The effect of dietary fatty acids on osteocyte-mediated mechanotransduction

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Rabindra Dhital

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Abstract

The bones in our skeleton are subjected to mechanical loading every day and are continuously remodelled in a process called “bone remodelling”. The capacity of the skeleton to adapt its mass and structure in response to mechanical loading has been intensively studied. Over the last few decades, much focus has been given to bone cells such as osteoblasts and osteoclasts but less so to osteocytes, even though these comprise almost 90% of the cellular space in bone [1]. The detailed way these cells function in bone mechanotransduction and therefore control bone remodelling is not well understood. Diet and especially dietary fatty acids (DFA) are an important aspect of the regulation of bone health. Strong evidence has been presented in the scientific literature to support the benefits of DFA in bone health, but the exact mechanism of how they benefit bone health is still unclear [2-6].

It has been shown that bone cells secrete various osteogenic molecules in response to fluid shear stress. The goal of this project was to explore osteocyte cellular mechanisms involved in their response to mechanical loading and dietary fatty acids. This project specifically focused on the osteocytic secretion of ATP in response to dietary fatty acids and fluid shear stress *in vitro*. This study showed for the first time that the increased ATP secretion induced by fluid shear stress due to DFA treatment possibly explore a new understanding of how DFA might benefit bone health and could be used in future experiments to help us understand their possible effects. This study might be set as a model experimental design to study the cell response to DFA treatment exposed to fluid shear stress.

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Abbreviations

ATP:	Adenosine triphosphate
AA:	Arachidonic acid
ALA:	Alpha linoleic acid
BMD:	Bone mineral density
BMU:	Basic multicellular unit
BSC:	Biological safety cabinet
CaSR:	Calcium-sensing receptor
CS:	Calf serum
DBP:	Vitamin D binding protein
DFA:	Dietary fatty acid
DMP1:	Dentin Matrix Acidic Phosphoprotein 1
DHA:	Docosahexanoic acid
DKK1:	Dickkopf WNT Signaling Pathway Inhibitor 1
EPA:	Eicosapentaenoic acid
FA:	Fatty acids
FBS:	Foetal bovine serum
FGF23:	Fibroblast growth factor 23
FSS:	Fluid shear stress
GIT:	Gastro-intestinal tract
HIF-1α:	Hypoxia-inducible transcription factor 1 alpha
IGF-1:	Insulin-like growth factor 1
LA:	Linoleic acid

LDL:	Low-density lipoprotein
LRP5/6:	Low-density lipoprotein receptor-related protein 5/6
M-CSF:	Macrophage colony-stimulating factor
MEMα:	Alpha-modified Minimum Essential Medium Eagle
MLO-Y4:	Murine Long bone Osteocyte-Y4
MSC:	Mesenchymal stem cells
NO:	Nitric oxide
OPG:	Osteoprotogerin
PA:	Palmitic acid
PG	Prostaglandins
PHEX:	Phosphate Regulating Endopeptidase Homolog X-Linked
PS:	Penicillin streptomycin
PTH:	Parathyroid hormone
PUFA:	Polyunsaturated fatty acids
RANK:	Receptor activator of nuclear factor kappa
RANKL:	Receptor activator of nuclear factor kappa-B ligand
TGFβ:	Transforming Growth Factor Beta
TNFα:	Tumour Necrosis Factor alpha

Chapter 1. Introduction

A good diet and exercise are very important to bone health and the prevention of osteoporosis [6-10]. The cliché "we are what we eat" appropriately defines the vital role food plays in overall health. The food we eat may have different fates inside the body. It may be catabolised to provide energy, regulate metabolism or be incorporated into structural elements of the body. The regulation of metabolic processes is primarily guided by hormones [11-13] and hormone production is altered depending on the nature of fatty acids (FA) present in the diet. For example, saturated and trans FAs are found to increase insulin resistance whereas unsaturated FAs decrease insulin resistance [14]. Similarly, growth hormone production is decreased when the diet is deficient of essential fatty acids [15]. Diets rich in omega-3 polyunsaturated fatty acids (PUFAs) from fish oil have a positive effect on steroid hormone production in testes of adult pigs [16]. Diets rich in omega-3 PUFAs were found to lower coronary heart disease risk factors such as atherosclerotic and thrombotic factors in humans [17]. This implies that an imbalance in such dietary fatty acid intake may lead to the disparity of hormone production leading to alteration of the metabolism.

It is a well-known fact that exercise is good for bone health. Those who exercise have better bone health and structure [18-20]. In combination with other lifestyle factors, an exercise that starts earlier in life has been shown to contribute greatly to bone mass and health [18-20]. Hormones such as oestrogen [21], parathyroid hormone [22], testosterone [23] and glucocorticoids [24], are promoted by exercise to regulate bone turnover and maintenance. It is believed that exercise provides mechanical stimuli to the bones and joints [25]. This further supported the claim, that diet and exercise have a very important role in bone health.

The osteocytes are the most abundant cell type, which comprises around 90-95% of total bone cells, in adult bone. They have dendritic processes that go from deep in the bone

matrix to the bone surfaces [1] and are in a most favourable position to detect mechanical stimuli [26]. These mechanical stimuli cause bone formation by releasing early mediators of bone formation such as nitric oxide and prostaglandins [27, 28]. These molecules are involved in early gene expression of c-fos and IGF-1 in osteocytes [29, 30]. Recently osteocytes were also found to be involved in bone mineral and phosphate homeostatic processes etc., as an endocrine cell [31]. These facts clearly support that osteocytes are one of the most important bone cells in the body for bone cell regulation.

Mechanical loading causes the movement of the interstitial fluid in the bone to provide more nutrients and transporting waste products [32]. These mechanical stimuli also influence osteocytes to increase secretion of osteoprotective molecules such as nitric oxide (NO) and prostaglandin E2 (PGE2). These osteoprotective biomolecules are expressed by the bone cells (osteocytes and osteoblasts) in the fluid collected from osteocytic processes [27, 28, 33]. Where there is no movement such as in the case of microgravity [34] and bedridden patients [35, 36], they were found to be osteoporotic, having lower bone mineral density. There are different cells identified that could induce adenosine triphosphate (ATP) release following mechanical stress [37, 38] and the osteocytes are among them which release ATP in response to mechanical stress [39, 40]. Mechanical loading is sensed by osteocytes which secrete ATP in response. ATP and other secreted factors affect other bone cells to regulate bone remodelling in response to mechanical load. Osteoblastic cell lines such as the murine MC3T3-E1 and HOBIT cells have been shown to induce ATP release following mechanical stress [37, 38]. Osteoblastic activity is increased by osteocytes and osteoblasts when they sense the alteration in the interstitial fluid flow pressure thus enhancing bone formation *in vivo* [41-43]. These osteoblastic activities are largely driven by several paracrine and autocrine factors released by osteocytes and osteoblasts. ATP is an important signalling molecule which acts on other adjacent bone cells during mechanotransduction [38, 44-46]. This released ATP is thought to be active in the

mobilization of intracellular calcium [47, 48], regulation of gene expression [49, 50] and control of proliferation of osteoblastic cells [45, 51].

Little is known about the role of dietary fatty acids on osteocyte metabolism. The studies so far have suggested that the dietary fatty acids have important roles in bone remodelling, in fact, they have been found to improve bone density and help maintain the bone structure [52]. The study was designed to explore the gap in the current understanding and knowledge about how nutrition and exercise interact to enhance or protect bone density at the cellular level. Dietary fatty acids might improve bone health by enhancing the sensitivity of osteocytes to mechanical loading and affect mechanotransduction by osteocytes. However, there is limited knowledge of how these dietary fatty acids influence osteocyte-mediated mechanotransduction.

This project aimed to increase the basic understanding of the cellular and molecular processes underlying the mechanotransduction process *in vitro* especially in the presence of omega 3 and 6 fatty acids.

Therefore, the main focus was to explore the role of the long-chain fatty acids docosahexaenoic acid (DHA) and arachidonic acid (AA) on osteocyte mediated mechanotransduction pathways that may affect the bone mineral mass. These fatty acids were chosen because they are two major FAs found predominantly on cell membranes. They are very important for neurite growth and signal transmission [53] These FA were also found to be good for bone health [54, 55]. These FAs supplements were shown to improve essential fatty acids deficiency symptoms in rodents [56]. The effect of these FAs on the release of ATP by MLO-Y4 cells exposed to fluid shear stress (FSS) and the result was analysed for any changes between different treatment groups. The MLO-Y4 osteocyte cell line has phenotypic similarities with primary osteocytes cells. This cell line is the selected cell line for assessing the function of human osteocytes. These cells exhibit identical characteristics of osteocytes *in vivo* in terms of secretion of various markers specific to

osteocytes. Details about the cell line are presented in Literature review, please refer to the heading 2.7.4. The cells were maintained at 37°C during all the experiment processes. Proper care was taken to create an *in vitro* environment suitable for bone cells specially osteocyte.

To achieve this aim, the objectives were:

- To set up an *in vitro* model of osteocyte mechanotransduction in which murine MLO-Y4 cells were subjected to fluid shear stress using the Flexcell Streamer experimental platform for mechanical fluid stress.
- To use this experimental system to test the hypothesis that specific omega-3 and 6 fatty acids found in the diet mediate their osteoprotective effect by directly modulating osteocyte mechanotransduction or the associated signalling pathways.
- To perform the different tests and experiments required for method validation prior to FSS experiments.

Chapter 2. Literature Review

2.1. Bone: An overview

Bone is a dynamic biological tissue, highly specialised to provide a rigid framework for the body. It is composed of metabolically active cells that are capable of continuous regeneration and repair under normal circumstances (Figure 2-1). Bones support and protect vital internal organs in the vertebrates. Bone stores minerals such as calcium and phosphorus, growth factors and cytokines and also participate in acid-base balance to maintain homeostasis [58]. Additionally, bone participates in haematopoiesis by housing bone marrow inside its internal cavities.

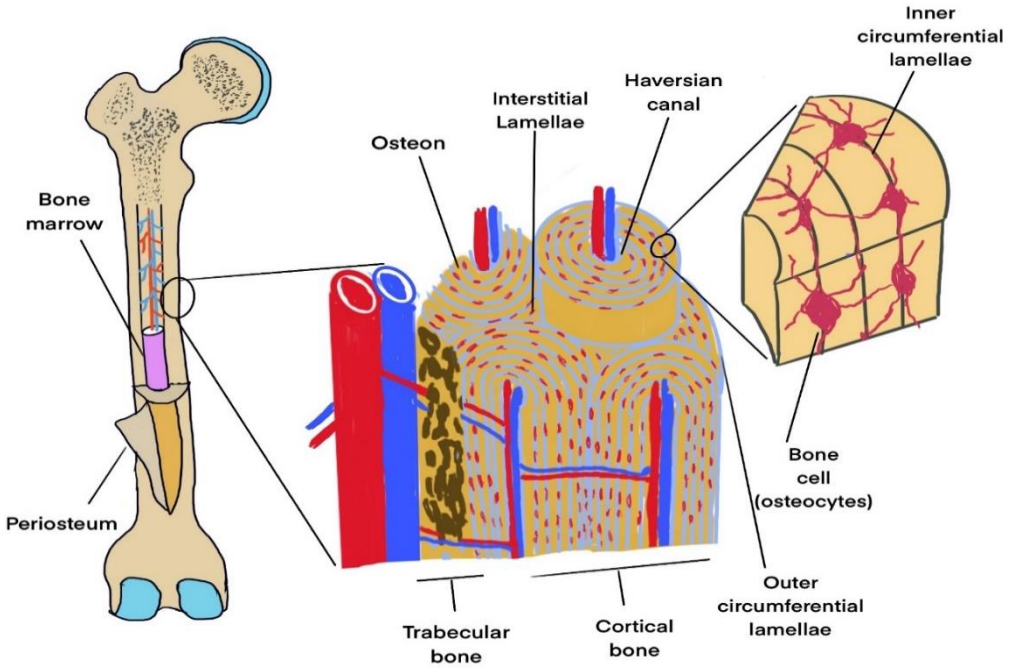

Figure 2-1: General structure of bone, depicting gross overview and cellular distribution (adapted from [57]).

Bone maintains its balance, shape and structure in response to physical load it experiences as well as hormonal influences. Bone formation and resorption are continuous processes in healthy bones. New bone forms during embryonic growth or fracture repairs. Hormones such as parathyroid hormone (PTH) causes bone resorption, releasing calcium and phosphate into the blood circulation, whereas the hormone calcitonin promotes bone formation by lowering the level of calcium in the blood. Vitamin D promotes calcium absorption and reabsorption leading to an increase in serum calcium concentration [59]. In this way, these hormones function simultaneously to help in the maintenance of bone homeostasis.

Recently, bone has been hailed as an endocrine organ influencing activities of various organs such as kidney, muscles, pancreatic cells and adipocytes [60] which affect bone homeostasis and health. These properties of bone made it unique and dynamic tissue.

2.2. Bone structure

Bone is the substance that builds the structural component of the skeleton. Structurally, all vertebrates possess two types of bone. More compact cortical bone and loosely arranged trabecular or cancellous bone (Figure 2-1). Cortical bone generally lines or covers the bone and it is much denser whereas trabecular bone resides in the inner part of the bone. Cortical bone can be found in the surfaces and shaft of the long bones while the trabecular bone is found in inner areas of flat bone.

2.2.1 Cortical bone

Around 80% of the total skeletal mass is cortical bone. This bone is comprised of highly organised and compact bone tissue. Cortical bone has low porosity but contains microscopic pores which accommodate blood vessels and nerves. Cortical bone is generally stronger and formed by densely packed collagen fibrils. This bone is found mostly in the periphery of long bones such as arms and legs and used primarily in physical activity. Four different levels of

lamellae organization have been identified which are found in cortical bone. 1) Haversian systems, wherein concentric circles of lamellae surround a central longitudinal cavity containing blood vessels and nerves; 2) outer circumferential lamellae near the periosteum; 3) inner-circumferential lamellae near the endosteum; and 4) interstitial lamellae, in between osteons and the inner or outer circumferential lamellae.

2.2.2 Trabecular or cancellous bone

Trabecular bone also termed cancellous bone, has a loosely organised porous matrix and honeycomb-like structure in the bone marrow compartment. This bone is found in ribs, skull, vertebrae, pelvic bones and the ends of long bones. It is generally weaker and readily fractured compared to cortical bone.

2.2.3 Matrix composition

The matrix of the bone consists of organic and inorganic components. The inorganic matrix of bone is primarily calcium and phosphate in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) whereas the organic matrix primarily consists of type 1 collagen, proteoglycans, growth factors and matrix proteins such as osteocalcin and osteopontin.

2.2.4 The cellular components of bone

Bone is composed of four different types of cells. Osteoblasts, osteoclasts and bone lining cells are present on bone surfaces, whereas, osteocytes reside deep inside in the mineralized matrix. The cellular components of bone can be categorized into two families. The first one is osteoblastic lineage which contains pre-osteoblasts, osteoblasts, osteocytes and bone lining cells all derived from mesenchymal stem cells whereas another being the osteoclastic lineage which contains osteoclasts, macrophages derived from haematopoietic stem cells [61].

