Pea Protein Derived Bioactive Peptides Stimulate Bone Health Promoting Effects

by

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ABSTRACT

Osteoporosis is a bone disease affecting 1 in 3 women and 1 in 5 men in Canada. One possible approach to prevent this disease is to stimulate the activity of osteoblasts (bone forming cells) using food derived bioactive peptides. As a sought-after pea protein, we previously identified a tripeptide LRW (Leu-Arg-Trp). Therefore, the 1st objective of this thesis was to investigate the effect of LRW on promoting osteoblastic activity using pre-osteoblast MC3T3-E1 cells.

LRW treatment (50 μM) caused a significant increase in cell proliferation (4-fold increase), stimulated differentiation by increased the levels of type 1 collagen (COL1 A2; 3-fold increase), alkaline phosphatase (2-fold increase), runt-related transcription factor 2 (RUNX2; 2-fold increase), as well as promoted mineralization evidenced by Alizarin-S red staining and nodule formation. LRW treatment also and increased osteoprotegrin levels (OPG; 2-fold increase), thereby decreasing bone resorption. Furthermore, LRW also significantly increased the wound healing based on cell migration assay.

Since LRW was identified from pea protein hydrolysate, the second objective of the thesis was to determine the osteoblastic activity of pea protein hydrolysates using human osteoblast cells (U-2OS). Among seven pea protein hydrolysates prepared, three (prepared by chymotrypsin, alcalase and thermolysin, respectively) showed better ability to increase the level of COL1 A2 and thus selected for further study. Pea protein hydrolysate up-regulated COL1 A2 (2-fold increase), procollagen (1.25-fold increase), nuclear factor erythroid 2- related factor 2 levels (NRF2; 1.35-fold increase), C-X-Chemokine receptor type 4 (CXCR4; 2-fold increase) and signal transducer and activator of transcription 3 (STAT3; 1.5-fold increase) in alcalase prepared hydrolysate. Furthermore, increased mRNA and protein expression of STAT3 (3.5-fold increase) and CXCR4 (4-fold increase) respectively in alcalase prepared sample were further validated by qRT-PCR. Pea

protein hydrolysate also decreased the levels of matrix metalloproteinase MMP-1 and MMP-9, indicating the inhibitory role on the degradation of bone matrix.

This research demonstrated the presence of bioactive peptides in pea protein that can positively modulate the activity of osteoblasts, indicating the potential of pea derived peptides for the prevention or treatment of osteoporosis.

PREFACE

This thesis is an original work by Harshita Arora and has been written as per the guidelines given by the Faculty of Graduate Studies and Research, University of Alberta. The concept of the research work in this thesis is originated from my supervisor Dr. Jianping Wu. The figures in this thesis is prepared and drawn by Harshita Arora.

The thesis consists of five chapters: Chapter 1 provides a general introduction and the objectives of the thesis; Chapter 2 is a literature review on several subjects, including bone health, osteoporosis, pea protein, pea protein hydrolysate, and pea protein derived tripeptide LRW; Chapter 3 reports an investigation of pea protein derived tripeptide LRW on the osteoblastic activity using murine mouse pre-osteoblast MC3T3-E1 cells; Chapter 4 evaluates the osteogenic activity of pea protein hydrolysate prepared using chymotrypsin, alcalase, and thermolysin on human osteoblast cells U-2OS; Chapter 5 provides overall conclusions and discussion with its significance in food and health management industry for osteoporosis treatment.

Dr. Jianping Wu greatly contributed to the experimental design, data interpretation, thesis preparation, and edits. I was responsible for literature search relevant for the above studies, designing and performing experiments, data collection and analysis, and drafting the thesis.

DEDICATIONS

This thesis is dedicated to my beloved family and friends.

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ABBREVIATIONS USED

ALP- Alkaline phosphatase

ANOVA- One-way analysis of variation

BCAAs- Branched chain amino acids

BFP- Bone forming peptides

BMD- Bone mineral density

BMP- Bone morphogenetic proteins

BMU- Basic multicellular units

BrdU- Bromodeoxyuridine

BSA: Bovine serum albumin

COL1 A2- Type 1 collagen

Cp- Cathepsin

CSF-1- Colony stimulating factor -1

CXCR4-C-X-C chemokine receptor type 4

DMEM- Dulbecco's modified eagle medium

DMEM: Dulbecco's modified eagle medium

DTT-Dithiothreitol

DXA- Dual X-ray absorptiometry

EDTA: Ethylenediaminetetraacetic acid

ELISA-Enzyme linked immunosorbent assay

Eph2- Ephrin B2

FBS: Fetal bovine serum

FRAX- Fracture risk assessment tool model

GAPDH-Glyceraldehyde 3phosphate dehydrogenase

HRP- Horseradish peroxidase

IgE: Immunoglobuline E

IL-6- Interleukine 6

IPP- Ile-Pro-Pro

IQW- Ile-Gln-Trp

IRW- Ile-Arg-Trp

LRW- Leu-Arg-Trp

M-CSF- Macrophage colony-stimulating factor

MAPK- Mitogen activated protein kinase

MMP-1 Matrix metalloproteinase 1

MMP-9- Matrix metalloproteinase 9

NAVPITPTL- Asn-Ala-Val-Pro-Ile-Thr-Pro-Thr-Leu

NF-KB- Nuclear factor kappa B

NSI-Nitrogen solubility index

OPG-Osteopetegrin

Os- Osterix

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PIP-Procollagen type 1- C peptide

PPI- Pea protein isolate

PTH- Para-thyroid hormone

qRT-PCR- Quantitative real time polymerase chain reaction

RANK- Receptor activator of nuclear factor kappa-B

RANKL- Receptor for activation of nuclear factor kappa-B ligand

RNA: Ribonucleic acid

RUNX2- Runt-related transcription factor 2

SDS: Sodium dodecyl sulfate

SEM: Standard error of mean

SERMs- Selective estrogen receptor modulators

STAT3- Signal transducer and activator transcription 3

TGF- Transforming growth factor

TMB- 3,3,5,5 tetramethylbenzidine

TNBS-Trinitrobenzesulfonic acid

TNF- Tumour necrosis factor

TRAP- Tartrate- resistant acid phosphate

VLPVQK- Val-Leu-Pro-Val-Glu-Lys

VPP- Val-Pro-Pro

WHO-World Health Organization

Wnt: Wingless

CHAPTER 1- LITERATURE REVIEW

1.1 Bone physiology ad metabolism

1.1.1 Bone as a tissue

Bone is a dynamic mineralized connective tissue that exhibits important functions in the body such as locomotion, support and protection to organs, storage site of minerals like calcium and phosphate [1]. It also provides a suitable environment for the bone marrow to harbour [2]. Bones are composed of mainly two types of tissues which includes cortical (compact) and trabecular (cancellous) bone [4]. Cortical or the compact bone makes up the diaphysis (shaft) of the long bones which comprises 80% of the skeletal mass. There function is to provide support, protection and mechanical strength to the organs [5]. Whereas trabecular bone makes up the ends of the long bones consisting of a network of plates and rods. Trabecular bone also has a larger surface area exposed to bone marrow with higher turnover than cortical bone [8]. They are present at the site of bone-forming cells and constitutes a reserve of calcium and phosphorus [7]. Due to the more total surface area of the trabecular bone, it is more metabolically active and largely covers the site for bone formation and resorption [9]. The two different compartments of bone differ in the material composition with lower calcium content in trabecular bone and more water content as compared to cortical bone. As we age or in case of any bone disease, the cortical bone becomes porous resulting in more surface area and less bone strength [6].

1.1.2 Bone matrix

Bones consist of both organic and inorganic components which forms the bone matrix. The organic compartment of the matrix includes 90% collagenous proteins mainly (type 1 collagen) and 10% non-collagenous proteins such as osteocalcin, osteopontin, bone sialoprotein II, osteonectin, fibronectin, bone morphogenetic proteins (BMPs) and growth factors [10]. However, the inorganic compartment of the bone contains calcium and phosphate ions with significant amounts of magnesium, carbonate, potassium, zinc, fluorite, citrate, strontium and barium [11]. In the bone matrix, the calcium and phosphate ions nucleate and form hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$]. Altogether with hydroxyapatite deposition, collagenous and non-collagenous proteins, the formed bone matrix provides stiffness and resistance to bone tissues [12]. With progressing changes in nutrition and age, the occurrence of bone disease has increased. Bone diseases and their treatment affect the concentration of bone matrix proteins which results in deformation and fractures [13].

1.1.3 Bone cells

Bone cells are present on both surface and in lacunae of bone matrix. There are mainly four types of cell types: osteoblast, bone lining cells, osteocytes, and osteoclasts. Each of these cells plays an important role in bone formation and remodeling [14].

1.1.3.1 Osteoblasts

Osteoblasts are cuboidal cells which occupy 4-6% of total resident cells onto the bone surface [3]. These are proliferative cells from mesenchymal cells [3]. They cover the surface of the trabecular and cortical bone as well as the alveolar bone. These are protein synthesizing cells, which further produce osteoid used for bone formation matrix. These cells are responsible for the formation of bone along with performing other functions [3].

1.1.3.2 Bone lining cells

Bone lining cells covers the bone surface and are flat-shaped [26]. They contribute to bone remodeling and play an important role in regulating the concentration of minerals. In any situation of mechanical stress, they stimulate the formation of bone by regulating themselves to form active osteoblasts. The bone lining cells affect both osteoblast and osteoclast activity. They digest non-mineralized collagen and also forms a thin deposition of fibrillar collagen on to the bone surface in order to facilitate osteoblastic activity during the process of bone resorption [27].

1.1.3.3 Osteocytes

Osteocytes are the most abundantly found bone cells comprising of total 90-95%. The osteocytes have a dendritic morphology and are generally found in the lacunae of the mineralized bone matrix [28]. Osteocytes are also derived from mesenchymal cells in four differentiation stages: osteoid-osteocytes, pre-osteocytes, young osteocytes and mature osteocytes [29]. Towards the last phase in bone formation, some osteoblasts from osteocytes with structural and morphological changes. During this transition, osteoblast biomarkers such as type 1 collagen (COL1A2) and alkaline phosphatase (ALP) are downregulated [30].

1.1.3.4 Osteoclasts

Osteoclasts are derived from hematopoietic stem cell lineage. They are stimulated with macrophage colony-stimulating factor (M-CSF) and receptor activator for nuclear factor K B (RANK) ligand which is mainly secreted from osteoblasts [31]. These two factors regulate the gene expression and activity of transcriptional factors [32,33]. They are terminally differentiated

cells which are responsible for the resorption of bone. They produce hydrogen ions which dissolve the mineral crystals of the matrix providing the appropriate pH for the activity of enzyme such as protease. Protease hydrolyses the organic compartment of the matrix.

Osteoclasts are characterized at the ruffled edge where bone resorption takes place digesting the matrix.

1.1.4 Osteoblast differentiation and bone formation

Osteoblasts are cuboidal in shape and are present at the bone surface. These cells maintain the skeletal architecture of bones by synthesizing bone matrix and regulating osteoclastogenesis. These are proliferative cells from mesenchymal cells with the timed commitment of synthesis of bone morphogenetic proteins (BMPs) and adherents of the Wingless pathway [16]. As osteoblast differentiates, they procure the ability to secrete bone matrix [15]. In the proliferation stage of osteoblasts, the expression of RUNX2 and COL1 A2 proceeds with differentiation. The expression of Runt-related transcription factor 2 (RUNX2) is the master key for osteoblast differentiation, as it upregulates osteoblast-related genes such as COL1 A2 and ALP. [17]. With progressing differentiation, the increase in the secretion of COL1 A2 reflects a transition of pre-osteoblasts to mature osteoblasts. Furthermore, these differentiated osteoblasts undergo morphological changes in structure to form large cuboidal cells [19]. Differentiating osteoblasts secrete collagen protein mainly type 1 collagen and proteoglycans such as decorin and biglycan which forms the organic matrix for mineralization of bone to take place [20].The formation of the bone matrix occurs in two phases, beginning with deposition of organic matrix and further mineralization of deposited bone matrix. Matrix vesicles are released from the osteoblast into the newly formed bone matrix bound to proteoglycans and other components. Vesicle calcification is often followed by collagen propagation and mineral deposition in spatially oriented nodules [25]. This phase is generally called as a vesicular phase. The negatively charged proteoglycans immobilize calcium ions [21]. The enzymes secreted by osteoblast degrade the proteoglycans and release calcium ions into the matrix vesicle membranes as calcium channels. However, the ALP secreted by osteoblasts degrade the phosphate-containing compounds inside the matrix vesicles. Thus, free calcium and phosphate ions nucleate into the matrix vesicle forming hydroxyapatite crystals [22]. The saturation of calcium and phosphate ions in the matrix vesicles ruptures the structure, leading to diffusion of hydroxyapatite crystals to the surrounding matrix [23]. The matrix vesicles are further degraded

during advanced stages of mineral propagation [24]. Throughout the mineral propagation, it forms radial nodules starting with the periphery of matrix vesicles and propagates out into the extravesicular matrix.

1.1.5 Osteoclast and bone resorption

The osteoclasts are formed by the fusion of the macrophage family. They are bone resorptive cells that regulates the total mass of skeleton [41]. Osteoclastogenesis is sufficiently regulated by two molecules: macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B (NF-KB) (RANK) ligand (RANKL). M-CSF generates signals for osteoclast activity by binding to its receptor (c-Fms) [42]. Osteoprotegerin (OPG) is known as “decoy” receptor which competes with RANK for RANKL [43]. RANK present on osteoclasts acts as a differentiating factor. Moreover, RANKL is also abundantly expressed by activated T lymphocytes which directly initiate the osteoclast activity and are widely seen in rheumatoid arthritis [44].

Therefore, an equilibrium in the expression of the regulator of osteoclast activity, RANKL, and the inhibitor, OPG controls the total bone resorption activity [45]. Bone resorption is a multivariate process which is triggered by the immature osteoclast, committing to the osteoclastic phenotype markers for degradation of the organic and inorganic bone matrix [46]. The binding of RANKL to its receptor RANK mediates osteoclastogenesis, which further activates the expression of some osteogenic transcription factors [47]. Upon activation, the osteoclasts are polarized in reorganization on the ruffled border and clear zone. The vacuolar-type ATPase in ruffled border dissolves hydroxyapatite crystals. H^+ and bicarbonate ions (HCO_3) are released from the degradation of bone matrix. The osteoclasts discharges cathepsin (Cp), matrix metalloproteinase-9 (MMP-9) and tartrate-resistant acid phosphate (TRAP) for degrading the bone organic matrix. The ephrin B2 (Eph2), which is present in the osteoclast membrane binds onto osteoblast membrane (ephrin B4), resulting in promoting the action of osteoclast differentiation [49]. Furthermore, osteoclasts also produce clastokines, which indirectly controls osteoblast activity [50].

1.1.6 Bone remodeling

Bone remodeling is a process which involves the replacement of old tissues by new bone to maintain the bone mass. The process is done within the bone cavities in which a temporary structure called basic multicellular units (BMU) are formed [34]. These structures constitute

osteoclasts and osteoblasts. Osteoclasts present forms the cutting cone and the osteoblasts form the closing cone, this coupled structure ensures the minimal net change in bone volume during the process of resorption [35]. Bone remodeling is a sequential process of activation, resorption, reversal, formation and termination. Bone remodeling begins with activation phase in which signal is generated resulting in structural or hormonal changes. In the situation of mechanical stress, osteocytes translate to initiate bone remodeling, and in adverse damages, osteoclasts result in apoptosis with increased osteoclastogenesis. Parathyroid hormone (PTH) generates signals to regulate calcium homeostasis and indirectly maintains serum calcium levels. Binding of PTH to G-protein coupled receptor results in activation of protein kinase A and calcium intracellular signaling pathways inducing transcriptional responses which recruit osteoclast precursors, inducing activation and differentiation of osteoclasts [36]. In response to the activation of osteoclasts, osteoblasts produce chemokine chemoattractant protein-1 which attracts precursors of osteoclast to function [37]. CSF-1 and RANKL expression are increased whereas OPG expression is reduced, promoting osteoclast activity. Osteocytes transition to osteoblasts generates signal for the activation of osteoclasts on the remodeling site [36]. On activation, they produce cytokines, CSF-1, RANKL and OPG. Hydrogen ions released to dissolve the mineralized matrix creates acidic conditions which degrade the remaining organic bone matrix [38]. After osteoclast resorption on the surface, the mononuclear cell removes collagen and prepares the surface for new bone formation mediated by osteoblast. These “reversal” cells are precursors from osteoblast generation which is represented by positive expression for ALP. The reversal cells reverse the conditions of localized micro-environment by degrading the bone matrix. These cells receive and produce coupling signals which translate the process of bone resorption to bone formation. The mature osteoblast and reversal cells are regulated by the bone formation signals. In this response, PTH and activated osteocyte allow the bone formation to occur via the Wnt pathway. This is also significantly observed with a decrease in sclerostin expression. After complete formation, the newly mineralized osteoid is deposited and the bone surface is in resting phase [39]. Termination is also expressed by the increase in sclerostin expression, which indicates the end of the remodeling cycle [40].

1.2 Osteoporosis

1.2.1 Prevalence of osteoporosis

Osteoporosis is a bone disease which is symbolized by low bone mass, disruption in bone architecture and deterioration in bone tissues. This is characterized by an increased risk of fractures with conceded bone strength [51]. In Canada, 2 million people are affected by osteoporosis including at least 1 in 3 women and 1 in 5 men. The fragility fractures represent 80% of menopausal women over the age of 50. Osteoporotic bone impairs mobility and quality of life. Worldwide, osteoporosis is considered as a serious health concern, with 200 million people are affected by this disease [52]. Ageing of populations is a major contribution to the incidence of osteoporosis in postmenopausal women [53]. The people who are already sustained with fractures represent an increased risk of 86% with any fracture in the future [54].

It is projected, that by 2050 the worldwide incidence of hip fractures in women will increase by 240% and 310% in men [55].

1.2.2 Foundations of Osteoporosis

Osteoporosis is regarded as "silent killer" as it is painless until the patient experiences a fracture. There are several reasons for osteoporosis such as ageing, hypogonadism and menopause, clinical risk factors and high bone turnovers which results in increased bone loss [88]. Inadequate peak bone mass (maximum amount of bone that can possessed by an individual) and increased bone loss results in low bone density and impaired bone quality which further leads to skeletal fragility [89]. The higher fall mechanics and propensity to fall concludes to falls resulting in excessive bone loading. All the factors together including skeletal fragility and excessive bone loading which further leads to fractures. These fractures result in chronic pain, disability and death followed by minor trauma, which is shared in osteoporotic patients [56].

1.2.3 Diagnosis of osteoporosis

Bone strength is determined using bone mass density (BMD) and bone quality. The prognosis of osteoporosis is defined by the measurement of BMD or by knowing the occurrence of fragility fractures. Bone mineral density is measured using the dual X-ray absorptiometry (DXA), which gives an expression of bone in terms of grams of mineral per square centimetre of scanned bone and is expressed as g/cm^2 of calcium [113]. The determination of BMD confirms and establishes the course of the disease. T-scores are calculated as the difference of the patient BMD and mean

BMD of a healthy referenced adult aged in the range of 20-29 years divided by the standard deviation [114]. According to the World Health Organization (WHO), osteoporosis is represented with T-score at or above -2.5 SD. Higher threshold describes "low bone mass" or osteopenia with a T-score between -1 and -2.5 SD. Whereas, for a normal healthy adult, T-score is at -1 or above, within 1 SD of the mean value of an adult reference population [56].

Similarly, the fracture risk assessment tool model (FRAX) is an algorithm, which predicts the risk of fractures. This takes in to account various predictors of fracture risk supplementing to BMD. This integrates all the clinical risk factors which are further expressed to calculate the probability of fractures in the next 10 years [115]. Clinical risk factors which are taken in consideration include age, sex, information of any prior fracture, BMD of femur neck, parental history, secondary causes of osteoporosis, past or current smoking habits, alcohol intake levels per day. [57].

1.2.4 Preventive measures of osteoporosis

Osteoporosis is considered normal with the course of ageing, but it is now possible to treat and prevent the occurrence of this disease [58]. Interventions such as reduced fracture risk can be said as primary prevention. Whereas, lifestyle factors such as diet, tobacco avoidance, moderate alcohol levels and physical activity greatly influence the bone mass and strength and can be regarded as secondary prevention. A nutritious diet with recommended amounts of calcium, protein and vitamin D can help to maintain the adequate levels for the bone formation and maintaining bone density [116]. The skeletons comprise of 99% of total calcium which further mobilizes serum levels, crucial for bone health and muscles. Supplementation of calcium after the 30s is required to maintain adequate levels for bone formation [117]. Physical activities including weight-bearing and muscle-strengthening activities are recommended as they improve postures, agility, strength for preventing falls and balance [118]. Apart from lifestyle factors, genetic factors also play an important role in determining the risk of osteoporosis [59]. Pharmacological strategies are necessary to decrease the ageing and postmenopausal bone loss, as this increases the risk for osteoporosis. Estrogen therapy maintains the bone strength, decelerates the bone loss and protecting against the risk of fracture [60].

1.2.5 Current treatment strategies

Prevention strategies of osteoporosis involves oral administration of calcium and vitamin D supplements or synthetic drugs. For the treatment of osteoporosis, drugs can be based on two approaches, one that decreases bone resorption and the second, one that increases bone formation [61]. Antiresorptive drugs act by decreasing the overall rate of bone turnover which further maintains a balance between bone formation and resorption [119]. Pharmacological drugs are generally used for treating osteoporosis such as calcium and vitamin D supplements, bisphosphonates, calcitonin, estrogen and selective estrogen receptor modulators [62]. Whereas other bone formation drugs such as teriparatide slow down the resorptive activity of osteoclast, thus improving the bone strength [62].

1.2.5.1 Calcium and vitamin D supplements

Calcium and vitamin D are fiercely obtained from diets. The recommended daily intake of calcium is 1200-1500 mg and for vitamin D, it is 400-800 IU [63]. Calcium supplements are available in the form of calcium citrate and calcium carbonate. Supplements are recommended with higher doses for elderly patients. Oral administration of supplements has shown to prevent fractures in elderly women [64].

1.2.5.2 Bisphosphonates

Bisphosphonates are the class of drugs which have pyrophosphate analogues, which binds to hydroxyapatite, thwarting the osteoclast activity [121]. There are currently four approved bisphosphonates: Alendronate, Etidronate, Risedronate and Zoledronic [122]. They tend to increase bone density and reduce the risk of fractures. Bisphosphonates also possess some common side effects like nausea, abdominal pain, loose bowel movements, joint pain. In extreme cases, it may also result in kidney dysfunction and breakdown of jawbone called as osteonecrosis [122].

1.2.5.3 Calcitonin

Calcitonin is used in the treatment of osteoporosis as it increases BMD [124]. Calcitonin is another hormone produced by thyroid gland, it reduces the risk of fractures and is used in treating bony pain subordinates to fractures, by slowing the absorbing rate of osteoblasts [125]. Women who have passed at least five years of their menopause are recommended to take calcitonin. It can be taken through injection or nasal spray [64]. Side effects from use of calcitonin depends on the mode of delivery. Injection side effects include nausea, vomiting and flushing of the skin.

Although, nasal irritation, backpain, bleeding nose and headaches can be seen via nasal administration [124].

1.2.5.4 Estrogen and selective estrogen receptor modulators (SERMs)

For a woman, a drop-in estrogen is observed as they reach menopause. This has a direct effect on their bones. The deteriorating rate in estrogen levels weaken the bones and become vulnerable for fractures [127]. After menopause, estrogen therapy is recommended which decrease the risk of getting osteoporosis, as they increase the estrogen levels, maintaining the bone mineral density [64]. Estrogen receptor modulators such as raloxifene enhances BMD. It has been shown that a daily dose of 60 mg of raloxifene can reduce the risk of fractures by 40% [65]. Fatigue, hot flushes, night sweats and vaginal discharge are common side-effects for SERMs [127].

1.2.5.5 Anabolic

Anabolic are those type of drugs which promote bone formation. PTH is an anabolic agent which efficaciously treats osteoporosis [129]. It increases bone resorption and as well as bone formation, thereby increasing BMD and improving micro-architecture. PTH increases the density of bone by increasing cortical thickness [66]. The synthetic form of PTH, teriparatide activates osteoblast to perform their action, which results in an increase in bone [67]. It has already been shown that 40 mg of teriparatide can result in an increase in total body bone mineral [64]. Adverse effects of teriparatide includes headache, nausea, dizziness and limb pain. Theoretically, it also possesses risk of osteosarcoma, an aggressive condition of tumor in the bone [130].

1.2.5.6 Non-pharmacological treatments

Non-pharmacological treatments focus on utilizing alternatives to synthetic drugs. They provide therapeutic interventions for osteoporotic patients. These treatments include vertebroplasty, hip protectors, posture training supports, balance and exercise training programs [64].

1.2.6 Emerging therapies

New medications and natural alternatives have now been widened. These therapies approach as the individualised treatment based on patient preference and specific clinical requirements. Sophisticated options for treating osteoporosis involve sequential intake of new agents, targeting specific therapeutic targets with minimal side-effects [68]. The side-effects and limited efficiency of the currently available treatment options have now exhilarated extensive research into the pathophysiology of osteoporosis with the introduction to newer drug treatments such as stem cell

therapy, osteoprotegerin, integrin inhibitors, chloride channel inhibitors, cathepsin K inhibitors, nitrates. [69]. Emerging anabolic agents include calcilytics, statins, antibodies against sclerostin and Dickkopf-1, endo-cannabinoid agonist and matrix extracellular phosphoglycoprotein fragments [69]. The upcoming treatment strategies focus on continuous refinement to optimize the efficiency, safety and patient adherence. One such example of the emerging technique includes strontium ranelate. In this technique, strontium is ceded on the surface of newly formed bone which decreases the activity of osteoclasts and thereby reduces bone resorption. Simultaneously, it also stimulates osteoblast differentiation and increases the expression of bone forming markers [131]. While the recent researches highlight the emerging treatment techniques for treating osteoporosis, it is difficult to know the common side effects of these drugs. Therefore, a transitional interest from synthetic to natural product is transpiring [132].

Thereby from using synthetic treatment, natural treatments are emerging which simultaneously acts at different target sites promoting physiological health benefits. However, the bioavailability of bioactive components and the levels required to possibly act for the cure is necessary. Therefore, intake levels and doses are required to optimize health benefits [70]. It has been reported that pomegranate extract 0.01mL/g body weight when fed to mice, shows beneficial effects on BMD [71]. Prunes (1g/day) have also been reported for inhibiting bone resorption, as a rich source of boron [72]. Boron is a magical mineral working in close harmony with calcium to strengthen bone and protect against osteoporosis. Similar effects were observed with soya, higher BMD and reduced bone turnover [73]. It has also been reported that garlic oil modulates oestrogen properties by reducing an increase in urinary levels of hydroxyproline, calcium, phosphate, TRAP and alkaline phosphatase [74]. Various traditional herbal combinations in herbal medicine have also shown stable effects on the pathological model of osteoporosis. Cannabinoids have shown stable and positive effects on osteoblast differentiation indicating bone health-promoting effects [133]. However, many epidemiological and pharmacological models support in prevention of osteoporosis providing wide options for the beneficial effect on bone mineralization involving a variety of foodstuffs with astounding nutritional supplementation [75].

1.3 Pea protein

1.3.1 General composition

Peas (Pisum sativum L.) are a rich source of proteins. The protein content in peas varies from 21-25% depending upon environmental conditions and genetic factors [76]. It contains a majority of storage proteins, globulins (55-65%) and albumins (18-25%), which makes it nutritious. The major pea proteins are legumin, vicilin and convicilin, which represent 65-85% of total pea protein [77]. Legumin is a hexamer with a molecular weight between 320 to 380 kDa. The digestibility is reduced due to the presence of anti-nutrients such as phytase, amylase inhibitors and phenolics. Pea contains a variety of phenolics, which can act as antioxidants [77].

