Effects of whole greenshell mussel (Perna canaliculus) powder on macrophage, osteoblast, and chondrocyte cell models of metabolic osteoarthritis

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Abstract

Osteoarthritis (OA) is a chronic, painful disorder of synovial joints in the hands, knees, hips, and spine. It is characterized by articular cartilage degeneration, subchondral bone sclerosis, and synovium inflammation. Obesity is an important risk factor, by exerting additional mechanical loading on the joints and by increasing the hormone leptin (LEP). LEP, an adipokine produced by white adipose tissue, is important in regulating the metabolic activities of inflammatory, cartilage and bone cells in OA pathogenesis. OA has no cure and is conventionally managed with painkilling medications. Oil from New Zealand Greenshell™ mussel (GSM) is also used to treat OA, but its mechanism of action is unclear, and it is not known whether peptides and other components in whole GSM may have additional health benefits in OA. This study investigated the effects of whole GSM using novel *in vitro* models of OA. LEP-stimulated J774A.1 macrophages, MC3T3-E1 pre-osteoblasts and differentiated osteoblasts, and ATDC5 pre-chondrocytes and differentiated chondrocytes modelled the behaviour of synovial macrophages, subchondral bone, and cartilage cells during OA. Blue mussel (BM) and GSM extracts were used to optimise macrophage assay conditions. Two whole GSM extracts were further tested in the *in vitro* models at non-cytotoxic concentrations. RT-qPCR was used to quantify biomarkers; chemical and staining assays were used to assess alkaline phosphatase activity and mineralization, proteoglycan, and collagen synthesis. LEP-induced inflammatory cytokine expression in macrophages peaked at 4 and was dose-dependent; neither mussel type ameliorated inflammation but BM alone significantly induced IL-1β, IL-6, TNF-α and IL-10 expression. GSM significantly increased osteoblast proliferation, mineral deposition, and expression of osteogenic markers *Alp, Osx, Col10α1 and Runx2*. In chondrocytes, GSM significantly blocked LEP-induced

hypertrophic differentiation by suppressing alkaline phosphatase, *Col10α1* and mineralized nodules and increasing *Sox9* expression. This project developed simple but effective *in vitro* models of inflammatory, bone, and cartilage cells that mimic the physiological response to LEP and the pathological changes observed in OA and demonstrated that whole GSM may prevent OA by acting directly on bone and cartilage rather than acting through well recognized anti-inflammatory pathway, indicating novel protective effects of whole GSM on all three cell types.

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List of abbreviations

%	*Percent*
°C	*Degree centigrade*
µg/ml	*Microgram per milliliter*
µm	*Micrometer*
AA	*Ascorbic acid*
Acan	*Aggrecan*
ACL	*Anterior cruciate ligament*
ADAMTS	*Disintegrin and metalloproteinase with thrombospondin motifs*
ADP	*Adenosine diphosphate*
AITS	*Ascorbic acid, Insulin, Transferrin, Sodium selenate*
ALP	*Alkaline phosphatase*
AMPs	*Antimicrobial peptides*
ANOVA	*Analysis of variance*
ARS	*Alizarin red staining*
ATCC	*American Type Culture Collection*
ATP	*Adenosine triphosphate*
BCA	*Bicinchoninic acid*
bFGF/FGF2	*Basic fibroblast growth factor*
BM	*Blue mussel*
BMI	*Body mass index*
BMP-7	*Bone morphogenetic protein 7*
BMSCs	*Bone marrow mesenchymal stem cells*
BSP	*Bone sialoprotein*
CD14	*Cluster of differentiation 14*
CD163	*Cluster of differentiation 163*
CDH11	*Cadherin 11*
cDNA	*Complementary DNA*
CHO	*Carbohydrates*
CHST11	*Carbohydrate Sulfotransferase 11*
CIOA	*Collagenase-induced OA*
CLEC3A	*C-Type Lectin Domain Family 3 Member A*
CLEC3B	*C-Type Lectin Domain Family 3 Member B*
CM	*complete medium*
CNS	*Central nervous system*
Col10α1	*Collagen type 10 chain alpha chain 1*
COL13A1	*Collagen type 13 alpha chain 1*
COL14A1	*Collagen type 14 alpha chain 1*
COL15A1	*Collagen type 15 alpha chain 1*
Col1α1	*Collagen type 1 alpha chain 1*
Col2α1	*Collagen type 2 alpha chain 1*
COL8A2	*Collagen type 8 alpha chain 2*

COX	*Cyclooxygenases*
CPC	*Cetylpyridinium chloride*
CPCs	*Chondrogenic progenitor cells CPCs*
CTX-II	*C-telopeptide fragments of type II collagen*
CXCR1	*C-X-C chemokine receptor type 1*
CXCR2	*C-X-C chemokine receptor type 2*
DAMPs	*Damage-associated molecular patterns*
DAP	*Damage associated patterns*
DHA	*Docosahexaenoic acid*
DMDs	*Disease-modifying drugs*
DMEM	*Dulbecco's Modified Eagle's Medium*
DMM	*Destabilization of the medial meniscus*
DMSO	*Dimethyl sulphoxide*
ECACC	*European Collection of Authenticated Cell Cultures*
ECM	*Extracellular matrix*
EDTA	*Ethylenediamine tetraacetic acid*
E-OH	*Ethanol*
EPA	*Eicosapentaenoic acid*
FCS	*Foetal calf serum*
FDA	*Food and Drug Administration*
FFA	*Free fatty acids*
FGF-18	*Fibroblast growth factor-18*
FGFs	*Fibroblast growth factors*
GADPH	*Glyceraldehyde 3-phosphate dehydrogenase*
GalNAc	*Gal-specific lectin*
GBD	*Global Burden of Disease study*
GC-MS	*Gas chromatography- mass spectrometry*
GDF	*Growth and differentiation factor*
GDP	*Gross domestic product*
GIT	*Gastrointestinal tract*
GLM	*Green-lipped mussel*
GPNMB	*Glycoprotein nonmetastatic melanoma protein B*
GSM	*Greenshell mussel*
HA	*Hyaluronic acid*
HAS2	*Hyaluronic acid synthase*
hBMSCs	*Human bone marrow stromal cells*
HCl	*Hydrochloric acid*
HDL	*High density lipoproteins*
HS3ST3A1	*Heparan sulphate 3 sulfotransferase 3A1*
HSA	*Human serum albumin*
IA	*Intra-articularly*
IFP	*Infrapatellar fat pad*
IL	*Interleukin*
IL-1	*Interleukin-1*

IL-10	*Interleukin-10*
IL-1β	*Interleukin-1 beta*
IL-6	*Interleukin-6*
IPFP	*Infrapatellar fat pad*
KOH	*potassium hydroxide*
LDL	*Low density lipoproteins*
LEP	*Leptin*
LEPR	*LEP receptor*
LIF	*Leukemic inhibitor factor*
LO	*Lipoxygenase*
LPS	*Lipopolysaccharide*
M. edulis	*Mytilus edulis*
MAP	*Mussel adhesive proteins*
MCP1	*Macrophage chemoattractant protein 1*
MEPE	*Matrix extracellular phosphoglycoprotein*
MetOA	*Metabolic OA*
MetS	*Metabolic syndrome*
MHC	*Major-Histocompatibility-Complex*
M-OH	*Methanol*
MPC	*Mesenchymal progenitor cells*
MPP-3	*Metalloproteinase-3*
MRI	*Magnetic resonance imaging*
mRNA	*Messenger RNA*
MSX1	*Msh Homeobox 1*
MSX2	*Msh Homeobox 2*
MTT	*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*
NaCl	*Sodium Chloride*
NAD	*Nicotinamide adenine dinucleotide*
NaOH	*Sodium hydroxide*
ng/ml	*nanogram per millilitre*
Nitric oxide	*NO*
NLT	*Not lower than*
NMT	*Not more than*
NOS2	*Type 2 nitric oxide synthase*
NSAIDS	*Non-steroidal anti-inflammatory drugs*
OA	*Osteoarthritis*
OCN	*osteocalcin*
OD	*Optical density*
Osx	*Osterix*
P. canaliculus	*Perna canaliculus*
PAMPs	*Pathogen-associated molecular patterns*
PAR2	*Proteinase-activated receptor-2*
PBS	*Phosphate buffer saline*
PGE2	*Prostaglandins E2*

pNPP	*Para-nitro-phenyl phosphate*
PRG4	*Proteoglycan 4*
PRRs	*Pattern recognition receptors*
PSG	*Penicillin-streptomycin-L-glutamine*
PTOA	*Post-traumatic OA*
PTP1B	*Protein tyrosine phosphatase 1B*
PUFAs	*Polyunsaturated fatty acids*
RA	*Rheumatoid arthritis*
RAGE	*Receptor for Advanced Glycation Endproducts*
RANKL	*Receptor activator of nuclear factor κ B ligand*
RCTs	*Randomized controlled trials*
RNA	*Ribonucleic acid*
ROAD	*Research on Osteoarthritis/Osteoporosis Against Disability*
RORα	*Retinoic acid-related orphan receptor alpha*
RT-qPCR	*Real time quantitative polymerase chain reaction*
Runx2	*Runt-related transcription factor 2*
SASP	*Senescence-associated secretory phenotype*
SBP	*Subchondral bone plate*
SCB	*Subchondral bone*
SD	*Standard deviation*
SEM	*Standard error of means*
SIRT	*Sirtuins*
Sox9	*Sex-determining region y-box9*
STB	*Subchondral trabecular bone*
TBS	*Tris Buffered Saline*
TGF-β	*Transforming growth factor-beta*
TIMPs	*Tissue inhibitors of metalloproteinases*
TLR4	*Toll-like receptor 4*
TNF-α	*Tumor necrosis factor α*
TRACP	*Tartrate-resistant acid phosphatase*
Tris	*Trisaminomethane*
US	*United states*
Wnts	*A term formed by combining the proto-oncogene names of wingless and integration-1)*
YLD	*Years lived with disability*
ZIP8	*The zinc (Zn2+) importer*
αMEM	*Alpha minimum essential medium*
βGP	*β-glycerophosphate*
β-NGF	*β-Nerve growth factor*

Chapter One: General introduction, aims and objectives

1.1 Research background

Osteoarthritis (OA) is an umbrella term that covers a group of prolonged, painful and potentially debilitating disorders of synovial joints, including joints of the hands, fingers, knees, hips and spine. It is the most common chronic degenerative disease of joints and typically involves degeneration of articular cartilage, sclerosis/thickening of subchondral bone and inflammation of the synovium (Ji and Zhang, 2019).

OA constitutes a significant and growing health burden and has serious consequences for afflicted people, health-care systems, as well as the wider socioeconomic costs (Hunter and Bierma-Zeinstra, 2019). OA affects 3.3 - 3.6% of the population globally and is responsible for causing moderate to severe disability in 43 million people, making it 11th in terms of disease prevalence worldwide. In the United States, 80% of people over 65 years of age are estimated to have radiographic evidence of OA, with 60% of this subset having symptoms. In 2011, approximately 1 million people in the US were hospitalized for OA with a cumulative cost of nearly US $15 billion, making it the second most expensive disease in the United States (Berenbaum et al., 2018, Bortoluzzi et al., 2018).

Risk factors relevant for OA include gender, older age, obesity, race and ethnicity, genetics, nutrition, smoking, anatomical factors, muscle weakness, and joint injury (occupation/sports activities) (Felson et al., 2000, Loughlin et al., 2002, Loeser, 2010). Based on its aetiology, OA can be classified as primary OA (idiopathic or non-traumatic) or secondary OA (usually due to trauma or mechanical misalignment). Primary OA is the

most common subset of the disease and the above-mentioned risk factors are sufficient to trigger the disease. It can be identified in the absence of predisposing trauma or any other disease. All of the above-mentioned risk factors can trigger primary OA; however, older age and obesity are the key factors in the development of OA. A genetic predisposition for the disease alone will not necessarily trigger the disease unless some other factor such as joint injury is present. Secondary OA, which is not a focus of this thesis, occurs with pre-existing joint anomalies such as trauma or injury, inflammatory arthritis, infectious arthritis, congenital joint disorders, avascular necrosis, osteopetrosis, osteochondritis dissecans, Paget disease, metabolic disorders (hemochromatosis, Wilson's disease), hemoglobinopathy, Ehlers-Danlos syndrome, or Marfan syndrome (Donahue, 2018, Krishnan and Grodzinsky, 2018).

OA is the most common form of arthritis among older people (Helmick et al., 2008, Loeser, 2010), and also the leading cause of disability in the ageing population (Li et al., 2013b). It has a strong link with aging; consequently, the incidence of the disease is increasing as a greater proportion of the population ages, as shown by the Arthritis Data Workshop report which states that the incidence of OA of the hands, hips or knee increased among older adults in the US from 21 million in 1995 to 27 million in 2005 (Lawrence et al., 2008b). Moreover, recent research (Loeser et al., 2016a) further highlights the role of aging in the pathogenesis of OA. The study involved 3 million individuals, scrutinized based on clinically diagnosed OA. It was reported that the incidence of hand OA peaked in women of ages between 60 to 64 years, while hip and knee OA continued to increase with increasing age. Aging changes the cellular

composition of joints and signalling mechanisms that could result in the progression of degenerative joint disease. Senescent/old cells are present both in aged and OA joints, which release senescent-associated mediators responsible for destroying the articular tissues. These changes along with the aging-related chronic pro-inflammatory environment might be responsible for the delay in the ability of the joint to repair, leading to onset of OA (O'Brien and McDougall, 2019).

Obesity is another risk factor for the disease. Although the link to OA has been demonstrated for a long time, the exact mechanism through which it contributes to OA is not fully understood. Obesity not only contributes to OA by exerting mechanical stress to the joints (mechanical hypothesis), but it also incites an inflammatory state by mechanisms such as increased leptin (LEP) expression, compromised gut mucosa, and/or gut microbiota disruption, which may contribute to the pathogenesis of OA (King et al., 2013, Raud et al., 2020). The main obesity-related signalling pathways involved in OA inflammation are protein tyrosine phosphatase 1B (PTP1B) and toll-like receptor 4 (TLR4) or damage associated patterns (DAP12) (Jiang et al., 2021).

A study in the United Kingdom on a cohort of 2,597 participants demonstrated a direct correlation between body mass index (BMI) and knee OA in men and women (Martin et al., 2013). Similar results were reported in another study involving African American and Caucasian American populations (Deshpande et al., 2016). Moreover, the ROAD (Research on Osteoarthritis/Osteoporosis Against Disability) study, comprising 1,690 participants, showed that frequency and intensity of knee OA were associated with significantly increased central obesity, high triglycerides, low HDL cholesterol, high

blood pressure and insulin resistance (Yoshimura et al., 2012), all of which are associated with metabolic syndrome. Studies have also shown that the incidence of the disease in obese women is four times greater than in non-obese women. This correlation is even higher in men, with obese men being at five times greater risk of OA than non-obese men (Anderson and Felson, 1988a). Obesity, therefore, is believed to be a primary risk factor for both the onset and progression of OA.

OA is a whole joint disease primarily due to the breakdown of the articular cartilage, which is the smooth, lubricated, shock-absorbing tissue covering the ends of joints. Though articular cartilage is considered the site of OA, it lacks neurons and cannot be the source of pain by itself (Goncharov, 2011). Structural modifications in the hyaline articular cartilage, subchondral bone, ligaments, capsule, synovium, and periarticular muscles together lead to OA and its debilitating symptoms (Brandt et al., 2006, Glyn-Jones et al., 2015). The complex pathogenesis of the disease involves genetic, metabolic, biochemical, biomedical and inflammatory factors, which eventually result in the structural destruction and collapse of the synovial joint. OA is therefore not a passive degenerative disease or so-called wear-and-tear disease as was once believed; rather, it is a dynamic and intense change in the joint that arises from a disproportion between anabolic and catabolic activities of joint tissues (Hassanali and Oyoo, 2011, Fu et al., 2017).

OA is generally considered a non-inflammatory condition, different from inflammatory arthritis disorders such as rheumatoid arthritis (RA) or seronegative spondyloarthropathies, but the recent interpretation of OA as a chronic injury highlights

the role of inflammation and the response of the body to repair the chronic damage. Inflammation is now recognised as an important contributor to OA pathogenesis. Inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor α (TNF-α), and surface-expressed pattern recognition receptors such as toll-like receptors 2 and 4, complement factors such as C5, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) all produce inflammatory signals, which drive the enzymatic cascade that eventually destroys the cartilage matrix as OA progresses. Degradation products formed as a result of cartilage breakdown, including cartilage fragments, soluble proteoglycan, and the fragment of c-terminal crosslinked telopeptide of type II collagen (CTX-II) may themselves be sources of further inflammation and can stimulate the release of inflammatory mediators from synovial macrophages (Wang et al., 2019a). During OA the pathogenic joint acts as an entire organ where cells residing within the synovium, such as macrophages and other immune cells, interact with each other and drive enzymatic activity in cartilage, which, in turn, sends signals back to the synovium that carries on the degradation in a feed-back circuit (Wojdasiewicz et al., 2014a, Woodell-May and Sommerfeld, 2020b).

Owing to the insufficient natural healing capacity of articular cartilage, it remains a challenge to find an adequate medication for OA. Analgesics are used to ease the pain and to improve the quality of life of the patient by enhancing mobility. Simple analgesics such as paracetamol and codeine-based drugs, or non-steroidal anti-inflammatory drugs (NSAIDS) that act by inhibiting the activity of cyclooxygenases (COX)-1 and 2, are usually recommended. They are effective in moderate to severe pain, but they can cause

gastrointestinal (GIT) problems as a side effect. In addition, a more specific COX-2 inhibitor, Vioxx (rofecoxib), with decreased risk of GIT disturbances was marketed but withdrawn not long after due to adverse cardiac effects. Intra-articular injection of corticosteroids can also relieve the pain for a short time. Hyaluronic acid (HA) injection is also used, and it is comparatively more effective in relieving pain compared to oral NSAIDs. It lubricates the joint by providing viscosity to synovial fluid. HA therapy is the most popular therapeutic option in many knee arthritis treatment centres (Roach and Tilley, 2007). Currently there is no therapeutic option that cures the disease.

Disease-modifying drugs (DMDs) could be a powerful therapeutic option for OA but these are still in the early stages of development. Progress in the domain of therapeutic agents requires a thorough understanding of the pathophysiology of OA progression. Cartilage metabolism (synthesis and breakdown), bone remodelling, and synovial inflammation could be possible targets of DMDs. The emerging OA drugs such as bone morphogenetic protein-7 (BMP-7), fibroblast growth factor-18 (FGF-18), human serum albumin (HSA), interleukin-1 (IL-1) inhibitor, β-Nerve growth factor (β-NGF) antibody, matrix extracellular phosphoglycoprotein (MEPE) and inverse agonist of retinoic acid-related orphan receptor alpha (RORα) etc. have shown success in modifying the progression of OA with minimum harmful effects. However, large-scale randomized controlled trials (RCTs) are required to explore their safety and efficacy before approval for treatment of the disease (Zhang et al., 2019b). These drugs are designed to target the components of the joint that directly participate in disease progression. For instance, cytokine (IL-1β & TNF-α) inhibitors to reduce inflammation, enzyme inhibitors or

synovial gene therapy to modify the production of cartilage matrix. Chondrocytes are thought to have the greatest potential to be explored as a therapeutic target for DMDs research. Researchers are aiming to design drugs that can target chondrocytes and prevent them from producing degradative enzymes by either inhibiting their expression at the gene level or preventing their phenotypic transformation into degradative chondrocytes.

As previously mentioned, there is no cure for OA at present, and its prevention or at least an effective treatment is needed. The first and most important step in preventing OA is maintaining a healthy lifestyle by managing one's body weight and other modifiable factors, while improving dietary choices with a focus on nutrients that may mitigate the disease or its symptoms. Nutraceuticals as treatment supplements are a possible choice for preventing or managing OA symptoms. The term nutraceutical is the combination of two words: *nutrition* and *pharmaceutical*. It refers to food or food products that provide both nutrients and medical benefits, including disease prevention and treatment (Henrotin et al., 2011). By definition and regulatory laws, they have no harmful side effects.

Nutraceuticals such as fish oil, chondroitin sulphate and glucosamine are the most commonly used non-prescription supplements for OA. The exact mechanisms of action of these supplements are unknown but they are thought to affect chondrocytes in different ways. For example, omega 3-fatty acids (present in fish oil) inhibit IL-1β induced expression of degradative enzymes and other inflammatory mediators, while chondroitin sulphate inhibits the expression of MPP-3 (metalloproteinase-3) synthesis

by chondrocytes (Roach and Tilley, 2007). Fish oil contains all the essential lipids that have to date been found to be effective in treating different types of arthritis including OA (Boe and Vangsness, 2015). These components include the marine n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Current aquaculture practice relies solely on fish to produce fish oil, which is unfortunate and is leading to overfishing, an escalating global concern. Currently, there are no means to produce long-chain n-3 PUFA or fish containing these fatty acids in an economically and environmentally sustainable way.

Shellfish including blue mussel (BM; *Mytilus edulis*) and Greenshell™ mussel (GSM; green-lipped mussel; *Perna canaliculus*) have macronutrient profiles comparable to fish. Both fish and mussels contain EPA and DHA. In addition to these, mussels are also rich in nutrients such as zinc, selenium, riboflavin, and carotenoids. Besides nutritional benefits, blue and greenshell mussel farming can also be undertaken in a environmentally friendly manner so that these shellfish remove excessive nitrogen at harvest and reduce the eutrophication of the sea. Fish and shellfish are often included in the same food group, although their nutrient composition varies significantly between species. Therefore, in most studies the nutritional and bioactive effects of different fish or shellfish species have not been distinguished.

There are no reported studies to date regarding joint health interventions in patients or healthy subjects using consumption of whole mussel meat or other shellfish intake. Yet, some animal- and cell-model studies show anti-arthritic or anti-inflammatory effects from lipid extracts of mussels (Stebbings et al., 2017). One such study demonstrated that

lipid extracts from BM had positive effects on arthritis in rats as compared to olive oil (McPhee et al., 2010). BM as well as GSM are identified as containing potential bioactive novel fatty acids in addition to EPA and DHA (Treschow et al., 2007, McPhee et al., 2010). Lipid extracts from BM and GSM also revealed anti-cyclooxygenase effects in *in vitro* studies (Treschow et al., 2007). In addition to the anti-inflammatory lipids, a water extract of BM consisting of substituent peptides and taurine, in addition to other nutrients, decreased inflammation in an *in vivo* zebrafish inflammation model (Cheong et al., 2017), as well as an *in vitro* model (Kim et al., 2016b). These results suggest that not only lipids but the whole mussel with its a combination of lipids and water-soluble components should be of interest as a potential anti-inflammatory dietary intervention.

GSM in particular has long been used as a nutraceutical to manage osteoarthritis in New Zealand and it has now gained worldwide attention. Interest in investigating its health benefits began with studies carried out in 1960 when it was tested in humans for its anti-cancer activity. It failed to be effective in the treatment of cancer but it was observed to have pain-relieving effects in cancer patients who suffered from joint stiffness and pain (Cobb and Ernst, 2006b). It was evident from the results that *P. canaliculus* might contain natural compounds that are effective in the treatment of osteoarthritis (Cobb and Ernst, 2006b) and clinical trials commenced in 1980.

Seatone® and Lyprinol® are two lipid derivations obtained from *P. canaliculus* that are commercially available as anti-inflammatory and anti-arthritic dietary supplements. Their synthesis is anecdotally reported to have been originally inspired by the fact that native Māori peoples living on New Zealand coastal lines who depended on *P.*

canaliculus for their diet were less likely to develop osteoarthritis than their inland counterparts. Seatone® was the first commercially available product of freeze-dried mussel extract marketed in 1976, but quality concerns due to instability arose among consumers and industry (Grienke et al., 2014b). The instability issues were solved using a new formulation called Lyprinol® in 1998. Lyprinol was synthesized as an oil from stabilized freeze-dried mussel powder and blended with vitamin E and olive oil as an antioxidant (Whitehouse et al., 1997, Singh et al., 2008).

Reports in the literature collectively demonstrate that GSM extracts such as Lyprinol® have proven anti-inflammatory effects. For some conditions, its anti-inflammatory mechanism is also understood; however, in osteoarthritis the exact mechanism of its action is unknown.

1.2 Research Project Proposal

GSM is reported to treat OA and other inflammatory conditions through its anti-inflammatory mechanism of action, with its biological activity being mostly attributed to its lipid fraction. However, the effects of the whole mussel and the mechanism of its action are unknown. This research project is primarily designed to gain insight into the mechanism of action of whole mussel powder extracts using *in vitro* cell models of OA. The following questions were addressed during this study:

Question 1 - What is the anti-inflammatory mechanism of action of GSM extracts?

Hypothesis: Being anti-inflammatory in nature, GSM should affect cytokine levels produced by macrophages.

Study design: We created a novel LEP-stimulated *in vitro* cell model of synovial inflammation by using J774A.1 macrophages and then tested GSM extracts at non-cytotoxic levels for their immunomodulatory potential. We further assessed both GSM and blue mussel extracts to identify optimal solvent and assay conditions, with the goal of establishing this cell model for future whole-meat-extract experimental use. We measured the expression of selected cytokines in response to the treatments using RT-qPCR.

Question 2 - Do GSM extracts alter cell function such as levels of mineralization in a bone model of OA?

Hypothesis: GSM extracts should have a direct protective effect on cell function, as fish oil-containing neutraceuticals have been shown to have protective effects on osteoblasts, osteoclasts, and osteocytes.

Study design: We created a novel *in vitro* model of OA subchondral bone remodeling by treating murine MC3T3-E1 osteoblasts with LEP. After treating the bone model of OA with the GSM extracts, we measured mineralization and production of extracellular matrix molecules, alkaline phosphatase, and other markers using specialized staining techniques and RT-qPCR.

Question 3 - How do GSM extracts affect cartilage metabolism *in vitro*?

Hypothesis: GSM extracts should have a direct protective effect on cartilage cell function, as fish oil-containing neutraceuticals have been shown to have protective effects on chondrocytes.

Study design: We created a novel *in vitro* model of OA cartilage by treating ATDC5 chondrocytes with LEP. After treating the cartilage model of OA with the GSM extracts, we measured mineralization and production of extracellular matrix molecules, alkaline phosphatase, and other markers using specialized staining techniques and RT-qPCR.

1.3. Study Outcomes and Funding

There is a lack of information on how whole GSM can affect immune, bone, and cartilage models of OA. This study has allowed us to test the hypotheses outlined above and demonstrated that GSM extracts, as a whole food, can indirectly alter the disease progression of OA induced by leptin by modifying the immune response as well as by directly providing protective effects to cartilage and bone. The study also provided novel insights into the mechanisms of action of GSM extracts, adding to the existing body of data describing how GSM extracts can affect different cell types. Finally, three new cell models to mimic the immune, bone, and cartilage changes that occur in OA were developed and assessed. These findings have aided in determining that whole GSM is a candidate for usage instead of or in addition to NSAIDS to treat OA.

This project is part of a collaboration between Massey University, the Cawthron Institute and Sanford Ltd. The research is supported by a High Value Nutrition grant (Ministry of Primary Industries) with funds of >$50,000 for running costs, and a three-year full doctoral scholarship (including tuition) from the Riddet Centre of Research Excellence.

1.4 Thesis layout

The thesis is divided into eight chapters, containing five experimental chapters (the sixth experimental chapter yet to be completed). Experimental chapters are written with the intention to be published in scientific journals and their publication details are mentioned in each chapter.

Chapter One briefly introduces osteoarthritis (OA), its prevalence and risk factors, the pathogenesis of the disease, currently available therapeutic options, and the importance of food supplements or nutraceuticals in disease management, with a special focus on green shell mussel (GSM) followed by aims and objectives of the project, funding information of the project and details about thesis layout at the end of this chapter. All these topics are discussed in more detail in chapter 2, which thoroughly reviews the literature on OA and GSM.

Chapter Two is a review of the literature regarding important aspects of OA such as prevalence, risk factors of the disease, aetiology, pathogenesis and pathophysiology. It reviews the literature on different *in vitro* models used for the study of OA including their advantages and limitations, as well as GSM and blue mussel (BM) composition and

bioactives present in them with a special focus on GSM. This chapter also highlights previous studies regarding the use and mechanism of action of GSM in different research studies.

Chapter Three aims to identify a suitable and more natural stimulant to mimic mild, subclinical inflammation related to MetS or OA in an *in vitro* macrophage cell model. To address this question, we compared LPS and LEP-stimulated J774A.1 models of inflammation for the mRNA expression of the proinflammatory cytokines *IL-1β, IL-6,* and *TNF-α* and the anti-inflammatory cytokine *IL-10*.

Chapter Four aims to compare the immune-modulating potential of unfractionated BM and GSM in LEP-stimulated macrophages using the murine J774A.1 cell line. Moreover, this study was designed to identify the optimal time point, dose, solvent, and culture conditions for peak cytokine production followed by a comparison of the immunomodulatory bioactivities of BM and GSM.

Chapter Five examines the role of GSM on the OA model of bone remodelling. This study was primarily designed to compare two freeze-dried whole GSM powders produced by different processing methods for their effects on mineralization in LEP-stimulated MC3T3-E1-cells as an *in vitro* bone model of OA. Biomarkers associated with different stages of bone mineralization were analysed in response to GSM treatment. Alizarin red staining assay, alkaline phosphatase activity assay and RT-qPCR were used as tools to study osteogenic markers.

Chapter Six explores the immunomodulatory and osteogenic effects of GSM in LEP-stimulated murine J774A.1 macrophages and MC3T3-E1 osteoblasts. The aim of this study was firstly to design and assess *in vitro* cell models by challenging J774A.1 macrophages and differentiated MC3T3-E1 osteoblasts with LEP, and secondly to determine whether whole GSM has protective effects in these models of synovial inflammation and bone remodelling. Alizarin red staining assay, alkaline phosphatase activity assay and RT-qPCR were used as a tool to study osteogenic and inflammatory biomarkers.

Chapter Seven further investigates the role of GSM extracts on chondrocyte metabolism. This study was aimed to mimic the OA-related pathophysiology of chondrocytes *in vitro* and then to explore the mechanism of action of GSM in the disease model. We used ATDC5 cells to create an LEP-stimulated *in vitro* chondrocytes model of OA and compared two whole GSM powders, conventionally processed GSM-A versus enzymatically processed GSM-B, for their effects on proliferation, differentiation, collagen, proteoglycan, and mineral deposition of the LEP-stimulated chondrocytes.

Chapter Eight reviews and describes the general findings of the whole thesis and discusses the study limitations, recommendations, and future perspectives.

Chapter Two: Review of literature

2.1 Introduction

Osteoarthritis (OA), also called “degenerative joint disease”, is the most common type of joint disorder found not only in humans but also in other mammals including horses and dogs (Henrotin et al., 2005, Wieland et al., 2005, McIlwraith et al., 2012). OA affects the joints of the hands, knees, feet, spine and hips (figure 2.1). Pain is a typical symptom that leads to the diagnosis of OA. OA is marked by considerable suffering and responsible for reduced quality of life and disability. In the population of adults aged 60+ years, OA is the primary cause of disability of the hip and knee (lower extremity disability), which is considered to account for 2.4% of all years lived with disability (YLD). In the Global Burden of Disease (GBD) studies, OA is constantly ranked among the chief contributors to global YLDs. OA has been classified as the second most rapidly growing condition among the top twenty contributors of YLDs from 1990 to 2016, with a 46% surge in YLDs, just behind diabetes, which is at 52%. This burden is predicted to increase as the aged and obese populations increase, and will be an increasing challenge for health care and public health systems (March and Cross, 2020).

OA ensues with chronic and progressive deterioration of cartilage, remodelling of subchondral bone, narrowing of the joint space and function, and osteophytosis on the joint margins (Roach and Tilley, 2007). The origin or aetiology of the disease is not completely understood and it may differ between species or individuals of the same species, but the course of development and some pathological components are conserved for the disease. The conserved features include a cascade of biochemical

processes facilitated by cytokines, proteolytic enzymes, and other pro-inflammatory mediators such as prostaglandins, leukotrienes, and nitric oxide. These biochemical pathways lead to the alteration of joint homeostasis including articular cartilage degeneration, osteolysis, subchondral bone sclerosis, the formation of osteophytes, and thickening of the synovial membrane and subchondral bone; regardless of the aetiology, all of the prior mentioned components are involved in the pathogenesis of the disease (Carmona and Prades, 2009).

OA is one of the most prevalent chronic health conditions in the western world; with a higher frequency amongst people aged 60 years or higher (Helmick et al., 2008, Loeser, 2010). OA has a strong link with aging, consequently, the incidence of the disease has increased as the population has aged, as shown by the Arthritis Data Workshop report which stated that the incidence of OA of the hands, hips, or knee (Figure 2.1) was increased among older adults of US from 21 million in 1995 to 27 million in 2005 (Lawrence et al., 2008b). Obesity is another risk factor for the disease, as previous research shows that the incidence of the disease in obese women is four times greater than in non-obese women. Likewise, obese men have five times greater risk of OA than non-obese men (Anderson and Felson, 1988a). Obesity, therefore, is considered as a primary risk factor for the pathogenesis of OA. Other risk factors include sex, race and ethnicity, genetics, nutrition, smoking, and injuries/trauma to the joint (Felson et al., 2000, Loughlin et al., 2002, Loeser, 2010).

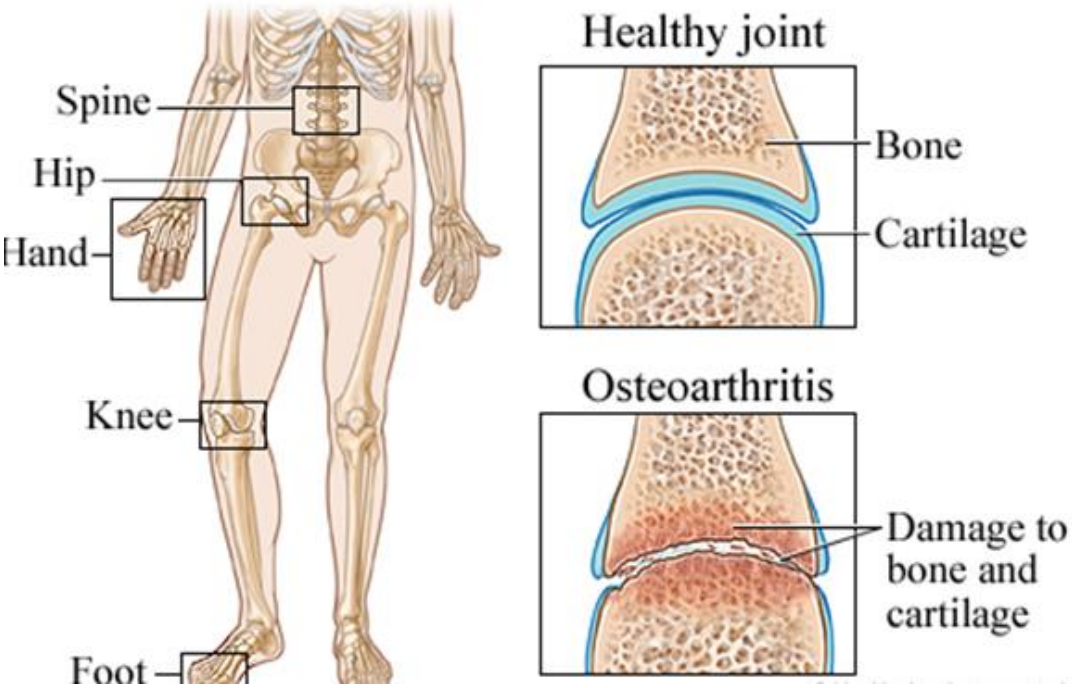

Figure 2.1: shows OA in different synovial joints of the body and pathological changes in OA knee joint vs healthy joint. Reproduced with the permission of www.healthwise.org (© Healthwise, 2019).

2.2 Epidemiology of osteoarthritis

The prevalence of OA varies across human clinical studies depending upon the definition used for diagnosis. For example, self-reported OA is called symptomatic OA, when it is diagnosed radiographically it is called radiographic OA and when defined clinically then it is doctor-diagnosed OA. Interestingly, not all patients with radiographic OA are shown to have symptomatic OA, therefore, overall, the prevalence for symptomatic OA tends to be lower than radiographic OA as its presence is defined by a combination of symptoms such as pain and stiffness in addition to radiographic features (Parsons et al., 2015, Li et al., 2020).

2.2.1 Incidence

The 2016 Global Burden of Disease study (GBD2016) estimates 199 cases of hip and knee OA per 100,000 population globally (Vos et al., 2017). The incidence of OA varies according to socioeconomic conditions in different regions of the world with estimates of 347 new cases per 100,000 in high-income areas, compared with 256 per 100,000 in Central Europe and 91 per 100,000 in sub-Saharan Africa. Also, in the United States, the incidence of OA is generally lower for African Americans when compared to Caucasians (Neogi and Zhang, 2013, Nelson, 2018b).(Neogi and Zhang, 2013, Nelson, 2018b). The incidence of OA increases with age and women have a higher incidence as compared to men. The frequency of symptomatic hand, hip, and knee OA increases rapidly around the age of 50 years and then stabilizes after 70 years of age (Zhang and Jordan, 2010).

2.2.2 Prevalence

Approximately 240 million people are affected by OA around the globe (Nelson, 2018a), including over 30 million in the United States, which has increased from 21 million in 1990 to 27 million in 2005 (Zhang and Jordan, 2008). The prevalence of OA of the hip and knee is increasing, affecting 5% of adults aged over 18 years, although prevalence varies due to differences between rural and urban areas, and between high- and low- to moderate-income regions. This rate is expected to increase with the increased incidence of aging and obesity (Hong et al., 2020).

Knee OA is the most widespread form of OA globally and accounts for around 80% of the disease's total burden (Vos et al., 2012). The age-standardized and radiographically confirmed symptomatic knee OA has been estimated at 3.8% globally, with the incidence being higher in women (4.8 percent) compared with men (2.8 percent). Knee OA has a similar prevalence in the United States and Europe and lower incidence being reported in southern Asia (Litwic et al., 2013, Cross et al., 2014).

Hand OA is the next most prevalent form of OA with the incidence of radiographic hand OA that is approximately 27 to over 80% in the United States (Litwic et al., 2013) and in Japan, its incidence is even higher at over 90% (Kodama et al., 2016). However, the percentage of symptomatic hand OA is much lower at around 8% (Dillon et al., 2007). The incidence of symptomatic hand OA is strongly correlated with age in the United States, with 13% of men and 26% of women over 70 years of age reported to have symptomatic hand OA, while in China symptomatic hand OA is rarely reported irrespective of age and gender (Litwic et al., 2013).

The incidence of hip OA is lower than knee or hand OA, with an age-standardized global prevalence of radiographically confirmed symptomatic hip OA of 0.85% in adults over 18 years of age, again considering the differences between rural and urban areas and socioeconomic status of the regions. Hip OA has equal rates in men and women with no gender differences and incidence increases with age. Rates of radiographic hip OA are reported to be higher than symptomatic OA, with a prevalence of 1 to 6%, based on the definition used (Litwic et al., 2013, Cross et al., 2014).

Limited data are available on the prevalence of spinal OA (Gellhorn et al., 2013). However, one community-based study in the United States reported the presence of cervical facet joint OA in 19% of adults of ages between 45 to 64 years, and in 57% of adults aged 65 years and older (Dodge et al., 1970). A similar age-related increase was seen in facet joint OA in the lumbar facet joints, with an incidence of 24% in adults younger than 40 years, which increased to 69% in people of age 70 years and older (Kalichman et al., 2008).

2.3 Risk factors for osteoarthritis

Osteoarthritis is a complex interplay of mechanical, cellular, and biomechanical factors (Johnson and Hunter, 2014). Multiple risk factors have been associated with OA, due to which it is considered as a spectrum of conditions or diseases of different phenotypes rather than a single disease. Some of the factors involved in the disease pathogenesis are discussed in detail below.

2.3.1 Aging

Half of the global population over the age of 65 suffer from OA (Bijlsma et al., 2011b). Age is considered a primary risk factor both for the onset and advancement of the disease. Aging can contribute to OA in many ways, including cumulative mechanical load over the years that results in clinical “wear and tear” and cartilage breakdown as a pathological hallmark (Aigner et al., 2004). Therefore, OA was viewed as a natural and irreversible phenomenon rather than a -defined and possibly treatable disease. It is only

in the last decade that the concept of the disease being purely a “mechanical problem” was changed and now it is clearer that inflammatory and metabolic processes are extensively involved in the pathogenesis of the disease and its progression. It is clear now that not only cartilage but almost all components of the joint are involved in the pathogenesis of OA. OA is now called a “whole joint disease” instead of merely one of cartilage degradation, and this concept, despite its greater complexity has not only broadened our understanding of the disease but has also increased the potential for new therapeutic strategies (Poole, 2012, He et al., 2020).

To understand the influence of aging on the progression of OA, the association between the mechanism of aging and OA related pathological changes in the joint needs to be understood. Aging induces changes in the proteins that make up the extracellular matrix (ECM) in and around the joint, i.e. collagen and proteoglycan, and as a result the collagen network becomes rigid and proteoglycans undergo increased glycosylation leading to functional impairment of cartilage and thus the joint (Verzijl et al., 2002). Nonenzymatic cross-linking in collagen results in tough and hard bones, which is further enhanced by an increase in osteon density. Together these changes decrease bone plasticity and the potential of bone repair (Zimmermann et al., 2011). In addition, aging profoundly affects cellular activities, for example, the rate of apoptosis is increased and age reduces the regeneration process (Martin et al., 1997).

Synovitis contributes to OA, particularly in the early phase (Benito et al., 2005) as proved both by histological studies and magnetic resonance imaging (MRI) analyses

(Benito et al., 2005). The synovial fluid of OA patients contains elevated proinflammatory cytokines, which can penetrate the subchondral space to induce an inflammatory response after microfracture of the subchondral bone (Rainbow et al., 2012). This happens in a similar fashion as observed in the case of a Baker's cyst rupture which spreads the inflammation and pain to surrounding soft tissue (Frush and Noyes, 2015).

The exact mechanism of how inflammation is produced in OA is unknown but it may be due to mechanical load over the years, which not only activates osteoblasts but also mast cells, which then recruit additional immune components (Wang et al., 2019b). For instance, the formation of calcium pyrophosphate crystals, which are a characteristic feature of OA can stimulate inflammasome that leads to the activation of interleukin-1 (IL-1) (Jin et al., 2011). Also, in the elderly the immune system is generally less efficient, and therefore cannot resolve inflammation by initiating an anti-inflammatory response, therefore, not only inflammation but also lack of an inflammatory response might cause or contribute to OA. Nevertheless, mechanical load is still considered as a primary risk factor for the pathogenesis of OA, particularly in weight-bearing joints, this is referred to as the "continuous loading theory". Muscles are also vital for the protection and support of joints. Therefore, muscular atrophy is a well-documented symptom in OA, which generally has a higher prevalence in elderly people due to sarcopenia, insufficient physical exercise, malnourishment, or arthogenic muscle inhibition as a result of OA (Valderrabano et al., 2006, Amirthalingam et al., 2019).

Also, during aging, the regenerative capability is reduced due to an increase in catabolic activities as compared to anabolic activities (Hügle et al., 2012), which results in loss of homeostasis in connective tissues as shown by reduced levels of stem cells and increased levels of presenescent in connective tissues (Shane Anderson and Loeser, 2010, Loeser et al., 2016b).

2.3.2 Obesity

An estimated 2.1 billion of the world's population is either overweight or obese (Ng et al., 2014). Obesity is a prevailing health issue of the 21st century in both developed and developing countries, and obesity now has an onset at a younger age. The exact relationship between obesity and OA is yet to be understood. Initially, higher prevalence of knee OA in obese individuals (Anderson and Felson, 1988b, Felson et al., 1988) led to the hypothesis that the linkage between obesity and OA is purely mechanical, i.e. overloading of the joints with increased weight causes wear and tear of joints (Radin et al., 1972) but obesity has also been revealed to cause OA in non-weight bearing joints such as the hand and wrist (Carman et al., 1994, Oliveria et al., 1999, Grotle et al., 2008), demonstrating that the link between excessive weight and joint loading is insufficient to explain the connection between obesity and OA.

Recent research reveals the role of adipose tissue as a dynamic endocrine metabolic organ. It maintains metabolic homeostasis in lean individuals but in obese individuals nutrient overload in adipose tissues stimulates them to activate macrophages and to produce a number of inflammatory mediators including adipokines (leptin, resistin, and

visfatin) and cytokines (tumour necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6)) (Ellulu et al., 2017), resulting in chronic low-grade local and systemic inflammation called metabolic or meta inflammation. Meta inflammation is a hallmark of all the inflammatory conditions associated with metabolic syndrome including OA (Gregor and Hotamisligil, 2011a). Furthermore, dyslipidemia (disturbed lipid metabolism) such as reduced systemic levels of high-density lipoproteins (HDLs) and increased levels of free fatty acids (FFA), triglycerides, and oxidized low-density lipoproteins (ox-LDLs) are also associated with obesity and might be the source of inflammation (Klop et al., 2013).

2.3.3 Joint Injury

Joint injury is another well-studied risk factor for OA, called post-traumatic OA (PTOA). Post-injury inflammatory processes are responsible for the development and progression of OA, and age at the time of injury also influences disease onset (Roos et al., 1995a). Injuries or fractures cause ligament or meniscal tears, which incite acute inflammation accompanied by joint swelling; this effect is more pronounced when a major ligament, such as the anterior cruciate ligament (ACL) is injured. Specific features of inflammation comprising cytokines, chemokines, and synovial reactions come into action after injury, studies have shown that there is a surge in the concentration of TNF-α (6-fold) and IL-6 (1,000-fold) immediately after injury (Struglics et al., 2015). The body reacts instantaneously to injury by producing a strong inflammatory response, which

persists afterward and is thought to contribute to cartilage erosion and thus cause PTOA (Lieberthal et al., 2015).2.3.4 Gender

Gender is also an important but non-modifiable risk factor for OA. Studies have shown that OA of the hands and knees is more prevalent among women than men, while hip OA is prevalent equally in both genders (Johnson and Hunter, 2014). Aging in women also plays a central role in OA pathogenesis, as OA is more frequent in the postmenopausal years, although some studies have shown loss of estrogen as a causative factor in postmenopausal women (Cirillo et al., 2006, Hussain et al., 2014).

2.3.5 Genetics

Genetics may or may not be a serious risk factor for OA pathogenesis, depending on the type and number of genes affected. Generally, a mutation in any individual gene provides only a very slight risk for OA (likelihoods ratios in the 1.2-1.4 range) (Richard, 2020), signifying the involvement of either multiple genes for a more substantial OA risk or importance of environmental factors and/or epigenetics in addition to mutant genes for the disease to be incited (Yucesoy et al., 2015). However, there are some severe forms of OA inherited due to rare mutations in the genes encoding structural collagens present in articular cartilage and the vitreous of the eye. These genes include collagen types II, IX, or XI. Mutations in these genes result in premature OA that can initiate as early as adolescence, resulting in a severe destructive form of arthritis that affects multiple joints (Snead and Yates, 1999, Kannu et al., 2010). As the vitreous fluid of the eye also contains these collagen types, some patients also have eye disease. These

mutations also cause Stickler syndrome which affects 1 in 7,500-9,000 new-borns (syndromes with craniofacial abnormalities) (Couchouron and Masson, 2011). Another less severe form of OA triggered due to alterations in joint shape is caused due to mutation (polymorphisms) of genes associated with expression of growth and differentiation factor (GDF)-5, which is a member of bone morphogenetic proteins that play a part in joint growth and development (Valdes and Spector, 2011).

2.3.6 Anatomic factors

Joint structure also plays a significant role in the onset of OA. Altered joint shape results in altered joint mechanics, i.e. excessive joint loads and activation of mechanotransduction pathways lead to increased production of inflammatory mediators and proteolytic enzymes (Andriacchi and Favre, 2014). The joint shape is particularly important in the case of the hip, where hip joint alteration due to Congenital acetabular dysplasia results in premature hip OA that often requires replacement of the (Morvan et al., 2013). Similarly, in the case of the knee joint lower extremity alignment is important concerning its role in the onset of OA. Individuals who have a Varus alignment (bow-legged) are at increased risk of medial tibial-femoral OA, while those with a valgus alignment (knocked-knee) are at risk for lateral tibial-femoral OA (Moisio et al., 2011).

2.3.7 Inflammation

Inflammation is the natural immune response of the body to many pathways that occur in the body such as aging, obesity, injury, etc. All these processes converge at

inflammation which can lead to a number of pathological conditions including OA depending upon the site affected. There is growing evidence that OA is manifested by low-grade local and systemic inflammation (Lopez-Otin et al., 2013). Similarly, aging is also correlated with chronic low-grade inflammation, occasionally called inflammaging (Franceschi et al., 2000). Inflammaging might be involved in OA but the exact mechanism that links OA with inflammation is not known yet. However, several features of aging can contribute to OA pathogenesis such as epigenetic modifications, mitochondrial dysfunction, cellular senescence, dysregulated nutrient sensing, and transformed intercellular communication (Loeser et al., 2016b). Increased production of proinflammatory mediators is associated with senescence-associated secretory phenotype (SASP) that may have an important role to play in pathogenesis of OA (Greene and Loeser, 2015). IL-6 is an important cytokine associated with age related diseases (Morrisette-Thomas et al., 2014) and systemic increase in its concentration might be considered as a strong risk factor for the development of OA (Livshits et al., 2009). Although the role of IL-6 in progression of rheumatoid arthritis (RA) is clear and IL-6 targeted therapy is found to be an effective treatment for RA (Smolen et al., 2008), its role in age associated OA has not yet been recognized, as *IL-6* gene deficient mice show even more severe age related OA. This reveals the involvement of other mediators in the disease pathogenesis and that the relation between age and OA is not determined by a single factor but by multiple cytokine profiles changing during aging (Morrisette-Thomas et al., 2014).

Inflammaging, the term for chronic low-grade inflammation that occurs with aging, is also attributed partly to the age-related deposition of visceral fat that replaces muscle mass (Smolen et al., 2008). Obesity is a well-recognized risk factor for OA in a population irrespective of age (Losina et al., 2013). An increase in fat mass increases the number of adipocytes and proinflammatory macrophages in adipose tissues, which increases adipokines, and proinflammatory cytokines that might contribute to OA (Sellam and Berenbaum, 2013). The exact link between these mediators and OA has not been established yet. However, obesity and increased fat mass could cause OA by causing a metabolic alteration called “meta inflammation. Although “meta inflammation” is primarily linked to augmented levels of cytokines and adipokines, there is a considerable increase in circulatory free fatty acids, hyperglycemia, and oxidative stress, all of which damage the joint tissues by promoting matrix destruction (Wang et al., 2015b). Moreover, obesity-related low muscle mass (sarcopenic obesity) is related to knee OA and contributes to the instability of joints and a higher risk of falls in elderly people especially in women (Lee et al., 2012, Scott et al., 2012).

Along with visceral fat, the infrapatellar fat pad also increases with age (Chuckpaiwong et al., 2010). This fat pad is closer to the knee joint and makes different inflammatory mediators such as TNF, and IL-6, adipokines, adiponectin, and leptin, basic fibroblast growth factor (bFGF; also known as FGF2), and vascular endothelial growth factor (Ushiyama et al., 2003). Therefore, it is quite reasonable to think that an age-linked increase in the volume of this fat pad might be a factor for OA pathogenesis. Conversely, *in vitro* studies involving conditioned media from infrapatellar fat pads (obtained from

end-stage knee arthritis patients) were shown to have protective effects on bovine cartilage explants rather than being catabolic (Bastiaansen-Jenniskens et al., 2012).

2.3.8 Proteases

Proteases (protein degrading enzymes) involved in the degradation of cartilage matrix proteins such as aggrecans and type II collagen are known to play an important role in OA pathogenesis. These proteases include numerous matrix metalloproteinases (MMPs), cysteine proteinases such as cathepsin K, and serine proteinases. Members of the ADAMTS (**a d**isintegrin and **m**etalloproteinase with **t**hrombospondin **m**otifs), a family called aggrecanases (ADAMTS-4 and -5), catalyse the breakdown of aggrecan in the early phases of OA, which is the large proteoglycan that provides plasticity to cartilage. (Thomas, 1956) Similarly, collagenases are MMPs; one example, MMP-13, is a major collagenase involved in the breakdown of type II collagen, the major collagen in cartilage that is responsible for the tensile strength of cartilage (Troeberg and Nagase, 2012). Cathepsin K is a cysteine proteinase that is primarily expressed in osteoclasts and is responsible for the degradation of type I collagen in bone but can also degrade type II collagen in cartilage (Salminen-Mankonen et al., 2007). Once collagen is broken down significantly, the repair of the damaged matrix is thought to be impossible, and the progression of matrix loss is obvious (Svelander et al., 2009). Considering the importance of aggrecanase-2 (ADAMTS-5) and MMP-13 in OA, it is logical to utilize them as potential targets for the development of disease-modifying drugs such as protease inhibitors (Tonge et al., 2014). MMPs are produced in their inactive or pro-forms, and are only activated into functional MMPs when cleaved by proteolytic enzymes such as serine

proteases including, HtrA1 and activated protein C (Visse and Nagase, 2003). Therefore, serine proteases can also be used as potential targets for treatment of OA in order to inhibit the production of functionally active MMPs.

2.3.9 Other pathways

Several studies have revealed potential mediators of OA other than pro-inflammatory in nature, which can contribute to OA either by activating pathways involved in joint tissue destruction or by inhibition of tissue repair. These mediators include:

2.3.9.1 FGF signalling

Fibroblast growth factors (FGFs) produced after joint injury can signal both catabolic and anabolic pathways in chondrocytes, depending on the specific receptor activated. FGF-18, for instance, is a potent cartilage anabolic factor that signals through FGF receptor-3 and is being explored as a potential intra-articular therapy for OA (Yu and Hunter, 2015). These findings have led to the concept that OA might reinforce developmental processes, leading to the investigation of other proteins such as bone morphogenetic proteins (BMPs) and Wnts (a term formed by combining the proto-oncogene names of wingless and integration-1) for their roles in OA (Loeser, 2013).

2.3.9.2 BMP and Wnt signalling

BMP and Wnt are proteins which act through cell surface receptors. These proteins are produced in subchondral bone and have receptors on cartilage; the interactions

between ligands and receptors regulate the interplay between bone and overlying cartilage during OA pathogenesis (Luyten et al., 2009). BMP-2 and TGF-β (transforming growth factor-beta) produced locally in the joint seem to be key mediators of osteophyte formation in OA (van der Kraan and van den Berg, 2007, Blaney Davidson et al., 2015). Excessive activation of the *Wnt* pathway (or *Wnt/β-catenin* pathway) in cartilage promotes chondrocyte hypertrophy and expression of matrix-degrading enzymes, possibly through Wnt-induced signalling protein (Blom et al., 2009, Luyten et al., 2009). The role of Wnt family members in facilitating the cartilage-bone connection is yet to be understood, as they have shown opposing outcomes from gene deletion and inhibitor studies depending on the animal model used and the target Wnt family member (Loeser, 2013). However, many studies suggest TGF-β plays a role in promoting the degradation of the overlying cartilage in mesenchymal stem cells of subchondral bone (Zhen et al., 2013). It has not yet been determined whether these findings will extrapolate to human OA, but overall research suggests that in some cases OA may originate in the bone rather than the articular cartilage.

2.3.9.3 Epigenetics

Epigenetics is another interesting aspect of the pathogenesis of OA. Epigenetics is a collection of post-transcriptional modifications such as histone modifications (acetylation and methylation), DNA methylation, micro-RNAs, and long noncoding RNAs. Several studies involving samples of cartilage or bone from normal and OA joints found significant differences in DNA methylation patterns and similarly a growing list of micro-

RNAs and long noncoding RNAs that differ between normal cartilage and OA cartilage (Gabay and Sanchez, 2012, Loeser, 2013, Tsezou, 2014). Yet, it is not clear which of the various epigenetic changes are more important regarding their role in the development of OA in humans.

2.3.9.4 Sirtuins

Sirtuins (SIRT) are a family of nicotinamide adenine dinucleotide (NAD)-dependent deacetylases involved in the regulation of gene expression in response to altered energy metabolism and serve as a key factor in the promotion of longevity in response to dietary restriction (Leibiger and Berggren, 2006). SIRT1 serves as a histone deacetylase and studies have shown that mice deficient in SIRT1 resulted in the cartilage to develop spontaneous OA (Gabay and Sanchez, 2012). Other SIRTs, including SIRT6, found in cartilage may also have an important role in maintaining cartilage homeostasis (Nagai et al., 2015).

2.4 Summary of the aetiology and pathogenesis of osteoarthritis

As discussed above, OA was initially considered as age-associated wear and tear of the cartilage in the joints of the hands, knees, and hips but modern research shows that it is a whole joint disease of complex aetiology involving the above-mentioned multiple risk factors. One of the multiple components of the disease are genetic and studies show that it is a polygenic disease with a substantial hereditary component. Data obtained from gene analyses and genome-wide screening of individuals with OA identify a

multitude of information about different aspects of the disease. For instance, gene studies have shown that multiple genes involved in bone formation *(CLEC3B, CDH11, GPNMB, CLEC3A, CHST11, MSX1, MSX2)* and genes encoding collagens *(COL13A1, COL14A1, COL15A1, COL8A2)* (Sun et al., 2015) are associated with the development of synovial joints, and mutations in these genes can directly cause OA. Moreover, they can also determine the age of the disease onset, site of the joint involved, severity of the disease, and rate of its progression (Sandell, 2012).

Altered metabolism in cartilage and synovial joints is also considered a significant factor in the onset and development of OA. According to this hypothesis mammalian cells, under adverse microenvironment (increased mechanical loading, trauma, or injury) undergo a shift from regulated metabolism to highly activated metabolic pathways to maintain energy homeostasis (Zheng et al., 2021). This change in metabolism is accompanied by increased production of metabolic intermediates which lead to biosynthesis of inflammatory mediators and other proteins which ultimately stimulate inflammatory pathways and transcription factors involved in catabolism of cartilage. The continuation of these processes culminates in the pathogenesis of OA (Mobasheri et al., 2017).

Injury or trauma (macro trauma or repeated microtrauma) is generally considered the cause of OA in many patients. In response to injury, chondrocytes produce degradative enzymes that result in poor repair responses. Cartilage defects are a less common origin of OA (Ding et al., 2005). Defective cartilage, due to a genetic mutation in type II collagen

or ochronotic cartilage with deleterious pigment deposition, fails under normal joint loading, resulting in the development of OA (Lotz, 2010, Lieberthal et al., 2015).

In short, OA can be initiated due to a failure of biomechanical forces and /or cartilage, but once it has triggered, the progression of the disease is an interplay between many other factors including protease and protease inhibitors, cytokines and inflammatory mediators, cartilage degradation, and many risk factors such as age, body weight, systemic hormones, mineral deposition, and abnormalities in neurogenic control. Finally, the damage extends beyond the cartilage and affects other components of the joint such as subchondral bone, synovial membrane, and ligaments (Hassanali and Oyoo, 2011), as shown in figure 2.2. Subsequently, the patient suffers from joint agony, stiffness, and limited movement. If left untreated, these symptoms slowly spread all over the joint, resulting in pain, failure of the joint and disability.

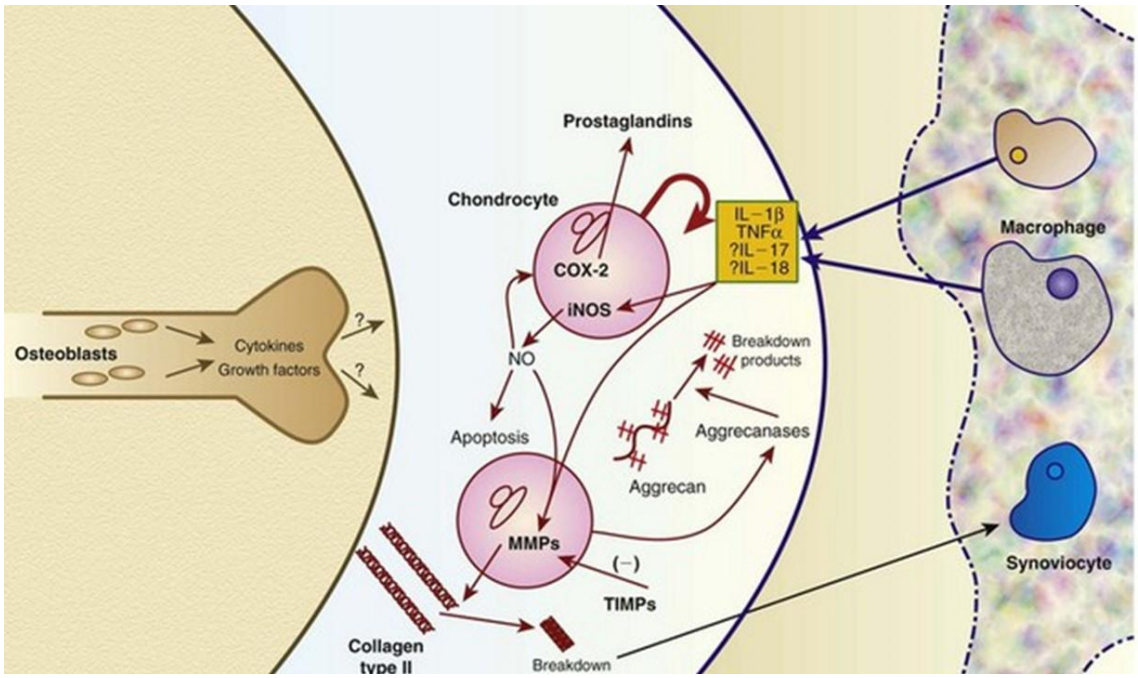

Figure 2.2*:* OA pathogenesis is an interplay between different components of joints i.e. synovial macrophages, chondrocytes of cartilage, and underlying subchondral bone. Cross talk is facilitated through cytokines. Reproduced with the permission of the Xuan cu Cao (Innes, 2016).

2.5 Pathophysiology of osteoarthritis

2.5.1 Joint anatomy & physiology

A typical synovial joint consists of different tissues such as articular cartilage, synovium, ligaments, joint capsules, subchondral and metaphyseal bone, and the muscles that act over the joint (Buckwalter et al., 2005). During OA disease progression, all of these components undergo modifications such as subchondral bone undergoes remodelling, osteophytes or bony projections are formed, synovial inflammation is caused, ligamentous laxity (loose ligaments) occurs, and the periarticular muscles deteriorate due to an imbalance between the catabolic and anabolic processes of joint tissue

(Brandt et al., 2006). Subsequently, the patient suffers from pain, stiffness, and limited joint movement, which ultimately lead to joint failure and disability (Martel-Pelletier, 2004).

2.5.1.1 Articular cartilage

Articular cartilage is primarily made up of extracellular matrix (ECM) with sparingly scattered, a particular type of cells called chondrocytes (Buckwalter and Mankin, 1998). It lacks nerves lymphatic vessels or blood vessels. Chondrocytes are the only cells present in cartilage. ECM contains tissue fluid and a background of structural macromolecules including collagens, non-collagenous proteins, proteoglycans, and glycoproteins, produced by chondrocytes. Each chondrocyte produces and preserves a particular microenvironment in its adjoining zone (Poole et al., 2001). Tissue fluid is the major component (80% of the wet weight of articular cartilage) of ECM and is mainly composed of water with minute amounts of gas, metabolites, small proteins, and a high concentration of cations to compensate for the anionic proteoglycans. ECM water is distributed in different fractions such as 30% in intrafibrillar space within collagen in the form of a gel, a small proportion in the intracellular space, and the remainder in the pore space of the matrix. Tissue fluid not only provides lubrication but also facilitates the transport of nutrients to chondrocytes.

The macromolecular portion of ECM consists of different macromolecules with the most abundant one being collagen (around 60% of the dry weight of articular cartilage). Different types of collagen are present in the ECM, such as collagen types I, II, IV, VI, IX,

X and XI, but the major one is type II collagen which interlinks the proteoglycan aggregates by forming a fibrillary network. This organization is maintained and stabilized by other forms of collagen. All these molecules together form a meshwork that encompasses the whole tissue and provides flexibility, cohesiveness, and strength to the articular cartilage (Eyre, 2002, Fox et al., 2009a). The second group of abundant macromolecules in the ECM are proteoglycans. Proteoglycans are of two types, aggrecans and other minor proteoglycans (decorin, biglycan, and fibromodulin) (Watanabe et al., 1998). Aggrecans are large aggregating molecules which intermingle with hyaluronic acid (also termed as HA, hyluronin) and proteins to produce huge proteoglycan aggregates, supporting the proteoglycans anchoring within the matrix. Aggrecans are also responsible for osmotic properties of cartilage, crucial for fighting compressive loads (Hardingham et al., 1994, Watanabe et al., 1998, Knudson and Knudson, 2001). Small proteoglycans (decorin, biglycan, and fibromodulin) are not directly involved in mechanical support, but they indirectly support cartilage function; for example, decorin and fibromodulin are involved in fibrillogenesis and interfibril connections by networking with collagen type II fibrils, while biglycan in the immediate surrounding of chondrocytes interact with collagen type IV (Hedlund et al., 1994, Poole et al., 1996, Buckwalter and Mankin, 1998).

Cartilage is divided into different zones and regions depending upon the structure, organization, morphology, and mechanical properties of the matrix. From the articular surface to subchondral zone there are four zones; the superficial zone, the transitional zone, the middle (radial or deep) zone, and the calcified cartilage zone. These zones

differ from one another with respect to composition, structure, and function of the matrix. The composition, structure and function of matrix even vary within the same zone depending upon the distance from the chondrocytes, resulting in different areas such as the pericellular area, the territorial area, and the interterritorial area (Bhosale and Richardson, 2008, Fox et al., 2009b).

The superficial zone is the thinnest zone close to the synovial fluid and is rich in flattened chondrocytes, types II and IX collagen. It is strongly packed and arranged parallel to the articular surface and protects deeper zones. It also provides flexibility and strength to the joint to stand compressive forces produced during joint use due to its highly packed collagen fibrils arranged parallel to the joint (Clark, 1990). The transitional zone constitutes 40-60% of the total cartilage and is composed of proteoglycans and thicker (having largest diameter) collagen fibrils, arranged perpendicularly. It acts as an anatomic bridge between the superficial and deeper zone and provides resistance against compressive forces. The deep zone is richest in proteoglycans and lowest in water and these properties enable it to resist compressive forces (Buckwalter et al., 2005, Fox et al., 2009a). Finally, the calcified cartilage zone is separated from the deep zone by a basophilic line. The calcified zone anchors the collagen fibrils of the deeper zone to the subchondral bone and thus safeguards the cartilage and bone, (Redler et al., 1975). Furthermore, calcified cartilage facilitates the movement of small molecules between the cartilage and subchondral bone and thus non-calcified tissues can biochemically interact with subchondral bone (Green et al., 1970, Arkill and Winlove, 2008).

2.5.1.2 Synovium

The synovium or synovial membrane is one of the multiple tissues of synovial joints and it is a thin vascular layer of connective tissue consisting of fibroblast-like (type B) cells and macrophage-like (type A) cells within an ECM called synovial fluid, mainly composed of HA, proteoglycans, and collagens (Hui et al., 2012). This distribution of synovial fluid is important with respect to its interactions within and across joint tissues. Synovial fluid is the refined form of a comparatively acellular part of the blood i.e. plasma, having less than 200 leukocytes per mm^3 compared to 3,540–9,060 per mm^3 in whole blood (Yehia and Duncan, 1975, Kratz et al., 2004). Synovial fluid also contains lymphocytes, macrophages and shed lining cells (Castor, 1960, Barland et al., 1962). It has biomechanical, metabolic, and regulatory functions (Mow et al., 1993, Hui et al., 2012a). Hyaluronan (HA) and proteoglycan 4 (PRG4, also called lubricin or superficial zone protein) are the two major biochemicals produced by the cells in the synovial lining and the cells within the synovial tissue cavity, which provide boundary lubrication to the joint and protect articular cartilage from damage by reducing friction during joint movement. HA provides viscosity to the synovial fluid and buffers the volume of synovial fluid while the mucinous glycoproteins, superficial zone protein, and lubricin lubricate the boundaries of articular cartilage (Schmid et al., 2002, Blewis et al., 2007).

