-radical scavenger and ferrous-ion chelator, while it successfully boosted the growth of *Lactobacillus rhamnosus* and *Bifidobacterium breve*. Molan et al. (2010) conducted an animal study to investigate the prebiotic effects of orally-gavaged water extracts of both Se-rich green tea and regular China green tea on caecal populations of beneficial bacteria species (i.e. *Lactobacillus* spp. and *Bifidobacterium* spp.) and pathogenic bacteria (i.e. *Bacteroides* spp. and *Clostridium* spp.) in the caecum of rats using fluorescence *in situ* hybridization (FISH). Subsequently, the effects of both the tea water extracts on the activity of two bacterial enzymes in the caecal content of rats were determined. The results of this animal study supported their previous findings (Molan et al., 2009a), with the authors concluding that administration of Se-rich green tea water extract for six days enhanced the number of beneficial bacteria significantly more effectively than regular green tea. Se-green tea also showed a strong antibacterial effect against *Bacteroides* spp. and *Clostridium* spp. in rats. The mechanisms of action behind the strong antioxidant effect and prebiotic potential of the Se-rich green tea are not known, however, is suggested to be partly attributed to both its polyphenol antioxidants and high selenium content (Weisburger, 1999; Molan et al., 2009a; Molan, 2013).

In general, tea (i.e. mainly of green and black teas, and increasingly teas with higher selenium content) contains a variety natural dietary antioxidants with wide-ranging mechanisms and has aroused a lot of interest from different groups throughout the world. This is not only due to the purported antioxidative properties that could protect against oxidative stress-related disorders, but also ascribed to their prebiotic ability that may positively modulate resident gut microbiota towards maintenance of metabolic homeostasis and improvement of immune system performance that leads to improved overall human health. Interestingly, several studies suggested that the dual

antioxidant effects of tea (i.e. green tea) and high selenium content in that tea, had a promising synergistic effect (Hu et al., 2001, 2003; Xu et al., 2003a, 2003b; Yu et al., 2007; Li et al., 2008, 2009a; Molan et al., 2009a). However, up to now, there is a lack of information on the understanding of possible interactions between Se and the complex mixture of polyphenolic compounds of tea, and their influence (if any) on the biological role of tea against excess reactive oxygen species and remains to be determined.

2.3 The effects of tea on bone health

The consumption of tea and its wide range of health benefits has been an active area of nutrition and phytochemical research over the past few decades. In view of this, one of the most recent discussions in published tea studies is the association between tea consumption and its benefits on postmenopausal osteoporosis, a multifaceted bone loss disease following oestrogen deficiency. A large and growing volume of scientific research has investigated the beneficial effects of tea and/or its bioactive constituents on bone health, as evident from both laboratory and human studies (Blumberg, 2013; Das et al., 2013; Shen et al., 2009a, 2011b, 2013b; Nash & Ward, 2015a, 2015b, 2016). Many studies have linked tea to protective effects on bone, mostly attributed to its abundant and powerful polyphenolic antioxidants (Dew et al., 2007; Shen et al., 2009a, 2011b, 2013b; Rao et al., 2012; Das et al., 2013; Pan et al., 2013; Lima et al., 2014). Interestingly, some polyphenolic antioxidants (i.e. flavonoids, lignans, stilbenes and coumestans) of tea may act as phytoestrogens that exert oestrogen-like effects; a role which is mainly linked to their polyphenol-derived colonic metabolites (Kuruto-Niwa et al., 2000; Chiang & Pan, 2013; Del Rio et al., 2013; Sirotkin & Harrath, 2014; Landete et al., 2015). Phytoestrogens are a group of plant-derived non-steroidal compounds that structurally resemble endogenous oestrogen 17 β -oestradiol (E₂), and exhibit relatively weak binding affinities toward oestrogen receptors (ERs) (Hu & Aizawa, 2003). Their chemical structure similarities enable phytoestrogens to mimic the physiological actions of endogenous oestrogen to some degree; hence, phytoestrogens are a potential alternative to hormone replacement therapy (HRT) to counteract oestrogen-deficiency bone loss in postmenopausal women (Al-Anazi et al., 2011; Chiang & Pan, 2013; Sirotkin & Harrath, 2014).

Additionally, there are other non-phenolic antioxidant compounds of tea that exhibit bone-associated benefits at minute amounts: carotenoids, vitamins (i.e. A, B complex, C, E and K) and minerals (i.e. fluoride, selenium) (Łuczaj & Skrzydlewska, 2005; Dew et al., 2007; Malinowska et al., 2008; Kohlmeier, 2012; Almeida & Figueira, 2013; Zeng et al., 2013a; Bailey et al., 2015; Nieves, 2015; Wicks & Mahady, 2015). In the present review, we summarise the recent existing literature regarding the impact of tea in the maintenance of bone mass and protection of postmenopausal bone loss, discuss the proposed mechanisms by which tea may mediate an effect, and finally identify the knowledge gaps in the existing research literature. Despite the purported effects of various tea types and constituents on bone health, this review primarily focuses on the impact of whole extracts of green and black teas, as well as their major group of active polyphenolic antioxidants (i.e. catechins and theaflavins, respectively) on bone health.

2.3.1 Laboratory studies

The efficacy of tea drinking and the major active polyphenolic compounds of tea in improving bone health has been extensively studied, particularly via *in vitro* and animal model approaches. Accordingly, the osteoprotective potential of tea and its active polyphenols on bone metabolism in both *in vitro* and animal models are summarised in **Appendices I** and **II**. These tables provide reliable scientific evidence that suggest promising regulatory effects of tea on bone metabolism. From the literature, the pronounced positive effects of tea and its polyphenols on the regulation of bone metabolism are mainly ascribed to enhanced osteogenesis (Choi & Hwang, 2003; Park et al., 2003; Chen et al., 2003, 2005b; Mount et al., 2006; Vali et al., 2007; Ko et al., 2009, 2011; Byun et al., 2014; Jin et al., 2014; Mah et al., 2014; Zeng et al., 2014; Kaida et al., 2015; Liu et al., 2016; Nash & Ward, 2016; Peng et al., 2016) and suppressed osteoclastogenesis (Nakagawa et al., 2002; Yun et al., 2004; Morinobu et al., 2008; Ko et al., 2009; Lin et al., 2009; Kamon et al., 2010; Lee et al., 2010a, 2010b; Nakamura et al., 2010; Oka et al., 2012; Irie et al., 2014; Zhao et al., 2014). These effects were reflected in a variety of osteoblast and osteoclast precursors that were cultured under different experimental conditions (e.g. pre-, co- or post-incubations; co-culture systems; study durations), and evaluated using various experimental techniques

(e.g. different types of cell staining and gene expression assays). Furthermore, it is important to note that tea and its active polyphenols showed hormetic properties in both anabolic and anti-resorptive effects, whereby the biphasic effect on bone metabolism is greatly dependent on time and/or concentrations as reported in some studies (Jin et al., 2014; Mah et al., 2014; Vester et al., 2014; Zhao et al., 2014; Kaidar et al., 2015; Liu et al., 2016; Nash & Wards, 2016).

Most *in vitro* studies have used single isolated tea polyphenols rather than a whole tea extract; these isolated compounds have especially been of the monomeric catechins that are present in green tea. A number of catechin subgroups have been investigated for their bone-benefit effects at a wide range of concentrations (as depicted in **Appendix I**), with EGCG being the most extensively studied (Choi & Hwang, 2003; Yun et al., 2004, 2007; Kamon et al., 2010; Ko et al., 2009; Lin et al., 2009; Shen et al., 2009a; Horcajada & Offord, 2012; Oka et al., 2012; Zeng et al., 2013b, 2014; Jin et al., 2014, 2015; Zhao et al., 2014; Đudarić et al., 2015). EGCG is the most abundant catechin in green tea and is thought to have wide-ranging health benefits due to its strong antioxidant properties. Therefore, EGCG is the most common focus of research into tea compounds for health (Perva-Uzunalic et al., 2006; Zaveri, 2006; Jin et al., 2014; Ghasemi-Pirbaluti et al., 2015; Chowdhury et al., 2016). Further, EGCG is also a known modulator of the activity of redox-sensitive transcription factors that are implicated in various vital cell processes including proliferation, differentiation and death (Bode & Dong, 2003; Wattel et al., 2004; Rahman et al., 2006; Na & Surh, 2008; Surh & Na, 2008; Trachootham et al., 2008; De Mejia et al., 2009; Shen et al., 2009a; Lorenz, 2013; Rein et al., 2013; Zhao et al., 2014).

Whilst black tea also contains a mixture of catechins, complex polymeric theaflavins (TFs) and thearubigins (TRs) are the largest by composition of the total phenolic content in black tea; whereby total theaflavins mainly determine black tea quality (Owuor & Obanda, 2001). These polyphenolic pigments are the oxidised derivatives of monomeric catechins yielded from enzyme-mediated fermentation during the black tea manufacturing process (Sutherland et al., 2006; Li et al., 2013; Kosińska & Andlauer, 2014). In contrast to green tea and its catechins, studies concerning the effects of black tea consumption on bone health are scarce (as observed in **Appendices I** and **II**); despite the fact that black tea is preferentially consumed around the world and

makes up the vast majority of tea production as compared to that of green tea (Li et al., 2013; Butt et al., 2014). It has earlier been thought that black tea is a less effective antioxidant than green tea due to its lower content of monomeric polyphenols (Del Rio et al., 2004), which may be the reason for less interest in the health effects of black tea and its polymeric polyphenols. However, recent evidence has suggested that black tea displayed comparable or greater antioxidative effect than green tea both *in vitro* and in animal studies, which is attributed to **1**) antioxidant actions (i.e. as free-radical scavengers and transition metal chelators) exerted by both TFs and TRs due to their catechin-like structure, and **2**) modifications in chemical structure of these polymeric polyphenols following xenobiotic metabolisms by resident gut microbiota into small, readily absorbable metabolites with more profound bioactivities; hence, supporting the notion that enzyme-mediated fermentation during black tea manufacturing processes did not lower the antioxidant properties of black tea (Leung et al., 2001; Rechner et al., 2002; Łuczaj & Skrzydleska, 2005; Karori et al., 2007; Yang et al., 2008a; Lorenz et al., 2009; Sharma & Rao, 2009; Chow & Hakim, 2011; Sang et al., 2011; Chen et al., 2012; Sun et al., 2012; Sen & Bera, 2013; Koech et al., 2013; Lorenz, 2013; Sinha & Ghaskadbi, 2013; Chiou et al., 2014).

Based on the findings of the *in vitro* experimental studies, there are different mechanisms that have been proposed for the osteoprotective effects of tea (mainly attributed to EGCG). In regards to stimulation of osteogenesis, tea may exert its effects, in part, by: **1**) enhancing proliferation and differentiation of osteoblasts through modulation of canonical wingless (Wnt) signalling pathway that regulates pluripotency and lineage speciation in stem cells (Mount et al., 2006), **2**) enhancing gene expression of certain osteogenic markers, such as alkaline phosphatase (ALP), bone morphogenetic protein-2 (BMP2), bone sialoprotein (BSP), core-binding factor alpha 1 (Cbfa1), collagen type I alpha 1 (COL1A1), osteocalcin (OC), osteonectin, osteopontin (OPN), runt-related transcription factor-2 (Runx2), and sclerostin (SOST) (Chen et al., 2005b; Vali et al., 2007; Ko et al., 2011; Byun et al., 2014; Jin et al., 2014, 2015; Nash & Ward, 2016), **3**) increasing differentiation and survival of osteoblasts through suppression of pro-inflammatory cytokines production in the cells, i.e. tumor necrosis factor alpha (TNF α) and interleukin-6 (IL6) that are involved in the regulation of bone homeostasis (Choi & Hwang, 2003; Nash & Ward, 2016), **4**) downregulating apoptotic pathway modulators (i.e. heat shock protein 27 (HSP27) and IL6) via suppression of the

stress-activated protein kinase/c-Jun N-terminal protein kinase (SAPK/JNK) pathway involved in apoptotic cell death signalling (Hayashi et al., 2008; Takai et al., 2008), **5**) downregulating HSP27 via suppression of p44/p42 mitogen activated protein kinase (p44/p42 MAPK) pathway that is implicated in the regulation of osteoclastic bone resorption (Yamauchi et al., 2007), **6**) enhancing synthesis of vascular endothelial growth factor (VEGF) required for bone remodelling by upregulating prostaglandin F_{2α}-mediated activation of SAPK/JNK pathway in osteoblasts (Tokuda et al., 2007), and finally **7**) enhancing matrix mineralisation through upregulation of the JNK and p38 MAPK pathways essential for late-stage differentiation and bone formation, respectively (Kaida et al., 2015).

Meanwhile, tea exerts *in vitro* antiosteoclastogenesis effects, in part, by: **1**) inhibiting osteoclast formation and activity through suppression of osteoblast-derived matrix metalloproteinases (MMPs) expression in osteoclasts; where MMPs are the enzymes responsible for extracellular bone matrix degradation (Yun et al., 2004; Oka et al., 2012; Irie et al., 2014), **2**) increasing apoptotic cell death in osteoclasts through activation of caspase-3, which is the key player for the majority of the apoptotic effects in cells (Yun et al., 2007), **3**) inhibiting RANKL-mediated osteoclastogenesis through suppression of RANKL-induced activation of NF-κB, a transcription factor crucially targeted by RANKL signalling during osteoclast development (Lin et al., 2009; Lee et al., 2010a; Nakamura et al., 2010), **4**) suppressing gene expression of: i) certain osteoclastic markers (i.e. c-Fos; calcitonin receptor (CTR); nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1); tartrate-resistant acid phosphatase (TRAP)), ii) apoptosis-related genes (i.e. Forkhead box O3 (FOXO3a)); Bcl-2-like protein 11 (Bim)), and iii) nuclear factor kappa beta (NF-κB)-related genes (i.e. intercellular adhesion molecule 1 (ICAM-1); nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (Nfkb2); TNFα) (Morinobu et al., 2008; Ko et al., 2009; Lee et al., 2010a; Zhao et al., 2014), **5**) downregulating RANKL-RANK signal through suppression of RANK and its key co-stimulator, i.e. osteoclast-associated receptor (OSCAR) (Zhao et al., 2014), **6**) suppressing RANKL-induced activation of c-Jun N-terminal protein kinase (JNK/c-Jun) pathway, a RANKL signalling pathway known to have an important role in osteoclast development (Lee et al., 2010a), **7**) directly suppressing osteoclast formation (Kamon et al., 2010; Zhao et al., 2014), **8**) inhibiting bone resorption and osteoclastogenesis through activation of 5'adenosine

monophosphate-activated protein kinase (AMPK) pathway, which is a negative regulator of RANKL (Lee et al., 2010b), and finally **9**) causing apoptotic cell death in osteoclasts through its ability to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), which in the process triggers a Fenton reaction that generates highly toxic hydroxyl radicals (OH^{\bullet}) from Fe^{2+} and the EGCG-generated hydrogen peroxide; the hydroxyl radicals then cause oxidative stress in osteoclast cultures, and further induces activation of caspase-3 and direct DNA breakage that leads to apoptotic cell death of the osteoclasts (Nakagawa et al., 2002, 2007).

While *in vitro* studies may provide a clear perception of the possible mechanisms by which tea consumption has effects on osteoclasts and osteoblasts, animal studies provide an intact system with consideration of any coupling effects between osteoblasts and osteoclasts, as well as their precursor cells, while at the same time providing a natural environmental setting by permitting metabolic activity, therefore probably affecting the performance of tea bioactive constituents on bone metabolism (Setchell & Lydeking-Olsen, 2003). In line with this, the use of an animal model is crucial to better understand the pathogenesis of osteoporosis, in an attempt to elucidate the protective mechanisms that tea may have against the progression of bone loss. The ovariectomised rat model is commonly used as it has been previously confirmed as suitable animal model to study postmenopausal bone loss, due to similarities in the pathophysiological bone loss responses to postmenopausal bone loss in women (Kalu, 1991; Lelovas et al., 2008).

As indicated in **Appendix II**, there are several types of animal models that have been used to determine osteoprotective effects of tea and its active polyphenols. Among all these, the ovariectomised (OVX) rat is the most commonly used model of postmenopausal osteoporosis, as they are a well-established model of the oestrogen-deficient condition following human menopause, which leads to a significant bone loss, skeletal changes and reduction in bone mineral density (BMD) (Kalu, 1991; Frost & Jee, 1992; Turner et al., 2001; Kharode et al., 2008). In a series of investigations on the effects of black tea extract (BTE) administration (2.5% aqueous, orally gavaged at a single dose per day) for 28 days in OVX female rats, BTE had both protective and restorative effects compared with sham-operated rats, which was evident by the reduction in; **1**) serum levels of bone resorption and pro-inflammatory cytokines, **2**)

oxidative stress, 3) urinary loss of minerals and organic components of bone, and 4) osteoclast (OCL) counts in the proximal tibia; while simultaneously showing an enhancement in: 1) levels of serum oestradiol (E_2), 2) intestinal calcium (Ca) absorption, 3) bone mineral density (BMD), 4) bone strength, and 5) bone ash mineral content (Das et al., 2004, 2005, 2009, 2013). In another research report with similar BTE administration for 30 days in an animal model of high-fat-diet (HFD)-induced non-alcoholic steatohepatitis (Karmakar et al., 2011), BTE also ameliorated skeletal alterations in calcium homeostasis, serum and urinary biomarkers of bone turnover, and bone quality parameters of the rats.

Overall, it is thought that the bone protective and restorative roles of the BTE in these animal studies could be part-mediated by enhanced activity of endogenous antioxidant enzymes (i.e. catalase (CAT)) (Das et al., 2009), enhanced intestinal calcium absorption via increased levels of intestinal mucosal Ca^{+} transference and Ca-transferring enzymes (i.e. ALP and Ca-activated adenosine triphosphatase (Ca-ATPase)) (Karmakar et al., 2011; Das et al., 2013), reduced bone resorption via increased levels of serum oestrogen (Das et al., 2005, 2013; Karmakar et al., 2011), reduced bone turnover via decreased levels of serum ALP and urinary calcium-to-creatinine ratio (Ca:Cr) (Das et al., 2004, 2013; Karmakar et al., 2011), decreased osteoclastic differentiation and activity via reduced levels of pro-inflammatory (i.e. $TNF\alpha$, IL6) and bone resorption cytokines (i.e. hydroxyproline (HPr), osteoprotegerin (OPG), RANKL, TRAP) (Das et al., 2004, 2005, 2009, 2013; Karmakar et al., 2011), and decreased production of reactive oxygen species (ROS) via reduced levels of superoxide dismutase (SOD) activity (Das et al., 2009).

Green tea polyphenol (GTP) administration for 16 weeks in 14-month-old OVX rats at 0.1% or 0.5% (w/v; in drinking water), which is equivalent to ~1 or ~4 cups (120 mL/cup) of green tea per day, significantly preserved bone microstructure and quality, enhanced bone mass, increased the liver antioxidant enzymes activity, and reduced oxidative damage (Shen et al., 2008a, 2009a). Using a similar experimental setting, Shao et al. (2011) reported that GTP attenuated OVX-induced bone loss through their antioxidative and oestrogen-associated effects, as indicated by increased levels of endogenous antioxidant enzymes (i.e. SOD1 and adenosine triphosphatase (ATP synthase)) and reduced gene expression of an oestrogen-degrading enzyme (i.e.

catechol-O-methyltransferase (COMT)). The osteoprotective effect showed by similar GTPs administration has also been evaluated (for 12- or 16-week interventions) using various bone-loss models, including lipopolysaccharide (LPS)-induced chronic inflammatory bone loss and bone microarchitecture deterioration (Shen et al., 2010a, 2010b, 2011d), orchidectomised (ORX)-induced deterioration of bone microstructure and quality in male rats (Shen et al., 2011a), and LPS-induced systemic chronic inflammatory deterioration of bone microarchitecture (Shen et al., 2011c). In these various animal models (Shen et al. 2010a, 2010b, 2011a, 2011c, 2011d), GTP consumption exerts osteoprotective effects as shown by: **1)** an ameliorated femoral BMD and strength, **2)** restored trabecular bone volume density (BV/TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N), as well as bone formation rate per bone surface of proximal tibia (BFR/BS), and **3)** reduced trabecular separation (Tb.Sp) and eroded surface per bone surface (ES/BS); of both the proximal tibia and the tibial shaft that eventually resulted in a larger net bone volume.

Altogether, it is thought that the protective and restorative roles of the GTP (a mixture of 480 mg (-)-epigallocatechin-3-gallate (EGCG), 160 mg (-)-epicatechin-3-gallate (ECG), 103 mg (-)-epigallocatechin (EGC), 60 mg (-)-epicatechin (EC), and 30 mg (-)-catechin (C) in 1000 mg GTP; with purity higher than 98%) in these animal models could be partly-mediated by enhanced activity of endogenous antioxidant enzymes (i.e. glutathione peroxidase (GSH-Px), SOD1, and ATP synthase) (Shen et al., 2008a, 2011a; Shao et al., 2011), attenuated inflammation via reduced levels of pro-inflammatory cytokines (i.e. TNF α , IL6, cyclooxygenase-2 (COX2)) (Shen et al., 2008b, 2010b; 2011c), reduced activity of oestrogen-degrading enzyme (i.e. COMT) (Shao et al., 2011), and decreased DNA damaging effect of oxidative stress via reduced levels of an oxidative damage biomarker, i.e 8-hydroxy-2-deoxyguanosine (8-OHdG) (Shen et al., 2008, 2010a).

Ryou et al. (2012) revealed the ability of green tea leaves (in a powder form) to restore some bone density in 10-week-old OVX female Sprague-Dawley rats, after being fed at 10% concentration of the diet during a four-week tea intervention; whereby the tea intervention took place after a four-week recovery period following ovariectomy. A study performed by Nakamura et al. (2010) indicated that topical application of 1% green tea catechins (GTC) into infected gingival tissue (i.e. once every 48 hours for 10

doses) effectively prevented alveolar bone resorption following LPS-induced inflammatory bone resorption in seven-week-old BALB/c mice, as shown by significant reduction of RANKL-induced osteoclastogenesis and interleukin-1 beta (IL-1 β). The GTC used in this study is a commercially available green tea extract, Sunphenon BG (i.e. 91.3% polyphenol and 76.6% catechins) from Taiyo Kagaku, Mie, Japan.

As seen in **Appendix II**, there are three studies that have used an OVX-induced bone loss model to determine the impact of the isolated compound EGCG, whereby certain doses of EGCG were injected into the OVX female rats in order to obtain consistent serum EGCG concentrations. According to Lee et al. (2012b), eight-week administration of EGCG (at 50 mg/kg BW, five times weekly) to nine-week-old C57BL/6 mice showed that tea acts as an AMPK activator that negatively regulates RANKL-induced osteoclastogenesis, as indicated via increased AMPK α expression, BMD, trabecular bone volume density (BV/TV) and trabecular number (Tb.N); while simultaneously decreased trabecular separation (Tb.Sp) and osteoclast surface per bone surface (OcS/BS) and number of osteoclast per bone surface (N.Oc/BS). Chen et al. (2013) reported that 12-week administration of low-dose EGCG (at 3.4 mg/kg/day result in serum concentrations of 10 uM, injected three months after ovariectomy) to 24-week-old Sprague-Dawley rats attenuated bone loss and ameliorated bone microarchitecture changes in OVX rats, which is part-mediated by enhanced osteogenesis; as observed in increased expression of Tb.Sp in the proximal tibial and increased BV/TV and trabecular thickness (Tb.Th) in the third lumbar vertebra (LV), along with increased bone morphogenetic protein-2 (BMP2) that is involved in major pathways for governing bone regeneration. A recent report by Song et al. (2014) also supports the bone-restorative effect of EGCG on OVX-induced bone loss. The authors found that 12-week administration of EGCG (at 10 mg/kg/day, injected three days after ovariectomy) to 12-week-old Sprague-Dawley rats attenuated bone loss via its effect on semaphorin4D (Sema4D), an axon guidance molecule that could inhibit bone formation; as indicated by reduced Tb.Sp and expression of Sema4D, as well as increased trabecular number, trabecular BMD and trabecular BV/TV.

While most *in vivo* studies (**Appendix II**) support the notion that tea and its polyphenols are effective at alleviating bone loss, only one study has reported contradictory finding. According to Iwaniec et al. (2009), tea may suppress skeletal accumulation during growth in young growing mice following GTE supplementation at

1% or 2% (w/w) concentration for six weeks, as shown by reduced femoral length, BMC, volumetric BMD, cortical volume and thickness, trabecular bone volume density and trabecular thickness. This could be due to some compounds in tea such as caffeine, phytic acid, and oxalic acid, that may negatively affect calcium absorption and/or excretion during the early stage of bone development in young rats, (Noonan & Savage, 1999; Gibson et al., 2010; Ross et al., 2011). Iwaniec et al. (2009) used a calcium-rich diet to feed the experimental mice, and there was still a negative impact on skeletal growth of the young growing mice. The authors suggested that the impact of green tea on bone metabolism may vary depending on the different phases of life (e.g. weaning *versus* aging).

Hence, the evidence obtained from both *in vitro* and animal studies suggests that low to moderate concentrations of tea may have a prophylactic role in alleviating bone loss and microarchitectural deterioration while at the same time improving bone strength and quality, which may stem from their antioxidant, anti-inflammatory and oestrogen-like effects. Nevertheless, despite the promising osteoprotective benefits of tea that have been offered by the literature, the definitive molecular targets and the mechanism responsible for the anabolic and anti-resorptive actions of tea are still elusive.

2.3.2 Human studies

Several epidemiological studies have been conducted to investigate the effect of tea consumption on bone health, most with a focus on postmenopausal osteoporosis. A number of human epidemiological studies have shown a positive association between tea consumption and bone health in different study populations of postmenopausal women from Southern Europe (Johnell et al., 1995), the United Kingdom (Hegarty et al., 2000), Denmark (Vestergaard et al., 2001), Iran (Hossien-Nezhad et al., 2007; Keramat et al., 2008), India (Keramat et al., 2008; Jha et al., 2010), Japan (Muraki et al., 2007), Taiwan (Wu et al., 2002), Australia (Devine et al., 2007; Myers et al., 2015), Canada (Hoover et al., 1996), and the United States (Chen et al., 2003).

In the Mediterranean Osteoporosis Study (MEDOS), tea was found to be an independent factor that exerted protection against hip fracture risk in women (Johnell et al., 1995) over the age of 50 years recruited in six European countries: France, Greece, Italy, Portugal, Spain and Turkey. The findings were further supported by a cross-sectional study of Hoover et al. (1996), who found that tea consumption was positively and significantly associated with BMD at both the lumbar spine and femoral neck in 62 postmenopausal Canadian women. In another study conducted in the United Kingdom, Hegarty et al. (2000) examined the association of tea consumption and BMD in 1,256 women aged 65-76 years. Tea drinkers were found to have a higher BMD assessed at multiple skeletal sites (i.e. lumbar spine, trochanter and Ward's triangle), than that of the non-tea drinkers even after adjustment for age and body mass index (BMI); these findings were independent of smoking, hormone replacement therapy, coffee intake, and whether tea was consumed with/without milk. Vestergaard et al. (2001) also reported a protective effect of tea consumption on femoral neck BMD in a cross-sectional study of 2,016 Danish perimenopausal women aged 45-58 years. A cross-sectional study conducted in Taiwan (Wu et al., 2002) showed that 1,037 habitual tea drinkers aged over 30 years had a higher BMD than non-habitual tea drinkers, after adjustments for age, BMI and other variables regarding lifestyle. The authors found that people who had consumed tea for a minimum length of 10 years had a higher BMD of the total body, lumbar spine and hip bone than that of the non-habitual tea drinkers; which was true for green, black and oolong teas.

In a prospective study including 91,465 multi-ethnic postmenopausal women aged 50-79 years in the United States, Chen et al. (2003) found a positive trend towards higher total BMD in habitual tea drinkers. Devine et al. (2007) reported a cross-sectional study that showed that tea consumption in 1,500 Australian women aged 70-85 years was independently associated with higher areal BMD at several skeletal sites (i.e. total hip and trochanter). In a subsequent investigation on the same women participants, Devine et al. (2007) also reported a prospective longitudinal study that showed tea consumption protected against bone loss, as shown by a lower reduction in the areal BMD at several skeletal sites (i.e. total hip, trochanter, and intertrochanter) over a four-year period. In a cross-sectional study, Muraki et al. (2007) reported that amongst 632 osteoporotic Japanese women aged over 60, habitual tea drinkers had higher BMDs than non-habitual tea drinkers after adjustment for age, BMI, and other

variables regarding lifestyle. In a cross-sectional study of 1,200 Iranian men and women aged 20-76, only women who were habitual tea drinkers had higher BMD at the hip when compared to non-habitual tea drinkers (Hossien-Nezhad et al., 2007). Keramat et al. (2008) reported a case-control study of 717 osteoporotic and non-osteoporotic Indian and Iranian women where habitual daily consumption of over four and seven cups of tea (respectively) was found to be significantly associated with reduced incidence of osteoporosis. Recently, Myers et al. (2015) reported that habitual consumption of three or more cups of black tea in 1,460 elderly Australian women (over the age of 70 years) was associated with a lower risk of fracture-related hospitalisation.

While these studies suggest an association between habitual tea consumption and better bone health (i.e. either improved BMD at different skeletal sites or reduced fracture risk) both in the elderly or gonadal hormone-deficient populations, there are other conflicting reports inconsistent with the findings of the previously described studies (Hernández-Avila et al. 1993; Chen et al., 2003; Hallström et al., 2006; Hamdi Kara et al., 2007; Jha et al., 2010). For example, a weak negative association between BMD of the ultradistal radius and tea consumption was found in a cross-sectional study of 281 pre- and peri-menopausal women aged 50-60 years recruited in the United States (Hernández-Avila et al. 1993). Meanwhile, Chen et al. (2003) reported that higher BMD in relation to higher tea consumption did not translate well to lower fracture risk in a US prospective study of 91,465 multi-ethnic postmenopausal women aged 50-79. This was further supported by Hallström et al. (2006), who recruited 31,527 Swedish women aged 40-76 for a prospective cohort study that examined the association between tea and osteoporotic fracture risk over a 10.3-year period. The authors found that no association between tea consumption (up to four cups per day) and osteoporotic fracture incidence was observed. Further, Hamdi Kara et al. (2007) reported that habitual tea consumption was not a significant protective factor for BMD in 724 postmenopausal Turkish women. In an Indian study, there was a negative association between tea consumption and fracture risk in a case-control study of 100 elderly men and women (Jha et al., 2010). The inconsistent findings concerning the association of tea consumption in humans and bone health may be due to the wide range differences in the different experimental designs and settings (e.g. study designs, targeted populations, tea types, amount of tea, and skeletal sites) and also due to non-inclusion of potential confounding variables in the analyses.

Of several recent meta-analyses examining the association between tea consumption and fracture risk (Modirian et al., 2011; Chen et al., 2014; Sheng et al., 2014; Yan et al., 2015), only the Sheng et al. (2014) study supported a bone-protective effect against osteoporotic fracture risk. The Sheng et al. (2014) meta-analyses examined the association between tea consumption and the risk of hip fracture for a total of 11 prospective cohort and case-control studies published between 1990 and 2010 that appeared to support a bone-protective effect of tea, whereby authors indicated that habitual daily tea consumption of one to four cups by an individual positively contributed to a lower hip fracture risk than those who were not a tea drinker. In contrast, Modirian et al. (2011) examined a similar association in eight studies published between 1980 and 2010; the authors found no significant protective effect of tea consumption on hip fracture risk. This is supported by Chen et al. (2014) and Yan et al. (2015), who examined the association between tea consumption and osteoporotic fracture risk in nine studies published between 1992 and 2013, where tea consumption was not associated with reduced osteoporotic-fracture risk. The reasons for the conflicting outcomes for bone health of these studies on tea consumption in different populations is unknown and outside the scope of this review, but may be related to the use of different approaches in meta-analysis design and the different studies that were included in the analyses.

Regardless of the importance of the laboratory and epidemiological studies, it is generally accepted that clinical intervention trials remain the most reliable way to evaluate the tea potential effects of tea on bone health (Henderson, 1992). At present, limited clinical intervention studies are available concerning the impact of tea consumption on bone health, especially in postmenopausal women with osteoporosis. In 2009, Shen et al. (2009c) conducted an intervention study, which focused on the impact of green tea polyphenol (GTP) supplementation and/or Tai Chi (TC) exercise on postmenopausal women with osteopenia. In their report, Shen et al. (2009c) described the rationale, design and methodology of the six-month placebo-controlled randomised clinical trial, with quantitative studies to determine individual and combination effects of the treatments. Later on, Shen et al. (2010c) released the first report of the trial concerning the safety data which indicated that the GTP supplementation of up to 500 mg/day (equivalent to four to six cups of green tea) in 171 postmenopausal women with osteopenia affected neither quality of life nor the liver and kidney functions of the

postmenopausal osteopenic participants, throughout the 24-week intervention period. In this study, the authors had used GTP in capsule form, with each containing 250 mg GTP (a mixture of 46.5% epigallocatechin-3-gallate (EGCG), 21.25% epigallocatechin (EGC), 10% epicatechin (EC), 9.5% gallocatechin gallate (GCG), 7.5% epicatechin-3-gallate (ECG), and 4.5% catechin (C); 99.25% purity); a formulation that is slightly different than that of the GTP assessed in their previous animal studies (reviewed in Shen et al., 2009a, 2011b, 2013a; Shao et al., 2011). Further in their subsequent investigation, Shen et al. (2012) determined the osteoprotective potential of GTP on bone turnover biomarkers in postmenopausal women with osteopenia. The influence of TC exercise was also investigated in the study. The study showed that GTP supplementation (500 mg per day) and/or TC exercise in 171 postmenopausal women with osteopenia resulted in higher values for serum bone formation biomarker (bone-specific alkaline phosphatase (BAP)), leading to an increased BAP/TRAP ratio that could be due to an increase in bone formation or a decrease in bone resorption. Noteworthy, GTP supplementation exerted its positive effects after 4- and 12-week study periods (respectively), while TC exercise exerted its effects after 12- and 24-week study periods (respectively). However, no effects by GTP or TC exercise were observed on serum and urinary biomarkers of bone resorption (TRAP, calcium, inorganic phosphate, and creatinine). Based on the study findings, Shen et al. (2012) concluded that GTP supplementation (and TC exercise) increased bone formation biomarkers and improved bone turnover rate in postmenopausal women with osteopenia. Subsequently, Qian et al. (2012) examined the same postmenopausal women participants for the efficacy of GTP (as well as TC exercise) on alleviating oxidative damage. It was found that GTP supplementation, either alone or together with TC exercise can decrease the DNA damaging effect of oxidative stress via reduced urinary levels of an oxidative damage biomarker, i.e 8-hydroxy-2-deoxyguanosine (8-OHdG).

Overall, the benefits of tea and its bioactive chemical compounds on bone health have been increasingly investigated and reviewed (Shen et al., 2009a, 2011b, 2013a, 2013b; Blumberg, 2013; Das et al., 2013; Nieves, 2013; Nash & Ward, 2015a, 2015b, 2016; Đudarić et al., 2015). From the review of literature, it can be pointed out that green and black teas are among the most promising natural dietary antioxidants able to mitigate bone loss and attenuate osteoporosis risk, considering its popularity as the most-consumed beverage worldwide next to water. Hence, consumption of tea as an

alternative or complementary therapy in the management and prevention of bone loss is relevant as a safe and economic option to deal with this growing public health crisis. While the outcomes of the laboratory and human studies regarding the impact of tea on bone health are encouraging, to the best of our knowledge, no information is available about the protective effect of Se-teas on bone health.

CHAPTER 3

GENERAL MATERIALS AND METHODS

This chapter describes the general materials and methods used throughout the research. More specific methods and statistical analyses can be found within the relevant chapters.

3.1 *In vitro* studies

3.1.1 Chemicals

All chemicals and reagents used in this study were analytical grade and supplied by Sigma-Aldrich Pty. Ltd., Australia, unless stated otherwise. Ultrapure Milli-Q (MQ) water was used for preparation of chemicals and of tea water extracts (Milli-Q system, Millipore, Milford, MA).

3.1.2 Equipment

Spectrophotometric measurements of total phenolic content (TPC), antioxidant activity and *in vitro* cell culture procedures (colorimetric assay, alkaline phosphatase activity assay, Crystal Violet staining assay, cetylpyridinium chloride extraction assay) were performed using an ELX 808 Ultra Microplate Reader (KC4 version 3.01 software Bio-Tek. Instruments Inc, USA). Centrifugation was performed using an Eppendorf Centrifuge MiniSpin® (Eppendorf, Germany) or Megafuge 1.0R centrifuge (D-37520 Osterode, Heraeus Instruments, Germany). Bacterial cell culture experimental work was carried out in a biological safety cabinet (Gelman Sciences BH120 Class 2, Singapore) and bacterial cell cultures incubated in anaerobic jars (BBL™ GasPak Pouch System, Becton Dickinson & Co., USA) placed in a humidified CO₂ incubator (Revco Ultima, Thermo-Fisher Scientific Ltd., USA). Bone cell culture experimental work was carried out in a laminar flow workstation (Class 2 1800, Email Westinghouse Pty. Ltd., NSW, Australia) and cell cultures were incubated in a humidified CO₂ incubator (Heraeus Hera Cell Incubator 150, Germany). The waterbath used in the cell culture experimental work was a Grant waterbath (SUB 6, Grant Instruments Ltd., Cambridge, UK). Microscopic observations of bone cell cultures were performed using an Olympus IX71 inverted microscope and XC50 digital camera (Olympus Corporation, Tokyo, Japan). All non-sterile lab consumables for bacterial and bone cell culture experimental work were steam-sterilised at 122 °C for 20 min using a Tomy High-Pressure Steam Sterilizer (Model ES-315, Tomy Kogyo Co. Ltd., Japan) before use.

3.1.3 Tea leaves

Dry leaves of regular green tea (R-GTE) of an unknown brand were purchased from a local food retailer in New Zealand (Dong Sheng Market, Palmerston North, New Zealand), and powdered black tea leaves (R-BTE) were of the Dilmah brand (100% Pure Ceylon). The dry leaves of selenium-rich green tea (Se-GTE) and selenium-rich black tea (Se-BTE) were obtained from a tea plantation in Enshi, Hubei province, China which is only 19 km away from a selenium mine. Prior to use, dry tea leaves were ground into fine powders using a grinder (CG2B, Breville Pty. Ltd., Australia).

3.1.4 Bacterial strains

Pure cultures of *Lactobacillus acidophilus* (NZRM 52, ATCC 11975) and *Lactobacillus rhamnosus* (NZRM 299^T, ATCC 7469) were obtained from the culture collection held by the Microbiology Lab, Massey Institute of Food Science and Technology (MIFST), College of Health, Massey University, Palmerston North, New Zealand. These strains were maintained in de Mann-Rogosa-Sharpe broth (MRS, BD DifcoTM, USA) under anaerobic conditions (BBLTM Gas Pak Pouch System, Becton Dickinson & Co., USA) in a CO₂ incubator at 37 °C in the presence of 10% CO₂.

3.1.5 Maintenance of bacterial cell cultures

The bacterial cell cultures were kept frozen in MRS broth as 20% glycerol stock (v/v, in MQ water) at -80 °C. For activation, 1 mL of each thawed cell stock was inoculated aseptically into 10 mL of MRS broth and incubated for 48 hours at 37 °C under anaerobic condition (BBLTM GasPak Pouch System, Becton Dickinson & Co., USA). Subsequently, 1% inoculums of the revived cells were sub-cultured into fresh MRS broth and anaerobically incubated for another 48 hours at 37 °C before they were ready to be used in the prebiotic assay. Aseptic technique was applied throughout the experimental studies, with all work being carried out in a biological safety cabinet. All non-sterile solutions used for cell culture maintenance were filter-sterilised prior to use using 0.20 µm syringe filters (Minisart, Sartorius AG, Goettingen, Germany).

3.1.6 Bone cell lines

Murine pre-osteoblast cells, MC3T3-E1 (Subclone 4) (ATCC CRL-2593, Manassas, VA, USA) and murine macrophage cells, RAW 264.7 (ATCC TIB-71, Manassas, VA, USA) were obtained from the cell culture collection held by the Bone Lab, Massey Institute of Food Science and Technology (MIFST), College of Health, Massey University, Palmerston North, New Zealand.

The MC3T3-E1 (Subclone 4) cells were grown in cell culture growth media Minimum Essential Media alpha modification (MEM α , Invitrogen, New Zealand) supplemented with 10% foetal calf serum (Heat-inactivated FCS, Invitrogen, New Zealand) and gentamicin (25 μ g/mL, Invitrogen, New Zealand). When grown in an osteogenic differentiation medium, MC3T3-E1 (Subclone 4) cells differentiate into mature osteoblasts and are capable of synthesising mineralised extracellular matrix. Osteogenic differentiation medium comprised the following: MEM α , 10% FCS, 50 μ g/mL ascorbic acid and 10 mM β -glycerophosphate, with the addition of gentamicin (25 μ g/mL).

The RAW 264.7 cells were grown in the Dulbecco's Modified Eagles Medium (DMEM, Gibco, Life Technologies, New Zealand) supplemented with 10% foetal calf serum (Heat-inactivated FCS, Invitrogen, New Zealand) and antibiotic-antimycotic (15240-062, Gibco, Life Technologies, New Zealand).

3.1.7 Maintenance of bone cell cultures

The bone cells were seeded at 1×10^5 cells/mL into 75 cm² flasks and maintained as continuous culture in an incubator with a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The cell culture growth medium was replaced with fresh medium every 3 to 4 days. The cells were passaged weekly when a confluent monolayer had formed, and all experiments were performed with early passage cells (less than passage 17). Aseptic technique was applied throughout all experimental studies, with all work being carried out in a laminar flow workstation. All non-sterile solutions used for

cell culture maintenance were filter-sterilised prior to use with a 0.20 µm syringe filter (Minisart, Sartorius AG, Goettingen, Germany).

The cells were kept frozen when required for future use by resuspension of the cell pellet at a cell density of 5×10^6 cells/mL with 1.5 mL of cold freezing medium-DMSO (1101-011, Gibco, Invitrogen, New Zealand) in a cryovial and allowed to freeze slowly with a decreasing temperature rate of approximately at 1 °C per min by placing the cryovials in an insulated box placed in a -85 °C freezer overnight. The frozen cells were later transferred into liquid nitrogen containment (Statebourne Cryogenics Ltd., UK) and stored at -196 °C. The different sub-culture procedures for the different cells are described below:

3.1.7.1 Murine pre-osteoblast MC3T3-E1 (Subclone 4) cells

Briefly, old medium was discarded and the confluent monolayer cells rinsed twice with 10 mL of phosphate buffered saline (PBS, pH 7.3) to remove traces of serum that might inhibit trypsin activity. Cells detached from the flask with addition of 3 mL of 0.05% Trypsin-EDTA (23500-054, Gibco, Invitrogen, New Zealand) at room temperature with gentle agitation. When all cells were dislodged (approximately 5 min), 7 mL of warm growth medium was added to neutralise the action of Trypsin-EDTA and gently mixed with a pipette to break up cell clumps. The cell suspension was subjected to centrifugation at 200 g for 5 min to pellet cells. The cell pellet was then resuspended in fresh growth medium and counted using a haemocytometer with trypan blue exclusion. Finally, the cells were plated into 75 cm² flasks at a seeding density of 1×10^5 cells/mL.

3.1.7.2 Murine macrophage RAW 264.7 cells

Briefly, old medium was discarded and 10 mL of warm fresh media was added into the flask of confluent cells. Cells were then gently scraped from the flask using a sterile rubber scraper and mixed with a pipette to break up cell clumps. The cell suspension was counted using a haemocytometer with trypan blue exclusion, and finally plated into 75 cm² flasks at a seeding density of 1×10^5 cells/mL.

3.1.8 Bone cell culture procedures

Trypan blue exclusion was used to determine the viability of the experimental cell population using a haemocytometer. Trypan blue solution was used at a concentration of 0.4% in PBS. This assessment was accomplished using microscopy using an improved Neubauer haemocytometer (Weber, England) to count trypsinised cells that excluded the vital dye trypan blue using an Olympus BX2 upright microscope (Olympus Corporation, Tokyo, Japan). Cell viability was determined to be greater than 98%. In all experimental work, cell concentrations for MC3T3-E1 (Subclone 4) and RAW 264.7 cells were finally adjusted to 2×10^4 cells/mL and 5×10^4 cells/mL, respectively. The cell suspensions were seeded in either 96-microwell plates or 24-microwell plates for their respective analyses as specified in **Chapter 5** and **Chapter 6**.

3.1.9 Experimental techniques

3.1.9.1 Evaluation of cell proliferation using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay

The MTT colorimetric assay was performed as described by Mosman (1983) with some modifications. Briefly, at the end of each experiment the old MEM α medium with 0.1% BSA was aspirated and replaced with 90 μ L of freshly made media. Then, 10 μ L of MTT (5 mg/mL in PBS, 0.20 μ m filtered) was added to each well and further incubated for three hours. Following incubation, the medium was carefully aspirated, and the reduced MTT dye in each well was solubilised with 100 μ L of dimethyl sulfoxide (DMSO). The 96-microwell plate was incubated for 5 to 10 min prior to optical density (OD) measurements being read at 550 nm on a microplate reader. All control wells were processed in exactly the same manner as the rest of the tea-treated wells. Cell proliferation was expressed as percentage of zero control (%).

3.1.9.2 Evaluation of cell differentiation using alkaline phosphatase (ALP) activity assay

The ALP activity assay was assessed according to Coetzee et al. (2009) with some modifications. At the end of the tea treatment period, the old osteogenic media

were removed and each well was washed once with 500 μ L PBS. Then, cells were fixed with 200 μ L of 1% formaldehyde per well for 15 min at room temperature. Cells were washed again with the same amount of PBS prior to a one-hour incubation at 37 °C with 300 μ L of ALP test reagent (N4645, Sigma-Aldrich Pty. Ltd., Australia), i.e. 0.05 M para-nitrophenyl phosphate (ρ -NPP) in tris buffered saline (TBS, pH 9.5). Thereafter, 100 μ L aliquots of reaction product from each well (in duplicate) were transferred to a 96-microwell plate and the absorbance was measured at 405 nm. All control wells were processed in exactly the same manner as the rest of the tea-treated wells. The remaining ALP test reagent was then washed from the wells with 500 μ L PBS to prepare the wells for cell number determination by Crystal Violet staining, in order to quantify ALP levels per cell number. For cell quantification of ALP activity, the total absorbance of ALP was normalised by the absorbance of Crystal Violet staining at 550 nm.

3.1.9.3 Cell quantification of ALP activity using Crystal Violet staining assay

ALP activity levels were quantified using Crystal Violet staining procedure of Coetzee et al. (2009) with some modifications. In brief, 300 μ L of 1% Crystal Violet in PBS (w/v) was added to each of the wells previously fixed and stained for ALP. Each plate was stained and incubated for one hour at 37 °C. Following incubation, cells were gently washed with the plate immersed in running tap water for 15 min. The plate was then air-dried overnight. On the following day, 500 μ L of 0.2% Triton-X 100 (in PBS) was added into each well to solubilise the dye. The plate was incubated for 90 min at room temperature. Duplicate measures of 150 μ L aliquots from each well were transferred to 96-microwell plates and the absorbance read at 550 nm against the reagent blank (0.2% Triton-X100) prepared under similar conditions. All control wells were processed in exactly the same manner as the rest of the tea-treated wells. ALP activity was expressed as an index by dividing absorbance levels of ρ -NPP by the intensity of the Crystal Violet absorbance.

3.1.9.4 Evaluation of cell mineralisation using the Alizarin Red S (ARS) staining assay

Mineralised bone nodule formation of MC3T3-E1 cells was measured using the ARS staining assay. This assay procedure was performed according to a modified method of Scutt et al. (2003). At the end of each treatment period (8, 16 and 24 days), each well was rinsed once with 500 μ L PBS and then fixed in 500 μ L of ice-cold

absolute ethanol (99.8%) in the dark at room temperature for one hour. Following cell fixation, absolute ethanol from each well was aspirated and the cells were allowed to dry at room temperature. Thereafter, cells were counterstained with 500 μ L/well of 0.1% ARS (1 mg/mL (w/v), pH 5.5) and allowed to sit overnight at room temperature. On the following day, cells were gently washed with the plate immersed in running tap water for 15 min. The plate was then air-dried at room temperature. Stained cultures were visually identified and the ARS-positive nodules on each plate photographed. All control wells were processed in exactly the same manner as the rest of the tea-treated wells.

3.1.9.5 Cell quantification of ARS-positive nodules using cetylpyridinium chloride (CPC) extraction procedure

ARS staining retained in mineralised bone nodules was quantified using CPC extraction procedure as described by Bodine et al. (1996), with some modifications. In brief, 500 μ L of 10% CPC (w/v) was added to each well to de-stain the cultures and culture plates were incubated overnight at room temperature. On the following day, duplicate measures of 150 μ L aliquots from each well were transferred to 96-microwell plates and the absorbance was read at 550 nm against the reagent blank (10% CPC) prepared under similar conditions. All control wells were processed in exactly the same manner as the rest of the tea-treated wells. ARS extraction was expressed as percentage of control wells (%).

3.1.10 Statistical analysis

Results are presented as means with their standard error of the mean (mean \pm SEM). All results were statistically analysed using SAS software for Windows version 9.3 (SAS Institute Inc., Cary, NC, USA). All data were tested for normality using the PROC UNIVARIATE, a procedure within SAS used mainly for examining the distribution of data. Homogeneity of group variances was estimated using Levene's test. The differences between group means were compared using one-way analysis of variance (ANOVA), followed by post-hoc Tukey's test for pair-wise multiple comparisons of the group means. The Welch's variance-weighted ANOVA was used in place of the simple one-way ANOVA when the assumption of homogeneity of

variances was not met and groups were unequal in size, which was followed by post-hoc Tukey-Kramer test for pair-wise multiple comparisons. The ANOVA analyses were conducted using the PROC GLM, a procedure within SAS that analyses data within the framework of general linear models. All measurements were carried out in triplicate across three independent experiments, unless otherwise stated. A difference was considered to be statistically significant when $p < 0.05$.

3.2. Animal Studies

3.2.1 Chemicals

All chemicals and reagents used in this study were of analytical grade and supplied by Sigma-Aldrich Pty. Ltd., Australia, unless stated otherwise. Ultrapure Milli-Q (MQ) water was used for preparation of chemicals (Milli-Q system, Millipore, Milford, MA).

3.2.2 Equipment

Spectrophotometric measurements of serum antioxidant capacity, serum biomarker of bone resorption, and bacterial enzyme activities were performed using an ELX 808 Ultra Microplate Reader (KC4 version 3.01 software Bio-Tek. Instruments Inc, USA). Centrifugations were performed using an Eppendorf Centrifuge MiniSpin® (Eppendorf, Germany) or Megafuge 1.0R centrifuge (D-37520 Osterode, Heraeus Instruments, Germany). The waterbath used in the fluorescence *in situ* hybridisation (FISH) experimental work was a Grant waterbath (Type VF, Grant Instruments Ltd., Cambridge, UK).

3.2.3 Animals and protocols

All male or female Sprague-Dawley (SD) rats were obtained from the Small Animal Production Unit (SAPU), Massey University, Palmerston North, New Zealand.

Upon arrival, each rat was weighed and housed individually in clean shoe-box cages, in a temperature (22 ± 2 °C) and humidity (45% – 50%) controlled room with a 12 hours light/dark (illumination cycle) schedule. All rats had *ad libitum* access to standard rat chow pellets and deionised water or tea water extracts throughout the study period. Rats were monitored daily for general health and changes in appearance or behaviour throughout the study. The rats were cared and maintained in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council (NRC), 1996) and Massey University guidelines. Ethical consent for the study protocols of male and female rats were obtained from the Massey University Animal Ethics Committee (Approval Numbers 11/28 and 11/29, respectively).

3.2.4 Diets

Standard rat chow pellets used in the study were a semi-synthetic diet containing 0.5% calcium. This was formulated to contain all nutrients that are necessary for rapid growth in growing male rats and reproduction in adult female rats based on AIN-93G and AIN-93M, respectively (NRC, 1995). The type of oil in the diet was changed from soybean oil to corn oil as soybean oil is a rich source of phytoestrogens, i.e. daidzein and genistein (Hodgson et al., 1998). Materials used for the preparation of standard rat chow pellets were supplied by the Feed Mill, Massey University, Palmerston North. The diet was prepared in bulk at the Feed Mill, Massey University, Palmerston North and stored at -20 °C until used.

3.2.5 Preparation of tea water extracts

Deionised water was used to prepare tea water extracts and also given to rats in the control group. Deionised water was used in the study to control calcium from water throughout the experimental period. The tea water extracts (1%, w/v) were freshly prepared by infusing 10 grams of powdered tea leaves in 1 L of boiling deionised water for 5 min. The tea solutions were filtered with sterile muslin cloth to produce a clear solution and allowed to cool to room temperature prior to serving. Tea water extracts or deionised water were supplied *ad libitum* in clean polyethylene water bottles, as the

only source of drinking fluid during the study duration. Deionised water or tea water extracts were replaced and served fresh once every second day during the feed intake measurement. Polyethylene water bottles were cleaned weekly. Water bottles for tea drinks were cleaned thoroughly once every second day during the fluid intake measurement to prevent spoilage from bacteria or traces of tea residues.

3.2.6 Experimental techniques

3.2.6.1 Ovariectomy and sham-operation

All rats were anaesthetised before ovariectomy surgery by inhalation of isoflurane. Female Sprague-Dawley rats were injected subcutaneously with an analgesic carprofen (Rimadyl®, Pfizer New Zealand Ltd, Auckland, New Zealand) at 0.1 mL/kg before the surgery to relieve pain. Thirty min after administration of the analgesic, the rats were anaesthetised by inhalation of 1.5% – 2% isoflurane. Once the rats reached the required depth of anaesthesia, the surgical site was prepared by shaving the hair and the skin was disinfected with povidine-iodine solution (Betadine® Surgical Scrub, Purdue Pharma L.P., Stamford, Connecticut, USA) followed by 70% ethanol. With the rat laying on its ventral surface, a midline incision was made approximately half way between the hump of the back and the base of the tail. The skin was pulled half way down one side of the body and an incision made through the peritoneal muscles. The ovary with the surrounding fat was exteriorised through the incision (by grasping the periovarian fat pad). The ovary was excised along with the ovarian blood vessels, uterine tube and uterine horn by a single cut through the distal part of the horn, and the horn was returned to the abdominal cavity. The procedure was then repeated for the other ovary. In order to maintain fluid levels, 2 mL of warm sterile saline were injected into the peritoneal cavity through the incision in the muscle layer. The skin incision is closed using 3 to 4 surgical clips which were removed after 7 days. An analgesic butorphanol tartrate (Butorphanol™ Injection, Lloyd Laboratories NZ Ltd, Auckland, New Zealand) at 0.2 mL/kg was administered subcutaneously after the surgery to relieve pain. The rats were kept warm under a heat lamp and on a heat blanket for about three hours post-surgery. The ambient temperature did not exceed 25 °C. A similar

procedure was used for sham surgery, except that the ovaries were exteriorised but left intact and returned to the abdominal cavity.

3.2.6.2 Evaluation of *in vivo* bone mineral measurements using dual energy X-ray absorptiometry (DEXA)

Female Sprague-Dawley rats were weighed and anaesthetised at an appropriate dose (0.06 mL/100 g body weight) before being scanned for bone mineral density (BMD), with an intraperitoneal injection (25G × 5/8" needle and 1 mL syringe) of anaesthetic mixture of 0.2 mL acepromazine (2 mg/mL; Delta Laboratories Pty Ltd, New South Wales, Australia), 0.5 mL ketamine (100 mg/mL; Parnell Laboratories New Zealand Ltd, Auckland, New Zealand), 0.1 mL xylazine (10%; Phoenix Pharm Distributors, Auckland, New Zealand), and 0.2 mL of sterile water. The rats attained a suitable level of anaesthesia approximately 5 to 10 min after injection and remained under anaesthesia for two hours.

Bone mineral measurements were assessed using a Hologic Discovery A bone densitometer (Bedford, MA, USA). On each day the DEXA was used, a quality control (QC) scan was taken to ensure precision. This QC scan was required to meet the DEXA manufacturer's standard coefficient of variation (CV) of 0.98% – 1.01%. The spine phantom was scanned according to manufacturer's guidelines at the start and end of the scanning session to verify proper system calibration. Anaesthetised rats were placed on an acrylic platform of uniform 3.81 cm thickness. Each rat underwent three regional high-resolution scans of the whole body. Rats were positioned supine with right angles between the spine and femur, and between the femur and tibia. The CV for the femurs ranged between 0.92% and 0.85%, with and without repositioning between scans. These values ranged between 1% and 0.98% for the spine.

After the DEXA scan, rats were given 2 mL of warm saline via an intraperitoneal injection and then placed on a purpose built thermostatically controlled heat pad, covered with paper towel and monitored closely until recovery. After each of DEXA scan (weeks -2, 3 and 7), the rats were returned to their shoe-box cages at SAPU.

3.2.6.3 Blood sample collection via terminal heart puncture

Fasted blood samples were drawn directly from the heart under anaesthesia at the completion of the studies. An overnight fasting applied in all animal models was aimed to reduce the diurnal variation in serum biomarkers carboxy-terminal crosslinks telopeptide of type I collagen (CTX-I) and osteocalcin (OC) (Nielsen et al., 1990; Hassager et al., 1992; Schlemmer & Hassager, 1999), whereby these markers typically peak during the night to early morning and gradually decrease reaching the lowest point (called the nadir) in the late afternoon. The rats were weighed and anaesthetised with an overdose of anaesthetic (0.12 mL/100 g body weight) via an intraperitoneal injection using a 25G × 5/8" needle and 1 mL syringe of the following mixture: 0.2 mL acepromazine (2 mg/mL; Delta Laboratories Pty Ltd, New South Wales, Australia), 0.5 mL ketamine (100 mg/mL; Parnell Laboratories New Zealand Ltd, Auckland, New Zealand), 0.1 mL xylazine (10%; Phoenix Pharm Distributors, Auckland, New Zealand), and 0.2 mL sterile water. Then, an 18G × 1 1/2" hypodermic needle and 10 mL syringe were used to withdraw the blood directly from the heart. A blood sample (~10 mL) was collected in a plastic serum vacutainer (Cat. #367895, Becton, Dickinson & Co., USA) and left to clot at room temperature for 60 min. Serum samples were separated from the clot by centrifugation at 1,200 g at room temperature for 10 min, and aliquoted into labelled microcentrifuge tubes within one hour of centrifugation. The serum samples were stored at -80 °C pending assays for serum antioxidant capacity, and serum bone resorption/formation markers.

3.2.6.4 Euthanasiation and dissection

Rats were euthanised by exsanguination following terminal heart puncture (section 3.2.6.3), and dissected. The peritoneal cavity was opened by a midline incision and the caecum was removed. The lumbar spine (LS1 – LS4) and both femurs of each rat were excised with some flesh remaining, kept in labelled plastic containers filled with PBS (pH 7.2) and frozen at -20 °C pending bone mineral and biomechanical analysis. The caeca with contents were transferred into labelled plastic bags and stored at -80 °C pending analysis for pH, bacterial enzyme activities of intestinal microbiota and fluorescence *in situ* hybridisation (FISH).

3.2.6.5 Evaluation of serum antioxidant capacity using ferric-reducing antioxidant power (FRAP) assay

The FRAP assay was performed as described by Benzie and Strain (1996), with some modifications (Molan et al., 2009c). In short, an aliquot of serum (8.5 μ L) was added to 275 μ L of pre-warmed (37 $^{\circ}$ C) diluted FRAP reagent in a 96-well microplate and incubated for 10 min at 37 $^{\circ}$ C, before absorbance was read at 595 nm against a MQ water blank sample as a reference. Only freshly prepared FRAP reagent (50%; v/v) was used, by mixing 30 mL sodium acetate buffer (pH 3.6; 300 mmol/L), 3 mL of 2,4,6-tripyridyl-*s*-triazine (TPTZ; 10 mmol/L) in hydrogen chloride (HCl; 40 mmol/L), and 3 mL of ferric chloride (FeCl_3 ; 20 mmol/L), in 43.2 mL of MQ water, pre-warmed to 37 $^{\circ}$ C. The results obtained were corrected for dilution and compared to a standard curve prepared using different concentrations (100 – 1,000 μ g/mL) of ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$). FRAP values were expressed as milligram of FeSO_4 equivalents per litre of serum (mg FeSO_4 E/L serum). The antioxidant measurements for each serum sample were conducted in triplicate.

3.2.6.6 Evaluation of serum biomarker of bone resorption

Fasted blood serum samples were tested for carboxy-terminal crosslinks telopeptide of type I collagen (CTX-I), a marker of bone resorption. The CTx-I concentration was measured by enzyme-linked immunosorbent assay using the RatLapsTM EIA kit (AC-06F1, Immunodiagnostic Systems Ltd., UK), according to the manufacturer's instructions. The absorbance was measured at 450 nm, with 650 nm as a reference using a microplate reader. The CTx-I measurements for each serum sample were conducted in duplicate.

3.2.6.7 Evaluation of serum biomarker of bone formation

Fasted blood serum samples were tested for osteocalcin (OC), a specific marker of bone formation. The osteocalcin concentration was measured by enzyme-linked immunosorbent assay using the RAT-MIDTM Osteocalcin EIA kit (AC-12F1, Immunodiagnostic Systems Ltd., UK), according to the manufacturer's instructions. The absorbance was measured at 450 nm, with 650 nm as a reference using a microplate reader. The osteocalcin measurements for each serum sample were conducted in duplicate.

3.2.6.8 Evaluation of *ex vivo* bone mineral measurements using dual energy X-ray absorptiometry (DEXA)

Bone mineral measurements were taken using a Hologic Discovery A bone densitometer (Bedford, MA, USA). On each day the DEXA was used, a QC scan was taken to ensure precision. This QC scan was required to meet the DEXA manufacturer's standard CV of 0.98% – 1.01%. The spine phantom was scanned according to manufacturer's guidelines at the start and end of the scanning session to verify proper system calibration. *Ex vivo* bone mineral measurements using DEXA were performed on excised bone obtained after the end of the treatment period. The frozen femurs and lumbar spines were thawed and dissected to a surrounding tissue depth of approximately 5 mm. All excised bones of each rat, i.e. left and right femurs, and lumbar spine (LS1 – LS4) were individually scanned using a small-animal regional high-resolution protocol. The CV for the femurs and lumbar spine scans ranged between 0.4% and 0.6%.

3.2.6.9 Evaluation of bone biomechanical properties of appendicular long bones using a three-point bending test

Right femurs were thawed and adherent soft tissues removed. The length of the femurs was measured between the proximal end of the intercondylar notch and the proximal limit of the base of the femoral neck using an electronic vernier caliper (± 0.02 mm, MitutoyoTM, Kanagawa, Japan). The midpoint of the femur was marked with a waterproof pen, and the width and thickness at this midpoint recorded using the calipers. The bones were kept at room temperature (22 ± 2 °C) throughout the testing procedure. Biomechanical testing was conducted using a Shimadzu texture analyser (Ezi-test series, Kyoto, Japan). The femurs were placed on the three-point bending jig with a fixed distance of 12 mm between the two supporting rods where the fulcrum was positioned midway between the supporting rods. The midpoint of the anterior surface of the femur was then subjected to a constant deformation rate of 50 mm/min until fracture. Values for maximum load (N), maximum stroke (mm), breaking load (N), breaking stress (N/mm^2), breaking stroke (mm), breaking strain (%), elasticity (N/mm^2) and energy (J), were obtained using Shimadzu WinAGSLite 2000 software.

Under three-point loading condition, femoral shafts were loaded with bending force until failure (bone break), where the width (mm) and thickness (mm) of the

femoral midshaft being recorded and integrated by Shimadzu WinAGSLite 2000 software into calculation of the results. The bending forces loaded on the bone to failure were recorded, yielding maximum load (N) and maximum stroke (mm); with these being respectively defined as the maximum force applied and the displacement value at which the bone maintains elastic integrity. Breaking load (N) and breaking stroke (mm) are defined as the bending force and the displacement value at failure, respectively. Breaking stress and strain are the amounts of stress and strain observed at the bending force, respectively. Breaking stress (N/mm^2) is the force per unit area which causes the bone to break. Breaking strain (%) is the percent deformation of the femur relative to its length, just prior to bone-breaking timepoint. The higher the breaking stress and strain, the stronger the bone is. The elasticity (elastic modulus) was defined as the force required to bend the bone in the elastic phase (reversible) of deformation. Being established from the breaking stress-strain curve, elastic modulus reflects the stiffness (resistance to deformation) of the bone. Thus, under continuous high enough bending force, permanent bone deformation (plasticity) occurs. An increase in elasticity of the bone indicates a decrease in bone stiffness, whereby stiffness reflects collagen quality and mineralisation. Breaking energy (J) is the ultimate measure of overall bone strength, where it was defined as the amount of energy required (energy that bone must absorb) to break a bone and represented as an integration value of force (area under the force/displacement curve). The breaking energy applied to deform the bone is stored in the bone material as potential energy, whereby this energy is available to the bone in order to recover from deformation once the applied bending force was removed. The higher is the breaking energy, the more energy a bone can absorb prior to breaking; thus, indicating how much stronger the bone is. Among all these parameters of bone strength, maximum load, maximum displacement, stiffness and energy are the parameters of interest for mechanical strength of bone (Turner & Burr, 1993; Mosekilde, 1995; Ferretti et al., 2001; Turner, 2006; Guede et al., 2013).

3.2.6.10 Evaluation of caecal pH

The pH of each caecal sample was determined at room temperature using an ISFET pH meter (KS701, Shindengen Electric Manufacturing Co. Ltd., Japan). The caecal pH was measured in triplicate by inserting the electrode of the ISFET pH meter directly into the middle portion of the thawed caeca. Caecal contents from each rat were divided into several portions for further bacterial enzymes and FISH analysis.

3.2.6.11 Evaluation of bacterial enzyme activities in *ex vivo* rat caecal contents to determine enzyme activities of intestinal microbiota

The metabolic activity of the intestinal microbiota for each rat was determined using a modified method of Goldin et al. (1980), as described by De Preter et al. (2008). Briefly, 0.3 g of caecal contents was suspended in 3 mL of cold 0.1 M potassium phosphate buffer (PBS, pH 7.2). The mixture was homogenised by vortexing for 3 min, and subjected to low-speed centrifugation at 700 g for 2 min to pellet the debris. The supernatant was transferred into a new microcentrifuge tube and exposed to a freeze (-70 °C)/thaw (37 °C) cycle thrice to disrupt the bacterial cells. The supernatants were then used for the assessment of bacterial enzyme activity, as shown in **Figure 3-1**. Activities of the enzymes β -glucosidase (β -GLU) and β -glucuronidase (β -GUS) were determined as the rate of release of para-nitrophenol (p -NP) and phenolphthalein from p -nitrophenyl β -pyranoside and phenolphthalein β -glucuronide, respectively.

Briefly, a sample of caecal supernatant (10 μ L) was added to 40 μ L of reaction mixture, consisting of 0.1 M PBS (pH 7.2) and 1 mM p -nitrophenyl β -pyranoside (for β -glucosidase) or 0.02 M potassium phosphate buffer, 0.1 mM ethylene diamine-tetra acetic acid (EDTA) and 0.05 mM phenolphthalein β -glucuronide (for β -glucuronidase) in a 96-well microplate. The enzyme reaction was incubated for 60 min at 37 °C and stopped by the addition of 250 μ L of 0.1 M NaOH (for β -glucosidase), or 250 μ L of 0.2 M glycine buffer (pH 10.4) containing 0.2 M NaCl (for β -glucuronidase). For each test sample, a control (caecal supernatant with a reaction mixture without p -nitrophenyl β -pyranoside substrate or phenolphthalein β -glucuronide substrate) was run at the same time. The amount of p -NP (for β -glucosidase) or phenolphthalein (for β -glucuronidase) released from the enzymatic reaction were quantified by measuring the ultraviolet absorption at 450 nm and 550 nm, respectively, using a microplate reader. The results were corrected for controls and calculated according to a calibration curve of p -NP (for β -glucosidase) or phenolphthalein (for β -glucuronidase). The results for both enzymes were expressed as one unit (U) of enzyme per gram caecal content. One unit (U) of enzyme was defined as “the amount of β -glucosidase or β -glucuronidase enzyme that releases 1 mg of p -NP or phenolphthalein per hour”.

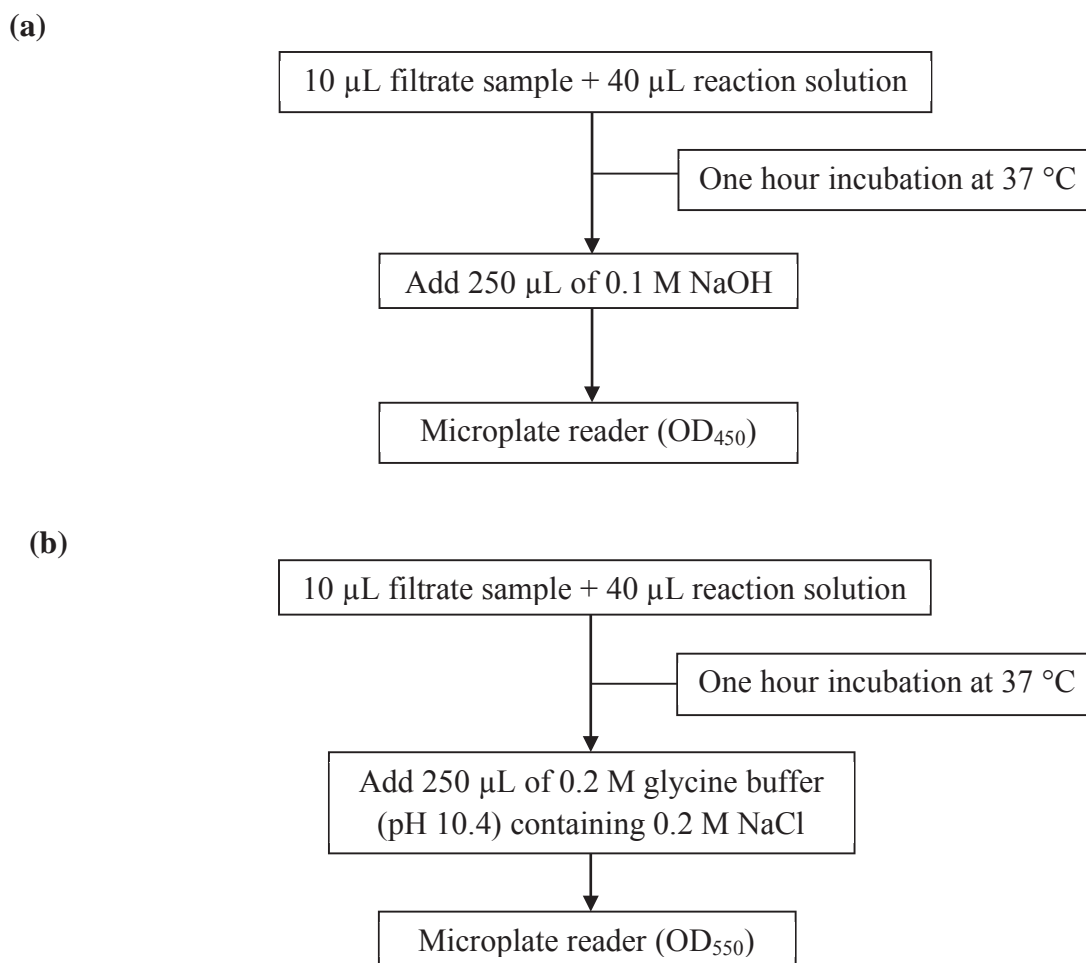


Figure 3-1. Assessment of bacterial enzyme activities. (a) β -glucosidase; β -GLU, and (b) β -glucuronidase; β -GUS.