2.2.4.1. Osteoclasts

Osteoclasts are large multinucleated cells originating from myeloid hematopoietic stem cells. These cells are generally formed by the fusion of monocyte progenitors. These cells resorb bone during the bone remodelling and turnover process. Osteoclasts have a special type of plasma membrane which is highly folded and participate in the bone resorption site by latching onto the bone surface and sealed by integrin [62].

Osteoclasts are activated by Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL; which binds to RANK receptors on its surface) and macrophage colony-stimulating factor (M-CSF) released from osteoblasts and osteocytes. These factors support the osteoclast to mature, differentiate and progress toward the bone resorption activity. Osteoclasts secrete acid phosphatase which can be used as an identifier or marker. Osteoclasts secrete hydrochloric acid which breaks down bone and then secrete metalloproteases which will dissolve the collagen [63]. Osteoclasts derive H^+ from the proton pump and Cl^- from the inner cell membrane of the ruffled border [64]. They resorb the bone at Howship lacunae where osteoclasts secrete tartrate-resistant acid phosphatase and cathepsin K [63, 65]. This will degrade the bone by lowering the pH and digesting the organic matrix.

Osteopetrosis, Paget's disease and Gorham-stout syndrome are some clinical conditions that arise due to osteoclast dysfunction [66]. These clinical conditions are the result of altered bone vasculature supply where the osteoclasts have a compromised role and cannot perform their normal activity.

2.2.4.2. Osteoblasts

Osteoblasts are cuboidal cells that are derived from undifferentiated mesenchymal cells. Osteoblasts secrete osteoid to produce bone matrix [67]. The major function of the osteoblast is the secretion of type 1 collagen. Osteoblasts control the formation of the bone matrix by secreting collagenous and non-collagenous proteins. They vary in size ranging

from 20-30 micrometres in diameter. Alkaline phosphatase which acts as osteoblast marker is found mostly in the cytoplasm and nucleus of this cell. They have parathyroid hormone receptors on their cell surface which signal osteoblasts to secrete RANKL. RANKL influences osteoclasts by binding to its receptor RANK and promoting differentiation and activation of osteoclasts. Osteoblasts also have receptors for oestrogen and vitamin D on their surface which suppresses osteoclastic functions and support bone formation.

2.2.4.3. Osteocytes

Osteocytes are stellate shaped mature osteoblasts which reside deep inside the bone matrix. They are formed when the ageing osteoblasts are trapped inside the bone matrix during ongoing bone formation. These are the most prevalent cell type in bone. Their shapes vary with the location in which they reside. Cells within trabecular bone have a rounded shape whereas the cells from cortical bone have an elongated form [68]. They are the most numerous type of bone cells occupying around 90-95% of the total cellular space [69, 70]. Osteocytes are connected to other osteocytes and bone cells through fusion of gap junction proteins of the dendritic processes within the lacuno-canalicular network to communicate with each other. These cells used to be regarded as inactive and dormant but are active and dynamic. Osteocytes can synthesize collagen, control bone mineralization within their lacunae and can carry out lacunar demineralization surrounding them (osteocytes). When osteocytes were exposed to PTH, these peri-lacunar demineralizations were found to be more prominent, which was recovered by the administration of calcitonin [71, 72].

2.2.4.4. Bone lining cells

Bone lining cells are flat and elongated cells found mostly on the bone surfaces. It had been speculated that bone lining cells were precursors of osteoblasts. These are generally inactive and possess few cytoplasmic organelles. Bone lining cells remove non-mineralised

collagenous material by secretion of the metalloproteinase from the bone surfaces. After clearing the surfaces, they smooth the layer by secreting collagen on top of it [73-75].

2.3. Regulation of Bone Homeostasis

Calcium and phosphate ions are so important in bone health that their homeostasis is maintained by two different mechanisms. These are hormonal (endocrine) and non-hormonal (local) mediators.

2.3.1 Endocrine regulation

Calcium and phosphate level regulation in the blood is a prerequisite condition for normal bone homeostasis. Calcium and phosphate concentration in the human body is controlled by three different types of hormones. These are parathyroid hormone (PTH), calcitonin and vit D3 (1, 25-OH vitamin D3). These hormones act on kidney, bone and the gastrointestinal tract (GIT) to regulate bone homeostasis.

2.3.1.1. Parathyroid hormone

Parathyroid hormone (PTH) is the most important hormone that regulates calcium and phosphate levels in the blood. It is an 84 amino acid polypeptide produced by the chief cells of the parathyroid gland. There are four parathyroid glands, two on each side of the dorsal surface of the thyroid gland. PTH increases calcium release from the bone by enhancing reabsorption of calcium from the distal nephron by ATP dependent and sodium gradient dependent mechanisms [76] and reducing the reabsorption of phosphate from kidney, resulting in phosphate excretion through the urine, usually resulting in bone loss [77].

Another factor controlling and lowering PTH secretion is a high concentration of 1, 25-dihydroxy vitamin D [78]. It increases circulating Ca^{2+} which activate the calcium-sensing receptor (CaSR) located at the chief cells, which gives negative feedback and decreases PTH production [79]. The number of calcium to CaSR bindings, trigger the activation or inhibition

of PTH secretion from chief cells. A study by Jono S et al showed that 1,25 vit D suppresses the PTH related peptide expression in bovine vascular smooth muscle cells (BVMC) and promote calcification [80]. The overall outcome of the 1,25 vit D effect is the suppression of PTH synthesis by the parathyroid cells [81, 82].

PTH's net effect in bone is an increase in bone resorption by activating osteoclasts. Osteoblasts respond to PTH by producing IL-1, IL-6 and other cytokines which trigger osteoclastic differentiation [83-86]. PTH also influences osteoblasts to produce more RANKL [87] and M-CSF [88] that induce differentiation and proliferation of osteoclasts. Another study suggests that PTH induced osteoblasts secrete monocyte chemoattractant protein 1 (MCP-1) which enhances the fusion of and multinucleation of pre-osteoclasts [88].

2.3.1.2. Calcitonin

Calcitonin sometimes referred to as thyrocalcitonin, is a 32 amino acid containing polypeptide hormone produced by the C-cells in the thyroid gland. Calcitonin increases the calcium excretion in urine, lowering the calcium and phosphate level in the blood. Calcitonin deficiency is associated with higher bone mass in mice suggested it to be a bone formation controller in the skeletal system [89-91].

2.3.1.3. Vitamin D3

The two major types of vitamin D are vit D2 or ergocalciferol and vit D3 or cholecalciferol. Cholecalciferol or vit D3 is an active form. Vit D3 can be taken in the diet, mainly from dairy products, fish and oils or synthesised in the body in the presence of sunlight. When ultraviolet rays hit the skin from the sun, vit D3 is produced. The precursor to vit D3 found in the skin is termed as 7-dehydrocholesterol. This 7-dehydrocholesterol is converted to cholecalciferol in the presence of ultraviolet light at the wavelength of 290-315nm. This cholecalciferol is transported by vitamin D binding protein (DBP) to the liver where it is converted to 25 hydroxyvitamin D3. Hydroxylation reaction occurs in the endoplasmic

reticulum in the presence of magnesium by the enzyme 25 hydroxylase a member of cytochrome p450. After this, 25 hydroxy Vit D3 again enters the circulation and is transported to the kidney by DBP. When reaching the kidneys this 25 hydroxyvitamin D3 is converted to 1, 25 hydroxyvitamin D3 or simply 1, 25 vitamin D3 which is an active form of vitamin D3. This process occurs in mitochondria of the renal proximal convoluted tubule in the presence of at least three enzymes called renal ferredoxin reductase, renal ferredoxin and cytochrome P450.

PTH also increases the serum calcium concentration by affecting the osteocytic osteolysis, pattern seen in the lacunae of the osteocyte and was first described morphological by Baud [71]. These lacunae where osteocytes reside were found to be enlarged with the continuous administration of PTH [92]. These resorptions of lacunae were found to be decreased with the administration of calcitonin [93, 94].

2.3.2 Local regulation

When the body recognises the low amount of calcium and phosphate ions in the blood plasma, a homeostatic mechanism allows these ions to move out from the hydroxyapatite crystal where they were situated and stored within the bones into the extracellular fluid. So this non-hormonal response is usually quick. Generally local regulation aids the hormonal regulation as it solely cannot fulfil the greater demand of the body.

The table below summarises the role of PTH, vit D3 and calcitonin in the various parts of the body to maintain bone homeostasis.

Table 1: Hormones involved in calcium and phosphate homeostasis

	PTH	Vitamin D3	Calcitonin
Kidney	Increase the activity of 1α hydroxylase to promote vit D3 synthesis Increase reabsorption of calcium in the distal tubule Decrease phosphate absorption in the proximal tubule.	Increase the reabsorption of calcium and phosphate in kidney tubule.	Causes decrease reabsorption of calcium and phosphate in kidney tubule.
Net effect	Decrease phosphate concentration but increase calcium concentration in plasma.	Increase calcium and phosphate concentration in the plasma.	The decrease in phosphate and calcium concentration in plasma
Bone	Increase the maturation of osteoclast and osteoclastic activity. A decrease in osteoblast collagen in the matrix.	Stimulates PTH which then increase calcium reabsorption only and decrease phosphate in the blood.	Decreases osteoclastic activity
Net effect	Decrease phosphate concentration but increase calcium concentration in plasma.	Decrease phosphate concentration but increase calcium concentration in plasma.	The decrease in phosphate and calcium concentration in plasma
GIT	Increase both calcium and phosphate absorption.	Increase the absorption of calcium and phosphate.	No action
Net effect	Increases calcium and phosphate concentration in plasma	Increase calcium and phosphate concentration in the plasma.	No effect

2.4. The Wnt signalling pathway in bone turnover

The purpose of this signalling pathway is to regulate cell proliferation, maturation, differentiation by initiating cellular and nuclear processes (Figure 2-2). Wnt signalling stimulates mesenchymal stem cells (MSC) to differentiation into osteoblast lineage cells rather than differentiating into adipocyte lineage cells, which supports bone formation [95]. Osteocytes sense and adapt to a different mechanical stimulus by the help of the wnt signalling pathway [96].

When wnt is available, β-catenin is not destroyed and proceed towards the nucleus and start gene activation. Canonical Wnt signalling mediates its effect by binding Wnt proteins to a receptor complex that includes a member of the frizzled family of seven transmembrane receptors and either LRP5 or LRP6 [97]. Wnt molecules bind with the receptor in the frizzled (Fz) and co-receptor LRP5/6, this activates the wnt receptor complex. Binding of wnt to receptor complex leads Dishevelled (DSH) to relocate to the membrane and Glycogen synthase Kinase (GSK3β) to displace from the destruction complex which generally consists of Glycogen Synthase Kinase 3β (GSK3β), the scaffold protein Axin, the tumour suppressor Adenomatous Polyposis Coli (APC), protein phosphatase 2A and casein kinase 1α (CK1α). The mechanism of displacement of GSK3β from the destruction complex by the action of wnt receptor complex is unclear. This binding of wnt to the receptor complex also causes Axin and destruction complex to relocate to the plasma membrane. Axin binds to LRP5/6 which is phosphorylated by GSK3β and CK1α. By already phosphorylating the LRP 5/6 receptor, GSK3β and CK1α cannot phosphorylate the β-catenin and therefore cannot signal it to ubiquitination and proteasomal degradation. Because of no destruction of β-catenin, the level of it increases and it enters the nucleus. It binds there with LEF/TCF family of transcription factors to initiate transcription of targeted genes.

When there is no wnt molecule present, the GSK3β and possibly CK1α phosphorylates the β-catenin molecules and signal it towards the proteasomal degradation. The

phosphorylation of β-catenin marks it for degradation and proceeds towards the destruction.

DKK1 and sclerostin block wnt molecules and prevent wnt from binding with LRP receptor molecules. These antagonists combine with LRP molecules and occupy the LRP receptor where wnt binds. GSK3β phosphorylates the β-catenin and send it for the proteasomal degradation and ubiquitination [98].

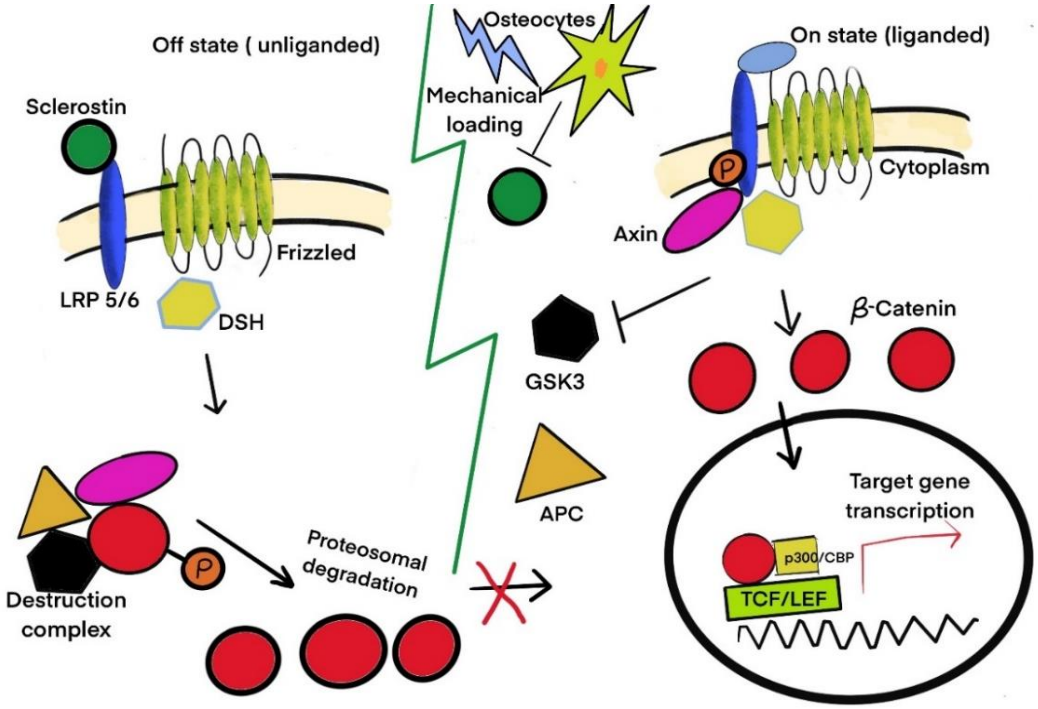

Figure 2-2: Wnt signalling pathway in osteoblasts.
Without wnt activation (off state), beta-catenin is tagged by destruction complex for proteasomal degradation. With wnt activation (on state), binding of wnt and receptor complex destroys the destruction complex which leaves beta-catenin in the cytosol. Then beta-catenin will enter the nucleus and start the expression of target genes for bone formation (adapted from [98]).