The emerging interest in plant proteins for food and animal feed has led to the evolution of valued crops such as field pea in the world market with Canada as a leader in the export of field pea [78]. Proteins fragments of pea are an important constituent of the pea seed. Protein from pea can be easily isolated using current knowledge for first concentrating protein fractions and then isolating, in order to maximise the use of protein fractions for promoting health benefits. The current knowledge procedures for isolation, purification and analysis are key for extraction. Extraction of protein fragments gives a heterogenous product known as pea protein isolate (PPI) [79]. It is of immense importance to advance the production process resulting in a protein isolate with an improved beneficial protein source. The enzymatic approach on pea protein isolate leads to increased protein fragments over 90% protein, contributing to health-promoting effects.

1.3.2 Production process of protein isolates and protein hydrolysate

Protein isolates are dietary supplements created by separating other constituting components other than protein [134]. They are commercially produced by alkaline extraction and precipitation at the isoelectric point or by using ultra filtration and reverse osmosis [135]. In the alkaline extraction method, the precipitated curd is then washed, and spray dried resulting in more soluble protein. This method utilises the difference of protein solubilisation in acids and alkali at specific pH conditions [136]. The pH for the solution is maintained until the isoelectric point of the protein solution is reached which separates out into protein isolates with the maximum attained protein level [80]. Another method for protein isolate production involves ultrafiltration and reverse osmosis. This system is based on the chemical nature and porous structure, which is governed by solute separation. The final composition of the protein isolate depends on the characteristics of

the membrane and operating conditions [137]. The ultrafiltration process is designed to retain the protein-rich stream which is dependent on the volume of permeate recovered from the original sample. The lower limit of protein isolates contains more than 90% protein [137]. To achieve the desired protein %, it is necessary to retentate efficiently prior to spray drying. Production and evaluation of pea protein isolate would necessarily increase the further food potential depending on the isolated quality, isolate management, water quality, integration process and quality assurance.

Hydrolysates are defined as an enzymatic and chemical combination of broken peptides in varying sizes [139]. They improve palatability and storage stability of the parent food protein. Protein hydrolysates provide beneficial effects in order to make functional protein readily available [140]. They can be prepared using chemical and biological methods. Chemical hydrolysis is generally used in industrial practices and biological methods include addition of enzymes which degrades the protein in smaller peptide fractions [141]. In the process, a product with high functionality and nutritive value is obtained. Although the production process for hydrolysates is massive, a proper control of enzyme catalysts, appropriate conditions and physiochemical mechanisms aids the final characteristics of the product [120].

1.3.3 Characterization of bioactive peptides

Bioactive peptides trigger specific physiological responses and further promote overall health and well-being [104]. Designing of bioactive peptides is a step-by-step process which involves selection of protein source and biocatalyst, selection of production technique and peptide production, separation and purification [143]. Selection of production technique depends on the targeted function of the peptide [143]. Food proteins are enzymatically digested with an enzyme followed by key stages of purification and identification [145]. The quantitative structure-activity relationship predicts the overall synthesizing of the bioactive peptides from parent protein. In the end, economic analysis is carried out to calculate the economic assessment of the entire process determining the amount of enzyme required, enzyme to substrate ratio, amount of substrate required to achieve the targeted production rate of effect [121].

1.3.4 Functional properties of pea protein

Functionality assessment of pea protein isolates is generally based on their solubility, water binding capacity, emulsifying properties, foaming abilities, gelation properties and its sensory

parameters such as colour, flavour. [146]. Solubility is generally assessed using a nitrogen solubility index (NSI), which is indirectly dependent on pH [147]. Pea proteins are highly soluble in acidic and alkali pH conditions. Solubility is the key characteristic for evaluating potential applications of proteins. Water binding ability of proteins are important for any incorporation in food products, pea protein is a hydrated protein which imparts structural properties. Similarly, the pea protein possesses good emulsifying properties [147]. Foaming and whipping properties of pea protein are considered less when compared with other plant protein such as chickpea and soy. Similar results for the viscosity of pea protein isolate dispersion is observed when compared to that of soy [148].

1.3.5 Bioactivities of pea protein

Apart from nutritional and functional contributions of pea proteins, it also provides offerings in the area of functional food and nutraceuticals. The performance and effectiveness of a food protein are evaluated using bioactive properties which positively influences human health [149]. Enzymatic hydrolysis modifies the conformational structure of the parent protein releasing peptides exhibiting various bioactivities which can improve health status. Multifunctional peptides target various pathological events and result in beneficial effects for that condition. Some of the recognized bioactivities from pea protein are discussed below.

1.3.5.1 Antioxidant activity

Phenolic compounds are regarded as the natural antioxidants, which help in protecting against chronic diseases such as cancer and other inflammatory-related diseases [150]. Phenolic compounds are present in the seed coat and cotyledon of peas from which it provides its antioxidant capacity. Antioxidant activity of pea protein-derived peptides represents good metal chelating capacity and improved linoleic acid oxidation inhibition [151].

1.3.5.2 ACE inhibitory properties

Blood pressure in the human body is regulated by the renin-angiotensin system in which angiotensin II acts as a vasoconstrictor with renin and angiotensin-converting enzyme as two important enzymes. Two fractions extracted from pea protein showed a significant ACE inhibitory property indicating the ability of pea protein derived peptide in treating hypertension [123].

1.3.5.3 Antimicrobial properties

Antimicrobial properties contribute to their capability of inhibiting the growth and existence of pathogenic microorganism. Due to this property, growing interest is emerging in the area of pharmacology and food industry [91]. Psdl, 46 amino acids residue isolated from pea protein appeared to exert an antibacterial effect by acting as K^+ channel inhibitor using surface charge distribution [90].

1.3.5.4 Hypolipidemic effect

Lipid-lowering effects of pea protein isolates have been observed, as they regulate the lipid metabolism by modifying genes regulating fatty acid and cholesterol homeostasis. Pea protein upregulate cellular lipid homeostasis involving gene controlling hepatic cholesterol by downregulating fatty acid synthesis [92].

1.3.5.5 Anticancer properties

Anti-cancer activity from the extracts of pea has been investigated showing the presence of chemo-preventive agent [126]. A potential cancer chemo preventive agent with protease inhibitors, rT11B and rT12B from pea pharmacologically induce anti-cancer activity. Pea is also rich in apigenin, hydroxybenzoic acid, hydroxycinnamic, acid luteolin and quercetin which all pays to remedial therapies including anticarcinogenic [93].

1.3.5.6 Ani-inflammatory

Apart from the bioactivities discussed above, pea protein hydrolysate also exhibits anti-inflammatory property. Inflammation is a pathological mechanism by an organism in order to defend themselves against infection and restoring homeostasis. Inflammation is the beginning of severe diseases [128]. Pea protein hydrolysate significantly reduces nitric oxide production by 20% on activated macrophages. Pea protein hydrolysates are also expected to inhibit the secretion of cytokine, IL-6 and TNFs [94]. Pea protein hydrolysate exhibits anti-inflammatory action by targeting with high specificity with innovative strategies [95].

1.3.5.7 Immunomodulatory properties

Oral intake of dietary antigens from pea protein hydrolysate stimulates the non-specific immunity of the host and forwards the systemic immune response by modulating the role of immunocompetent cells [138]. Pea protein hydrolysate signals the stimulation of the innate

immune system by enhancing the activity of peritoneal macrophages. Therefore, pea protein can help immunostimulation for the regulation of IL-6 production [95].

1.4 Bioactivities of food-derived peptides

Food-derived bioactive peptides have significant biological activities regenerating substantial responses. On ingestion of bioactive components in the form of peptides, hydrolysate or non-active metabolites it produces specific responses [96]. The collagen derived bioactive peptides such as Pro-Hyp and Hyp-Gly, increases the growth of cultured fibroblast and thereby stimulates hyaluronic acid levels [97]. Wheat gluten hydrolysate derived peptide Pro-Glu-Leu attenuates the D-glucosamine induced acute hepatitis when fed to rats at 20mg/kg BW. Similar effects of wheat gluten hydrolysate were observed in mice fed at 0.1mg/kg BW in reducing colitis- induced dysbiosis [98]. In the spontaneously hypertensive rat model, a pyro-Gly peptide from elastin shows an increase in the production of elastin by protecting the aortic endothelial cells against hypertensive endothelial injury [99]. Bioactive peptides derived from milk such as VPP and IPP, exhibits angiotensin-converting enzyme inhibitory effects, stimulates nitric oxide levels and vasorelaxant pathways associated with atherosclerosis and hypertension [100]. Enzymatic digestion of casein protein results in a mixture of peptide fragments generating anti-inflammatory effects [101]. Bioactive peptide lactoferricin derived from lactoferrin, a milk protein exhibits anti-inflammatory activity on human cartilage and synovial cells indicating finest arthritis management [102]. Egg derived IRW and IQW from ovotransferrin down-regulates cytokine-induced expression stimulating via NF-KB pathway [103]. Soybean derived peptides exerts inflammatory activities by TGF-beta signaling [104].

These example for bioactivities of the food-derived peptides indicate their substantial responses in *in vitro* and *in vivo* for promoting health benefits [104]. Food-derived peptides and peptide-rich protein hydrolysate ranges with potential health benefits based on various physiological conditions.

1.5 Bioactive peptides and osteoporosis

Protein intake plays an important role in maintaining bone quality, bone strength, fracture repair and bone formation [144]. Due to the side effects of pharmacological drugs, a growing interest is emerging in dietary intrusions with food-derived bioactive peptides on preventing and treating osteoporosis [142]. Especially in the ageing population, protein intake has crucial standing on bone

health. The first derived bioactive peptides were casein phosphopeptides which acted upon enhancing vitamin D to independent bone calcification [107]. Synthetic peptides from endogenous parent proteins have been utilised as a key element in the regeneration of bone for treating osteoporosis. They generate osteogenic effects in producing potent osteoinductive growth factors which mediates bone formation [105]. The bone morphogenetic protein (BMP-2) peptide stimulates the highest expression of ALP and osteocalcin and induce bone regeneration [106]. Bone forming peptide-1 (BFP-1) induces osteogenic differentiation and enhance ALP activity with increased calcium deposition. With our current knowledge of the important role of type1 collagen in the formation of bone matrix, the literature suggests that hydrolysate or peptides from collagen enhance osteoblast differentiation, improving BMD in OVX rats by regulating ALP levels and expression of RUNX2 [108]. MPDW, peptide derived from marine microalgae *Nannochloropis* oculate stimulates differentiation of osteoblasts via Mitogen activated protein kinase (MAPK) pathway [109]. NAVPITPTL increases osteoblast differentiation through p-Akt pathways. Furthermore, VLVPQK peptide derived from buffalo casein is an antioxidant peptide exhibiting osteoporotic effects acts by inhibiting oxidative damage and bone-resorbing cytokine in OVX rats [110].

Food derived bioactive peptides provide safe conditions as compared to synthetic drugs. They provide bioactive benefits with nutritional and functional value [104]. Enzymatic hydrolysis provides easy production of bioactive food peptides from parent food protein. These peptides play a synergistic effect which results in osteogenic activity [158]. Therefore, the development of more food-based bioactive peptides can help in enhancing safety and bone health-promoting effects.

1.6 Potential challenges

With an emergent interest in the area of bioactive peptides, safety risk factors are also involved such as efficacy. In order to evaluate the efficacy of the derived peptide, it first requires an early stage *in vivo* experiment before applying it towards the benefit of human health. An associated risk system after *in vivo* studies also requires substantial pharmacokinetic studies for proper dosage and frequency of administration, which is difficult to determine the result in huge variability in biological activity and intake levels [111].

Regular intake of these peptides might possess a risk to health, as daily consumption of some vitamins at high dose suggests potential toxic possibilities upon consumption. The presence

of immunogenic proteins and peptides within the protein hydrolysate are another factor of impending risk which can exert aggravate sensitized side effects.

Therefore, appropriate screening such as *in vivo* experiments and pharmacokinetic studies prior to consumption of explicit peptide fragments which can potentially have a beneficial effect is necessary. While with the lack of preliminary study about the bioactivity of the peptide, its potential health-promoting effects can act as a limitation imitating optimistic opportunity for future research [112].

1.7 Future perspectives

Bioactive peptides and protein hydrolysates have now emerged as a new direction in functional foods and nutraceuticals. Various scientific and technological issues about human health and nutrition has been resolved with the occurrence of many biologically active compounds. Molecular and epigenetic studies have assessed the mechanistic pathways of bioactive compounds to exert their activities. Bioactive peptides and protein hydrolysates possess valuable functional components in nutritious diet. Through bioinformatic tools, empirical approach to the functioning of bioactive peptides and protein hydrolysates have become easier with better understanding at peptide-structure activity relationship. Standardized clinical trials assist in evaluating efficacy and metabolic fate of bioactive compounds in gastro-intestinal tract in order to investigate its effect are essential.

1.8 General conclusion

There is a growing interest in the use of bioactive components (food-derived bioactive peptides) for health management and in preventing occurrence of severe health diseases. Osteoporosis is chronic disease that affects millions of people with its silent aggressive nature. Bone matrix quality and bone strength are important factors for bone health, which depends on the bone remodeling cycle; therefore, opportunities of identifying food derived bioactive components in maintaining the bone matrix quality and bone strength can largely play an important role in preventing osteoporosis. A few scientific studies suggest the potential of various food-derived components in promoting bone health. However, health benefits from bioactive components such as food peptides and hydrolysates are dependent on their fate during gastrointestinal digestion (GI). Due to their smaller peptide sequence, they present a strong chance for easy absorption in gastrointestinal system. Thus, the bioavailability and efficacy of orally administrated bioactive component further

influence its response. Furthermore, it is important to study and examine the underlying pathways elucidating its signalling mechanism.

Apart from all the breakthroughs, food-derived bioactive components present a promising approach in benefitting the human health.

1.9 References

[1] Florencio-Silva, R., Sasso, G.R.D.S., Sasso-Cerri, E., Simões, M.J. and Cerri, P.S., 2015. Biology of bone tissue: structure, function, and factors that influence bone cells. BioMed Research International, 2015.

[2] Shiozawa, Y., Havens, A.M., Pienta, K.J. and Taichman, R.S., 2008. The bone marrow niche: habitat to hematopoietic and mesenchymal stem cells, and unwitting host to molecular parasites. Leukemia, 22(5), p.941.

[3] Putnam, S.E., Scutt, A.M., Bicknell, K., Priestley, C.M. and Williamson, E.M., 2007. Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 21(2), pp.99-112.4 3, Kumar and Clark, 2002.

[4] Kozielski, M., Buchwald, T., Szybowicz, M., Błaszczak, Z., Piotrowski, A. and Ciesielczyk, B., 2011. Determination of composition and structure of spongy bone tissue in human head of femur by Raman spectral mapping. Journal of Materials Science: Materials in Medicine, 22(7), pp.1653-1661.

[5] Todd, J.C., Sanford, A.H., Davidsohn, I. and Henry, J.B., 1979. Todd-Sanford-Davidsohn clinical diagnosis and management by laboratory methods. WB Saunders Company.

[6] Ott, S.M., 2018. Cortical or trabecular bone: What's the difference? American Journal of Nephrology, 47(6), pp.373-375.

[7] Niall Ferguson, 2004. Osteoporosis in focus. Pharmaceutical Press.

[8] Parfitt, A.M., 2002. Misconceptions (2): turnover is always higher in cancellous than in cortical bone. Bone (NY), 30, pp.807-809.

[9] Brot, C., Jørgensen, N., Madsen, O.R., Jensen, L.B. and Sørensen, O.H., 1999. Relationships between bone mineral density, serum vitamin D metabolites and calcium: phosphorus intake in healthy perimenopausal women. Journal of Internal Medicine, 245(5), pp.509-516.

[10] Christoffersen, J. and Landis, W.J., 1991. A contribution with review to the description of mineralization of bone and other calcified tissues *in vivo*. The Anatomical Record, 230(4), pp.435-450.

[11] Boskey, A.L., Spevak, L., Paschalis, E., Doty, S.B. and McKee, M.D., 2002. Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. Calcified Tissue International, 71(2), pp.145-154.
[12] Datta, H.K., Ng, W.F., Walker, J.A., Tuck, S.P. and Varanasi, S.S., 2008. The cell biology of bone metabolism. Journal of Clinical Pathology, 61(5), pp.577-587.
[13] Vashishth, D., 2005. Collagen glycation and its role in fracture properties of bone. Journal of Musculoskeletal and Neuronal Interactions, 5(4), p.316.
[14] Florencio-Silva, R., Sasso, G.R.D.S., Sasso-Cerri, E., Simões, M.J. and Cerri, P.S., 2015. Biology of bone tissue: structure, function, and factors that influence bone cells. BioMed Research International, 2015.
[15] Caetano-Lopes, J., Canhao, H. and Eurico Fonseca, J., 2007. Osteoblasts and bone formation. Acta reumatológica portuguesa, 32(2).
[16] Grigoriadis, A.E., Heersche, J.N. and Aubin, J.E., 1988. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. The Journal of Cell Biology, 106(6), pp.2139-2151.
[17] Fakhry, M., Hamade, E., Badran, B., Buchet, R. and Magne, D., 2013. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. World Journal of Stem Cells, 5(4), p.136.
[18] Capulli, M., Paone, R. and Rucci, N., 2014. Osteoblast and osteocyte: games without frontiers. Archives of Biochemistry and Biophysics, 561, pp.3-12.
[19] Damoulis, P.D. and Hauschka, P.V., 1997. Nitric oxide acts in conjunction with proinflammatory cytokines to promote cell death in osteoblasts. Journal of Bone and Mineral Research, 12(3), pp.412-422.
[20] Pawelec, K. and Planell, J.A. eds., 2018. Bone Repair Biomaterials: Regeneration and Clinical Applications. Woodhead Publishing.
[21] Anderson, H.C., 2003. Matrix vesicles and calcification. Current Rheumatology Reports, 5(3), pp.222-226.
[22] Yoshiko, Y., Candeliere, G.A., Maeda, N. and Aubin, J.E., 2007. Osteoblast autonomous Pi regulation via Pit1 plays a role in bone mineralization. Molecular and Cellular Biology, 27(12), pp.4465-4474.

[23] Glimcher, M.J., 1998. The nature of the mineral phase in bone: biological and clinical implications. In Metabolic bone disease and clinically related disorders (pp. 23-52e). Academic Press.

[24] Wu, L.N., Genge, B.R., Lloyd, G.C. and Wuthier, R.E., 1991. Collagen-binding proteins in collagenase-released matrix vesicles from cartilage. Interaction between matrix vesicle proteins and different types of collagen. Journal of Biological Chemistry, 266(2), pp.1195-1203.

[25] Arsenault, A.L., Frankland, B.W. and Ottensmeyer, F.P., 1991. Vectorial sequence of mineralization in the turkey leg tendon determined by electron microscopic imaging. Calcified Tissue International, 48(1), pp.46-55.

[26] Miller, S.C., Bowman, B.M., Smith, J.M. and Jee, W.S., 1980. Characterization of endosteal bone-lining cells from fatty marrow bone sites in adult beagles. The Anatomical Record, 198(2), pp.163-173.

[27] Miller, S.C., Bowman, B.M. and Jee, W.S., 1989. Bone lining cells: structure and function. Scanning Microscopy, 3(3), pp.953-60.

[28] Schaffler, M.B., Cheung, W.Y., Majeska, R. and Kennedy, O., 2014. Osteocytes: master orchestrators of bone. Calcified Tissue International, 94(1), pp.5-24.

[29] Florencio-Silva, R., Sasso, G.R.D.S., Sasso-Cerri, E., Simões, M.J. and Cerri, P.S., 2015. Biology of bone tissue: structure, function, and factors that influence bone cells. BioMed Research International, 2015.

[30] Komori, T., 2009. Regulation of osteoblast differentiation by Runx2. In Osteoimmunology (pp. 43-49). Springer, Boston, MA.

[31] Xu, F. and Teitelbaum, S.L., 2013. Osteoclasts: new insights. Bone Research, 1, p.11.

[32] Xie, W., Ji, L., Zhao, T. and Gao, P., 2015. Identification of transcriptional factors and key genes in primary osteoporosis by DNA microarray. Medical science monitor: International Medical Journal of Experimental and Clinical Research, 21, p.1333.

[33] Ralston, S.H. and de Crombrugghe, B., 2006. Genetic regulation of bone mass and susceptibility to osteoporosis. Genes & Development, 20(18), pp.2492-2506.

[34] Hauge, E.M., Qvesel, D., Eriksen, E.F., Mosekilde, L. and Melsen, F., 2001. Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers. Journal of Bone and Mineral Research, 16(9), pp.1575-1582.

[35] Salgado, P.C., Sathler, P.C., Castro, H.C., Alves, G.G., De Oliveira, A.M., De Oliveira, R.C., Maia, M.D., Rodrigues, C.R., Coelh, P.G., Fuly, A. and Cabral, L.M., 2011. Bone remodeling, biomaterials and technological applications: revisiting basic concepts. Journal of Biomaterials and Nanobiotechnology, 2(03), p.318.
[36] Swarthout, J.T., D'Alonzo, R.C., Selvamurugan, N. and Partridge, N.C., 2002. Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. Gene, 282(1-2), pp.1-17.
[37] Li, X., Qin, L., Bergenstock, M., Bevelock, L.M., Novack, D.V. and Partridge, N.C., 2007. Parathyroid hormone stimulates osteoblastic expression of MCP-1 to recruit and increase the fusion of pre/osteoclasts. Journal of Biological Chemistry, 282(45), pp.33098-33106.
[38] Teitelbaum, S.L., 2000. Bone resorption by osteoclasts. Science, 289 (5484), pp.1504-1508.
[39] Manolagas, S.C., 2006. Choreography from the tomb: an emerging role of dying osteocytes in the purposeful, and perhaps not so purposeful, targeting of bone remodeling. IBMS BoneKEy, 3, p.5.
[40] Florencio-Silva, R., Sasso, G.R.D.S., Sasso-Cerri, E., Simões, M.J. and Cerri, P.S., 2015. Biology of bone tissue: structure, function, and factors that influence bone cells. BioMed Research International, 2015.
[41] Teitelbaum, S.L., 2000. Bone resorption by osteoclasts. Science, 289(5484), pp.1504-1508.
[42] Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T.J. and Suda, T., 1990. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proceedings of the National Academy of Sciences, 87(18), pp.7260-7264.
[43] Lacey, D.L., Timms, E., Tan, H.L., Kelley, M.J., Dunstan, C.R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S. and Hsu, H., 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell, 93(2), pp.165-176.
[44] Kong, Y.Y., Yoshida, H., Sarosi, I., Tan, H.L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A.J., Van, G., Itie, A. and Khoo, W., 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature, 397(6717), p.315.

[45] Hofbauer, L.C., Gori, F., Riggs, B.L., Lacey, D.L., Dunstan, C.R., Spelsberg, T.C. and Khosla, S., 1999. Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. Endocrinology, 140(10), pp.4382-4389.

[46] Blair, H.C., Teitelbaum, S.L., Ghiselli, R. and Gluck, S., 1989. Osteoclastic bone resorption by a polarized vacuolar proton pump. Science, 245(4920), pp.855-857.

[47Matsumoto, M., Kogawa, M., Wada, S., Takayanagi, H., Tsujimoto, M., Katayama, S., Hisatake, K. and Nogi, Y., 2004. Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU. 1. Journal of Biological Chemistry, 279(44), pp.45969-45979.

[48Florencio-Silva, R., Sasso, G.R.D.S., Sasso-Cerri, E., Simões, M.J. and Cerri, P.S., 2015. Biology of bone tissue: structure, function, and factors that influence bone cells. BioMed Research International, 2015.

[49] Arana-Chavez, V.E. and Bradaschia-Correa, V., 2009. Clastic cells: mineralized tissue resorption in health and disease. The International Journal of Biochemistry & Cell biology, 41(3), pp.446-450.

[50] Charles, J.F. and Aliprantis, A.O., 2014. Osteoclasts: more than 'bone eaters. Trends in Molecular Medicine, 20(8), pp.449-459.

[51] https://www.bones.nih.gov/health-info/bone/osteoporosis/overview July 12, 2091.

[52] Cooper, C., Campion, G. and Melton, L.3., 1992. Hip fractures in the elderly: a world-wide projection. Osteoporosis International, 2(6), pp.285-289.

[53] Reginster, J.Y. and Burlet, N., 2006. Osteoporosis: a still increasing prevalence. Bone, 38(2), pp.4-9.

[54] De Laet, C., Kanis, J.A., Odén, A., Johanson, H., Johnell, O., Delmas, P., Eisman, J.A., Kroger, H., Fujiwara, S., Garnero, P. and McCloskey, E.V., 2005. Body mass index as a predictor of fracture risk: a meta-analysis. Osteoporosis International, 16(11), pp.1330-1338.

[55] Gullberg, B., Johnell, O. and Kanis, J.A., 1997. World-wide projections for hip fracture. Osteoporosis International, 7(5), pp.407-413.

[56] Sözen, T., Özışık, L. and Başaran, N.Ç., 2017. An overview and management of osteoporosis. European Journal of Rheumatology, *4*(1), p.46.

[57] Kanis, J.A., McCloskey, E.V., Johansson, H., Oden, A., Ström, O. and Borgström, F., 2010. Development and use of FRAX® in osteoporosis. Osteoporosis International, 21(2), pp.407-413.
[58] World Health Organization, World Health Organization. Ageing and Life Course Unit, 2008. WHO global report on falls prevention in older age. World Health Organization.
[59] Notelovitz, M., 1993. Osteoporosis: screening, prevention, and management. Fertility and Sterility, 59(4), pp.707-725.
[60] Robinson, R.F. and Nahata, M.C., 2001. Prevention and treatment of osteoporosis in the cystic fibrosis population. Journal of Pediatric Health Care, 15(6), pp.308-315.
[61] Pavone, V., Testa, G., Giardina, S., Vescio, A., Restivo, D.A. and Sessa, G., 2017. Pharmacological therapy of osteoporosis: a systematic current review of literature. Frontiers in Pharmacology, 8, p.803.
[62] Sunyecz, J.A., 2008. The use of calcium and vitamin D in the management of osteoporosis. Therapeutics and Clinical Risk Management, 4(4), p.827.
[63] Mitchell, D.G., Rao, V.M., Dalinka, M.K., Spritzer, C.E., Alavi, A., Steinberg, M.E., Fallon, M. and Kressel, H.Y., 1987. Femoral head avascular necrosis: correlation of MR imaging, radiographic staging, radionuclide imaging, and clinical findings. Radiology, 162(3), pp.709-715.
[64] Chapuy, M.C., Arlot, M.E., Duboeuf, F., Brun, J., Crouzet, B., Arnaud, S., Delmas, P.D. and Meunier, P.J., 1992. Vitamin D3 and calcium to prevent hip fractures in elderly women. New England Journal of Medicine, 327(23), pp.1637-1642.
[65] Morello, K.C., Wurz, G.T. and DeGregorio, M.W., 2002. SERMs: current status and future trends. Critical reviews in Oncology/Hematology, 43(1), pp.63-76.
[66] Reeve, J., Meunier, P.J., Parsons, J.A., Bernat, M., Bijvoet, O.L., Courpron, P., Edouard, C., Klenerman, L., Neer, R.M., Renier, J.C. and Slovik, D., 1980. Anabolic effect of human parathyroid hormone fragment on trabecular bone in involutional osteoporosis: a multicentre trial. The British Medical Journal, 280(6228), pp.1340-1344.
[67] Neer, R.M., Arnaud, C.D., Zanchetta, J.R., Prince, R., Gaich, G.A., Reginster, J.Y., Hodsman, A.B., Eriksen, E.F., Ish-Shalom, S., Genant, H.K. and Wang, O., 2001. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. New England Journal of Medicine, 344(19), pp.1434-1441.

[68] Clarke, B.L., 2009. New and emerging treatments for osteoporosis. Clinical Endocrinology, 71(3), pp.309-321.

[69] Bhutani, G. and Gupta, M.C., 2013. Emerging therapies for the treatment of osteoporosis. Journal of Mid-life Health, 4(3), p.147.