3.2.6.12 Evaluation of intestinal microbiota populations in *ex vivo* rat caecal contents using fluorescence *in situ* hybridisation (FISH) analysis

Caecal contents obtained from the young growing male rats were analysed by FISH analysis to assess the effect of tea water extracts on intestinal microbiota populations. The FISH analysis was used for enumeration of selected groups of bacterial components of the intestinal microbiota using a modified procedure of Dinoto et al. (2006), as described by Molan et al. (2009c). In this study, commercially synthesised genus-specific 16S rRNA-targeted oligonucleotide probes labelled with the Cy3 fluorescent dye (GeneWorks, Australia) were utilised for enumeration of selected bacterial groups: *Bacteroides-Prevotella* group (Bac 303), *Bifidobacterium* spp. (Bif 164), *Clostridium perfringens/histolyticum* subgroup (Chris 150) and *Lactobacillus/Enterococcus* spp. (Lab 158). The DNA sequences of Bac 303, Bif 164, Chris 150 and

Lab 158 were: 5'-CC AAT GTG GGG GAC CTT-3', 5'-CA TCC GGC ATT ACC ACC C-3', 5'-TT ATG CGG TAT TAA TCT CCC TTT-3' and 5'-GG TAT TAG CAT CTG TTT CCA-3', respectively.

The caecal samples were prepared by mixing 0.3 g of the digesta collected from the middle portion of the caeca with 3 mL of sterile-filtered potassium phosphate buffer (PBS, pH 7.2). The caecal debris were removed by centrifugation at low speed (700 g) for 2 min, and 200 μ L of the supernatants (containing bacteria) were fixed in 600 μ L of 4% (w/v) paraformaldehyde in 0.1 M PBS (pH 7.2) at 4 °C overnight. Bacterial cells were pelleted by centrifugation (10,000 g) for 5 min and washed twice in filter-sterilised PBS. Fixed bacterial cells were suspended in a known volume of 50% (v/v) ethanol-PBS prior to hybridisation.

Following hybridisation, 5 μ L aliquots of fixed bacterial cells were applied to the wells on Teflon-coated microscopic slides (10 well, 6 mm; Biolab Scientific, Auckland, New Zealand) and air-dried to prevent cells detaching in subsequent steps. The bacterial cells were then dehydrated with an ethanol series of 50%, 80% and 99.5% concentrations, allowing 3 min incubation for each concentration. The bacterial cells fixed on the glass slides were hybridised by addition of 8 μ L of hybridisation buffer (0.9 M NaCl, 0.01% sodium dodecyl sulphate, 20 mM Tris-HCl, 20% deionised formamide; pH 7.2) with 0.5 μ L of Cy3-labeled oligonucleotide specific probes (50 ng/ μ L). The slides were hybridised for two hours in a covered plastic box containing wet tissue paper (as a moist chamber) and placed horizontally into a hybridisation oven (46 °C). The wet tissue paper was previously soaked in hybridisation buffer to prevent evaporation of buffer in the wells of the Teflon-coated slides during hybridisation. After hybridisation, the slides were rinsed with freshly made hybridisation buffer pre-warmed at 48 °C, washed in pre-warmed (48 °C) washing buffer (225 mM NaCl, 0.01% sodium dodecyl, 20 mM Tris-HCl; pH 7.2) and finally incubated for 20 min at 48 °C (in a waterbath). The slides were then rinsed with ice-cold MQ water and thoroughly dried before being mounted in DPX mountant (BDH Chemicals Ltd., Poole, UK) and examined with an Olympus BX51 microscope, under 400 \times magnification. The bacterial cell images were captured using an Optronics MagnaFIRE SS99802 digital camera with MagnaFIRE frame-grabbing software on a Pentium IV computer (Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North, New Zealand).

Fluorescent bacterial cells were counted automatically in five randomly selected fields per slide using ImageJ software (Abramoff et al., 2004), which is freely downloadable from <https://imagej.nih.gov/ij/>. Bacterial populations for each species were expressed as log number of bacterial cells per g wet weight caecal content (Log_{10} cells/g caecal content).

3.2.7 Statistical analysis

Results are presented as means with standard error of the mean (mean \pm SEM). All statistical analyses were conducted using SAS software for Windows version 9.3 (SAS Institute Inc., Cary, NC, USA). The numbers of animals used in the studies were based on a statistical power calculation for sample size, using the method of Snedecor and Cochran (1989). All data were tested for normality using the PROC UNIVARIATE procedure. Homogeneity of group variances was estimated using Levene's test. For the FRAP assay, simple linear regression analysis was performed using Microsoft Excel (2007) to calculate the dose-response relationship of standard ferrous sulphate (FeSO_4) solution used for calibration. Changes in rat body weight over time were analysed with repeated measures analysis of variance (ANOVA) for comparison of means using the PROC MIXED, a procedure within SAS that fits a variety of mixed linear models. The differences between group means of rats were compared using one-way ANOVA, followed by post-hoc Tukey's test for pair-wise multiple comparisons of the group means. Welch's variance weighted ANOVA were used in place of the simple one-way ANOVA when the assumption of homogeneity of variances was not met and groups were unequal in size, which was followed by post-hoc Tukey-Kramer test for pair-wise multiple comparisons. The one-way ANOVA analyses were conducted using the PROC GLM, a procedure within SAS that analyses data within the framework of general linear models. A difference was considered statistically significant when $p < 0.05$.

CHAPTER 4

TOTAL PHENOLIC CONTENT, ANTIOXIDANT ACTIVITIES AND PREBIOTIC POTENTIAL OF SELENIUM-RICH GREEN AND BLACK TEAS *VERSUS* REGULAR GREEN AND BLACK TEAS

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Abstract

Four different tea extracts of *Camellia sinensis* were assessed for their total phenolic content (TPC) and antioxidant properties. The tea extracts included selenium-rich green tea (Se-GTE), selenium-rich black tea (Se-BTE) extracts, regular green tea (R-GTE) and regular black tea (R-BTE) extracts. Aqueous tea extracts at 10 mg/mL (1% concentration; w/v) were prepared using different extraction temperatures and times. The total phenolic content (TPC) was measured using the Folin-Ciocalteu method, while antioxidant activity was measured using the ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The Se-GTE contained the highest TPC, followed by R-BTE, R-GTE and Se-BTE. The Se-GTE also exhibited the highest antioxidant activity compared to other tea extracts. The R-BTE showed significantly higher TPC and antioxidant activity than the Se-BTE, which may be due to the fact that these are different cultivars. The extraction temperature and time were found to be important determinants of extracted TPC and antioxidant activities of the tested teas. The optimal method for maximising TPC was found to be extracting tea in a water temperature of 90 °C for 5 min; this was then used as the standard preparation of the tea water extract for the *in vitro* prebiotic study. All tea extracts at concentrations of 5% and 10% (v/v) showed a positive effect on the bacterial cell growth of both *Lactobacillus acidophilus* and *L. rhamnosus* populations during the exponential and stationary phase, and exerted a survival effect during the death phase. However, all tea extracts at a 10% (v/v) concentration exerted a greater effect than the 5% (v/v) concentrations in maintaining the bacterial cell viability after 72 hours of incubation and onwards. The effect among various teas at the 10% (v/v) concentration were observed to be significantly different after 72 hours of incubation and onwards, with Se-GTE exhibiting the highest count of lactobacilli followed by R-BTE, R-GTE and Se-BTE. This decreasing order of prebiotic potential represents a similar pattern as that previously shown by the TPC values and antioxidant activities of the tea extracts. In conclusion, these findings confirmed that the antioxidant properties and prebiotic potential of the tea extracts are not necessarily dependent on the high level of selenium that the tea contains. This study provides new information on the antioxidant properties and prebiotic function of tea that may provide health benefits beyond its consumption as the most widely consumed beverage.

4.1 Introduction

Tea is an infusion beverage made from the leaves of the plant *Camellia sinensis* and has been consumed worldwide for centuries. Tea is served in a diverse range of varieties, with the most common forms being black, green, oolong and white teas, all categorised according to their method of production (Karori et al., 2007). Tea has a high polyphenol content, with catechins and their polymerised forms such as theaflavins and thearubigins being responsible for most of the health-giving properties. The main tea catechins are (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin (EC) and (-)-epicatechin-3-gallate (ECG), which are found abundantly in green tea. The theaflavins and thearubigins are complex phenolic compounds derived from the enzymatic oxidation of catechins during the fermentation stage of tea production (Ostadalova et al., 2011) and are only present in oolong and black teas. The four major theaflavins identified in black and oolong teas, include theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-gallate. However, the chemical structure of thearubigins is not well-defined and remains unclear (Ho et al., 1997; Wang & Ho, 2009; Bandyopadhyay et al., 2012).

Tea polyphenols have potent antioxidant activity and these antioxidative properties have been described extensively in *in vitro* and *in vivo* studies (Frei & Higdon, 2003; Das et al., 2009; Molan et al., 2009a, 2009b; Lambert & Elias, 2010). Tea polyphenols act as antioxidants by scavenging reactive oxygen and nitrogen species free radicals, as well as chelating redox-active transition metal ions (Weisburger, 1999; Frei & Higdon, 2003; Das et al., 2009). Epidemiological and experimental studies have demonstrated that tea polyphenols may have a role in reducing the risk of oxidative-related diseases such as cancers (Lambert, 2011; Yuan et al., 2011), diabetes (Bansal et al., 2011; Islam, 2011), degenerative diseases (Holzer et al., 2012; Jose & Marco, 2012; Mandel et al., 2012; Oka et al., 2012) and obesity (Grove & Lambert, 2010; Grove et al., 2011). Due to their strong antioxidant activities, tea catechins are utilised in the food industry as a source of natural antioxidants to extend the shelf life of food products and enhance food quality (Brewer et al., 2011; Perumalla & Hettiarachchy, 2011; Vuong et al., 2011b; Carrizo et al., 2015).

There has been increased interest in the antioxidant properties of selenium-rich teas, particularly in response to purported health claims surrounding their use, as well as the potential use of tea extracts as nutraceuticals. Selenium (Se) is an important trace mineral found in meats and seafood, with Se levels in agricultural products solely dependent on soil selenium concentrations and the bioavailability to plants (Cressey et al., 2000; Hu et al., 2001). Importantly, Se is an essential component of selenoenzymes, which are selenium-dependent antioxidant enzymes (e.g. glutathione peroxidase (GSH-Px)) that play an antioxidant role in cellular membrane protection (Rayman, 2000). Tea plants grown in Se-rich soils are high in Se, the tea brewed from these plants is thought to be more antioxidant rich when compared to regular tea, possibly due to a higher Se content (Xu et al., 2003), and may act synergistically with other polyphenolic compounds (Molan et al., 2009a, 2009b). Previous research has shown that Se-enriched tea can significantly enhance the antioxidant activity of tea (Yu et al., 2007; Li et al., 2008, 2009a).

At present, there is growing interest in the prebiotic potential of tea to promote the growth of beneficial gut microbiota such as lactobacilli and bifidobacteria, which are known for their role in exerting health-promoting properties (Gibson & Roberfroid, 1995; Ruxton, 2013). Gut microbiota have complex interactions with their hosts, where a balanced gut microbiota composition not only confers vital benefits for nutrition and metabolism of the non-digestible food substances, but is also crucial for the development of gut functions and the immune system of the host (Di Mauro et al., 2013; Purchiaroni et al., 2013). Some *in vitro* work has demonstrated the promising effect of tea and/or its polyphenols in enhancing beneficial gut microbiota (Duda-Chodak et al., 2008; Tzounis et al., 2008; López de Felipe et al., 2010; Vodnar et al., 2012; Gaudreau et al., 2013). Recent work by Molan et al. (2009a, 2010) demonstrated the prebiotic effect of Se-green tea and China-green tea water extracts in stimulating the growth of lactobacilli and bifidobacteria under *in vitro* and *in vivo* conditions. However, the mechanism by which tea increases the growth of beneficial bacteria remains unclear.

Despite the large amount of literature documenting the antioxidant and prebiotic activity of tea, to the best of our knowledge, there is limited information concerning Se-

rich green tea, and indeed there has been no characterisation of the antioxidant and prebiotic properties of Se-rich black tea. Promising reports on the antioxidant and prebiotic effects of tea, as well as recent work on Se-rich green tea, have led to the hypothesis that Se-rich teas might have greater antioxidant properties and prebiotic activity than regular teas. Therefore, this study will determine the total phenolic content, antioxidant activity and prebiotic effect of Se-rich green and black teas to test this hypothesis.

The aim of the present study was to assess the total phenolic content and antioxidant activities of Se-rich green and black teas in comparison with regular green and black teas, as well as the prebiotic potential of the tea extracts to enhance the growth and viability of beneficial bacteria using pure bacterial cultures under *in vitro* conditions. In addition, the effect of different tea extraction conditions (water temperature and extraction time) on polyphenolic content and antioxidant activities was evaluated. The correlation between the total phenolic content (TPC) and antioxidant properties of the tea extracts was also investigated.

4.2 Materials and methods

4.2.1 Mineral analysis of dry tea leaves

Dry leaves of selenium-rich teas (Se-GTE and Se-BTE) and powdered leaves of regular teas (R-GTE and R-BTE) were ground into fine powders and sent to Hill Laboratories Ltd. (Hamilton, New Zealand) for mineral analysis. The Se, Mn, Zn and Cu concentrations were determined by inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7700 Series, Agilent Technologies, United Kingdom), while Fe, P, K, Ca and Na concentrations were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES, Varian Vista MPX CCD, Agilent Technologies, United Kingdom).

4.2.2 Preparation of tea water extracts

Tea leaves obtained for the present study have been described in **Chapter 3**, section 3.1.3. Tea water extracts (1%, w/v) were prepared using a modified infusion method of that used by Molan et al. (2009a). Briefly, 1 g of each of the powdered tea leaves was infused with 100 mL of Milli-Q (MQ) water at different brewing temperatures (50, 70, 90 and 100 °C) and allowed to brew for different lengths of time (2, 5 and 10 min) with continuous stirring. The infusions were decanted and filtered through Whatman No. 4 filter paper (0.45 mm) to remove insoluble solid matter. For the *in vitro* prebiotic assays, 1% tea extracts were prepared at 90 °C for 5 min and then filter-sterilised using a 0.20 µm syringe filter (Minisart, Sartorius AG, Goettingen, Germany) before use.

4.2.3 Determination of total phenolic content

The total phenolic content (TPC) of each tea sample was quantified using Folin-Ciocalteu's reagent, as described previously by Molan et al. (2009a). Briefly, aliquots of tea extracts (12.5 µL) were mixed with 250 µL of 2% Na₂CO₃ solution in a 96-microwell plate and allowed to sit at room temperature (22 ± 2 °C) for 5 min. Then, 12.5 µL of 50% Folin-Ciocalteu's phenol reagent (diluted 1:1 in MQ water) was added, and the plate was allowed to sit at room temperature for a further 30 min prior to absorbance being read at 650 nm. Absorbance was measured against blank incubations containing MQ water instead of tea extracts. The TPC of the tea extracts was calculated using a calibration curve for gallic acid (µg gallic acid mL⁻¹ vs. nm absorbance), and expressed as mg gallic acid equivalents per gram of tea leaves on a dry basis (mg GAE/g DW). Results were also expressed as mg of TPC estimated to be held in a typical serving of a common tea strength consumed (1%, w/v).

4.2.4 Measurement of antioxidant activities (AOA)

Antioxidant assays are based on different reaction mechanisms, therefore two different methods of antioxidant activity were engaged for a comparison of antioxidant

function measurement in tea extracts, i.e. ferric-reducing antioxidant power (FRAP) and diphenyl-picrylhydrazyl (DPPH) radical scavenging activity assays.

4.2.4.1 Ferric-reducing antioxidant power (FRAP) assay

The AOA of the tea extracts was determined using a modified FRAP assay (Molan et al., 2009a). This assay depends upon the reduction of the colourless ferric tripyridyltriazine (Fe^{3+} -TPTZ) complex to the blue-coloured ferrous tripyridyltriazine (Fe^{2+} -TPTZ) by action of electron donating reductants (antioxidants) at low pH. As previously described in **Chapter 3** (section 3.2.6.5), freshly prepared FRAP reagent (50%) consisted of sodium acetate buffer (pH 3.6; 300 mmol/L), TPTZ (10 mmol/L) in hydrochloric acid (40 mmol/L), FeCl_3 (20 mmol/L), and MQ water. Briefly, aliquots of tea extract (8.5 μL) were added to 275 μL of diluted FRAP reagent in a 96-microwell plate and incubated for 10 min at 37 °C, before the absorbance was read at 595 nm. Absorbance was measured against blank incubations containing MQ water instead of tea extracts, where the absorbance readings of tea samples and standard were corrected for the blank and dilutions before FRAP values were determined. The FRAP values were expressed as mg FeSO_4 equivalents per gram tea leaves on a dry basis (mg FeSO_4 E/g DW), where the calculations were based on a calibration curve of ferrous sulphate ($\mu\text{g FeSO}_4 \cdot 7\text{H}_2\text{O mL}^{-1}$ vs. nm absorbance). The ferric-reducing antioxidant power results are also expressed as mg of FRAP estimated to be held in a typical serving of a common tea made to a typical strength for consumption (1%, w/v).

4.2.4.2 Diphenyl-picrylhydrazyl (DPPH) radical scavenging activity assay

The AOA of the tea extracts was assessed on the basis of the radical scavenging effect of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical activity according to a modification of the method used by Molan et al. (2009a). In the DPPH test, the scavenging of DPPH radicals was measured by the decrease in absorbance of ethanolic DPPH solution at 515 nm in the presence of tea extracts. Twenty five μL of diluted tea extracts (diluted 1:9 in MQ water) were allowed to react with 250 μL of 0.2 mM DPPH in absolute ethanol in a 96-microwell plate, and incubated in the dark at 22 ± 2 °C for 30 min. The absorbance was measured at 550 nm. The absorbance of tea samples was measured against control incubations containing MQ water and corrected for dilution. The DPPH scavenging activity was expressed as mg ascorbic acid (Vitamin

C) equivalent antioxidant capacity per gram dry weight of tea leaves (mg VCEAC/g DW), calculated from the calibration curve of ascorbic acid standard solutions (μg ascorbic acid mL^{-1} vs. nm absorbance). Results are also expressed as mg of DPPH estimated to be held in a typical serving of a common tea strength (1%, w/v).

4.2.5 *In vitro* prebiotic assay using pure cultures of two strains of lactic acid bacteria (LAB)

The growth response of two different strains of lactic acid bacteria (i.e. *Lactobacillus acidophilus* and *L. rhamnosus*) was monitored in the presence of tea water extracts, using a modified method used by Molan et al. (2009b). These LAB strains were grown in Mann-Rogosa-Sharpe (MRS) medium under anaerobic condition to stationary phase for 48 hours. Then, aliquots of these cultures (30 μL) were introduced into tubes of 11.85, 11.37 and 10.77 mL of fresh MRS broth containing different concentrations of filter-sterilised tea water extracts (1%, 5% and 10%; v/v) to obtain a final volume of 12 mL for each tube. The tubes were incubated at 37 °C for 4, 8, 24, 48, 72, 96 and 120 hours. For each incubation time period (8, 24, 48, 72, 96 and 120 hours), the growth of each LAB strain was measured using the plate methods for viable bacterial enumeration. Briefly, the broths from blank and extract-containing incubations were serially diluted (10-fold) in fresh MRS broth. An aliquot of each dilution (100 μL) was spread in duplicate on the surface of the plates containing MRS agar and further incubated for 48 hours at 37 °C in CO_2 incubator for bacterial enumeration of both LAB strains. Controls used in the study included a positive control (MRS media containing 1%, 5% or 10% sterile MQ water + LAB), and a negative control (blank; MRS media containing 1%, 5% or 10% sterile MQ water). Colony populations for each LAB strain were expressed as log colony-forming units per volume of medium (CFUs/mL). The measurement was conducted with duplicate incubations of two independent experiments.

4.2.6 Statistical analysis

The detailed statistical analysis of the data have been described in **Chapter 3**, section 3.1.10. Simple linear regression analysis was performed using Microsoft Excel (2007) to calculate the dose-response relationship (coefficient of determination (R^2)) of standard solutions used for calibration, i.e. ferrous sulphate (FeSO_4), gallic acid and ascorbic acid. One-way analyses of variance (ANOVA) were used to test for significance between group means. Factorial ANOVA was performed to analyse the effect of tea extract, extraction temperature and time on the total phenolic contents. The ANOVA analyses were done using the PROC GLM, a procedure within SAS that analyses data within the framework of general linear models. The ANOVA tests employed Tukey's post-hoc analysis for pair-wise multiple comparisons of the group means. Pearson correlation coefficients (R) between assays were performed using the PROC CORR procedure to determine the correlation between phenolic contents and antioxidant activity assays. A difference was considered statistically significant when $p < 0.05$.

4.3 Results

4.3.1 Mineral composition of dry tea leaves

The analysed mineral composition of the tea leaves is shown in **Table 4-1**. The total selenium concentration of Se-GTE (1.40 mg of Se/kg) and Se-BTE dry leaves (1.42 mg of Se/kg) was higher than R-GTE (0.05 mg of Se/kg), and R-BTE (0.08 mg of Se/kg). Se-GTE contained higher concentrations of copper, zinc, iron, manganese, phosphorus and potassium than R-GTE, but R-GTE contained more calcium than Se-GTE. Whereas, Se-BTE contained higher concentrations of copper, zinc, iron, calcium and potassium than R-BTE, but R-BTE contained more manganese than Se-BTE. By using the data for the total selenium content of Se-GTE and Se-BTE, we estimated that a cup of brewed Se-GTE may provide 2.8 μg Se, while a cup of brewed Se-BTE may provide 2.84 μg Se; assuming that a cup of tea equates to 200 mL of a 1% tea infusion (w/v; 2 g tea in 200 mL water). Thus, in relation to the Recommended Dietary

Allowance for daily selenium intake by an adult and a child, we found that approximately ~20 cups of tea/day per adult (aged over 14 years) and ~9 cups of tea/day per child (aged 1 to 3 years) would be able to fulfil the RDA of 55 µg Se/day/adult and 20 µg Se/day/child (USA Institute of Medicine, 2000).

Table 4-1. Mineral composition of Se-rich green and black teas in comparison to regular green and black teas.

Mineral	Unit	Concentration			
		Se-GTE	R-GTE	Se-BTE	R-BTE
Selenium	mg/kg	1.40	0.05	1.42	0.08
Copper	mg/kg	25.5	21.0	48.0	22.0
Zinc	mg/kg	49.1	42.0	67.0	45.0
Iron	mg/kg	407.0	120.0	163.0	146.0
Manganese	mg/kg	1,159.0	950.0	620.0	1,350.0
Calcium	g/100 g	0.33	0.42	0.60	0.57
Phosphorus	g/100 g	0.46	0.28	0.31	0.31
Potassium	g/100 g	1.78	1.60	1.90	1.70
Sodium	g/100 g	< 0.02	< 0.01	< 0.01	< 0.01

4.3.2 Total phenolic content (TPC) of Se-rich teas and regular teas

The TPC of different tea extracts (**Figure 4-1**) were calculated from the regression equation of the gallic acid calibration curve ($y = 0.0024x + 0.0042$, $R^2 = 0.9922$, as shown in **Appendix III**) and expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). The results showed that TPC of the tested teas varied significantly ($p < 0.05$) ranging from 34.7 to 116.9 mg GAE/g dry weight. The Se-GTE showed the highest TPC (106.3 ± 0.4 to 116.9 ± 0.6 mg GAE/g DW) followed by R-BTE (57.6 ± 0.3 to 82.2 ± 0.4 mg GAE/g DW), R-GTE (35.3 ± 0.4 to 57.2 ± 0.3 mg GAE/g DW), and then Se-BTE (34.7 ± 0.6 to 44.1 ± 0.2 mg GAE/g DW). The TPC values of Se-GTE were significantly higher ($p < 0.05$) than that of the R-GTE, whereas the R-BTE showed significantly ($p < 0.05$) higher TPC values than Se-BTE. The TPC of tea extracts in one cup servings are presented in **Tables 4-2** and **4-3**.

4.3.3 Antioxidant activities (AOA) of the tea extracts

The FRAP values of different tea extracts (**Figure 4-2**) were determined from the regression equation of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ calibration curve ($y = 0.0035x + 0.2305$, $R^2 = 0.9999$, as shown in **Appendix III**) and expressed as mg FeSO_4 E/g DW, whereas the DPPH values were evaluated from the regression equation of the ascorbic acid calibration curve ($y = -0.0039x + 1.2358$, $R^2 = 0.9750$, as shown in **Appendix III**).

The FRAP of the tea extracts varied significantly between different teas. The Se-GTE showed the highest ($p < 0.05$) antioxidant power (137.6 ± 0.3 to 147.1 ± 0.3 mg FeSO_4 E/g DW), followed by R-BTE (86.8 ± 0.2 to 139.7 ± 0.3 mg FeSO_4 E/g DW), R-GTE (76.2 ± 0.6 to 127.5 ± 0.4 mg FeSO_4 E/g DW), and Se-BTE (44.1 ± 0.2 to 67.0 ± 0.4 mg FeSO_4 E/g DW).

For the DPPH-radical scavenging abilities (**Figure 4-3**), Se-GTE also showed the highest values ($p < 0.05$) ranging from 298.6 ± 4.1 to 306.6 ± 4.5 mg VCEAC/g DW, followed by R-BTE (129.8 ± 2.4 to 192.0 ± 4.2 mg VCEAC/g DW), R-GTE (100.5 ± 2.6 to 129.6 ± 2.5 mg VCEAC/g DW) and Se-BTE (88.1 ± 1.2 to 113.0 ± 1.7 mg VCEAC/g DW). The FRAP and DPPH estimated amounts of tea extracts in one cup servings are presented in **Tables 4-2** and **4-3**.

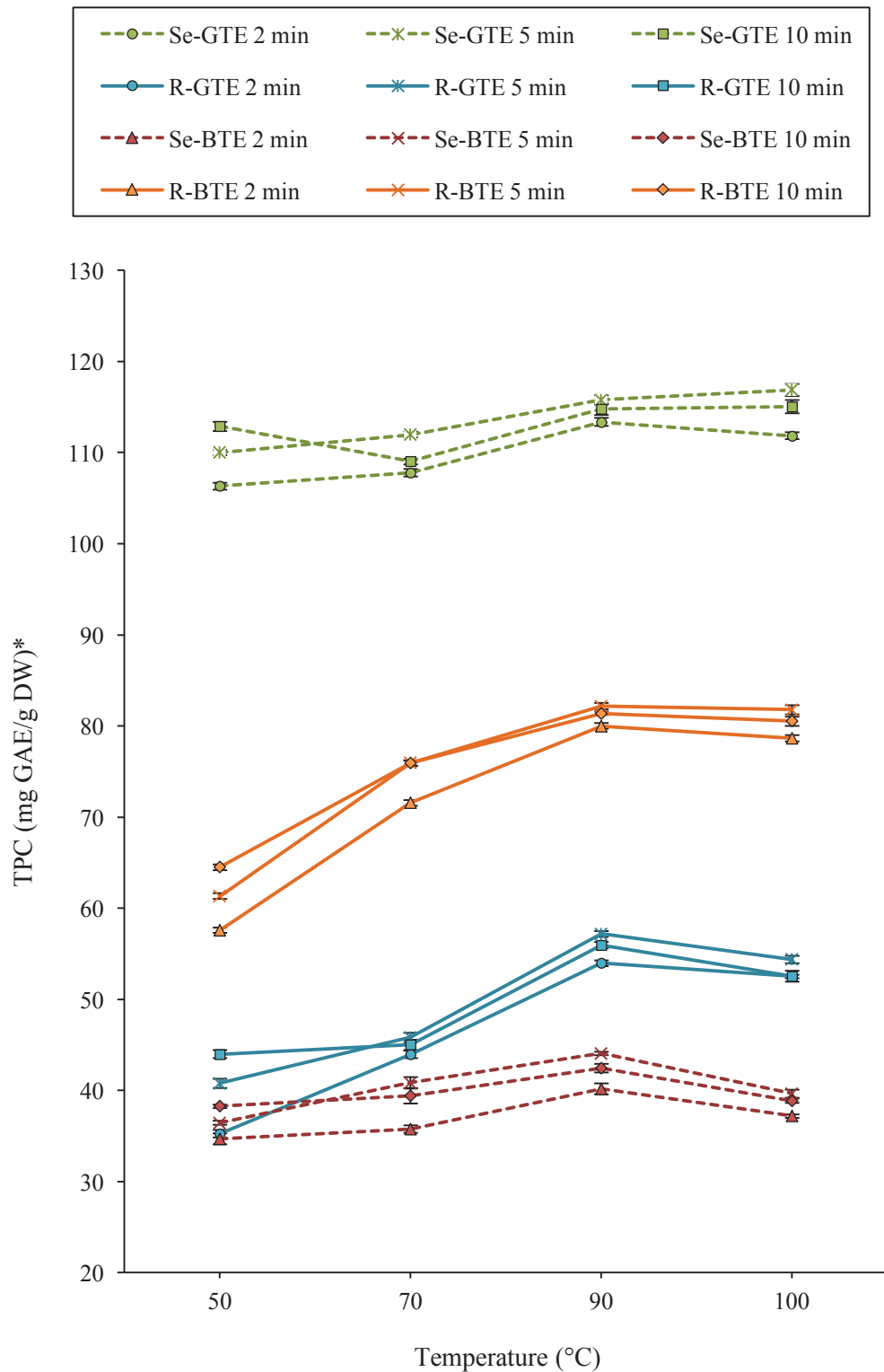


Figure 4-1. Total phenolic content (TPC) of Se-GTE, R-GTE, Se-BTE and R-BTE, steeped at different temperatures (50, 70, 90 and 100 °C) and for different times (2, 5 and 10 min). Data are mean \pm SEM. *Total phenolic content (mg gallic acid equivalent/g dry weight).

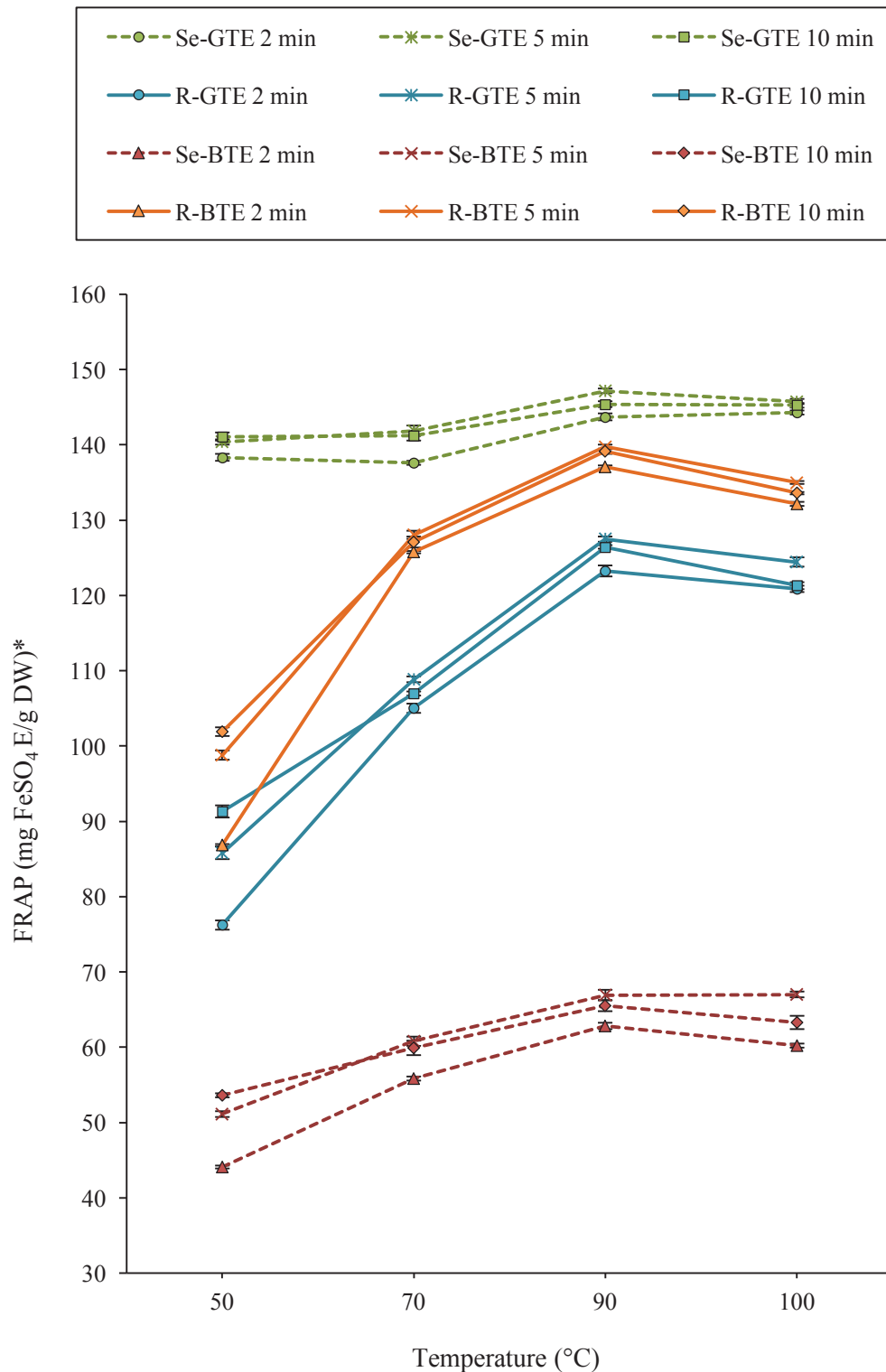


Figure 4-2. Ferric-reducing antioxidant power (FRAP) values of Se-GTE, R-GTE, Se-BTE and R-BTE steeped at different temperatures (50, 70, 90 and 100 °C) and for different times (2, 5 and 10 min). Data are mean ± SEM. *FRAP (mg FeSO₄ equivalent/g dry weight).

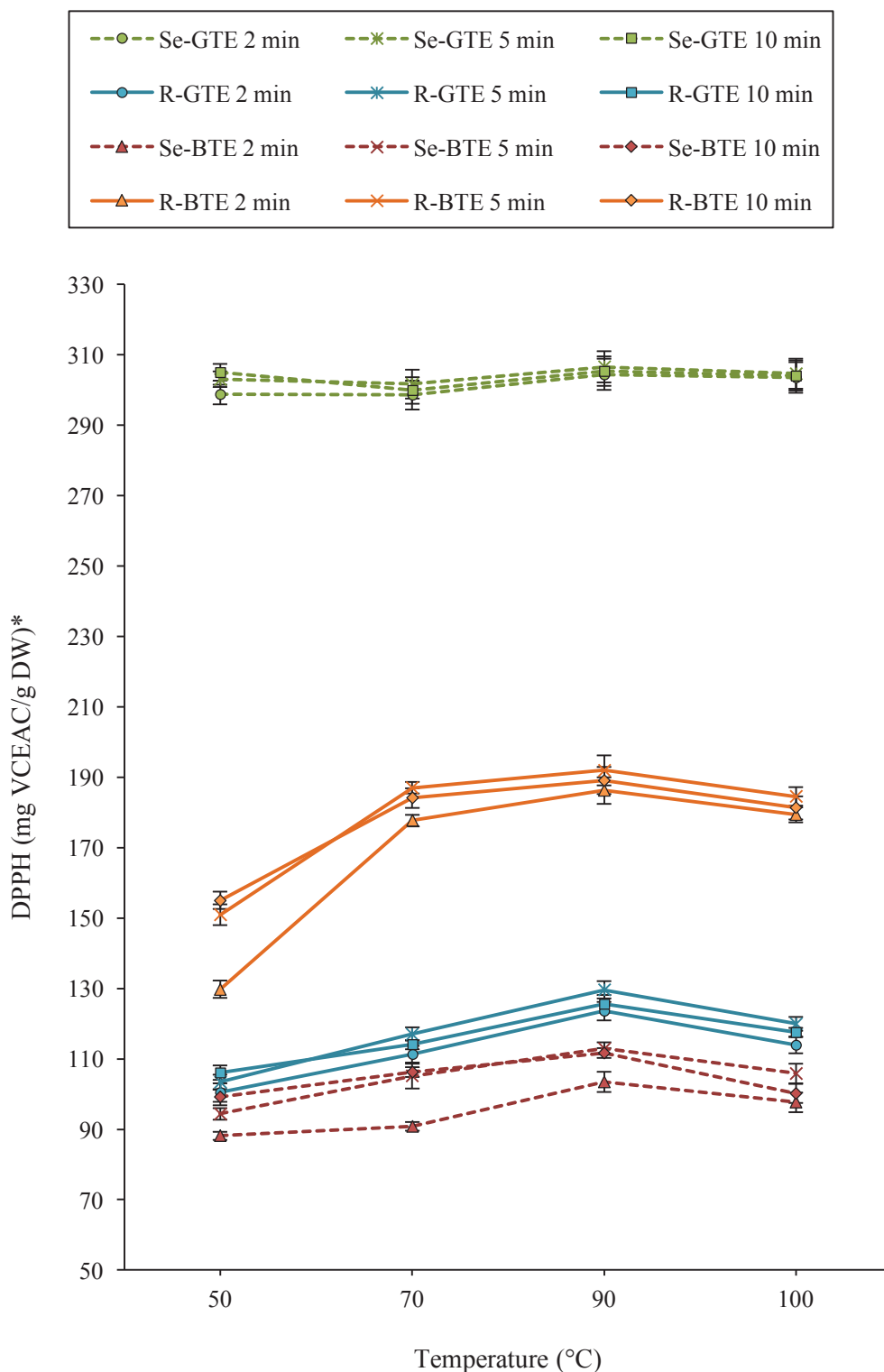


Figure 4-3. DPPH scavenging activities of Se-GTE, R-GTE, Se-BTE and R-BTE steeped at different temperatures (50, 70, 90 and 100 °C) and for different times (2, 5 and 10 min). Data are mean \pm SEM. *1,1-diphenyl-2-picrylhydrazyl radical scavenging activity (mg vitamin C equivalent antioxidant capacity/g dry weight).

Table 4-2. The estimated amounts of total phenolic content (TPC), ferric-reducing antioxidant power (FRAP), and DPPH scavenging activities of Se-GTE and R-GTE, per cup serving (2 g/serving)*. One serving is 200 mL of a 1% (w/v) tea infusion.

Tea	Temperature (°C)	Time (min)	TPC ^A estimation in 1 cup of tea	FRAP ^B estimation in 1 cup of tea	DPPH ^C estimation in 1 cup of tea
Se-GTE	100	2	223.7 ± 1.1 ^{cd}	288.4 ± 0.6 ^b	607.1 ± 17.3 ^{abc}
		5	233.7 ± 2.3 ^a	291.4 ± 1.1 ^{ab}	609.3 ± 17.2 ^{ab}
		10	230.1 ± 2.5 ^{ab}	290.6 ± 0.9 ^{ab}	608.2 ± 16.9 ^{ab}
	90	2	226.7 ± 1.6 ^{bc}	287.4 ± 1.5 ^{bc}	608.9 ± 17.7 ^{ab}
		5	231.6 ± 1.7 ^a	294.3 ± 0.4 ^a	613.2 ± 17.9 ^a
		10	229.5 ± 1.8 ^{ab}	290.8 ± 1.0 ^{ab}	610.8 ± 16.5 ^{ab}
	70	2	215.6 ± 1.4 ^{ef}	275.2 ± 0.8 ^f	597.2 ± 16.2 ^{de}
		5	223.9 ± 0.5 ^{cd}	283.7 ± 2.7 ^{cd}	603.4 ± 16.6 ^{bcd}
		10	218.1 ± 1.2 ^e	282.4 ± 2.3 ^d	599.8 ± 15.1 ^{cd}
	50	2	212.7 ± 1.2 ^f	276.6 ± 1.8 ^{ef}	597.4 ± 10.7 ^f
5		220.0 ± 0.6 ^{de}	280.7 ± 0.3 ^{de}	606.1 ± 8.5 ^e	
10		225.8 ± 2.0 ^{bc}	282.2 ± 1.5 ^d	610.0 ± 9.1 ^{de}	
R-GTE	100	2	105.1 ± 2.3 ^c	241.7 ± 1.3 ^d	227.8 ± 8.6 ^{cde}
		5	108.8 ± 1.6 ^{bc}	248.8 ± 2.3 ^{bc}	240.0 ± 6.9 ^{abcd}
		10	105.0 ± 0.3 ^c	242.6 ± 1.4 ^d	235.1 ± 4.4 ^{bcd}
	90	2	107.9 ± 1.1 ^{bc}	246.5 ± 2.8 ^{cd}	247.1 ± 10.3 ^{abc}
		5	114.4 ± 0.3 ^a	255.0 ± 1.0 ^a	259.2 ± 9.8 ^a
		10	111.9 ± 1.1 ^{ab}	252.8 ± 0.4 ^{ab}	251.1 ± 10.0 ^{ab}
	70	2	88.0 ± 1.5 ^d	210.1 ± 2.1 ^f	222.6 ± 7.5 ^{def}
		5	91.7 ± 1.9 ^d	217.7 ± 1.2 ^e	234.1 ± 6.5 ^{bcd}
		10	90.0 ± 2.1 ^d	213.9 ± 0.6 ^{ef}	228.1 ± 4.5 ^{cde}
	50	2	70.5 ± 0.9 ^f	152.4 ± 1.8 ⁱ	201.0 ± 10.3 ^g
5		81.6 ± 1.5 ^e	171.6 ± 3.2 ^h	206.9 ± 7.8 ^{fg}	
10		88.0 ± 0.6 ^d	182.6 ± 3.0 ^e	212.3 ± 8.0 ^{efg}	

*Values are mean ± SEM. ^ATotal phenolic content (mg gallic acid equivalent/g dry weight). ^BFerric-reducing antioxidant power (mg FeSO₄ equivalent/g dry weight). ^CDiphenyl-1-picrylhydrazyl radical scavenging activity (mg vitamin C equivalent antioxidant capacity/g dry weight). ^{a-i}Values with different letters in each group of tea are significantly different at $p < 0.05$.

Table 4-3. The estimated amounts of total phenolic content (TPC), ferric-reducing antioxidant power (FRAP), and DPPH scavenging activities of Se-BTE and R-BTE, per cup serving (2 g/serving)*. One serving is 200 mL of a 1% (w/v) tea infusion.

Tea	Temperature (°C)	Time (min)	TPC ^A estimation in 1 cup of tea	FRAP ^B estimation in 1 cup of tea	DPPH ^C estimation in 1 cup of tea
Se-BTE	100	2	74.4 ± 0.5 ^{efg}	120.5 ± 0.6 ^{de}	195.3 ± 11.0 ^{bcde}
		5	79.3 ± 1.1 ^{cd}	134.1 ± 1.5 ^a	211.7 ± 10.4 ^{ab}
		10	77.7 ± 0.8 ^{cde}	126.5 ± 3.4 ^{bc}	200.4 ± 10.7 ^{bed}
	90	2	80.3 ± 1.7 ^{cd}	125.6 ± 1.3 ^{cd}	206.9 ± 11.2 ^{abc}
		5	88.1 ± 0.6 ^a	133.9 ± 2.4 ^a	225.9 ± 6.4 ^a
		10	84.9 ± 1.7 ^{ab}	131.0 ± 2.8 ^{ab}	223.3 ± 5.3 ^a
	70	2	71.5 ± 0.9 ^{gh}	111.6 ± 0.5 ^f	181.7 ± 4.1 ^{de}
		5	81.7 ± 2.1 ^{bc}	121.6 ± 2.3 ^{cde}	210.3 ± 14.0 ^{abc}
		10	78.9 ± 3.2 ^{cd}	119.8 ± 3.4 ^e	212.4 ± 5.1 ^{ab}
50	2	69.3 ± 2.0 ^h	88.2 ± 0.1 ^h	176.3 ± 2.9 ^e	
	5	72.9 ± 0.2 ^{fgh}	102.2 ± 0.9 ^g	188.8 ± 5.6 ^{cde}	
	10	76.5 ± 0.4 ^{def}	107.2 ± 0.7 ^{fg}	198.2 ± 8.3 ^{bcd}	
R-BTE	100	2	157.3 ± 0.7 ^d	264.3 ± 1.0 ^d	358.6 ± 8.2 ^{ab}
		5	163.6 ± 1.8 ^{ab}	270.0 ± 0.5 ^c	369.0 ± 9.9 ^{ab}
		10	161.0 ± 1.8 ^{bc}	267.2 ± 0.2 ^{cd}	362.6 ± 12.4 ^{ab}
	90	2	160.0 ± 1.0 ^{cd}	274.1 ± 0.2 ^b	372.4 ± 14.7 ^{ab}
		5	164.4 ± 1.1 ^a	279.5 ± 0.9 ^a	384.0 ± 16.8 ^a
		10	162.7 ± 0.6 ^{abc}	278.3 ± 0.7 ^a	378.3 ± 14.5 ^{ab}
	70	2	143.1 ± 0.5 ^f	251.5 ± 0.2 ^f	355.5 ± 6.4 ^b
		5	151.9 ± 0.8 ^e	256.0 ± 1.8 ^e	374.0 ± 6.4 ^{ab}
		10	151.9 ± 1.1 ^e	254.2 ± 2.9 ^{ef}	368.3 ± 9.0 ^{ab}
50	2	115.2 ± 1.0 ⁱ	173.7 ± 0.3 ⁱ	259.5 ± 9.1 ^d	
	5	122.7 ± 0.7 ^h	197.5 ± 2.4 ^h	301.7 ± 10.4 ^c	
	10	129.0 ± 0.5 ^g	203.9 ± 2.1 ^g	310.0 ± 8.7 ^c	

*Values are mean ± SEM. ^ATotal phenolic content (mg gallic acid equivalent/g dry weight). ^BFerric-reducing antioxidant power (mg FeSO₄ equivalent/g dry weight). ^CDiphenyl-1-picrylhydrazyl radical scavenging activity (mg vitamin C equivalent antioxidant capacity/g dry weight). ^{a-i}Values with different letters in each group of tea are significantly different at $p < 0.05$.

4.3.4 Effect of extraction temperature and time on TPC of the tea extracts

Table 4-4 shows the factorial ANOVA summary for the main effects (tea, temperature, time) and the interaction effects on TPC. It was confirmed that the extraction efficiency of polyphenols is significantly affected by tea type ($F_{3,384} = 67873.2, p < 0.0001$), extraction temperature ($F_{3,384} = 1716.3, p < 0.0001$) and extraction time ($F_{2,384} = 300.0, p < 0.0001$). The interaction effects of tea \times temperature ($F_{9,384} = 232.1$), temperature \times time ($F_{6,384} = 33.3$) and tea \times temperature \times time ($F_{18,384} = 4.96$), were also significant ($p < 0.0001$).

Table 4-4. Analysis of variance for the effect of tea variety, extraction temperature, extraction time and their interactions on total phenolic content by Folin-Ciocalteu’s phenol reagents.

Source of variance	DF ^a	Total polyphenol	
		MS ^b	F value
Tea	3	115,605.9	67,873.2*
Temperature	3	2,923.4	1,716.3*
Time	2	511.0	300.0*
Tea \times Temperature	9	395.3	232.1*
Tea \times Time	6	2.5	1.47
Temperature \times Time	6	56.7	33.3*
Tea \times Temperature \times Time	18	8.45	4.96*
Error	384	1.7	

^aDegree of freedom; ^bMeans squares; * $p < 0.0001$.

4.3.5 Correlation between TPC and AOA of the tea extracts

The correlation coefficient (R) values of both black teas and green teas are shown in **Table 4-5** and its strength was referred to guidelines provided by Jackson (2009). A weak to strong positive relationship was found between the total phenolic content and antioxidant activities (FRAP and DPPH).

Table 4-5. Correlation coefficients between total phenolic content (TPC) and antioxidant properties of water extracts from Se-rich green and black teas and regular teas. Antioxidant activity was measured by the ferric-reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays.

Sample	Correlation coefficients (R)		
	TPC vs FRAP	TPC vs DPPH	FRAP vs DPPH
Se-GTE	0.7611*	0.3187*	0.2367*
R-GTE	0.9498*	0.7496*	0.7444*
Se-BTE	0.7173*	0.6984*	0.5761*
R-BTE	0.9772*	0.8705*	0.8877*

*indicates significance, $p < 0.05$.

4.3.6 Effect of tea extracts on growth of *Lactobacillus acidophilus* NZRM 52 under *in vitro* conditions

For the *in vitro* prebiotic assay, tea leaves were extracted using the optimal extraction condition (90 °C for 5 min) as reported in section 4.3.3. The addition of 1% (v/v) concentration of the tea extracts (10 mg/mL) to the MRS broth with *L. acidophilus* showed no difference in the numbers of the bacterial cells after 8, 24 and 48 hours of incubation (**Figure 4-4**). There was slight but significant increases in the numbers of bacteria in the tea-containing incubations after 72 ($p = 0.0008$), 96 ($p < 0.0001$) and 120 hours ($p < 0.0001$), as opposed to the control incubation (MRS media + *L. acidophilus* + 1% sterile MQ water). However, the effect of 1% tea extracts on *L. acidophilus* at 72 hours of incubation showed no difference among the teas following post-hoc Tukey's test. Interestingly, Se-GTE showed the highest counts of *L. acidophilus*, followed by R-BTE, R-GTE and Se-BTE after 96 and 120 hours of incubation.

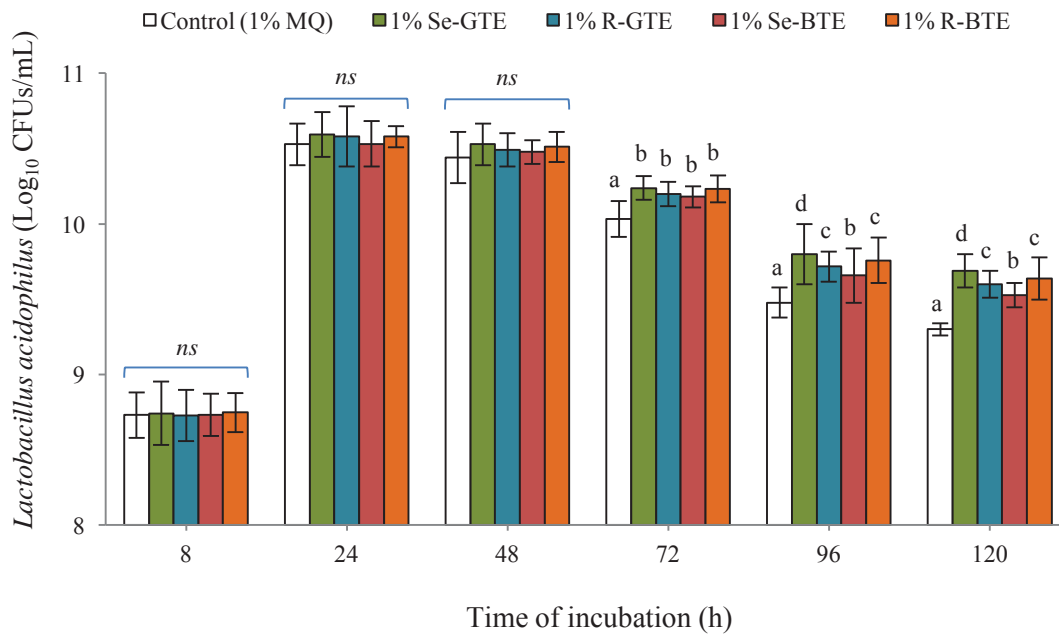


Figure 4-4. Effect of different tea water extracts at 1% concentration (v/v) on *Lactobacillus acidophilus* NZRM 52 grown anaerobically (37 °C) for 8, 24, 48, 72, 96 and 120 hours in MRS broth. Data are mean ± SEM. Different letters indicate significant differences between groups within the same period of incubation.

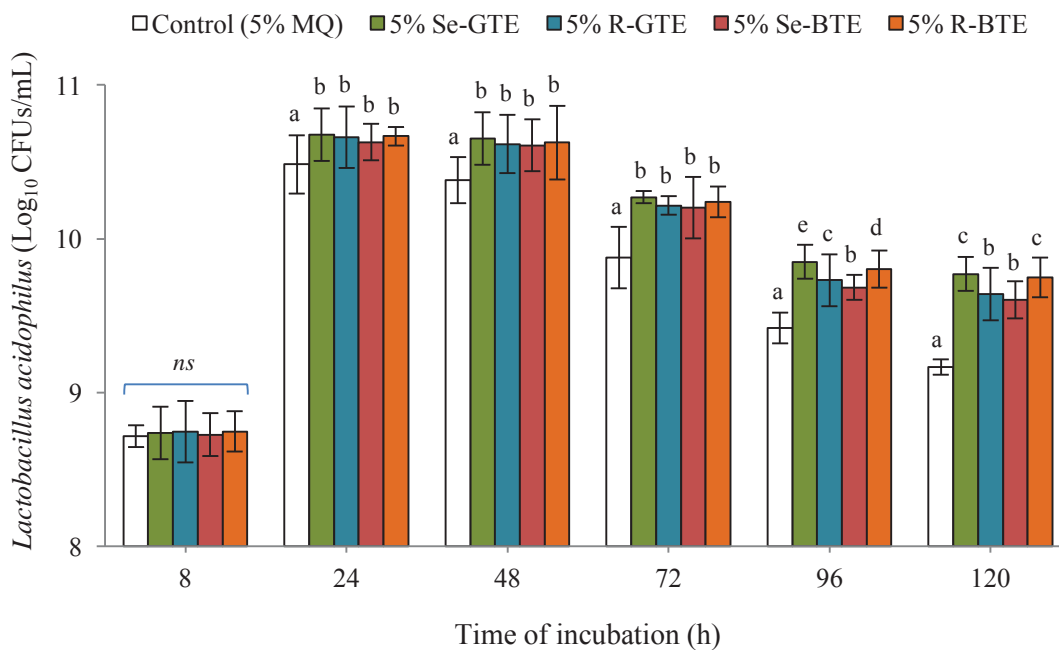


Figure 4-5. Effect of different tea water extracts at 5% concentration (v/v) on *Lactobacillus acidophilus* NZRM 52 grown anaerobically (37 °C) for 8, 24, 48, 72, 96 and 120 hours in MRS broth. Data are mean ± SEM. Different letters indicate significant differences between groups within the same period of incubation.

The addition of a 5% concentration of the tea extracts had no influence on the bacterial cells after an eight hour incubation, but was significant after 24 ($p < 0.0001$), 48 ($p < 0.0001$), 72 ($p < 0.0001$), 96 ($p < 0.0001$) and 120 ($p < 0.0001$) hours (**Figure 4-5**), the increments in time were also significantly different. The effects of 5% tea extracts on *L. acidophilus* after 24, 48 and 72 hours of incubation were significant in comparison to the control incubation (MRS media + *L. acidophilus* + 5% sterile MQ water). However, the effect of 5% tea extracts showed no difference among various teas after post-hoc Tukey's test. In addition, Se-GTE showed the highest counts of *L. acidophilus*, followed by R-BTE, R-GTE and Se-BTE after 96 and 120 hours of incubation with the tea extracts at 5% concentration. As seen in **Figure 4-6**, the effect of the tea extracts at 10% concentration on *L. acidophilus* was significant after 8 ($p = 0.0005$), 24 ($p < 0.0001$), 48 ($p < 0.0001$), 72 ($p < 0.0001$), 96 ($p < 0.0001$) and 120 ($p < 0.0001$) hours of incubation when compared to the control incubation (MRS media + *L. acidophilus* + 10% sterile MQ water). However, the effect of 10% tea extracts on *L. acidophilus* at 8, 24 and 48 hours of incubation showed no difference among the teas following post-hoc Tukey's test. Again, Se-GTE showed the highest counts of *L. acidophilus*, followed by R-BTE, R-GTE and Se-BTE after 72, 96 and 120 hours of incubation.

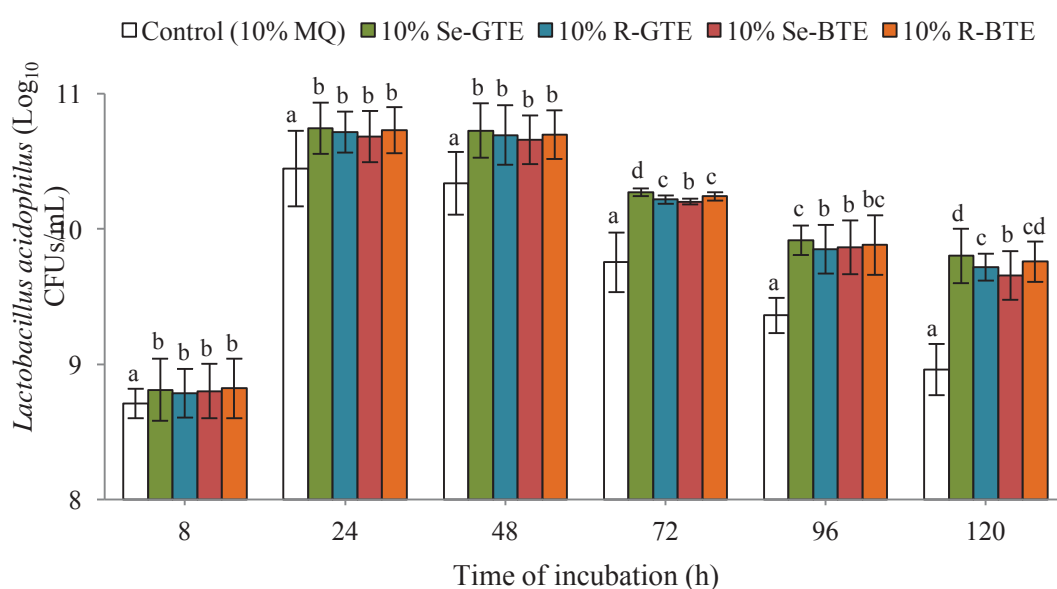


Figure 4-6. Effect of different tea water extracts at 10% concentration (v/v) on *Lactobacillus acidophilus* NZRM 52 grown anaerobically (37 °C) for 8, 24, 48, 72, 96 and 120 hours in MRS broth. Data are mean ± SEM. Different letters indicate significant differences between groups within the same period of incubation.

4.3.7 Effect of tea extracts on growth of *Lactobacillus rhamnosus* NZRM 299^T under *in vitro* conditions

The tea extracts at 1% concentration showed no difference in the numbers of the *L. rhamnosus* bacterial cells after 8, 24, 48, 72, 96 and 120 hours of incubation (**Figure 4-7**). The addition of tea extracts at 5% concentration to the MRS broth containing *L. rhamnosus* resulted in a significant difference in the numbers of bacterial cells after 8 ($p < 0.0001$), 24 ($p = 0.0058$), 48 ($p = 0.0065$), 72 ($p < 0.0001$), 96 ($p < 0.0001$) and 120 ($p < 0.0001$) hours of incubation (**Figure 4-8**). Although the effect of 5% tea extracts on *L. rhamnosus* differed significantly from the control incubation (MRS media + *L. rhamnosus* + 5% sterile MQ water) in all incubation periods, no differences were observed among the teas following post-hoc Tukey's test.

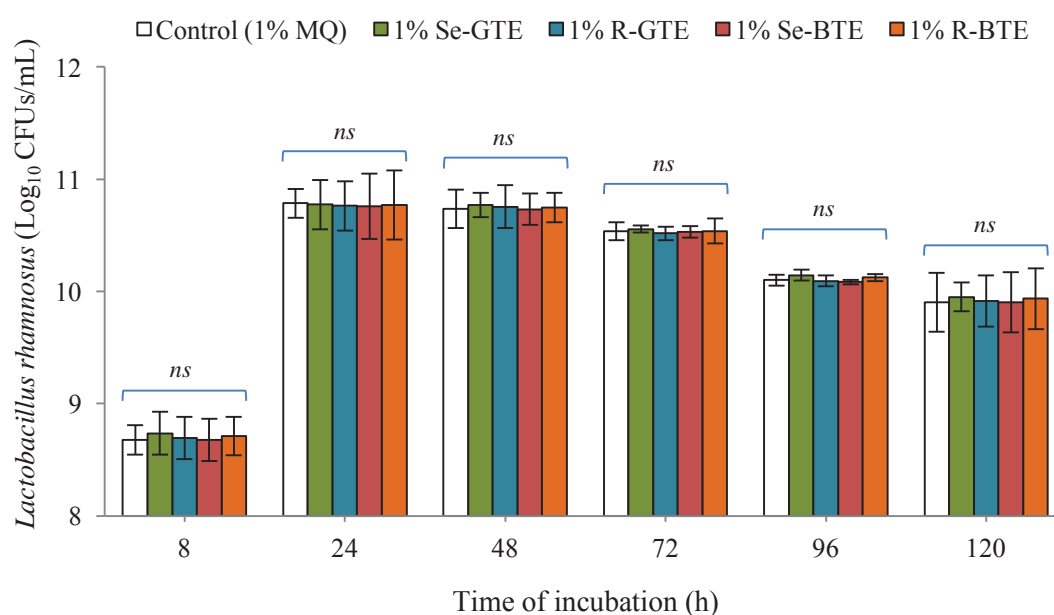


Figure 4-7. Effect of different tea water extracts at 1% concentration (v/v) on *Lactobacillus rhamnosus* NZRM 299^T grown anaerobically (37 °C) for 8, 24, 48, 72, 96 and 120 hours in MRS broth. Data are mean ± SEM. Different letters indicate significant differences between groups within the same period of incubation.

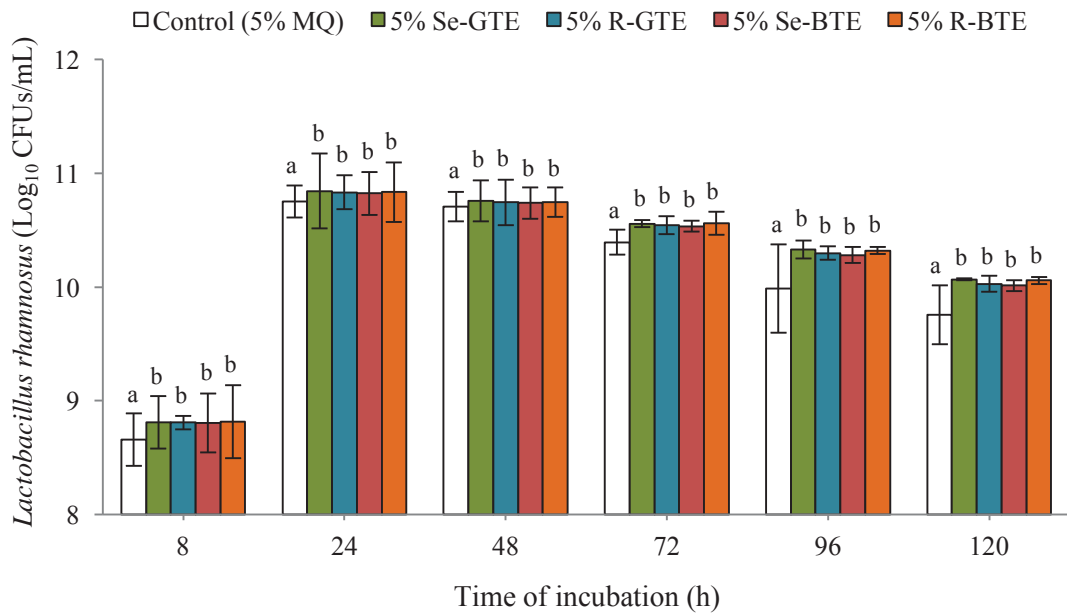


Figure 4-8. Effect of different tea water extracts at 5% concentration (v/v) on *Lactobacillus rhamnosus* NZRM 299^T grown anaerobically (37 °C) for 8, 24, 48, 72, 96 and 120 hours in MRS broth. Data are mean ± SEM. Different letters indicate significant differences between groups within the same period of incubation.

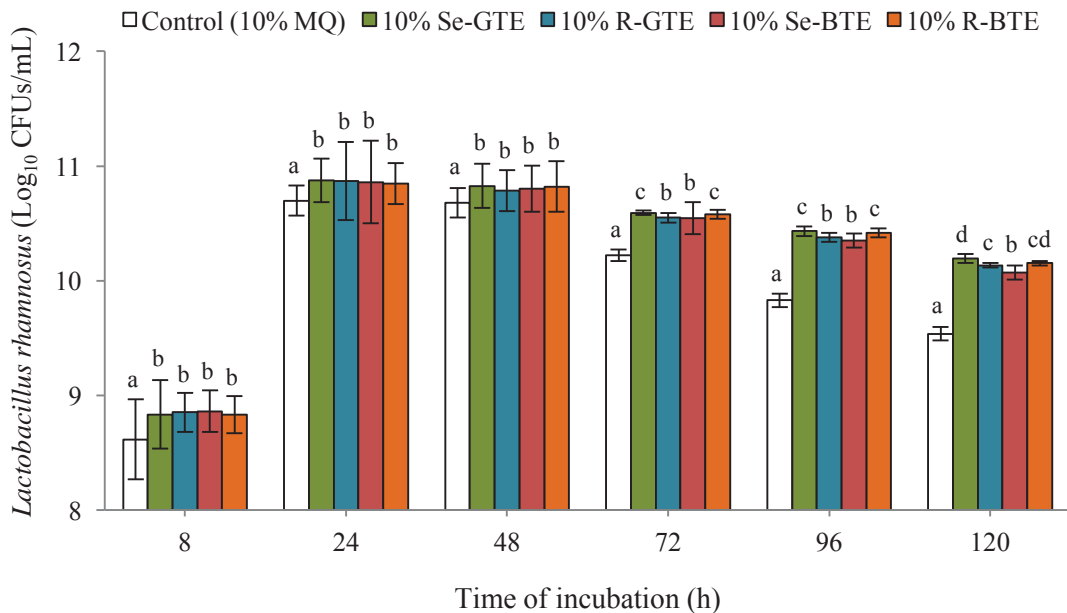


Figure 4-9. Effect of different tea water extracts at 10% concentration (v/v) on *Lactobacillus rhamnosus* NZRM 299^T grown anaerobically (37 °C) for 8, 24, 48, 72, 96 and 120 hours in MRS broth. Data are mean ± SEM. Different letters indicate significant differences between groups within the same period of incubation.

As seen in **Figure 4-9**, the tea extracts at 10% concentration significantly increased the numbers of *L. rhamnosus* after 8, 24, 48, 72, 96 and 120 hours of incubation, as opposed to the control incubation (MRS media + *L. rhamnosus* + 10% sterile MQ water) (p values of 0.0001 for all incubation periods). However, the effect of 10% tea extracts on *L. rhamnosus* at 8, 24 and 48 hours of incubation showed no difference among the teas following post-hoc Tukey's test. In fact, after 72, 96 and 120 hours of incubation, Se-GTE showed the highest counts of *L. rhamnosus* followed by R-BTE, R-BTE and Se-BTE.

4.4 Discussion

4.4.1 Total phenolic content (TPC) of various tea extracts

Phenolic compounds have drawn the attention of researchers because of their significant antioxidant activities (Molan et al., 2009a, 2009b; Cartea et al., 2011). They have an important role in stabilising lipid oxidation, scavenging reactive oxygen species and free radicals, breaking radical chain reaction and chelating metals (Chang et al., 2007; Jayathilakan et al., 2007). In the present study, the Folin-Ciocalteu method was used to determine the content of phenolic compounds in the tested tea extracts. This method is based on the oxidation/reduction reaction due to the redox properties of phenolic groups with phosphomolybdic and phosphotungstic acids (Verzelloni et al., 2007). When the tea extracts react with Folin-Ciocalteu reagent and sodium carbonate, a blue-colored phosphomolybdic – phosphotungstic – phenol complex is formed, which indicates the presence of phenolic compounds in the extracts and enhances the measurement of phenolic concentration (Prior et al., 2005).

Green tea has previously been reported to have greater amount of TPC than black tea due to its high catechin content (Graham, 1992; Astill et al., 2001; Novotny & Baer, 2013); this is in accordance with our results where Se-GTE and R-GTE are higher in TPC than Se-BTE. However, the results of TPC also found that R-BTE unexpectedly had higher TPC compared to R-GTE. While this may seem contrary to the previous statement where green tea is higher in TPC than black tea, our explanation is that this

finding may be due to differences in polyphenol profile of different varieties of teas used for production of the black teas. Generally, all teas are derived from the *Camellia* plant (*Camellia sinensis*), which has two sub-varieties: *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica* (Sealy, 1958). The *sinensis* variety originates from China, while *assamica* originates mainly from India (Chan et al., 2007; De la Paix et al., 2010). In this study, the Dilmah brand R-BTE was obtained from Sri Lanka, where *C. sinensis* var. *assamica* is mostly cultivated and have been used in black tea production (Harbowy & Balentine, 1997; Anesini et al., 2008). This tea cultivar is known to have higher polyphenols than the *C. sinensis* var. *sinensis*, a variety of tea that is mostly cultivated in China and used in production of various types of tea (Takeda, 1994; Chu, 1997; Harbowy & Balentine, 1997; Astill et al., 2001; Karori et al., 2007; Kaur et al., 2014); including production of R-GTE and Se-BTE used in this study. Furthermore, according to Karori et al. (2007), the green, black and white teas processed from Kenyan tea cultivars (originally used for black tea production) had shown significantly higher antioxidant activity than green tea processed from tea cultivars originated from Japan and China. Their results clearly suggest that the cultivar type plays an essential role in determination of antioxidant ability of the tea product due to phenolic contents, and that the black teas processed from suitable cultivars could be more potent in antioxidant activity than green teas.