Wnt/β catenin signalling is very important to the bone development and regulation of bone mass. Wnt signalling in bone leads to osteoblast differentiation, proliferation, function and survival and hence increased bone mass [99]. Studies have revealed that mutations in LRP5 lower the bone mineral density implying the important role of LRP5 in mechanical signalling [100, 101]. The mechanism underlying the effect of LRP5 mutations on bone mass is yet unclear. Sclerosteosis and van Buchem disease [102, 103] are some of the bone disorders associated with an impaired SOST gene which encodes for sclerostin. These diseases are associated with thicker and denser bone mass affecting mostly the skull, jaw and long bones [104].

2.5. Role of different bone cells in bone remodelling

Bone remodelling is a process in which continuous removal of old bone and formation of new bone simultaneously occurs in the skeleton. Bone remodelling takes place within the Basic multicellular unit (BMU), where these bone cells participate simultaneously in the bone formation and resorption process. The bone remodelling process ensures proper bone structure, properties and characteristics of bone. Osteoblasts, osteoclasts and osteocytes are the key participants of the bone remodelling process.

Osteocytes: The osteocytes comprise around 90-95% of the total cellular space. They are also interconnected with other bone cells such as osteoblasts, osteoclasts and other osteocytes with a dendritic structure that allows cell communication and viability [105]. Osteocytes regulate osteoblasts and osteoclasts during bone remodelling process to ensure stable bone structure [106, 107] and because of this, the osteocytes are called the “master orchestrators of bone’’ [108]. Osteocytes can regulate osteoblasts and osteoclasts either by promoting or inhibiting their activity. Osteocytes control the osteoclasts by stimulating or promoting their differentiation by providing signalling molecules, RANKL [109-111] and M-CSF. Xiong et al selectively deleted RANKL in their osteocytes and measured bone

resorption. They have found that the RANKL produced by osteocytes is responsible for osteoclastogenesis [111]. Osteocytes secrete PGE2, NO and ATP, which stimulates osteoblastic activity and inhibits the maturation of osteoclasts [112-114]. Transforming growth factor β-1 (TGFβ-1) released by osteocytes and the bone matrix inhibits osteoclastic activity and prevent bone resorption whereas it also stimulates osteoblasts to recruit at the site of bone resorption to form new bone [113, 115]. At these sites, osteoblast and osteocytes also secrete osteoprotegerin (OPG) which inhibits osteoclast differentiation. OPG acts as a decoy receptor by binding with RANKL thereby preventing RANKL to interact with RANK on the osteoclasts [107].

Mature osteocytes secrete a signalling molecule called Sclerostin encoded by the SOST gene, which is a significant regulator to inhibit bone formation [116]. Sclerostin binds with the low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) and prevents it from binding with Wnt molecules [117]. This sclerostin and LRP5 binding negatively regulates the canonical Wnt signalling pathway in osteoblasts and inhibits osteoblast activation [118, 119] and bone formation. Mechanical loading supports bone formation by decreasing the expression of sclerostin in osteocytes [120]. The excessive bone formation may arise due to disorders of sclerostin synthesis in osteocytes or from the mutation of LRP5 [121, 122]. By this process, osteocytes act as a mediator and regulate the osteoclast and osteoblast activity during the bone remodelling process [123, 124].

Apart from controlling the function of osteoblasts, osteocytes can undergo de-differentiation back to osteoblasts. This was found to be occurring during the healing of injuries which require migration and proliferation of osteoblasts. The exact mechanism by which osteocytes sense these signals to de-differentiate back to osteoblasts is not yet clear and more study in this area is required [125, 126].

The osteocyte also releases soluble factors that regulate phosphate homeostasis. The osteocyte secretes fibroblast growth factor 23 (FGF23) to inhibit the renal reabsorption of

inorganic phosphate which will ultimately decrease the bone mineralization. However, when dentin matrix protein-1 (DMP1) and phosphate-regulating gene with homologies to endo-peptidases on the X chromosome (PHEX) are expressed, FGF23 [127] expression is decreased leading to high renal absorption of phosphate, allowing mineralization of the bone matrix.

Osteoclasts: Osteoclasts usually accumulate by chemotaxis in resorption space[1]. These resorption spaces are also called Howship's lacunae where bone resorption occurs. Osteoclasts clear and resorb the bone, which creates spaces for osteoblasts to form bones in the Howship's lacunae. The osteoclasts form an extensively folded structure called the ruffled border which increases the surface area to cover the bone pit to facilitate bone resorption (Figure 2-3). After settling onto the surface of the bone, osteoclasts release several enzymes such as acid phosphatase, tartrate-resistant acid phosphatase and cathepsin K, which breakdown bone into calcium and phosphorus ions which causes a breakdown of bone or bone resorption.

Osteoblasts: After osteoclastic bone resorption, the osteoblast takes the lead by secreting type 1 collagen and mineralizes the area to build bones on top of it [128, 129]. Like osteocytes, it also secretes RANKL [130] which helps osteoclasts to proliferate and differentiate. Osteoblasts primarily lay down new bones during remodelling and skeletal development process [131].

[1] It is the space where osteoclasts remove bone. Osteoblast need resorption spaces to proceed toward the bone formation.

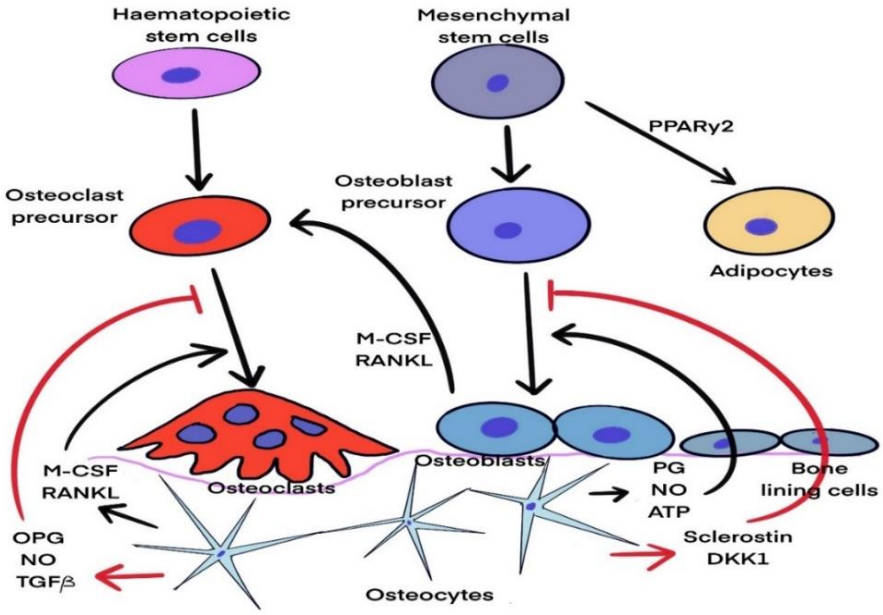

Figure 2-3: Interaction of bone cells: osteocytes, osteoblasts and osteoclasts during bone remodelling.
Osteocytes secrete M-CSF and RANKL to support osteoclast differentiation and maturation. Osteocytes also secrete OPG, NO and TGFβ inhibit the osteoclastic activity and support osteoblastic activity. It also secretes sclerostin and DKK1 to inhibit bone formation (adapted from [31, 132]).

2.6. Osteocytes: Response to nutrition and exercise

Physical activity and good nutrition contribute significantly to the prevention of osteoporosis, a significant problem in an ageing population [7-10, 133, 134]. The positive effect of diets supplemented with omega fatty acids and regular exercise on improving bone density in the young and old people is well known [7-10, 133-136].

Bone turnover is influenced strongly by the nutritional status of the individual [133]. Recent studies show that the incidence of osteopenia and osteoporosis was improved by dietary supplementation of omega-3 fatty acids (FA), although the exact mechanism is not understood yet [2, 6]. Dietary supplementation with other foods such as blueberries has

also been found to inhibit bone loss by lowering RANKL production in the stromal cells [137]. Deficiency in dietary omega-3 FA is linked with decreased bone density [2]. A Mediterranean diet, rich in olive oil and oleic acid results in a lower incidence of bone fractures and correlates with higher bone mass [138, 139] whilst a ketogenic diet (high fat, fewer carbohydrates) lowers the bone mass [140]. Omega-3 and omega-6 FA are shown to have a beneficial effect on bone health [5] with a lower fracture rate [141], and a higher bone mineral density [142] in various studies.

Mechanical loading and exercise positively affect bone remodelling. It was demonstrated that mechanical loading increases osteocytes viability [143] and that physiological fluid shear stress is important in osteocyte survival [144]. Osteocyte apoptosis was linked to experimental mice and rats having low [145, 146] and or excessive [147, 148] mechanical loading. Local skeletal micro injuries resulting from a bone fracture can also lead to osteocyte necrosis or apoptosis. Necrosis and apoptosis of osteocytes promote osteoclast bone resorption [108, 149]. To show this, Cardoso et al [150] completely inhibited the resorptive activity of osteoclasts by blocking osteocyte apoptosis They have used pan-caspase inhibitor Q-VD-OPh (quinolyl-valyl-O-methylaspartyl-[-2,6-difluorophenoxy]-methylketone to inhibit the osteocyte apoptosis. They have found that apoptosis inhibition has suppressed the intracortical resorption. This study further supports the work of Tatsumi et al [151], in which they have shown that gene-targeted ablation of osteocytes causes rapid bone loss. These studies firmly support the idea of osteocytes regulating osteoclasts in the bone turnover process.

At zero gravity and in microgravity environments, astronauts experience a higher loss of bone mineral density [152-154]. This bone loss is associated with higher bone resorption and lower calcium absorption [155] due to decreased loading in zero gravity. Bone mass significantly increases after the space flight (post-flight) due to reloading of the bone which appears to induce normal bone turnover by increasing calcium absorption and reducing

bone resorption [155]. In other studies, exercise such as strength training, resistance training had reduced bone loss in elderly and postmenopausal women [156-159]. Similar results were obtained in a study where mice had shown improved bone and structure in the region where mechanical loading was applied, possibly due to enduring the applied stresses [160, 161]. These reports suggest that the absence of loading decreases bone mass and vice versa. Tatsumi et al [151] also prevented the unloading induced bone loss by selective ablation of 70-80% of the osteocytes in mice. They showed that osteocytes can sense mechanical unloading and induce subsequent bone resorption.

Mechanical unloading has been reported to induce osteocytes hypoxia *in vivo* [162]. The metabolic needs and nutrient demands of the deeply embedded osteocytes are met when forces generated by mechanical loading and fluid shear stress [163] act on them. It is shown that the disuse of bone leads to the diminished nutrient supply to the deeply embedded osteocytes through the vasculature. This disuse induced hypoxia upregulates the hypoxia-inducible transcription factor (HIF-1α) and osteopontin in osteocytes [162, 164]. Since the hypoxia-induced osteocyte expression of osteopontin has been suggested to induce osteoclast chemotaxis and adhesion [165], it can be speculated that mechanical unloading plays a crucial part in osteoclastic bone resorption although the actual mechanism is not fully understood yet.

2.7. Mechanical loading and bone

2.7.1 History

For a long time, people have been fascinated by the fact that lifestyle, diet and environment affect bone structures. In ancient literature, Greeks and Italians have mentioned the effect of different lifestyles on skeletal mass. In 1638, Galileo first indicated that the shape of bones is determined by the load they bear, but the scientific study started only at the beginning of the 19th century [166]. In 1867, Von Meyer noticed the distorted trabecular arrangement in the sagittal section of bone which Karl Culmann claimed as Culmann's 'crane', these are results of the load they bear [167]. At the beginning of 1869, Julius Wolff suggested a novel Wolff's law [168], which states that the structure of bone is the result of the loading and direction of the load it faced or carried [169]. He described bone as an organ capable of producing new bone or removing bone. He suggested that the area of increased loading builds new bone and regions of decreased loading removes bone. This law was subsequently developed by Roux as the idea of functional adaptation in bone [170]. Harold Frost [171] has explained in his popular mechanostat hypothesis that the loading is the controlling factor which determines the bone structure and shape.

2.7.2 Mechanical loading and role of osteocytes

Mechanical loading affects bone development and differentiation. Mechanical loading increases bone mass [172, 173] and unloading or immobilisation of the bone leads to a loss in the bone mass [174, 175]. The bone cells which can sense this loading include osteoblasts and osteocytes.

The osteocyte is the most sensitive cell that can experience shear stress compared to osteoblasts or fibroblasts [26] and it also comprises 90-95% of the total cellular mass in bone [176]. Due to their sheer number and interconnectivity, osteocytes are likely the major player in the mechanosensation process [177]. Reduced mechanical loading has been found

to cause osteocyte apoptosis *in vivo* [145], which ultimately leads to a reduction in its mechanosensory activity in bone.

Osteocytes communicate with other bone cells by two types of mechanisms. One mechanism is a diffusion of molecules about 1 kDa through gap junctions (connexin 43 is the most common gap junction protein in the osteocyte). Another mechanism allows communication through paracrine signalling in which molecules such as prostaglandins (PG), Adenosine triphosphate (ATP), Fibroblast growth factor 23 (FGF23) and RANKL are secreted by osteocytes and transported to other bone cells [7]. The dendrites can reach to the bone marrow and interconnect with other marrow cells [178] which indicates that osteocytes have far greater range compared to the other bone cells.

The most potent mechanical stimulus that the osteocytes respond to is interstitial fluid flow. The mechanical stimulus is primarily received by the body in the form of muscle contraction. First, muscle contraction leads to bone deformation. These mechanical load experienced by the bone is directly proportional to the mass of muscle that is contracting. Mechanical loading causes compression of the calcified matrix and the region of lacuna-canalicular space which house osteocytes and their dendritic processes [179-181]. This extravascular pressure drives the extracellular fluid to low-pressure regions [182]. The exact mechanism by which osteocytes sense the mechanical loading is still unclear, but it is believed that the osteocytes experience shear stress because of fluid flow pressure that distorts these cells and dendrites within the lacunar canalicular system. Three different hypotheses are suggested based on three different parts of the osteocytes. These are responsible for processing mechanical stimuli into the biochemical signals that regulate various downstream signalling pathways.

The effect of loading on bone cells varies greatly depending on the location at which these cells exist [183]. These cells experience fluid shear stress in the interstitial fluid ranging from 8 to 30 dyne/cm^2 [41].

Osteocytes evolved from the mature osteoblast entrapped inside the osteoid matrix [184], but they are also more sensitive than the osteoblast precursor, responding profoundly to shear forces in the interstitial fluid compartment [185]. Osteocytes sense mechanical stimuli received from mechanical loading using three different anatomical features of the cells. After receiving mechanical signals, osteocytes convert them into biochemical messages directing the differentiation and recruitment of osteoblasts and osteoclasts. The exact mechanism by which osteocytes sense different stimuli and secrete or express different types of molecules remains unclear. It is believed that they may sense through deformation of the cell body [186], through dendritic processes (dendrites) [187] or bending of specific solitary ciliary extensions [177].