Synovial fluid also contains cytokines and growth factors which act as regulatory factors not only for the cells within the synovium but also for chondrocytes (Hui et al., 2012). Cytokines can be classified as pro-inflammatory and anti-inflammatory based on their

effects in specific tissues. Pro-inflammatory cytokines present in synovial fluid include interleukins i.e. IL-1α, IL-1β, IL-6, IL-8, IL-17, and IL-18; tumour necrosis factor-α (TNF-α), and leukaemia inhibitory factor (LIF) (Futani et al., 2002, Kapoor et al., 2011b) while anti-inflammatory cytokines present in synovial fluid are IL-4, IL-10, and IL-13 (Goldring, 2000). Growth factors present in synovial fluid are transforming growth factor-beta 1 (TGF-β1) and insulin-like growth factor (IGF-1), which are anabolic (Denko et al., 1996). The concentrations of most pro-inflammatory cytokines and growth factors are significantly increased in osteoarthritis compared to their concentrations under normal conditions (Goldring and Otero, 2011a, Hui et al., 2012a).

Additionally, synovial fluid has highly regulated levels of proteolytic enzymes for degradation of the matrix such as matrix metalloproteinases (MMPs). MMPs are a group of Zn^{+2}-dependent enzymes that have an ability to degrade all the components of the ECM and remodel normal and diseased tissue (Woessner, 1991). MMPs are further classified as collagenases (MMP-1, MMP-8, MMP-13), gelatinases, stromelysins (MMP-3), and membrane-type MMPs depending on their structure and type of their substrate (Konttinen et al., 1999). In addition, disintegrin, a metalloproteinase with thrombospondin motifs (ADAMTS) proteinases that degrades aggrecan, is another type of MMPs also present in synovial fluid (Jones and Riley, 2005, Zhang et al., 2013a).

MMPs are released from chondrocytes in normal as well as in injured joints but their levels are elevated in joint injury as evident from elevated levels of their mRNAs in tissue and proMMPs in synovial fluid. These are secreted as zymogens (proMMPs) and

activated after cleavage of propeptide domains in ECM (Roos et al., 1995b, Knäuper et al., 2002, Tchetverikov et al., 2005). The activity of MMPs is regulated by different molecules such as tissue inhibitors of metalloproteinases (TIMPs) and proteinase inhibitors that activate proMMPs. These molecules control the changes in the activity and level of these enzymes; therefore, it can be concluded that MMPs, their inhibitors, and activators together maintain homeostasis by controlling the anabolic and catabolic processes in the joint (Martel-Pelletier et al., 1994, Yoshihara et al., 2000).

The synovial membrane is a size-dependent molecular sieve for the components of synovial fluid. HA and PRG4 lubricants, being larger, are retained by the membrane, while smaller molecules such as metabolic substrates and by-products including cytokines, and growth factors are not (Pejovic et al., 1995, Sabaratnam et al., 2005).

2.5.1.3 Subchondral Bone

Previously, OA was considered to be the outcome of cartilage degeneration but it has now been established that this is not the only cause of disease, with all components of the joints are involved in its pathology. Remodeling of the subchondral bone is accompanied by articular cartilage damage and subchondral sclerosis and together these conditions contribute to OA (Burr and Gallant, 2012, Henrotin et al., 2012).

Subchondral bone is a membrane-like structure that lies away from calcified cartilage (Madry et al., 2010, Li et al., 2013c). Subchondral bone has two layers i.e. subchondral bone plate and subchondral trabecular bone (Goldring and Goldring, 2010). The

subchondral bone plate is somewhat spongy and lies underneath the calcified cartilage that connects the articular cartilage and trabecular bone through the arterial and venous blood vessels that enter into the networks present in the subchondral bone plate and split into small branches to connect with calcified cartilage (Holmdahl and Ingelmark, 1950, Clark and Huber, 1990, Madry et al., 2010). Subchondral trabecular bone is more porous and metabolically more dynamic, containing sensory nerves, blood vessels, and bone marrow formed by supporting trabeculae that emerge from the subchondral bone plate in addition to deeper zone structure (Inoue, 1981). It is shock-absorbing and provides support to the joint and may be involved in delivering nutrients to the cartilage and their metabolism (Li et al., 2013c). Subchondral bone is specially designed to tolerate mechanical stress applied across the joint (Inoue, 1981, Li et al., 2013c) and modifies its shape accordingly, through bone modeling and remodeling (Martin, 2007, Goldring, 2012). Analogous to the "tidemark" that separates two different cartilage zones, the "cement line" is a clear boundary between subchondral bone and calcified cartilage (Li et al., 2013c). The deeper layer of non-calcified cartilage, the tidemark, calcified cartilage, the cement line, and subchondral bone are closely connected, forming the osteochondral junction, a compound structural and functional unit of the joint (Imhof et al., 1999). Biomechanical and biochemical crosstalk through this region is very important in the preservation or disintegration of the joint because changes in any of these components will modify the characteristics and roles of other regions of the osteochondral junction (Suri and Walsh, 2012).

2.5.1.4 Infrapatellar fat pad

The infrapatellar fat pad (IFP) or Hoffa's fat pad is a unique structure that is present only in the knee joint (most affected joint by OA) (Felson et al., 1995, Saddik et al., 2004, Sharma et al., 2006). This intracapsular and extra synovial adipose structure is made up of a fibrous framework which is surrounded by fat tissue and it is found beneath the patella, between the patellar tendon, femoral condyle, and tibial plateau, closely connected to the synovium, articular cartilage, and the bone (Jacobson et al., 1997, Vahlensieck et al., 2002). Little is known about its function, except that it assists the supply of synovial fluid and captures the forces through the knee joint (Clockaerts et al., 2010). Previous studies have shown that the IFP remains intact during life-threatening starvation even though subcutaneous adipose tissues are depleted (Smillie, 1980, Ioan-Facsinay and Kloppenburg, 2013), which suggests its critical physiological role in the knee. The IFP has a massive number of fibroblasts, adipocytes, macrophages, leukocytes, and other immune cells that are competent to produce inflammatory cytokines, suggesting its potential defensive and/or offensive role in the inflammatory response during OA. The IFP also has pain-sensitive nerves, which are peptidergic and substance-P positive. This stimulates IFP and has been shown to increase C-fibers in the IFP of patients with chronic frontal knee pain (Bohnsack et al., 2005b, Lehner et al., 2008). Anterior knee pain normally present in patients with OA is triggered due to the pathology of the IFP (Bohnsack et al., 2005a).

2.5.2 Joint pathophysiology during OA

Joint homeostasis is maintained by the entire joint together with articular cartilage, synovium, subchondral bone, and IFP, all of these components are involved in the onset and pathogenesis of OA. However, the critical component is articular cartilage, which loses the ability to absorb shock produced as a result of mechanical forces imposed on the joint. This failure of cartilage to withstand mechanical force is due to the destruction of the extracellular matrix (Roach and Tilley, 2007).

2.5.2.1 Altered cartilage homeostasis

During OA, chondrocytes progressively experience a variety of pressures including biomechanical stress, pro-inflammatory cytokines and chemokines, and altered ECM. As a result, chondrocytes undergo phenotypic modification and cartilage homeostasis is disturbed (Goldring and Otero, 2011b). Several pathways maintain these phenotypic changes; these are discussed in detail below.

2.5.2.1.1 mRNA decay in healthy vs OA chondrocytes

The mRNA levels in a cell are not only regulated by the rate at which it is produced, but also the rate at which it is degraded (post-transcriptional regulation); the combination of the two determines the cell mRNA levels. Tew and colleagues (Tew et al., 2014) compared chondrocyte transcriptomes of healthy and osteoarthritic patients, using microarray analysis and actinomycin D chase. They stated that most of the transcripts were unaffected in both healthy and OA chondrocytes with only a small division of short-lived transcripts. These transcripts with decreased half-life were high in OA

chondrocytes and were identified as expressing genes that are involved in the regulation of transcription, found in the nucleus, or the genes involved in the regulation of apoptosis. In addition, some other genes involved in the turnover of ECM, such as ADAMTS-1, ADAMTS-5, ADAMTS-9, the hyaluronic acid synthase (HAS2), the heparan sulphate sulfotransferase (HS3ST3A1), and the NFkB complex component RELA were also identified during the study. The higher expression of these short-lived transcripts suggests their association with the processes that depend on instant and flexible gene responses in OA chondrocytes.

2.5.2.1.2 A homeostatic role for CXCR2 signalling in articular cartilage

The chemotactic role of heparin binding CXC (C-X-C Motif) chemokines with ELR motifs through their C-X-C Motif Chemokine receptors CXCR1 and CXCR2 is already known and are being studied as potential areas for intervention in inflammatory OA (Hou et al., 2020). However, Sherwood and co-workers discovered an unusual role of CXCR2 in maintaining the homeostasis of cartilage. Their study revealed that CXCL6, the ligand of CXCR1 and CXCR2, is locally present in the healthy human cartilage matrix in association with heparin sulphate proteoglycans. However, it not detected in OA. In *in vivo* studies in mice, in which CXCR2 deficient mice and wild types were used and underwent destabilization of the medial meniscus (DMM), it was observed that cartilage damage was more severe after 8 weeks of DMM in CXCR2 deficient mice as compared to wild types. Studies show that *in vitro* disruption of CXCR2 signalling is associated with the loss of *Sox9* (a transcription factor), *COL2α1*, and aggrecan mRNAs in human primary

chondrocytes. From these findings, it can be suggested that CXCL6 has a possible role in the stability of chondrocyte phenotype by using Sox9 as a transcription factor, and its loss during OA may contribute to the phenotypic changes of OA chondrocytes (Sherwood et al., 2015).

2.5.2.1.3 The zinc-ZIP8-MTF1 axis as an essential regulator of the catabolic cascade in cartilage

The zinc (Zn^{2+}) importer, ZIP8, was reported to be elevated in murine and human cartilage resulting in increased intracellular Zn^{2+} levels in OA chondrocytes (Kim et al., 2014). This ZIP8 derived influx of Zn^{2+} upregulates the production of catabolic enzymes such as MMP3, MMP9, MMP12, MMP-13, and ADAMTS-5. In a study in mice with overly expressed ZIP8, using Col2αl promoter, cartilage damage, and subchondral bone (SCB) sclerosis was observed without evident synovitis (Malfait, 2016). These mice established quicker cartilage destruction and SCB modifications after DMM surgery with no effect on synovitis and osteophytes. In contrast, chondrocyte specific conditional ZIP8 knockout mice presented opposite results i.e. fewer cartilage and SCB changes and more synovitis and osteophyte growth after 8 weeks of DMM. In addition, a transcription factor, MTF1 was acknowledged as an important factor to mediate Zn^{2+}/ZIP8 driven catabolic activity in the first study (Kim et al., 2014). Furthermore, the zinc-ZIP8-MTF1 axis was recognized as a novel therapeutic target in OA. The first study mentioned (Kim et al., 2014)above also authenticates the idea that cartilage damage initiates changes in

other parts of the joint, SCB for example, without any effect on synovium and osteophytes.

2.5.2.1.4 Catabolic and pro-inflammatory effects of an aggrecan fragment mediated through TLR2

Matrix particles and fragments including tenascin C, fibronectin, and hyaluronan fragments have long been documented to act as Damage Associated Molecular Patterns (DAMPs). These DAMPs can stimulate locally expressed Pattern Recognition Receptors (PRR) in the joint such as Toll-like receptors (TLR) and Receptor for Advanced Glycation Endproducts (RAGE), commencing a cascade for the synthesis of inflammatory cytokines (Liu-Bryan and Terkeltaub, 2015). The biological activity of the inborn glycosylated 32 amino acid long peptide fragment was confirmed by Lees and co-workers who performed an *in vitro* study using mouse and human chondrocytes in which they produced aggrecan fragments by treating it with ADAMTS-4/5, which cleaved the interglobular domain of the aggrecan core protein at the 374ARGS cleavage site. The remaining leftover G1-EGE373 is subsequently cleaved by MMPs at DIPEN341, resulting in a 32-amino acid fragment (Lees et al., 2015). A synthetic replicate of this fragment was observed to be pro-catabolic, anti-anabolic, and pro-inflammatory when tested *in vitro* using human and murine chondrocytes (Malfait, 2016). The fragment increased mRNA expression for several proteases, including *MMP-13* and *ADAMTS-5*, and decreased the mRNA for matrix molecules, including *Col2α1* and aggrecan through the TLR2 and NFkB-dependent pathways (Malfait, 2016). This was the first study

demonstrating that TLR ligand can be generated from one of the primary macromolecules of cartilage during OA when cartilage undergoes destruction. Aggrecan fragments may interact with the existing pool of DAMPs but their role within the immune system *in vivo* is yet to be established.

2.5.2.2 The pathogenic role of synovium

Data obtained from clinical and imaging studies of OA provides strong evidence that synovitis is related to cartilage loss and other symptoms of OA (Scanzello and Goldring, 2012). Described below are the synovial pathways that may cause OA.

2.5.2.2.1 The role of the alarmins, S100A8, and S100A9, in OA with pronounced synovitis

The alarmins known as S100A8 and S100A9, whose production are elicited by cellular damage, are copiously found in OA joints (Zreiqat et al., 2010). They have pro-catabolic activity in chondrocytes and they act through TLR4 (Blom et al., 2020). They add to OA pathogenesis in the collagenase-induced OA (CIOA) model, having prominent synovial inflammation, but not in the DMM model which reveals low-grade synovitis (van Lent et al., 2012). The van Lent research group found that intra-articularly (IA) deposited stem cells derived from adipose tissue are effective in minimizing the cartilage impairment and osteophytes in CIOA but not in DMM, which shows that synovial provocation stimulates the protective effects of adipose-derived stem cells when administered locally (Schelbergen et al., 2014). Adipose-derived stem cells were effective in CIOA

because they suppress S100A8/A9 and IL-1 in the joint and S100A8/A9 levels in serum, ultimately blocking synovial activation (Schelbergen et al., 2014). These findings further validate the idea that alarmins can be involved in the pathogenesis of OA in subgroups having synovitis as a prominent feature and can be explored as a therapeutic target.

Along with S100 proteins, there are many other DAMPs in the OA joint such as products of ECM degradation that signal through various PRRs. But more recent studies involving female mice deficient in TLR1, TLR2, TLR4, TLR6, and MyD88 have reported contradicting results; these DAMPs were found not to defend against cartilage destruction and synovial inflammation 8 weeks after the medial meniscus was partially removed (Nasi et al., 2014). Further research on the role of the innate immune system in different subtypes of OA at different phases of the disease are needed.

2.5.2.2.2 PAR2 ablation modulates synovial macrophage activation 1 week after DMM and results in cartilage protection at 8 weeks

Proteinase-activated receptor-2 (PAR2), which is a pro-inflammatory G protein-coupled receptor, is increased in concentration in OA synovium and cartilage and thought to be involved in the progression of OA (Boileau et al., 2007). This idea was confirmed by Jackson et al (Jackson et al., 2014) who compared the advancement of OA in wild type and PAR2 -/- mice up to 8 weeks after DMM and found that chondrocytes were protected in PAR2 -/- mice. Wild type mice revealed synovitis and increased expression of synovial pro-inflammatory cytokines and metalloproteases and increased numbers of CD4þ T-lymphocytes and activated macrophages, while PAR2-deficient mice had fewer

macrophages one week after DMM. These findings suggest that this early-stage synovitis after DMM, though minor as compared to other inflammatory models, is key to the progression of the OA. *In vitro* studies, however, have not proved the chondroprotective effects of PAR2 ablation (Jackson et al., 2014), suggesting the involvement of extra cartilaginous pathways may be important in the protective mechanism of PAR2 ablation.

2.5.2.2.3 Synovitis and pain

The histopathology of knee OA was studied by Stoppiello and co-workers (Stoppiello et al., 2014), who collected medial tibial plateaux and synovia from 29 post-mortem asymptomatic donors and 29 symptomatic donors having a total knee replacement. They compared the samples for macroscopic tibiofemoral chondropathy between the two groups and analysed similarities and differences in their histological features. Their findings suggested that there was more loss of cartilage and chondrocyte modifications in the symptomatic chondropathy group as compared to the asymptomatic chondropathy group. But the feature which was more strongly linked to the pain was synovitis, and 8 out of 29 symptomatic knees displayed severe inflammation. Pain relating nerves called the pro-algesic neurotrophin nerve growth factor (NGF) stained positive in the different areas of the synovium; most of NGF were localized with synovial fibroblasts but some CD68þ macrophages in the sub lining of the synovium also contained NGF. The presence of NGF in synovitis highlights the relationship between

synovitis and pain and also provides a basis to perform anti-NGF therapies in subsets of patients.

2.5.2.3 Pathophysiology of bone

Bone undergoes different modifications during OA pathogenesis, which include thickening of the subchondral bone (bone sclerosis) due to increased production of collagen and its improper mineralization, the formation of osteophytes (bony spurs) at the margins of the joints, and also bone cysts that occur at an advanced stage of the disease (Goldring, 2009). However, bone erosions are not generally observed in OA except in erosive OA, which are most commonly seen in the distal joints of the hands (distal interphalangeal and proximal interphalangeal) and are located centrally (Rothschild, 2013). These erosions are different from marginal erosions usually associated with rheumatoid arthritis (RA) and gout. MRI shows bone marrow lesions also in some cases and in the areas where overlying cartilage is damaged and where mechanical loads are greatest. Pathologically, these focal lesions consist of microstructural damage to bone accompanied by localized necrosis and fibrosis (Taljanovic et al., 2008).

2.5.2.4 Pathophysiology of the infrapatellar fat pad

Knee OA involves cellular changes in IFP such as penetration of immune cells, which produce various inflammatory mediators that contribute to disease progression (Klein-Wieringa et al., 2011, Ioan-Facsinay and Kloppenburg, 2013). These inflammatory

mediators stimulate the disease progression in different ways; for example, inflammatory cytokines may increase the sensitivity of joint nociceptor nerve fibres and thus worsening the pain sensation (Witonski et al., 2010). Levels of leukocytes including neutrophils, eosinophils, basophils, and monocytes are also raised in the IFP of patients with OA (Clements et al., 2009). Neutrophils contribute to the breakdown of cartilage and necrosis of adipose tissues by producing cytokines such as IL-1, IL-8, and MMP-8 (Abbink et al., 1991, Clements et al., 2009, Clockaerts et al., 2010). Eosinophils and basophils increase the production of matrix-degrading enzymes and pro-inflammatory mediators in synovial fibroblasts and cartilage by releasing histamine (Tetlow and Woolley, 2004). Lymphocytes are also present in IFP expressing Th1 cytokines thought to be involved in cartilage degradation either directly or indirectly by interacting with macrophages, which are stimulated to produce mediators, involved in cartilage degradation (Jedrzejczyk et al., 1996, Sakkas and Platsoucas, 2007). Consequently, inflammatory cells within IFP are involved in inflammation and destruction of osteoarthritic knees.

Adipocytes present in the IFP, on the other hand, are involved in the secretion of factors called adipokines which include leptin and adiponectin (Dumond et al., 2003b, Fain, 2006, Lago et al., 2008). Leptin increases inflammatory responses by stimulating different types of inflammatory mediators such as IL-β, other pro-inflammatory cytokines and MMPs, and by activating immune cells including macrophages, neutrophils, dendritic cells, natural killer cells, and Th1 cells in OA cartilage (Matarese et al., 2007, Toussirot et al., 2007, Koskinen et al., 2011). Adiponectin generally provides

protection against obesity and vascular diseases but in joint diseases like knee OA, it acts as a pro-inflammatory agent (Ehling et al., 2006, Gomez et al., 2009). Adiponectin induces the production of IL-6 and MMP-1 in the cells having adiponectin receptors such as synovial fibroblasts and chondrocytes (both normal and OA), as proved by the production of IL-6 and MMP-3, MMP-9 and monocyte chemoattractant protein 1 (MCP1) in adiponectin-treated chondrocytes (Ehling et al., 2006, Lago et al., 2008).

2.5.2.5 Inflammatory cytokines

Current findings advocate the involvement of pro-inflammatory cytokines in the catabolic events occurring in damaged tissues. Initially, the cytokines are formed by the synovial membrane after which they move to the cartilage through the synovial fluid. In cartilage, the cytokines stimulate chondrocytes to make additional pro-inflammatory cytokines.

Synovial lining cells in the OA synovial membrane act as effectors to produce many inflammatory cytokines, of which IL-1β, TNF-α, leukemic inhibitor factor (LIF), IL-17, and IL-6 are most important with respect to disease pathology (Kapoor et al., 2011a). Recent research confirms the catabolic role of IL-1β and TNF-α in OA pathogenesis (Molnar et al., 2021)but whether they work independently or in coordination with each other is still unknown. Animal studies proved that blocking IL-1β can significantly prevent cartilage degradation (van de Loo et al., 1995) while TNF-α blockage significantly reduced inflammation (Kollias et al., 1999), and both of these cytokines are found in higher levels in the synovial membrane, synovial fluid and cartilage undergoing OA. IL-6 contributes

to OA pathogenesis by increasing the inflammatory effectors in synovial tissue, increasing the production of chondrocytes, inhibiting the proteoglycan production, and increasing the synthesis of MMPs indirectly by amplifying the effects of IL-1 (Wiegertjes et al., 2020). However, the effect of IL-6 on MMPs is controlled by feedback regulation (Akeson and Malemud, 2017). IL-6 also inhibits the production of MMPs by stimulating the production of tissue inhibitor of MMPs (Lotz and Guerne, 1991). LIF, a cytokine of the IL-6 family, is produced in high levels in OA in response to IL-6 and other cytokines (Lotz and Guerne, 1991, Liu et al., 2002), but its role in disease progression is still unknown. IL-17 is a family of cytokines that affects cell activation and which has recently been implicated in OA (Snelling et al., 2017).

2.5.2.6 Nitrous Oxide

Nitrous oxide is an inorganic free radical, though the exact mechanism of its action in OA is yet to be understood. Primarily, it is believed to be involved in cartilage degradation (Zhou et al., 2018) However, there is also evidence indicating a protective role of nitrous oxide on the cartilage (Abramson, 2008, Hsu et al., 2017).

2.5.2.7 Calcium crystals

Calcium pyrophosphate has been found in some OA patients along with damaged cartilage (Ivory and Velázquez, 2012); however, it is still unclear whether these are the result or the cause of cartilage damage. Whichever is the case, these crystals have been

found to have stimulatory effects on the production of MMPs, COX-1 and 2, PGE2, IL-10, and NO (Rosenthal, 2011).

2.5.2.8 Angiogenesis

The process of formation of new blood vessels is referred to as angiogenesis. Angiogenesis and inflammation have proved to play important roles in OA. Both processes reinforce each other: inflammation stimulates angiogenesis and angiogenesis, in turn, contributes to inflammation and other processes such as chondrocyte hypertrophy and endochondral ossification (Ashraf and Walsh, 2008).

2.5.2.9 Leptin

Leptin (LEP) is a metabolic factor produced by white adipose tissue (Zhang et al., 2015). There is an immense body of evidence that endorses the involvement of this adipokine in the pathophysiology of OA, and it provides a link between obesity and OA (Yan et al., 2018). Its level is found to be elevated in serum, synovial tissues, infrapatellar fat pad (IPFP), and cartilage of OA patients compared to healthy individuals (Conde et al., 2014, Scotece and Mobasheri, 2015). Some studies involving LEP-deficient or LEP receptor (LEPR)-deficient mice have revealed the onset of extreme obesity in such mice without increased incidence of OA, emphasising the imperative role of LEP signalling in the development of OA (Griffin et al., 2009). Moreover, a longer form of LEPR (which has a long intracellular domain with full capacity for intracellular signal transduction) was

identified to be expressed in human chondrocytes (Tsiotra et al., 2000, Figenschau et al., 2001).

LEP contributes to OA pathogenesis by affecting almost all the parts of the joint such as immune cells, cartilage, and bone cells (figure 2.3), where it influences the pro-inflammatory status, cartilage catabolic activity, as well as cartilage and bone remodelling (Francisco et al., 2018). LEP has been reported to have contradictory roles in maintaining cartilage homeostasis. Early research demonstrated LEP to have an anabolic activity in cartilage when administrated exogenously (30 μg) in rat knee cartilage as indicated by the increase in production of proteoglycans and growth factors (Dumond et al., 2003b). However, most of the research identifies LEP to play a catabolic role in OA pathogenesis. A recent study involving rats with LEP induced articular cartilage showed increased expression of genes associated with increasing the production of matrix metalloproteinases (MMPs), inflammatory factors, growth factors, and osteogenic genes in microarray analysis (Fan et al., 2018). Similarly, other research including cultured human and murine chondrocytes induced with LEP (400 or 800 nM) and IFNγ or IL-1β were found to have increased activity of type 2 nitric oxide synthase (NOS2) via JAK2, PI3K and MAPK (MEK1 and p38) pathways. Nitric oxide (NO) is well-known for its pro-inflammatory role during OA pathogenesis. It leads to cartilage damage by inducing the loss of chondrocyte phenotype, apoptosis, and activity of metalloproteinase (Rahmati et al., 2016).

LEP (800 nM) alone or in together with IL-1β, facilitated the induction of a pro-inflammatory environment in cartilage explants of OA patients and human primary chondrocytes by stimulating the expression of COX-2 and the production of PGE2, IL-6, and IL-8 in cartilage (Vuolteenaho et al., 2009, Gomez et al., 2011). LEP at a concentration of 500 ng/ml was also found to stimulate the production of IL-6 by mediating chondrocyte-synovial fibroblast interaction in OA patients (Pearson et al., 2017). Furthermore, LEP also contributed to the pro-inflammatory environment of the OA joint by altering the production of inflammatory mediators by immune cells. Specifically, IL-6, IL-8, and CCL3 were produced in higher concentrations by LEP-stimulated CD4+ T cells in OA patients, but not in healthy subjects; hence, demonstrating the role of LEP in the immune system and pathophysiology of OA (Scotece et al., 2017).

LEP can contribute to joint destruction directly by inducing the expression of MMPs such as MMP-1 (interstitial collagenase), MMP-3 (stromelysin), MMP-13 (collagenase), by activating PKC, NF-κB, and MAPK pathways (Bao et al., 2010, Koskinen et al., 2011, Hui et al., 2012b). Likewise, LEP also increased the production of MMP-2 (type IV collagenase), MMP-9, disintegrin, and metalloproteinase with thrombospondin motifs ADAMTS4 and ADAMTS5, while the production of fibroblast growth factor 2 and proteoglycan was decreased (Bao et al., 2010). LEP at 800 nM perpetuated the process of cartilage breakdown by inducing the production of VCAM-1, an adhesion molecule involved in chemotaxis and infiltration of leukocyte and monocytes to inflamed joints, through JAK2 and PI3K pathways in chondrocytes (Conde et al., 2011, Vestweber, 2015).

The roles of LEP are believed to be associated with microRNAs, which are small single-stranded non-coding types of RNA, known to regulate the progression of diseases such as inflammation, OA, and obesity (Marques-Rocha et al., 2015, Deiuliis, 2016, Nugent, 2016). The miR-27, which is a subtype of microRNAs that directly target the 3'-untranslated region of leptin, was found to be reduced in OA chondrocytes (Zhou et al., 2017). This finding was further reinforced by the results of an assay where miR-27 lentiviral overexpression vector injection to OA rats decreased the levels of IL-6 and -8, as well as MMP-9 and -13, hence signifying the protective role of miR-27 in OA, probably by regulating the synthesis of LEP.

LEP (50 ng/ml) was found to change the differentiation fate of chondrogenic progenitor cells (CPCs). The CPCs, which under normal conditions are supposed to differentiate into the chondrocytes critical to preserve cartilage homeostasis and repair injured tissue, instead undergo osteogenic transformation due to leptin induction (Seol et al., 2012). Leptin also induces CPCs cell cycle arrest and senescence (Zhou et al., 2015).

Likewise, the role of LEP in bone metabolism is also very interesting (figure 2.3). Elevated levels of LEP in OA subchondral osteoblasts were found to be associated with *in vitro* increased levels of alkaline phosphatase, osteocalcin, collagen type 1, and TGF-β1, all being indicators of the dysregulated function of the osteoblasts (Mutabaruka et al., 2010c). Furthermore, LEP was found to influence the regulatory mechanisms of bone metabolism by inducing abnormal osteoblast function, which leads to joint destruction in OA patients (Conde et al., 2011, Conde et al., 2014, Frasca et al., 2016). Also, bone

morphogenic protein (BMP)-2 level was found to be elevated in LEP-stimulated human primary chondrocytes (Chang et al., 2015). LEP suppressed the formation of bone *in vivo,* but there are some inconsistencies with *in vitro* results (Dumond et al., 2003b).

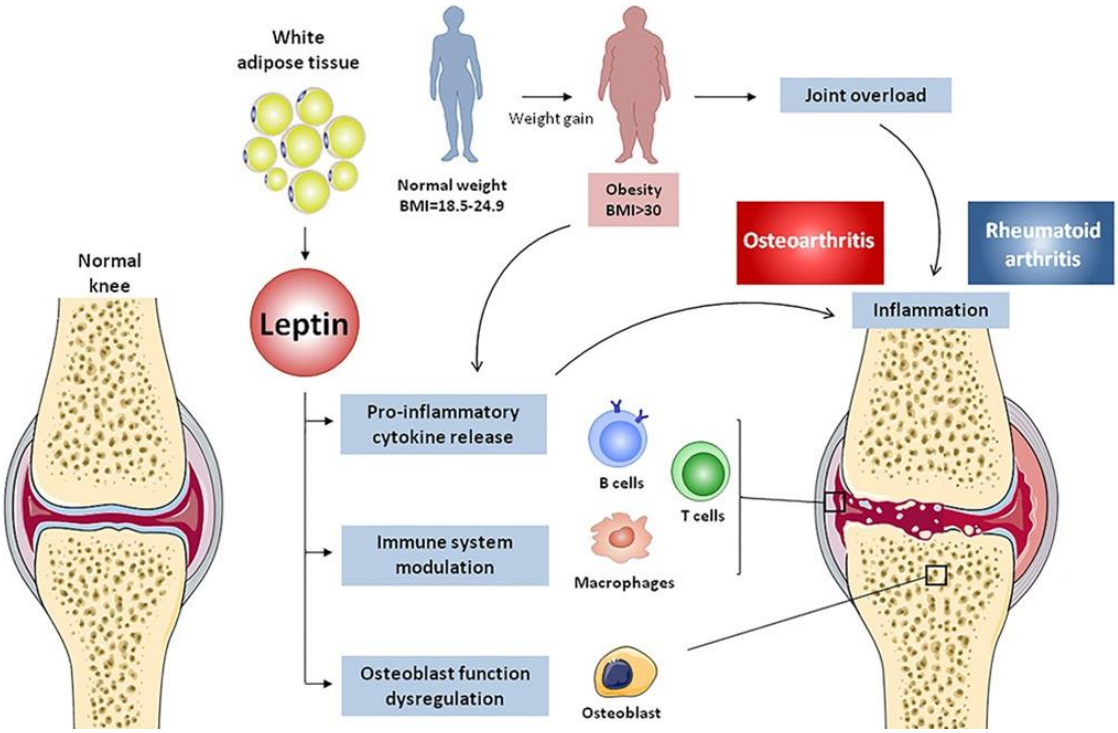

Figure 2.3: Effects of adipose tissue-derived leptin on osteoarthritis and rheumatoid arthritis. Bodyweight gain, accompanied by white adipose tissue enlargement, leads to obesity followed by the mechanical load, resulting in cartilage degradation and osteoarthritis onset. Adipose tissue-derived leptin causes osteoblast dysregulation in subchondral bone, thus promoting joint destruction. Additionally, leptin induces pro-inflammatory cytokine release from innate and adaptive immune cells, generating an inflammatory environment that prompts cartilage damage (Francisco et al., 2018).

2.6 Suitable *in vitro* models for osteoarthritis

Numerous *in vitro* and *in vivo* models have been utilized in research involving inflammatory diseases and related biomaterials/biocompatibility preceding human

clinical trials (Johnson et al., 2016a, Salgado et al., 2021). Murine models, both *in vivo* and *in vitro,* are employed most often due to the availability of reagents and species-specific probes, low cost, and copious gene knock-in and knock-out variants. *In vivo* murine models are generally used in pre-clinical research as a primary phase in the transition between *in vitro* models and pre-clinical trials, and the prevalent use of *in vivo* murine models in pre-clinical trials has led to the widespread application of murine cells as *in vitro* models (Chamberlain et al., 2009).

In vitro models are key to study molecular mechanisms involved in the physiology and pathophysiology of OA. Due to the complex aetiology and pathogenesis of OA, there is no single validated model. Different models are preferred depending on the specific pathological aspects of the disease being investigated. Previously reported models are comprised of *ex vivo* tissue samples, primary cell cultures, and cell lines. Also, more complex formats of co-cultures of different cells or cells with tissues that enable the study of the complex crosstalk between different cells and tissues within the joint have also been reported (Li et al., 2021). Cytokine-based models are most frequently used to study the pathology of OA in response to a single stimulus (Johnson et al., 2016b). J774A.1 is an immortalized, relatively stable, mature, and adherent cell line with a macrophage phenotype that has been used as a model of macrophage activation in numerous studies (Gutting et al., 2005, Vigo et al., 2005). Therefore, to assess the behaviour of macrophages during OA pathogenesis, the mouse macrophage cell line J774A.1 can be used (Scotece et al., 2012).

The mouse pre-chondrocyte ATDC5 cell line is an established model to assess changes regarding chondrocyte development in OA (Newton et al., 2012). The ATDC5 pre-chondrocyte, a mouse teratocarcinoma derived cell line, is categorized as a chondrogenic cell line that undergoes a series of developmental phases that correspond to the differentiation of chondrocytes. Therefore, it is regarded as a relevant *in vitro* model to understand the features that affect cell behaviours during chondrogenesis. It also helps to understand the signalling pathways associated with the development of the skeletal system as well as interactions with innovative materials. There are more than 200 studies to date that have utilised ATDC5 cells to gain substantial information regarding the pathology of OA, which have been reviewed in part elsewhere (Yao and Wang, 2013b).

Bone-related pathological changes can be assessed using the mouse bone cell line Mc3t3-E1 sub-clone 4 for mineralization studies (Mochida et al., 2003). The MC3T3-E1 cell is a clonal non-transformed cell line derived from new-born mouse calvaria and is generally used to study osteoblast differentiation *in vitro*, as MC3T3-E1 cells mature into mineralizing osteoblasts when cultured in a standard osteogenic medium containing ascorbic acid and β-glycerophosphate (Tarkkonen et al., 2017).

OA has a strong link with obesity and it is a cytokine, LEP, that provides a link between obesity and inflammation-induced OA. LEP, secreted by adipose tissue, induces synovial macrophages, cartilage, and bone to produce inflammatory cytokines and other pathological biomolecules, discussed in detail under the subheading “LEP”, which in turn

are responsible for the chronic inflammatory response and destruction of cartilage and thus joints during OA pathogenesis (Bondeson et al., 2006, Francisco et al., 2018). LEP is also classified as a hormone with endocrine functions including influencing bone metabolism (La Cava, 2017). LEP has not previously been used to induce an OA-type phenotype in J774A.1 macrophages, ATDC5 chondrocytes, or MC3T3 osteoblasts. However, it is the factor most closely aligned with metabolic syndrome and OA, and there is limited evidence that it will induce changes in cellular behaviour in these cell lines. LEP was therefore selected as a stimulant to create the *in vitro* models of MetOA used in this study.

2.7 New therapeutics for osteoarthritis

To design a single effective drug for the treatment of OA is challenging; firstly due to its complex etiology and multifactorial nature, and secondly because of its late diagnosis. Imaging techniques such as MRI & radiography can diagnose the disease, but these methods can only detect existing structural changes of the joint and are poor at monitoring disease progression status. Therefore, OA is diagnosed at the stage where it is irreversible. Although progression may be slowed or stopped, the disease can not be cured or the damage repaired. Therefore researchers are trying to establish biological markers for the disease which can be monitored for earlier diagnosis and targetted for therapy (Mobasheri, 2013).

To date, different therapeutic options, including simple analgesics, non-steroidal anti-inflammatory drugs (NSAIDS), corticosteroids, and hyaluronic acid injections have been

used to reduce pain and stiffness in OA; however, due to low efficacy and multiple side effects (Roth and Anderson, 2011, Wongrakpanich et al., 2018), recently researchers have shifted their attention to disease modifying drugs (DMDs), which can be useful in preventing disease progression by inhibiting the structural changes in the OA joint, thereby providing symptomatic relief. There are no effective disease-modifying osteoarthritis drugs (DMOADs) globally approved by regulatory bodies to date (Oo et al., 2018). Researchers have used different DMDs such as IL-1β receptor antagonist, the soluble truncated receptor of IL-1β and TNF-α, as well as anti-inflammatory cytokines such as TGF-β, IL-4, IL-10, and IL-13 (Oo et al., 2021). Some of these have been shown to have a positive effect on other forms of arthritis such as rheumatoid arthritis and psoriatric arthritis (Benjamin et al., 2022).

2.7.1 Nutraceuticals in OA pain management

In addition to clinical drug treatments, some nutraceuticals have also been found to be useful in managing the symptoms of OA including olive oil, marine oils, and botanical extracts (Castrogiovanni et al., 2016). These nutraceuticals could play an important part in balancing the anabolic and catabolic pathways in joints. Due to their regulatory role in chondrocyte homeostasis, their use is focused not only on disease management but also on disease prevention. Therefore, it is highly recommended to integrate these non-pharmacologic compounds within the diet to increase treatment options for patients with recognized OA, rather than limiting treatment to the traditional restoration, surgical interventions, and medications, and further to encourage their integration into the diet of people at risk of developing OA.

Fish oil contains all the essential nutrients found to be effective in treating different types of arthritis including OA. These components include the marine n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, they are not present in high concentrations in the oil of most wild-caught fish, which still make up the majority of products from global fisheries (Azra et al., 2021). Overfishing has led overexploitation and depletion of many ocean species, and many consumers may choose to avoid even effective nutraceuticals if their production has a significantly detrimental environmental impact (Yaghubi et al., 2021). Salmon is a good source of PUFAs and is one of the few species of farmed fish worldwide, but salmon produced by aquaculture are high-value and result in products expensive for consumers. Furthermore, high-intensity salmon farming can be detrimental to the health of the animals. Currently, there is no economically and environmentally sustainable system to produce marine creatures with high levels of long-chain n-3 PUFA other than green-lipped mussels.

2.7.2 Shellfish/Mussels as Nutraceuticals for OA

Shellfish constitute a main source of aquatic food worldwide. They are broadly classified as crustaceans and molluscs. Crustaceans are invertebrates with segmented bodies protected by a chitin-made exoskeleton, and this group includes shrimp, crayfish, crab, lobster, and krill. Molluscs are invertebrates with soft bodies consisting of foot and visceral portions, and are further classified as bivalves, gastropods, and cephalopods. Mussels, oysters, clams, and scallops are commercially important bivalves. The

gastropod group contains abalone, sea snail, cockle, and whelks, while cephalopods include squid, cuttlefish, and octopus (Venugopal and Gopakumar, 2017).

Shellfish including blue mussel (*Mytilus edulis*) and green lipped mussel (*Perna canaliculus*) are bivalves, with nutrient compositions comparable to fish. Like fish, mussels contain the marine n-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). They also are abundant in essential nutrients like zinc, riboflavin, selenium, and carotenoids (Venugopal, 2018). Mussel farming has important environmental advantages as these creatures reduce eutrophication of the sea by removing excessive nitrogen (Clements and Comeau, 2019).

Fish and shellfish are often included in the same nutrition group, as their general nutrient compositions are similar. Therefore, in many studies, a variety of fish and shellfish have been assessed as a single intervention (Menon and Gopakumar, 2017). However, there are a few studies related specifically to anti-arthritic or anti-inflammatory lipid extracts of mussels (Stebbings et al., 2017). Along with EPA and DHA, potentially bioactive novel fatty acids have been identified in blue (McPhee et al., 2010) and green-lipped mussels (Treschow et al., 2007). In one study, lipid extracts from blue mussel had positive effects on arthritic rats as compared to olive oil (McPhee et al., 2010). Lipids extracts from a green-lipped mussel or blue mussel have been identified to have anti-cyclooxygenase effects in *in vitro* studies (Treschow et al., 2007). In addition to the anti-inflammatory lipid fraction, water extracts from the blue mussel, containing substituent peptides and taurine in addition to other nutrients, reduced inflammation

in an *in vivo* zebrafish model (Cheong et al., 2017), as well as an *in vitro* model (Kim et al., 2016b). More recently, blue mussel intake in a clinical trial with rheumatoid arthritis patients was found to reduce disease symptoms and improve quality of life (Lindqvist et al., 2018b). These findings suggest that both the lipids and the water fraction, i.e., the whole food/mussel may also be of interest to study as an anti-inflammatory food.

2.8 Blue mussel

Mytilus edulis (*M. edulis*) is a filter-feeding marine mollusc belonging to the family Bivalvia (Mytilidae). Black mussel, blue mussel, and common or edible mussel are alternative names used for this mussel. The average size of the mollusc varies between 90 mm to 133 mm, and it is common to many marine environments including the temperate waters of Europe, Asia, and America and the coastal waters off the southern coast of Australia (McPhee et al., 2010, Utermann et al., 2018). Blue mussel (BM) is one of the major aquaculture shellfish species cultured in the USA, Canada, Europe, and Africa, and in many countries, it is considered as a valuable food and a primary industry similar to other food industries such as beef, wheat and dairy Canada's shellfish industry is primarily based on the BM, which constitutes 70% of their shellfish industry (Beaulieu et al., 2013). BM are reported to contain many nutritionally and pharmacologically important bioactive components with health benefits, although these have sometimes been described in generic terms such as liver and kidney nourishing, regulating blood pressure, or curing night sweats, dizziness, and impotence (Qiao et al., 2018b).(Qiao et al., 2018b).

2.8.1 Bioactive metabolites of BM

M. edulis is mostly composed of proteins, which constitute about 55% of its dry mass (Park et al., 2014). On the basis of their solubility, these proteins are classified as water-soluble protein fraction (sarcoplasmic protein), salt-soluble protein fraction (myofibrillar protein) and water-insoluble protein fraction (matrix protein). All these proteins can be processed by different methods to produce biologically active peptides, which are functionally more important than proteins because of their smaller size and simpler structures (Qiao et al., 2018a). These peptides are reported to have multiple beneficial roles such as regulation of gastrointestinal motility and the immune system (Feng et al., 2012), anti-bacterial properties (Liao et al., 2013), converting enzyme inhibitory activity (Qu et al., 2012), as well as anti-cancer, anti-oxidant and antihypertensive activity (Zhang et al., 2013b, Kleekayai et al., 2015). To date, most of the research involving the health potential of BM is focused on bioactive peptides (Park et al., 2014, Qiao et al., 2018a, Xu et al., 2019). Moreover, recently, water-soluble protein hydroxylates (bioactive peptides) from BM were found to have osteogenic properties on bone, in both *in vivo* and *in vitro* models (Xu et al., 2019). These findings are encouraging to explore the benefits of water-soluble BM proteins further as a functional food for bone health.

Lipids from *Mytilus* species have been less studied as compared to the *Perna* species. Generally, *Mytilus* and *Perna* oils have a similar composition with respect to the main long-chain omega-3 PUFAs (EA, EPA, and DHA); however, *Mytilus* have significantly lower yields of these PUFAs. Both nonhydrolyzed crude lipid extracts and hydrolysed triglyceride fractions from BM have anti-inflammatory effects, particularly the crude

extracts having 37% of EPA and DHA of the total fatty acids (McPhee et al., 2010). These crude lipid extracts on saponification inhibited the leukotriene production in a neutrophil 5-LO assay *in vitro* and in an AIA rat model (McPhee et al., 2010). The total free fatty acid fractions also showed a selective *in vitro* inhibition of COX-II along with production of 5-lipoxygenase by neutrophils, reducing leukotriene activity (Grienke et al., 2014b). Carbohydrates are the least studied group among the three major biomolecules or primary metabolite groups from mussels. To date, no biologically active carbohydrate has been identified in *M. edulis (Grienke et al., 2014b).*

2.9 Greenshell Mussel

Green lipped mussel, *Perna canaliculus*, is called kuku in Māori (Grienke et al., 2014b) and is trademarked as greenshell mussel (GSM) by the New Zealand seafood industry. GSM is characterized by the bright green streak around the posterior abdominal portion and inside the lip of its shell (Wolyniak et al., 2005), as shown in figure 2.4. The taxonomic hierarchy comprises the phylum *Mollusca*, class *Bivalvia*, family *Mytilidae*, and the genus *Perna*, which includes species of green as well as brown mussels, the latter being found primarily in the southern hemisphere but also present in North Africa and the northern shores of South America (Siddall, 1980, Gosling, 2008). The paleontological records of *Perna* date back to a 60 million year old Eocene period (Wood et al., 2007). GSM has been sustainably cultivated in New Zealand on a commercial scale since 1970 (Wood et al., 2007).

Figure 2.4: Pictures of M. edulis (A, whole mussel; B, shells) and P. canaliculus (C, whole mussel; D shells). Reproduced with the permission of Elsevier Science, Ltd. (Grienke et al., 2014b)

GSM is endemic to New Zealand and was used as a staple food by indigenous Māori, especially those living in coastal zones (Ulbricht et al., 2009). Māori populations with high GSM intake were anecdotally reported to have a lower incidence of osteoarthritis than comparative inland and European populations. Although undocumented, this claim drove researchers to explore GSM's potential health benefits (Ferreira, 2005, Ulbricht et al., 2009). Interests in investigating its health benefits increased following a human clinical trial in 1960 assessing GSM for potential anti-cancer activity. Although it failed to be effective in the treatment of cancer it was observed to relieve pain in cancer patients who had joint stiffness and pain (Cobb and Ernst, 2006b). It was evident from

the results that GSM might contain effective natural compounds that were effective in the treatment of osteoarthritis (OA) (Cobb and Ernst, 2006b). Since then GSM has been studied for its anti-inflammatory activities *in vitro, in vivo*, and in clinical trials and suggested as a supplement to treat many inflammatory conditions including asthma, osteoarthritis, and rheumatoid arthritis (Ferreira, 2005). GSM has been used as a non-prescription supplement for treating arthralgias in both humans and animals, but existing products tend to be lipid extracts and/or unstandardised (Coulson et al., 2015).

2.9.1 Bioactive metabolites of GSM

Metabolites identified in mussel meat are classified into three main fractions: lipids, proteins, and carbohydrates. These fractions have been recognized to contain various antimicrobial, anti-inflammatory, antihypertensive, and bioadhesive activities (Grienke et al., 2014) but their therapeutically effective role in OA is yet to be understood. Previous studies demonstrated that the lipid fraction has the predominant anti-inflammatory activity (Ulbricht et al., 2009). Gibson and Gibson (Gibson and Gibson, 1998) compared a lipid fraction versus a whole mussel stabilized powder extract in treating joint pain caused in OA and rheumatoid arthritis (RA). Both mussel formulations showed significant therapeutic activity in relieving joint pain with no noticeable variance between either formulation.

The metabolites present in mussel meat are variables that depends on many factors including season, life cycle, nutrition, and mussel habitat and environment, all of which can differ between harvests (Fearman et al., 2009, Narváez et al., 2008). A metabolomics

study comparing Australian BM (*Mytilus galloprovincialis*) and GSM demonstrated that taurine, glycine, lactate, succinate, homarine, ATP, ADP, valine, and leucine were higher in GSM while betaine, isoleucine, acetoacetate, and glucose were prominent in the BM (Rochfort et al., 2013). Likewise, investigation of lipid methyl ester products indicated marked differences between the species. The palmitic acid methyl ester (C16:0), cis-5, 8,11,14,17 eicosapentaenoic acid methyl ester (C20:5n3), and palmitoleic acid methyl esters (C16:1) obtained from GSM were significantly higher with overall higher lipid content. These differences are owed partially to the difference in the environmental differences between species, as a lower temperature of the water is correlated with a higher degree of unsaturated lipids (Rochfort et al., 2013). The typical nutrient composition of an extract made from whole GSM meat is presented in Table 2.1.

Table 2.1: Whole GSM (P. canaliculus) extract: A typical nutritional evaluation. Reproduced with the permission of Elsevier Science, Ltd. (Coulson et al., 2015).

General			**Amino acids**	
Crude protein (g/100 g)	56–61 (40–70)		Aspartic acid	42.8–44.0
Carbohydrate (g/100 g)	9.6–12 (NLT		Glutamic acid	51.8–58.8
Lipids (g/100 g)	10–10.8 (6–15)		Serine	18.9–19.5
Omega 3 fatty acids (EPA/DHA per	2.8–4.5 (NLT		Histidine	7.8–8.5
Saturated fat %	3.3		Glycine	40.9–43.8
Glycosaminoglycans %	3.0 (NLT 1.0)		Threonine	18.9–21.2
Ash (g/100 g)	18–21 (4–25)		Arginine	27.0–35.9
Moisture (g/100 g)	0.6–4 (0–5)		Alanine	18.3–24.5
			Valine	14.6–16.3
Vitamins			Methionine	8.5–9.5
Vitamin A (IU/100 g)	131.5–329		Phenylalanine	14.8–16.2
Vitamin D3 (IU/100 g)	272–1640		Isoleucine	16.0–17.7
Vitamin E (IU/100 g)	2.8–10.6		Lysine	29.2–51.3
Vitamin B12 (µg/100 g)	116		Leucine	17.9–23.6
			Proline	14.5–19.7
Minerals			L cysteine	6.1–6.6
Copper (mg/kg)	4.5–5.6		Tyrosine	13.9–15.4
Zinc (mg/kg)	57–62		Tryptophan	4.9–5.2
Manganese (mg/kg)	15–24			
Boron (mg/kg)	28		Heavy metals	
Chromium (mg/kg)	1.4		Lead	0.89 (NMT
Iron (mg/kg)	380–670		Cadmium	0.56 (NMT
Calcium (g/100 g)	1.3–1.5		Mercury	0.08 (NMT
Phosphorus (g/100 g)	0.84–1.25		Total arsenic	11 (NMT
Sodium (g/100 g)	3.6–4.8			
Potassium (g/100 g)				
Magnesium (g/kg)	4.9–6.8			
Nickel (ppm)	1.3			
Selenium (mg/kg)	2.5			
Iodine (mg/kg)	15.4			
Sulphur (g/100 g)	3.9			

(**Note:** NLT Not lower than; NMT Not more than; EPA Eicosapentaenoic acid; DHA docosahexaenoic acid)

Most studies to date have focused on the lipid fraction of GSM for its therapeutic activity, with no experimental evidence available demonstrating bioactivity of its carbohydrate content (Ovodova et al., 1992). Only one study assessed glycogen in carrageenan-induced OA in rats, and although glycogen from GSM was found to be significantly effective, the authors confirmed after treating glycogen with potassium hydroxide (KOH) and proteinase K that it was a protein moiety associated with glycogen rather than glycogen itself that had the anti-inflammatory activity, as after enzymatic treatment the protein was lost and glycogen showed no activity (Miller et al., 1993).

2.9.2 Bioactive proteins, peptides, and amino acids

Proteins constitute about 70% of the solids in mussel meat. Pernin, which circulates in *P. canaliculus* haemolymph, is the only bioactive protein identified in GSM to date. Pernin is a glycosylated, non-pigmented protein capable of self-aggregation. It is made up of 497 amino acids, with histidine and aspartic acid residues being predominantly high. Pernin can inhibit serine proteases but demonstrated a weak anti-thrombin activity in an *in vitro* chemical assay (Scotti et al., 2001). The whole mussel meat homogenate contains an average of 0.2 mg pernin per mussel and pernin has not been shown to have any bioactivity in humans or animal models (Scotti et al., 2001).

Antimicrobial peptides (AMPs) are found in marine mussels and have a very important immune defensive function for the host; for example, they have effective bactericidal, antifungal, antiviral, antihypertensive, anticoagulant, anti-thrombin, antioxidant, and anti-inflammatory effects (Grienke et al., 2014a). GSM powder extract prevented the

production of prostaglandins in rats (Miller and Wu, 1984) but existing evidence is not sufficient to prove the presence of AMPs in Perna species (Grienke et al., 2014). Overall, mussel protein and peptide fractions have not yet been explored for their therapeutic activity in humans.

2.9.3 Bioactive lipid fractions

Of the three major classes of metabolites in mussels, the lipid portion has been most widely explored for its health benefits, both as a nutritional and bioactive dietary supplement (Grienke et al., 2014b). The lipid fraction of GSM is subdivided into five major types: sterol esters (cholesterol and desmosterol/brassicasterol), triglycerides, free fatty acids (FFAs), sterols and phospholipids (Coulson et al., 2015) as shown in figure 2.5. The fatty acid and sterol components of GSM lipid vary with both the temperature of water in which the mussel lives and the nutrients it ingests such as marine phytoplankton, dinoflagellates and zooplankton (Murphy et al., 2003, Rochfort et al., 2013).

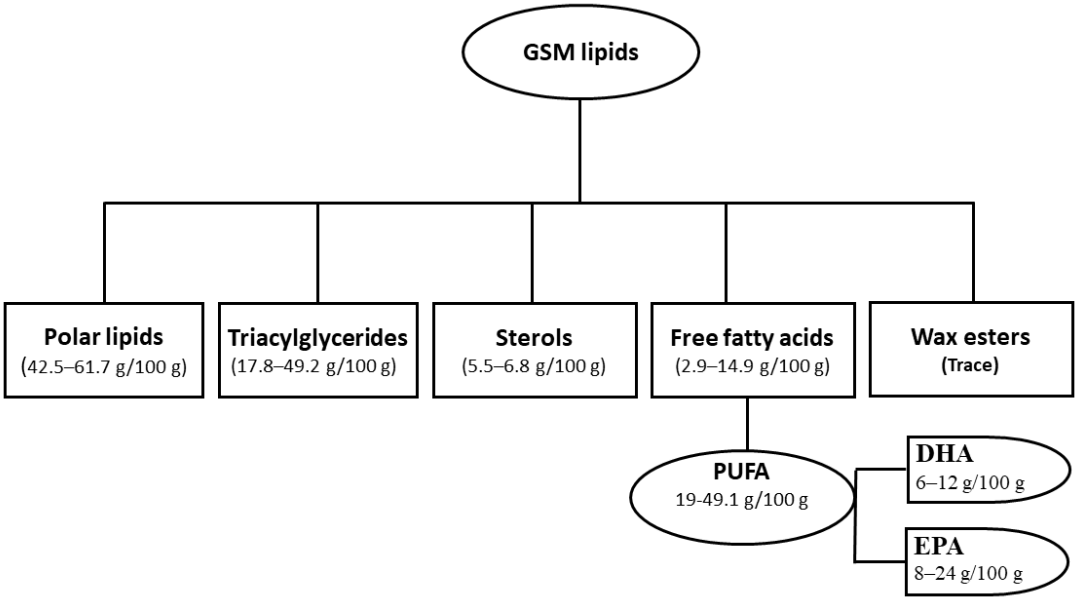

Figure2.5: Different types of lipids found in the lipid fraction of GSM (P. canaliculus)

Different methods and biochemical techniques are used for lipid extraction such as solvent extraction and chromatographic separation using fresh or freeze-dried mussel meat. Complex lipids are then subjected to enzymatic (proteases or lipases) or chemical (KOH) hydrolysis to obtain single biologically active fatty acids (McPhee et al., 2010). Whole GSM meat extracts can be used as a food ingredient, whereas the highly valuable lipid extracts are marketed as stand-alone products.

Seatone® and Lyprinol® are two GSM lipid derivations commercially available as anti-inflammatory and anti-arthritic dietary supplements. Seatone®, marketed in 1976, was the first commercially available product of freeze-dried GSM extract. The initial product's instability prompted concerns (Grienke et al., 2014b)m as these issues affected the anti-inflammatory activity of the product. This problem was solved by adding 3% tartaric acid as an antioxidant and metal chelator to the preparation directly

after shucking the mussel, which improved the consistency of the anti-inflammatory activity of the mussel (Whitehouse et al., 1997). As a result, a mussel product was introduced in a new formulation called Lyprinol® in 1998. Lyprinol® was synthesized as a GSM oil from stabilized freeze-dried mussel powder blended with vitamin E and olive oil as an antioxidant (Whitehouse et al., 1997, Singh et al., 2008). A total of 90 fatty acids, including free fatty acids (FFA) and polyunsaturated fatty acids(PUFAs) with anti-inflammatory properties, are reported to be present in Lyprinol® (Murphy et al., 2002).

PUFAs are broadly classified as omega-6 or omega-3 class, based on the position of the double bonds. Omega-6 occurs primarily in plants and is restricted to vegetable oils, while omega-3 occurs primarily in fish and shellfish but is also found in plants to a smaller extent. The omega-3 PUFAs are considered biologically more active than omega-6 (Chan and Cho, 2009). Omega-3 PUFAs with 13% eicosapentaenoic acid (EPA) and 21% docosapentaenoic acid (DHA) are the main constituents (Murphy et al., 2002) of Lyprinol®. Additionally, novel anti-inflammatory omega-3 PUFAs (5,9,12,15-octadecatetraenoic acid, 5,9,12,16-nonadecatetraenoic acid, 7,11,14,17-eicosatetraenoic acid, and 5,9,12,15,18-heneicosapentaenoic acid) have also been isolated from Lyprinol® by normal and reversed-phase chromatography and verified by GC–MS (Singh et al., 2008).

2.10 Anti-inflammatory mode of action of *Perna canaliculus*

The anti-inflammatory activity of Lyprinol® is mainly attributed to the FFAs (McPhee et al., 2007b) and it is marketed for the treatment of chronic inflammatory disorders such

as asthma, OA, and rheumatoid arthritis (RA). The anti-inflammatory mechanisms through which its activities are mediated have not been assessed and identified in clinical trials to date, although *in vitro* studies have provided some information.

Cyclo-oxygenase (COX)-II is primarily inhibited by Lyprinol® (25%), while COX-I is inhibited to a lesser extent (12%) (McPhee et al., 2007b). Therefore, it is likely that one of Lyprinol®'s therapeutic pathways involves the COX enzyme, which is implicated in the pathogenesis of asthma. Being a structural analogue of arachidonic acid (AA), a natural substrate of COX to produce prostaglandin 2 is employed in inflammatory pathways associated with asthma. It competitively replaces AA and binds to COX, thus inhibiting the production of the inflammatory mediator. This selective inhibition makes Lyprinol® suitable for treating chronic inflammatory conditions including asthma, OA, and RA, as COX-II is employed in chronic inflammation and COX-I is involved in acute inflammation (Whitehouse et al., 1997). For this reason, Lyprinol® rarely induces side effects as compared to most commonly used therapeutic options for arthritic conditions i.e. NSAIDs, which have an adverse effect on the gastrointestinal tract (GIT) due to their non-selective inhibition of both COX-I and COX-II in the stomach. Chronic NSAID use may lead to the deficiency of prostaglandins which are anti-inflammatory and cytoprotective in nature and also which also reduce the production of mucous, which can result in gastric ulcers (Toki et al., 2007).

In a study (Lee et al., 2008) involving rats induced with adjuvant arthritis, splenocyte metabolism was altered and malate dehydrogenase, an important enzyme of glucose

metabolism, was upregulated in the rats orally administered with Lyprinol®. Increased expression of malate dehydrogenase is associated with low levels of glucose in splenocytes. At a set level of glucose in these cells, formation of MHC-I (Major-Histocompatibility-Complex-I) occurs; therefore, production of active MHC-I is inhibited under conditions of low glucose, consequently reducing the progress of autoimmune diseases including arthritis. In patients suffering from RA and OA, orally ingested mussel preparations are effective in treating pain and stiffness of bones (Hyung et al., 2018). Moreover, mussel preparations are also applied topically on the affected area of the body to treat pain and stiffness (Mulye and Assoulin, 2011), but to date there have been no studies conducted to compare the results of topical application and oral use of mussel preparation.

Thus, reports in the literature collectively demonstrate that GSM lipid extracts such as Lyprinol® have proven anti-inflammatory effects. For some conditions, its anti-inflammatory mechanism is understood; however, in OA the exact mechanism of its action is unknown. Further, it is not known what cell types GSM may have bioactivity in, whether non-lipid components of GSM are bioactive, or whether the lipid in GSM retains bioactivity when it is present in an unfractionated GSM product. Therefore, studies of whole GSM in multiple cell types are warranted.

2.11 Summary of aims for studies to be performed

This research project is primarily aimed to gain insight into the mechanisms of action of whole greenshell mussel powder extracts using *in vitro* cell models of OA. The first step

in achieving this goal is to create effective *in vitro* cell models that mimic the pathophysiology of OA. Therefore, we aim to design and assess *in vitro* cell models by challenging J774A.1 macrophages, differentiated MC3T3-E1 osteoblasts and ATDC5 chondrocytes with leptin. The second step is to determine whether whole GSM-B has protective effects in these models of synovial inflammation, bone remodelling and OA chondrocytes. The expression of selected cytokines, osteogenic and chondrogenic markers in response to the treatments will be measured by using RT-qPCR. Mineralization and production of extracellular matrix molecules and alkaline phosphatase will be measured using specialized staining techniques.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Saima Rizwan
Name/title of Primary Supervisor:	Dr. Frances M Wolber
In which chapter is the manuscript /published work:	chapter Two

Please select one of the following three options:

○ The manuscript/published work is published or in press

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- Describe the contribution that the candidate has made to the manuscript/published work:

Designed and carried out all experiments; curated, analysed and interpreted data; prepared figures and text for original draft; reviewed and co-edited revisions

◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Saima Rizwan Digitally signed by Saima Rizwan Date: 2021.04.03 14:33:33 +13'00'
Date:	03-Apr-2021
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Date:	4-Apr-2021

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Chapter Three: Comparison between lipopolysaccharide and leptin as a more appropriate stimulus to mimic metabolic syndrome and osteoarthritis in murine J774A.1 macrophages

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Lipopolysaccharide (LPS) is the most common stimulant to model physiologic inflammation *in vitro*, however as this endotoxin can elicit a significant cytokine response, its use in mimicking meta-inflammation is arguable and we propose that leptin (LEP), which is an adipokine produced by adipose tissues in the body, could be a more natural stimulant to mimic mild, subclinical inflammation related to MetS or OA. To address this question, we compared LPS and LEP-stimulated J774A.1 models of inflammation for the expression of the proinflammatory cytokines *IL-1β, IL-6,* and *TNF-α* and the anti-inflammatory cytokine *IL-10*.

Abstract

Meta-inflammation is an important feature of Metabolic syndrome (MetS) and Osteoarthritis (OA). Macrophages are key players in inflammation, they can adopt M1 or M2 phenotypes depending upon the type of stimulus and secrete the pro-inflammatory cytokines; tumour necrosis factorα (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) and, anti-inflammatory cytokines interleukin-10 (IL-10) respectively. A higher M1/M2 ratio is the hallmark of MetS and OA, and a variety of factors during MetS/OA can cause the polarization of macrophages into the M1/proinflammatory phenotype, leptin (LEP) being one of them.

Macrophage based inflammatory models have long been used *in vitro* to study inflammatory conditions and related aspects. Lipopolysaccharide (LPS) is the most common stimulant to model physiologic inflammation *in vitro*, however as this endotoxin can elicit a significant cytokine response, its use in mimicking meta-inflammation is arguable and we propose that LEP, which is an adipokine produced by adipose tissues in the body, could be a more natural stimulant to mimic mild, subclinical inflammation related to MetS or OA. To address this question, we compared LPS and LEP-stimulated J774A.1 models of inflammation for the expression of the proinflammatory cytokines *IL-1β, IL-6,* and *TNF-α* and the anti-inflammatory cytokine *IL-10*.LEP and LPS both increased cytokine mRNA expression, and this peaked four hours after treatment. LPS-induced cytokine expression was significantly higher ($p<0.001$) than LEP particularly after 4 and 6 hours for all the cytokines except IL-6 which had a

significant ($p<0.01$) difference of after 6 hours between the two stimuli. LPS induced expression of IL-6 and TNF-α was significantly higher ($p<0.001$) in 0.1% DMSO as compared to CM control. The effect of LEP was dose dependent. LEP (10 μg/ml) in DMSO polarized the J774A.1 macrophages into the M1 phenotype analogous to the low-grade inflammation related to MetS and OA.

Finally, we report the successful creation of a novel LEP-stimulated *in vitro* model of inflammation to study MetS/OA related low-grade inflammation, which is compatible to be used with 0.1% DMSO as a carrier for novel test factors.

Keywords: Metabolic syndrome, inflammation, macrophages, lipopolysaccharide, leptin

3.1 introduction

Metabolic syndrome (MetS) is a major obesity-related health issue of the modern world. Once thought to be limited to a few countries, it is now considered part of the global obesity epidemic (Grundy, 2008, Saklayen, 2018). MetS includes a range of metabolic disorders, such as hypertension, insulin resistance, central obesity, atherogenic dyslipidemia, cardiovascular disease, stroke, fatty liver, cancer, and many other diseases (Rochlani et al., 2017, Saklayen, 2018). Osteoarthritis (OA), was initially believed to be associated with age-related wear and tear of articular cartilage due to mechanical stress, but is now considered to be aassociated with MetS as obesity-related metabolic factors are involved in the pathogenesis of the disease (Berenbaum et al., 2017, Dickson et al., 2019). The pathogenesis of MetS is complex and yet to be fully understood but chronic

inflammation, also called meta-inflammation, is one of the key players in the initiation, progression, and transition of MetS into a disease condition (Gregor and Hotamisligil, 2011b, Rochlani et al., 2017). Increased adiposity/obesity and systemic low-grade inflammation are the common links between MetS and OA (Dickson et al., 2019).

A key player in inflammatory processes are macrophages, which are highly plastic and can adopt different phenotypes depending upon the nature of the stimuli. M1 and M2 are the two important phenotypes that are associated with the production of the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and anti-inflammatory cytokine interleukin-10 (IL-10) respectively. These cytokines are often studied because they are linked to each other, as well to thousands of genes associated with inflammation, through the common denominator of being regulated by NF-κB signalling (add this reference https://www.frontiersin.org/articles/10.3389/fimmu.2019.00705/full). A multitude of studies has proved the importance of activated macrophages in OA (Berenbaum, 2013, Woodell-May and Sommerfeld, 2020a) and their number is significantly related to pain and joint space narrowing. Additionally, biomarkers of activated macrophages can be used to predict the severity of disease progression in OA patients (Daghestani et al., 2015, Dickson et al., 2019). Early insult to cartilage releases damage-associated molecular patterns (DAMPs), which are recognized by the pattern recognition receptors (PRRs) on macrophages and result in the subsequent production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) through activation of the NF-κB pathway. Pro-inflammatory cytokines finally degrade cartilage by targetting different components of

the cartilage (Bondeson et al., 2010). A higher M1/M2 ratio is considered as an important indicator of MetS and OA. Macrophages are present in several metabolic tissues in the body including adipose tissues and synovium. Various features of MetS can alter proliferation, plasticity, and polarization of macrophages from M2 to M1, such as dietary factors (disturbance in cellular metabolites), and systemic factors where adipokines are the most important (Lumeng et al., 2007).