Therefore, the cultivar type may be the main reason behind the significant variation in TPC found in the tested teas, and consequently influences the antioxidant activity of these teas; a finding that has been confirmed by others (Wachira & Kamuya, 2005; Katalinic et al., 2006; Anesini et al., 2008; Gulati et al., 2009) as reviewed by Wachira et al. (2013). Other contributing factors behind the TPC variation of teas may be differences in the age of tea leaf (Lin et al., 2003; Song et al., 2012; Lee et al., 2014), degree of fermentation during tea manufacturing (Lin et al., 1998; Kosińska & Andlauer, 2014), climate and region of production (Shishikura & Khokhar, 2005; Owuor et al., 2008, 2010), particle size (Astill et al., 2001), genetic diversity between tea varieties (Owuor & Obanda, 1995; Magoma et al., 2000), fermentation conditions of tea processing (Obanda et al., 2001), geographical areas the teas were sourced from (Bhattacharya & Sen-Mandi, 2011; Jayasekera et al., 2011), environmental conditions and the agricultural practices (Song et al., 2012; Kaur et al., 2014), as well as

differences in the harvesting season (Erkturk et al., 2010; Jayasekera et al., 2011, 2014; Liu et al., 2015a).

Herein, our findings primarily emphasise that one should not generalise that all teas of similar variety or of similar type of final tea product have comparable biochemical substances, since differences in biochemistry profile may be significant even within a particular type of tea. In fact, there are other contributing factors that greatly influence the chemical composition of tea, right from the freshly harvested leaf of tea to its final infusions.

4.4.2 Effects of different extraction conditions on TPC and AOA

The results of the present study show that TPC varies for a given tea depending on the temperature of the water and infusion time, where TPC was found to increase with increasing extraction temperature and time, as previously reported (Liebert et al., 1999; Shishikura & Khokhar, 2005; Molan et al., 2009b; Jayasekera et al., 2011). TPC increased significantly ($p < 0.05$) as extraction time increased from 2 to 5 min at all temperatures. However, after 10 min of infusion with extraction temperatures higher than 50 °C, TPC decreased slightly but not significantly. Dai and Mumper (2010) reported that the use of high water temperatures for tea infusion increased TPC extraction, as well as reducing viscosity and surface tension. Indeed, the use of high water temperature has also been confirmed as one of the six factors that had an impact on the yield of catechins extracted from green tea (Vuong et al., 2011a), as previously explained by Vuong et al. (2010, 2011b). Therefore, the extraction of total phenolics, total flavonoids, catechins, and theaflavins from tea using water at boiling point are relatively inefficient at infusion times less than 2 min (Lakenbrink et al., 2000). However, TPC extraction using high temperatures at longer times may lead to the loss of thermally unstable polyphenolic components in the tea extracts, as the result of thermal degradation, oxidation, epimerisation, and polymerisation of phenolics (Kim et al., 2007; Dai & Mumper, 2010; Sharma et al., 2010; Vuong et al., 2011a; Samaniego-Sánchez et al., 2011). This finding supports the results of previous studies conducted by Su et al. (2007) which demonstrated a loss of phenolic contents when teas were extracted at 100 °C for 10 min. There is further evidence that catechins, the major

polyphenolic compounds in tea, tend to degrade when extracted in water at high temperatures (Robinson et al., 1997; Wang et al., 2008; Saklar et al., 2015) and for prolonged extraction times (Cheong et al., 2005; Perva-Uzunalić et al., 2006; Komes et al., 2010).

The infusion of teas at 50 °C for 2 to 5 min yielded lower TPC extraction than that obtained at higher temperatures (70 to 100 °C). The TPC extracted at 50 °C gradually increased after 5 to 10 min but this increase was not significant and was not observed at other temperatures. The increase in TPC extraction at 50 °C for 10 min might be due to low saturation of phenolics in the tea extracts, which allows for the maximum solubilisation of the compounds (Dai & Mumper, 2010). In addition, low extraction temperature (50 °C) may prevent the degradation of phenolic compounds (Dai & Mumper, 2010). Appropriate infusion times and temperatures for tea brewing have been generally considered the critical factors for maximising the extraction of polyphenols from tea leaves, such as catechins and theaflavins (Astill et al., 2001; Khokhar & Magnusdottir, 2002; Molan et al., 2009b; Vuong et al., 2010, 2011a, 2011b; Samaniego-Sánchez et al., 2011; Hajiaghaalipour et al., 2015; Saklar et al., 2015). Therefore, a combination of high temperature with short extraction time or low temperature by long extraction time should be used to maximise the polyphenolic content.

There are numerous assays for measuring the antioxidant capacity of food and biological samples. However, there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively (Prior et al., 2005). The FRAP method is a simple assay that can measure the ability of the test extracts to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) by donating an electron to Fe^{3+} (Benzie & Strain, 1996). The higher the FRAP value, the greater the antioxidant activity. Whereas, DPPH is a common free radical assay used to test the radical scavenging activity of a sample, with this test being the most effective method for evaluating the radical-scavenging activity by a chain-breaking mechanism (Maisuthisakul et al., 2005). The DPPH assay measures the ability of the tea extracts to donate hydrogen to the DPPH radical resulting in bleaching of the purple colour of the DPPH solution to a bright yellow which is stoichiometrically related to the number of free radical electrons

captured (Khamsah et al., 2006), causing a decrease in absorbance, which can be measured spectrophotometrically (Blois, 1958). The greater the bleaching action, the greater the hydrogen-donating ability and thus the higher the radical scavenging activity of the tea extracts. The DPPH scavenging activity is expressed as an ascorbic acid equivalent to represent the antioxidant capacity of tea through a commonly accepted measure (Kim et al., 2002a), since ascorbic acid is an antioxidant compound usually found in nutritional labelling on food products. In both assays, the tea extracts ranked as follow: Se-GTE > R-BTE > R-GTE > Se-BTE, which is a similar pattern as that shown by the TPC values of the tea extracts.

In general, AOA was significantly increased from 2 to 5 min in all tested teas, as measured by FRAP and DPPH assays ($p < 0.05$). In line with the TPC results, teas extracted for 10 min at temperatures higher than 50 °C had slightly decreased AOA. In contrast, AOA increased in teas extracted at 50 °C temperature for 5 to 10 min, although not significantly, which may be closely related to the extraction of polyphenol compounds from the leaves as previously described by Dai and Mumper (2010). The present study shows that all teas have high DPPH-radical scavenger activity even after a 10-fold dilution of the tea from 10 mg/mL (1%, w/v) stock solution, which indicates a promising potential for their use in human health. Overall, using both antioxidant methods, the highest AOA was exhibited by the Se-GTE when extracted at 90 °C for 5 min, whereas the Se-BTE extracted at 50 °C for 2 min had the lowest AOA ($p < 0.05$).

The potential antioxidant properties of tea polyphenols have been previously discussed in a number of reviews (Frei & Higdon, 2003; Rietveld & Wiseman, 2003; Perron & Brumaghim, 2009). Benzie and Szeto (1999) reported that antioxidant activities are well-correlated with their respective total phenolic content and this finding is also supported by other researchers (Molan et al., 2009b; Venditti et al., 2010; Samaniego-Sánchez et al., 2011). In addition, Graham (1992), Katalinic et al. (2006) and Lin et al. (2008), found that polyphenol compounds, such as catechins, are important antioxidant ingredients in teas. Importantly, most of the antioxidant properties are well correlated with the corresponding polyphenolic compounds rather than ascorbic acid, tocopherol or β -carotene (Lee et al., 2007; Vamanu & Nita, 2013), and the

relationship is closely dependent on the extraction conditions used in the study (Liebert et al., 1999; Komes et al., 2010; Venditti et al., 2010).

In this study, the optimal extraction condition for the tested teas was found to be at 90 °C for 5 min. The tea extracts prepared under these conditions showed higher TPC than other extraction temperatures and times. Furthermore, the statistical analysis indicates that the effect of extraction temperature on TPC strongly depends on the extraction time (and *vice versa*). The present results were close to the findings of Komes et al. (2010), which suggest that aqueous extraction at 80 °C for 5 min is the optimal extraction condition of bioactive compounds from powdered green tea. Moreover, recent study performed by Saklar et al. (2015) indicated that the yield of epistructured catechins (i.e. EGCG, EGC, ECG, and EC) increased rapidly between the first 3 to 5 min of tea brewing at 85 °C; however, prolonged brewing time at the same temperature (85 °C) and at brewing temperature higher than 95 °C resulted in a decreased yield of these catechins. The discrepancies observed in these studies could be due to diverse extraction conditions, where different times, temperatures and forms of tea were used.

The AOA of all teas correlated well with their TPC, which implies that TPC are the major contributors to the antioxidant activity of the tea extracts. The strong relationships found between TPC and FRAP of all tea extracts point to TPC being highly associated with FRAP capacity for the tested teas due to the fact that these assays having similar chemical reactions towards compounds with redox potential (Prior et al., 2005). However, moderate and strong correlations were found between the TPC and DPPH of the Se-rich teas and regular teas, respectively, suggesting that the DPPH scavenging activity of Se-rich teas might be attributed not only to phenolic compounds, but also to non-phenolic compounds, where the possible explanation lies in the presence of high Se content and its well-known contribution as an antioxidant agent (Subramanyam et al., 2013). Whereas, the weak to moderate correlations between FRAP and DPPH observed in the Se-rich teas are probably related to the phenolic and non-phenolic compounds reaction in both assays which express different mechanisms. Although these two assays have identical mechanism towards redox potential, DPPH

assay reactions involve both electron- and hydrogen atom-transfers compared to FRAP that involves only electron-transfer reactions (Huang et al., 2005; Prior et al., 2005).

4.4.3 Prebiotic potential of the tea extracts

Results of the *in vitro* prebiotic study showed that the lactobacilli pure cultures used in the study responded differently to the tea extracts. All tea extracts at 1% concentration showed no effect on the population size of *L. acidophilus* and *L. rhamnosus* after 8, 24 and 48 hours of incubation in comparison to the control incubation. This indicates that these LAB strains were not affected by the tea extracts during exponential and stationary phases of growth. The present results agree with an *in vitro* study conducted by Lee et al. (2006a) who found that lactobacilli were not affected by tea phenolic compounds after 24 hours exposure, compared to other pathogenic bacteria such as *Bacteroides* spp. and *Clostridium perfringens*. Michalczyk and Zawislak (2008) have also reported that black and green teas showed no effect on the growth rate of *L. rhamnosus* under *in vitro* condition when measured between two to six hours of incubation after inoculation into MRS broth media, but they demonstrated antimicrobial activity against pathogenic bacteria such as *Staphylococcus aureus*. Interestingly, in incubations containing tea extracts (1%) the numbers of *L. acidophilus* were significantly higher ($p < 0.05$) after 72 hours incubation and onwards, in comparison to the control incubation. Whereas, no effect of 1% tea extracts was observed on *L. rhamnosus* during the same incubation periods; the increased counts of *L. acidophilus* suggested that this LAB strain is more sensitive towards 1% tea extracts than *L. rhamnosus*.

All tea extracts at 5% concentration further increased the counts of *L. acidophilus* after 24 hours and onwards, in comparison to the control incubation. However, the effect among various teas were observed to be significantly different only after 96 hours of incubation and onwards, with Se-GTE causing the highest increase followed by R-BTE, R-GTE and Se-BTE. On the other hand, results observed for the effect of 5% tea extracts on the population size of *L. rhamnosus* were different, with bacterial counts significantly higher in the tea-treated groups than the control after eight

hours of incubation and onwards. However, no differences among various tea extracts were found in any of the incubation periods.

It is noteworthy that all tea extracts at 10% concentrations showed similar effects on the population size of both LAB strains used in the study, where bacterial counts of both LAB strains were significantly increased by the tea extracts after eight hours of incubation and onwards as opposed to the control incubation. In addition, the effect of various teas at 10% concentrations were observed to be significantly different after 72 hours of incubation and onwards, with Se-GTE causing the highest increase followed by R-BTE, R-GTE and Se-BTE. This decreasing order of prebiotic potential represents a similar pattern as that shown by the TPC values and antioxidant activities of the tea extracts found in the previous results. The present results, in part, are in contrast to the *in vitro* data of Molan et al. (2009a) who reported that Se-green tea extracts (Se-GTE) of different concentrations (10% and 25%, v/v) were able to increase the growth of a *L. rhamnosus* pure culture when measured after a 48-hour incubation. However, the experimental procedure described by the authors differed from our method in points such as tea concentration and the preparation of the tea water extract, all things that might exert significant impact on the results.

Nevertheless, the present results support previous findings that showed significantly greater prebiotic effect of Se-GTE over China green tea (CH-GTE). Molan et al. (2010) investigated the prebiotic activity of Se-GTE and CH-GTE on lactobacilli and bifidobacteria using a fluorescence *in situ* hybridisation (FISH) method to determine their effects on the activity of two bacterial enzymes in the caeca of rats. This *in vivo* study supported their previous *in vitro* results (Molan et al., 2009a), where the authors concluded that Se-GTE is more potent as a growth promoter of lactobacilli and bifidobacteria than the CH-GTE. There is other evidence that supports the prebiotic potential of tea, which comes from human intervention studies, animal trials and *in vitro* data (Ankolekar et al., 2011; Axling et al., 2012; Jin et al., 2012; Vodnar & Socaciu, 2012; Gaudreau et al., 2013; Zhang et al., 2013). However, the available literature does not provide data on the prebiotic effect of tea extracts via *in vitro* models against lactobacilli for long incubation periods up to 120 hours, thus the data obtained are therefore difficult to compare.

Although the mechanism of tea as a prebiotic under *in vitro* conditions is unclear, it has been suggested that the possible mechanism, in part, could be due to its polyphenolic compounds directly acting as an antioxidant by scavenging singlet oxygen and free radicals (Molan et al., 2009a, 2009b; Bancirova, 2010; Gyawali & Ibrahim, 2012). Tea polyphenols may modulate the reactive oxygen species that are naturally produced in the cell culture medium as a by-product of cellular metabolism by the LAB strains (*L. acidophilus* and *L. rhamnosus*), thus, reducing the redox potential of the growth media. Eventually, tea may render an optimal environment for the growth and multiplication of the beneficial bacterial strains, since these bacteria grow better in the absence of oxygen (Molan et al., 2009a; Gaudreau et al., 2013; Marhamatizadeh et al., 2013). Additionally, the tea polyphenolic compounds may also be taken up directly as growth substrates by the lactic acid bacteria (LAB), thus increasing the population size of both cultured LAB strains used in the present study (Gibson & Roberfroid, 1995; Hervert-Hernández & Goñi, 2011).

4.5 Conclusions

Se-GTE contained significantly higher TPC, and exhibited significantly higher ferric-reducing/antioxidant power and DPPH scavenging activities than Se-BTE and both regular green and black teas. These findings may be due to differences in polyphenol profile of the different tea cultivars. The optimal method for maximising TPC was found to be extracting the tea samples at a temperature of 90 °C for 5 min. All tea extracts at 5% and 10% concentrations showed positive effects on the growth of both *L. acidophilus* and *L. rhamnosus* populations during the exponential and stationary phase, and exerted a survival effect during the death phase. However, all tea extracts at a 10% concentration exerted a greater effect than their 5% concentrations in maintaining the bacterial cell viability after 72 hours of incubation and onwards. The effect of various teas at 10% concentrations were observed to be significantly different after 72 hours of incubation and onwards, with Se-GTE exhibiting the highest count of lactobacilli followed by R-BTE, R-GTE and Se-BTE. This decreasing order of prebiotic potential represents a similar pattern as that shown by the TPC values and antioxidant activities of the tea extracts found in this study. Taken together, these findings show that

the antioxidant properties and prebiotic potential of the tea extracts are not necessarily dependent on the high level of selenium that the tea contains. In addition, this study provides new information on the antioxidant properties and prebiotic function of tea that may provide health benefits beyond current knowledge. Tea beverages can be important dietary sources of antioxidants for prevention of bone diseases caused by oxidative stress. Therefore, further work that aims to determine the potential bone-stimulating effect of these tested teas on bone cell culture using *in vitro* assays will be conducted in our next investigation.

CHAPTER 5

THE OSTEOGENIC AND OSTEOPROTECTIVE EFFECTS OF VARIOUS TEA EXTRACTS ON MURINE PRE-OSTEOBLASTIC MC3T3-E1 (SUBCLONE 4) CELLS

Part of this chapter was presented at the International Conference on Food Science and Nutrition, 2 to 4 April, 2012, Kota Kinabalu, Sabah, Malaysia.

Abstract

There is increasing interest in the potential of tea as a bone-protective agent to mitigate the risk of developing osteoporosis. Selenium-rich teas are thought to have added health benefits and little is known about the osteogenic and osteoprotective effect of these selenium-rich teas in comparison to regular teas. In this study, four different teas (i.e. selenium-rich green tea (Se-GTE), selenium-rich black (Se-BTE) teas, regular green tea (R-GTE) and regular black tea (R-BTE)), were investigated for their *in vitro* effect on osteogenic proliferation and differentiation in murine pre-osteoblastic MC3T3-E1 (Subclone 4) cells. The osteogenic effects of freeze-dried tea extracts on osteoblast proliferation, differentiation and mineralisation were determined by treating the cell lines with various tea concentrations. Osteoblast proliferation was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay after 24, 48 and 72 hours of tea treatment. Osteoblast differentiation was evaluated using the alkaline phosphatase (ALP) activity assay after 9 days of tea treatment, and matrix mineralisation was measured using the Alizarin Red S (ARS) staining procedure after 8, 16 and 24 days of tea treatment. To determine the osteoprotective effect of the tea extracts against the damaging effects of reactive oxygen species (ROS) on osteoblast differentiation, MC3T3-E1 cells were pre-treated with various tea concentrations for 7 days followed by H₂O₂ incubation (300 µM) for three hours, prior to ALP activity measurement. Findings of the study were irrespective of the levels of selenium content, and had a concentration- and time-dependent effect. All tea extracts, when used in low concentrations, were not cytotoxic to the MC3T3-E1 osteoblasts and stimulated mineralised bone nodule formation. This indicated an anabolic effect of teas in that they can stimulate bone formation by enhancing mineralisation in MC3T3-E1 cells. However, there was an inhibitory effect shown by the black teas (Se-BTE and R-BTE) irrespective of selenium content during differentiation of the osteoblasts. The mechanism by which black tea acts on osteoblast differentiation is presumably different from that of green tea. Moreover, only the green tea extracts were capable of protecting and restoring the differentiated osteoblasts against the dysfunctional effects of H₂O₂-induced oxidative stress. The exact mechanism of action associated with these findings is unknown and needs to be elucidated. In conclusion, the present findings provide

preliminary cellular evidence that the green tea used in the study has osteogenic and osteoprotective properties.

5.1 Introduction

Osteoporosis is a progressive bone disease characterised by a reduction in bone mass and a deterioration of bone microarchitecture that subsequently leads to increased bone fragility and susceptibility to fracture (National Institute of Health, 2001). As a result of osteoporosis, over half of the elderly population will face the risk of a hip or vertebral fracture, which is a major cause of morbidity and mortality (Looker et al., 1995; Boonen et al., 2008). The development of osteoporosis is influenced by both non-modifiable (e.g. genetic, gender, and age) and modifiable (e.g. nutrition, alcohol consumption, smoking, and excessive sedentary lifestyle) risk factors (Mundy, 2006; Sarkis et al., 2012). Many drug therapies are available for the treatment of osteoporosis including anti-resorptive and anabolic agents, such as hormone replacement therapy (HRT), parathyroid hormone (PTH), selective oestrogen receptor modulators (SERMs) and vitamin D. However, concerns about their adverse effects and efficacy have been a major issue (Sharif et al., 2011; Reginster et al., 2013; Reginster, 2014). There has been an increase in research looking into the most efficient and affordable complementary (adjunctive) and alternative therapies for the prevention and management of osteoporosis which may have less detrimental side effects than pharmacological agents (Jia et al., 2012). Natural complementary and alternative therapies including nutrients and dietary components independent from calcium and vitamin D, can influence bone metabolism by various mechanisms (Cashman, 2007; Nieves, 2013; Rondanelli et al., 2013; Welch & Hardcastle, 2014), therefore, providing alternative treatments for osteoporosis and maintenance of bone health throughout life (Stránský & Rysavá, 2009; Schulman et al., 2011; Horcajada & Offord, 2012; Sacco et al., 2012; Rao & Rao, 2013).

Reactive oxygen species (ROS) are oxygen-containing molecules which play a dual role in having both beneficial and deleterious effects on cellular metabolism (Djordjević et al., 2008; Filaire & Toumi, 2012). Under normal physiological

conditions, ROS are constantly produced by mitochondria in living cells at low levels during metabolism, and are scavenged by endogenous antioxidant systems to keep the intracellular ROS level under control (Almeida, 2012). Recent studies have demonstrated that ROS generation is particularly involved in regulating bone cell functions and mediating intracellular signals (Sontakke & Tare, 2002; Altindag et al., 2008; Wauquier et al., 2009). However, the intracellular ROS level increases with age and quicken by gonadal hormone deficiency, due to depletion of endogenous antioxidant defence systems and/or excessive accumulation of ROS (Almeida et al., 2007a; Pandey & Rizvi, 2010; Zhang et al., 2011; Bellanti et al., 2013). Overproduction of ROS disrupts the intracellular redox balance in cells and tissues, causing oxidative stress that can be an important mediator of damage to cell structures (lipids, membranes, proteins, and DNA) (Djordjević et al., 2008; Augusto & Miyamoto, 2011; Schieber & Chandel, 2014). An increasing body of evidence has linked bone biology and the regulation of redox balance, indicating that excessive ROS production may lead to the initiation and progression of osteoporosis (Portal-Núñez & Esbrit, 2013; Holmström & Finkel, 2014; Philippe & Wittrant, 2014), in part by inhibiting pre-osteoblastic differentiation (Bai et al., 2004; Maiese et al., 2010). The close relationship between oxidative stress and the development of osteoporosis in humans has been suggested in a number of studies (Shen et al., 2009; Wauquier et al., 2009; Almeida, 2012).

There has been increasing interest in the health giving potential of tea as a bone-protective agent to mitigate the risk of developing osteoporosis. Tea drinking has been shown to be positively associated with bone mineral density (Chen et al., 2003; Devine et al., 2007; Hossein-nezhad et al., 2007) which may indicate a positive effect of tea as a nutritional strategy for the prevention and management of osteoporosis. Indeed, several studies have suggested beneficial effects of tea on bone cells *in vitro* (Nakamura et al., 2010; Holzer et al., 2012; Oka et al., 2012), and on bone turnover in an animal model (Shen et al., 2008, 2011; Chen et al., 2013; Das et al., 2013), postmenopausal women (Hamdi Kara et al., 2007; Shen et al., 2012), as well as in men (Wu et al., 2002). Most of these studies have focused on green and black teas and/or their polyphenolic compounds. To the best of our knowledge, no information is available about the *in vitro* osteogenic effect of tea with naturally high selenium content on bone cell culture.

Selenium (Se) is the main ingredient in the production of selenoenzymes, which act as antioxidants that help prevent cellular damage due to oxidative stress, and are essential for bone metabolism (McKenzie et al., 1998; Sandukji et al., 2011). It has been suggested that Se deficiency in humans is associated with osteoporosis (Ebert & Jakob, 2007). Moreover, Se is essential for regulation of thyroid hormone secretion, which is necessary for bone formation (Kaprra & Krassas, 2006; Zagrodzki & Ratajczak, 2008).

The purpose of this study was to determine the osteogenic effect of Se-rich green and black teas on *in vitro* bone formation in comparison with regular varieties of green and black teas. The present study used murine pre-osteoblastic MC3T3-E1 (Subclone 4) cells, an established and widely used *in vitro* model of osteogenesis. This cell line was chosen as it represents a pre-osteoblastic phenotype with a growth rate comparable to human osteoblasts, exhibits a high alkaline phosphatase (ALP) activity, and is capable of forming a mineralised extracellular bone matrix (Quarles et al., 1992; Kartsogiannis & Ng, 2004; Czekanska et al., 2012). Four different teas (i.e. Se-rich green tea (Se-GTE), Se-rich black tea (Se-BTE), regular green tea (R-GTE) and regular black tea (R-BTE)), were investigated for their influence on osteogenic proliferation, differentiation and mineralisation in MC3T3-E1 cells. Apart from these, the osteoprotective effect of various teas against hydrogen peroxide (H₂O₂)-induced oxidative stress in differentiated MC3T3-E1 was also evaluated.

5.2 Materials and methods

5.2.1 Preparation of sterile tea water extracts

The tea leaves used for the present study were described in **Chapter 3**, section 3.1.3. Tea water extracts (1%, w/v) were prepared at 90 °C for 5 min. Briefly, 1 g of each of the powdered tea leaves was infused with 100 mL of Milli-Q (MQ) water at 90 °C and allowed to brew for 5 min with continuous stirring. The infusions were decanted and filtered through Whatman No. 4 filter paper (0.45 mm) to remove the solid matter. Tea extracts were then freeze-dried using a freeze-dryer (FD18LT, Cuddon Ltd., New Zealand) and stored at -20 °C until further use. On the day of the experiment, sterile

stock solutions of tea extracts and the tea phenolic compound, i.e. (-)-epigallocatechin-3-gallate (EGCG), were freshly made by dissolving 1 mg of freeze-dried tea extracts, or 1 mg of EGCG ($\geq 80\%$, HPLC), into 1 mL of cell culture media Minimum Essential Media alpha modification (MEM α , Invitrogen, New Zealand). The 1 mg/mL EGCG stock solution was then diluted 100-fold to obtain a final concentration of 10 $\mu\text{g/mL}$, and then filter-sterilised using a 0.20 μm syringe filter (Minisart, Sartorius AG, Goettingen, Germany). A 100-fold dilution was made from the 1 mg/mL tea stock solution to obtain a concentration of 10 $\mu\text{g/mL}$, which was then filter-sterilised (0.20 μm). Finally, a five point 10-fold serial dilutions from the 10 $\mu\text{g/mL}$ tea extracts were made into aliquots of decreasing concentration (10, 1, 0.1, 0.01 and 0.001 $\mu\text{g/mL}$).

5.2.2 Preparation of sterile 1 mM hydrogen peroxide (H_2O_2) stock solution in osteogenic differentiation media

A 1 mM H_2O_2 (30% in H_2O_2 , 9.8 M) stock solution was freshly prepared in the osteogenic differentiation media (MEM α + 10% FCS + 50 $\mu\text{g/mL}$ ascorbic acid + 10 mM β -glycerophosphate + 25 $\mu\text{g/mL}$ gentamicin), immediately before use on the day of the experiment to avoid degradation of the solution. The stock solution was filter-sterilised (0.20 μm) and kept in the dark at 4 $^\circ\text{C}$ until treatment. To obtain the concentrations of interest (100, 200, 300, 400, 500, 600 and 700 μM), the stock solution was further diluted in sterile osteogenic differentiation media.

5.2.3 Cell culture

Cell culture and maintenance of the murine pre-osteoblast cells, MC3T3-E1 (Subclone 4) have been described in **Chapter 3**, sections 3.1.6 and 3.1.7.1.

5.2.4 Experimental procedures and techniques

Cell culture procedures for the experimental work have been described in **Chapter 3**, section 3.1.8.

5.2.4.1 Proliferation of MC3T3-E1 cells after 24, 48 and 72 hours of exposure to tea extracts

The effect of various tea extracts on osteoblast proliferation was determined by treating the MC3T3-E1 cell lines with each of the tea extracts at varying concentrations (0.001, 0.01, 0.1, 1.0 and 10 µg/mL) for 24, 48 and 72 hours of incubation. The tea extract concentrations were chosen based on a pilot experiment (data are not included). In this experiment, cell suspensions were seeded into flat-bottomed 96-microwell plates (100 µL/well) in Minimum Essential Media alpha modification (MEM α , Invitrogen, New Zealand) supplemented with 10% foetal calf serum (Heat-inactivated FCS, Invitrogen, New Zealand) and gentamicin (25 µg/mL, Invitrogen, New Zealand), and incubated for 24 hours at 37 °C, prior to exposure to tea extracts. After 24 hours, cell growth was arrested by replacing the growth medium with 10% FCS to fresh MEM α medium containing 0.1% bovine serum albumin (BSA, Invitrogen, New Zealand) and the plates were further incubated for 24 hours at 37 °C. After 24 hours, the media was replaced with fresh growth media with varying concentrations of sterile tea solutions. There were eight replicate wells for each treatment per experiment, and each experiment was repeated on three separate occasions. The EGCG (10 µg/mL) was used as a positive control, whereas MEM α medium with 0.1% BSA was used as the zero control (blank). Cell proliferation was quantified with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay in cells after 24, 48 or 72 hours of tea extract incubation (different plates for each time point), as previously described in **Chapter 3**, section 3.1.9.1.

5.2.4.2 Differentiation of MC3T3-E1 cells after 9 days of exposure to tea extracts

In this experiment, the MC3T3-E1 cell suspensions were seeded into 24-microwell plates (1 mL/well) in MEM α with 10% FCS and incubated at 37 °C for 72 hours until cells reached approximately 80% confluence. Following incubation, the old MEM α with 10% FCS media was aspirated and replaced with fresh osteogenic differentiation media, with or without tea extracts of four different concentrations (0.01, 0.1, 1, and 10 µg/mL). The tea extract concentrations were chosen based on previous experimental study (**Chapter 5**, section 5.2.4.1). There were four replicate wells for each treatment per experiment, and each experiment was repeated on three separate

occasions. EGCG (10 µg/mL) was used as a positive control, whereas MEM α with 10% FCS was used as the zero control (blank). The plates were then further incubated for 9 days. On the last day of the tea treatment, cell differentiation was quantified with ALP activity assay and the quantification of ALP activity was further evaluated using the Crystal Violet staining assay, as previously described in **Chapter 3**, section 3.1.9.3.

5.2.4.3 Mineralisation of MC3T3-E1 cells after 8, 16 and 24 days of exposure to tea extracts

In this experiment, the MC3T3-E1 cell suspensions were seeded into 24-microwell plates (1 mL/well) in MEM α with 10% FCS and incubated at 37 °C for 72 hours until cells reached approximately 80% confluence. Following incubation, the old MEM α with 10% FCS media was aspirated and replaced with fresh osteogenic differentiation media, with or without tea extracts of four different concentrations (0.01, 0.1, 1, and 10 µg/mL); these concentrations were chosen based on a previous experimental study (**Chapter 5**, section 5.2.4.2). There were four replicate wells for each treatment per experiment, and each experiment was repeated on three separate occasions. EGCG (10 µg/mL) was used as a positive control, whereas MEM α with 10% FCS was used as the zero control (blank). The plates were then further incubated for 8, 16 and 24 days. On the last day of each treatment period (day 8, 16 and 24), cell mineralisation was assessed using the Alizarin Red S (ARS, pH 5.5) staining assay and the quantification of ARS-positive nodules was further analysed using cetylpyridinium chloride (CPC), as previously described in **Chapter 3**, sections 3.1.9.4 and 3.1.9.5.

5.2.4.4 Evaluation of proliferation of differentiated MC3T3-E1 cells after three hours of exposure to H₂O₂ at different concentrations

The aim of this experiment was to determine the concentration ranges for H₂O₂ that would be used to induce oxidative stress in differentiated osteoblasts without damaging the growth and functions of the osteoblast cells. Briefly, the MC3T3-E1 cell suspensions were seeded into 24-microwell plates (1 mL/well) in MEM α with 10% FCS and incubated at 37 °C for 72 hours until cells reached approximately 80% confluence. Following incubation, the old MEM α with 10% FCS media was aspirated and replaced with fresh osteogenic differentiation media and the plates were further incubated for seven days. Seven days incubation was used as a sufficient time period to attain a fully

differentiated state of the osteoblastic cells, which is based on data reported by Fatokun et al. (2006). On day 7 of culture, cells were treated with various concentrations of H₂O₂ (100 to 700 μM) for three hours. The H₂O₂ concentrations were chosen based on several pilot experiments (data are not included), for which the range of concentrations were estimated from study conducted by Fatokun et al. (2006). There were four replicate wells for each treatment per experiment, and each experiment was repeated on three separate occasions. The H₂O₂-free osteogenic differentiation media was used as the H₂O₂-untreated control (blank). After three hours of exposure to the H₂O₂ concentrations, the old osteogenic differentiation media was aspirated and replaced with 900 μL of freshly made H₂O₂-free osteogenic differentiation media. Cell proliferation was quantified with the MTT assay as described in **Chapter 3** (section 3.1.9.1), but was slightly modified to suit the higher volumes of MTT reagent (100 μL/well) and dimethyl sulfoxide (DMSO, 1 mL/well) due to the use of 24-microwell plate. Duplicate measures of 200 μL aliquots from each well were transferred to 96-microwell plates and the absorbance was read at 550 nm against the H₂O₂-untreated control (blank) wells prepared under similar conditions.

5.2.4.5 Evaluation of the osteoprotective effect of various tea extracts on H₂O₂-induced oxidative stress in differentiated MC3T3-E1 cells after three hours of exposure to 300 μM H₂O₂

The aim of this experiment was to determine the osteoprotective effect of tea extracts on the function of differentiated osteoblast incubated for three hours in 300 μM H₂O₂. H₂O₂ was used at 300 μM to induce oxidative stress in differentiated osteoblasts based on previous pilot experiments, which showed that H₂O₂ at concentrations of 400 μM and higher significantly decreased viability of the differentiated osteoblasts. Briefly, MC3T3-E1 cells were seeded into 24-microwell plates (1 mL/well) in MEMα with 10% FCS and incubated at 37 °C for 72 hours until cells reached approximately 80% confluence. Following incubation, the old MEMα with 10% FCS media was aspirated and replaced with fresh osteogenic differentiation media, with or without tea extracts of four different concentrations (0.01, 0.1, 1, and 10 μg/mL); these concentrations were chosen based on a previous experimental study (**Chapter 5**, section 5.2.4.2). There were four replicate wells for each treatment per experiment, and each experiment was repeated on three separate occasions. The EGCG (10 μg/mL) was used

as a positive control, whereas H₂O₂-free osteogenic differentiation media was used as the H₂O₂-untreated control (blank). The plates were then further incubated for seven days. On day 7 of culture, cells were treated with or without 300 µM H₂O₂ for three hours. After three hours of exposure to the H₂O₂ concentrations, the old osteogenic differentiation media was aspirated from each well and replaced with 1 mL of freshly made H₂O₂-free osteogenic differentiation media. Differentiation of the osteoblasts was quantified with the ALP activity assay and ALP activity per cell number was further evaluated using Crystal Violet staining assay, as previously described in **Chapter 3**, section 3.1.9.3.

5.2.5 Statistical analysis

The detailed information about general statistical analyses has been described in **Chapter 3**, section 3.1.10. All experiments were performed in 8 or 4 replicates per condition, and independently repeated three times. One-way analyses of variance (ANOVA) were used to test for significance between experimental groups. Nested ANOVA was used to test for significance between H₂O₂-treated and H₂O₂-untreated cells, with tea concentrations being the experimental factors. The ANOVA tests employed Tukey's post-hoc analysis for pair-wise multiple comparisons of the group means. All analyses were done using the PROC GLM, a procedure within SAS that analyses data within the framework of general linear models. Student's *t*-tests were used to test for overall significance between zero control and EGCG. A difference was considered to be statistically significant when $p < 0.05$.

5.3 Results

5.3.1 Effects of tea extracts on viability and proliferation of MC3T3-E1 cells

Figure 5-1 shows the effect of various concentrations of each tea extract on the viability of proliferating MC3T3-E1 cells as measured by the MTT assay. In the present study, most of the tea extracts did not affect cell viability within the first 24-hours of exposure to varying concentrations (0.001 to 10 $\mu\text{g/mL}$). However, there was an exception with R-GTE at 10 $\mu\text{g/mL}$, which significantly decreased cell viability after a 24-hour incubation ($91.8 \pm 1.4\%$), in comparison to the zero control incubation ($p = 0.0257$). Following 48-hours exposure to the tea extracts, the highest concentration (10 $\mu\text{g/mL}$) of both Se-GTE and R-GTE significantly reduced cell viability to $83.1 \pm 2.4\%$ and $78.5 \pm 2.1\%$ of the zero control, respectively (both p values < 0.0001). On the other hand, no single concentration of Se-BTE and R-BTE was different from the zero control after 48-hours exposure. When the osteoblasts were cultured for 72 hours in presence of the tea extracts, there was a significant decrease in viability of osteoblasts ($p < 0.0001$) observed in a range between $90.3 \pm 2.0\%$ to $67.4 \pm 0.9\%$ of the zero control, in the presence of high concentrations of Se-GTE (1 and 10 $\mu\text{g/mL}$; $p = 0.0003$ and $p < 0.0001$, respectively), R-GTE (1 and 10 $\mu\text{g/mL}$; $p = 0.0003$ and $p < 0.0001$, respectively), Se-BTE (0.01, 0.1, 1 and 10 $\mu\text{g/mL}$; $p = 0.0021$, $p = 0.0033$, $p = 0.0015$ and $p < 0.0001$, respectively) and R-BTE (0.1, 1 and 10 $\mu\text{g/mL}$; $p = 0.0012$, $p = 0.0003$ and $p < 0.0001$, respectively). Overall, the viability of osteoblasts was significantly increased with the administration of EGCG within 24 ($114.8 \pm 1.4\%$) and 48 ($109.1 \pm 1.5\%$) hours of incubation when compared to the zero control ($100.0 \pm 1.3\%$ and $100.0 \pm 1.7\%$; $p < 0.0001$ and $p = 0.0001$, respectively). However, after 72 hours of exposure, the viability of cells was significantly decreased by EGCG to $69.0 \pm 0.9\%$ of the zero control ($100.0 \pm 1.7\%$, $p < 0.0001$).

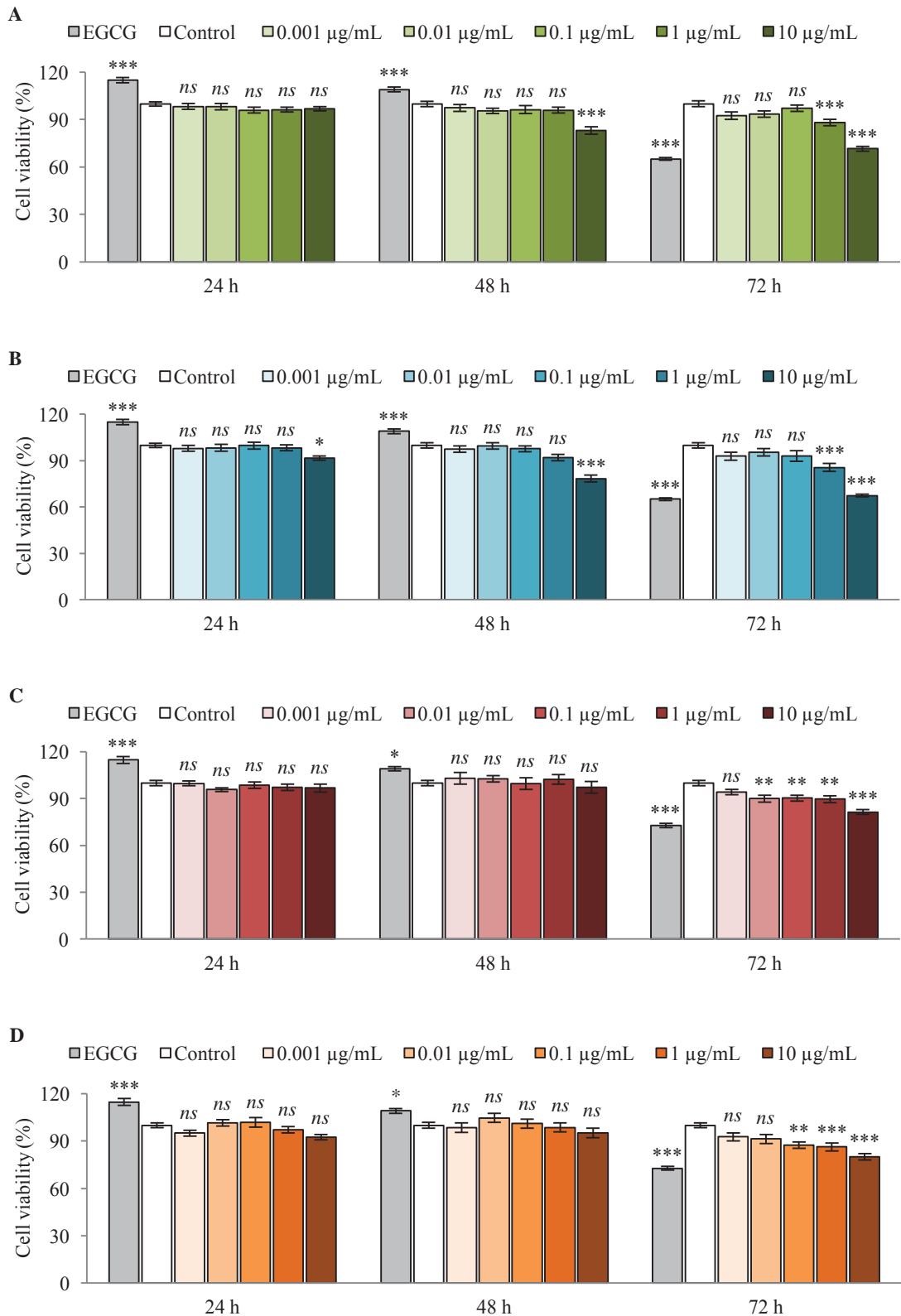


Figure 5-1. The effect of varying concentrations of tea extracts (0.001, 0.01, 0.1, 1 and 10 µg/mL) and EGCG (10 µg/mL) on viability of MC3T3-E1 cells after 24, 48 and 72 hours of incubation, as measured by MTT assay. (A) Se-GTE, (B) R-GTE, (C) Se-BTE, and (D) R-BTE. Data are mean ± SEM. *ns* = not significant. *indicates significance from the zero control: *ns* = not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

5.3.2 Effect of tea extracts on differentiation of MC3T3-E1 cells

After 9 days of incubation, ALP activity was significantly affected by Se-GTE ($p = 0.0090$), R-GTE ($p = 0.0454$), Se-BTE ($p < 0.0001$) and R-BTE ($p = 0.0134$). As depicted in **Figure 5-2A**, ALP activity was significantly increased at the highest concentration (10 $\mu\text{g/mL}$) of Se-GTE (1.113 ± 0.018 ; $p = 0.0028$) and R-GTE (1.058 ± 0.022 ; $p = 0.0148$). On the other hand, Se-BTE concentrations of 0.1, 1 and 10 $\mu\text{g/mL}$ significantly decreased osteoblast differentiation (0.920 ± 0.024 , 0.918 ± 0.021 and 0.906 ± 0.019 , respectively), as compared to the zero control (p values of 0.0287, 0.0237 and 0.0066, respectively). Although there was a significant difference among means in R-BTE, no single concentration was significantly different from the zero control (**Figure 5-2D**). Overall, there was a tendency towards a stimulatory effect of EGCG (10 $\mu\text{g/mL}$) on osteoblast differentiation (1.032 ± 0.016) when compared to the zero control (1.000 ± 0.006), but the increase did not reach significance ($p = 0.0673$).

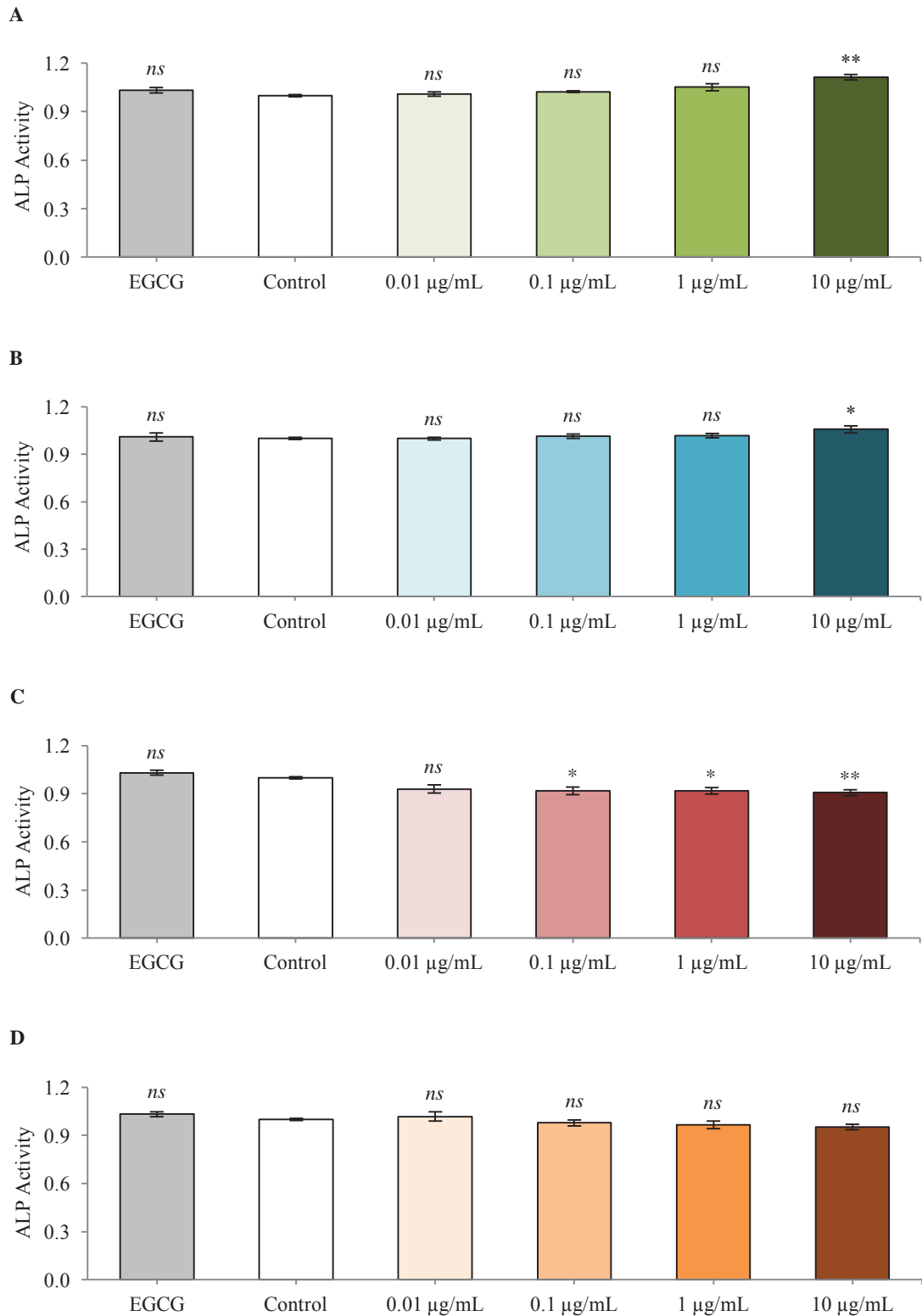


Figure 5-2. The effect of varying concentrations of tea extracts (0.01, 0.1, 1 and 10 µg/mL) and EGCG (10 µg/mL) on MC3T3-E1 osteoblastic differentiation after 9 days of incubation in osteogenic differentiation media, as measured by ALP enzyme activity. (A) Se-GTE, (B) R-GTE, (C) Se-BTE, and (D) R-BTE. Data are mean ± SEM. *ns* = not significant. *indicates significance from the zero control: *ns* = not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

5.3.3 Effect of tea extracts on mineralisation of MC3T3-E1 cells

Figures 5-3 and **5-4** show the effect of various concentrations of each tea extract on the mineralisation of MC3T3-E1 cells as measured by the ARS assay. Microscopic observations of ARS-positive mineralised bone nodules formation (**Figure 5-3**) clarify the bone-stimulating effect of tea extracts on MC3T3-E1 cells during differentiation (**Figure 5-4**). In the present study, there was no matrix mineralisation in MC3T3-E1 cells within the 8-day exposure to varying concentrations (0.01 to 10 µg/mL). The effect of tea extracts on MC3T3-E1 mineralisation was positively observed when the cultures stained with ARS on the 16 and 24 days of incubation. As represented in **Figure 5-4A**, the lowest concentrations (0.01 and 0.1 µg/mL) of Se-GTE significantly increased cell mineralisation to $110.3 \pm 2.0\%$ and $107.7 \pm 3.0\%$ of the zero control ($p < 0.0001$ and $p = 0.0085$, respectively) after 16 days of incubation. Not a single concentration of R-GTE was significantly different from the zero control after 16 days of incubation (**Figure 5-4B**). Meanwhile, both Se-BTE and R-BTE at 0.01 µg/mL significantly increased cell mineralisation to $106.9 \pm 2.7\%$ and $107.4 \pm 1.9\%$ of the zero control ($p = 0.0273$ and $p = 0.0093$, respectively), after 16 days of incubation.

When the osteoblasts were further incubated for 24 days, cell mineralisation was significantly affected by all tea extracts. After 24 days of incubation, all tea extracts at concentrations of 0.01 and 0.1 µg/mL significantly increased cell mineralisation ($p < 0.0001$), when compared to the zero control. However, all teas at higher concentrations (1 and 10 µg/mL) seemed to decrease the levels of cell mineralisation after 24 days in comparison to the zero control, with a significant effect seen in Se-GTE (1 and 10 µg/mL; $p < 0.0001$ and $p < 0.0001$, respectively) and Se-BTE (10 µg/mL, $p < 0.0001$). Overall, cell mineralisation in cells pre-treated with EGCG at 10 µg/mL was significantly decreased after 8 ($95.3 \pm 1.1\%$), 16 ($92.9 \pm 1.1\%$), and 24 ($45.1 \pm 1.3\%$) days of incubation when compared to the zero control (p values of 0.0041, 0.0001 and < 0.0001 , respectively).

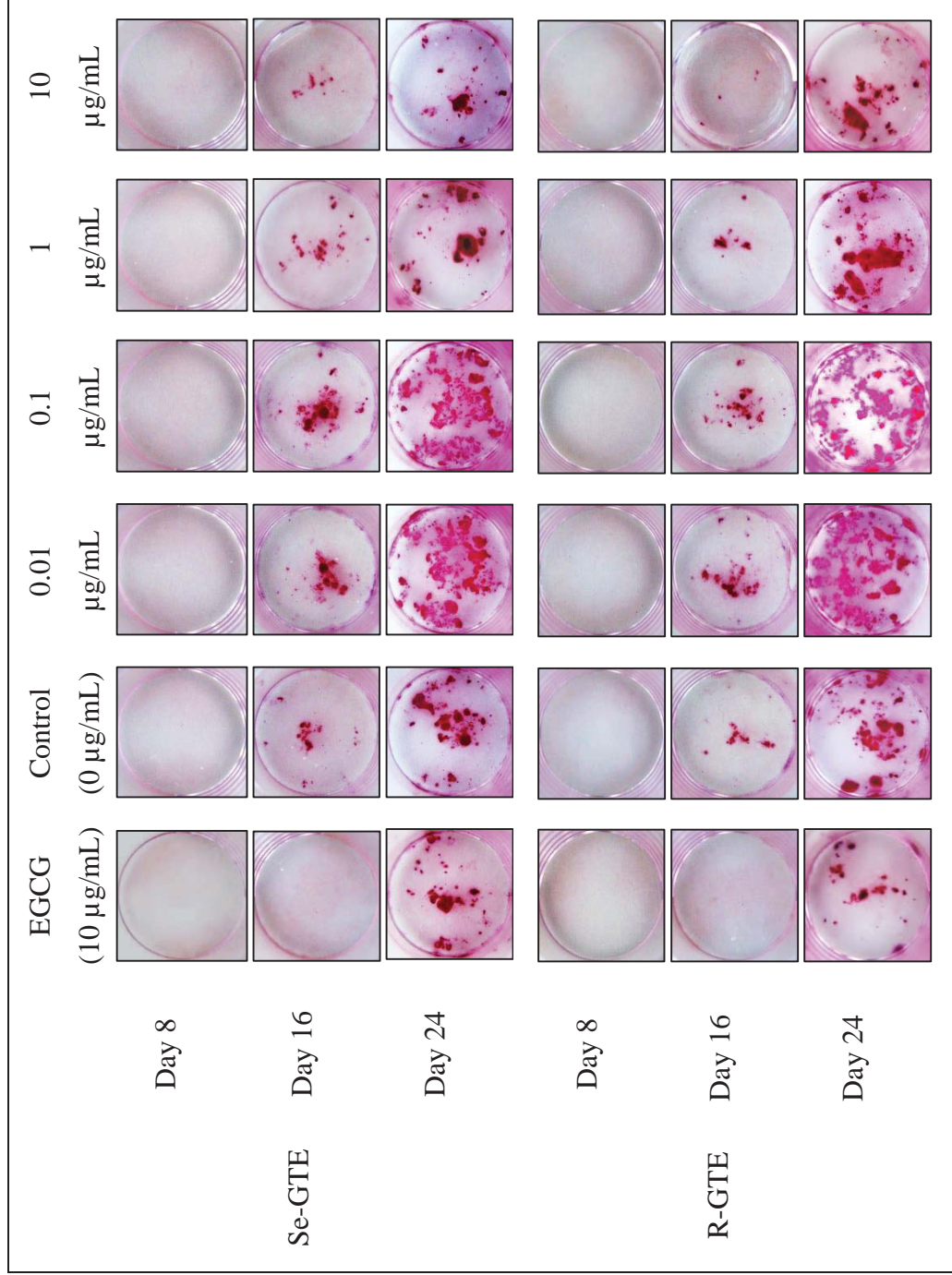


Figure 5-3. Assessment of extracellular matrix mineralisation characterised as mineralised bone nodules formation in MC3T3-E1 cells using Alizarin Red S staining procedure. Cells were seeded in 24-microwell plates (2×10^4 cells/well) until 80% confluent, followed by incubation with varying concentrations of Se-GTE, R-GTE, Se-BTE and R-BTE in osteogenic differentiation media for 8, 16 and 24 days.

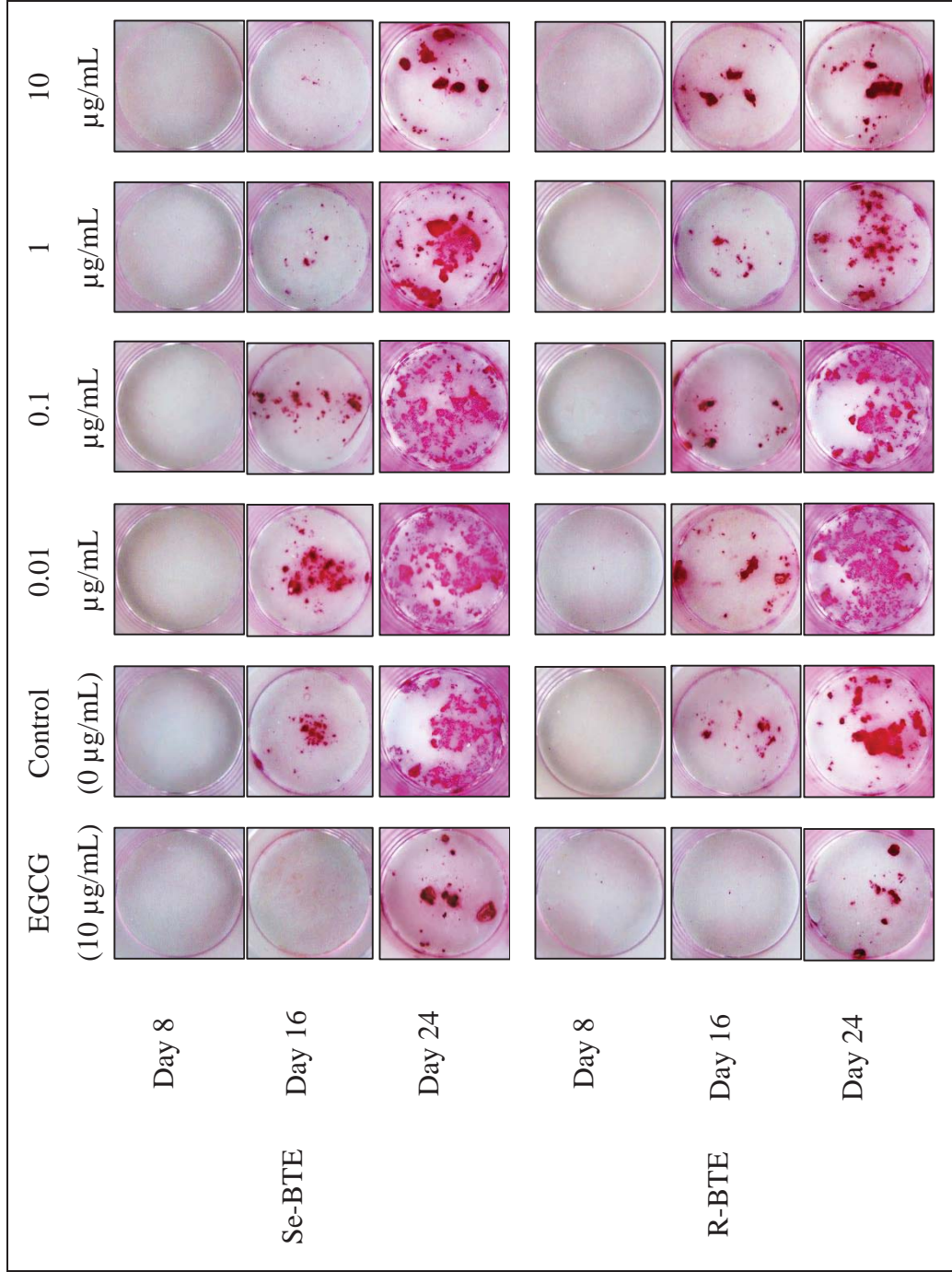


Figure 5-3. (continued)

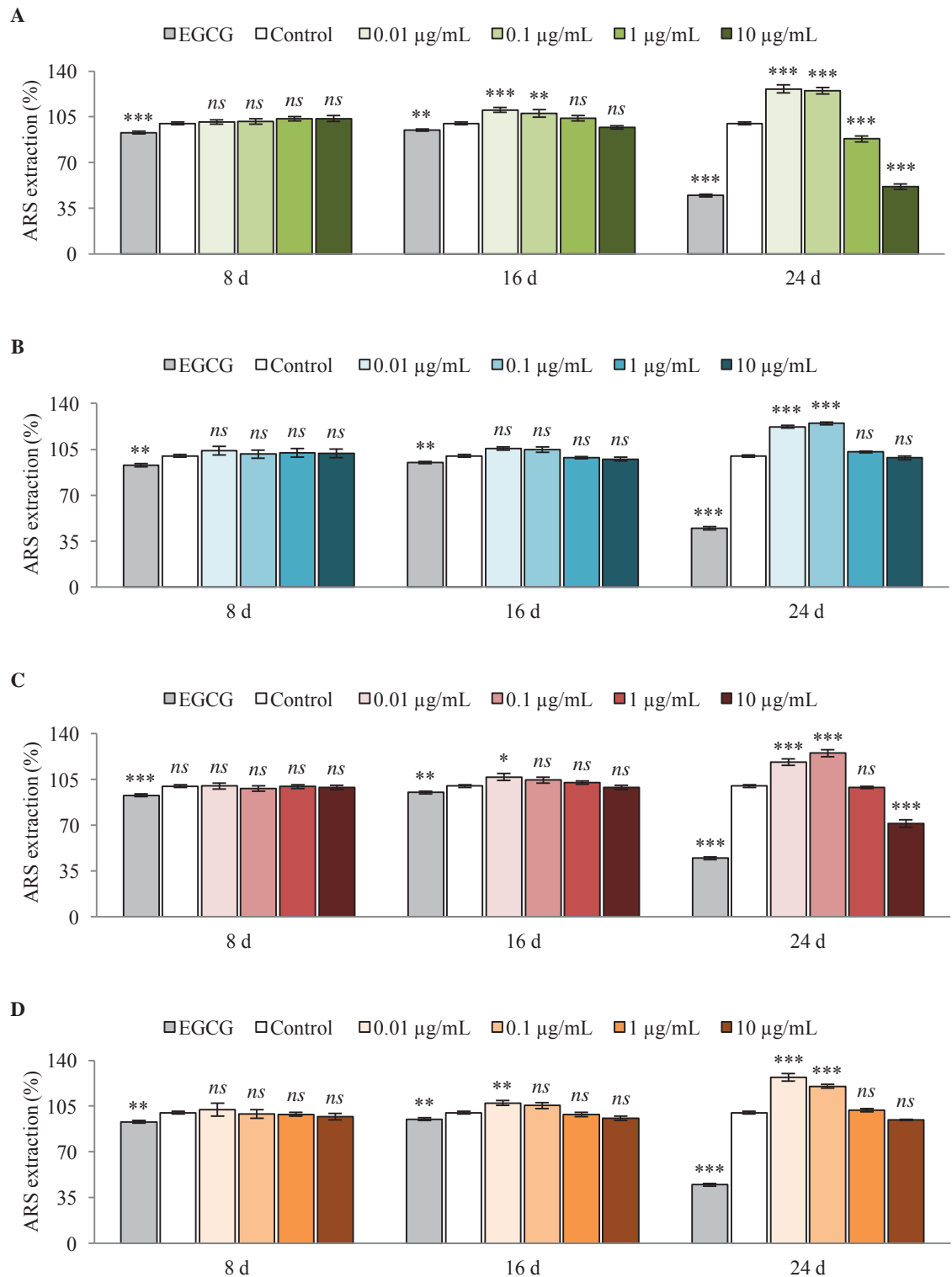


Figure 5-4. The effect of varying concentrations of tea extracts (0.01, 0.1, 1 and 10 µg/mL) and EGCG (10 µg/mL) on MC3T3-E1 osteoblastic mineralisation after 8, 16 and 24 days of incubation in osteogenic differentiation media, as measured by ARS staining. (A) Se-GTE, (B) R-GTE, (C) Se-BTE, and (D) R-BTE. Data are mean ± SEM. *ns* = not significant. *indicates significance from the zero control: *ns* = not significant, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

5.3.4 Effect of different concentrations of H₂O₂ on growth in differentiated osteoblastic MC3T3-E1 cells

Figure 5-5 shows the effect of three hours exposure to H₂O₂ (100 to 700 μM) on the cellular viability of differentiated osteoblasts ($p < 0.0001$). Addition of H₂O₂ at concentrations of 400, 500, 600 and 700 μM resulted in significant decreases in cell viability in a concentration-dependent manner ($89.8 \pm 0.9\%$, $89.2 \pm 0.7\%$, $84.0 \pm 1.4\%$ and $74.4 \pm 1.0\%$, respectively), when compared to the H₂O₂-untreated control cells ($100.0 \pm 0.5\%$, $p < 0.0001$). Interestingly, the lowest concentration of H₂O₂ at 100 μM resulted in a significant increase in the cell viability ($106.7 \pm 0.8\%$), in comparison to the H₂O₂-untreated control ($p < 0.0001$). Low concentrations of H₂O₂ at 200 and 300 μM had no effect on cell viability ($101.0 \pm 1.4\%$ and $97.0 \pm 0.9\%$, respectively), as compared to the H₂O₂-untreated control cells (p values of 0.9949 and 0.3641, respectively). Therefore, a H₂O₂ concentration at 300 μM was chosen as a safe concentration to induce oxidative stress in subsequent experiments.

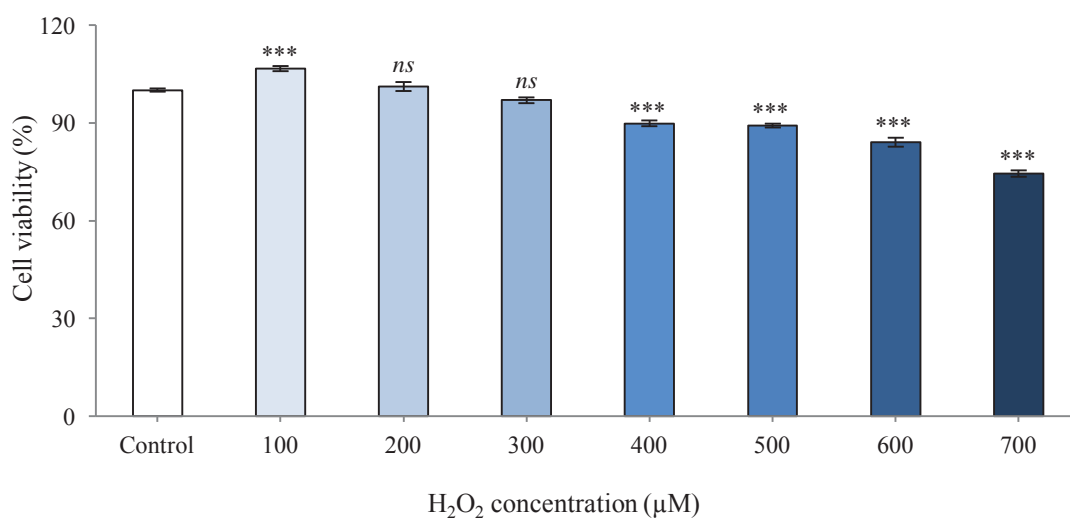


Figure 5-5. The effect of varying concentrations of H₂O₂ (100, 200, 300, 400, 500, 600 and 700 μM) on differentiated MC3T3-E1 cells after three hours of exposure, as measured by MTT assay. The cells were incubated in osteogenic differentiation media for 7 days prior to H₂O₂ incubation. Data are mean ± SEM. *indicates significance from the H₂O₂-untreated control cells (cells without exposure): *ns* = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

5.3.5 Osteoprotective effect of various tea extracts on H₂O₂-induced oxidative stress in differentiated MC3T3-E1 cells after three hours of exposure to 300 μM H₂O₂

Alkaline phosphatase (ALP) activity was analysed to determine the osteoprotective effect of various tea extracts on the response of differentiated MC3T3-E1 cells to oxidative stress induced by H₂O₂. The ALP activity of differentiated osteoblast cells treated with H₂O₂ in cells pre-treated with varying concentrations of tea extracts during differentiation is shown in **Figure 5-6**. Overall, the presence of 300 μM H₂O₂ for three hours significantly decreased ($p < 0.0001$) cellular ALP activity (0.932 ± 0.009) of differentiated osteoblasts when compared to the H₂O₂-untreated control cells (1.000 ± 0.007). Altogether, there were no significant changes in ALP activity in the differentiated osteoblasts pre-treated with EGCG (10 μg/mL) followed by H₂O₂ exposure (0.948 ± 0.008) in comparison to H₂O₂-treated control cells (0.932 ± 0.009 , $p = 0.3594$). Furthermore, EGCG administration during osteoblast differentiation in the presence of H₂O₂ did not have an effect on recovery of the ALP activity when compared to the H₂O₂-untreated cells. This was confirmed by the significant differences in the ALP activity between H₂O₂-treated cells and H₂O₂-untreated cells observed for EGCG treatment that were included as positive control in each tea treatment (Se-GTE, R-GTE, Se-BTE and R-BTE; $p = 0.0373$, 0.0192 , 0.0118 and 0.0258 , respectively).

The increased ALP activity in cells pre-treated with 0.01 μg/mL of Se-GTE (0.971 ± 0.010) followed by H₂O₂ exposure was not significantly different ($p = 0.4334$) to H₂O₂-treated control cells (**Figure 5-6A**). However, when cells were pre-incubated with Se-GTE at higher concentrations (0.1, 1 and 10 μg/mL), ALP activity significantly increased (1.006 ± 0.008 , 1.086 ± 0.020 and 1.116 ± 0.018 , respectively) compared to the H₂O₂-treated control cells ($p = 0.0007$, < 0.0001 and < 0.0001 , respectively). Interestingly, addition of the Se-GTE at all concentrations (0.01, 0.1, 1 and 10 μg/mL) followed by H₂O₂ exposure had restored the ALP activity to a level comparable to their respective H₂O₂-untreated cells (1.035 ± 0.009 , 1.075 ± 0.016 , 1.135 ± 0.008 and 1.160 ± 0.025 , respectively), made evident by the non-significant differences in the ALP activity between H₂O₂-treated cells and H₂O₂-untreated cells, as observed for each of the Se-GTE concentrations ($p = 0.0930$, 0.0521 , 0.4430 and 0.6247 , respectively).

No significant differences were found in the ALP activity of cells pre-treated with R-GTE at 0.01 (0.939 ± 0.024), 0.1 (0.964 ± 0.027) and 1 $\mu\text{g/mL}$ (0.987 ± 0.034) followed by H_2O_2 exposure (**Figure 5-6B**), as compared to H_2O_2 -treated control cells ($p = 1.000$, 0.9126 and 0.2434 , respectively). However, addition of R-GTE at 10 $\mu\text{g/mL}$ followed by H_2O_2 exposure, resulted in significantly increased ALP activity (1.010 ± 0.023 ; $p = 0.0095$) in comparison to the H_2O_2 -treated control cells. It is noteworthy that the addition of R-GTE at 0.01, 0.1 and 1 $\mu\text{g/mL}$ to the cells followed by H_2O_2 exposure also restored the ALP activity to a level comparable to their respective H_2O_2 -untreated cells (0.01, 0.1 and 1 $\mu\text{g/mL}$; 1.022 ± 0.013 , 1.044 ± 0.007 and 1.065 ± 0.016 , respectively), which was indicated by non-significant differences between H_2O_2 -treated cells and H_2O_2 -untreated cells, that were observed in each of the R-GTE concentrations ($p = 0.0730$, 0.0963 and 0.1243 , respectively). However, the addition of R-GTE at 10 $\mu\text{g/mL}$ could not provide protection to the cells from the damaging effect of H_2O_2 -induced OS, as there was a significant decrease in ALP activity when compared to cells not treated with H_2O_2 ($p = 0.0220$).

As shown in **Figure 5-6C**, there were no significant differences found in the ALP activity of cells pre-treated with Se-BTE concentrations at 0.01 (0.939 ± 0.018) and 0.1 $\mu\text{g/mL}$ (0.879 ± 0.021) followed by H_2O_2 exposure, as compared to H_2O_2 -treated control cells ($p = 1.000$ and 0.2736 , respectively). However, ALP activity significantly decreased in cells pre-treated with Se-BTE at higher concentrations, compared to H_2O_2 -treated control cells (1 and 10 $\mu\text{g/mL}$; 0.862 ± 0.024 and 0.828 ± 0.026 ; $p = 0.0317$ and $p < 0.0001$, respectively).

From the data shown in **Figure 5-6D**, no significant differences were also found in the ALP activity of cells pre-treated with R-BTE concentrations at 0.01 (0.876 ± 0.023), 0.1 (0.902 ± 0.013) and 1 (0.884 ± 0.013) $\mu\text{g/mL}$ followed by H_2O_2 exposure, as compared with H_2O_2 -treated control cells ($p = 0.9859$, 0.4739 and 0.7144 , respectively). Meanwhile, ALP activity significantly decreased in cells pre-treated with R-BTE at 10 $\mu\text{g/mL}$ in comparison to the H_2O_2 -treated control cells (0.765 ± 0.034 , $p < 0.0001$).

