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

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NOOR HAZARINA NORDIN

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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 H_2O_2-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 H_2O_2-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
ß	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
$CO_2^{\bullet-}$	carbon dioxide radical

$CO_3^{\bullet-}$	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; 17ß-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
$FeSO_4.H_2O$	ferrous sulphate
FISH	fluorescence *in situ* 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	(-)-gallocatechin
GCG	(-)-gallocatechin-3-gallate; gallocatechin 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_2O	water; dihydrogen monoxide
H_2O_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
$HO_2^{\bullet}$	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-1ß	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_2CO_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
$O_2^{\bullet -}$	superoxide anion radical
O_2NOO^-	peroxynitrate
O_3	ozone
OC	osteocalcin
OCLs	osteoclasts
OcS/BS	osteoclast surface per bone surface
OD	optical density
-OH	hydroxyl groups
$OH^{\bullet}$	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
p	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
R	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
$RO^{\bullet}$	alkoxyl
$RO_2^{\bullet}$	peroxyl
ROOH	organic peroxides
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
Runx2	runt-related transcriptor 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
TGFß	transforming growth factor beta
TNFα	tumor necrosis factor alpha
TPC	total phenolic content
TPTZ	tripyridyltriazine; 2,4,6-tripyridyl-*s*-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
vs	*versus*
w/v	weight per volume
w/w	weight per weight
Wnt	wingless-type mammary tumor virus integration site
$1,25(OH)_2D_3$	calcitriol
$^1O_2\Delta g$	singlet oxygen, electronically excited ^{1}Deltag state
$^1O_2\Sigma g^+$	singlet oxygen, electronically excited $^1Sigmag^+$ state
8-OHdG	8-hydroxy-2-deoxyguanosine
14-kDa protein	a protein with a mass (molecular weight) of 14 kilo daltons