Leptin (LEP) is the most extensively studied of adipokines produced by white adipose tissues that regulate a multitude of physiological processes in mammals including lipid metabolism, appetite, growth, reproduction, stress, immune function (Deck et al., 2017) and also regulate energy homeostasis by acting on the hypothalamus (Koskinen et al., 2011). It has receptors in many cell types including monocytes and macrophages (Loffreda et al., 1998, Frühbeck, 2006), where it increases the expression of proinflammatory cytokines such as *TNF-α* and *IL-6*; however, the underlying mechanisms are not defined. LEP is known for its pro-inflammatory role in the activation of macrophages. It promotes the proinflammatory phenotype by increasing the secretion of TNF-α and IL-1β in macrophages by acting through JAK2-STAT3 and PI3K-AKT-mTOR pathways (Monteiro et al., 2019).

The pro-inflammatory cytokines IL-1, TNF-α, and IL-6 are the most important cytokines with respect to their role both in obesity and OA pathogenesis. During OA, their levels are elevated in synovial fluid, cartilage, and subchondral bone and can directly damage the cartilage or indirectly disturb cartilage homeostasis by activating other cytokines and

catabolic factors including LEP (Wang and He, 2018). In contrast, the anti-inflammatory cytokine IL-10 inhibits the production of pro-inflammatory cytokines specifically IL-1β and TNF-α in stimulated cells (Alaaeddine et al., 1999), and in addition to IL-10 also regulates osteogenesis and chondrogenesis by enhancing osteogenic and chondrogenic proliferation and differentiation and by inhibiting bone resorption (Jung et al., 2013, Wojdasiewicz et al., 2014b, Yuan et al., 2021).

OA related inflammation is mild and chronic, which is quite different from rheumatoid arthritis (RA) which is a classical inflammatory disease. The immune response during OA not only varies in its degree of severity but also with respect to the cellular and molecular mechanisms involved (de Lange-Brokaar et al., 2012). OA primarily involves innate inflammatory response unlike to prototypical inflammatory RA. Moreover, levels of inflammatory plasma proteins and synovial fluid are higher in RA as compared to OA (Haseeb and Haqqi, 2013, Robinson et al., 2016). That is why RA related anti-inflammatory therapies are not successful in targeting OA related inflammation, and novel mechanisms are required to target inflammation in OA (Robinson et al., 2016).

In vitro research is an important tool to study disease-related mechanisms and macrophages are an important target of anti-inflammatory therapy in MetS and related conditions (Philp et al., 2016). Murine macrophages J774A.1 are widely reported as a model used in inflammation research (Rajaram et al., 2014, Seyedi et al., 2018, Yamazaki et al., 2020), and of particular interest are the critical functional and survival activities that are jointly controlled by NFkB signalling (Liu et al., 2017)

In many *in vitro* studies, lipopolysaccharide (LPS) is used as a stimulus to activate the M1 phenotype in macrophages and to model physiological inflammation to gain insights into various inflammatory conditions, and to evaluate the anti-inflammatory potential of novel compounds *in vitro* (Rabe et al., 2015, Hung et al., 2019b). LPS is a heat-resistant endotoxin and a component of the gram-negative bacterial cell wall, known for inducing systemic inflammatory response by acting through Toll-like receptor 4 (TLR4), which may lead to cell damage, shock, and possibly multiple organ failure (Chae, 2018b).

Very low doses of LPS, i.e. up to 5 pg/ml in blood are normally present in healthy individuals without producing any harmful effects (Wassenaar and Zimmermann, 2018). While low doses are considered beneficial in generating host immune responses and the removal of invading pathogens, high doses can initiate a cascade of inflammatory responses, which can ultimately lead to cell death, tissue injury, and organ failure (Pestka and Zhou, 2006). LPS is the most commonly used stimulus for macrophages and monocytes both *in vivo* and *in vitro*, used in varying doses depending upon the aims of the studies and other experimental conditions (Fang et al., 2004, Li et al., 2014b, Monguió-Tortajada et al., 2018, Kamperschroer et al., 2019). Data regarding the optimal LPS dose needed to induce the M1 macrophage phenotype are inconsistent in the literature; however, doses $\leq$5 pg/ml were unable to induce an inflammatory response while a higher dose (~100 ng/ml) of LPS is generally considered optimal to simulate a robust and physiologically relevant model of inflammation *in vitro* (Xiong and Medvedev, 2011, Deng et al., 2013, Martin et al., 2015). Therefore, based on previous literature we selected 100 ng/ml of LPS to induce an inflammatory response. Although

100 ng/ml can induce inflammation in *in vitro* cell lines, we hypothesized that physiologically it is not an appropriate stimulus to mimic the low-grade inflammatory response associated with metabolic syndrome and OA, as physiologically high doses of LPS in the body are correlated to sepsis (Meng and Lowell, 1997).

Fasting serum LEP levels of healthy individuals are highly variable across different populations and are typically higher in women than men, however, <15 ng/ml is generally considered normal, and >15 ng/ml is considered high (Askari et al., 2010). Physiologic levels of LEP are reported to exert anti-inflammatory rather than pro-inflammatory effects (Flatow et al., 2017). LEP is the most important adipocyte-derived adipokine with respect to its regulatory roles in energy metabolism and controlling the interplay between metabolism and the immune system. It is known to have receptors throughout the immune system and regulates both innate and adaptive immunity (Francisco et al., 2018). It is reported to stimulate the production of pro-inflammatory cytokines in macrophages and natural killer cells During OA, LEP levels are found to be elevated by adipose tissues and due to local production in both the cartilage and joint (Dumond et al., 2003a, Simopoulou et al., 2007). A study in a group of middle-aged women, which revealed that LEP serum levels, 10 years before an MRI diagnosis of OA, were associated with cartilage defects, bone marrow abrasions, osteophytes, meniscal anomalies, synovitis, and joint effusion, further supports the clinical relevance of LEP in OA (Karvonen-Gutierrez et al., 2014).

An appropriate model is key to understand the molecular mechanisms related to disease and for the development of new therapies. Due to the damaging attributes of LPS and the inflammatory cytokine "storm" that follows LPS cell activation *in vitro*, we propose that the use of LPS to mimic mild, subclinical inflammation related to MetS or OA is questionable and low-grade inflammation followed by LEP induction is physiologically more relevant to model meta-inflammation in OA and MetS. This study was designed to mimic meta-inflammation or OA related low-grade inflammation *in vitro* and we hypothesized that using LEP rather than LPS to activate J774A.1 cells will induce a pattern of cytokine production that is more closely related to what is seen in humans with obesity, metabolic syndrome, chronic inflammation, and osteoarthritis.

There is currently a scarcity of studies that examine the *in vitro* use of LEP to stimulate an immune response. Therefore, this study aimed to compare the cytokine response of murine macrophages J774A.1 treated with LPS or LEP-induced. Moreover, there is no standardized protocol for LEP treatment, so, this study alsod compare the temporal and dose-dependent responses of cytokine expression in LEP- stimulated J774A.1 macrophages in complete medium (CM) and CM containing 0.1% DMSO, a well-known vehicle in cell culture studies.

3.2 Materials and methods

3.2.1 Reconstitution of LPS and leptin for *in vitro* use

Leptin (recombinant mouse leptin OB) was purchased from Biomyx (San Diego, California) and LPS from Sigma Aldrich (Merck, NZ). Both were reconstituted in sterile phosphate buffer saline (PBS; Sigma Aldrich, Merck, NZ) according to manufacturer's instructions, aliquoted, and stored at -20°C until further use.

3.2.2 Reagents and cell culture

The murine macrophage cell line J774A.1 was purchased from American Type Culture Collection (ATCC Virginia, USA). The cells were grown and maintained in complete medium (CM), i.e. DMEM (Gibco, Life Technologies, Auckland, NZ) and 10% heat-inactivated foetal calf serum (FCS) (Gibco, Life Technologies, Auckland, NZ) supplemented with 1% penicillin-streptomycin and glutamine (Invitrogen, Life Technologies, Auckland, NZ). Subcultures were obtained twice a week by scrapping. At confluence, cells were transferred to culture plates for treatment. Cells were grown and maintained at 37°C in a humidified atmosphere of 5% CO_2.

3.2.3 Cell viability assay

The MTT assay is a commonly used colorimetric assay for evaluating the NAD(P)H-dependent cellular oxidoreductase dependent metabolic activity of viable cells. The assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan. The assay was performed as described previously (Michel et al., 2013a), to determine the effects of LPS and LEP with 0.1% DMSO (Sigma Aldrich, Merck, NZ) on the viability of metabolically active cells. 100 ng/ml LPS, a dose reported to be effective and non-cytotoxic by multiple research groups (Mohamad et

al., 2014, Kim et al., 2015, Lv et al., 2021) and four different concentrations of leptin (0.05-100 µg/ml), were assessed for their effect on the viability of metabolically active cells in CM and CM+0.1% DMSO. Briefly, J774A.1 were plated at 50,000 cells/well in standard tissue-culture plastic 96-well plates, left for 2 hours to adhere, and then treated (4 replicates per treament group). After 24 hours of incubation, 10 µl of MTT (Sigma Aldrich, Merck, NZ) was added to each well at a final concentration of 0.5 mg/ml and incubated for three hours at 37°C in 5% CO_2 until purple crystals were microscopically visible, which were solubilized by adding DMSO to a final concentration of 50%. Absorbance was quantified by measuring optical density (OD) at a wavelength of 550 nm using a microplate reader (Multiskan Fc, Thermo Scientific).

3.2.4 Macrophage stimulation and cytokine mRNA quantification

J774A.1 macrophage mRNA was assessed for pro-inflammatory cytokines (*IL-1β, TNF-α*, and *IL-6)* and the anti-inflammatory cytokine *IL-10*. J774A.1 were plated in 6-wells plates at 2 x10^6 cells/well. After a 2 hour incubation, cells were treated with LPS (100 ng/ml) as described previously (Huang, 2012; Martin, 2015) and LEP (10 µg/ml) as described above; total RNA was extracted at 4, 6, 16, 24, or 48 hours. For the 48 hour time point, treatment was applied to the cells at the 0 and 24 hour time points, to see the cumulative effect of the repeated treatments on the cytokine response over a relatively longer period.

The solvent and the dose-dependent response of the LEP were measured concomitantly in a single experiment and J774A.1 were treated with 100 ng/ml of LPS and 0.05, 0.1, 10

and100 µg/ml of LEP in CM and CM+0.1% DMSO. Total RNA was extracted from the cells after 4 hours of incubation using an RNA extraction kit (PureLink RNA Mini Kit, Thermo Fisher, Auckland, NZ). 2 µg of RNA was reverse transcribed using SuperScript® IV First-Strand Synthesis System (Invitrogen, Thermo Fisher, Auckland, NZ). RT-qPCR was done with gene-specific primers (Invitrogen, Life Technologies, Australia) using a PCR kit (LightCycler® 480 SYBR Green I Master, Roche Life Science, Thermo Fisher, Auckland, NZ). Relative expression of the genes was quantified by the E-method of advanced relative quantification. Each test condition was assessed in a single well within an experiment, and each experiment was carried out two or three times. Finally, 10 µl of the PCR products were separated on a 2% agarose gel and visualized under UV light. *GADPH* (Glyceraldehyde 3-phosphate dehydrogenase) was used as an internal reference gene. A list of primers and their annealing temperatures is given in table 3.1.

Table 3.1: List of primers and their annealing temperatures

Primer	Forward sequence (5'→3')	Reverse sequence (5'→3')	Ta (°C)
GADPH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	55
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	65
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA	60
IL-10	TGAGGCGCTGTCGTCATCGATTTCTCCC	ACCTGCTCCACTGCCTTGCT	65
TNF-α	CACGTCGTAGCAAACCACCAAGTGGA	TGGGAGTAGACAAGGTACAACCC	60

(**Note:** *GADPH* = glyceraldehyde-3-phosphate dehydrogenase, *IL-1β* = Interleukin beta, *IL-6* = Interleukin 6, *IL-10* = Interleukin 10, *TNF-α* = Tumour necrosis factor alpha), Ta = annealing temperature

3.2.5 Data Analysis

All the experiments were performed two or three times and data were presented as mean ± standard error. Treatment groups were compared with the control group (unstimulated) by one-way and two-way ANOVA. GraphPad Prism (San Diego, USA) was used for statistical analysis and statistical significance was considered at $p \leq 0.05$.

3.3 Results

3.3.1 Effects of LPS and LEP on the viability of the cells

Cell viability of J774A.1 macrophages treated with LPS and LEP in CM or medium containing 0.1% DMSO was evaluated by the MTT assay. As shown in figure 3.1, there was no significant effect of any treatment on the viability of cells except 100 µg/ml LEP, which was cytotoxic both in CM and 0.1% DMSO and significantly suppressed the

viability of murine macrophages both in CM and 0.1% DMSO as compared to the 0.1% DMSO control. Lower doses of LEP i.e. 10, 0.1, and 0.05 µg/ml of LEP did not alter the viability of the cells significantly. A small, dose-dependent reduction in the viability of cells in both CM and 0.1% DMSO was observed, with the effect being more pronounced in 0.1% DMSO, but these did not reach statistical significance. Overall, no significant solvent effect was observed in any of the treatment groups.

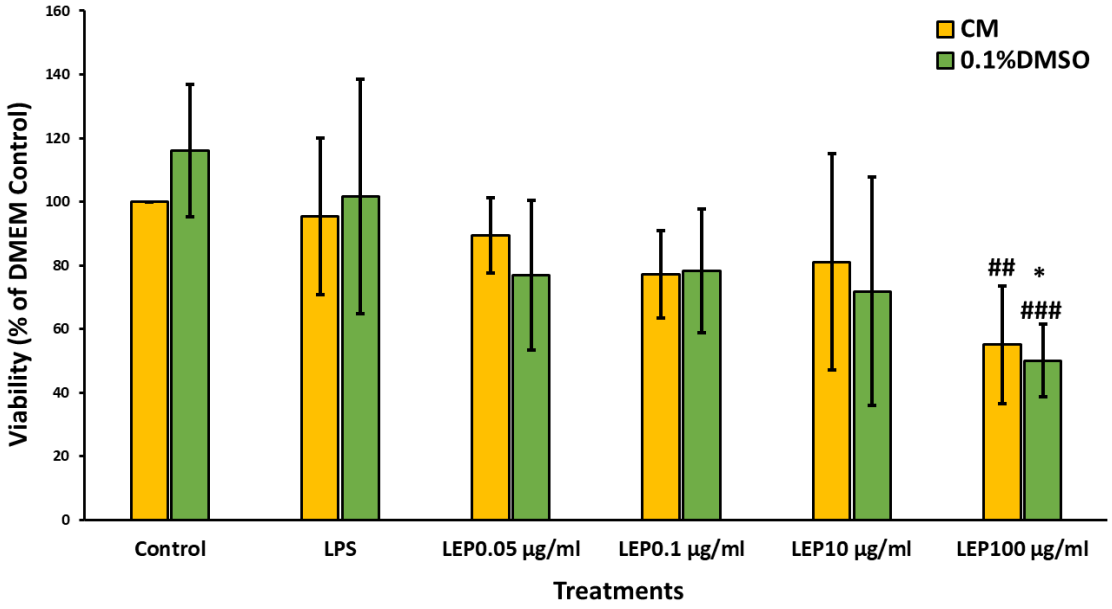

Figure 3.1: Effect of LPS and LEP treatment on the viability of metabolically active J774A.1 macrophages. Viability was evaluated by MTT assay after 24 hours incubation of 50,000/well of J774A.1 cells with 100µg/ml-0.05 µg/ml of leptin and 100 ng/ml LPS. The values represent the mean ± SD of two independent experiments, (n=8). Data were analysed by one-way ANOVA followed by Tukey's test, *p<0.05 signifies difference from CM control, ##p<0.01, and ###p<0.001 signifies difference from 0.1% DMSO control.

3.3.2 Temporal effects of LEP and LPS on cytokine mRNA expression

The effects of LPS (100 ng/ml) and LEP (10 µg/ml) on cytokine expression were measured at 4, 6, 16, 24, and 48 hours by RT-qPCR, with the LEP dose chosen being the highest dose that had no significant cytotoxicity. Cytokine expression peaked at 4 and 6 hours (figure 3.2) and declined thereafter, with a second increase observed at 48 hours when the cells were re-stimulated at the 24 hour time point.

LPS, as expected, induced the greatest production of all four cytokines assessed. LPS significantly increased ($p<0.001$) expression of all cytokines after 4 hours with the response persisting up to 6 hours while *TNF-α* continued to show a small but significant increase in expression at all time points (figure 3.2). LEP also had a significant ($p<0.001$) effect, upregulating the expression of all cytokines after 4 hours.

LPS increased expression of all cytokines to levels significantly higher ($p<0.001$) than with LEP particularly after 4 and 6 hours. Cytokine expression decreased to near-baseline by 24 hours with both LPS and LEP treatments. After restimulation and an additional 24 hours of incubation, LPS again stimulated a significant increase in the expression of only the pro-inflammatory cytokines measured at the 48 hour time point. Moreover, at 48 hours the difference between LPS and LEP-induced expression of pro-inflammatory cytokines was further magnified and LPS induced expression of *IL-1β* and *TNF-α* was significantly higher than LEP. LEP-induced expression appeared to be slightly increased after 48 hours but the effect was not significant except for *IL-10* which was significantly increased after the second induction.

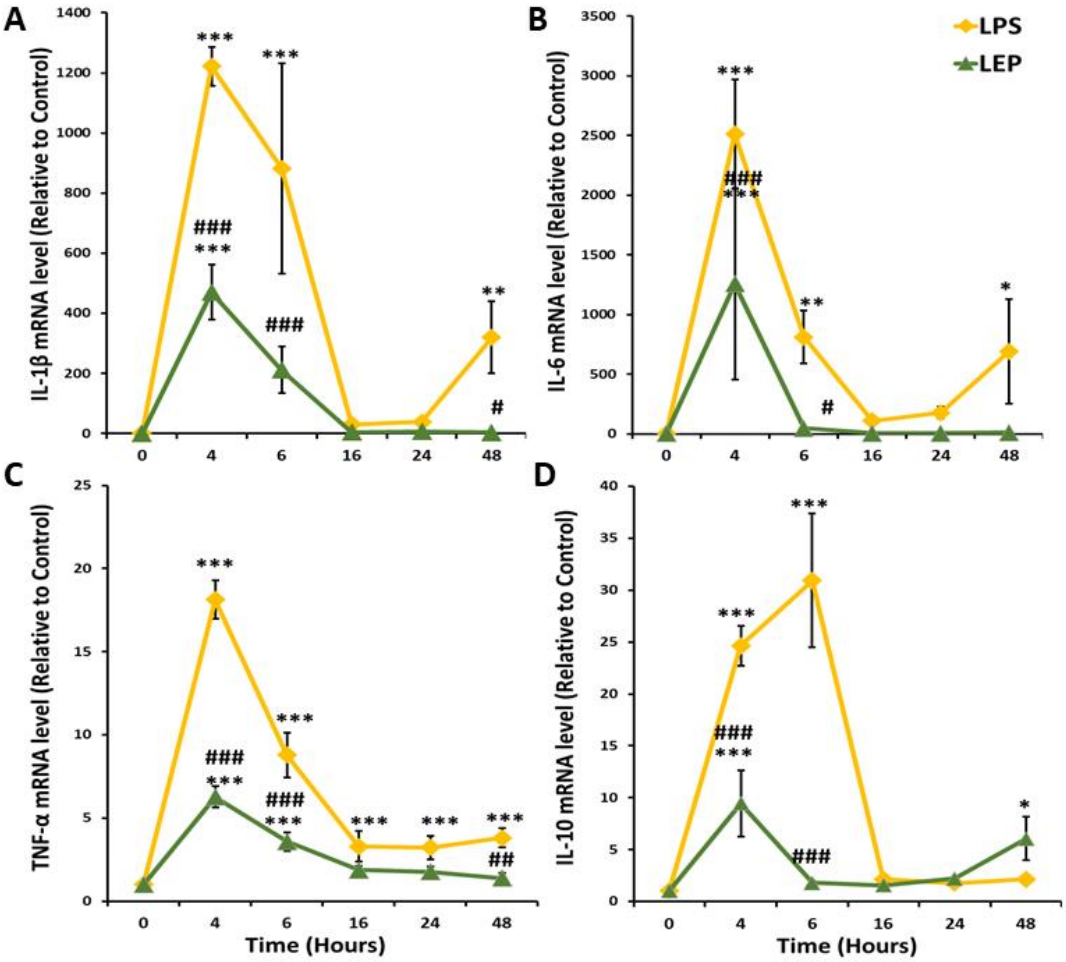

Figure 3.2: Effect of LPS and LEP on the expression of cytokines (IL-1β, IL-6, TNF-α and IL-10) (panels A-D respectively) in J774A.1 macrophages. Cytokine expression was evaluated using RT-qPCR at 0, 4, 6, 16, 24 and 48 hours after incubation of leptin (LEP; 10 µg/ml) or lipopolysaccharide (LPS; 100 ng/ml) with J774A.1 cells ($1x10^6$ cells/well in 6 well plates). The data were analysed by two-way ANOVA (repeated measures) followed by Bonferroni's multiple comparisons. All values represent mean ± SEM of three independent experiments (n=3). #p<0.05, ##p<0.01, and ### p<0.001 signifies a statistical significance difference between LPS and LEP at a given time point; *p<0.05, **p<0.01, and ***p<0.001 signifies significant from the control.

3.3.3 Solvent and the dose-dependent response of cytokine mRNA expression

The effects of LPS (100 ng/ml) or each of four doses of LEP (0.05, 0.1, 10, and 100 µg/ml) on cytokine expression were measured by RT-qPCR following 4 hours of incubation with J774A.1 macrophages in CM or CM containing 0.1% DMSO. As shown in figure 3.3, CM+0.1% DMSO slightly stimulated the production of cytokines as compared to the CM control (which is normalized to one). LPS induced the largest increase in expression in all four cytokines assessed. It significantly increased the expression of all the cytokines in CM as well as in CM+0.1% DMSO; however, in CM+0.1% DMSO the LPS-induced expression was higher compared to CM only and the effect was statistically significant ($p<0.001$) for the expression of IL-6 and *TNF-α*. LPS-induced expression of *IL-6* was markedly high in CM+0.1% DMSO.

LEP induced expression of cytokines was dose-dependent and relatively reduced in CM+0.1% DMSO apart from IL-6, which was expressed more in 0.1% DMSO with 100 µg/ml of LEP. Although CM+0.1% DMSO seems to have an obvious reduction in cytokine expression in the cells induced with 10 µg/ml of LEP, the effect was not statistically significant in either of the LEP treatments. At higher doses i.e. 10 and 100 µg/ml LEP, the cytokine response was high and 100 µg/ml of LEP produced a cytokine response comparable to LPS in cells grown in medium only (CM). Both the higher doses produce a significant increase in the expression of *IL-1β, TNF-α,* and *IL-10.* Lower doses i.e. 0.05 and 0.1 µg/ml did not produce any noticeable change in cytokine expression and the effect of 0.05 µg/ml LEP was almost equal to CM+0.1% DMSO. No significant increase in the expression of any cytokine was observed with 0.05 or 0.1 µg/ml of LEP in the presence or absence of 0.1% DMSO; however, cytokine expression was considerably

higher with 10 and 100 μg/ml to represent the activated/stimulated state of J774A.1 macrophages.

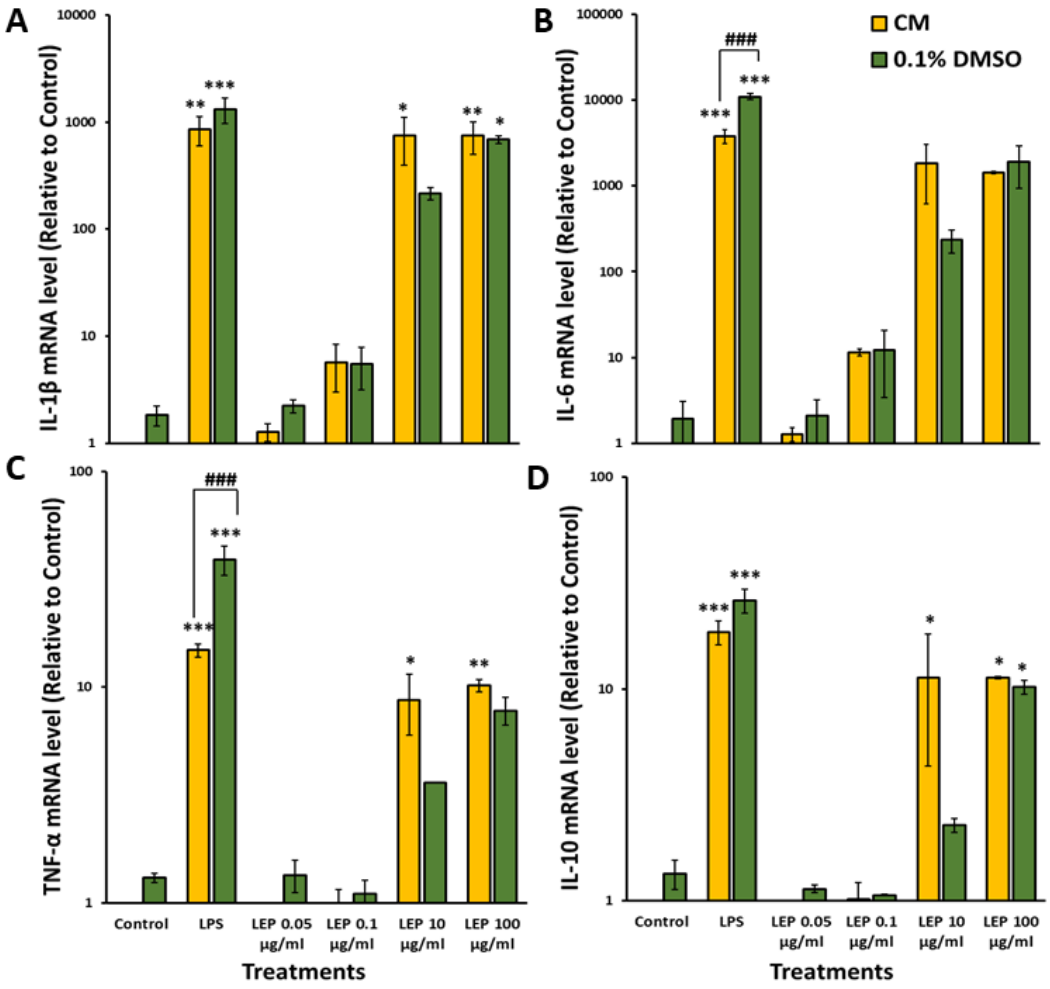

Figure 3.3: Effects of LPS and LEP on the expression of cytokines in J774A.1 macrophages. Cytokine expression was evaluated by RT-qPCR in response to LPS (100 ng/ml) and four doses of LEP (100 µg/ml-0.05 ng/ml) in medium only (CM) and CM+ 0.1% DMSO. 1x106/well of J774A.1 cells were incubated in 12 well plates with 100 ng/ml LPS and 100 µg/ml-0.05 µg/ml leptin (LEP) for 4 hours followed by RNA extraction. Data were analysed by Two-way ANOVA followed by Bonferroni's multiple comparisons and the values represent mean ± SEM of two independent experiments, (n=2). *p<0.05, **p<0.01, and ***p<0.001 signifies difference from CM control and ### p<0.001 signifies difference between CM and 0.1% DMSO. Values are represented on a logarithmic scale.

3.4 Discussion

This is the first study to our knowledge that models meta-inflammation in J774A.1 macrophages using LEP as a stimulus. Also, it is first to compare LPS and LEP for their ability to polarize macrophages into the M1 phenotype. We observed that LEP produced a less pronounced and more physiologically representative inflammatory response compared to LPS.

Macrophages detect a wide variety of inflammatory signals, including those produced by the body and those derived from microbes, such as LPS (Borregaard, 2010). LPS is a strong activator of monocytes and macrophages, triggering a cytokine cascade that includes the production of TNF-α, β, and IL-6, leading to sepsis (Meng and Lowell, 1997). However, LPS may not be a physiologically relevant stimulant in osteoarthritis. We hpothesised that LEP would be a more relevant stimulus to simulate obesity-related inflammation and MetS, as LEP is an adipokine whose levels are correlated with excessive body fat and are reported to have implications in obesity and related complications (Al-Amodi et al., 2018).

As little is known about the *in vitro* use of LEP to induce inflammation this study also aimed to optimize a protocol for *in vitro* LEP treatment. Two high and two low doses of LEP were used for this purpose, i.e. 10 and 100 versus 0.05 and 0.1 µg/ml and studied for their effects on the expression of the selected cytokines preceded by the MTT assay to assess cytotoxicity on J774A.1 macrophages.

3.4.1 Effects of LPS and LEP on the viability of the cells

We found that except for 100 µg/ml of LEP, neither LPS nor LEP with or without DMSO negatively impacted the viability of cultured macrophages (fig 3.1) according to the norms of the International Organization of Standardization, which considers a substance to be cytotoxic when it reduces cell viability to less than 70% (Iso, 2009). In the published literature, the effects of LPS and LEP on viability are dose, time, and cell-type dependent (Stone et al., 2003, Haque et al., 2018). Moreover, at lower doses, LEP is reported to increase cell proliferation (Kabadere et al., 2007, Haque et al., 2018). In contrast to this, our results showed a dose-dependent decrease in cell proliferation with all the doses of LEP in CM as well as in 0.1% DMSO. In the literature 100 ng/ml of LPS is also reported to be non-cytotoxic (Stone et al., 2003). As shown by the MTT assay in this study, none of the treatments showed any significant cytotoxic effect except the highest dose of LEP (100 µg/ml), which significantly reduced the viability of the cells both in CM and 0.1% DMSO as compared to unstimulated cells cultured in complete medium containing 0.1% DMSO. Although 0.1% DMSO did not produce any significant cytotoxic effects, it did reduce the viability of LEP treated cells and the effect was correlated with the dose of LEP, as a significant decrease in viability was observed in the cells treated with the highest dose (100 µg/ml). The addition of 0.1% DMSO decreased the viability of the cells treated with 100 µg/ml of LEP by 50% as compared to their CM counterpart. Similarly 10, 0.1 and 0.05 µg/ml of LEP reduced the cell viability by 28%, 22% and 22% respectively in 0.1% DMSO as compare to CM. Though DMSO slightly affected the viability of cells, there was no solvent effect observed overall, except when combined with 100 µg/ml of

LEP. In the literature, cytotoxic effects of DMSO are controversial and mostly depend on the dose, type of cells, and other experimental conditions (Timm et al., 2013, Jamalzadeh et al., 2016) but 0.1% DMSO is usually well tolerated with no observable toxic effects to cells at a final concentration of 0.1% and thus it is widely used as a solvent for various pharmacological agents at concentrations of 0.05–1.5% (Jamalzadeh et al., 2016, Villarroel et al., 2020). Moreover, it is placed in the safest category of class 3 solvents according to the Q3C solvent classification system, which means they are less toxic and have less risk to human health (Jamalzadeh et al., 2016).

3.4.2 Temporal profile of cytokine mRNA expression

Results from our temporal response experiment showed that time was a critical factor for analysing cytokine mRNA expression in J774A.1 macrophages. mRNA expression of all the cytokines peaked at 4 hours after induction with both LPS and LEP as shown in the figure 3.2. Both stimuli induced significant expression of cytokines after 4 hours but as expected LPS-induced cytokine expression was the highest. LPS induced cytokine expression lasted longer and remained significantly elevated up to 6 hours. The difference between LPS and LEP induced cytokine expression was significantly different after 4 and 6 hours of induction, LPS significantly augmented the expression of all the cytokines after 4 and 6 hours as compared to LEP. This is the first study to characterise LEP in this manner *in vitro*, and our findings with LPS are supported by a previous study, which reports 4 hours as the optimal time point for the induction of cytokines in J774A.1 macrophages (Huang, 2012).

Repeated stimulation of J774A.1 was performed for a relatively long period (48 hours) in such a way that cells were exposed twice to the stimuli without changing the existing medium over 48 hours to determine whether there may be cumulative effects of the stimuli on the expression of cytokines. This was done to best mimic what occurs *in vivo*, where continuous or periodic exposure to LPS and/or LEP would be expected. LPS diffuses into the blood from the bacteria residing in the gut and exogenous sources including food and food supplements (Wassenaar and Zimmermann, 2018). Similarly, LEP is secreted into the blood by adipose tissue and its concentration also changes in response to different stimuli such as food, BMI, and different pathophysiological conditions (Cammisotto and Bukowiecki, 2002, Romon et al., 2003, Lin et al., 2011). We found that after 48 hours of induction created by two serial stimulations 24 hours apart, the cumulative/chronic effect of two treatments was more noticeable in the LPS-stimulated group. A significant increase in the expression of only inflammatory cytokines was observed after 48 hours of exposure to LPS. A previous study reported similar findings where pre-exposure of RAW 264.7 macrophages and peritoneal macrophages with 100 ng/ml LPS made them more sensitive to induce proinflammatory cytokines upon secondary exposure to other toxins (Pestka and Zhou, 2006). A similar study described that repeated exposure to LPS is required to maintain the expression of pro-inflammatory cytokines and the activated state of blood cells can be restored to normal after LPS neutralization with plasma components (Dedrick and Conlon, 1995). The neutralization time of LPS is different under different conditions depending on the cell types, LPS dose, plasma proteins, and LPS receptors and co-receptors (Dawson, 1998).

The decline in cytokine response of J774A.1 macrophages in this study might be due to the neutralization of LPS over time until they received a second dose of LPS. The first exposure of cells to LPS for 24 hours increased *IL-1β, IL-6, TNF-α,* and *IL-10* by 39.7-, 117-, 3.2-, and 1.7-fold compared to baseline respectively. Although these were not statistically significant, this exposure may have made the cells more responsive to secondary exposure, as the cumulative effects of the two LPS treatments resulted in a significant increase of pro-inflammatory cytokines after 48 hours. By 48 hours, *IL-1β, IL-6, TNF-α,* and *IL-10* mRNA levels raised to 706%, 290%, 17%, and 20% respectively as compared to their mRNA levels after 24 hours. LPS induced *TNF-α* expression remained low overall but was significantly elevated at all the time points and persisted after 48 hours. After 48 hours, the difference between the cytokine profiles of LPS and LEP stimulated cells increased. LPS induced cytokine expression seemed to be more augmented as compared to LEP, since repeated exposure did not produce a similar hyper-responsive state in LEP treated cells as observed in LPS treated cells. The decline in LEP-induced cytokine expression was more marked after the 4 hour peak compared to LPS, and thus is more representative of the physiological acute low-grade inflammatory response. After four hours LEP induced cytokines were significantly elevated, followed by a gradual decrease at 6 hours after which the expression remained near baseline and even a second exposure for 24 hours to LEP did not change the profile.

However, it must be acknowledged that cytokine levels were not tested at 48 hours in cells that did not receive re-stimulation at 24 hours, so it is possible that the effects seen

at 48 hours occurred solely due to the initial stimulation at time 0. It would be of interest to conduct a comparative study to verify or rule out this alternative explanation.

LEP effects on cytokine expression appeared to have a narrower timeframe than LPS. This could be due to a shorter half-life of LEP. Mouse plasma LEP has been found to have a half-life of 40.2 (+/− 2.2) minutes and mice injected with LEP showed a maximum increase in plasma LEP concentration after 1 hour, and decreased to baseline after 4 hours (Burnett et al., 2017). Hence, continued exposure to LEP is necessary to achieve a persistent inflammatory response with LEP. Studies employing animal models have introduced LEP every hour to induce a systemic inflammatory response (Flatow et al., 2017). In our study, the effects of LEP persisted for longer (6 hours) likely because the *in vitro* system does not exactly recapitulate the *in vivo* physiology. The second exposure of LEP did not greatly affect pro-inflammatory cytokines while anti-inflammatory cytokine expression was relatively increased after 48 hours as compared to their levels after 24 hours. The proportional change between the cytokine expression of the LPS induced expression between 24 and 48 hours, following the second stimulatory treatment, was very high. LPS induced expression of cytokines at 48 hours was increased by 7590% for IL-1β, 5306% for IL-6, and 175% for *TNF-α,* while *IL-10* expression was decreased by -65%, compared to their levels at the 24 hour time point. This large increase in the inflammatory response induced by LPS resembles septic shock rather than chronic low-grade inflammation and further confirms our hypothesis of LEP being the more appropriate stimulant to mimic meta-inflammation.

3.4.3 Solvent and the dose-dependent response of cytokine mRNA expression

To further optimize the LEP treatment protocol we compared the cytokine expression of J774A.1 macrophages induced with LPS and multiple doses of LEP in CM and 0.1% DMSO. 0.1% DMSO alone had no significant effect on the expression of cytokines as compared to the CM control and therefore can be considered to be neither cytotoxic nor a functional stimulant and is therefore a suitable solvent for these assay conditions. LPS-induced expression of the cytokines was the highest under both conditions. LPS significantly induced the expression of all the cytokines in CM as well as in 0.1% DMSO but the response was more prominent in the latter. 0.1% DMSO significantly increased the LPS induced expression of *IL-6* and *TNF-α*. There is conflicting evidence in the literature regarding the effects of DMSO on the expression of cytokines. Some studies report that DMSO decreases cytokine expression (Kelly et al., 1994, Elisia et al., 2016b, de Abreu Costa et al., 2017), while Xing and Remick (2005) reported that DMSO augmented the LPS stimulated production of *IL-1β* without affecting *IL-6* and *TNF-α* (Xing and Remick, 2005). LEP on the other hand has shown dose-dependent effects on the increase in the expression of chemokines in J774A.1 cells (Kiguchi et al., 2009) both in CM and 0.1% DMSO, but overall expression was reduced in 0.1% DMSO. Our findings are in line with previous studies listed above which report that DMSO decreases inflammation.

LEP (100 µg/ml) had the maximum effect on the expression of cytokines both in CM and 0.1% DMSO but the effect was more prominent in CM, followed by LEP (10 µg/ml), which significantly upregulated the expression of *IL-1β, TNF-α,* and *IL-10*. Our results agree

with Shivahare et al, who reproted that LEP significantly increased *TNF-α* mRNA expression in J774A.1 macrophages at 10 and 15 μg/ml (Shivahare et al., 2015). In the current study, no significant increase in the expression of any cytokine was observed with 10 μg/ml of LEP in the medium containing 0.1% DMSO; however, with both 100 and 10 μg ml of LEP the cytokine expression was markedly elevated. 100 μg/ml of LEP was relatively cytotoxic especially in 0.1% DMSO (figure 3.1). The lower doses of LEP (0.1 and 0.05 μg ml) may be physiologically more relevant, but they did not produce inflammation *in vitro*; this may be due to a macrophage-only *in vitro* system not containing all of the complex intercellular interactions that are present in blood and tissues *in vivo*. It has been demonstrated that *in vivo* and in *ex vivo* primary cell cultures are more sensitive to stimuli as compared to established cell lines of matching lineages (Welser, 2015). The established cell lines require a higher concentration of a treatment to produce effects observed at lower concentrations *in vivo* or with primary cells. For this reason, we chose to proceed further with the higher concentration of 10 μg/ml of LEP. The effects stimulated by this dose represented low-grade inflammation, which is the hallmark of MetS and OA (Gregor and Hotamisligil, 2011a). In medium containing 0.1% DMSO 10 μg/ml of LEP increased the mRNA expression of *IL-1β, IL-6, TNF-α*, and *IL-10* up to 217-, 234-, 4-, and 2-fold respectively.

3.5 Conclusion

In conclusion, this study is the first to establish that LEP is a physiologically more relevant stimulus to mimic MetS/OA related low-grade inflammation *in vitro* as compared to LPS,

which induces a nonspecific, general, and exaggerated inflammatory response. Time is a crucial consideration for mRNA analysis and 4 hours was the optimal time point for the expression of all cytokines measured. Furthermore, it is safe to use 0.1% DMSO, as this concentration produced no significant effects on cell viability and or cytokine expression. LEP affected cytokine expression dose-dependently and we found 10 μg/ml of LEP in 0.1% DMSO to be appropriate to mimic physiologic inflammation related to MetS and OA *in vitro*.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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Name of candidate:	Saima Rizwan
Name/title of Primary Supervisor:	Dr. Frances M Wolber
In which chapter is the manuscript /published work:	Chapter Three

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Designed and carried out all experiments; curated, analysed and interpreted data; prepared figures and text for original draft; reviewed and co-edited revisions

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Date:	03-Apr-2021
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Chapter Four: Comparison of mussel extracts and assay optimisation to assess effects on immune response modulation in leptin stimulated J774A.1 macrophages

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This study aimed to compare the immune-modulating potential of unfractionated blue mussel (BM) and greenshell mussel (GSM) in LEP-stimulated macrophages using the murine J774A.1 cell line. However, there are no standardized protocols for assessing whole-food extracts in this model. Therefore, this study was designed to identify the optimal time point, dose, solvent, and culture conditions for peak cytokine production, followed by a comparison of BM and GSM for immunomodulatory bioactivity.

Abstract

Mytilus edulis (blue mussel, BM) and *Perna canaliculus* (greenshell mussel, GSM) are two commercially important species of marine mussels studied for their anti-inflammatory lipids such as omega-3 polyunsaturated fatty acids. However, their bioactivity in an immunomodulatory context has not been directly compared, and optimal *in vitro* assay conditions for testing this have not yet been established.

The pro-inflammatory hormone leptin (LEP) was used to stimulate the murine macrophage cell line J774A.1 as an *in vitro* model of obesity-driven low-grade chronic inflammation. Unstimulated and LEP-stimulated macrophages were treated with whole freeze-dried BM (conventionally processed BM-A and mineral rich BM-C) and GSM (conventionally processed GSM-A and enzymatically processed GSM-B) or control in various solvents at multiple time points. The expression of the pro-inflammatory cytokines interleukin-1β *(IL-1β),* interleukin-6 *(IL-6),* and tumour necrosis factor-α *(TNF-α)* and the anti-inflammatory cytokine interleukin-10 *(IL-10)* was quantified by RT-qPCR.

The maximum expression of cytokine mRNA was observed after 4 hours; however, BM-A and GSM-A significantly increased ($p<0.001$ and $p<0.05$ respectively) the expression of IL-10 after 48 hours. BM extracts were found to have higher overall immunomodulatory activity than GSM extracts. Mineral-rich BM-C significantly increased the production of IL-1β, IL-6 ($P <0.01$), TNF-α and IL-10 ($p<0.001$) independently of LEP after 4 hours. BM-C affected cytokine expression also in a dose-

dependent manner. A solvent effect was also observed, as DMSO reduced the overall pro-inflammatory activity and enhanced the anti-inflammatory activity of this extract.

Our results showed that GSM alone did not significantly induce cytokine expression, but BM induced transient expression of both pro- and anti-inflammatory cytokines. This is likely due to multiple, competing bioactive factors in BM and possibly also in GM. Mussel type, production method, solvent, and time point all impacted the assay results.

Keywords: inflammation, macrophages, Blue mussels, Greenshell mussels, leptin

4.1 Introduction

Mammals have a complex system of immunity including an innate and acquired immune system. The innate immune system is a rapid, nonspecific, first line of host defence against pathogens and is mediated by phagocytes including macrophages and neutrophils (Akira et al., 2006). Macrophages are vital for host immune defence and inflammation (Zhang et al., 2017). They recognize a variety of micro-environmental signals and differentiate accordingly into two different phenotypes called M1, associated with the secretion of proinflammatory cytokines: interleukins-1β, interleukin-6 and tumour necrosis factor-α (IL-1β, IL-6, and TNF-α) and M2, signatured by the secretion of anti-inflammatory cytokines: interleukin-10 (IL-10) (Sica et al., 2015). Under normal physiological conditions, M1 and M2 phenotypes are in relative balance with respect to their killing and repairing functions; however, an imbalance in M1-M2 polarization may result in disease development (Zhang et al., 2017). The diseases

associated with the M1 phenotype, which is a characteristic feature of chronic inflammation, include allergic reactions, cardiovascular diseases, arthritis and other joint disorders, diabetes, inflammatory bowel disease, and chronic obstructive pulmonary disease (Ashley et al., 2012). Since macrophages are vital in maintaining tissue homeostasis and fighting disease, immunostimulation of macrophages that specifically contribute to the improvement of various diseases present themselves as attractive targets for therapeutic intervention (Chan-Zapata et al., 2018).

Functional foods have been extensively explored globally for possible immunomodulatory properties and can be used as an alternative to or adjunct to conventional therapy, which can potentialize the immune function for a variety of diseases (Zhang et al., 2019c). Omega-3 polyunsaturated fatty acids, especially eicosapentaenoic (EPA) and docosahexaenoic (DHA), are important micronutrients that can protect against a variety of inflammatory conditions. Marine foods have high levels of EPA and DHA, in addition to other micronutrients such as peptides, essential amino acids, carotenoids, vitamins including vitamin B_{12}, and minerals such as calcium, copper, zinc, sodium, potassium, selenium, and iodine; this makes them well-placed to be explored as functional foods (Soccol and Oetterer, 2003, Venugopal, 2017). Fish are the most common source of EPA and DHA, but consumer demand has led to overfishing and sustainability issues; therefore, additional dietary sources of EPA and DHA must be explored. Mussels, a bivalve mollusc, are an excellent alternative source as they are rich in omega-3 polyunsaturated fatty acids (PUFAs) and a good source of essential amino acids (Venugopal, 2017). They are rich in nutrients such as zinc,

selenium, riboflavin, and carotenoids. Mussels are also well-suited to sustainable and environmentally friendly but intensive aquaculture production.

Mytilus and *Perna* are two commercially important genera of marine mussels. *Mytilus* species are found in temperate waters of Europe, Asia, and America, whereas *Perna* species are cultivated in warmer waters such as Thailand, the Philippines, China, and New Zealand. *M. edulis* is the most commonly cultivated species within the genus *Mytilus* are commonly known as blue or black mussels due to its shell colour. It is mostly cultured in Canada, the USA, Europe, and Africa, although some blue mussels (BM) are cultured in New Zealand. Popular species belonging to the genus *Perna* include *P. viridis*, the Asian green mussel, and *P. canaliculus*, the green-shelled mussel (GSM) that is endemic to New Zealand. The latter is a traditional food in the indigenous Polynesian people of New Zealand, the Māori (Māori name: kuku) and is the basis of an important aquaculture and processing industry serving both export and domestic markets.

In addition to EPA and DHA, novel potentially bioactive fatty acids have been identified in BM (McPhee et al., 2010) as well as in GSM (Treschow et al., 2007). *In vitro* studies have also revealed anti-cyclooxygenase effects of lipids extracted from BM and GSM (McPhee et al., 2007a, Treschow et al., 2007). In addition to the lipid fraction that has anti-inflammatory effects, water extracts from BM, which contain peptides and taurine in addition to other nutrients, decreased inflammation in an *in vivo* zebrafish model (Cheong et al., 2017), as well as an *in vitro* model (Kim et al., 2016b). These findings suggest that a combination of the lipids and the water fraction, i.e., the whole food, may

be a superior source of anti-inflammatory bioactive factors compared to isolated individual components. However, whole BM and GSM have never been directly compared in any published study.

Lipopolysaccharide (LPS) is a major component of the bacterial cell wall. It is widely used in models studying inflammation as it mimics the effects of cytokines, such as TNF-α, IL-1β, or IL-6 (Sweet and Hume, 1996) and is considered an effective activator of monocytes/macrophages that can alter the production of key inflammatory mediators (Tucureanu et al., 2018). However, the inflammatory response generated by LPS is nonspecific and generalized. In contrast, leptin (LEP), which is produced by adipose tissue, induces a more selective and targeted inflammatory response in a variety of immune cells including macrophages; *in vivo*, high LEP levels can lead to chronic inflammation and related diseases including osteoarthritis (Bondeson et al., 2006). LEP is therefore a physiologically more relevant stimulant to mimic a macrophage-driven inflammatory response *in vitro* and is an appropriate model to assess modulation of the expression of cytokines including *TNF-α, IL-1β, IL-6,* and *IL-10*.

This study aimed to compare the immune-modulating potential of unfractionated BM and GSM in LEP-stimulated macrophages using the murine J774A.1 cell line. However, there are no standardized protocols for assessing whole-food extracts in this model. Therefore, this study was designed to identify the optimal time point, dose, solvent, and culture conditions for peak cytokine production, followed by a comparison of BM and GSM for immunomodulatory bioactivity.

4.2 Materials and Methods

4.2.1 Materials and reagents

The murine macrophage cell line J774A.1 was purchased from the American Type Culture Collection (ATCC; Manassas, Virginia, United States). Foetal calf serum (FCS), and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco (Thermo Fisher, Auckland, NZ). Penicillin-streptomycin-L-glutamine (PSG), 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), and lipopolysaccharide (LPS, *E. coli* 0111:B4) were obtained from Sigma-Aldrich (Merck, Auckland, NZ). Leptin (recombinant mouse leptin OB) was purchased from Biomyx (San Diego, California, USA). Dimethyl sulfoxide (DMSO), ethanol, and methanol were purchased from Sigma-Aldrich (Merck, Auckland, NZ)). RNA extraction kits (PureLink RNA Mini Kit) and SuperScript® IV First-Strand Synthesis System for RT-PCR were purchased from Thermo Fisher. PCR primers were from Invitrogen (Life Technologies, Melbourne, Australia) and PCR kits (LightCycler® 480 SYBR Green I Master) from Roche Life Science (Thermo Fisher).

4.2.2 Mussel Extracts

The mussel extracts used in this study were kindly provided by Sanford Ltd, New Zealand. Two GSM and two BM extracts were used; proximal analyses were carried out by Cawthron Institute (Nelson, NZ) and are shown in Table 1. Both GSM products were from the same harvest but were manufactured using two different processing methods; GSM-A was blended, freeze-dried, and milled, whereas GSM-B included an added enzymatic digestion step. The two BM extracts were likewise from a single harvest. BM-

A underwent the same processing method as GSM-A. BM-C processing was incomplete and resulted in a lower protein, higher ash product.

Table 4.1: Composition of mussel extracts

Extract	Source location	Source material	Proprietary (Sanford Ltd) Extraction process	% protein	% CHO	% fat	% ash
BM-A	"1"	Whole BM meat	A	49.7	19.9	5.5	14.5
BM-C	"1"	Whole BM meat	C	43.7	20.0	3.2	27.0
GSM-A	"2"	Whole GSM meat	A	50.8	24.2	6.5	15.3
GSM-B	"2"	Whole GSM meat	B	47.3	20.3	7.9	20.1

4.2.3 Mussel extracts preparation

All the mussel extracts were provided in powdered form, which were dissolved in DMEM or DMEM plus one of the following solvents: 10% DMSO, ethanol, or methanol; the rationale for these choices is explained in the Discussion. Samples were sonicated briefly to ensure solubility, sterilized through a 0.20 µm pore syringe filter, aliquoted, and stored at -20 ^{0}C until further use.

4.2.4 Cell culture

The murine macrophage cell line J774A.1 was cultured in 75cm^2 flasks in a complete growth medium ("CM") comprising DMEM supplemented with 10% FCS and 1% PSG

(penicillin, streptomycin, and L-glutamine) at 37°C under 5% CO_2 humidified air. Subcultures were obtained twice a week by scraping. At confluence, cells were transferred to culture plates for experimental work.

4.2.5 Macrophage metabolic activity and survival

MTT assays were performed, as described previously (Michel et al., 2013a) with minor modifications to determine the effects of LEP, mussel extracts, and various solvents on the survival of metabolically active cells. Briefly, J774A.1 were plated at 50,000 cells/well in 96-well plates, left for 2 hours to adhere, and then treated. After 24 hours of incubation, 10 µg/ml of 5 mg/ml MTT (in PBS) was added to each well at a final concentration of 0.5 mg/ml and incubated for three hours until purple crystals were microscopically visible, which were solubilized by adding a volume of DMSO equal to that in the wells. Absorbance was quantified by measuring optical density (OD) at a wavelength of 550 nm using a microplate reader (Multiskan Fc, Thermo Scientific).

To determine optimal extract concentration, cells were treated with each of the four extracts at 1000, 100 or 10 µg/ml (Raso et al., 2002a), with medium alone serving as the negative control. Next, LEP at 10 µg/ml and mussel extracts (100 µg/ml) $\pm$ LEP were assessed. Finally, LEP, the BM-C extract (100 µg/ml), and LEP+BM-C were compared using four solvents to solubilize the BM extract: medium alone, 0.1% DMSO, 0.1% ethanol, or 0.1% methanol. Each test condition was assessed in 4 replicate wells within an experiment, and each experiment was carried out twice.

4.2.6 Macrophage stimulation and cytokine mRNA quantification

J774A.1 were plated in 12-wells plates at 2 x10^6 cells/well. After 2 hours of incubation, cells were treated with various extracts and stimuli as described above; at various time points thereafter (4, 6, 16, 24 or 48 hours; for the 48 hour time point, the treatment was repeated after 24 hours to measure the cumulative effect of the treatments on cytokine response over a longer period), total RNA was extracted from the cells using the PureLink RNA Mini Kit as per the manufacturer's instructions. Each test condition was assessed in a single well within an experiment, and each experiment was carried out two or three times.

From each sample, 2 µg of total RNA was reverse transcribed to cDNA by using SuperScript® IV First-Strand Synthesis System. Real-time PCR with gene-specific primers was performed for four genes *(TNF-α, IL-1β, IL-6,* and *IL-10)* with 5 µl of cDNA by using LightCycler® 480 SYBR Green I Master, according to the manufacturer's protocol. Finally, 10 µl of the PCR products were separated on a 2% agarose gel and visualized under ultraviolet light. Glyceraldehyde 3-phosphate dehydrogenase *(GADPH)* was used as an internal reference gene. Primer sequences and their annealing temperatures are provided in Table 4.2.

Table 4.2: List of primers and their annealing temperatures

Primer	Forward sequence (5'→3')	Reverse sequence (5'→3')	Ta (°C)
GADPH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	55
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	65
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA	60
IL-10	TGAGGCGCTGTCGTCATCGATTTCTCCC	ACCTGCTCCACTGCCTTGCT	65
TNF-α	CACGTCGTAGCAAACCACCAAGTGGA	TGGGAGTAGACAAGGTACAACCC	60

(**Note:** *GADPH* = glyceraldehyde-3-phosphate dehydrogenase, *IL-1β* = Interleukin beta, *IL-6* = Interleukin 6, *IL-10* = Interleukin 10, and *TNF-α* = Tumour necrosis factor alpha)

4.2.7 Statistical analyses

All the experiments were performed two or three times and data are presented as mean ± standard error or standard deviation. Treatment groups were compared with the control group (unstimulated) and/or LEP stimulated group by One-way and Two-way ANOVA, followed by Bonferroni's multiple comparisons. Graph Pad Prism San Diego, USA was used for statistical analysis and statistical significance was considered at $p \leq 0.05$.

4.3 Results

4.3.1 Metabolic activity of J774A.1 macrophages in response to treatments

The potential cytotoxicity effects of the mussel extracts on the J774A.1 macrophages were assessed using the MTT assay. As shown in Figure 4.1A, each of the extracts

increased the macrophage metabolic activity by 20 – 50% above control, although the effect did not appear to be dose-dependent. This increase was statistically significant ($p<0.05$) for BM-A at all the doses. As none of the extract concentrations were cytotoxic, the middle dose of 100 µg/ml was used for the next experiments.

Leptin (LEP) alone at 10 µg/ml (Figure 4.1B) or combined with mussel extracts at 100 µg/ml caused a 10 – 20% reduction in metabolically active macrophages; this was statistically significant for LEP + BM-A ($p<0.01$), LEP + BM-C ($p<0.05$) and LEP + GSM-A $p<0.01$). Next, the effects of different solvents: DMEM, 0.1% DMSO, 0.1% ethanol, and 0.1% methanol (Figure 4.1C) were assessed. The solvents alone had no significant effect, although 0.1% DMSO increased the viability of untreated cells by approximately 40% as compared to the DMEM control. The inclusion of LEP with each of the solvents caused a reduction in macrophage metabolic activity, with the decrease being statistically significant for LEP in DMSO ($p<0.001$). BM-C had no effect regardless of solvent type. When LEP was combined with BM-C, LEP again caused a reduction which was statistically significant ($p<0.01$) when DMSO was used as the solvent.

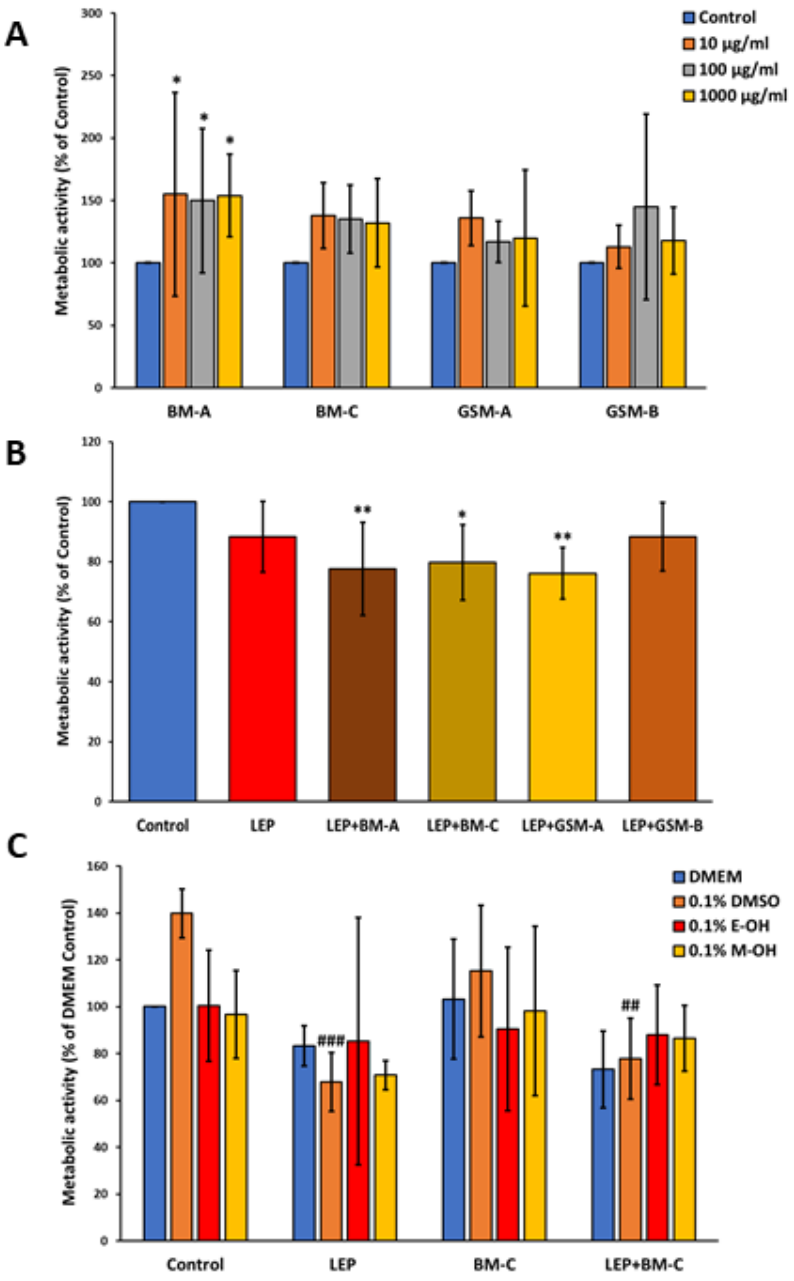


Figure 4.1: Effects of unfractionated mussel extracts on the survival of metabolically active J774A.1 macrophages. Activity was evaluated by MTT assay after 24 hours treatment with A) each of four extracts at 1000, 100 or 10 µg/mL; B) with 100 ug/ml mussel extracts, 10 ug/ml leptin (LEP) ± extracts at 100 µg/mL; C) with 100 ug/ml of BM-C dissolved in four different solvents i.e. DMEM, 0.1% DMSO, 0.1% ethanol (E-OH), and 0.1% methanol (M-OH),with or without 10 ug/ml leptin (LEP). Data are shown as mean ± SD of two independent experiments, each assessed in quadruplicate or duplicate. * signifies ($p<0.05$) difference from control (cells with DMEM alone), ##$p<0.01$, and ###$p<0.001$ signifies difference from 0.1% DMSO control.

4.3.2 Temporal response of cytokine mRNA expression

The effects of the LEP and mussel powders on cytokine expression were measured at 4, 6, 16, 24 and 48-hour time points by RT-qPCR. Because LEP, although not the solvents or mussel extracts, had a slight cytotoxic effect, all cytokine mRNA levels were normalised to the housekeeping gene GAPDH to control for potential differences in the number of viable cells per well. The extracts were assessed in the most benign solvent, DMEM.

LEP significantly induced ($p<0.05$) high levels of *IL-1β* mRNA at 4 hours, which declined at 6 hours and returned to baseline at 16 hours (Figure 4.2A). BM-A alone induced much lower levels of *IL-1β* mRNA; when combined with LEP the significant effects appeared additive ($p<0.001$). BM-C induced *IL-1β* mRNA as strongly as LEP ($p<0.01$) and the levels remained high ($p<0.01$) at 6 hours, declining thereafter. When combined with LEP the effects again appeared to be additive ($p<0.001$). In contrast, GSM-A did not elicit *IL-1β* mRNA expression, and GSM-B had a very minimal effect. When either GSM was combined with LEP, the stimulation of *IL-1β* mRNA expression was similar to that of LEP alone ($p<0.001$).

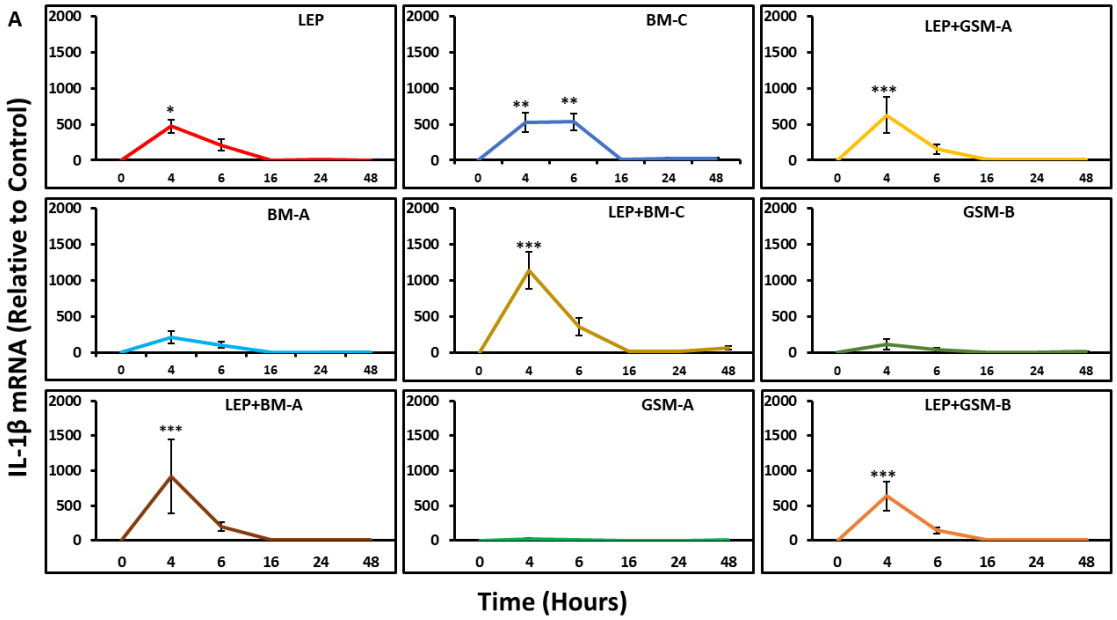
LEP also induced *IL-6* mRNA (Figure 4.2B) but only at the 4 hour time point, and there was a high degree of variability. BM-A, GSM-A and GSM-B did not elicit *IL-6* expression. Only BM-C significantly upregulated ($p<0.01$) *IL-6* at both 4 and 6 hours, with similar levels and patterns with BM-C alone versus combined with LEP.

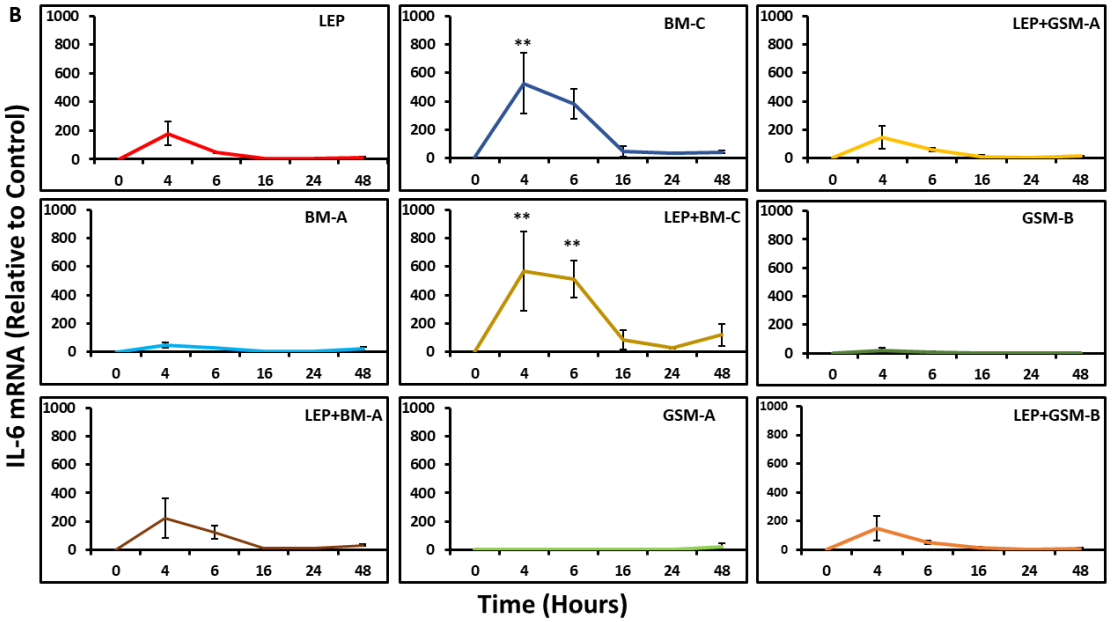
LEP induced low but measurable levels of *TNF-α* mRNA at 4 ($p<0.05$) and 6 hours (Figure 4.2C). BM-A induced similar levels. Interestingly, BM-A treatment resulted in little *TNF-α* mRNA after 24 hours, but re-application of the extract at that time caused a subsequent spike ($p<0.001$) in *TNF-α* another 24 hours later, at the 48 hour time point. BM-C induced the highest level of *TNF-α* mRNA, with significant rises at both 4 ($p<0.001$) and 6 hours ($p<0.05$). When either BM was combined with LEP, there did not appear to be additive effect. In contrast, neither GSM induced *TNF-α*; when combined with LEP, the effect was similar to LEP alone.

LEP also induced low but measurable mRNA levels of the anti-inflammatory cytokine *IL-10* (Figure 4.2D), peaking ($p<0.01$) at 4 hours but with a small amount of *IL-10* mRNA found at 48 hours due to the re-application of the stimulus at the 24 hour time point. BM-A and GSM-A induced *IL-10* only at 48 hours with $p<0.001$ and $p<0.05$ respectively, or at 4 and 48 hours when combined with LEP, whereas GSM-B induced no *IL-10* at all. BM-C induced higher levels of *IL-10* than LEP with significant increases at both 4 and 6 hours ($p<0.001$); this effect was reproduced when BM-C was combined with LEP, although the effect appeared to be due to BM-C alone rather than having an additive effect with LEP.

Taken together, expression of the pro-inflammatory cytokines *IL-1β* and *IL-6* showed higher expression with all the treatments as compared to pro-inflammatory *TNF-α* and anti-inflammatory *IL-10*. The mRNA expression of all cytokines peaked at 4 hours and declined thereafter. LEP significantly induced the production of all the cytokines

assessed, verifying its use as an immunomodulatory stimulant. Blue mussel extracts, in general, showed higher immunomodulatory potential than green shell mussel extracts, with BM-C being the strongest immunomodulator. The effects of LEP and mussel extracts appeared to be additive only for *IL-1β*.