In contrast to the significant reduction in ALP activity in H₂O₂-treated control cells when compared to H₂O₂-untreated control cells, the addition of Se-BTE and R-BTE (0.01, 0.1, 1 and 10 µg/mL) followed by H₂O₂ exposure demonstrated no significant change to ALP activity (Se-BTE: $p = 0.5952, 0.0732, 0.3039$ and 0.6712 , respectively; R-BTE: $p = 0.3216, 0.5266, 0.9997$ and 1.000 , respectively). This indicates that black teas were surprisingly as capable as green tea to recover the ALP activity reduction in the osteoblasts following exposure to H₂O₂-induced oxidative stress. It is important to note that pre-treatment of the osteoblast cells with 1 and 10 µg/mL of Se-BTE (0.928 ± 0.023 and 0.880 ± 0.013 ; $p = 0.0198$ and < 0.0001 , respectively) and R-BTE (0.908 ± 0.018 and 0.753 ± 0.051 ; $p = 0.0115$ and < 0.0001 , respectively) during the period of differentiation without exposure to H₂O₂, caused a significant decrease in ALP activity when compared to the H₂O₂-untreated control cells. The results of both black teas demonstrated a trend towards a concentration-dependent decrease of ALP activity that were similar to the results of our previous investigation on ALP activity as shown in **Figures 5-2C** and **5-2D**. The unfavourable effect of both black teas (Se-BTE and R-BTE) on ALP activity of osteoblasts, however, was opposite to the enhancing effect of the green teas (Se-GTE and R-GTE) in ALP activity of the osteoblasts.

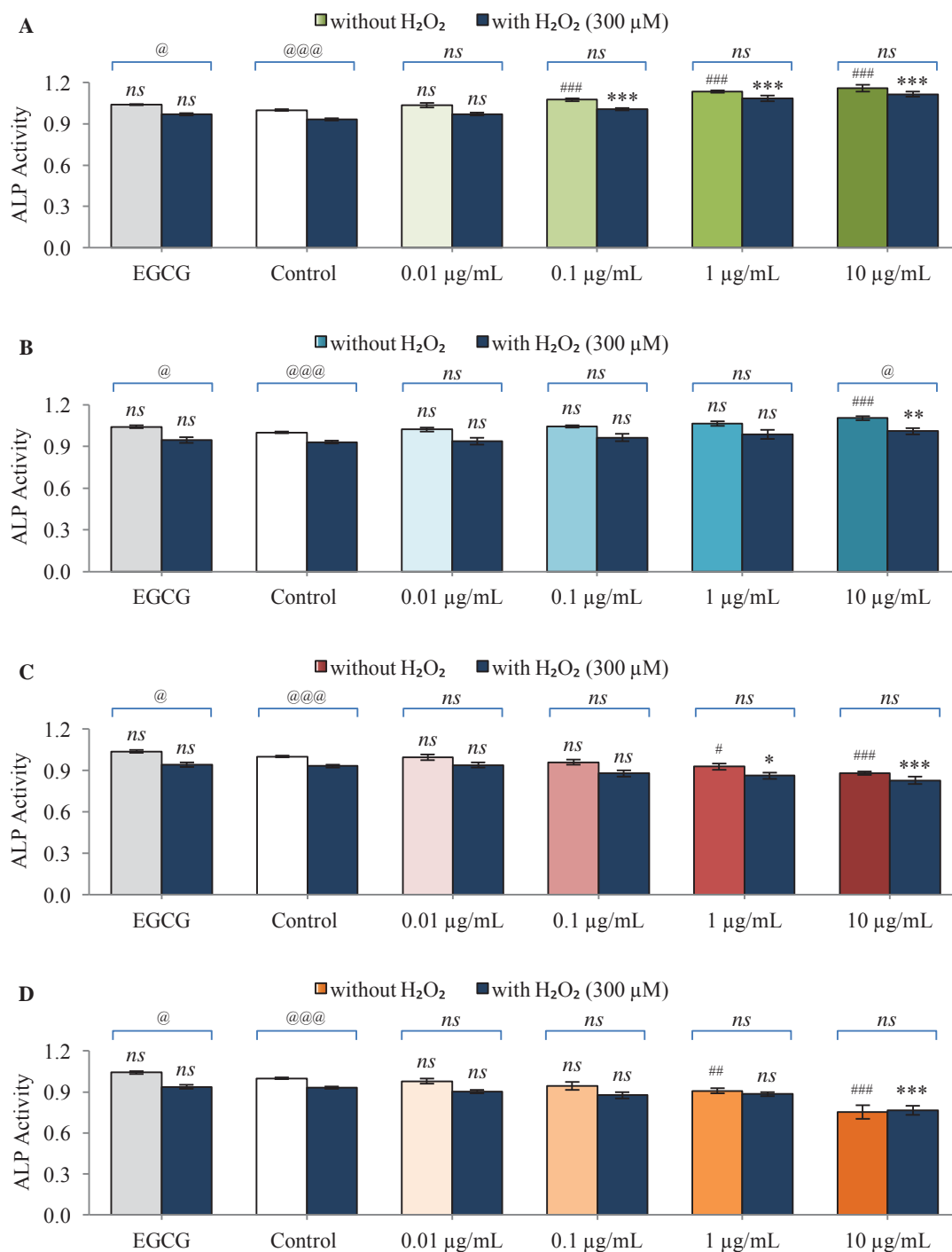


Figure 5-6. The osteoprotective effect of various tea extracts on the ALP activity of differentiated MC3T3-E1 cells with/without the presence of 300 µM H₂O₂. The cells were incubated in osteogenic differentiation media and pre-treated with/without varying tea concentrations for 7 days prior to H₂O₂ incubation. (A) Se-GTE, (B) R-GTE, (C) Se-BTE, and (D) R-BTE. Data are mean ± SEM. # indicates significance from the H₂O₂-untreated control. * indicates significance from the H₂O₂-treated control. @ H₂O₂-treated cells versus H₂O₂-untreated cells. Statistical analysis was conducted by nested ANOVA followed by post-hoc Tukey's test: ns = not significant, #/*/@ p < 0.05, ##/**/@@ p < 0.01, ###/***/@@@ p < 0.001).

5.4 Discussion

5.4.1 Effects of various tea extracts on the proliferation, differentiation and mineralisation of pre-osteoblastic MC3T3-E1 cells

The present study demonstrates that all tea extracts had different effects on cell proliferation of MC3T3-E1 (Subclone 4) osteoblasts in concentration- and time-dependent manners. The results of this study suggest that the addition of varying concentrations of green tea extracts had no effect on cell growth when measured at 24 and 48 hours, except for the significant decrease of growth in those cells incubated with the highest concentration at 10 µg/mL. Our results are in accordance with the findings of Vester et al. (2014), who reported that 24-hour administration of green tea extract Sunphenon LG90 (GTE) at low concentrations (0.01 to 1 µg/mL) to primary human osteoblasts had no detectable effects on the cell viability, but high concentrations (100 and 200 µg/mL) significantly reduced the viability of cells as measured by the MTT assay. However, this is contrary to the recent findings of Nash and Ward (2016), who indicated that green tea aqueous extract (normalised to 1 and 10 µg polyphenols/mL culture media) positively influenced viability of human osteogenic sarcoma (SaOS-2) cells in time- and concentration-dependent manners following 24 and 48 hours of incubation. In contrast to green tea, black tea treatments in the present study had no effect on cell proliferation at 24 and 48 hours, even at the highest concentration (10 µg/mL). However, at 72 hours, decreased proliferation of cells incubated with high tea concentrations was observed for all teas used in the study. Again, the present finding is contrary to Nash and Ward (2016), who found that black tea aqueous extracts (i.e. English Breakfast and Golden Monkey brands) increased viability of human SaOS-2 cells after 24 and 48 hours of incubation following administration at 1 and 10 µg polyphenols/mL, as assessed using MTT assay.

Epigallocatechin-3-gallate (EGCG) is the most abundant tea catechin and also the main bioactive component in green tea with high antioxidant activity, but is present in smaller amounts in black tea (Sutherland et al., 2006). In the present study, the proliferation of osteoblast cells was increased when treated with this pure phenolic compound at 10 µg/mL for 24 to 48 hours. Recent studies conducted by Jin et al. (2014,

2015) also showed significantly increased proliferation of cells that were grown both in osteogenic and non-osteogenic media in concentration- and time-dependent manners following administration of EGCG. However, it is important to note that the authors used lower EGCG concentrations (2.5, 5 and 10 μM); these were administered during an incubation period between 3 to 21 days to a primary cell line (human bone-marrow-derived mesenchymal stem cells, i.e. hBMSCs) that has slower rate of growth than commercially obtained cell lines (Sengers et al., 2007; Pendleton et al., 2013). Further, Liu et al. (2016) also reported an increase in the viability of hBMSCs after administration of 5 μM EGCG for 5 days. On the contrary, Peng et al. (2016) found no changes in viability of human osteoblast-like MG-63 cells after 3 days of incubation with EGCG at concentrations between 7.5 and 30 μM . Apart from EGCG, there is another type of tea compound, i.e. (+)-catechin, that has been previously reported to increase the viability of MC3T3-E1 cells which was evaluated using the MTT assay after 48-hours exposure to concentrations ranging between 10^{-10} to 10^{-4} M (Choi & Hwang, 2003). However, the present finding of the EGCG is in contrast to those of other authors. For example, Takai et al. (2008) found that viability and proliferation of MC3T3-E1 cells were not affected after 24-hour incubation with 30 μM EGCG, which was assessed using the trypan blue dye exclusion assay. More recently, Kamon et al. (2010) reported that EGCG (1 to 20 μM) exposure for 48 hours did not significantly affect the viability and proliferation of MC3T3-E1 cells when measured using MTT assay and bromodeoxyuridine (BrdU) proliferation assay. The result of the present study is also different to the findings of the study conducted by Chen et al. (2005b), who found a significant inhibitory effect of EGCG (1 and 10 $\mu\text{mol/L}$) on the proliferation of a murine bone marrow mesenchymal stem cell line (D1), when exposed to EGCG for 24 hours and evaluated using the thymidine incorporation assay. On the other hand, the effect of EGCG in the present study resulted in a marked reduction in osteoblast proliferation after a 72-hour incubation period, a response that was similarly observed in cells treated with various tea extracts under the same incubation period. Based on the present results, it is concluded that the inhibitory effect of tea and EGCG on MC3T3-E1 cells was most profound after a 72-hour incubation, suggesting a possible cytotoxic effect of tea and EGCG on cell proliferation at high concentrations and after prolonged incubation.

In the present study, differentiation of MC3T3-E1 cells into functional mature osteoblasts was induced, by the addition of ascorbic acid and β -glycerophosphate into the cell culture medium (Quarles et al., 1992). The phenotype of functional mature osteoblasts is characterised by their ability to synthesise alkaline phosphatase, followed by mineralisation of the bone extracellular matrix (Neve et al., 2010). Alkaline phosphatase (ALP) is an important enzyme in the early stages of osteoblast differentiation that plays a role in bone mineralisation, and is used as a biomarker of the osteoblastic phenotype (Bellows et al., 1991; Golub & Boesze-Battaglia, 2007). In this study, the results of the ALP enzyme activity assays showed differences in the response of the MC3T3-E1 osteoblasts to the various teas. Both green teas (Se-GTE and R-GTE) enhanced osteoblast differentiation, as indicated by the significant increase in ALP activity at 10 $\mu\text{g}/\text{mL}$ concentration. Most importantly, Se-GTE and R-GTE demonstrated a distinct concentration-dependent enhancement of osteoblast differentiation, although the enhancing effect at lower concentrations (0.01 to 1 $\mu\text{g}/\text{mL}$) was not significant. This finding is in agreement with the findings of Ko et al. (2011), which showed significant increases of ALP activity in a concentration-dependent manner upon the addition of green tea extract (10 to 50 $\mu\text{g}/\text{mL}$) to rat mesenchymal stem cells (MSCs) after 7 days of incubation. On the other hand, both black teas (Se-BTE and R-BTE) tested in the present study demonstrated an inhibitory effect on osteoblast differentiation, even though the effects shown by R-BTE concentrations were found to be non-significant. These results showed that irrespective of selenium content, green tea extracts stimulated osteoblast differentiation, which may indicate an ability to stimulate bone-forming activity, whereas black teas suppressed the differentiation of murine pre-osteoblastic MC3T3-E1 cells. However, the present study do not support the recent report by Nash and Ward (2016), which indicated that all green and black tea aqueous extracts (normalised to 1 μg polyphenols/ mL culture media) investigated in their study have greatly increased ALP activity in human SaOS-2 cells following 3 and 7 days of incubation. Whilst previous reports have shown that green tea polyphenols can stimulate differentiation of osteoblast cells, the effect of whole green tea water extracts on osteoblast differentiation in MC3T3-E1 cells has never been performed. To the best of our knowledge, no such work has also been conducted for the black tea water extract or its bioactive compounds. Accordingly, no comparison to published work is possible. Therefore, the underlying mechanism behind the stimulatory or

inhibitory effect of these tea extracts on osteoblast differentiation is unknown and should be further investigated.

The cellular effects of EGCG at 10 µg/mL against osteoblastic differentiation of MC3T3-E1 cells resulted in an increase in ALP activity after 9 days of incubation, although this was not statistically significant. Thus, our results show that the effects of green tea extracts (consisting of a complex mixture of polyphenolic compounds) were more potent than that of EGCG (major green tea catechin), in terms of the osteoblast differentiation. The present result of EGCG on osteoblast differentiation corroborates the findings of Ko et al. (2011), who reported that EGCG (20 µM) showed no effect on ALP activity in rat bone marrow-derived MSCs, which was measured after 7 days of incubation. The present result of EGCG, however, is not in agreement with the findings of Kamon et al. (2010) and Zhao et al. (2014), which indicated that EGCG (10 µM) significantly reduced both the number of ALP-positive cells, and ALP activity during differentiation of osteoblastic MC3T3-E1 cells when measured after 10 days of incubation. In addition to this, an earlier study conducted in 2001 by Yamaguchi and Jie also demonstrated a significant reduction in ALP activity in rat femoral tissue *in vitro* following 24-hours incubation with 10^{-4} M EGCG, whereas no alterations in ALP activity was observed when the tissue was incubated for a similar time period with another type of tea catechin, i.e. (+)-catechin at concentrations ranged between 10^{-7} to 10^{-4} M. The present result of EGCG is also contrary to the increase in ALP activity after EGCG treatments (1 and 10 µmol/L) on mesenchymal D1 stem cells and differentiated human osteoblast-like SaOS-2 cells (EGCG concentrations at 1 to 5 µM), as previously studied by Chen et al. (2005b) and Vali et al. (2007), respectively. Additionally, Choi and Hwang (2003) described an increase of ALP activity in MC3T3-E1 cells when incubated for 48 hours in cell culture media containing (+)-catechin at 10^{-5} M. The study findings of Jin et al. (2014) also showed significant increase in ALP activity following administration of various concentrations of EGCG (2.5, 5 and 10 µM) to human primary bone-marrow-derived mesenchymal stem cells (hBMSCs), after 7 days of incubation. Likewise, recent study by Peng et al. (2016) demonstrated a concentration-dependent increase in the ALP activity of human MG-63 cells following 4-day incubation with EGCG at various concentrations (7.5 to 30 µM). In addition, therapeutic effect of another major green tea catechin, (-)-epicatechin-3-gallate (ECG)

has been revealed by different researchers for its promising stimulation effect on osteoblast differentiation in a concentration-dependent manner; the ALP activity was assessed in rat UMR-106 cells (5 to 20 μM) and in murine mesenchymal cell line C3H10T1/2 (1 to 10 μM) after certain days of treatment, i.e. 4 days and 2 to 6 days, respectively (Ko et al., 2009; Byun et al., 2014).

Cellular bone mineralisation is characterised by the formation of mineralised bone nodules resulting from extracellular calcium deposition by functional mature osteoblasts. This effect indicates the final stages of osteoblast differentiation that can be analysed and quantified by direct staining using Alizarin Red S (ARS). The addition of various teas did not have any effect or show any visual evidence on mineralisation after 8 days of incubation in comparison to the control, but only when observed at 16-days incubation. These results mirror those of the previous studies that the MC3T3-E1 cells are differentiated into mature osteoblast phenotype after 14 days incubation with ascorbic acid and β -glycerophosphate (Yazid et al., 2010), as the osteoblasts enter the terminal differentiation stage where extracellular matrix mineralisation is produced (Hoemann et al., 2009). Following 24-days incubation, all teas affected the cell mineralisation with a biphasic effect, whereby Se-rich teas indicated significantly elevated mineralisation in the cells at low concentrations and significantly decreased mineralisation at high concentrations. In contrast to our findings, Nash and Ward (2016) have recently reported that green tea aqueous extract (normalised to 1 and 10 μg polyphenols/mL culture media) positively influenced matrix mineralisation in human SaOS-2 cells when determined using ARS staining assay after 7 days of incubation. Under similar experimental settings, the authors also found that both black tea aqueous extracts used in their study (English Breakfast and Golden Monkey brands) increased mineralisation only at low concentrations (1 μg polyphenols/mL); which is consistent with our findings where black teas demonstrated a concentration-dependent biphasic effect on extracellular matrix mineralisation.

In contrast to the results of the whole tea extracts, EGCG at 10 $\mu\text{g}/\text{mL}$ significantly decreased the mineralisation of osteoblasts after each of the incubation periods (8, 16 and 24 days), as significantly observed in **Figure 5-4**. Since all tea extracts at all concentrations have no influence on cellular mineralisation after 8 days of

incubation, it is suggested that mineralisation after a short incubation period is suppressed specifically by EGCG alone. This could be due to the nature of tea extracts as a complex mixture of polyphenolic compounds that may synergistically suppress the specific effect of any EGCG compound that they may contain (Ulrich-Merzenich et al., 2010). Moreover, the tea extracts only showed significant inhibition of mineralisation at higher concentrations (1 and 10 $\mu\text{g/mL}$) after a long incubation period (24 days). Thus, it is suggested that the concentration of EGCG (10 $\mu\text{g/mL}$) as a pure concentrated single compound is incomparably higher than whole tea extract at similar concentration; since its presence in tea is only as a part of a whole mixture of compounds (Rusak et al., 2008; Rosen, 2012; Yang & Wang, 2013). The present results showing EGCG does decrease osteoblast mineralisation is in contrast to the findings reported by Kamon et al. (2010), which reported that EGCG at 1 to 10 μM had no effect on mineralisation of MC3T3-E1 cells after 10 days of incubation. However, it is important to note that their study was conducted by treating the differentiated osteoblasts with EGCG; a method that was different from the present study where EGCG was added into the osteogenic differentiation media at the start of the experiment. Meanwhile, Vali et al. (2007) demonstrated increased formation of mineralised bone nodules in differentiated human SaOS-2 osteoblasts after 7 days of incubation with EGCG (1 to 5 μM). Additionally, a recent report by Peng et al. (2016) indicated a concentration-dependent increase in matrix mineralisation of human MG-63 cells following treatment with EGCG at various concentrations (7.5 to 30 μM) for 6 days. The increase of mineralisation due to treatment with various tea catechins was also observed in other types of cell line, such as murine stem cells, human stem cells, deer antler stem cells, human alveolar bone cells, primary rat osteoblasts and dedifferentiated fat cells (Chen et al., 2005b; Bickford et al., 2006; Mount et al., 2006; Jin et al., 2014; Mah et al., 2014; Zeng et al., 2014; Kaida et al., 2015).

In the literature, many cellular studies investigating the osteogenic effect of tea were performed on a variety of cell lines, and have used single compound rather than whole tea extract. To date, data on the *in vitro* osteogenic effect of tea water extracts and/or its polyphenol compounds remain scarce and inconclusive, thus the obtained results showing the effects of tea water extracts and EGCG on MC3T3-E1 cells need careful interpretation. The present study is the first to evaluate the effect of tea extracts

on proliferation, differentiation and mineralisation in MC3T3-E1 cells. There are two theories that could be associated with the decrease of proliferation and mineralisation in MC3T3-E1 cells following prolonged treatment with tea extracts at high concentrations and EGCG at 10 µg/mL.

Firstly, it is suggested that the undesirable decrease of proliferation and mineralisation in cells after prolonged treatment with high tea concentrations are due to excess ROS formation affecting cell growth and function, which was tightly dependent upon tea concentration. Accumulating evidence suggests that tea supplemented at high concentrations can either trigger its pro-oxidant nature and further increase ROS production (Bouayed & Bohn, 2010; Forester & Lambert, 2011; Kapetanovic, 2013), or disrupt the cellular redox homeostasis and leads to cellular dysfunction (Martin & Barret, 2002). Tea polyphenols have been reported to exert antioxidant properties at low concentrations, and pro-oxidant properties at high concentrations *in vitro* (Bouayed & Bohn, 2010; Yordi et al., 2012; Kapetanovic, 2013). The pro-oxidant effect of tea may be due to high concentration of catechins having strong reducing power (reduce Fe³⁺ to Fe²⁺) that can generate ROS (Forester & Lambert, 2011) either by autoxidation (Sang et al., 2007; Ishii et al., 2008), or through redox-cycling process involving the Fenton reaction (the main biochemical source of highly reactive hydroxyl radicals) in the presence of metal ions (Hayakawa et al., 2004). Several tea polyphenolic compounds that have been reported to act as pro-oxidants include (but not limited to) epigallocatechin-3-gallate (EGCG), epicatechin (EC), theaflavin-3-gallate, theaflavin-3'-gallate, theaflavin-3,3'-digallate, gallic acid, caffeine, and quercetin (Azam et al., 2003, 2004; Galati et al., 2006; Robaszkiewicz et al., 2007; Babich et al., 2008; Schuck et al., 2008; Kim et al., 2014). Nevertheless, limited information has been reported for black tea and its compounds acting as pro-oxidants (Babich et al. 2008; Schuck et al., 2008; Babich et al., 2013), with Yen et al. (1997) finding weak *in vitro* pro-oxidant effects of black tea extracts at low concentrations on DNA when compared to green, pouchong and oolong tea.

Secondly, some researchers have speculated that tea catechin compounds, especially EGCG are unstable under *in vitro* cell culture conditions, thus are subjected to autoxidation resulting in the formation of ROS that may be responsible for the

inhibition of cellular growth and function (Buoayed & Bohn, 2010; Forester & Lambert, 2011; Sang et al., 2011). The stability of EGCG *in vitro* is influenced by several factors such as pH, temperature, oxygen levels, antioxidant levels, metal ions, EGCG concentration, and the presence of other tea compounds (Sang et al., 2005). Furthermore, it has been suggested that the EGCG-induced ROS formation was not only concentration-dependent, but also cell-type dependent which was due to the degree of cellular sensitivity towards EGCG, and different regulation of cellular signalling pathways (Yang et al., 2000; Hsu et al., 2001; Yamamoto et al., 2004; Kim et al., 2014).

The conflicting reports regarding the *in vitro* effect of EGCG on pre-osteoblast MC3T3-E1 cells obtained from the present study in comparison to other published research, could be due to the use of different methodological approaches including cell seeding density, osteogenic differentiation media, different sources of EGCG, range of EGCG concentrations, and either EGCG preparation or its storage conditions (Golden et al., 2009; Jin et al., 2014). Moreover, it has been suggested that the physiological functions exerted by EGCG are highly varied depending upon type of cell lines (with varied sensitivity towards EGCG) and optimal physiological doses of EGCG at different cell stages (Hsu et al., 2001; Yamamoto et al., 2004; Zaveri, 2006; Golden et al., 2009; Jin et al., 2014; Kim et al., 2014).

Overall, the present results of proliferation and differentiation of MC3T3-E1 cells indicate that long-term *in vitro* treatment of differentiating MC3T3-E1 osteoblastic cells with various teas at high concentrations can either increase or decrease ALP activity of the osteoblasts, in spite of the teas inhibitory effect on proliferation. This effect is highly dependent on the tea concentration and is irrespective of selenium content. Meanwhile, incubation with high concentrations of the tea extracts suppressed mineralisation of the osteoblasts after 24 days. It is noteworthy that low concentrations of the tea extracts significantly enhanced mineralisation after 24 days of incubation, although these concentrations did not exert any effect on the proliferation and differentiation of the osteoblasts.

5.4.2 Proliferation of differentiated MC3T3-E1 cells in response to H₂O₂-induced oxidative stress

H₂O₂ was used in this study as an exogenously added reactive oxygen species (ROS) to induce oxidative stress (OS) in MC3T3-E1, a method based on previous work by Fatokun et al. (2006). Moreover, Mody et al. (2001) also showed that H₂O₂ caused an increase in cellular OS levels in MC3T3-E1 cells as measured using a cell permeable dye, i.e. 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), which eventually caused inhibition of osteoblast differentiation and mineralisation. In the present study, hydrogen peroxide (H₂O₂) concentrations of 400 µM and higher significantly reduced cell viability in a concentration-dependent manner. This finding is in line with Li et al. (2009d) who reported that cell proliferation of murine MC3T3-E1 and human MG63 cells was affected by H₂O₂ in a concentration- (5 to 200 µM) and time- (24, 48 and 72 hours) dependent manner. Interestingly, H₂O₂ at 100 µM concentration tended to cause a significant increase in viable cell numbers. However, H₂O₂ at 400 µM decreased the numbers of viable cells.

This finding confirms earlier reports by Fatokun et al. (2006), who demonstrated that H₂O₂ concentrations lower than 100 µM are generally stimulatory to growth of differentiated MC3T3-E1 cells, whilst concentrations of 200 µM and upwards have a significantly lethal effect on the cells. Further, ROS have also been reported for their role in stimulating growth in MC3T3-E1 cells by stimulating the expression of early growth-regulated genes such as *c-fos* and *c-jun*, suggesting that ROS might stimulate mitogenesis in bone cells via growth factor-signalling pathways (Burdon, 1995; Gough & Cotter, 2011). Indeed, ROS also function as signalling messengers in receptor-mediated signalling pathways (Dröge, 2002). Since a H₂O₂ concentration of 300 µM caused no significant decrease in cellular proliferation of the differentiated osteoblast MC3T3-E1 cells, this concentration was used to investigate the ALP activity of differentiated osteoblasts pre-treated with tea extracts in response to OS.

5.4.3 Osteoprotective effect of various tea extracts on differentiated MC3T3-E1 cells in response to H₂O₂-induced oxidative stress

Other studies have used higher concentrations and longer time periods for H₂O₂ exposure to initiate oxidative stress in cells (Mody et al., 2001; Park et al., 2005). However, recent studies by Fatokun et al. (2006) and Arai et al. (2007) have shown that three hours exposure to similar doses of H₂O₂ were enough to induce oxidative stress, without causing cell death that may eventually cause misleading results in the differentiation biomarker evaluation. Therefore in this study, differentiated osteoblasts were treated with H₂O₂ at 300 µM for three hours to induce oxidative stress (OS). The H₂O₂ treatment inhibited differentiation of the osteoblast cells as shown by the significant reduction of the early differentiation biomarker, alkaline phosphatase (ALP) activity in H₂O₂-treated control cells relative to the H₂O₂-untreated control cells. This study finding matches those observed in earlier studies that demonstrated a decrease in ALP activity after H₂O₂ exposure (Lee et al., 2006b; Arai et al., 2007).

The present study showed for the first time that pre-treatment of differentiated MC3T3-E1 cells with green tea extracts (Se-GTE and R-GTE) not only benefits the differentiated osteoblasts by enhancing ALP activity important for mineralisation (Golub & Boesze-Battaglia, 2007), but could also provide concentration-dependent protection to the cells from the suppression of ALP activity due to oxidative stress following H₂O₂ exposure. Many studies on modulation of the oxidative stress response by tea polyphenols have been performed in different *in vitro* models such as hepatic cells (Fernández-Iglesias et al., 2013), microvascular endothelial cells (Rah et al., 2005), bladder urothelial cells (Coyle et al., 2008), colorectal epithelial cells (Rodríguez-Ramiro et al., 2011; Kalaiselvi et al., 2013), and myoblast cells (Sivakumar & Hwang, 2015), but little is known with regards to osteoblastic cells. The only data available for osteoblastic cells are from Park et al. (2003), Ehnert et al. (2012), Holzer et al. (2012) and Vester et al. (2014). According to Park et al. (2003), green tea polyphenols protect rat calvarial osteoblast cells against oxidative stress-induced toxicity by preserving the viability of cells following 24 hours of H₂O₂ exposure at different concentrations. Meanwhile, both Ehnert et al. (2012) and Holzer et al. (2012) suggested green tea extract (GTE) and/or catechins may protect primary human

osteoblasts from smoke-induced oxidative injury after four hours incubation with GTE (three different settings of incubation conditions: pre- (prophylactic), co- (acute), and post- (therapeutic)) by increasing cell viability and inhibiting ROS formation in a concentration-dependent manner. A recent study by Vester et al. (2014) found that four-hour stimulation of GTE at high doses exerted osteoprotective effects on cell viability following H₂O₂-induced oxidative stress in primary human osteoblasts, as performed in three different experimental settings including pre-incubation, co-incubation, and post-incubation of the GTE. Furthermore, GTE administration at low concentrations under long-term oxidative stress conditions in primary human osteoblasts significantly enhanced matrix mineralisation and increased the expression of pro-osteogenic genes, as evident after 21 days of osteogenic differentiation with concurrent treatment of 50 µM H₂O₂ (Vester et al., 2014).

In response to H₂O₂-induced oxidative stress, the bone-stimulatory effect on ALP activity shown by differentiated MC3T3-E1 cells pre-treated with green teas was not seen in those cells pre-treated with black tea. In fact, the present study showed that pre-treatment of differentiated osteoblasts with the black teas (Se-BTE and R-BTE) alone without H₂O₂ exposure caused a significant concentration-dependent reduction in ALP activity. Although the black teas seem to exert a reversal effect on the reduction of ALP activity due to oxidative stress, the protective role of black teas against the suppression of ALP activity in differentiated osteoblasts exposed to oxidative stress is uncertain, which may be mediated by a different mechanism compared to the green teas.

There is little evidence regarding the *in vitro* effect of black tea on bone health. Oka et al. (2012) recently reported that the black tea polyphenol (theaflavin-3,3'-digallate) inhibited formation and differentiation of bone-resorbing osteoclasts *in vitro*. In regards to oxidative stress-related studies of black tea, Das et al. (2009) demonstrated that black tea extracts have both a protective and a restorative role against ovariectomy-induced oxidative stress of mononuclear cells in female albino rats and the progression of bone loss. This was evident by the reduced number of active osteoclasts in the decalcified proximal tibia excised from the ovariectomised rats following 28-days oral treatment with 2.5% aqueous black tea extract when compared to the ovariectomised

rats without treatment, as assessed through histochemical and histomorphometric analyses. This positive effect of a black tea extract, in part, was directly linked with the reduced oxidative stress of the mononuclear cells (Das et al., 2009). A previous study by Letchuomy et al. (2007) revealed the protective effect of Polyphenon B, a mixture of black tea polyphenols against 7,12-dimethylbenz[a]-anthracene (DMBA)-induced genotoxicity and oxidative stress by increasing bone marrow antioxidant defence mechanisms in male Syrian hamsters. According to Letchuomy et al. (2007), the protective role of Polyphenon B against DMBA-induced OS was reflected by a significantly reduced both the frequency of bone marrow micronuclei and the extent of lipid peroxidation in bone marrow plasma, thought to be due to the significant enhancement of antioxidant status following four weeks of Polyphenon B supplementation at 0.05% in the diet.

To the best of our knowledge, *in vitro* effects of black tea on bone metabolism in osteoblast cells or against oxidative stress-induced in osteoblasts has never been reported. Thus, the mechanism responsible for the effect of black teas on osteoblast cells in response to oxidative stress is not clear and poorly understood. The present findings suggests that green tea extracts used in the study may be capable to improve bone recovery under pathologic conditions due to oxidative stress. Green tea appeared to be superior to black tea due to its ability to increase the antioxidant capacity and decreased oxidative stress as previously suggested by Shen et al. (2008). It is also suggested that different types of tea may have different anabolic efficacy on the osteoblasts and also may exert their protective effect by different mechanisms (Das et al., 2009; Shen et al., 2009, 2013; Shen & Chyu, 2013).

5.5 Conclusions

The present study demonstrates that all teas used in the study can stimulate bone formation by enhancing mineralisation in MC3T3-E1 cells, indicating an anabolic effect. All tea extracts, when used in low concentrations, were not cytotoxic to the MC3T3-E1 pre-osteoblasts, and were able to stimulate mineralised bone nodule formation under normal conditions. Furthermore, under oxidative stress-induced conditions, the green tea extracts were capable of protecting and restoring the cells against the dysfunctional effects of H₂O₂-induced oxidative stress during cell differentiation into mature osteoblasts. All these *in vitro* findings are irrespective of the levels of selenium content, and are in a time- and concentration-dependent manner. There is limited information available regarding the effect of tea or its polyphenol compounds on osteoblast cells. Additionally, most *in vitro* studies of tea use individual phenolic compounds, but not tea as a common beverage mixture. Due to the lack of literature on the osteogenic and osteoprotective effect of tea as a whole mixture of compounds, the present findings are difficult to place in the literature. Although all tea extracts used in the present study may be good candidates for the protection against oxidative stress-induced dysfunction in osteoblasts, the inhibitory effect of the black teas (Se-BTE and R-BTE) on the differentiation of osteoblasts is unusual and the mechanism need to be characterised. Also, the mechanism by which black tea acts on osteoblast differentiation appears to be different from that of green tea. In summary, our results provide preliminary cellular evidence to support the osteogenic and osteoprotective properties of the tea extracts used in the study. However, further studies of the effect of the tea extracts on bone-resorption cells are required to provide useful information on the bone-protective effects of the teas.

CHAPTER 6

THE EFFECT OF VARIOUS TEA EXTRACTS ON RANKL-INDUCED OSTEOCLASTOGENESIS IN MURINE MACROPHAGE RAW 264.7 CELLS

Part of this chapter was presented at the International Conference on Antioxidant and Degenerative Diseases, 3 to 4 June, 2015, Kuala Lumpur, Malaysia.

Abstract

Osteoclasts are bone-resorbing cells that are crucial for bone resorption. Therapeutic strategies of degenerative bone diseases such as osteoporosis are now focusing on reducing osteoclast generation and activity by decreasing osteoclast differentiation and its bone-resorption activity. Teas made from the plant *Camellia sinensis* are widely considered to be safe for long-term consumption and have been extensively studied for their purported bone protective effects. However, the effect of selenium-rich teas on osteoclasts has never been investigated. In the present study, selenium-rich green tea (Se-GTE), selenium-rich black tea (Se-BTE), regular green tea (R-GTE) and regular black tea (R-BTE) were investigated for their effect on osteoclastogenesis using murine RAW 264.7 cells, a macrophage cell line that can be stimulated to differentiate into a bone resorbing osteoclast cell when incubated in the presence of receptor activator of nuclear factor kappa beta ligand (RANKL). The anti-osteoclastogenic effects of different teas on osteoclast differentiation and formation were determined by treating osteoclast precursor cells with various tea concentrations for six days. Osteoclastogenesis was assessed by measuring tartrate-resistant acid phosphatase (TRAP) activity in RANKL-treated RAW 264.7 cells, and the number of TRAP-positive osteoclasts with five or more nuclei were quantified using a microscope. All teas effectively suppressed RANKL-induced osteoclastogenesis in a concentration-dependent manner, with significant effects observed at the higher concentrations (1 to 10 $\mu\text{g/mL}$). The positive control, (-)-epigallocatechin-3-gallate (20 μM) inhibited osteoclastogenesis in RANKL-treated cells, but simultaneously initiated osteoclastogenesis in the absence of RANKL stimulation. The black teas (Se-BTE and R-BTE) suppress osteoclastogenesis more effectively than the green teas (Se-GTE and R-GTE) at concentrations as low as 0.01 $\mu\text{g/mL}$. The antiosteoclastogenic effect showed by all the tested teas are irrespective of their selenium content. Taken together, the present results demonstrate for the first time the anti-osteoclastogenic effects of Se-rich green and black teas (Se-GTE and Se-BTE) in comparison to regular green and black teas (R-GTE and R-BTE), as demonstrated by the decrease in TRAP activity and suppression in TRAP⁺ OCL formation from macrophage precursor RAW 264.7 cells. The data suggest that these teas may serve as effective nutritional factors to reduce bone loss and possibly prevent the development of osteoporosis.

6.1 Introduction

Bone is a dynamic tissue that is constantly being formed and resorbed throughout life by means of remodelling process, that is essential for the achievement and maintenance of peak bone mass (Raggat & Partridge, 2010). Bone mass is maintained through the complementary actions of bone-resorbing activity by activated osteoclasts, and the subsequent synthesis of new extracellular bone matrix by osteoblasts (Boyle et al., 2003). Osteoclasts are giant multinucleated cells developed from haemopoietic stem cells and are formed by fusion of the monocyte-macrophage cell line. The main function of the osteoclast is to degrade mineralised extracellular bone matrix. The bone-forming osteoblasts are involved in osteoclast development as they produce two major cytokines, i.e. receptor activator of nuclear factor kappa beta ligand (RANKL) and macrophage-colony stimulating factor (M-CSF), that regulate osteoclast differentiation, function and survival (Matsuo & Irie, 2008).

The RANKL and its receptor (RANK) are known to be key factors in bone remodelling, where they are vital for the differentiation and activation of osteoclasts (Theill et al., 2002; Wittrant et al., 2004). The binding of RANKL to its RANK receptor on precursor macrophage cells stimulates the generation of intracellular reactive oxygen species (ROS) (Thannickal & Fanburg, 2000; Väänänen et al., 2000). At physiological levels, the RANKL-induced ROS act as intracellular signal mediators to regulate RANK signalling pathways which are not only crucial to induce osteoclast differentiation (Garrett et al., 1990; Ha et al., 2004; Lee et al., 2005; Sasaki et al., 2009a, 2009b; Goettsch et al., 2013), but also to stimulate osteoclast resorption activity (Armstrong et al., 2002; Huh et al., 2006). Tartrate-resistant acid phosphatase (TRAP) also generates ROS (Väänänen et al., 2000; Vääräniemi et al., 2004) that are involved in the degradation of mineralised extracellular bone matrix (Halleen et al., 1999; Halleen et al., 2003). TRAP is an enzyme highly expressed by osteoclasts and is often used to reflect the number of osteoclasts and the likely bone resorption rate. Thus, TRAP is widely accepted as a useful marker for monitoring the efficacy of an anti-resorptive treatment in clinical research (Halleen et al., 2006; Rissanen et al., 2008, Civitelli et al., 2009).

However, excessive intracellular ROS production associated with aging may cause an imbalance between bone resorption and formation due to increased osteoclast-mediated bone resorption activity that exceeds the osteoblast-mediated bone formation, which often results in net bone loss that further contributes to pathological bone diseases such as osteoporosis (Manolagas, 2010; Almeida, 2012). Hence, a variety of anti-resorptive agents have become a therapeutic strategy in management of osteoporosis (Waalén, 2010; Feng & McDonald, 2011). A number of anti-resorptive agents are currently in place for prevention and treatment of osteoporosis, including selective oestrogen receptor modulators (SERMs), bisphosphonates and calcitonin (Jules et al., 2010; Waalen, 2010; Feng & McDonald, 2011). However, each has issues with efficacy and adverse effects, warranting the investigation and development of more effective and/or safer anti-resorptive agents (Waalén, 2010; Sharif et al., 2011; Reginster et al., 2013; Reginster, 2014).

Tea is increasingly being studied for its benefits in bone health, and is widely regarded as safe for long-term consumption (Fujiki et al., 2002; Sarma et al., 2008; Abdel-Rahman et al., 2011). Tea is worth investigating as a nutritional approach for management and prevention of osteoporosis (Schulman et al., 2011; Nieves, 2013). There is cellular evidence suggesting that tea or its polyphenols may exert both anabolic and anti-resorptive effects *in vitro*, by enhancing osteoblastogenesis (Chen et al., 2005b; Mount et al., 2006; Vali et al., 2007), and the suppression of osteoclastogenesis (Yun et al., 2007; Das et al., 2009; Lin et al., 2009). Furthermore, our previous findings in **Chapter 5** indicated an anabolic effect by various teas on osteoblastogenesis in murine pre-osteoblast MC3T3-E1 cells and mineralisation in functional osteoblasts.

The purpose of the present study was to investigate the effects of various teas on osteoclast formation from macrophage precursor cells. In this study, Se-rich green tea (Se-GTE), Se-rich black tea (Se-BTE), regular green tea (R-GTE), and regular black tea (R-BTE) were investigated for their influence on RANKL-induced osteoclast differentiation in RAW 264.7 cells. The present study employed murine macrophage RAW 264.7 cells, an established and widely used *in vitro* model for osteoclastogenesis (Collin-Osdoby et al., 2003; Collin-Osdoby & Osdoby, 2012). This macrophage cell

line was chosen for its unique ability to differentiate into osteoclasts upon stimulation with RANKL (Kartsogiannis & Ng, 2004; Wattel et al., 2004).

6.2 Materials and methods

6.2.1 Preparation of sterile tea water extracts

The tea leaves used for the present study are described in **Chapter 3**, section 3.1.3. Tea water extracts (1%, w/v) were prepared at 90 °C for 5 min. Briefly, 1 g of each of the powdered tea leaves was infused with 100 mL of Milli-Q (MQ) water at 90 °C and allowed to brew for 5 min with continuous stirring. The infusions were decanted and filtered through Whatman No. 4 filter paper (0.45 mm) to remove the non-soluble solid matter. Tea extracts were then freeze-dried using a freeze-dryer (Model FD18LT, Cuddon Ltd., New Zealand) and stored at -20 °C until further use. On the day of the experiment, sterile stock solutions of tea extracts and a known tea phenolic compound, i.e. (-)-epigallocatechin-3-gallate (EGCG), were made by dissolving 1 mg of freeze-dried tea extracts and 9.1674 mg of EGCG ($\geq 80\%$, HPLC), into 1 mL and 10 mL of cell culture media Dulbecco's Modified Eagles Medium (DMEM, Gibco, Life Technologies, New Zealand), respectively, and supplemented with 10% foetal calf serum (Heat-inactivated FCS, Invitrogen, New Zealand) and antibiotic-antimycotic (Gibco, Life Technologies, New Zealand). The 2000 μM EGCG stock solution was then diluted 100-fold in cell culture media containing FCS to obtain a final concentration of 20 μM , and then filter-sterilised using a 0.20 μm syringe filter (Minisart, Sartorius AG, Goettingen, Germany). A 100-fold dilution was made from the 1 mg/mL tea stock solution to obtain a concentration of 10 $\mu\text{g/mL}$, which was then filter-sterilised (0.20 μm). Finally, a five point 10-fold serial dilutions from the 10 $\mu\text{g/mL}$ tea extracts were made into aliquots of decreasing concentration (10, 1, 0.1, 0.01 and 0.001 $\mu\text{g/mL}$).

6.2.2 Cell culture

Cell culture and maintenance of the murine macrophage RAW 264.7 cell line have been described in **Chapter 3**, sections 3.1.6 and 3.1.7.2.

6.2.3 Experimental procedures

Cell culture procedures for the experimental work have been described in **Chapter 3**, section 3.1.8.

6.2.4 Experimental techniques

To investigate the effect of tea on osteoclastogenesis, RAW 264.7 cells were cultured with tea extracts at a range of concentrations while treated with receptor activator of nuclear factor kappa beta ligand (RANKL, 462-TEC-010, R & D Systems). Briefly, RAW 264.7 cells were seeded at 5×10^4 cells/mL into 24-microwell plates (1 mL/well) in cell culture media DMEM (10% FCS) with 15 ng/mL of RANKL, supplemented with or without tea extracts at four different concentrations (0.01, 0.1, 1, and 10 $\mu\text{g/mL}$), and incubated for 6 days at 37 °C. The tea extract concentrations were chosen based on a previous experimental study (**Chapter 5**, section 5.2.4.2). Each plate included treatments which did not receive RANKL, these served as a negative control for the RAW 264.7 RANKL model. There were three replicate wells per tea concentration for RANKL treatment and one well per tea concentration for non-RANKL treatment, per experiment. Each experiment was repeated on three separate occasions to provide experimental replication. EGCG (20 μM) was used as a positive control and DMEM (10% FCS) was used as the zero control (blank). In this study, an EGCG concentration of 20 μM was used. Yun et al. (2007) and Lin et al. (2009) showed that EGCG concentrations up to 50 μM had no cytotoxic effect on RAW 264.7 cells. The cell culture media were changed every 72 hours with freshly-made growth media DMEM containing their respective RANKL and/or tea treatment. After 6 days exposure to the tea extracts, differentiation into mature osteoclasts was analysed by

measuring tartrate-resistant acid phosphatase (TRAP) activity, and by counting the number of TRAP-positive multinucleated osteoclast cells.

6.2.4.1 Tartrate-resistant acid phosphatase (TRAP) activity assay

On day 6, TRAP activity of the cells was measured from the cell culture supernatants using a leukocyte acid phosphatase kit (387-A, Sigma-Aldrich, Sydney, Australia). Briefly, 30 μ L aliquots of cell culture media from each well were transferred to 96-microwell plates in duplicate. Then, 170 μ L of freshly prepared TRAP solution was added to each cell culture sample and incubated in the dark for three hours at 37 °C. The optical absorbance was read at 550 nm using a microplate reader against the zero control (blank) wells prepared under similar conditions. TRAP activity was expressed as percentage (%) of zero control.

6.2.4.2 Tartrate-resistant acid phosphatase (TRAP) staining assay

RAW 264.7 cells were fixed and stained using a leukocyte acid phosphatase kit (Sigma-Aldrich, Sydney, Australia). Briefly, the remaining cell culture media was aspirated and the culture plates were allowed to air-dry at room temperature. Cells were then fixed with 300 μ L of fixative solution (25 mL of citrate solution + 65 mL of acetone + 8 mL of 37% formaldehyde) for 30 seconds and washed three-times with warm MQ water. Five hundred μ L of freshly prepared TRAP staining solution was then immediately added to each well and incubated for one hour at 37 °C, while protected from light throughout the incubation period. Following incubation, cells were rinsed three-times with warm MQ water. Cells were counterstained with haematoxylin solution (Sigma-Aldrich, Sydney, Australia) for two min, followed by rinsing with tap water for two min and then allowed to air-dry at room temperature overnight while protected from light. TRAP-positive osteoclast cells were visualised and photographed at 100 \times magnification, using an inverted microscope equipped with a digital camera (refer to **Chapter 3**, section 3.1.2). The number of TRAP-positive osteoclasts (TRAP⁺ OCLs) with five or more nuclei were manually counted in three fields per well using the Cell Counter plugin for ImageJ (Rasband, 1997 – 2014). Each tea treatment concentration was performed in three replicate wells, and each tea treatment was independently repeated three times. The number of TRAP⁺ OCLs formed per well were expressed as percentage (%) of zero control.

6.2.5 Statistical analysis

The detailed information about general statistical analyses has been described in **Chapter 3**, section 3.1.10. The differences between group means were compared using one-way analysis of variance (ANOVA), followed by post-hoc Tukey's test for pair-wise multiple comparisons of the group means. The Welch's variance weighted analysis of variance (ANOVA) were used in place of the simple one-way ANOVA when the assumption of homogeneity of variances is not met and groups are unequal in size, which was followed by post-hoc Tukey-Kramer test for pair-wise multiple comparisons. All analyses were done using the PROC GLM, a procedure within SAS that analyses data within the framework of general linear models. A difference was considered to be statistically significant when $p < 0.05$.

6.3 Results

6.3.1 Effect of tea extracts on TRAP activity of RANKL-induced osteoclast (OCL) formation from murine macrophage RAW 264.7 cells

Figure 6-1 shows the effect of tea extracts at various concentrations and EGCG on TRAP activity of osteoclast (OCL) differentiated from the precursor RAW 264.7 cells, and also in the undifferentiated cells. TRAP is an enzyme secreted by OCLs and commonly used as a marker of functional mature OCLs (Minkin, 1982). Overall, TRAP activity of RAW 264.7 cells induced by RANKL was significantly higher ($p < 0.0001$) than undifferentiated cells, indicating successful differentiation of precursor RAW 264.7 cells into mature osteoclast-like cells. The TRAP activity decreased with addition of the tea extracts in a concentration-dependent manner. As seen in **Figure 6-1**, significant effects shown by the R-BTE against TRAP activity of mature OCLs were observed at concentrations as low as $0.01 \mu\text{g/mL}$ ($93.0 \pm 1.7\%$, $p = 0.0020$), in comparison to the zero control. Meanwhile, Se-GTE, R-GTE and Se-BTE markedly decreased TRAP activity at concentrations as low as $0.1 \mu\text{g/mL}$ ($93.2 \pm 1.1\%$, $93.4 \pm 1.3\%$ and $95.3 \pm 1.0\%$, respectively; p values of < 0.0001 , < 0.0001 and 0.0080 , respectively), in comparison to their zero control. Undifferentiated RAW 264.7 cells not treated with RANKL demonstrated no significant difference in TRAP activity between control and various tea concentrations after 6 days of treatment, which was observed in all tea extracts. EGCG at $20 \mu\text{M}$ showed a significant inhibitory effect ($p < 0.0001$) on TRAP activity ($57.6 \pm 0.5\%$, $p < 0.0001$) when compared to the zero control, as observed in all tea extracts.

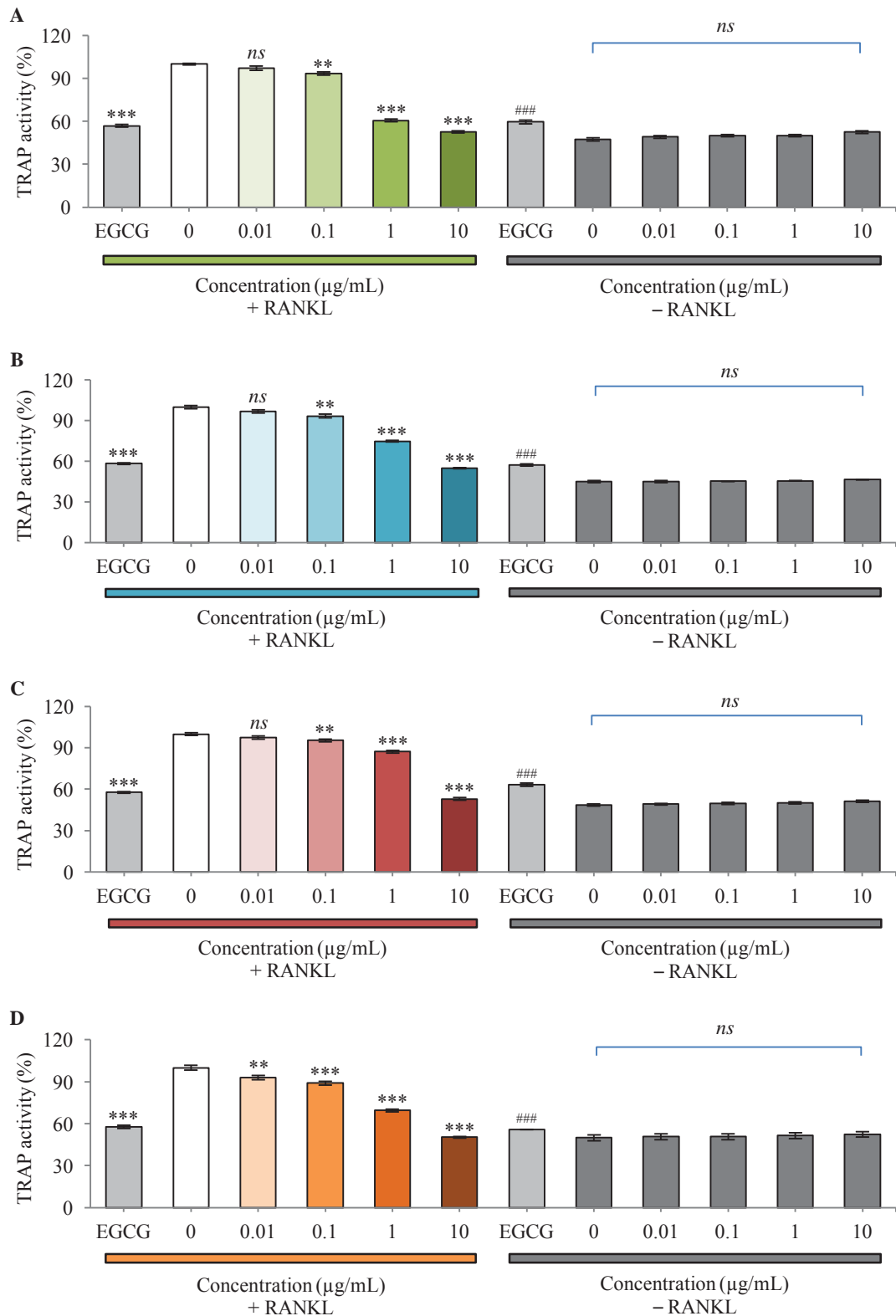


Figure 6-1. Tartrate-resistant acid phosphatase (TRAP) activity of RANKL-stimulated RAW 264.7 cells after 6 days exposure to tea extracts at varying concentrations and EGCG (20 μ M). (A) Se-GTE, (B) R-GTE, (C) Se-BTE, and (D) R-BTE. Data are mean \pm SEM. * indicates significance from the zero control (+ RANKL). # indicates significance from the zero control (- RANKL). ns = not significant, */# $p < 0.05$, **/# $p < 0.01$, ***/### $p < 0.001$.

6.3.2 Morphological features of RANKL-induced OCL formation from murine macrophage RAW 264.7 cells

Following TRAP staining, RAW 264.7 cells not treated with RANKL did not show any activity and retained an undifferentiated morphology appearing as small, round mononuclear cells which were uniform in size (**Figure 6-2A**). After 6 days of RANKL treatment, macrophage precursor cells differentiated into large osteoclast-like cells. TRAP-positive osteoclasts (TRAP⁺ OCLs) are those cells containing dark red-purple granules in the faintly staining cytoplasm with a specific cell morphology exhibiting multinucleation (**Figure 6-2B**) distinct to the blue colour of TRAP-undifferentiated RAW 264.7 cells (**Figure 6-2A**). Morphologically different to the undifferentiated macrophages, OCLs were larger, heterogenous in size, and had multiple nuclei. The cytoplasm of OCLs appears densely granular with presence of several vacuoles.

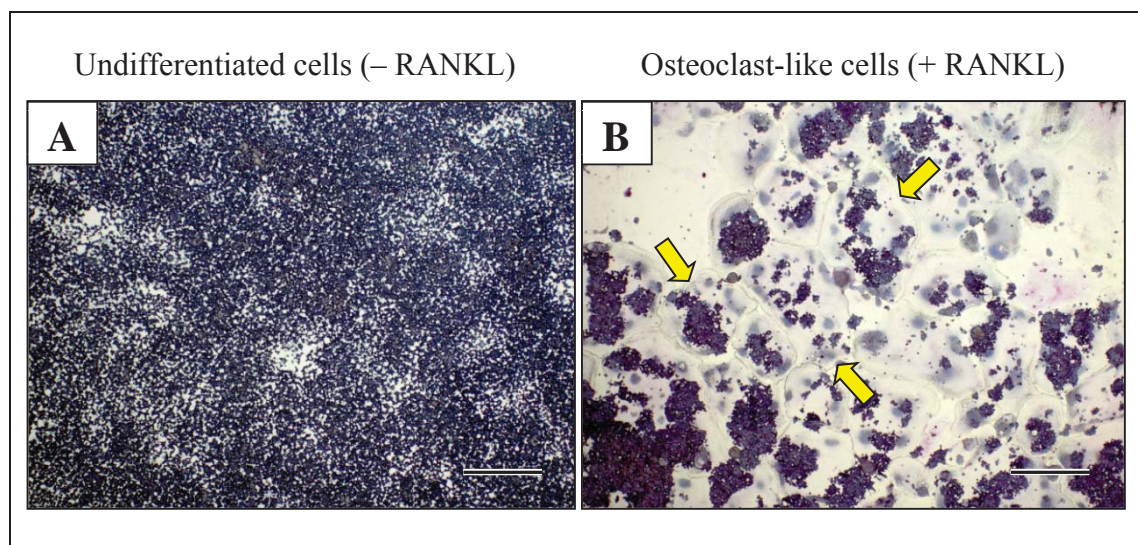


Figure 6-2. Representative photomicrographs of RANKL-induced osteoclast formation from RAW 264.7 cells, as confirmed by tartrate-resistant acid phosphatase (TRAP) staining (40 \times magnification, scale bars = 200 μ m). TRAP staining allows the visualisation of undifferentiated cells and osteoclasts (yellow arrows), with haematoxylin counterstain. Cells were seeded in 24-microwell plates (5×10^4 cells/mL) and incubated for 6 days, in the absence (-) or presence (+) of RANKL (15 ng/mL). (A) Undifferentiated cells which are small, round mononuclear cells that were not stained by the TRAP staining assay. (B) Osteoclast cells which are large, multinucleated cells stained in dark red-purple colour. Micrograph representative of 3 independent experiments, conducted in 3 replicate wells per plate, with 3 fields per well.

6.3.3 Effect of tea extracts on RANKL-induced OCL formation from murine macrophage RAW 264.7 cells

The effect of the tea extracts on OCL formation from macrophage precursor RAW 264.7 cells are shown in **Figures 6-3, 6-4 and 6-7**, where TRAP staining confirmed the presence of TRAP-positive osteoclasts (TRAP⁺ OCLs) in wells treated with 15 ng/mL of RANKL. Microscopically, pre-treatment of RAW 264.7 cells with Se-GTE and R-GTE at 0.01 and 0.1 µg/mL resulted in the formation of OCLs comparable to control cells (**Figure 6-3**). However, pre-treatment of the RAW 264.7 cells with higher concentrations of Se-GTE and R-GTE (1 and 10 µg/mL) decreased the number of TRAP⁺ OCLs and caused dramatic changes in the OCL morphology, where OCLs (≥ five nuclei) appeared smaller with fewer nuclei when compared to control cells. Furthermore, this was reflected in the number of TRAP⁺ OCLs (**Figure 6-7**), with significantly less OCLs due to the Se-GTE treatment at 1 and 10 µg/mL ($25.0 \pm 2.0\%$, $p < 0.0001$ and $5.7 \pm 1.7\%$, $p < 0.0001$, respectively) and R-GTE treatment at 1 and 10 µg/mL ($32.7 \pm 12.0\%$, $p < 0.0001$ and $1.4 \pm 0.6\%$, $p < 0.0001$, respectively), when compared to their respective control.

Se-BTE and R-BTE treatment at concentrations as low as 0.01 µg/mL resulted in a decrease in the number of TRAP⁺ OCLs in comparison to control cells (**Figure 6-4**). Moreover, as the Se-BTE and R-BTE concentrations increased, the number of TRAP⁺ OCLs further declined, which was accompanied with a decrease in OCL size and the number of nuclei per cell as compared to control cells. This observation was in line with the results of TRAP⁺ OCLs quantified in the cultured cells, as depicted in **Figure 6-7**. There was a decreased number of TRAP⁺ OCLs formed from RAW 264.7 cells following Se-BTE exposure at 0.01 ($90.1 \pm 5.6\%$, $p = 0.6168$) and 0.1 µg/mL ($86.9 \pm 6.5\%$, $p = 0.3205$) concentrations, which was statistically non-significant compared to the control cells. However, Se-BTE at concentrations of 1 ($60.6 \pm 6.5\%$, $p < 0.0001$) and 10 µg/mL ($2.1 \pm 1.7\%$, $p < 0.0001$) significantly inhibited osteoclastogenesis in RAW 264.7 cells. Meanwhile, all concentrations of R-BTE significantly inhibited OCL formation; 0.01 ($70.7 \pm 5.3\%$, $p < 0.0001$), 0.1 ($65.1 \pm 8.5\%$, $p = 0.0002$), 1 ($10.4 \pm 3.2\%$, $p < 0.0001$) and 10 µg/mL ($0.0 \pm 0.0\%$, $p < 0.0001$).

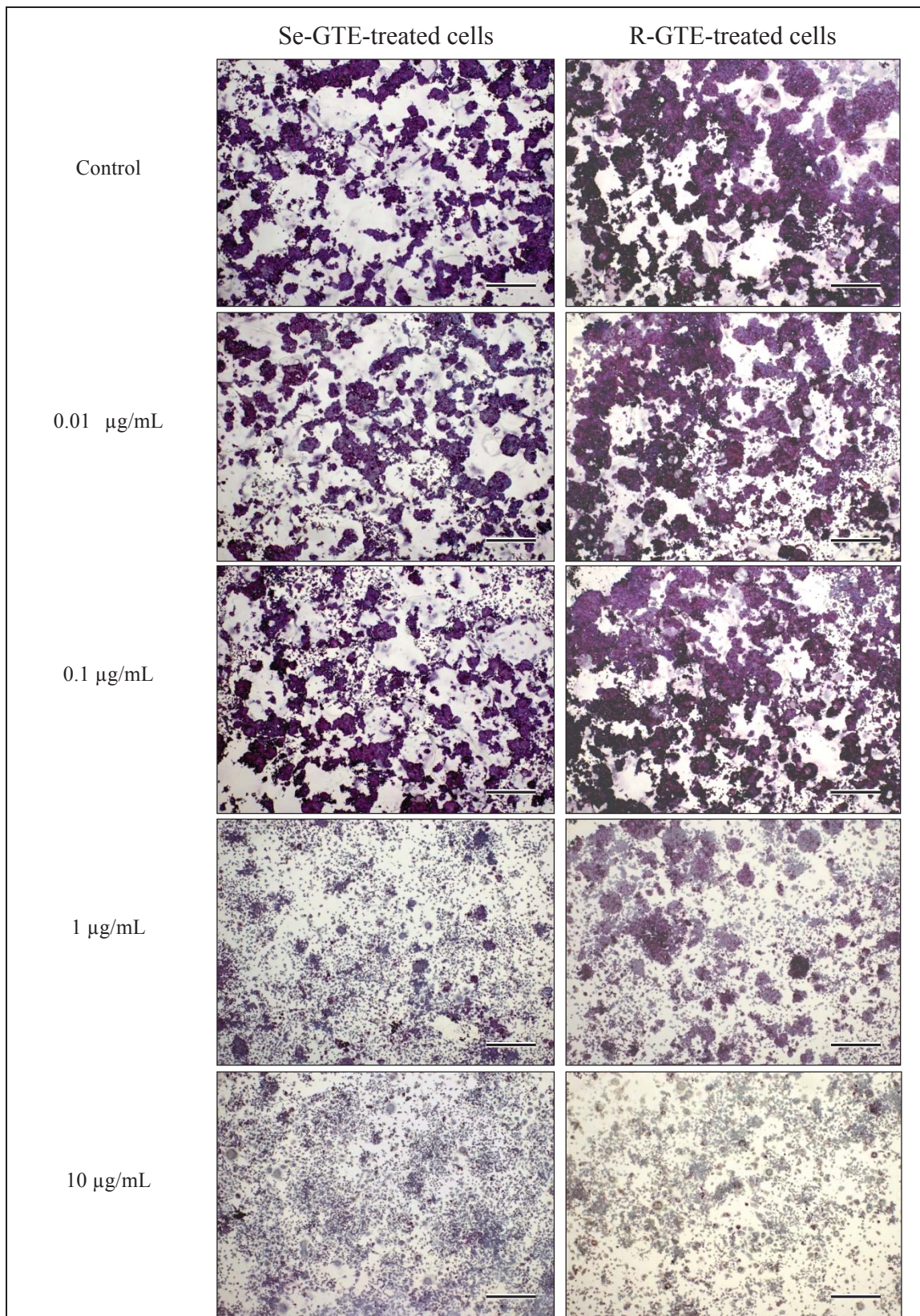


Figure 6-3. Representative photomicrographs of RANKL-induced osteoclast formation from RAW 264.7 cells, following incubation with Se-GTE and R-GTE (40× magnification, scale bar = 200 µm). Photomicrographs representative of 3 independent experiments, conducted in 3 replicate wells per plate, with 3 fields per well.

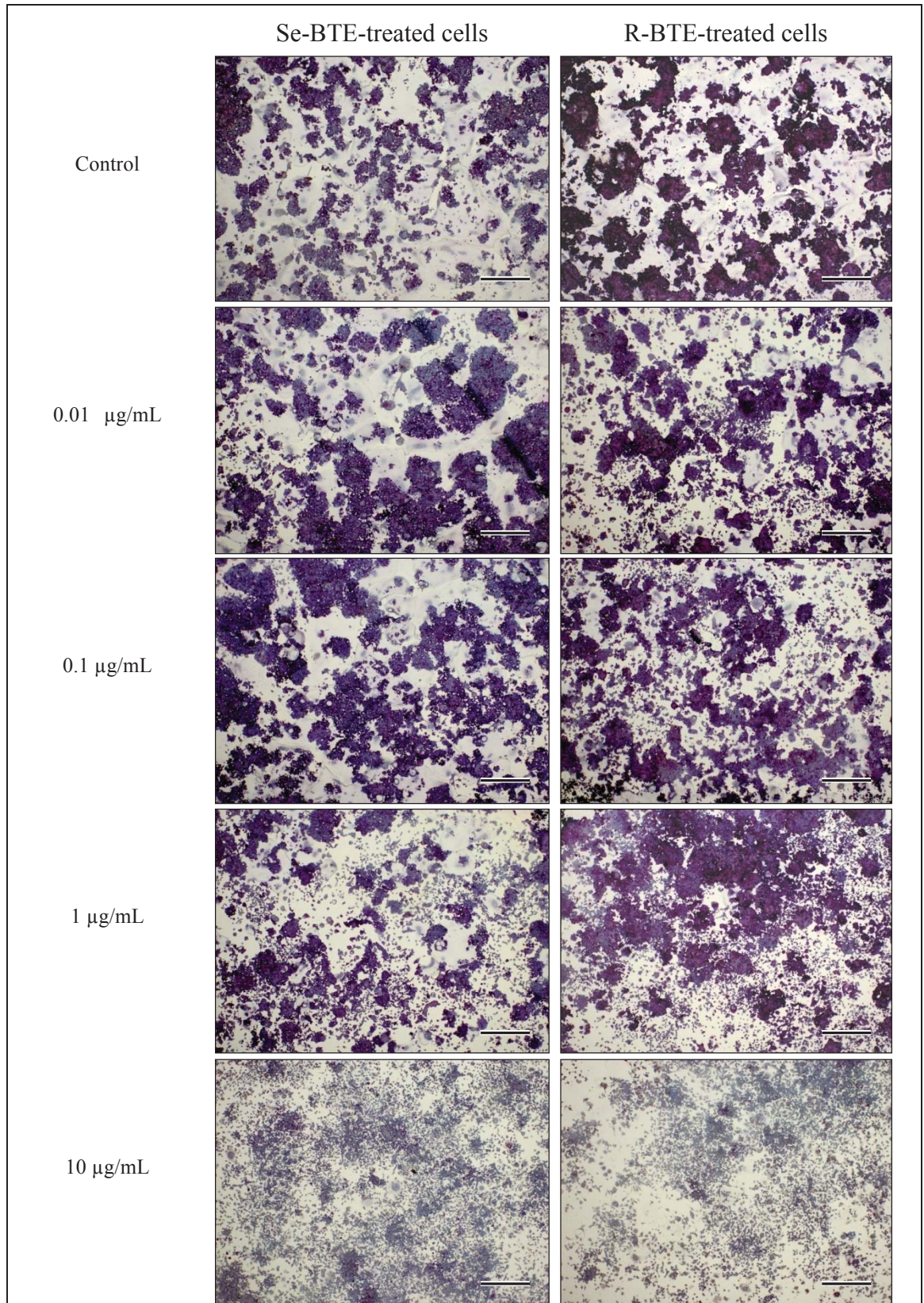


Figure 6-4. Representative photomicrographs of RANKL-induced osteoclast formation from RAW 264.7 cells, following incubation with Se-BTE and R-BTE (40× magnification, scale bars = 200 µm). Photomicrograph representative of 3 independent experiments, conducted in 3 replicate wells per plate, with 3 fields per well.

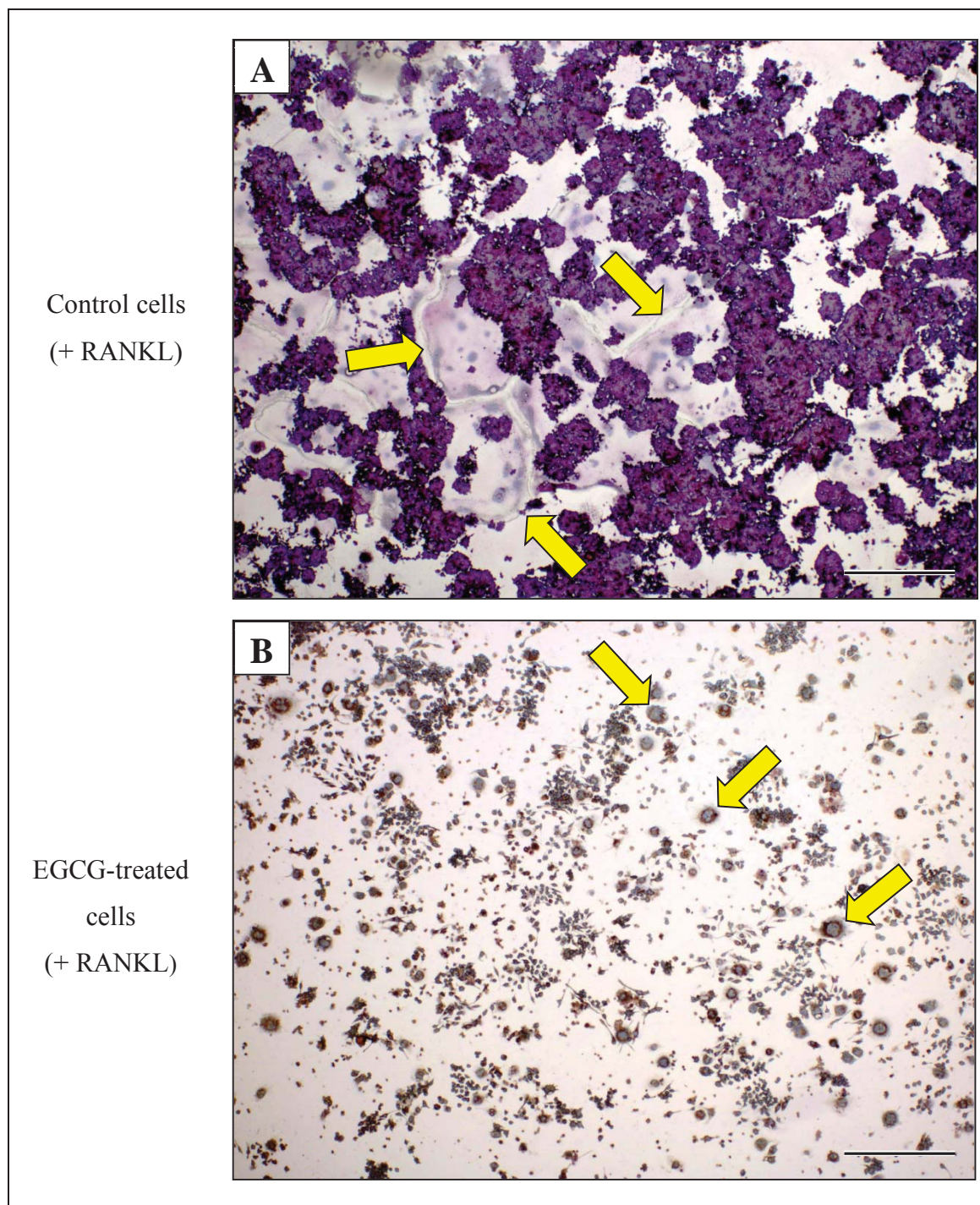


Figure 6-5. Representative photomicrographs of RANKL-induced osteoclast formation from RAW 264.7 cells, following incubation with EGCG (40× magnification, scale bar = 200 μm). TRAP staining allows the visualisation of osteoclasts (yellow arrows), with haematoxylin counterstain. Cells were seeded in 24-microwell plates (5×10^4 cells/mL) and exposed to EGCG (20 μM) for 6 days, in the presence (+) of RANKL (15 ng/mL). (A) Osteoclast cells which are large, multinucleated cells (\geq five nuclei) stained in dark red-purple colour. (B) Osteoclasts which are small, round-shaped cells with less than 5 nuclei that were faintly stained by TRAP staining assay. Photomicrograph representative of 3 independent experiments, conducted in 3 replicate wells per plate, with 3 fields per well.