- Integrins: It has been found that there are some extensions from the cytoskeleton to the extracellular matrix that sense the signal from fluid shear stress. The cell extensions then amplify this signal from FSS, ten times inside the cell [188]. Later these cell extensions were confirmed as Integrins [189]. It is thought that fluid flow stress induces mechanosensation in osteocytes through perturbation of integrins which when excited by shear stress, open hemichannel (connexion 43 (CX43) in osteocytes) to release prostaglandin E2 [190-192]. It is postulated that Integrins are mechanosensors responsible for converting the mechanical signal into a cellular response [193, 194]. Integrins are major receptors and transducers that help in communication with the outside of cells and various cell membrane-bound proteins. Studies [195, 196] indirectly support the idea that integrin conformation is modulated by applied force.
- Cell body: Fluid shear stress is experienced by the cell processes and cell body similarly [197] but Anderson et al [198] refuted this by presenting a finite element model of the osteocyte. He showed that while shear stress exists on the cell process, the cell body is affected by fluid pressure only within the lacuna and the highest stress exists where the

process joins the cell body. However, it is not known yet, whether the cell body or the cell process is stimulated *in vivo* that results in mechanotransduction.

- Cilium: Cells extend a non-motile primary cilium that protrudes from the cell surface. Cilium has been proposed to work as signal receivers and sensors [199], by acting as a mechanosensory organelle [200]. Bending of this cilium by the response to fluid shear stress has been shown to increase intracellular calcium level [201] however it is also shown to work individually in bone cells [202]. Malone et al. demonstrated that the cilia in osteoblasts and osteocytes are required for responses to dynamic fluid flow independently from Ca^{2+} influx [202]. Mutation in the cilia-related gene, Pkd1 displayed a defect in bone development and decreased runx2 expression in mice [203].

2.7.3 Osteocyte in bone mechanotransduction

Converting mechanical signals into cellular biochemical reactions in a series of signalling pathways is called mechanotransduction. The osteocytes respond to these mechanical signals by releasing signalling molecules which will control the activity of osteoblasts and osteoclasts in the bone. Due to their sheer number, interconnection and location they are considered a major player in mechanotransduction.

Mechanical loading is a controlling factor responsible for the molecular and physical adaptation of bone. Short term loading induces (seconds to minutes) ATP secretion, increases intracellular calcium whereas long term loading (minutes to an hour) causes the secretion of NO and PG [204, 205] in osteocytes and osteoblasts. There are numerous factors secreted by osteocytes in response to mechanotransduction. These are

- ATP: Chloride-conducting channels [206, 207], gap junctional hemichannels [37, 208], and vesicular transport [209, 210] are suggested for the mechanism of secretion of ATP, however, it is not understood properly yet.

- PG: The release of prostaglandins (PG) is essential for cell proliferation, differentiation and survival.
- NO: Nitric oxide (NO) has a different action in different systems as such in the neuronal system, in blood pressure control, during inflammation and bone mass regulation [211-215].

2.7.4 MLO-Y4 cells as a model for osteocytes

The murine long bone osteocyte Y4 (MLO-Y4) cell line has been selected for our experimental work. This cell line is the selected cell line for assessing the function of human osteocytes. MLO-Y4 cells exhibit identical characteristics of osteocytes *in vivo* in terms of secretion of various markers specific to osteocytes. MLO-Y4 cells express a low amount of alkaline phosphatase (ALP) activity but a high secretion of osteocalcin. Similarly, this cell line shows high expression of connexion 43 (CX43), a type of gap junction protein and the antigen E11 [216]. This cell line also possesses dendrites which are identical to osteocytes [217]. These cells secrete a very low amount of sclerostin, compared to osteocytes [218]. It has been confirmed that these cells are highly sensitive and useful for studying the release of signalling molecules such as ATP, NO, PGE2 and calcium in response to fluid flow experiments [184, 219]. These cells are used in our fluid shear stress experiments detailed in the methodology section (Chapter 3).

2.8. Fatty acid overview

Fatty acids (FA) are the simplest form of lipids. These are a long chain of hydrocarbon with a carboxylic acid at the terminal end. Glycerol or other compounds serves as a backbone structure and incorporate fatty acid in it. There is rarely a fatty acid found in a free state. Most naturally occurring fatty acids except in marine animals are even numbers of carbon atoms because the synthesis of fatty acids occurs in a sequential addition of two-carbon units.

Fatty acids can be classified into saturated and unsaturated based on the double bonds it contains in their carbon chain. If all the carbon bonds are single, then the fatty acid is termed as saturated and if it contains one or more double bonds then it is called an unsaturated fatty acid. Unsaturated fatty acids are more prevalent in plants compared to saturated fatty acid. The saturated fatty acid can pack closely to form a rigid structure but unsaturated fatty acids prevent such close structure and tend to be more fluidic and random.

The unsaturated fatty acids can be further divided into omega-3 and omega-6 fatty acids. First double bond occurring to carbon unit number is counted from the methyl end to determine the nature of unsaturated fatty acid. In omega-3 fatty acid double bond starts at the number three-carbon units, whereas if the double bond starts at carbon number 6, then the fatty acid is termed as an omega-6 fatty acid. Linoleic acid (LA; 18:2n-6) and arachidonic acid (AA; 20:4n-6) are the examples of omega-6 fatty acids whereas α-Linolenic acid (ALA; 18:3n-3), stearidonic acid (SDA; 18:4n-3), Docosapentaenoic acid (DPA; 22:5n-3), Docosahexaenoic acid (DHA; 22:6n-3) and Eicosapentaenoic acid (EPA; 20:5n-3) are examples of omega-3 fatty acids. Omega-3 FA is found mostly in oily fish and green leafy vegetables. Vegetable oils, nuts, seeds and beans are the best sources for omega-6 FA.

2.9. Fatty acid metabolism

General fatty acid (FA) oxidation and synthesis are both metabolic processes of FA but reaction steps are reverse to each other.

In FA oxidation, long-chain aliphatic carbon chains are broken down into activated acetyl CoA which is utilised in the Krebs cycle. Whereas in synthesis, monomeric activated acyl CoA (acetyl CoA) and Malonyl CoA condense to form long-chain aliphatic carbon compounds in a series of steps. The formation of Malonyl CoA is the prerequisite step or rate-limiting step in the fatty acid synthesis. Fatty acids are synthesized in the cytosol, while fatty acids are broken down in the mitochondrial matrix. Fatty acids are synthesized by the repetition of condensation, reduction, dehydration and reduction reaction sequences. The reductant in FA synthesis is NADPH, but the oxidant in FA degradation are NAD^+ and FAD.

2.10. Metabolism of omega-3 and 6 fatty acids

Lipid is taken in the form of triacylglycerols [220] in larger amount whereas cholesterols, phospholipids etc. are taken in a smaller amount in the diet. Fatty acids which are essential to vertebrates, cannot be synthesised inside the body so needed to be provided in the diet are called essential fatty acids [221]. Humans cannot produce alpha-linolenic acid (ALA) or linoleic acid (LA) which are the precursor molecules for the omega-3 and omega-6 fatty acids respectively because the body cannot insert double bonds beyond carbon number 9 [222]. Vegetable oils and egg yolk are a good source of linoleic acid whereas linolenic acids are found in oils, fish and liver.

The desaturation and elongation reactions occur in both omega-6 FA and omega-3 FA families (Figure 2-4). The LA and ALA have an opposing function and generally compete for the same enzyme involved in the elongation desaturation process [223-225]. LA and ALA act as the precursors which convert to various eicosanoids and docosanoids through different elongation and desaturation steps by help of various enzymes [226].

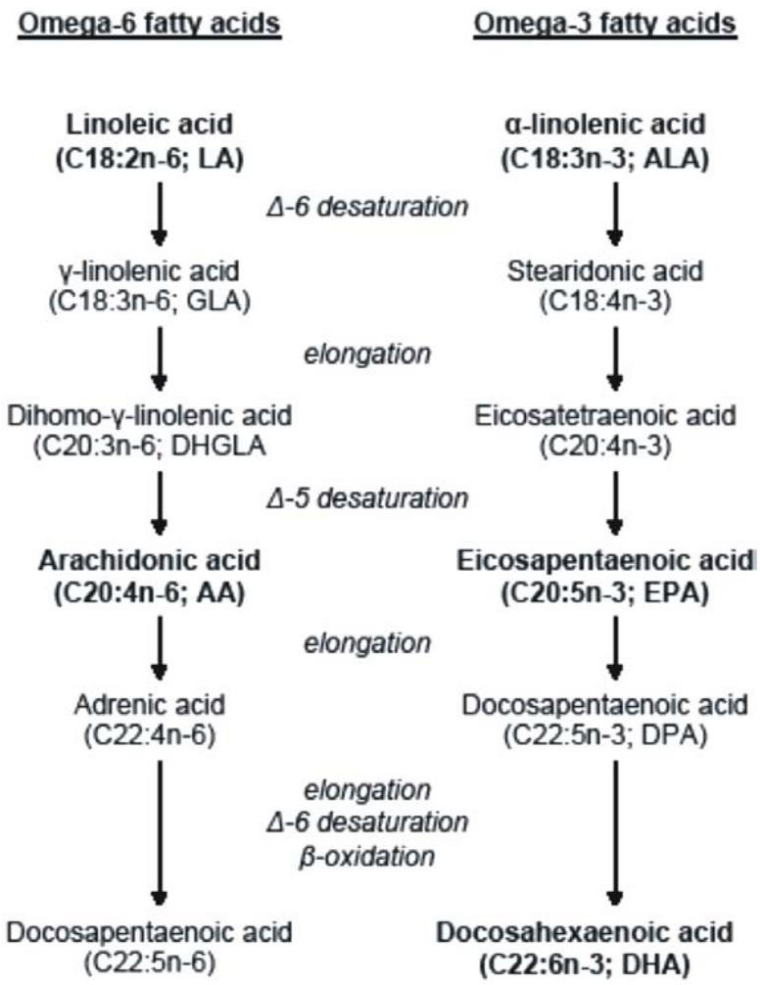

Figure 2-4: Metabolic pathway of the omega 3 and omega 6 fatty acids [227].

2.11. Effect of dietary fatty acids on bone health and diseases

Dietary intake of omega-3 and omega-6 FA has proved to be a crucial factor in promoting good bone health in animals and humans. A diet rich in omega-3 FA leads to greater bone density and mineral content in free-range laying hens [54] and young piglets were found to have improved bone mineral density when fed with a diet rich in omega-6 FA [55]. Improved supplementation of omega-3 FA decreases N-telopeptides (NTx) which is a bone turnover marker which indicates bone loss in humans [228]. Omega-3 and 6 FA supplementation are found to have a beneficial effect in bone mineral density of postmenopausal women [229-232]. Omega-6 fatty acid together with omega-3 fatty acids show a protective effect on bone in postmenopausal women compared to the placebo group [233]. However an increased ratio of omega-6/omega-3 food intake is shown to increase PGE2 [234, 235], lower BMD [236], possibly due to the modulation of mesenchymal cells towards adipogenesis inhibiting osteoblastogenesis [237].

Long-chain polyunsaturated fatty acids are very important lipid components in all tissue membranes. They are responsible for the membrane fluidity, flexibility and permeability, which will allow transportation of various molecules [238]. Anomalies in the fatty acid constituents of the cell membrane are found to cause decreased membrane fluidity in NP-C fibroblasts cells [239]. Irregular metabolism of EFAs has been associated with diseases such as cystic fibrosis [240], Crohn's disease [241], acrodermatitis enteropathica [242], Sjogren syndrome [243], cirrhosis and Reyes syndrome. There is evidence that omega -6 fatty acids such as linoleic acid, gamma-linoleic acid and arachidonic acid enhance cancer and metastasis [244] whereas DHA and EPA are shown to inhibit the growth of tumours and metastasis [245] so proper dietary intervention is required [246].

2.12. The effects of DHA and AA on bone cells

Maurin et al [247] studied the effect of dietary fatty acids, DHA and AA on human primary osteoblastic cells. They showed that these fatty acids inhibit osteoblastic proliferation through cell cycle arrest in the G1 phase. They further discovered that these FAs directly activate PPARy and disturb osteoblast function in age-related bone loss patients [248].

Coetzee et al [249] studied the effect of AA and DHA on osteoblastic cell lineage MG63 and MC3T3-E1 and found a similar trend, that these fatty acids inhibited the proliferation of MG63 and MC3T3-E1 cells. Kruger et al [2] suggested that omega-3 FA improves bone mass by increasing the number of mesenchymal stem cells and committing them towards osteoblasts formation. Omega-3 FA also inhibits osteoclastogenesis by downregulation of Nuclear Factor-KappaB-Inducible Nitric Oxide Synthases (NF-κB-iNOS) signalling [250], suppressing TNFα [251] and RANKL signalling [252] whereas omega-6 improves bone mass by inhibiting inflammatory cytokines [253].

Nakanishi et al [254] claimed in their studies that dietary omega 3 fatty acids cause suppression of TNF, NFkB, MCSF and RANKL which inhibit the function of the osteoclasts and prevent excessive bone resorption. Similarly, Boeyens [255] has shown that AA and DHA are both effective in the inhibition of RANKL induced osteoclast formation in RAW264.7 cells with DHA having the strongest inhibitory effect. They claimed that these polyunsaturated fatty acids can be important in controlling bone loss resulting from hyperactivity of osteoclasts. Another study by Kasonga et al [256] showed that AA and DHA inhibit various osteoclastogenic markers such as CTSK, TRAP and MMP-9 affecting osteoclast differentiation and activity. In a study by Casado-Diaz indicated that the omega 6 AA fatty acid favours mesenchymal cells to commit towards adipogenesis more readily than omega-3 EPA and DHA fatty acid. This adipogenic activity of AA is independent and the presence of EPA and DHA together does not affect it [257].

Chapter 3. Materials and methods

3.1. Reagents

Minimum Essential medium α (α-MEM) containing sodium pyruvate, Phenol red & L-glutamine (Gibco Cat# 12571-063), Collagen I rat protein (3 mg/ml; Gibco Cat# A1048301), heat-inactivated Fetal Bovine Serum (FBS; Gibco Cat# 10082-147), Penicillin-Streptomycin (10,000 U/ml; Gibco Cat# 15140-122), and Trypsin EDTA (0.05%; Gibco Cat# 25300-054) were all purchased from Thermo Fisher Scientific, Auckland, New Zealand. Iron-enriched bovine calf serum (CS; Hyclone Cat# SH30072) was purchased from Invitro Technologies, Auckland, New Zealand.

An ATP bioluminescent assay kit (FLAA, Sigma-Aldrich, Sydney, Australia) was used for estimation of ATP secretion in cell culture media samples.

The sterile PBS solution used in all experiments consisted of 136.89 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4, 1.76 mM KH_2PO_4 (all Sigma-Aldrich, Sydney, Australia) and was adjusted to pH 7.4. Sterile filtered water was sourced from a Milli-Q system (Millipore, Milford, MA, United States of America) and autoclaved at 121°C for 40 min.

A cell lysis buffer (all reagents Sigma-Aldrich, Sydney, Australia) was used to lyse the cells at the end of the fluid shear stress experiment. The buffer comprised 10 mM Tris, 25 mM β-glycerophosphate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X100, 1 mM DTT, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonyl fluoride (PMSF), and a Protease Inhibitor Cocktail (Sigma, Cat# P2714) and adjusted to pH 7.5.