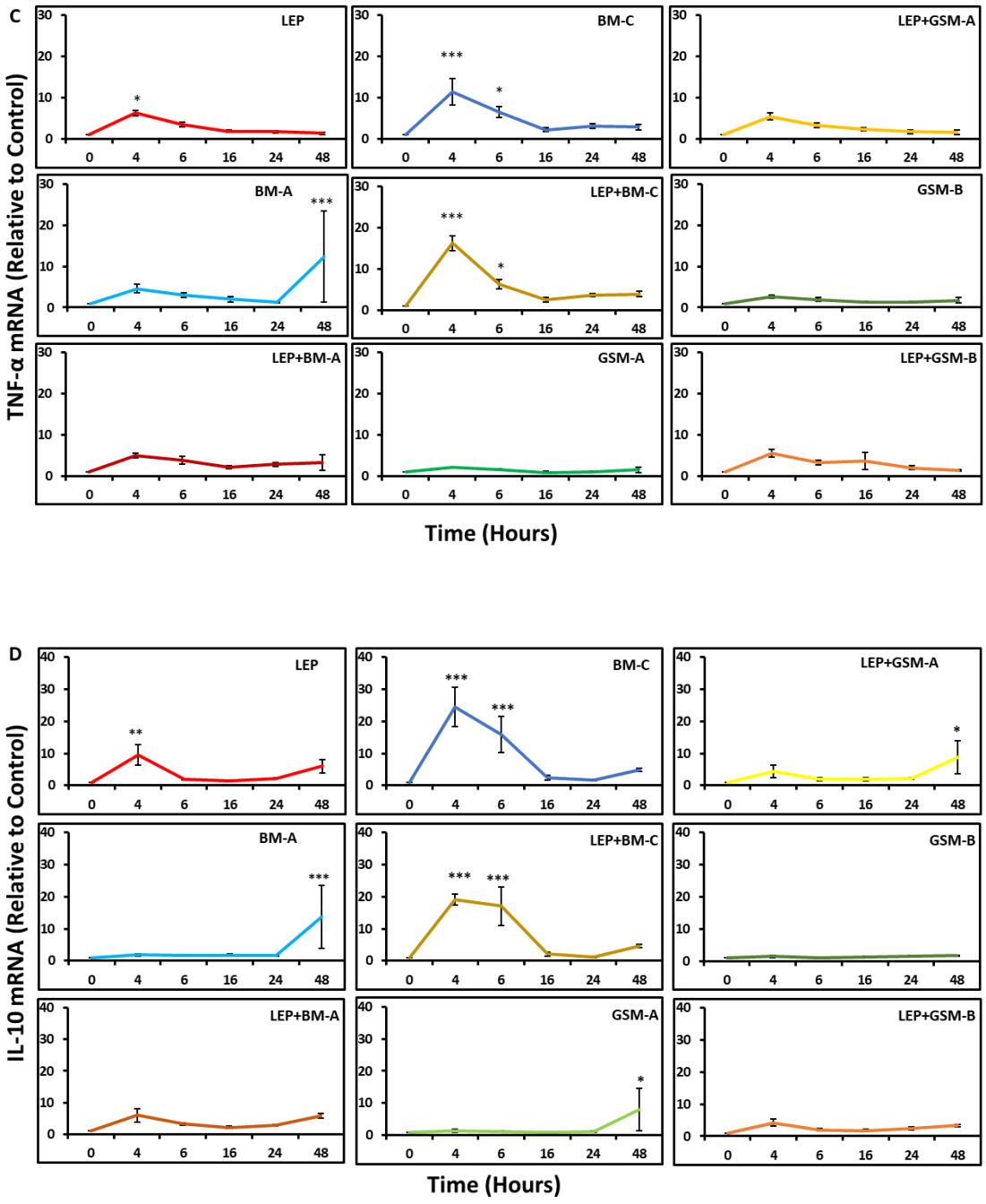


Figure 4.2 (A-D): Effects of crude mussel extracts on expression of cytokines in J774A.1 macrophages. Cytokine expression was evaluated by RT-qPCR at different time points i.e. 0, 4, 6, 16, 24, and 48 hours after incubation of at least 1x106/well of J774A.1 cells in 12 well multiwell plate with 100 µg/ml mussel extracts and 10 µg/ml leptin (LEP). The values represent the mean ± SE of three independent experiments (n=3). * signifies ($p<0.05$), **$p<0.01$, ***$p<0.001$ difference from control.

4.3.3 Dose-dependent response of cytokines mRNA expression

Having established 4 hours as the peak time point and BM-C as the most bioactive extract, the dose-dependent response was next determined. J774A.1 macrophages were treated with 10, 100, or 1000 µg/ml of BM-C with or without 10 µg/ml LEP for four hours. LEP again induced high mRNA levels of *IL-1β* and *IL-6,* and low levels of *TNF-α* and *IL-10* (Figure 4.3). BM-C induced expression of all four cytokines in a dose-dependent manner, with 1000 µg/ml inducing the highest levels of IL-1β and *TNF-α* with a significance of $p<0.01$ and $p<0.001$ respectively, and 100 µg/ml inducing the highest levels of *IL-6* and significantly higher levels ($p<0.001$) of *IL-10*. As observed previously, LEP and BM-C had an additive effect in upregulating *IL-1β*, and an additive effect was also observed for *TNF-α* and *IL-10* when BM-C was used at the lowest dose.

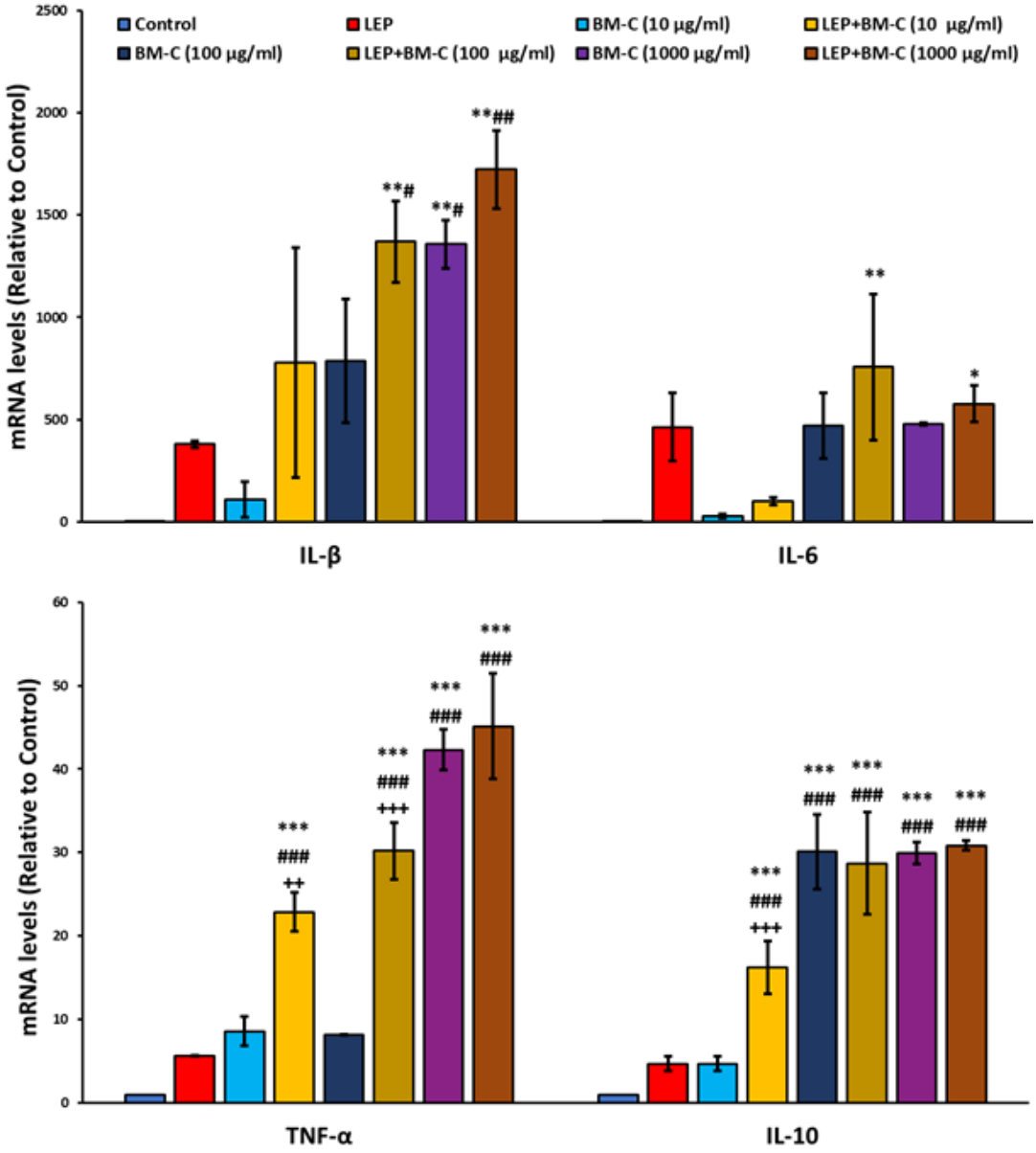


Figure 4.3: Effects of blue mussel (BM-C) extract on the expression of cytokines in J774A.1 macrophages. Cytokine expression was evaluated by RT-qPCR in response to three different doses of BM-C, i.e. 10, 100, and 1000 µg/ml. $1x10^6$ cells/well were incubated in 12 well multiwell plates with 10, 100, and 1000 µg/ml of BM and 10µg/ml leptin (LEP) followed by RNA extraction after 4 hours. The values represent the mean ± SEM of two independent experiments (n=2). * signifies ($p<0.05$), **$p<0.01$, ***$p<0.001$ difference from control group, # signifies ($p<0.05$), ##$p<0.01$, ###$p<0.001$ difference from LEP stimulated group and ++$p<0.01$, and +++$p<0.001$ signifies difference between BM-C and LEP+BM-C.

4.3.4 Solvent dependent response of cytokine mRNA expression

To determine the effect of solvent type on cytokine induction, J774A.1 cells were treated with 100 µg/ml of BM-C dissolved in four different solvents i.e. DMEM, 0.1% DMSO, 0.1% ethanol, 0.1% methanol. As shown in Figure 4.4, solvents did alter the cytokine response of BM-C in cells treated with LEP, BM-C, or both. As observed previously, LEP in DMEM stimulated high levels of *IL-1β* and *IL-6,* and low levels of *TNF-α* and *IL-10*, but these were partially supressed with other solvents for all but *TNF-α*. BM-C in DMEM activated cytokine expression similar to LEP. BM-C stimulated *IL-6* expression in LEP-stimulated cells was significantly higher ($P < 0.01$) in 0.1% methanol as compared to DMEM, 0.1% DMSO, and ethanol, while in the absence of LEP, BM-C stimulated cytokine response of methanol was statistically higher ($P < 0.05$) than 0.1% DMSO only. The pro-inflammatory potential of BM-C generally seemed to be higher in 0.1% ethanol and methanol but 0.1% methanol significantly upregulated the expression of the pro-inflammatory cytokines *IL-1β, IL-6,* and *TNF-α* with and without LEP as compared to the respective control and LEP-stimulated groups. The 0.1% DMSO though did not show any general solvent effect overall, but it did reduce the expression of IL-6 in all the treatment groups and significantly augmented ($P < 0.05$) the expression of the anti-inflammatory cytokine (IL-10) both in unstimulated and LEP-stimulated cells as compared to control as well as LEP-stimulated group.

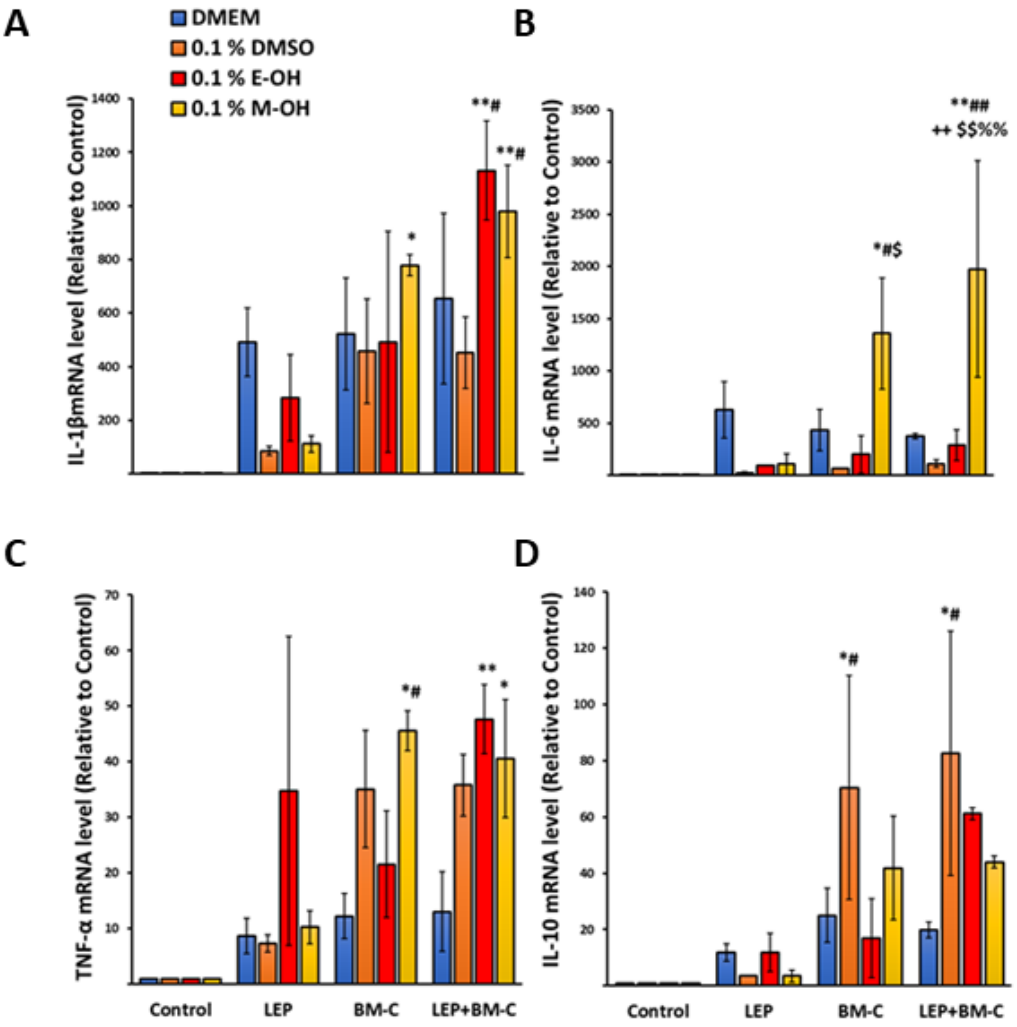


Figure 4.4: Effects of blue mussel C (BM-C) extract on the expression of cytokines in J774A.1 macrophages. Cytokine expression was evaluated by RT-qPCR in response to BM-C dissolved in four different solvents i.e. DMEM, 0.1% DMSO, 0.1% ethanol, and 0.1% methanol. $1x10^6$ cells/well were incubated in 12 well multiwell plates with 100 µg/ml BM-C and 10µg/ml leptin (LEP) for 4 hours followed by RNA extraction. The values represent the mean ± SEM of two independent experiments (n=2). * signifies ($p<0.05$), **$p<0.01$, ***$p<0.001$ difference from the respective solvent controls, # signifies ($p<0.05$), ##$p<0.01$, ###$p<0.001$ difference from the respective solvent LEP stimulated groups. ++$p<0.01$ (DMEM), $ ($p<0.05$), and $$$p<0.01$ (0.1% DMSO) and %% $p<0.01$ (0.1% ethanol) indicate the solvent differences among the same treatment groups.

4.4 Discussion

This study was planned to compare the cytokine profile of LEP stimulated J774A.1 macrophages in response to mussel extracts at the transcription level. Currently, there is a scarcity of data regarding *in vitro* use of mussels and especially whole mussel extracts; therefore, we also aimed to develop an optimized protocol for *in vitro* use of blue mussel, greenshell mussel, and other similar whole-meat extracts for the treatment of macrophages *in vitro*. In the context of optimizing the treatment protocol, temporal response experiments identified the optimal time point for cytokine expression as being 4 hours. Dose and solvent response experiments identified the concentrations of mussel extracts that were not cytotoxic but had immunomodulatory activity, and compared the effect of solvents on both mussel and LEP bioactivity. Comparing extracts from two mussel types demonstrated species-specific and process-specific differences in bioactivity. Finally, we confirmed that LEP is a suitable stimulant for J774A.1 murine macrophages to induce immunoactivity that mimics the physiological response.

Instead of using LPS, which is commonly used *in vitro* to simulate an acute physiologic inflammatory response, we experimented with an alternative stimulus. LEP was used to stimulate the immune response in murine macrophages J774A.1 to mimic the low-grade inflammatory state associated with many disorders such as cardiovascular disease, type II diabetes, or degenerative diseases such as osteoarthritis (La Cava, 2012). Results of MTT assays indicated that mussel extracts produced no cytotoxic effects on J774A.1 cells at concentrations ranging from 10 – 1,000 µg/ml (figure 4.1). All of the extracts at all the

concentrations slightly increased the proliferative activity of the cells as compared to untreated cells, particularly BM-A which produced a small but statistically significant increase in the number of cells at all the doses. This might be due to extra nutrients present in the whole mussel extracts. Similar effects are reported in a study which demonstrated that growth regulatory effects of shellfish are differential and depend on the type of cells and type of shellfish, but many shellfish extracts had growth-promoting effects for macrophage cell lines and growth inhibitory effects on cancer cell lines (Kong et al., 1997). Likewise, (Kim et al., 2016a) also found blue mussel hydrolysates had no cytotoxic effects when tested on RAW264.7 cells.

None of the mussel extracts negatively affected the viability of J774A.1 cells when used alone but they did reduce the cell viability when used together with LEP. LEP independently slightly reduced cell viability but the effect was enhanced when used together with mussel extracts especially BM-A and GSM-A, which caused a small but significant reduction in the viability of the cells when used together with LEP. Unlike our results, previous studies regarding the effect of LEP on macrophages report contradictory findings. Santos-Alvarez et al., and Cannon et al. reported that LEP increased the proliferation of human circulatory monocytes *in vitro* (Santos-Alvarez et al., 1999, Cannon et al., 2014) while another study reported that LEP at lower concentrations (50-1,600 ng/ml) had no effects on the viability of RAW264.7 cells (Hsieh et al., 2017). This discrepancy may be species-specific (human vs murine cells), cell line specific or due to differences in the test format including the LEP doses and other experimental conditions.

Both LEP and the most bioactive extract, BM-C, did behave differently based on which of the four solvents/delivery systems was used, i.e. DMEM, 0.1% DMSO, 0.1% ethanol, or 0.1% methanol. When LEP and BM-C were used together, the slight reduction in the viability of treated cells became statistically significant in the case of DMSO. 0.1% DMSO alone considerably increased the growth of untreated cells; this phenomenon has been reported previously (Rodríguez-Burford et al., 2003), where 0.1% DMSO was found to significantly increase the number of ovarian carcinoma cells. In our study, none of the solvents had any significant cytotoxic effects as reported previously (Nemudzivhadi and Masoko, 2014, Jamalzadeh et al., 2016) and none of the solvents altered the effects of the LEP or BM-C test subtances.

Inflammation is an important physiological response necessary for the maintenance of tissue homeostasis by enabling the body to get rid of harmful and pathogenic stimuli. A successful immune response, however, involves the resolution of an inflammatory response by anti-inflammatory and reparative cytokines, followed by tissue repair, regeneration, and restoration (Loi et al., 2016). Cytokine expression induced by LEP or mussel extract peaked after four hours with all the treatments, with a slight decline after 6 hours and eventually a return to baseline as shown in figure 4.2. This compares well with a previous report (Huang et al., 2012) where LPS-induced cytokine expression by J774A.1 cells peaked at 4 hours. The J774A.1 cell line can be stimulated to display a pro-inflammatory M1 phenotype (He et al., 2018, Seyedi et al., 2018, Lin et al., 2020). The presence of M1 macrophages in culture can be verified by the cells' expression of pro-inflammatory cytokines such as *IL-6* or *TNF-α,* and the presence of M2 macrophages by

the anti-inflammatory cytokine *IL-10* (Bartosh and Ylostalo, 2014, Lin et al., 2020). In the current study, we demonstrated that LEP alone rapidly induced the presence of both M1 and M2 macrophages. This has not been demonstrated before in J774A.1 or other cell lines *in vitro*, although in a single report human peripheral blood monocytes were found to produce both IL-1β (M1) and IL-10 (M2) cytokines after being stimulated in culture for five days with leptin (Acedo et al., 2013). Macrophage infiltration into adipose tissue increases with obesity (Li et al., 2016b), as does LEP production (Guzik et al., 2017), and mice with diet-induced obesity have increased LEP levels that correlate with increases of both M1 and M2 type cytokines in the adipose tissue (Enos et al., 2013). LEP has also been shown to potentiate cytokine expression in macrophages already induced by other stimuli to assume an M1 or M2 phenotype *in vitro* (Kredel et al., 2013). This suggests LEP is a complex inflammatory mediator that can manipulate the behaviour of both polarised macrophages and non-polarised monocytes and macrophages. The cytokine induction effects by LEP are consistent with true, multifactorial immunomodulation rather than simply inflammation or anti-inflammation when used in combination to treat J774A.1 cells. LEP and the mussel extracts neither interfered with nor augmented each other's bioactivity, further validating this adipokine as being a more relevant *in vitro* stimulant than LPS.

Neither GSM-A nor GSM-B induced a pro-inflammatory M1 phenotype in treated cells. Repeated treatment with GSM-B induced at least some of the cells to express the M2 phenotype as identified by *IL-10* production. The increase in the expression of *IL-10* after 48 hours of treatment with GSM-A *in vitro* would, if extrapolated to *in vivo*, suggest the

possibility that long-term use of GSM-A could potentialize the immune system to fight chronic inflammatory conditions by altering the polarization of macrophages from M1 to M2. This may be the mechanism occurring in reported *in vivo* studies where regular consumption of GSM produced anti-inflammatory results in rats and humans. For instance, a lipid fraction (Lyprinol®) from GSM was found to have anti-inflammatory activity in rats (Whitehouse et al., 1997). Similarly, whole BM reduced disease symptoms in women with rheumatoid arthritis (Lindqvist et al., 2018a). However, neither GSM extract was able to reduce the pro-inflammatory effects of LEP when the treatments were combined. It would be of interest to determine whether pre-treating J774A.1 macrophages with GSM could alter subsequent LEP treatment effects.

In contrast, BM-A and BM-C increased M1 cytokine production within 4 hours of treatment. This novel finding suggests that blue mussels have a significant pro-inflammatory component. BM-A also induced M2 macrophage activity; this activity was likely due to multiple, competing bioactive factors in the extract, and BM has been reported elsewhere to reduce inflammation (Lindqvist et al., 2018b). BM-C induced a much stronger pro-inflammatory response compared to BM-A but failed to produce any anti-inflammatory response. The significant differences between the bioactivity of the two BM extracts are ascribed to the production processes.

BM-A and GSM-A administered at 0 and 24 hours significantly increased the expression of *TNF-α* and/or *IL-10* after 48 hours. This repeat-stimulatory effect of the mussel extracts on cytokine expression has been observed in macrophages where priming with

small doses of LPS or particulate matter may lead to exacerbation of the immune response upon second exposure to LPS (Chae, 2018a, Gawda et al., 2018). There is little research regarding the *in vitro* use of blue or green mussel extracts, particularly the whole mussel product; however a GalNAc/Gal-specific lectin (CGL) from the edible mussel *Crenomytilus grayanus* has been found to increase the production of proinflammatory cytokines *(IL-1β, IL-6, and TNF-α)* in RAW264.7 macrophages independent of LPS (Chernikov et al., 2017). In another study a protein hydrolysate from BM was found to promote differentiation of mesenchymal stem cells into osteoblasts (Hyung et al., 2018), showing their osteogenic potential. This suggests that mussels may have bioactivity in multiple physiological systems, and that this activity may be attributed to components other than lipids.

Interestingly the production process of BM-A and GSM-A was the same. Both BM-A and GSM-A were made by process A "traditional mussel powder process" which involves manual shucking followed by blending, freeze-drying, and finally milling. Therefore, the differences in bioactivity between these two extracts are due to the mussel species rather than the production process. In contrast, GSM-B is produced by using process B "new enzymatic mussel powder process" in which GSM undergo preconditioning, enzymatic extraction, filtration followed by freeze-drying and milling. GSM-A induced *IL-10* expression while GSM-B did not. The traditional production process may preserve the integrity of anti-inflammatory biomolecules present in the mussels while an additional enzymatic step in process B may disrupt the activity of that specific component.

The pro-inflammatory potential of BM-C was generally higher in 0.1% ethanol and methanol but BM-C in 0.1% methanol significantly upregulated the expression of the pro-inflammatory cytokines *IL-1β, IL-6,* and *TNF-α* in the unstimulated as well as LEP-stimulated cells as compared to the respective control and LEP-stimulated groups (figure 4.4). Similar findings were reported by Désy et al, who found that methanol synergizes with the activating stimuli to augment cytokine production (Désy et al., 2010). Ethanol, on the other hand, has contradictory data regarding its role in modulating the immune response: it is considered to have an inhibitory role on cytokine production (Pruett and Fan, 2009) but was also found to augment LPS stimulated cytokine response in the liver and brain of mice (Qin et al., 2008).

The 0.1% DMSO did not show any overall solvent effect, but it did reduce the expression of *IL-6* in all the treatment groups and significantly augmented the expression of the anti-inflammatory cytokine *(IL-10)* both in unstimulated and LEP-stimulated cells as compared to control as well as the LEP-stimulated group. Although many studies have revealed that DMSO exhibits anti-inflammatory potential and may suppress the expression of inflammatory cytokines (Elisia et al., 2016a, Costa et al., 2017, Mehta et al., 2019), in our case this effect may not be responsible for its increased anti-inflammatory activity, because solvent effects of each treatment group were normalized with the untreated control (0.1% DMSO). Instead, its anti-inflammatory effects might be due the bioactives present in the mussel extracts, which had greater access into the cells because of excellent solvate capability of DMSO. Being amphiphilic, DMSO is a common solvent of choice as it can solubilize both polar and nonpolar components and can

transpose through hydrophobic cell membranes of the cells and is generally not cytotoxic when used at a final concentration of 0.1% in the culture medium (Tunçer et al., 2018). Moreover, it is placed in the safest category of class 3 solvents according to the Q3C solvent classification system, which means they are less toxic and are of less risk to human health (Jamalzadeh et al., 2016). However, use of any solvent is not physiologically relevant, as macrophages *in vivo* would be exposed to bioactive mussel compounds in the bloodstream in an aqueous rather than solvent carrier form.

4.5 Conclusion

We conclude that all the mussel extracts were non-cytotoxic to J774A.1 macrophages, with and without LEP and in all the solvents according to the norms of the International Organization of Standardization, which considers a substance to be cytotoxic when it reduces cell viability to less than 70% (Iso, 2009). Time is crucial for cytokine expression and 4 hours was the optimal time point for all the treatment groups with the exception of BM-A and GSM-A, which induced the expression of TNF-*α/ IL-10* and *IL-10* respectively after 48 hours when administered at 24 hour intervals. BM-C was found to be the strongest immunomodulator, which independently increased the expression of both inflammatory and anti-inflammatory cytokines. Also, immunomodulation by BM-C was dose dependent and the highest immunomodulation was observed with the highest dose of BM-C. Solvents also affected the cytokine response of BM-C; 0.1% DMSO was noticed to augment anti-inflammatory cytokine *(IL-10)* production probably because of its excellent solvate capability.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Saima Rizwan
Name/title of Primary Supervisor:	Dr. Frances M Wolber
In which chapter is the manuscript /published work:	Chapter Four

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Date:	07-Apr-2021
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Chapter Five: Green shell mussel promotes proliferation, differentiation, and mineralization in an *in vitro bone* model of osteoarthritis

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Blue mussel (BM-C) was an imprecisely prepared extract whose production method could not be replicated; therefore, further work with BM was not pursued. The main goal of the HVN-funded project was to study the mechanism of action of greenshell mussel (GSM) in osteoarthritis. Therefore, we proceeded with conventionally processed (GSM-A) and enzymatically processed (GSM-B) in different *in vitro* models of OA. There is a scarcity of data regarding *in vitro* use of whole GSM and there are no published studies to date exploring the effects of GSM on subchondral bone. This study is therefore primarily designed to compare two freeze-dried whole GSM powders (GSM-A and GSM-B) produced by different processing methods for their effects on mineralization in LEP-stimulated MC3T3-E1-cells as an *in vitro* bone model of OA.

Abstract

Osteoarthritis (OA) is a metabolic disorder, rather than simply a disease of cartilage degeneration, involving local and systemic factors that modify the formation and biosynthetic activity of cells of mesenchymal origin, leading to changes in skeletal tissues. Leptin (LEP) is an adipokine that acts as a genetic, proinflammatory, local, and systemic factor in OA pathogenesis, and its levels are elevated in serum, infrapatellar fat pad, synovial tissues, osteoblasts, and cartilage of OA patients. Due to the multifactorial and complex nature of the disease, identifying disease-modifying OA drugs (DMOADs) that demonstrate efficacy and reliable safety is still a challenge. Enzymes, growth factors, and cytokines involved in regulating cartilage differentiation and destruction, subchondral bone remodelling, and synovial inflammation are attractive targets due to their critical role in OA pathogenesis. New Zealand's green shell mussel (GSM) has already been proven to treat OA related symptoms by modulating the immune response and by preventing or reducing cartilage damage. However, there is no research so far regarding the effects of GSM on subchondral bone. The aim of this study was to mimic OA-related pathophysiology of subchondral trabecular bone (STB) *in vitro* and then to evaluate the effects of GSM treatments in the disease model. We used murine MC3T3-E1 cells to create a LEP-stimulated *in vitro* bone model of OA and compared two whole GSM powders, conventionally processed GSM-A versus enzymatically processed GSM-B, for their effects on proliferation, differentiation, and mineralization of LEP-stimulated MC3T3-E1 osteoblasts. We found inconsistent results with LEP, it increased osteoblast

proliferation and expression of osteogenic markers but decreased alkaline phosphatase (ALP) activity and mineral deposition. Both GSM extracts promoted bone mineralization by regulating every step of bone formation, but GSM-B was more effective. It significantly upregulated proliferation, osteogenic markers expression *(Alp, Osx, Col10α1 and Runx2),* ALP activity and mineral deposition of pre-differentiated osteoblasts as compared to control and/or LEP-stimulated group. GSM promoted bone formation in unstimulated as well in the LEP-stimulated group, however, the efficacy of GSM extracts was higher in the LEP-stimulated group, where it significantly increased proliferation ($p<0.001$, expression of *Alp, Osx* ($p<0.05$)*, Col10α1 and Runx2* ($p<0.001$) and mineral deposition ($p<0.001$) as compared to control and/or LEP-stimulated group, indicating GSM's potential to prevent bone loss and improve bone mineral density in patients with metabolic OA.

Keywords: Metabolic OA**,** Leptin**,** Subchondral bone, Greenshell mussel, *In vitro* OA model

5.1 Introduction

Osteoarthritis (OA) is a chronic disease of the whole joint, characterized by progressive degeneration of the cartilage and underlying bone, which may be caused by genetic, environmental, or mechanical factors (Wang et al., 2015a). Recent advancements in the field of OA have led researchers to redefine it as a metabolic disorder rather than merely a disease of cartilage. According to the modern definition, it is a metabolic disorder

involving local and systemic factors that modify the formation and biosynthetic activity of the cells of mesenchymal origin, leading to changes in skeletal tissues (Aspden et al., 2001). Obesity is a well-established risk factor for OA in both weight-bearing and non-weight-bearing joints (Chen and Yang, 2015). Leptin (LEP), a hormone produced by adipose tissue, is thought to be the linking factor between obesity and OA. Elevated levels of LEP in obese individuals and in females, who have a higher proportion of body fat than men, make them more susceptible to OA (Lajeunesse et al., 2005, Gualillo, 2007, Hu et al., 2011). LEP is considered to act as a genetic, proinflammatory, local, and systemic factor in OA pathogenesis (Chen and Yang, 2015), and its levels are found to be elevated in serum, infrapatellar fat pad (IPFP), synovial tissues, osteoblasts, and cartilage of OA patients compared to healthy individuals (Dumond et al., 2003a, Mutabaruka et al., 2010b, de Boer et al., 2012, Conde et al., 2014).

The skeletal system is imperative for body support as it provides a platform for tissue attachment, shields vital body organs, and facilitates movement (Li, 2013, Luo et al., 2016). Bone homeostasis is maintained by osteoblasts (bone-forming cells) and osteoclasts (bone-resorbing cells) (Luo et al., 2016) and any imbalance between these cells can lead to metabolic diseases such as osteoporosis, osteopetrosis, and Paget's diseases (Li et al., 2013a). Although, OA is generally not considered a disease related to bone metabolism (Gevers et al., 1989), research indicates that bone alterations occur even before cartilage deterioration. In recent years, subchondral bone has emerged as a dynamic structure involved in the pathogenesis of OA (Li et al., 2013b), and it plays an important role in the onset and progression of OA (Castañeda et al., 2012). Subchondral

bone is an intricate structure, which has a biomechanical and biochemical relationship with overlying cartilage. Initially, it was believed that OA was involved in new bone formation and sclerosis, however, it is now understood that bone resorption or hypomineralization is an early sign of progressive OA (Jia et al., 2018). Subchondral bone is anatomically divided into two parts: a layer of corticalized subchondral bone plate (SBP) and the underlying subchondral trabecular bone (STB). These two parts are important with respect to their architectural and mechanical changes during OA pathogenesis; as they undergo different pathological changes during the disease progression (Dedrick et al., 1993). SBP sclerosis or thickening is certainly considered a hallmark of late-stage non-progressive OA but the underlying mechanism is yet to be understood (Goldring and Goldring, 2016). STB, on the other hand, experiences hypomineralization and thus reduced mechanical strength due to increased bone remodelling during the progression of OA (Bailey et al., 2004, Funck-Brentano and Cohen-Solal, 2011). Cellular signalling for micro-damage repair, stimulation of vascular invasion, and bone-cartilage crosstalk via channels in the SBP could be the possible mechanisms for the alterations in STB (Burr and Gallant, 2012). Moreover, recent studies in mice showed that OA progression could be prevented by modifying osteoblastic gene expression or inhibiting osteoclastic bone resorption (Findlay and Kuliwaba, 2016).

Bone formation involves three major steps: osteogenesis, modelling, and remodelling. All of these processes are controlled by osteoblasts (Papachroni et al., 2009), which are mononuclear cells differentiated from mesenchymal stem cells through osterix (Osx), Runt-related transcription factor, and Wnt signalling transcription pathways (Neve et al.,

2011). Mature osteoblasts synthesize bone matrix constituents such as type I collagen and non- collagen proteins such as alkaline phosphatase (ALP), osteocalcin (OCN), and bone sialoprotein (BSP), which are involved in bone mineralization (Corrado et al., 2017).

OA research is designed to discover new therapeutic strategies that can prevent, stop, reduce, or restore the damage in the joints, but due to the multifactorial complex nature of the disease, it is still challenging to bring to market disease-modifying OA drugs (DMOADs) with sufficient efficacy and reliable safety (Bijlsma et al., 2011a, Martel-Pelletier et al., 2012). Enzymes, growth factors, and cytokines involved in regulating cartilage differentiation and destruction, subchondral bone remodelling, and synovial inflammation could be the most attractive targets due to their critical role in OA pathogenesis (Thysen et al., 2015).

The bivalve mollusc *Perna canaliculus* (P. *canaliculus*), commonly called the green-lipped mussel (GLM) or greenshell™ mussel (GSM), is endemic to New Zealand (Cobb and Ernst, 2006a). It has an extensive history of being used as a traditional medicine to treat various arthralgias in both humans and animals. As a major species of the New Zealand aquaculture industry (Coulson et al., 2015) it accounts for a value of over US$ 200 million. Also, a large proportion of mussels are processed into biopharmaceuticals and health supplements, which are used to treat inflammation, joint pain, and other disorders. According to Professor Andrew Jeffs of the Leigh Marine Laboratory at the University of Auckland, New Zealand, this sector of the mussel industry could be worth over US$ 40 million (Pūtaiao, 2013). Interest in investigating the health benefits of GSM

started in 1960 when it was found to relieve pain in cancer patients. Since then it has been extensively studied for its anti-inflammatory activities *in vitro* and *in vivo*. Clinical trials have evaluated GSM as a supplement for the treatment of many inflammatory conditions including asthma, osteoarthritis, and rheumatoid arthritis (Ferreira, 2005).

Conflicting findings in these studies are due in part to the variability of mussel quality and composition. In addition to sex, season, life cycle, nutrition, environment, and habitat (Teshima and Kanazawa, 1974, Narváez et al., 2008, Fearman et al., 2009), processing methods may also affect the bioavailability of bioactive components in GSM. The method involving enzymatic processing is shown to better preserve the bioactive composition of GSM as compared to conventional mechanical shucking of the shelled mussel. Lipids, proteins, and carbohydrates are the major constituents of GSM, consisting of several components that have various antimicrobial, anti-inflammatory, antihypertensive, and bioadhesive activities (Grienke et al., 2014b). As far as their role in OA is concerned, which of the fractions is therapeutically effective is yet to be understood; previous claims, however, suggest the lipid fraction has the prevailing anti-inflammatory activity (Ulbricht et al., 2009).

A multitude of studies have demonstrated that GSM is effective in treating OA-related symptoms and most of these studies have been done in animal models such as dogs, cats, and horses (Eason et al., 2018a). Less is known about the mechanism of action of GSM or whether all of its bioactivity can be attributed to its lipid fraction, which is thought to relieve OA symptoms by reducing inflammation (McPhee et al., 2007b). More

recently, Siriarchavatana *et al* found whole GSM prevented OA in a rat model of metabolic osteoarthritis by reducing cartilage degradation (Siriarchavatana et al., 2019b). However, there is a scarcity of data regarding *in vitro* use of whole GSM and there are no published studies to date exploring the effects of GSM on subchondral bone. This study is therefore primarily designed to compare two freeze-dried whole GSM powders produced by different processing methods for their effects on mineralization in LEP-stimulated MC3T3-E1-cells as an *in vitro* bone model of OA.

5.2 Materials and Methods

5.2.1 Mussel extracts

Whole green shell mussel (GSM) powder extracts were kindly provided by Sanford Ltd, New Zealand. Proximate analyses were carried out by the Cawthron Institute (Nelson, NZ) and are shown in Table 5.1. Both GSM products were from the same harvest but were manufactured using two different process methods; GSM-A was blended, freeze-dried, and milled, whereas GSM-B included an added enzymatic digestion step.

Table 5. 1: Composition of mussel extracts

Extract	**Source material**	**Extraction process**	**% protein**	**% CHO**	**% fat**	**% ash**
GSM-A	Whole GSM meat	"A"	50.8	24.2	6.5	15.3
GSM-B	Whole GSM meat	"B"	47.3	20.3	7.9	20.1

5.2.2 Mussel extract preparation and leptin reconstitution for *in vitro* use

The freeze-dried powder was dissolved at a concentration of 10 mg/mL in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies, Auckland, NZ) containing 10% DMSO (Sigma Aldrich, Merck, NZ), sonicated briefly to maximise solubility, sterilized through a 0.20 µm pore syringe filter, aliquoted, and stored at -20°C until further use.

Leptin (recombinant mouse leptin OB) was purchased from Biomyx (San Diego, California) and reconstituted in PBS (phosphate buffer saline, pH 8) (Sigma Aldrich, Merck, NZ) according to the manufacturer's instructions, then aliquoted and stored at -20°C until further use.

5.2.3 Reagents and cell culture

Murine MC3T3-E1 cells (MC3T3-E1 Subclone 4, ATCC, Manassas, VA, USA) were grown and maintained in alpha minimum essential medium (αMEM) (Gibco, Life Technologies, Auckland, NZ), supplemented with 10% foetal calf serum (FCS; Gibco, Life Technologies, Auckland, NZ) and 0.27% gentamicin (Invitrogen, Life Technologies, Auckland, NZ) at 37°C in a humidified atmosphere of 5% CO_2. Subcultures were obtained twice a week by trypsinization using 0.25% trypsin/EDTA solution (Sigma-Aldrich, Merck, NZ). At confluence, cells were transferred to culture plates, where differentiation was induced by growing cells in an osteogenic medium of αMEM supplemented with 10% FCS and ascorbic acid (AA, 50 µg/ml; Sigma-Aldrich, Merck, NZ) and β-glycerophosphate (βGP, 10mM; Sigma-Aldrich) as differentiating factors. Cells were treated with control (medium + carrier only), GSM-A or GSM-B (100 µg/ml)) and/or LEP (100 ng/ml) for 7 to

21 days depending on the type of the assay. 0.1% DMSO/unstimulated cells were used as a negative control (control group) and LEP-stimulated cells were used as a model group in all the experiments.

5.2.4 Cell metabolic activity/viability (MTT) assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed as described earlier (Van Meerloo et al., 2011) with some modifications for MC3T3-E1 cells to check the metabolic activity of viable cells against GSM and LEP.

MC3T3-E1 were plated at a density of 4×10^3 cells/well in 96 well plates and allowed to adhere and grow for 24 hours. Cells were then cultured in osteogenic medium and GSM-A or GSM-B (100 µg/ml) and/or LEP (100 ng/ml) with 6 wells/treatment for the next 6 days with the medium including factors changed twice a week. The MTT assay was performed on day 7 of incubation; 10 µL of MTT (Sigma Aldrich, Merck, NZ) in PBS was added to each well at a final concentration of 0.5 mg/mL and incubated for three hours until purple crystals were microscopically visible, which were then solubilized by adding a volume of DMSO equal to that of the cell culture media in the well. Absorbance was quantified by measuring optical density (OD) at a wavelength of 550 nm using a microplate reader (Multiskan Fc, Thermo Fisher Scientific).

5.2.5 RNA extraction, reverse transcription and RT-qPCR

MC3T3-E1 cells were seeded at a density of 2×10^4 cells/well in 24 well plates. At approximately 80% confluence, they were induced to differentiate in osteogenic medium for 14 days, which was refreshed twice a week. Cells were then treated with GSM and/or LEP in osteogenic medium which was refreshed twice a week for the next 7 days. On day 21, total RNA was extracted using an RNA extraction kit (PureLink RNA Mini Kit, Thermo Fisher, Auckland, NZ). 1 µg of RNA was reverse transcribed using SuperScript® IV First-Strand Synthesis System (Invitrogen, Thermo Fisher, Auckland, NZ). RT-qPCR was done with gene-specific primers (Invitrogen, Life Technologies, Australia) using a PCR kit (LightCycler® 480 SYBR Green I Master, RochLife Science, Thermo Fisher, Auckland, NZ). GADPH was used as an internal control. Relative expression of the genes was quantified by the E-method of advanced relative quantification.

MC3T3-E1 osteoblast mRNA was assessed for alkaline phosphatase *(Alp),* runt-related transcription factor 2 *(Runx2),* bone sialoprotein *(BSP),* osterix *(Osx),* osteocalcin *(OCN),* collagen type 1 alpha chain 1 *(Col1α1),* and collagen type 10 chain alpha chain 1 *(Col10α1).* The specific primers for the genes are listed in Table 5.2.

Table 5.2: List of primers used for RT-qPCR

Primer	Forward sequence (5'→3')	Reverse sequence (5'→3')	Ta (°C)
GADPH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	55
Alp	GTGACTACCACTCGGGTGAAC	GTGACTACCACTCGGGTGAAC	60
Runx2	GACTGTGGTTACCGTCATGGC	ACTTGGTTTTTCATAACAGCGGA	60
BSP	CAGGGAGGCAGTGACTCTTC	AGTGTGGAAAGTGTGGCGTT	60
Osx	TCCCTGGATATGACTCATCCCT	CCAAGGAGTAGGTGTGTTGCC	60
OCN	CTGACCTCACAGATCCCAAGC	TGGTCTGATAGCTCGTCACAAG	60
Col1α1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG	60
Col10α1	TTCTGCTGCTAATGTTCTTGACC	GGGATGAAGTATTGTGTCTTGGG	60

(**Key:** GADPH = glyceraldehyde-3-phosphate dehydrogenase, Alp = alkaline phosphatase, Runx2 = runt-related transcription factor 2, BSP = *bone sialoprotein, Osx = osterix, OCN = osteocalcin, Col1α1 = collagen type 1 alpha chain 1, and Col10α1 = collagen type 10 alpha chain)*

5.2.6 ALP activity assay

Alkaline phosphatase (ALP) activity and total protein content were determined as described previously (Yodthong et al., 2018) with some modifications. 4×10^4 cells per well were seeded into 24 well plates. When confluent, cells were induced to differentiate in osteogenic medium and treated with GSM and/or LEP in single or duplicate wells. ALP activity was determined in the whole cell lysate using reagents and the protocol as described in a TRACP & ALP assay kit (TaKaRa, Otsu, Japan). Reagents included: physiological/0.9% saline (NaCl, Sigma Aldrich) for washing the cells, an

extraction buffer (1% Triton-X100 in 1× TBS) (Tris, HCl, NaCl, Triton X100, Sigma Aldrich), ALP assay solution (Tris, HCl, $MgCl_2$, Sigma Aldrich), ALP substrate, para-nitro-phenyl phosphate (pNPP, Sigma Aldrich), stop solution (1N NaOH, Sigma Aldrich), and 4-Nitrophenol (Sigma Aldrich) for the standard curve.

ALP activity was then normalized by total protein content, which was measured by using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, Auckland, NZ). The assay was done on MC3T3-E1 cells treated either during or after the early osteoblast differentiation procedure. For the former, MC3T3-E1 pre-osteoblasts were induced to differentiate in osteogenic medium together with GSM and/or LEP for the full incubation period of 14 days and thus were exposed to GSM and/or LEP during both as pre-osteoblasts and as developing, proliferating osteoblasts. For the latter, MC3T3-E1 pre-osteoblasts were first partially differentiated in an osteogenic medium for 7 days and then the developing osteoblasts were retained in the osteogenic medium in combination with GSM and/or LEP for the next 7 days.

5.2.7 Mineralization (Alizarin red staining)

Alizarin red staining was done to measure the effects of GSM on extracellular mineralization of pre-differentiated MC3T3-E1 osteoblasts. For this purpose, MC3T3-E1 cells were seeded at a density of 2×10^4 cells/well in 24 well plates. At approximately 80% confluence, they were induced to differentiate in osteogenic medium for 14 days, which was refreshed twice a week, and then treated in duplicate wells with GSM and/or LEP, which were refreshed twice a week for the next 7 days in osteogenic medium. On day

21, mineralized nodules were stained with Alizarin red (Sigma Aldrich) as described previously with some modifications (Yingyu et al., 2016). The mineralized nodules were photographed, and the stain was solubilized using cetylpyridinium chloride (CPC; Sigma Aldrich) and quantified by measuring optical density at 550 nm using a microplate reader (Multiskan Fc, Thermo Fisher Scientific). The timeline for the development of MC3T3-E1 preosteoblasts into functioning osteoblasts is shown in figure 5.1:

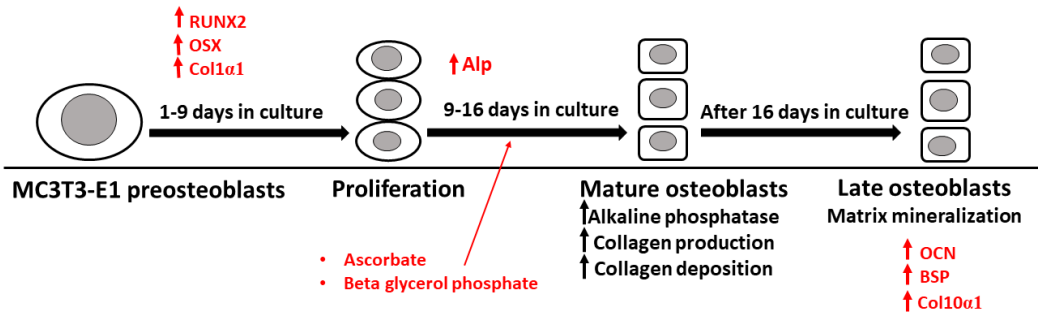

Figure 5.1: Schematic representation of MC3T3-E1 developmental stages.

5.2.8 Statistical analysis

All experiments were repeated at least three times and results are presented as mean ± SEM. Statistical analysis was done using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, N.Y., USA). Different groups were compared by one-way analysis of variance (ANOVA). $p<0.05$ was considered to indicate a statistically significant difference.

5.3 Results

5.3.1 Cell metabolic activity and survival

Potential cytotoxicity of LEP and the GSM extracts on MC3T3-E1 pre-osteoblasts was determined by MTT assay. As shown in figure 5.2, none of the treatments negatively affected the viability of the cells. LEP and both the GSM extracts increased the metabolic activity of the viable cells. LEP at 100 ng/ml significantly increased ($p<0.05$) the metabolic activity of the cells, GSM-A also slightly increased the cell viability of both the unstimulated and the LEP-stimulated cells. GSM-B or the combination of LEP + GSM-B significantly increased ($p<0.01$, $p<0.001$ respectively) the number of metabolically active cells present. GSM-A with or without LEP at 100 ng/ml had no significant effect as shown in figure 5.2.

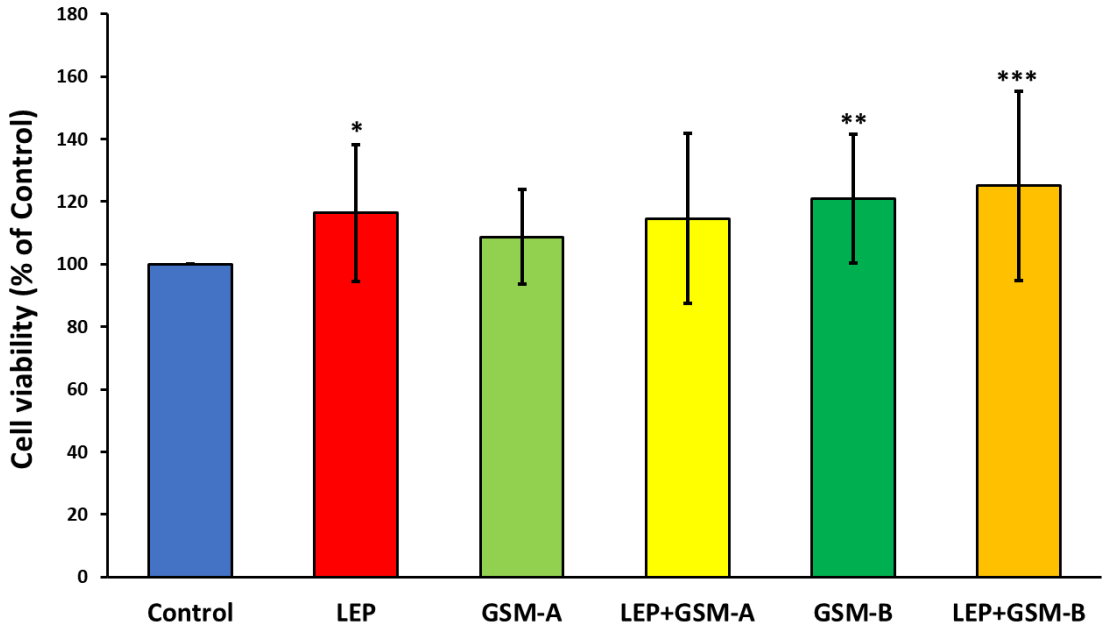

Figure 5.2: Effect of LEP (100 ng/ml), GSM-A and GSM-B (100 µg/ml) was assessed by MTT on the metabolic activity of MC3T3-E1 osteoblasts after 7 days. Cells were treated with 100 µg/ml of GSM-A and GSM-B, and/or 100 ng/ml of LEP. The data were analysed by single-factor ANOVA. *p<0.05, **p<0.01, ***p<0.001 indicates difference from 0.1% DMSO carrier control (unstimulated) cells. The values represent the mean ± SD of three independent experiments each conducted in six replicates (n=18)

5.3.2 RT-qPCR analysis

RT-qPCR was performed after extracting mRNA from MC3T3-E1 cells following treatment with GSM and/or LEP to assess their effects on transcription levels of specific genes related to osteogenesis. All PCR results were normalized to a housekeeping gene to account for potential variations among the samples, extraction, and RNA quality and efficiency in cDNA synthesis, internal controls, and the treatment groups. Differentiated MC3T3-E1 osteoblasts were assessed for mRNA expression for the osteoblast differentiation initiator osterix (*Osx*); the pro-mineralization biomarkers alkaline phosphatase *(Alp)* and osteocalcin *(OCN);* the osteoblast proliferation-initiator runt-

related transcription factor 2 *(Runx2);* and the biomarkers of healthy trabecular bone formation bone sialoprotein *(BSP),* collagen type 1 alpha chain 1 *(Col1α1)* and collagen type 10 alpha chain 1 *(Col10α1).*

Figure 5.3 compares LEP, GSM-A and the GSM-B for their effects on the expression of osteoblast mineralization markers and as shown, there was no significant difference between unstimulated control and LEP stimulated cells. Both GSM-A and GSM-B increased the expression of the selected markers; however, GSM-B appeared more effective as compared to GSM-A especially when used together with LEP. GSM-A alone increased the expression of all osteogenic/mineralization markers except *OCN,* with *Alp* (+83%) being significantly increased ($p<0.001$). When combined with LEP, GSM-A also significantly increased ($p<0.01$) *Col1α1* and *Osx.* GSM-B also increased the expression of all genes except *OCN*, with Alp being significantly increased ($p<0.05$). When GSM-B was combined with LEP*, Alp*($p<0.05$)*, RUNX2* ($p<0.01$), *Osx* ($p<0.05$) and *Col10α1* ($p<0.01$) were significantly increased.

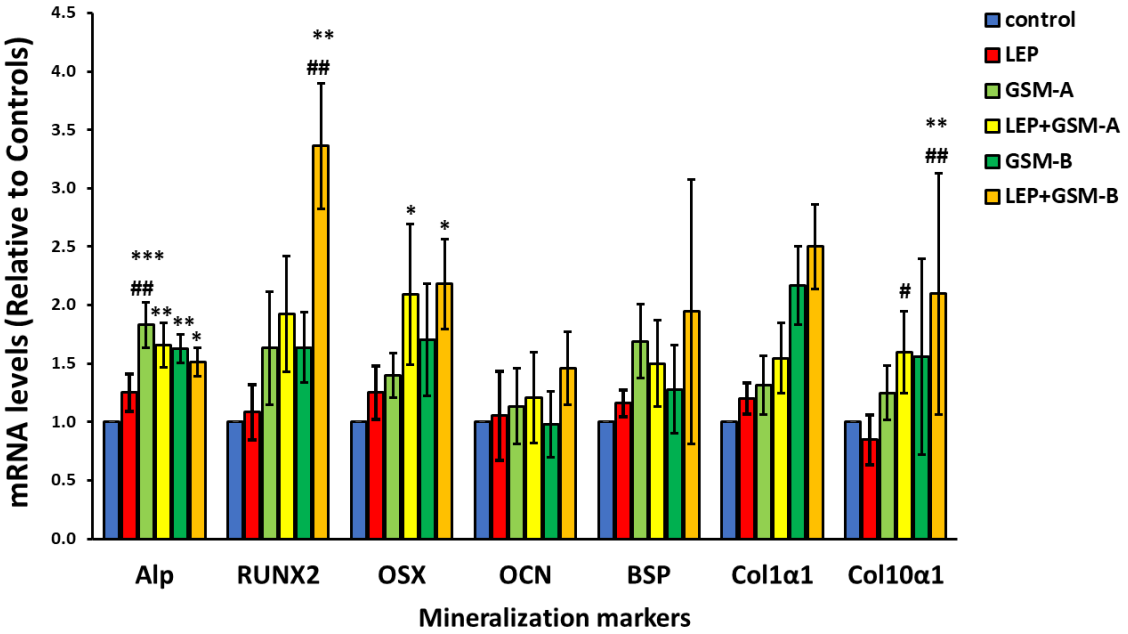

Figure 5.3: Advanced relative quantification (RT-qPCR) of early markers of osteoblast differentiation (*Alp, RUNX2, Osx, and Col1α1)* and osteogenic markers *(BSP, OCN,* and, *Col10α1)* in MC3T3-E1 cells pre-differentiated for 14 days and then treated with 100 µg/ml GSM-A or GSM-B and/or 100 ng/ml LEP for 7 days, *GADPH* was used as a reference gene for normalization. Each column represents the mean of seven biological samples. The data were analysed by single-factor analysis of variance, *p<0.05, **p<0.01 and ***p<0.001 signifies difference from 0.1% DMSO carrier control (unstimulated), and #p<0.05, ##p<0.01 and ###p<0.001 represents a difference from the model group (LEP-stimulated); values were normalized to the matching unstimulated control and represent mean ± SEM of seven independent biological samples.

5.3.3 ALP activity

Alkaline phosphatase (ALP) is an early marker of osteoblast differentiation. ALP activity was first measured for both GSM extracts in MC3T3-E1 treated during both pre-osteoblast and developing osteoblast periods. For this, MC3T3-E1 pre-osteoblasts were induced to differentiate in osteogenic medium concurrently with GSM and/or LEP treatment during a total incubation period of 14 days. As shown in figure 5.4A, all the

treatment groups significantly suppressed ($p<0.001$) the ALP activity and thus the differentiation of pre-osteoblasts as compared to control.

Seven days pre-differentiated MC3T3-E1 osteoblasts (figure 5.4B) on the other hand, behaved differently when subsequently treated with GSM-A and GSM-B (100 µg/ml) and/or LEP during the proliferation period from days 7-14. LEP appeared to slightly (22%) decrease ALP activity compared to 0.1% DMSO control, but this did not reach statistical significance and the coefficient of variance for this parameter was high. Neither of the mussel extracts affected the ALP activity of pre-differentiated osteoblasts and both GSM extracts appeared to negate the slight decrease caused by LEP when used in combination.

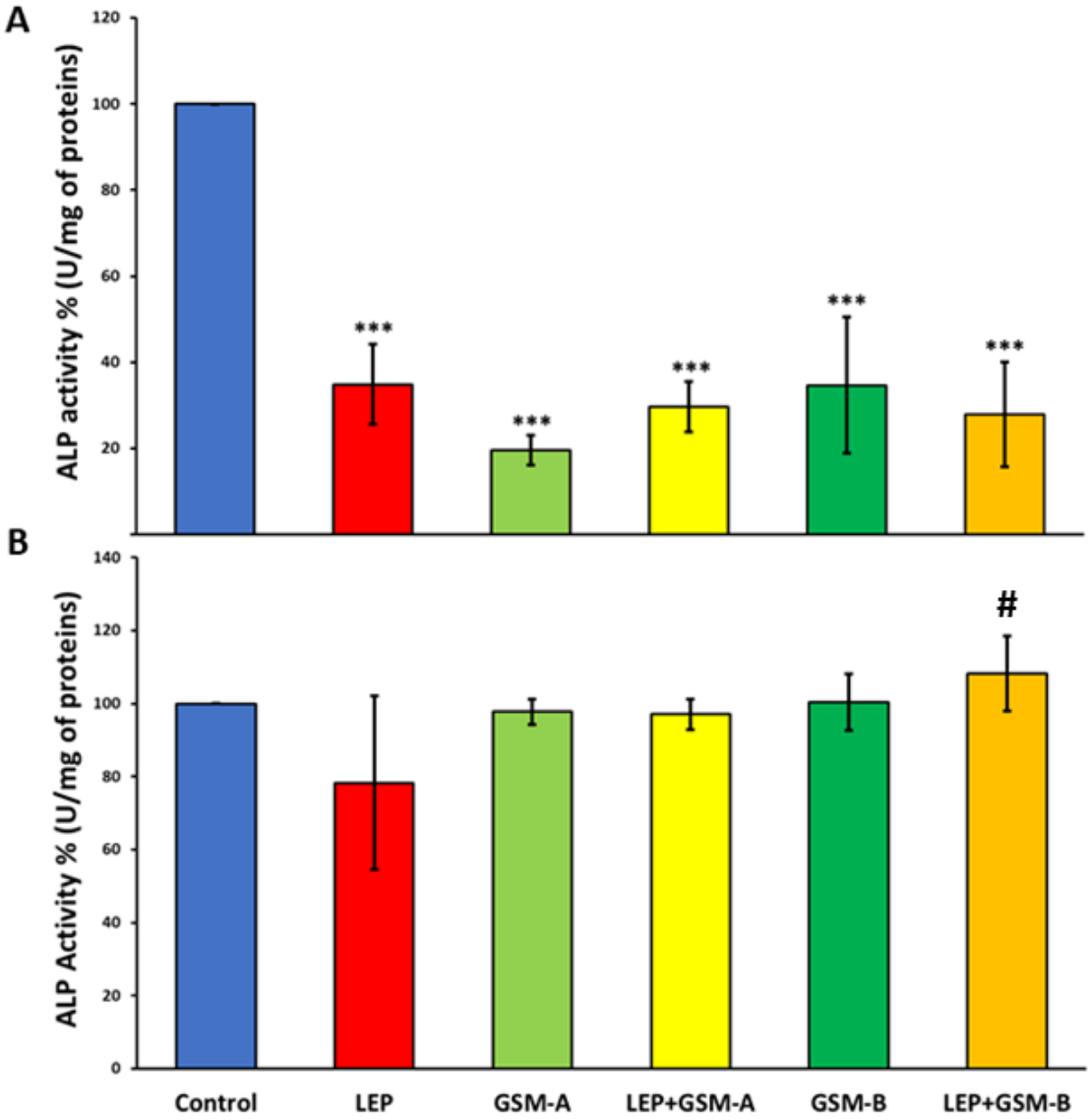

Figure 5.4: Effect of LEP and GSM on ALP activity in MC3T3-E1 osteoblasts. A) MC3T3-E1 osteoblasts treated with GSM-A and GSM-B (100 µg/ml) and LEP (100 ng/ml) for 14 days; B) 7 days pre-differentiated MC3T3-E1 osteoblasts treated with GSM-A and GSM-B (100µg/ml) and/or LEP (100 ng/ml) for 7 days. The data were normalized to total protein content and analysed by single-factor analysis of variance. ***p<0.001 signifies difference from 0.1% DMSO carrier control group (unstimulated). The values represent the mean ± SEM of three independent experiments.

5.3.4 Osteoblast mineralization (Alizarin red staining)

Alizarin red staining (ARS) to identify calcium deposition and mineralisation signifying *de novo* bone formation was performed after treating 14 days pre-differentiated osteoblasts with GSM-A and GSM-B (100 µg/ml) and or LEP (100 ng/ml). Staining was quantified by measuring the optical density of the solubilized stain and verified visually in photomicrographs. As shown in figure 5.5, none of the treatments except LEP+GSM-B produced any significant effects on mineralization. Although neither LEP nor GSM-B independently altered mineralization, the combination resulted in a synergistic stimulus that significantly increased ($p<0.001$) the mineralization/calcium deposition of MC3T3-E1 osteoblasts as compared to all the other treatment groups.

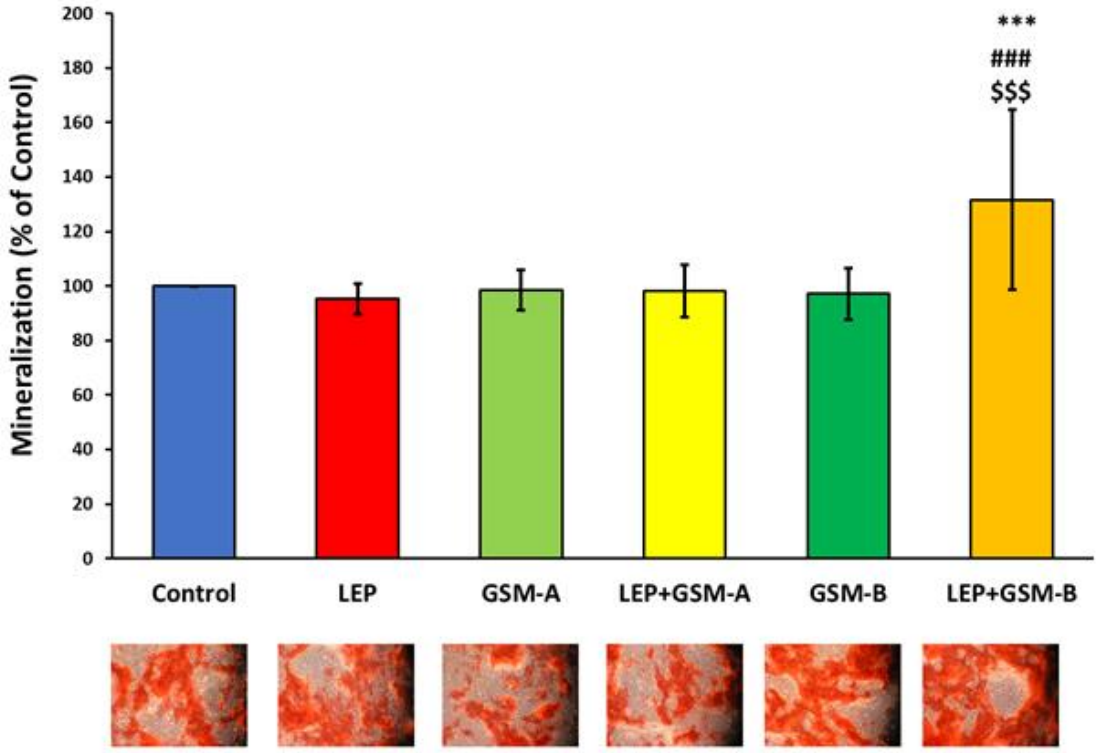

Figure 5.5: Effect of LEP and GSM extracts on mineralization of 14 days pre-differentiatedMC3T3-E1 osteoblasts. Cells treated with 100 µg/ml of GSM-A and GSM-B and/or 100 ng/ml LEP, for 7 days and formation of mineralized nodules was evaluated by ARS, followed by the representative images (400× magnification) of each treatment. The data were analysed by single-factor analysis of variance. ***p<0.001 signifies difference from 0.1% DMSO carrier control group (unstimulated), ###p<0.001 denotes difference from model group (LEP) and $$$ denotes difference from LEP+GSM-A. The values represent the mean ± SD of three independent experiments (n=12).

5.4 Discussion

This study aimed to first mimic the OA-related subchondral trabecular bone (STB) changes *in vitro* and then to study the effects of two GSM extracts on the STB model of OA. MC3T3-E1 cells are commonly used to assess osteogenic differentiation and mineralization of bone *in vitro* (Li et al., 2019a). Keeping in view the role of LEP in OA pathogenesis, we stimulated MC3T3-E1 cells with LEP to mimic OA related changes *in*

vitro. Interestingly, both GSM extracts and LEP independently increased cell proliferation under normal growth conditions (figure 5.1) although not all treatment differences were significantly significant, but they actively suppressed differentiation of some pre-osteoblasts into functional osteoblasts (figure 5.4A). LEP alone had no significant effect on the gene expression by mature osteoblasts of any osteoblast proliferation markers (figure 5.2). In contrast, GSM significantly increased expression of *Alp*, and GSM when combined with LEP acted synergistically to increase expression of *Runx2, Osx* and *Col10α1*. GSM-B demonstrated a higher overall level of bioactivity compared to GSM-A, and in particular acted synergistically with LEP to significantly increase mineralisation by post-differentiated osteoblasts; this was not observed with any other treatment condition. GSM appears to be a strong candidate as a dietary supplement to improve bone health as related to OA, although efficacy is significantly impacted by the processing procedure used for the source mussel material.