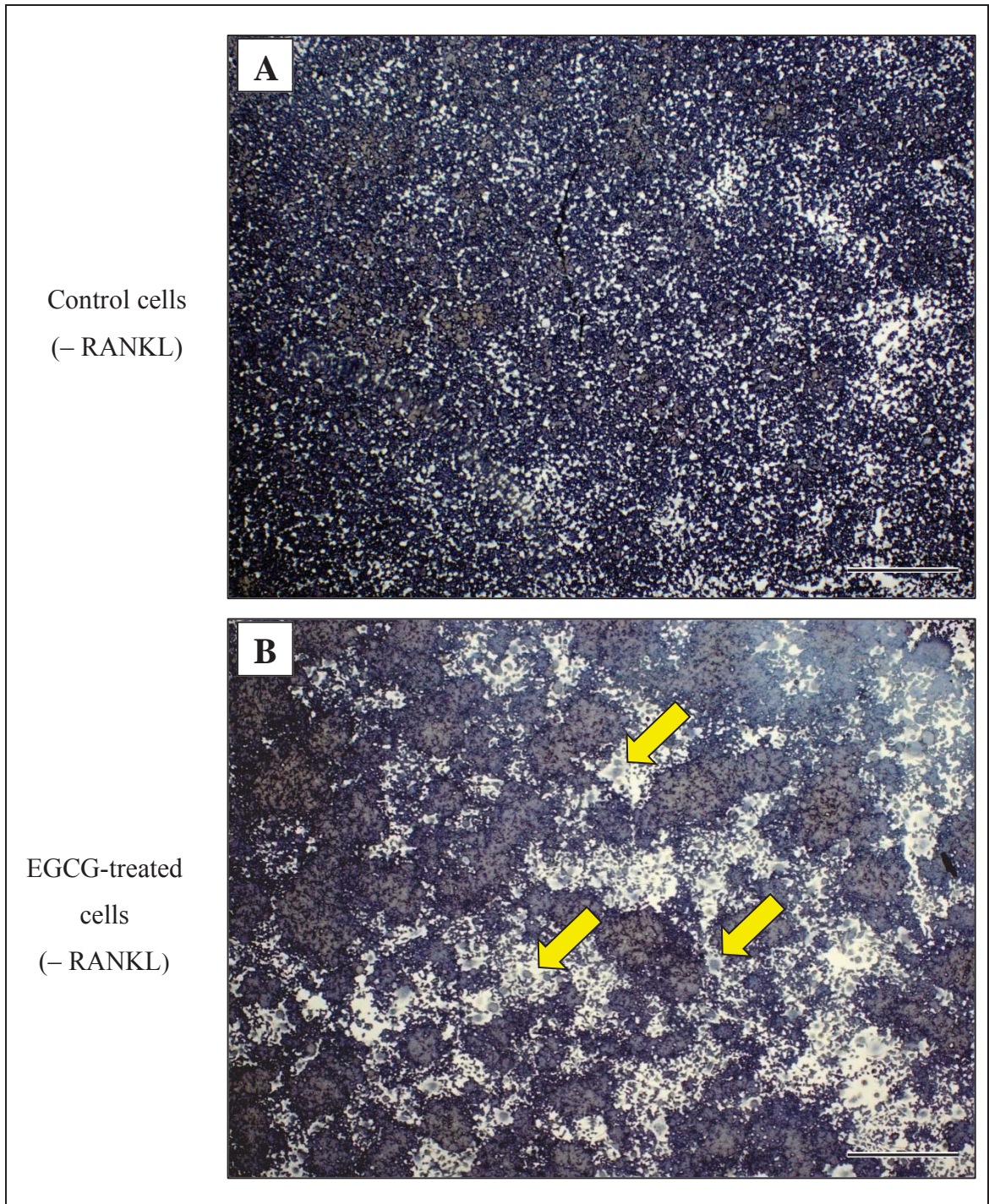


Figure 6-6. Representative photomicrographs of osteoclast formation from RAW 264.7 cells, following incubation with EGCG (40× magnification, scale bar = 200 μm). TRAP staining allows the visualisation of undifferentiated cells and multinucleated osteoclast-like cells (yellow arrows), with haematoxylin counterstain. Cells were seeded in 24-microwell plates (5×10^4 cells/mL) and exposed to EGCG (20 μM) for 6 days, in the absence (-) of RANKL. (A) RAW 264.7 cells remained small, undifferentiated and did not stain for TRAP. (B) Arrows indicate small multinucleated osteoclast-like cells (≤ 5 nuclei) that showed some TRAP stain. Photomicrograph representative of 3 independent experiments, conducted in 4 replicate wells, with 3 fields per well.

6.3.4 Effect of EGCG on RANKL-induced OCL formation from murine macrophage RAW 264.7 cells

Figures 6-5, 6-6 and 6-7 show the inhibitory effect of EGCG on RANKL-induced osteoclastogenesis in RAW 264.7 cells in comparison to control cells. In the control differentiated cells (**Figures 6-5A**), there were numerous and large TRAP-positive multinucleated osteoclast cells (TRAP⁺ OCLs, \geq five nuclei) formed from RAW 264.7 cells following the addition of 15 ng/mL of RANKL to the cell culture media. However, treatment with 20 μ M EGCG during RANKL-induced osteoclastogenesis in RAW 264.7 cells caused inhibition of OCL formation (**Figure 6-5B**), with fewer OCLs formed and with morphological features that were different than those in the control cells, i.e. smaller in size with less than five nuclei (yellow arrows). The microscopy observations of the inhibition effect exerted by EGCG were supported by the results of counting the TRAP⁺ OCLs, as shown in **Figure 6-7**. There were no TRAP⁺ OCLs with more than five nuclei formed from RAW 264.7 cells following EGCG exposure at 20 μ M, which was significantly less than the control treatment (**Figure 6-6**).

In the absence of RANKL, RAW 264.7 cells treated with EGCG, in the absence of RANKL, resulted in the formation of a small number of osteoclast-like multinucleated cells (**Figure 6-6B**). This observation is consistent with our previous results of TRAP activity following EGCG exposure to RAW 264.7 cells that were not pre-treated with RANKL, as described in **Figure 6-1**. While EGCG treatment alone caused an increase in TRAP activity and multinucleated cell formation compared to the zero control (–RANKL), all of these TRAP⁺ OCLs were small and had less than five nuclei (**Figure 6-6B**).

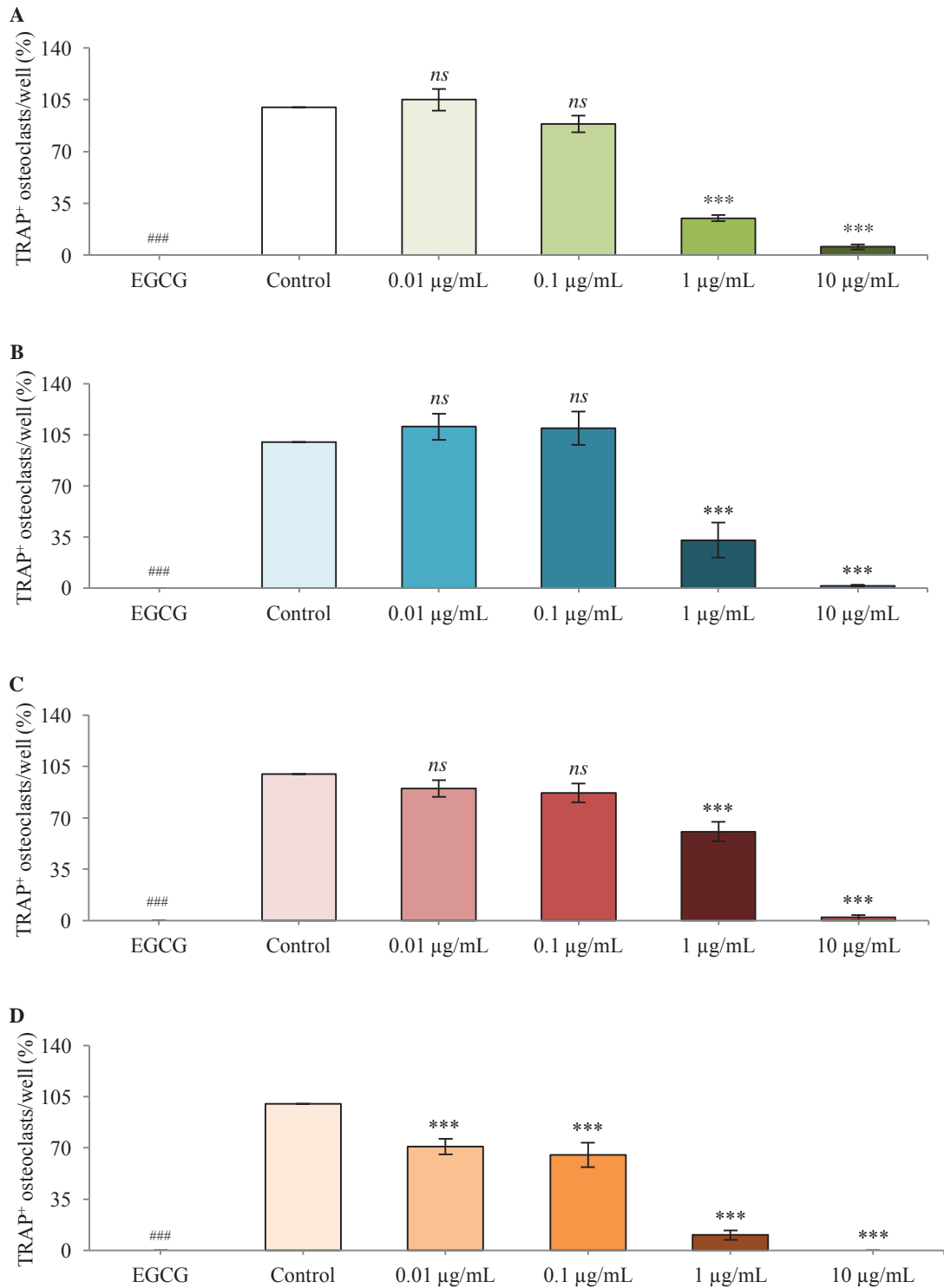


Figure 6-7. Number of TRAP-positive multinucleated osteoclast (\geq five nuclei) cells after 6 days exposure to varying concentrations of tea extracts (0.01, 0.1, 1 and 10 $\mu\text{g/mL}$) and EGCG (20 μM) in RANKL (15 ng/mL)-treated RAW 264.7 cells. (A) Se-GTE, (B) R-GTE, (C) Se-BTE, and (D) R-BTE. Data are mean \pm SEM and expressed as a percentage of total cells per well. Significant differences between zero control and varying tea concentrations or EGCG are indicated by asterisks (One-way ANOVA followed by post-hoc Tukey's test: *ns* = not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

6.4 Discussion

Preventive and therapeutic strategies in the management of bone diseases commonly take the approach of regulating bone homeostasis, either by increasing bone formation by osteoblasts and/or by decreasing the activity of bone resorbing osteoclasts. A decrease in bone-resorption activity could be achieved by inhibiting osteoclastogenesis or by increasing the apoptotic death rate of the osteoclasts (OCLs), which involve either direct action on osteoclast precursors, or indirect action on RANKL-RANK signalling pathways (Hughes & Boyce, 1997; Tanaka et al., 2005; Jules et al., 2010).

The present study is the first to report an inhibitory effect of selenium-rich green and black teas against RANKL-induced osteoclastogenesis in RAW 264.7 cells, in comparison to regular green and black teas. In this study, osteoclastogenesis was assessed by tartrate-resistant acid phosphatase (TRAP) activity in RANKL-treated RAW 264.7 cells, and also by the number of TRAP-positive osteoclasts (TRAP⁺ OCLs) formed from the macrophage precursor cells. The findings show that all teas suppressed RANKL-induced osteoclastogenesis in a concentration-dependent manner, reaching significance at high concentrations (1 and 10 µg/mL). The findings of the present study show that the black teas (Se-BTE and R-BTE) suppress osteoclastogenesis more effectively than the green teas (Se-GTE and R-GTE) at concentrations as low as 0.01 µg/mL. The present findings are in accordance with those observed by Oka et al. (2012) who found that the anti-osteoclastogenic effect of black tea bioactive polyphenol, theaflavin-3,3'-digallate (TFDG), against osteoclast differentiation in osteoclast precursor cells was greater than that of the major green tea polyphenol EGCG. The greater effect of black teas over green teas might be explained by black teas greater absorption into the cells as previously reported by Henning et al. (2006), who demonstrated that the relative absorption of theaflavins into mouse prostate tissue was 70% greater than that of EGCG. We suggest that the suppression of osteoclastogenesis may be due to direct anti-osteoclastogenic effect of the tea, and part mediated by indirect antioxidant properties of tea that improve endogenous antioxidant defences to combat RANKL-induced ROS production by the cells (Shen et al., 2009). However, the precise mechanisms by which the different teas used in the present study inhibited the

RANKL-induced osteoclastogenesis in RAW 264.7 cells are not clear and remain to be determined.

Additionally, our data showed that EGCG at 20 μ M effectively suppressed osteoclastogenesis following RANKL stimulation in RAW 264.7 cells, which is in agreement to the report of other researchers (Yun et al., 2007; Ko et al., 2009; Lin et al., 2009), and as reviewed by Shen et al. (2013a, 2013b). Moreover, the inhibitory effect of the teas and EGCG investigated in our study occurred at the early stage of osteoclastogenesis when cell fusion and multinucleated cell formation occur, which is also in line with work done by Yun et al. (2007), Lin et al. (2009) and Zhao et al. (2014). Interestingly, our study is also the first to report that EGCG simultaneously initiates osteoclastogenesis in the absence of RANKL stimulation; although the multinucleated cells formed were small in size and had less than five nuclei. Here, we suggest that the spontaneous osteoclast formation following administration of pure EGCG (20 μ M) on RAW 264.7 cells in the absence of RANKL stimulation may probably be due to the stimulatory effect of EGCG on expression of a number of inflammatory cytokines (i.e. tumor necrosis factor-alpha (TNF α) and interleukin-1 (IL1)) that have previously been reported to directly influence osteoclastogenesis in a RANKL-independent manner (Jimi et al., 1999; Azuma et al., 2000; Kobayashi et al., 2000; Suda et al., 2001; Fuller et al., 2002; Wei et al., 2005). However, the exact mechanisms by which EGCG affects these cytokines that activates osteoclastogenesis remains to be elucidated. To the best of our knowledge, no previous studies have tested EGCG in an approach similar to the one adopted in the present study. Therefore, further studies are needed to confirm these findings and to explore the mechanism by which the EGCG initiates the osteoclastogenesis in the absence of RANKL stimulation.

Previous reports of the anti-osteoclastogenic effect of tea has been mostly conducted using tea bioactive compounds such as (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-gallocatechin (GC), (-)-gallocatechin-3-gallate (GCG), (+)-catechin and theaflavin-3,3'-digallate (TFDG); either in RAW 264.7 cells (Yun et al., 2007; Ko et al., 2009; Lin et al., 2009; Kamon et al., 2010) or in a different *in vitro* model of osteoclastogenesis, including crude murine osteoclast-like multinucleated cells (Nakagawa et al., 2002), co-culture system of mouse bone marrow

cells and calvarial primary osteoblastic cells (Yun et al., 2004; Kamon et al., 2010; Lee et al., 2010a), human peripheral blood mononuclear CD14⁺ cells (Morinobu et al., 2008) and primary mouse bone marrow-derived macrophages (Lee et al., 2010b; Nakamura et al., 2010; Oka et al., 2012; Irie et al., 2014). Furthermore, EGCG was also reported to induce apoptotic cell death of OCLs in a concentration-dependent manner (Nakagawa et al., 2002; Yun et al., 2007; Zhao et al., 2014).

The most recent work on tea bioactive compounds and osteoclastogenesis was conducted by Zhao et al. (2014), who investigated the effect of various tea polyphenol compounds, namely EGCG, (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC) on pre-osteoclast RAW 264.7 cells, pre-osteoblast MC3T3-E1 cells, as well as on a co-culture model of bone marrow cells and mouse calvaria-derived osteoblasts. According to Zhao et al. (2014), EGCG was found to be the most potent tea bioactive compounds among all tested polyphenols, whereby EGCG inhibited formation of RANKL-induced TRAP⁺ OCLs, and directly inhibited OCL formation in a co-culture of differentiated osteoblasts (i.e. neonatal mouse calvarial osteoblasts) and the osteoclast precursor cells (i.e. mouse bone marrow cells of tibiae and femurs). To the best of our knowledge, very little information is available on the anti-osteoclastogenic effect of tea as a complex mixture of compounds. The only data available for tea is from Das et al. (2009), who reported an inhibitory effect of a black tea water extract (2.5%, w/v) against osteoclastic differentiation and activity, as assessed by the number of TRAP⁺ OCLs through histochemical observation and histomorphometric analysis of decalcified proximal tibia excised from ovariectomised rats.

6.5 Conclusions

Tea beverages are thought to have a beneficial effect on bone health but very few *in vitro* studies have been conducted to gain insight into their possible mechanism of action. The findings of the present study add to the current knowledge regarding the effect of tea at the cellular level that may assist development of complementary and alternative therapeutic strategies for the treatment of bone resorption and osteoporosis management. In summary, the present results provide the first evidence of the anti-osteoclastogenic action of Se-rich green and black teas (Se-GTE and Se-BTE) in comparison to regular green and black teas (R-GTE and R-BTE), as demonstrated by a decrease in TRAP activity and suppression in TRAP⁺ OCL formation from macrophage precursor RAW 264.7 cells. The antiosteoclastogenic effect showed by all the tested teas are irrespective of their selenium content. Interestingly, black teas inhibited osteoclastogenesis more effectively than the green teas. The inhibitory effects of the tested teas on osteoclast formation indicate its anti-resorptive effect, suggesting that these teas may serve as an effective nutritional factor to minimise bone loss. Our next investigation is to evaluate the bone-stimulating effect of the tea water extracts in a young growing animal model using rats. This will test the role of tea consumption during the rapid growth phase, which is a critical period for bone development and the attainment of peak bone mass.

CHAPTER 7

THE EFFECTS OF VARIOUS TEA EXTRACTS ON BONE PROPERTIES IN SKELETAL GROWTH OF YOUNG GROWING MALE RATS

Part of this chapter was presented at the International Conference on Pharmaceuticals, Nutraceuticals and Cosmetic Sciences (IPNaCS), 12 to 13 November, 2015, Malacca, Malaysia.

Abstract

Tea consumption has been shown to be effective in minimising age-related bone loss. However, it is not known whether tea consumption has a positive effect on bone mass and strength during the rapid growth phase. The role of tea may be associated with its antioxidant and prebiotic effects in bone health. The aim of this study was to investigate the potential bone-stimulating and prebiotic effect of tea that could have an impact on bone mass in young growing rats. The effect of four-week tea consumption on bone mass and strength were examined in young growing male Sprague-Dawley rats. Four-week-old rats were randomised into five groups: control (deionised water), Se-GTE (1% selenium-rich green tea), Se-BTE (1% selenium-rich black tea), R-GTE (1% regular green tea), and R-BTE (1% regular black tea). Rats were fed standard rat chow pellets containing 0.5% calcium, and provided with deionised water or tea water extracts, *ad libitum* throughout the study period. At the end of the study, animals were euthanased and blood, caecum with contents, femurs and lumbar spine were harvested. Serum antioxidant capacity, the serum bone resorption biomarker carboxy-terminal crosslinks telopeptide of type I collagen (CTX-I), caecal pH, bacterial enzymatic activities (β -glucosidase and β -glucuronidase) and selected intestinal microbiota populations were analysed. *Ex vivo* bone mineral density (BMD) of femurs and lumbar spine were measured using dual energy X-ray absorptiometry (DEXA). The right femurs were tested biomechanically using the three-point bending test. Fluid and feed intake varied significantly among the groups ($p < 0.0001$), with the highest mean fluid and feed intake observed in Se-BTE group and the lowest mean values in the Se-GTE group. Serum CTX-I concentrations were significantly affected by the tea ($p = 0.0459$), although no tea groups were different from control rats. In addition, bone mass and strength parameters were not affected by tea consumption. Longer tea intervention could possibly allow the tea extracts to exert a significant effect on bone parameters. Se-GTE showed the greatest prebiotic-like effects among the tested teas in the modulation of intestinal microbiota composition; as evidenced by low caecal pH, low bacterial count of *Clostridium* spp. (*perfringens/histolyticum* subgroup), and high bacterial β -glucosidase enzyme activity. Taken together, all tea water extracts showed some prebiotic potential in the modulation of intestinal microbiota composition,

although these teas did not significantly improve the bone parameters evaluated in the study.

7.1 Introduction

Most of the health benefits conferred by tea consumption are related to its high levels of bioactive phytochemical compounds, namely polyphenols. Polyphenols are strong antioxidants that neutralise free radicals by breaking the free radical chain through donation of an electron or hydrogen atom (Tsao, 2010). Tea polyphenols may exert direct and indirect antioxidant activity to reduce oxidative stress that results from increased levels of free radicals (Lorenz, 2013). However the antioxidant roles and mechanisms of tea polyphenols *in vivo* may be different from what would occur *in vitro* with the complicating factor of low bioavailability (Forester & Lambert, 2011; Thilakarathna & Rupasinghe, 2013). The chemical structure and degree of polymerisation of tea polyphenols are the main factors that influence their bioavailability. Tea polyphenols may exist as monomers (e.g. catechins), dimers (e.g. theaflavins), and polymers (e.g. thearubigins), with most existing as polymers or in glycosylated forms that cannot be absorbed in the small intestine and must therefore be hydrolysed by intestinal bacterial enzymes in the colon to allow fermentation and metabolism of the compound (Cardona et al., 2013) for further absorption into the systemic circulation.

Prebiotics have been proposed as the most promising substances for their potential to promote bone health, when compared with probiotics and synbiotics (Scholz-Ahrens et al., 2001, 2007). Prebiotics enhance the bioavailability and uptake of bone-relevant minerals, such as calcium, magnesium, and zinc (Scholz-Ahrens et al., 2007, Slavin, 2013), and may further affect mineral absorption by stimulating the growth of selected beneficial saccharolytic bacteria in the intestine. Beneficial intestinal microbiota also have a role on bone accretion independent of that of prebiotics (Scholz-Ahrens et al., 2007), through synthesis of bone-relevant vitamins (vitamin C, D or K), and short-chain fatty acids (SCFAs) production from intestinal bacterial fermentation (Lopez et al., 1998; Trinidad et al., 1999; Topping & Clifton, 2001). Bioactive

polyphenol-derived metabolites obtained from the microbial fermentation and metabolism of complex tea polyphenolic substances, serve as a growth substrate for the intestinal microbiota that eventually modulate population composition of the intestinal microbiota and benefits host health (Gibson & Roberfroid, 1995; Hervert-Hernández & Goñi, 2011; Wallace et al., 2011). In fact, intestinal microbiota are not only crucial for nutrition and metabolism of non-digestible food substances, but also for the development of gastrointestinal function and the immune system of the host (Di Mauro et al., 2013; Purchiaroni et al., 2013). Tea polyphenols and their metabolites have been reported to exhibit a positive effect on the intestinal environment by acting as a metabolic prebiotic (Lee et al., 2006a).

Currently, research efforts have been put into prebiotic studies on intestinal microbiota and their importance in bone health (Sjögren et al., 2012), at least in part due to their primary role in the development of the immune system (Di Mauro et al., 2013; Purchiaroni et al., 2013). Tea consumption has been identified to be positively associated with bone mineral density in humans, which may optimise bone health (Hosseini-nezhad et al., 2007). While numerous epidemiological studies reporting on tea have shown health-protective potential against cancer and cardiovascular disease (Bøhn et al., 2012; Yuan, 2013), the actual benefits of tea consumption on bone health are uncertain. Therefore, the interaction between tea as antioxidant and prebiotic agent on bone health requires further investigation. To date, the potential bone-protecting effects of tea consumption in minimising bone loss have been reported, mostly for green tea (Shen et al., 2013). Tea consumption may improve bone mineral density (BMD), reducing the risk of bone fracture, actively enhancing osteoblasts, while at the same time suppressing osteoclast activity, as evaluated by *in vitro* cell culture, in animal models and epidemiological studies (Shen et al., 2009; Shen et al., 2011). However, most of the research on tea conducted in animal models and epidemiological studies have focused on the prevention of bone loss due to aging, not on a bone-stimulating effects during the rapid growth phase.

Bone integrity and bone health may be greatly affected by nutrition in at least two stages of the lifecycle: during growth and aging. During the rapid phase of growth, enhanced calcium absorption assists an individual in reaching a peak bone mass at

maturity that may lower the risk of developing osteoporosis later in life (Kruger et al., 2003). Peak bone mass is the maximum bone size and strength that is largely determined by genes, and influenced by lifestyle factors (e.g. diet and exercise), hormonal status and body mass index (Eisman, 1999; Heaney et al., 2000). Low bone mass and a low peak bone mass may lead to an increased chance of developing osteoporosis and the associated risks of osteoporotic fractures (Kanis, 2002). The young growing rat model can provide valuable information on nutritional factors that can affect peak bone mass, and their respective effects on bone accrual (Bonjour et al., 1999).

To date, no studies have been conducted to examine whether there is a bone-stimulating effect of tea in young growing animals. In the present study, the effect of consumption of a variety of teas on bone mass in weanling male rats were assessed during four weeks of skeletal growth, in order to clarify the association between tea and bone health. We hypothesised that some of these teas may promote bone mineralisation through their antioxidative role and/or prebiotic activity.

7.2 Materials and methods

7.2.1 Animals and protocols

Sixty three-week-old weanling male Sprague-Dawley rats (mean body weight 50.8 ± 0.9 g) were obtained from the Small Animal Production Unit (SAPU), Massey University, Palmerston North, New Zealand. Ethical approval, and care and maintenance of the animals have been described in **Chapter 3**, section 3.2.3.

7.2.2 Diets

Standard rat chow pellets used in the study was formulated to contain all nutrients that are necessary for normal growth in rats based on AIN-93G (**Table 7-1**). Preparation of rat chow pellets based on AIN-93G (NRC, 1995) is described in

Chapter 3, section 3.2.4. Rats had *ad libitum* access to the standard rat chow pellets throughout the study period from week -1 to the end of week 4, except for an overnight period of fasting prior to blood collection on the last day of the study. The diet was randomly sampled for chemical analysis to confirm the formulated nutrient contents.

Table 7-1. Diet formulation for the growing rats*.

Ingredients	Amount (g/kg diet)
Macronutrients:	
Wheat	421.3
Barley	300.0
Skim milk powder	90.0
Broll	50.0
Lucerne	50.0
Corn oil	39.0
Meat and bone	36.0
Micronutrients:	
Methionine	5.0
Premix	4.0
Lysine	3.2
Limestone	1.5
Total	1,000.0

*Formulated according to NRC nutrient requirements for laboratory animals (AIN-93G) (NRC, 1995).

7.2.3 Preparation of tea water extracts (1%, w/v)

All tea water extracts or deionised water were replaced and served fresh once every second day, and fluid intake was measured at this point. Preparation of the tea water extracts is described in **Chapter 3**, section 3.2.5.

7.2.4 Experimental design

The rats underwent a 7-day acclimatisation period before the study commenced. During this period of acclimatisation (week -1), they were weaned onto a standard rat chow pellets diet and provided with deionised water, *ad libitum*. At week 0, rats were weighed and assigned randomly into five experimental treatment groups ($n = 12/\text{group}$) to ensure balance for average initial body weight across the treatment groups; control (deionised water), selenium-rich green tea (Se-GTE), selenium-rich black tea (Se-BTE), regular green tea (R-GTE), and regular black tea (R-BTE). The treatment course of all groups was four weeks (**Figure 7-1**). Tea extracts or deionised water was given *ad libitum* throughout the study, as the only source of drinking fluid (Gu et al., 2009). The feed and fluid intake fluid intake (water/tea) were measured once every second day, while body weight was measured weekly. All measurements were done at a specific time in the morning. Feed intake was determined as the difference in weights of standard rat chow pellets before and after each feeding period. Similarly, the fluid intake was determined by measuring the weight difference of the water bottles containing deionised water or tea water extracts. At the end of the treatment period (week 4), rats (eight weeks of age) were weighed and fasted overnight. Fasted blood samples were drawn directly from the heart under anaesthesia. Rats were euthanised by exsanguination following terminal heart puncture, and then dissected. The lumbar spine (LS1 – LS4), both femurs, and the caecum with contents were harvested.

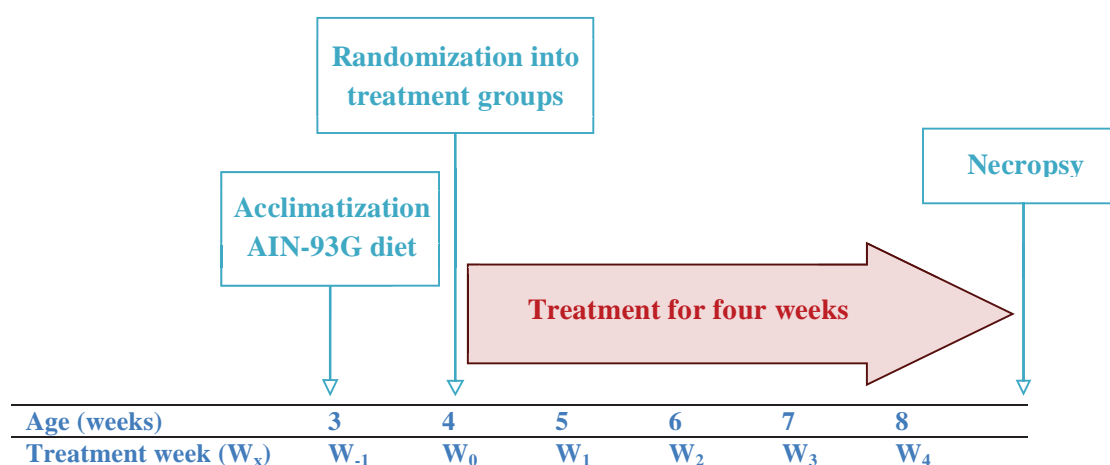


Figure 7-1. Experimental design for assessing the effects of tea extracts on bone properties in young growing male rats. Rats were fed standard rat chow pellets (AIN-93G) starting at three weeks old. Tea water extracts were given for four weeks.

7.2.5 Experimental techniques

7.2.5.1 Blood sample collection via terminal heart puncture

Fasted blood samples were drawn directly from the heart under anaesthesia at the completion of the studies, as described in **Chapter 3**, section 3.2.6.3.

7.2.5.2 Euthanasia and dissection

Rats were euthanised by exsanguination following terminal heart puncture, and dissected, as described in **Chapter 3**, section 3.2.6.4.

7.2.5.3 Evaluation of serum antioxidant capacity

The fasted blood serum samples were evaluated for antioxidant capacity using ferric-reducing antioxidant power (FRAP) assay, as described in **Chapter 3**, section 3.2.6.5.

7.2.5.4 Evaluation of serum biomarker of bone resorption

The fasted blood serum samples were evaluated for carboxy-terminal crosslinks telopeptide of type I collagen (CTX-I), as described in **Chapter 3**, section 3.2.6.6.

7.2.5.5 Evaluation of *ex vivo* bone mineral measurements using dual energy X-ray absorptiometry (DEXA)

Bone mineral measurements of excised lumbar spine and femurs were performed using DEXA, as described in **Chapter 3**, section 3.2.6.8

7.2.5.6 Evaluation of bone biomechanical properties of appendicular long bones using a three-point bending test

Bone biomechanical properties were performed on right femurs using a three-point bending test, as described in **Chapter 3**, section 3.2.6.9.

7.2.5.7 Evaluation of caecal pH and bacterial enzyme activities (β -glucosidase and β -glucuronidase) in *ex vivo* rat caecal contents

The pH measurement of each caecal sample and the bacterial enzymatic activities of the intestinal microbiota were performed using method as described in **Chapter 3**, sections 3.2.6.10 and 3.2.6.11.

7.2.5.8 Evaluation of selected intestinal microbiota populations in *ex vivo* rat caecal contents using fluorescence *in situ* hybridisation (FISH) analysis

Ex vivo caecal contents obtained from the young growing male rats were analysed using FISH analysis, as described in **Chapter 3**, section 3.2.6.12.

7.2.6 Statistical analysis

A total of sixty rats ($n = 12/\text{group}$) were used in the study, and detailed statistical analysis of the data have been described in **Chapter 3**, section 3.2.7. On calculation of power and sample size on BMD measurements using reference data from Kruger et al. (2003), a sample size of 12 is necessary for a power of 0.91 to detect a significant difference between the control and test group. The expected difference that could be detected between a sham control and OVX rat was set at 0.0138 g/cm^2 for BMD measurements. Simple linear regression analysis was performed using Microsoft Excel (2007) to calculate the dose-response relationship of standard ferrous sulphate (FeSO_4) solution used for calibration. Changes in rat's body weight over time were analysed with repeated measures analysis of variance (ANOVA) for comparison of means using the PROC MIXED, a procedure within SAS that fits a variety of mixed linear models. The differences between group means of rats were compared using one-way ANOVA, followed by post-hoc Tukey's test for pair-wise multiple comparisons of the group means. The one-way ANOVA analyses were done using the PROC GLM, a procedure within SAS that analyses data within the framework of general linear models. A difference was considered statistically significant when $p < 0.05$.

7.3 Results

7.3.1 Dietary analysis

The standard rat chow pellets used in the study were made according to AIN-93G specifications for the laboratory rats, to provide minimal requirements for growth. The analysed dietary composition of the rat chow is shown in **Table 7-2**. All key dietary components, i.e. protein, fat and calcium were slightly higher than the specified levels of AIN-93G. The Ca/P ratio in the diet met the requirements both for maximal body growth and maximal mineralisation (1.1 *versus* 1.1 and 1.1 *versus* 1.4, for body growth and mineralisation, respectively) (Benhart et al., 1969).

Table 7-2. Dietary composition analysis of standard pellet rat chow^a.

Component*	Unit	Amount
Dry matter	%	89.30
Carbohydrate	%	61.30
Protein	%	17.10
Moisture	%	10.70
Fat	%	6.90
Ash	%	4.00
Calcium	mg/kg	6,300
Phosphorus	mg/kg	5,700
Magnesium	mg/kg	1,420
Zinc	mg/kg	104

^aIngredients: wheat, barley, broil, lucerne, meat and bone, limestone, methionine, lysine, skim milk powder, corn oil and premix. *Nutrient composition was analysed by the Nutrition Laboratory, Massey University; fat by acid hydrolysis using Mjonner extraction (AOAC 922.06); protein by LECO total combustion method (AOAC 968.06); total carbohydrate by difference; minerals by Plasma Emission Spectrometry.

7.3.2 General observations

The tea drinks were well tolerated by the weaned male rats during the four weeks of the study. There were no adverse side effects such as behavioural changes, illness or death due to the tea treatments. There were no signs of deteriorating health observed in rats given tea extracts and all rats were healthy for the duration of the study.

7.3.3 Effects of tea extracts on weight gains, fluid intake and feed intake

Body weight (BW), total weight gain, fluid intake and feed intake are shown in **Table 7-3**. The initial BW at the start of the study ranged from 78.9 ± 2.3 to 79.8 ± 2.2 g. As expected, the statistical analysis indicating that there were no differences in the initial body weight ($p = 0.9973$) among the experimental groups, since the rats were randomly assigned by body weight at the beginning of the study (week 0). At the endpoint (week 4), no difference was found in the final BW between control and experimental tea groups, even when calculated as total weight gain ($p = 0.3345$ and $p = 0.2476$, respectively). Moreover, a repeated measure analysis on BW by week of the rats indicated no influence of tea drinks on BW during the study ($p = 0.3068$). Fluid and feed intake varied significantly among the experimental groups ($p = 0.0002$ and $p = 0.0024$, respectively), with the highest mean fluid and feed intake in the Se-BTE group, whereas the lowest mean values were in the Se-GTE group (**Table 7-3**). Only differences in mean fluid and feed intake between control and Se-BTE were found to be significant ($p = 0.0006$ and $p = 0.0166$, respectively).

Table 7-3. Body weight, total weight gain, fluid intake and feed intake of young growing male rats following four weeks receiving deionised water or tea drinks^a.

Group	Control	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^b
Body weight (g):						
Initial	78.9 (2.3)	78.7 (2.3)	79.4 (2.3)	79.8 (2.2)	79.2 (2.3)	0.9973
Final	307.4 (9.2)	303.8 (7.9)	307.5 (7.3)	323.6 (6.6)	317.9 (7.6)	0.3345
Total weight gain (g)	228.5 (8.6)	225.1 (6.8)	228.1 (6.5)	243.8 (5.4)	238.8 (6.4)	0.2476
Fluid intake (mL/day)	22.9 (1.2)	22.8 (0.7)	23.0 (0.8)	27.9*** (0.6)	25.4 (0.7)	0.0002
Feed intake (g/day)	22.6 (0.8)	22.6 (0.7)	22.4 (0.6)	25.8* (0.7)	25.4 (0.7)	0.0024

^aData represent mean (\pm SEM), $n = 12$ rats/group. ^bSignificant differences between control and experimental tea groups are indicated by asterisks (One-way ANOVA followed by post-hoc Tukey's test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

7.3.4 Effect of tea extracts on serum antioxidant capacity

The mean serum FRAP values in mg/L (\pm SEM) were: 150.5 ± 4.1 (control), 131.8 ± 2.1 (Se-GTE), 128.5 ± 2.2 (R-GTE), 144.2 ± 2.1 (Se-BTE) and 148.0 ± 2.0 (R-BTE), as shown in **Figure 7-2**. The differences between serum FRAP levels among treatment groups were significant ($p < 0.0001$). Although the FRAP values in all tea drink groups were lower than the control group, only Se-GTE and R-GTE groups showed significantly lower FRAP values when compared to the control group ($p < 0.0001$ and $p < 0.0001$, respectively).

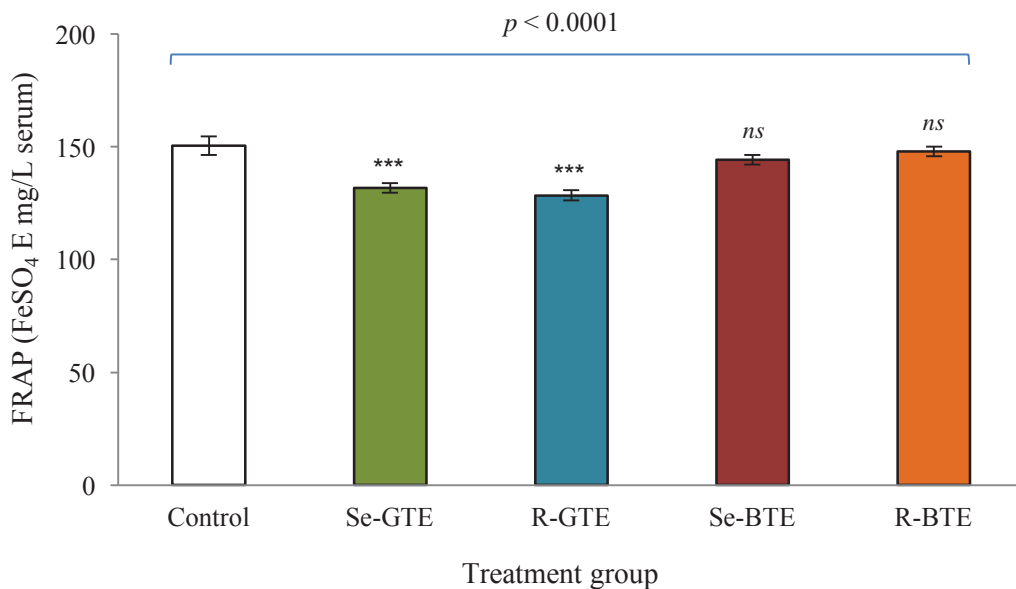


Figure 7-2. Serum ferric-reducing antioxidant power (FRAP) of young growing male rats following four-week consumption of deionised water or tea drinks. Data are mean (\pm SEM) of triplicate measurements, $n = 12$ rats/group. *indicates significance from the control: *ns* = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

7.3.5 Effect of tea extracts on serum CTx-I concentration

CTx-I levels were significantly different amongst the treatment groups ($p = 0.0459$), however no difference was found between the control and any of the experimental tea groups (**Figure 7-3**).

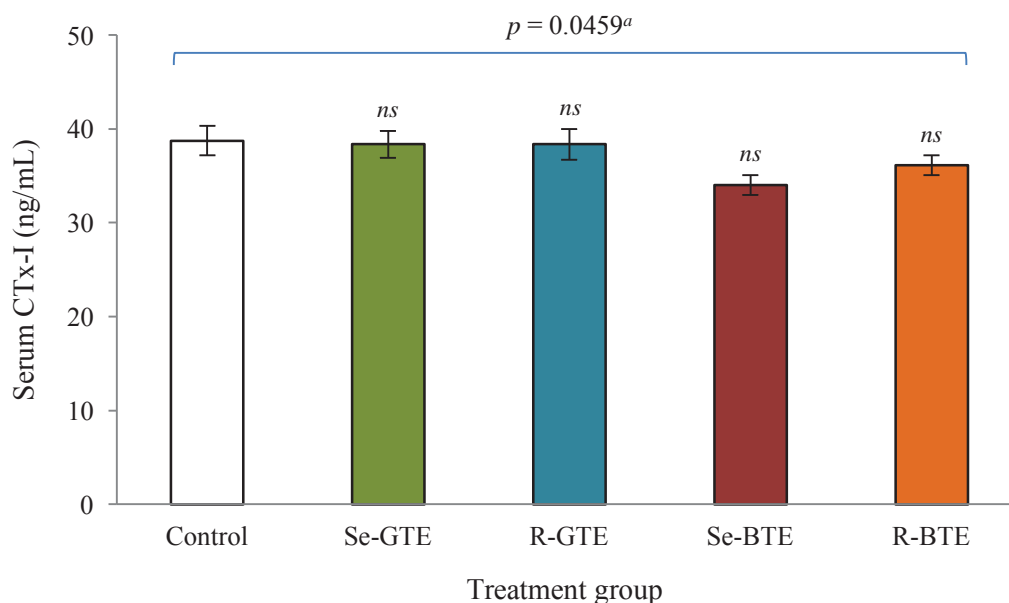


Figure 7-3. Effect of tea consumption on serum CTx-I concentrations in eight-week-old male Sprague-Dawley rats following four-week consumption of deionised water or tea drinks. Data are mean (\pm SEM) of triplicate measurements, $n = 12$ rats/group. ^aOne-way ANOVA followed by post-hoc Tukey’s test.

7.3.6 Effects of tea extracts on *ex vivo* bone mineral measurements

7.3.6.1 Lumbar spine DEXA

Table 7-4 shows *ex vivo* bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) values for the lumbar spine (LS1 – LS4) from each treatment group. Together, tea drinks had an effect on BA ($p = 0.0164$) and BMC of the lumbar spine ($p = 0.0443$), but not BMD of the spine ($p = 0.2861$). Both black teas, i.e. Se-BTE and R-BTE, showed a trend towards higher values of BA (Se-BTE, 1.730 ± 0.025 cm²; R-BTE, 1.709 ± 0.023 cm²) and BMC (Se-BTE, 0.294 ± 0.009 g; R-BTE, 0.289 ± 0.009

g) of the lumbar spine relative to control group (BA, $1.640 \pm 0.023 \text{ cm}^2$; BMC, $0.264 \pm 0.008 \text{ g}$). However, these increased BA and BMC values of the lumbar spine were not significant.

7.3.6.2 Femoral bone DEXA

There was a significant effect of tea on femur BA ($p = 0.0286$), but not on BMC ($p = 0.2654$) and BMD ($p = 0.2070$) when these bones were scanned *ex vivo* (Table 7-4). Even though the femur bone area differed significantly among treatment groups, there were no significant differences in treatment effects between the control group and any of the experimental tea groups.

Table 7-4. *Ex vivo* bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) in the lumbar spine and femoral bone of eight-week-old male Sprague-Dawley rats^a.

Group	Control	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^b
Lumbar spine						
Bone area (cm ²)	1.640 (0.023)	1.631 (0.023)	1.666 (0.024)	1.730 (0.025)	1.709 (0.023)	0.0164
Bone mineral content (g)	0.264 (0.008)	0.267 (0.008)	0.276 (0.007)	0.294 (0.009)	0.289 (0.009)	0.0443
Bone mineral density (g/cm ²)	0.161 (0.003)	0.163 (0.003)	0.165 (0.003)	0.170 (0.003)	0.169 (0.003)	0.2861
Femur						
Bone area (cm ²)	1.638 (0.019)	1.611 (0.027)	1.652 (0.019)	1.702 (0.019)	1.672 (0.017)	0.0286
Bone mineral content (g)	0.289 (0.007)	0.285 (0.006)	0.286 (0.005)	0.300 (0.006)	0.287 (0.004)	0.2654
Bone mineral density (g/cm ²)	0.176 (0.003)	0.171 (0.001)	0.173 (0.002)	0.177 (0.002)	0.176 (0.002)	0.2070

^aData are mean (\pm SEM), $n = 12$ rats/group. ^bOne-way ANOVA followed by post-hoc Tukey's test.

7.3.7 Effect of tea extracts on femoral bone strength

Right femur midpoint width and thickness did not differ between control and any of the experimental tea groups (**Table 7-5**).

Table 7-5. Effect of tea on the three-point bending biomechanical test of the right femur^a.

Group	Control	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^b
Midpoint width (mm)	4.20 (0.06)	4.30 (0.05)	4.34 (0.03)	4.32 (0.05)	4.40 (0.07)	0.1302
Midpoint thickness (mm)	3.07 (0.04)	3.04 (0.06)	3.04 (0.04)	3.02 (0.05)	3.07 (0.02)	0.8725
Maximum load (N)	70.8 (2.4)	68.0 (2.5)	69.8 (2.8)	79.3 (4.0)	77.7 (3.3)	0.0373
Maximum stroke (mm)	1.39 (0.08)	1.30 (0.07)	1.38 (0.06)	1.39 (0.07)	1.34 (0.06)	0.8593
Break load (N)	36.7 (5.7)	36.7 (3.6)	36.5 (5.7)	53.1 (6.3)	50.8 (5.3)	0.0573
Break stress (N/mm ²)	21.2 (3.5)	20.7 (2.0)	20.5 (3.2)	30.9 (4.3)	27.4 (2.8)	0.0816
Break stroke (mm)	2.25 (0.13)	2.46 (0.15)	2.47 (0.12)	2.16 (0.12)	2.12 (0.14)	0.1925
Break strain (%)	18.5 (1.1)	20.1 (1.5)	20.0 (1.0)	17.5 (1.1)	17.4 (1.2)	0.2919
Elasticity (N/mm ²)	497 (30)	492 (33)	505 (37)	578 (33)	534 (30)	0.4054
Energy (J)	0.10 (0.01)	0.11 (0.01)	0.11 (0.01)	0.11 (0.01)	0.11 (0.01)	0.9503

^aData are mean (\pm SEM), *n* = 12 rats/group. ^bOne-way ANOVA followed by post-hoc Tukey's test.

The differences in some of the biomechanical measures, such as maximum stroke, break stress, break stroke, break strain, elasticity and energy were not statistically significant (**Table 7-5**) between group means. Break load was marginally significant (**Table 7-5**; $p = 0.0573$). Only maximum load differed significantly (**Table 7-5**; $p = 0.0373$), although no significant differences were found between the control group and all experimental tea groups.

7.3.8 Effects of tea extracts on caecal pH, bacterial enzyme activities and selected intestinal microbiota populations

Caecal pH values ranged from 6.63 ± 0.05 to 7.23 ± 0.06 and were significantly influenced by tea treatments ($p < 0.0001$), as shown in **Table 7-6**. The caecal pH values of rats given Se-GTE, R-GTE and R-BTE as the only source of fluid were significantly lower ($p = 0.0001$, 0.0120 and 0.0423 , respectively) compared to the control group.

As shown in **Table 7-6**, caecal β -glucosidase (β -GLU) activity was significantly affected by tea consumption ($p < 0.0001$). However, only β -GLU enzyme activity in Se-GTE was significantly increased compared with the control ($p = 0.0009$). In contrast, tea consumption showed no effect on enzymatic activity of β -glucuronidase (β -GUS) ($p = 0.4032$).

Fluorescence *in situ* hybridisation (FISH) was performed to quantify the target bacterial populations in rat caecal samples, and the results are depicted in **Table 7-6**. The bacterial counts of *Bacteroides* spp. were not significantly different across all experimental groups ($p = 0.2098$). On the other hand, tea consumption had significantly influenced numbers of *Bifidobacterium* spp. in rat caecal samples ($p = 0.0032$), although none of the experimental tea groups differed from the control. Significant differences were found between group means for *Clostridium* spp. (*perfringens/histolyticum* subgroup) ($p = 0.0287$), with lower bacterial counts seen in all experimental tea groups in comparison to the control group. The Se-GTE caused significantly reduced levels of *Clostridium* spp. as compared to the control group ($p =$

0.0385). Numbers of *Lactobacillus/Enterococcus* spp. were not significantly different among all experimental groups ($p = 0.1592$).

Table 7-6. Caecal parameter measurements in eight-week-old male Sprague-Dawley rats^a.

Group	Control	Se-GTE	R-GTE	Se-BTE	R-BTE	p value ^b
Caecal pH	7.23 (0.05)	6.63*** (0.06)	6.82* (0.06)	7.11 (0.10)	6.88* (0.14)	< 0.0001
β -GLU (U/g caecal content) ^c	0.59 (0.06)	0.92*** (0.05)	0.80 (0.08)	0.60 (0.05)	0.76 (0.05)	< 0.0001
β -GUS (U/g caecal content) ^c	1.43 (0.06)	1.29 (0.05)	1.32 (0.06)	1.40 (0.06)	1.39 (0.05)	0.4032
<i>Bacteroides</i> spp. ^d	7.78 (0.07)	7.52 (0.10)	7.50 (0.08)	7.67 (0.12)	7.63 (0.08)	0.2098
<i>Bifidobacterium</i> spp. ^d	7.70 (0.07)	7.92 (0.08)	7.73 (0.06)	7.57 (0.02)	7.59 (0.06)	0.0032
<i>Clostridium</i> spp. ^d	7.96 (0.08)	7.61* (0.10)	7.67 (0.09)	7.69 (0.09)	7.86 (0.06)	0.0287
<i>Lactobacillus</i> / <i>Enterococcus</i> spp. ^d	7.80 (0.05)	7.99 (0.06)	7.85 (0.03)	7.92 (0.07)	7.89 (0.06)	0.1592

^aData are mean (\pm SEM), $n = 12$ rats/group. ^bOne-way ANOVA followed by post-hoc Tukey-Kramer's test. *indicates significance from the control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ^cU is defined as mg/h. ^dData are presented as log number of bacterial cells per g wet weight caecal content (Log_{10} cells/g).

7.4 Discussion

This study was conducted to determine the beneficial effect of four different teas on bone metabolism and skeletal growth of young growing male rats, as well as to evaluate the potential relationship between tea antioxidant capacity, the intestinal microbiota and bone properties of these rats. Weanling rats were used in this study, in order to investigate the short-term effect of tea consumption on attainment of peak bone mass (Bonjour et al., 1999). Male rats were used because their bone mass increments are greater than that of female rats (Iwaniec et al., 2009). In general, observations from the present study showed that tea consumption for four weeks had no detrimental effect on young growing male rats.

7.4.1 Tea intake and feed intake in young growing male rats

Rats in all experimental groups had *ad libitum* access to standard rat chow pellets and deionised water or tea water extracts as the sole source of fluid. Only rats drinking black teas exhibited higher levels of fluid and feed intake throughout the four-week period of study when compared to the control group. The increased feed and fluid intake could be due to the caffeine level in the black teas that resulted in a simultaneous increase of thirst and appetite in the rats throughout the study. Black tea has been reported to contain a higher amount of caffeine than green tea (Khokhar & Magnusdottir, 2002; Lin et al., 2003), due to full fermentation of black tea as described by Lin et al. (1998) and Lin et al. (2003). Caffeine, an alkaloid, belongs to the methylxanthine family and is one of the main active compounds in tea other than catechins (Lin et al., 1998). Caffeine has been reported to have a diuretic effect (Maughan & Griffin, 2003) that affects hydration status, thus increasing thirst and therefore fluid intake. Caffeine may heighten the level of thirst, so the rats that drank black tea would be thirstier and drink more black tea throughout the study. In addition to caffeine, theophylline and theobromine are other compounds of the methylxanthine family found in tea that could also exert a diuretic effect (Maughan & Griffin, 2003; Kasabe & Badhe, 2010). On the other hand, recent evidence has demonstrated the appetite-stimulating effect of caffeine in rats (Retzbach et al., 2014; Sweeney et al., 2016). This effect could be due to caffeine antagonising adenosine A2A receptors in the

brain, leading to inactivation of the adenosine receptors that consequently increase palatable food consumption. Furthermore, caffeine can also activate agouti-related peptide neurons that modulate feeding behavior, as well as increasing levels of dopamine and activate dopamine neurons in the brain region (nucleus accumbens) that control motivation and feeding behaviours (reviewed by Sweeney et al., 2016).

Selenium (Se) supplementation has been reported to stimulate appetite (Ewan, 1976; Bunk & Combs Jr., 1980). Thus, high Se content in Se-BTE might have stimulated the appetite of the rats, and eventually resulting in a higher final body weight (BW) compared to the other experimental groups, though not significant. Ewan (1976) demonstrated that male Holtzman rats from Se-depleted dams given *ad libitum* access to selenium-supplemented diets, consumed more feed and grew faster compared to rats given control diet. Bunk and Combs Jr. (1980) also indicated that the short-term ration of orally-administered Se in the form of seleno-DL-methionine and sodium selenite stimulated appetite in Se-treated chicks compared to Se-adequate control chicks; resulted in a higher feed intake within two to four hours, and an increased body weight gain after four to six hours from the time of Se administration in the Se-treated chicks.

However, the explanation for the role of Se increasing appetite in the Se-BTE group cannot be used to interpret the opposite effects of Se-GTE in this study, which statistically showed no effect on feed intake when compared to the control. A possible explanation could be attributed to a synergistic effect between catechins and caffeine promoting satiety in rats (Rains et al., 2011; Hursel & Westerterp-Plantenga, 2013), that may have counteracted the appetite-stimulating effect of Se in green tea.

7.4.2 Effect of various tea extracts on serum antioxidant capacity

In comparison to the control, Se-GTE and R-GTE consumption reduced serum antioxidant capacity levels in young growing rats by 12% and 14%, respectively. These results contradict the previous findings in **Chapter 4**, in which both Se-GTE and R-GTE were found to be high in polyphenolic compounds and showed *in vitro* antioxidant activity. Indeed, the findings contrast with the results of other researchers (Leenen et al.,

2000; Serafini et al., 2000; Rietveld & Wiseman, 2003; Erba et al., 2005), which demonstrated an improved *in vivo* antioxidant capacity in human studies after tea administration, although data in some studies are inconsistent (Freese et al., 1999; Miura et al., 2000; Frei & Higdon, 2003; Henning et al., 2004).

In contrast to the green teas, serum antioxidant capacity levels in the Se-BTE and R-BTE remained unchanged as compared to the control. This result is consistent with previous human studies conducted on black tea consumption (Van het Hof et al., 1997; Princen et al., 1998; Davies et al., 2003; Higdon & Frei, 2003; Henning et al., 2004). At present, very limited amount of data is available in regard to *in vivo* antioxidant capacity of black tea or its complex polyphenols such as theaflavins and thearubigins (Sun et al., 2012). The effectiveness of black teas in the present results are also in agreement with the work done by Sun et al. (2012), who found that black tea showed greater *in vivo* antioxidant capacity than green tea, which could be due to structural changes to its polyphenolic compounds during digestion and absorption following tea ingestion (Serafini et al., 2000; Sun et al., 2012).

Several factors might possibly contribute to the significantly reduced serum antioxidant capacity levels in Se-GTE and R-GTE groups, as well as the unchanged level of serum antioxidant in Se-BTE and R-BTE after the tea intervention in comparison to the control rats. First, the tea itself could be the factor that accounts for the difference found in the results of serum antioxidant capacity, where the biochemical profiles of phenolic compounds that exert the antioxidant effects are highly variable in tea leaves (as discussed in **Chapter 4**). The differences in biochemical profiles of phenolic compounds is based on several factors such as age of tea leaf, type of tea cultivars, climate and region of production, genetic make-up, geographical areas, harvesting season, environmental conditions and agricultural practices used in the production of these teas (Owuor & Obanda, 1995; Magoma et al., 2000; Lin et al., 2003; Shishikura & Khokhar, 2005; Owuor et al., 2008, 2010; De la Paix et al., 2010; Erkturk et al., 2010; Bhattacharya & Sen-Mandi, 2011; Jayasekera et al., 2011; Song et al., 2012; Wachira et al., 2013; Lee et al., 2014; Kaur et al., 2014).

Second, data from the other *in vivo* studies showed mixed results probably due to differences in methodological approaches, including experimental protocols for serum antioxidant capacity, design of the study (Van het Hof et al., 1997; Serafini et al., 2000; Frei & Higdon, 2003; Rietveld & Wiseman, 2003), preparation of the tea water extracts, and also variation in the amount of tea consumed by the rats due to *ad libitum* access to fluids. Thus, the current findings are not completely comparable nor expected to reflect the *in vivo* antioxidant results of green tea and black tea presented by others.

Third, limitations of the FRAP assay may also contribute to the present results. The direct antioxidant effects of tea polyphenols *in vitro* are as radical scavengers and transition metal chelators. However, Frei and Higdon (2003) suggested that tea polyphenols may exert indirect antioxidant effects *in vivo*, which in part, involves enzymatic antioxidants that unfortunately cannot be identified by the FRAP assay. In general, FRAP assay measures the total antioxidant capacity of serum as determined by non-enzymatic antioxidants (e.g. phenolic compounds, vitamin E, carotenoids, ascorbic acid, uric acid, bilirubin). However, the FRAP assay cannot detect non-enzymatic antioxidant compounds that act by radical quenching (hydrogen atom transfer), particularly low molecular weight sulfhydryl (SH)-containing compounds such as thiol antioxidants (e.g. glutathione, thioredoxin, lipoic acid), and also enzymatic antioxidants (e.g. superoxide dismutase, catalase, glutathione peroxidase) (Cao & Prior, 1998; Prior & Cao, 1999; Frei & Higdon, 2003). Furthermore, FRAP is a time-dependent assay, which may be associated with different rates of ferric tripyridyltriazine (Fe^{3+} -TPTZ)–phenol reaction as previously discussed by Pulido et al. (2000), Prior et al. (2005) and Henríquez et al. (2011). These different rates of reaction could be due to the presence of phenolic compounds or metabolites with different reactivity towards the Fe^{3+} -TPTZ complex, where longer incubation times should be allowed for the FRAP assay to allow for complete reactions of all antioxidants contained in the serum samples. In addition, the use of ferrous ion (Fe^{2+}) as a final indicator in the FRAP assay may also contribute to some issues since some antioxidants, such as ascorbic acid, not only reduce ferric ion (Fe^{3+}) to Fe^{2+} but can also react with Fe^{2+} , a potential pro-oxidant to further generate free radicals (Cao & Prior, 1998; Gorąca & Skibska, 2006; Gáspárová et al., 2010). These limitations of the FRAP assay, therefore may explain the result of serum antioxidant capacity obtained in the experimental tea groups.

Fourth and finally, it is difficult to relate *in vitro* data to animal studies where experimental *in vitro* and *in vivo* studies have been reported to produce contrasting results due to the complexity of an *in vivo* system and pharmacokinetic processes (Hermans et al., 2007). In this regard, the limited bioavailability of tea phenolics, different tea concentrations, interactions between different polyphenol antioxidants, as well as their interaction with other nutrient molecules *in vivo* must be taken into account when evaluating the *in vivo* antioxidant capacity of tea water extracts (Serafini et al., 2000; Shen et al., 2007).

Generally, there are several chemical assays used to measure antioxidant activities *in vitro* and *in vivo*. These assays are classified according to their reaction mechanisms with free radicals, namely single electron transfer (SET)-based assays and hydrogen atom transfer (HAT)-based assays. The SET-based assays spectrophotometrically measure redox potential of an antioxidant agent against an oxidant (including radicals, carbonyls, and metal ions) through electron donation. The FRAP, DPPH, Folin-Ciocalteu assays that were performed in **Chapter 4** are among the SET-based reactions; other assays are including Trolox equivalent antioxidant capacity (TEAC) and cupric reducing antioxidant capacity (CUPRAC) (Prior et al., 2005; Apak et al., 2013; Prior & Wu, 2013; Prior, 2015).

On the other hand, HAT-based assays measure the free-radical quenching ability of an antioxidant through hydrogen donation, which leads to the chain-breaking mechanism of an antioxidant. Examples of HAT-based assays include oxygen-radical antioxidant capacity (ORAC), total radical-trapping antioxidant parameter (TRAP) and crocin bleaching assays. Most HAT-based assays spectrophotometrically or fluorometrically measure antioxidant capacity based on kinetic curves obtained from competitive reaction kinetics. Among all these assays, the ORAC assay has been the most popular method to evaluate radical-scavenging ability of antioxidant substances (Prior et al., 2005; Apak et al., 2013; Prior & Wu, 2013; Prior, 2015). This is due to its high specificity, the use of peroxy radicals known as physiologically relevant to *in vivo* conditions, and for being the only method that is able to combine both inhibition time-length and percentage of the free radical quenching activity by an antioxidant into a single quantity.

Nevertheless, all these chemical assays do not account for the complex *in vivo* system and pharmacokinetic processes, hence the *in vitro* antioxidant activity of Se-rich teas are not necessarily reflected in the *in vivo* effects (Prior et al., 2005; Apak et al., 2013; Prior & Wu, 2013; Prior, 2015). Accordingly, *in vivo* biomarkers of oxidative stress status have been widely used to assess the efficacy of dietary antioxidants against oxidative damage to lipids, proteins and DNA. The most commonly used biomarkers of oxidative stress are including malondialdehyde (MDA), 3-nitrotyrosine (NTyr), 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Additionally, assessments of blood levels of both enzymatic antioxidants (e.g. selenoenzymes, SOD, CAT) and non-enzymatic antioxidants (e.g. GSH, Trx) are among the methods of choice able to provide substantial information and help measure antioxidant status *in vivo* (McKenzie, 2000; Frei & Higdon, 2003; Hermans et al., 2007; Cimen, 2008; Pandey & Rizvi, 2010, 2011; Erkekoğlu et al., 2013).

7.4.3 Effects of various tea extracts on a serum bone-resorption biomarker, bone mineral density and bone strength in young growing rats

Body weight is directly associated with bone mass, which can be partly attributed to the altered hormonal environment and greater mechanical loading (Shapses & Sukumar, 2012). Thus, the absence of significant differences in final body weight and total weight gain between experimental groups suggests that the observed differences in the bone biomarker and biomechanical strength are independent of body weight factors. Serum carboxy-terminal crosslinks telopeptide of type I collagen (CTx-I) is a major bone biomarker for assessment of the bone resorption rate, with high levels indicating increase bone resorption and high osteoclast activity (Coleman, 2002). Serum CTx-I concentrations in Se-BTE and R-BTE were lower (*p* values of 0.1139 and 0.6705, respectively) than that of the control group after four weeks of tea consumption, whereas no changes were observed in Se-GTE and R-GTE when compared with the control group. Although the decrease of serum CTx-I was not significant following the post-hoc Tukey's test, the results suggest that Se-BTE and R-BTE consumption led to a reduction of serum CTx-I levels in young growing rats and may indicate an anti-resorptive effect by the tea.

It is important to note that the evaluation of bone formation biomarker osteocalcin (OC) in serum may complement CTx-I and enables a more accurate indication of elevated bone turnover (Lukaszkiwicz et al., 2008). However, OC was not measured in this study due to its low sensitivity in the animal model of young growing rats (M. Kruger, personal communication, April 21, 2011). Furthermore, according to Modrowski et al. (1992), the maximum levels of OC in rats are achieved at three weeks of age and decrease precipitously through to 11 weeks of age, which was confirmed by Watkins et al. (2000). Thus, suggesting that examining serum osteocalcin in young growing rats at 8 weeks of age might not give a fruitful result.

Lumbar spine showed a trend of higher BA, BMC and BMD values during skeletal growth in young rats given Se-BTE and R-BTE compared to the control. There was also a slight increase in femoral BA in Se-BTE rats. Although none of these increases were significant, the results indicate that Se-BTE and R-BTE consumption for four weeks may exert subtle increases in bone mass. The lumbar spine and femur are the best sites for measuring changes in bone density in pre-clinical animal models (Thompson et al., 1995). However, the lumbar spine and femur responded differently to the tea treatment, which could be due to different proportions of cancellous and cortical bone at each site. Lumbar spine vertebrae are mainly composed of cancellous bone, whereas femurs are largely composed of cortical bone (Clarke, 2008). Cancellous bone has greater metabolic activity and mineral uptake than cortical bone (Mathias et al., 2000; Downey & Siegel, 2006), which may explain the rapid increase in bone mass of the lumbar spine with Se-BTE and R-BTE interventions, as compared to the change in the femurs. The Se-BTE and R-BTE also provided an increase in bone strength in the femurs subjected to biomechanical testing. Even though the maximum load and break load values were not statistically significant from the control, the increases in both measures indicate a change in biomechanical properties possibly due to ingestion of Se-BTE and R-BTE over the control and other tea experimental drinks.

7.4.4 Effects of various tea extracts on caecal pH and bacterial enzyme activities

The results of the present study showed that the caecal pH in all experimental tea groups was lower than that of the control rats, even though the decrease in caecal pH in the Se-BTE group was not significant. The decrease of caecal pH in the experimental tea groups may be due to the increased synthesis of organic acids (e.g. lactate, succinate, pyruvate, butyrate, fumarate) and short-chain fatty acids (SCFA) from the fermentation activity carried out by intestinal microbiota (Hernández-Hernández et al., 2011; Van Dorsten et al., 2012). It has been shown that low pH levels are favorable to beneficial intestinal microbiota by increasing their growth rate (Gibson & Roberfroid, 1995; Guarner & Malagelada, 2003). On the other hand, low pH suppresses the growth of selected pathogenic bacteria, such as *Bacteroides* and *Clostridium* (Gibson & Roberfroid, 1995; Guarner & Malagelada, 2003; Both et al., 2011), leading to the decrease in numbers of these bacteria as observed in the present study. Moreover, the growth suppression of these pathogenic bacteria may also be due to direct inhibition by immunostimulants or antimicrobial action of the fermentation end-product secreted by the beneficial intestinal microbiota (Gibson & Wang, 1994; Gibson & Roberfroid, 1995; Guarner & Malagelada, 2003; De Vuyst & Leroy, 2007; Both et al., 2011). The reduction of caecal pH is important to maintain a balanced intestinal microbiota composition in order to support the intestinal physiology, prevention of intestinal diseases, and also in the metabolism of phytonutrients (e.g. polyphenols) (Coxam, 2007).

In the present study, there was a significant positive effect of various teas on changes in caecal β -glucosidase enzyme activity ($p < 0.001$). However, the increase was only significant in the Se-GTE group when compared to the control group (following post-hoc Tukey-Kramer's test). In contrast, no significant changes in the activity of β -glucuronidase was observed in any experimental tea groups when compared to the control, although there was a slight reduction in the activity of this enzyme in all tea groups. β -glucosidase is an intestinal bacterial enzyme that catalyses the hydrolysis of plant glycosides (e.g. flavonoid glycosides) escaping digestion in the small intestine to glycones and aglycones. Aglycones further degrade into simple phenolic acids that are

readily absorbed and more bioavailable (Robertson et al., 1982; Pool-Zobel et al., 2002; Manach et al., 2004). An increase in β -glucosidase was likely a consequence of elevated numbers of bifidobacteria and lactobacilli, which have been reported to produce this enzyme at a higher rate than other bacteria (Saito et al., 1992; Rowland & Tanaka, 1993; Tsangalis et al., 2002; Otieno et al., 2005). β -glucuronidase is an intestinal bacterial enzyme involved in deconjugation of hepatic metabolites (methylated, sulphated, or glucuronidated conjugates) obtained from plant glycosides absorbed in the small intestine. These are regulated by biliary secretions (Manach et al., 2004) for further reabsorption in the large intestine. The enzymatic activity of β -glucuronidase is considered to affect host health due to its ability to convert the glucuronide conjugates to a carcinogenic form, in addition to its important role in the enterohepatic circulation of drugs and exogenous compounds (Salminen et al., 1998; Thilakarathna & Rupasinghe, 2013). There is a wide range of intestinal microbiota including *Escherichia coli*, *Staphylococcus* spp., *Eubacteria* spp., *Bacteroides* spp. and some strains of *Clostridium* that have been reported to produce high levels of β -glucuronidase, while bifidobacteria and lactobacilli possess lower levels of this enzyme (Nakamura et al., 2002; Benjdia et al., 2011; Gluox et al., 2011).