3.2. Culture of MLO-Y4 cells

All cells were handled in a biological safety cabinet (BSC; Hera Safe 2020 1.2, Thermo Fisher Scientific, Auckland, New Zealand) in a physical containment level 2 (PC2) laboratory at Massey University, Palmerston North.

Murine long bone osteocytes Y4 (MLO-Y4) cells, derived from transgenic mice (kindly gifted by Prof. Lynda Bonewald; Indiana Center for Musculoskeletal Health, Indianapolis, USA), were seeded on sterile collagen-coated 150 mm culture dishes (Nunc TM 168381, Thermo Fisher Scientific, Auckland, New Zealand) suspended in α-MEM medium, supplemented with 2.5% FBS, 2.5% CS and penicillin/streptomycin (PS) at 1%. Cells were grown in an incubator at 37°C with 5% CO_2 until cells reached 70-75% confluence. If growing cells from frozen stocks, α-MEM medium with 5% CS, 5% FBS with 1% PS was used. Cells were sub-cultured when the cell culture reached 70-75% confluence and undertaken at a 1:5 sub-culture of the trypsinised cell suspension. For regular cell passaging (sub-culturing) and maintenance, 30 ml of α-MEM containing 1.5×10^4 cells per ml was added to each 150 mm dish. Cells were not used when the passage number exceeded 45.

For fluid shear stress experiments requiring cells on collagen-coated glass slides, cells were seeded onto slides as follows. The ”same” batch of cells were used for each experiment containing test and control cells. Forty-five ml of α-MEM supplemented with 2.5% FBS, 2.5% CS and PS at 1% containing 1.5×10^4 cells per ml was added to a 150 mm culture dish containing four collagen-coated slides (75 × 25 × 1 mm, Cat# 180626, Flexcell International Corporation, Burlington, NC, USA). Dishes were then covered and left in an incubator at 37°C with 5% CO_2 until cells reached 70-75 % confluence (6-8 days).

Cell cultures were observed using an inverted microscope when required (Leica DM IL LED, Leica, Germany).

3.3. Collagen coating of culture dishes and glass slides

MLO-Y4 cells require a substrate coated with collagen I to express an osteocyte-like phenotype. Sterile 150 mm culture dishes were coated with collagen by adding 15 ml of sterile collagen solution (Collagen I at 0.15 mg/ml in 0.02 M acetic acid). For FSS experiments, collagen-coated glass slides were placed inside sterile 150 mm culture dishes. One ml of collagen solution (same as for 150 mm plates) was added to each glass slide to cover the exposed top surface of each slide.

Culture dishes and slides were kept in the BSC for one hour at room temperature. After one hour, any excess collagen was aspirated, and the dishes or slides were left to air dry in the BSC. When completely dry, coated dishes or those containing slides were sealed with paraffin tape, labelled and stored at 4°C for 1-2 months or used immediately. Prior to use, coated dishes and slides were washed with sterile PBS to remove any trace amounts of acetic acid.

3.4. Lipid treatment studies for MLO-Y4 cells

In order to expose MLO-Y4 cells to dietary fatty acids (FAs), FAs were solubilised in a solvent that could be added to the cell culture medium. Docosahexaenoic acid (DHA) and arachidonic acid (AA) were solubilised in 100% ethanol while palmitic acid (PA) was solubilised in 10 mM NaOH, heated to 70°C, with 10% BSA added when cool. Appropriate FA test concentrations that the MLO-Y4 cells were exposed to were previously determined by Léa Ribet (2017; unpublished results). Briefly, cell viability was measured in MLO-Y4 cells exposed to a wide range of concentrations of either DHA, AA or PA. Concentrations equal to or less than 20 μg/ml did not have any detrimental effect on cell viability and were therefore deemed appropriate for use with the cells.

For the treatment of cells, DHA and AA were added at a concentration of 20 μg/ml to cells grown on slides (in culture dishes) while PA was added at a concentration of 0.2 mM. The

cells in control groups were treated with vehicle alone (solvent without FA). The amount of solvent vehicle present in culture with the cells was well below that previously documented as causing cytotoxicity or a loss of viability of the cells. All the cell groups with or without DFA containing media were cultured for five days in an incubator at 37°C with 5% CO_2 prior to FSS experiment.

3.5. Exposing MLO-Y4 cells to fluid shear stress (FSS)

All fluid shear stress (FSS) experiments were conducted using a Flexcell Streamer™ device (Flexcell International Corporation, Burlington, NC, USA). The Streamer™ device, Masterflex L/S peristaltic pump (Cole-Palmer, USA), and associated tubing were purchased from Flexcell International Corporation and the standard configuration of the fluid flow experiment can be seen in Figure 3-1.

The associated tubing and their measurements were as follows: two lengths of silicone tubing (length 0.6 m), one length (0.9 m) of Phar Med tubing and one length of silicone tubing (0.076 m).

Fluid shear stress experiments were conducted in a cell culture incubator (Heracell 150i, Thermo Fisher Scientific, Auckland, New Zealand). The Streamer device and Masterflex pump were placed in the incubator (37°C, 5% CO_2) for at least 20 minutes before starting an experiment to equilibrate the temperature and gas environment of the system and solutions. Prior to this, sterile PBS was run through the fluid circuit in the apparatus for several minutes to flush the system before the PBS was discarded, and the device was run with fresh medium to displace the PBS.

Six slides covered with adherent MLO-Y4 cells on one side were inserted into the Streamer™ device in the following manner. Briefly, the flow of the medium in the circuit was reversed so that the top 20% of the streamer was void of the medium. This minimised air bubbles being trapped inside the device during the slide insertion process. After all six

slides were inserted into the device, the lid of the device was replaced and bolted in place, and fluid flow was restored in the correct direction. This method was found to be effective in eliminating any bubbles trapped inside the inlet or outlet ports on the Streamer^{TM}. Fluid shear stress values of 0 to 20 dyn/cm^2 were used for two hours duration for the dose and time response experiment, whereas 1 to 20 dyn/cm^2 were used for dietary fatty acid treatment experiments. Cells at 0 dyn/cm^2 were placed in culture dish with fresh media and same environment similar to test for duration of time and were not exposed to fluid shear stress. The 0 dyn/cm^2 treatment was also replaced in dietary fatty acids treatment experiments with 1 dyn/cm^2 as we discovered a minimal amount of fluid movement in the Streamer^{TM} circuit was important to collect media that the cells had been exposed to. We found that 30 minutes of time was the optimal duration time for the lipid treatment study so that sampling time was used for subsequent experiments. Cells treated with FA and in the absence of FA were exposed FSS of 1 or 20 dyn/cm^2. Samples of cell culture media circulating in the Flexcell Streamer^{TM} device were collected at 0, 5 and 30 minutes after the commencement of FSS.

Samples of cell culture medium were collected at the time points described above to allow quantification of ATP secretion by the MLO-Y4 cells. Briefly, 1 ml samples of cell culture medium were collected at each sample time during fluid shear stress experiment using a sterile 1 ml syringe inserted into the length of silicone draining the Streamer^{TM} device, this was done for both test and control treatments. All samples of cell culture medium were kept on ice and centrifuged at 10,000 g for 2 min at 4°C to remove any cellular debris and the supernatant was snap-frozen and stored at -80°C until further analysis.

At the end of each experiment, MLO-Y4 cells on all slides were either treated with 1ml of 0.05% Trypsin EDTA for cell counting or treated with 500 µl of ice-cold lysis buffer for protein extraction. An aliquot of the cell suspension was diluted (10-fold) in 0.2% Trypan Blue (Sigma-Aldrich, Sydney, Australia) and the cell number quantified using a haemocytometer.

Lysed cells were scraped off the slides and collected along with the lysis buffer into individual Eppendorf tubes for each treatment. Sample tubes were kept on ice and then centrifuged at 15,000 g for 15 min at 4°C. The supernatants were collected and frozen at -80°C for further analysis.

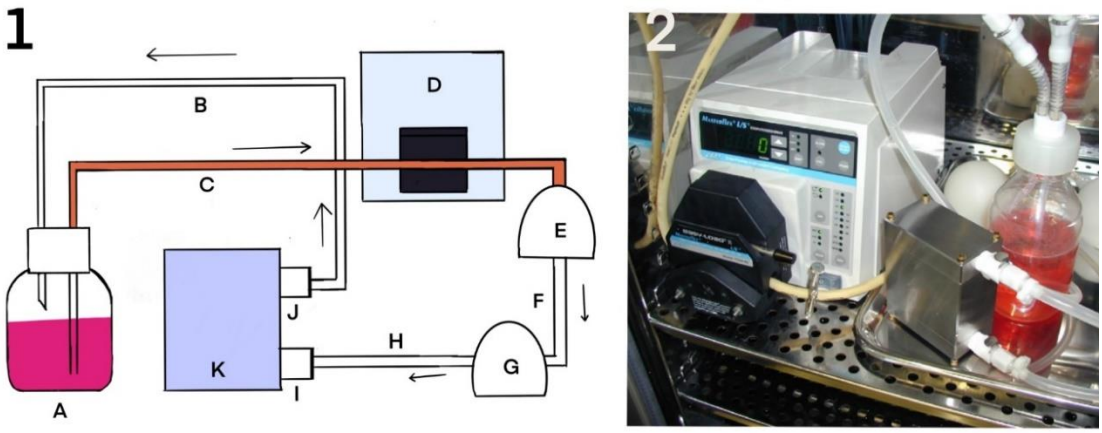

Figure 3-1: 1) Outline of the streamer device system.
A. Cell culture media reservoir bottle, B. Silicone tubing, C. Phar Med tubing, D. Masterflex peristaltic pump, E. Pulse Dampner #1, F. Silicone Tubing, G. Pulse Dampner #2, H. Silicone tubing, I. Inlet port, J. Outlet port, K. StreamerTM device. Arrow indicates the direction of fluid flow. 2) StreamerTM device in operation. (Adapted from: Flexcell Streamer Users Manual, April 2011).

3.6. ATP assay

ATP was measured in cell culture medium samples collected from MLO-Y4 cells exposed to FSS or controls as has been previously described [38, 135, 258]. The method of quantifying ATP uses an assay that is based on the Luciferin-Luciferase reaction. This assay technique is based on the luciferases requirement for ATP in producing light (emission maximum ~560 nm at pH 7.8).

The concentration of ATP secreted into the cell culture media was measured using a commercially available ATP bioluminescent assay kit (FLAA, Sigma-Aldrich, Auckland, New Zealand). The kit contained (1) an ATP standard (FL-AAS), an (2) ATP assay mix (FL-AAM)

consisting of lyophilized powder containing luciferase, luciferin, magnesium sulphate ($MgSO_4$), 1,4-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), and tricine buffer salts and (3) an ATP assay buffer (FL-AAB): lyophilized powder containing $MgSO_4$, DTT, EDTA, BSA, and tricine buffer salts.

Briefly, one vial of ATP assay mix was mixed with 5 ml of sterile Milli-Q water and mixed gently until dissolved and then kept on ice for at least one hour. The ATP assay mix solution was then aliquoted into smaller volumes and frozen for future use. This solution was stored at 4°C for up to 2 weeks.

In preparation for measuring ATP in samples, an undiluted vial of ATP assay dilution buffer was dissolved in 50 ml of sterile Milli-Q water and aliquoted into five 10 ml glass test tubes. A suitable volume of thawed ATP assay mix was then mixed with the ATP assay dilution buffer in a 25-fold dilution. This dilution rate permits an ATP detection range of 2×10^{-10} to 2×10^{-7} moles/litre (as recommended in the manufacturer's protocol). An ATP standard curve was created by making a serial dilution of the supplied ATP standard (3.90, 7.81, 15.625, 31.25, 62.5, 125, 250, 500, 1000 nM).

ATP assays were conducted in flat-bottomed 96 well plates. One hundred µl of ATP assay mix diluted in ATP dilution buffer was dispensed to the required number of wells. Following this, 100 µl of each ATP standard (including a blank) and unknown samples were added to wells containing ATP assay mix. All standard and unknown samples were analysed using four replicates including a blank containing ATP assay dilution buffer. The assay plate was then placed on an orbital shaker and allowed to gently mix at room temperature (RT) for 3 min. After 3 min, the luminescence signal of the plate was measured using a luminometer plate reader (Fluoroskan Ascent FL, Thermo Fisher Scientific, Auckland, New Zealand). The luminance values were used to generate a standard curve which could be used to determine the ATP concentration of the unknown samples. The standard curve and determination of

ATP from unknown samples were conducted using Microsoft Excel (2010) and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

3.7. Counting of cells

Cells were briefly washed using the 1XPBS while still on the slides. Cells were treated with 1 ml of 0.05% Trypsin EDTA and placed in an incubator at 37°C in 5% CO_2 for 5 min. The detached cells were collected in a vial and diluted (10-fold) with 0.2% Trypan Blue. Cell numbers were then measured using a haemocytometer. Using the cell number for each sample, the amount of ATP secreted was expressed per million cells.

3.8. Protein assay

The protein concentrations of the cell lysates collected from FSS experiments was measured using the Bio-Rad DC protein assay kit (Bio-Rad, Cat# 500-0116). This is a colourimetric assay for protein concentration following detergent solubilisation. The overall principle is similar to the Lowry assay [259] but with a few modifications. Briefly, the assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps which lead to colour development: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein [259]. Colour development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine [259, 260]. Proteins affect the reduction of the Folin reagent by loss of one, two or three oxygen atoms, thereby producing one or more of several possible reduced species which have a characteristic blue colour with maximum absorbance at 750 nm and minimum absorbance at 405 nm.

For determining the protein content of cell lysates, serial dilutions (31.25, 125, 250, 500, 1000, 2000 µg/ml) of a protein standard (2 mg/ml; Bio-Rad) were prepared and mixed with

the assay reagents before the absorbance was measured using a Nanophotometer p300, Implen. Colourimetric data was plotted for the concentration of protein against absorbance and used to create standard curves. Then the protein content of unknown cell lysate samples was determined using this standard curve. Normalization of ATP values measured in cell culture medium samples was undertaken using the protein values obtained for each sample. Any variations in protein content in each sample due to differences in total cell lysate volume were corrected by multiplying protein concentration with the volume of lysate collected. Then the amount of ATP was divided by the amount of protein to get an amount of ATP per mg of protein.

3.9. Statistical analysis

The statistical analysis was done using Microsoft Excel (2010) and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Student T-tests and analysis of variance (ANOVA)were performed to compare between fatty acid treatment, fluid shear stress and time. Means and standard errors were calculated and analysed between different treatment groups at various fluid shear stress and time points.