[70] Abuajah, C.I., Ogbonna, A.C. and Osuji, C.M., 2015. Functional components and medicinal properties of food: a review. Journal of Food Science and Technology, 52(5), pp.2522-2529.

[71] Mori-Okamoto, J., Otawara-Hamamoto, Y., Yamato, H. and Yoshimura, H., 2004. Pomegranate extract improves a depressive state and bone properties in menopausal syndrome model ovariectomized mice. Journal of Ethnopharmacology, 92(1), pp.93-101.

[72] Mühlbauer, M., Bosserhoff, A.K., Hartmann, A., Thasler, W.E., Weiss, T.S., Herfarth, H., Lock, G., Schölmerich, J. and Hellerbrand, C., 2003. A novel MCP-1 gene polymorphism is associated with hepatic MCP-1 expression and severity of HCV-related liver disease. Gastroenterology, 125(4), pp.1085-1093.

[73] Branca, C., Giudicianni, P. and Di Blasi, C., 2003. GC/MS characterization of liquids generated from low-temperature pyrolysis of wood. Industrial & Engineering Chemistry Research, 42(14), pp.3190-3202.

[74] Putnam, S.E., Scutt, A.M., Bicknell, K., Priestley, C.M. and Williamson, E.M., 2007. Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 21(2), pp.99-112.

[75] Hernlund, E., Svedbom, A., Ivergård, M., Compston, J., Cooper, C., Stenmark, J., McCloskey, E.V., Jönsson, B. and Kanis, J.A., 2013. Osteoporosis in the European Union: medical management, epidemiology and economic burden. Archives of Osteoporosis, 8(1-2), p.136.

[76] Hood-Niefer, S.D., Warkentin, T.D., Chibbar, R.N., Vandenberg, A. and Tyler, R.T., 2012. Effect of genotype and environment on the concentrations of starch and protein in, and the physicochemical properties of starch from, field pea and fababean. Journal of the Science of Food and Agriculture, 92(1), pp.141-150.

[77] Dahl, W.J., Foster, L.M. and Tyler, R.T., 2012. Review of the health benefits of peas (Pisum sativum L.). British Journal of Nutrition, 108(S1), pp. S3-S10.

[78] Small, E., 1999. New crops for Canadian agriculture. Perspectives on new crops and new uses. ASHS Press, Alexandria, VA. USA, pp.15-52.
[79] Adebiyi, A.P. and Aluko, R.E., 2011. Functional properties of protein fractions obtained from commercial yellow field pea (Pisum sativum L.) seed protein isolate. Food Chemistry, 128(4), pp.902-908.
[80] Pergande, M. and Cologna, S., 2017. Isoelectric point separations of peptides and proteins. Proteomes, 5(1), p.4.
[81] Hasegawa, T., Yamamoto, T., Tsuchiya, E., Hongo, H., Tsuboi, K., Kudo, A., Abe, M., Yoshida, T., Nagai, T., Khadiza, N. and Yokoyama, A., 2017. Ultrastructural and biochemical aspects of matrix vesicle-mediated mineralization. Japanese Dental Science Review, 53(2), pp.34-45.
[82] Rucci, N., 2008. Molecular biology of bone remodelling. Clinical cases in Mineral and Bone Metabolism, 5(1), p.49.
[83] Tsuboi, H., Matsui, Y., Hayashida, K., Yamane, S., Maeda-Tanimura, M., Nampei, A., Hashimoto, J., Suzuki, R., Yoshikawa, H. and Ochi, T., 2003. Tartrate resistant acid phosphatase (TRAP) positive cells in rheumatoid synovium may induce the destruction of articular cartilage. Annals of the Rheumatic Diseases, 62(3), pp.196-203.
[84] Raggatt, L.J. and Partridge, N.C., 2010. Cellular and molecular mechanisms of bone remodeling. Journal of Biological Chemistry, 285(33), pp.25103-25108.
[85] Shaker, J.L. and Deftos, L., 2018. Calcium and phosphate homeostasis. In Endotext [Internet]. MDText. com, Inc.
[86] Lerner, U.H., Kindstedt, E. and Lundberg, P., 2019. The critical interplay between bone resorbing and bone forming cells. Journal of Clinical Periodontology, 46, pp.33-51.
[87] Bonewald, L.F. and Johnson, M.L., 2008. Osteocytes, mechanosensing and Wnt signaling. Bone, 42(4), pp.606-615.
[88] Lindsay, R., Hart, D.M., Forrest, C. and Baird, C., 1980. Prevention of spinal osteoporosis in oophorectomised women. The Lancet, 316(8205), pp.1151-1154.
[89] Leali, P.T., Muresu, F., Melis, A., Ruggiu, A., Zachos, A. and Doria, C., 2011. Skeletal fragility definition. Clinical cases in Mineral and Bone Metabolism, 8(2), p.11.

[90] Almeida, M.S., Cabral, K.M., Kurtenbach, E., Almeida, F.C. and Valente, A.P., 2002. Solution structure of Pisum sativum defensin 1 by high resolution NMR: plant defensins, identical backbone with different mechanisms of action. Journal of Molecular Biology, 315(4), pp.749-757.

[91] Larios-Cruz, R., Londoño-Hernández, L., Gómez-García, R., García-Galindo, I., Sepulveda, L., Rodríguez-Herrera, R. and Aguilar, C.N., 2019. Extraction of Bioactive Molecules through Fermentation and Enzymatic Assisted Technologies. High Value Fermentation Products: Human Health, 1, pp.27-59.

[92] Rigamonti, E., Parolini, C., Marchesi, M., Diani, E., Brambilla, S., Sirtori, C.R. and Chiesa, G., 2010. Hypolipidemic effect of dietary pea proteins: Impact on genes regulating hepatic lipid metabolism. Molecular Nutrition & Food Research, 54(S1), pp. S24-S30.

[93] Rungruangmaitree, R. and Jiraungkoorskul, W., 2017. Pea, Pisum sativum, and its anticancer activity. Pharmacognosy Reviews, 11(21), p.39.

[94] Ndiaye, F., Vuong, T., Duarte, J., Aluko, R.E. and Matar, C., 2012. Antioxidant, anti-inflammatory and immunomodulating properties of an enzymatic protein hydrolysate from yellow field pea seeds. European Journal of Nutrition, 51(1), pp.29-37.

[95] La Manna, S., Di Natale, C., Florio, D. and Marasco, D., 2018. Peptides as Therapeutic Agents for Inflammatory-Related Diseases. International Journal of Molecular Sciences, 19(9), p.2714.

[96] Chakrabarti, S., Guha, S. and Majumder, K., 2018. Food-derived bioactive peptides in human health: Challenges and Opportunities. Nutrients, 10(11), p.1738.

[97] Ohara, H., Iida, H., Ito, K., Takeuchi, Y. and Nomura, Y., 2010. Effects of Pro-Hyp, a collagen hydrolysate-derived peptide, on hyaluronic acid synthesis using *in vitro* cultured synovium cells and oral ingestion of collagen hydrolysates in a guinea pig model of osteoarthritis. Bioscience, Biotechnology, and Biochemistry, 74(10), pp.2096-2099.

[98] Zhang, C., Li, J., Hu, C., Wang, J., Zhang, J., Ren, Z., Song, X. and Jia, L., 2017. Antihyperglycemic and organic protective effects on pancreas, liver and kidney by polysaccharides from Hericium erinaceus SG-02 in streptozotocin-induced diabetic mice. Scientific reports, 7(1), p.10847.

[99] Sato, Y., Ren, X.S., Harada, K., Sasaki, M., Morikawa, H., Shiomi, S., Honda, M., Kaneko, S. and Nakanuma, Y., 2012. Induction of elastin expression in vascular endothelial cells relates to

hepatoportal sclerosis in idiopathic portal hypertension: possible link to serum anti-endothelial cell antibodies. Clinical & Experimental Immunology, 167(3), pp.532-542.

[100] Pujiastuti, D.Y., Amin, G., Nur, M., Alamsjah, M.A. and Hsu, J.L., 2019. Marine Organisms as Potential Sources of Bioactive Peptides that Inhibit the Activity of Angiotensin I-Converting Enzyme: A Review. Molecules, 24(14), p.2541.

[101] Marcone, S., Belton, O. and Fitzgerald, D.J., 2017. Milk-derived bioactive peptides and their health promoting effects: a potential role in atherosclerosis. British Journal of Clinical Pharmacology, 83(1), pp.152-162.

[102] Bruni, N., Capucchio, M., Biasibetti, E., Pessione, E., Cirrincione, S., Giraudo, L., Corona, A. and Dosio, F., 2016. Antimicrobial activity of lactoferrin-related peptides and applications in human and veterinary medicine. Molecules, 21(6), p.752.

[103] Liao, W., Chakrabarti, S., Davidge, S.T. and Wu, J., 2016. Modulatory effects of egg white ovotransferrin-derived tripeptide IRW (Ile-Arg-Trp) on vascular smooth muscle cells against angiotensin II stimulation. Journal of Agricultural and Food Chemistry, 64(39), pp.7342-7347.

[104] Chakrabarti, S., Jahandideh, F. and Wu, J., 2014. Food-derived bioactive peptides on inflammation and oxidative stress. BioMed Research International, 2014.

[105] Ahn, C.B. and Je, J.Y., 2019. Bone health-promoting bioactive peptides. Journal of Food Biochemistry, 43(1), p.e12529.

[106] Tian, G., Zhang, G. and Tan, Y.H., 2013. Calcitonin gene-related peptide stimulates BMP-2 expression and the differentiation of human osteoblast-like cells *in vitro*. Acta Pharmacologica Sinica, 34(11), p.1467.

[107] Bhat, Z.F., Kumar, S. and Bhat, H.F., 2015. Bioactive peptides of animal origin: a review. Journal of Food Science and Technology, 52(9), pp.5377-5392.

[108] Li, J., Hao, L., Wu, J., Zhang, J. and Su, J., 2016. Linarin promotes osteogenic differentiation by activating the BMP-2/RUNX2 pathway via protein kinase A signaling. International Journal of Molecular Medicine, 37(4), pp.901-910.

[109] Carson, M. and Clarke, S., 2018. Bioactive compounds from marine organisms: Potential for bone growth and healing. Marine Drugs, 16(9), p.340.

[110] Mada, S.B., Reddi, S., Kumar, N., Kumar, R., Kapila, S., Kapila, R., Trivedi, R., Karvande, A. and Ahmad, N., 2017. Antioxidative peptide from milk exhibits antiosteopenic effects through

inhibition of oxidative damage and bone-resorbing cytokines in ovariectomized rats. Nutrition, 43, pp.21-31.

[111] Turner, R.M., Park, B.K. and Pirmohamed, M., 2015. Parsing interindividual drug variability: an emerging role for systems pharmacology. Wiley Interdisciplinary Reviews: Systems Biology and Medicine, 7(4), pp.221-241.

[112] Lax, R., 2010. The future of peptide development in the pharmaceutical industry. PharManufacturing: The International Peptide Review, 2, pp.10-15.

[113] Katikaneni, R., Ponnapakkam, A., Miller, E., Ponnapakkam, T. and Gensure, R.C., 2009. A new technique for precisely and accurately measuring lumbar spine bone mineral density in mice using clinical dual energy X-ray absorptiometry (DXA). Toxicology Mechanisms and Methods, 19(3), pp.225-231.

[114] Sözen, T., Özışık, L. and Başaran, N.Ç., 2017. An overview and management of osteoporosis. European Journal of Rheumatology, 4(1), p.46.

[115] Azagra, R., Roca, G., Encabo, G., Aguyé, A., Zwart, M., Güell, S., Puchol, N., Gene, E., Casado, E., Sancho, P. and Solà, S., 2012. FRAX® tool, the WHO algorithm to predict osteoporotic fractures: the first analysis of its discriminative and predictive ability in the Spanish FRIDEX cohort. BMC Musculoskeletal Disorders, 13(1), p.204.

[116] Jones, G., Winzenberg, T.M., Callisaya, M.L. and Laslett, L.L., 2014. Lifestyle modifications to improve musculoskeletal and bone health and reduce disability–A life-course approach. Best Practice & Research Clinical Rheumatology, 28(3), pp.461-478.

[117] Beto, J.A., 2015. The role of calcium in human aging. Clinical Nutrition Research, 4(1), pp.1-8.

[118] Giangregorio, L.M., Papaioannou, A., Macintyre, N.J., Ashe, M.C., Heinonen, A., Shipp, K., Wark, J., McGill, S., Keller, H., Jain, R. and Laprade, J., 2014. Too fit to fracture: exercise recommendations for individuals with osteoporosis or osteoporotic vertebral fracture. Osteoporosis International, 25(3), pp.821-835.

[119] Pellico, L.H. and Bautista, C., 2012. Focus on adult health medical-surgical nursing.

[120] Kristinsson, H.G. and Rasco, B.A., 2000. Fish protein hydrolysates: production, biochemical, and functional properties. Critical Reviews in Food Science and Nutrition, 40(1), pp.43-81.

[121] https://www.nlm.nih.gov/nichsr/hta101/ta10107.html, July 13, 2019

[122] Drake, M.T., Clarke, B.L. and Khosla, S., 2008, September. Bisphosphonates: mechanism of action and role in clinical practice. In Mayo Clinic Proceedings (Vol. 83, No. 9, pp. 1032-1045). Elsevier.

[123] Aluko, R.E., 2019. Food protein-derived renin-inhibitory peptides: *in vitro* and *in vivo* properties. Journal of Food Biochemistry, 43(1), p.e12648.

[124] Muñoz-Torres, M., Alonso, G. and Raya, P.M., 2004. Calcitonin therapy in osteoporosis. Treatments in Endocrinology, 3(2), pp.117-132.

[125] Tuchendler, D. and Bolanowski, M., 2014. The influence of thyroid dysfunction on bone metabolism. Thyroid Research, 7(1), p.12.

[126] Islam, M., Rahi, M., Jahangir, C.A., Rahman, M.H., Jerin, I., Amin, R., Hoque, K.M. and Reza, M.A., 2018. *In vivo* Anticancer Activity of Basella alba Leaf and Seed Extracts against Ehrlich's Ascites Carcinoma (EAC) Cell Line. Evidence-Based Complementary and Alternative Medicine, 2018.

[127] Väänänen, H.K. and Härkönen, P.L., 1996. Estrogen and Bone Metabolism. Maturitas, 23, pp. S65-S69.

[128] Pahwa, R. and Jialal, I., 2018. Chronic Inflammation. In StatPearls [Internet]. StatPearls Publishing.

[129] Lane, N.E. and Kelman, A., 2003. A review of anabolic therapies for osteoporosis. Arthritis Res Ther, 5(5), p.214.

[130] Stroup, J., Kane, M.P. and Abu-Baker, A.M., 2008. Teriparatide in the treatment of osteoporosis. American Journal of Health-System Pharmacy, 65(6), pp.532-539.

[131] Marie, P.J., 2006. Strontium ranelate: a dual mode of action rebalancing bone turnover in favour of bone formation. Current Opinion in Rheumatology, 18, pp. S11-S15.

[132] Awasthi, H., Mani, D., Singh, D. and Gupta, A., 2018. The underlying pathophysiology and therapeutic approaches for osteoporosis. Medicinal Research Reviews, 38(6), pp.2024-2057.

[133] Bab, I. and Zimmer, A., 2008. Cannabinoid receptors and the regulation of bone mass. British Journal of Pharmacology, 153(2), pp.182-188.

[134] Patel, S., 2015. Emerging trends in nutraceutical applications of whey protein and its derivatives. Journal of Food Science and Technology, 52(11), pp.6847-6858.

[135] Dong, X.Y., Guo, L.L., Wei, F., Li, J.F., Jiang, M.L., Li, G.M., Zhao, Y.D. and Chen, H., 2011. Some characteristics and functional properties of rapeseed protein prepared by ultrasonication, ultrafiltration and isoelectric precipitation. Journal of the Science of Food and Agriculture, 91(8), pp.1488-1498.

[136] Surasani, V.K.R., 2018. Acid and alkaline solubilization (pH shift) process: a better approach for the utilization of fish processing waste and by-products. Environmental Science and Pollution Research, 25(19), pp.18345-18363.

[137] Alberts, B., Bray, D., Hopkin, K., Johnson, A.D., Lewis, J., Raff, M., Roberts, K. and Walter, P., 2013. Essential Cell Biology. Garland Science.

[138] Kiewiet, M., Faas, M. and de Vos, P., 2018. Immunomodulatory protein hydrolysates and their application. Nutrients, 10(7), p.904.

[139] Wang, J., Su, Y., Jia, F. and Jin, H., 2013. Characterization of casein hydrolysates derived from enzymatic hydrolysis. Chemistry Central Journal, 7(1), p.62.

[140] Rao, Q., Klaassen Kamdar, A. and Labuza, T.P., 2016. Storage stability of food protein hydrolysates—a review. Critical Reviews in Food Science and Nutrition, 56(7), pp.1169-1192.

[141] Chakrabarti, S., Guha, S. and Majumder, K., 2018. Food-derived bioactive peptides in human health: Challenges and Opportunities. Nutrients, 10(11), p.1738.

[142] Senevirathne, M. and Kim, S.K., 2012. Development of bioactive peptides from fish proteins and their health promoting ability. In Advances in Food and Nutrition Research (Vol. 65, pp. 235-248). Academic Press.

[143] Raveschot, C., Cudennec, B., Coutte, F., Flahaut, C., Fremont, M., Drider, D. and Dhulster, P., 2018. Production of bioactive peptides by Lactobacillus species: from gene to application. Frontiers in Microbiology, 9.

[144] Bonjour, J.P., 2011. Protein intake and bone health. International Journal for Vitamin and Nutrition Research, 81(2), p.134

[145] Robinson, P.K., 2015. Enzymes: principles and biotechnological applications. Essays in Biochemistry, 59, pp.1-41.

[146] Barać, M., Čabrilo, S., Pešić, M., Stanojević, S., Pavlićević, M., Maćej, O. and Ristić, N., 2011. Functional properties of pea (Pisum sativum, L.) protein isolates modified with chymosin. International Journal of Molecular Sciences, 12(12), pp.8372-8387.

[147] Căpriţă, R., Căpriţă, A. and Creţescu, I., 2010. Protein solubility as quality index for processed soybean. Scientific Papers Animal Science and Biotechnologies, 43(1), pp.375-378.
[148] Tömösközi, S., Lasztity, R., Haraszi, R. and Baticz, O., 2001. Isolation and study of the functional properties of pea proteins. Food/Nahrung, 45(6), pp.399-401.
[149] Weaver, C.M., Dwyer, J., Fulgoni III, V.L., King, J.C., Leveille, G.A., MacDonald, R.S., Ordovas, J. and Schnakenberg, D., 2014. Processed foods: contributions to nutrition. The American Journal of Clinical Nutrition, 99(6), pp.1525-1542.
[150] Zhang, Y.J., Gan, R.Y., Li, S., Zhou, Y., Li, A.N., Xu, D.P. and Li, H.B., 2015. Antioxidant phytochemicals for the prevention and treatment of chronic diseases. Molecules, 20(12), pp.21138-21156.
[151] Socorro López-Cortez, M., Rosales-Martínez, P., Arellano-Cárdenas, S. and Cornejo-Mazón, M., 2016. Antioxidants Properties and Effect of Processing Methods on Bioactive Compounds of Legumes. Grain Legumes, p.103.
[152] Kong, E., Hinds, P.W. and Agarwal, M., 2012. The Retinoblastoma protein in osteosarcomagenesis. In Osteosarcoma. INTECH Open Access Publisher.
[153] Trouvin, A.P. and Goëb, V., 2010. Receptor activator of nuclear factor-κB ligand and osteoprotegerin: maintaining the balance to prevent bone loss. Clinical Interventions in Aging, 5, p.345.
[154] Kapinas, K. and Delany, A.M., 2011. MicroRNA biogenesis and regulation of bone remodeling. Arthritis Research & Therapy, 13(3), p.220.
[155] Depraetere, V., 2001. G-protein activation live. Nature Cell Biology, 3(5), pp.E107-E107.
[156] Cojoc, M., Peitzsch, C., Trautmann, F., Polishchuk, L., Telegeev, G.D. and Dubrovska, A., 2013. Emerging targets in cancer management: role of the CXCL12/CXCR4 axis. OncoTargets and Therapy, 6, p.1347.

Figure 1.1 The osteoblasts originate from mesenchymal stem cells in a sequential order of different phenotypic transcription factors. The mesenchymal stem cells further differentiate in osteoprogenitor, to pre-osteoblasts and finally matured osteoblast. The different stages of differentiation are accompanied by the expression of specific genes/proteins. This figure was prepared using (Kong, et al., 2012).

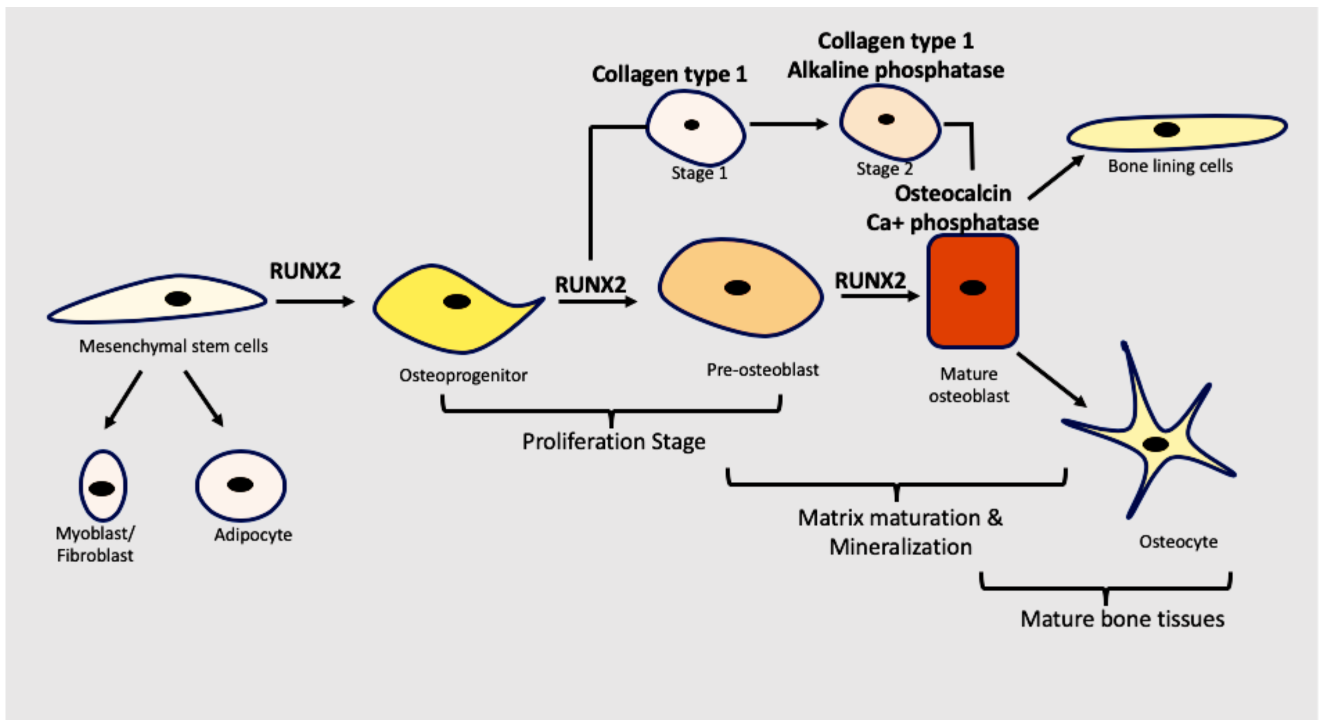


Figure 1.2 Before activation, the resting bone is covered with bone lining cells. Bone remodeling starts with resorption of bone mineral and matrix by osteoclasts. The mononuclear cells prepared the resorbed surface for osteoblasts generating newly synthesized bone matrix as they differentiate. The matrix mineralization and the differentiation of some osteoblasts into osteocytes completes the remodeling cycle. The figure is prepared using (Kapinas and Delany 2011).

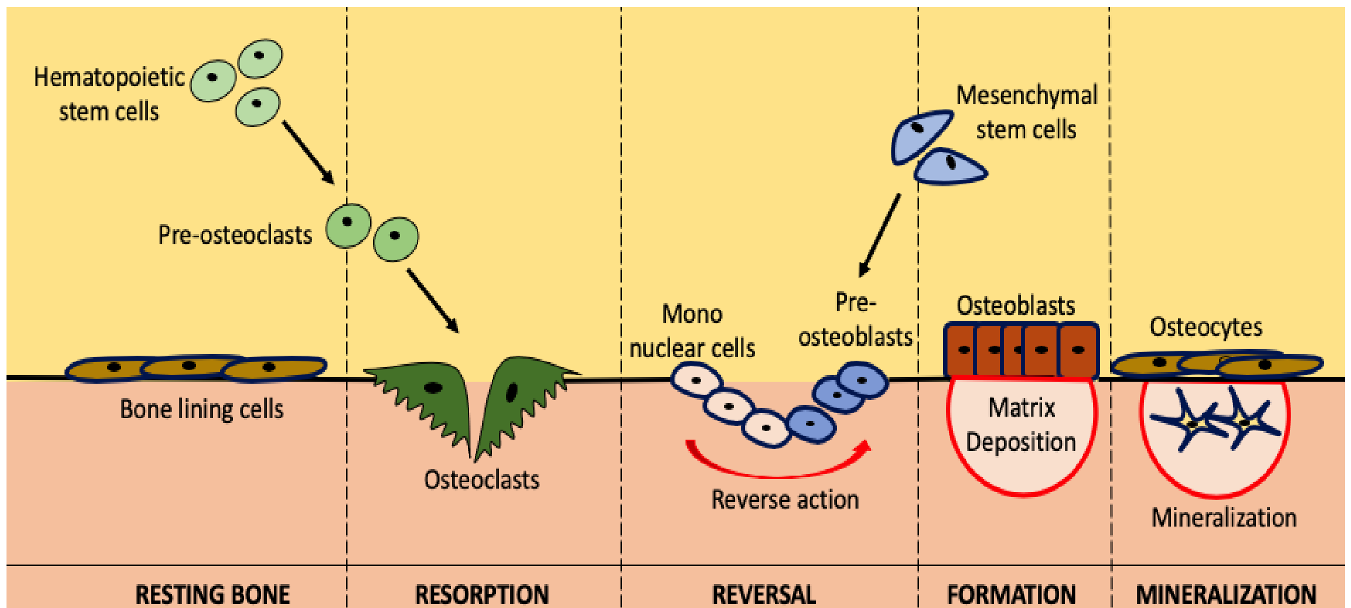


Figure 1.3 OPG/RANK/RANKL pathways depicting bone turnover. The precursor osteoclasts cells mature into a multinucleated osteoclast under the stimulating action of cytokines, hormones and growth factors. The matured multinucleated cell further differentiates into activated osteoblasts in the presence of MCSF and RANKL. Upon activation, the osteoclasts degrade the bone surface. OPG the decoy receptor of RANKL, inhibits binding of RANKL on RANK, leading to apoptosis of the osteoclast. Furthermore, pre-osteoblasts begin with bone formation by further depositing the mineral matrix. The figure was prepared using (Trouvin and Goëb 2010).