The effects of Se-GTE and R-GTE on bacterial β -glucosidase enzyme activity in the present study are in agreement with the results of the study done by Molan et al. (2010), who reported that selenium-rich green tea was significantly more effective than China green tea at increasing the activity of β -glucosidase in adult male rats, which were gavaged orally with 10 mL/kg body weight of 1% tea water extract for six consecutive days. Regarding the activity of β -glucuronidase, the present study showed no changes, which is contradictory to the significant decrease of β -glucuronidase enzyme activity in the adult male rat study of Molan et al. (2010). The differences between the activity of these bacterial enzymes in the tea-supplemented rats and the control animals may be due to the changes in the composition of intestinal microbiota and influenced by the age of rats (Woods & Gorbach, 2001; Both et al., 2011; Molan et al., 2010, 2013).

7.4.5 Effects of various tea extracts on intestinal microbiota compositions

The results of the present study indicate that green tea consumption (Se-GTE and R-GTE) did not influence caecal bifidobacteria and lactobacilli counts in young growing rats, which supports previous *in vitro* and *in vivo* studies showing that green tea extracts or their constituents do not significantly affect the growth of *Bifidobacterium* spp. (Lee et al., 2006a; Ankolekar et al., 2011; Gaudreau et al., 2013) or *Lactobacillus* spp. (Lee et al., 2006a; Jaziri et al., 2009; Cueva et al., 2010; Ankolekar et al., 2011). However, this contradicts an *in vivo* study by Molan et al. (2010), who showed selenium-rich green tea had bifidogenic and lactogenic effects. The authors also suggested that the selenium-rich green tea had a larger prebiotic potential than regular teas. Indeed, results from other laboratory studies were also inconsistent, which observed that green tea extracts or their constituents show positive effects on the growth of *Bifidobacterium* spp. (Tzounis et al., 2008; Molan et al., 2009b; Vodnar & Socaciu, 2012; Parkar et al., 2013) and *Lactobacillus* spp. (Tzounis et al., 2008; Molan et al., 2009b; López de Felipe, 2010; Vodnar et al., 2012; Gaudreau et al., 2013).

The consumption of black teas (Se-BTE and R-BTE) also showed no effect on caecal bifidobacteria and lactobacilli counts in the young growing rats, which is not in agreement to previous *in vitro* studies reported by Banerjee et al. (2010) and Vodnar et al. (2012) who showed positive effects of black tea extract on the growth of bifidobacteria and lactobacilli. Most recently, Kemperman et al. (2013) used an *in vitro* model of the intestinal microbial ecosystem, namely the Simulator of Human Intestinal Microbial Ecosystem (SHIME) to determine the potential effects of complex polyphenol mixtures of black tea on human colonic microbiota, and they found that the black tea extract also caused a reduction in bifidobacterial count. Notably, the results of the present study showed a reduction in the bacterial count of pathogenic *Bacteroides* spp. and *Clostridium* spp. (*perfringens/histolyticum*) in all experimental tea groups, although the mean differences between groups was only significant for *Clostridium* spp. (*perfringens/histolyticum*) after four weeks of Se-GTE consumption. The reduction in the numbers of *Clostridium* spp. (*perfringens/histolyticum*) agree well with the previous *in vitro* and *in vivo* studies that explored the antimicrobial effect of green tea and/or its polyphenolic compounds (Lee et al., 2006a; Tzounis et al., 2008; Molan et al.,

2009b; Molan et al., 2010) and black tea (Banerjee et al., 2010). However, an *in vitro* study done by Kakuda et al. (1991) found no changes in the population sizes of *Clostridium perfringens* in the presence of green tea water extracts. We suggest that the discrepancy between these results with the reports of other researchers may be due to differences in concentrations of tea and/or its polyphenol, diverse methodological approaches, and a wide range of compositional microbiota analysis techniques of animal fecal samples employed in their study (Dueñas et al., 2015a, 2015b).

7.5 Conclusions

The present work indicates that tea consumption did not cause any harm to bone properties in the young growing rats. This study reports for the first time the effect of the consumption of various teas, particularly selenium-rich green and black teas on bone mass and strength during the rapid growth phase using an animal model of young growing rats. Even though all teas did not improve the bone parameters significantly, it might be possible that longer tea intervention could give more time for the tea extracts to be effective and to significantly affect the bone parameters evaluated in this study. The present study is also the first to evaluate the prebiotic-like effects of the consumption of various teas on caecal parameters in a young growing rat model. Selenium-rich green tea (Se-GTE) showed a promising effect on intestinal microbiota composition; by decreasing caecal pH, reducing the numbers of *Clostridium* spp. (*perfringens/histoliticum* subgroup) and increasing the activity of bacterial β -glucosidase enzyme. In summary, this study provides fundamental knowledge on the bone-stimulating and prebiotic effects of the tested teas.

CHAPTER 8

THE EFFECTS OF VARIOUS TEA EXTRACTS ON BONE LOSS IN THE OVARIECTOMISED RAT MODEL OF POSTMENOPAUSAL OSTEOPOROSIS

Abstract

The consumption of tea (*Camellia sinensis*) has been reported to have potential in reducing bone loss associated with oestrogen deficiency following menopause. However, no work has been done to study the effect of consumption of tea with naturally high selenium content. In addition, research efforts have recently been focused on the role of prebiotics in influencing bone health in postmenopausal women. This study aimed to investigate the bone-protective effects of a variety of teas against ovariectomy-induced bone loss in mature adult female rats, and to determine if any protective effects showed by the teas involved their antioxidative role and/or prebiotic potential. Three-month-old rats were randomised into six groups: sham-operated control (deionised water) and five groups of ovariectomy-operated (OVX) rats that were given either deionised water (control), selenium-rich green tea (Se-GTE), regular green tea (R-GTE), selenium-rich black tea (Se-BTE), or regular black tea (R-BTE). Tea water extracts (1%, w/v), as the only sourced fluids for an eight-week course of intervention, were given immediately after the OVX surgery at week 0. *In vivo* bone mineral density of femurs and the lumbar spine were measured using dual energy X-ray absorptiometry (DEXA) at weeks -2 (baseline), 3 and 7. At the end of the study, animals were euthanased and blood, the caecum with contents, uterus and right femurs were collected. The effects of various teas were determined by analysing bone biochemical markers, serum antioxidant capacity, caecal pH, bacterial enzymatic activities, and populations of selected intestinal microbiota. The excised right femurs were tested biomechanically using the three-point bending test. Our data show, for the first time, that consumption of R-BTE for eight weeks significantly ($p = 0.0052$) suppressed bone turnover in OVX rats. No significant effects were shown by any of the teas on bone density and femur biomechanics as compared to the OVX control rats. Selenium-GTE and R-BTE showed promising modulating effect on intestinal microbiota, as shown by a significant decrease ($p = 0.0061$ and $p = 0.0294$; respectively) in caecal pH and a significant increase ($p < 0.0001$ and $p = 0.0030$; respectively) in the activity of β -glucosidase bacterial enzyme. The results suggested that the protective effect of teas against bone loss is possibly associated with their prebiotic-like effects, but is not due to a direct antioxidant effect. However, the exact mechanism underlying such effects needs to be further investigated.

8.1 Introduction

Osteoporosis is one of the most common degenerative diseases of the elderly. Postmenopausal osteoporosis is a type I osteoporosis, and is mainly caused by oestrogen deficiency in postmenopausal women, which results in accelerated bone remodelling due to bone resorption exceeding bone formation (Baldock et al., 1999; Raisz, 2005; Clarke & Khosla, 2010; Feng & McDonald, 2011). While increased bone resorption activity following menopause is linked to increased bone formation, the amount of bone formation is inadequate and results in a loss of bone mass (Clarke & Khosla, 2010; McNamara, 2010).

Low bone mass resulting from postmenopausal osteoporosis can progress over several years without symptoms, and unfortunately is often detected only after the first fracture occurs (Elliot-Gibson et al., 2004; Rachner et al., 2011). Osteoporosis-related fractures in postmenopausal women can happen at most skeletal sites, with the most common being vertebral compression fractures in the spine, as well as fractures of the ribs, hip, and wrists (Khosla, 2010). Osteoporotic-related fractures are a major cause of morbidity and mortality worldwide and have become an important public health problem bringing social and economic burdens on both individuals and health service resources (Harvey et al., 2010; Ström et al., 2011; Cauley, 2013).

The pharmacotherapy drugs that are available for the management of osteoporosis mostly exert their effects by restoring oestrogen concentrations or the balance of bone remodelling, although they may not entirely reverse the effects of established osteoporosis (Jules et al., 2010; Jia et al., 2012). The currently available pharmacotherapy drugs include bisphosphonates, selective oestrogen receptor modulators (SERMs) (i.e. raloxifene), strontium ranelate, parathyroid hormone (PTH), tibolone, denosumab, calcitonin and hormone replacement therapy (HRT), as well as other emerging drugs under investigation such as cathepsin K inhibitors and new SERMs (i.e. lasofoxifene, bazedoxifene and arzoxifene) (Sandhu & Hampson, 2011; Singh et al., 2013). While some of these drugs are effective in the prevention and treatment of postmenopausal osteoporosis, some of them may not be affordable to some

patients and/or cause serious adverse effects that limit their use and efficacy (Rossouw et al., 2002; Reginster et al., 2013; Reginster, 2014).

In **Chapter 5**, it was briefly mentioned that the development of osteoporosis is influenced by both non-modifiable and modifiable factors. Indeed, lifestyle modifications (e.g. physical exercise) and nutritional supplementations (e.g. calcium, vitamin D, dietary antioxidants) are usually used for the prevention and treatment of osteoporosis rather than pharmacotherapy (Rachner et al., 2011; Jia et al., 2012; Singh et al., 2013). Due to the controversy surrounding the safety and efficacy of current pharmacotherapy drugs, some researchers have turned to alternative treatments, where effective and safer nutritional supplementation, independent of calcium and vitamin D, to reduce bone loss in postmenopausal women is receiving more attention as an alternative natural approach for the prevention and management of osteoporosis (Horcajada & Offord, 2012; Sacco et al., 2013; Rao & Rao, 2013).

The generation of reactive oxygen species (ROS) at physiological levels is particularly crucial in the maintenance of mineral homeostasis and mediating intracellular signals involved with cell growth and differentiation. Regarding skeletal function, ROS mainly involve with bone remodelling by enhancing resorption activity (Wauquier et al., 2009; Filaire & Toumi, 2012; Kanzaki et al., 2013). In parallel with aging, oestrogen deficiency may lead to increased levels of ROS, where loss of oestrogen elevates production of ROS due to depletion of endogenous antioxidant cellular defences (Muthusami et al., 2005; Almeida et al., 2007a; Wauquier et al., 2009; Manolagas, 2010; Bellanti et al., 2013). Due to the important roles of ROS and oestrogen deficiency in bone resorption, the use of dietary nutritional antioxidants as a natural approach for management of postmenopausal osteoporosis has received a lot of attention (Rao & Rao, 2013; Weaver & Hohman, 2013). Tea beverages from leaves of *Camellia sinensis* contain dietary antioxidants rich in polyphenols that are increasingly investigated for their potential to promote bone health, specifically in the prevention and treatment of osteoporosis (Trzeciakiewicz et al., 2009; Rao & Rao, 2013; Shen et al., 2013). Despite the safety of tea consumption, there is a limited amount of information on the role of tea as a whole mixture of compounds in bone health *in vivo*,

and many pre-clinical studies only provide evidence for the skeletal benefit of isolated compounds from tea (Shen et al., 2013).

To date, no studies have been conducted to examine whether there is a bone-protective effect of selenium-rich tea on ovariectomy-induced bone loss using an animal model of osteoporosis. Our previous research using cell lines (**Chapters 5 and 6**) and young growing rats (**Chapter 7**) has demonstrated that some of the tested teas have a bone-protecting effect. Based on these positive effects, we hypothesised that these teas would mitigate bone loss and improve bone strength in ovariectomised rats via an increase of antioxidant capacity and/or prebiotic activity. In the present study, the effect of 8-week consumption of a variety of teas (i.e. Se-GTE, R-GTE, Se-BTE and R-BTE) on bone loss in bilaterally ovariectomised mature adult female Sprague-Dawley rats were assessed to determine the antioxidant and prebiotic effects of the teas. This study was performed to investigate whether these teas may provide protection against ovariectomy-induced bone loss in rats, as well as to determine whether prevention of bone loss by the teas, if any, involves their antioxidative role and/or prebiotic potential.

8.2 Materials and methods

8.2.1 Animals and protocols

Ninety 3-month old female Sprague-Dawley (SD) rats (mean body weight 272.8 ± 2.0 g) were obtained from the Small Animal Production Unit (SAPU), Massey University, Palmerston North, New Zealand. Ethical approval, and care and maintenance of the animals have been described in **Chapter 3**, section 3.2.3.

8.2.2 Diets

Standard rat chow pellets used in the study was formulated to contain all nutrients that are necessary for normal reproduction in female rats based on AIN-93M (**Table 8-1**). Preparation of rat chow pellets is described in **Chapter 3**, section 3.2.4.

Rats had *ad libitum* access to the standard rat chow pellets throughout the study period from week -3 to the end of week 8, except for an overnight period of fasting prior to blood collection on the last day of the study. The diet was randomly sampled for chemical analysis to confirm the formulated nutrient contents.

Table 8-1. Diet formulation for the female Sprague-Dawley rats*.

Ingredients	Amount (g/kg diet)
Macronutrients:	
Wheat	420.0
Barley	300.0
Skim milk powder	90.0
Broll	50.0
Lucerne	50.0
Corn oil	39.0
Meat and bone	46.0
Micronutrients:	
Methionine	1.0
Premix	4.0
Total	1000.0

*Formulated according to NRC nutrient requirements for laboratory animals (AIN-93M) (NRC, 1995).

8.2.3 Preparation of tea water extracts (1%, w/v)

All tea water extracts or deionised water were replaced and served fresh once every second day and fluid intake was measured at this point. Preparation of the tea water extracts is described in **Chapter 3**, section 3.2.5.

8.2.4 Experimental design

The rats underwent a 7-day acclimatisation period before the study commenced. During this period of acclimatisation (week -3), they were fed a standard rat chow pellet diet and provided with deionised water, *ad libitum*. All rats were scanned for baseline

measurements of bone mineral density (BMD) using dual-energy X-ray absorptiometry (DEXA) at week -2, before ovariectomy surgery at week 0. The rats were scanned again for BMD at weeks 3 and 7 after the surgery.

At week -1, rats were weighed and assigned randomly into six experimental groups ($n = 15/\text{group}$) to ensure balance for average initial body weight across the treatment groups. At week 0, seventy-five rats were ovariectomised (OVX) and fifteen rats were sham-operated. Following ovariectomy surgery, the OVX rats were divided into 5 equal groups ($n = 15/\text{group}$), i.e. OVX control (deionised water), Se-GTE (selenium-rich green tea), Se-BTE (selenium-rich black tea), R-GTE (regular green tea), and R-BTE (regular black tea). All rats were fed standard rat chow pellets. The OVX rats were given their respective tea extracts, whereas the sham-operated rats were given deionised drinking water. The treatment course of all groups was eight weeks (**Figure 8-1**).

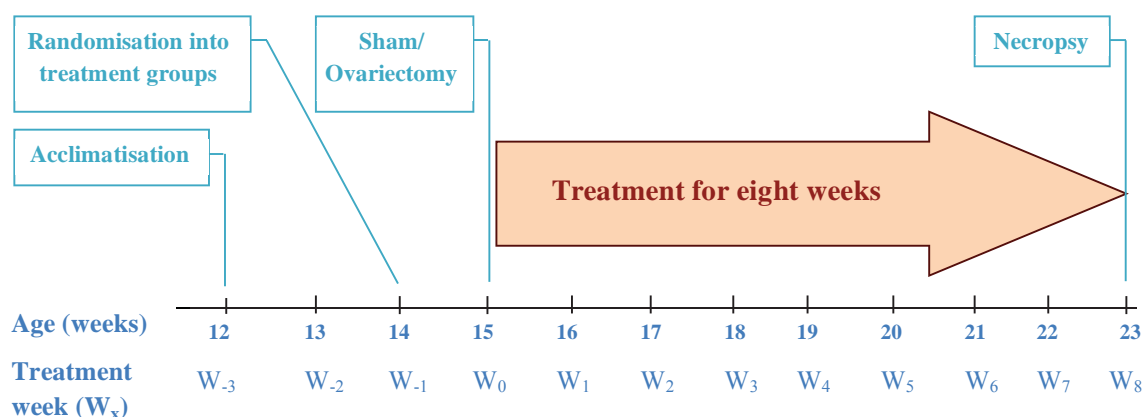


Figure 8-1. Experimental design for evaluation of bone properties in ovariectomised female rats. Rats were fed standard rat chow pellets (AIN-93M) starting at 12 weeks of age. Tea water extracts were given for eight weeks.

Tea extracts or deionised water were given *ad libitum* throughout the study (week 0 to the end of week 8), as the only source of drinking fluid (Gu et al., 2009). The feed and fluid intake (water/tea) of the rats were measured every second day. Body weight of the rats was recorded weekly. All measurements were done at a specific time in the morning. Feed intake was determined as the difference in weights of standard rat

chow pellets before and after each feeding period. Similarly, the fluid intake was determined by measuring the weight difference of the water bottles containing deionised water or tea water extracts. At the end of the treatment period (week 8), rats (23 weeks of age) were weighed and fasted overnight. Fasted blood samples were drawn directly from the heart under anaesthesia. Rats were euthanised by exsanguination following terminal heart puncture, and then dissected. Right femur, uterus and the caecum with contents were harvested.

8.2.5 Experimental techniques

8.2.5.1 Ovariectomy and sham-operation

At 15 weeks of age, all rats were subjected to ovariectomy or sham surgery under anaesthesia, as described in **Chapter 3**, section 3.2.6.1.

8.2.5.2 Evaluation of *in vivo* bone mineral measurements using dual energy X-ray absorptiometry (DEXA)

Bone mineral measurements of rat whole body at weeks -2 (baseline), 3 and 7 were performed using DEXA under anaesthesia, as described in **Chapter 3**, section 3.2.6.2. Due to the effect of body weight on bone mineral density, data of *in vivo* DEXA were corrected for body weight (Zhao et al., 2008) during statistical analysis.

8.2.5.3 Blood sample collection via terminal heart puncture

Fasted blood samples were drawn directly from the heart under anaesthesia at the completion of the studies, as described in **Chapter 3**, section 3.2.6.3.

8.2.5.4 Euthanisation and dissection

Rats were euthanised by exsanguination following terminal heart puncture, and dissected, as described in **Chapter 3**, section 3.2.6.4.

8.2.5.5 Evaluation of serum antioxidant capacity

The fasted blood serum samples were evaluated for antioxidant capacity using ferric-reducing antioxidant power (FRAP) assay, as described in **Chapter 3**, section 3.2.6.5.

8.2.5.6 Evaluation of serum biomarker of bone resorption

The fasted blood serum samples were evaluated for carboxy-terminal crosslinks telopeptide of type I collagen (CTX-I), as described in **Chapter 3**, section 3.2.6.6.

8.2.5.7 Evaluation of serum biomarker of bone formation

The fasted blood serum samples were evaluated for osteocalcin (OC) concentration, as described in **Chapter 3**, section 3.2.6.7.

8.2.5.8 Evaluation of bone biomechanical properties of the appendicular long bones using a three-point bending test

Bone biomechanical properties were performed on right femurs using a three-point bending test, as described in **Chapter 3**, section 3.2.6.9.

8.2.5.9 Evaluation of caecal pH and bacterial enzymatic activities (β -glucosidase and β -glucuronidase) in *ex vivo* rat caecal contents

The pH measurement of caecal samples and the bacterial enzymatic activities of the intestinal microbiota were performed using the same methods described in **Chapter 3**, sections 3.2.6.10 and 3.2.6.11.

8.2.5.10 Evaluation of selected intestinal microbiota populations in *ex vivo* rat caecal contents using fluorescence *in situ* hybridisation (FISH) analysis

Ex vivo caecal contents obtained from the female rats were analysed using FISH analysis, as described in **Chapter 3**, section 3.2.6.12.

8.2.6 Statistical analysis

A total of ninety rats ($n = 15/\text{group}$) were used in the study, and detailed statistical analysis of the data have been described in **Chapter 3**, section 3.2.7. On calculation of power and sample size on BMD measurements using reference data from Kruger et al. (2005), a sample size of 15 was necessary for a power of 0.94 to detect a significant difference between the control and test group. The expected difference that could be detected between a sham control and OVX rat was set at 0.02 g/cm^2 for BMD measurements. For the FRAP assay, simple linear regression analysis was performed using Microsoft Excel (2007) to calculate the dose-response relationship of standard ferrous sulphate (FeSO_4) solution used for calibration. A mixed models approach for repeated measures analysis of variance (ANOVA) was used for the weekly body weight and repeated measurements of *in vivo* DEXA (lumbar spine, femur, whole body composition), with treatment group as the fixed variable. The reported p values for the effects tested were based on a covariance pattern model (unstructured). The main analysis focused on the raw data, and body weights at each time point were included as a covariate. A secondary analysis looked at the percentage change in *in vivo* DEXA results for each time point compared with the baseline (i.e. week 3 *versus* -2, and week 7 *versus* -2). Both main and secondary analyses were comprised of two independent repeated measures ANOVA which tests for sham *versus* OVX control, and OVX control *versus* experimental tea groups that subsequently followed by Tukey-Kramer's post-hoc tests for comparisons. The repeated measures analyses were performed using the PROC MIXED, a procedure within SAS that fits a variety of mixed linear models. Uterus weight, fluid intake, feed intake, serum FRAP, serum CTx-I, serum osteocalcin, three-point bending biomechanical test of the right femur, caecal pH, bacterial enzymatic activities and FISH analysis were analysed using a one-way ANOVA with treatment groups as the fixed effect. In each one-way ANOVA analyses, the sham control group (non-OVX) was excluded, where a student's t -test was used for comparisons between sham *versus* OVX control groups.

8.3 Results

8.3.1 Dietary analysis

The analysed dietary composition of the rat chow is shown in **Table 8-2**. The standard rat chow pellets used in the study were made according to AIN-93M specifications for the laboratory rats, to provide minimal requirements for reproduction in adult female rats. All key dietary components, i.e. protein, fat, and calcium were slightly higher than the specified levels of AIN-93M. The Ca/P ratio in the diet met the requirements both for maximal body growth and maximal mineralisation (1.17 *versus* 1.1 and 1.17 *versus* 1.4 for body growth and mineralisation, respectively) (Benhart et al., 1969).

Table 8-2. Dietary composition analysis of standard pellet rat chow^{a*}.

Component	Unit	Amount
Dry matter	%	90.00
Carbohydrate	%	62.50
Protein	%	15.70
Moisture	%	10.0
Fat	%	7.6
Ash	%	4.2
Calcium	mg/kg	7,000
Phosphorus	mg/kg	6,000
Magnesium	mg/kg	1,400
Zinc	mg/kg	50

^aIngredients: wheat, barley, broll, lucerne, meat and bone, methionine, skim milk powder, corn oil and premix. *Nutrient composition was analysed by the Nutrition Laboratory, Massey University; fat by acid hydrolysis using Mjonner extraction (AOAC 922.06); protein by LECO total combustion method (AOAC 968.06); total carbohydrate by difference; minerals by Plasma Emission Spectrometry.

8.3.2 General observations

The tea extract infused water was well tolerated by the rats during the eight weeks of the study. There were no adverse effects such as behavioral changes, illness or death due to the tea consumption. There were no signs of deteriorating health observed in rats given tea extracts and all were healthy throughout the study period. Apart from this, two healthy rats died (rat 65 from the control group, and rat 76 from the Se-BTE treatment group) following intraperitoneal anaesthesia for DEXA scan at different time points (week -2, week 3). These rats died unexpectedly and the results of the post-mortem examination were inconclusive. The deaths were likely unrelated to the treatment (**Appendix IV**).

8.3.3 Effect of ovariectomy surgery on uterus weight of female Sprague-Dawley rats

Sham and ovariectomy surgery was conducted when the rats were 15 weeks of age. At the end of the feeding trial, the uterus of each rat was resected and weighed. The uterus is a highly oestrogen-dependent organ and maintaining its size is highly dependent on oestrogen production from the ovaries. The uteri weights of the sham-operated (sham) group were greater ($p < 0.0001$) than the ovariectomised (OVX) control group (**Figure 8-2**). Bilateral ovariectomy in rats caused atrophy of the uterus resulting in decreased weight of uterine tissue which indicates successful surgical procedure. As seen in **Figure 8-2**, uterus weight did not differ between the five OVX groups ($p = 0.6545$). However, the uteri weights were higher in two OVX rats (i.e. rat 84 = 0.4709 g, and rat 89 = 0.5230 g) compared to the rest of the OVX rats, indicating incomplete resection of the ovaries during the ovariectomy procedure. On these grounds, rats 84 and 89 (both from Se-BTE treatment group) were excluded from all future analyses, thus, leaving only 12 rats available for future analyses involving the Se-BTE treatment group.

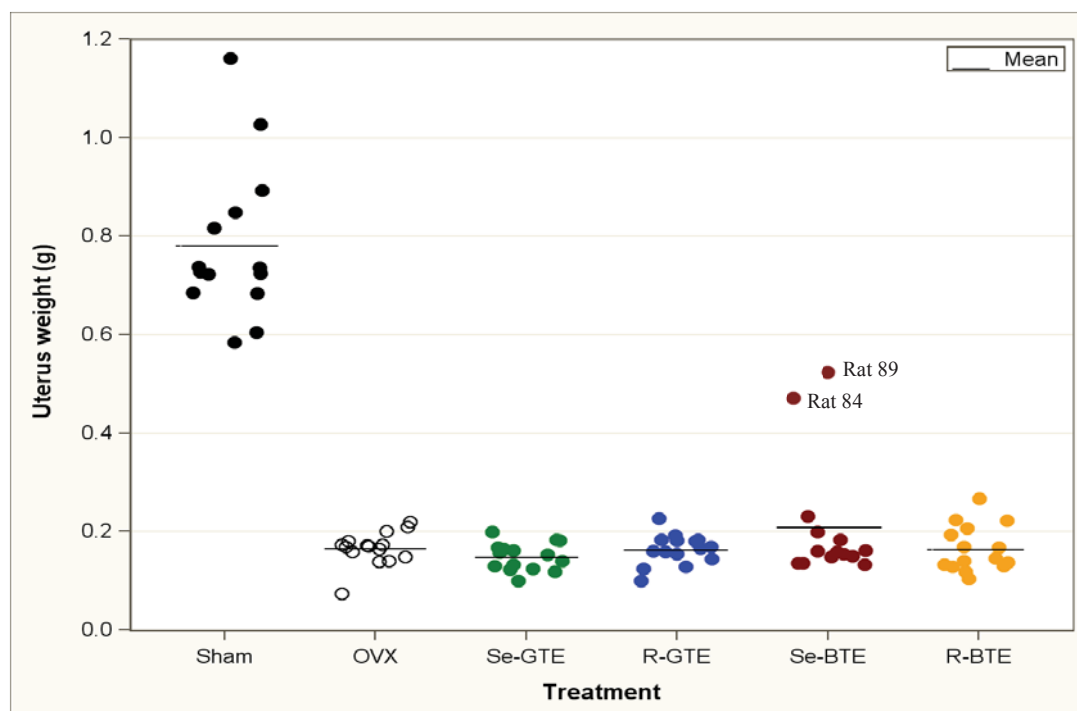


Figure 8-2. Uterus weights of 23-week-old female Sprague-Dawley rats. Rats were given either deionised water or tea drinks (Se-GTE, R-GTE, Se-BTE, R-BTE) following ovariectomy surgery at week 0 until the rats were sacrificed at week 8. The coloured points represent each individual rat whilst the group mean is indicated by the solid horizontal line.

8.3.4 Effects of tea extracts on body weight, fluid intake and feed intake

The weekly body weight (BW) measurements of female Sprague-Dawley rats are shown in **Figure 8-3**. The initial BW at the start of the study (week -3) ranged from 270.2 ± 4.8 to 273.4 ± 5.2 g, with statistical analysis indicating no difference ($p = 0.8741$) among the rats. As expected, the statistical analysis indicated that there was no difference in the body weight ($p = 0.8741$) among the OVX groups at ovariectomy (week 0), due to the rats being randomly assigned by body weight at week -1. No difference in BW ($p = 0.2246$) was also found among the OVX groups at the endpoint BW (week 8). All OVX rats including sham-operated (ovaries intact) rats gained body weight in a time-dependent manner throughout the tea intervention study. The body weight of OVX rats increased at a faster rate than sham-operated rats following OVX surgery at week 0 and reached a significant value at week 2 ($p = 0.0007$), with no difference ($p = 0.5433$) found between any of the OVX groups throughout the eight-

week tea intervention (**Figure 8-3, Table 8-3**). As depicted in **Table 8-3**, there was also no interaction between tea and week indicating no tea effect on body weight of OVX rats throughout the tea intervention study (week 0 to week 8). Although body weight increased at a greater rate in rats given R-BTE over that of the other OVX groups during eight-week tea intervention, there were no significant differences ($p = 0.6648$).

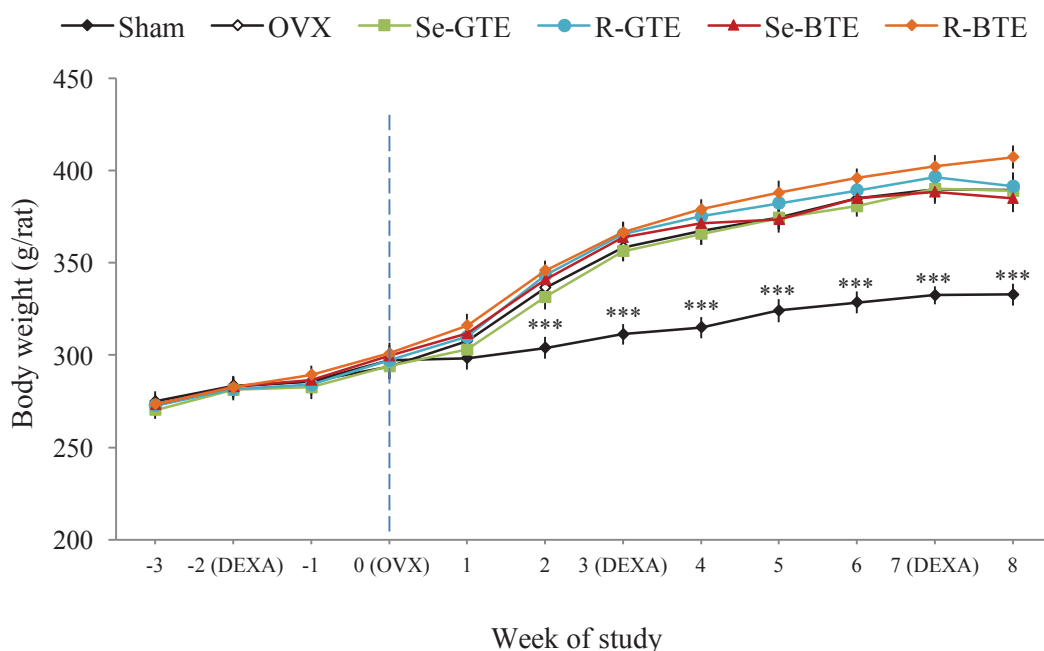


Figure 8-3. Mean weekly body weight measurements in female Sprague-Dawley rats. Rats were given deionised water (OVX, sham) or tea drinks (Se-GTE, R-GTE, Se-BTE, R-BTE) for eight weeks following ovariectomy surgery at week 0 until the rats were sacrificed at week 8. Data are mean (\pm SEM), $n = 12 - 15$ rats/group. *indicates significance in comparison with the OVX control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 8-3. Effect of experimental tea drinks on body weight^a.

OVX groups	p value ^b
Tea	$p = 0.5433$
Week	$p < 0.0001$
Tea \times Week	$p = 0.1538$

^aWeekly body weight measurements in Sprague-Dawley rats were made during eight-week tea intervention, i.e. prior to ovariectomy surgery (week 0) until the study endpoint (week 8). ^bRepeated measures ANOVA, $n = 12 - 15$ rats/group.

The mean fluid intake and feed intake are shown in **Table 8-4**. Fluid intake throughout the intervention study varied significantly among the OVX groups ($p < 0.0001$), with the highest fluid consumption observed in the Se-BTE group (26.3 ± 0.7 mL/day), and the lowest fluid consumption by the OVX control group (21.6 ± 1.0 mL/day). However, among the OVX rats, only differences in mean fluid intake between OVX control *versus* Se-BTE, and between OVX control *versus* R-BTE were found to be significant ($p = 0.0010$ and $p = 0.0085$, respectively). There was no difference in feed intake among the OVX groups ($p = 0.1176$).

Table 8-4. Mean values of fluid intake and feed intake of female Sprague-Dawley rats during eight-week tea intervention study following ovariectomy surgery at week 0^a.

Group	Sham ^b	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^c
Fluid intake (mL/day)	24.4 (1.7)	21.6 (1.0)	21.7 (0.7)	22.5 (0.7)	26.3** (0.7)	25.8** (1.1)	< 0.0001
Feed intake (g/day)	20.0 (0.4)	21.3 (0.5)	21.4 (0.4)	21.8 (0.4)	21.8 (0.4)	22.6 (0.2)	0.1176

^aData represent mean (\pm SEM), $n = 12 - 15$ rats/group. ^bSham *versus* OVX control rats (student's *t*-test). ^cOne-way ANOVA followed by post-hoc Tukey-Kramer's test. *indicates significance from the OVX control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

8.3.5 Effect of tea extracts on serum antioxidant capacity

The mean serum FRAP values expressed as FeSO₄ mg equivalent (E)/L serum (\pm SEM) were: 124.9 ± 1.8 (sham), 117.2 ± 1.8 (OVX control), 134.0 ± 3.1 (Se-GTE), 114.5 ± 1.6 (R-GTE), 119.0 ± 2.1 (Se-BTE) and 108.1 ± 1.4 (R-BTE), as shown in **Figure 8-4**. The differences between serum FRAP levels among OVX groups were significant ($p < 0.0001$), where the FRAP values in the Se-GTE group were the highest ($p < 0.0001$), while the FRAP values in the R-BTE group were the lowest ($p = 0.0182$). There was no significant effect shown by R-GTE ($p = 0.8881$) and Se-BTE ($p = 0.9775$) on serum FRAP when compared to the OVX control. Serum FRAP in the sham-operated rats was significantly higher than the OVX control rats ($p = 0.0032$).

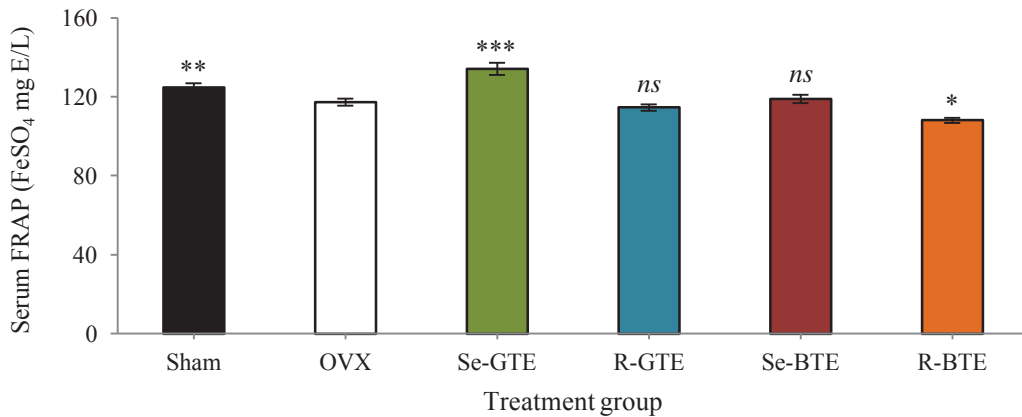


Figure 8-4. Serum ferric-reducing antioxidant power (FRAP) of 23-week-old female Sprague-Dawley rats following eight-week consumption of deionised water or tea drinks. Data are the mean (\pm SEM), $n = 12 - 15$ rats/group. *indicates significance versus the OVX control: *ns* = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

8.3.6 Effect of tea extracts on serum CTx-I concentration

CTx-I levels were significantly different amongst the OVX groups ($p = 0.0057$) (**Figure 8-5**). The mean values of serum CTx-I in ng/mL were: 6.08 ± 0.26 (sham), 10.01 ± 0.43 (OVX), 9.36 ± 0.43 (Se-GTE), 9.83 ± 0.50 (R-GTE), 9.09 ± 0.47 (Se-BTE), and 7.91 ± 0.21 (R-BTE). Only rats on R-BTE showed a significant decrease in CTx-I levels ($p = 0.0052$) in comparison to the OVX control. Serum CTx-I in the sham-operated rats was significantly lower ($p < 0.0001$) than the OVX control.

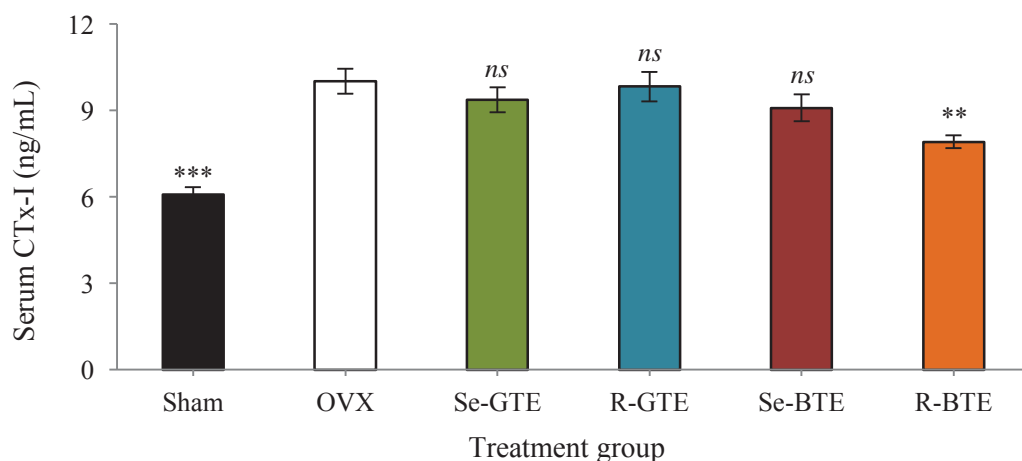


Figure 8-5. Effect of tea consumption on serum CTx-I concentrations in 23-week-old female Sprague-Dawley rats following eight-week consumption of deionised water or tea drinks. Data are mean (\pm SEM), $n = 12 - 15$ rats/group. *indicates significance versus the OVX control: *ns* = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

8.3.7 Effect of tea extracts on serum OC concentration

As depicted in **Figure 8-6**, the serum OC concentration in 23-week-old female rats ranged from 90.1 ± 4.9 to 188.1 ± 9.0 ng/mL, with statistical analysis indicating no significant differences ($p = 0.0517$) among the OVX groups. Thus, none of the experimental tea groups showed significant changes in the levels of serum OC (Se-GTE: 177.1 ± 9.7 ng/mL, R-GTE: 188.1 ± 9.0 ng/mL, Se-BTE: 170.8 ± 4.7 ng/mL, R-BTE: 172.1 ± 5.3 ng/mL), when compared to the OVX control (179.9 ± 6.3 ng/mL). Serum OC in the sham rats (90.1 ± 4.9 ng/mL) was significantly lower than that of the OVX control rats ($p < 0.0001$).

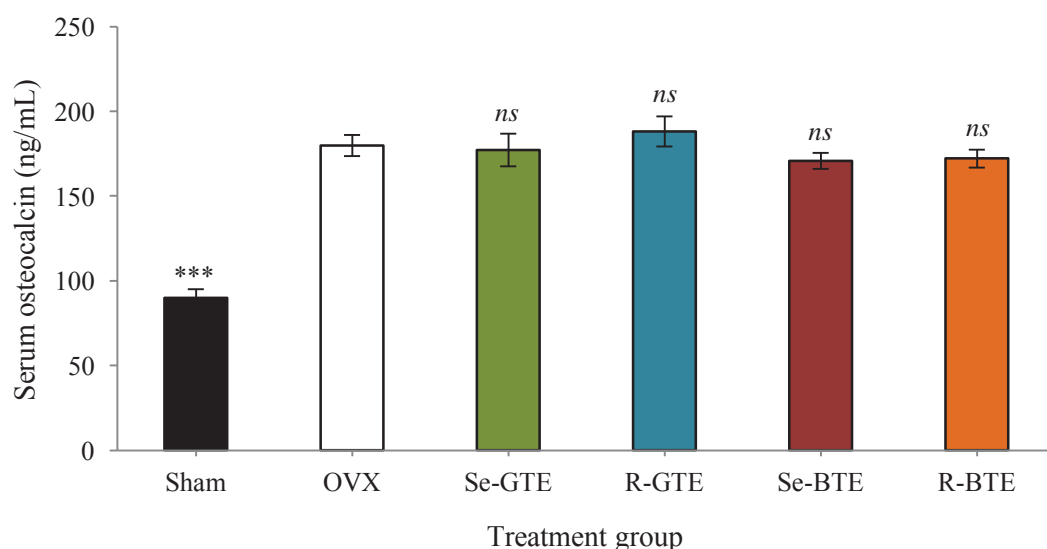


Figure 8-6. Effect of tea consumption on serum osteocalcin concentrations in 23-week-old female Sprague-Dawley rats following eight-week consumption of deionised water or tea drinks. Data are mean (\pm SEM) of triplicate measurements, $n = 12 - 15$ rats/group. *indicates significance *versus* the OVX control: ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

8.3.8 Effects of tea extracts on *in vivo* bone mineral measurements

8.3.8.1 Lumbar spine DEXA

The main analysis of the raw data from *in vivo* DEXA of lumbar spine is provided in full as an appendix (**Appendix VI**), where body weights at each time point were included as a covariate. As summarised in **Table 8-5**, there was no effect of tea on

lumbar spine bone area (BA, $p = 0.7949$), bone mineral content (BMC, $p = 0.8820$) and bone mineral density (BMD, $p = 0.8646$) or any interaction between tea and week that indicating any increase in the lumbar spine BA, BMC and BMD over the course of the tea consumption study in the OVX rats ($p = 0.3554$, 0.0957 and 0.2049 , respectively).

Table 8-5. Effect of experimental tea drinks on lumbar spine bone area (BA), bone mineral content (BMC) and bone mineral density (BMD)^a.

OVX groups	Lumbar spine ^b		
	BA	BMC	BMD
Tea	$p = 0.7949$	$p = 0.8820$	$p = 0.8646$
Week	$p = 0.0010$	$p < 0.0001$	$p < 0.0001$
Tea × Week	$p = 0.3554$	$p = 0.0957$	$p = 0.2049$

^aMeasurements were made *in vivo* by DEXA at week -2 (baseline), week 3 and week 7.
^bRepeated measures ANOVA, $n = 12 - 15$ rats/group.

A secondary analysis that looked at the percentage (%) change in *in vivo* lumbar spine DEXA of each time point compared with baseline (i.e. week 3 vs -2, and week 7 vs -2) is depicted in **Figure 8-7**. There were no significant between-group differences observed for % change in the lumbar spine DEXA measures after three- and seven-weeks tea consumption when compared with baseline. The OVX model was successful with the lumbar spine BMD of the OVX control rats being significantly lower than the sham control group at both three ($p = 0.0095$) and seven ($p = 0.0015$) weeks after ovariectomy. ~~Significant differences between sham and OVX control groups were only observed in the decrease of % change from baseline for lumbar spine BMD in OVX control after three weeks ($p = 0.0095$) and seven weeks ($p = 0.0015$) of tea consumption study.~~

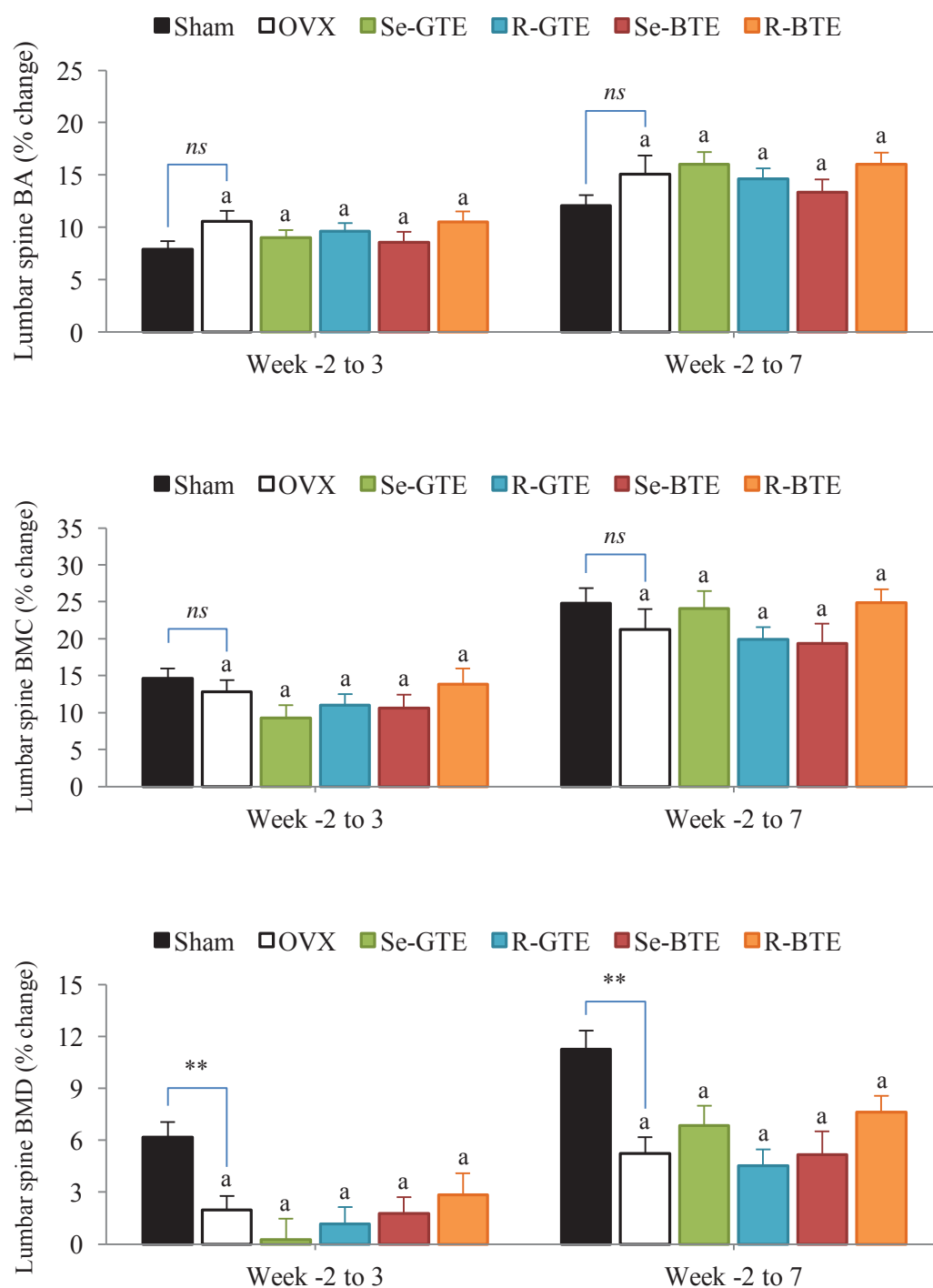


Figure 8-7. Between-group differences of percentage (%) change for lumbar spine bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) over time in female Sprague-Dawley rats. Rats were given their respective deionised water or tea drinks (Se-GTE, R-GTE, Se-BTE, R-BTE) for eight weeks following ovariectomy surgery at week 0 until the rats were sacrificed at week 8. Differences in the letters indicate statistical significance between the OVX groups for each time period. Data are mean (\pm SEM), $n = 12 - 15$ rats/group. *indicates significance versus the OVX control: *ns* = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

8.3.8.2 Femoral bone DEXA

The main analysis of the raw data from *in vivo* DEXA of femoral bone is provided in full as an appendix (**Appendix VII**), where body weights at each time point were included as a covariate. As shown in **Table 8-6**, there was a positive effect of tea on BA ($p = 0.0352$), but not on the BMC ($p = 0.0854$) and BMD ($p = 0.7037$). In addition, there was an interaction between tea and week on BMC ($p = 0.0024$) but none was observed for BA ($p = 0.3554$) and BMD ($p = 0.1205$) of femoral bone over the course of tea consumption in the OVX rats.

Table 8-6. Effect of experimental tea drinks on femur bone area (BA), bone mineral content (BMC) and bone mineral density (BMD)^a.

OVX groups	Femurs ^b		
	BA	BMC	BMD
Tea	$p = 0.0352$	$p = 0.0854$	$p = 0.7037$
Week	$p = 0.0279$	$p < 0.0001$	$p < 0.0001$
Tea × Week	$p = 0.1593$	$p = 0.0024$	$p = 0.1205$

^aMeasurements were made *in vivo* by DEXA at week -2 (baseline), week 3 and week 7.

^bRepeated measures ANOVA, $n = 12 - 15$ rats/group.

A secondary analysis that looked at the % change in *in vivo* DEXA of femurs for each time point *versus* the baseline (i.e. week 3 *versus* -2, and week 7 *versus* -2) is depicted in **Figure 8-8**. No significant between-group differences were observed for % change in femur BA, BMC and BMD of OVX rats from baseline (week -2), after three- and seven-week tea consumption. Again, the OVX model was successful with the femur BMD of the OVX control rats being significantly lower than the sham control group at both three ($p < 0.0001$) and seven ($p < 0.0001$) weeks after ovariectomy. ~~Meanwhile, significant differences between sham and OVX control groups were only observed in the decrease of % change from baseline for femur BMD in OVX control after three weeks ($p < 0.0001$) and seven weeks ($p < 0.0001$) of tea consumption study.~~

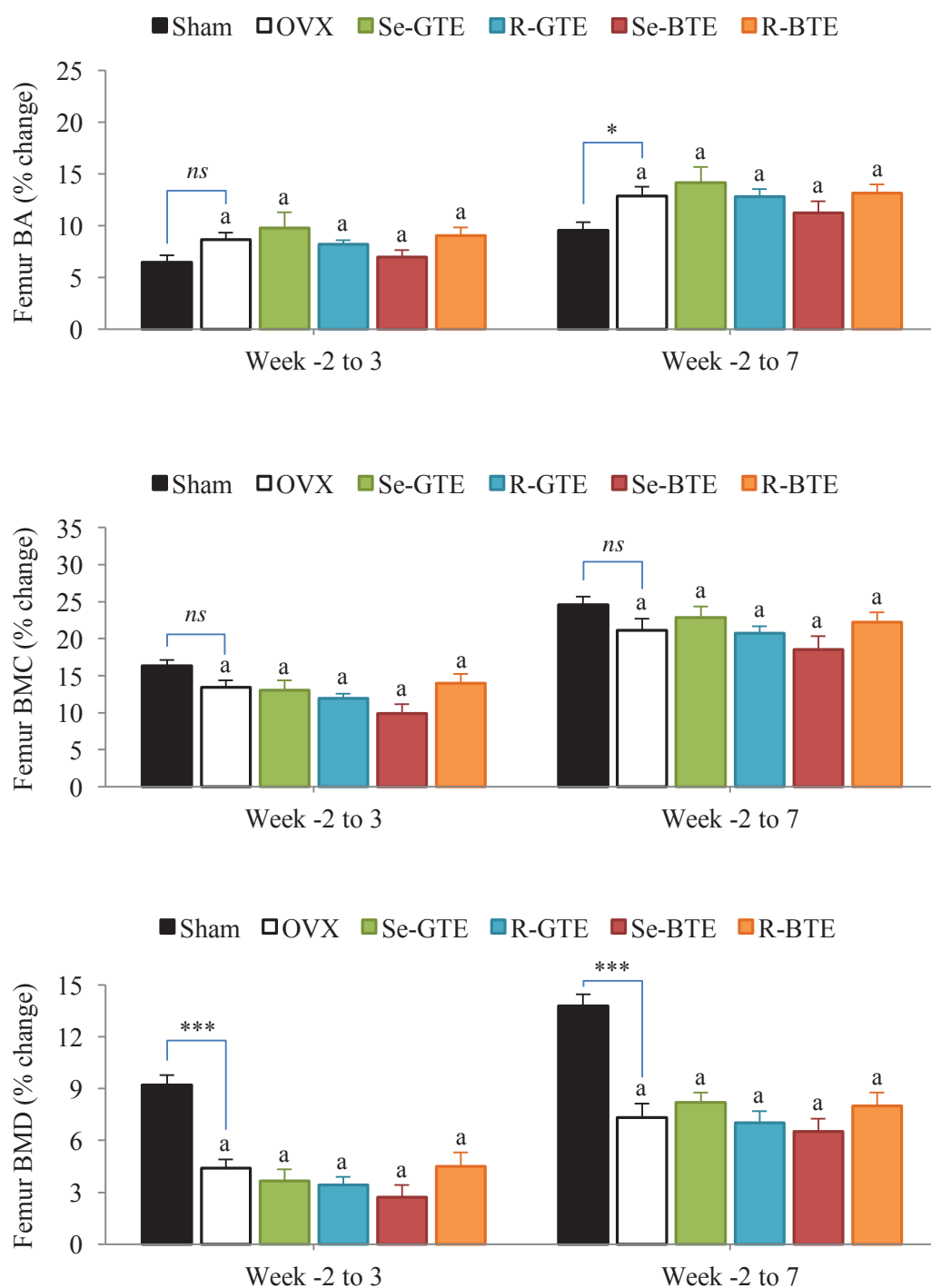


Figure 8-8. Between-group differences of percentage (%) change for femur bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) between successive time points in female Sprague-Dawley rats. Rats were given either deionised water or tea drinks (Se-GTE, R-GTE, Se-BTE, R-BTE) for eight weeks following ovariectomy surgery at week 0 until the rats were sacrificed at week 8. Differences in the letters indicate statistical significance between the OVX groups for each time period. Data are mean (\pm SEM), $n = 12 - 15$ rats/group. *indicates significance *versus* the OVX control: *ns* = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

8.3.8.3 Whole body bone mineral measurements

The main analysis of the raw data from *in vivo* DEXA of whole body bone mineral is provided in full as an appendix (**Appendix VIII**), where body weights at each time point were included as a covariate. As shown in **Table 8-7**, there was a positive effect of tea on whole body BA ($p = 0.0494$) and BMC ($p = 0.0131$), but not on the BMD ($p = 0.2884$). Moreover, no interaction between tea and week was observed on BA, BMC and BMD (p values of 0.9476, 0.8701 and 0.1320, respectively) of the whole body over the course of tea consumption in the OVX rats.

Table 8-7. Effect of experimental tea drinks on whole body bone area (BA), bone mineral content (BMC) and bone mineral density (BMD)^a.

OVX groups	Whole body ^b		
	BA	BMC	BMD
Tea	$p = 0.0494$	$p = 0.0131$	$p = 0.2884$
Week	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Tea × Week	$p = 0.9476$	$p = 0.8701$	$p = 0.1320$

^aMeasurements were made *in vivo* by DEXA at week -2 (baseline), week 3 and week 7.

^bRepeated measures ANOVA, $n = 12 - 15$ rats/group.

A further analysis to examine % change in *in vivo* whole body bone mineral DEXA for each time point compared with baseline (i.e. week 3 *versus* -2, and week 7 *versus* -2) is depicted in **Figure 8-9**. No significant between-group differences were observed for whole body BA, BMC and BMD of OVX rats at any of the time points compared with baseline (week -2) following tea consumption. For the efficacy of the rat OVX model, significant differences between the sham and OVX control group were observed:

- 1) increased % change of baseline of whole body BA in the OVX control rats after three ($p = 0.0064$) and seven ($p = 0.0004$) weeks of tea consumption,
- 2) increased % change of baseline of whole body BMC in OVX control rats after seven weeks of tea consumption ($p = 0.0063$), and
- 3) a decrease in the % change of baseline of whole body BMD in OVX control rats after three ($p = 0.0003$) and seven ($p = 0.0010$) weeks tea consumption.

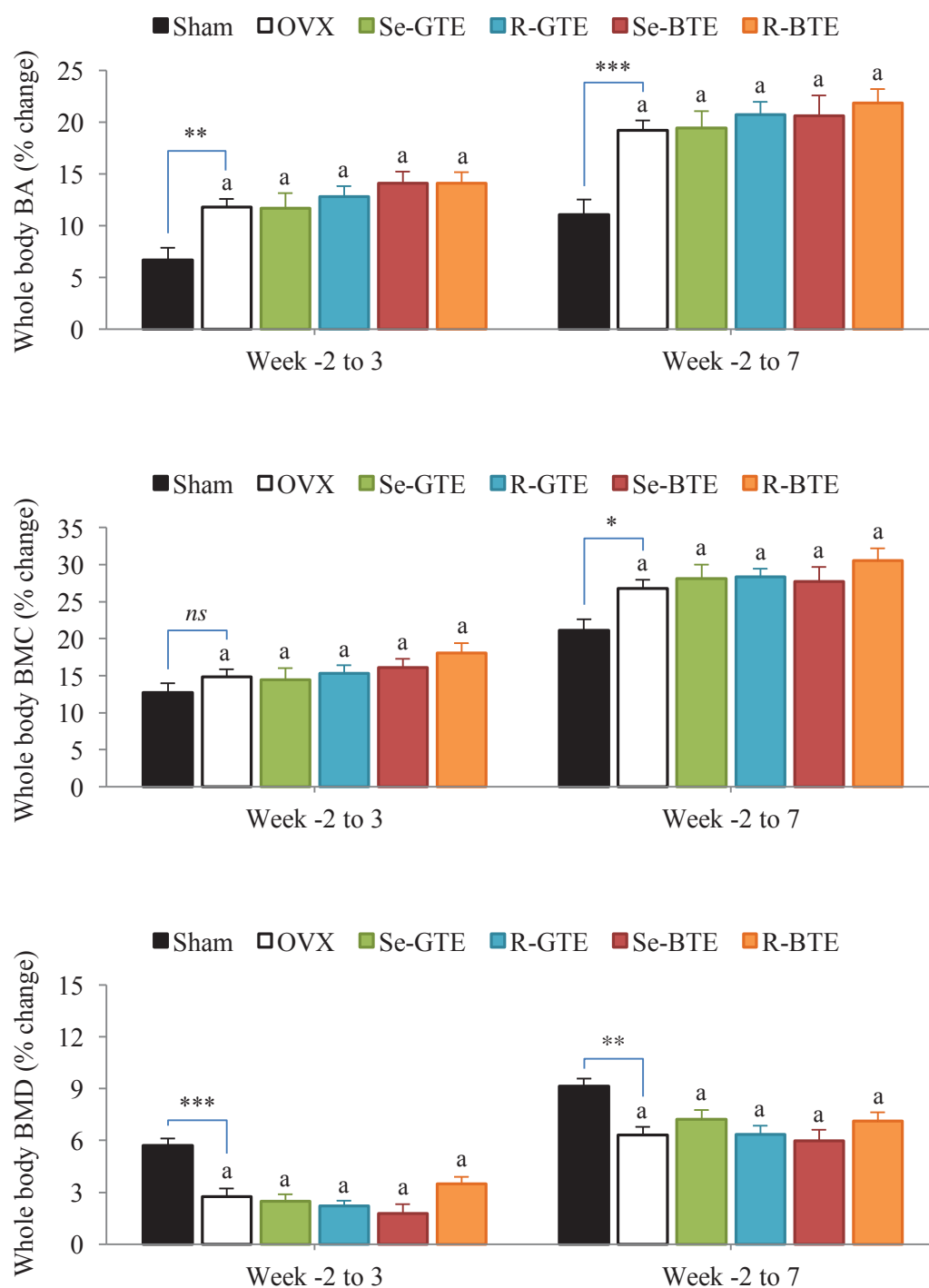


Figure 8-9. Between-group differences of percentage (%) change for whole body bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) between successive time points in female Sprague-Dawley rats. Rats were given either deionised water or tea drinks (Se-GTE, R-GTE, Se-BTE, R-BTE) for eight weeks following ovariectomy surgery at week 0 until the rats were sacrificed at week 8. Differences in the letters indicate statistical significance between the OVX groups for each time period. Data are mean (\pm SEM), $n = 12 - 15$ rats/group. *indicates significance versus the OVX control: *ns* = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

8.3.8.4 Whole body composition

The main analysis of the raw data from *in vivo* DEXA of whole body composition was provided in full as an appendix (**Appendix VIII**), where body weights at each time point were included as a covariate. **Table 8-8** showed that there was no effect of tea on whole body lean mass, fat mass and percentage fat. However, an interaction between tea and week was observed on lean mass, fat mass and percentage fat of the whole body composition over the course of tea consumption in the OVX rats (*p* values of 0.0289, 0.0152 and 0.0143, respectively).

Table 8-8. Effect of experimental tea drinks on whole body lean mass, fat mass and percentage fat^a.

OVX groups	Whole body ^b		
	Lean mass	Fat mass	Percentage fat
Tea	<i>p</i> = 0.3552	<i>p</i> = 0.2776	<i>p</i> = 0.3301
Week	<i>p</i> < 0.0001	<i>p</i> < 0.0001	<i>p</i> < 0.0001
Tea × Week	<i>p</i> = 0.0289	<i>p</i> = 0.0152	<i>p</i> = 0.0143

^aMeasurements were made *in vivo* by DEXA at week -2 (baseline), week 3 and week 7.

^bRepeated measures ANOVA, *n* = 12 – 15 rats/group.

A secondary analysis that looked at the % change in *in vivo* DEXA of whole body composition for each time point compared with the baseline (i.e. week 3 *versus* -2, and week 7 *versus* -2) is depicted in **Figure 8-10**. No significant between-group differences were observed for % change in whole body lean mass of OVX rats from baseline (week -2), after three- and seven-week tea consumption. However, significant between-group differences between OVX control and Se-GTE groups were observed for the decrease of % change in whole body fat mass (*p* = 0.0038) and whole body percentage fat of Se-GTE group after three-week tea consumption (from baseline). Meanwhile, significant differences between sham and OVX control groups were only observed in the:

- 1) increase of % change from baseline for whole body lean mass in OVX control after three weeks (*p* < 0.0001) and seven weeks (*p* < 0.0001) of tea consumption, and
- 2) increase of % change from baseline for whole body fat mass in OVX control after seven weeks of tea consumption (*p* < 0.0001).

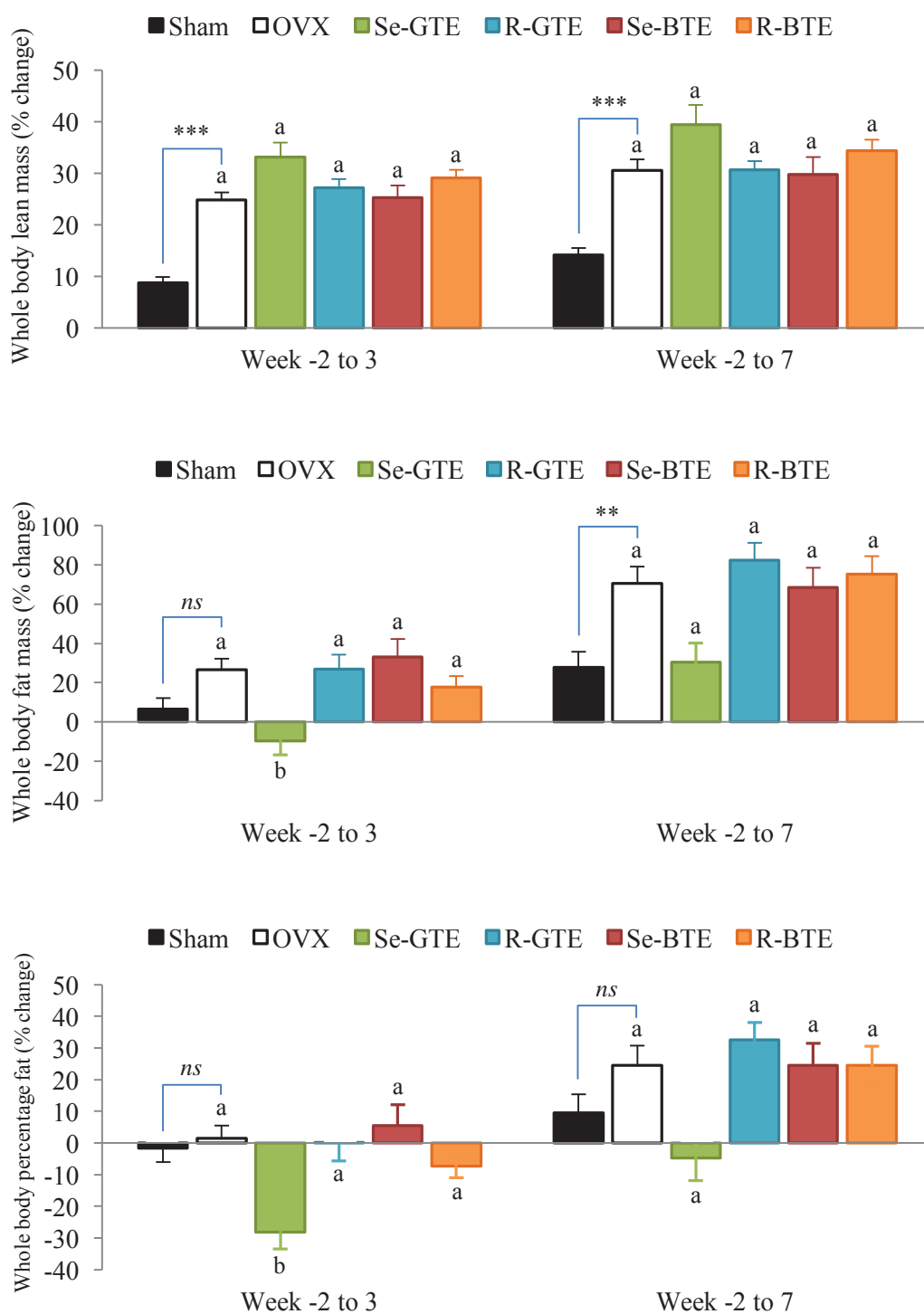


Figure 8-10. Between-group differences of percentage (%) change for whole body composition measures of lean mass, fat mass, and percentage fat between successive time points in female Sprague-Dawley rats. Rats were given either deionised water or tea drinks (Se-GTE, R-GTE, Se-BTE, R-BTE) for eight weeks following ovariectomy surgery at week 0 until the rats were sacrificed at week 8. Differences in the letters indicate statistical significance between the OVX groups for each time period. Data are mean (\pm SEM), $n = 12 - 15$ rats/group. *indicates significance from the OVX control: *ns* = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

8.3.9 Effect of tea extracts on femoral bone strength

Table 8-9 summarises the mechanical properties of bone for the various groups of OVX rats given either deionised water or tea drinks.

Table 8-9. Effect of tea drinks on the three-point bending biomechanical test of the right femur excised from female Sprague-Dawley rats^a.

Group	Sham ^b	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^c
Midpoint width (mm)	4.28** (0.05)	4.49 (0.05)	4.36 (0.04)	4.47 (0.06)	4.52 (0.07)	4.54 (0.06)	0.1546
Midpoint thickness (mm)	2.98 (0.04)	3.02 (0.06)	2.93 (0.04)	3.11 (0.06)	3.18 (0.06)	3.18 (0.04)	0.0026
Maximum load (N)	144.48* (3.68)	162.52 (5.94)	158.01 (4.34)	166.19 (6.04)	161.48 (6.08)	162.26 (5.21)	0.8849
Maximum stroke (mm)	1.32** (0.05)	1.58 (0.08)	1.56 (0.06)	1.60 (0.07)	1.69 (0.12)	1.53 (0.08)	0.7589
Break load (N)	143.14* (3.87)	161.35 (6.04)	156.79 (4.72)	165.76 (6.00)	159.10 (6.37)	158.06 (4.76)	0.7988
Break stress (N/mm ²)	85.0 (2.0)	89.3 (3.1)	94.6 (2.4)	86.7 (2.9)	78.5 (2.6)	77.7 (2.2)	< 0.0001
Break stroke (mm)	1.34** (0.00)	1.60 (0.08)	1.60 (0.06)	1.61 (0.08)	1.73 (0.12)	1.56 (0.08)	0.6747
Break strain (%)	10.6* (0.4)	13.0 (0.8)	12.5 (0.6)	13.5 (0.9)	14.8 (1.2)	13.3 (0.9)	0.4592
Elasticity (N/mm ²)	1239 (43)	1180 (59)	1251 (44)	1065 (63)	977 (52)	980 (38)	0.0008
Energy (J)	0.12** (0.01)	0.18 (0.01)	0.17 (0.01)	0.17 (0.01)	0.19 (0.02)	0.17 (0.02)	0.8937

^aData are mean (\pm SEM), $n = 12 - 15$ rats/group. ^bSham versus OVX control rats (student's *t*-test). ^cOne-way ANOVA followed by post-hoc Tukey-Kramer's test. *indicates significance from the OVX control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

All of the biomechanical measures assessed in the excised right femur obtained from sham-operated rats were significantly lower ($p < 0.05$) than the OVX control rats (**Table 8-9**), except for midpoint thickness ($p = 0.5710$), break stress ($p = 0.2518$) and elasticity ($p = 0.4341$). No significant differences were detected between various groups of OVX rats following eight weeks of tea consumption, except for midpoint thickness ($p = 0.0026$), break stress ($p < 0.0001$) and elasticity ($p = 0.0008$). However, of these three significant measures, no experimental tea group was significantly different from the OVX control group.

8.3.10 Effects of tea extracts on caecal pH, bacterial enzyme activities and selected intestinal microbiota populations

Caecal pH values in OVX rats given either deionised water or tea drinks ranged from 7.41 ± 0.07 to 7.68 ± 0.03 and were significantly influenced by tea type ($p = 0.0077$), as shown in **Table 8-10**. The caecal pH values of OVX rats given Se-GTE and R-BTE as the only source of fluid were significantly lower ($p = 0.0061$ and $p = 0.0294$, respectively) than those of the OVX control group. As presented in **Table 8-10**, tea consumption significantly affected enzymatic activity of β -glucosidase (β -GLU) in the OVX rats ($p < 0.0001$), with all teas except Se-BTE showing significant differences from the OVX control (Se-GTE, $p < 0.0001$; R-GTE, $p = 0.0097$; Se-BTE, $p = 0.1485$; R-BTE, $p = 0.0030$). However, caecal β -glucuronidase (β -GUS) activity was not affected by eight weeks of tea consumption in OVX rats ($p = 0.9321$). **Table 8-10** also summarises the bacterial populations in rat caecal samples as quantified by fluorescence *in situ* hybridisation (FISH) assay. The bacterial counts of all four target bacterial populations (i.e. *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium* spp. (*perfringens/histolyticum* subgroup) and *Lactobacillus/Enterococcus* spp.) were not significantly different across OVX groups (p values of 0.9085, 0.1662, 0.1554 and 0.1545, respectively). However, data obtained from this study showed no significant difference between sham and OVX rats for caecal pH ($p = 0.2178$), and bacterial enzymatic activities (β -glucosidase, $p = 0.5904$; β -glucuronidase, $p = 0.9380$).