In the cell model used in this study, 100 ng/ml of LEP was used to stimulate MC3T3-E1 osteoblasts. There are no published reports of this cell line being treated with LEP to study its effects on osteoblast mineralisation or production of the osteogenesis genes measured in the current study, and thus these novel findings cannot be directly compared with other studies. Our results showed that LEP at this concentration increased the number of metabolically active cells as well as mRNA expression of osteogenic/differentiation markers, although only the effect on cell number was statistically significant. LEP downregulated the ALP activity in pre-osteoblasts and appeared to slightly decrease ALP in pre-differentiated osteoblasts.

Previous literature regarding the effects of LEP on the skeleton are contradictory: Ducy *et al* described LEP as a potent inhibitor of bone formation (Ducy et al., 2000), while in another study LEP was found to increase bone formation (Iwaniec et al., 2007). Numerous studies have shown that in addition to acting through the CNS, where LEP binds to ventromedial hypothalamus and activates noradrenergic signalling at the osteoblasts that results into increased bone mass (Upadhyay et al., 2015), LEP can also act directly on peripheral tissues, including bone marrow mesenchymal stem cells (BMSCs) and increases osteoblast proliferation and mineralization (Burguera, 2001, Gordeladze et al., 2002, Mutabaruka et al., 2010a). Mutabaruka et al (2010b) also demonstrated that local production of LEP increases in OA osteoblasts which may also increase certain osteoblast differentiation markers. Moreover, the exogenous administration of LEP was shown to increase ALP activity of MC3T3-E1 cells but failed to further affect the release of OCN and collagen type 1 production. In another study (Lamghari et al., 2006), 200 ng/ml of LEP was found to decrease ALP activity as well as the production of the bone resorption protein receptor activator of nuclear factor kappa-B ligand (RANKL); however a lower dose of LEP had the opposite effect on ALP activity. Xu *et al* also found that 160 ng/ml LEP significantly increased the expression of genes related to bone cell differentiation in human bone marrow stromal cells (hBMSCs) (Xu et al., 2016). Together these data suggest that the role of LEP with respect to bone metabolism is complex; it has been proven to play an important role in the abnormal physiology of OA osteoblasts but the exact mechanism is yet to be explored (Mutabaruka et al., 2010a). It is possible that its effects *in vivo* are dose-dependent and

thus the level of body adiposity, correlating with the amount of LEP produced and the circulating LEP concentration, may result in different effects on bone cell function.

GSM-A and GSM-B used in this study are different with respect to their processing. GSM-A as mentioned earlier was prepared by the conventional method that involves blending, freeze-drying, and milling, whereas GSM-B was made by a method that included an additional enzymatic digestion step. Our results showed GSM-B to be more potent regarding its effects on growth, differentiation, and mineralization of the osteoblasts particularly in the LEP-stimulated/model group (figures 5.2, 5.3, 5.4B, 5.5); these results are in agreement with the literature, which says that enzymatic processing may preserve the bioactive composition of GSM as compared to conventional mechanical shucking (Tian Hong, 2018).

A variety of transcription factors and proteins are involved at every stage of osteoblast development. Runx2 is an early differentiation factor that regulates the differentiation of mesenchymal stem cells into osteoblast precursors (pre-osteoblasts). Osx is produced in pre-osteoblasts and controls the further maturation of osteoblasts and finally the formation of osteocytes and bone (Sinha and Zhou, 2013). Alp and Col1α1 are proteins produced during early matrix formation in osteoblasts while OCN, Col10α1, and BSP are the osteogenic markers representing the mineralization of the osteoblast matrix and thus maturation of osteoblasts in bone (Kirkham and Cartmell, 2007). GSM-B, but not GSM-A, significantly increased expression of OSX, RUNX2 and Col10α1. Overall, the findings suggested that both extracts have the potential to upregulate mRNA levels of

osteogenic markers, but the effect was more prominent with GSM-B and the effect was greater in LEP-stimulated cells. These results show that GSM-B in particular assists every step of bone maturation from proliferation through late-stage differentiation to finally mineralization both independently and together with LEP, and clearly demonstrate that the manufacturing process for GSM-B produced bioactive factors not present in GSM-A.

The effects of GSM on ALP activity showed a very interesting trend in MC3T3-E1 pre-osteoblasts and 7 days pre-differentiated osteoblasts. The pre-osteoblasts, which were exposed to the differentiating factors and the treatments simultaneously for 14 days showed significantly reduced ALP activity in response to both the GSM extracts alone or together with LEP. However, the pre-differentiated cells, which were first differentiated for seven days in the differentiating medium and then treated with GSM and/or LEP for the next 7 days showed a totally opposite trend. Under these conditions, GSM significantly increased the ALP activity of LEP stimulated cells when compared to the LEP-stimulated group. No study has so far compared the response of non-differentiated and differentiated MC3T3-E1 osteoblasts to any treatment; however, a similar study explored the effects of LEP on mesenchymal progenitor cells (MPC) and bone marrow stomal cells (BMCs) and demonstrated that LEP maintained MPC in an undifferentiated state and promoted the mineralization of more differentiated osteoblasts. This showed LEP has multiple peripheral roles depending on the differentiation state of MPC (Scheller et al., 2010). In another study involving osteoclasts, LEP negatively affected the development of osteoclast precursors but had no effect on mature osteoclasts (Cornish et al., 2002). The clinical relevance of these findings is yet to be understood.

GSM-B also promoted calcium deposition in the extracellular matrix (ECM) of LEP-stimulated osteoblasts. Although, there is no study so far reporting the effects of GSM on subchondral bone in OA, it is already proved that GSM can treat OA related symptoms by controlling inflammation and preventing cartilage damage (Li et al., 2014a, Siriarchavatana et al., 2019a). Furthermore, Siriarchavatana also found that GSM improved bone mineral density in rats with diet- and ovariectomy-induced metabolic OA (Siriarchavatana et al., 2020). Also, a water-soluble protein from blue mussel was shown to increase the growth and osteogenic activity of MC3T3-E1 cells (Xu et al., 2019).

5.5 Conclusion

In summary, the present study establishes that GSM and particularly, enzymatically processed GSM-B has anabolic effects on bone formation *in vitro* especially when osteoblasts are also stimulated with LEP. The higher efficacy of GSM-B specifically in the LEP-stimulated group indicates that it has the potential to prevent the damage to subchondral bone that occurs with OA, and further may be of use in improving bone mineral density and structure in osteoporosis.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Saima Rizwan
Name/title of Primary Supervisor:	Dr. Frances M Wolber
In which chapter is the manuscript /published work:	Chapter Five

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Chapter Six: Immunomodulation and osteogenesis induced by whole green shell mussel *(Perna canaliculus)* powder in macrophage and osteoblast cell models of osteoarthritis

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Enzymatically processed greenshell mussel (GSM-B) was found to be more effective in the previous studies (chapter 5) in providing protective health benefits. Therefore, in this chapter only GSM-B was further studied in models of synovial inflammation and bone. There was no information in the literature regarding *in vitro* use of LEP or GSM so we tried multiple concentrations and cast a wide net so that we would not miss any possible effects. The aims of this study, therefore, were first to design and assess *in vitro* cell models by challenging J774A.1 macrophages and differentiated MC3T3-E1 osteoblasts with leptin, and second to determine whether whole GSM-B has protective effects in these models of synovial inflammation and bone remodelling.

Abstract

Osteoarthritis (OA) is an incurable joint disease characterized by gradual loss of articular cartilage. Synovial macrophages and subchondral bone remodelling play important roles in the pathogenesis of this condition. Leptin, produced by adipocytes, exacerbates the pathogenesis of OA. Green shell mussel (GSM) oil can treat inflammatory conditions including OA but the effects of the whole mussel are unknown, and GSM has never been assessed using bone osteoblasts. We created novel cell assays using leptin (LEP), murine J774A.1 and MC3T3-E1 subclone 4 cells as *in vitro* OA models of synovial inflammation in macrophages and subchondral bone remodelling by osteoblasts respectively and investigated the role of GSM in these models. LEP significantly upregulated pro-inflammatory *IL-1β* ($p<0.01$) and *TNF-α* ($p<0.05$) but not anti-inflammatory *IL-10* mRNA in macrophages, and significantly suppressed osteoblast proliferation ($p<0.001$). GSM however significantly upregulated ($p<0.05$) macrophage *IL-10* mRNA, an effect not inhibited by LEP. GSM also significantly upregulated *IL-1β* and *TNF-α* mRNA ($p<0.05$); when combined with LEP the effects were synergistic and additive ($p<0.001$) respectively. In osteoblasts, GSM significantly upregulated alkaline phosphatase *(Alp)* mRNA ($p<0.05$), ALP enzyme activity ($p<0.05$), and subsequent mineralisation ($p<0.05$). Inclusion of LEP did not inhibit GSM-induced ALP mRNA or enzyme activity but did block mineralisation. In the presence of LEP, GSM also significantly increased osteoblast mRNA levels of the osteogenic markers bone sialoprotein($p<0.05$), osteocalcin ($p<0.05$) and collagen type 10 ($p<0.05$). These results describe the novel use of LEP with J774A.1 and

MC3T3-E1 cells to model synovial inflammation and subchondral bone damage in OA and demonstrate that whole GSM can provide protective effects in both macrophages and osteoblasts challenged with LEP.

Key words: osteoarthritis, leptin, osteoblast, macrophage, inflammation

6.1. Introduction

Osteoarthritis (OA) is a painful and debilitating deterioration of the whole synovial joint accompanied by a progressive and steady loss of articular cartilage, sclerotic changes to the subchondral bone, osteophytosis, and synovial inflammation (Dickson et al., 2019a). Despite its widespread prevalence, the exact mechanism of its pathogenesis is not fully understood. However, it is no longer thought of as a purely non-inflammatory or biomechanical process, an increasing body of evidence suggests it is accompanied by chronic low-level, often subclinical inflammation (Orlowsky and Kraus, 2015).

Although OA is primarily considered a disorder of articular cartilage, the role of subchondral bone has gained increasing attention and osteoblasts are believed to play a vital role in the pathogenesis of OA (Suri and Walsh, 2012). Morphological changes in bone during OA are well characterized and include sclerosis, hypomineralization, osteophytosis, and subchondral cyst formation (Li et al., 2013). Macrophages are key players during inflammation (Dickson et al., 2019a) and a multitude of studies have proved the importance of activated macrophages in OA and their number is significantly related to pain and joint space narrowing. In addition, biomarkers of activated

macrophages could be used to predict the severity of disease progression in OA patients (Kraus et al., 2016, Wu et al., 2020, Zhang et al., 2018). Early insult to cartilage releases damage-associated molecular patterns (DAMPs), which are recognized by the pattern recognition receptors (PRRs) on macrophages and results in the subsequent production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) through activation of the NF-κB pathway. Pro-inflammatory cytokines finally degrade cartilage by targetting different components of the cartilage (Bondeson et al., 2010). Macrophages are a heterogeneous and functionally diverse class of the cells that play an important role in homeostasis and immune responses. They have two important phenotypes: M1, which initiates and sustains the inflammatory response and is characterized by the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6), and M2 (the anti-inflammatory or pro-resolving), the phenotype which is associated with the release of anti-inflammatory mediators/cytokines (IL-10) meant to resolve inflammation (Viola et al., 2019). A higher M1/M2 ratio is considered as an important hallmark of OA. Macrophages are present in several metabolic tissues in the body including adipose tissues and synovium. Various factors can alter proliferation, plasticity, and polarization of macrophages from M2 to M1, dietary factors (disturbance in cellular metabolites), and systemic factors such as adipokines are the most important (Lumeng et al., 2007).

Osteoblasts are cells of mesenchymal origin and serve as the building blocks for bone formation. Pre-osteoblasts are the precursors of osteoblasts which typically pass through three stages of development i.e. proliferation, matrix maturation, and mineralization (Rutkovskiy et al., 2016). A variety of transcription factors and proteins

are involved at every stage of osteoblast development. Runt related transcription factor 2 (Runx2) is an early differentiation factor that regulates the differentiation of mesenchymal cells into osteoblasts precursors (pre-osteoblasts). Osterix (Osx) is produced in pre-osteoblasts and controls the further maturation of osteoblasts and finally the formation of osteocytes and bone (Sinha and Zhou, 2013). Alkaline phosphatase (ALP) and collagen type 1 alpha 1 (Col1α1) are the proteins which, act as markers of early matrix formation in osteoblasts while osteocalcin (OCN), collagen type 10 alpha 1 (Col10α10), and bone sialoprotein (BSP) are the osteogenic markers representing the mineralization of the osteoblasts matrix and thus maturation of osteoblasts finally into the bone (Kirkham and Cartmell, 2007). Moreover, OCN is used as a marker of successful bone regeneration therapy (Saffarian Tousi et al., 2013).

Leptin (LEP), produced by adipocytes and other cells, is a multifunctional adipokine known for its pro-inflammatory role in the activation of macrophages. It promotes a proinflammatory phenotype by increasing the secretion of TNF-α and IL-1β in macrophages by acting through JAK2-STAT3 and PI3K-AKT-mTOR pathways (Monteiro et al., 2019). During OA, LEP levels are found to be elevated due to both the increased adipose tissue mass associated with aging and increased localised production in cartilage and tissues surrounding the joint (Dumond et al., 2003, Simopoulou et al., 2007). A study in a group of middle-aged women further supports the clinical relevance of LEP with OA, which revealed that LEP serum levels, 10 years before OA dignosis by MRI, were associated with cartilage defects, bone marrow abrasions, osteophytes,

meniscal anomalies, synovitis, and joint effusion (Karvonen-Gutierrez et al., 2014, Francisco et al., 2018).

Owing to the complex pathology of OA and the poor regenerative properties of the cartilage tissue (Karuppal, 2017), this disease currently has no cure. All existing treatments, which include exercise, weight management, physical therapy, medications, and surgery are meant to reduce pain and symptoms, as well as to improve the functional capacity of the joint (Zhang et al., 2019). The most commonly used medication for the treatment of OA are painkillers and anti-inflammatory medicines. Although these help relieve OA symptoms, they are sometimes associated with adverse side effects including nausea, vomiting, dizziness, constipation, sleepiness, tiredness, headache, potential cardiovascular risk, damage to liver, and gastrointestinal problems (Zhang et al., 2016). Therefore, alternative treatments that are safe and effective are needed to prevent or treat OA (McAlindon et al., 2014).

Nutrition is a possible alternative treatment that is increasingly recognized to have disease preventive potential along with fulfilling the basic nutritional requirements (German et al., 1999). OA is a classic example of a chronic disease amenable to being addressed by nutrition. Cartilage degradation in OA is a complex and multifactorial process, but pharmacologic compounds often target a single pathway, and this could be a reason for their partial ineffectiveness in the treatment of OA. Nutritional compounds on the other hand may contain multiple bioactive compounds and, therefore, could target multiple pathways. Moreover, chronic diseases including OA require long-term

pharmacological interventions often associated with significant adverse effects. Nutraceuticals and functional foods could be a better option because they are less likely to cause adverse physiological effects, addiction, or liver damage (Ameye and Chee, 2006).

Perna canaliculus or Greenshell mussels (GSM) are a bivalve mollusc long used as a source of nutraceuticals and are mostly recognized because of their bioactive lipids; including EPA (eicosapentaenoic acid), DHA (docosahexaenoic acid) and furan fatty acids (F-acids), sphingolipids, phytosterols, diacylglycerols, diterpenes, sesquiterpenes, and saponins, alongside anti-oxidants such as carotenoids, xanthophylls, and anthocyanins. Bioactives such as EPA and DHA are sourced not just from GSM but also from other sources that have already been proven to have anti-inflammatory effects in a variety of inflammatory diseases including arthritis (Eason et al., 2018).

Research into OA to better understand the initiating factors, disease progression, and potential intervention strategies in humans and animal models has been invaluable, but also time-consuming and costly. There are no established macrophage or bone osteoblast cell models designed to mimic the events that occur in osteoarthritis, particularly those driven by LEP. And while GSM lipids have been shown to have protective effects in OA, there is no published research to date regarding the use of GSM as a whole food including both water-soluble and fat-soluble components. The aims of this study were to design and assess *in vitro* cell models by challenging J774A.1 macrophages and differentiated MC3T3-E1 osteoblasts with leptin, and subsequently to

determine whether whole GSM has protective effects in these models of synovial inflammation and bone remodelling.

6.2 Materials and methods

6.2.1 Preparation of GSM extract and reconstitution of leptin for *in vitro* use

Whole Green shell mussel (GSM) powder extract was kindly provided by Sanford Ltd, New Zealand. The freeze-dried powder was dissolved in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies, Auckland, NZ) containing 10% DMSO (Sigma Aldrich, Merck, NZ), sonicated briefly to ensure solubility, sterilized through a 0.20 µm pore syringe filter, aliquoted, and stored at -20°C until further use.

Leptin (recombinant mouse leptin OB) was purchased from Biomyx (San Diego, California) and reconstituted in PBS (phosphate buffer saline, pH 8) (Sigma Aldrich, Merck, NZ) according to manufacturer's instructions, aliquoted and stored at -20°C until further use.

6.2.2 Reagents and cell culture

J774A.1 murine macrophage cell line and MC3T3-E1 subclone 4 murine pre-osteoblasts were purchased from American Type Culture Collection (ATCC, Virginia, USA).

J774A.1 cells were grown and maintained in DMEM (Gibco, Life Technologies, Auckland, NZ) and 10% heat-inactivated foetal calf serum (FCS) (Gibco, Life Technologies, Auckland, NZ) supplemented with 1% penicillin-streptomycin and glutamine (Invitrogen,

Life Technologies, Auckland, NZ). Subcultures of J774A.1 were obtained twice a week by scraping. At confluence, cells were transferred to culture plates where they were treated with LEP (10 µg/ml) and GSM (100 µg/ml). Cells were grown and maintained at 37°C in a humidified atmosphere of 5% CO_2.

MC3T3-E1 cells were grown and maintained in alpha minimum essential medium (αMEM) (Gibco, Life Technologies, Auckland, NZ), supplemented with 10% FCS (Gibco, Life Technologies, Auckland, NZ) and 0.27% gentamicin (Invitrogen, Life Technologies, Auckland, NZ) at 37°C in a humidified atmosphere of 5% CO_2. Subcultures were obtained twice a week by trypsinization using 0.25% trypsin/EDTA solution (Sigma-Aldrich, Merck, NZ). At confluence, cells were transferred to the culture plates, where differentiation was induced by growing cells in osteogenic media i.e. αMEM supplemented with 10% FCS and differentiating factors ascorbic acid (AA, 50 µg/ml) (Sigma-Aldrich, Merck, NZ) and β-glycerophosphate (βGP, 10mM) (Sigma-Aldrich, Merck, NZ) and treated with GSM (100 µg/ml and 1000 µg/ml) and/or LEP (10 µg/ml) for 7 to 21 days depending on the type of the assay. 0.1% DMSO/unstimulated cells were used as a negative control (control group) and LEP-stimulated cells were used as the test (model group) in all the experiments.

6.2.3 Cells metabolic activity/viability (MTT)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay were performed as described earlier (van Meerloo et al., 2011) with some modifications, both

for J774A.1 and MC3T3-E1 cells to check the metabolic activity of viable cells against GSM and LEP.

J774A.1 cells at a density of $5x10^4$ cells/well were plated in 96 well plates and allowed to adhere for 2 hours and then treated with 100 µg/ml GSM and/or 10 µg/ml LEP, GSM and/or LEP. After 24 hours, 10uL of MTT (Sigma Aldrich, Merck, NZ) in PBS was added to each well at a final concentration of 0.5 mg/mL and incubated for three hours until purple crystals were microscopically visible, which were then solubilized by adding a volume of DMSO equal to that of the cell culture media in the well. Absorbance was quantified by measuring optical density (OD) at a wavelength of 550 nm using a microplate reader (Multiskan Fc, Thermo Fisher Scientific).

MC3T3-E1 were plated at a density of $4x10^3$ cells/well in 96 well plates and allowed to adhere and grow for 24 hours. Cells were then cultured in osteogenic medium and GSM and/or LEP for the next 6 days and the medium was changed twice a week. The MTT assay was performed on day 7 of incubation as described for J774A.1 above.

6.2.4 RNA extraction, reverse transcription and RT-qPCR

J774A.1 cells were plated at a density $1x10^6$/well in 6 well plates and treated with 100 µg/ml GSM and/or 10 µg/ml LEP for 4 hours and total RNA was extracted using an RNA extraction kit (PureLink RNA Mini Kit, Thermo Fisher, Auckland, NZ). 1 µg of RNA was reverse transcribed using SuperScript® IV First-Strand Synthesis System (Invitrogen, Thermo Fisher, Auckland, NZ). RT-qPCR was performed with gene-specific primers

(Invitrogen, Life Technologies, Australia) using a PCR kit (LightCycler® 480 SYBR Green I Master, RochLife Science, Thermo Fisher, Auckland, NZ). *GADPH* was used as an internal control. Relative expression of the genes was quantified by advanced relative quantification. J774A.1 macrophage mRNA was assessed for changes in gene expression levels of pro-inflammatory cytokines interleukin 1-beta (*IL-1β*), tumour necrosis factor alpha (*TNF-α*) and interleukin 6 (*IL-6*), and anti-inflammatory cytokine interleukin 10 (*IL-10*), stimulated by various treatments.

MC3T3-E1 cells were seeded at a density of $2x10^4$ in 24 well plates. At approximately 80% confluence, they were induced to differentiate in osteogenic medium for 14 days, which was refreshed twice a week and then treated with GSM and/or LEP which was refreshed twice a week for the next 7 days in osteogenic medium. On the 21st day total RNA was extracted as described above for J774A.1

MC3T3-E1 osteoblast mRNA was assessed for changes in gene expression levels of alkaline phosphatase (Alp), runt-related transcription factor 2 (Runx2), Bone sialoprotein *(BSP), Osterix (Osx), O*steocalcin *(OCN),* Collagen type 1 alpha chain 1 *(Col1α1),* and Collagen type 10 chain alpha chain 1 *(Col10α1).* The specific primers for the genes are listed in Table 6.1:

Table 6.1: List of primers used

Primer	Forward sequence (5′→3′)	Reverse sequence (5′→3′)	Ta (°C)
GADPH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	55
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	65
IL-6	GACAAAGCCAGAGTCCTTCAGAGAG	CTAGGTTTGCCGAGTAGATCTC	60
IL-10	TGAGGCGCTGTCGTCATCGATTTCTCCC	ACCTGCTCCACTGCCTTGCT	65
TNF-α	CACGTCGTAGCAAACCACCAAGTGGA	TGGGAGTAGACAAGGTACAACCC	60
Alp	GTGACTACCACTCGGGTGAAC	GTGACTACCACTCGGGTGAAC	60
Runx2	GACTGTGGTTACCGTCATGGC	ACTTGGTTTTTCATAACAGCGGA	60
BSP	CAGGGAGGCAGTGACTCTTC	AGTGTGGAAAGTGTGGCGTT	60
Osx	TCCCTGGATATGACTCATCCCT	CCAAGGAGTAGGTGTGTTGCC	60
OCN	CTGACCTCACAGATCCCAAGC	TGGTCTGATAGCTCGTCACAAG	60
Col1α1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG	60
Col10α1	TTCTGCTGCTAATGTTCTTGACC	GGGATGAAGTATTGTGTCTTGGG	60

(**Note:** *GADPH* = glyceraldehyde-3-phosphate dehydrogenase, *IL-1β* = Interleukin beta, *IL-6* = Interleukin 6, *IL-10* = Interleukin 10, *TNF-α* = Tumour necrosis factor alpha, *Alp* = Alkaline phosphatase, *Runx2* = runt-related transcription factor 2, *BSP = Bone sialoprotein, Osx = Osterix, OCN = Osteocalcin, Col1a1 = Collagen type 1 alpha chain 1, and Col10a1 = Collagen type 10 alpha chain 1)*

6.2.5 ALP activity assay

Alkaline phosphatase (ALP) activity and total protein content were determined as described previously (Yodthong et al., 2018) with some modifications. $4x10^4$ cells per

well were seeded in 24 well plates, when confluent cells were induced to differentiate in osteogenic medium and treated with GSM in the presence or absence of LEP. ALP activity was determined in the whole cell lysate using reagents and protocol as described in the TRACP & ALP assay kit (TaKaRa, Otsu, Japan). Reagents include; physiological/0.9% saline (NaCl, Sigma Aldrich) for washing the cells, extraction buffer (1% Triton-X100 in 1 x TBS) (Tris, HCl, NaCl, Triton X100, Sigma Aldrich), ALP assay solution (Tris, HCl, $MgCl_{2,,}$ Sigma Aldrich), ALP substrate, para-nitro-phenyl phosphate (pNPP, Sigma Aldrich), stop solution (1N NaOH, Sigma Aldrich), and 4-Nitrophenol (Sigma Aldrich) for the standard curve.

ALP activity was then normalized by total protein content, which was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, Auckland, NZ). ALP activity was measured in both MC3T3-E1 pre-osteoblasts and pre-differentiated MC3T3-E1 osteoblasts. MC3T3-E1 pre-osteoblasts were induced to differentiation in osteogenic medium together with GSM and/or LEP and the total incubation period was seven days. MC3T3 osteoblasts on the other hand were first differentiated in osteogenic medium for seven days and then treated with GSM and/or LEP for the next seven days.

6.2.6 Mineralization (Alizarin red staining)

Alizarin red staining was performed to measure the effects of GSM on extracellular mineralization of pre-differentiated MC3T3 osteoblasts. For this purpose, MC3T3-E1 cells were seeded at a density of $2x10^4$ in 24 well plates. At approximately 80% confluence, they were induced to differentiate in osteogenic medium for 14 days, which

was refreshed twice a week and then treated with GSM and/or LEP which was refreshed twice a week for the next 7 days in osteogenic medium. On the 21st day, mineralized nodules were stained with Alizarin red (Sigma Aldrich) as described previously with some modifications (Yingyu et al., 2016). The mineralized nodules were photographed, and the stain was solubilized using cetylpyridinium chloride (CPC) (Sigma Aldrich) and the stain was quantified by measuring optical density at 550 nm using a microplate reader (Multiskan Fc, Thermo Fisher Scientific).

6.2.7 Statistical Analysis

All experiments were repeated at least two times and results are presented as mean ± SEM/SD. Statistical analysis was done using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, N.Y., USA). Different groups were compared by one-way analysis of variance (ANOVA). $p<0.05$ was considered to indicate a statistically significant difference.

6.3 Results

6.3.1 Cell metabolic activity and survival

The MTT assay is a common bioassay for determining total cell viability where optical density of formazan crystals corresponds to the relative number of metabolically active cells, which is often used as a proxy for total cell viabililty. GSM (100 µg/ml) and LEP (10 µg/ml) treatments each caused a small but statistically significant reduction ($p<0.01$ and $p<0.001$ respectively) in J774A.1 cells compared to the untreated control (Figure 6.1A).

A significant reduction ($p<0.001$ and $p<0.01$) was also observed when GSM was used in combination with LEP compared to both the control and the model group (LEP alone) respectively, although the effect of the two individual treatments did not appear additive.

In differentiated MC3T3-E1 osteoblasts, LEP significantly reduced ($p<0.001$) the number of metabolically active cells. GSM at 100 or 1,000 µg/ml in the absence of LEP had no significant effect compared to control. When the two treatments were combined, GSM at 100 µg/ml partially mitigated the cytotoxic effects of LEP (Figure 6.1B). GSM at 1,000 µg/ml completely mitigated the effect of LEP and resulted in a significantly higher ($p<0.01$ and $p<0.001$) number of MC3T3-E1 cells compared to control and model group repectively. Thus LEP had a small but significant cytotoxic effect in both cell lines, and GSM reduced J774A.1 cell numbers but resulted in no effect or a slight increase in cell numbers in MC3T3-E1 cells.

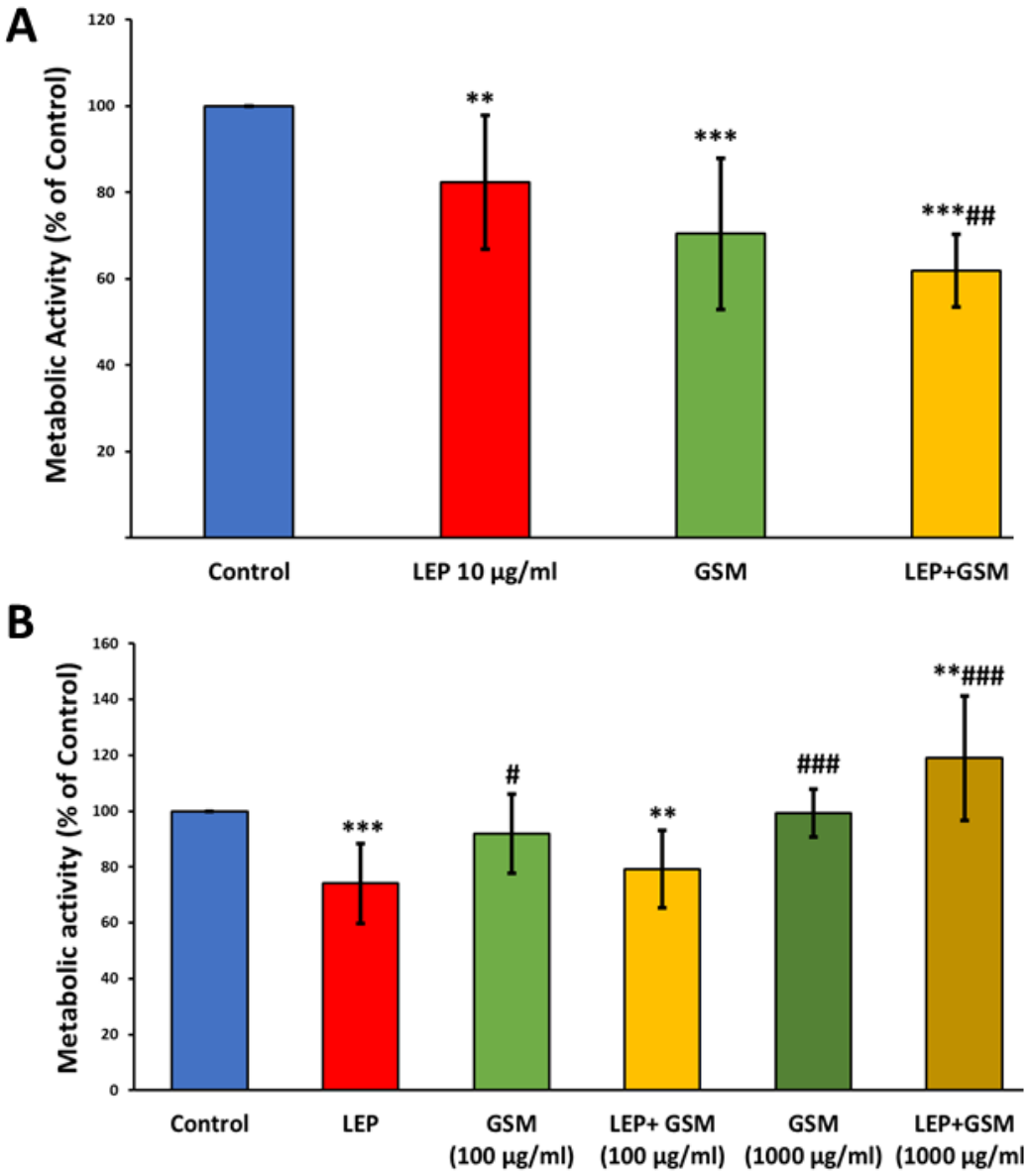

Figure 6.1: Effect of LEP (10 µg/ml) and GSM (100 or 1000 µg/ml) assessed by MTT on the cell metabolic activity of (A) J774A.1 macrophages with after 24 hours and (B) on MC3T3-E1 osteoblasts after 7 days. The data were analysed by single-factor ANOVA; **p<0.01, ***p<0.001 signifies significant difference with respect to 0.1% DMSO carrier control (unstimulated) and #p<0.05, ##p<0.01, ###p<0.001 signifies significant difference with respect to model group (LEP). The values represent mean ± SD of two to three independent experiments each conducted in 4 replicates (n=4).

6.3.2 RT-qPCR analysis

RT-qPCR was performed after extracting mRNA from J774A.1 and MC3T3-E1 cells following treatment with GSM and/or LEP to assess their effects on transcription levels of specific genes related to immunomodulation in macrophages and osteogenesis. As LEP and GSM each had small but significant effects on the number of metabolically active cells, albeit after 1 – 7 days treatment (figure 6.1), all PCR results were normalised to a housekeeping gene to account for potential differences in total cell numbers between treatment groups.

In J774A.1 macrophages, LEP alone upregulated the mRNA expression of the pro-inflammatory cytokines *IL-6* and *TNF-α* approximately three-fold, and of *IL-1β* approximately 40-fold (Figure 6.2). GSM alone at 100 µg/mL had a similar effect on these cytokines. As GSM at 100 µg/mL and LEP at 10 µg/mL had small but significant cytotoxic effects on these cells, higher concentrations were not assessed. The increases induced by either LEP or GSM were statistically significant for both *TNF-α* ($p<0.05$) and *IL-1β* ($p< 0.01$ and $p<0.05$). The effect of combining GSM and LEP treatments was additive for *TNF-α* and synergistic for *IL-1β*, with mRNA levels being nearly 140-fold higher than control for the latter. The combination of GSM and LEP did not upregulate *IL-6* mRNA above the levels seen with either treatment alone.

LEP alone did not significantly alterJ774A.1 mRNA levels of the anti-inflammatory cytokine *IL-10*. GSM alone did significantly increase ($p<0.05$) *IL-10*, approximately doubling the mRNA level. The combination of LEP and GSM retained the GSM effect,

significantly increasing (p<0.05) *IL-10* above both basal level and the level induced by LEP alone (Figure 6.2).

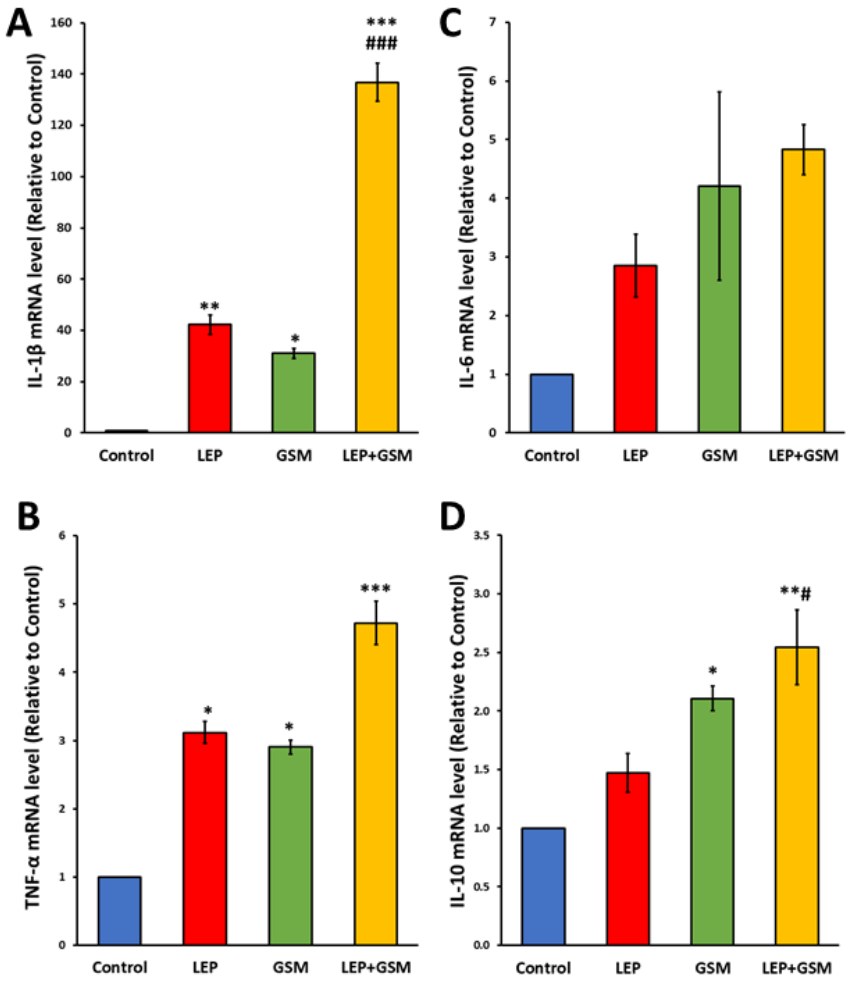

Figure 6.2: mRNA quantification (RT-qPCR) of pro-inflammatory (IL-1β, TNF-α & IL-6) and anti-inflammatory (IL-10) cytokines in J774A.1 cells treated with 100µg/ml of GSM extract and 10µg/ml leptin for 4 hours. GADPH was used as a reference gene for normalization. The data were analysed by single-factor analysis of variance, *p<0.05, **p<0.01, ***p<0.001 signifies difference from 0.1% DMSO carrier control (unstimulated), and #p<0.05, ###p<0.001 represents difference with respect to the model group (LEP); values were normalised to the matching unstimulated control and represent mean ± SE of two independent biological samples each tested in duplicate.

Differentiated MC3T3-E1 osteoblasts were assessed for mRNA expression for the osteoblast differentiation initiator osterix *(Osx);* the pro-mineralisation biomarkers alkaline phosphatase *(Alp)* and osteocalcin *(OCN);* the osteoblast proliferation-initiator runt-related transcription factor 2 *(Runx2);* and the biomarkers of healthy trabecular bone formation bone sialoprotein (BSP), collagen type 1 alpha chain 1 *(Col1α1)* and collagen type 10 alpha chain 1 *(Col10α1).* As shown in Figure 6.3, LEP alone (10 µg/mL) did not significantly affect these markers; LEP did suppress *Alp* mRNA by approximately 40% but this did not reach statistical significance due to higher variability between repeat measurements in this cytokine compared to the others measured. GSM at 100 µg/mL with or without LEP had no significant effect on these markers. GSM at a ten-fold higher concentration caused a significant increase ($p<0.05$) in *Alp* mRNA, and this effect was not blocked by LEP despite LEP alone markedly reducing *Alp* mRNA. Interestingly, osteoblasts treated with GSM at 1,000 µg/mL in the presence of LEP challenge significantly upregulated (250 – 320%) *BSP* ($p<0.05$), *OCN* ($p<0.05$) and *Col10α1* ($p<0.05$),and induced non-significant (50 – 100%) increases in *Col1α1, Osx* and *Runx2*.

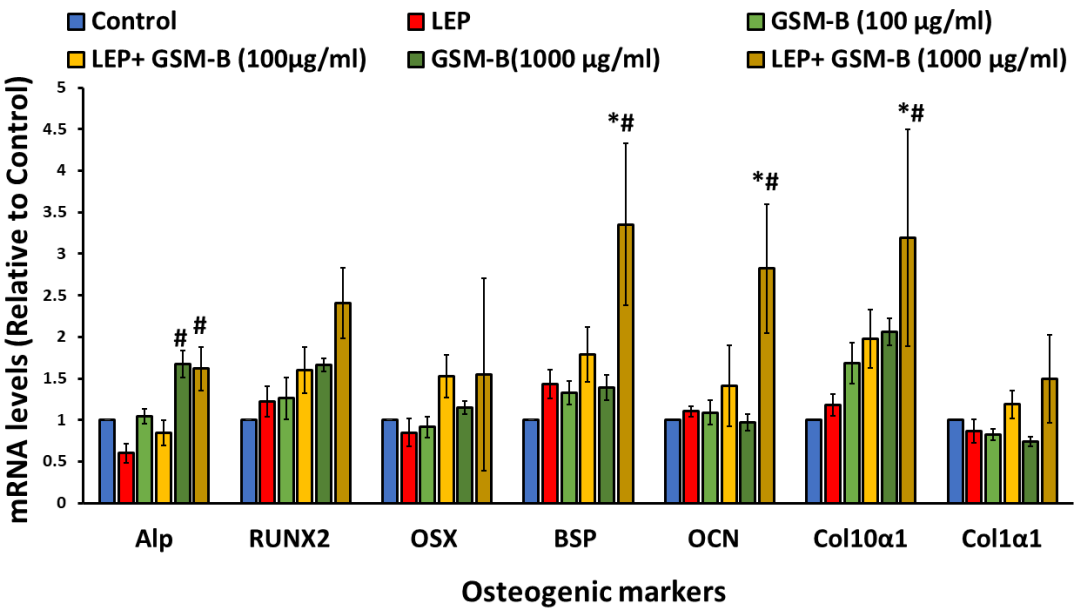

Figure 6.3: Advanced relative quantification (RT-qPCR) of early markers of osteoblast differentiation (*Alp, Runx2, Osx,* and *Col1α1*) and osteogenic markers (*BSP, OCN*, and *Col10α1*) in MC3T3-E1 cells pre-differentiated for 14 days and then treated with two concentrations of GSM extract (100µg/ml and 1000 µg/ml) and/or 10µg/ml leptin for 7 days. *GADPH* is used as a reference gene for normalization. Each column represents the mean of three replicates of each of the two biological samples. The data were analysed by single-factor analysis of variance, *p<0.05, signifies difference from 0.1% DMSO carrier control (unstimulated), and #p<0.05, represents a difference from the model group (LEP-stimulated); values were normalised to the matching unstimulated control and represent mean ± SEM of three independent biological samples each tested in duplicate.

6.3.3 ALP activity

As Alp mRNA was upregulated in 14 days differentiated osteoblasts treated for an additional 7 days with GSM both with and without LEP, ALP activity was also assessed in cells that underwent 7 days of differentiation followed by 7 days of treatment with GSM and LEP. GSM and LEP were previously observed to cause small changes in cell number, so ALP activity was normalised to total protein per well corresponding to total cells per

well. As expected, LEP alone caused a small but not significant reduction in ALP activity, similar to the earlier effect observed for Alp mRNA (Figure 6.4A). GSM at 100 µg/mL with or without LEP did not significantly alter ALP activity. GSM at 1000 µg/mL did significantly increase ($p<0.05$) ALP activity compared to either the control or LEP alone treatments, and this increase in ALP was not blocked by the addition of LEP stimulation, thus reproducing the effect observed on Alp mRNA.

ALP activity in non-differentiated but proliferating pre-osteoblasts was several log-fold lower in undifferentiated MC3T3-E1 cells. All the treatment groups suppressed the ALP activity and thus the differentiation of pre-osteoblasts as compared to control, and this was not significantly altered by LEP (Figure 6.4B). However, GSM significantly reduced ($p<0.05$) ALP activity in these cells, and this effect was not lost with the inclusion of LEP.

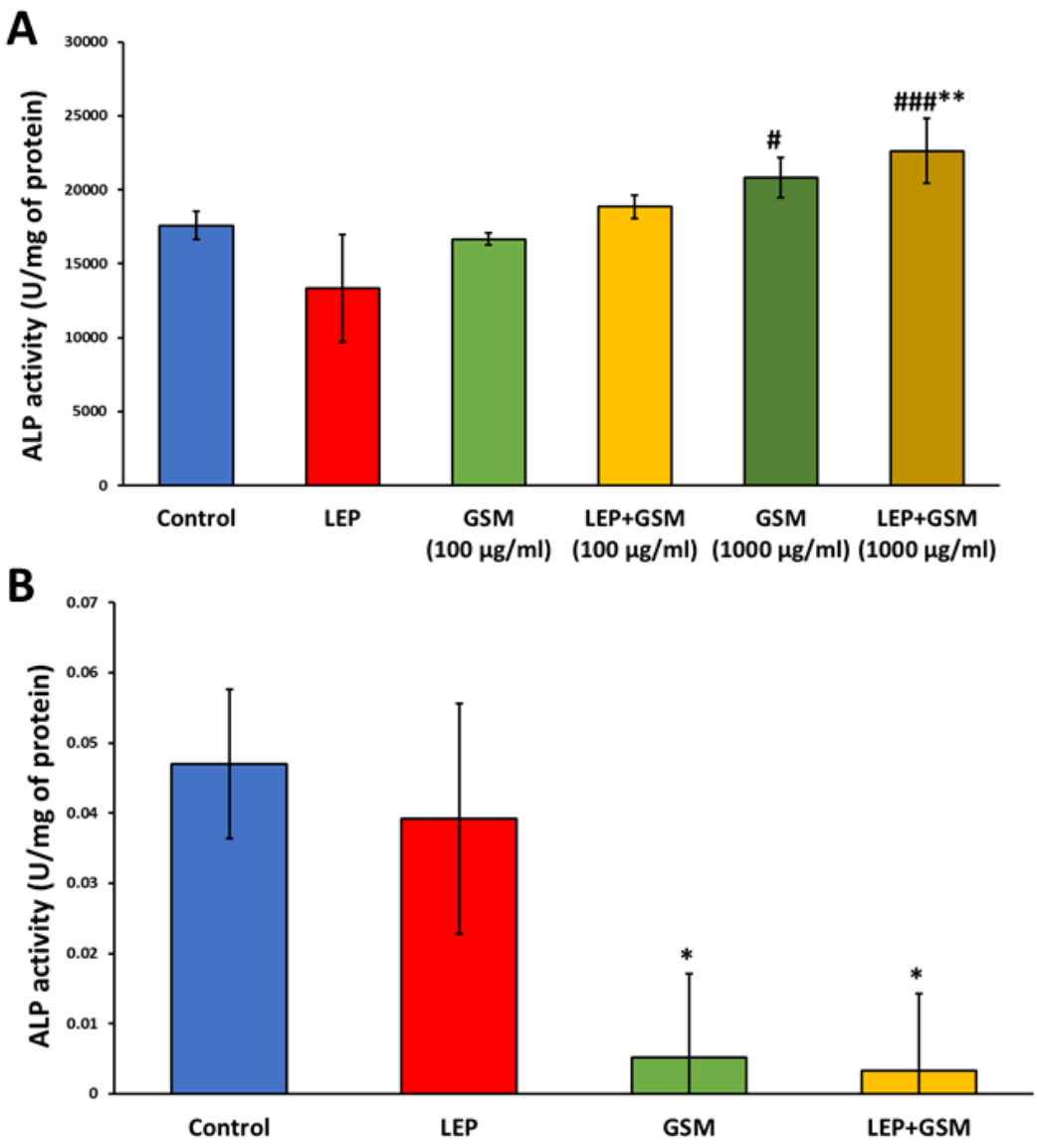

Figure 6.4: Effect of LEP and GSM on ALP activity A) in 7-day pre-differentiated MC3T3-E1 osteoblasts treated for an additional 7 days with GSM (100 µg/ml and 1000 µg/ml) and/or LEP (10 µg/ml) and B) in non-differentiated pre-osteoblasts treated with GSM (100 µg/ml) and /or LEP (10 µg/ml) for 7 days. The data were normalised to total protein content and analysed by single-factor analysis of variance *p<0.05, **p<0.01 signifies difference with respect to 0.1% DMSO carrier control group (unstimulated) and #p<0.05, ###p<0.001, denotes difference with respect to model group (LEP). The values represent the mean ± SEM of three independent experiments each assessed in duplicate wells.

6.3.4 Alizarin red staining (ARS)

As ALP leads to mineralisation in bone, a functional assay was performed to measure the effect of GSM on mineralisation. MC3T3-E1 cells were differentiated for 14 days into functional osteoblasts, and then for an additional 7 days with GSM and LEP treatments. The deposition of calcium and formation of mineralised nodules was identified by Alizarin red staining. Staining was quantified by measuring the optical density of the solubilised stain and verified visually in photomicrographs. LEP alone caused a small but nonsignificant decrease in mineralisation (Figure 6.5). GSM at 100 µg/mL had no effect, but when combined with LEP a small nonsignificant decrease matching that seen with LEP alone was observed. GSM at 1000 µg/mL significantly increased ($p<0.05$) mineralisation by osteoblasts, but this effect was lost when LEP was included, indicating that even the highest concentration of GSM could not overcome the hypomineralisation effect induced by LEP.

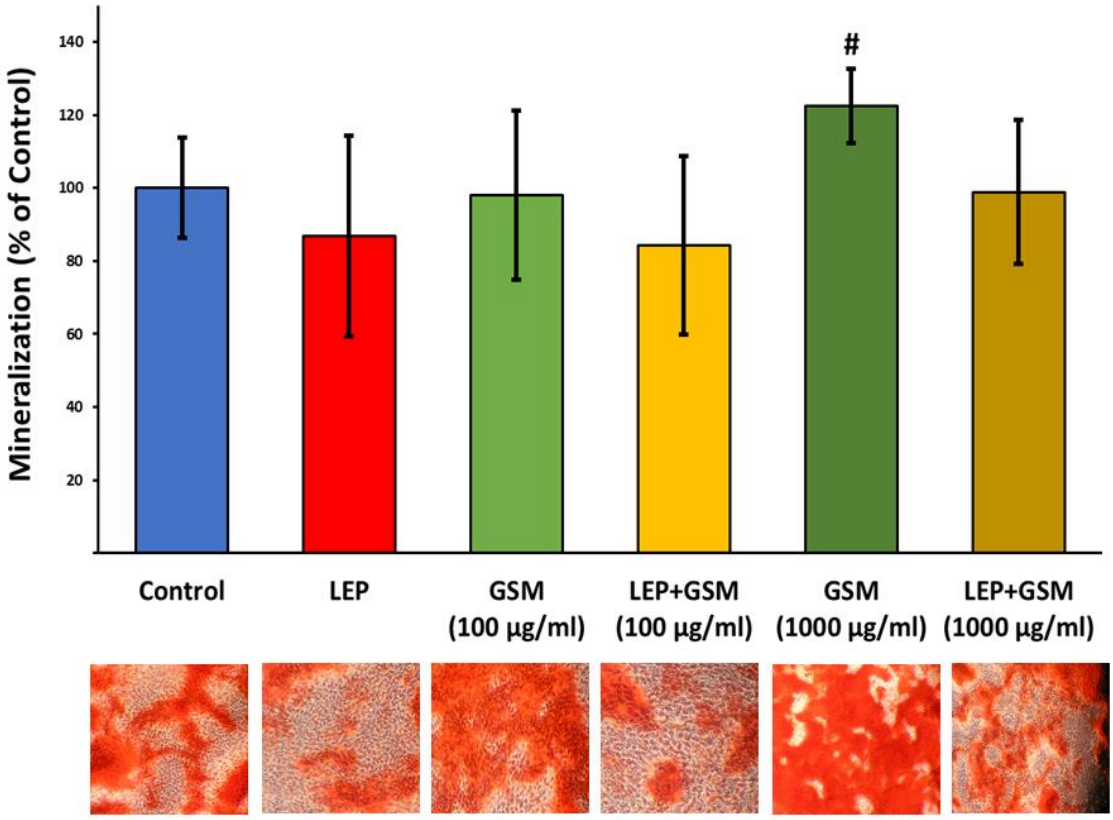

Figure 6.5: Effect of LEP and GSM on mineralization of 14 days pre-differentiated MC3T3-E1 osteoblasts. The formation of mineralized nodules was evaluated by ARS after 7 days incubation with GSM (100 µg/ml and 1000 µg/ml) and/or LEP (10 µg/ml) versus 0.1% DMSO carrier control, followed by the representative images (400X magnification) of each treatment. The data were analysed by single-factor analysis of variance, #$p<0.05$ denotes difference from the model group (LEP-stimulated group). The values represent the mean ± SD of three to six independent experiments (n=6-18).

6.4 Discussion

In this study, we report the successful creation of novel cell models of LEP-activated J774A.1 and differentiated MC3T3-E1 cells to simulate the effects of this adipokine on macrophages and osteoblasts in OA. Treatment of these cells with whole GSM powder was found to significantly increase pro-inflammatory cytokine mRNA expression in macrophages, particularly IL-1β and TNF-α, at levels similar to those observed with LEP

treatment. However, GSM with or without LEP also significantly increased expression of the anti-inflammatory cytokine *IL-10* (figure 6.2). In osteoblasts, a high concentration of GSM increased mRNA levels of *Alp* and similarly increased ALP enzymatic activity and the formation of mineralised nodules, with only mineralisation activity blocked by the inclusion of LEP. GSM when combined with LEP also increased mRNA expression of other osteoblast differentiation and osteogenic markers including *BSP, OCN* and *Col10α1* (figure 6.3).

As expected, the inflammatory adipokine LEP induced significant increases in pro-inflammatory but not anti-inflammatory cytokine expression in this OA macrophage model. GSM upregulated expression of the anti-inflammatory cytokine *IL-10,* and this effect was not blocked by the inclusion of LEP. However, in contrast to a previous study that reported the health benefits of a GSM lipid extract are based on its anti-inflammatory potential (Li et al., 2014), this study observed that whole GSM product significantly upregulated the expression of pro-inflammatory *IL-1β* and and *TNF-α*. Furthermore, when GSM and LEP were combined the increase in these cytokines was additive or synergistic, suggesting that GSM has potent pro-inflammatory qualities as well as anti-inflammatory capability.

These conflicting results can be partly explained by differences in both test format and model system. A combination of GSM oil, vitamin E and olive oil was reported to reduce LPS-induced expression of *IL-1β, IL-6,* and *TNF-α* in Raw264.7 macrophages, but the GSM product was not assessed in the absence of LPS (Chen et al., 2017) so any inherent pro-

inflammatory activity would not have been identified. In addition, whole GSM powder is predominantly protein with smaller proportions of carbohydrates, lipids, and minerals. One class of proteins known as mussel adhesive proteins (MAP) are reported to have anti-inflammatory activity in keratinocytes, but in murine RAW264.7 macrophages had no effect on LPS-stimulated production of IL-1β, IL-6 or TNF-α; however in this study also, the MAP was not tested in the absence of LPS so any potential pro-inflammatory effect of this MAP remains unknown (Ahn et al., 2019). A lectin isolated from a different mussel species, assessed in both THP-1 and RAW264.7 macrophages, was shown to significantly upregulate IL-1β and TNF-α (Chernikov et al., 2017); thus it is possible that a lectin in GSM was responsible for the similar effect observed in our study.

There are no published reports of any mussel product being assessed for cell-altering activity J774A.1 cells or being assessed in LEP-stimulated rather than LPS-stimulated macrophages so direct comparisons cannot be made. However, as discussed in 6.1, M1 and M2 macrophages are induced by different means: inflammatory signals drive the polarization of macrophages in the M1 phenotype while anti-inflammatory agents cause polarization in the M2 phenotype (Viola et al., 2019). As macrophages in OA are skewed to the M1 phenotype (Dickson et al., 2019b) and their pro-inflammatory activity in this disorder is largely attributed to leptin and other adipokines (Wang and He, 2018, Xie and Chen, 2019), this validates the novel design of the LEP-J774A.1 assay used in this study to model OA.

Inflammation is an important physiological response necessary for the maintenance of tissue homeostasis by enabling the body to expel harmful and pathogenic stimuli. A successful immune response, however, involves the resolution of an inflammatory response by anti-inflammatory and reparative cytokines, followed by tissue repair, regeneration, and restoration (Loi et al., 2016). Our findings with GSM showed bi-directional cytokine effects. In addition to pro-inflammatory activity GSM induced a significant increase in IL-10 expression which was increased rather than blocked with concurrent LEP stimulation. This is likely due to multiple and potentially competing bioactive factors in the whole GSM product, and indicates that whole GSM can induce multifactorial immunomodulation and its effects in this area are not negated by LEP.

Pro-inflammatory cytokines produced during chronic inflammatory diseases such as rheumatoid arthritis or after implantation of biomedical devices are generally accepted to induce damaging effects on tissues and joints (Mountziaris et al., 2011). However, a multitude of studies have not only determined the anabolic role of controlled inflammatory signals on bone regeneration and repair but also suggest that immunomodulation can be exploited as a strategy in the field of bone tissue engineering for the successful utilization of biomaterials in humans for bone regeneration (Lee et al., 2019, Maruyama et al., 2020, Goodman et al., 2019, Mountziaris et al., 2011). This is because pro-inflammatory cytokines display paradoxical roles in different physiological contexts. High circulatory levels of pro-inflammatory cytokines such as IL-1 and TNF-α are associated with bone and joint destruction in rheumatoid arthritis but the same molecules are involved in fracture healing in bones, and altered levels of these cytokines

have a major impact on bone healing and regeneration (Tsiridis et al., 2007). For instance, anti-TNF-α antibodies were found to block bone formation triggered by TNF-α *in vitro* (Sivonová et al., 2006). Drugs which act through the same signaling pathways triggered by pro-inflammatory cytokines such as IL-1 and TNF- α can also enhance bone healing; synthetic peptide TP508 has been found to stimulate the same signaling pathways stimulated by pro-inflammatory mediators and to have bone healing potential in a variety of animal models (Fife et al., 2007). Similarly, corticosteroids, prostaglandins, and non-steroidal anti-inflammatory drugs (NSAIDs) which suppress inflammatory responses have negative effects on bone healing and regeneration (Mountziaris and Mikos, 2008). Taken together, this suggests that the pro-inflammatory cytokines induced by GSM in macrophages may be able to indirectly aid in the repair or reduction of bone damage in OA rather than exacerbating the condition, amplifying the positive effects of the concurrent induction of anti-inflammatory IL-10 by GSM.

Osteoblasts are cells of mesenchymal origin and serve as the building blocks for bone formation. Pre-osteoblasts are the precursors of the osteoblasts and typically pass through three stages of development i.e. proliferation, matrix maturation, and mineralization (Rutkovskiy et al., 2016). There are no published reports indicating that GSM lipids can increase ALP expression or function in differentiated osteoblasts, and leptin has not previously been used to treat differentiated MC3T3-E1 cells to study OA markers, other than a single report noting that brief (24 hour) exposure of these cells to leptin caused a dose-dependent biphasic effect on ALP activity (Lamghari et al., 2006).

The evaluation of this novel osteoblast cell model as well as the osteogenic effects of GSM both gave positive results in the current study and indicate that GSM may directly aid in reducing bone damage. LEP significantly decreased the number of metabolically active cells in the early stages of differentiated osteoblasts, but this effect was mitigated by the inclusion of GSM at 1,000 µg/ml (figure 6.1). In addition, GSM significantly increased ALP mRNA and enzyme activity in osteoblasts on a per-cell basis in both the absence and presence of LEP, and also significantly increased mineralisation by osteoblasts although GSM could not overcome the hypomineralisation effect induced by LEP (figure 6.4A). There is one report of a blue mussel protein hydrolysate increasing ALP in differentiated osteoblasts (Hyung et al., 2018), suggesting that the ALP-promoting activity observed in the current study was due to the protein rather than lipid components of the GSM. However, more research to verify this is needed.

Finally, GSM significantly suppressed virtually all ALP activity on a per-cell basis in undifferentiated precursor cells. and this effect was not lost with the inclusion of LEP stimulation. In contrast, a peptide and a water-soluble protein isolate from blue mussels have been shown to increase ALP in undifferentiated MC3T3 cells in two different studies (Xu et al., 2019a, Xu et al., 2019b). In both of these studies the mussel treatment also significantly increased total cell numbers, and as ALP activity was not reported normalised to cell number or total protein the effect of their test treatments on ALP on a per-cell basis was not determined. However, it is possible that proteins in mussels promote early differentiation of mesenchymal cells into osteoblasts in the absence of the osteogenic differentiation medium normally used for MC3T3-E1 cells; it would be of

interest to further explore this possibility. No study has so far compared the response of non-differentiated and differentiated MC3T3-E1 osteoblasts to any treatment, however a similar study explored the effects of LEP on mesenchymal progenitor cells (MPC) and bone marrow stomal cells (BMCs) and demonstrated that LEP maintained MPC in an undifferentiated state and promoted the mineralization of more differentiated osteoblasts. This showed LEP has multiple peripheral roles depending on the differentiation state of MPC (Scheller et al., 2010). In another study involving osteoclasts, LEP negatively affected the development of osteoclast precursors but had no effect on mature osteoclasts (Cornish et al., 2002). The clinical relevance of these findings is yet to be understood.

A variety of transcription factors and proteins are involved at every stage of osteoblast development. Runx2 is an early differentiation factor that regulates the differentiation of mesenchymal stem cells into osteoblast precursors (pre-osteoblasts). Osx is produced in pre-osteoblasts and controls the further maturation of osteoblasts and finally the formation of osteocytes and bone (Sinha and Zhou, 2013). Alp and Col1α1 are proteins produced during early matrix formation in osteoblasts while OCN, Col10α1, and BSP are the osteogenic markers representing the mineralization of the osteoblasts matrix and thus maturation of osteoblasts in bone (Kirkham and Cartmell, 2007). Moreover, OCN is used as a marker of successful bone regeneration therapy (Saffarian Tousi et al., 2013).

LEP alone did not significantly affect the expression of these differentiation and osteogenic markers. GSM alone also did not have significant effects, but at a high dose

combined with LEP caused significant increases in mRNA levels of *BSP, OCN* and *Col10α1. Col1α1, Osx* and Runx2 expression also increased but did not reach statistical significance. These are the first reported findings of GSM promoting osteoblast function, although there is a single report of a blue mussel protein hydrolysate having similar effects on Col1α1 and OCN production (Hyung et al., 2018).

6.5 Conclusion

In conclusion, we have established that leptin is an appropriate stimulant for both macrophages and osteoblasts to mimic aspects of synovial inflammation and subchondral bone remodelling that occur in OA. This study also reports for the first time novel inflammomodulatory and osteogenic protective effects of a whole GSM extract. These cell models will be of use in future both to study the pathogenesis of OA and to screen potential intervention treatments.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Saima Rizwan
Name/title of Primary Supervisor:	Dr. Frances M Wolber
In which chapter is the manuscript /published work:	Chapter Six

Please select one of the following three options:

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Designed and carried out all experiments; curated, analysed and interpreted data; prepared figures and text for original draft; reviewed and co-edited revisions

◉ It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Saima Rizwan Digitally signed by Saima Rizwan Date: 2021.04.07 11:25:07 +12'00'
Date:	07-Apr-2021
Primary Supervisor's Signature:	Fran Wolber Digitally signed by Fran Wolber Date: 2021.04.07 12:43:30 +12'00'
Date:	7-Apr-2021

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

Chapter Seven: Comparison of mechanically processed green shell mussel, GSM-A versus enzymatically processed GSM-B for their effects on the metabolism of leptin-stimulated chondrogenic ATDC5 cells

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This study was aimed to mimic the OA-related pathophysiology of chondrocytes *in vitro* and then to explore the mechanism of action of GSM in the disease model. We used ATDC5 cells to create an LEP-stimulated *in vitro* chondrocyte model of OA and compared two whole GSM powders, conventionally processed GSM-A versus enzymatically processed GSM-B, for their effects on proliferation, differentiation, collagen, proteoglycan, and mineral deposition of the LEP-stimulated chondrocytes.

Comparison of mechanically processed green shell mussel, GSM-A versus enzymatically processed GSM-B for their effects on the metabolism of leptin-stimulated chondrogenic ATDC5 cells. 195

Abstract

Greenshell mussels (GSM; *Perna canaliculus)* are a bivalve marine mollusc endemic to New Zealand and have been a part of the indigenous Māori diet for centuries. In more recent times, GSM have been used to treat osteoarthritis (OA) and other inflammatory conditions. However, *in vitro* and *in vivo* studies have revealed conflicting evidence regarding the efficacy of GSM, possibly due to variations in the retention of bioactive components due to differences in processing. Enzymatic processing may preferentially preserve the bioactive composition of GSM as compared to conventional mechanical shucking. Further, GSM is believed to treat OA and other health conditions through anti-inflammatory pathways but its actions are not fully understood. This study aimed to mimic the OA-related pathophysiology of chondrocytes *in vitro* and then to explore the effects of GSM in this disease model whilst comparing two GSM extracts produced through different processes. ATDC5 cells stimulated with leptin (LEP) were treated with conventionally processed GSM-A versus enzymatically processed GSM-B and assessed for proliferation, differentiation, collagen, proteoglycan, and mineral synthesis. LEP increased hypertrophic differentiation of chondrocytes by promoting the expression of chondrogenic genes and by increasing the mineral and proteoglycan content of the chondrocytes. GSM extracts, particularly GSM-B, prevented the hypertrophic differentiation of chondrocytes by suppressing the hypertrophic cartilage related markers alkaline phosphatase ($p<0.05$), Col10α1 and mineralized nodules in LEP-stimulated chondrocytes ($p<0.001$). Moreover, it suppressed endochondral ossification

by decreasing *Runx2* and *Col1α1* expression and maintained a chondrogenic phenotype by increasing ($p<0.01$) *Sox9* expression. GSM-A also favoured the production of hyaline cartilage by increasing the expression of *Col2α1*, which provides shape and tensile strength to articular cartilage and prevents hypertrophic differentiation of chondrocytes. Together these findings suggest that both GSM-A and GSM-B were able to suppress LEP-stimulated hypertrophic differentiation of OA chondrocytes, but GSM-B was found more potent regarding its effects in maintaining the chondrogenic phenotype and suppression of hypertrophic differentiation of OA chondrocytes.

Keywords: Greenshell mussel, ATDC5 chondrocytes, chondrogenesis, leptin, osteoarthritis

7.1 Introduction

Osteoarthritis (OA) is the most common degenerative disease of old age. It involves metabolic changes to the whole joint structure including loss of cartilage matrix, synovial inflammation, osteophytosis, and subchondral bone sclerosis (Lawrence et al., 2008a, Clouet et al., 2009). These pathological changes cause severe pain, limit the ability to move, and end up in disability (Ji et al., 2017). OA is a multifactorial disease and though age, obesity, trauma, joint deformity, and inherited joint anomaly are considered the important risk factors for the disease pathogenesis, the exact aetiology of the disease is unknown (Li et al., 2019b). The prevalence of OA is increasing due to the rapid increase in the aging population and increased incidence of obesity in recent years, and it is

estimated to affect 10-15% of the world population over 60 years of age (Ji et al., 2017). The cost of the disease is about 0.25-0.50% of the country's gross domestic product (GDP) (Puig-Junoy and Ruiz Zamora, 2015). Various pain management strategies including non-pharmacological and pharmacological are available to support osteoarthritic patients; for example, lifestyle changes may be helpful in obese/overweight patients and medications for others depending upon the symptoms present in individual patients (Cibulka et al., 2017). Currently, there is no FDA-approved drug for the treatment of OA due to a lack of a thorough understanding of OA related pathology. Pain management drugs provide symptomatic relief but have multiple side effects, which demand more effective treatment options (Jin et al., 2018).

Although OA is the disease of the whole joint, progressive cartilage loss remains a central feature of the disease (Musumeci et al., 2015). To date, there is no successful therapy to restore or regenerate the damaged cartilage partly because of the complex structure and physiology of the tissue (Johnstone et al., 2013, Madry et al., 2014, Armiento et al., 2018). Articular cartilage is a shock-absorbing tissue lining the surface of the bones and facilitates pain-free movement by minimizing friction (Johnstone et al., 2013). Normal adult cartilage has two compartments, the extracellular matrix (ECM) made up of water, collagen, proteoglycans, and a trace amount of calcium and the chondrocytes (Goldring and Marcu, 2009). Under normal physiological conditions, the turnover rate of ECM components is slow and proteoglycans have a higher turnover than collagen (Mow, 1997). Normally chondrocytes regulate the balance between anabolic and catabolic

changes occurring in ECM by synthesizing ECM components as well as by synthesizing the degradative enzymes involved in the breakdown of these components (Sandell and Aigner, 2001, Man and Mologhianu, 2014b). During OA, chondrocytes fail to maintain this balance between the anabolic and catabolic activity of ECM leading to an initial increase in both anabolic and catabolic activity and culminating in an increase in catabolic activity and loss of ECM (Sandell and Aigner, 2001). Different factors like growth factors, cytokines, physical and structural stimuli, and the components of ECM themselves are considered to influence the regulatory role of chondrocytes during OA pathogenesis (Man and Mologhianu, 2014a).