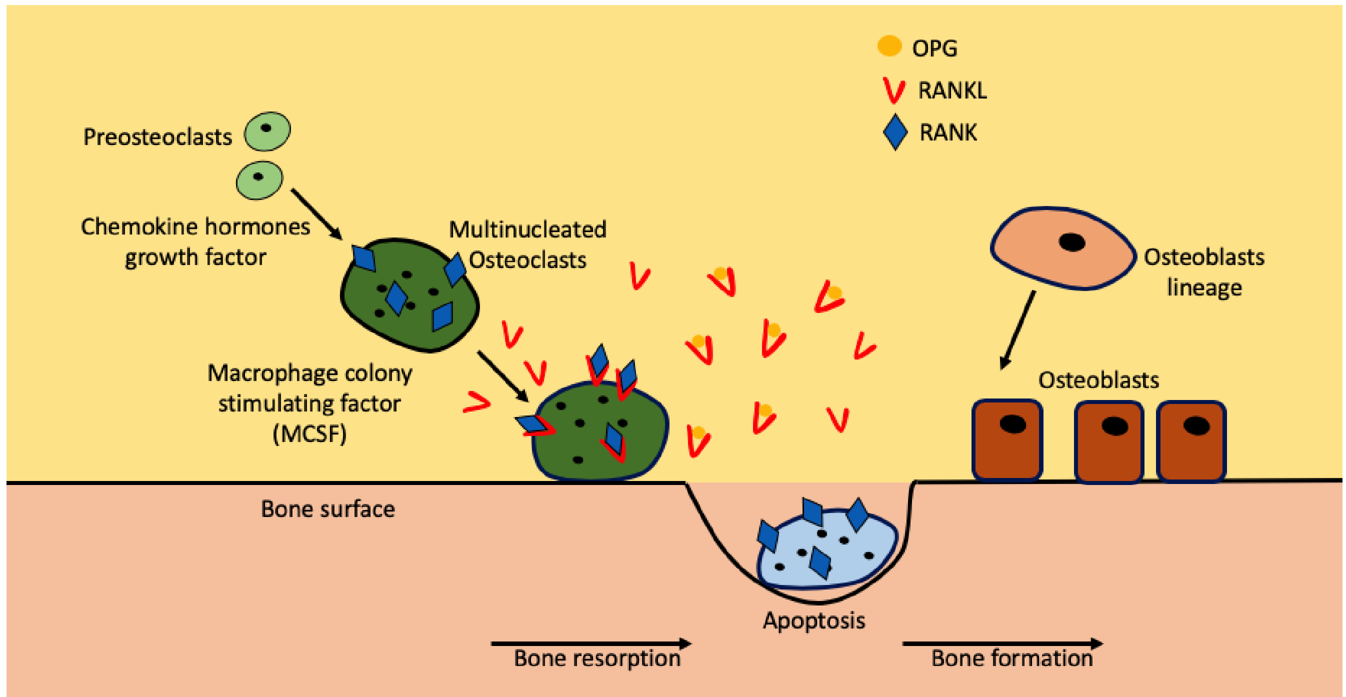


Figure 1.4 The G-protein coupled receptor (GPCRs). On binding of ligand to GPCRs, the receptor undergoes conformational change by activating G-protein in exchange of GDP for GTP. The alpha and beta-gamma subunit dissociates and activate downstream signalling cascades. This figure was prepared using (Depraetere, 2001).

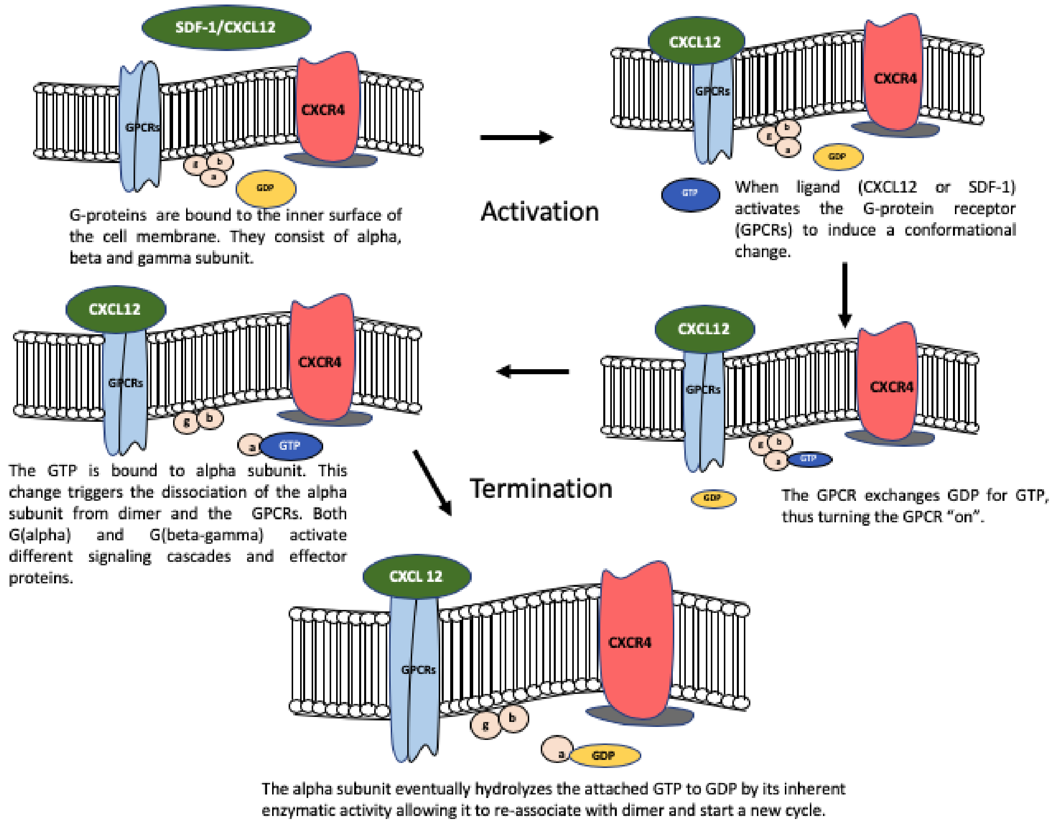


Figure 1.5 Upon activation of GPCRs, the SDF-1/STAT3 binds, resulting in downregulation of CXCR4 using various mechanism. This figure was prepared using (Cojoc et al., 2013).

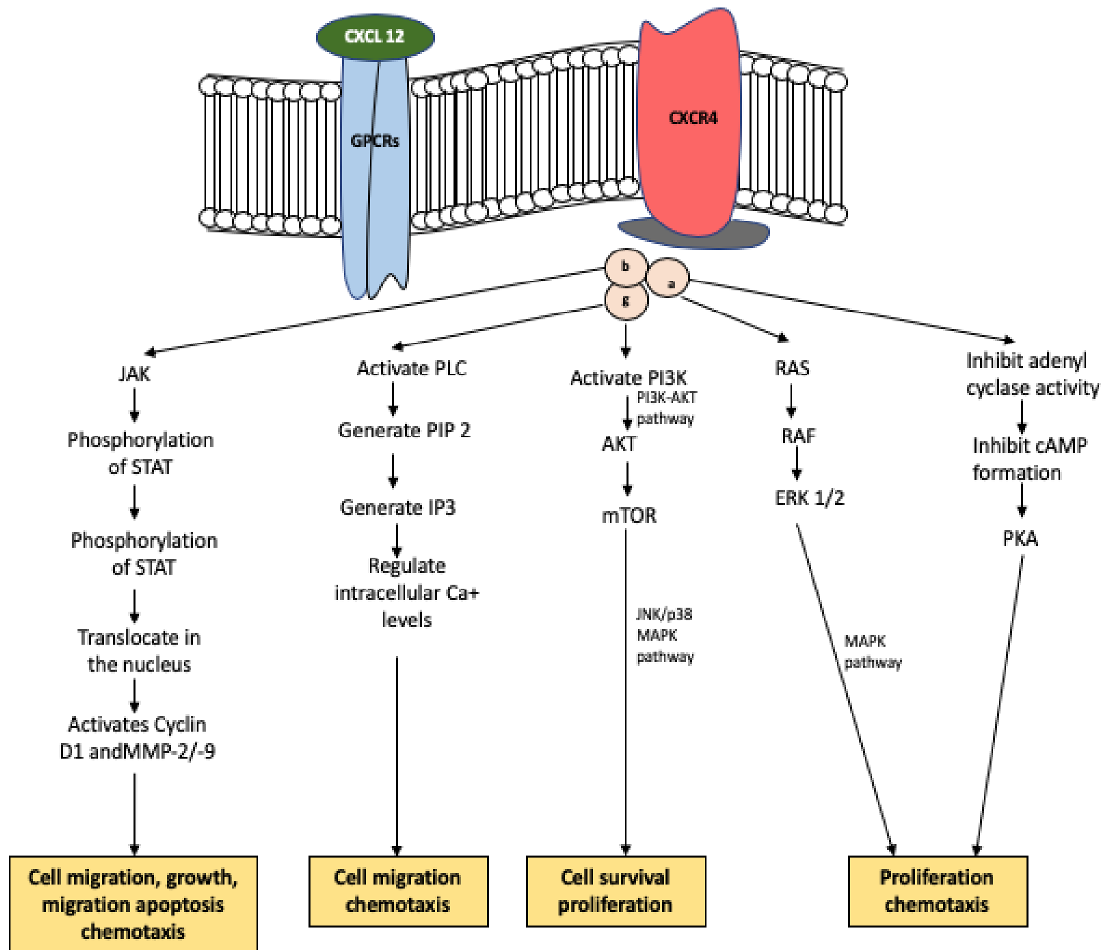


Figure 1.6 Cytokine binding to cytokine receptors results in JAK activation and subsequent phosphorylation of tyrosine residue of the cytokine receptor. Phosphorylated tyrosine residues serve as a docking sites for STATs, which allows dimerization. STAT translocate to the nucleus, in which they recognize specific sequences. Along with transcriptional co-activators and enhancer, STAT direct the transcription of their target genes. This figure was prepared using (Pedranzini et al., 2004).

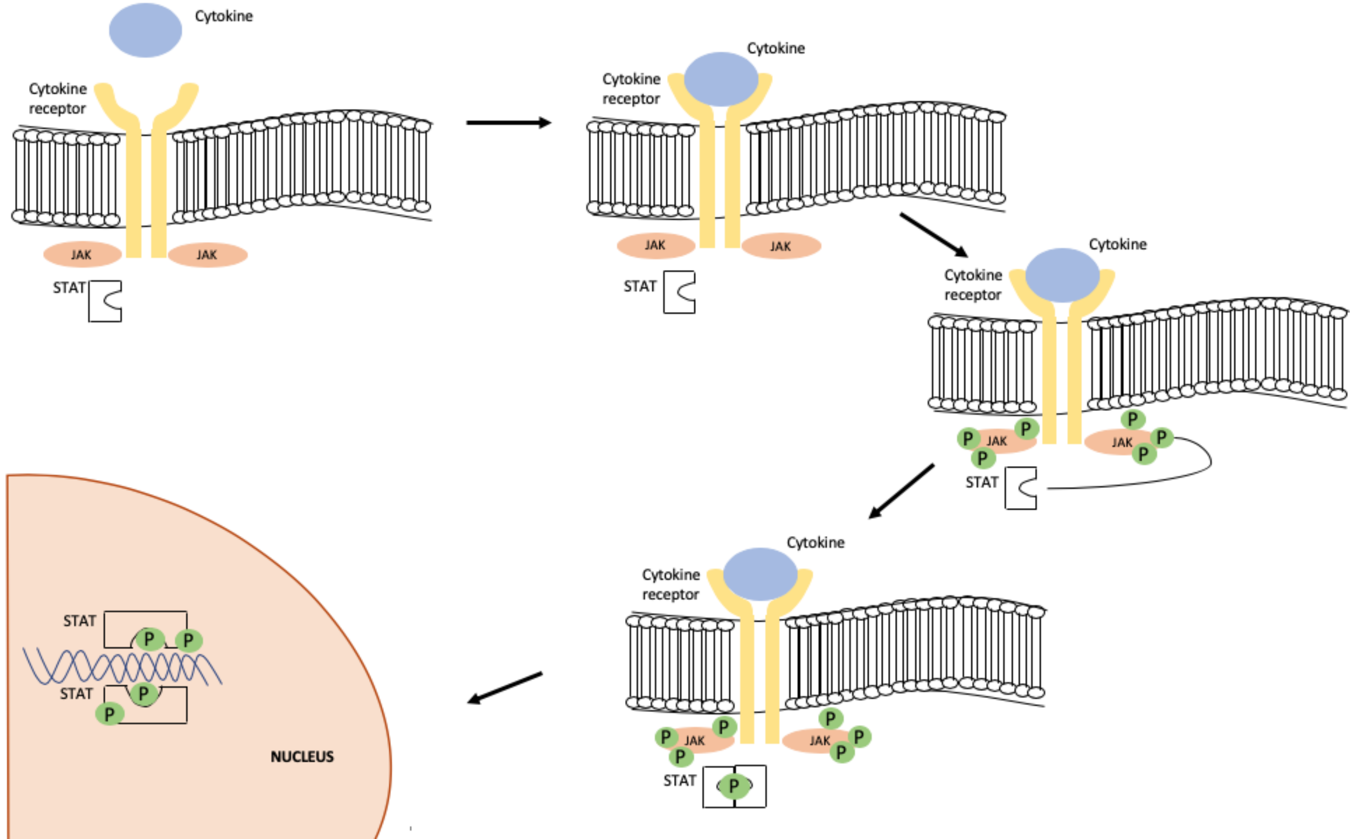


Table 1.1 Food and Drug Administration approved medication for the treatment of osteoporosis.

Drug	Dosing	Route	Adverse effects
Bisphosphonates			
Alendronate (Fosamax)	Treatment: 10 mg once daily or 70 mg once weekly	Oral	Dyspepsia, abdominal pain, musculoskeletal pain
	Prevention: 2.5 mg once daily or 150 mg once a month		
	IV: 3 mg every 3 months		
Ibandronate (Boniva)	Oral: 2.5 mg once daily or 150 mg once a month	Oral, IV	Dyspepsia, abdominal pain, musculoskeletal pain, headache
	IV: 3 mg every 3 months		
Risedronate	IR: 5 mg once daily or 35 mg once weekly or 150 mg once a month	Oral	Rash, abdominal pain, dyspepsia, diarrhea, arthralgia
	DR: 35 mg once weekly		
Zoledronic acid (Reclast)	IR: 5 mg once daily or 35 mg once weekly or 150 mg once a month	IV	Acute reaction (flulike symptoms, fever, myalgia) may occur within 3 days of infusion; hypertension, fatigue, eye inflammation, nausea, vomiting, abdominal pain
	DR: 35 mg once weekly		
Calcitonin			
Calcitonin (Fortical)	200 IU in 1 nostril alternating each day	Intranasal	Rhinitis, nasal irritation, dizziness, nasal dryness
Calcitonin (Miraculin)	100 IU every other day 200 IU in 1 nostril daily alternating each day	SC, IM, Intranasal	Injection site reactions, nausea, vomiting, abdominal cramping, flushing

Selective estrogen receptor modulator (SERMs)			
Raloxifene (Forteo)	60 mg once daily	Oral	VTE, arthralgia, leg cramps, flu syndrome, peripheral edema, hot flushes
Parathyroid hormone analogue			
Teriparatide (Forteo)	20 mg once daily	SC, IM, Intranasal	Transient hypercalcemia, nausea, rhinitis, arthralgia, pain
Monoclonal antibody			
Denosumab (Prolia)	60 mg every 6 months	SC, IM, Intranasal	Dermatitis, rash, mild bone/muscle pain, UT's

Table 1.2 Bioactivities of pea protein components

Sno.	Pea protein components	Enzyme used	Functional property identified	Source
1	Pea protein hydrolysate	Trypsin	Oxidative stability	Tamm, et al., 2016
			Emulsion droplet stability	
2	Pea protein hydrolysate	Thermolysin	Antihypertensive activity in SHRs	Girgih et al., 2016
3	Pea protein hydrolysate	Alcalase	ACE inhibitory	Li, H. and Aluko, R.E., 2010
			Calmodium-dependent phosphodiesterase 1 inhibitory	
4	Pea protein hydrolysate	Alcalase	ACE inhibitory	Humiski, L.M. and Aluko, R.E., 2007
		Flavourzyme		
		Papain	Free radical scavenging activity	
		Chymotrypsin		
5	Pea protein hydrolysate	Trypsin	high alpha-amino nitrogen	Panasiuk et al., 1998
	Pea protein isolate			
6	Pea protein hydrolysate	Thermolysin	High Antioxidant	Ndiaye et al., 2012
			High Anti-inflammatory	
			Immunomodulating properties	
7	Pea protein hydrolysate (F1-F5 peptide)	Thermolysin	Antioxidant activity	Pownall et al., 2011

CHAPTER 2- GENERAL INTRODUCTION AND OBJECTIVES

2.1 General Introduction

Bone is a specialized connective tissue that exhibits numerous significant functions in human body such as providing support and location for attachment of muscle leading to movement [1]. It also acts as a reservoir of minerals such as calcium and phosphate that promote bone health and prevent bone loss [5]. A healthy diet and regular physical activity with adequate nutrition is required for maintaining the strength and integrity of bones [1]. Bone is a mineralized connective tissue that comprises of four main types of bone cells: osteoblast, bone lining cells, osteocytes and osteoclast [2]. The dynamic nature of bone enables it to continuously be resorbed and reformed by bone cells through the process called bone remodeling [5]. Bone remodeling is a convoluted process that occurs in stages, beginning with bone resorption by osteoclasts, followed by reversing the action for proliferation and differentiation of osteoblast and final stage characterized by depositing mineralized matrix for formation of new bone [2]. It is a vital process for fracture healing, mineral homeostasis and skeleton adaptation for mechanical use [6,5,15]. Any disproportion in the process of bone remodeling can result in several bone diseases such as osteopetrosis, osteoporosis, rickets [7].

Osteoporosis is one such disease that happens with excessive resorptive action of osteoclasts. Osteopenia is a conditional precursor to osteoporosis accompanied with a fracture of bone but retains normal bone strength. Osteoporosis is often called as “silent killer” as it progresses painlessly from thinning of bone to the point of their breakability [8]. With our progressing life, our bones become inefficient and tend to lose tissues, making the bone more prone to fractures. Bone disease such as osteoporosis can be prevented with a healthy lifestyle embraced with wholesome nutrition, physical exercise and adequate intake of calcium. For the finest defense against the development of osteoporosis, it is important to build strong bones before the age of 30 [8]. After the mid-30s, the balance between the activity of osteoblast and osteoclast is shifted that results in loss of more bone than what is replaced. According to Health Canada, two million Canadians are affected by osteoporosis including 1 in 3 women and 1 in 5 men [9]. The people at risk aims to assess the risk points and carry on with drug treatment. The recommended drug therapy includes antiresorptive agents and bone formation techniques. Antiresorptive agents are those agents that reduce the rate of bone loss and include bisphosphonates, denosumab, raloxifene and estrogen. They act by binding on the bone surface which results in slowing down the resorbing

action of osteoclast and thereby improves bone strength [10]. However, with bone formation therapies, medication teriparatide is used which helps to build up bones. Teriparatide is an analogue to para-thyroid hormone (PTH), which acts by increasing the bone forming tissues and provide remediation of skeleton's architectural defects [11]. With their health benefits, these synthetic drugs have some common side effects like heart burn, nausea, abdominal pain, loose bowel movement. In extreme cases, these may also result in kidney dysfunction [6].

Therefore, it is critical to recognize the potential secondary cause, detection and treatment technique to avoid fractures and crippling deformities [17]. Considering all the side effects of osteoporosis medication, recent studies indicate several natural products can provide an alternative treatment for bone disorders [12]. For example, it has been reported that essential oils from sage, thyme, rosemary and other herbs inhibit osteoclastic activity *in vitro* commanding to an increase in bone mineral density [13]. Similarly, peptide VLVPQK derived from buffalo casein exhibits osteoporotic effects by inhibiting oxidative damage and bone-resorbing cytokine in ovariectomized rat model of osteoporosis (OVX) [110]. Likewise, soya has also shown assuring epidemiological evidence supporting bone health [15]. Traditional medicines have also demonstrated a potential effect in the pharmacological model of osteoporosis [14]. Recently, tripeptide IRW derived from egg white have shown similar bone health-promoting effects in MC3T3-E1 cell line [16].

2.2 Objectives

LRW is a tripeptide identified from pea protein hydrolyzed by thermolysin enzyme [19] ; this peptide differs only one amino acid residue from IRW, furthermore Leu (L) is the isomer of Ile (I); therefore, we hypothesized that LRW, an isomer of IRW, can have the osteoblastic activity on MC3T3-E1 cells. Since this peptide is identified from thermolysin pea protein hydrolysate, we further hypothesized pea protein hydrolysate can have the osteoblastic activity.

Therefore, the overall objectives of the research were to explore the potential application of pea protein derived peptide peptides LRW and hydrolysate in promoting bone health. The specific objectives of the research are:

1. To investigate the effect of pea protein derived tripeptide LRW on osteoblastic activity on pre-osteoblast MC3T3-E1 cells.
2. To study the *in vitro* regulatory role of pea protein hydrolysate on osteoblast and osteogenesis.

2.3 Chapter format

There are five chapters in the thesis and a brief description of each chapter is given as follows:

Chapter 1 provides an insight strategy based on the literature review of the current knowledge on bone physiology, osteoporosis, pea protein and its bioactive components (hydrolysate and tripeptide LRW). In the end, the perspective on developing bioactive proteins on bone health including potential challenges are briefly summarized.

Chapter 2 gives a brief introduction to the bone physiology with the current status of osteoporosis. The growing interest in emerging food-derived natural alternatives which possibly promote bone health is briefly discussed. Following with the general introduction, the thesis hypothesis and objectives are described.

Chapter 3 investigates the *in vitro* effect of pea protein derived tripeptide LRW on stimulating osteoblastic activity. Murine pre-osteoblast MC3T3-E1 cells were used to investigate the stimulatory effects of tripeptide LRW on cell proliferation, cell differentiation and mineralization. The effects on cell viability and cell proliferation were determined by AlamarBlue assay and BrdU incorporation assay. The effects on cell differentiation were tested by expression of differentiation biomarkers using the western blot. The effects on mineralization were evaluated by Alizarin Red assay. First objective of the thesis is addressed in this chapter.

Chapter 4 explores the *in vitro* effect of pea protein hydrolysate prepared with various enzymes on stimulating LRW osteogenic activity. Human osteoblast U-2OS cells were used to explore the osteogenic activity of pea hydrolysates, using western blot and qRT-PCR. The effects of matrix metalloproteinases were also examined using MMP-1 and MMP-9 assay. The second objective of the thesis is addressed in this chapter.

Chapter 5 provides overall conclusions and discussions of the thesis. It also relates the significance of pea protein derived bioactive peptides in the food and health management industry in treatment alternatives for osteoporosis disease. The limitations and future recommendations of this research work is also discussed.

2.4 References

[1] Baron, R., 2008. Anatomy and ultrastructure of bone–histogenesis, growth and remodeling. MDText. com, Inc.

[2] Florencio-Silva, R., Sasso, G.R.D.S., Sasso-Cerri, E., Simões, M.J. and Cerri, P.S., 2015. Biology of bone tissue: structure, function, and factors that influence bone cells. BioMed Research International, 2015.

[3] Capulli, M., Paone, R. and Rucci, N., 2014. Osteoblast and osteocyte: games without frontiers. Archives of Biochemistry and Biophysics, 561, pp.3-12.

[4] Marks Jr, S.C. and Popoff, S.N., 1988. Bone cell biology: the regulation of development, structure, and function in the skeleton. American Journal of Anatomy, 183(1), pp.1-44.

[5] Office of the Surgeon General (US, 2004. The Basics of Bone in Health and Disease. In Bone Health and Osteoporosis: A Report of the Surgeon General. Office of the Surgeon General (US).

[6] Huang, Q.Y., Recker, R.R. and Deng, H.W., 2003. Searching for osteoporosis genes in the post-genome era: progress and challenges. Osteoporosis International, 14(9), pp.701-715.

[7] Dawson-Hughes, B., Tosteson, A.N.A., Melton, L.3., Baim, S., Favus, M.J., Khosla, S. and Lindsay, R.L., 2008. Implications of absolute fracture risk assessment for osteoporosis practice guidelines in the USA. Osteoporosis International, 19(4), pp.449-458.

[8] https://www.cysticfibrosis.ca/osteoporosis. July 10, 2019

[9] https://osteoporosis.ca/about-the-disease/, July 10, 2019

[10] http://www.osteoporosis.ca/multimedia/pdf/publications/Drug_Treatments_EN.pdf

[11] Ma, Y.L., Zeng, Q., Donley, D.W., Ste-Marie, L.G., Gallagher, J.C., Dalsky, G.P., Marcus, R. and Eriksen, E.F., 2006. Teriparatide increases bone formation in modeling and remodeling osteons and enhances IGF-II immunoreactivity in postmenopausal women with osteoporosis. Journal of Bone and Mineral Research, 21(6), pp.855-864.

[12] Putnam, S.E., Scutt, A.M., Bicknell, K., Priestley, C.M. and Williamson, E.M., 2007. Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 21(2), pp.99-112.

[13] Mühlbauer, R.C., Lozano, A., Palacio, S., Reinli, A. and Felix, R., 2003. Common herbs, essential oils, and monoterpenes potently modulate bone metabolism. Bone, 32(4), pp.372-380.

[14] Jia, M., Nie, Y., Cao, D.P., Xue, Y.Y., Wang, J.S., Zhao, L., Rahman, K., Zhang, Q.Y. and Qin, L.P., 2012. Potential antiosteoporotic agents from plants: a comprehensive review. Evidence-based Complementary and Alternative Medicine, 2012.

[15] Messina, M. and Messina, V., 2000. Soyfoods, soybean isoflavones, and bone health: a brief overview. Journal of Renal Nutrition, 10(2), pp.63-68.

[16] Shang, N., Bhullar, K.S., Hubbard, B.P. and Wu, J., 2019. Tripeptide IRW initiates differentiation in osteoblasts differentiation via the RUNX2 pathway. Biochimica et Biophysica Acta (BBA)-General Subjects, 1863(6), pp.1138-1146.

[17] Office of the Surgeon General (US, 2004. Bone health and osteoporosis: a report of the Surgeon General.

[18] Hanley, D.A., Adachi, J.D., Bell, A. and Brown, V., 2012. Denosumab: mechanism of action and clinical outcomes. International Journal of Clinical Practice, 66(12), pp.1139-1146.

CHAPTER 3 - PEA PROTEIN DERIVED TRIPEPTIDE LRW SHOWS OSTEOBLASTIC ACTIVITY ON MC3T3-E1 CELLS

3.1 Introduction

Bone is a connective tissue that provides protection to various organs, stores and regulates homeostasis of minerals, and maintains overall structural integrity [1]. It also plays a vital role in providing the structural framework of the skeleton to withstand mechanical stress [2]. The bone matrix constitutes the organic part, which includes collagenous (such as type 1 collagen) and non-collagenous proteins, and the inorganic part comprising of various minerals [3]. The skeleton is a warehouse of two vital minerals: calcium and phosphorous, which combine to form hydroxyapatite crystals. The hydroxyapatite crystals incorporate with the collagen fibrils to form extracellular bone matrix [4]. The bones are continuously resorbed and formed by a process called bone remodeling, mainly by osteoblasts and osteoclasts [5]. Bone remodeling is self-regeneration process which replaces old bone with new bones. It is continuous process in maintaining mineral homoeostasis in regulating bone marrow [5].

The osteoblasts are the cells responsible for bone formation whereas osteoclasts are the cells capable of resorbing bone tissue [6]. The regulation of osteoblast differentiation is mainly hinged on two major transcription factors i.e. runt-related transcription factor (RUNX2) and osterix (Os), which is necessary for early and late osteoblast differentiation [7]. The osteoblastogenesis can sequentially express several explicit biomarkers such as type 1 collagen (COL1A2) and alkaline phosphatase (ALP) at the early differentiation stage [9]. The final stage of the osteoblast differentiation is characterized by the increased expression of the osteocalcin, osteopontin with calcium and phosphate deposition. Besides this, osteoblast differentiation is also required for osteoclastogenesis and osteoclastic bone resorption [8]. The osteoblasts express two important proteins, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL), which binds to its receptor, RANKL. RANK is expressed on the surface of osteoclasts, binding of RANK to RANKL stimulates the differentiation and activation of osteoclasts resulting in increased bone resorption [9]. In this situation, excessive bone resorption by RANKL binding is prevented by osteoprotegerin (OPG), a decoy receptor secreted by osteoblasts. Thus, RANK/RANKL/OPG system is crucial in maintaining bone health [10].[9].