Table 8-10. Caecal parameter measurements in female Sprague-Dawley rats^a.

Group	Sham ^b	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^c
Caecal pH	7.73 (0.02)	7.68 (0.03)	7.41** (0.07)	7.64 (0.04)	7.65 (0.04)	7.45* (0.07)	0.0077
β-GLU (U/g caecal content) ^d	1.06 (0.06)	1.11 (0.08)	1.72*** (0.12)	1.53** (0.07)	1.40 (0.08)	1.57** (0.09)	< 0.0001
β-GUS (U/g caecal content) ^d	1.51 (0.06)	1.52 (0.06)	1.45 (0.06)	1.50 (0.06)	1.50 (0.06)	1.46 (0.06)	0.9321
<i>Bacteroides</i> spp. ^e	7.58 (0.15)	7.73 (0.12)	7.61 (0.08)	7.61 (0.08)	7.70 (0.08)	7.66 (0.14)	0.9085
<i>Bifidobacterium</i> spp. ^e	8.11 (0.07)	8.01 (0.05)	8.07 (0.05)	8.01 (0.08)	7.89 (0.04)	7.87 (0.08)	0.1662
<i>Clostridium</i> spp. ^e	7.61 (0.07)	7.66 (0.09)	7.39 (0.08)	7.38 (0.09)	7.50 (0.08)	7.54 (0.09)	0.1554
<i>Lactobacillus</i> / <i>Enterococcus</i> spp. ^e	8.08 (0.03)	7.90 (0.08)	7.89 (0.05)	7.78 (0.06)	7.96 (0.06)	8.01 (0.07)	0.1545

^aData are mean (± SEM), *n* = 12 – 15 rats/group. ^bSham versus OVX control rats (student's *t*-test). ^cOne-way ANOVA followed by post-hoc Tukey-Kramer's test. *indicates significance versus the OVX control (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). ^dU is defined as mg/h. ^eData are presented as log number of bacterial cells per g wet weight of caecal contents (Log₁₀ cells/g).

8.4 Discussion

This study was conducted to determine the effect of four different tea extracts on bone loss in ovariectomised (OVX) mature adult female rats, as well as to evaluate the potential link between tea antioxidant capacity, the intestinal microbiota and bone properties of these rats following consumption of these tea extracts. Ovariectomised rats were used in this study as a model of postmenopausal osteoporosis, they are a well-established model of the oestrogen-deficient condition following human menopause, which leads to a significant bone loss, skeletal changes and reduction in bone mineral density (BMD) (Kalu, 1991; Frost & Jee, 1992; Kimmel et al., 1996; Turner et al., 2001; Kharode et al., 2008). Further, the OVX rat model is recommended by the American Food and Drug Administration (FDA) as a pre-clinical animal model for the evaluation of potential therapeutic agents for the prevention of osteoporosis; where the proximal tibia, distal femur, and lumbar spine of the OVX rats emulates cancellous bone turnover in the postmenopausal woman when evaluated over relatively short-time periods (Thompson et al., 1995). Sham-operated (intact ovaries) rats were used to represent oestrogen-replete women (Kalu, 1991; Shen et al., 2008, 2013). Overall, observations from the present study showed that tea consumption had no detrimental effect on the health of the female rats throughout the eight weeks of tea intervention.

8.4.1 Body weight, tea intake and feed consumptions following ovariectomy in mature adult female rats

Body weight gain for the OVX rats used in this study was significantly higher than that of the sham rats from week 2 to 8 post-ovariectomy, suggesting that the increased body weight in OVX rats is due to oestrogen deficiency as previously reported (Landau & Zucker, 1976; Ezzat-Zadeh et al., 2012; Hayatullina et al., 2012; Al-Rahbi et al., 2013; Das et al., 2013; Tousen et al., 2014). It is well documented that oestrogen is positively associated with leptin (Shimizu et al., 1997; Pelleymounter et al., 1999; Olstad et al., 2011), a 14-kDa protein secreted by adipose tissues that regulates food intake by inhibiting hunger and stimulating energy expenditure (Anubhuti & Arora, 2008). Hence, oestrogen deficiency causes a decrease in the production of leptin,

where low leptin levels will signal the body to increase food intake, leading to hyperphagia and increased adiposity and rapid weight gain (McElroy & Wade, 1987; Silva et al., 2010; Eckel et al., 2011).

All female rats had *ad libitum* access to deionised water or tea water extracts as the sole source of fluids. However, only OVX rats given Se-GTE and R-GTE showed levels of fluid intake comparable to the OVX control rats given deionised water throughout the eight-week period of study. There were increased levels of fluid intake by rats given the black teas (Se-BTE and R-BTE), which was in accordance with those seen in our previous study on young growing male rats (**Chapter 7**, section 7.3.3). As described in **Chapter 7** (section 7.4.1), explanation for the increased consumption of the black tea water extracts was speculated to be due to the effect of caffeine in these black teas causing an increase in thirst among the experimental rats given black tea drinks throughout the study. Thus, resulting in an increased intake of the black teas compared to the green teas. Meanwhile, feed intakes in all OVX rats were comparable and not significantly different to the sham rats, which are different to the results obtained from our previous tea consumption study using young growing rat model (**Chapter 7**, section 7.3.3). One possible explanation for this result is that the OVX rats may develop tolerance against appetite-stimulating effect of caffeine over long-term consumption (Retzbach et al., 2014).

8.4.2 Effect of various tea extracts on serum antioxidant capacity

Previously in **Chapter 7**, we demonstrated that four weeks of tea consumption significantly influenced serum antioxidant capacity in young growing male rats causing a significant reduction in serum ferric-reducing antioxidant power (FRAP) levels in rats given Se-GTE and R-GTE, whereas serum FRAP in rats given Se-BTE and R-BTE remained unchanged, when compared to the control rats given deionised water. This unexpected finding, in comparison with the *in vitro* results and the results of other studies, directed us to speculate that these differences could be attributed to several factors, some of which were discussed in **Chapter 7** including: **1)** differences in polyphenolic profile of the different cultivar type of the tested teas, **2)** differences in

experimental methodological approaches, 3) limitations of the FRAP assay, and 4) complexity of an *in vivo* systems and pharmacokinetic processes that make comparisons with *in vitro* data difficult. However, as an attempt to further understand the role of tea consumption in the enhancement of the endogenous antioxidant defence system against free radicals, we further evaluated the serum antioxidant capacity in ovariectomy-induced oxidative stress in mature adult female rats.

Results of the present study showed that the serum FRAP levels in OVX control rats significantly decreased following ovariectomy surgery in comparison to the sham rats. Hence, confirming previous claims that the loss of oestrogen lowered intracellular antioxidant defence in the OVX rats (Muthusami et al., 2005; Almeida et al., 2007a; Wauquier et al., 2009; Manolagas, 2010; Bellanti et al., 2013). The results of the present study showed that OVX control rats responded in a similar manner to the findings of Tang et al. (2012), who observed increased oxidative stress in Sprague-Dawley female rats following ovariectomy surgery, using different assays to measure the antioxidative and oxidative parameters (i.e. ferric-reducing capacity (FRC), malondialdehyde (MDA), and assessment of DNA damage by comet assay in lymphocytes).

Serum FRAP levels in OVX rats given R-GTE and Se-BTE remained unchanged when compared to the OVX control rats. Of particular interest, Se-GTE consumption significantly enhanced and restored serum FRAP in the OVX rats up to a level well above that of the sham rats. In contrast, R-BTE consumption resulted in a significantly decreased level of serum FRAP. These varying tea effects on OVX rats contradict those seen in our previous study on young growing male rats. Although several studies have investigated the effect of tea and/or its polyphenols on antioxidant activity *in vivo* (Frei & Higdon, 2003; Shen et al., 2008; Sun et al., 2012), to the best of our knowledge, there have been no reports regarding serum antioxidant capacity in OVX rats following tea consumption, as measured using FRAP assay. In previous studies using the ovariectomy-induced bone loss model (Shen et al., 2008; Shao et al. 2011), green tea polyphenols supplementation (in drinking water) has benefited the antioxidant defence system, where there was enhanced liver antioxidant enzyme activity (i.e. glutathione peroxidase and superoxide dismutase-1) in 14-month-old OVX rats following a 16-week intervention study. The discrepancies between our previous young

growing male rat study (**Chapter 7**) and the present ovariectomised female rat study, as well as other studies reporting antioxidant activity of tea polyphenols *in vivo* (Frei & Higdon, 2003; Sun et al., 2012), may possibly be due to differences in the age of the rats (Kim et al., 2002b), different concentrations of tea and/or its polyphenols and methods of assessment of serum antioxidant capacity (Sun et al., 2012), inter-individual diversity in absorption and metabolism of polyphenolic compounds of tea (Lee et al., 2002; Van Duynhoven et al., 2011), and gender differences in antioxidant capacity (Katalinic et al., 2005).

The current study demonstrated that all the tested teas contributed to the serum FRAP results but with different modalities. The increased level of serum FRAP, after eight weeks of Se-GTE consumption by OVX rats, may reflect the difference in absorption of the tea polyphenolic antioxidant compounds through the intestinal barrier (Serafini et al., 1996; Van het Hof et al., 1999; Scalbert et al., 2002) and up-regulation of endogenous antioxidant defence systems (Cao et al., 1998a, 1998b). While Se-GTE consumption may restore *in vivo* antioxidant defences eight weeks post-ovariectomy, the effects of other teas against ovariectomy-induced oxidative stress are not fully understood. The most likely explanation for the lack of effect exerted by R-GTE and Se-BTE is that their circulating polyphenolic concentrations following ingestion are too low to raise serum antioxidant capacity (Stangl et al., 2006; Pecorari et al., 2010). Circulating polyphenolic concentrations may also be dependent on the length of tea intervention and polyphenolic antioxidant concentrations of these teas (Pellegrini et al., 2000; Van der Berg et al., 2001). Also, we speculate that the decrease in serum FRAP in R-BTE rats may be due to a disturbance in serum antioxidant and pro-oxidant balance during the FRAP assay measurement. The disturbed balance may result from accumulation and/or overproduction of ferrous ion (Fe^{2+}) as a final product of the FRAP assay that plays a role as a potential pro-oxidant that can further generate free radicals in the serum when reacting with certain antioxidants (Cao & Prior, 1998b; Gorąca & Skibska, 2006; Gáspárová et al., 2010).

Furthermore, the serum antioxidant capacity was measured after a fasting period of at least 12 hours. Fasting serum may not represent the accumulative antioxidant potential due to fluctuations in the serum concentrations of various tea bioactive

compounds after oral ingestion, and the different elimination half-lives of the different tea constituents (Van het Hof et al., 1999; Khan & Mukhtar, 2007; Marnewick et al., 2011; Ajuwon et al., 2013). It is important to note that the elimination half-life of most polyphenols in tea is less than eight hours (Warden et al., 2001; Van het Hof et al., 1999), although polyphenol elimination half-life have been reported to range between 1 and 28 hours (Manach et al., 2005). The circulating polyphenolic compounds in serum or plasma are very dependent on the consumption pattern throughout the day. Thus, the timing of blood collection for antioxidant capacity measurement is critical, fasted serum concentrations will be much lower than postprandial values. In addition to being poorly absorbed, the lack of antioxidant effect of tea polyphenolic compounds could also be due to the fact that these compounds were extensively metabolised to conjugated forms by the time the antioxidant capacity was measured after the fasting period (Kaur et al., 2014). The process of conjugation may reduce the antioxidant capacity of the metabolites with respect to parental compounds (Natsume et al., 2004; Manach et al., 2005).

With a view to overcome the imprecise assessment of serum antioxidant capacity relating to fasting serum, the acute ingestion model has been suggested as a more reliable approach to investigate the effects of antioxidant-rich beverages on the endogenous antioxidant defence *in vivo* (Wang et al., 2013c). This experimental model assesses acute changes in plasma or serum antioxidant capacity following ingestion of food or beverages (Serafini et al., 2000; Pecorari et al., 2010; Fernández-Arroyo et al., 2012; Rubió et al., 2014). Indeed, there is evidence that acute studies consistently demonstrated significant increases in plasma antioxidant capacity several hours after tea consumption, both in animals and human participants (Benzie et al., 1999; Sung et al., 2000; Henning et al., 2004, 2005; Koutelidakis et al., 2009).

8.4.3 Effects of various tea extracts on bone turnover, bone mineral density and bone strength

In the present study, bone mass evaluation using several methods such as biochemical markers of bone turnover, bone densitometry, and biomechanical analysis of bone strength was assessed in sham-operated and OVX rats to investigate whether consumption of four different teas exerted a bone protective effect against ovariectomy-induced bone loss in mature adult female Sprague-Dawley rats.

There are various biochemical markers of bone turnover that can provide information on the state of bone metabolic activity; carboxy-terminal crosslinks telopeptide of type I collagen (CTx-I) and osteocalcin (OC) are among the sensitive and specific biomarkers that are well-documented and commonly used (Eastell & Hannon, 2008; Wheeler et al., 2013). The CTx-I is a reliable indicator of bone resorption, which increases upon oestrogen deficiency, and reflects elevated levels of bone resorption activity (Coleman, 2002; Garnero, 2008). Meanwhile, OC is a non-collagenous extracellular bone matrix protein exclusively synthesised by osteoblasts, reflects osteoblast activity, and is considered a sensitive and specific bone formation marker (Garnero, 2008). However, OC is also released from the bone matrix during bone resorption. Thus, OC is also regarded as a bone turnover marker (Ivaska et al., 2004; Clarke, 2008; Herrman, 2011).

In comparison to the sham-operated group, ovariectomy resulted in a significant increase in the concentration of serum CTx-I ($p < 0.0001$) and OC ($p < 0.0001$). This result is in line with the anticipated increase in bone turnover induced by ovariectomy in 12-week-old Sprague-Dawley rats (Srivastava et al., 2000), and also in accordance with results of human studies reported by Boskey et al. (2005), who stated that the high bone turnover state is indicated by an increase in osteoclast activity along with either normal or increased osteoblast activity. Interestingly, serum CTx-I concentrations in OVX rats supplemented with R-BTE were significantly lower ($p = 0.0052$) than that of the OVX control rats after the eight-week tea intervention. This observation suggests that R-BTE consumption may reduce bone resorption in OVX rats following the eight-week nutritional intervention study. There was also a decrease in serum CTx-I concentrations

in OVX rats following Se-GTE and Se-BTE consumption compared with the OVX control group, although this effect was not significant. On the other hand, the levels of serum OC in all tea intervention groups were comparable to the OVX control rats.

Our findings are in agreement with other studies which have assessed the effect of tea on bone turnover biomarkers in bone loss animal model. For example, several reports by Das et al. (2004, 2005, 2009, 2013) have consistently provided evidence of bone-protective effect of black tea against increased bone turnover in ovariectomised rats, as indicated by a significant decrease in a bone formation marker (serum alkaline phosphatase (ALP)) and a bone resorption marker (serum tartrate-resistant acid phosphatase (TRAP)), following oral administration of aqueous black tea extract (2.5% (w/v)) at a single dose of 1 mL/100 g of body weight daily for 28 days. For studies on the bone-protective effect of green tea, Shen et al. (2010a, 2010b) reported that 12-week supplementation of 0.5% (w/v, in drinking water) green tea polyphenols (GTP) to lipopolysaccharide (LPS)-treated rats significantly decreased serum TRAP, but had no effect on serum OC concentration when compared to the LPS control rats in a chronic inflammation-induced bone loss model. Meanwhile, in an animal study using a male model of osteoporosis conducted by Shen et al. (2011a), a significant decrease in serum TRAP concentration was observed in 15-month-old androgen-deficient aged male rats, following 16 weeks of GTP supplementation (0.5% (w/v) in drinking water. Although the reports on the *in vivo* bone-protective effect of tea are promising (Shen et al., 2011b, 2013, 2014; Mitra et al., 2013; Shen & Chyu, 2013; Sacco et al., 2013), the effect of tea consumption on bone turnover biomarkers in ovariectomy-induced bone loss models have been poorly investigated (Shen et al., 2008, 2009).

The present bone turnover marker results indicate that among all tested teas, only R-BTE consumption had a pronounced effect on bone metabolic activity by suppressing high bone turnover in OVX rats. In addition, data obtained from R-BTE supplementation in OVX rats showed a more rapid response in the reduction of serum CTx-I concentration than in serum OC concentration, indicating that the bone resorption markers were more sensitive than the bone formation markers in response to the nutritional intervention. This could be due to the fact that the length of the resorption phase is shorter (2 to 12 weeks) than the phase of bone formation (3 to 6 months), thus,

resorption markers responded faster to changes in remodelling than formation markers (Stein & Lian, 1993; Watts, 1999; Manolagas, 2000; Eriksen, 2010).

The *in vivo* DEXA of the present study was not sufficiently sensitive to detect any significant difference in bone density after eight weeks of tea intervention. Thus, the percentage (%) change in bone density over the eight-week intervention study were utilised to provide useful indications of differences between experimental tea groups after ovariectomy surgery, as this has also been previously employed by others (Poulsen et al., 2006; Dontas et al., 2011; Tousen et al., 2014). In this study, we found that the OVX control rats demonstrated a marked decrease in % change for BMD values of lumbar spine, femoral bone and whole body when compared to the sham rats, which is consistent with other studies (Shen et al., 2008; Ryou et al. 2012; Tousen et al., 2012, 2014). This is expected, since the significant decrease of % change for BMD values in the OVX control rats reflects accelerated bone turnover following ovariectomy-induced oestrogen deficiency (Kalu, 1991; Frost & Jee, 1992; Kimmel et al., 1996; Turner et al., 2001; Kharode et al., 2008). Apart from the significant decrease in % change of BMD values of lumbar spine, femoral bone and whole body in OVX control rats when compared to the sham rats, there was no detectable increase in bone density in response to tea consumption.

Oestrogen deficiency in rats following ovariectomy increases bone turnover, which causes a rapid decrease in the strength of cortical and trabecular bone (Seeman et al., 2010). The tendency of bone to fracture is determined by its mechanical strength, hence, direct biomechanical testing is the most accurate evaluation of bone strength to assess direct bone fracture resistance rather than the commonly used imaging techniques and bone densitometry (Kim et al., 2004; McGee-Lawrence et al., 2013). The biomechanical properties of bone determine the bone capacity to resist external forces and bone susceptibility to fracture (Martin & Correa, 2010; Havill et al., 2014). Being well-known for its wide application and simplicity (Guede et al., 2013; Karim et al., 2013), the three-point bending test were used to assessed the whole bone strength of excised femurs obtained from the rats following termination of the tea intervention study. Further explanation regarding outcome definitions of bone strength parameters has been mentioned in **Chapter 3**, section 3.2.6.9.

In terms of biomechanical properties of the femurs of rats fed tea as measured by three-point bending test, sham rats showed lower values in most of the biomechanical parameters than that of the OVX control rats, except for the midpoint thickness, break stress and elasticity. The present finding is in agreement with expectations regarding the effects of ovariectomy-induced bone loss on femoral bone strength in mature rats (Jee & Yao, 2001; Zhao et al., 2003). Indeed, there are several studies that have reported an initial temporary increase in femoral bone strength at three months post-ovariectomy followed by a decrease at nine months post-ovariectomy, as reviewed by Jee and Yao (2001), where the ovariectomy surgery was carried out on rats between three and six months of age (Aeressens et al., 1993, 1996; Danielsen et al., 1993). It has been suggested that the increase in bone strength in OVX rats during the first 3 months post-ovariectomy in comparison to the sham rats was due to the improved geometric properties and strength of the bone; where ovariectomy stimulated periosteal apposition of the femoral bone that further compensates for the net increase in endocortical resorption (Aeressens et al., 1993; Zhao et al., 2003). Additionally, OVX rats experienced an increase in body weight following ovariectomy; and greater body weight increases mechanical loading to the bones (Ribot et al., 1994; Douchi et al., 2000; Zhao et al., 2003). Therefore, we suggest that the confounding influence of the mechanical loading of body weight may contribute to the increased femoral bone strength in OVX rats used in this study, in spite of the observed decrease in femur BMD (mainly of cortical bone). The enhanced femoral bone strength in mature adult female rats at eight-week post-ovariectomy in the current study, contrasts with the findings of others (Kruger et al., 2005, 2006, 2008, 2009). The most likely reason for the discrepancies found in the literature are differences in the degree of osteopenia (low bone mass) established in rats following ovariectomy, which can be explained by the use of aged rats at the time the ovariectomy surgery was performed, and/or the long post-ovariectomy period employed in their studies (Patlas et al., 2000).

In the present study, none of the tested teas had any significant effect on femur biomechanics when compared to the OVX control rats. To the best of our knowledge, limited information is available on the bone-restoring effect of tea or its bioactive compounds on femoral strength in ovariectomy-induced bone loss in animal models, as assessed using three-point bending test (Shen et al., 2011b, 2013). In an animal study

that utilised a male model of osteoporosis, Shen et al. (2011a) found a positive impact of 16 weeks of GTP supplementation (0.5% (w/v) in drinking water) on femoral strength in 15-month-old orchidectomy-induced androgen-deficient aged male rats. Meanwhile, in a different study using a chronic inflammation-induced bone loss model, Shen et al. (2011c, 2011d) provided evidence that GTP supplementation at 0.5% (w/v) improved femoral bone strength in LPS-treated three-month-old rats following a 12-week intervention period compared to the LPS control rats. Furthermore, six weeks of GTP supplementation at 0.1 and 0.5% concentrations (w/v) resulted in enhanced bone mass and bone strength in alcohol-treated adolescent rats, where Shen et al. (2014) had been assessing the effect of GTP on bone loss by employing a rat model of adolescent repeated binge-like alcohol exposure.

The lack of changes in biomechanic parameters following tea drinking by OVX rats in comparison to OVX control rats could be due to the fact that the biomechanical tests were conducted in the diaphysis of long bones (i.e. femurs); an area mainly composed of cortical bone, and no trabecular (cancellous) bone (Clarke, 2008). Generally, the turnover rate for cortical bone is slower than that of trabecular bone (Jee & Yao., 2001; Ma et al., 2012), and according to Jee and Yao (2001), a decrease in bone strength was not evident in their study until 15 months post-ovariectomy. Based on this fact, we suggest that any alterations in biomechanical strength of femoral bone after eight weeks of tea drinking as assessed by three-point bending test, is not influenced by the degree of cortical osteopenia that occurs during the eight weeks post-ovariectomy. Therefore, a longer study intervention period is needed in order to achieve cortical osteopenia and to observe a significant effect of tea on femoral bone strength after post-ovariectomy.

8.4.4 Effects of various tea extracts on caecal pH, bacterial enzyme activities and intestinal microbiota compositions in OVX rats

The intestinal microbiota is crucial for the nutritional, metabolic, physiological, and immunological functions of the human body (Gerritsen et al., 2011; Kabat et al., 2014; Lin et al., 2014). Thus, modulation of the composition and metabolic parameters

of the intestinal microbiota may be taken to confer a health benefit on the host organism. The composition and metabolic parameters of intestinal microbiota are affected by endogenous and environmental factors, of which diet has the most influence (Ley et al., 2008; Gong & Yang, 2012). There is emerging evidence for the potential of tea, as tea is considered a good source of non-digestible nutrients that may selectively modulate the composition and/or metabolic activity of the intestinal microbiota, as reviewed by Etxeberria et al. (2013) and Ruxton (2013).

The metabolic activities of the intestinal microbiota are achieved through the colonic fermentation of non-digestible nutrients that escape digestion in the small intestine; resulting in a decline in colonic pH levels and production of essential nutrients such as short-chain fatty acids (SCFAs), vitamins (e.g. vitamin B1, vitamin K and folic acid), and amino acids (Wong et al., 2006; Hamer et al., 2008; Macfarlane & Macfarlane, 2012). The present study demonstrated that there were no differences between the levels of caecal pH, and metabolic activities between sham-operated and OVX control rats. This may indicate that ovariectomy did not cause any changes to the intestinal microbiota metabolism in the Sprague-Dawley female rats used in the study. The caecal pH levels in rat given Se-GTE and R-BTE were lower than that of the OVX control rats. This result was further supported by the observed increase in caecal β -glucosidase enzyme activity in all experimental tea groups when compared to the OVX control group, although this was not significant for the Se-BTE group. In contrast, no significant changes in the activity of β -glucuronidase were detected in any experimental tea group when compared to the OVX control group.

In the present study, none of the tea water extracts had a significant effect on caecal microbiota composition in the Sprague-Dawley female rats, after eight weeks of tea consumption. Most of the results related to the tea intervention are in agreement with those obtained on caecal microbiota counts of *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroides* spp. and *Clostridium* spp. (*perfringens/histolyticum*) from previous animal study using a growing rat model (**Chapter 7**). In line with our report in **Chapter 7**, we observed a decreased number of pathogenic *Clostridium* spp. (*perfringens/histolyticum*) counts in all experimental tea groups, although these changes were not significant in comparison to the OVX control rats. The findings of our current study are

contrary to other studies that have seen mostly positive results due to the prebiotic-like effect and antimicrobial activities of tea, although some are mixed (Kakuda et al., 1991; Lee et al., 2006a; Tzounis et al., 2008; Jaziri et al., 2009; Molan et al., 2009b, 2010; Banerjee et al., 2010; Cueva et al., 2010; López de Felipe, 2010; Ankolekar et al., 2011; Jin et al., 2012; Vodnar & Socaciu, 2012; Vodnar et al., 2012; Gaudreau et al., 2013; Kemperman et al., 2013), as previously discussed in **Chapter 7**. The discrepancies encountered between our previous (**Chapter 7**) and present animal studies, and other studies regarding the effect of tea and/or its polyphenols on intestinal microbiota, may be explained by differences in the age of the rats (Lees et al., 2014), various concentrations of tea and/or its polyphenols, diverse methodological approaches, and a wide range of compositional microbiota analysis techniques of animal fecal samples employed in these studies (Dueñas et al., 2015a, 2015b). In the current study, the lack of significant changes in intestinal microbiota composition following tea consumption, could be due to low signal intensity and low sensitivity of the FISH analysis, where the detection threshold of FISH analysis is estimated at 10^7 bacteria cells per gram of caecal sample (Jansen et al., 1999; Mai et al., 2004; Gong & Yang, 2012).

Based on the present results for Se-GTE, we suggest that Se-GTE may positively influence the metabolism and composition of the intestinal microbiota, as shown by the reduction in caecal content pH and increased activity of bacterial β -glucosidase enzyme. These findings are in line with findings of an *in vivo* study performed by Molan et al. (2010), who demonstrated that administration of green tea with high selenium content significantly increased the activity of bacterial β -glucosidase enzyme in adult male rats in comparison with China green tea, following six consecutive days of oral gavage (10 mL/kg body weight of 1% tea water extract). To the best of our knowledge, there are no other studies that have investigated the effect of selenium-rich teas on the composition and metabolic activity of the intestinal microbiota.

8.5 Conclusions

The consumption of R-BTE by ovariectomised rats affected bone metabolic activity by suppressing serum bone resorption marker CTx-I following eight-week tea intervention study. However, no significant effect was shown by any of the teas on bone density and femur biomechanics as compared to the OVX control rats. Based on these findings, we suggest that a longer period of tea intervention may allow more time to fully assess these tea extracts and whether or not they exert a significant protective effect on bone parameters following ovariectomy. Our study is the first to investigate the prebiotic-like effects of the consumption of a variety of different tea types in ovariectomy-induced bone loss in mature adult female rats. Data obtained from the study showed a potential effect of Se-GTE and R-BTE in modulating intestinal microbiota composition, as seen by a marked decrease in caecal pH and enhanced activity of bacterial β -glucosidase enzyme which is mainly generated by lactic acid bacteria. These results suggest that the tested teas may not benefit bone health, but have potential prebiotic-like effects on intestinal microbiota. However, the exact mechanism(s) underlying prebiotic-like effect of tea need to be further investigated. Taken together, these findings add to our basic understanding and insight into the possible mechanisms of the bone-protective effects of tea against bone loss in ovariectomised rats.

CHAPTER 9

GENERAL DISCUSSION AND FUTURE RESEARCH DIRECTIONS

This chapter brings the results together and highlights significant research findings obtained from a series of in vitro and in vivo studies. This chapter also provides a discussion of the results from all experimental work pertaining to the main objectives outlined in Chapter 1. Study limitations and implications are acknowledged, and recommendations for future directions are presented.

9.1 Summary of the principal findings

This thesis represents several significant research findings, which are summarised below:

1. Irrespective of tea selenium content, the *in vitro* results obtained for total phenolic content (TPC), antioxidant properties and prebiotic-like potential of the green teas (Se-GTE, R-GTE) and black teas (Se-BTE, R-BTE) were highly varied depending on the type of the tea used. In addition, the optimal time and temperature of tea infusion for maximising TPC was determined to be 90 °C for 5 min, which was then used as the standard method of preparation for tea water extracts for the *in vitro* and *in vivo* experimental work. Interestingly, the prebiotic-like potential of each individual tea also reflected the TPC and antioxidant activity (**Chapter 4**).
2. At low concentrations, all tea extracts were not cytotoxic to the murine pre-osteoblastic MC3T3-E1 cells and exerted a stimulatory effect on mineralised bone nodule formation. This indicated that the anabolic effect of these teas on bone formation *in vitro* was achieved by direct enhancement of matrix mineralisation of pre-osteoblastic cells. In addition, green teas (Se-GTE, R-GTE) stimulated osteoblast differentiation at high concentrations, thus demonstrating their ability to stimulate osteogenesis. On the contrary and surprisingly, black teas (Se-BTE, R-BTE) of similar concentrations suppressed osteoblast differentiation. Furthermore, all teas showed an osteopromoting effect by protecting osteoblasts against consequences of H₂O₂-induced oxidative stress. These *in vitro* activities were irrespective of the selenium content, and were in a time- and concentration-dependent manner. Additionally, the mechanism by which black tea affects osteoblast differentiation is presumably different from that of green tea (**Chapter 5**).
3. All teas effectively suppressed RANKL-induced osteoclastogenesis *in vitro* in a concentration-dependent manner, with significant anti-osteoclastogenic effects observed at the higher concentrations (**Chapter 6**). This effect was independent of selenium levels in the tea extracts. Interestingly, black teas (Se-BTE, R-BTE)

had stronger anti-osteoclastogenic effects than green teas (Se-GTE, R-GTE). Furthermore, the present study is the first to report that the positive control, (-)-epigallocatechin-3-gallate (EGCG, 20 μ M) not only inhibited RANKL-induced osteoclastogenesis in RAW 264.7 cells, but also unexpectedly initiated osteoclastogenesis in the absence of RANKL; with the formation of small multinucleated cells of less than five nuclei.

4. All teas showed no significant osteostimulative effect on improving bone mass and bone strength parameters in young growing rats during the rapid growth phase, a period that is critical for bone development and the attainment of peak bone mass. However, as there were subtle but statistically non-significant increases in other measured bone properties of the young growing rats, it may be possible that a longer period of tea consumption could exert significant effects on bone parameters (**Chapter 7**).
5. Among all the tested teas, only R-BTE significantly suppressed bone turnover in ovariectomised mature adult rats. In addition, Se-GTE and R-BTE showed non-significant positive changes in bone density and femur biomechanical properties as compared to the ovariectomised control rats. Hence, a longer period of tea intervention may allow more time to fully assess these tea extracts and as to whether or not they exert significant protective effects against bone loss following ovariectomy in mature adult rats (**Chapter 8**).
6. The assessment of serum antioxidant capacity conducted in both young growing rats (**Chapter 7**) and ovariectomy-induced bone loss in mature female rats (**Chapter 8**) following consumption of a variety tea extracts gave inconsistent results when compared to *in vitro* antioxidant activities of the teas shown in **Chapter 4**. All teas had no influence on populations of beneficial bacteria (bifidobacteria and lactobacilli) in both animal models. However, Se-GTE demonstrated the greatest prebiotic-like effect among all teas in both animal studies, significantly improving the composition and metabolic activity of the intestinal microbiota, as illustrated by decreased caecal pH, reduced counts of *Clostridium* spp. (*perfringens/histolyticum*) (significant effect observed only in young growing rat model), and increased bacterial β -glucosidase enzyme

activity. Overall, the significant positive effect exerted by certain teas on some of the bone parameters may not be caused by direct antioxidant properties, but in part, mediated by their prebiotic-like effect on intestinal microbiota. However, the exact mechanism underlying such an association remains unclear and requires further investigation.

9.2 Overall discussion

This research was conducted due to growing interest in natural non-pharmacotherapy approaches for the strategic management and prevention of postmenopausal osteoporosis, which resulted from emerging issues over the safety, efficacy and accessibility of pharmacotherapy drugs that are currently being used to treat this bone disease. Furthermore, it is well established that the development of osteoporosis is influenced by a variety of both non-modifiable and modifiable risk factors (Mundy, 2006; Sarkis et al., 2012). There are a range of natural dietary interventions aimed at minimising the incidence of osteoporosis and risk of osteoporotic fractures, including dietary and nutritional supplementation independent of calcium and vitamin D (Cashman, 2007; Levis & Lagari, 2012; Nieves, 2013; Rondanelli et al., 2013; Suntar & Akkol, 2013; Welch & Hardcastle, 2014). The promising impact of tea consumption on bone health as a dietary antioxidant has gained attention from both the general public and the scientific community due to the accessibility and presumed safety of teas, with recent literature providing extensive evidence on the beneficial effects of tea on bone loss (Das et al., 2009, 2013; Chen et al., 2013; Rao & Rao, 2013; Shen et al., 2013a, 2013b; Song et al., 2014). Recently, Se-rich tea has become of interest due to its beneficial role in various oxidative stress-related diseases (Moghadaszadeh & Beggs, 2006; Zeng et al., 2013a; Santos et al., 2014), and the positive effect of Se-rich foods on endogenous antioxidant enzyme activity (Finley et al., 2005; Bermingham et al., 2014; Liang et al., 2014).

To the best of our knowledge, no studies have yet investigated the osteopromoting potential of selenium-rich teas on bone health. Hence, the primary aim of this study was to determine the osteopromoting properties of a variety of teas with different levels of Se content; i.e. Se-GTE, Se-BTE, R-GTE and R-BTE. This study was

also undertaken to elucidate whether the antioxidative effect and/or prebiotic-like potential of tea are actually associated with benefits on bone, as well as to identify the potential underlying mechanisms. This is the first study that investigates the beneficial effects of a variety of teas with different levels of Se content on bone health through a series of *in vitro* and *in vivo* assays. The objectives of the present study were met and the results obtained contradicted our initial expectations, showing that these teas could affect bone health irrespective of Se content.

9.2.1 Prebiotic-like potential and osteopromoting effect of tea extracts *in vitro*: In relation to TPC and antioxidant properties

The results described in **Chapter 4** suggested that the total phenolic content (TPC) of the tested teas was the main factor responsible for the *in vitro* antioxidant activities (Dai & Mumper, 2010), and was well-matched with their *in vitro* prebiotic-like potential (Shi & Schlegel, 2012; Etxeberria et al., 2013). The results of the TPC, antioxidant activities, and prebiotic-like potential of the teas are all comparable when arranged in decreasing order: Se-GTE > R-BTE > R-GTE > Se-BTE. Indeed, the TPC of the tested teas was highly varied, which may mainly be due to differences in both the varietal origins and processing methods of each of the tested teas (as discussed in **Chapter 4**). In addition, it is speculated that teas exhibit a prebiotic-like effect *in vitro* partly due to their polyphenolic compounds; **1**) as antioxidants act on free radicals that result in an optimal oxygen-free environment for the growth and multiplication of lactic acid bacteria (LAB), and **2**) as growth substrate for the LAB (Molan et al., 2009b; Bancirova, 2010; Gyawali & Ibrahim, 2012; Gaudreau et al., 2013; Marhamatizadeh et al., 2013). These possible mechanisms may explain our findings, where the prebiotic-like effect of the tested teas is well-matched with their respective TPC and antioxidant activities.

Previous *in vitro* and *in vivo* studies on Se-rich green tea have successfully demonstrated its greater antioxidative impact compared to regular green teas (as discussed in **Chapter 4**). Surprisingly, our present study did not exhibit similar findings for Se-rich black tea when compared to regular black tea. In our study, Se-GTE had the

highest antioxidant activities, while Se-BTE had the lowest. We suggest that the high antioxidative properties seen in Se-GTE could be due to a synergistic reaction between its catechins and Se that enhances antioxidant activity, as previously proposed by others (Hu et al., 2001, 2003; Xu et al., 2003a, 2003b; Yu et al., 2007; Li et al., 2008, 2009a; Molan et al., 2009b), although there is no definitive description of the mechanism exerted by Se in increasing antioxidant activities. Meanwhile, to the best of our knowledge, no study has been conducted to investigate the health-promoting effect of black tea with high Se content, or to determine a synergistic action between Se and complex polyphenolic compounds of black tea. Most importantly, it is noteworthy that the polyphenolic profile of black tea is very different from green tea (Harold & Graham, 1992; Harbowy & Balentine, 1997), thus, suggesting that Se-BTE may not have the same synergistic reactions resembling that of the catechins and selenium present in Se-GTE.

The *in vitro* work that was performed to investigate the osteogenic and osteoprotective effects of a variety of teas using murine pre-osteoblastic MC3T3-E1 cells (**Chapter 5**), produced results that were inconclusive. For instance, the low concentration of tea extracts at 0.01 µg/mL showed no influence on proliferation and differentiation of the pre-osteoblastic MC3T3-E1 cells, but significantly increased cell mineralisation. These teas caused an undesirable decrease in osteoblast proliferation after a long period of intervention, and a biphasic effect during matrix mineralisation that was highly dependent upon tea concentration. Possible explanations have been given in **Chapter 5**, but these effects could be due to the pro-oxidant nature of tea polyphenolic compounds at high concentrations. It is clear that all the tested teas may directly promote matrix mineralisation when supplemented at low concentrations (0.01 and 0.1 µg/mL), which considered a dose range that is safe for growth and differentiation of osteoblasts *in vitro*. Therefore, we suggest that low concentrations of teas are considered safe and may be able to maintain and/or modulate ROS at the physiological effective levels required for fundamental cellular processes in bone metabolism.

Interestingly, green teas (Se-GTE and R-GTE) showed more potent concentration-dependent actions on osteoblastogenesis *in vitro* than black teas (**Chapter 5**), including greater mineralised bone nodule formation, and greater

osteoprotective impact on differentiated osteoblast cells against the dysfunctional consequences of H₂O₂-induced oxidative stress. However, in contrast to the stimulatory effects exerted by green teas on osteogenesis, black teas suppressed osteogenesis. We suggest that black tea extracts may indirectly limit the formation of bone-resorbing osteoclasts through a suppressive effect on the differentiation of pre-osteoblastic cells (Kamon et al., 2010; Zhao et al., 2014); whereby mature osteoblast cells are importantly involved in regulation of osteoclast formation and function through RANKL production on the surface of osteoblastic cells (Hofbauer & Heufelder, 2001). The suppression of cell proliferation by black teas corresponds to the observed response in bone activity of some isolated polyphenolic compounds (i.e. EGCG and apigenin), which inhibited osteoblast differentiation in addition to their detrimental impact on the formation and function of osteoclasts (Kamon et al., 2010; Goto et al., 2015). These compounds are also present in green tea (Savić et al., 2014), however, the osteogenic impact exhibited by green teas may be a result of synergistic and additive interactions (Williamson, 2001; Chen et al., 2002; Lila & Raskin, 2005; Hu et al., 2011) between these compounds and other constituents of the green tea that are different from those of black tea.

Although the Se-GTE and R-GTE demonstrated effective anti-osteoclastogenic potential against RANKL-induced osteoclastogenesis in macrophage cells (**Chapter 6**), the anti-osteoclastogenic effect shown by Se-GTE was not as strong as that of the black teas (Se-BTE and R-BTE). In addition to the effective absorption of black tea as mentioned in **Chapter 6**, we suggest that the greater anti-osteoclastogenic effect of black teas compared to green tea may be attributed to the biochemical profiles of the black tea. Black tea contains mainly theaflavins and thearubigins that have been reported for their antioxidant properties and health-promoting role *in vitro* (Leung et al., 2001; Lorenz et al., 2009; Sun et al., 2012; Lorenz, 2013; Sen & Bera, 2013; Sinha & Ghaskadbi, 2013). Furthermore, bioactive compounds in black tea may exert biological properties either by additive and/or synergistic effects (Pan et al., 2013).

While there are several mechanisms that have been proposed by different researchers for the suppressive effect of green tea polyphenols (i.e. EGCG) on RANKL-induced osteoclastogenesis *in vitro* (Nakagawa et al., 2002; Yun et al., 2004, 2007; Morinobu et al., 2008; Lin et al., 2009; Lee et al., 2010a, 2010b; Nakamura et al., 2010; Oka et al., 2012; Shen et al., 2009a, 2013a, 2013b; Irie et al., 2014; Zhao et al., 2014),

to the best of our knowledge, only a few studies have explored the potential mechanism behind the *in vitro* anti-osteoclastogenic effect of black tea (Das et al., 2009) and/or its polyphenolic compounds (Lin et al., 1999; Sarkar & Bhaduri, 2001; Frei & Higdon, 2003; Łuczaj & Skrzydlewska, 2005). In relation to the research done on black teas, authors have proposed that the health benefits of black tea are attributed to the antioxidant properties of their polyphenolic compounds against oxidative stress and its subsequent cellular damage.

Of the many mechanisms proposed for the osteopromoting effects of the polyphenolic compounds of both green and black teas, we suggest that the overall osteogenic, osteoprotective and anti-osteoclastogenic effects reported in the present *in vitro* studies may not only be due to direct osteogenic and anti-osteoclastogenic properties of the tested teas, but partly mediated by the direct antioxidant ability of the tea phenolic compounds acting as reducing agents and free radical scavengers against intracellular ROS production (Higdon & Frei, 2003; Shen et al., 2009a, 2011b, 2013a, 2013b; Das et al., 2009; Sacco et al., 2013), as supported by the data represented in **Chapters 4, 5 and 6**. In addition, tea may also have indirect antioxidative effects (Higdon & Frei, 2003) through: *1*) up-regulation of total intracellular antioxidant defence system to overcome excess ROS, achieved via the activation of endogenous antioxidant enzymes and accompanied with tea antioxidant vitamins (vitamins A, C, E and K) (Meng et al., 2008; Abdul-Raheim et al., 2009; Hu et al., 2011), and *2*) modulation of activity of redox-sensitive transcription factors that regulate cell proliferation, differentiation, and death (Wattel et al., 2004; Rahman et al., 2006; Trachootham et al., 2008; De Mejia et al., 2009; Lorenz, 2013).

It is important to note that both the osteoblastogenic (osteogenesis) and anti-osteoclastogenic properties of the tested teas do not correlate with their respective levels of TPC and antioxidant effects *in vitro* as indicated in **Chapter 4**. Therefore, we also suggest that all teas used in this study may each exert *in vitro* osteopromoting effects through mechanism(s) other than their direct antioxidant action including:

- 1*) diverse bioactive compounds of tea (other than the major catechins) that have been reported to influence bone health, which include Se (Zeng et al., 2013a), caffeine (Tsuang et al., 2006; Su et al., 2013; Reis et al., 2015), fluoride (Palmer

- & Gilbert, 2012), lignans and isoflavonoids (Mazur, 1998; Mazur et al., 1998), flavanes (Zeng et al., 2014), kaempferol (Prouillet et al., 2004; Choi, 2011), myricetin (Hsu et al., 2007; Ying et al., 2014), complex polyphenols and proanthocyanidins (tannins) (Chang et al., 1994; Das et al., 2005, 2009; Oka et al., 2012; Nishikawa et al., 2015), rutin and its aglycone quercetin (Horcajada-Molteni et al., 2000; Wattel et al., 2004; Tsuji et al., 2009; Braun et al., 2011; Yamaguchi & Weitzmann, 2011);
- 2) synergistic, additive, and/or antagonistic interactions between the various bioactive compounds in tea (Williamson, 2001; Chen et al., 2002; Lila & Raskin, 2005; Hu et al., 2011; Colon & Nerin, 2015);
 - 3) synergistic and/or additive interactions between different mechanisms of action of tea (Williamson, 2001; Wagner & Ulrich-Merzenich, 2009; Rasoanaivo et al., 2011; Kim et al., 2014); and
 - 4) interactions between tea bioactive compounds and cellular components of target tissues, which may involve modulation of multiple molecular targets (i.e. transcriptional factors and cellular signal-transduction pathways) that are associated with various vital cell processes (Bode & Dong, 2003; Na & Surh, 2008; Surh & Na, 2008; Shen et al., 2009a; Lorenz, 2013; Rein et al., 2013; Zhao et al., 2014).

In the cellular studies, we used various concentrations (10, 1, 0.1, 0.01 and 0.001 µg/mL) of four different teas (Se-GTE, R-GTE, Se-BTE and R-BTE). These tea aliquots of decreasing concentrations were prepared from freeze-dried tea solids sourced from tea water extracts at 1% concentration (w/v; 10 mg/mL); where 0.2 g of freeze-dried tea solids were derived from 1 g of dry ground tea leaves (data shown in **Appendix X**). Therefore, at 1% concentration, a cup of brewed tea (2 g of tea in 200 mL water) may contain 400 mg of tea solids. Additionally, for a positive control, we used a pure isolated compound EGCG ($\geq 80\%$ purity), at concentrations of 10 µg/mL (21.8 µM) (**Chapter 5**) and 20 µM (**Chapter 6**). The concentrations of EGCG used in the present *in vitro* studies were initially chosen due to previous reports regarding their non-cytotoxicity (Yun et al., 2007; Lin et al., 2009; Li et al., 2010), as well as these being the circulating concentration achievable in rats following EGCG supplementation *in vivo* (Cai et al., 2002; Ahmed et al., 2004).

According to Balentine et al. (1997), catechins make up around 30% – 42% and 3% – 10% of solids in brewed green and black teas, respectively; with EGCG constituting approximately 65% of the total catechins (Perva-Uzunalić et al., 2006; Zaveri, 2006). Thus, based on the literature, at the highest concentration (10 µg/mL), green and black teas used in this study correspond to approximately 1.95 – 2.73 µg/mL and 0.195 – 0.65 µg/mL of EGCG, respectively (data shown in **Appendix XI**). The concentration of EGCG used in the *in vitro* studies is far above the estimated amount of EGCG in each of the tested teas at the highest concentration (i.e. approximately 5- and 50-fold higher than that of the green and black teas at 10 µg/mL, respectively). Most importantly, this calculation explains the *in vitro* findings presented here, where tea as a whole mixture of compounds (at low concentrations) is more effective and harmless as bone anabolic and anti-catabolic agents than the pure isolated EGCG at ~20 µM.

9.2.2 Tea effect on bone health *in vivo*: In relation to serum antioxidant capacity and prebiotic-like effect of tea

For the *in vivo* studies, we used whole tea extracts to reflect the usual manner in which tea beverages are prepared and consumed by the general public. The way in which tea is brewed varies between different types of tea, individuals, cultures and countries; this may result in diverse amounts of tea polyphenolic compounds in the resultant cups of tea (Astill et al., 2001; Peterson et al., 2004; Komes et al., 2010; Ahmed & Stepp, 2013; Henning et al., 2003; Zimmermann & Gleichenhagen, 2013; Kosińska & Andlauer, 2014; Fernando & Soysa, 2015). Hot water extraction of tea provides the most cost-effective and accessible way for consumers to have their polyphenolic antioxidant compounds from dietary tea intake (Bahorun et al., 2013). Furthermore, hot water extraction of tea has been reported to provide the highest extraction yield of phenolic compounds and antioxidant activity compared to that by ethyl acetate and methanolic extractions (Khokhar & Magnusdottir, 2002; Farhoosh et al., 2007; Nkubana & He, 2008). Additionally, the complex mixture of constituents in whole extracts have been consistently reported to have multiple bioactivities that may lead to greater beneficial health impacts, as a result of the synergistic effects of several

bioactive compounds in the whole extract (Williamson, 2001; Chen et al., 2002; Lila & Raskin, 2005; Schmidt et al., 2008; Wagner & Ulrich-Merzenich, 2009; Cravotto et al., 2010; Hu et al., 2011; Rasoanaivo et al., 2011; Carmona & Pereira, 2013; Kim et al., 2014).

The inconsistent results from the serum antioxidant capacity in the two animal models (**Chapters 7 and 8**) following consumption of teas with different levels of selenium content is puzzling and this may be due to several contributing factors that have been outlined in these chapters. Most importantly, the health-promoting properties of both selenium and tea phenolic compounds as a separate entity are greatly dependent upon their bioavailability and not only on their total content in tea (Manach et al., 2005; Fairweather-Tait et al., 2010; Rein et al., 2013), hence likely the main reason for the differences in the present findings between *in vitro* and *in vivo* studies.

It is noteworthy that Se-GTE had the most significant prebiotic-like impact compared with the other teas investigated in both *in vivo* studies. Following tea consumption, tea constituents come into contact with the gastrointestinal tract (GIT), where they are normally present in concentrations higher than that of circulating plasma or other body tissues (Scalbert & Williamson, 2000; Koo & Cho, 2004). The presence of tea constituents in high concentrations in the lumen of the gut is ascribed to their poor absorption across the gastrointestinal epithelium, hence allowing for the antioxidant activity of tea to take effect in the gastrointestinal tract (Halliwell et al., 2000, 2005; Scalbert et al., 2002). Indeed, it has been suggested that the selective modulation effect exerted by tea on the resident gut microbiota composition may be attributed to the direct antioxidant action of polyphenols against excess ROS formation, thus providing a better *in vivo* environment in the GIT for the growth of the beneficial gut microbiota (Molan et al., 2009b; Bancirova, 2010; Gyawali & Ibrahim, 2012). Furthermore, tea may also impart antimicrobial effects against selective species of gastrointestinal pathogenic microbiota (Van Dyunhoven et al., 2013; Das et al., 2014). In support of this finding, there is a growing body of evidence that tea and/or its polyphenols may exert potential prebiotic-like effects, which lead to positive changes in the gut microbiota composition and their metabolic activity that ultimately benefits the host (Tzounis et al., 2008; Landete, 2012; Exteberria et al., 2013; Ruxton, 2013). In fact, Lee et al. (2006a) suggested that tea phenolics exert significant effects on the gut

microbiota compositions by acting as metabolic prebiotics, which result from the colonic fermentation activity, and the complex two-way interactions between gut microbiota and tea phenolic compounds (Hervert-Hernández & Goñi, 2011; Van Duynhoven et al., 2013).

The prebiotic-like effects of tea are highly dependent not only on the dietary intake of tea polyphenols, but also on the bioavailability of the tea polyphenols, the chemical structure of the tea polyphenolic compounds, the strain of resident gut microbiota, and colonic fermentation of the undigested tea polyphenols by resident gut microbiota (Laparra & Sanz, 2010; Hervert-Hernández & Goñi, 2011; Shi & Schlegel, 2012; Cardona et al., 2013; Del Rio et al., 2013; Dueñas et al., 2015a, 2015b). With regard to the bioavailability of dietary tea polyphenols, it is generally known that tea polyphenolic compounds are poorly absorbed (Yang et al., 2008b; Chow & Hakim, 2011), only a small proportion of consumed tea polyphenols (i.e. monomeric and dimeric compounds) can be absorbed in the small intestine (Clifford, 2004; Gao et al., 2006), while a major undigestible proportion (i.e. polymeric compounds) is metabolised by gut microbiota and converted into simplified polyphenol metabolites to ease their absorption into the systemic circulation (Van Duynhoven et al., 2011; Da Silva Pinto, 2013). However, among the diverse species of gut microbiota (Mariat et al., 2009; Arumugam et al., 2011), only a few bacterial species are reported to have the ability to metabolise polyphenolic compounds (Wang et al., 2001; Blaut & Clavel, 2007; Selma et al., 2009; Kutschera et al., 2011; Jin & Hattori, 2012; Chen & Sang, 2014), which may account for the high degree of inter-individual differences in the gut microbiota composition (Cardona et al., 2013).

While the antioxidant activity of the fermentation metabolites may be lower than their respective parental compounds (Natsume et al., 2004; Manach et al., 2005), these polyphenol metabolites are potentially more pharmacologically active than their parent compounds. Polyphenol metabolites are involved in the growth and/or activity of resident gut microbiota as they serve as growth substrates for particular beneficial strains (Gibson & Roberfroid, 1995; Hervert-Hernández & Goñi, 2011; Wallace et al., 2011). The tea polyphenol-derived metabolites may also modulate composition of the gut microbiota through growth-stimulating effects and antimicrobial activity against selective resident gut microbiota, which is achieved via functional interactions between

gut microbiota and polyphenolic compounds (Lee et al., 2006a; Selma et al., 2009; Van Duynhoven et al., 2013; Parkar et al., 2013; Duda-Chodak et al., 2015; Dueñas et al., 2015a, 2015b). Additionally, it has been suggested that both polyphenols and polyphenol-derived metabolites, as well as the bacterial fermentation end-products (i.e. short-chain fatty acids (SCFAs)) could contribute to protection against oxidative stress (Gonthier et al., 2003; Wong et al., 2006; Hamer et al., 2008, 2009; Pandey et al., 2009; Ruijters et al., 2013; Christensen & Christensen, 2014). There has been evidence that the positive health-promoting effects shown by dietary polyphenols are mostly ascribed to the polyphenol-derived metabolites generated during the fermentation activity of resident gut microbiota (Del Rio et al., 2010, 2013; Monagas et al., 2010; Williamson & Clifford, 2010; Chen & Sang, 2014).

As described in **Chapter 7**, the enhanced growth of the beneficial gut microbiota may increase production of organic acids and short-chain fatty acids (SCFAs) and potentiate antimicrobial activity, as organic acids and SCFAs decrease pH levels of the gut and suppress growth of pathogenic bacteria (Both et al., 2011; Hervert-Hernández & Goñi, 2011; Van Dorsten et al., 2012). Therefore, we can speculate that the impact of tea supplementation on resident gut microbiota may lead to improved bone health by increasing absorption of minerals (e.g. calcium, magnesium, iron), as previously reported to be enhanced by the fermentation end-products (e.g. SCFAs) and through the benefits of synergism of prebiotics and probiotics (Cashman, 2003; Abrams et al., 2005; Scholz-Ahrens & Schrezenmeir, 2007; Scholz-Ahrens et al., 2007; Su et al., 2008; Sharma & Rao, 2009; Legette et al., 2012; Slavin, 2013). This is supported by the recent reports of Karmakar et al. (2011) and Das et al. (2013), where aqueous black tea supplementation (2.5%, w/v) positively influenced calcium absorption in both rat models of non-alcoholic steatohepatitis (NASH)-induced skeletal dysfunction and early menopausal bone loss, respectively, by modulating the activities of intestinal mucosal calcium-transferring enzymes, i.e. alkaline phosphatase (ALP) and calcium adenosine triphosphatase (Ca-ATPase).

Bacterial fermentation end-products (e.g. SCFA) may directly influence mineral absorption through two different mechanisms: **(1)** by forming complexes with minerals, thereby increasing their uptake by the intestinal cells (Trinidad et al., 1999), and **(2)** by stimulating the intestinal epithelium and increasing its absorptive capacity (Topping &

Clifton, 2001). Furthermore, a lower gut pH following consumption of teas (which was demonstrated by testing in **Chapters 7 and 8**) may not only offer an optimal growth environment for beneficial gut microbiota, but may also enhance absorption of minerals through improved mineral solubility, as previously discussed by other researchers (Younes et al., 1996; Coudray et al., 1997; Lopez et al., 1998). We suggest that tea and/or its polyphenols may contribute to the positive modulation of the host's gut microbiota through the end-products generated from fermentation activity of the undigested tea polyphenols by resident gut microbiota (Lambert et al., 2007; Parkar et al., 2013; Chiou et al., 2014), thus potentially allowing tea to enhance bone properties *in vivo*.

Despite promising evidence of the likely osteopromoting potential of these teas in the previous *in vitro* cellular studies, consumption of a variety of teas in both animal models (**Chapters 7 and 8**) showed no significant changes in bone parameters (i.e. serum bone turnover biomarkers, bone mass, and bone biomechanical). It is possible that the study was too short in duration to show a significant bone-beneficial effect of tea. Thus, we strongly suggest that a longer intervention period is needed to achieve a significant effect of tea on bone parameters, as well as other measures of interest.

It is worth noting that the amount of tea consumed by the rats in these animal models was highly varied. In the young growing rat model (**Chapter 7**), the amount of each tea beverage (prepared at 1% concentration; 10 mg dry tea leaves/mL) consumed by the rats was quite high, where the intake of four different teas was 29 to 35 mL tea/day (i.e. approximately 58 to 70 mg tea solids/day) per 100 g body weight (BW); the BW at the start of the study ranged from 78.9 ± 2.3 to 79.8 ± 2.2 ; $p = 0.9973$ (**Appendix XII and Chapter 7**). However, a lower volume of tea was consumed by the ovariectomised rats (**Chapter 8**) ranging from 7 to 9 mL tea/day (i.e. approximately 15 to 18 mg tea solids/day) for every 100 g BW; the BW at the start of the study ranged from 293.9 ± 7.0 to 300.9 ± 6.0 ; $p = 0.8741$ (**Appendices V and XII**). It is important to note that, due to the steady amount of tea intake in each experimental tea group throughout the study, these initial volumes of tea intake were decreased in parallel to the increased BW of the rats during the four- and eight-week intervention periods. Based on our data, the initial consumption of tea by the young growing rats and ovariectomised rats, may translate to a human intake of approximately 87 to 105 cups of

tea/day per person, and 22 to 26 cups of tea/day per person, respectively (**Appendix XII**); assuming a cup of tea equates to 200 mL of a 1% (w/v) tea infusion. Obviously, the levels of tea intake in both animal studies were substantially higher than the typical volume of tea consumption in human; typically at 1 mL tea/day per 100 g BW, an amount equivalent to moderate daily consumption of three cups of tea beverage per person (Arab & Lieberskind, 2010; Molan et al., 2010).

However, according to Reagen-Shaw et al. (2007), the Food and Drug Administration (FDA) has suggested that the body surface area (BSA) normalisation method is crucial for a more accurate extrapolation of an animal dose to a human equivalent dose (Food and Drug Administration, 2005). In fact, the authors strongly recommend the use of BSA normalisation as the most appropriate method for extrapolating doses between species when compared to the simple conversion using body weight. Indeed, the BSA-based conversion seems logical for allometric scaling of drug dosages between two different species due to BSA correlating well with several biological parameters, such as circulating plasma proteins, basal metabolism and renal function. Therefore, in **Appendix XII**, we further calculate the appropriate conversion of daily intakes of tea obtained from the animal studies to their approximate human equivalent intakes by using the given formula (Reagen-Shaw et al., 2007). Based on these extra calculations, the consumption of various tea drinks by the young growing rats and ovariectomised rats, may translate to an approximate intake of 7 to 9 cups of tea/day per human child and 3 to 5 cups of tea/day per human adult (**Appendix XII**); by taking into account that a cup of tea equates to 400 mg tea solids of a 1% (w/v) tea infusion.

The present data supports the report of others (Isbrucker et al., 2006; Hsu et al., 2011; Saleh et al., 2013; Sur et al., 2015) for consumption of tea as a safe alternative approach for health and wellness. In fact, the low toxicity of compounds in tea is thought to be due to their poor bioavailability and short elimination half-life following oral ingestion, which resulted from rapid and extensive metabolism of tea compounds (Chow et al., 2003; Manach et al., 2005; Liu & Hu, 2007). In the previous cellular *in vitro* studies, tea water extracts were physiologically active even though they were administered at low μM concentrations, since most of bioactive compounds in tea are commonly available as parent compounds (i.e. predominantly of glycosylated

derivatives and polyphenol aglycones) (Del Rio et al., 2013, Marín et al., 2015) that directly interact with the cultured cells. However, following consumption of tea water extracts in the animal studies, tea bioactive compounds undergo xenobiotic biotransformation (via phase II metabolism that serve as a detoxifying step in the metabolism of xenobiotics) within the gastrointestinal tract and the liver (Liu & Hu, 2007; Jančová & Šiller, 2012; Visioli, 2015), thus explaining the presence of both tea compounds and associated metabolites at low nM to μ M circulating plasma concentrations (Nakagawa et al., 1997; D'Archivio et al., 2010; Sang et al., 2011; Van Duynhoven et al., 2011; Del Rio et al., 2013). Furthermore, different tea polyphenolic compounds are widely dispersed into various body tissues/fluids through diverse metabolism and transportation routes. Thus, high concentrations of both polyphenols and their metabolites do not commonly accumulate in their common target organs (Suganuma et al., 1998; Kim et al., 2000; Chen & Lin, 2015).

To our knowledge, only one study has so far shown a detrimental effect of tea consumption on bone growth; this was observed in growing mice following administration of dried green tea extracts at 1% and 2% concentrations in powdered diets for six weeks (Iwaniec et al., 2009). We suggest that the use of dried green tea extracts, and different types of animals (i.e. lean, wild type (C57BL/6) and genetically obese, leptin-deficient (ob/ob) male mice), are the reasons behind the discrepancy. It has previously been reported that different preparations of green tea (i.e. dried green tea extracts *versus* typical brewed tea infusion) can influence the safety characteristics of the green tea (Speijers et al., 2010). Furthermore, the absolute bioavailability of EGCG is higher in mice than in rats (Kim et al., 2000; Lambert et al., 2003), thus, potentially explaining the effect of tea toxicity in the study findings of Iwaniec et al. (2009).

Above all, the discrepancies between the results of *in vitro* cellular assays and *in vivo* animal models, and the lack of significant impact of tea on bone health *in vivo* should be interpreted with caution. This is due to the fact that the data obtained *in vivo* is complicated by other physiological functions in the body, and also depends upon pharmacokinetic processes (i.e. absorption, distribution, metabolism, and excretion) of both selenium and polyphenolic compounds in tea infusion following oral ingestion (Thomson, 2004b; Hermans et al., 2007; D'Archivio et al., 2010; Martin & Appel, 2010; Poljsak et al., 2013; Pompella et al., 2014).

9.3 Implications of the research study

This study provides new information and leads for research on the ability of tea to confer bone benefits due to its common consumption as a beverage. Additionally, the study findings add to our basic understanding of the potential mechanisms of the bone-stimulative effect of tea. Most importantly, the effect of tea cannot be explained solely on the basis of their antioxidant properties. Obviously, more research work connecting tea and bone is warranted to confirm the current findings on tea and its potential as a nutritional approach to minimise bone loss in order to prevent the development of osteoporosis. Although some of the findings of this research are inconclusive, this study supports the nutritional non-pharmacotherapy strategy for the management and prevention of postmenopausal osteoporosis through consumption of teas as inexpensive and safe dietary modifications. The study findings are expected to generate enthusiasm to further explore tea as a potential source of prebiotics that are beneficial to maintain and restore skeletal health, and to clarify its mechanism(s) of action in association to gut microbiota.

9.4 Limitations of the research study

There are several limitations encountered in this study that deserve mention.

- 1) The dried tea leaves used throughout the study were obtained from different sources. Both Se-rich green and black teas were obtained from the same plantation in Enshi, Hubei province, China (19 km away from a Se mine). Meanwhile, R-GTE (an unknown brand from Dong Sheng Market, Palmerston North, New Zealand) and R-BTE (Dilmah, 100% Pure Ceylon) were a sample of convenience, using tea products that were commercially available in New Zealand. Despite using two groups of teas with either low or high levels of Se, this study essentially compared four unrelated types of tea from different sources. Therefore, to avoid issues relating to the high variation in chemical profile between each tea, it is strongly suggested that both green and black teas with different levels of Se should be obtained from the same plantation if at all

possible, although this may not possible as Se content is a reflection of the content of this element in the soil.

- 2) The scope of the present study did not include a full chemical characterisation of the tea samples constituents, only the total phenolic content and total Se content. Therefore, it is strongly suggested that this study should be accompanied with a full chemical characterisation of each tea, in order to use it as another factor for comparison and interpretation of the obtained results. Furthermore, all teas (Se-GTE, R-GTE, Se-BTE, and R-BTE) investigated in the present study were not subjected to Se speciation analysis. This is due to the fact that the determination of Se species in food involves a difficult technique, only performed in a limited number of laboratories worldwide (Fairweather-Tait et al., 2010).
- 3) The *in vitro* antioxidant activity of the tested teas was measured using the FRAP and DPPH assays, which are SET-based assays that assess redox potential and utilise a non-physiologically relevant radical source. In contrast, the ORAC assay is a HAT-based assay that assesses quenching activity against physiologically relevant peroxy radicals (Prior et al., 2005; Apak et al., 2013; Prior, 2015). Thus, it would have been a better decision to have used the ORAC assay to determine antioxidant activity/capacity of both the tea extracts and plasma/serum of the tea-supplemented rats.
- 4) The FRAP assay was the only method used to evaluate serum antioxidant capacity in rats. Unfortunately, this assay has several limitations and does not detect all antioxidant compounds present in serum (as discussed in **Chapter 7**, section 7.4.2), thus, their estimates are unlikely to indicate completely the resultant changes in serum antioxidant capacity (Cao & Prior, 1998; Lotito & Frei, 2006; Halliwell, 2009). Due to its limitations and the disputed differences among various *in vitro* methods available for determination of serum antioxidant capacity, it is suggested that at least two different assays should be used to get more robust results (Schlesier et al., 2002; Apak et al., 2013; Jansen & Ruskovska, 2013).

- 5) Standard chemical antioxidant assays are not ideal for the measurement of the antioxidant capacity of Se-rich teas *in vivo*, as the assays do not account for endogenous antioxidant enzyme activities and unable to reflect the complexity of cellular physiological conditions. This may contribute to the inconclusive effects of Se-rich teas on antioxidant status in the animal models used in this study. Therefore, it is rather the effect of Se-rich teas on activity of endogenous antioxidant enzymes (both selenoenzymes and non-selenoenzymes) in erythrocytes (e.g. GSH-Px1, GST, TrxR, CAT, SOD) and plasma levels of GSH-Px and thiol antioxidants (e.g. thioredoxin and glutathione) that can be evaluated (McKenzie, 2000; Pandey & Rizvi, 2010, 2011; Erkekoğlu et al., 2013).

- 6) The serum samples used for evaluating antioxidant capacity were obtained from blood collected at exsanguination following heart puncture on the day of necropsy, after an overnight fast that aimed to reduce the diurnal variation in serum biomarkers carboxy-terminal crosslinks telopeptide of type I collagen (CTX-I) and osteocalcin (OC) (as described in **Chapter 3**). However, the fasting serum may not represent the accumulative antioxidant potential of tea (as discussed in **Chapter 8**). Thus, to maximise the sensitivity of the assay in detecting changes in serum antioxidant capacity without compromising serum bone biomarkers, our study protocol should include a model of acute tea ingestion (deionised water for sham and OVX control groups) following an overnight fasting at the completion of the studies, preceding blood sampling at exsanguination by heart puncture. In addition, the amount of tea/deionised water provided to all rats after an overnight fast should have been standardised and given by gavage (on an empty stomach, in order to enhance systemic absorption of polyphenols) one to two hours before exsanguination (Sung et al., 2000); the gavage procedure would minimise inter-individual variation in tea intake amongst the OVX rats.

- 7) The biomechanical tests were conducted at a single sample site of cortical bone, i.e. the femoral diaphysis (as discussed in **Chapter 8**). Hence, the use of other biomechanical tests to assess trabecular bone, which is more sensitive to oestrogen deficiency, on several parts of the skeleton (Bono & Einhorn, 2003)

may complement the bone biomechanical data obtained in the present study. Currently, there are several biomechanical tests that allow mechanical strength assessment in trabecular bone of rats, such as vertebrae compression test and femoral head cantilever bending test; all bone sites of frequent osteoporotic fractures in humans (Turner et al., 2001; Jia et al., 2013; Yang et al., 2014b).

- 8) Low signal intensity and limited sensitivity of the FISH analysis may be the reason behind the non-significant effect of the tested teas on intestinal microbiota composition (as described in **Chapter 8**). Therefore, it may be useful to include another method for the gut bacterial enumeration in order to validate the results of FISH assay. For example, multiplex quantitative real-time polymerase chain reaction (qPCR) is a highly sensitive and specific method with a detection limit approximately at 2.5×10^3 bacteria cells per gram of fecal sample (Cleusix et al., 2008).
- 9) The length of the tea intervention period employed in both animal models may have not been long enough for a significant treatment effect to be detected. Polyphenolic compounds in tea generally have low bioavailability and have short elimination half-life period, thus, requiring repeated intake over time (Van het Hof et al., 1999; Manach et al., 2005) in order to maintain high accumulation of both phenolic compounds and its metabolites in systemic circulation, and to exhibit benefits in human health.
- 10) Finally, the rats were fed a diet that ensures maximal health and well-being, such as lucerne, barley, vitamins and minerals. This could mask the beneficial effects of the tea extracts.

9.5 Recommendations for future research directions

Several recommendations can be made based on the findings of the present study, these include:

- 1) An improved and extended experimental research design that overcomes all the study limitations and further investigates cellular and molecular targets of tea could be expected to unveil the osteoinductive effect of the tested teas, and cast light on possible prebiotic mechanism(s) of tea in relation to its claimed bone benefits. In addition, the evaluation of changes in bone microstructure using higher resolution imaging techniques such as micro-computed tomography (μ CT) and peripheral quantitative computed tomography (pQCT) could further clarify the effect of tea consumption in rats.
- 2) The underlying *in vitro* mechanisms of the osteoprotective effects shown by the tea extracts against H_2O_2 -induced oxidative stress in MC3T3-E1 cells should be investigated by assessing the accumulation of intracellular reactive oxygen species (ROS) following H_2O_2 stimulation. This evaluation can be done using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay, in order to further confirm whether the tested teas scavenge intracellular ROS formation and cause a reduction in the levels of ROS accumulation in the cells.
- 3) The daily amount of tea water extracts provided to the rats during the study should be standardised to an amount that is achievable in human consumption and given by gavage; the latter aimed to minimise inter-individual variation in tea intake among the rats throughout the tea intervention period, in order to avoid variable findings attributed to the differences in amounts of tea consumed by the rats.
- 4) The evaluation of the current serum antioxidant capacity needs to be supplemented with other measures that employ sensitive and specific biomarkers of both endogenous antioxidant enzymes (e.g. GSH-Px, CAT) and oxidative damage to lipids (e.g. malondialdehyde), proteins (e.g. 3-nitrotyrosine), and DNA (e.g. 8-hydroxy-2'-deoxyguanosine) (Frei & Higdon,

2003; Hermans et al., 2007), in order to gain reliable results and complete the understanding of the obtained *in vivo* data.