Chapter 4. Results

4.1. Introduction

Mechanical loading is a crucial factor in the maintenance of bone homeostasis and structure. Bone cells, in response to mechanical loading release signalling molecules and participate in the bone remodelling process. These signalling molecules stimulate a range of other bone cells such as osteoblasts, which enhances the bone formation process. Osteocytes are one of the key players in this process as they have a larger population and deeply embedded habitat. These cells are sensitive to mechanical loading and secrete different signalling molecules in response to mechanical loading. Depending on the level of physical and mechanical activity or shear stress, these secreted molecules activate cell signalling pathways in osteoblasts and osteocytes in a process called mechanotransduction. People who are physically inactive or in induced microgravity have shown diminished bone structure because of reduced mechanical loading they bear in their skeleton [261, 262]. Studies show that dietary supplementation of omega 3 and 6 fatty acids is beneficial in maintaining bone health but the exact mechanism is unclear [236, 263]. The role of these fatty acids in the release of ATP by fluid shear stressed were studied on MLO-Y4 cells. The main objective of the study was to check the effect of different dietary fatty acids, mainly DHA and AA on the secretion of ATP by MLO-Y4 cells in response to fluid shear stress. The general hypothesis of the study was that the dietary fatty acids, more specifically omega-3 (DHA) and omega-6 (AA) might have a positive effect on bone health when combined with physical activities. To test this we treated MLO-Y4 cells with different fatty acids and measured the level of ATP secreted by these cells due to increased mechanotransduction in response to fluid shear stress.

4.2. Method development

It is very difficult to create an environment similar to that experienced by osteocytes *in vitro.* However, the MLO-Y4 osteocytic cell line was chosen and treated with different FA in a simulated environment *in vitro*. MLO-Y4 cells were exposed to different degrees of fluid shear stress and the cell culture medium collected for evaluation of secreted ATP. By doing this, this study tried to find out if the treatment of MLO-Y4 cells with different fatty acids under fluid shear stress had any role in the modulation of ATP secretion.

4.3. Calibration of flow rate

Calibration of the flow rate through the Flexcell Streamer fluid circuit by a peristaltic pump was done every time before starting a new experiment. It was done to evaluate the accuracy of the pump. The accuracy of the pump to deliver a known flow rate through the fluid circuit was checked. The volume of water passing through the circulation and pump for a set time point was measured with a certain flow rate. We then cross-checked the volume of water with expected volume specified in the instrument manual. Manual suggested a list of flow rate based on the amount of shear stress required. Any fluctuation in the volume was corrected by adjusting the flow rate in the pump. For example, we needed 10 ml per minute of flow rate to get 5 dyn/cm^2 of shear stress as suggested by the manual. We set pump for 10 ml/min and checked the volume. We found out that the device was pumping 13 ml per min. When we set the flow rate to 8 ml/min, the device was pumping 10 ml/min which was required to get 5 dyn/cm^2 of shear stress. In such a manner other shear stress values were established by correcting the flow rate and used for our study.

4.4. Dilution adjustment

Initial experiments showed that the alpha MEM medium contained Phenol red and other unknown inhibitory substances that affected the ATP assay so the MEM medium was diluted with Milli-Q water to determine the optimal concentration that could be used in

the assay as shown in Figure 4-1. For the 1,000 nM ATP standard, the assay luminance increased with the increasing dilution with water. Standard is not diluted but the medium on which standard prepared is diluted. In our case, we prepared 1000 nM of standard in several tubes with different dilutions of the medium with water. We found that increasing the water composition in the medium increased the luminance. Which implied that there was some unknown substance that inhibited luminance. In subsequent experiments, samples were diluted 2:8 (or 1:4) as this dilution had luminance similar to the 1:9. As this experiment was mostly based on detecting ATP in the collected medium, diluting 1:9 would have possibly made it too dilute and 2:8 would fairly represent the amount of ATP. A new standard curve (Figure 4-2) was created every time during an ATP assay.

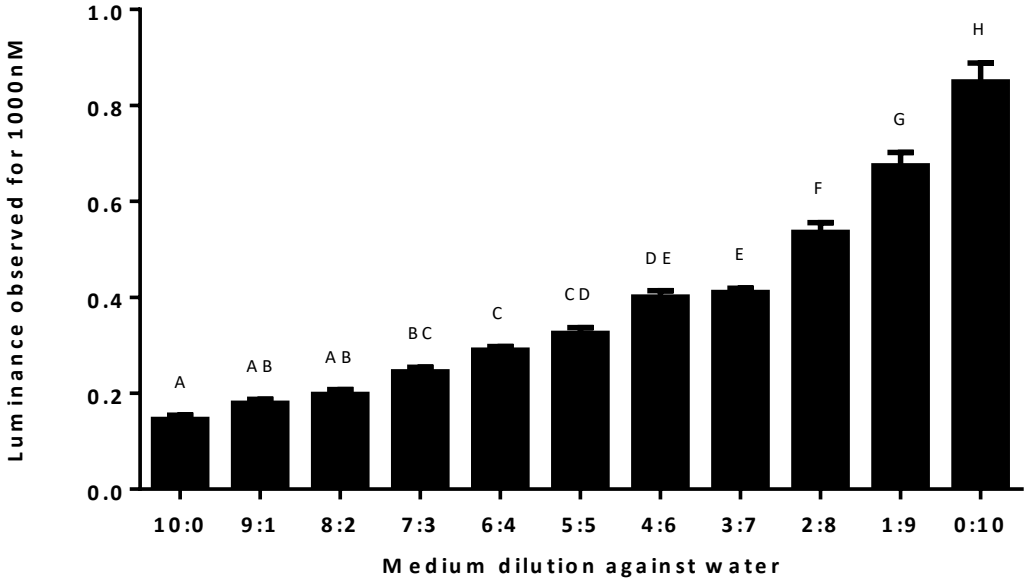

Figure 4-1: ATP standard with concentration 1000 nanoMolar (nM) at various dilutions of medium.

The 1000 nM ATP standard prepared with various dilutions of cell culture medium with water. Then the luminance of the ATP of the various 1000 nM solutions was measured. From left to right on the X-axis is the ascending ratio of the water content. The luminance was seen lowest in the medium solution where there is no water content and highest in the dilution where there is no medium. Levels not connected by the same letter were significantly different. All data were mean ± SEM, n=3.

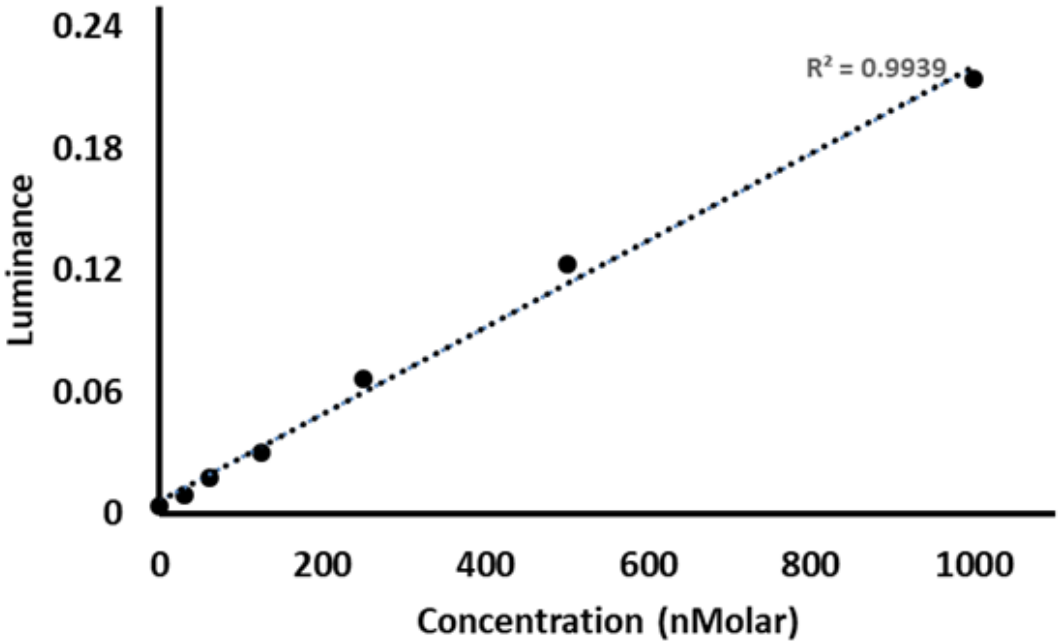

Figure 4-2: A typical standard curve for ATP assay.
Various concentrations of ATP were prepared by diluting a 1,000 nM standard into various concentrations. Then these standard solutions were checked for the luminance using a luminometer. A standard curve was prepared every time during a new ATP assay. Luminance was seen highest in the highest concentration of ATP standard and vice versa.

4.5. Effect of fluid shear stress on the secretion of ATP by MLO-Y4 cells

Weinbaum et al [41] estimated the magnitude of the mechanical loading-induced fluid shear stress sensed by osteocytic processes to be in the range between 8 and 30 dyn/cm^2. A fluid movement system was created *in vitro* similar to fluid shear stress experienced by the osteocytes *in vivo* in a normal and loading condition. This part of the study sought to determine how the magnitude of fluid shear stress might influence ATP secretion in the chosen test system using MLO-Y4 cells. So the effect of fluid shear stress on ATP secretion in the medium by MLO-Y4 cells was checked. Fluid shear stress (FSS) *in vivo* experienced by the osteocytic processes ranges from 0-30 dyn/cm^2 [41], so MLO-Y4 cells were exposed to FSS and ATP secretion by MLO-Y4 cells in the medium exposed to FSS ranging from 0, 5, 10, 20 and 30 dyn/cm^2 was measured (Figure 4-3). The amount of ATP measured in the cell culture medium increased with the magnitude of FSS. ATP amounts in the medium were relatively constant in the control cells not exposed to FSS (0 dyn/cm^2) for 30 minutes. ATP increased within 5 minutes of FSS in cells exposed to 5-30 dyn/cm^2. ATP increased 3.75 - 4.94 nmol/10^6 cells at 2, 15 and 30 min in cells exposed to 5 dyn/cm^2. ATP increased to the range of 5.20 to 6.51 nmol/10^6 cells at 2, 15 and 30 min in cells exposed to 10 or 20 dyn/cm^2. ATP amounts were significantly higher in medium collected from cells exposed to 30 dyn/cm^2 increasing to 6.71 nmol/10^6 cells at 2 min and then to 8.83 and 9.07 nmol/10^6 cells at 15 and 30 min respectively.

There were no significant differences in the ATP in the medium collected at 10 and 20 dyn/cm^2 at any time point. The highest amount of ATP was seen in the medium collected from the cells exposed to 30 dyn/cm^2 which is significantly higher compared to ATP collected from 5 and 10 dyn/cm^2 at 2, 15 and 30 minutes. The ATP collected from the cells exposed to 30 dyn/cm^2 were also higher compared to 20 dyn/cm^2 in all time points except at 0 and 2 mins.

The amount of ATP in the medium across the FSS treatments at 2 min after the onset of FSS is shown in Figure 4-4. ATP amount were significantly elevated above the control at 5, 10, 15 and 30 min in a dose-dependent manner.

In these results, fluid shear stress was shown to stimulate the MLO-Y4 cells to secrete ATP. ATP was released within 2 minutes of the cells being exposed to fluid shear stress and the amount released remained steady for the duration of the exposure. Increasing the rate of fluid flow was found to increase the amount of ATP. At 2 minutes, amount of ATP at 0 dyn/cm^2 was significantly lower than the amount of ATP at 10, 20 and 30 dyn/cm^2 ($P<0.05$). The amount of ATP was increased in 5 dyn/cm^2 but the difference was not found to be statistically significant compared to 0 dyn/cm^2.

4.6. Time course of ATP release

In the dose-response experiment, the amount of ATP released was found to be greatest in cells exposed to 30 dyn/cm^2 and lowest in 0 dyn/cm^2. Weinbaum et al [37] estimated the magnitude of the mechanical loading-induced fluid shear stress sensed by osteocytic fluid movement processes to be in the range between 8 and 30 dyn/cm^2. The similar fluid movement comparable to the osteocytic environment in vitro was replicated and ATP secretion was measured in media collected from MLO-Y4 cells exposed to fluid shear stress. A fluid flow rate of 20 dyn/cm^2 was used for fluid shear experiments and not 30 dyn/cm^2 as this is at the upper borderline of the range that the osteocytic processes can experience in vivo. Also, 20 dyn/cm^2 gave a robust response but not at the maximum of the physiological range, thus allowing us to determine if cell treatments with fatty acids can decrease or increase the response.

The effect of exposure time on fluid shear stress on the ATP secretion by MLO-Y4 cells is shown in (Figure 4-5). The amount of ATP measured in the cell culture medium increased within a few minutes of the osteocytes being exposed to fluid shear stress and remained

at an 11.73 ± 4.57 nmol/10^6 cells throughout the two hours of FSS. ATP secretion did not change and remained constant at 1.59 ± 0.79 nmol/10^6 cells in the medium collected from control cells (0 dyn/cm^2). Higher amounts of ATP were observed at the 0 time point for the test. The reason for this was collection of the media at the start of the streamer device. It is possible to assume that immediately after running the device, fluid shear stress causes the MLO-Y4 cells to secrete the ATP. Analysis of the data showed that there were no significant differences in the amount of ATP secreted by the cells exposed and not exposed to FSS.

This was the primary experiment conducted to standardize the system and subsequent experiments. This experiment helped us to choose an optimal dose (20 dyn/cm^2) and time (30 minutes) for the subsequent experiments.

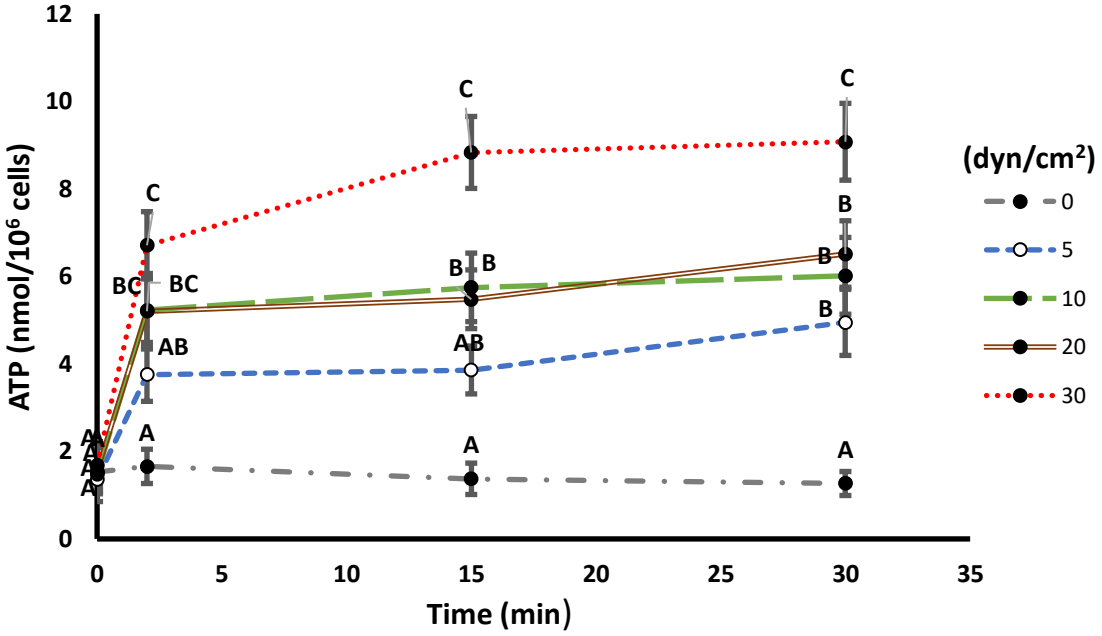

Figure 4-3: Figure showing the effect of various dosages of shear stress on ATP secretion in MLO-Y4 cells.