Equilibrium in the activity of osteoblasts and osteoclasts is necessary to maintain the structural integrity and normal functioning of the skeleton [20]. During aging, bone composition, mass and function is impaired or deteriorated. The decrease in the bone mass and the weakening

of bone structure is a usual course with aging, which contributes to bone diseases such as osteoporosis [21]. Osteoporosis is characterized by low bone mass which leads to increased risk of bone fractures [7]. In Canada, 2 million people are affected by osteoporosis comprising of about 1 in 3 women and 1 in 5 men [22]. Medications used in treating osteoporosis include antiresorptive drugs [14] such as bisphosphonates, calcitonin, and bone-forming therapies using para-thyroid hormone [15]. These pharmacological therapies are associated with some common and adverse side effects such as nausea, abdominal pain, headache, and malignant hypercalcemia [16].

Thus, there is a growing interest in transition towards food-derived alternatives such as bioactive peptides. Casein phosphopeptides have shown to stimulate bone calcification in rachitic infants [45] and have also demonstrated reduced bone loss in aged ovariectomized rat (OVX, a model of osteoporosis) [46]. The bioactive peptides IPP and VPP identified from *Lactobacillus helveticus*-fermented milk showed an increase in bone mineral density in growing rats and calcium absorption rate in postmenopausal women [47]. A peptide NAVPITPTL, derived from buffalo casein hydrolysate, stimulated osteoblast differentiation via activating Akt signaling pathway [48]. Therefore, food protein-derived peptides show promise in promoting bone health. These food-derived bioactive peptides are encrypted within parent proteins that positively impact the human health [17]. The pharmaceutical and nutraceutical benefits of food-derived bioactive peptides have now emerged as a novel food strategy, exerting health benefits [25]. We recently showed that an egg-derived tripeptide, IRW (Ile-Arg-Trp), positively regulates osteogenesis and collagen synthesis [18]. Interestingly, an analog of LRW, differing in only one amino acid residue, LRW (Leu-Arg-Trp) was identified from pea protein hydrolysate [49]. Therefore, the objective of this study was to explore the osteoblastic activity of tripeptide LRW using mouse pre-osteoblast cells MC3T3-E1.

3.2 Materials and Methods

3.2.1 Reagents

The tripeptide LRW was synthesized by GenScript (Piscataway, NJ, U.S.A) with a purity of 99.8%. Minimum essential medium-alpha (MEM-α), fetal bovine serum (FBS), penicillin-streptomycin, 0.25% trypsin-EDTA, and phosphate buffered saline (PBS) were purchased from Gibco/Invitrogen (Carlsbad, CA, U.S.A). AlamarBlue reagent, BrdU labeling agent, and Hoechst 33342 were purchased from Thermofischer Scientific, Inc. (Waltham, MA, U.S.A). Dithiothreitol

(DTT), Triton-X-100, Alizarin Red S, ascorbic acid, β-glycerophosphate, and cetylpyridium chloride and bovine serum albumin (BSA) were purchased from Sigma Aldrich, (St. Louis, MO, U.S.A). The primary antibody against BrdU, Col1A2, GAPDH and Runt-related transcription nuclear factor 2 (RUNX2) were purchased from Abcam (Toronto, ON, Canada). The primary antibody against receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) were purchased from Santa Cruz Biotechnology (Mississauga, ON, Canada). Primary antibody against phosphor-Akt and total-Akt were purchased from Cell Signaling technology (Danvers, MA, U.S.A). Goat anti-rabbit IgG (H+L) secondary antibody Alexa Fluor 546 and rabbit anti-mouse IgG (H+L) secondary antibody Alexa Fluor 594 was procured from Molecular probes (Waltham, MA, U.S.A).

3.2.2 Cell culture

The mouse pre-osteoblast cell MC3T3-E1 (ATCC CRL-2593) was purchased from American type culture collection (ATCC) (Manassas, VA, U.S.A). The cells were cultured in α-MEM medium with 10% FBS, and 1% penicillin-streptomycin and incubated at 37°C, 95% O_2 and 5% CO_2. After 80-90% confluency, the cells were subculture into cell culture plates using 0.25% trypsin-EDTA. To determine the osteoblastic effect of LRW peptide, the cells were treated with the different LRW concentrations (10, 25 and 50 μM) for 24 h prior to experimental analysis.

3.2.3 Cell cytotoxicity

Cell cytotoxicity was determined using the AlamarBlue assay. Cells were seeded in 96-well plate and were incubated until 80-90% confluency. The tripeptide LRW at a concentration of 50 μM was added into the culture medium for another 24 h. After treatment with LRW, the old medium was removed and fresh medium containing AlamarBlue was added. The cells were then incubated for 4 h at 37°C. The medium for blank, control and sample were transferred in opaque 96well plate and read at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The cell viability was then calculated using the following formula.

$$\text{Cell viability} = \frac{(\text{Absorbance of treated sample} - \text{Absrbance of blank})\ \text{X}\ 100}{\text{Absorbance of control} - \text{Absorbance of blank}}$$

3.2.4 Bromodeoxyuridine (BrdU) incorporation assay

Cell proliferation was determined using BrdU incorporation assay, in which cells were seeded in 48 well plates. Cells were treated for 24 h with 10, 25 and 50 μM LRW after 4 h of seeding. After treatment, the medium containing peptide was removed, and the cells were rinsed with PBS once.

Fresh medium containing 1% BrdU labeling agent was added and the cells were incubated for 1 h at 37°C. The cells were fixed using 70% ethanol. After fixing, the cells were treated with 1N HCl for 20 min, permeabilized with 0.1% Triton-x-100 in PBS for 5 min, and blocked with 1% BSA in PBS for 60 min. Mouse monoclonal primary antibody against BrdU (0.1%) was added and the plate was incubated overnight at 4 °C. The plate was rinsed with 1X PBS and incubated with 0.2% anti-mouse IgG secondary antibody Alexa Fluor 594 for 30 min in dark. Cell nuclei were then stained with Hoechst 33342 dye for 10 min. After incubation, the cells were washed and observed under a fluorescent microscope (Olympus, Tokyo, Japan). Images were obtained using Metamorph imaging software (Molecular Devices, Sunnyvale, CA, U.S.A). Three random fields were chosen for each data point. The number of positively stained nuclei with BrdU was calculated and was subsequently used for data analysis.

3.2.5 Cell migration assay

Cell migration or commonly called as wound healing assay was determined with LRW concentrations (10, 25, and 50 μM). The wound was superficially casted on 12 well culture plates using inserts. The culture-inserts were fully placed in each well of cell culture plates and the cells were then seeded. After 24 h of incubation, the culture inserts were removed casting a superficial wound. The cells were then treated with different concentrations of LRW for 24 h. After 24 h, the cells were washed with PBS and fixed with 70% ethanol for 20 min. After fixation, the cells were treated with 1N HCl for 20 min, permeabilized using 0.1% Triton-X-100 in PBS for 5 min. The cells were then blocked in 1% BSA in PBS for 1 h. The nuclei were then stained using Nuclei stain Hoechst 33342 for 10 min in dark. The cells were washed with PBS and observed under a fluorescent microscope (Olympus, Tokyo, Japan). The images were clicked using Metamorph imaging software (Molecular Devices, Sunnyvale, CA, U.S.A). For each data point, three random fields were chosen. The images were further analyzed using ImageJ software, the area of the wound was measured and was used to calculate the rate of cell migration and wound closure using the following formula.

$$\text{Rate of cell migration } \left(\frac{\text{cm}}{\text{h}}\right) = \frac{\text{Initial wound width} - \text{Final wound width (cm)}}{\text{Duration of migration(h)}}$$

$$\text{Wound closure \%} = \frac{\text{Area of the wound for (control} - \text{treatment)}}{\text{Area of the wound for control}} \text{X } 100$$

3.2.6 Western blot

The cells were seeded in 6-well culture plates with MEM-α containing 10% FBS and 1% penicillin-streptomycin. After 90% confluency, the cells were treated with 10, 25 and 50 µM LRW concentrations for 24 h. After 24 h treatment, the culture medium was removed, and the cells were lysed in boiling Laemmle's buffer prepared with 50 µM DTT and 0.2% Triton-X-100. The prepared samples were run in SDS-PAGE, transferred to a nitro-cellulosic membrane, blocked with 5% TPBS in TPBS, and incubated with antibodies against COL1A2, RUNX2, RANKL, OPG, Phospho-Akt, total-Akt with the protein loading control GAPDH. The protein bands were detected by Licor Odyssey Bio Imager and were quantified by densitometry using corresponding software (Licor Biosciences, Lincoln, NE, U.S.A). Each band was normalized to its corresponding band of loading control and the results were expressed as fold change to the corresponding untreated control (control).

3.2.7 Alkaline Phosphatase Assay

The activity of alkaline phosphatase (ALP) was determined using the ALP assay kit (Abcam) and was followed according to instruction protocol. The cells were grown in 24 well culture plate and were treated with 10, 25 and 50 µM LRW for 24 h. After treatment, the culture medium and cell lysate were collected separately. The culture medium and the cell lysate sample were prepared using 5 mM p-NPP solution. Each sample was incubated for 1 h at 25°C in dark. After incubation, the reaction was stopped using stop solution and the plate was read at 405 nm using SpectraMax 340 plate reader (Molecular Devices, San Jose, CA, U.S.A). The standard curve was plotted, and net alkaline phosphatase activity was calculated.

3.2.8 Mineralization

The degree of mineralization was determined by Alizarin S red staining. The medium used in mineralization study was prepared by adding ascorbic acid (5 µg/mol) and beta-glycerophosphate (10 mM) in MEM-α. The cells were treated with different concentrations of LRW (10, 25 and 50 µM) and were incubated for 10, 15 and 20 days. After 80-90%confluency of the cells, the medium was removed, and the cells were washed with PBS. The cells were fixed using 70 % ethanol for 1 h, subsequently washed with Milli-Q water. After washing, the cells were stained with Alizarin S red stain (1% w/v in water). The stained cells were observed under white light and the pictures were taken. The cells were then destained using 10 % cetylpyridinium chloride (w/v) and the

absorbance for extracted stain was observed at 562 nm using SpectraMax 340 plate reader (Molecular Devices, San Jose, CA, U.S.A).

2.9 Statistical Analysis

All data presented in this study as the mean± SEM (standard error of the mean) of three to six independent experiments. One-way analysis of variation (ANOVA) was used in data analysis with Dunnett's posthoc test for comparison to the control using PRISM 6 statistical software (GraphPad Software, San Diego, CA) with $P<0.05$ as statistically significant.

3.3 Results

3.3.1 Tripeptide LRW shows no cell cytotoxicity in MC3T3-E1 cells.

To investigate the cytotoxicity of tripeptide LRW, cells were incubated for 24 h with 50 μM LRW. Cell viability was not affected by adding 50 μM LRW (Figure 1), indicating that tripeptide LRW preserve no cytotoxicity at the highest LRW concentration (50 μM) used in this study.

3.3.2 Tripeptide LRW stimulates cell proliferation

Effect of peptide LRW on cell proliferation was determined by measuring incorporation of bromodeoxyuridine (BrdU). BrdU is a structural analog of thymidine, which gets incorporated into the newly synthesized DNA of proliferating cells [25]. The percentage of BrdU positive cells was significantly increased from 2.0±0.4 (control) to 4.5±0.3, 5.3±0.7 and 8.4±0.6 for 10, 25 and 50 μM LRW concentrations ($p<0.001$), respectively (Figure 2). The increase in the percentage of positive BrdU cells suggests that tripeptide LRW could promote the cell proliferation in osteoblast.

3.3.3 Tripeptide LRW promotes cell migration

Cell migration ability was determined by calculating rate of cell migration and wound closure %. The migration rate of the cells increased from 1 cm^2/h (control) to 1.89 cm^2/h, 2.54 cm^2/h and 2.69 cm^2/h for LRW concentrations 10, 25 and 50 μM ($p<0.5$), respectively (Figure 3 B). The wound area decreased after treatment with LRW for 24 h (Figure 3A). Correspondingly, the closure percentage was normalized with control and was increased to 12, 22.5 and 34% for LRW treatments (10, 25 and 50 μM), indicating collective cell migration ability of the tripeptide LRW *in vitro*.

3.3.4 Tripeptide LRW promotes osteoblasts differentiation

Alkaline phosphatase (ALP) and type 1 collagen (COL1A2) expressions are considered as an essential indicator for osteoblasts differentiation. ALP activity was measured for both cultured cell

lysates and growth medium. Upon treatment with tripeptide LRW, a significant increase in dose dependent manner was observed in cell cultured lysates; 2-fold, 2.8-fold and 3-fold ($p<0.05$) increased was observed in the LRW treated concentrations of 10, 25 and 50 μM as compared to that of control (Figure 4 A). In the case of culture medium, a significant increase was observed in 25 and 50 μM LRW concentrations treated group (Figure 4 B).

Whereas, type 1 collagen is an eminent structural component of bone extracellular matrix, which plays an important role in osteoblastic bone formation. Thus, the expression of COL1A2 was studied in this study to investigate the effect of tripeptide LRW on cell differentiation. As presented in Figure 5, COL1A2 expression increased in all three different LRW concentrations (10, 25 and 50 μM) when compared to control. The highest significant effect was observed in 50 μM LRW treatment showing 2.8-fold ($p<0.001$) increase as compared with the control.

3.3.5 Tripeptide LRW activates the Akt pathway and increase the expression of transcription factor RUNX2

Osteoblast differentiation is mainly mediated by various signaling pathways, such as Akt pathway, which thereby regulate the expression of major transcription factor RUNX2. Akt expression is considered as a potent player in regulating bone mass and turnover. Therefore, an increased Akt expression suggest osteoblast differentiation [29]. Tripeptide LRW (50 μM) concentration showed significant 1.8-fold increase ($p<0.05$), whereas 10 and 25 μM induced no significant change in phosphorylation and total-Akt (Figure 7). Akt pathway regulates the expression of RUNX2, with only significant effect in 50 μM LRW treatment group. The RUNX2 expression was increased with LRW treatment, with highest 2.8-fold increase at 25 μM LRW concentration (Figure 6), as compared to control group, suggesting a stimulating role on cell differentiation.

3.3.6 Tripeptide LRW promotes mineralization

Bone mineralization is an index of bone mineral density or deposited mineral matrix. This is the final stage and conclusive indicator of bone formation. Osteoblasts were treated with tripeptide LRW for 10, 15 and 20 days. Subsequent matrix formation was observed, an increase in the absorbance of extracted stain was measured which is directly relative to the degree of matrix formation (Figure 8B). Furthermore, the deposition of calcium nodules in osteoblasts were clearly visible on day 15 and day 20 in LRW treated group (Figure 8A). A 0.6-fold, 1.2-fold and 1.4-fold

increase was observed in the absorbance of extracted strain of LRW (10, 25 and 50 μM) treatment groups respectively at 20th day as compared to control.

3.3.7 Tripeptide LRW did not decreases RANKL expression

RANKL is a marker for osteolysis [28]. It is expressed on the surface of osteoblast, which further binds to its receptor (RANK) and promotes bone resorption. Thus, RANKL expression is precarious in affecting osteoblast activity. Figure 9 shows that the expression of RANKL was not significantly affected by LRW as compared with the control.

3.3.8 Tripeptide LRW promotes OPG expression in osteoblasts

Osteoblast also releases OPG to restrain the binding of RANKL with RANK, preventing bone resorption mediated through osteoclast. Similarly, the effect of different concentrations of tripeptide LRW was investigated on preventing bone resorption. After treatment with tripeptide LRW, 1.3-fold ($p<0.05$),1.5-fold ($p<0.01$) and 1.6-fold ($p<0.001$) increase was observed in 10 μM, 25 μM and 50 μM LRW treated group, respectively, compared with the control group (Figure 10).

3.4 **Discussion**

Osteoporosis is categorized as a bone disease with decreased bone mass and strength, which can be critically regulated by epigenetic mechanisms emphasizing on the fate of cells. While, genetic dynamics is an imperative factor in determining bone compositional strength, thus it is analytically important to promote bone health status [5].

Recent studies have given a new edge to the transcriptional regulation of osteoblast differentiation [31]. The cascade of transcriptional events in the differentiation of osteoblast is largely controlled by a series of phenotypic gene expressions such as RUNX2 and type 1 collagen [32]. Osteoblast (MC3T3-E1) cells are widely used *in vitro* model to study osteogenesis in a harmonized approach beginning with the differentiation of osteoblast cells on the bone surface to initiate bone formation. The differentiation of osteoblasts is generally characterized in three stages: cell proliferation, cell differentiation and matrix formation. Following this systematic approach, our results showed that tripeptide LRW promoted osteoblast proliferation, differentiation and matrix mineralization with no cellular cytotoxicity.

Osteoblasts treated with LRW showed an increased number of BrdU positive cells after 24 h, suggesting that LRW enhanced cell proliferation. The differentiating stages of osteoblasts is presented as the chronological process deriving from transcriptional factors such as COL1A2,

ALP, and RUNX2 [33]. These bone-cell specific markers are regarded as functional detection characteristic for cell differentiation. COL1A2 is an extracellular matrix component essentially required for maintaining bone mineral density. The increasing ALP levels are associated with vascular calcification. It is a marker for bone formation and bone turnover which are linked to the skeletal strength [33]. The molecular swift of the transcriptional factor RUNX2 has multiple upstream regulators and an extensive variability of targets. RUNX2 is expressed as an osteoblastic-specific protein controlled by post-translational effects on bone formation [34]. On treatment with LRW, RUNX2 expression levels were increased, suggesting differentiation of osteoblast lineage. The Akt- controlled pathway is a signaling conduit promoting differentiation of osteoblasts. The Akt activity leads to bone growth and development. Treatment with tripeptide LRW also shown to increase the phosphorylation of Akt. Therefore, tripeptide LRW represented a significant increasing trend in the activity of ALP and COL1A2 expression, suggesting osteoinductive activity of tripeptide LRW.

All together these transcriptional factors are accessory components required for intracellular signaling in response to physiological bone development and differentiation [35]. Furthermore, osteoblast differentiation is forwarded under permissive conditions of ascorbic acid and glycerol-2-phosphate [40]. Glycerophosphate belongs to the family of serine-threonine phosphatase inhibitor and acts as a simple phosphate donor. Altogether with ascorbic acid, it stimulates mineralization of matrix by increasing neutral metalloproteinase in vesicles and degrading proteoglycans resulting in precipitation of minerals in the form of crystals. Binding of alizarin's red stain to the crystals indicates the deposition of calcium nodules, which further leads to the formation of bone matrix as shown in Figure 8 [41]. Hence, the levels of COL1A2, ALP and mineralized nodules in our study demonstrate the stimulatory effect of LRW on osteoblast differentiation. Thus, it is feasible for investigating *in vitro* osteoblast activity and relating to bone -markers.

The RANK/RANKL/OPG system regulates bone modeling and remodeling. RANK/RANKL stimulates osteoclast activation by reducing the number of osteoclasts, resulting in pathological conditions characterized by increased bone turnover [36]. Besides the function of osteoclast stimulation, OPG protects bone from disproportionate resorption by acting as a receptor enabling RANKL to bind. It has also been reported in the literature, that RANKL may also act by

reducing the number of osteoclasts which thereby increase the cancellous bone mass [51]. Nonetheless, an increase in OPG levels suggests a protective effect against excessive resorption by RANKL binding. Thus, the relative increase in OPG expression suggests major determinant of bone mass and strength in maintaining balanced bone mass by inhibiting osteoclast-mediated bone resorption [37]. Therefore, it is also possible that RANKL, does not positively induce bone mass or bone structure via osteoblasts stimulation but may reduce the number of osteoclasts for osteoclastogenesis.

In addition to the osteogenic activity, peptide LRW also showed an increased cell migration rate. The role of cell migration is associated with the collective movement of cells which is mainly associated with rate of cell migration and wound closure %. The cells migrate to the bone surface to heal the superficial wound, which is created by a trans-well insert. After subsequent removal of the insert, movement of cells is regulated to maintain intracellular adhesions [43]. During the repairing stages of the cells, the movement of cells happens over time. Therefore, an increased rate of cell migration and wound closure % factors suggest fastened cell movement in the presence of LRW peptide [44]. The movement of cells on to the injury site participates in bone formation. The cell migration is a key implication in bone formation and bone disease treatment. The migration of osteoblasts to bone injury sites indicates the therapeutic efficacy in the treatment of osteoporosis or fractures. These results also suggest a conclusive association in increased movement of cells at the resorption site under the influence of LRW.

Tripeptide LRW presented similar profound effects on RUNX2, ALP, COL1A2 and OPG expressions as that of IRW peptide derived from egg protein. However, LRW-stimulated osteogenic activity of bone cells enhanced collagen synthesis for extracellular matrix leading to bone health-promoting effects and subsequent bone formation.

The treatment and preventive actions against bone mass deterioration have now been focused greatly on naturally derived compounds such as polyphenols. Moreover, some food bioactive compounds such as genistein, an isoflavone isolated from various plants have also been projected as a preventive drug against bone mass in postmenopausal women [52]. Interactions among other available food macro-nutrients affect the bioavailability of the single bioactive compound. The genetic aspects act as a controlling factor for contributing pathogenesis of osteoporosis is, however, small but also reflects the individual relationship between candidate

biomarker and osteoporosis. Therefore, it is interesting to investigate the effectual dose with the appropriate valuation of the potent bioactive components.

3.5 Conclusion

Osteoporosis is bone disease with reduced bone mass, consequently resulting in increased risk of fractures. Due to the increased prevalence of this progressive disease, we provided a potential bioactive tripeptide. We demonstrated that tripeptide LRW increased the proliferation and differentiation of mouse pre-osteoblast cells MC3T3-E1. Moreover, LRW also stimulated the expressions of phenotypic markers which control the activity of osteoblasts. The formation of mineralized nodules suggests LRW-stimulated roles in bone formation. Therefore, LRW peptide have bone health promoting and osteoprotective effects. Our results may support the possibilities to investigate the development of medical alternative based on therapeutic potential of LRW in preventing or treating osteoporosis.

3.6 References

[1]. Florencio-Silva, R., Sasso, G.R.D.S., Sasso-Cerri, E., Simões, M.J. and Cerri, P.S., Biology of bone tissue: structure, function, and factors that influence bone cells, BioMed research international, 2015.

[2]. Gillies AR, Lieber RL., Structure and function of the skeletal muscle extracellular matrix, Muscle & nerve. 2011, 44(3), pp. 318-331.

[3]. Feng X., Chemical and biochemical basis of cell-bone matrix interaction in health and disease, Current chemical biology. 2009, 3(2), pp.189-96.

[4]. Green J., The physicochemical structure of bone: cellular and noncellular elements, Mineral and electrolyte metabolism, 1994, 20(1-2), pp.7-15.

[5]. Office of the Surgeon General (US) The Basics of Bone in Health and Disease, In Bone Health and Osteoporosis: A Report of the Surgeon General, Office of the Surgeon Generall (USA), 2004.

[6]. Rucci N. Molecular biology of bone remodelling. Clinical cases in mineral and bone metabolism. 2008, 5(1), 49.

[7]. Komori, T., Regulation of osteoblast differentiation by transcription factors, Journal of Cellular Biochemistry, 2006, 99(5), pp.1233-1239.

[8]. Chen X, Wang Z, Duan N, Zhu G, Schwarz EM, Xie C., Osteoblast–osteoclast interactions, Connective tissue research. 2018, 59(2), pp.99-107.

[9]. Kim HK, Cho SG, Kim JH, Doan TK, Hu QS, Ulhaq R, Song EK, Yoon TR, Mevinolin enhances osteogenic genes (ALP, type I collagen and osteocalcin), CD44, CD47 and CD51 expression during osteogenic differentiation, Life sciences. 2009, 84(9-10), pp.290-5.

[10]. Boyce BF, Xing L., The Rankl/Rank/Opg pathway, Current osteoporosis reports. 2007, 5(3), pp. 98-104.

[11]. Buckwalter JA, Cooper RR, Bone structure and function. Instructional course lecture, 1987, 36, pp. 27-48.

[12]. Wojda SJ, Donahue SW. Parathyroid hormone for bone regeneration. Journal of Orthopaedic Research®. 2018, 36(10), pp. 2586-94.

[13]. Clarke, B., Normal bone anatomy and physiology, Clinical Journal of the American Society of Nephrology, 2008, (Supplement 3), pp. S131-S139.

[14]. Tabatabaei-Malazy, O., Salari, P., Khashayar, P. and Larijani, B., New horizons in treatment of osteoporosis, DARU Journal of Pharmaceutical Sciences, 2008, 25(1), pp.2.

[15]. Bensaha, T. and El Mjabber, H., Evaluation of new bone formation after sinus augmentation with two different methods, International Journal of Oral and Maxillofacial Surgery, 2016, 45(1), pp.93-98.

[16]. Ukon, Y., Makino, T., Kodama, J., Tsukazaki, H., Tateiwa, D., Yoshikawa, H. and Kaito, T., Molecular-Based Treatment Strategies for Osteoporosis: A Literature Review, International Journal of Molecular Sciences, 2019, 20(10), pp.2557.

[17]. Chakrabarti, S., Guha, S. and Majumder, K., Food-derived bioactive peptides in human health: Challenges and opportunities, Nutrients, 2018, 10(11), pp.1738.

[18]. Shang, N., Bhullar, K.S., Hubbard, B.P. and Wu, J., Tripeptide IRW initiates differentiation in osteoblasts differentiation via the RUNX2 pathway, Biochimica et Biophysica Acta (BBA)-General Subjects, 2019, 1863(6), pp.1138-1146.

[19]. Office of the Surgeon General (US)., Prevention and treatment for those who have bone diseases. In Bone Health and Osteoporosis: A Report of the Surgeon General, 2004.

[20]. Feng, X. and McDonald, J.M., Disorders of bone remodeling, Annual Review of Pathology: Mechanisms of Disease, 2011, 6, pp.121-145.

[21]. https://www.bones.nih.gov/health-info/bone/osteoporosis/overview, July 2, 2019

[22]. Meesters, D., Wijnands, K., Brink, P. and Poeze, M., Malnutrition and Fracture Healing: Are specific deficiencies in amino acids important in nonunion development? Nutrients, 2018, 10(11), pp.1597.

[23]. Perez, J.R., Kouroupis, D., Li, D.J., Best, T.M., Kaplan, L. and Correa, D., Tissue engineering and cell-based therapies for fractures and bone defects, Frontiers in Bioengineering and Biotechnology, 2018, 6, pp.105.

[24]. Mogensen, T.H., Pathogen recognition and inflammatory signaling in innate immune defenses, Clinical Microbiology Reviews, 2009, 22(2), pp.240-273.

[25]. Korhonen, H. and Pihlanto, A., Food-derived bioactive peptides-opportunities for designing future foods, Current Pharmaceutical Design, 2003, 9(16), pp.1297-1308.

[26]. Sharma, S., Singh, R. and Rana, S., Bioactive peptides: a review, International Journal of Bioautomation, 2011, 15(4), pp.223-250.

[27]. Eming, S.A., Martin, P. and Tomic-Canic, M., Wound repair and regeneration: mechanisms, signaling, and translation, Science Translational Medicine, 2014, 6(265), pp.2656.

[28]. Ramage, S.C., Urban, N.H., Jiranek, W.A., Maiti, A. and Beckman, M.J., Expression of RANKL in osteolytic membranes: association with fibroblastic cell markers, Journal of bone and joint surgery, 2007, 89(4), pp.841-848.

[29]. Heaney, R.P. and Layman, D.K., Amount and type of protein influences bone health, The American Journal of Clinical Nutrition, 2008, 87(5), pp.1567S-1570S.

[30]. Bonjour, J.P., Dietary protein: an essential nutrient for bone health, Journal of the American College of Nutrition, 2005, 24(sup6), pp.526S-536S.

[31]. Parra-Torres, A.Y., Valdés-Flores, M., Orozco, L. and Velázquez-Cruz, R., Molecular Aspects of Bone Remodeling, In Topics in Osteoporosis, 2013.