- 5) Future research of tea bioactivities will require a more complete understanding of the pharmacokinetics of the various tea bioactive components (both in animal and humans), in order to determine their bone-promoting effects and overall usefulness of tea in the management of postmenopausal osteoporosis.
- 6) Due to the balanced and complete diet that these rats were fed, many of the antioxidant effects were possibly masked. In future studies, it would be of benefit to feed the rats with pellets that fulfil their caloric intake, but have a low polyphenol and Se content. This is important and especially related to Se, as the Se-rich green and black teas have been specifically developed to address a poor antioxidant status due to low Se intake.

In conclusion, these studies provide new insights into the potential antioxidant and prebiotic roles of teas with different levels of selenium, and their possible impact on bone health. While this study offers cellular evidence that both green and black teas, regardless of selenium content, may affect bone health directly on osteogenic and anti-osteoclastogenic activity, the data are insufficient to confirm any conclusive effects of these teas on bone mass following consumption in rats.

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APPENDICES

Appendix I | The effects and proposed molecular mechanisms of whole tea extracts and/or active polyphenols from *in vitro* studies.

<i>In vitro</i> model(s)	Type(s) of tea /polyphenols	Assay concentration(s)	Method(s) of assessment	Study period	Finding(s)/Mechanism(s)	Reference
A. TEA VERSUS OSTEOCLAST DIFFERENTIATION (OSTEOCLASTOGENESIS)						
1) Murine RAW264.7 cells	EGCG; EGC; ECG; EC EGCG EGCG EGCG	10 and 100 μ M 100 μ M 0 to 50 μ M 10 to 100 μ M 10 μ M	TRAP staining assay MTT viability assay Hoechst 33258 staining assay TRAP staining assay TRAP staining assay	5 d 1 to 3 d 3 d 5 d 0 to 5 d	\downarrow TRAP ⁺ MNCs; EGCG most effective. \downarrow cell viability. Cell viability unaffected. \downarrow osteoclastogenesis dose-dependently. Showed biphasic effects: low dose \downarrow TRAP ⁺ MNCs between 1 to 2 days and <i>vice versa</i> . \downarrow the expression of RANK, OSCAR, osteoclastic genes (CTR, MMP9, NFATc1), and apoptosis-related genes (FOXO3a, Bim). Directly \downarrow osteoclastogenesis in co-culture of OBLs and bone marrow cells.	Zhao et al., 2014
2) Primary murine co-cultures	EGCG EGCG	10 μ M 5 to 50 μ M	RT-PCR analysis; Western blotting TRAP staining assay	3 and 5 d 6 d	\downarrow the expression of RANK, OSCAR, osteoclastic genes (CTR, MMP9, NFATc1), and apoptosis-related genes (FOXO3a, Bim). Directly \downarrow osteoclastogenesis in co-culture of OBLs and bone marrow cells.	Irie et al., 2014
Primary murine BMCs	EGCG	1, 10 and 100 μ M	F-actin and TRAP staining assays	6 d	\downarrow osteoclastogenesis, cell-cell fusion and BMCs proliferation dose-dependently. BMC counts unaffected. \downarrow OCL resorption activity. \downarrow mRNA expressions of MMP9, MMP13, TIMP1 and MTI-MMP; dose-dependently.	Oka et al., 2012
1) Primary murine OCL precursors	EGCG; TFDG	10 and 100 μ M	TRAP staining assay; RT-PCR analysis; Gelatin zymography (MMP2, MMP9).	Up to 7 d	EGCG and TFDG (most effective) \downarrow osteoclastogenesis dose-dependently; through downregulation of MMP2 and MMP9.	Oka et al., 2012
2) Primary murine OCLs	EGCG; TFDG	10 and 100 μ M	F-actin and TRAP staining assays	48 h	EGCG and TFDG (most effective) \downarrow multinucleated OCL formation.	
Primary murine BMMs	Green tea catechin	2, 5 or 10 μ g/mL	Trypan blue dye exclusion assay; TRAP staining assay.	4 d	Cell viability unaffected. EGCG \downarrow osteoclastogenesis dose-dependently.	Nakamura et al., 2010

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A. TEA VERSUS OSTEOCLAST DIFFERENTIATION (OSTEOCLASTOGENESIS) – continued						
1) Primary murine co-cultures	EGCG	20 µM	TRAP staining assay	6 d	↓osteoclastogenesis in co-culture of OBLs and bone marrow cells.	Lee et al., 2010a
2) Primary murine BMMs	EGCG	20 µM 1 to 50 µM 5 to 20 µM; 20 µM	TRAP staining assay XTT viability assay RT-PCR analysis; Western blot analysis	4 d 48 h 12 h	↓osteoclastogenesis in precursor BMMs. Cell viability unaffected. Pre-treatment ↓expression of c-Fos and NFATc1 dose-dependently. Pre-treatment ↓activation of JNK in BMMs. Pre-treatment ↓upregulation of NF-κB-related genes (ICAM-1, Nfkb2 and TNFα), with DNA binding activity unaffected.	
Primary murine BMMs	EGCG	25 µM	TRAP staining assay; Western blot analysis	2 d 30 min	↓bone resorption and osteoclastogenesis via AMPK activation dose-dependently.	Lee et al., 2010b
1) Primary murine co-cultures	EGCG	10 µM	TRAP staining assay	6 d	↓numbers of TRAP ⁺ OCLs in co-cultures (OBLs and bone marrow cells), and ↓OCL formation in bone marrow cells. mRNA expression of RANKL and CSF1 were unaffected.	Kamon et al., 2010
2) RAW264.7 cells	EGCG	1 to 10 µM	TRAP staining assay	5 d	↓osteoclastogenesis dose-dependently.	
1) RAW264.7 cells;	EGCG	10 to 100 µM	TRAP staining and activity assays	5 d	↓osteoclastogenesis in both cell types dose-dependently; exert inhibition (100 µM) at early stage of osteoclastogenesis.	Lin et al., 2009
2) Primary murine BMMs		10 to 100 µM 10 to 100 µM 10 to 100 µM 100 µM	Functional bone resorption assay NF-κB luciferase assay Western blot analysis Fluorescence study of NF-κB	10 d 4 d 2 h 2 h	↓pit formation in both cell types dose-dependently. ↓transcriptional activity of NF-κB dose-dependently. The levels of p65 subunit of NF-κB in the total lysate were unaffected. ↓nuclear localisation of NF-κB.	

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A. TEA VERSUS OSTEOCLAST DIFFERENTIATION (OSTEOCLASTOGENESIS) – continued						
Murine RAW264.7 cells	EGC, GC, GCG EGC, GC, GCG	5 to 20 μ M	MTT viability assay TRAP staining assay	48 h 4 d	Cell viability unaffected. All catechins (EGC most potent) \downarrow osteoclastogenesis dose-dependently. EGC \downarrow TRAP expression dose-dependently.	Ko et al., 2009
Primary human peripheral blood mononuclear CD14 ⁺ cells	EGCG	3.2 to 50 μ M	TRAP staining; WST-8 viability assay	6 d	\downarrow osteoclastogenesis dose-dependently, but cell viability unaffected.	Morinobu et al., 2008
Murine RAW264.7 cells	EGCG	20 and 50 μ M	Bone resorption assay; RT-PCR analysis; Western blot analysis.	6 d	\downarrow osteoclast function. \downarrow mRNA expression of all the molecules in osteoclasts. Selectively \downarrow mRNA expression of NFATc1.	
Murine RAW264.7 cells	EGCG	5 to 50 μ M 50 μ M	TRAP staining Caspase activity assay; Western blot analysis; DNA-fragmentation assay	5 d 24 h	\downarrow osteoclast survival dose-dependently through EGCG-induced activation of caspase-mediated apoptosis.	Yun et al., 2007
Primary murine co-cultures	EGCG	5 to 100 μ M	MTT viability assay	5 d	Cell viability unaffected; \downarrow cell viability only at the highest concentration tested (100 μ M).	
Primary murine co-cultures	EGCG	20 μ M	TRAP staining assay RT-PCR analysis MTT viability assay	4 d 24 h 3 d	\downarrow osteoclastogenesis via inhibition of MMP9 expression in osteoblasts. Cell viability unaffected.	Yun et al., 2004
Primary murine OCL-like multi-nucleated cells	EGCG	12.5 to 100 μ M 100 μ M 25 or 100 μ M; 100 μ M; 100 μ M	Crystal Violet, TRAP and Hoechst 33258 staining assays. Resorption pit staining assay Caspase-3-like protease activity; TRAP and Hoechst 33258 staining assays.	24 h 24 h 6 h; 24 h; 14 h	\downarrow osteoclastogenesis and viability of OCLs dose-dependently. \downarrow size of resorption pits, not OCL counts. \uparrow caspase-mediated apoptosis dose-dependently. \uparrow apoptosis – nuclear disintegration.	Nakagawa et al., 2002
		12.5 to 100 μ M 0.01 to 100 μ M	Fe(II) production assay; <i>In vitro</i> DNA damage assay	20 s 30 min	EGCG-induced Fenton reaction implicated in apoptotic cell death.	

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B. TEA VERSUS OSTEOBLAST DIFFERENTIATION (OSTEOGENESIS/OSTEOBLASTOGENESIS)						
Human MG-63 cells	EGCG	7.5 to 30 μ M	MTT viability assay; ALP activity assay; ARS staining assay; RT-PCR analysis.	3 d 4 d 6 d	Cell viability is unaffected. \uparrow ALP activity and matrix mineralization dose-dependently. \uparrow expression of osteogenic genes (β -catenin) dose-dependently. EGCG-induced osteoblastic activities may involve Wnt signalling through ER pathway.	Peng et al., 2016
Human SaOS-2 cells	GT and BTs (English Breakfast and Golden Monkey)	2% (w/v) aqueous extract normalised to 1 and 10 μ g PPs/mL 1 μ g PPs/mL	MTT viability assay LDH activity assay ALP activity assay	24 and 48 h 24 and 48 h 3 and 7 d	GT \uparrow cell viability time- and dose-dependently. BTs showed biphasic effects: low dose \uparrow cell viability and <i>vice versa</i> . GT and BTs \downarrow cellular LDH activity, time-dependently. GT and BTs showed biphasic effects: short-incubation period \uparrow ALP activity and <i>vice versa</i> .	Nash & Ward, 2016
		1 and 10 μ g PPs/mL	ARS staining assay	7 d	GT \uparrow mineralisation dose-dependently. BTs showed biphasic effects: low dose \uparrow mineralisation and <i>vice versa</i> .	
		1 μ g PPs/mL	ELISA-based multiplex kit	3 and 7 d	GT and BTs \downarrow proinflammatory markers (IL6 and TNF α) and \uparrow osteoblast regulatory markers (OPN and SOST) time-dependently.	
Primary hBMSCs	EGCG	5 to 40 μ M	MTT viability assay; LDH activity assay; Flow cytometry analysis; ALP and ARS staining assays; RT-PCR analysis; Western blot analysis	5 d 3 to 16 d	Biphasic effects on cell viability time- and dose-dependently. Low dose \uparrow cell viability and \downarrow cell death, and <i>vice versa</i> . Biphasic effects on osteogenesis time- and dose-dependently. Protects against inhibition of osteogenesis at 5 μ M.	Liu et al., 2016

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B. TEA VERSUS OSTEOBLAST DIFFERENTIATION (OSTEOGENESIS/OSTEOBLASTOGENESIS) – continued						
Primary human	EGCG	1.25 and 10 µM	MTT viability assay	3 to 12 d	↑cell proliferation in time- and dose-dependent (biphasic) manners.	Kaida et al., 2015
DFAT cells		1.25 and 10 µM	RT-qPCR assay	1 and 6 d	↑mRNA expression of certain osteogenic markers in time- and dose-dependent (biphasic) manners.	
		1.25 and 10 µM	ALP staining and activity assays	6 d	↑ALP activity in dose-dependent biphasic manner.	
		1.25 and 10 µM	ARS staining	6 and 12 d	↑mineralisation in time- and dose-dependent (biphasic) manners.	
		1.25 µM	ARS staining; Inhibitory assay of signalling pathways.	12 d	↑mineralisation via upregulation of JNK and p38 MAPK pathway.	
Primary hBMSCs	EGCG	2.5, 5 and 10 µM	MTT viability assay	3 to 21 d	↑cell proliferation of hBMSCs grown in non-osteogenic media; time- and dose-dependent manners.	Jin et al., 2015
			ALP activity assay		ALP activity of hBMSCs grown in non-osteogenic media was unaffected.	
			RT-PCR analysis; Immunohistochemical staining for BMP2.		↑osteogenesis only in the presence of osteoinductive agents via upregulation of BMP2 expression; time- and dose-dependent manners.	
1) Primary rat OBLs from neonatal calvariae	Flavanes: (+)-catechin (-)-catechin (-)-epicatechin (-)-afzelechin (-)-epiafzelechin	3.125 to 50 µg/mL	MTT viability assays; ALP activity; Hydroxyproline assay	48 h 7 to 21 d	All compounds ↑cell viability, ↑ALP and ↑collagen content in a dose-dependent manner.	Zeng et al., 2014
			ARS staining assay	20 d	All compounds ↑mineralisation dose dependently, except (+)-catechin.	
2) Mouse myoblast C2C12 cells			H ₂ O ₂ -induced cytotoxicity assay (MTT viability assay; Flow-cytometry analysis).	2 d	All compounds ↑cell viability and protect against H ₂ O ₂ -induced cytotoxicity dose-dependently.	

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B. TEA VERSUS OSTEOBLAST DIFFERENTIATION (OSTEOGENESIS/OSTEOBLASTOGENESIS) – continued						
Primary human OBLs from femoral head	Decaffeinated	0.01 to 1 µg/mL;	LDH activity assay; MTT	1 to 24 h	Biphasic effects on cell viability in time- and dose-dependent manners.	Vester et al., 2014
	GTE	100 to 200 µg/mL	viability assay			
Primary hABCs	EGCG	100 to 200 µg/mL	LDH activity assay; MTT	Up to 4 h	Pre- and co-incubation ↓cellular damage and ↑cell viability.	Mah et al., 2014
		100 to 200 µg/mL	Flow cytometry; MTT	Up to 4 h	Post-incubation ↓intracellular ROS formation and ↑cell viability.	
		0.01 to 1 µg/mL	ARS and Von Kossa staining	7 and 14 d	↑mineralisation and ↑expressions of OC and COL1A1 during H ₂ O ₂ -induced oxidative stress.	
Murine MC3T3-E1 cells	EGCG	10 µM	ALP activity and staining assays	10 d	↓number of ALP ⁺ cells and ↓ALP activity during cell differentiation.	Zhao et al., 2014
Primary hABCs	EGCG	1 to 50 µM	Cell proliferation assay	1 to 9 d	↓cell viability dose-dependently.	Mah et al., 2014
			Flow cytometry for apoptosis evaluation	5 d	↑the number of apoptotic cells at the highest concentration.	
		Cell migration assay	6 to 54 h	↓migration area dose-dependently.		
		ARS staining	4 wk	↑mineralisation in a dose-dependent biphasic manner.		
Primary hBMSCs	EGCG	10 µM	RT-PCR analysis	7 to 21 d	↑expressions of ALP and BSP in a time-dependent biphasic manner.	Jin et al., 2014
		2.5, 5 and 10 µM	ALP staining and activity assays	3 to 21 d	↑ALP activity after 7 days in time- and dose-dependent manners.	
Primary hBMSCs	EGCG	2.5, 5 and 10 µM	MTT viability assay		Biphasic effects: ↑cell proliferation between 7 to 14 days in time- and dose-dependent manners.	Jin et al., 2014
			ARS staining		↑cell mineralisation in mature OBLs in time- and dose-dependent manners.	
			RT-PCR analysis; Immunohistochemical staining (BMP2, COL1).		Biphasic effects: ↑mRNA expression of BMP2, Runx2, ALP, BSP, OC and COL1 time- and dose-dependently.	

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B. TEA VERSUS OSTEOBLAST DIFFERENTIATION (OSTEOGENESIS/OSTEOBLASTOGENESIS) – continued						
1) Murine MSCs (C3H10T1/2)	EGC	1 to 10 μ M	ALP staining and activity assays; RT-PCR analysis; Luciferase assay; Endogenous Runx2 immunoprecipitation; Chromatin immunoprecipitation; Immunoblot analysis; Immunocytochemistry; MAPK activity assay; PP1A siRNA transfection.	2 to 6 d	<p>↑Runx2- mediated osteoblastogenesis, dose-dependently; via upregulation of TAZ and ↑interaction between TAZ and Runx2.</p> <p>↑OC expression via recruitment of TAZ at Runx2-binding site of OC promoter in differentiating cells.</p> <p>↑nuclear localisation of TAZ and Runx2 and ↑TAZ-mediated gene transcription.</p> <p>↑PP1A expression, dephosphorylation of TAZ and p38 MAPK.</p>	Byun et al., 2014
2) Primary hBMSCs	EGC	1 to 10 μ M	ALP staining and activity assays; RT-PCR analysis.		<p>↑MSCs differentiation into OBLs dose-dependently; via upregulation of osteoblastic genes (DLX5, MSX2, Runx2 and TAZ) dose-dependently.</p>	
Primary human OBLs from femoral head	Catechin	200 μ M	Resazurin viability assay	4 h	Pre-, co- and post-incubations of cigarette smoke medium-treated OBLs.	Ehnert et al., 2012
Primary human OBLs from femoral head	Decaffeinated GTE and various catechins	50 to 200 μ M	Resazurin viability assay; Flow cytometry	4 h	<p>No effects on cell viability.</p> <p>Pre- and co-incubations ↓intracellular ROS formation in cigarette smoke medium-treated OBLs dose-dependently.</p> <p>Pre- and co-incubations ↑viability of cigarette smoke medium-treated OBLs dose-dependently.</p>	Holzer et al., 2012

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B. TEA VERSUS OSTEOBLAST DIFFERENTIATION (OSTEOGENESIS/OSTEOBLASTOGENESIS) – continued						
Primary rat BMSCs	Decaffeinated GTE; Catechins (C, EC, GC, EGC, GCG, EGCG) EGC	10 to 50 µg/mL; 20 µM 20 µM 2 to 20 µM 20 µM	ALP activity assay ALP activity assay ALP activity assay; ARS staining assay RT-PCR analysis (Runx2, Osteonectin, OPN, ALP)	7 d 7 d 7 and 14 d 7 d	GTE ↑ALP activity in a concentration-dependent manner. Only EGC ↑ALP activity. EGC ↑ALP activity and ↑Ca deposition dose-dependently. EGC ↑mRNA expression of bone formation markers.	Ko et al., 2011
Murine MC3T3-E1 cells	EGCG EGCG EGCG and ECG	1 to 20 µM 1 to 10 µM 1 to 10 µM	MTT viability assay BrdU proliferation assay ALP staining and activity assays; RT-PCR analysis.	48 h 48 h 4 to 10 d	No effects on cell viability. No effects on cell proliferation. Both catechins ↓the number of ALP-positive cells and ↓ALP activity at 10 µM, during cell differentiation. No effects on cell mineralisation in mature (differentiated) OBLs.	Kamon et al., 2010
Rat UMR-106 cells	EGCG, GC and GCG	5 to 20 µM	MTT viability assay ALP activity assay ARS staining	2 and 6 d 4 d 6 d	No effects on viability of OBL precursors. EGC ↑ALP activity dose-dependently. EGC ↑matrix mineralisation at 20 µM.	Ko et al., 2009
1) MC3T3-E1 cells 2) Primary OBLs and MC3T3-E1 cells	EGCG EGCG	30 µM 1 to 30 µM	Trypan blue dye exclusion assay; ELISA-based kit. RT-PCR analysis; Western blot analysis.	24 h	No effects on cell viability and differentiation. ↓IL6 expression in both cells, and ↓PDGF-BB-stimulated IL6 synthesis via inhibition of SAPK/JNK pathway.	Takai et al., 2008
Murine MC3T3-E1 cells	EGCG	30 µmol/L	Western blot analysis	60 min	↓both TGFβ effect and TGFβ-mediated HSP27 induction; via EGCG-induced inhibition of SAPK/JNK pathway.	Hayashi et al., 2008
Murine MC3T3-E1 cells	EGCG	100 µmol/L	Western blot analysis	60 min	↓prostaglandin D ₂ effect and ↓prostaglandin D ₂ -mediated HSP27 induction; via EGCG-induced inhibition of p44/p42 MAPK pathway.	Yamauchi et al., 2007

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B. TEA VERSUS OSTEOBLAST DIFFERENTIATION (OSTEOGENESIS/OSTEOBLASTOGENESIS) – continued						
Murine MC3T3-E1 cells	EGCG	10 to 100 µmol/L	VEGF assay; Western blot analysis	60 min	↑prostaglandin F _{2α} -stimulated VEGF synthesis via enhancing activation of SAPK/JNK among the MAPK superfamily in OBLs.	Tokuda et al., 2007
Human SaOS-2 cells	EGCG	1 to 5 µM	Von Kossa and ARS staining ALP activity assay Western blot analysis	17 d 2 to 8 d 6 to 48 h	↑mineralisation dose-dependently. ↑ALP activity in time and dose-dependent manners. ↓Runx2 expression after 48 h of treatment dose-dependently.	Vali et al., 2007
Primary antler progenitors	EGCG	25 µM	TUNEL staining ALP activity; Immuno-histochemical staining.	24 h 5 to 7 d	↑number and % of apoptotic cells. ↑ALP activity via Wnt signalling pathway.	Mount et al., 2006
1) Human BMCs	GTE; Catechin	0.3125 to 20 µM	MTT viability assay	72 h	Both ↑cell proliferation of human bone marrow cells dose-dependently.	Bickford et al., 2006
2) Human CD34 ⁺ cells	GTE; Catechin	500 ng/mL; 20 µM	MTT viability assay	72 h	Both ↑cell proliferation of CD34 ⁺ cells; effects were lower than human BMCs.	
3) Human CD133 ⁺ cells	GTE; Catechin	500 ng/mL; 20 µM	MTT viability assay	72 h	Both ↑cell proliferation of CD133 ⁺ cells; effects were lower than human BMCs.	
1) Murine BMSCs (D1)	EGCG	1 and 10 µmol/L	Thymidine incorporation assay RT-PCR analysis	24 h 48 h	↓cell proliferation dose-dependently. ↑mRNA expression of bone formation markers (ALP, OC, Osterix and Cbfa1/Runx2) dose-dependently.	Chen et al., 2005
2) Murine 3T3-E1 cells	EGCG	1 and 10 µmol/L	ALP activity assay Alizarin Red S staining Von Kossa staining	4 to 14 d Wk 2 to 4 14 d	↑ALP activity in time- and dose-dependent manners. ↑cell mineralisation in time- and dose-dependent manners. ↑ALP activity in time- and dose-dependent manners.	

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B. TEA VERSUS OSTEOBLAST DIFFERENTIATION (OSTEOGENESIS/OSTEOBLASTOGENESIS) – continued						
Murine BMSCs (D1)	EC, ECG, EGC and EGCG	10^{-6} , 10^{-5} and 10^{-4} M	RT-PCR analysis	24 and 48 h	↑mRNA expression of OPG time- and dose-dependently.	Chen et al., 2003
Primary rat OBLs from neonatal calvariae	GtPP	200 µg/mL	MTT assay; Flow cytometry analysis; ALP activity assay.	1 h	Pre-incubation ↓ the H ₂ O ₂ - and XO-induced loss of cell viability. Pre-incubation ↓ H ₂ O ₂ -induced alteration in ALP activity. Pre-incubation alone showed no effect on cell viability and ALP activity.	Park et al., 2003
Murine MC3T3-E1 cells	(+)-catechin	10^{-10} to 10^{-4} M 10^{-5} M 10^{-5} M	MTT viability assay ALP activity assay Apoptosis quantification; Cytokine enzyme immune-assay systems	48 h	↑cell viability dose-dependently. ↑ALP activity. Showed no effect on apoptosis in the basal state, but able to ↓TNFα-induced apoptosis. Able ↓ both constitutive and TNFα-induced IL6 production in cells.	Choi & Hwang, 2003
Primary rat femoral tissues (diaphyseal and metaphyseal)	(+)-catechin and EGCG	10^{-7} to 10^{-4} M	ALP activity assay; Calcium content determination using atomic absorption spectrophotometry.	24 h	(+)-catechin exerts no effect on ALP activity and Ca content in both femoral tissues. EGCG showed no effect on bone Ca content, but ↓ALP activity dose-dependently; significantly ↓ALP at the highest concentration (10^{-4} M).	Yamaguchi & Jie, 2001

Abbreviation: ALP, alkaline phosphatase activity; AMPK, 5' adenosine monophosphate-activated protein kinase; ARS, Alizarin Red S; Bim, Bcl-2-like protein 11; BMCs, bone marrow cells; BMMs, bone marrow-derived macrophages; BMP2, bone morphogenetic protein-2; BMSCs, bone marrow stromal osteoprogenitor cells; BrdU, 5-bromo-2'-deoxyuridine; BSP, bone sialoprotein; BTs, black teas; C, catechin; Ca, calcium; Cbfa1, core-binding factor 1/Runx2; c-Fos, proto-oncogene that is the human homolog of the retroviral oncogene v-fos; COL1, anticollagen I antibody; COL1A1, collagen type I alpha 1; CSF1, colony stimulating factor 1; CTR, calcitonin receptor; DFAT, dedifferentiated fat; DLX5, distal-less homeobox 5; DNA, deoxyribonucleic acid; EC, epicatechin; ECG, epigallocatechin-3-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; ELISA, enzyme-linked immunosorbent assay; ER, oestrogen receptor; Fe(II), ferrous ion; FOXO3a, forkhead homeobox type O 3a; GC, gallic acid; GCG, gallic acid; gallic acid; gallic acid; Gt, green tea; GTE, green tea extract; GtPP, green tea polyphenols; H₂O₂, hydrogen peroxide; hABCs, human alveolar bone-derived cells; hBMSCs, human bone-marrow-derived mesenchymal stem cells; HSP27, heat shock protein 27; ICAM-1, intercellular adhesion molecule 1; IL6, interleukin-6; JNK, c-Jun N-terminal protein kinase; LDH, lactate dehydrogenase; M, molar; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; MMP, matrix

Appendix I | The effects and proposed molecular mechanisms of whole tea extracts and/or active polyphenols from *in vitro* studies.

metalloproteinase; mRNA, messenger ribonucleic acid; MSX2, Msh homeobox 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NFATc1, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1; Nfkb2, nuclear factor of kappa light polypeptide gene enhancer in B-cells 2; NF- κ B, nuclear factor kappa beta; ng/mL, nanogram per milliliter; OBL, osteoblast; OC, osteocalcin; OCL, osteoclast; OPG, osteoprotegerin; OPN, osteopontin; OSCAR, osteoclast-associated receptor; P38 MAPK, p38 mitogen activated protein kinase; p44/p42 MAPK, p44/p42 mitogen activated protein kinase; PGDF-BB, platelet-derived growth factor-BB; PPIA, protein phosphatase 1 alpha; PPs/mL, polyphenols per milliliter; RANK, receptor activator of nuclear factor kappa beta; RANKL, receptor activator of nuclear factor kappa beta ligand; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; Runx2, runt-related transcription factor-2; s, second; SaOS, osteosarcoma cell line; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; siRNA, short interfering ribonucleic acid; SOST, sclerostin; TAZ, WW domain-containing transcription regulator 1; TFDG, theaflavin-3,3'-digallate; TGF- β , transforming growth factor- β ; TIMP1, tissue inhibitor of metalloproteinase 1; TNF α , tumor necrosis factor alpha; TRAP, tartrate-resistant acid phosphatase; TRAP⁺ MNCs, TRAP-positive multinucleated cells; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; μ g, microgram; μ M, micromolar; μ mol/L, micromoles per litre; UMR, osteosarcoma cell line; VEGF, vascular endothelial growth factor; Wnt, wingless-type mammary tumor virus integration site; WST-8, water soluble tetrazolium salt [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]; XO, xanthine oxidase; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

Appendix II | The effects and proposed mechanisms of whole tea extracts and/or active polyphenols from animal studies.

Animal model (s)	Animal(s)	Type(s) of tea /polyphenols	Assay concentration(s)	Finding(s)/Mechanism(s)	Reference
OVX-induced bone loss	Virgin 12-week-old female Sprague-Dawley rats	EGCG	10 mg/kg/d, intraperitoneally injected (3 d post-OVX), for 12 wk.	Exerts bone-restoration effect on bone mass: <ul style="list-style-type: none"> ● \uparrowBMD^c and BV/TV^c, and \downarrowTb.Sp^c in the trabecular bone of the distal femur ● \uparrowdense distribution^e and Tb.N^e of the proximal tibia; non-significant ● \downarrowSema4D expression along the bone surface 	Song et al., 2014
OVX-induced early menopausal bone loss	140 to 150 g female Wistar rats ^a	BTE	2.5% (25 g/L) aqueous BTE, gavaged orally at a single dose daily (1 mL/100 g BW) for 28 d.	Promotes intestinal absorption of Ca: <ul style="list-style-type: none"> ● \uparrowactivities of intestinal mucosal Ca-transferring enzymes: ALP and Ca-ATPase Ameliorates changes in: <ul style="list-style-type: none"> ● urinary parameters (\downarrowurinary loss of both minerals (Ca, P) and organic components (Cr, HPr) of bone) ● bone parameters (\uparrowbone densities^b, BMC and breaking force) ● serum parameters (\uparrowE₂; (\downarrowALP and TRAP) ● bone histological features^c (\uparrowcortical thickness and bone marrow content at the proximal tibia; \uparrowtrabecular BV and thickness at the 3rd LV) 	Das et al., 2013
OVX-induced deterioration of bone microarchitecture	12-week-old female Sprague-Dawley rats	EGCG	3.4 mg/kg/d (peak serum ~10 μ mol/L), intraperitoneally injected (3 mos post-OVX), for 12 wk.	Attenuates OVX-induced bone loss via: <ul style="list-style-type: none"> ● ameliorates changes in parameters of trabecular bone region of proximal tibiae (\uparrowBMD^b, BV/TV^c, Tb.Th^c, Tb.N^c,^e and \downarrowTb.Sp^c), 3rd LV (\uparrowBV/TV^c and Tb.Th^c) and tibial cortex (\uparrowBV^c) ● \uparrowBMP2 expression 	Chen et al., 2013

Appendix II | The effects and proposed mechanisms of whole tea extracts and/or active polyphenols from animal studies.

Animal model (s)	Animal(s)	Type(s) of tea /polyphenols	Assay concentration(s)	Finding(s)/Mechanism(s)	Reference
OVX-induced bone loss	10-week-old female Sprague Dawley rats	GT powdered leaves	10% of total diet, <i>ad libitum</i> for 4 wk.	↑femoral BMD	Ryou et al., 2012
OVX-induced bone loss	9-week-old female C57BL/6 mice	EGCG	50 mg/kg BW, intraperitoneally injected (1 wk post-OVX), 5x weekly (1 mL/100 g BW) for 8 wk.	Attenuates OVX-induced bone loss via: <ul style="list-style-type: none"> ● ↑BMD^b, BV/TV^c and Tb.N^c ● ↓Tb.Sp^c ● ↓OcS/BS^d and N.Oc/BS^d ● ↓MAR^d ● ↑phosphorylated AMPKα expression 	Lee et al., 2012b
OVX-induced bone loss	14-month-old (middle-aged) F344 x BN/N1/NIA female rats	GTP	0.5% (w/v) GTP in drinking water daily for 16 wk.	Attenuates OVX-induced bone loss via: <ul style="list-style-type: none"> ● antioxidative (↑SOD1 and ATP synthase) ● E₂-associated effects (↓COMT expression) 	Shao et al., 2011
High-fat-diet-induced non-alcoholic steatohepatitis skeletal dysfunction	4 to 5-month-old male and female Wistar rats	BTE	2.5% (25 g/L) aqueous BTE, gavaged orally at a single dose daily (1 mL/100 g BW) for 30 d.	Ameliorates skeletal changes in: <ul style="list-style-type: none"> ● calcium homeostasis (↑intestinal mucosal Ca⁺ transference and Ca-transferring enzymes ALP and Ca-ATPase) ● urinary parameters (↓urinary loss of both minerals and organic components of bone), serum parameters (↓ALP, TRAP and RANKL; ↑E₂ and OPG) ● bone parameters (↑bone densities, BMC and breaking force; ↑cortical thickness and bone marrow content at the proximal tibia and 4th LV^e; ↑trabecular BV and Tb.Th at the 4th LV^e) 	Karmakar et al., 2011

Appendix II | The effects and proposed mechanisms of whole tea extracts and/or active polyphenols from animal studies.

Animal model (s)	Animal(s)	Type(s) of tea /polyphenols	Assay concentration(s)	Finding(s)/Mechanism(s)	Reference
LPS-induced chronic inflammatory deterioration of bone microstructure.	Virgin 3-month-old CD female rats	GTP	0.5% (w/v) GTP in drinking water daily for 12 wk	Both GTP and alphacalcidol reversed skeletal changes: <ul style="list-style-type: none"> ● ↑BMC^b and breaking force in femur ● ↑trabecular BV/TV^d, and ↓ BFR/BS^d in proximal tibia ● ↑Ps-MS/BS^d and Ps-BFR/BS^d in periosteal tibial shaft ● ↓Ec-MS/BS^d and Ec-ES/BS^d in endocortical tibial shaft ● ↓N.Oc/B.Pm and Oc.Pm/B.Pm ● ↓TNFα expressions in proximal tibia 	Shen et al., 2011d
LPS-induced systemic chronic inflammatory deterioration of bone microarchitecture.	Virgin 3-month-old Sprague Dawley female rats	GTP	0.5% (w/v) GTP in drinking water daily for 12 wk.	Exerts bone-restoration effect on bone mass via: <ul style="list-style-type: none"> ● ↑trabecular BV/TV^c, Tb.N^c and breaking force in femur ● ↑trabecular BV/TV^d, ↑Tb.N^d, and ↓Tb.Sp^d in proximal tibia ● ↑Ps-BFR/BS^d in periosteal tibial shaft ● ↓ES/BS^d in endocortical tibial shaft ● ↓TNFα expressions in proximal tibia 	Shen et al., 2011c
ORX-induced deterioration of bone microstructure and quality.	Virgin 15-month-old aged F344 x BFN1/NIA male rats	GTP	0.5% (w/v) GTP in drinking water daily for 16 wk.	Exerts bone-restoration effect on bone mass via: <ul style="list-style-type: none"> ● ↑BMD^b, trabecular BV/TV^c, Tb.N^c and breaking force in femur; ↑liver GSH-Px ● ↑trabecular BV/TV^d, ↑Tb.Th^d, ↑BFR/BS^d, and ↓ES/BS^d in proximal tibia ● ↑trabecular BV/TV^d, ↑Tb.Th^d and ↑Ps-BFR/BS^d in periosteal tibial shaft ● ↓ES/BS^d in endocortical tibial shaft ● ↓serum TRAP only in ORX rats 	Shen et al., 2011a

Appendix II | The effects and proposed mechanisms of whole tea extracts and/or active polyphenols from animal studies.

Animal model (s)	Animal(s)	Type(s) of tea /polyphenols	Assay concentration(s)	Finding(s)/Mechanism(s)	Reference
LPS-induced chronic inflammatory bone loss.	Virgin 3-month-old CD female rats	GTP	0.5% (w/v) GTP in drinking water for 12 wk.	Exerts synergistic effect with alphacalcidol on bone mass restoration: <ul style="list-style-type: none"> ● ↑femoral BMC^b and BMD^b ● Urinary parameters (↑EGC and EC; ↓8-OHdG); ↓serum TRAP ● ↓mRNA expressions of TNFα and COX2 	Shen et al., 2010b
LPS-induced chronic inflammatory bone loss.	Virgin 3-month-old CD female rats	GTP	0.5% (w/v) GTP in drinking water daily for 12 wk.	Exerts bone-restoration effect on bone mass: <ul style="list-style-type: none"> ● ↑femoral BMC^b and BMD^b ● Urinary parameters (↑EGC and EC; ↓8-OHdG) ● ↓serum TRAP ● ↓mRNA expressions of TNFα and COX2 	Shen et al., 2010a
LPS-induced inflammatory alveolar bone resorption.	7-week-old male BALB/c mice	GTC	Injected into infected gingival tissue, once every 48 h for 10 doses (i.e. 20 d).	Attenuates LPS-induced bone loss via: <ul style="list-style-type: none"> ● ↓osteoclastogenesis ● ↓IL-1β expression 	Nakamura et al., 2010
Bone mass and architecture in young growing lean and genetically obese mice.	5-week-old lean (C57BL/6 wild type) and leptin-deficient (ob/ob) male mice.	GTE	1% or 2% (w/w) in diet for 6 wk.	Reduce bone mass during skeletal growth: <ul style="list-style-type: none"> ● ↓length, BV/TV^c, BMC^c, cortical volume, marrow volume and cortical thickness in femur ● ↓trabecular BV/TV^c and Tb.Th^c in LV 	Iwaniec et al., 2009
1) Aging-induced deterioration of bone microarchitecture.	Virgin 14-month-old middle-aged F344 x BFN1/NIA female rats	GTP	0.1% or 0.5% (w/v) in drinking water daily for 16 wk.	Exerts bone-restoration effect on: <ul style="list-style-type: none"> ● proximal tibia^d (↑BV/TV, Tb.N, Tb.Th and BFR/BS; ↓Tb.Sp and ES/BS) ● cortical bone of tibia shaft^d (↑Ct.Ar; ↑Ps-MAR and Ps-BFR/BS; ↓Ec-ES/BS) ● cortical bone of femur^c (↑thickness) ● 4th LV^c (↑Tb.Th) 	Shen et al., 2009
2) Aging- and OVX-induced deterioration of bone microarchitecture.					

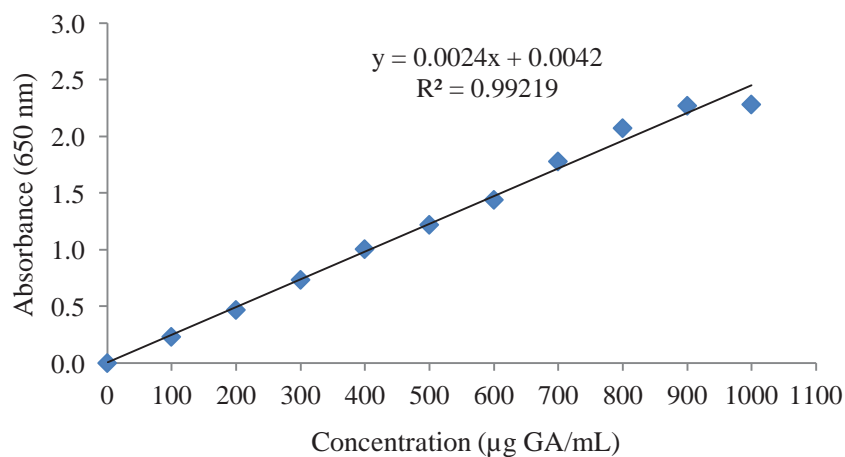
Appendix II | The effects and proposed mechanisms of whole tea extracts and/or active polyphenols from animal studies.

Animal model (s)	Animal(s)	Type(s) of tea /polyphenols	Assay concentration(s)	Finding(s)/Mechanism(s)	Reference
OVX-induced bone loss	Virgin 14-month-old middle-aged F344 x BFN1/NIA female rats	GTP	0.1% or 0.5% (w/v) in drinking water daily for 16 wk.	Exerts bone-restoration effect on bone mass (↑ femur BMD ^b and ↓urinary Ca) and activates antioxidant enzymes (↑liver GSH-Px) that effectively mitigate oxidative damage (↓urinary 8-OHdG).	Shen et al., 2008a
OVX-induced oxidative stress of mononuclear cells and its associated progression of bone loss.	6-month-old female adult Wistar albino rats	BTE	2.5% aqueous BTE, gavaged orally at a single dose per d (1 mL/100 g BW) for 28 d.	Exerts protective and restorative effects against OVX-induced bone loss: <ul style="list-style-type: none"> ● ↓specific bone resorption markers (serum IL6, TNFα, RANKL, TRAP and urinary HPr) ● ↓oxidative stress in peritoneal macrophages and PBMC (↑CAT, ↓SOD) ● ↓osteoclastogenesis (↓numbers of active OCLs in the proximal tibia^c) ● ↑femoral breaking force ● ↑bone densities (↑C.Th^d at both tibia and LV, ↑BV/TV^d and Tb.Th^d at the 4th LV) 	Das et al., 2009
OVX-induced bone loss	120 – 130 g female albino rats ^a	BTE	2.5% aqueous BTE, gavaged orally at a single dose daily (1 mL/100 g BW) for 28 d.	Ameliorates oestrogen deficient-bone loss: <ul style="list-style-type: none"> ● ↓serum TRAP and urinary HPr ● ↑serum E₂; ↑BMD; ↑Ca and P in femur thoracic rib, thoracic vertebra and lumbar vertebra 	Das et al., 2005
OVX-induced bone loss	95 – 100 days female albino rats	BTE	2.5% aqueous BTE, gavaged orally at a single dose daily (1 mL/100 g BW) for 28 d.	Suppresses the rate of bone turnover as shown by: <ul style="list-style-type: none"> ● ↑bone densities (right femur, 8th thoracic rib, 8th thoracic vertebra and 4th LV) ● ↓biomarkers of bone turnover (serum ALP) and urinary loss of bone minerals and organic components) ● ↓osteoclastic activity markers, i.e. serum TRAP and urinary HPr 	Das et al., 2004

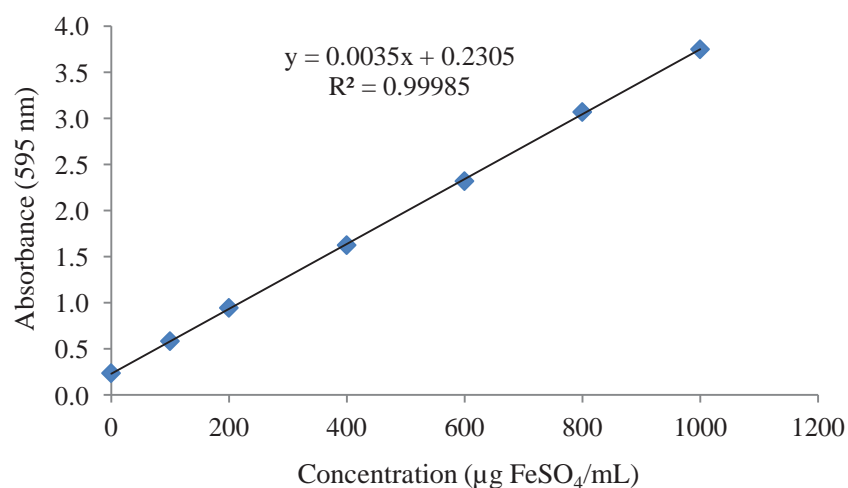
Appendix II | The effects and proposed mechanisms of whole tea extracts and/or active polyphenols from animal studies.

Abbreviations: ALP, alkaline phosphatase; AMPK α , 5'adenosine monophosphate-activated protein kinase alpha; ATP, adenosine triphosphatase; BFR/BS, bone formation rate per bone surface of proximal tibia; Bim, Bcl-2-like protein 11; BMC, bone mineral content; BMD, bone mineral density; BMP2, bone morphogenetic protein-2; BV, bone volume; BTE, black tea extract; BV/TV, bone volume density per bone volume per total volume; BW, body weight; C.Th, cortical thickness; Ca, calcium; Ca⁺, calcium ion; Ca-ATPase, calcium-activated adenosine triphosphatase; CAT, catalase; COMT, catechol-O-methyltransferase; COX2, cyclooxygenase-2; Cr, creatinine; Ct.Ar, cortical area; d, day; DPPH, 2,2-diphenyl-1-picrylhydrazyl; E₂, oestrogen/17 β -oestradiol; oestradiol; EC, epicatechin; Ec-Es/BS, endocortical eroded surface per bone surface; EGC, epigallocatechin-3-gallate; ES/BS, eroded surface per bone surface; FRAP, ferric-reducing antioxidant power; g/L, gram per litre; GSH-Px, glutathione peroxidase; GT, green tea; GTE, green tea extract; GTC, green tea catechins; GTP, green tea polyphenols; IL-1 β , interleukin-1 beta; HPr, hydroxyproline; IL6, interleukin-6; LPS, lipopolysaccharide; LV, lumbar vertebrae; Ps-MAR, periosteal mineral apposition rate; mL/100 g, milliliter per hundred gram; mRNA, messenger ribonucleic acid; N.Oc/B.Pm, number of osteoclasts per bone perimeter; N.Oc/BS, number of osteoclasts per bone surface; ob/ob, obese leptin-deficient; Oc.Pm/B.Pm, osteoclasts perimeter per bone perimeter; OCLs, osteoclasts; Ocs/BS, osteoclast surface per bone surface; OPG, osteoprotegerin; ORX, orchidectomised; OVX, ovariectomised; P, phosphorus; PBMC, peripheral blood mononuclear cell count; Ps-BFR/BS, periosteal bone formation rate; Ps-MAR, periosteal mineral apposition rate; Ps-MS/BS, periosteal mineralised surface per bone surface; p-value, probability value; RANKL, receptor activator of nuclear factor kappa beta ligand; Sema4D, semaphorin4D; Sham, sham-operated; SOD, superoxide dismutase; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TNF α , tumor necrosis factor alpha; TPC, total phenolic content; TRAP, tartrate-resistant acid phosphatase; μ mol/L, micromoles per litre; w/v, weight per volume; wk, week; 8-OHdG, 8-hydroxy-2-deoxyguanosine.

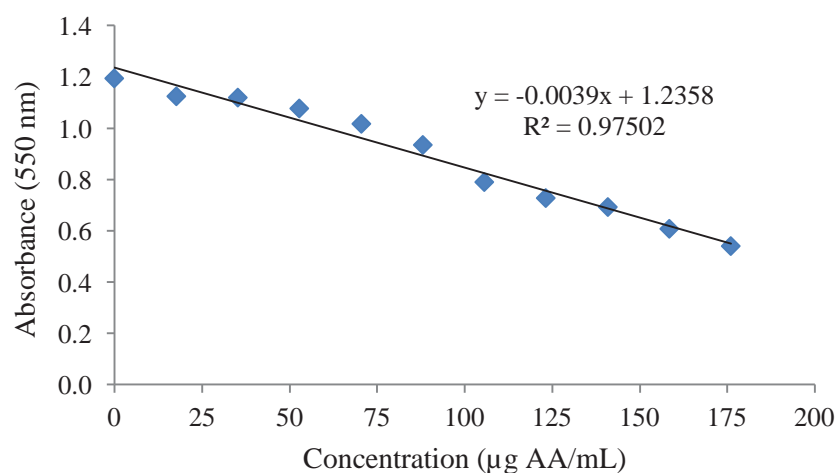
^aage of rats is not provided; ^bassessed via Dual-energy X-ray Absorptiometry (DeXA); ^cassessed by micro-computed tomography (μ CT); ^dassessed by histomorphometry; ^eassessed by histology.



Calibration curve of gallic acid for determination of TPC at 650 nm.



Calibration curve of ferrous sulphate for determination of FRAP at 595 nm.



Calibration curve of ascorbic acid for determination of DPPH at 550 nm.



MASSEY UNIVERSITY
COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Institute of Veterinary, Animal and Biomedical Sciences

PATHOLOGY REPORT

Submitter's Ref.:	Date Sent: 10/06/2011	Accession No.: 46352
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TO: Noor Nordin
IFNHH

Species: Rodent (1)	Sex: Female	Age: 3 Months	Breed: Sprague Dawley Ra
ID: 65	At Risk:	Affected:	Dead:
Owner:	Prev. Accn.:	Type: Post Mortem	

HISTORY

Died after anaesthetic injection. The animal reportedly died on 9/6/2011 at 9:30 am.

GROSS FINDINGS

This is a 361g female albino rat in good postmortem and nutritional condition. There are two ill-defined circumferential red foci on the serosa of the jejunum. Peyer's patches are prominent. The vasculature of the reproductive tract is prominent.

HISTOPATHOLOGY

Slide 1: Brain (cerebrum, thalamus, hippocampus, cerebellum, and brainstem): No significant findings (NSF)

Lung: There is widespread congestion and atelectasis with patchy mild oedema.

Slide 2: Lung: The changes are similar to those described above, with the addition of multifocal acute perivascular haemorrhage and oedema.

Heart: There is a very small perivascular accumulation of lymphocytes within the interventricular septum.

Kidney: NSF

Slide 3: Salivary glands: NSF

Lymph node (submandibular): NSF

Liver: The hepatocytes have expanded clear cytoplasm (glycogen accumulation). There are small random foci of hyperchromatic nuclei in the sinusoids (extramedullary haematopoiesis).

Ileum: NSF

Colon (with gut-associated lymphoid tissue): NSF

Slide 4: Jejunum: NSF (some sections are more autolysed than others)

MORPHOLOGICAL DIAGNOSIS

Moderate diffuse acute pulmonary congestion and atelectasis

Mild multifocal acute pulmonary oedema and haemorrhage

Minimal focal lymphocytic myocarditis

Mild diffuse hepatocellular glycogen accumulation

Minimal multifocal hepatic extramedullary haematopoiesis

DIAGNOSIS

Anaesthetic death

COMMENTS

There are no gross or histologic lesions to explain the death of this rat following anaesthetic injection.

File Nos.:

Students:

Date:

Pathologist: L E Craig/ V Lashley

Copy to:

Te Kunenga
ki Pūrehuroa

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COLLEGE OF SCIENCES
TE WĀHANGA PŪTAIAO

Institute of Veterinary, Animal and Biomedical Sciences

PATHOLOGY REPORT

Submitter's Ref.:	Date Sent: 13/07/2011	Accession No.: 46449
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TO: Abdul Molan
IFNHH
Massey University
Palmerston North

Species: Rodent (1)	Sex: Female	Age:	Breed: Rattus
ID: 76	At Risk:	Affected:	Dead:
Owner: IFNHH	Prev. Accn.:	Type: Post Mortem	

HISTORY

The animal was reportedly anaesthetised for a DEXA scan and died.

GROSS FINDINGS

The female rat presented dead in good preservation and body condition. The animal weighed 0.34 kg and there was a 5cm x 8cm area of clipped/ shaved hair in the thoracolumbar area. There were no obvious gross lesions.

HISTOPATHOLOGY

Sections of kidney, liver, heart, spleen, thymus, intestines, pancreas and salivary gland were examined. Sections display varying degrees of autolysis. Within one lung section there are rare foci of alveolar collapse with increased numbers of lymphocytes, distended foamy alveolar macrophages and mild type II pneumocyte hyperplasia. Another section displayed moderate congestion and approximately 30% of alveoli contained eosinophilic proteinaceous fluid. Changes within peribronchiolar lymphoid tissue were minimal to absent. Other tissues examined were within normal histological ranges.

DIAGNOSIS

Anaesthetic death

COMMENTS

There were no gross or microscopic lesions to indicate a pre-existing disease predisposing to death during anaesthesia.

File Nos.:

Students:

Date: 27 July 2011

Pathologist: K G Thompson / V Lashley

Copy to:

Mean values of body weight of female Sprague-Dawley rats^a.

Group	Sham ^b	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^c
Body weight (g)							
Week -3	274.9 (5.5)	272.7 (4.7)	270.2 (4.8)	272.6 (5.4)	273.4 (5.2)	273.3 (5.0)	0.9895
Week -2	283.4 (5.1)	282.9 (4.8)	281.1 (5.3)	281.5 (4.9)	283.1 (4.9)	282.5 (5.2)	0.9996
Week -1	285.9 (4.8)	285.6 (5.4)	282.5 (6.0)	283.8 (5.4)	286.5 (3.9)	289.3 (5.0)	0.9268
Week 0	297.2 (5.5)	293.7 (6.9)	293.9 (7.0)	297.0 (5.0)	299.7 (5.6)	300.9 (6.0)	0.8741
Week 1	298.3 (6.0)	307.7 (7.2)	302.9 (7.2)	310.0 (7.4)	311.8 (5.2)	316.0 (6.3)	0.6942
Week 2	303.9*** (5.9)	336.4 (6.7)	331.4 (6.5)	343.2 (6.3)	341.0 (3.2)	345.8 (5.4)	0.3952
Week 3	311.3*** (5.6)	358.2 (7.0)	356.2 (5.4)	365.4 (6.5)	363.6 (4.0)	366.3 (5.8)	0.6458
Week 4	314.9*** (5.7)	367.4 (7.7)	365.5 (5.8)	375.2 (6.6)	371.4 (4.0)	378.9 (5.5)	0.4837
Week 5	324.1*** (6.3)	374.6 (8.2)	374.2 (6.1)	382.2 (7.9)	373.6 (5.4)	388.0 (6.4)	0.4720
Week 6	328.5*** (5.9)	384.8 (7.5)	380.6 (5.5)	389.1 (7.1)	384.9 (4.8)	395.8 (5.1)	0.4420
Week 7	332.4*** (4.9)	389.7 (7.7)	390.0 (5.9)	396.4 (7.1)	388.3 (5.1)	402.2 (6.0)	0.4920
Week 8	332.7*** (5.9)	389.5 (8.8)	388.9 (6.9)	391.3 (7.6)	384.8 (7.2)	407.2 (6.3)	0.2246

^aData represent mean (\pm SEM), $n = 12 - 15$ rats/group. Analysis was conducted using repeated measure ANOVA. ^bSham *versus* OVX control. ^cOVX control *versus* experimental groups. *indicates significance from the OVX control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Appendix VI | *In vivo* lumbar spine DEXA of female Sprague-Dawley rats.

In vivo bone area (BA, cm²), bone mineral content (BMC, g) and bone mineral density (BMD, g/cm²) in the lumbar spine of female Sprague-Dawley rats^a.

Group	Sham ^b	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^c
BA (cm²)							
Week -2	1.898 (0.033)	1.860 (0.023)	1.860 (0.011)	1.894 (0.036)	1.915 (0.034)	1.879 (0.035)	0.6472
Week 3	2.047 (0.035)	2.055 (0.024)	2.027 (0.018)	2.075 (0.035)	2.077 (0.028)	2.073 (0.026)	0.6321
Week 7	2.126 (0.036)	2.140 (0.038)	2.157 (0.022)	2.170 (0.037)	2.169 (0.036)	2.176 (0.032)	0.9423
BMC (g)							
Week -2	0.439 (0.015)	0.423 (0.009)	0.416 (0.006)	0.431 (0.015)	0.436 (0.013)	0.422 (0.015)	0.8232
Week 3	0.502 (0.015)	0.476 (0.009)	0.454 (0.009)	0.476 (0.012)	0.481 (0.013)	0.478 (0.012)	0.4552
Week 7	0.546 (0.017)	0.513 (0.016)	0.515 (0.010)	0.515 (0.016)	0.519 (0.017)	0.525 (0.014)	0.9804
BMD (g/cm²)							
Week -2	0.230 (0.004)	0.227 (0.003)	0.224 (0.003)	0.227 (0.004)	0.227 (0.003)	0.224 (0.004)	0.8986
Week 3	0.244** (0.004)	0.231 (0.002)	0.224 (0.003)	0.229 (0.003)	0.231 (0.004)	0.230 (0.003)	0.4208
Week 7	0.256** (0.004)	0.239 (0.003)	0.239 (0.003)	0.237 (0.004)	0.239 (0.004)	0.241 (0.003)	0.9630

^aData represent mean (\pm SEM), $n = 12 - 15$ rats/group. ^bSham *versus* OVX control rats (student's *t*-test). ^cOne-way ANOVA followed by post-hoc Tukey-Kramer's test. *indicates significance from the OVX control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In vivo bone area (BA, cm²), bone mineral content (BMC, g) and bone mineral density (BMD, g/cm²) in the femoral bone of female Sprague-Dawley rats^a.

Group	Sham ^b	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^c
BA (cm²)							
Week -2	1.307 (0.013)	1.328 (0.009)	1.279 (0.010)	1.329 (0.016)	1.338 (0.013)	1.326 (0.015)	0.0910
Week 3	1.392 (0.014)	1.443 (0.012)	1.398 (0.011)	1.437 (0.015)	1.430 (0.010)	1.446 (0.019)	0.4017
Week 7	1.432* (0.016)	1.499 (0.012)	1.454 (0.010)	1.498 (0.016)	1.487 (0.014)	1.500 (0.019)	0.4348
BMC (g)							
Week -2	0.369 (0.007)	0.373 (0.004)	0.355 (0.004)	0.378 (0.008)	0.379 (0.007)	0.377 (0.009)	0.3341
Week 3	0.430 (0.008)	0.422 (0.005)	0.401 (0.004)	0.423 (0.008)	0.416 (0.006)	0.429 (0.010)	0.3462
Week 7	0.460 (0.009)	0.451 (0.007)	0.436 (0.004)	0.456 (0.008)	0.448 (0.007)	0.460 (0.010)	0.5610
BMD (g/cm²)							
Week -2	0.282 (0.003)	0.280 (0.002)	0.277 (0.002)	0.284 (0.003)	0.283 (0.003)	0.283 (0.004)	0.6732
Week 3	0.308** (0.003)	0.293 (0.002)	0.287 (0.002)	0.294 (0.003)	0.290 (0.003)	0.296 (0.004)	0.5337
Week 7	0.321** (0.003)	0.301 (0.003)	0.300 (0.002)	0.304 (0.003)	0.301 (0.003)	0.306 (0.003)	0.8084

^aData represent mean (\pm SEM), *n* = 12 – 15 rats/group. ^bSham *versus* OVX control rats (student's *t*-test). ^cOne-way ANOVA followed by post-hoc Tukey-Kramer's test. *indicates significance from the OVX control: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

In vivo whole body bone area (BA, cm²), bone mineral content (BMC, g) and bone mineral density (BMD, g/cm²) in female Sprague-Dawley rats^a.

Group	Sham ^b	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^c
BA (cm²)							
Week -2	59.90 (0.92)	59.99 (0.64)	58.84 (0.78)	59.85 (0.98)	59.48 (1.06)	59.84 (1.01)	0.8895
Week 3	63.81* (0.90)	67.07 (0.82)	65.61 (0.82)	67.42 (0.92)	67.76 (0.87)	68.16 (0.89)	0.2808
Week 7	66.41*** (0.94)	71.53 (1.00)	70.18 (0.89)	72.17 (1.06)	71.60 (1.23)	72.78 (0.90)	0.4367
BMC (g)							
Week -2	9.58 (0.23)	9.57 (0.16)	9.20 (0.13)	9.58 (0.23)	9.47 (0.25)	9.48 (0.23)	0.6667
Week 3	10.79 (0.25)	10.99 (0.19)	10.51 (0.13)	11.03 (0.25)	10.97 (0.21)	11.17 (0.24)	0.2134
Week 7	11.59 (0.25)	12.13 (0.24)	11.77 (0.17)	12.28 (0.28)	12.07 (0.30)	12.35 (0.24)	0.4798
BMD (g/cm²)							
Week -2	0.160 (0.002)	0.159 (0.001)	0.156 (0.001)	0.160 (0.002)	0.159 (0.002)	0.158 (0.002)	0.4797
Week 3	0.169* (0.002)	0.164 (0.001)	0.160 (0.001)	0.163 (0.002)	0.162 (0.001)	0.164 (0.002)	0.2960
Week 7	0.174* (0.002)	0.169 (0.001)	0.168 (0.001)	0.170 (0.002)	0.168 (0.002)	0.170 (0.002)	0.8202

^aData represent mean (\pm SEM), *n* = 12 – 15 rats/group. ^bSham *versus* OVX control rats (student's *t*-test). ^cOne-way ANOVA followed by post-hoc Tukey-Kramer's test. *indicates significance from the OVX control: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

In vivo whole body lean mass (g), fat mass (g) and percentage fat (%) in female Sprague-Dawley rats^a.

Group	Sham ^b	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^c
Lean mass (g)							
Week -2	236.38 (3.89)	237.91 (4.50)	233.83 (3.61)	243.39 (4.25)	243.83 (2.96)	238.58 (4.45)	0.4018
Week 3	257.35*** (5.67)	296.94 (6.39)	310.18 (3.90)	309.88 (7.72)	305.16 (5.14)	307.79 (6.08)	0.5063
Week 7	269.50*** (4.22)	310.06 (5.97)	324.52 (6.10)	317.84 (6.57)	315.90 (6.83)	320.22 (6.36)	0.5726
Fat mass (g)							
Week -2	49.97 (2.96)	45.65 (2.54)	49.44 (4.34)	41.79 (2.70)	41.99 (3.35)	48.20 (3.03)	0.3456
Week 3	51.46 (2.01)	57.08 (3.14)	44.23 (4.78)	51.00 (2.56)	53.92 (4.08)	55.55 (3.09)	0.2220
Week 7	61.69* (3.12)	77.93 (5.77)	63.50 (6.80)	73.58 (2.61)	70.16 (6.04)	82.16 (4.66)	0.2309
Percentage fat (%)							
Week -2	17.37 (0.89)	16.05 (0.78)	17.22 (1.26)	14.57 (0.82)	14.51 (0.94)	16.72 (0.94)	0.1848
Week 3	16.70 (0.66)	16.08 (0.77)	12.33 (1.24)	14.22 (0.79)	15.01 (1.12)	15.30 (0.85)	0.1543
Week 7	18.58 (0.84)	19.91 (1.27)	16.21 (1.62)	18.81 (0.62)	18.16 (1.55)	20.40 (1.12)	0.2979

^aData are mean (\pm SEM), $n = 12 - 15$ rats/group. ^bSham *versus* OVX control rats (student's *t*-test). ^cOne-way ANOVA followed by post-hoc Tukey-Kramer's test. *indicates significance from the OVX control: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Ex vivo bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) in the lumbar spine and femoral bone of 23-week-old female Sprague-Dawley rats^a.

Group	Sham ^b	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE	<i>p</i> value ^c
Lumbar spine							
Bone area (cm ²)	2.084 (0.037)	2.119 (0.038)	2.129 (0.024)	2.148 (0.033)	2.165 (0.031)	2.147 (0.032)	0.8729
Bone mineral content (g)	0.531 (0.017)	0.514 (0.014)	0.510 (0.012)	0.510 (0.014)	0.512 (0.016)	0.517 (0.014)	0.9964
Bone mineral density (g/cm ²)	0.254* (0.004)	0.242 (0.003)	0.240 (0.004)	0.237 (0.004)	0.236 (0.004)	0.240 (0.003)	0.7808
Femur							
Bone area (cm ²)	1.919* (0.023)	1.986 (0.022)	1.990 (0.014)	2.042 (0.027)	2.058 (0.024)	2.051 (0.026)	0.0628
Bone mineral content (g)	0.499 (0.010)	0.486 (0.007)	0.477 (0.004)	0.489 (0.010)	0.486 (0.007)	0.499 (0.011)	0.4165
Bone mineral density (g/cm ²)	0.259*** (0.002)	0.244 (0.002)	0.240 (0.002)	0.239 (0.002)	0.236 (0.002)	0.243 (0.003)	0.0555

^aData are mean (\pm SEM), *n* = 12 – 15 rats/group. ^bSham *versus* OVX control rats (student's *t*-test). ^cOne-way ANOVA followed by post-hoc Tukey-Kramer's test. *indicates significance from the OVX control: **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Based on the equation formula of Erol et al. (2009), the freeze-drying process of tea water extracts at 1% concentration (10 mg/mL) resulted in 200 mg of tea solids per g dry weight of ground tea leaves.

$$\text{Yield (\%)} = (W_1/W_2)$$

Where, Yield was the tea solids obtained from tea water extract (in percentage).

W_1 was the weight of tea solids after freeze-drying of solvent.

W_2 was the dry weight of the ground tea leaves.

1% (w/v) tea water extract = 1 g dry weight of tea in 100 mL water = **10 mg tea/mL**

1 cup of tea = 2 g tea in 200 mL water

Yield percentage (%) of freeze-dried tea water extracts.

Tea	Freeze-dried tea extract (W_1 , g)	Dry weight (W_2 , g)	Yield (%)	Yield (%) mean	SEM
Se-GTE	0.40	2	20.0	20.2	0.4
	0.39		19.5		
	0.42		21.0		
R-GTE	0.43	2	21.5	20.2	0.7
	0.39		19.5		
	0.39		19.5		
Se-BTE	0.42	2	21.3	20.0	0.6
	0.38		19.0		
	0.40		20.0		
R-BTE	0.39	2	19.5	20.3	0.6
	0.40		20.0		
	0.43		21.5		
Mean of tea solids in mg per 2 g dry weight of ground tea leaves (equates to 1 cup of tea)			= 400 (± 0.2)		

Estimated amount and percentage (%) of EGCG in a typical cup of green and black teas.

A cup of tea (400 mg tea solids)	Green tea (GTE)	Black tea (BTE)
Catechins	120 – 168 mg (30% – 42% of tea solids)	12 – 40 mg (3% – 10% tea solids)
EGCG in tea (65% of catechins)	78 – 109.2 mg (19.5% – 27.3% of tea solids)	7.8 – 26 mg (1.95% – 6.5% of tea solids)

Estimated amount and percentage (%) of EGCG in each concentration of green and black teas used in the *vitro* cell culture works.

Tea concentrations (µg/mL)	EGCG (µg/mL) in GTE (19.5% – 27.3% of tea solids)	EGCG (µg/mL) in BTE (1.95% – 6.5% of tea solids)
10	1.95 – 2.73	0.195 – 0.65
1	0.195 – 0.273	0.0195 – 0.065
0.1	0.0195 – 0.0273	0.00195 – 0.0065
0.01	0.00195 – 0.00273	0.000195 – 0.00065
0.001	0.000195 – 0.000273	0.0000195 – 0.000065

Appendix XII | Consumption of tea in rats, and its approximate translation to tea-daily drinking in humans.

Tea consumption by young growing male rats and its approximate equivalents to tea-drinking in humans.

Group	Control	Se-GTE	R-GTE	Se-BTE	R-BTE
Fluid intake (mL/day)^a	22.9 (1.2)	22.8 (0.7)	23.0 (0.8)	27.9 (0.6)	25.4 (0.7)
Initial body weight (g)^a	78.9 (2.3)	78.7 (2.3)	79.4 (2.3)	79.8 (2.2)	79.2 (2.3)
Animal daily tea intake (mL/100 g BW)	29.0	29.0	29.0	34.9	32.1
Tea solids/day (mg)	58.0	58.0	58.0	69.8	64.2
Approximate daily tea intake in humans (60 kg BW)	87 cups	87 cups	87 cups	104.7 cups	96.3 cups

^aData represent mean (± SEM), *n* = 12 rats/group. ^bA cup of tea = 200 mL water.

Tea consumption by ovariectomised rats and its approximate equivalents to tea-drinking in humans.

Group	Sham	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE
Fluid intake (mL/day)^a	24.4 (1.7)	21.6 (1.0)	21.7 (0.7)	22.5 (0.7)	26.3 (0.7)	25.8 (1.1)
Initial body weight (g)^a	297.2 (5.5)	293.7 (6.9)	293.9 (7.0)	297.0 (5.0)	299.7 (5.6)	300.9 (6.0)
Animal daily tea intake (mL/100 g BW)	8.2	7.4	7.4	7.6	8.8	8.6
Tea solids/day (mg)	16.4	14.8	14.8	15.2	17.6	17.2
Approximate daily tea intake in humans (60 kg BW)	24.6 cups	22.2 cups	22.2 cups	22.8 cups	26.4 cups	25.8 cups

^aData represent mean (± SEM), *n* = 15 rats/group. ^bA cup of tea = 200 mL water.

These approximate daily intakes of tea in human are based on the calculation below:

$$\text{Daily tea intake (cups/60 kg person)} = \frac{\left[\frac{\text{Daily fluid intake (mL)} \times 10 \text{ mg DW tea (per mL)}}{\text{BW of rat}} \right] \times 60 \text{ kg BW}}{2 \text{ g tea per cup}}$$

We further calculate the appropriate conversion of daily intakes of tea obtained from the animal studies to their approximate human equivalent intakes, by using the formula shown below (Reagent-Shaw et al., 2007):

Human Equivalent Dose (mg/kg) = Animal dose (mg/kg) × Animal K_m /Human K_m

Tea consumption by young growing male rats and its approximate equivalents to tea-drinking in human child (with body surface area normalisation).

Group	Control	Se-GTE	R-GTE	Se-BTE	R-BTE
Fluid intake (mL/day)^a	22.9 (1.2)	22.8 (0.7)	23.0 (0.8)	27.9 (0.6)	25.4 (0.7)
Initial body weight (g)^a	78.9 (2.3)	78.7 (2.3)	79.4 (2.3)	79.8 (2.2)	79.2 (2.3)
Animal daily dose (mL/kg BW)	290	290	290	349	321
Human (child) equivalent dose (mg tea solids/kg)	580×(6/25) ^b = 139.2	580×(6/25) ^b = 139.2	580×(6/25) ^b = 139.2	700×(6/25) ^b = 168	642×(6/25) ^b = 154.1
Human (child) daily tea intake (mg tea solids/20 kg)	2784	2784	2784	3360	3082
Approximate daily tea intake in human child	2784/400 ^c = 7 cups	2784/400 ^c = 7 cups	2784/400 ^c = 7 cups	3360/400 ^c = 8.4 cups	3082/400 ^c = 7.7 cups

^aData represent mean (± SEM), *n* = 12 rats/group. ^bAnimal K_m /Human K_m = rat K_m /human child K_m . ^cA cup of tea = 400 mg tea solids.

Tea consumption by ovariectomised rats and its approximate equivalents to tea-drinking in human adult (with BSA normalisation).

Group	Sham	OVX	Se-GTE	R-GTE	Se-BTE	R-BTE
Fluid intake (mL/day) ^a	24.4 (1.7)	21.6 (1.0)	21.7 (0.7)	22.5 (0.7)	26.3 (0.7)	25.8 (1.1)
Initial body weight (g) ^a	297.2 (5.5)	293.7 (6.9)	293.9 (7.0)	297.0 (5.0)	299.7 (5.6)	300.9 (6.0)
Animal daily dose (mL/kg BW)	82	74	74	76	88	86
Human (adult) equivalent dose (mg tea solids/kg)	$164 \times (6/37)^b$ = 26.6	$148 \times (6/37)^b$ = 24.0	$148 \times (6/37)^b$ = 24.0	$152 \times (6/37)^b$ = 24.7	$176 \times (6/37)^b$ = 28.5	$172 \times (6/37)^b$ = 27.9
Human (adult) daily tea intake (mg tea solids/60 kg)	1596	1440	1440	1482	1710	1674
Approximate daily tea intake in human adult	$1596/400^c$ = 4 cups	$1440/400^c$ = 3.6 cups	$1440/400^c$ = 3.6 cups	$1482/400^c$ = 3.7 cups	$1710/400^c$ = 4.3 cups	$1674/400^c$ = 4.2 cups

^aData represent mean (\pm SEM), $n = 15$ rats/group. ^bAnimal K_m /Human $K_m = \text{rat } K_m/\text{human adult } K_m$. ^cA cup of tea = 400 mg tea solids.

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