These cells were divided into five groups on the fluid shear experiment days based on the various fluid shear stress applied on MLO-Y4 cells, such as 0, 5, 10, 20 and 30 dyn/cm^2. Media samples were collected at 0, 2, 15 and 30 minutes and ATP was measured using the Luciferin-Luciferase assay. Letters at each time point indicate significance, where differences between letters at two points indicate significance ($P<0.05$). All data were mean ± SEM, n=3.

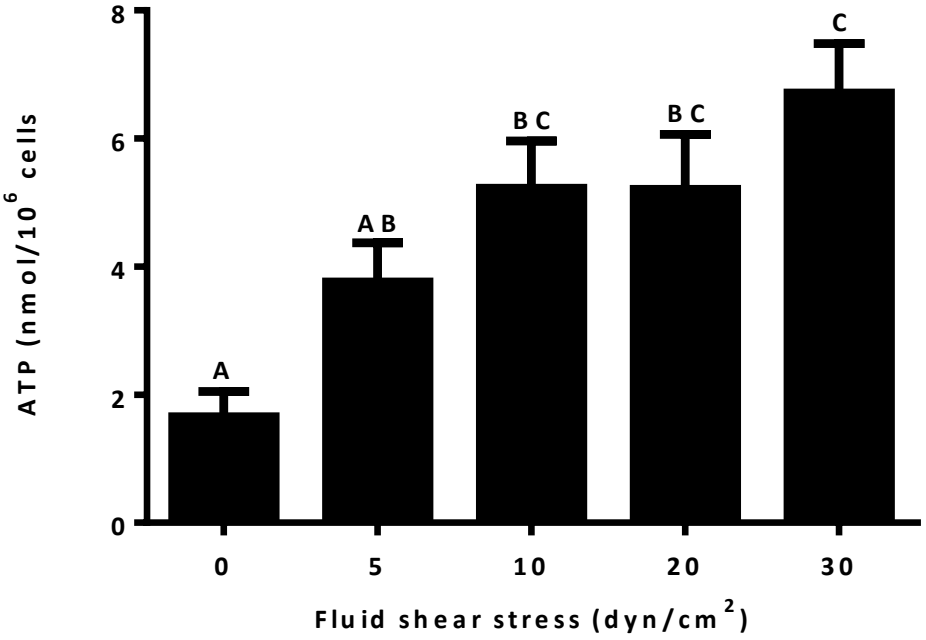

Figure 4-4: The effect of fluid shear stress on ATP secretion by MLO-Y4 cells in two minutes time point.
MLO-Y4 cells were divided into five groups on the basis of fluid shear stress they experienced, such as 0, 5, 10, 20 and 30 dyn/cm^2. ATP was measured using Luciferin-Luciferase assay in the collected media samples. Levels not connected by the same letters were significantly different ($P<0.05$). All data were mean ± SEM, n=3.

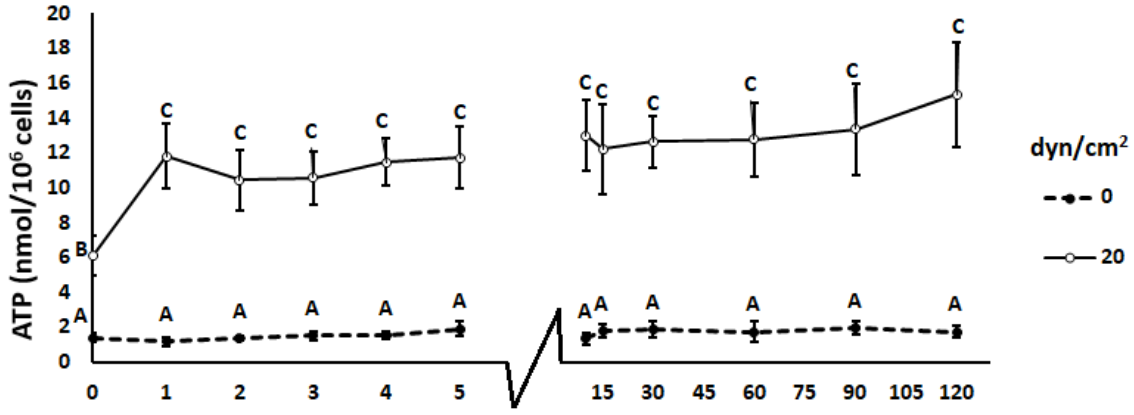

Figure 4-5: Detailed and extended time course graph showing the effect of fluid shear stress on MLO-Y4 cells for 120 minutes on ATP secretion.
MLO-Y4 cells were divided into two groups and exposed to either 0 dyn/cm^2 or 20 dyn/cm^2 for two hours. Samples of the medium were collected at the time points indicated in the figure. The medium was analysed for secreted ATP using the Luciferin-Luciferase assay. ATP values were normalised to the number of cells. All data were mean ± SEM, n=3.

4.7. Effect of Docosahexaenoic acid (DHA) on ATP secretion from MLO-Y4 cells exposed to fluid shear stress.

Omega 3 fatty acids, mainly DHA, are good for bone health in animals. There are studies on the effect of DHA and AA on other bone cells but no information on the secretion of ATP by osteocytes. To determine if the DHA has any effect on ATP secretion by osteocytes, osteocytes were treated with DHA at a concentration of 20 μg/ml, and its effect on ATP secretion was measured. MLO-Y4 cells cultured on slides were treated with DHA or without DHA containing medium for five days. The cells were exposed to fluid shear stress for thirty minutes on the day of the experiment as described previously in the methods section (Chapter 3). Both cell groups with or without DHA were exposed to 1 dyne/cm^2 and 20 dyn/cm^2 of FSS and medium was collected at 0, 5 and 30 minutes. The amount of ATP was determined by Luciferase assay. The effect of DHA on ATP secretion from MLO-Y4 cells exposed to fluid shear stress is shown in Figure 4-6.

At 0 minutes no significant differences were found among the DHA treated and non-DHA treated cells whether they were exposed to fluid shear stress or not.

ATP secretion in DHA treated cells exposed to 1 dyn/cm^2 rose from 0.77 ± 0.39 nmol/mg of ATP at 0 minutes to 2.47 ± 0.56 nmol/mg at 5 minutes. ATP secretion in DHA treated cells exposed to 20 dyn/cm^2 rose from 2.38 ± 1.63 nmol/mg at 0 minutes to 20.23 ± 9.33 nmol/mg protein in 5 minutes. Similarly ATP secretion in control cells treated to 1 dyn/cm^2 found to be 0.58 ± 0.55 nmol/mg of ATP at 0 minutes which rose to 1.51 ± 0.31 nmol/mg of ATP at 5 minutes whereas ATP secretion in control cells exposed to 20 dyn/cm^2 rose from 1.23 ± 1.13 nmol/mg at 0 minutes to 6.64 ± 0.38 nmol/mg of ATP in 5 minutes. The DHA treated cells exposed to 20 dyn/cm^2 had significantly higher ($P<0.05$) ATP secretion compared to all controls and DHA 1 dyn/cm^2 treated cells at 5 minutes. There were no differences among control 1 dyn/cm^2, control 20 dyn/cm^2 and DHA 1 dyn/cm^2 cells.

At 30 minutes DHA treated cells exposed to 1 dyn/cm^2 remained steady at 2.58 ± 0.99 nmol/mg whereas DHA treated cells exposed to 20 dyn/cm^2 remained steady at 20.28 ± 10.74 nmol/mg. Control cells exposed to 1 dyn/cm^2 settled at 1.17 ± 0.03 nmol/mg from 1.51 ± 0.31 nmol/mg at 5 min similarly Control cells exposed to 20 dyn/cm^2 settled at 6.00 ± 0.77 nmol/mg from 6.64± 0.38 nmol/mg at 5 min. DHA treated cells exposed to 20 dyn/cm^2 had significantly higher ($P<0.05$) ATP secretion compared to all controls and DHA 1 dyn/cm^2 treated cells at 30 minutes time point similar to 5 minutes. There were no differences among control 1 dyn/cm^2, control 20 dyn/cm^2 and DHA 1 dyn/cm^2 cells at 30 minutes.

Interestingly there were no significant differences in ATP secretion in DHA treated cells exposed to 1 dyn/cm^2 and control cells exposed to 20 dyn/cm^2 at any point of time. Similarly, there were differences between control cells exposed to 1 dyn/cm^2 and 20 dyn/cm^2 but they were found to be not statistically significant ($P>0.05$).

In conclusion, the treatment of DHA significantly enhanced the ATP secretion from MLO-Y4 cells after fluid shear stress.

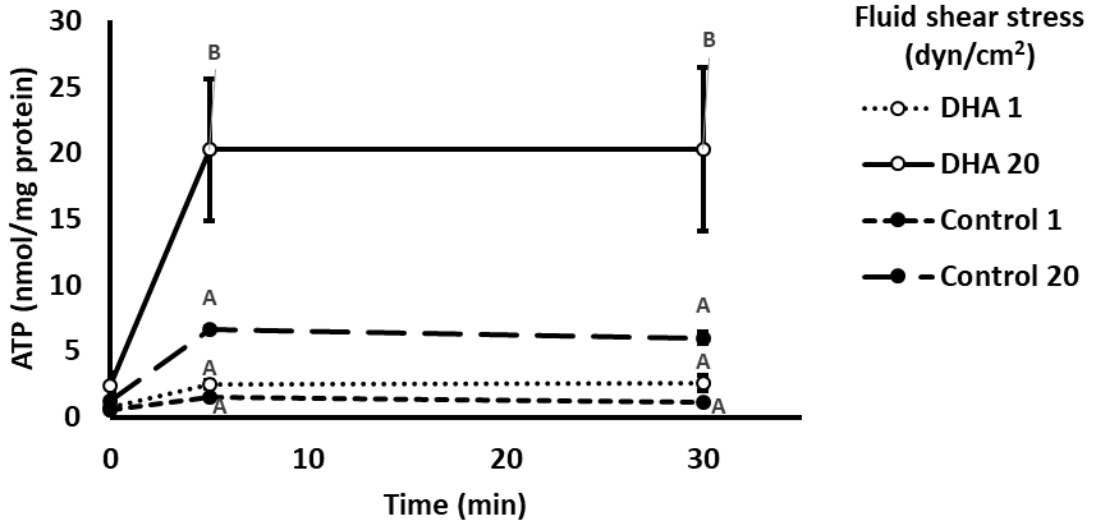

Figure 4-6: Figure showing the effect of docosahexaenoic acid (DHA) on ATP secretion by MLO-Y4 cells exposed to fluid shear stress.

MLO-Y4 cells were divided into two groups. The test cells, which were treated with 20 µg/ml of DHA and control cells which were treated with vehicle control. Then MLO-Y4 cells were exposed to fluid flow for 30 minutes at 1 dyn/cm^2 and 20 dyn/cm^2. The medium was collected at various time points 0, 5 and 30 minutes. After the experiment, the collected medium was assayed for ATP using the Luciferin-Luciferase assay. Levels not connected by the same letters were significantly different ($P<0.05$). All data were mean ± SEM, n=3.

4.8. Effect of Arachidonic acid (AA) on ATP secretion

After investigating the effect of an omega 3 fatty acid on ATP secretion, the effect of an omega 6 fatty acid was compared. The effect of the omega 6 FA arachidonic acid (AA) on ATP secretion in MLO-Y4 cells exposed to fluid shear stress is shown in Figure 4-7. MLO-Y4 cells were categorised to control (AA non-treated) or test (AA treated) cells which were both exposed to 1 dyne/cm^2 and 20 dyn/cm^2.

At 0 minutes no significant differences were found among the AA treated and control cells at any shear stress. The fluid shear stress did not affect ATP secretion at 0-minute time point.

At 0 minutes, 0.10 ± 0.03 nmol/mg of protein of ATP was secreted by AA treated cells exposed to 1 dyn/cm^2 which rose to 0.12 ± 0.07 nmol/mg of protein at 5 minutes and settled at 0.16 ± 0.12 nmol/mg of protein at 30 minutes. ATP secretion in AA treated cells exposed to 20 dyn/cm^2 rose from 0.55 ± 0.21 nmol/mg at 0 minutes to 5.42 ± 3.88 nmol/mg in 5 minutes which then settled at 4.08 ± 3.05 nmol/mg at 30 minutes. Similarly, 0.19 ± 0.29 nmol/mg of protein of ATP was secreted by control cells exposed to 1 dyn/cm^2 which rose to 1.10 ± 0.54 nmol/mg of protein and settled to 0.53 ± 0.32 nmol/mg of protein at 30 minutes. Whereas ATP secretion in control cells exposed to 20 dyn/cm^2 rose from 0.24 ± 0.31 nmol/mg of protein at 0 minutes to 2.43 ± 1.33nmol/mg of protein in 5 minutes which settled to 2.88 ± 1.87 nmol/mg of protein at 30 minutes.

There were no significant differences in ATP secretion between AA treated cells exposed to 1 dyn/cm^2 and control cells exposed to 1 dyn/cm^2 at any point in time.

When compared between ATP secretion in control cells treated with 1 dyn/cm^2 and 20 dyn/cm^2, significant differences (<0.05) were observed at the 30 minute time point but no differences at 0 and 5 minutes. Also a significant difference ($P<0.05$) in ATP secretion

between AA treated cells exposed to 20 dyn/cm^2 and control 20 dyn/cm^2 was observed at 5 minutes but not at 0 and 30 minutes.

A significant difference ($P<0.05$) in ATP secretion was observed at 5 and 30 minutes for groups of AA treated cells exposed to 1 dyn/cm^2 and 20 dyn/cm^2. ATP secretion among AA treated cells exposed to 1 dyn/cm^2 and control cells exposed to 20 dyn/cm^2 were also found to be significantly different ($P<0.05$) at 5 minutes and 30 minutes. A similar trend was seen when comparing ATP secretion in AA treated cells exposed to 20 dyn/cm^2 and control cells exposed to 1 dyn/cm^2 which were significantly different ($P<0.05$) at 5 and 30 minutes time points. However, at the 0-minute time point, there were no significant differences.

In conclusion, AA treatment enhanced ATP secretion in MLO-Y4 cells exposed to fluid shear stress similar to DHA.