[32]. Rojas, A., Aguilar, R., Henriquez, B., Lian, J.B., Stein, J.L., Stein, G.S., Van Wijnen, A.J., Van Zundert, B., Allende, M.L. and Montecino, M., Epigenetic control of the bone-master Runx2 gene during osteoblast-lineage commitment by the histone demethylase JARID1B/KDM5B, Journal of Biological Chemistry, 2015. 290(47), pp.28329-28342.

[33]. Rhee, C., Kim, J. and Tucker, H.O, Transcriptional Regulation of the First Cell Fate Decision, Journal of Developmental Biology & Regenerative Medicine, 2017, 1(1).

[34]. Lee, K.S., Kim, H.J., Li, Q.L., Chi, X.Z., Ueta, C., Komori, T., Wozney, J.M., Kim, E.G., Choi, J.Y., Ryoo, H.M. and Bae, S.C., Runx2 is a common target of transforming growth factor β1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12, Molecular and Cellular Biology, 2000, 20(23), pp.8783-8792.

[35]. Cheng, S.L., Zhang, S.F., Nelson, T.L., Warlow, P.M. and Civitelli, R., Stimulation of human osteoblast differentiation and function by ipriflavone and its metabolites, Calcified Tissue International, 1994, 55(5), pp.356-362.

[36]. Boyce, B.F. and Xing, L., Functions of RANKL/RANK/OPG in bone modeling and remodeling, Archives of Biochemistry and Biophysics, 2008, 473(2), pp.139-146.

[37]. Rucci, N., Rufo, A., Alamanou, M. and Teti, A., Modeled microgravity stimulates osteoclastogenesis and bone resorption by increasing osteoblast RANKL/OPG ratio, Journal of Cellular Biochemistry, 2007, 100(2), pp.464-473.

[38]. Thomford, N., Senthebane, D., Rowe, A., Munro, D., Seele, P., Maroyi, A. and Dzobo, K., Natural products for drug discovery in the 21st century: innovations for novel drug discovery, International Journal of Molecular Sciences, 2018, 19(6), p.1578.

[39]. Mukherjee, A. and Rotwein, P., Akt promotes BMP2-mediated osteoblast differentiation and bone development, Journal of cell science, 2009, 122(5), pp.716-726.

[40]. Torii, Y., Hitomi, K. And Tsukagoshi, N., L-ascorbic acid 2-phosphate promotes osteoblastic differentiation of MC3T3-E1 mediated by accumulation of type I collagen, Journal of Nutritional Science and Vitaminology, 1994, 40(3), pp.229-238.

[41]. Blair, H.C., Larrouture, Q.C., Li, Y., Lin, H., Beer-Stoltz, D., Liu, L., Tuan, R.S., Robinson, L.J., Schlesinger, P.H. and Nelson, D.J., Osteoblast differentiation and bone matrix formation *in vivo* and i*n vitro,* Tissue Engineering Part B: Reviews, 2017, 23(3), pp.268-280.

[42]. Guo, S.A. and DiPietro, L.A., Factors affecting wound healing, Journal of Dental Research, 2010, 89(3), pp.219-229.

[43]. Li, L., He, Y., Zhao, M. and Jiang, J., Collective cell migration: Implications for wound healing and cancer invasion, Burns & Trauma, 2013, 1(1), p.21.

[44]. Kiryushko, D., Bock, E. and Berezin, V., Pharmacology of cell adhesion molecules of the nervous system, Current Neuropharmacology, 2007, 5(4), pp.253-267.

[45]. Mellander, O.L.O.F., The physiological importance of the casein phosphopeptide calcium salts. II. Peroral calcium dosage of infants. Some aspects of the pathogenesis of rickets, Acta Societatis Botanicorum Poloniae, 1950, *55*, pp.247-257.

[46]. Tsuchita, H., Goto, T., Shimizu, T., Yonehara, Y. and Kuwata, T., Dietary casein phosphopeptides prevent bone loss in aged ovariectomized rats, The Journal of nutrition, 1996, 126(1), pp.86-93.

[47]. Narva, M., Collin, M., Lamberg-Allardt, C., Kärkkäinen, M., Poussa, T., Vapaatalo, H. and Korpela, R., Effects of long-term intervention with Lactobacillus helveticus-fermented milk on bone mineral density and bone mineral content in growing rats, Annals of nutrition and metabolism, 2004, 48(4), pp.228-234.

[48]. Reddi, S., Kumar, N., Vij, R., Mada, S.B., Kapila, S. and Kapila, R., Akt drives buffalo casein-derived novel peptide-mediated osteoblast differentiation, The Journal of Nutritional Biochemistry, 2016, 38, pp.134-144.

[49]. Wu, J., Yuan, L. and Aluko, R.E., Restriction of the *in vitro* formation of angiotensin II by leucinyl-arginyl-tryptophan, a novel peptide with potent angiotensin I-converting enzyme inhibitory activity, Bioscience, Biotechnology, and Biochemistry, 2006, 70(5), pp.1277-1280.
[50]. Jennings, A., MacGregor, A., Spector, T. and Cassidy, A., Amino acid intakes are associated with bone mineral density and prevalence of low bone mass in women: evidence from discordant monozygotic twins, Journal of Bone and Mineral Research, 2016, 31(2), pp.326-335.
[51]. Xiong, J., Cawley, K., Piemontese, M., Fujiwara, Y., Zhao, H., Goellner, J.J. and O'Brien, C.A., Soluble RANKL contributes to osteoclast formation in adult mice but not ovariectomy-induced bone loss, Nature communications, 2018, 9 (1), pp.2909.
[52]. Arcoraci V, Atteritano M, Squadrito F, D'Anna R, Marini H, Santoro D, Minutoli L, Messina S, Altavilla D, Bitto A., Antiosteoporotic activity of genistein aglycone in postmenopausal women: Evidence from a post-hoc analysis of a multicenter randomized controlled trial, Nutrients. 2017, 9(2), pp. 179.
[53]. McGonnell IM, Grigoriadis AE, Lam EW, Price JS, Sunters A, A specific role for phosphoinositide 3-kinase and AKT in osteoblasts? Frontiers in endocrinology, 2012, 3, pp. 88.

Figure 3.1- The in vitro effect of peptide LRW on cell cytotoxicity/viability in pre-osteoblast cell line MC3T3-E1. The cells were treated with 50µM LRW for 24 h by AlamarBlue assay. The absorbance showed ~100% viability, indicating no observed toxicity in MC3T3-E1 cells. The data presented is mean ± SEM of 3 independent experiments.

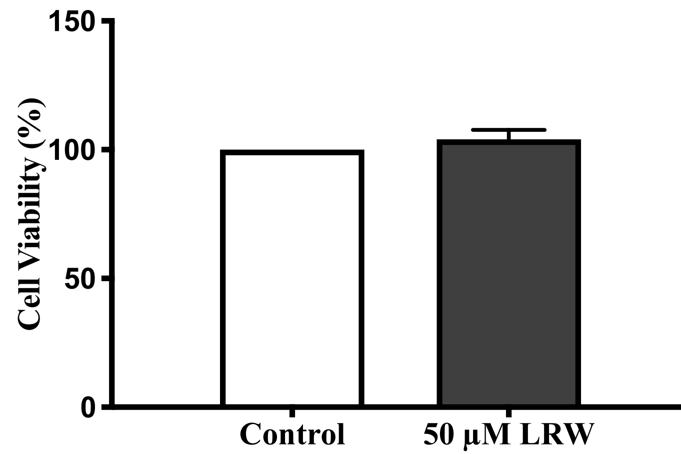
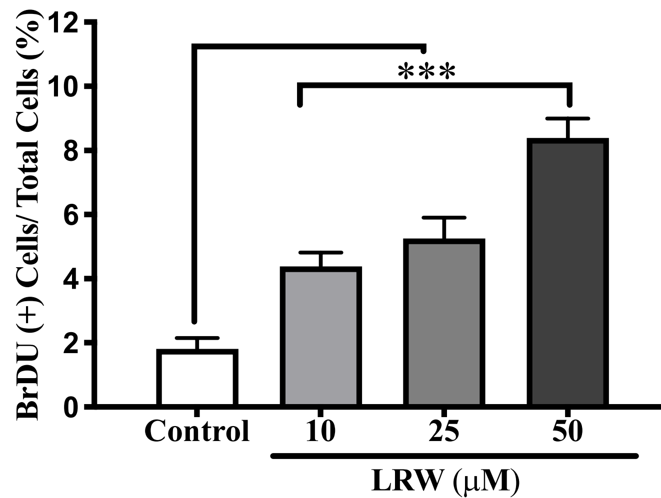
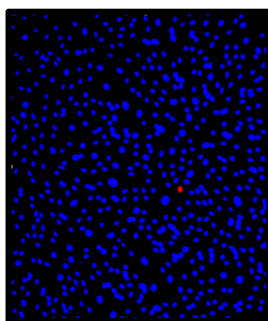
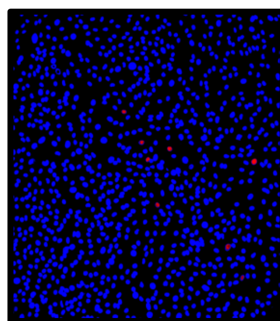
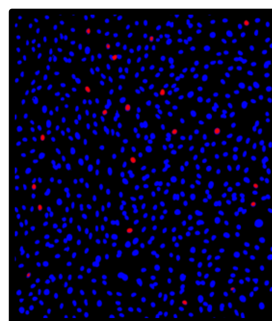


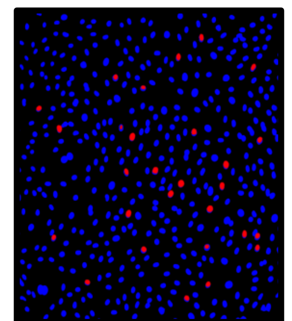
Figure 3.2- The in vitro effect of peptide LRW on cell proliferation in pre-osteoblast cell line MC3T3-E1. The cells were treated with 10, 25 and 50 µM LRW for 24 h prior to BrdU labelling agent. The cells were further immuno- stained and observed under florescence microscope (10X). The increasing LRW concentration, showed an increase in the percentage of the BrdU positive cells suggesting proliferation of cells under the influence of drug. The data presented is mean ± SEM of 6 independent experiments. *, **, and * indicate p<0.05, p<0.01 and p<0.001 respectively as compared to control.**





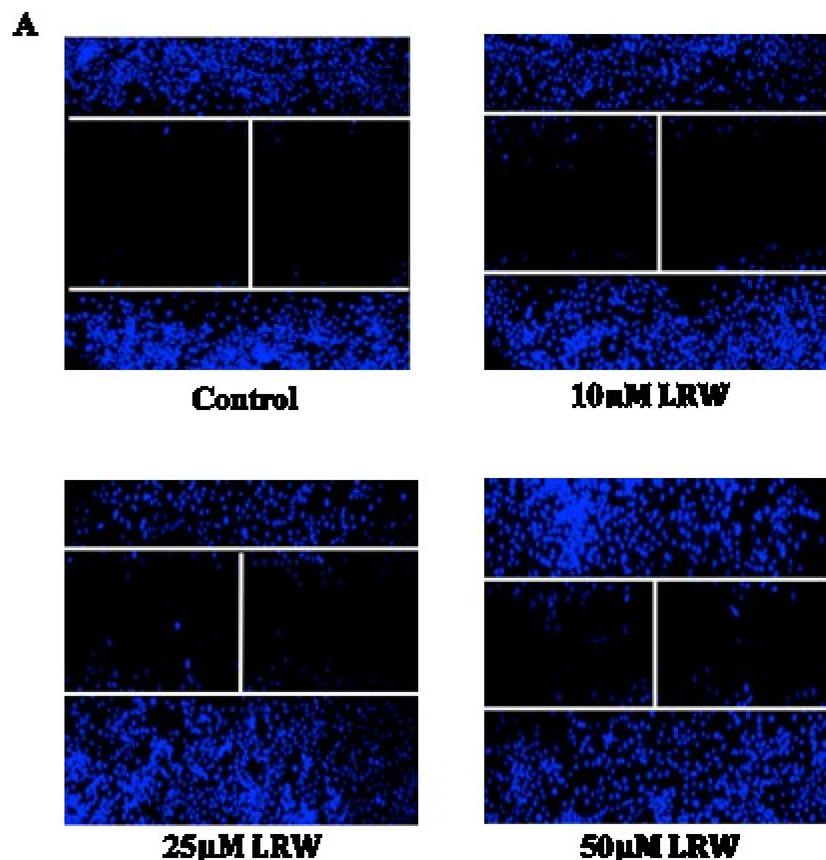






Control 10 25 50

Figure 3.3-The in vitro effect of peptide LRW on mineralization in pre-osteoblast cell line MC3T3-E1. The cells were cultured with insert, and then treated with 10, 25 and 50 µM of LRW for 24 h. Representative images of migrating movement of cells were taken using fluorescence microscopy (A). The rate of migration of cells (B) and wound closure % (C) was increased with increased LRW concentration. The data represented is mean ± SEM with 4 independent experiments. *, **, and * indicate p<l0.05, p<l0.01 and p<0.001 respectively as compared to control.**



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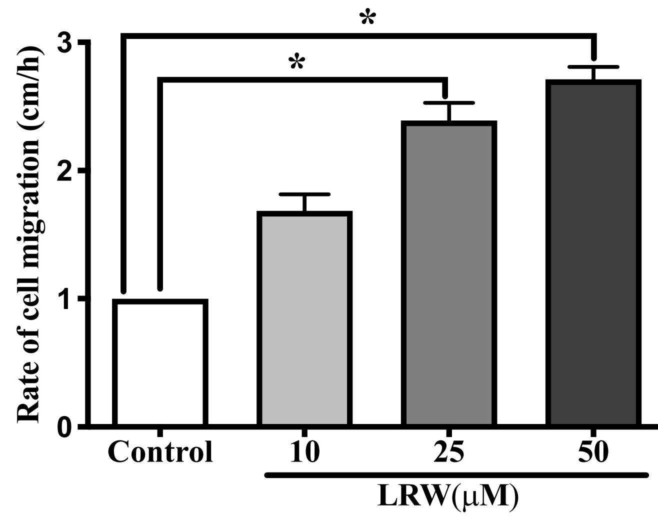

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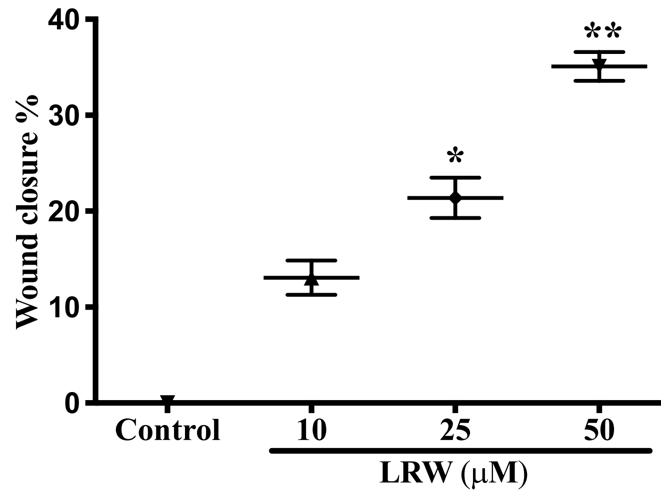


Figure 3.4-The in vitro effect of peptide LRW on ALP activity in pre-osteoblast cell line MC3T3-E1. The cells were treated with 10, 25 and 50 µM LRW. After 24 h, ALP activity was determined in both cell lysates (A) and culture medium (B). An increase in ALP activity was observed with LRW concentrations. The data presented is mean ± SEM with 3 independent experiments. *, **, and * indicate p <0.05, p<0.01 and p<0.001 respectively as compared to control.**

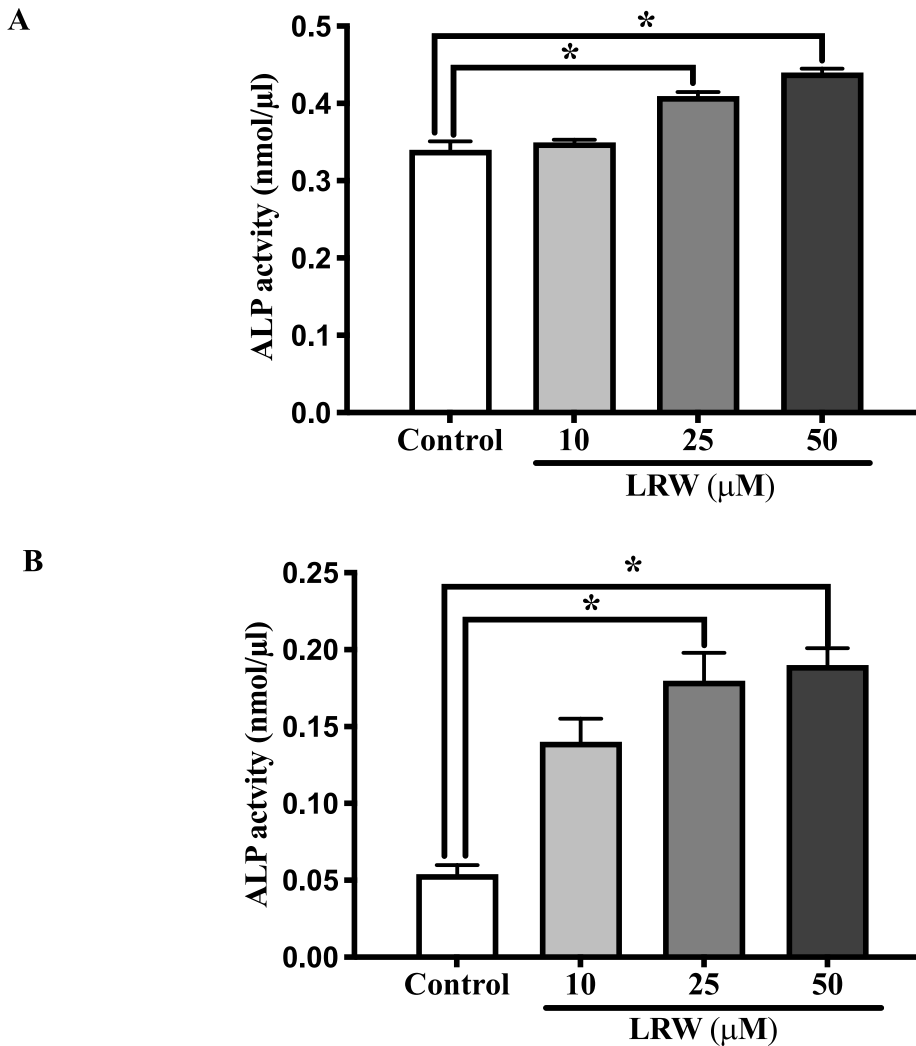
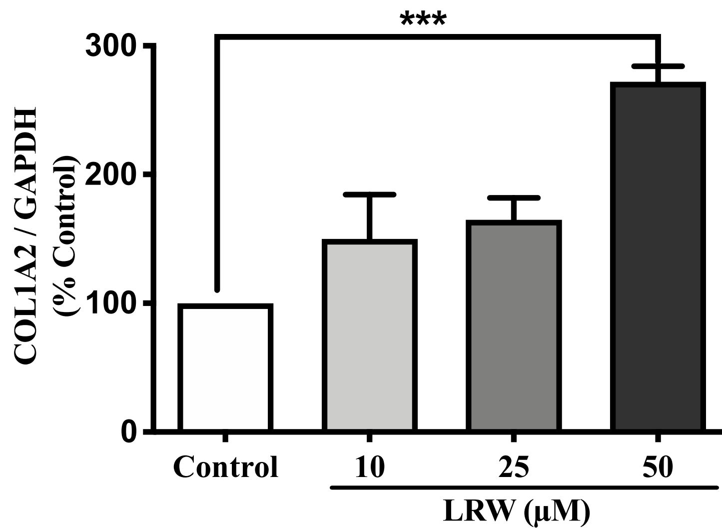


Figure 3.5- The in vitro effect of peptide LRW on COL1A2 expression in pre-osteoblast cell line MC3T3-E1. The cells were treated with 10, 25 and 50 µM LRW. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further immunoblotted for COL1A2 and loading control (GAPDH) expressions. An increase in COL1A2 levels was observed. The data presented is mean ± SEM of 6 independent experiments. *, **, and * indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively as compared to control.**



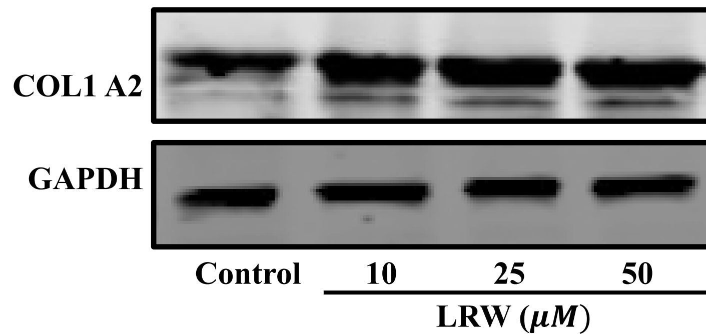
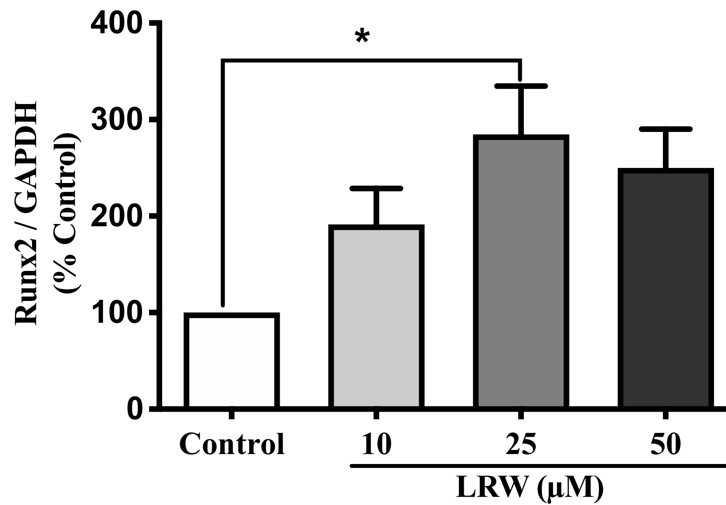


Figure 3.6- The in vitro effect of peptide LRW on RUNX2 expression in pre-osteoblast cell line MC3T3-E1. The cells were treated with 10, 25 and 50 µM LRW. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further immunoblotted for RUNX2 and loading control (GAPDH) expressions. An increase in RUNX2 levels was observed. The data presented is mean ± SEM of 6 independent experiments. *, **, and * indicate p <0.05, p<0.01 and p<0.001 respectively as compared to control.**



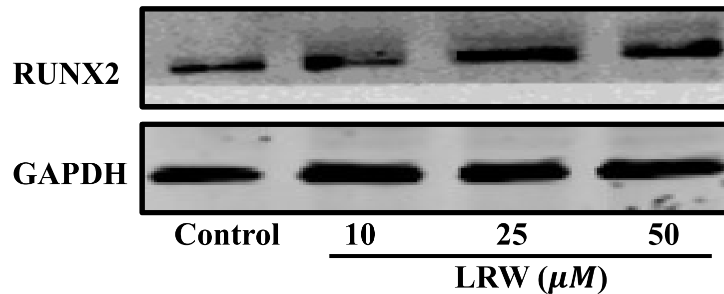


Figure 3.7- The in vitro effect of peptide LRW on p-Akt and t-Akt expression in pre-osteoblast cell line MC3T3-E1. The cells were treated with 10, 25 and 50 µM LRW. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further immunoblotted for p-Akt, t-Akt and loading control (GAPDH) expressions. An increase in expression for p-Akt whereas no change for t-Akt. The data presented is mean ± SEM with 5 independent experiments. *, **, and * indicate p<0.05, p<0.01 and p<0.001 respectively as compared to control.**

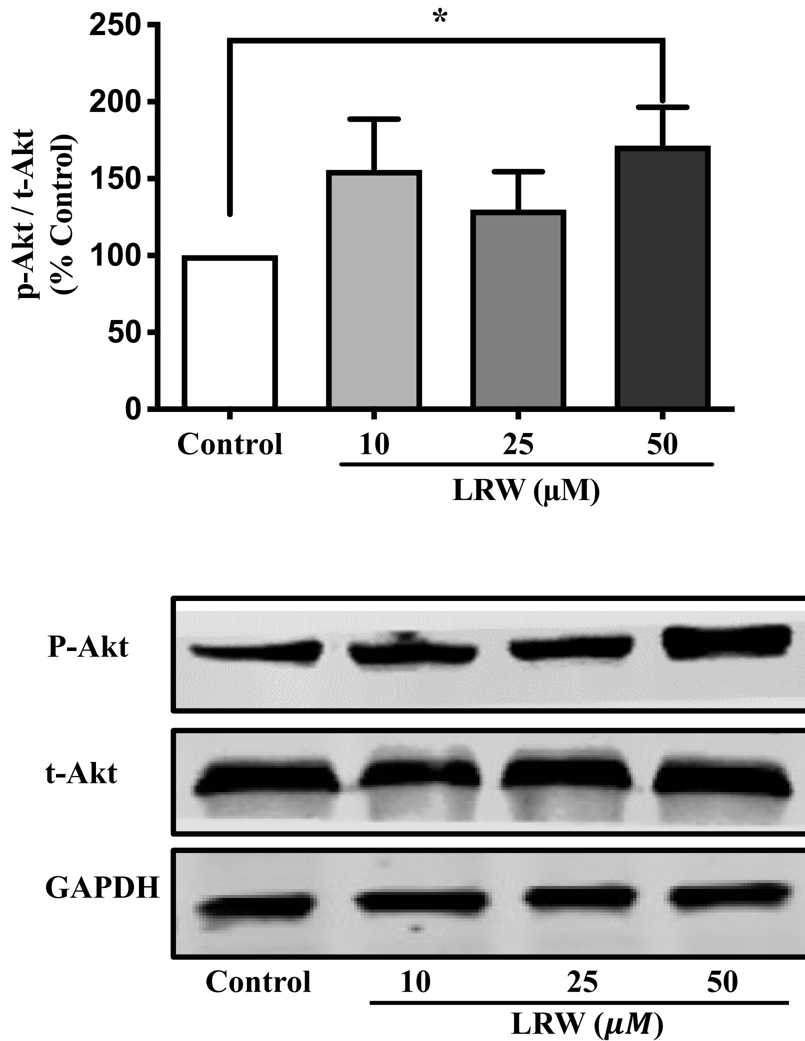


Figure 3.8- The in vitro effect of peptide LRW on mineralization in pre-osteoblast cell line MC3T3-E1. The cells were treated with 10, 25 and 50 µM of LRW in a medium containing ascorbic acid and beta—glycerophosphate for 10, 15 and 20 days. Representative images of Alizarin red S stain were taken (A). Absorbance of the extracted stain depicted an increase in bone matrix in higher LRW (25 and 50 µM) concentration (B). The data presented is mean ± SEM with 3 independent experiments. *, **, and * indicate $p<0.05$, $p<0.01$ and $p<0.001$ respectively as compared to control in their respective group.**