Leptin (LEP) is an important cytokine-like hormone, which was primarily considered an adipokine secreted by adipose tissues and has a primary role in energy metabolism, but numerous studies have proven that it is also secreted by tissues other than adipose (Dumond et al., 2003b, Iliopoulos et al., 2007) and its levels are elevated in OA chondrocytes as compared to healthy chondrocytes (Simopoulou et al., 2007). LEP has been considered to play an important role in cartilage degeneration and OA pathogenesis by increasing the production of degradative enzymes (matrix metalloproteinases and disintegrins) and pro-inflammatory cytokines (NO, IL-6, IL-1β, and many others).

Green shell mussels (GSM) *(Perna canaliculus)* are endemic to New Zealand and have been a part of staple food for the native Māori population for centuries (Halpern, 2000,

Cobb and Ernst, 2006b). The observation that the incidence of arthritis was lower in the Māori people consuming GSM as a staple food as compared to their European counterparts led researchers to explore it further for its health benefits (Halpern, 2000, Cobb and Ernst, 2006b). Research exploring the anti-inflammatory potential of GSM started in the early 1970s and since then it has been studied *in vitro, in vivo,* and in clinical trials. There is now stronger but sometimes conflicting evidence about the efficacy of GSM in improving the symptoms of inflammation and OA *in vitro* and *in vivo* trials (Szechiński and Zawadzki, 2011). The conflicting evidence regarding the efficacy of freeze-dried GSM powder might be due to different factors including variations in the ratio of bioactive components, which may result from different processing methods. Enzymatic processing has recently been demonstrated to preserve the bioactive composition of GSM as compared to conventional mechanical shucking (Tian Hong, 2018). Lipids, proteins, and carbohydrates are the major fractions of GSM, consisting of several metabolites, which have various antimicrobial, anti-inflammatory, antihypertensive, and bio-adhesive activities (Grienke et al., 2014b). The role of these components in OA and if these are therapeutically effective, however, still needs to be clarified. Previous claims suggest that the lipid fraction alleviates OA-related symptoms via regulating inflammatory pathways (Ulbricht et al., 2009, Eason et al., 2018b). Also, Siriarchavatana et al reported that the flash dried whole GSM extract has preventive effects against cartilage damage in a rat model of metabolic OA (Siriarchavatana et al., 2019a).

Comparison of mechanically processed green shell mussel, GSM-A versus enzymatically processed GSM-B for their effects on the metabolism of leptin-stimulated chondrogenic ATDC5 cells. 200

ATDC5 prechondrogenic cells are derived from differentiating AT805 teratocarcinoma cells of mice and are considered an excellent *in vitro* model to study chondrogenesis (Yao and Wang, 2013a). This cell line mimics the chondrogenic condensation over a relatively small period due to its rapid proliferation, thereby providing an excellent tool to study different steps of chondrogenic differentiation of cartilage and different factors influencing the process. This study was aimed to mimic the OA-related pathophysiology of chondrocytes *in vitro* and then to explore the mechanism of action of GSM in the disease model. We used ATDC5 cells to create a LEP-stimulated *in vitro* chondrocyte model of OA and compared two whole GSM powders, conventionally processed GSM-A versus enzymatically processed GSM-B, for their effects on proliferation, differentiation, collagen, proteoglycan, and mineral deposition of the LEP-stimulated chondrocytes. We studied the effect of GSM extracts on the metabolism of LEP-stimulated ATDC5 cells by measuring different biomarkers of chondrocyte metabolism such as chondrogenic genes, alkaline phosphatase (ALP) enzyme activity, and calcium, collagen and proteoglycan synthesis, which are characteristics of mature functional chondrocytes. Chondrocyte differentiation markers/genes include alkaline phosphatase (*Alp*), runt-related transcription factor 2 (*Runx2*), sex-determining region y-box9 *(Sox9),* aggrecan *(Acan),* collagen type 1 alpha chain 1 *(Col1α1),* collagen type 2 alpha chain 1 *(Col2α1*) and collagen type 10 chain alpha chain 1 *(Col10α1).*

7.2 Materials and Methods

7.2.1 Mussel extracts

Whole green shell mussel (GSM) powder extracts were kindly provided by Sanford Ltd, New Zealand. Proximal analyses were carried out by the Cawthron Institute (Nelson, NZ) and are shown in Table 7.1. Both GSM products were from the same harvest but were manufactured using two different process methods; GSM-A was blended, freeze-dried, and milled, whereas GSM-B included an added enzymatic digestion step which resulted in a product with slightly less carbohydrate and slightly more ash.

Table 7.1: Composition of mussel extracts

Extract	Source material	Extraction process	% protein	% CHO	% fat	% ash
GSM-A	Whole GSM meat	"A"	50.8	24.2	6.5	15.3
GSM-B	Whole GSM meat	"B"	47.3	20.3	7.9	20.1

7.2.2 Mussel extract preparation and leptin reconstitution for *in vitro* use

The freeze-dried powder was dissolved at a concentration of 10 mg/ml in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technologies, Auckland, NZ) containing 10% DMSO (Sigma Aldrich, Merck, NZ), sonicated briefly to ensure solubility, sterilized through a 0.20 µm pore syringe filter, aliquoted, and stored at -20°C until further use.

Leptin (recombinant mouse leptin OB) was purchased from Biomyx (San Diego, California) and reconstituted in PBS (phosphate buffer saline) (Sigma Aldrich, Merck, NZ) according to manufacturer's instructions, aliquoted, and stored at -20 ^{0}C until further use.

7.2.3 Cell culture

Murine ATDC5 cells (Sigma-Aldrich, Merck, NZ) were grown and maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 plus GlutaMAX (DMEM/F-12, Gluta*MAX*(TM)) (Gibco, Life Technologies, Auckland, NZ), supplemented with 5% foetal calf serum (FCS, Gibco) and 1% penicillin-streptomycin (Invitrogen, Life Technologies, Auckland, NZ). The cells were maintained at 37°C in a humidified incubator containing 5% CO_2. Subcultures were obtained using 0.25% trypsin/EDTA solution (Gibco) every 3 days. Once confluent, pre-chondrogenic ATDC5 cells were transferred to multiwell culture plates where they were induced to differentiate into functional chondrocytes by culturing the cells in the medium containing differentiating factors (Sigma-Aldrich) 50mg/ml ascorbic acid, 10mg/ml insulin, 10mg/ml transferrin and 5ng/ml sodium selenite ("AITS") for 7 to 14 days; the medium was changed twice weekly. 1% DMSO/unstimulated cells were used as a negative vehicle control (control group) and LEP-stimulated cells were used as a model group in all the experiments.

7.2.4 Cells metabolic activity/viability (MTT) assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were performed as described earlier (Van Meerloo et al., 2011) over a 7 day culture period with ATDC5 cells to check the metabolic activity of viable cells against GSM and LEP.

ATDC5 cells were plated at a density of $5x10^3$ cells/well as 6 replicates/treatment in 96 well plates and allowed to adhere and grow for 24 hours. Cells were then cultured in medium containing AITS with GSM-A or GSM-B (100 µg/ml), and/or LEP (100 ng/ml), for 6 days; the medium was changed twice a week. The MTT assay was performed on day 7 of incubation: 10µl of MTT (Sigma Aldrich, Merck, NZ) in PBS was added to each well at a final concentration of 0.5 mg/mL and incubated for three hours until purple crystals were microscopically visible, which were then solubilized by adding a volume of DMSO equal to that of the cell culture media in the well. Absorbance was quantified by measuring optical density (OD) at a wavelength of 550 nm using a microplate reader (Multiskan Fc, Thermo Fisher Scientific).

7.2.5 RNA extraction, reverse transcription and RT-qPCR

ATDC5 cells were seeded at a density of $5.4x10^5$ cells/well in one replicate/treatment in 6 well plates. At confluence, they were induced to differentiate in medium containing AITS for 7 days; the medium was refreshed twice a week. Cells were then treated with GSM and/or LEP which was again refreshed twice a week for the next 7 days in AITS

medium. On the 14th day, total RNA was extracted using an RNA extraction kit (PureLink RNA Mini Kit, Thermo Fisher, Auckland, NZ). 1 μg of RNA was reverse transcribed using SuperScript® IV First-Strand Synthesis System (Invitrogen, Thermo Fisher, Auckland, NZ). RT-qPCR was performed with gene-specific primers (Invitrogen, Life Technologies, Australia) using a PCR kit (LightCycler® 480 SYBR Green I Master, RochLife Science, Thermo Fisher, Auckland, NZ). Relative expression of the genes was quantified by the E-method of advanced relative quantification, and mRNA was assessed for *Alp*, *Runx2*, *Sox9, Acan, Col1α1, Col2α1,* and *Col10α1.* Glyceraldehyde 3-phosphate dehydrogenase *(GADPH*) was used as an internal control. The specific primers for the genes are listed in Table 7.2.

Table 7.2: List of primers used

Primer	Forward sequence (5'→3')	Reverse sequence (5'→3')	Ta (°C)
GADPH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	55
Alp	GTGACTACCACTCGGGTGAAC	GTGACTACCACTCGGGTGAAC	60
Runx2	GACTGTGGTTACCGTCATGGC	ACTTGGTTTTTCATAACAGCGGA	60
Sox9	AGTACCCGCATCTGCACAAC	ACGAAGGGTCTCTTCTCGCT	60
Acan	AGGTGTCGCTCCCCAACTAT	CTTCACAGCGGTAGATCCCAG	60
Col1α1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG	60
Col2α1	ATCTTGCCGCATCTGTGTGT	CTCCTTTCTGCCCCTTTGGC	60
Col10α1	TTCTGCTGCTAATGTTCTTGACC	GGGATGAAGTATTGTGTCTTGGG	60

(Note: *GADPH* = glyceraldehyde-3-phosphate dehydrogenase, *Alp* = Alkaline phosphatase, *Runx2* = runt-related transcription factor 2, *Sox9* = sex-determining region y-box9*, Acan*= Aggrecan, *Col1α1,* = Collagen type 1 alpha chain 1, , *Col2α1*= Collagen type 2 alpha chain 1 and *Col10α1* = Collagen type 10 alpha chain 1)

7.2.6 ALP activity assay

Alkaline phosphatase (ALP) activity and total protein content were determined as described previously (Yodthong et al., 2018) with some modifications. $2x10^4$ cells per well were seeded as duplicate wells/treatment in 12 well plates. Confluent ATDC5 cells were induced to differentiate in medium containing AITS for 7 days and the medium was changed twice a week. After 7 days, differentiating chondrocytes were then treated with GSM ± LEP for the next 7 days. ALP activity was determined in the whole cell lysate using reagents and protocol as described in the TRACP & ALP assay kit (TaKaRa, Otsu, Japan).

Reagents included: physiological/0.9% saline (NaCl, Sigma Aldrich) for washing the cells, extraction buffer (1% Triton-X100 in 1 x TBS) (Tris, HCl, NaCl, Triton X100, Sigma Aldrich), ALP assay solution (Tris, HCl, $MgCl_2$, Sigma Aldrich), ALP substrate, para-nitro-phenyl phosphate (pNPP, Sigma Aldrich), stop solution (1N NaOH, Sigma Aldrich), and 4-Nitrophenol (Sigma Aldrich) for the standard curve. ALP activity was normalized to total protein content, which was measured by using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, Auckland, NZ).

7.2.7 Histochemical procedures

Alizarin red, Sirius red, and Alcian blue staining were performed to evaluate calcium, collagen, and proteoglycan content of ATDC5 chondrocytes respectively in response to treatment with GSM and/or LEP. For this purpose, cells were seeded in 12 well plates at a density of $2x10^4$ per well as duplicates/ treatment. At confluence, cells were induced to differentiate in the medium containing AITS for 7 days and the medium was changed twice a week. 7 days differentiated ATDC5 cells were then treated with GSM in the presence or absence of LEP for the next 7 days and on the 14^{th} day cells were stained for mineralized nodules using Alizarin red (Sigma Aldrich) as described previously with some modifications (Huitema et al., 2006). Cells were washed twice with phosphate buffer saline (PBS), fixed for 1 hour with 100% ethanol and incubated with 500 µl of 0.1% alizarin red solution overnight. Excess stain was removed by washing twice with PBS. The stain was subsequently solubilized using cetylpyridinium chloride (CPC) (Sigma Aldrich). Similarly, on the 14^{th} day cells were stained for collagen production using Sirius

red/fast green collagen staining kit (Chondrex, Inc; Sigma), as described previously (Staines et al., 2012) with some modifications. Cells were washed twice with PBS and fixed overnight with 2% formaldehyde followed by staining using Sirius red/fast green collagen staining kit according to manufacturer's instructions. The stain was solubilized in 0.1N NaOH and optical density was read at 550nm using a microplate reader (Multiskan Fc, Thermo Fisher Scientific). Proteoglycan production was also measured following the above-mentioned 14 day treatment protocol. Cells were stained with Alcian blue stain as described previously (Staines et al., 2012) with some modifications; Cells were washed twice with PBS, fixed with 100% methanol for 20 minutes at -20^{0}C and incubated with 0.1% alcian blue stain in water overnight. The stain was solubilized in 6M Guanidine HCl and optical density was measured at 620nm using a microplate reader (Multiskan Fc, Thermo Fisher Scientific).

7.2.8 Statistical Analysis

All experiments were repeated at least three times and results are presented as mean ± SEM or, for experiments carried out with single wells per treatment, as mean ± SD. Statistical analyses were done using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, N.Y., USA). Different groups were compared by one-way analysis of variance (ANOVA). $p<0.05$ was considered to indicate a statistically significant difference.

7.3 Results

7.3.1 Cells metabolic activity/viability (MTT) assay

MTT assay was done to assess potential cytotoxicity of the GSM extracts and LEP on ATDC5 cells following 7 days of treatment. The individual treatments had no effect on the number of metabolically active cells as compared to unstimulated control (figure 7.1). Neither GSM-B alone nor LEP alone produced a significant effect on the viability of the cells and even the combination reduced cell viability by <10%.

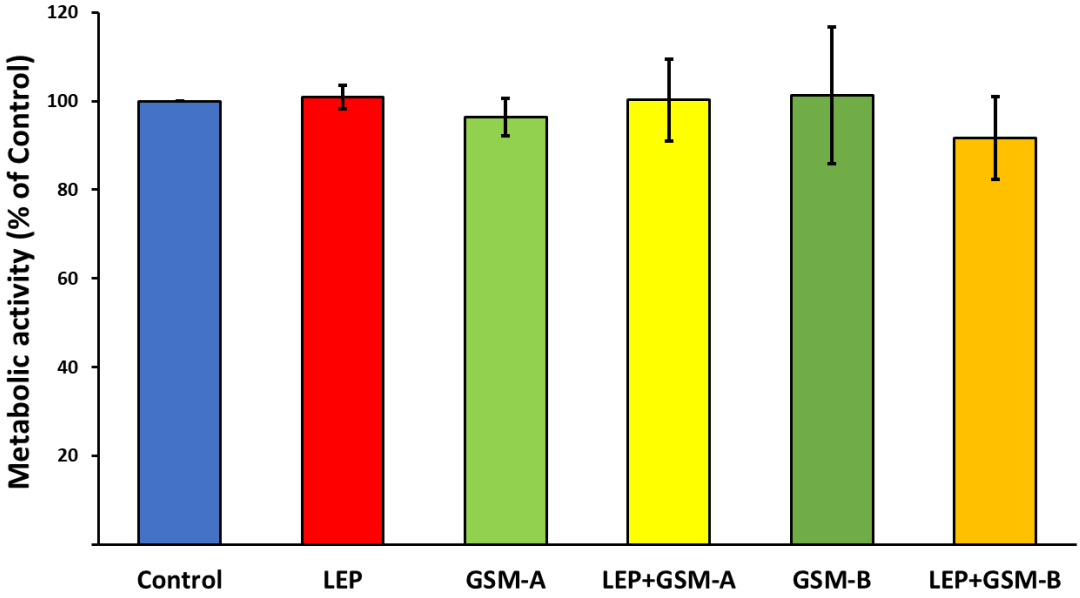

Figure 7.1: Effect of LEP (100 ng/ml), GSM-A, and GSM-B (100 µg/ml) was assessed by MTT on the metabolic activity of ATDC5 cells after 7 days. Cells were treated with 100 µg/ml of GSM-A, GSM-B, and/or 100 ng/ml of LEP. The data were analysed by single-fact factor ANOVA. There is no significant difference among any of the treatment groups. The values represent the mean ± SEM of three independent experiments each conducted in six replicates (n=18).

7.3.2 RT-qPCR analysis

RT-qPCR was performed after extracting mRNA from ATDC5 cells following treatment with GSM and/or LEP to assess their effects on transcription levels of specific genes related to the chondrogenic differentiation. All PCR results were normalized to a housekeeping gene to account for potential variations among the number of viable cells present in the samples, extraction efficiency, RNA quality, and efficiency in cDNA synthesis in the internal controls and the treatment groups. Differentiated ATDC5 cells were assessed for mRNA expression for the pro-mineralization biomarkers *Alp*; the chondrogenic initiator and maintenance factor *Runx2*; the chondrocyte differentiation initiator *Sox9* that commits multipotent skeletal progenitors to chondrocyte lineage; the major proteoglycan *Acan* that makes up the hydrated gel structure important for weight-bearing; and *Col1α1, Col2α1,* and *Col10α1*, which make up 60% of cartilage ECM and provide tensile strength.

As shown in Figure 7.2, *Runx2* (Figure 7.2B) was the only gene unaffected by any treatment. LEP had no significant effect on mRNA expression of any genes measured, although *Alp, Acan, Col1α1* and *Col2α1* appeared to increase slightly. GSM-A caused slight but non-significant increases in most genes; however, *Acan* was significantly decreased by GSM-A compared to LEP alone, and this also occurred when GSM-A was combined with LEP (Figure 7.2D). The combination of GSM-A and LEP also decreased *Col10α1* (Figure 7.2G), although this did not reach statistical significance; neither treatment alone affected *Col10α1* levels. In contrast, GSM-B significantly increased

Sox9, but this effect was negated when LEP was included in the treatment (Figure 7.2C), and *Col2α1* was decreased by GSM-B with or without LEP although this was not statistically significant.

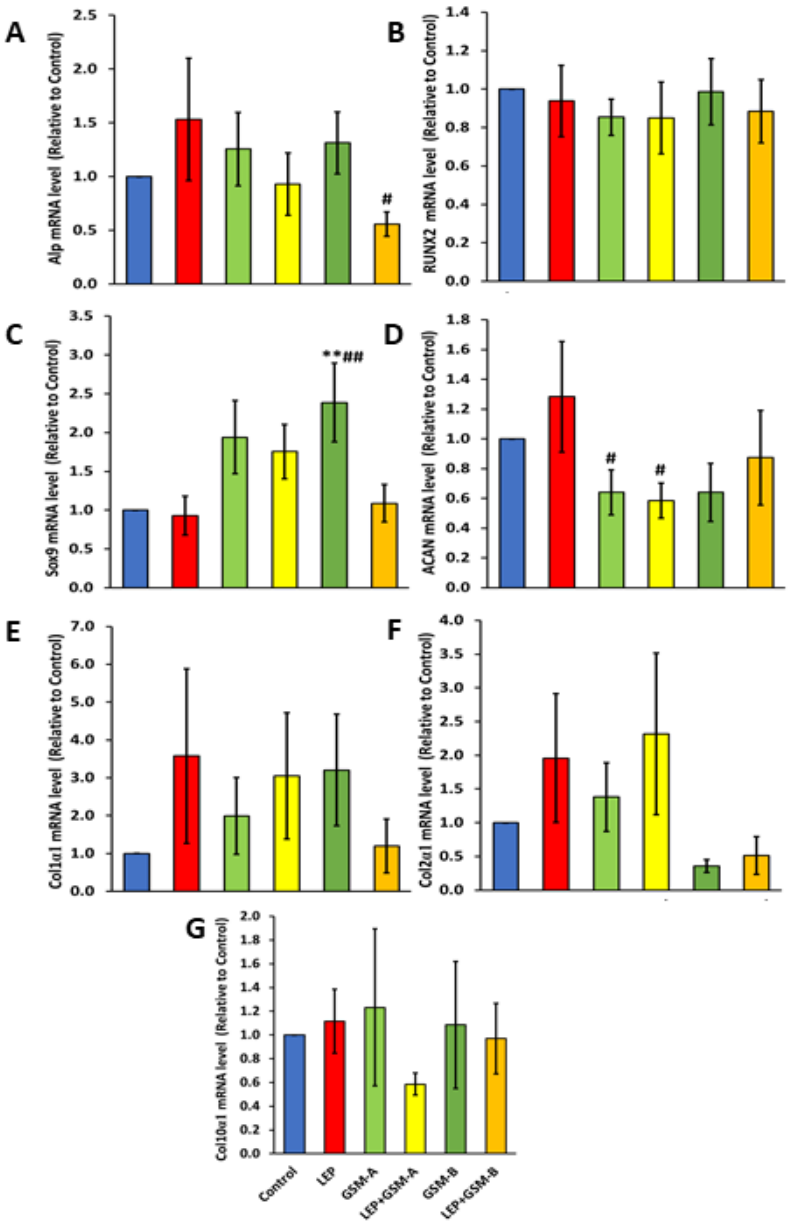

Figure 7.2: Advanced relative quantification (RT-qPCR) for markers of chondrocytes differentiation (*Alp, Runx2, Sox9, Col1α1, Acan, Col2α1* and *Col10α1*) in ATDC5 cells pre-differentiated for 7 days and then treated with 100µg/ml of GSM-A and GSM-B and/or 100 ng/ml LEP for 7 days. *GADPH* was used as a reference gene for normalization. The data were analysed by single-factor analysis of variance; **p<0.01 signifies difference from 0.1% DMSO carrier control (unstimulated), and #p<0.05 and ##p<0.01 represents a difference from the model group (LEP-stimulated); values were normalized to the matching unstimulated control and represent mean ± SE of three to six independent biological samples (n=3-6).

7.3.3 ALP activity

Alkaline phosphatase (ALP) enzyme activity was measured in ATDC5 chondrocytes differentiated and treated as described above for mRNA analysis. LEP, GSM-A, and the combination of these two had no apparent effect on ALP activity. GSM-B slightly increased ALP (30%), and GSM-B + LEP slightly decreased ALP (36%), compared to control or to LEP alone; these differences were not statistically significant but followed a similar pattern to the changes observed in *Alp* mRNA with these two treatments.

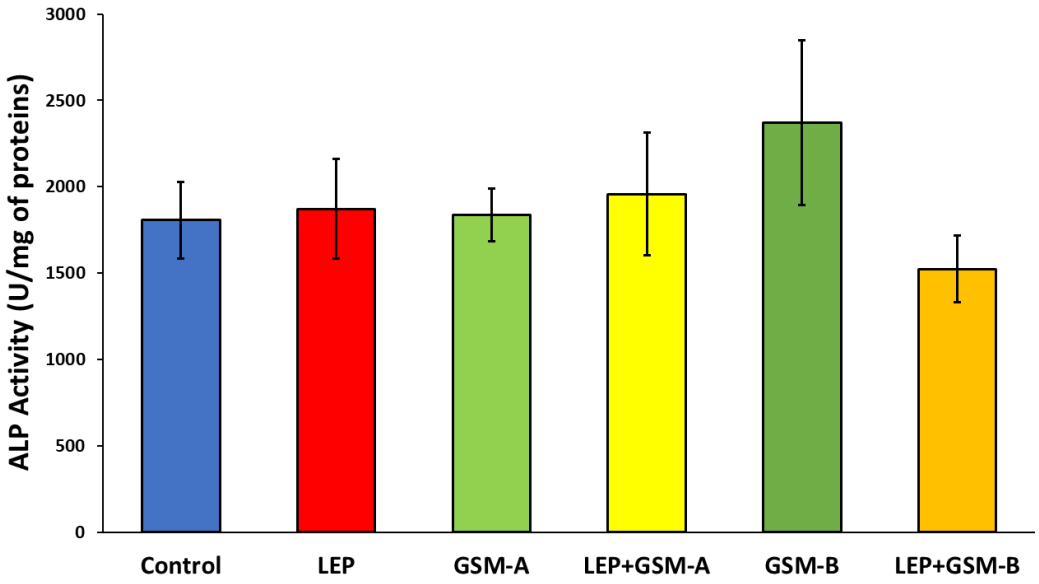

Figure 7.3: Effect of LEP and GSM on ALP activity in ATDC5 chondrocytes. 7 days pre-differentiated ATDC5 cells treated with GSM-A and GSM-B (100 µg/ml) and/or LEP (100 ng/ml) for 7 days. The data were normalized to total protein content and analysed by single-factor analysis of variance. There was no significant difference between the treatments and the 0.1% DMSO carrier control group (unstimulated). The values represent the mean ± SEM of three independent experiments each conducted in duplicates (n=6).

7.3.4 Histochemical analysis

Alizarin red staining (ARS), Sirius red staining, and Alcian blue staining were done to evaluate calcium, collagen, and proteoglycan content of ATDC5 chondrocyte cultures respectively in response to treatment with GSM and/or LEP. As shown in figure 7.4, LEP caused a non-significant increase in mineralisation as measured by calcium content. However, this detrimental change was significantly reduced by both GSM-A and GSM-B, even in the presence of LEP. LEP, GSM-A and GSM-B each independently caused slight decreases in collagen production, with the effect of GSM-B being statistically significant. None of the treatments had any effect on proteoglycan secretion.

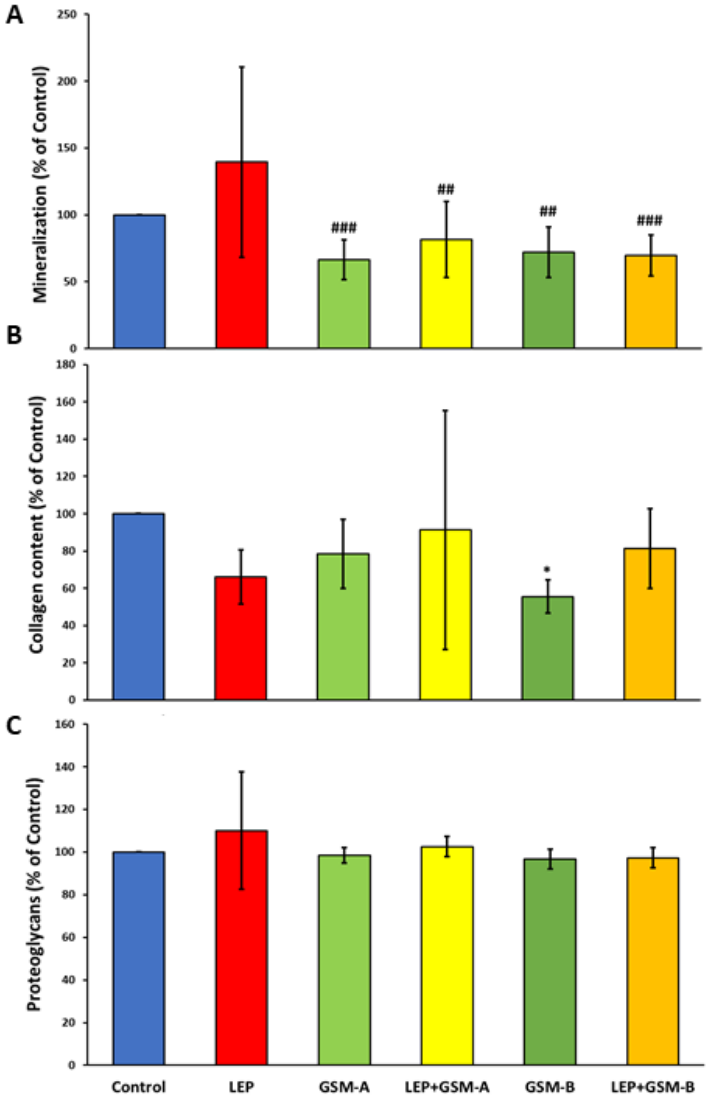

Figure 7.4: Quantitative histochemical staining for A) calcium/mineral deposition, B) total collagen and C) proteoglycan content of 7 days pre-differentiated ATDC5 chondrocytes. Cells were treated with GSM-A and GSM-B (100 µg/ml) and/or LEP (100 ng/ml) for 7 days. The data were analysed by single-factor analysis of variance and normalised to Control, which was set to 100%. *p<0.05 signifies difference from 0.1% DMSO carrier control (unstimulated), ##p<0.01 and ###p<0.001 represent a difference from the model group (LEP-stimulated) and data represent mean ± SD of 6 independent biological samples of three independent experiments each conducted in duplicates (n=6).

7.4 Discussion

Although GSM has already been proven to be effective in the treatment of OA, its exact mechanisms of action are unknown, and chondrocytes are a possible therapeutic target for this bioactive material. This study is the first to explore both the role of LEP on the OA-related metabolism of ATDC5 chondrocytes and the chondrogenic potential of GSM extracts in LEP-stimulated ATDC5 chondrocytes used to mimic OA chondrocytes *in vitro*. We determined that LEP stimulation for 7 days induced an OA-like phenotype evidenced by increased mineralisation and expression of *Alp*, *Acan*, *Col1α1* and *Col2α1.* These changes were largely reversed by inclusion of GSM, which significantly decreased mineralisation. The two GSM extracts tested did produce differing results, indicating that the processing procedures used, created products with different types or levels of bioactive components.

7.4.1 Effect of LEP on biomarkers of chondrocytes metabolism

LEP is a well-established contributing factor in OA pathogenesis and its levels are elevated in serum, infrapatellar fat pad, synovial tissues, osteoblasts, and cartilage of OA patients compared to healthy individuals (Dumond et al., 2003a, Mutabaruka et al., 2010b, de Boer et al., 2012, Conde et al., 2014). Both primary chondrocytes and ATDC5 cells have been shown to express the receptor for LEP (Figenschau et al., 2001, Wang et al., 2012a, Deng et al., 2017). Although the use of LEP with ATDC5 cells is to date limited largely to assessing its effect on the expression of surface receptors, adipokines and

intracellular signal transducers (Figenschau et al., 2001, Ben-Eliezer et al., 2007, Conde et al., 2012, Wang et al., 2012b). LEP has been shown to induce inflammation and catabolism in primary chondrocytes (Hui et al., 2012b, Bao et al., 2017). The use of LEP with ATDC5 cells in this study to simulate OA chondrocytes and assess effects on cell function was partly exploratory but the findings described below validated this to be an effective cell model.

100 ng/ml of LEP was used to activate ATDC5 chondrocytes, a dose reported to be effective with this cell line and other chondrocytes (Li et al., 2013d, Fu et al., 2019, Zhang et al., 2019a). No effect of LEP on cell viability was observed. LEP did not alter the mRNA levels of *Runx2*, a transcription factor necessary to commit pre-chondrocytes to the osteoblast lineage (Chen et al., 2014) or of *Sox9*, which initiates differentiation of pre-chondrocytes into chondrocytes (Jiang et al., 2018) and whose expression peaks in this cell line at approximately day 14 of differentiation (Naito et al., 2015). The effects of LEP on these parameters have not previously been measured in ATDC5 chondrocytes, but these findings indicate that LEP at this concentration neither blocks nor promotes the survival, proliferation or differentiation of these cells.

Alkaline phosphatase and mineralised nodules, characteristic features of calcified/hypertrophic cartilage and during *in vitro* cell differentiation assays, are considered an undesirable form of cartilage (Armiento et al., 2019) and are associated with OA. LEP induced slight but non-significant increases in mRNA expression of *Alp,* a

marker of hypertrophic chondrocytes that initiates the mineralisation of ECM (Newton et al., 2012). This was matched by an increase in mineralisation measured by Sirius red stain, corroborating a similar finding reported previously (Zhang et al., 2019a) and confirming the anabolic role of LEP regarding hypertrophic differentiation of chondrocytes. An earlier study reported that 100 ng/mL LEP completely abolished matrix calcification by ATDC5 cells (Naito et al., 2015), but in that study the LEP was present for 5 – 6 weeks of culture and calcium measured by ortho-cresolphthalein complexone method. ALP enzyme activity appeared unaffected in our results; however, it has been shown that when *Alp* mRNA is upregulated ten-fold by an external stimulant, concurrent ALP enzyme activity is less than doubled (Naito et al., 2015), likely due to differences in assay sensitivities.

LEP also induced visible although non-significant increases in *Col1α1* and *Col2α1*, but not *Col10α1*, mRNA expression. The first two are markers of fibrocartilage and hyaline cartilage respectively, whereas the latter is a differentiation marker of hypertrophic cartilage. Type II collagen has been shown to be induced by LEP in ATDC5 cells during chondrogenic differentiation (Li et al., 2013d), although this group also observed a significant decrease in type X collagen. The disparity between these reported findings and the current study may be due to the period of LEP stimulation (2 days vs 7 days) and the time point in chondrogenic differentiation selected for assessment (day 16 vs day 14). Another study found *Col2* significantly reduced and *Col10α1* significantly increased in LEP-treated ATDC5 cells (Zhang et al., 2019a), but in this report the cells were exposed

to LEP for only the final 24 hours of the 14 day culture. LEP was found to modulate type 2 collagen expression in primary human chondrocytes at 100 ng/mL but not 20 ng/mL (Pallu et al., 2010), suggesting that dose is important and this should be further explored to better optimise our model.

LEP induced slight but non-significant increases in mRNA expression of *Acan*, one of the proteoglycans that makes hydrogel and acts as a shock absorber in the articular joints (Roughley and Mort, 2014); however, levels of secreted proteoglycans were unchanged. LEP has similarly been shown to slightly increase *Acan* mRNA but not Alcian Blue staining in primary rat chondrocytes (Fu et al., 2019). *Runx2,* an essential regulatory factor for chondrocyte proliferation and maturation (Ding et al., 2012, Chen et al., 2014), regulates the expression of genes encoding matrix degradation enzymes (Chen et al., 2017). *Runx2* was unchanged by LEP or by GSM in this study, suggesting that collagen and proteoglycan staining levels were not affected by upstream changes in metalloproteinases or other degradative enzymes.

These results are consistent with previous studies which indicate that LEP plays a role as a growth factor in the chondrocytes of the skeletal growth centres and promotes the formation of bones through endochondral ossification (Maor et al., 2002). Our findings verify that LEP plays a pathological role in the progression of OA by changing the phenotype of cartilage, and thus is a suitable stimulant to activate chondrocytes *in vitro*. However, the results from our model when put into the context of the literature

demonstrate that LEP may have varying effects on chondrocytes depending on cell source, stage of differentiation, duration of exposure, and LEP concentration.

7.4.2 Effect of GSM extracts on biomarkers of chondrocytes metabolism

The conventionally processed GSM-A and enzymatically processed GSM-B extracts induced similar but not identical effects including decreasing *Acan* mRNA expression with no change in aggrecan production, and increasing expression of the differentiation initiator *Sox9,* a major transcription factor required for differentiation of prechondrogenic progenitors into chondrogenic cells (Haag et al., 2008). *Sox9* is crucial for the formation of hyaline/normal cartilage and prevents transitioning of normal proliferative chondrocytes into hypertrophic or osteoblastic states (Dy et al., 2012, Lefebvre and Dvir-Ginzberg, 2017). A recent study showed that upregulation of *Sox9* showed therapeutic effects on surgically induced OA in mice (Ouyang et al., 2019). Thus, the effects we observed with GSM in both unstimulated and LEP-stimulated chondrocytes suggest that GSM has the potential to prevent hypertrophic differentiation in chondrocytes.

Importantly, both extracts decreased mineralisation in unstimulated ATDC5 chondrocytes *in vitro*, which mimic normal *in vivo* chondrocytes. Further, in LEP-stimulated ATDC5 chondrocytes used to model in vivo OA chondrocytes, both GSM extracts overcame the detrimental effect of LEP, and significantly decreased mineralisation as measured by Sirius red staining. Lipids in GSM such as polyunsaturated

fatty acids, which are known to be effective in reducing OA symptoms, have not been reported to reduce mineralisation in chondrocytes; thus, this finding in the current study can more realistically be attributed to non-lipid components of GSM. There are a number of compounds reported to reduce mineralisation in this cell line. Most are pharmacological preparations unlikely to be present in GSM (Kirimoto et al., 2005, Okada et al., 2005, Hojo et al., 2010, Nasi et al., 2021). Individual ions can also decrease mineralisation by chondrocytes, but the supplemental ions provided by GSM in the current study as calculated from analysis of a similar powder (Nutrizing, 2021) were one hundred-fold and ten-fold lower than concentrations shown to be effective for magnesium (Nakatani et al., 2006) or iron (Ohno et al., 2012) respectively, and thus are unlikely to be responsible for the effects observed in this study. However, glucosamine (Nakatani et al., 2007), collagen-derived peptide (Nakatani et al., 2009), chondroitin sulphate and hyaluronic acid (Kudo et al., 2017) have been shown to have similar effects, although high concentrations (1 mg/ml) of each are required to be effective compared to unfractionated GSM used in the current study at 100 µg/ml. GSM extracts are likely to contain most or all of these compounds (Miller M, personal communication) as well as minerals, which may interact synergistically. It is also possible that GSM contains one or more novel bioactive factors that inhibit mineralisation of articular cartilage and play a protective role in OA.

GSM-B behaved comparatively differently from GSM-A when several parameters were measured in LEP-stimulated ATDC5 chondrocytes. GSM-B, but not GSM-A, significantly

decreased Alp expression; this may indicate a protective effect as *Alp* is associated with mineralisation by hypertrophic chondrocytes (Newton et al., 2012). GSM-B, but not GSM-A, also significantly decreased total collagen production and decreased *Col2α1,* a component of the calcified cartilage where hypertrophic chondrocytes reside (Korpayev et al., 2020). Conversely, GSM-A, but not GSM-B, significantly decreased expression of *Acan* and *Col10α1*; Acan has been shown to be required for production of *Col10α1,* and both are involved in hypertrophic differentiation of chondrocytes (Hodax et al., 2019). Chondrocyte hypertrophy and *Col10α1,* in turn, are critical in the process of endochondral ossification (Li et al., 2016a), and an epitope of type 10 collagen has been proposed as a diagnostic marker for OA (He et al., 2019). GSM extracts may therefore protect against OA by slowing the progression of the chondrocytes towards the hypertrophic state.

7.5 Conclusion

Together these findings indicate that LEP is a suitable stimulant to induce a mild OA-like phenotype in ATDC5 chondrocytes, and that OA chondrocytes are a potential target for GSM. A similar whole GSM powder used in an OA rat model *in vivo* was found to be effective in reducing knee joint damage (Siriarchavatana et al., 2019a). Both GSM extracts had effects on some aspects of hypertrophic differentiation of OA chondrocytes. GSM-B appeared to be more effective, possibility because enzymatic processing produced additional bioactive components. We hypothesise that factors

other than lipids in GSM may contribute to its bioactivity, and that whole GSM may be beneficial as a dietary supplement in OA patients. Given the novel direct effects of GSM observed in a model of OA chondrocytes, it would be of interest to identify its active components as well as to explore a wider panel of genes whose expression is modulated by GSM.

Comparison of mechanically processed green shell mussel, GSM-A versus enzymatically processed GSM-B for their effects on the metabolism of leptin-stimulated chondrogenic ATDC5 cells. 223

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Chapter Eight: Discussion, conclusions, and future directions

8.1 Overall discussion

The work described in this thesis was designed to gain insight into the mechanisms of action of whole green shell mussel (GSM) on osteoarthritis (OA) using *in vitro* cell models. OA is a multifactorial disease of complex pathogenesis and involves collaborative breakdown and restoration processes of cartilage, bone, and synovium. A complex interaction between genetic, metabolic, biochemical, and biomedical factors, combined with inflammation, initiates joint pain (Hassanali and Oyoo, 2011). The enzymes, growth factors, and cytokines involved in regulating cartilage differentiation and destruction, subchondral bone remodelling, and synovial inflammation could be the most attractive targets for therapy due to their critical role in OA pathogenesis (Thysen et al., 2015). Leptin (LEP), a hormone produced by adipose tissue, is thought to be the linking factor between obesity and OA. Elevated levels of LEP in obese individuals and in females, who have a higher proportion of body fat than men, make them more susceptible to OA (Lajeunesse et al., 2005, Gualillo, 2007, Hu et al., 2011). LEP is considered to act as a genetic, proinflammatory, local, and systemic factor in OA pathogenesis (Chen and Yang, 2015), and its levels are found to be elevated in serum, infrapatellar fat pad (IPFP), synovial tissues, osteoblasts, and cartilage of OA patients compared to healthy individuals (Dumond et al., 2003a, Mutabaruka et al., 2010b, de Boer et al., 2012, Conde et al., 2014).

GSM has known efficacy in treating pain and stiffness in joints. Reports in the literature collectively demonstrate that GSM extracts such as Lyprinol® have proven anti-inflammatory effects. For some conditions, its anti-inflammatory mechanisms are also

understood; however, in osteoarthritis the exact mechanism of its action are unknown. This study was conducted to understand the mechanism of action of GSM in OA. For this purpose, *in vitro* disease models representing the different parts of OA-joint were created and then GSM whole mussel extracts were assessed for their efficacy in these diseased models. Lipopolysaccharide (LPS) from bacterial cell wall was used as a control stimulant as this is the substance most commonly used for to initiate inflammation in J774A.1 macrophages (Michel et al., 2013b, Jin et al., 2018, Hung et al., 2019a) and other cell line *in vitro* models. ATDC5 chondrocytes have previously been used to model OA related inflammation and degeneration (Jin et al., 2018, Wang et al., 2019c), and MC3T3-E1 osteoblasts to model bone damage (Guo et al., 2013, Liu et al., 2016). However, we hypothesized that LEP would be a more natural stimulant to mimic mild, subclinical inflammation related to MetS or OA, none of which are directly correlated with bacterial infection. To address this question, we compared LPS and LEP-stimulated J774A.1 models of inflammation for the gene expression of the proinflammatory cytokines IL-1β, IL-6, and TNF-α and the anti-inflammatory cytokine IL-10. Moreover, different doses of LEP were compared with 100ng/ml of LPS at different time points and in different solvents in order to optimize experimental conditions. Finally, we reported the successful creation of a novel LEP-stimulated *in vitro* model of inflammation to study MetS/OA related low-grade inflammation, which is compatible to be used with 0.1% DMSO as a carrier for novel test factors **(Chapter Three)**. As shown in figure 3.2, both stimuli induced significant ($p<0.001$) expression of cytokines after 4 hours but as expected LPS-induced cytokine expression was the highest than LEP-induced. LPS induced cytokine expression that lasted longer and remained significantly elevated up

to 6 hours. The difference between LPS- and LEP-induced cytokine expression was significantly different after 4 and 6 hours of induction, as LPS significantly augmented the expression of all the cytokines after 4 and 6 hours as compared to LEP. This is the first study to report LEP characterization *in vitro*, and our findings with LPS are supported by a previous study, which reports 4 hours as the optimal time point for the induction of cytokines in J774A.1 macrophages (Huang, 2012).

To further optimize the LEP treatment protocol we compared the cytokine expression of J774A.1 macrophages induced with LPS and multiple doses of LEP in CM with no added solvent versus 0.1% DMSO (figure 3.3). LPS significantly induced the expression of all the cytokines in CM as well as in 0.1% DMSO but the response was more prominent in the latter. 0.1% DMSO significantly increased ($p<0.001$) the LPS-induced expression of *IL-6* and *TNF-α*. There is conflicting evidence in the literature regarding the effects of DMSO on the expression of cytokines. Some studies report that DMSO decreases cytokine expression (Kelly et al., 1994, Elisia et al., 2016b, de Abreu Costa et al., 2017), while Xing and Remick (2005) reported that DMSO augmented the LPS stimulated production of *IL-1β* without affecting *IL-6* and *TNF-α* (Xing and Remick, 2005). The effect of DMSO may be stimulus-dependent. LEP on the other hand has shown dose-dependent effects in the increase in the expression of chemokines in J774A.1 cells (Kiguchi et al., 2009) both in CM and 0.1% DMSO but the overall expression was reduced in 0.1% DMSO; these findings are in line with previous studies as listed above which report that DMSO decreases inflammation.

LEP (100 µg/ml) had the maximum effect on the expression of cytokines both in CM and 0.1% DMSO but the effect was more prominent in CM, followed by LEP (10 µg/ml), which significantly upregulated ($p<0.05$) the expression of *IL-1β, TNF-α,* and *IL-10*. In agreement to our results, Shivahare et al also reprted that LEP significantly increased *TNF-α* mRNA expression in J774A.1 macrophages at 10 and 15 µg/ml (Shivahare et al., 2015). No significant increase in the expression of any cytokine was observed with 10 µg/ml of LEP in the medium containing 0.1% DMSO; however, with both 100 and 10 µg ml of LEP the cytokine expression was quite high. 100 µg/ml of LEP was relatively cytotoxic especially in 0.1% DMSO (figure 3.1). The lower doses of LEP (0.1 and 0.05 µg ml) may be physiologically more relevant but they did not produce inflammation *in vitro*; this is likely due to *in vivo* versus *in vitro* differences. This has been demonstrated *in vivo* and in *in vitro* primary cell cultures as they are more sensitive to stimuli as compared to cell lines (Welser, 2015). The established cell lines require a higher concentration of a treatment to produce effects observed at lower concentrations *in vivo* or with primary cells. Therefore, we chose to proceed further with 10 µg/ml of LEP. This expression represented the low-grade inflammation, which is the hallmark of MetS and OA (Gregor and Hotamisligil, 2011a). In medium containing 0.1% DMSO 10 µg/ml of LEP increased the mRNA expression of *IL-1β, IL-6, TNF-α*, and *IL-10* up to 217-, 234-, 4-, and 2-fold respectively.

Next, we compared two whole blue mussel (BM) with green shell mussel extracts (GSM) for their immunomodulatory potential in the LEP-stimulated J774A.1 murine macrophage model. Moreover, the protocol for assessing whole-food extracts in this

model was also optimized. A series of experiments were conducted to identify the optimal time point, dose, solvent, and culture conditions for peak cytokine production, followed by a comparison of BM and GSM for immunomodulatory bioactivity. The maximum expression of cytokine mRNA was observed after 4 hours; however, BM-A and GSM-A induced a second round of cytokine expression after 48 hours. BM extracts were found to have higher overall pro-inflammatory activity than GSM extracts. A mineral-rich BM significantly increased cytokine production independently of LEP in a dose-dependent manner. A solvent effect was also observed, as DMSO reduced the overall pro-inflammatory activity and enhanced the anti-inflammatory activity of this extract **(Chapter Four)**. While the findings related to BM are of interest, the production method used to create BM-C could not be repeated due to introduced technical errors and its composition was found to be not representative of BM extracts in general. Therefore, further research with BM was not pursued and subsequent studies were carried out using only GSM.

Cytokine expression induced by LEP or mussel extract in macrophages peaked after four hours with all the treatments, with a slight decline after 6 hours and eventually a return to baseline as shown in figure 4.2. This compares well with a previous report (Huang et al., 2012) where LPS-induced cytokine expression by J774A.1 cells peaked at 4 hours. The J774A.1 cell line can be stimulated to display a pro-inflammatory M1 phenotype (He et al., 2018, Seyedi et al., 2018, Lin et al., 2020). The presence of M1 macrophages in culture can be verified by the cells' expression of pro-inflammatory cytokines such as *IL-6* or *TNF-α,* and the presence of M2 macrophages by the anti-inflammatory cytokine *IL-*

10 (Bartosh and Ylostalo, 2014, Lin et al., 2020), all of which are dependent on the NFκB pathway as noted in the literature review of this thesis.

In the current study, we demonstrated that LEP alone rapidly induced the presence of both M1 and M2 macrophages. This has not been demonstrated before in J774A.1 or other cell lines *in vitro*, although in a single report human peripheral blood monocytes were found to produce both IL-1β (M1) and IL-10 (M2) cytokines after being stimulated in culture for five days with LEP (Acedo et al., 2013). Macrophage infiltration into adipose tissue increases with obesity (Li et al., 2016b), as does LEP production (Guzik et al., 2017), and mice with diet-induced obesity have increased LEP levels that correlate with increases of both M1 and M2 type cytokines in the adipose tissue (Enos et al., 2013). LEP has also been shown to potentiate cytokine expression in macrophages already induced by other stimuli to assume an M1 or M2 phenotype *in vitro* (Kredel et al., 2013).

These findings suggest LEP is a complex inflammatory mediator that can manipulate the behaviour of both polarised macrophages and non-polarised monocytes and macrophages. The cytokine induction effects by LEP are consistent with true, multifactorial immunomodulation rather than solely pro-inflammation or anti-inflammation in J774A.1 cells. LEP and the mussel extracts neither interfered with nor augmented each other's bioactivity, further validating this adipokine as being a more relevant *in vitro* stimulant than LPS.

Neither GSM-A nor GSM-B induced a pro-inflammatory M1 phenotype in treated cells. Repeated treatment with GSM-B induced at least some of the cells to express the M2 phenotype as identified by *IL-10* mRNA production. The increase in the expression of *IL-10* after 48 hours of treatment with GSM-A *in vitro* would, if extrapolated to *in vivo*, suggest the possibility that long-term use of GSM-A could potentialize the immune system to fight chronic inflammatory conditions by altering the polarization of macrophages from M1 to M2. This may be the mechanism occurring in reported *in vivo* studies where regular consumption of GSM produced anti-inflammatory results in rats and humans. For instance, a lipid fraction (Lyprinol®) from GSM was found to have anti-inflammatory activity in rats (Whitehouse et al., 1997). Similarly, whole BM reduced disease symptoms in women with rheumatoid arthritis (Lindqvist et al., 2018a). However, neither GSM extract was able to reduce the pro-inflammatory effects of LEP when the treatments were combined. It would be of interest to determine whether pre-treating J774A.1 macrophages with GSM could alter subsequent LEP treatment effects.

In contrast, BM-A and BM-C increased M1 cytokine production within 4 hours of treatment. This novel finding suggests that blue mussels have a significant pro-inflammatory component. BM-A also induced M2 macrophage activity; this activity was likely due to multiple, competing bioactive factors in the extract, and BM has been reported elsewhere to reduce inflammation (Lindqvist et al., 2018b). BM-C induced a much stronger pro-inflammatory response compared to BM-A but failed to produce any anti-inflammatory response. The significant differences between the bioactivity of the two BM extracts are ascribed to the production processes.

BM-A and GSM-A administered serially at 0 and 24 hours significantly increased the expression of *TNF-α* and/or *IL-10* after 48 hours. This repeat-stimulatory effect of the mussel extracts on cytokine expression has been observed in macrophages where priming with small doses of LPS or particulate matter may lead to exacerbation of the immune response upon second exposure to LPS (Chae, 2018a, Gawda et al., 2018). There is not much research so far regarding the *in vitro* use of blue or green mussel extracts, particularly the whole mussel product; however a GalNAc/Gal-specific lectin (CGL) from the edible mussel *Crenomytilus grayanus* has been found to increase the production of proinflammatory cytokines *(IL-1β, IL-6, and TNF-α)* in RAW264.7 macrophages independent of LPS (Chernikov et al., 2017). In another study protein hydrolysate from BM was found to promote differentiation of mesenchymal stem cells into osteoblasts (Hyung et al., 2018), showing their osteogenic potential. This suggests that mussels may have bioactivity in multiple physiological systems, and that this activity may be attributed to components other than lipids.

Interestingly the production process of BM-A and GSM-A was the same. Both BM-A and GSM-A were made by process A "traditional mussel powder process" which involves manual shucking followed by blending, freeze-drying, and finally milling, and both mussel types underwent this process with exactly matching specifications by the same producer. Therefore, the differences in bioactivity between these two extracts are due to the mussel species rather than the production process. In contrast, GSM-B is produced by using process B "new enzymatic mussel powder process" in which GSM underwent preconditioning, enzymatic extraction, and filtration followed by freeze-

drying and milling. Details available for this process are limited due to a current patent pursuit but enzymatic treatment is likely to digest large proteins and produce novel peptides. GSM-A induced *IL-10* expression while GSM-B did not. The traditional production process may preserve the integrity of anti-inflammatory biomolecules present in the mussels while an additional enzymatic step in process B may disrupt the activity of that specific component.

The pro-inflammatory potential of BM-C was generally higher in 0.1% ethanol and methanol but BM-C in 0.1% methanol significantly upregulated the expression of the pro-inflammatory cytokines *IL-1β, IL-6,* and *TNF-α* in the unstimulated as well as LEP-stimulated cells as compared to the respective control and LEP-stimulated groups (figure 4.4). Similar findings were reported by Désy et al, who found that methanol synergizes with activating stimuli to augment cytokine production (Désy et al., 2010). Ethanol, on the other hand, has contradictory data regarding its role in modulating the immune response: it is considered to have an inhibitory role on cytokine production (Pruett and Fan, 2009) but was also found to augment LPS stimulated cytokine response in the liver and brain of the mice (Qin et al., 2008).

The 0.1% DMSO did not show any overall solvent effect, but it did reduce the expression of *IL-6* in all treatment groups and significantly augmented the expression of the anti-inflammatory cytokine *(IL-10)* both in unstimulated and LEP-stimulated cells as compared to control as well as the LEP-stimulated group. Although many studies have revealed that DMSO exhibits anti-inflammatory potential and may suppress the expression of inflammatory cytokines (Elisia et al., 2016a, Costa et al., 2017, Mehta et

al., 2019), in our case this effect may not be responsible for its increased anti-inflammatory activity because solvent effects of each treatment group were normalized with the untreated control (0.1% DMSO). Instead, its anti-inflammatory effects might be due to bioactives present in the mussel extracts, which had greater access into the cells because of excellent solvate capability of DMSO. Being amphiphilic, DMSO is a common solvent of choice as it can solubilize both polar and nonpolar components and can transpose through hydrophobic cell membranes of the cells and is generally not cytotoxic when used at a final concentration of $\leq$0.1% in the culture medium (Tunçer et al., 2018). Moreover, it is placed in the safest category of class 3 solvents according to the Q3C solvent classification system, which means they are less toxic and are of less risk to human health (Jamalzadeh et al., 2016). However, use of any solvent is not physiologically relevant, as macrophages *in vivo* would be exposed to bioactive mussel compounds in the bloodstream in an aqueous rather than solvent carrier form.

There is no research so far regarding the effects of GSM on subchondral bone. We tested two GSM extracts (GSM-A and GSM-B) for their effects in a subchondral trabecular bone model of OA *in vitro*. We created the novel *in vitro* model of OA bone by stimulating MC3T3-E1 cells with LEP and identifying how this stimulus altered osteoblast function. We then compared two whole GSM powders, conventionally processed GSM-A versus enzymatically processed GSM-B, for their effects on proliferation, differentiation, and mineralization of normal and LEP-stimulated MC3T3-E1 osteoblasts. We found results with LEP presented a dichotomy, as it increased the proliferation and expression of osteogenic markers but decreased ALP activity and mineral deposition of osteoblasts.

Both GSM extracts promoted bone mineralization by regulating every step of bone formation, but GSM-B was more effective. It significantly upregulated the proliferation, osteogenic markers expression (*Alp, Osx, Col10α1* and *Runx2*), ALP activity and mineral deposition by pre-differentiated osteoblasts as compared to control and/or LEP-stimulated group. GSM promoted bone formation both in unstimulated as well in the LEP-stimulated group; however, the efficacy of GSM extracts was higher in the LEP-stimulated group indicating its potential to prevent bone loss and improve bone mineral density in patients with metabolic OA **(Chapter Five)**.

The results presented in **chapter Five** verified the dualistic results of LEP in MC3T3-E1 cells, as it increased the proliferation and expression of osteogenic markers but decreased ALP activity and mineral deposition of osteoblasts. In the cell model used in this study, 100 ng/ml of LEP was used to stimulate MC3T3-E1 osteoblasts. There are no published reports of this cell line being treated with LEP to study its effects on osteoblast mineralisation or production of the osteogenesis genes measured in the current study, and thus these novel findings cannot be directly compared with other studies. Our results showed that LEP at this concentration increased the number of metabolically active cells as well as mRNA expression of osteogenic/differentiation markers, although only the effect on cell number was statistically significant. LEP downregulated the ALP activity in both the pre-osteoblasts and pre-differentiated osteoblasts.

Previous literature regarding the effects of LEP on the skeleton are contradictory: Ducy *et al* described LEP as a potent inhibitor of bone formation (Ducy et al., 2000), while in another study LEP was found to increase bone formation (Iwaniec et al., 2007)both via

the central nervous system (CNS). Numerous studies have shown that in addition to acting through the CNS, LEP can also act directly on peripheral tissues, including bone marrow mesenchymal stem cells (BMSCs) in which it increases osteoblast proliferation and mineralization (Burguera, 2001, Gordeladze et al., 2002, Mutabaruka et al., 2010a). Mutabaruka et al also demonstrated that local production of LEP increases in OA osteoblasts which may also increase some osteoblast differentiation markers. Moreover, the exogenous administration of LEP was shown to increase the ALP activity of MC3T3-E1 cells but failed to further affect the release of osteocalcin (OCN) and collagen type 1 production. In another study 200 ng/ml of LEP was found to decrease the ALP activity as well as the production of the bone resorption protein receptor activator of nuclear factor kappa-B ligand (RANKL); however, a lower dose of LEP had the opposite effect on ALP activity (Lamghari et al., 2006). Xu *et al* also found that 160 ng/ml LEP significantly increased the expression of genes related to bone cell differentiation in human bone marrow stromal cells (Xu et al., 2016). Together these data suggest that the role of LEP with respect to bone metabolism is complex; it has been proven to play an important role in the abnormal physiology of OA osteoblasts but the exact mechanism is yet to be explored (Mutabaruka et al., 2010a). It is possible that its effects *in vivo* are dose-dependent and thus the level of body adiposity, correlating with the amount of LEP produced and the circulating LEP concentration, may result in different effects on bone cell function.

GSM and particularly enzymatically processed GSM-B has anabolic effects on bone formation *in vitro* especially when osteoblasts are also stimulated with LEP. The higher

efficacy of GSM-B specifically in the LEP-stimulated group indicates that it has the potential to prevent the damage to subchondral bone that occurs with OA, and further may be of use in improving bone mineral density and structure in osteoporosis. Although, there is no study so far reporting the effects of GSM on subchondral bone in OA, it has already been shown that GSM can treat OA-related symptoms by controlling inflammation and preventing cartilage damage (Li et al., 2014a, Siriarchavatana et al., 2019a). Furthermore, Siriarchavatana also found that GSM improved bone mineral density in rats with diet- and ovariectomy-induced metabolic OA (Siriarchavatana et al., 2020). Similarly, a water-soluble protein from blue mussel was shown to increase the growth and osteogenic activity of MC3T3-E1 cells (Xu et al., 2019).

Our next set of experiments were planned to understand the effects of GSM-B in the *in vitro* bone model of OA and to study its immunomodulatory effects in the *in vitro* model of synovial inflammation. We interpret the immunomodulatory and osteogenic roles of GSM-B in a broader context of physiological significance. LEP significantly upregulated pro-inflammatory *IL-1β* and *TNF-α* but not anti-inflammatory *IL-10* mRNA in macrophages, and significantly suppressed osteoblast proliferation. GSM however significantly upregulated macrophage *IL-10* mRNA, an effect not inhibited by LEP. GSM also significantly upregulated *IL-1β* and *TNF-α* mRNA; when combined with LEP the effects were synergistic and additive respectively. In osteoblasts, GSM significantly upregulated alkaline phosphatase (*Alp*) mRNA, ALP enzyme activity, and subsequent mineralisation. Inclusion of LEP did not inhibit GSM-induced *Alp* mRNA or enzyme activity but did block mineralisation. In the presence of LEP, GSM also significantly

increased osteoblast mRNA levels of the osteogenic markers bone sialoprotein, osteocalcin and collagen type 10. These results describe the novel use of LEP with J774A.1 and MC3T3-E1 cells to model synovial inflammation and subchondral bone damage in OA and demonstrate that whole GSM can provide protective effects in both macrophages and osteoblasts challenged with LEP **(Chapter Six)**.

In **Chapter Six** we report the successful creation of novel cell models of LEP-activated J774A.1 and differentiated MC3T3-E1 cells to simulate the effects of this adipokine on macrophages and osteoblasts in OA. 10 μg/ml of LEP was used to stimulate both the cell lines. Treatment of these cells with whole GSM powder was found to significantly increase pro-inflammatory cytokine mRNA expression in macrophages, particularly IL-1β and TNF-α, at levels similar to those observed with LEP treatment. However, GSM with or without LEP also significantly increased expression of the anti-inflammatory cytokine IL-10. In osteoblasts, a high concentration of GSM increased mRNA levels of ALP and similarly increased ALP enzymatic activity and the formation of mineralised nodules, with only mineralisation activity blocked by the inclusion of LEP. GSM when combined with LEP also increased mRNA expression of other osteoblast differentiation and osteogenic markers including BSP, OCN and Col10α1. As expected, the inflammatory adipokine LEP induced significant increases in pro-inflammatory but not anti-inflammatory cytokine expression in this OA macrophage model. GSM upregulated expression of the anti-inflammatory cytokine IL-10, and this effect was not blocked by the inclusion of LEP. However, in contrast to a previous study that reports the health benefits of a GSM lipid extract are based on its anti-inflammatory potential (Li et al.,

2014), this study observed that the whole GSM product significantly upregulated the expression of pro-inflammatory IL-1β and TNF-α. Furthermore, when GSM and LEP were combined, the increase in these cytokines was additive or synergistic, suggesting that GSM has potent pro-inflammatory qualities as well as anti-inflammatory capability.

These conflicting results can be partly explained by differences in both test format and model system. A combination of GSM oil, vitamin E and olive oil was reported to reduce LPS-induced expression of IL-1β, IL-6, and TNF-α in Raw264.7 macrophages, but the GSM product was not assessed in the absence of LPS (Chen et al., 2017), so any inherent pro-inflammatory activity would not have been identified. In addition, whole GSM powder is predominantly protein with smaller proportions of carbohydrates, lipids, and minerals. One class of proteins known as mussel adhesive proteins (MAP) are reported to have anti-inflammatory activity in keratinocytes, but in murine RAW264.7 macrophages MAP had no effect on LPS-stimulated production of IL-1β, IL-6 or TNF-α (Ahn et al., 2019). However in that study MAP was not tested in the absence of LPS, so any potential pro-inflammatory effect of this MAP remains unknown. A lectin isolated from a different mussel species, assessed in both THP-1 and RAW264.7 macrophages, was shown to significantly upregulate IL-1β and TNF-α (Chernikov et al., 2017), thus it is possible that a lectin in GSM was responsible for the similar effect observed in our study.

A variety of transcription factors and proteins are involved at every stage of osteoblast development. *Runx2* is an early differentiation factor that regulates the differentiation of mesenchymal stem cells into osteoblast precursors (pre-osteoblasts). *Osx* is produced in pre-osteoblasts and controls the further maturation of osteoblasts and finally the

formation of osteocytes and bone (Sinha and Zhou, 2013). *Alp* and *Col1α1* code for proteins produced during early matrix formation in osteoblasts while *OCN*, *Col10α1*, and *Bsp* are the osteogenic markers representing the mineralization of the osteoblast matrix and thus maturation of osteoblasts in bone (Kirkham and Cartmell, 2007). Moreover, OCN is used as a marker of successful bone regeneration therapy (Saffarian Tousi et al., 2013). LEP alone did not significantly affect the expression of these differentiation and osteogenic markers. GSM alone also did not have significant effects, but at a high dose combined with LEP caused significant increases in mRNA levels of *Bsp*, *OCN* and *Col10α1*. *Col1α1*, *Osx* and *Runx2* expression also increased but did not reach statistical significance. These are the first reported findings of GSM promoting osteoblast function, although there is a single report of a blue mussel protein hydrolysate having similar effects on *Col1α1* and *OCN* production (Hyung et al., 2018).

There are no published reports of any mussel product being assessed in J774A.1 cells or being assessed in LEP-stimulated rather than LPS-stimulated macrophages so direct comparisons cannot be made. However, macrophages are a heterogeneous and functionally diverse class of cells with two main phenotypes: M1 which initiates and sustains the inflammatory response and is characterized by the secretion of pro-inflammatory cytokines, and M2 which is associated with the release of anti-inflammatory mediators/cytokines meant to resolve inflammation. Inflammatory signals drive the polarization of macrophages in the M1 phenotype while anti-inflammatory agents cause polarization in the M2 phenotype (Viola et al., 2019). As macrophages in OA are skewed to the M1 phenotype (Dickson et al., 2019b) and their

pro-inflammatory activity in this disorder is largely attributed to leptin and other adipokines (Wang and He, 2018, Xie and Chen, 2019), this validates the novel design of the LEP-J774A.1 assay used in this study to model OA. In conclusion, we have established that LEP is an appropriate stimulant for both macrophages and osteoblasts to mimic aspects of synovial inflammation and subchondral bone remodelling that occur in OA. This study also reports for the first time novel inflammomodulatory and osteogenic protective effects of a whole GSM extract. These cell models will be of use in future both to study the pathogenesis of OA and to screen potential intervention treatments. Next, GSM-A and GSM-B were tested for their effects in an *in vitro* OA model of cartilage. First, we created the novel LEP-stimulated *in vitro* chondrocyte model of OA using ATDC5 cells and characterised the effects of LEP. We then compared two whole GSM powders, conventionally processed GSM-A versus enzymatically processed GSM-B, for their effects on cell proliferation and differentiation, and on collagen, proteoglycan, and mineral synthesis by the LEP-stimulated chondrocytes. LEP increased the hypertrophic differentiation of chondrocytes by promoting the expression of chondrogenic genes and by increasing the mineral and proteoglycan content of the chondrocytes. GSM extracts, particularly GSM-B, prevented the hypertrophic differentiation of chondrocytes by suppressing the hypertrophic cartilage-related markers (*Alp*, *Col10α1* and production of mineralized nodules) in LEP-stimulated chondrocytes. Moreover, it suppressed endochondral ossification and maintained chondrogenic phenotype by decreasing *Runx2* and *Col1α1* expression and by increasing *Sox9* expression respectively. GSM-A also favoured the production of hyaline cartilage by increasing the expression of *Col2α1*, which provides shape and tensile strength to articular cartilage and prevents

hypertrophic differentiation of chondrocytes. Together these findings suggest that both GSM-A and GSM-B were able to suppress LEP-stimulated hypertrophic differentiation of OA chondrocytes, but GSM-B was found more potent regarding its effects in maintaining chondrogenic phenotype and suppression of hypertrophic differentiation of OA chondrocytes **(Chapter Seven)**.

Chapter Seven presents the first studies to explore both the role of LEP on the OA-related metabolism of ATDC5 chondrocytes and the chondrogenic potential of GSM extracts in LEP-stimulated ATDC5 chondrocytes used to mimic OA chondrocytes *in vitro*. We determined that LEP stimulation for 7 days induced an OA-like phenotype evinced by increased mineralisation and expression of *Alp*, *Acan*, *Col1α1* and *Col2α1*. These changes were largely reversed by inclusion of GSM, which significantly decreased mineralisation. The two GSM extracts tested did produce different results, indicating that the processing procedures used created products with different types or levels of bioactive components.

100 ng/ml of LEP was used to activate ATDC5 chondrocytes, a dose reported to be effective with this cell line and other chondrocytes (Zhang et al., 2019a). No effect of LEP on cell viability was observed. LEP did not alter the mRNA levels of *Runx2*, a transcription factor necessary to commit pre-chondrocytes to the osteoblast lineage (Chen et al., 2014) or of *Sox9*, which initiates differentiation of pre-chondrocytes into chondrocytes (Jiang et al., 2018) and whose expression peaks in this cell line at approximately day 14 of differentiation (Naito et al., 2015). The effects of LEP on these parameters have not previously been measured in ATDC5 chondrocytes, but these

findings indicate that LEP at this concentration neither blocks nor promotes the survival, proliferation or differentiation of these cells.