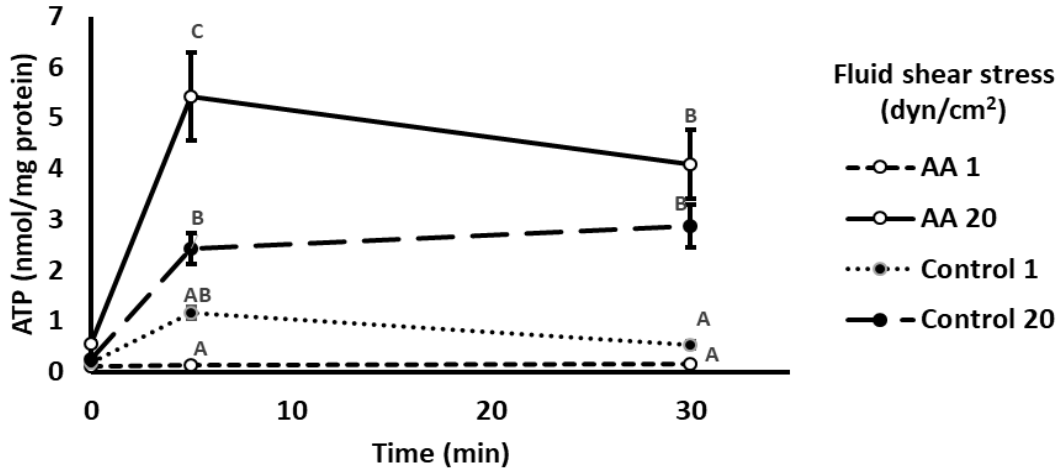

Figure 4-7: Figure showing the effect of arachidonic acid (AA) on ATP secretion by MLO-Y4 cells exposed to fluid shear stress.

MLO-Y4 cells were divided into two groups, test cells which were treated with 20 µg/ml of AA and control cells which were treated with vehicle control. Then MLO-Y4 cells were exposed to fluid flow for 30 minutes at 1 dyn/cm^2 and 20 dyn/cm^2. The medium was collected at various time points 0, 5 and 30 minutes. After the experiment, ATP was measured in the collected medium using the Luciferin-Luciferase assay. Levels not connected by the same letters were significantly different ($P<0.05$). All data were mean ± SEM, n=3.

4.9. Effect of Palmitic acid (PA) on ATP secretion.

MLO-Y4 cells were also treated with palmitic acid to see whether the enhanced amount of ATP seen on treatment with DHA and AA was due to the addition of the carbon chain or the nature of the fatty acids in the culture medium. Figure 4-8 shows the effect of palmitic acid on ATP secretion in MLO-Y4 cells exposed to fluid shear stress. The experiment suggests that there were differences in the ATP secretion between higher fluid shear stress (20 dyn/cm^2) and lower shear stress groups (1 dyn/cm^2) however, there was no effect of palmitic acid on ATP secretion. Both PA treated and the non-treated group had a similar trend in ATP secretion in both higher shear stress and lower shear stress.

At 0 minutes, 0.18 ± 0.22 nmol/mg of protein of ATP was secreted by PA treated cells exposed to 1 dyn/cm^2 which rose to 0.72 ± 0.67 nmol/mg of protein at 5 minutes and settled at 0.81 ± 0.75 nmol/mg of protein at 30 minutes. ATP secretion in PA treated cells exposed to 20 dyn/cm^2 rose from 0.26 ± 0.07 nmol/mg at 0 minutes to 6.40 ± 4.56 nmol/mg in 5 minutes which then settled at 5.93 ± 6.81 nmol/mg at 30 minutes. Similarly, 0.19 ±0.19 nmol/mg of protein of ATP was secreted by control cells exposed to 1 dyn/cm^2 which lowered to 0.89 ± 1.26 nmol/mg of protein and settled to 1.13 ± 1.19 nmol/mg of protein at 30 minutes. Whereas ATP secretion in control cells exposed to 20 dyn/cm^2 rose from 0.39 ± 0.82 nmol/mg of protein at 0 minutes to 4.77 ± 3.61 nmol/mg of protein in 5 minutes which settled to 5.88 ± 3.77 nmol/mg of protein at 30 minutes.

There were significant differences ($P<0.05$) between PA treated cells exposed to 20 dyn/cm^2 and 1 dyn/cm^2. There was also a significant difference ($P<0.05$) between control cells exposed to 20 dyn/cm^2 and 1 dyn/cm^2. There were no significant differences among the PA treated cells and control cells at 20 dyn/cm^2 or 1 dyn/cm^2.

The fact that the differences remained in the groups of cells between higher and lower shear stress but not between controls of PA treated cells, indicated that the ATP release

was the result of fluid shear stress and not the PA treatment. Thus, palmitic acid didn't affect ATP secretion in MLO-Y4 cells exposed to fluid shear stress, unlike AA or DHA which enhanced the secretion of ATP in response to FSS. These results indicated that the release of ATP observed in DHA and AA treated cells in response to FSS are specific and not general effect from addition of any fatty acids.

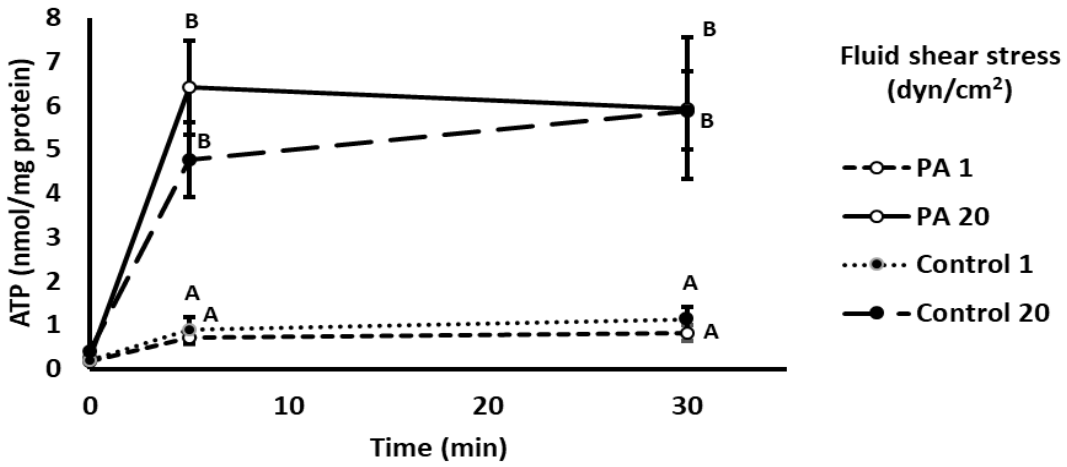

Figure 4-8: Figure showing the effect of Palmitic acid on ATP secretion by MLO-Y4 cells exposed to fluid shear stress.
MLO-Y4 cells were divided into two groups, test cells which were treated with 0.2 mM PA and control cells which were treated with vehicle control. MLO-Y4 cells were exposed to fluid flow for 30 minutes at 1 dyn/cm^2 and 20 dyn/cm^2. The medium was collected at various time points 0, 5 and 30 minutes. After the experiment, ATP was measured using the Luciferin-Luciferase assay. Levels not connected by the same letters were significantly different ($P<0.05$). All data were mean ± SEM, n=3.

Chapter 5. Discussion

Mechanical loading, in general, plays a very important role in the maintenance of the skeleton. It induces various biochemical signals at the molecular and cellular levels. The distribution and number of osteocytes in bone is such that it is considered the principal mechanosensor in the bone. It communicates the signal received by bone due to exercise to the various cells such as osteoblasts, osteoclasts, bone lining cells and other osteocytes in a form of biochemical response. It is believed that the release of ATP is one of the responses by which osteocytes respond to the mechanical signal.

5.1. Does fluid shear stress affect ATP secretion?

Fluid shear stress stimulated ATP secretion by MLO-Y4 cells when compared to the non-fluid shear stress treated MLO-Y4 cells. The relationship between the applied FSS and ATP secretion in MLO-Y4 cells is shown in Figure 4-3, which indicated that as the fluid shear stress increased, the amount of ATP secreted also increased. Minimal ATP secretion was observed at 0 dyn/cm^2 whereas a higher amount of ATP was secreted at 30 dyn/cm^2. Similarly, increased ATP secretion was observed between 10 and 20 dyn/cm^2. The amount of ATP released was significantly higher in 30 dyn/cm^2 compared to 0 dyn/cm^2 (Figure 4-4). Although not statistically significant, cells exposed to 5 dyn/cm^2 also had higher ATP secretion compared to the 0 dyn/cm^2. The mechanism for the secretion of higher ATP in MLO-Y4 cells exposed to higher fluid shear stress is yet unknown however an earlier study suggested a role for membrane permeability. A study by Genetos et al., showed FSS induced ATP release in MC3T3-E1 cells was found to be dependent on ca^{2+} channel and the transport was vesicular release and not through hemichannel [38]. In the study by Berthiaume et al., shear stress caused an increase in membrane permeability of human endothelial cells which caused the uptake of amphipathic dye merocyanine 540 by a mechanism different than endocytosis. This may suggest a fluid flow exposure induced

expansion in membrane fluidity [264]. Analysing these facts, enhanced ATP secretion from the cells might be due to vesicular transport which might be improved by the membrane fluidity. However, there is no evidence for this yet. Cell viability assay and alkaline phosphatase test might have further added strength to the results indicating that the ATP release was due to fluid shear stress and not because of cell destruction.

5.2. Does the duration of fluid shear stress affect ATP secretion?

ATP secretion by MLO-Y4 cells exposed to fluid shear stress for 120 minutes is shown in Figure 4-5. Within one minute of exposure, the ATP was secreted by MLO-Y4 and remained constant for the entire period. The peak ATP was observed at 120 minutes, but the overall amount remained similar throughout the period. This might be due to the cell membrane permeability that was opened after the first few minutes of the fluid shear stress remained entirely similar and fluctuated only after the prolonged exposure period. A relatively higher amount of ATP was detected at 120 minutes possibly due to the constant opening of the hemichannels leading to increases in the pore sizes and allowing more secretion of ATP at this point.

5.3. Does treating cells with different types of fatty acids affect the ATP secretion?

Under conditions considered optimal for cell growth, DHA and AA at a concentration of 20 µg/ml had a significant effect on the secretion of ATP by MLO-Y4 cells during the fluid shear stress however PA had no significant effect on the same process. ATP secretion in MLO-Y4 cells was enhanced by the treatment with DHA and AA following the fluid shear stress (Figure 4-6 and Figure 4-7). But PA had no significant effect on the secretion of ATP after fluid shear stress (Figure 4-8). It indicated that there was some relationship between the nature of the fatty acid added to the culture medium and its effect on ATP secretion by MLO-Y4 cells exposed to FSS. It appeared that FA composition of the membrane had been changed by the FA treatment during cellular growth. This FA alteration in the

membrane resulted in differences in the secretion pattern of ATP after the fluid shear stress by different treatment groups. Saturated FA had no differences on ATP secretion in FSS treated osteocytes whereas unsaturated FA increased the ATP secretion in FSS treated osteocytes.

PUFAs in general, have two main functions in human cells. Apart from being precursors for eicosanoids which can act as autocrine and paracrine regulators in the body, they contribute to the synthesis of phospholipids in the lipid bilayer of the cell membrane which results in a fluid mosaic structure. In a cell membrane, hydrophilic head and hydrophobic tails of phospholipids arrange in such a way that they appear as a fluid mosaic which gives the membrane a fluid-like structure. Membrane permeability is influenced by various factors, including changes in lipid composition, the concentration of cell membrane cholesterol, the density of lipid packing, relative water content, ion concentration, pH, and temperature, and shear stress may alter membrane lipid order via any of these factors or combinations of them. Dietary fat modification can lead to changes in the fatty acid composition of membrane phospholipids [265]. The nature of the lipids in the membrane has an impact on the fluidity of the particular membrane. Salem et al showed that polyunsaturated FA are present in the plasma membrane and around membrane-bound protein [266]. High concentrations of PUFA in membranes has a significant effect on membrane fluidity [267]. Unsaturated FA have several double bonds which make them loosely packed in the fluid mosaic model of the cell membrane which increases the ability of the membrane to bend readily due to cis bonds and prevents the molecular interaction among the FA chains in the membrane. This helps to maintain the fluidity in the cell membranes. Studies on living cells and membrane models have shown that the cholesterol content of the membrane has a major impact on the physical properties of plasma membranes, including on their membrane fluidity [268, 269]. On the other hand, a saturated fatty acid having no double bond, remain fairly straight, stacked more tightly and appeared more solid in the

membrane. Alam et al showed that trans FA in the diet decreased membrane fluidity in the plasma membrane of lacrimal glands [270]. Cooper et al modified red blood cell membrane structure by incorporating cholesterol-rich lipids [271] and found it to have several abnormalities including decreased membrane fluidity [272]. However, an increase in phosphatidylcholine/sphingomyelin ratio was associated with increased erythrocyte membrane fluidity [273, 274]. Incorporating hydrophobic cholesterol in the membrane might have decreased membrane fluidity and incorporating more hydrophilic phosphatidylcholine increased membrane fluidity. Concerning these studies, the saturated FA having hydrophobic carbons tightly packed with each other prevent other compounds to move into the membrane. On the other hand, unsaturated FA having one or multiple double bonds, might have allowed it to be less hydrophobic compared to saturated FA.

Genetos et al demonstrated that fluid shear stress-induced the opening of the hemichannels responsible for ATP release in osteocytes [275]. This might be one of the possible reasons as to why there was higher ATP secretion in PUFA treated MLO-Y4 cells compared to non-treated MLO-Y4 cells. Administration of fatty acid in the cell medium might have influenced the cell membrane fluidity causing variation in the ATP secretion among different fatty acids group. There was some association of FA treatment with ATP release [276, 277].

There is clearly no data available that present the effect of FA on mechanotransduction of osteocytes. There are some studies which treated the cells with mechanical loading and assessed their biochemical response [29, 33, 278-280] but not in combination with the FAs. This present study presented a novel approach to study the behaviour of cells in response to FSS after the treatment of the cells. These results indicate that the ATP secretion in cells was affected by the nature of FA treatment and these FA might be used to study biochemical response in the mechanotransduction studies. These results showed for the first time that the PUFA affect extracellular ATP secretion by osteocytes after fluid shear

stress. These extracellular ATP has been shown to positively influence PGE2 and nitric oxide production in the cells [38, 281]. These markers are an important factor in the regulation of osteoblastic activity and bone formation, which indirectly implies that the modulation by PUFA of FSS induced osteocytes may have a positive impact on bone health. In a general term, these results presented a hypothesis that regular dietary supplementation of PUFA may modulate the osteocytic membrane and exercise increases the cellular osteocytic responses which collectively have a positive effect on bone health.

There were some limitations to our experiment designs. It was not possible to collect the media in smaller time frames. It would be nice to see the ATP secretion pattern of MLO-Y4 cells in the first few seconds at the start of the experiments. The closed circuit system of our equipment hindered our evaluation of the real time release of ATP by the MLO-Y4 cells. Cell viability assays after the end of each experiment could have presented us with more robust information about the obtained results as to whether any increase in ATP release reflects possible cell destruction in response to FSS.

Further examinations of other molecules such as PGE2, NO in the collected medium may widen up our understanding and give a clear understanding about the mechanism behind ATP release and different pathways involved in osteocyte mechanosensation. Future work should focus on the effect of these PUFAs on different osteocytic markers such as RANKL, OPG, c-fos etc so that these loading and FA treatment studies could establish the osteocytic response in other signalling pathways. This work may lead to a novel mechanism of action by the PUFAs on osteocytes.

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