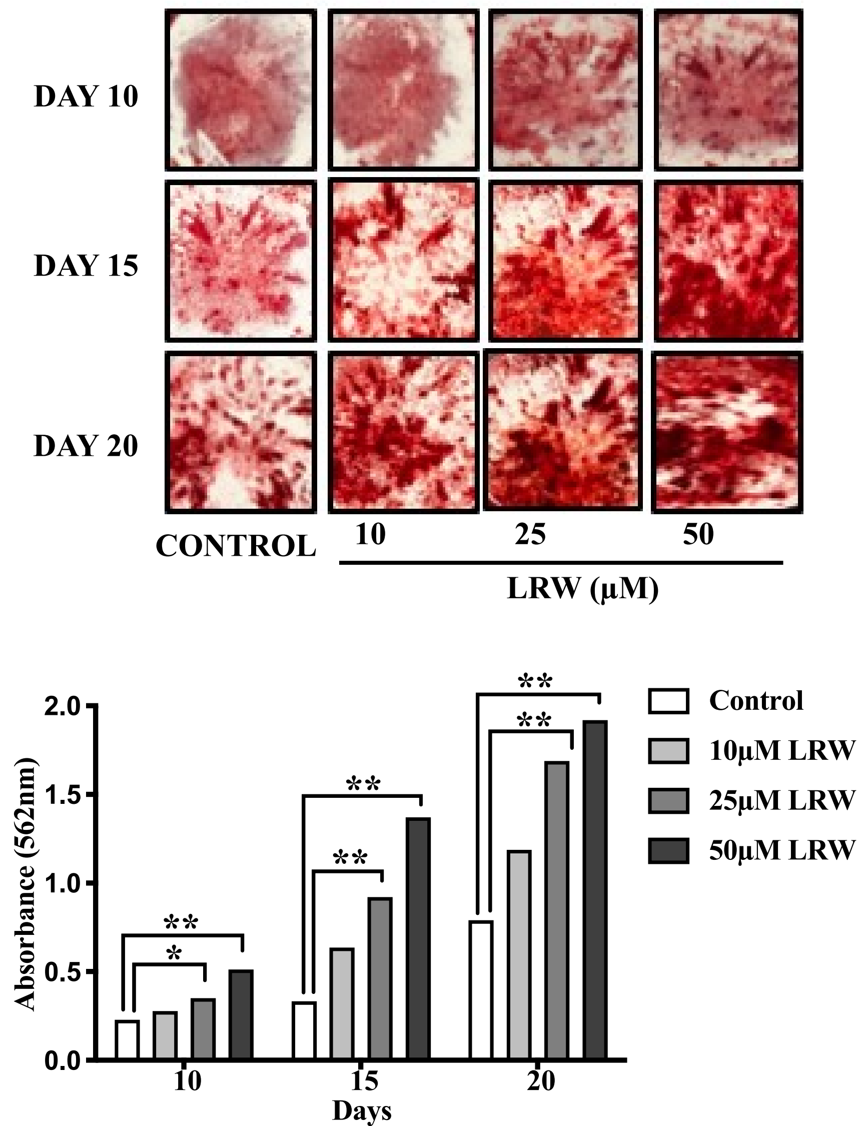


Figure 3.9- The in vitro effect of peptide LRW on RANKL expression in pre-osteoblast cell line MC3T3-E1. The cells were treated with 10, 25 and 50 µM LRW. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further immunoblotted for RANKL and loading control (GAPDH) expressions. A decrease in RANKL levels was observed. The data presented is mean ± SEM of 6 independent experiments *, **, * and ns indicate p <0.05, p<0.01, p<0.001 and not significant respectively as compared to control.**

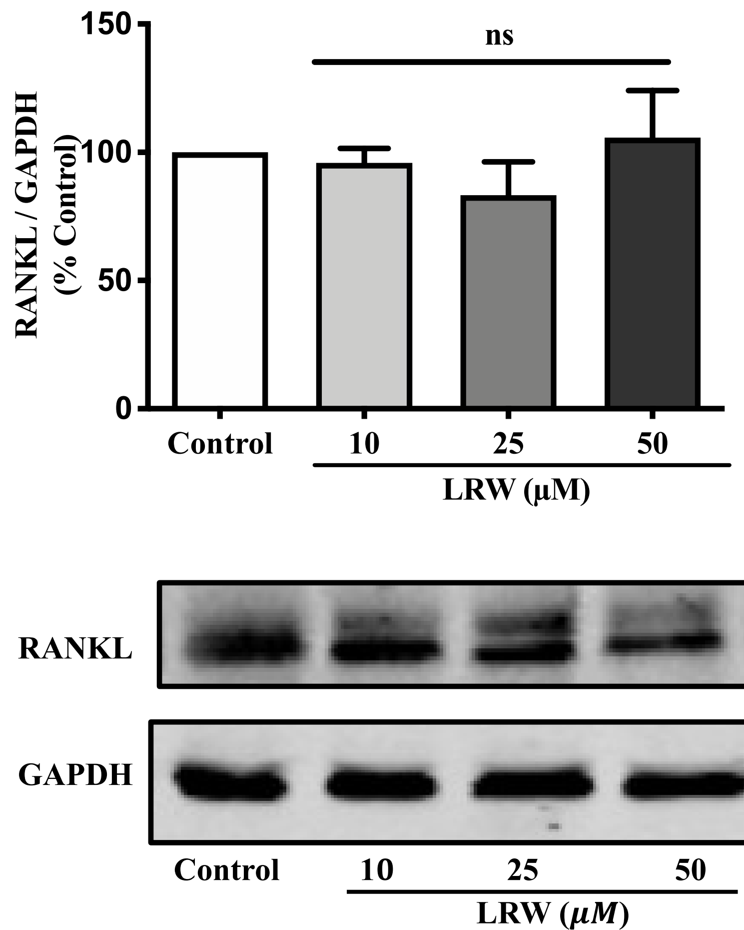
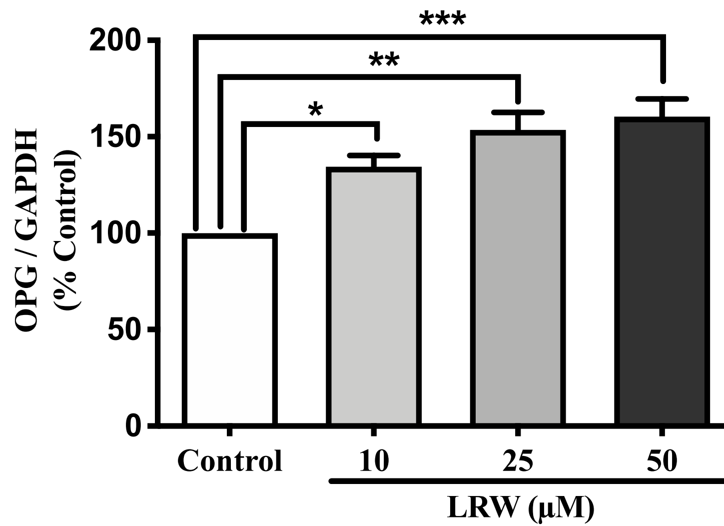
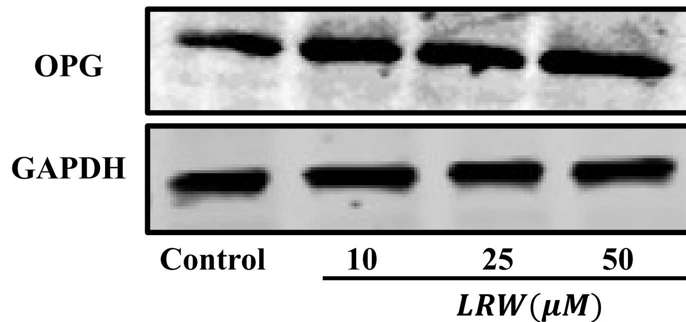


Figure 3.10- The in vitro effect of peptide LRW on OPG expression in pre-osteoblast cell line MC3T3-E1. The cells were treated with 10, 25 and 50 µM LRW. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further immunoblotted for OPG and loading control (GAPDH) expressions. An increase in OPG levels was observed. The data presented is mean ± SEM of 5 independent experiments. *, **, and * indicate p<0.05, p<0.01 and p<0.001 respectively as compared to control.**





CHAPTER 4 - THE *IN VITRO* EFFECT OF PEA PROTEIN HYDROLYSATE ON BONE REMODELING

4.1 Introduction

Osteoporosis, affecting 1 in 3 women and 1 in 5 men, is characterized by low bone mass and deteriorated microarchitecture of bone tissues [1]. As the most common bone disease, osteoporosis increases the risk of fractures, which leads to substantial increase in physical impairments and a decrease in quality of life, contributing to over $4.6 billion economic burdens in Canada [32]. In addition to aging and genetic factors, nutrients such as calcium, vitamin D and food protein are key in determining bone health [29]. Dietary protein has been clinically associated with increased bone strength and mineral mass, and reduced risk of osteoporotic fractures [28]. Recent studies indicate that an appropriate amount of food protein intake from either plant or animal sources can attenuate the risk of fractures by promoting bone strength, reducing bone loss and increasing bone density [5]. A clinical trial of 291 females aged between 50 and 65 y suggested that the intake of basic milk protein increases bone mineral density maintaining balanced bone remodeling [38]. Similarly, another clinical study of 35 healthy young women has reported that supplementation of 40 mg/day basic milk protein for 6 months can effectively increase bone mineral density mediated via the bone formation process [26]. Likewise, 0.01% and 0.1% of basic milk protein diet fed to an ovariectomized model of rats (OVX) for 17 weeks suppresses osteoclast-mediated bone resorption and prevents bone loss [30]. A cross-sectional study focusing on the consumption of whole egg reported a positive influence on the bone mineral content of cortical bone, promoting pediatric bone development and prevention of fractures [27]. Likewise, plant proteins have also sparked potential bone health properties. Increased intake of plant-based high protein foods incorporated in a vegetarian diet (comprising of beans, nuts, and vegetable meat analogs) significantly decreased the risk of wrist fractures in a cohort study of 65 peri- and postmenopausal women [36]. It has also been reported that an adequate amount of dietary proteins can positively impact bone health by increasing muscle mass, calcium absorption, and production of insulin growth factor-1 [41]; association of all these contributing factors highlights the importance of protein intake on bone health. Thus, adequate intake of proteins can help regulate bone turnover and bone mineral density [31].

In comparison to intact food proteins, protein hydrolysates are more rapidly absorbed by the human body. This results in an efficient supply of amino acids for building muscle strength and increasing bone turnover [8]. Often prepared by proteolysis, protein hydrolysates are the

cleaved products of the parent food protein that are composed mainly of low molecular peptides and free amino acids [7]. Recent studies also indicate that protein hydrolysates contribute to bone health-promoting effects via different metabolisms in the human body [10]. For example, soy protein hydrolysates were reported to exhibit positive effects in promoting the formation of calcium complexes on bone mass [49]; hydrolysates prepared using lentils and chickpea also exhibited bone health-promoting effects by increasing bone mass and slowing down the loss of bone density [50].

Pea protein is recognized as a high-quality and sustainable plant protein containing all essential amino acids [13]. Further, pea protein contains a relatively higher content of branched-chain amino acids (BCAAs) such as leucine, isoleucine, and valine [14], which are associated with higher bone mineral density and required for muscle synthesis. Unlike other plant protein sources, pea protein doesn't retain gluten and its allergenicity is comparatively lower [15]. Pea protein is also regarded as a rich source of iron [42]. Pea protein hydrolysates or its derived peptides were reported to exhibit ACE-inhibitory, antioxidant, anti-inflammatory and immunomodulatory properties [12]. LRW, a tripeptide derived from pea protein, was shown to have osteogenic activity in our previous chapter. We demonstrated that pea-protein derived tripeptide LRW can positively regulate bone formation biomarkers (such as RUNX2, COL1 A2, ALP, p-Akt), which represents sequential osteoblast proliferation and differentiation, followed by deposition of calcium nodules for bone matrix formation. As LRW was identified using pea protein hydrolysate and thermolysin enzyme, we hypothesized that upon enzymatic hydrolysis using different enzymes can release LRW or different peptides that can produce similar or increased osteogenic activity by stimulating osteoblastic activity. Therefore, our research objectives were to explore the in vitro regulatory role of pea protein hydrolysate in enhancing the activity of osteoblasts leading to managing bone remodeling.

4.2 Materials and Methods

4.2.1 Reagents

The commercial pea protein isolate, Propulse^{TM} was gifted from Nutri-Pea Ltd. (Portage la Prairie, Manitoba, Canada). The enzymes such as trypsin, chymotrypsin, alcalase, pepsin and thermolysin and, trifluoroacetic acid (TFA), Alizarin-S red stain, cetylperidium chloride, anti-STAT3 primary antibody, anti-CXCR4 primary antibody, dithiothreitol (DTT), Trito-X-100, β-glycerophosphate,

bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, Mo, USA). The enzyme Protex 6L was purchased from Amano Enzyme Inc. (Nagoya, Japan). Dulbecco's modified eagle media (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, 0.25% trypsin-EDTA, and phosphate buffer saline (PBS) were purchased from Gibco/Invitrogen (Carlsbad, CA, U.S.A). The primary antibody against COL1A2 and GAPDH was purchased from Abcam (Toronto, ON, Canada). Rabbit anti-mouse IgG (H+L) secondary antibody Alexa Fluor 594 was procured from Molecular probes (Waltham, MA, U.S.A).

4.2.2 Preparation of pea protein hydrolysate

Pea protein isolate was hydrolyzed using different enzymes (trypsin, chymotrypsin, Protex 6L, alcalase, pepsin, and thermolysin) under conditions presented in Table 1. For preparing pea protein hydrolysate, a 5% pea protein isolate slurry was prepared with distilled water and digested at specific pH and temperature conditions for 3 h. The digests were then heated to 95°C for 15 min to inactivate the enzyme. The hydrolysate was then collected after centrifugation of the prepared slurry at 10 000g for 25 min. The hydrolysates were then desalted using C18 cartridge column (Sep-Pak® Vac, 35 cc, C18 cartridges, Waters Corporation, Milford, Massachusetts, U.S.A). The hydrolysate was first dissolved in 10 mL distilled water and then buffer salts present in hydrolysates were removed in an exchange system with water and further lyophilized by freeze-drying.

4.2.3 Degree of Hydrolysis (DH)

The degree of hydrolysis was determined using the TNBS (Trinitrobenzesulfonic) method [39]. The hydrolysate samples were first acid hydrolyzed with 4 M methanesulfonic acid (0.5 mg/200µL) at 1115°C for 24 h and was then cooled to room temperature. The aliquots of 2mL from the samples were withdrawn followed by mixing with 10 mL of 1% SDS solution. The sample was then incubated for 15 min in a water bath maintained at 75°C. After incubation, the samples were top up with a 1% SDS solution in a 50 mL volumetric flask. Aliquots of 250 µL from the prepared samples were mixed with 2 mL of 0.2 M sodium phosphate buffer (pH 8.2) in a test tube. Thereafter, 2 mL of 0.1% TNBS solution was then added to each tube, followed by 1 h incubation in a water bath maintained at 50°C. The reaction was then terminated using 0.1 M HCl, and the samples were then cooled to room temperature. The sample absorbance was measured

at 340 nm using SpectraMax 340 plate reader (Molecular Devices, San Jose, CA, U.S.A) and the DH was calculated using following formula.

$$\text{Degree of Hydrolysis (DH) \%} = \frac{\text{Ls-Lo}}{(\text{Lmax-Lo})} \text{X100}$$

Where, Ls is the amount of amino acids released by hydrolysis, Lo is the amount of amino acids in original sample without enzymatic hydrolysis and Lmax is the amount of amino acids after acid hydrolysis.

4.2.4 Size-exclusion chromatography using FPLC

The prepared hydrolysates were then analyzed for the size-exclusion chromatography using fast protein liquid chromatography (FPLC). The Superdex peptide 10/300 GL column (GE Healthcare, Piscataway, NJ, U.S.A) was attached FPLC system AKTA explorer 10S system (GE Healthcare, Piscataway, NJ, U.S.A). The samples were prepared at 0.5 mg/mL in 30% acetonitrile containing 0.1% trifluoracetic acid (TFA). The injection volume was 100 µL and the samples were eluted in 30% acetonitrile buffer prepared with 0.1% TFA at a flow rate of 0.5 mL/min, monitored at 214 nm. The column was calibrated with vitamin B12 (1355 Da) [A], oxidized glutathione (660 Da) [B], reduced glutathione (310 Da) [C], triglycine (189 Da) [D] and glycine (75 Da) [E] as the standards. Peptides present in the sample were then estimated from the plot between the log of molecular weight and elution volume as compared to the standards.

4.2.5 Cell culture

The human osteoblast cell line U-2OS (ATCC HTB-96) was purchased from ATCC (Manassas, VA, U.S.A). Cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS and penicillin-streptomycin in an incubator under 95% air and 5% CO2. Cells were then sub-cultured using 0.25% trypsin every 2-3 days until 80-90% confluence. To examine the activity of pea protein hydrolysates, the cells were incubated with the different hydrolysates at a concentration of 1000 µg/mL for 24 h before any experimental analysis.

4.2.6 Cell viability using XTT

Cell viability was determined using the XTT assay kit as per the manufacturer's instruction protocol. The cells were grown in 96-well culture plate and were treated with 1000 ug/mL of hydrolysates prepared with chymotrypsin, alcalase, and thermolysin for 24 h. After treatment, the culture media was removed, and the cells were washed with PBS. The XTT reagent component A

and B in the 50:1 ratio was then added to the well containing treated cells for 1 h at 37°C. After 1 h of incubation, the plate was then read at Excitation wavelength 560 nm and emission wavelength 590 nm. The cell viability in terms of fold change from the control was then calculated.

4.2.7 Western blot

The cells were seeded in 6-well culture plates in DMEM containing 10% FBS and 1% penicillin-streptomycin. After ~90% confluence, the cells were treated with the prepared hydrolysates at 1000 µg/ml for 24 h. After then, the culture media was removed, the cells were lysed in boiling Laemmle's buffer containing 50 µM DTT and 0.2% Triton-X-100. The boiled sample was then run in SDS-PAGE and then transferred to a nitro-cellulosic membrane, blocked with antibodies against COL1A2, STAT 3, CXCR 4, NRF2 and the protein loading control GAPDH. The protein bands were then probed with specific secondary antibodies, and finally detected using Licor Odyssey Bio Imager (Lincoln, NE, U.S.A), and quantified using densitometry. Each band was normalized to its corresponding band of loading control. The results were expressed as fold change corresponding to its untreated sample.

4.2.8 Procollagen type I C-peptide assay

Procollagens are known as the precursor molecules of collagen type I, II, III, IV, and V. The procollagen type I C-peptide (PIP) EIA kit was used for quantifying the procollagen levels in the cell culture extracts using in ELISA. The procollagen levels were determined using Takara's PIP assay kit as per the manufacturer's instruction protocol. The cells were grown in a 6-well plate and were treated with hydrolysates at 1000 µg/mL for 24 h. After treatment, the cell lysates were collected. The antibody cocktail solution was added into the sample and standards wells and incubated for 3 h at 37°C. After incubation, the antibody solution was removed, the plate was then washed three times with 1X PBS buffer. Following the binding of the antibody, the reaction was initiated between peroxidase and TMB substrate resulting in color development with color intensities indicating the amount of PIP present in the samples and standards. The proportional amount was measured using a spectrophotometer. The number of procollagen levels in the samples were calculated using the standards obtained by plotting a standard curve and were further expressed as fold change to the control.

4.2.9 Matrix metallopeptidases (MMPs) assay

Matrix metalloproteinase 1 (MMP-1) and matrix metalloproteinase 9 (MMP-9) levels in the samples were determined using abcam's Human MMP-1 and MMP-9 ELISA kit as per the manufacturer's instruction manuals. The cells were seeded in a 6-well plate and incubated until 90% confluence, treated with pea protein hydrolysates at a concentration of 1000 µg/mL for 24 h, and the protein was extracted after 24 h using radioimmunoprecipitation assay buffer (RIPA). The cells were collected using a cell scrapper and resuspended in RIPA buffer.

The samples and standards were added to the precoated wells followed by respective antibody cocktail solutions and then incubated for 3 h at 37°C. The cells were then washed with 1X PBS to remove any unbound material. The TMB (3,3,5,5- tetramethylbenzidine) development solution was then added which was catalyzed by HRP (Horseradish peroxidase) generating blue coloration, and the color intensity was measured at 450 nm. The MMP-1 and MMP-9 levels in the samples were expressed as the fold change to that of untreated samples (control).

4.2.10 Quantitative RT-PCR

Human osteoblast cells were cultured in 6-well plates and were treated after 80-90% confluence with hydrolysates at a concentration of 1000 µg/mL. The total RNA was extracted using TriZol reagent (Ambion, Carlsbad, CA, U.S.A). The RNA was precipitated using chloroform, followed by isopropanol. The obtained RNA pellets were washed with 75% ethanol and resuspended in 20 µl of nuclease free water. The extracted mRNA was then quantified using nanodrop (Agilent technologies, Santa Clara, CA) to determine the concentration of mRNA in ng/µL. The mRNA was then reversely transcribed to cDNA using Superscript II reversely transcriptase (Invitrogen). The qRT-PCR was performed using SteponePlus Real Time PCR systems (Applied Biosystems, Foster City, CA, U.S.A). The real-time PCR was performed using the Fast SYBR Green PCR Master Mix (Applied Biosystem, Burlington, ON, Canada). The conditions used are 95°C for 20 sec, 40 cycles at 95°C for 1 sec, followed by annealing/extension for 20 sec at 60°C. The fold change of CXCR4 and STAT 3 was expressed by using ß-actin as the reference gene based on the cycle of threshold (CT).

4.2.11 Statistical Analysis

All data presented in this study as the mean ±SEM (standard error of the mean) of three to six independent experiments. One-way analysis of variation (ANOVA) was used in analyzing data

with Dunnette's posthoc test for comparison to the control using PRISM 6 statistical software (GraphPad Software, San Diego, CA) with $P<0.05$ as statistically significant.

4.3 Results

4.3.1 Hydrolysate preparation

Pea protein hydrolysates were prepared using the conditions listed in Table 1. The degree of hydrolysis ranged between 15-20%. The size-exclusion chromatogram of hydrolysates differed as per the enzyme used for hydrolysis (Figure 4.1); all hydrolysates showed peptides ranged from 70 Da to 1500 Da, with mostly under 600 Da. Based on the eluting peak of hydrolysate in the chromatograms, the sample prepared with alcalase showed more low molecular weight peptides as compared to others. Pea protein hydrolysates presented no cytotoxicity when compared to control, suggesting that the hydrolysates do not possess any cellular toxicity at the concentration of 1 mg/mL (Figure 4.3). Thus, further experiments were performed at a concentration of 1mg/mL.

4.3.2 Pea protein hydrolysates stimulates collagen levels

Type 1 collagen is a biomarker of osteogenesis and the increase in the secretion of COL1A2 reflects a transition of pre-osteoblasts to mature osteoblasts. As shown in Figure 4.2, cells treated with hydrolysates prepared by chymotrypsin, alcalase, and thermolysin increased COL1A2 level by 1.8-fold, 1.6-fold and 1.3-fold with $P<0.05$ respectively, compared with the control. Therefore, these three hydrolysates were chosen for further study (Figure 4.2).

Procollagens are known as the precursor molecules of synthesized collagen types I, II, III, IV, and V. The number of procollagens is directly related to the synthesis of the collagen molecules in the extracellular matrix, leading to bone matrix formation and regulating bone development. The procollagen levels were increased as 1.3-fold, 1.1-fold and 1.1-fold with $P<0.05$ for alcalase, chymotrypsin, and thermolysin hydrolysates, respectively (Figure 4.4 B). The trend of procollagen levels corroborated with the western blot expressions of COL1 A2 (Figure 4.4 A).

4.3.3 Pea protein hydrolysates increase intracellular NRF2 level

The nuclear factor erythroid 2- related factor 2 (NRF2) is the marker of antioxidant responses of genes regulating pathological pathways of oxidant exposure. The level of NRF2 increased after treatment with pea protein hydrolysate indicating a possible antioxidant activity. Our results indicated 1.4-fold, 1.2-fold with $P<0.05$ increase in pea protein hydrolysates prepared by alcalase

and chymotrypsin, respectively, compared with the control (Figure 4.5). Thus, increased NRF2 expression relates to the antioxidative response of pea protein hydrolysates.

4.3.4 Pea protein hydrolysates increase Intracellular CXCR4 level

C-X-C chemokine receptor type 4 (CXCR4) is the cytokine receptor which play an important role in regulating bone remodeling mechanisms [23]. The 2-fold ($P<0.05$) increase in the CXCR4 protein level was observed in the cells treated with alcalase hydrolysate. Similar 1.6 and 1.4-fold ($P<0.05$) increase was also witnessed in the cells treated with chymotrypsin and thermolysin hydrolysates, respectively (Figure 4.6). mRNA expression of CXCR4 was significantly increased in alcalase and thermolysin, but not chymotrypsin hydrolysate treated samples (Figure 4.8). Therefore, increased CXCR4 level suggests a regulatory role of pea protein hydrolysates towards the osteogenic activity.

4.3.5 Pea protein hydrolysates augment Intracellular STAT3 level

Signal transducers and activators of transcription 3 (STAT3) is a transcription factor secreted by osteoblasts, which maintains bone homeostasis [46]. Activation of STAT3 results in the regulation of bone formation leading to increased bone mass. Upon treatment with pea protein hydrolysate, the protein level of STAT3 was increased by 1.5-fold ($P<0.05$) and 1.45-fold ($P<0.05$), in cells treated with alcalase and chymotrypsin, hydrolysates, respectively, compared with the control (Figure 4.7). The mRNA expression of STAT3 was also upregulated in alcalase and thermolysin hydrolysates (Figure 4.9). An increased level of STAT3 suggests a possible increased bone mass, which may result in amplified bone formation.

4.3.6 Pea protein hydrolysates decrease MMP-1 and MMP-9 levels

Matrix metalloproteinases are the endopeptidase that is responsible for degrading extracellular matrix including collagen via physiological pathways leading to bone resorptive activity. The degradation of extracellular components of the bone matrix acts as the down-regulator of bone health. MMP-9 was reduced by 28% ($p<0.05$) (Figure 4.10) in alcalase and thermolysin prepared hydrolysates while MMP-1 was reduced by ~20% ($p<0.05$) (Figure 4.11) in cells treated with chymotrypsin hydrolysate, compared with the control.

4.4 Discussion

The influence of dietary proteins in maintaining bone structures have spurred the pharmacological methodology in preventing or treating osteoporosis. Protein supplementation increases muscle

strength, which retraces over the individual's life span, making bone health an important skeletal issue. The consumption of dietary proteins exerts direct relation with bone health conforming to the overall skeletal mobilization. Therefore, protein hydrolysates aim at nutritional aspects in providing adequate rich levels of protein leading to positive health effects such as promoting muscle mass and bone mineral density [16].

In this study, we showed that pea protein hydrolysates prepared using different enzymes stimulated osteogenic markers for bone matrix formation (COL1A2 and procollagen) and remodeling (CXCR4, STAT3, NRF2, MMP-9 and MMP-1) in human osteoblast cells. Enzymatic hydrolysis of food proteins in the process of making protein hydrolysates releases smaller peptides which delivers different physiological health benefits. Therefore, protein hydrolysates have emerged as a practical food strategy to improve human wellbeing in conjunction with the health effects [41]. We presented a positive connotation of pea protein hydrolysates with the phenotypic markers regulating bone health. Thus, it is reasonable to observe the possible anabolic effects of pea protein hydrolysates in bone formation.

The extent of enzymatic degradation of food proteins in producing hydrolysates is often reflected by its physiochemical properties [43]. The proteolytic effect of enzymes modulated the molecular size and structure of pea proteins; with degree of hydrolysis ranging between 15-20% (Table 4.1) for all the prepared hydrolysates. Such physiochemical properties of the hydrolysates indicate the affinity of pea protein hydrolysates in representing low molecular weight protein fragments; which was validated using size-exclusion chromatogram (Figure 4.1). The results from this study suggested dominant peaks of smaller peptide fractions from 70 to 1500 Da, showing the broad specificity of enzymes during protein hydrolysis. The low molecular weight peptides stand a virtuous fate in getting absorbed into the gastrointestinal system and facilitating the easy action in delivering its physiological health benefits [44].

Essentially, the physiochemical and transcriptional responses are both required in analyzing any induced monitoring effects on bone health [20]. The prognosis of age-related osteoporosis is the resulted outcome of reduced bone mineral density and impaired bone cell activity [21]. Therefore, we established new convincing evidences that pea protein hydrolysates promote bone health by supplementing quality source of proteins. To validate the clinical

applicability of the pea protein hydrolysates in treating osteoporosis; we used the human osteoblast cell model, eliminating any interspecies differences.

The cells treated with pea protein hydrolysates showed no presence of toxicity caused from free amino acids and peptide sequences after proteolysis. Therefore, the use of pea protein hydrolysates suggests no cytotoxicity.