Alkaline phosphatase and mineralized nodules, characteristic features of calcified/hypertrophic cartilage and during *in vitro* cell differentiation assays, are considered an undesirable form of cartilage (Armiento et al., 2019) and are associated with OA. LEP induced slight but non-significant increases in mRNA expression of *Alp,* a marker of hypertrophic chondrocytes that initiates the mineralization of the extracellular matrix (Zhang et al., 2019a). This was matched by an increase in mineralisation measured by Sirius red staining, corroborating a similar finding reported previously (Zhang et al., 2019a) and confirming the anabolic role of LEP regarding hypertrophic differentiation of chondrocytes. An earlier study reported that 100 ng/mL LEP completely abolished matrix calcification by ATDC5 cells (Naito et al., 2015), but in this study the LEP was present for 5 – 6 weeks of culture and calcium was measured using the ortho-cresolphthalein complexone method. ALP enzyme activity appeared unaffected in our results; however, it has been shown that when *Alp* mRNA is upregulated ten-fold by an external stimulant, concurrent ALP enzyme activity is less than doubled (Naito et al., 2015), likely due to differences in assay sensitivities.

LEP also induced visible although non-significant increases in *Col1α1* and *Col2α1*, but not *Col10α1*, mRNA expression. The first two are markers of fibrocartilage and hyaline cartilage respectively, whereas the latter is a differentiation marker of hypertrophic cartilage. Type II collagen has been shown to be induced by LEP in ATDC5 cells during chondrogenic differentiation (Li et al., 2013d), although this group also observed a

significant decrease in type X collagen. The disparity between these reported findings and the current study may be due to the period of LEP stimulation (2 vs 7 days) and the time point in chondrogenic differentiation selected for assessment (day 16 vs 14). Another study found *Col2* significantly reduced and *Col10α1* significantly increased in LEP-treated ATDC5 cells *(Zhang et al., 2019a),* but in this report the cells were exposed to LEP for only the final 24 hours of the 14 day culture. LEP was found to modulate type 2 collagen expression in primary human chondrocytes at 100 ng/ml but not 20 ng/ml (Pallu et al., 2010).

LEP induced slight but non-significant increases in mRNA expression of *Acan*, one of the proteoglycans that makes hydrogel and acts as a shock absorber in the articular joints (Roughley and Mort, 2014); however, levels of secreted proteoglycans were unchanged. LEP has similarly been shown to slightly increase *Acan* mRNA but not Alcian Blue staining in primary rat chondrocytes *(Fu et al., 2019). Runx2,* an essential regulatory factor for chondrocyte proliferation and maturation (Ding et al., 2012), regulates the expression of genes encoding matrix degradation enzymes (Chen et al., 2017). *Runx2* was unchanged by LEP or by GSM in this study, suggesting that collagen and proteoglycan staining levels were not affected by upstream changes in metalloproteinases or other degradative enzymes.

These results are consistent with previous studies which indicate that LEP plays a role as a growth factor in the chondrocytes of the skeletal growth centres and promotes the formation of bone through endochondral ossification (Maor et al., 2002). Our findings verify that LEP plays a pathological role in the progression of OA by changing the

phenotype of cartilage, and thus is a suitable stimulant to activate chondrocytes *in vitro.* However, the results from our model when put into the context of the literature demonstrate that LEP may have varying effects on chondrocytes depending on cell source, stage of differentiation, duration of exposure, and LEP concentration.

The effects of conventionally processed GSM-A and enzymatically processed GSM-B extracts showed similarities including decreasing *Acan* mRNA expression with no change in aggrecan production, and increasing expression of the differentiation initiator *Sox9,* a major transcription factor required for differentiation of prechondrogenic progenitors into chondrogenic cells (Haag et al., 2008). *Sox9* is crucial for the formation of hyaline/normal cartilage and prevents transitioning of normal proliferative chondrocytes into hypertrophic or osteoblastic states (Dy et al., 2012, Lefebvre and Dvir-Ginzberg, 2017). A recent study showed that upregulation of *Sox9* showed therapeutic effects on surgically induced OA in mice (Ouyang et al., 2019). Thus, GSM demonstrated in both unstimulated and LEP-stimulated chondrocytes the potential to prevent hypertrophic differentiation.

Importantly, both extracts decreased mineralisation in unstimulated ATDC5 *c*hondrocytes *in vitro*, which mimic normal *in vivo* chondrocytes. Further, in LEP-stimulated ATDC5 chondrocytes used to model in vivo OA chondrocytes, both GSM extracts overcame the detrimental effect of LEP and significantly decreased mineralisation as measured by Sirius red stain. Lipids in GSM such as polyunsaturated fatty acids, which are known to be effective in reducing OA symptoms, have not been reported to reduce mineralisation in chondrocytes; thus, this finding in the current study

can more realistically be attributed to non-lipid components of GSM. There are a number of compounds reported to reduce mineralization in this cell line. Most are pharmacological preparations unlikely to be present in GSM (Kirimoto et al., 2005, Okada et al., 2005, Hojo et al., 2010, Nasi et al., 2021). Individual ions can also decrease mineralisation by chondrocytes, but the supplemental ions provided by GSM in the current study as calculated from analysis of a similar powder (Nutrizing, 2021) were one hundred- and ten-fold lower than concentrations shown to be effective for magnesium (Nakatani et al., 2006) or iron (Ohno et al., 2012) respectively, and thus are unlikely to be responsible for the effects observed in this study. However, glucosamine (Nakatani et al., 2007), collagen-derived peptide (Nakatani et al., 2009), chondroitin sulphate and hyaluronic acid (Kudo et al., 2017) have been shown to have similar effects, although high concentrations *(*1 mg/ml) of each are required to be effective compared to unfractionated GSM used in the current study at 100 µg/ml. GSM extracts are likely to contain most or all of those compounds (Miller M, personal communication) as well as minerals, which may interact synergistically; it is also possible that GSM contains one or more novel bioactive factors that inhibit mineralisation of articular cartilage and play a protective role in OA.

GSM-B behaved comparatively differently from GSM-A when several parameters were measured in LEP-stimulated ATDC5 chondrocytes. GSM-B, but not GSM-A, significantly decreased *Alp* expression; this may indicate a protective effect as *Alp* is associated with mineralization by *hypertrophic chondrocytes (Newton et al., 2012)*. GSM-B, but not GSM-A, also significantly decreased total collagen production and decreased *Col2α1,* a

component of the calcified cartilage where hypertrophic chondrocytes reside (Korpayev et al., 2020). Conversely, GSM-A, but not GSM-B, significantly decreased expression of *Acan* and *Col10α1; Acan* has been shown to be required for production of Col10α1, and both are involved in hypertrophic differentiation of chondrocytes (Hodax et al., 2019). Chondrocyte hypertrophy and *Col10α1,* in turn, are critical in the process of endochondral ossification (Li et al., 2016a), and an epitope of type 10 collagen has been proposed as a diagnostic marker for OA (He et al., 2019). GSM extracts may therefore protect against OA by slowing the progression of the chondrocytes towards the hypertrophic state.

Together these findings indicate that LEP is a suitable stimulant to induce a mild OA-like phenotype in ATDC5 chondrocytes, and that OA chondrocytes are a potential target for GSM. A similar whole GSM powder used in an OA rat model in vivo was found to be effective in reducing knee joint damage (Siriarchavatana et al., 2019a). Both GSM extracts had effects on some aspects of hypertrophic differentiation of OA chondrocytes. GSM-B appeared to be more effective, possibly because enzymatic processing produced additional bioactive components. We hypothesise that factors other than lipids in GSM may contribute to its bioactivity, and that whole GSM may be beneficial as a dietary supplement in OA patients. Given the novel direct effects of GSM observed in a model of OA chondrocytes, it would be of interest to identify its active components as well as to explore a wider panel of genes of which expression is modulated by GSM.

8.2 Overall Conclusions

One aspect of OA research is designed to discover new therapeutic strategies that can prevent, stop, reduce, or restore the damage to the joints, but due to the multifactorial complex nature of the disease, it is still challenging to bring to market disease-modifying OA drugs (DMOADs) with promising results and reliable safety (Bijlsma et al., 2011a, Martel-Pelletier et al., 2012). Enzymes, growth factors, and cytokines involved in regulating cartilage differentiation and destruction, subchondral bone remodelling, and synovial inflammation could be the most attractive targets due to their critical role in OA pathogenesis (Thysen et al., 2015). As currently there is no cure for the disease, OA needs to be prevented, and for many people the first and most important step towards prevention is maintaining a healthy lifestyle. Weight loss and nutrition could help to achieve this goal. Bioactive factors in foods can be alternative and preventive treatments, as many are now increasingly recognized to have health-promoting benefits along with fulfilling basic nutritional requirements (German et al., 1999).

OA is a chronic disease whose initiation, progression and symptoms may be partly addressed by nutrition. Cartilage degradation in OA is a complex and multifactorial process but pharmacologic compounds tend to target a single specific pathway, which may explain why they are only partially effective in the treatment of OA. Nutritional compounds and whole foods on the other hand have multiple bioactives and therefore can target multiple pathways. In addition, their potential to prevent disease onset makes them a more attractive option to be explored further. Moreover, chronic diseases including OA require long-term pharmacological interventions that are often associated

with significant adverse effects. Nutraceuticals and functional foods may be better options because they are less likely to cause adverse effects (Ameye and Chee, 2006).

Mussels/molluscs are potential functional foods as they are a rich source of omega-3 polyunsaturated fatty acids (PUFAs) as well as essential amino acids (Venugopal, 2017). They are rich in nutrients such as zinc, selenium, riboflavin, and carotenoids, making them nutritionally desirable. Mussels are also well-suited to sustainable and environmentally friendly but intensive aquaculture production. The mollusc *Perna canaliculus* (P. *canaliculus*), generally called green-lipped mussel or Greenshell^{TM} mussel (GSM), is endemic to New Zealand (Cobb and Ernst, 2006a). This species has an extensive history of being used as a traditional medicine to treat various arthralgias in both humans and animals. A multitude of studies have demonstrated that GSM are effective in treating OA-related symptoms and most of these studies have been done in animal models such as dogs, cats, and horses (Eason et al., 2018a). Less is known about the mechanism of action of GSM and most of its bioactivity is attributed to its lipid fraction, which is thought to relieve the OA symptoms by reducing inflammation (McPhee et al., 2007b). More recently, Siriarchavatana *et al* found that a whole GSM extract prevented OA in a rat model of metabolic osteoarthritis by reducing cartilage degradation (Siriarchavatana et al., 2019b).

However, few studies have assessed whole GSM bioactivity *in vitro* and its actions at the cellular level are largely unknown. Therefore, this study was primarily designed to gain insights into the mechanism of action of GSM in cells with three distinct phenotypes. Novel LEP-stimulated *in vitro* models of J774A.1 macrophages, MC3T3-E1 osteoblasts,

and ATDC5 chondrocytes corresponding to OA-related synovial inflammation, subchondral bone remodelling, and cartilage metabolism respectively were created as tools to study the effects of GSM on different parts of the OA-joint. A series of *in vitro* assays were performed to evaluate the activity of different biological markers (genes, enzymes, proteins) in response to GSM treatment. Moreover, we optimized a protocol for *in vitro* use of unfractionated whole mussel extracts in LEP-stimulated J774A.1 macrophages by identifying the optimal time point, solvent and dose for cytokine production, characterising this model well enough for it to be useful to test any kind of whole-meat extract in future.

We also compared two freeze-dried whole GSM powders produced by different processing methods i.e. conventionally processed GSM-A and enzymatically processed GSM-B for their efficacy on different OA targets. We established that GSM extracts, as a whole food, can indirectly alter the disease progression of OA induced by LEP by modifying the immune response as well as by directly providing protective effects to cartilage and bone. The study also provided novel insights into the mechanisms of action of GSM extracts and added to the existing body of data describing how GSM extracts can affect different cell types. Finally, three new cell models to mimic the immune, bone, and cartilage changes that occur in OA were developed and assessed. These findings have aided in determining that whole GSM is a strong candidate for further study in animal models and humans with the goal of optimising the use of GSM instead of or in addition to NSAIDS to treat OA.

8.3 Recommended future research

The following research can be recommended for the future:

- In this study, we treated J774A.1 macrophages with LEP and mussel extracts and measured cytokine expression at 4, 6, 16, 24- and 48-hour time points by RT-qPCR **(chapter Four)**. The 48 hour time point accompanied by re-application of the treatments after 24 hours, was the longest time of the treatments, after which GSM-A was shown to increase the expression of anti-inflammatory *IL-10* mRNA. Therefore, in future it would be worth investigating the effect of chronic exposure of J774A.1 cells to multiple applications of the treatments to explore the anti-inflammatory potential of mussel extracts.

- In the present study, J774A.1 cells were treated with an inflammatory stimulus (LEP) and the mussel extracts simultaneously **(chapter Four)** and neither of the mussel extracts was able to reduce the pro-inflammatory effects of LEP when the treatments were combined. It would be of interest to determine whether pre-treating J774A.1 macrophages with GSM could alter subsequent LEP treatment effects.

- In this study, we compared the effects of the conventionally processed GSM-A and enzymatically processed GSM-B and also assessed the dose-dependent effects of more potent GSM-B on LEP-stimulated MC3T3-E1 cells (OA model of subchondral bone) **(chapter Five & Six).** We measured ALP activity, mineral deposition (Alizarin red staining), and expression of osteogenic markers (RT-

qPCR) in response to GSM treatment as markers of mineralization. The same was done for LEP-stimulated ATDC5 (OA-model of chondrocytes). It would be desirable to confirm the changes in the expression of osteogenic and chondrogenic markers at the protein level in the future.

- The effects of GSM in all three cell types has been studied in a limited fashion. To assess genes modulated by GSM, the use of RNA-sequencing would provide a much wider scope of I nformation and identify potential pathways for further study.
- Although in vitro research is an excellent tool for the initial screening of the products, isolated cells cannot portray the complex physiological system particularly related to multifaced OA, which is a whole joint disease where all the components of the joint interact. Therefore, conditioned medium or co-culture models of either subchondral bone and chondrocytes or macrophages and chondrocytes are highly recommended in the future to understand complex pathological processes of OA and the interaction of multicomponent GSM with multiple OA targets.
- Present findings related to chondroprotective and osteogenic effects of GSM were obtained using in vitro cell models backed by in vivo research by (Siriarchavatana et al., 2019b, Siriarchavatana et al., 2020). However, the effects of GSM need to be further investigated in OA patients.

Appendix A

Materials and Methods

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1.1 Materials:

1.1.1 Chemical reagents

The list of the chemicals and reagents used in this work and their manufacturer details are given below:

Reagent	Manufacturer
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco, Life Technologies, Auckland, NZ
Fetal calf serum (FCS)	Gibco, Life Technologies, Auckland, NZ
Alpha minimum essential medium (αMEM)	Gibco, Life Technologies, Auckland, NZ
Penicillin-streptomycin and glutamine	Invitrogen, Life Technologies, Auckland, NZ
Gentamicin	Invitrogen, Life Technologies, Auckland, NZ
Trypsin/EDTA solution	Sigma-Aldrich, Merck, NZ
Ascorbic acid	Sigma-Aldrich, Merck, NZ
β-glycerophosphate	Sigma-Aldrich, Merck, NZ
MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)	Sigma Aldrich, Merck, NZ
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 plus GlutaMAX ((DMEM/F-12, GlutaMAX™)	(Gibco, Life Technologies, Auckland, NZ

Insulin-transferrin-sodium selenite	Sigma Aldrich, Merck, NZ
Dimethyl sulphoxide (DMSO)	Sigma Aldrich, Merck, NZ
Leptin (recombinant mouse leptin OB)	Biomyx (San Diego, California)
Lipopolysaccharide (LPS)	Sigma Aldrich, Merck, NZ
Tris aminomethane (Tris)	Sigma Aldrich, Merck, NZ
Hydrochloric acid (HCl)	Sigma Aldrich, Merck, NZ
Magnesium chloride ($MgCl_2$)	Sigma Aldrich, Merck, NZ
Triton-X100	Sigma Aldrich, Merck, NZ
Agarose powder	Mediray, NZ
Ethidium bromide	Invitrogen, Life technologies, NZ
Bromophenol blue	BDH Laboratory supplies, Australia
2-Mercaptoethanol	BDH Laboratory supplies, Australia
Xylene Cyanol	Merck, NZ
DNA ladder (EasyLadder I)	Meridian Bioscience, Total Lab Systems, NZ
Ethanol	Sigma Aldrich, Merck, NZ
Nitrogen hydroxide (NaOH)	Sigma Aldrich, Merck, NZ
Para-nitro-phenyl phosphate (pNPP)	Sigma Aldrich, Merck, NZ
4-Nitrophenol	Sigma Aldrich, Merck, NZ
Trypan blue	Sigma Aldrich, Merck, NZ

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Disodium phosphate (Na_2HPO_4)	Sigma Aldrich, Merck, NZ
Monosodium phosphate (NaH_2PO_4)	Sigma Aldrich, Merck, NZ
Alizarin red stain	Sigma Aldrich, Merck, NZ
Alcian blue stain	Sigma Aldrich, Merck, NZ
Cetylpyridinium chloride (CPC)	Sigma Aldrich, Merck, NZ
Formaldehyde	Sigma Aldrich, Merck, NZ
Methanol	Sigma Aldrich, Merck, NZ
Ammonium hydroxide	Sigma Aldrich, Merck, NZ
Guanidine hydrochloride	Sigma Aldrich, Merck, NZ

1.1.2 Commercially purchased kits:

PureLink™ RNA Mini Kit	Thermo Fisher, Auckland, NZ
SuperScript® IV First-Strand Synthesis System	Invitrogen, Thermo Fisher, Auckland, NZ
Gene-specific primers	Invitrogen, Life Technologies, Australia
PCR kit LightCycler® 480 SYBR Green I Master	RochLife Science, Thermo Fisher, Auckland, NZ
Pierce™ BCA Protein Assay Kit	Thermo Fisher, Auckland, NZ
Sirius red/fast green collagen staining kit	Chondrex, Inc; Sigma, Merck, NZ

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1.1.3 Mussel extracts:

The mussel extracts used in this study were kindly provided by Sanford Ltd, New Zealand. Two Greenshell mussel (GSM) and two Blue mussel (BM) extracts were used; proximal analyses were carried out by Cawthron Institute (Nelson, NZ).

1.1.4 Other materials and equipment:

Weighing Balance	Mettler toledo, NZ
(AG204, PB 3001-S, XS105)	
Cell culture flasks (Corning, T-25 and T-75)	Thermofisher Scientific, NZ
Cell scrapers (Biologix)	Thermofisher Scientific, NZ
Centrifuge tubes (Cellstar® greiner bio-one, 15 ml, 50 ml)	Thermofisher, Scientific, NZ
Syringe filters (0.2 µm, 0.45 µm)	Labsupply, NZ
(ReliaPrep™ Ahlstorm-Munksjo)	
Microcentrifuge tubes (Multimax)	Labsupply, NZ
(2 , 1.5, 0.5, 0.2 ml)	
PCR 0.2 ml tubes (Multimax)	Labsupply, NZ
PCR 96 well plates	Roche, NZ
PCR plate sealers	Roche, NZ
Jenway pH meter (3510)	Acorn Scientific, NZ

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Serological pipettes (5,10, 25 ml)	Thermofisher Scientific, NZ
Troughs	Labsupply, NZ
Reservoir troughs	Labsupply, NZ
Cell culture plates (Cellstar® greiner bio-one, 96, 48, 24, 12, 6 well plates)	Thermofisher Scientific, NZ
Micropipettes (Tarsons, Biohit, Jencons)	Thermofisher Scientific, NZ
(P-1000, P-200, and P-20, P-10)	
Multichannel pipettes	Thermofisher Scientific, NZ
Micropipette tips (Multimax)	Labsupply, NZ
Microscope (BH2)	Olympus
Cell culture microscope (MHRC 3036)	Olympus
Biosafety Cabinet	Biostrategy, NZ
(Esco NordicSafe® Class II)	
Fume cupboard	Thermoplastic, NZ
Incubator (Contherm)	Thermofisher Scientific, NZ
Microcentrifuges (Labnet spectrofuge), (Eppendroff centrifuge 54150)	Total Lab Systems, NZ
Vortex (Labnet-VX100)	Total Lab Systems, NZ
Spinner/mini centrifuge (Biocote)	Total Labs Systems, NZ

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Centrifuge (148R-Gyrozen),	Biostrategy, NZ
Beckman Coulter's Allegra 64R	Beckman Coulter, NZ
PCR machine (Biometra T-advanced)	Total Lab Systems, NZ
Roche Lightcycler 480 real-time PCR machine	Roche, NZ
Gel electrophoresis apparatus	Bio rad, NZ
Gel doc system (MiniBIS Pro)	Total Lab Systems, NZ
UV transilluminator (UVitec)	Total Lab Systems, NZ
Orbital shaker (Bioline)	Alphatech Ltd, NZ
Heating block	Labortechnik
Microplate Photometer (Multisaken FC)	Thermofisher Scientific, NZ
Nanophotometer (Implen 4269)	Total Lab Systems, NZ
RNAStable tubes	Biomatrica, San Diego, USA
Fridge	Fisher and Paykel, NZ
-20 freezer	Fisher and Paykel, NZ
-80 freezer	Thermofisher Scientific, NZ
Autoclave (Tuttnauer 3850 ELV)	Thermofisher Scientific, NZ
Hotbox oven (GALLENKAMP)	Watson Victor Ltd, NZ

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Sonicator (Virtis Virsonic 100 sonicator sonicator)	Global Science, NZ
Water bath (Grant)	Global Science, NZ

1.1.5 Buffers and solutions:

All solutions and buffers were prepared using highly purified millique (milli-Q) water and autoclaved where required. Chemicals were properly weighed (weighing balance), pH was properly adjusted using pH calibration standards (pH 4.0 and 7.0). Solutions were stored in autoclaved bottles and autoclaved again where appropriate.

1. **Phosphate buffer saline (10XPBS):**

Dissolved the following in 800ml milli-Q water.

- 80g of NaCl
- 2.0g of KCl
- 14.4g of Na2HPO4
- 2.4g of KH2PO4
- Adjusted pH to 7.4.
- Adjusted volume to 1L with additional milli-Q water.
- Sterilized by autoclaving.

2. **Tris-acetate EDTA (TAE) Buffer:**

- **TAE Buffer 50x Stock Recipe:**
- 242 g tris base in Milli-Q H_2O
- 57.1 ml glacial acetic acid

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100 ml 0.5 M EDTA solution (pH 8.0)

- **10x TAE Recipe:**

For 1L of 10x solution,

- 48.5 g tris
- 11.4 mL glacial acetic acid
- 20 mL 0.5M EDTA (pH 8.0)
- **1x TAE Recipe:**

Dilute 1:10

- 0.4 M tris acetate (pH approximately 8.3)
- 0.01 M EDTA

using Milli-Q water.

3. 100 µg/ml LPS stock

LPS stock was prepared at a concentration of 100 ug/ml with PBS.

4. 1 mg/ml Leptin stock

Leptin stock was prepared at a concentration of 1 mg/ml with PBS.

5. Mussel extracts preparation (10 or 100 mg/ml stocks)

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The freeze-dried powder was dissolved at a concentration of 10 or 100 mg/ml in Dulbecco's Modified Eagle's Medium or DMEM containing 10% DMSO, sonicated briefly to ensure solubility, sterilized through a 0.20 µm pore syringe filter, aliquoted, and stored at -20°C until further use.

BM-C was dissolved into four different solvents i.e. DMEM, DMEM+10% DMSO, DMEM+10% Ethanol, DMEM+10% Methanol, syringe filtered through 0.2 µm filter, aliquoted, and stored at -20°C until further use.

6. 5 mg/ml MTT stock solution

5 mg MTT powder dissolved in 1 ml sterile PBS, Mix by vortexing or sonication until dissolved. particulate material that will not dissolve can be removed by filtration using a 0.2 µm syringe filter.

7. Differentiating solutions (Osteogenic media)

- **1 M β-Glycerophosphate**

  For 10 mL:

- 2.16 g β-Glycerophosphate (216.04 g/mol; G9891)
- Add 7 mL αMEM (SH30265)/DMEM: F12 (no FBS)
- Adjust volume to 10 mL
- Filter sterilise in TC hood (0.2 µm), aliquot and store at -20°C
- Use at 10 mM (1:100)
- **10 mg/mL Ascorbic acid (AA; Vitamin C)**

  For 10 mL:

- 100 mg Ascorbic acid (176.12 g/mol; A4544 4°C)

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- Add 9 mL PBS
- Adjust volume to 10 mL
- Filter sterilise in TC hood (0.2 μm), aliquot and store at -20°C
- Use at 50 μg/mL (1:200)

8. <u>Differentiating solutions (Chondrogenic media)</u>

- **<u>10 mg/mL Ascorbic acid (AA; Vitamin C)</u>**

For 10 mL:

- 100 mg Ascorbic acid
- Add 9 mL PBS
- Adjust volume to 10 mL
- Filter sterilise in TC hood (0.2 μm), aliquot and store at -20°C
- Use at 50 μg/mL (1:200)
- **<u>2 mg/mL Insulin</u>**

For 10 mL:

- 20 mg Insulin
- Add 9 mL PBS
- Adjust volume to 10 mL
- Filter sterilise in TC hood (0.2 μm), aliquot and store at -20°C
- Use at 10 μg/mL (1:200)
- **<u>2 mg/mL Transferrin</u>**

For 10 mL:

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- 20 mg Transferrin,
- Add 9 mL PBS
- Adjust volume to 10 mL
- Filter sterilise in TC hood (0.2 μm), aliquot and store at -20°C
- Use at 10 μg/mL (1:200)
- **20 μg/mL Sodium selenite**

For 50 mL:

- 1 mg Sodium selenite
- Add 40 mL PBS
- Adjust volume to 50 mL
- Filter sterilise in TC hood (0.2 μm), aliquot and store at -20°C
- Dilute it further to 1 μg/ml (working stock)
- Use at 5 ng/mL (1:200)

9. ALP assay buffers and solutions

- **5 M NaCl**

For 50 mL:

- 14.61 g NaCl (58.44 g/mol)
- Add 30 mL dH_2O
- Bring final volume to 50 mL and stir until dissolved (5 M = near solubility limit)
- Store at RT
- **Physiological Saline (0.9%, 154 mM NaCl)**

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For 50 mL:

- 1.54 mL 5 M NaCl (10.10.18)
- 48.46 mL dH_2O

- **0.5 M Tris-HCl pH 9.5**

For 50 mL:

- 3.03 g Tris (121.14 g/mol)
- Add 40 mL dH_2O
- Adjust pH to 9.5 using HCl (conc; drops)
- Adjust volume to 50 mL with dH_2O
- Filter sterilise and store at 4°C

- **10 x TBS (10 x = 200 mM Tris pH 9.5, 1.54 M NaCl)**

For 20 mL:

- 8 mL 0.5 M Tris pH 9.5 (18.07.18)
- 6.16 mL 5 M NaCl (10.10.18)
- 5.84 mL dH_2O
- Store at 4°C

- **Extraction TBS (1% Triton-X100 in 1 x TBS)**

For 30 mL (# wells x 0.5 mL = 24 mL):

- 26.7 mL Water
- 3 mL 10 x TBS pH 9.5 (17.10.18/09.04.19)
- 300 µL Triton X100 (T8787)

- **1 M Tris-HCl pH 9.5**

For 50 mL:

- 6.03 g Tris (121.14 g/mol)
- Add 40 mL dH_2O
- Adjust pH to 9.5 using HCl (conc; drops)
- Adjust volume to 50 mL with dH_2O
- Filter sterilise (0.2 µm) and store at 4°C
- **1 M $MgCl_2$**

  For 25 mL:
- 2.38 g $MgCl_2$ (95.211 g/mol
- Add ~15 mL dH_2O
- Adjust volume to 25 mL with dH_2O
- Filter sterilise (0.2 µm) and store at RT
- **ALP Assay Buffer**
- **Tris-HCl pH 9.5, 1 mM $MgCl_2$)**

  For 10 mL:
- 4 mL 1 M Tris-HCl pH 9.5 (01.05.19)
- 10 µL 1 M $MgCl_2$ (18.07.18)
- 5.99 mL dH_2O
- **pNPP solution**

(10 mM pNPP in ALP Assay Buffer)

For 10 mL:

- 37.114 mg pNPP (371.14 g/mol, N4645-5g)

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- Add 10 mL (10 mL) ALP Assay Buffer
- **5 M NaOH**

  For 50 mL:
- 10 g NaOH (40.00 g/mol)
- Add 35 mL dH_2O
- djust volume to 50 mL
- Store at RT
- **Stop Solution (1 N NaOH = 1 M NaOH)**

  For 50 mL:
- 10 mL 5 M NaOH
- 40 mL dH_2O

10. 0.1% (w/v; 1mg/ml) Alizarin Red S

1 mg of Alizarin Red S dissolved in 1 ml of Milli-Q water, adjusted to pH 5.5 with ammonium hydroxide (freshly made).

11. 10% Cetylpyridinium Chloride

Made up a 10% solution (w/w) in Milli-Q water.

12. 0.1% (w/v, 1mg/ml) Alcian Blue stain

1 mg of Alcian blue dissolved in 1 ml of Milli-Q water

1.2 Cell cultures

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Different cell lines were used to assess the *in vitro* effects of GSM extracts on different biochemical markers. All these cell lines were from the mouse and included J774A.1, Mc3t3-E1 subclone-4 (purchased from ATCC or the American Type Culture Collection) and ATDC5 (purchased from ECACC or European Collection of Authenticated Cell Cultures). All these cell lines are already reported in the literature to be used in osteoarthritis and inflammation research.

1.3 Cell Culture Media

Cell lines were cultured in growth media, according to the formulation recommended, with 10% fetal bovine serum. J774A.1 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) + 10% fetal bovine serum (FBS); Mc3t3-E1 subclone-4 in Alpha Minimum Essential Medium (MEM) with ribonucleosides, deoxyribonucleosides, 1 mM sodium pyruvate, and 10% FBS; ATDC5 in DMEM: Ham's F12 (1:1) + 5% Foetal Bovine Serum (FBS). 1% PSG (Penicillin, Streptomycin and L-glutamine) or 0.27% gentamicin in case of Mc3t3-E1 subclone-4 were added to all the media to prevent microbial contamination of the cell cultures.

1.4 LPS and Leptin

LPS (LPS, *E. coli* 0111:B4) purchased from Sigma-Aldrich (St. Louis, MO, USA), and leptin (recombinant mouse leptin OB), obtained from Biomyx (San Diego, California) was used to stimulate an inflammatory response in the cell lines under conditions of simulated obesity or to make an inflammatory model of osteoarthritis *in vitro*, as reported previously (Raso et al., 2002b).

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1.5 Mussel Extracts

Sanford Ltd and Cawthorn Institute have provided us all the extracts and fractions needed in the research. Two blue mussel extracts, i.e. BM-A and BM-C and two GSM extracts were used in the study i.e. GSM-A and GSM-B. The composition of these extracts is given in table 1.1:

Table 1.1: Composition of mussel extracts

Extract	Moisture	Protein	Ash	Fat	Carbohydrate
BM-A	10.4	49.7	14.5	5.5	19.9
BM-C	6.1	43.7	27	3.2	20
GSM-A	3.2	50.8	15.3	6.5	24.2
GSM-B	4.4	47.3	20.1	7.9	20.3

1.6 Cell thawing

All the cells used in this study were stored in the liquid nitrogen vapor phase. Thawing was done according to the protocol mentioned in the respective product sheet provided by ATCC or ECACC. Cells were rapidly defrosted (2 minutes approximately) in a water bath at 37°C, quickly transferred to centrifuge tubes containing 9 ml of complete medium and centrifuged at 1500 rpm for 5 minutes. After centrifugation supernatant was discarded and the pellet was resuspended in 25 cm^2 or a 75 cm^2 culture flask according to recommended dilution.

1.7 Cell culturing and subculturing

Cells were cultured and subcultured in 75 cm^2 culture flasks according to respective protocols of each cell line. J774A.1 are loosely adherent and their sub-cultures were prepared by scraping with a subcultivation ratio of 1:3 to 1:6. Mc3t3-E1 subclone-4 and ATDC5 cells on the other hand are adherent cells and were dislodged by trypsinization with 0.25% trypsin or trypsin/EDTA preceded by rinsing twice with 10 ml PBS (phosphate buffer saline). The medium was changed or replaced twice or thrice a week. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37℃.

1.8 Cryopreservation

Cryopreservation was done over a range of passage numbers, ranging from passage 6 to passage 30 according to the protocols in respective product sheets. Cells were first dislodged from the flasks and cell suspensions were spun at 1500 rpm for 5 minutes to get cell pellets which were resuspended into 95% complete medium + 5% DMSO (dimethyl sulphoxide). One ml of the suspended cells were shifted to 2 ml cryovials and frozen at -80℃ for 24 hours and then transferred to liquid nitrogen.

1.9 Storage of reagents, extracts, and samples

All the reagents and samples are stored at the recommended temperatures for optimum performance. Extracts and most of the reagents and samples were stored at -20℃, buffers and solutions at 4℃ or room temperature, and samples containing proteins and RNA at -80℃.

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2.Experiment Design

The experimental design of the thesis is summarized in figure 1.1

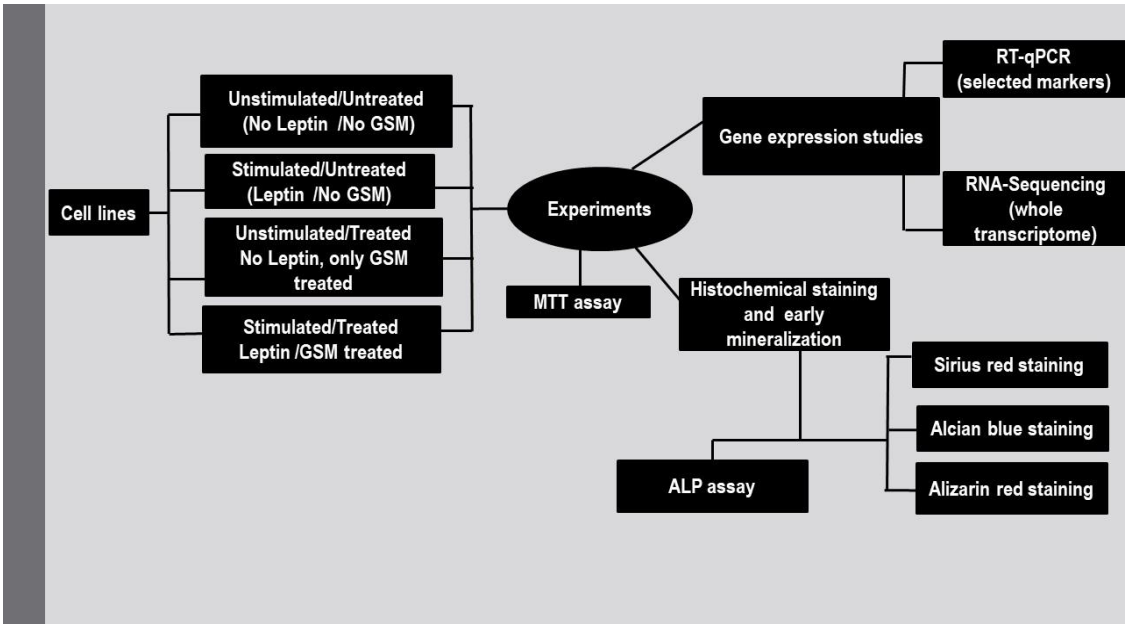

Figure 2.1: Overall experimental design of research

2.1 Plating out the cells

Before starting any assay, the first step is to plate the cells out in a suitable plate. Cells were seeded at different densities and in different plates depending upon the nature of the assay. For instance, MTT assay was performed in 96 well plates with different cell densities depending upon the cell line. RNA extraction was done by growing the cells in 6 wells, 12 wells, or 24 wells plates depending on the cell line and the number of treatment groups. Similarly, other staining assays were performed in 12 or 24 well plates. Cells were used at different densities in different assays. Cell density was determined after counting the cells with trypan exclusion assay using the

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hemocytometer. 10 µl of cells were mixed thoroughly with 10 µl of trypan blue dye and 10 µl of the mixture was then transferred to the hemocytometer cell counting chamber, observed under the microscope and counting took place in four corner squares of counting chamber.

Blue stained cells were excluded from counting, for being dead and white cells were considered alive and counted by the formula,

$$\frac{\text{Number of viable cells in 4 squares}}{4} \times \text{Dilution factor} \times 10{,}000 = \text{Number of cells in 1 ml}$$

Cells densities or cell concentration were then customized according to the requirement of the experiment by using formula M1V1 = M2V2

Where,

- M1 (Concentration of the stock)
- V1 (Volume of the solution to be taken from stock)
- M2 (Concentration of the solution to be made)
- V2(Volume of the solution to be made)

2.2 Cell treatments

All the cells used in the study were cultured in 75cm^2 flasks and subcultured according to instructions given by ATCC or ECACC. The pre-chondrocyte cell line ATDC5 was

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differentiated into functional chondrocytes by culturing the cells for 7-14 days with AITS (sodium selenite (5ng/ml), Transferrin (10µg/ml), ascorbic acid (50µg/ml), and insulin (10µg/ml)). Pre-osteoblasts Mc3t3-E1 subclone-4 were also cultured for 7 to 21 days and differentiating factors i.e. B-glycerophosphate (10mM) and ascorbic acid (50µg/ml) were added to differentiate them into osteoblasts. All of the cells were divided into four experimental groups for each extract as control group without leptin and GSM, Leptin treated (LEP), GSM treated and GSM+LEP treated.

2.3 MTT assay

MTT assay is named after a dye called "3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide" used to assess cell viability in response to different treatments. It is a colorimetric assay, based on the principle that metabolically active or viable cells have NAD(P)H-dependent cellular oxidoreductase enzymes that convert MTT yellow colour solution) into insoluble, purple colour formazan crystals. The intensity of the colour reflects the number of metabolically active cells in a particular treatment group or well. To measure the intensity, formazan crystals need to be solubilized into a coloured solution, which can be done by either dimethyl sulfoxide (DMSO), or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. The OD (optical density) is plotted against cell viability, and cell viability is directly proportional to the OD value.

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For our experiments, Cells at different densities were incubated with different treatments for different lengths of time ranging from one to seven days depending on the cell line, followed by 2-5 hours treatment with MTT until the appearance of purple formazan crystals. Formazan crystals were solubilized into colour solutions by adding 100% DMSO. OD values were measured after 10 minutes of incubation with DMSO, at 550nm using a plate reader photometer (Multiskan Fc, Thermo Scientific SkanIt Software). This assay gave us an idea about the cytotoxic levels of different treatments to be used in this study. With survival value/metabolic activity ≥ 70% for a given treatment we proceeded further to other experiments i.e. Gene expression analysis, gene sequencing or cell staining experiments.

2.4 Gene expression analysis

Gene expression analysis constitutes the following steps:

1. **Cells treatment and RNA extraction**

All cell lines were divided into four treatment groups for each extract. After incubating these cells with the given treatments for an optimal incubation time (each cell line has different incubation time) RNA was extracted from the cells according to manufacturer's protocols by using "Invitrogen PureLink RNA Mini Kit" which provides rapid column-based purification of total RNA from a wide range of cell and tissue types.

2. **Quality and quantity assurance**

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The quality and quantity of RNA were determined by using “Implen nanophotometer”. 3 µl of the RNA samples were poured on the lens and measured for RNA concentration with dilution factor 10. The quality of RNA was revealed by absorbance (OD values) at 260/280 and 260/230. Samples with a 260/280 ratio of 1.9-2.0 and A260/230 of 2.0-2.2 are considered pure. The volumes of all the samples to be used in RT-PCR (reverse transcription-polymerase chain reaction) were adjusted to have 1-5 ug RNA.

3. cDNA synthesis

RNA was converted into cDNA, using “SuperScript® IV First-Strand Synthesis System” for RT-PCR, according to manufacturer’s instructions. This is an optimized system for the synthesis of first-strand, cDNA from purified poly (A) + or total RNA. Oligodt was used for cDNA synthesis. 20-40 ul of cDNA was made depending upon the quantity required for downstream applications. cDNA was diluted by factor 5x or 10x for further use depending upon the concentration of RNA used

4. Primer design

Real-time quantitative PCR (RT-qPCR) was done with gene-specific primers. Therefore, primer designing is the pre-requisite for RT-qPCR. Already reported gene primers were used from “Primer Bank”.

Primer Bank is a public resource for PCR primers. These primers are designed for gene expression detection or quantification (real-time PCR). It contains over 306,800 primers covering most known human and mouse genes.

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The most important characteristic of primer pair to be considered for RT-qPCR is its amplicon size, which should be in the range of 70-200 bp. Greater amplicon size may end up with low PCR efficiency and smaller size will be difficult to be detected on the gel for being confused with primer dimers. The list of primer pairs used in the study are given in the table 2.1.

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Table 2.1: List of primers for cytokines, osteogenic markers, and chondrogenic markers, their annealing temperatures and amplicon size

Primer	Forward sequence (5'→3')	Reverse sequence(5'→3')	Ta (°C)	Amplicon size (bp)
GADPH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	55	123
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA	65	152
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA	60	141
IL-10	TGAGGCGCTGTCGTCATCGATTTCTCCC	ACCTGCTCCACTGCCTTGCT	65	61
TNF-α	CACGTCGTAGCAAACCACCAAGTGGA	TGGGAGTAGACAAGGTACAACCC	60	140
Alp	GTGACTACCACTCGGGTGAAC	CTCTGGTGGCATCTCGTTATC	60	96
Runx2	GACTGTGGTTACCGTCATGGC	ACTTGGTTTTTCATAACAGCGGA	60	84
BSP	CAGGGAGGCAGTGACTCTTC	AGTGTGGAAAGTGTGGCGTT	60	158
Osx	TCCCTGGATATGACTCATCCCT	CCAAGGAGTAGGTGTGTTGCC	60	187
OCN	CTGACCTCACAGATCCCAAGC	TGGTCTGATAGCTCGTCACAAG	60	93
Col1α1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG	60	103
Col2α1	ATCTTGCCGCATCTGTGTGT	CTCCTTTCTGCCCCTTTGGC	60	170
Col10α1	TTCTGCTGCTAATGTTCTTGACC	GGGATGAAGTATTGTGTCTTGGG	60	115
Acan	AGGTGTCGCTCCCCAACTAT	CTTCACAGCGGTAGATCCCAG	60	96
SOX9	AGTACCCGCATCTGCACAAC	ACGAAGGGTCTCTTCTCGCT	60	88

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5. Optimization of RT-qPCR

Before starting Rt-qPCR for the specific genes, the number of cycles, annealing temperature, and other reaction conditions was optimized for each gene by changing one variable at a time and keeping other conditions constant. In this way conditions for PCR were set and applied to all PCR reactions during the study. *GADPH* was used as a housekeeping gene and to normalize respective genes during expression analysis.

Standard curves and melting curves

The standard curve indicates the efficiency of PCR and the melting curve gives an idea about primer specificity; therefore, these are important steps of optimization. In addition, standard curves are a pre-requisite for "E-Method/ Advanced relative quantification", which was the method of our choice to compare the gene expression among different treatment groups. The relationship between PCR efficiency and the slope is given in table 2.2:

For calculation of the standard curve, an adequate number of cells were cultured in a 75 cm^2 flask, subjected to lysis and RNA extraction by the recommended protocols of respective kits, as mentioned earlier. Quality and quantity were checked and 1-5 ug was reverse transcribed to get cDNA, cDNA was serially diluted to get at least 5 dilutions by the factor 10, 5, or 2 depending upon the concentration of cDNA. Standard curves were calculated for each primer pair used in the research.

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Efficiency %	Fold increase per cycle	Slope
110	2.1	**-3.10**
100	2	**-3.32**
90	1.9	**-3.59**
80	1.8	**-3.92**
70	1.7	**-4.34**
50	**1.5**	**-6.68**

Table 2.2: A good qPCR run will have an efficiency ranging from 90%-110%. The relationship between slope and efficiency follows the equation: Slope = -1 x $1/\log_{10}$ Fold increase per cycle (http://tools.invitrogen.com/).

6. Real-time quantitative PCR (RT-qPCR)

Finally, cDNA was amplified with gene-specific primers by using "Light Cycler® 480 SYBR Green I Master kit" and "LightCycler ® 480 Real-Time PCR System" from Roche Life Science. RT-qPCR was done for four basic cytokines (inflammatory and anti-inflammatory) involved in inflammatory response *(IL-1β, IL-6, IL-10,* and *TNF-α)*, for the genes involved in chondrogenesis i.e. chondrogenic markers such as collagen *(Col1α1, Col2α1, Col10α1),* aggrecan *(Acan),* alkaline phosphatase *(Alp)* and *Sox9* (transcription factor involved in chondrogenesis) and genes involved in osteogenesis i.e. osteogenic markers such as, *Alp, Runx2* (Runt related transcription factor), *BSP* (bone sialoprotein), *OCN* (osteocalcin) *Osx* (osterix), collagen *(Col1α1, Col10α1).*

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7. Relative quantification

All these genes involved in the pathogenesis of OA were compared for their expression in different experimental groups of respective cell lines by using Light Cycler ® 480 Real-Time PCR System. Relative gene quantification was done by E-method/ advanced relative quantification which is highly accurate and employs standard curves for gene quantification, therefore, the first step towards gene quantification was the construction of standard curves for each gene using different standard dilutions of cDNA.

8. Gel electrophoresis & PCR product sequencing

primer pairs specificity was confirmed by agarose gel electrophoresis and sequencing of the PCR products.

A) Gel electrophoresis

Gel electrophoresis was done to separate products based on their size while moving in the direction of the electric field. 2% agarose gel was used for this purpose.

Forming the gel

2% agarose gel was prepared by dissolving agarose in TAE buffer (Tris acetate-EDTA) with a ratio of 4g to 200ml, the mixture was shaken well and heated until the solution becomes transparent. Then the solution was allowed to cool at room temperature and ethidium bromide (Et-Br) was added at a concentration of 0.5µg/ml to the slightly warm

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solution, again shaken thoroughly, and poured into gel casting tray to be solidified properly. The casting tray had combs of proper size fixed at one side for the formation of wells. After the solidification tray was placed in an electrophoresis tank having the gel being submerged in the same buffer in which the gel was formed i.e. TAE. Combs are carefully removed without damaging the wells.

Procedure

PCR products (10µl) including *GADPH* were loaded into the well along with xylene, bromophenol blue mixture (10µl) and allowed to run on the gel together with 5µl of 1000 bp ladder for about 30-40 min at 90 volts, this is enough time to separate the products. Bands were then made visible due to ethidium bromide staining which is an intercalator and insert itself between the base pairs of nucleic acids and made visible by absorbing UV at 300-360nm when seen in the gel documentation system. Gel images were captured at super-resolution in the gel doc system and saved for further analysis.

9 Cell staining studies

Cell staining studies were performed to assess other biochemical markers related to bone and cartilage cell function.

Experiment design for ATDC5 cell staining

For cell staining studies of ATDC5, cells were seeded at a concentration of 30,000 cells/2ml of the medium in a 12-welled multiwell plate and let to be confluent for about

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2-4 days. Once confluent, the old medium was replaced with fresh medium in such a way that half of the wells were receiving medium only (DMEM: Ham's F12 (1:1) + 5% Foetal Bovine Serum (FBS). 1% PSG (penicillin, streptomycin, and L-glutamine)) and half of the wells were receiving medium with AITS. On day 7 of differentiation different treatments such as, 100ng/ml LEP and 100ug/ml of extracts (GSM-A & GSM-B) were also added to both differentiating and undifferentiated cells in such a way that each well of the plate corresponded to a different treatment group. Treatment was done for 14 days accompanied by a change of medium twice a week. Both RNA extraction and staining assays were performed on day 14 of differentiation. Following Cell staining studies were done for ATDC5 cells:

1. Sirius red staining

This technique is used to measure collagen production by chondrocytes. Sirius red dye stains the collagen in the cell cultures, which can then be measured spectrophotometrically after dissolving the dye in an appropriate solvent. Sirius red/fast green collagen staining kit (Chondrex, Inc) was used for Sirius red staining according to the manufacturer's instructions:

Material required

Phosphate buffered saline

Formaldehyde, 2%

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Sirius Red staining kit

NaOH, 0.1 N

Method

1. The medium was removed from the wells.
2. PBS, 500 microliters per well was added twice to wash the cells.
3. 500 microliters of 2% formaldehyde was added to each well.
4. Incubated at room temperature overnight to fix the cells.
5. Formaldehyde was removed (rinsed down the sink with copious amounts of water to dilute the formaldehyde).
6. Plates were rinsed with water by dunking them into a container of water, then dumping the water out.
7. Staining was done with Sirius Red as per the manufacturer's instructions.
   Washed the plates three times with PBS as in Step 2.
8. Photographed for staining images
9. Added 500 microliters of 0.1N NaOH to each well to solubilise the stain.
10. Allowed the plates to sit for ten minutes.
11. Optical density was read at 550 nanometers, ensuring the wells have been well mixed (manually or by plate shaking option on the reader).

2. Alcian blue staining

Alcian blue stain was used to assess proteoglycan production in treated vs untreated chondrocytes (ATDC5 cells) in a manner similar to Sirius red staining. Alcian blue dye

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(Sigma Aldrich) was used for staining. Reagents used and the protocol followed is as under:

Material required

Phosphate buffered saline

Methanol

Alcian Blue stain, 0.1% in water

Guanidine HCl, 6M

Method

1. The medium was removed from the wells.
2. PBS, 500 microliters per well was added twice to wash the cells.
3. 500 microliters of Methanol was added to each well.
4. Incubated at -20°C for twenty minutes fix the cells.
5. Methanol was removed (rinsed down the sink with copious amounts of water to dilute the Methanol).
6. Plates were rinsed with water by dunking them into a container of water, then dumping the water out and plates were air-dried.
7. Staining was done with 500 ul of 0.1% alcian blue dye in each well overnight.
8. Washed the plates three times with PBS as in Step 2.
9. Photographed for staining images.

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10. 500 microliters of Guanidine HCl, 6M was added to each well to solubilise the stain.
11. Plates were allowed to sit overnight.
12. Optical density was read at 650 nanometres, ensuring the wells have been well mixed (manually or by plate shaking option on the reader).

3. Alizarin Red Staining.

Mineralization studies were performed on chondrocytes as well as Mc3t3-E1 subclone-4 cells, using alizarin red staining, Assay details are described below:

Material required

Phosphate buffered salin

Ice cold 100% Ethanol

Alizarin Red stain (Sigma Aldrich) ,0.1% in water, pH 5.5

Cetylpyridinium chloride (CPC) (Sigma Aldrich), 10% in water

Method

1. The medium was removed from the wells.
2. PBS, 500 microliters per well was added twice to wash the cells
3. 500 microliters of ice-cold 100% ethanol was added to each well.
4. Incubated at room temperature for one hour to fix the cells.

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5. Ethanol was removed (rinsed down the sink with copious amounts of water to dilute the ethanol).
6. Plates were rinsed with water by dunking them into a container of water, then dumping the water out and plates were air-dried.
7. Staining was done with 500 ul of 0.1% alizarin red blue dye in each well and allowed to sit overnight.
8. Washed the plates three times with PBS as in Step 2.
9. Photographed for staining images
10. 500 microliters of CPC was added to each well to solubilise the stain.
11. Plates were allowed to sit overnight.
12. Optical density was read at 550 nanometres, ensuring the wells have been well mixed (manually or by plate shaking option on the reader).

Experiment design for MC3T3-E1 cell staining

For cell staining studies of MC3T3-E1, cells were seeded at a concentration of 20,000 cells/ml of the medium in a 24-welled multiwell plate and let to be 80% confluent for about 2-4 days. Once confluent, the old medium was replaced with fresh medium in such a way that half of the wells were receiving medium only α- MEM + 10% FBS supplemented with 0.27% gentamicin and half of the wells were receiving medium with differentiating factors i.e. ascorbic acid (AA, 50 ug/ml) and β-glycerophosphate (βGP, 10mM). On day 14 of differentiation, different treatments such as, 100ng/ml LEP and 100ug/ml of extracts (GSM-A & GSM-B) were also added to both differentiating and undifferentiated cells in such a way that each treatment group had duplicate wells.

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Treatment was done for 21 days accompanied by a change of medium twice a week. Both RNA extraction and staining assays were performed on day 21 of differentiation, while ALP assay was done on day 14 of differentiation. For ALP assay both differentiation and addition of other treatments were started together on the day cells became confluent.

Alkaline phosphatase activity (ALP) Assay

ALP activity is considered an early marker of mineralization in bone and cartilage cells. ALP activity assay is a colorimetric assay based on the principle that ALP in the test samples catalyses the conversion of colourless phosphate substrate "p-nitrophenyl phosphate (*p*NPP)" into the yellow-coloured product "p-nitrophenol", which has different colour intensity depending on the activity of ALP. The more the activity is, the intensity is the colour of the product measured by a spectrophotometer at 405nm.

TRACP & ALP Assay Kit (Takara) protocol was followed for the assay. The same treatment protocol was followed as that for staining and RNA extraction. Treatment continued fo

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14 days both for ATDC5 and MC3T3 cells after which ALP assay was performed. The summary of the protocol is demonstrated in figure 2.2:

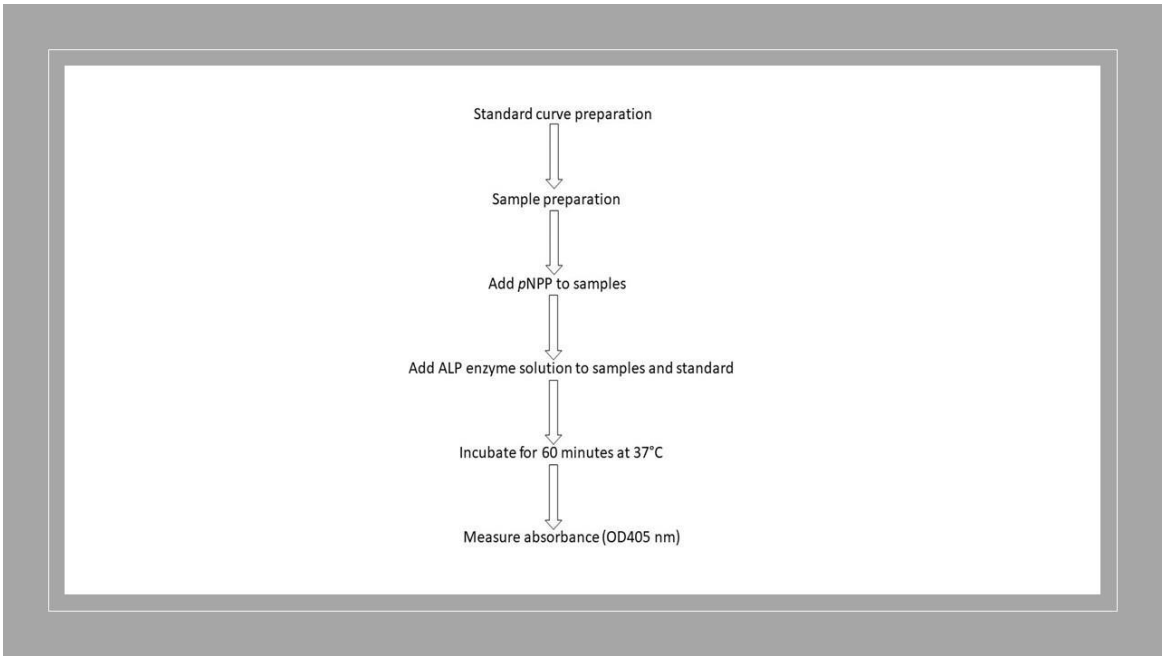

Figure 2.2: Summary of ALP assay Protocol

Standard curve preparation

A standard curve is a pre-requisite for ALP assay. A standard curve is calculated with different dilutions of 4-nitrophenol, which is produced as a yellow coloured product as a result of ALP activity in the test samples.

Material required

ALP assay buffer (400 mM Tris-HCl pH 9.5, 1 mM $MgCl_2$)4-nitrophenol

NaOH, 1N (stop solution)

Method

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1. First, 13.9 mg of 4-nitrophenol was dissolved in 10 ml of ALP buffer to make 10 mM solution.
2. 10 mM solution was diluted 10 times to make 1 mM stock solution, which was used to make further dilutions for standard curve calculation.
3. The scheme of dilution is given in figures 2.3 a & b. 50 ul of the standard dilutions were made in 96 well plates and incubated at 37°C for 60 minutes.
4. 50 ul of stop solution was added after 60 minutes and absorbance was recorded at 405 nm by using a spectrophotometer (Multiskan Fc, Thermo Scientific SkanIt Software).
5. Finally, standard concentration was plotted against OD values to get a standard curve using Ms excel as shown in figures 2.3 c & d.
6. This standard curve with a known concentration of product (4-nitrophenol) was used as a reference to calculate the concentration of 4-nitrophenol in test samples.
7. Linear fitted trend line was drawn to show a relationship between absorbance (OD) and concentration and was used to calculate unknown concentration of test samples by the formula; $y = ax+b$ or $x = y-b/a$ (where y is OD value of test sample,
8. x is the unknown concentration of test sample a and b are the values on the trend line e.g. a=3.5043 and b=0.0458 in 2.3c graph).

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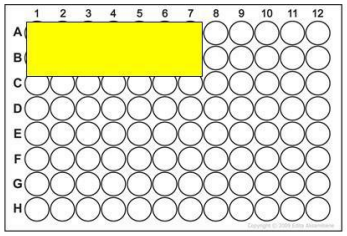
a



c

Standard 4-nitrophenol		
Dilution	Concentration (mM)	Absorbance
1	1	3.4835
2	0.75	2.7195
3	0.5	1.8765
4	0.4	1.4505
5	0.2	0.7285
6	0.1	0.354
7	0	0.046

b

Dilution	Concentration mM(50ul)	1mM 4-nitrophenol stock(ul)	ALP Buffer (uL)
1	1	50	0
2	0.75	37.5	12.5
3	0.5	25	25
4	0.4	20	30
5	0.2	10	40
6	0.1	5	45
7	0	0	50

d

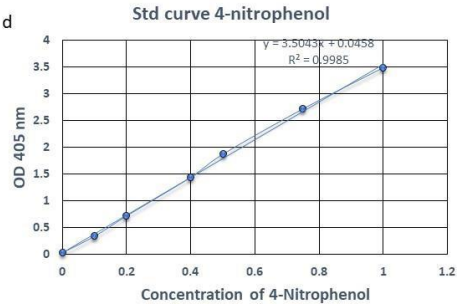


Figure 2.3: a) Dilution duplicates in 96 well plate, b) Scheme of dilutions, c) absorbance at 405 nm and d) scatter chart with line and markers to represent relationship between concentration and absorbance (OD) at 405 nm.

Sample preparation/Harvesting the cells

Material required

Extraction buffer (Extraction TBS (1% Triton-X100 in 1 x TBS)), pre-warmed at 37°C.

0.9% NaCl pre-warmed at 37°C.

Method

The steps of cell harvesting are as follows:

1. First, cells were washed with 1ml of 0.9% NaCl.
2. 500ul of extraction buffer was added to each well for lysis.
3. Incubated on an orbital shaker for 15 minutes at room temperature.

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4. Well contents were transferred in a 1.5 ml tube.
5. Incubated on ice for 15 minutes with brief vertexing after every 5 minutes.
6. The lysate was centrifuged at 13000 rpm for 5 minutes and transferred to a new 1.7ml tube on ice
7. Stored at -80^{O}C for ALP activity.
8. ALP Assay (Addition of pNPP to the samples-measuring OD at 405)

Material required

ALP assay buffer (400 mM Tris-HCl pH 9.5, 1 mM $MgCl_2$.

pNPP solution (10 mM pNPP in ALP Assay Buffer) on ice

NaOH, 1N (stop solution)

Method

1. ALP activity was performed within 1-5 days of cell harvesting.
2. Samples were retrieved from -80^{O}C and thawed immediately at 37^{O}C.
3. 50ul of sample lysate and TBS solution as a blank (in triplicate) was added in 96 well plates.
4. 50ul of pNPP substrate solution was added to each sample.
5. Incubated the plate at 37^{O}C for 60 minutes (15-60min).
6. Added 50ul of stop solution after 60 minutes.

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7. The plate was read at 405nm two times (read at 405nm then shake and again read at 405nm) and OD values were documented.

Measuring the concentration of 4-nitrophenol

1. Measuring the concentration of 4-nitrophenol (ALP product) is the first step towards the calculation of ALP activity. The concentration of 4-nitrophenol in the test samples was measured by using the 4-nitrophenol standard curve.
2. The concentration of product in test samples was calculated by the formula.
3. y = ax+b or x = y-b/a (where y is OD value of test sample, x is the unknown concentration of test sample a, and b are the values on the trend line e.g. a=3.5043 and b=0.0458 in 3.13c graph).
4. In the excel worksheet, the concentration of test sample (x) was calculated by the following steps:
5. First, the cell, where we want to insert the concentration of the test sample, was selected e.g. C12 in figure 3.14, followed by entering the "=" sign.
6. typed the word "trend"
7. . Next, all the cells having OD values of standards(a) i.e. B3:B9 were selected.
8. Followed by entering comma (,) sign.
9. Cells having a concentration of standards (b) i.e. C3:C9 were selected next
10. Followed by entering comma (,) sign.
11. Finally, the OD value of test sample (y) was entered and pressed the enter key to
12.
13. show the result. The final equation or the formula in the formula bar was looked like;

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14. =trend (B3:B9, C3:C9, B12).

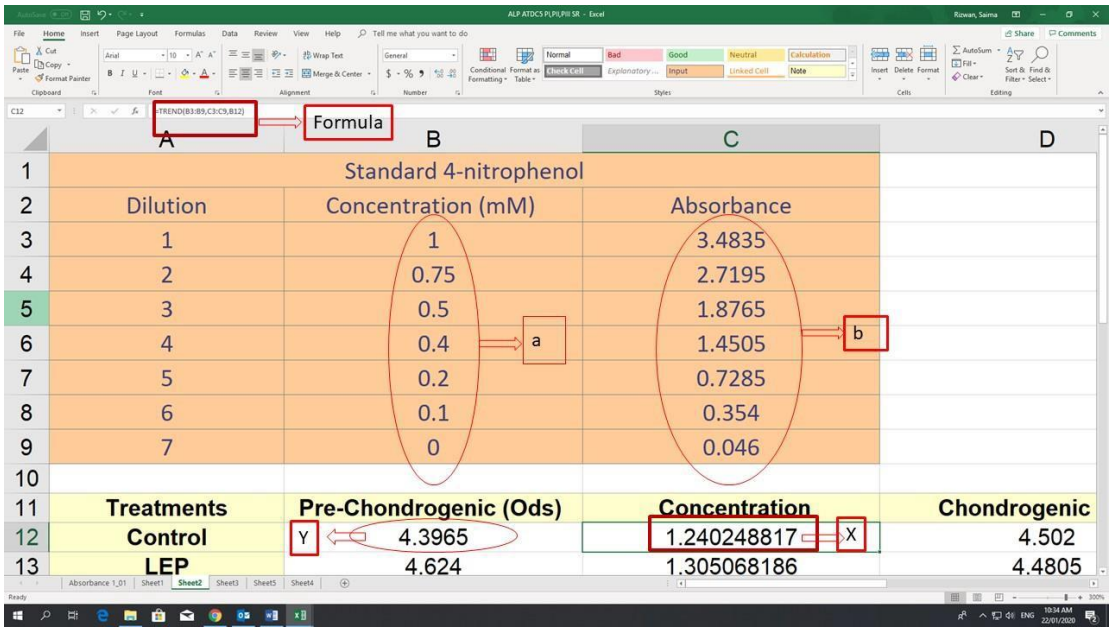

Figure 2.4: Calculation of ALP product (4-nitrophenol) concentration in excel worksheet

Measuring the ALP activity

Enzyme activity (EA) "ALP activity" can be defined as, the rate at which ALP catalyses the production of 4-nitrophenol in a given volume of sample. The formula can be written as,

ALP activity (U/ml) = A/V/T

Where U/ml is the unit for measurement of EA,

A is the amount of product in micromoles,

V is the volume of the sample in ml &T is the time of incubation of sample for product formation.

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ALP activity was calculated from the concentration of 4-nitrophenol in test samples by using the above-mentioned equation.

Normalization of the ALP activity

ALP activity was normalized to total protein content of the test samples to eliminate the possibility of error in the results. To get an accurate measurement and to eliminate the probability that, more product concentration in the test samples was due to more cell number and not due to more enzyme activity was eliminated by normalization i.e. by dividing EA to total protein concentration (mg/ml) of the test samples. Normalized ALP activity can also be defined as specific ALP activity

The formula can be written as,

Specific ALP activity (U/mg) = A/V/T/Total proteins (mg/ml)

Formulas for calculation of ALP activity and Specific ALP activity are highlighted in figure 2.5

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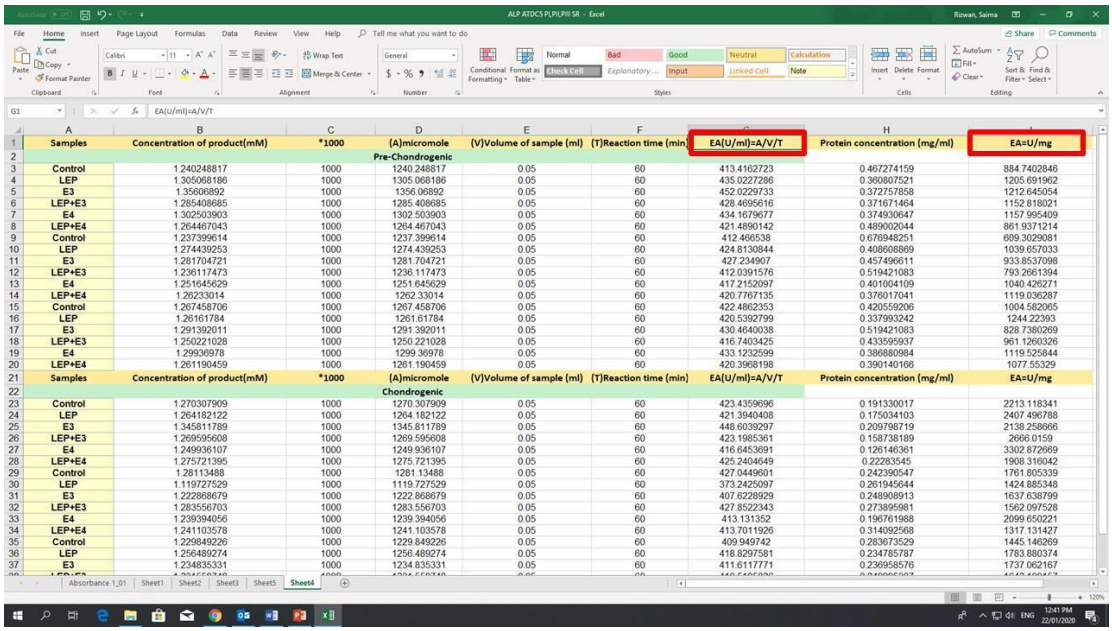

Samples	Concentration of product(mM)	*1000	(A)micromole	(V)Volume of sample (ml)	(T)Reaction time (min)	EA(U/ml)=A/V/T	Protein concentration (mg/ml)	EA=U/mg
			Pre-Chondrogenic					
Control	1.240248817	1000	1240.248817	0.05	60	413.4162723	0.467274159	884.7402846
LEP	1.305068186	1000	1305.068186	0.05	60	435.0227286	0.360807521	1205.691962
E3	1.35606892	1000	1356.06892	0.05	60	452.0229733	0.372757858	1212.645054
LEP+E3	1.285408685	1000	1285.408685	0.05	60	428.4695616	0.371671464	1152.818021
E4	1.302503903	1000	1302.503903	0.05	60	434.1679677	0.374930647	1157.995409
LEP+E4	1.264467043	1000	1264.467043	0.05	60	421.4890142	0.489002044	861.9371214
Control	1.237399614	1000	1237.399614	0.05	60	412.466538	0.676948251	609.3029081
LEP	1.274439253	1000	1274.439253	0.05	60	424.8130844	0.408608869	1039.657033
E3	1.281704721	1000	1281.704721	0.05	60	427.234907	0.457496611	933.8537098
LEP+E3	1.236117473	1000	1236.117473	0.05	60	412.0391576	0.519421083	793.2661394
E4	1.251645629	1000	1251.645629	0.05	60	417.2152097	0.401004109	1040.426271
LEP+E4	1.26233014	1000	1262.33014	0.05	60	420.7767135	0.376017041	1119.036287
Control	1.267458706	1000	1267.458706	0.05	60	422.4862353	0.420559206	1004.582065
LEP	1.26161784	1000	1261.61784	0.05	60	420.5392799	0.337993242	1244.22393
E3	1.291392011	1000	1291.392011	0.05	60	430.4640038	0.519421083	828.7380269
LEP+E3	1.250221028	1000	1250.221028	0.05	60	416.7403425	0.433595937	961.1260326
E4	1.29936978	1000	1299.36978	0.05	60	433.1232599	0.386880984	1119.525844
LEP+E4	1.261190459	1000	1261.190459	0.05	60	420.3968198	0.390140166	1077.55329
Samples	Concentration of product(mM)	*1000	(A)micromole	(V)Volume of sample (ml)	(T)Reaction time (min)	EA(U/ml)=A/V/T	Protein concentration (mg/ml)	EA=U/mg
			Chondrogenic					
Control	1.270307909	1000	1270.307909	0.05	60	423.4359696	0.191330017	2213.118341
LEP	1.264182122	1000	1264.182122	0.05	60	421.3940408	0.175034103	2407.496788
E3	1.345811789	1000	1345.811789	0.05	60	448.6039297	0.209798719	2138.258666
LEP+E3	1.269595608	1000	1269.595608	0.05	60	423.1985361	0.158738189	2666.0159
E4	1.249936107	1000	1249.936107	0.05	60	416.6453691	0.126146361	3302.872669
LEP+E4	1.275721395	1000	1275.721395	0.05	60	425.2404649	0.22283545	1908.316042
Control	1.28113488	1000	1281.13488	0.05	60	427.0449601	0.242390547	1761.805339
LEP	1.119727529	1000	1119.727529	0.05	60	373.2425097	0.261945644	1424.885348
E3	1.222868679	1000	1222.868679	0.05	60	407.6228929	0.248908913	1637.638799
LEP+E3	1.283556703	1000	1283.556703	0.05	60	427.8522343	0.273895981	1562.097528
E4	1.239394056	1000	1239.394056	0.05	60	413.131352	0.196761988	2099.650221
LEP+E4	1.241103578	1000	1241.103578	0.05	60	413.7011926	0.314092568	1317.131427
Control	1.229849226	1000	1229.849226	0.05	60	409.949742	0.283673529	1445.146269
LEP	1.256489274	1000	1256.489274	0.05	60	418.8297581	0.234785787	1783.880374
E3	1.234835331	1000	1234.835331	0.05	60	411.6117771	0.236958576	1737.062167

Figure 2.5: Calculation of ALP activity and ALP specific activity in excel worksheet

Total Protein quantification

The total protein content of the test samples was determined by protein assay, using Pierce™ BCA Protein Assay Kit (Thermofisher Scientific), according to the manufacturer's instructions. BCA protein assay uses bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method is based on the reduction of Cu+2 to Cu+1 by protein in an alkaline medium (the biuret reaction). cuprous cation (Cu+1) are sensitive to colorimetric detection by using a unique reagent containing bicinchoninic acid, which selectively detects (Cu+1). The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000µg/mL).

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The process followed for protein assay is quite similar to ALP assay.

Calculation of Standard curve

Calculation of standard curve is the first step in protein assay. protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration are prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown is determined based on the standard curve.

Material required:

ALP assay buffer (400 mM Tris-HCl pH 9.5, 1 mM $MgCl_2$)

BSA

BCA working reagent (BCA reagent A 50ml+ BCA reagent B 1ml i.e. 50:1, Reagent A: B)

Method

1. The first BCA working reagent is prepared from BCA reagents A and B, provided with the kit in the ratio of 50:1.
2. Next, 2mg/ml vial of BSA provided with the kit was diluted to half (1mg/ml) with ALP buffer and was used as a stock to make series of dilutions with known protein concentration.
3. The scheme of dilution is given in Figures 2.6 a & b.

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4. 100 ul of the standard BSA dilutions were made in duplicates in 96 well plates.
5. 200 ul of the working solution was added to each well. and incubated at 37°C for 30 minutes.
6. Absorbance was recorded at 550 nm by using a spectrophotometer (Multiskan Fc, Thermo Scientific SkanIt Software).
7. Finally, standard concentration was plotted against OD values to get a standard curve using Ms excel as shown in figure 2.6c & d.
8. This standard curve with known BSA concentration was used as a reference to calculate the concentration of proteins in the test samples.
9. Linear fitted trend line was drawn to show a relationship between absorbance (OD) and concentration and was used to calculate unknown concentration of test samples by the formula; $y = ax+b$ or $x = y-b/a$ (where y is OD value of test sample, x is the unknown concentration of test sample a and b are the values on the trend line e.g. a=32.298 and b=0.273 in 2.6d graph).

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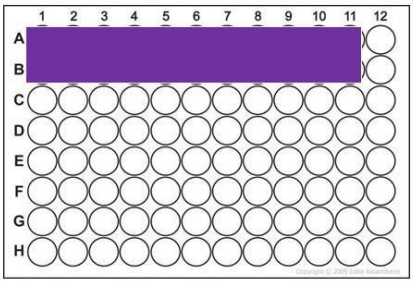
a



b

Dilutions	Concentrations (mg/ml)	BSA Volume(ul)	ALP buffer Volume (ul)
1	0.1	100	0
2	0.09	90	10
3	0.08	80	20
4	0.07	70	30
5	0.06	60	40
6	0.05	50	50
7	0.04	40	60
8	0.03	30	70
9	0.02	20	80
10	0.01	10	90
11	0	0	100

c

Dilutions	BSA Concentrations (mg/ml)	ODs(550nm)
1	0.1	3.3805
2	0.09	3.1945
3	0.08	2.864
4	0.07	2.5495
5	0.06	2.26
6	0.05	1.8905
7	0.04	1.59
8	0.03	1.374
9	0.02	0.9745
10	0.01	0.605
11	0	0.0845

d

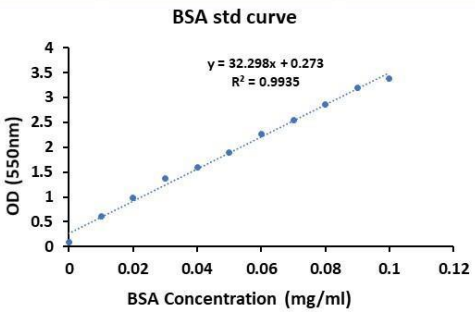


Figure 2.6: a) Dilution duplicates in 96 well plate, b) Scheme of dilutions, c) absorbance at 550 nm and d) scatter chart with line and markers to represent a relationship between concentration and absorbance (OD) at 550 nm.

Protein Assay (Addition of BCA working reagent to the samples-measuring OD at 550nm)

Material required

Frozen test samples

BCA working reagent

Method

1. Protein assay was performed alongside ALP assay.
2. Samples were retrieved from -80^{O}C and thawed immediately at 37^{O}C.

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3. 10ul of sample lysate and TBS solution as a blank (in triplicate) was added in 96 well plate.
4. 200ul of BCA working solution was added to each sample.
5. Incubated the plates at 37^{O}C for 30 minutes.
6. Plate was read at 550nm two times (read at 550nm then shake and again read at 550nm) and OD values were documented.
7. Measuring the concentration of proteins in test samples
8. The concentration of proteins in the test samples was measured by using the BSA standard curve (figure 2.6d).
9. The concentration of product in test samples was calculated by the formula;
10. y = ax+b or x = y-b/a (where y is OD value of test sample, x is the unknown concentration of test sample a, and b are the values on the trend line e.g. a=32.298 and b=0.273 in 2.6d graph).
11. In the Excel worksheet, the concentration of the test sample (x) was calculated by the same procedure we followed for 4-nitrophenol concentration.
12. Finally, ALP activity was normalized by dividing with a protein concentration of that sample to measure specific enzyme activity in that sample. The workflow is shown in figure 2.6.

3.3.6. Statistical Analysis

For multi, group comparison of dependent variables one-way ANOVA was used. Dependent variables include cytokines expression, cell viability, transcriptome comparison; mineralization, etc. software used for statistical analysis IBM SPSS Statistics

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for Windows, version 25 (IBM Corp., Armonk, N.Y., USA). Different groups were compared by one-way analysis of variance (ANOVA). $p<0.05$ was considered to indicate a statistically significant difference.

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Appendix B

Supplementary figures

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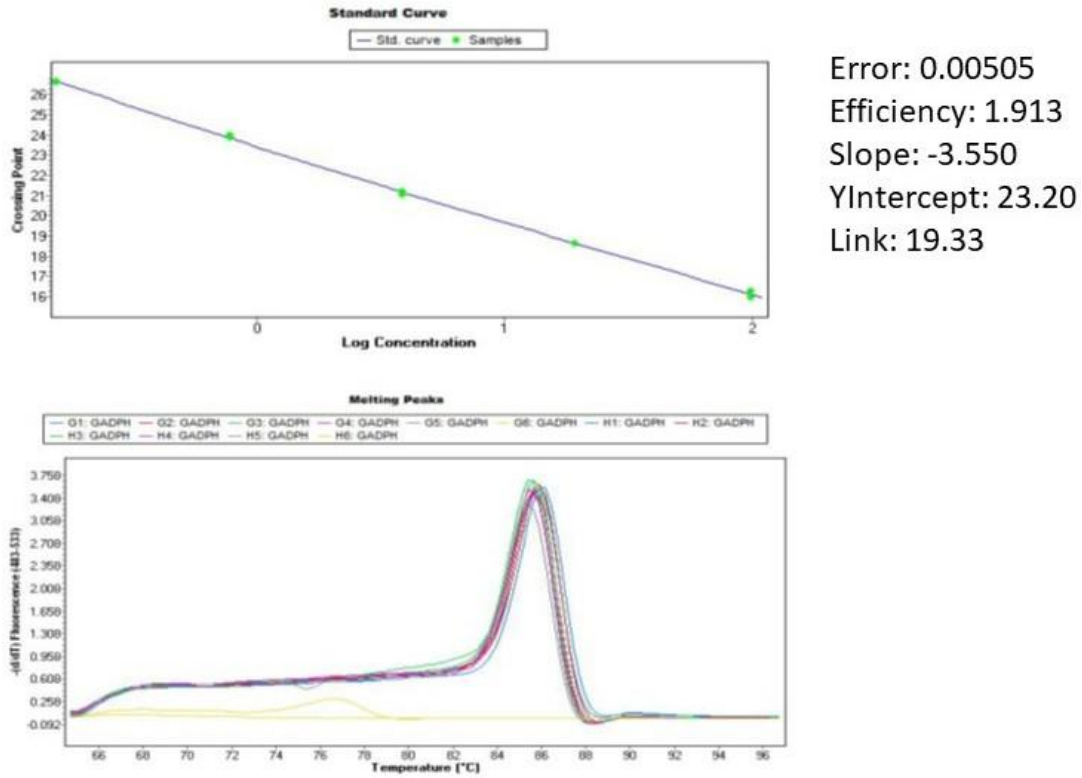

Figure 1A & B: Standard curve and melting curve for *GADPH*. five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,1/15……) to generate a standard curve and melting curve

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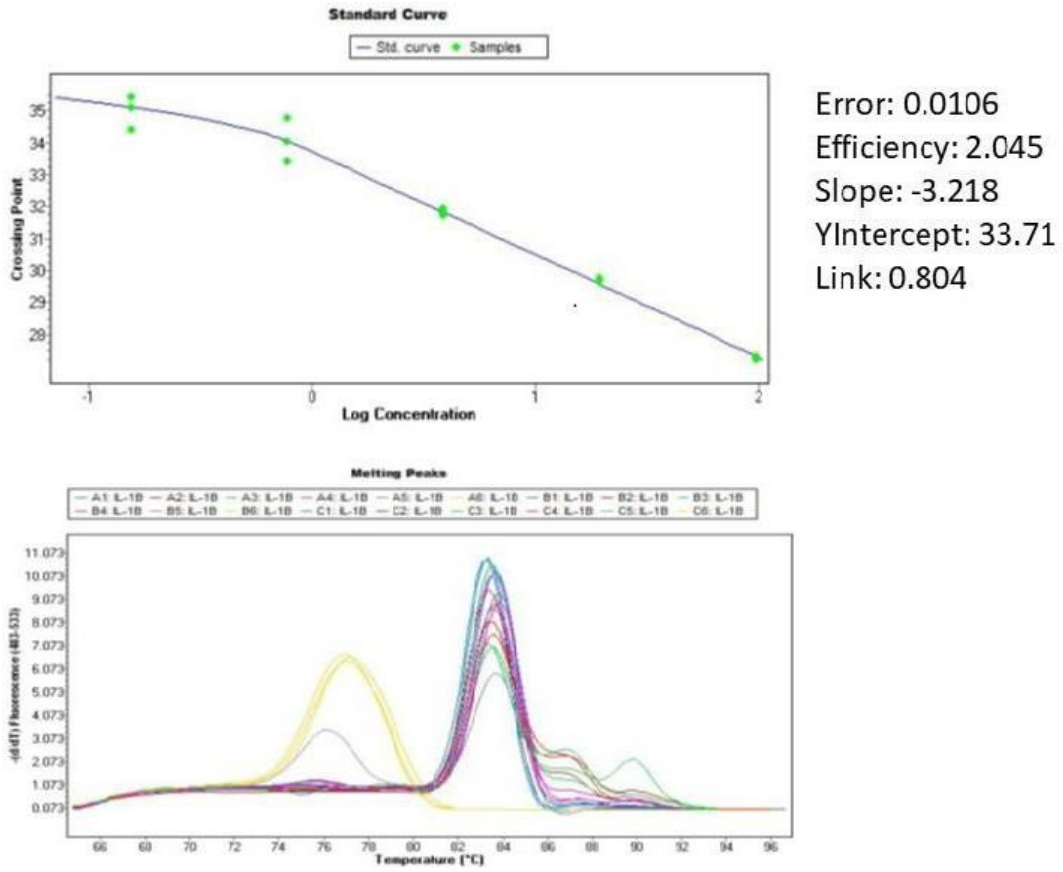

Figure 2A &B: Standard curve and melting curve for *IL-1β*. five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,1/15……) to generate a standard curve and melting curve.

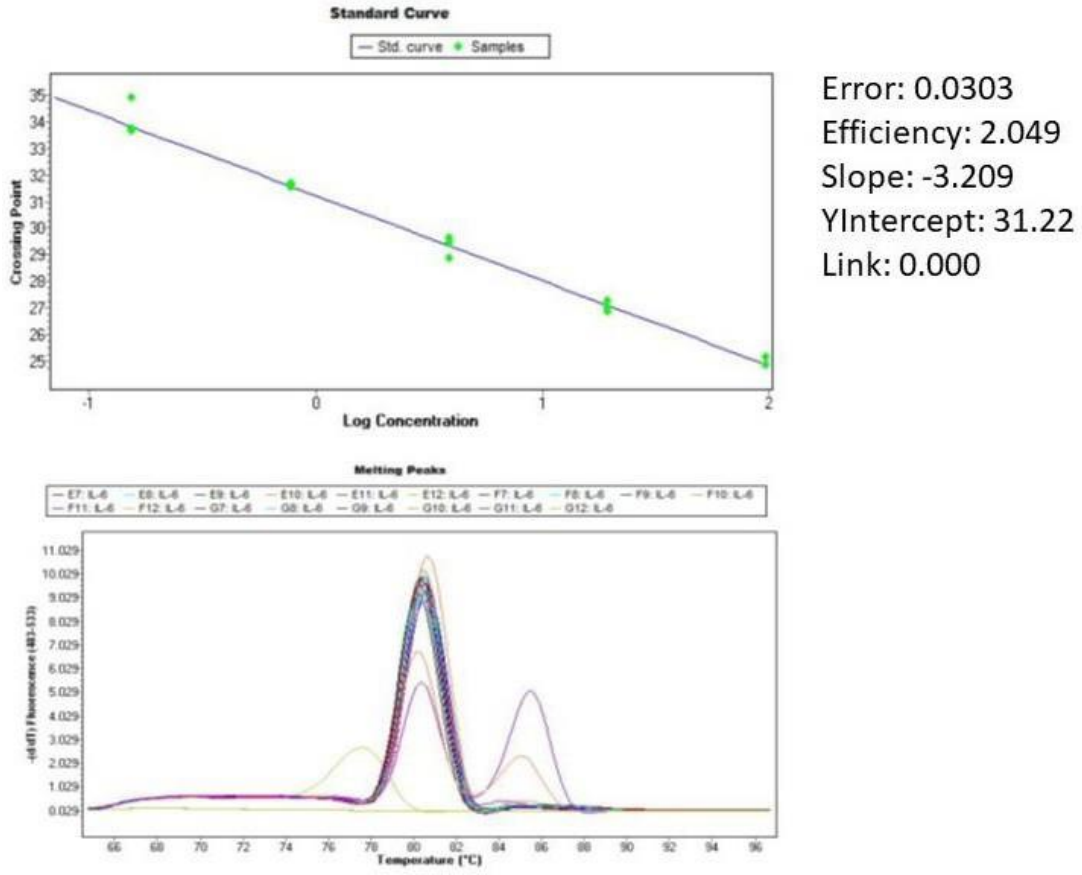

Figure3A & B: Standard curve and melting curve for *IL-6*. five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,1/15……) to generate a standard curve and melting curve.

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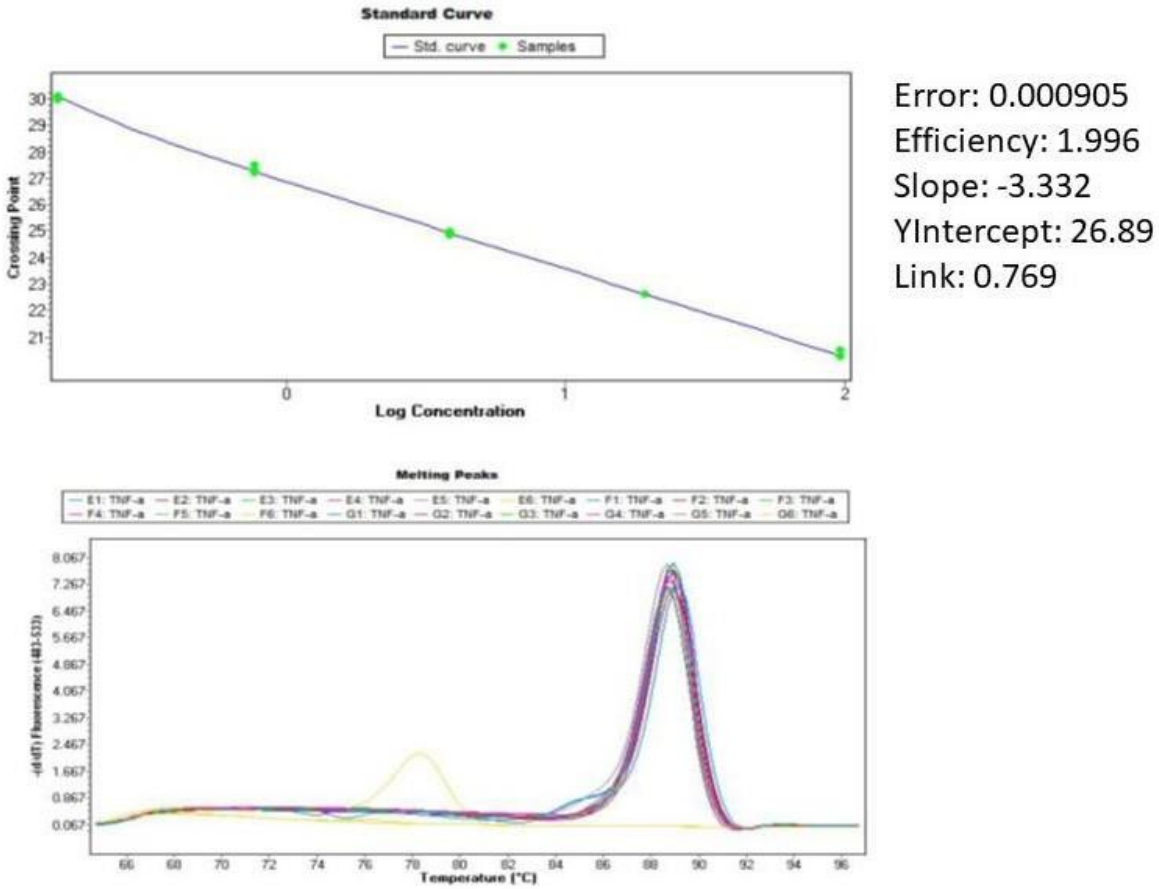

Figure 4A & B: Standard curve and melting curve for *TNF-α.* five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,1/15……) to generate a standard curve and melting curve.

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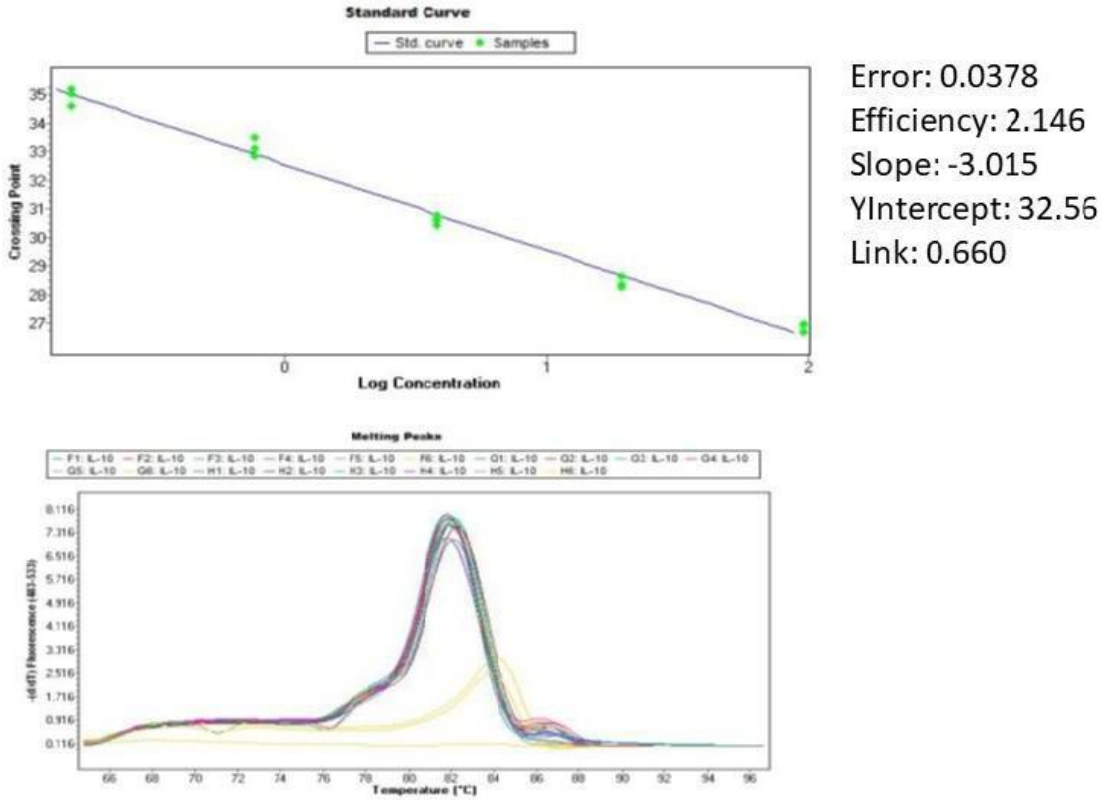

Figure 5A & B: Standard curve and melting curve for *IL-10.* five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

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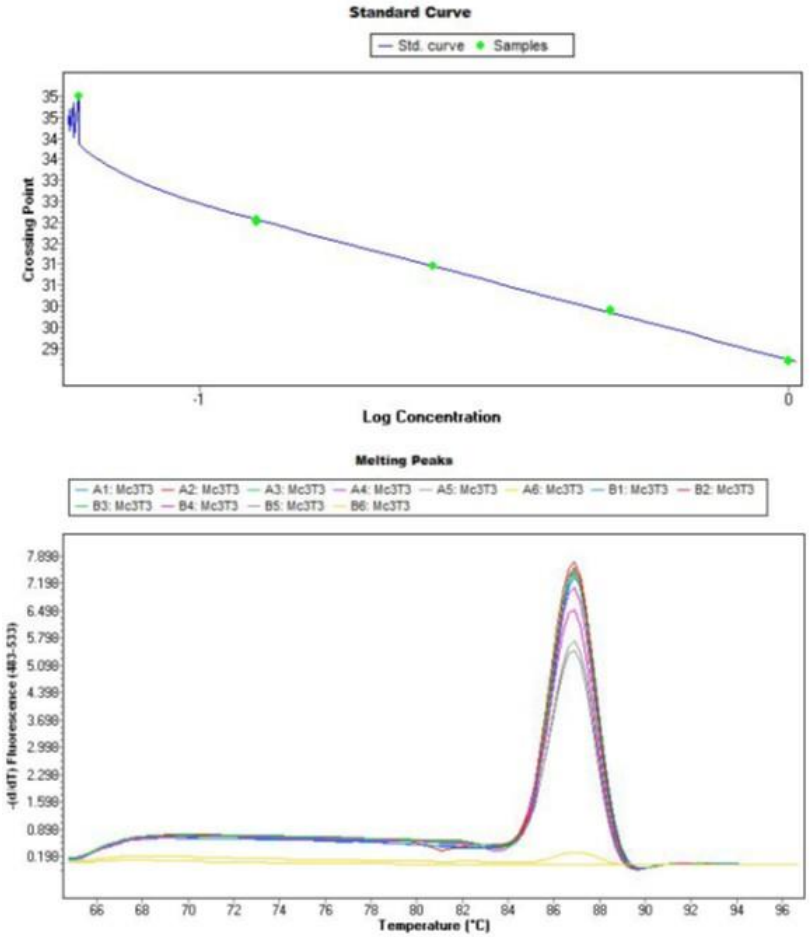

Figure 6A & B: Standard curve and melting curve for *Alp*. five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

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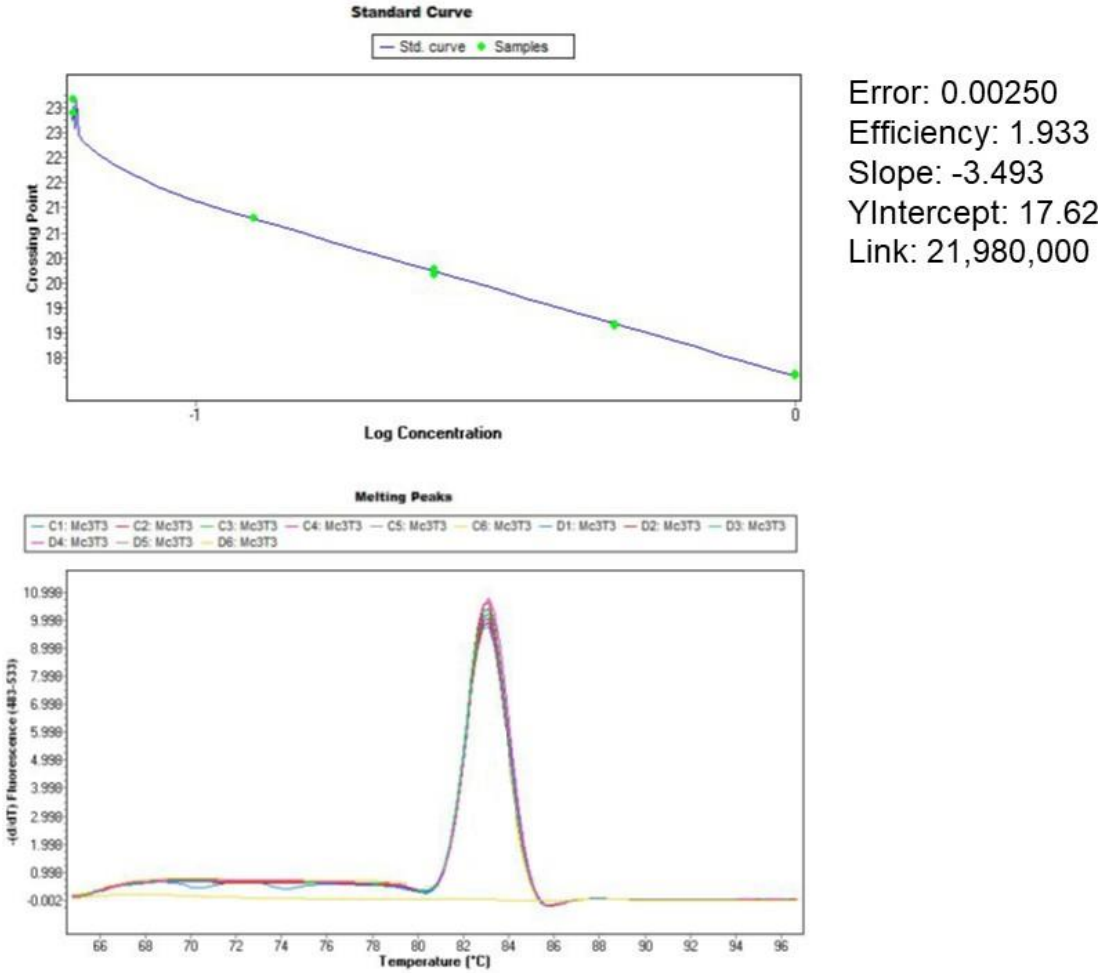

Figure 7A & B: Standard curve and melting curve for *BSP.* five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

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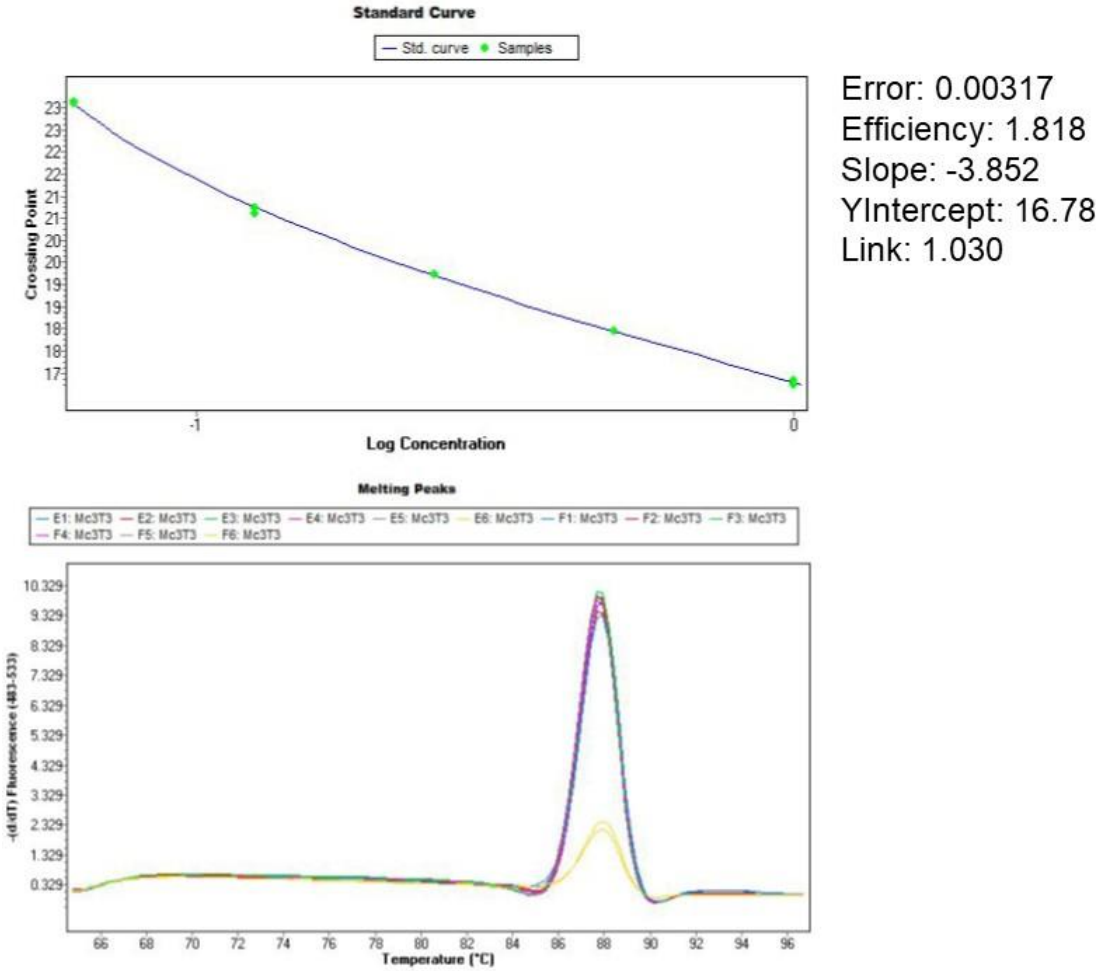

Figure 8A & B: Standard curve and melting curve for *OCN.* five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

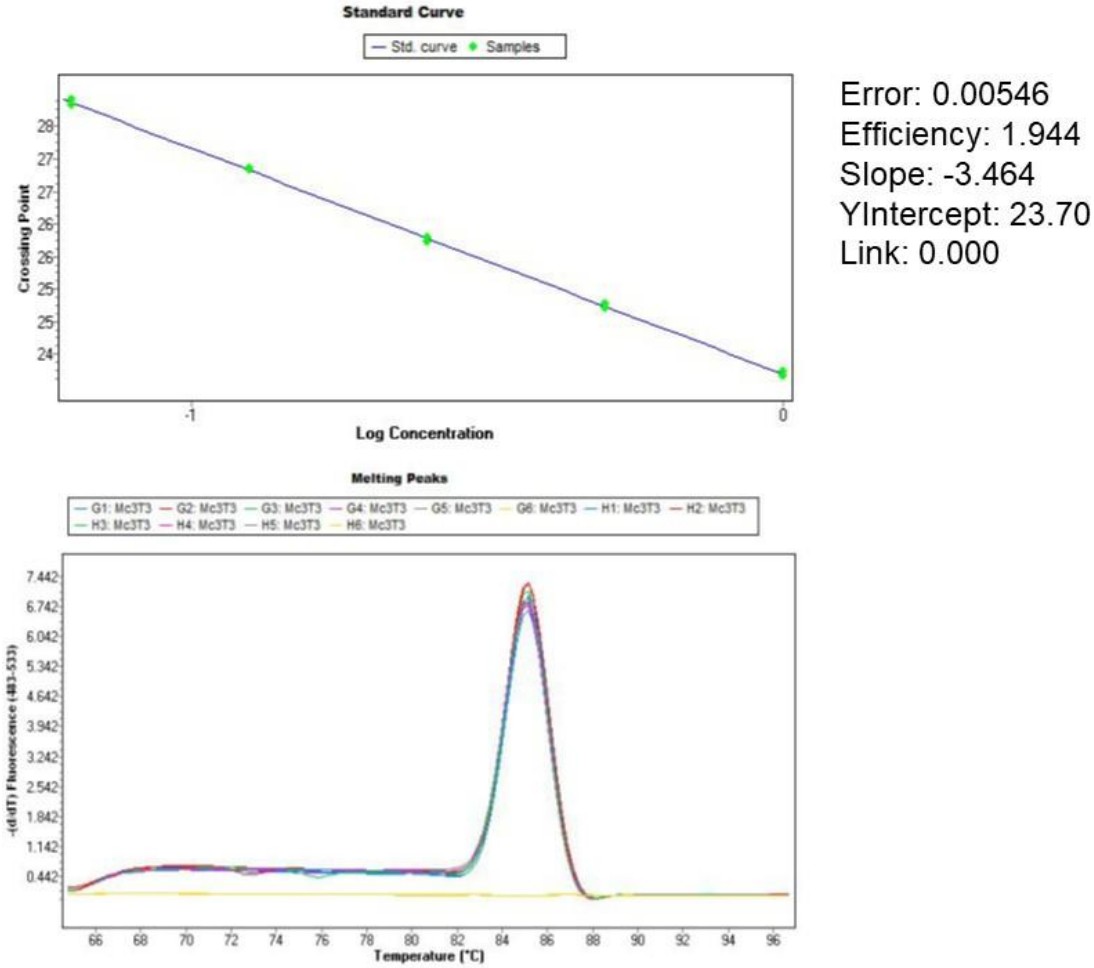

Figure 9A & B: Standard curve and melting curve for *Osx*. five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

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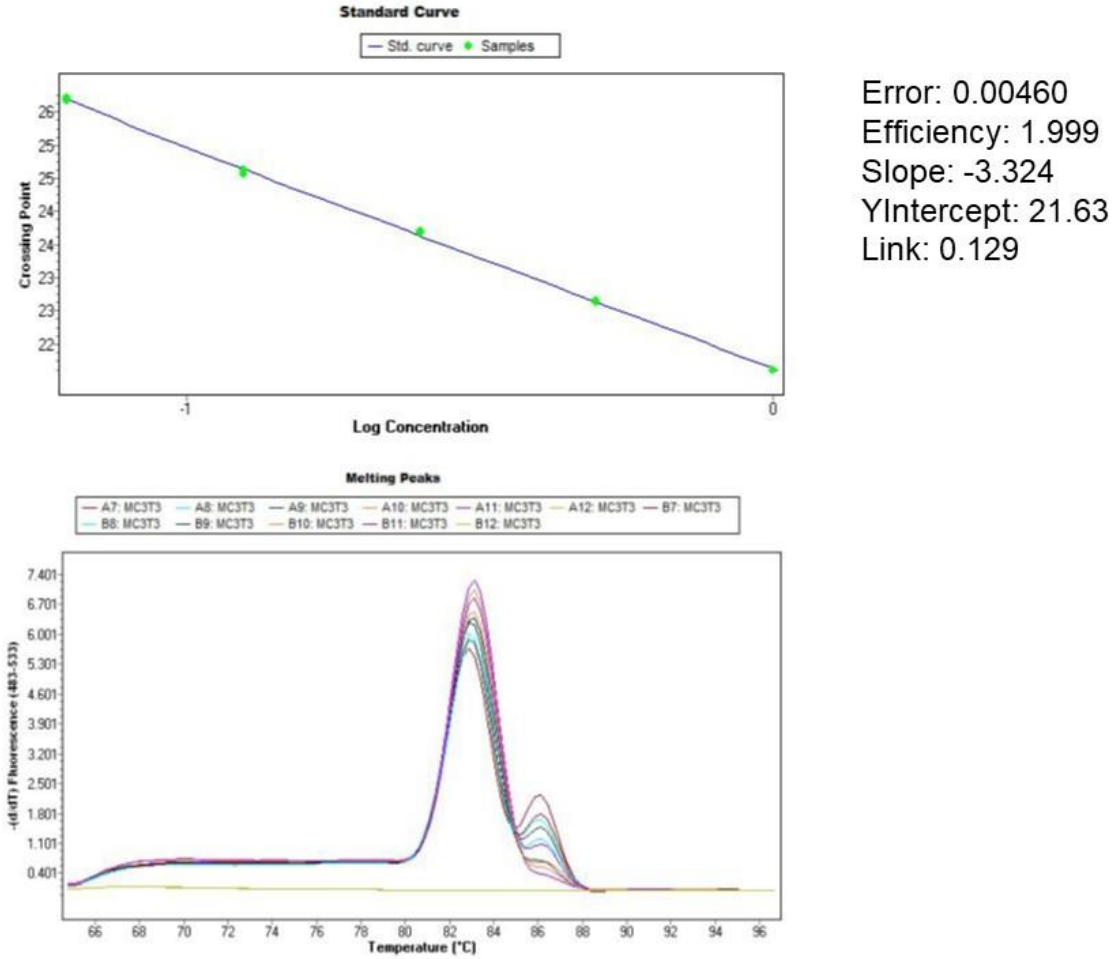

Figure 10A & B: Standard curve and melting curve for *Runx2*. five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

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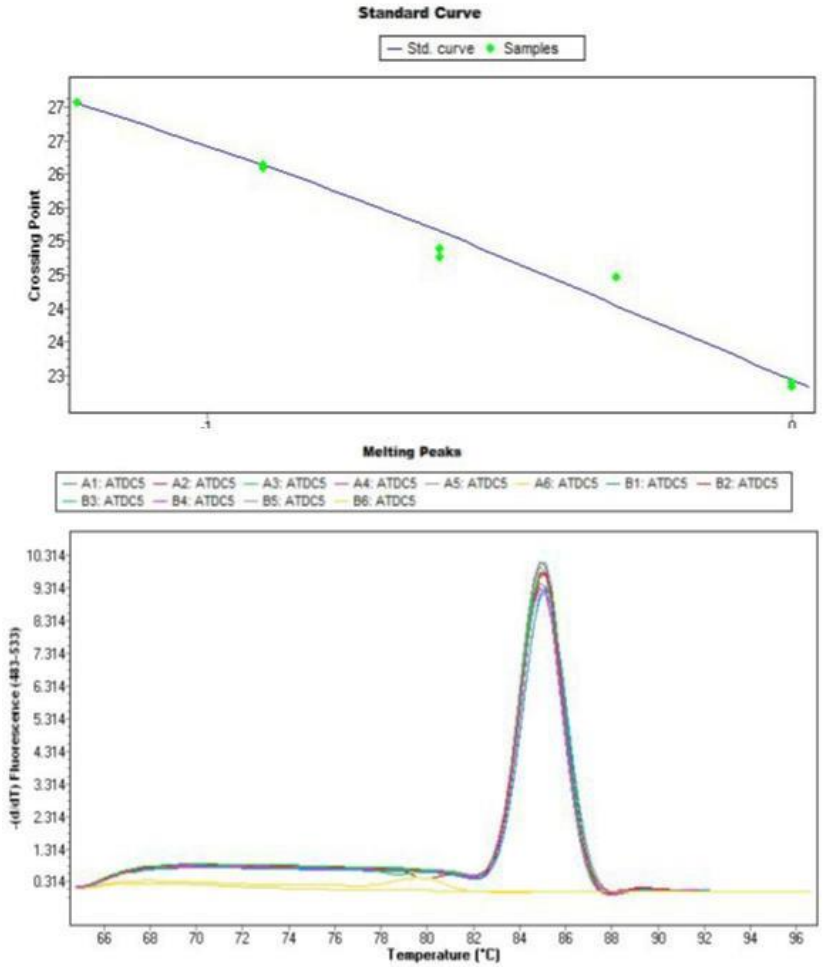

Figure 11A & B: Standard curve and melting curve for *Col1α1*. five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

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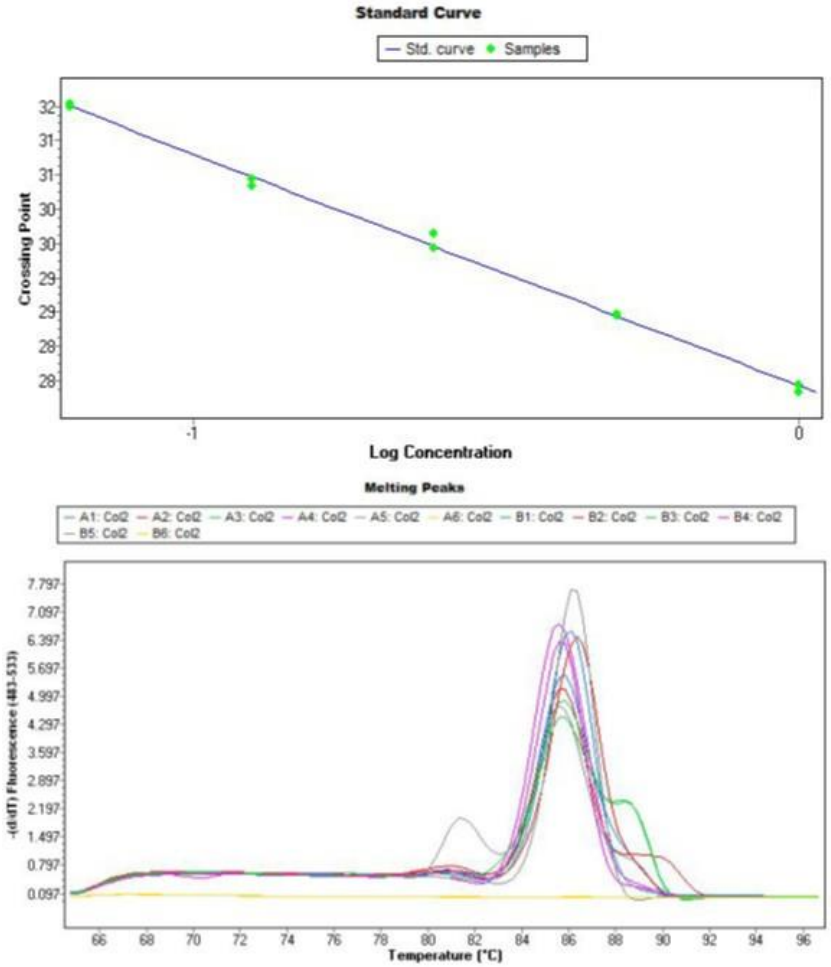

Figure 12A & B: Standard curve and melting curve for *Col2α1.* five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

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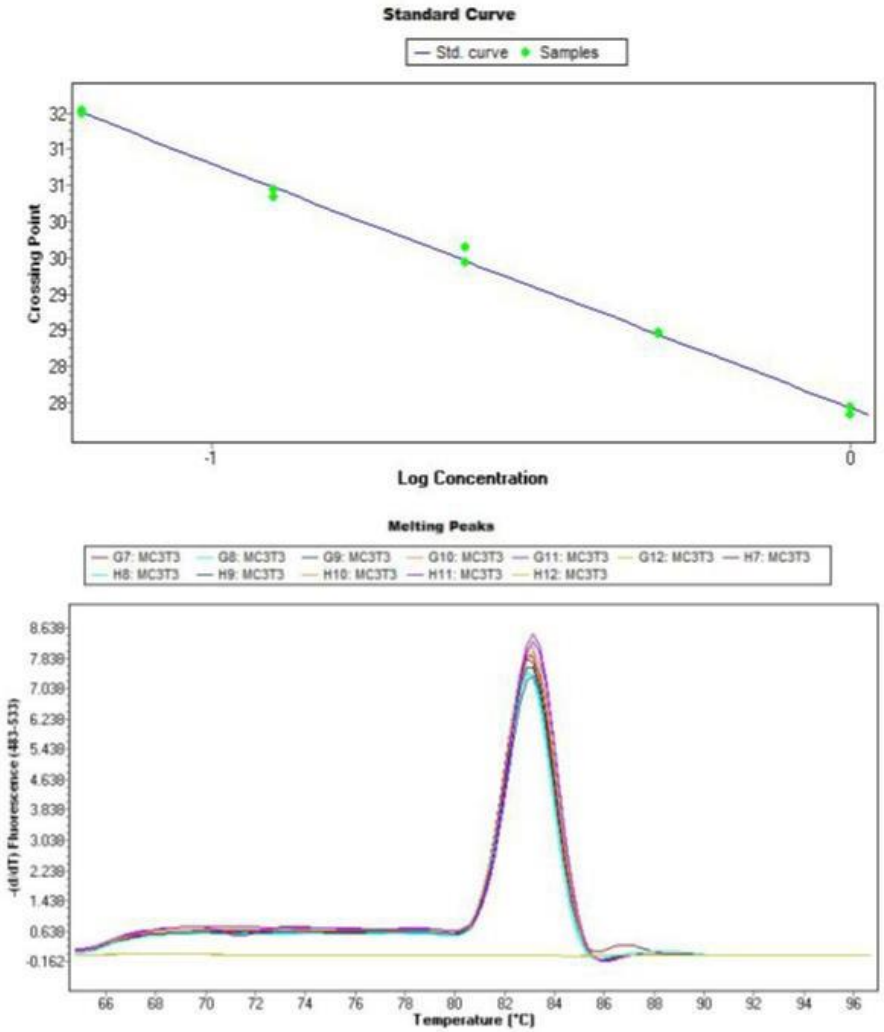

Figure 13A & B: Standard curve and melting curve for *Col10α1*. five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

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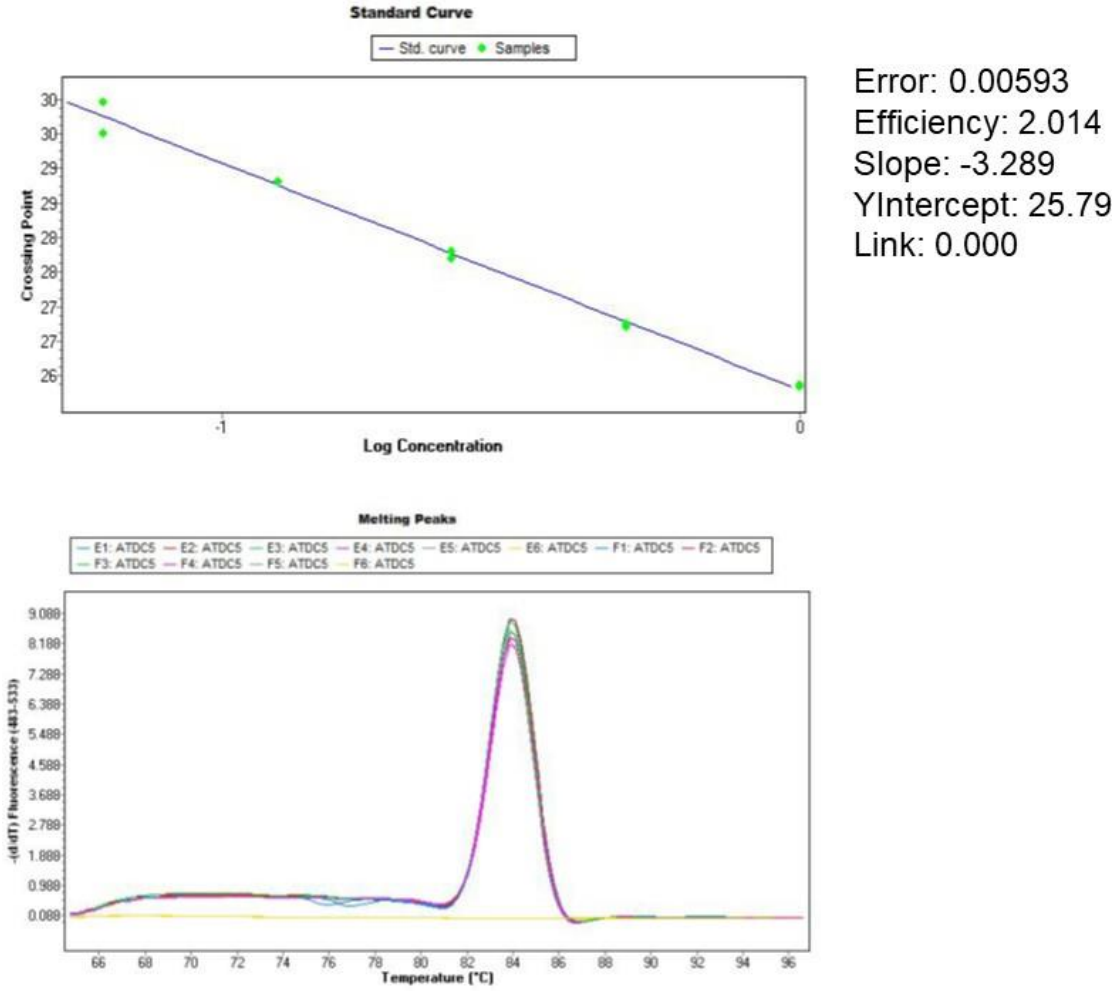

Figure 14A & B: Standard curve and melting curve for *Acan*. five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

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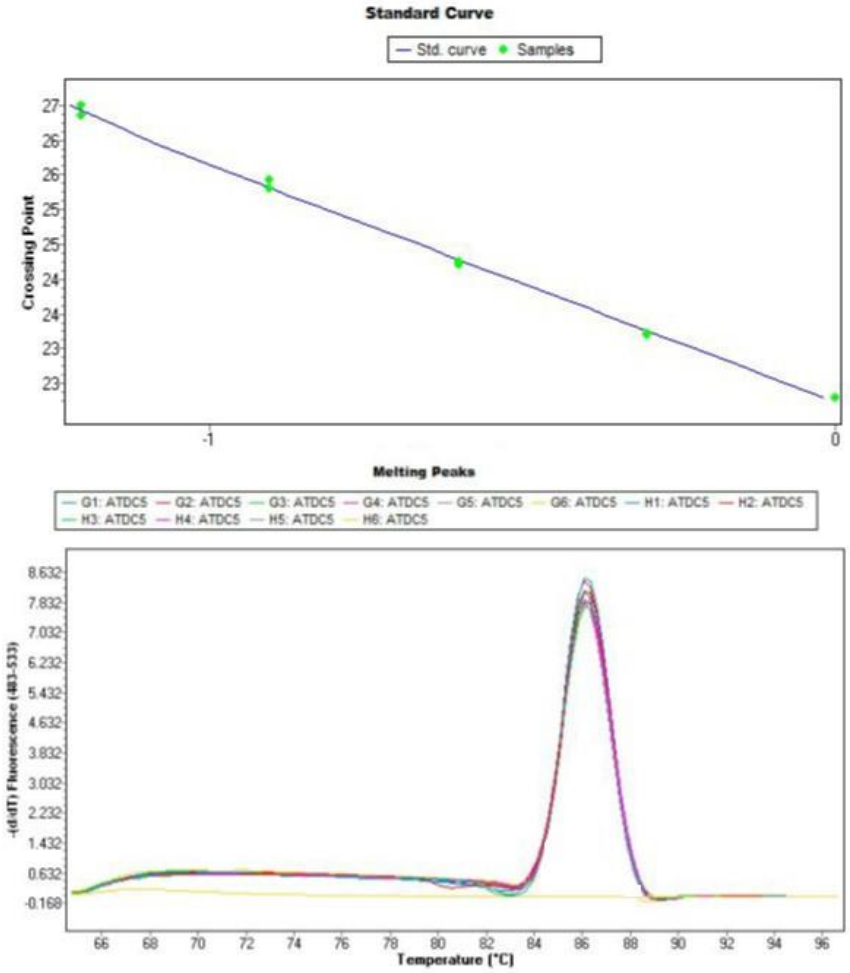

Figure 15A & B: Standard curve and melting curve for *Sox9.* five serial dilutions of a single sample with three replicates are used with dilutions of 1:5 (for example, 1/5, 1/10,3/15……) to generate a standard curve and melting curve.

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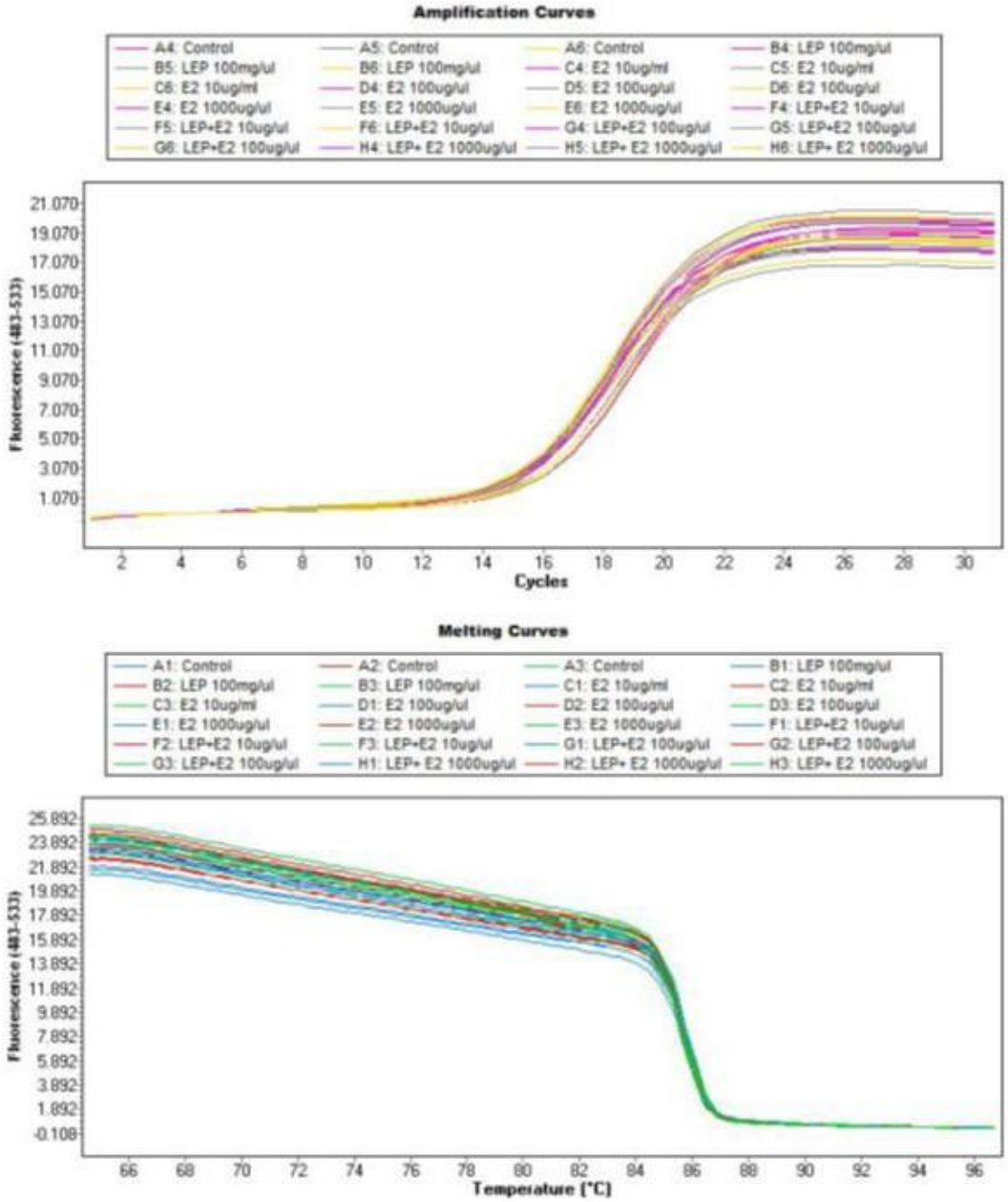

Figure16A & B: Amplification and melting curve for *GADPH* in J774A.1 macrophages. Amplification curves of the housekeeping gene *GADPH*. In the X-axis are the number of amplification cycles and in the Y-axis, the specific fluorescence. The C T of all samples (treated and untreated) was similar; showing that *GADPH* expression was the same in all the experimental groups irrespective of the treatment.

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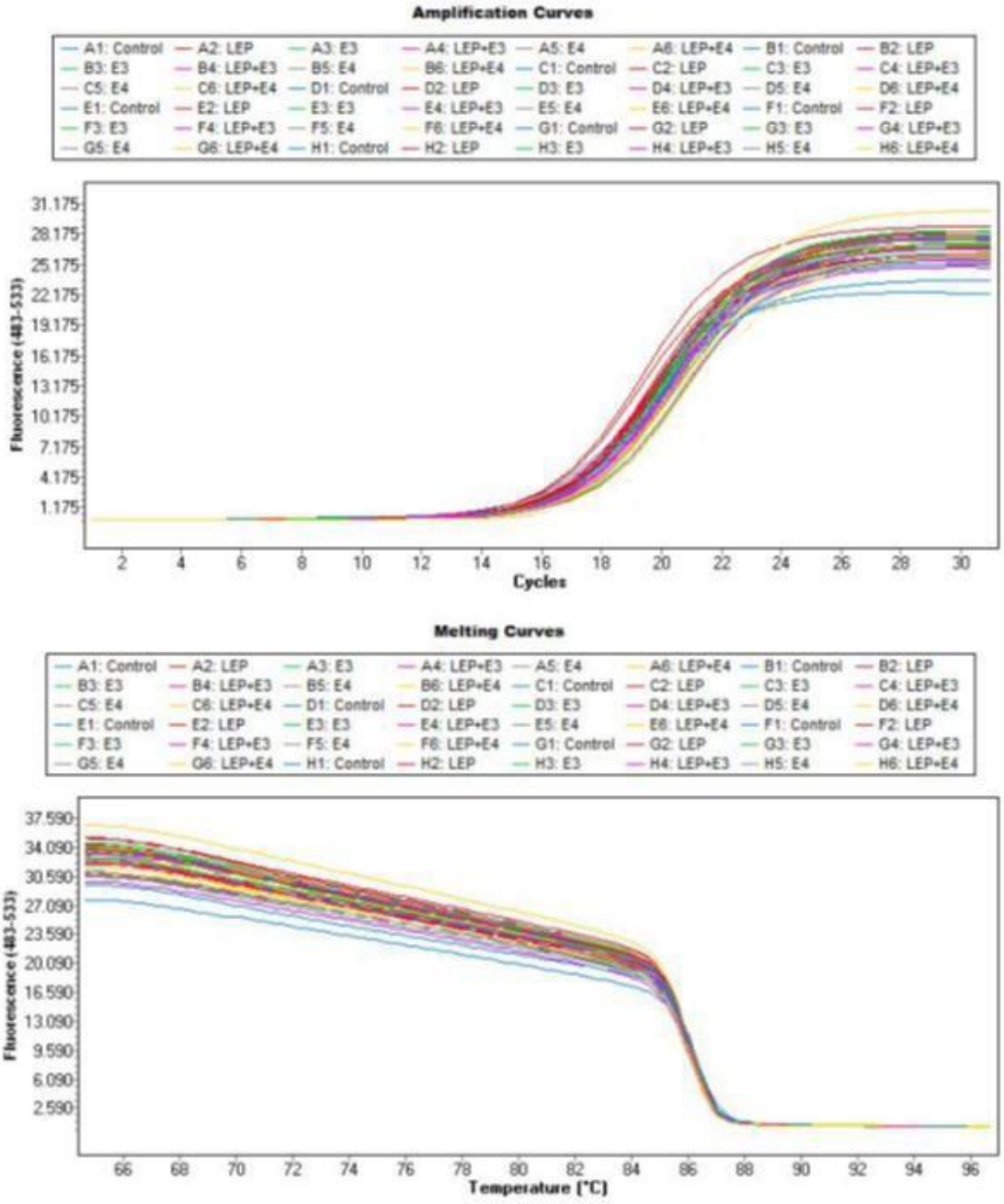

Figure17A & B: Amplification and melting curve for *GADPH* in MC3T3-E1 osteoblasts. Amplification curves of the housekeeping gene *GADPH*. In the X-axis are the number of amplification cycles and in the Y-axis, the specific fluorescence. The CT of all samples (treated and untreated) was similar; showing that *GADPH* expression was the same in all the experimental groups irrespective of the treatment.

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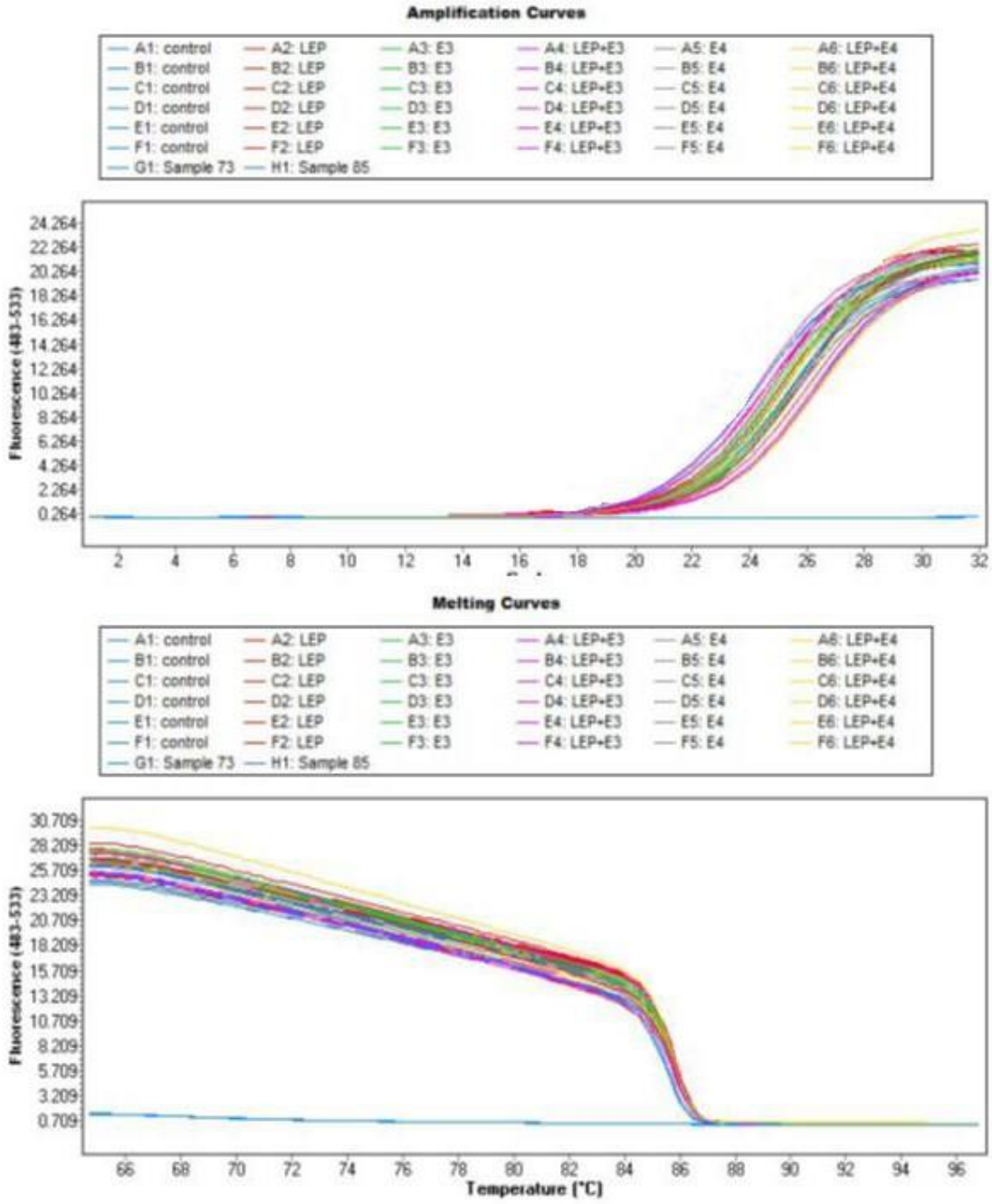

Figure18A & B: Amplification and melting curve for *GADPH* in ATDC5 chondrocytes. Amplification curves of the housekeeping gene *GADPH*. In the X-axis are the number of amplification cycles and in the Y-axis, the specific fluorescence. The CT of all samples (treated and untreated) was similar; showing that *GADPH* expression was the same in all the experimental groups irrespective of the treatment.

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Appendix C

Permission for copyright figures and table

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Sent via email to biochemisthu@gmail.com

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By: 
Printed Name: Saima
Title: PhD student
Date: 18.03.2021

Permission letter for figure 2.2

Rizwan, Saima

From: Cao Xuan cu <clinicalpub@gmail.com>
Sent: Thursday, 21 January 2021 3:55 PM
To: Rizwan, Saima
Subject: Re: Permission to reuse figure in my thesis

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Vào Th 3, 19 thg 1, 2021 vào lúc 11:34 Rizwan, Saima <S.Rizwan@massey.ac.nz> đã viết:

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>
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> Regards,
>
> Saima

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Rizwan, Saima

From: Vera Francisco <vlgfrancisco@gmail.com>
Sent: Thursday, 21 January 2021 9:03 PM
To: Rizwan, Saima
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Best regards,
Vera Francisco, PhD

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IDIS: Instituto de Investigación Sanitaria de Santiago, Spain
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