The known scientific evidences of collagen as an important organic component of bone micro-architecture and metabolism have established the definite actualities for wholesome bone wellbeing. Whereas, Type 1 collagen (COL1A2) and procollagen are the abundant forms found in bone matrix and are known as the sensitive markers to determine bone strength in medical application. On treatment with pea protein hydrolysate, expression of COL1A2 and procollagen levels increased, suggesting enhanced effects on new bone matrix formation.

The osteoblast cells also express transcription factor, NRF2 (Nuclear factor erythroid 2-related factor) which is considered as the main regulator for cytoprotective genes along with oxidative effects [40]. NRF2 mediates bone formation by regulating remodeling of bone. It is also regarded as a master regulator for protective cellular effects in conditions of inflammation and tissue damage by regulating responses at the antioxidant response elements (ARE) sites [45]. Apart, from its antioxidant activity, NRF2 also controls the pathophysiology of osteoporotic conditions by regulating antioxidant endogenous and bone accrual responses [40]. In this work, pea protein hydrolysate presented increased NRF2 expressions indicating their antioxidant and bone health-promoting effects.

The regulating pathway of CXCR4 is governed by the G-protein activation mechanism [23]. The binding of stromal cell-derived factor (SDF-1/CXCL 12, ligand) to C-X-C Chemokine 4 (CXCR4, receptor) activates cellular effector molecule resulting in the regulation of different signaling pathways stimulating bone cell activity [23]. The CXCR4 is also involved at the site of bone remodeling via proliferation and differentiation of osteoblasts, and regulation of blood calcium homeostasis [23]. It also adjusts bone matrix composition by increasing the movement of cells at the site of injury in case of disease or inflammation [24]. Likewise, Signal-transducer and activator of transcription (STAT3) plays an important role in bone development and homeostasis, mediated through the signaling of cytokines (CXCR4/SDF-1) [23].This results in the activation and phosphorylation of tyrosine residue of STAT3, which further dimerizes and enter nucleus to

transcribe genes resulting in cell proliferation and differentiation [46]. The anabolic increased expressions of CXCR4 and STAT3 were observed in three pea protein hydrolysates (chymotrypsin, alcalase, and thermolysin) implying to its positive effects. The anabolic effects of pea protein hydrolysates on human osteoblast cells are closely associated with an analogous increase in CXCR4 mRNA and protein expressions, parallelly coupled with the augmented expression of transcription factor STAT3 (Figure 4.7) Likewise, the regulatory signaling of CXCR4 and STAT3 have wider bone- health-promoting roles evidenced with mRNA and protein levels.

Furthermore, CXCR4 also directly interacts with the cells to regulate bone remodeling, which thereby encourages the signaling events in activating the promoters of type 1 collagen (COL1A2) and procollagen [47]. It has also been evidenced in literature, that CXCR4 is regarded as the contributing factor to collagen deposition leading to enhanced deposition of extra cellular matrix [48].

Matrix metalloproteinases (MMPs) are regarded as proteases that cleave the extracellular matrix. They are also associated with the inhibitors of osteoblast activity. The dynamic degradation of the fibrillar collagen is inversely related to the low bone mineral density, thus it is considered as a biochemical bone resorption marker [49]. Therefore, a decrease in the levels of MMP-9 suggested inhibitory role of pea protein hydrolysates on bone resorption. Notably, the present study suggest that pea protein hydrolysate may have some bioactive peptide sequences that can mechanistically regulate bone remodeling process by augmenting the expressions of STAT3 and CXCR4 mediated through the JAK-STAT pathway. Moreover, MMP also inactivates the regulation of STAT 3 and CXCR4 [25]. However, with the treatment of pea protein hydrolysates, a decrease in MMP-1 and MMP-9 was observed (Figures 10 and 11). In this work, pea protein hydrolysates were shown to regulate the expression of extra cellular matrix formation (COL1 A2 and procollagen) and bone remodeling markers (MMP-9, CXCR4, STAT3 and NRF2) expressions which mediates bone formation.

Hence, the new approach focusing on pea protein hydrolysates and bone phenotypic markers on bone formation have been substantiated. As well, pea protein hydrolysate reveals inhibition of bone resorption markers). Therefore, pea protein hydrolysate after the appropriate animal and clinical trials can be a potential natural treatment alternative for osteoporosis.

4.5 Conclusion

In this work, we established positive and compelling evidences showing the ability of pea protein hydrolyzed samples to regulate phenotypic markers of bone health. The activities of these hydrolysates prepared using various enzymes increased osteoblast proliferation and differentiation, possibly due to the action of constituent bioactive peptides [20]. The prepared pea protein hydrolysates increased COL1 A2, procollagen, NRF2 expressions, suggesting overall improvement in bone physiological function. The effect of pea protein hydrolysate on osteoblastogenesis was further elucidated at the molecular level by increased STAT3 and CXCR4 mRNA expressions. However, future *in vivo* studies followed by clinical trials can confirm the safety and efficacy of pea protein hydrolysates towards the prevention or possible mitigation of osteoporosis.

4.6 References

[1] Sözen, T., Özışık, L. and Başaran, N.Ç., 2017. An overview and management of osteoporosis. European Journal of Rheumatology, 4(1), p.46.

[2] Chen, J.S. and Sambrook, P.N., 2012. Antiresorptive therapies for osteoporosis: a clinical overview. Nature Reviews Endocrinology, 8(2), p.81.

[3] An, J., Yang, H., Zhang, Q., Liu, C., Zhao, J., Zhang, L. and Chen, B., 2016. Natural products for treatment of osteoporosis: The effects and mechanisms on promoting osteoblast-mediated bone formation. Life Sciences, 147, pp.46-58.

[4] Bonjour, J.P., 2011. Protein intake and bone health. International Journal for Vitamin and Nutrition Research, 81(2), p.134.

[5] Sahni, S., Mangano, K.M., McLean, R.R., Hannan, M.T. and Kiel, D.P., 2015. Dietary approaches for bone health: lessons from the Framingham Osteoporosis Study. Current Osteoporosis Reports, 13(4), pp.245-255.

[6] Sunyecz, J.A., 2008. The use of calcium and vitamin D in the management of osteoporosis. Therapeutics and Clinical Risk Management, 4(4), p.827.

[7] Manninen, A.H., 2009. Protein hydrolysates in sports nutrition. Nutrition & Metabolism, 6(1), p.38.

[8] Koopman, R., Crombach, N., Gijsen, A.P., Walrand, S., Fauquant, J., Kies, A.K., Lemosquet, S., Saris, W.H., Boirie, Y. and van Loon, L.J., 2009. Ingestion of a protein hydrolysate is accompanied by an accelerated *in vivo* digestion and absorption rate when compared with its intact protein. The American Journal of Clinical Nutrition, 90(1), pp.106-115.

[9] Ahn, C.B. and Je, J.Y., 2019. Bone health promoting bioactive peptides. Journal of Food Biochemistry, 43(1), p.e12529.

[10] Chakrabarti, S., Guha, S. and Majumder, K., 2018. Food-derived bioactive peptides in human health: Challenges and opportunities. Nutrients, 10(11), p.1738.

[11] Mohanty, D.P., Mohapatra, S., Misra, S. and Sahu, P.S., 2016. Milk derived bioactive peptides and their impact on human health–A review. Saudi Journal of Biological Sciences, 23(5), pp.577-583.

[12] Inan, S., 2019. The Potential Role of Nutraceuticals in Inflammation and Oxidative Stress. In Nutraceuticals-Past, Present and Future. IntechOpen.

[13] https://www.accessdata.fda.gov/scripts/InteractiveNutritionFactsLabel/protein.html, August 22, 2019.
[14] Holeček, M., 2018. Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. Nutrition & Metabolism, 15(1), p.33.
[15] Schultz, N.D., Giannini, A.V., Chang, T.T. and Wong, D.C., 1994. Food allergy. In the Best Guide to Allergy (pp. 111-135). Humana Press, Totowa, NJ.
[16] Hoffman, J.R. and Falvo, M.J., 2004. Protein–which is best? Journal of Sports Science & Medicine, 3(3), p.118.
[17] Cintineo, H.P., Arent, M.A., Antonio, J. and Arent, S.M., 2018. Effects of Protein Supplementation on Performance and Recovery in Resistance and Endurance Training. Frontiers in Nutrition, 5.
[18] Sunyecz, J.A., 2008. The use of calcium and vitamin D in the management of osteoporosis. Therapeutics and Clinical Risk Management, 4(4), p.827.
[19] Ridiandries, A., Tan, J. and Bursill, C., 2018. The role of chemokines in wound healing. International Journal of Molecular Sciences, 19(10), p.3217.
[20] Bhat, Z.F., Kumar, S. and Bhat, H.F., 2015. Bioactive peptides of animal origin: a review. Journal of Food Science and Technology, 52(9), pp.5377-5392.
[21] Center, J. and Eisman, J., 1997. The epidemiology and pathogenesis of osteoporosis. Baillière's clinical Endocrinology and Metabolism, 11(1), pp.23-62.
[22] Kukreja, P., Abdel-Mageed, A.B., Mondal, D., Liu, K. and Agrawal, K.C., 2005. Up-regulation of CXCR4 expression in PC-3 cells by stromal-derived factor-1α (CXCL12) increases endothelial adhesion and trans endothelial migration: role of MEK/ERK signaling pathway–dependent NF-κB activation. Cancer Research, 65(21), pp.9891-9898.
[23] Tang, C.H., Chuang, J.Y., Fong, Y.C., Maa, M.C., Way, T.D. and Hung, C.H., 2008. Bone-derived SDF-1 stimulates IL-6 release via CXCR4, ERK and NF-κB pathways and promotes osteoclastogenesis in human oral cancer cells. Carcinogenesis, 29(8), pp.1483-1492.
[24] Van Der Vorst, E.P., Peters, L.J., Müller, M., Gencer, S., Weber, C. and Döring, Y., 2019. G-Protein coupled receptor targeting on myeloid cells in atherosclerosis. Frontiers in Pharmacology, 10, p.531.

[25] Chetram, M.A. and Hinton, C.V., 2013. ROS-mediated regulation of CXCR4 in cancer. Frontiers in Biology, 8(3), pp.273-278.
[26] Sahni, S., Mangano, K.M., McLean, R.R., Hannan, M.T. and Kiel, D.P., 2015. Dietary approaches for bone health: lessons from the Framingham Osteoporosis Study. Current Osteoporosis Reports, 13(4), pp.245-255.
[27] Noma, T., Takasugi, S., Shioyama, M., Yamaji, T., Itou, H., Suzuki, Y., Sakuraba, K. and Sawaki, K., 2017. Effects of dietary gelatin hydrolysates on bone mineral density in magnesium-deficient rats. BMC musculoskeletal disorders, 18(1), p.385.
[28] Behera, P., Kumar, R., Sandeep, I.V.R., Kapila, R., Dang, A.K. and Kapila, S., 2013. Casein hydrolysates enhance osteoblast proliferation and differentiation in mouse bone marrow culture. Food Bioscience, 2, pp.24-30.
[29] Moskowitz, R.W., 2000, October. Role of collagen hydrolysate in bone and joint disease. In Seminars in Arthritis and Rheumatism (Vol. 30, No. 2, pp. 87-99). WB Saunders.
[30] Ono-Ohmachi, A., Ishida, Y., Morita, Y., Kato, K. and Nara, T.Y., 2017. Milk Basic Protein Facilitates Increased Bone Mass in Growing Mice. Journal of Nutritional Science and Vitaminology, 63(5), pp.315-322.
[31] Jabłońska-Trypuć, A., Matejczyk, M. and Rosochacki, S., 2016. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. Journal of Enzyme Inhibition and Medicinal Chemistry, 31(sup1), pp.177-183.
[32] Hopkins, R.B., Burke, N., Von Keyserlingk, C., Leslie, W.D., Morin, S.N., Adachi, J.D., Papaioannou, A., Bessette, L., Brown, J.P., Pericleous, L. and Tarride, J., 2016. The current economic burden of illness of osteoporosis in Canada. Osteoporosis International, 27(10), pp.3023-3032.
[33] Massey, L.K., 2003. Dietary animal and plant protein and human bone health: a whole foods approach. The Journal of Nutrition, 133(3), pp.862S-865S.
[34] Thorpe, M., Mojtahedi, M.C., Chapman-Novakofski, K., McAuley, E. and Evans, E.M., 2008. A positive association of lumbar spine bone mineral density with dietary protein is suppressed by a negative association with protein sulfur. The Journal of Nutrition, 138(1), pp.80-85.

[35] Thorpe, D.L., Knutsen, S.F., Beeson, W.L., Rajaram, S. and Fraser, G.E., 2008. Effects of meat consumption and vegetarian diet on risk of wrist fracture over 25 years in a cohort of peri- and postmenopausal women. Public Health Nutrition, 11(6), pp.564-572.
[36] Heaney, R.P. and Layman, D.K., 2008. Amount and type of protein influences bone health. The American Journal of Clinical Nutrition, 87(5), pp.1567S-1570S.
[37] Study of Osteoporotic Fractures Research Group Sellmeyer Deborah E dsellmeyer@ psg. ucsf. edu Stone Katie L Sebastian Anthony Cummings Steven R, 2001. A high ratio of dietary animal to vegetable protein increases the rate of bone loss and the risk of fracture in postmenopausal women. The American Journal of Clinical Nutrition, 73(1), pp.118-122.
[38] Uenishi, K., Ishida, H., Toba, Y., Aoe, S., Itabashi, A. and Takada, Y., 2007. Milk basic protein increases bone mineral density and improves bone metabolism in healthy young women. Osteoporosis International, 18(3), pp.385-390.
[39] Rutherfurd, S.M., 2010. Methodology for determining degree of hydrolysis of proteins in hydrolysates: a review. Journal of Association of Official Agricultural Chemists International, 93(5), pp.1515-1522.
[40] Ferrándiz, M.L., Nacher-Juan, J. and Alcaraz, M.J., 2018. NRF2 as a therapeutic target for rheumatic diseases. Biochemical Pharmacology, 152, pp.338-346.
[41] Kerstetter, J.E., Kenny, A.M. and Insogna, K.L., 2011. Dietary protein and skeletal health: a review of recent human research. Current Opinion in Lipidology, 22(1), p.16.
[42] Bajwa, S.K., Bajwa, S.J.S. and Singh, A., 2012. Nutritional facts and menopausal symptomatology: The role of nutraceuticals. Journal of Medical Nutrition and Nutraceuticals, 1(1), p.42.
[43] Daliri, E.B.M., Oh, D.H. and Lee, B.H., 2017. Bioactive peptides. Food*s*, 6(5), p.32.
[44] Renukuntla, J., Vadlapudi, A.D., Patel, A., Boddu, S.H. and Mitra, A.K., 2013. Approaches for enhancing oral bioavailability of peptides and proteins. International Journal of Pharmaceutics, 447(1-2), pp.75-93.
[45] Sun, Y.X., Xu, A.H., Yang, Y. and Li, J., 2015. Role of Nrf2 in bone metabolism. Journal of Biomedical Science, 22(1), p.101.
[46] Li, J., 2013. JAK-STAT and bone metabolism. Jak-Stat, 2(3), p.e23930.

[47] Zhu, W., Liang, G., Huang, Z., Doty, S.B. and Boskey, A.L., 2011. Conditional inactivation of the CXCR4 receptor in osteoprecursors reduces postnatal bone formation due to impaired osteoblast development. Journal of Biological Chemistry, 286(30), pp.26794-26805.

[48] Karsdal, M.A., Nielsen, M.J., Sand, J.M., Henriksen, K., Genovese, F., Bay-Jensen, A.C., Smith, V., Adamkewicz, J.I., Christiansen, C. and Leeming, D.J., 2013. Extracellular matrix remodeling: the common denominator in connective tissue diseases possibilities for evaluation and current understanding of the matrix as more than a passive architecture, but a key player in tissue failure. Assay and Drug Development Technologies, 11(2), pp.70-92.

[49] Lv, Y., Liu, H., Ren, J., Li, X. and Guo, S., 2013. The positive effect of soybean protein hydrolysates—calcium complexes on bone mass of rapidly growing rats. Food & Function, 4(8), pp.1245-1251.

[50] Mangano, K.M., Sahni, S. and Kerstetter, J.E., 2014. Dietary protein is beneficial to bone health under conditions of adequate calcium intake: an update on clinical research. Current Opinion in Clinical Nutrition and Metabolic Care, 17(1), p.69

Table 4.1- The degree of hydrolysis of pea protein hydrolysates prepared with various enzyme at different pH and temperature combinations.

Sno.	Enzyme used	pH conditions	Temperature (℃)	Degree of Hydrolysis
1.	Trypsin	8	37	15.5
2.	Chymotrypsin	8	37	18
3.	Protex 6L	8	60	16
4.	Alcalase	8	60	20.5
5.	Pepsin	2	37	18
6.	Thermolysin	8	60	18.5
7.	Pepsin+Thermolysin	2, 8	37, 60	19

Figure 4.1- The size exclusion chromatogram of pea protein hydrolysates prepared with enzymes at different pH and temperature combinations was calibrated and performed using vitamin B12 (1355 Da) [A], oxidized glutathione (660 Da) [B], reduced glutathione (310 Da) [C], triglycine (189 Da) [D] and glycine (75 Da) [E] as standards.

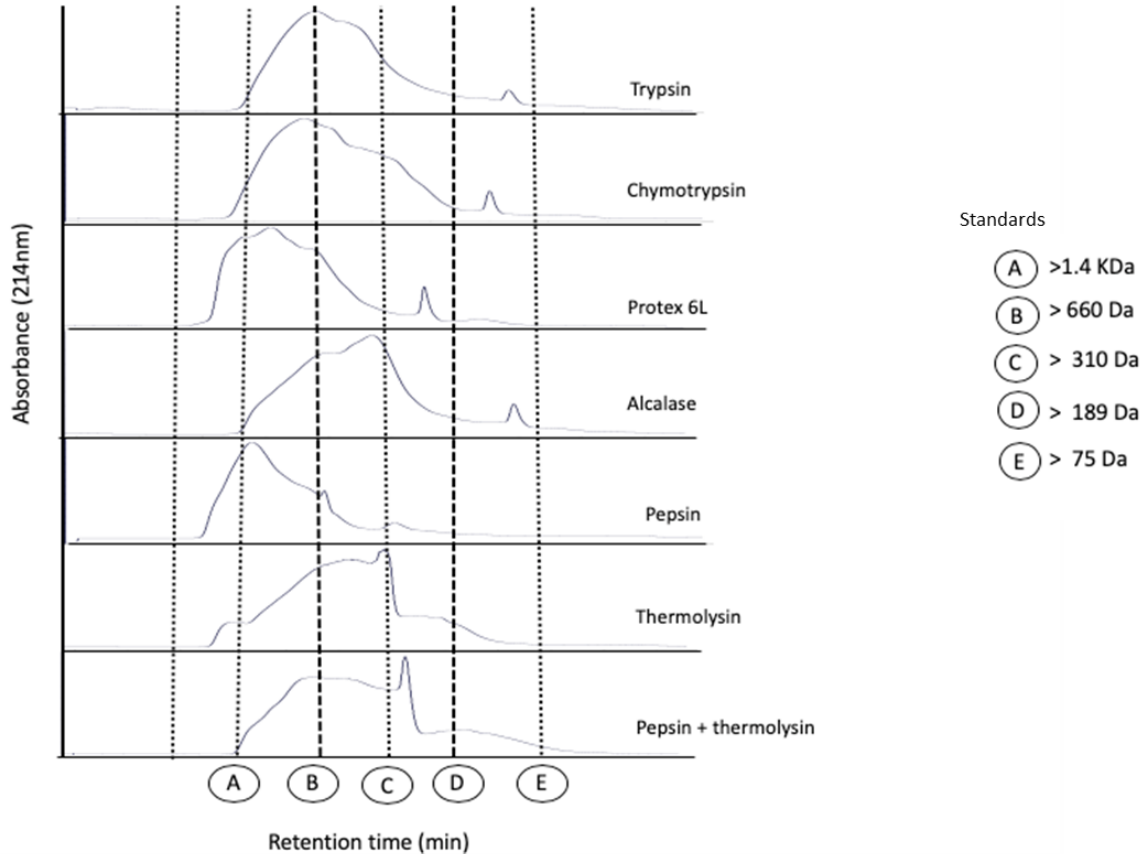
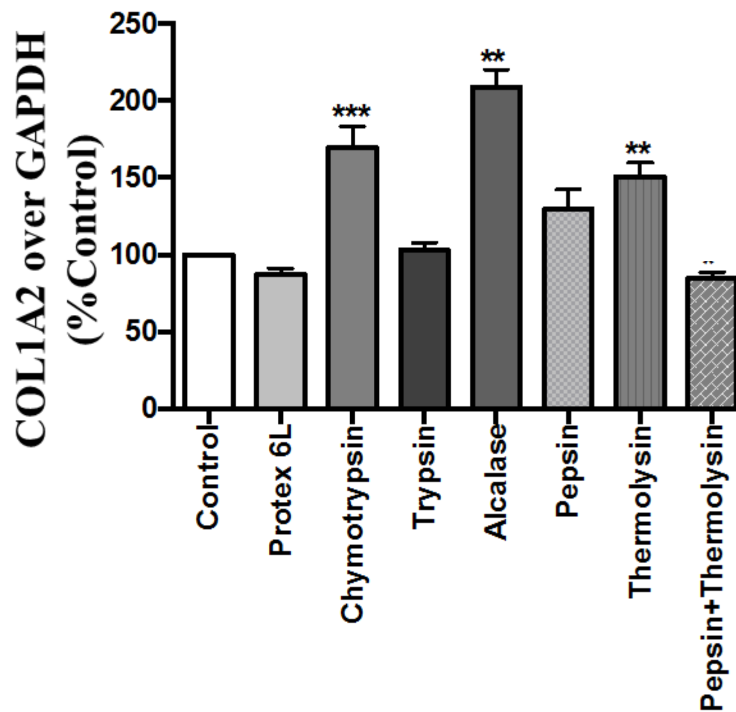


Figure 4.2- The *in vitro* effect of pea protein hydrolysates prepared with Protex 6L[P6L], Chymotrypsin [CT], Alcalase [A] and Thermolysin [Th], Pepsin [P], Pepsin+Thermolysin [P+Th], Trypsin [T] on COL1 A2 expression in human osteoblast cell line U-2OS. The cells were treated with 1000 µg/mL of hydrolysate. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further immunoblotted for STAT 3 and loading control (GAPDH) expressions. An increase in STAT 3 levels was observed. The data presented is mean ± SEM of independent experiments. *, **, and * indicate p <0.05, p<0.01 and p<0.001 respectively as compared to control [C].**



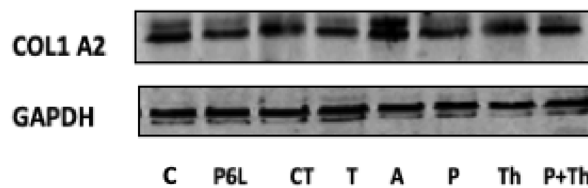


Figure 4.3- The *in vitro* effect of pea protein hydrolysates prepared with chymotrypsin, alcalase and thermolysin on cell viability in human osteoblast cell line U-2OS. The cells were treated with 1000µg/mL of hydrolysate. After 24 h, the cells were lysed, and protein was extracted. The absorbance showed viability, indicating no observed cytotoxicity in U-2OS cells. The data presented is mean ± SEM of 3 independent experiments.

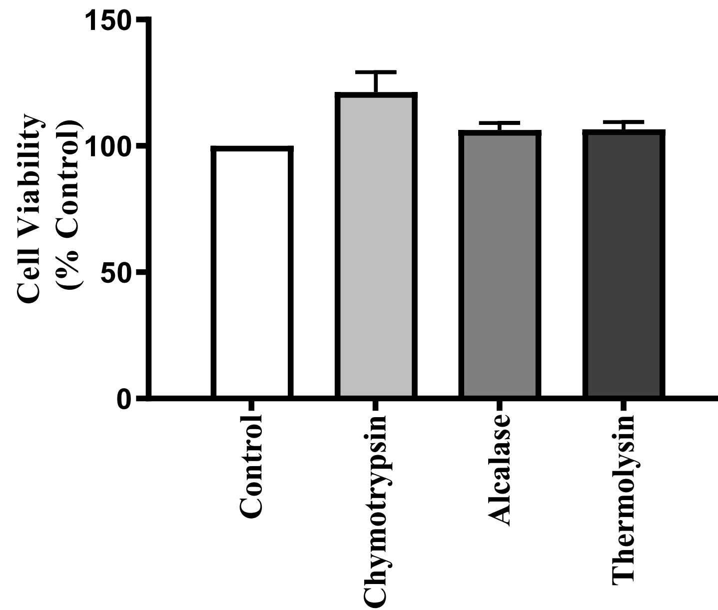
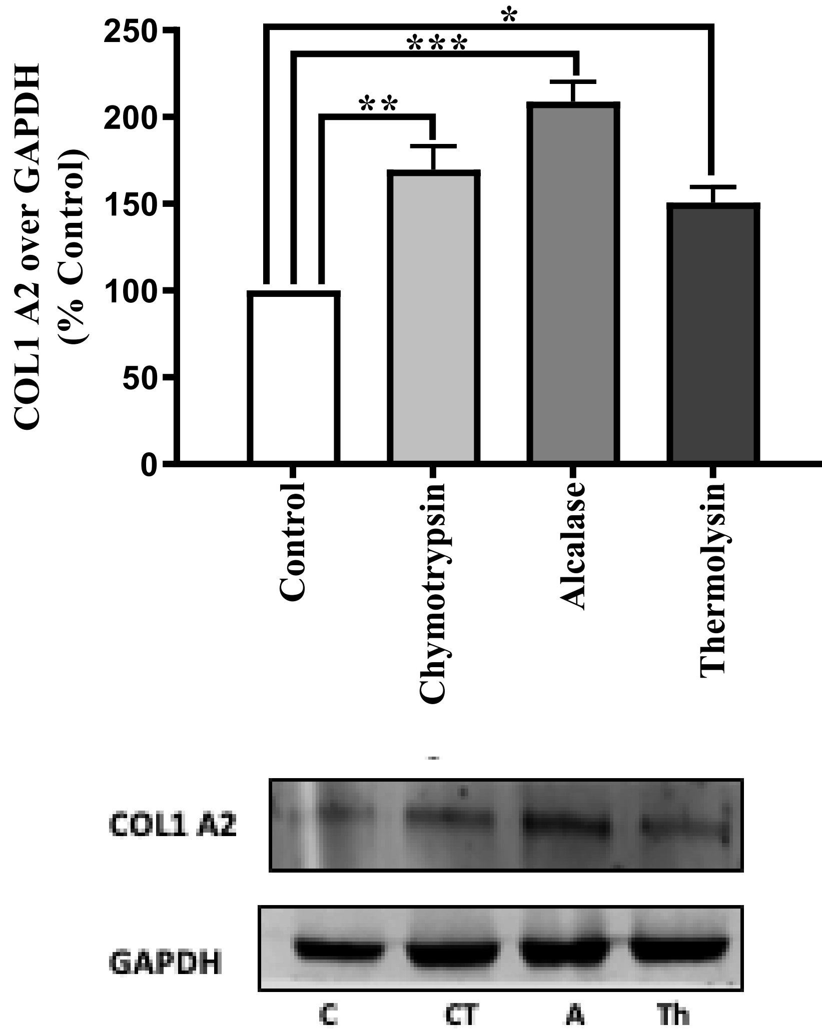


Figure 4.4- The *in vitro* effect of pea protein hydrolysates prepared with chymotrypsin [CT], alcalase [A] and thermolysin [Th] on COL1 A2 expression in human osteoblast cell line U-2OS (A) and collagen levels using collagen kit (B). The cells were treated with 1000µg/mL of hydrolysate. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further immunoblotted for STAT 3 and loading control (GAPDH) expressions. An increase in STAT 3 levels was observed. The data represented is mean ± SEM of 5 independent experiments. *, **, and * indicate p <0.05, p<0.01 and p<0.001 respectively as compared to control [C].**

A



B

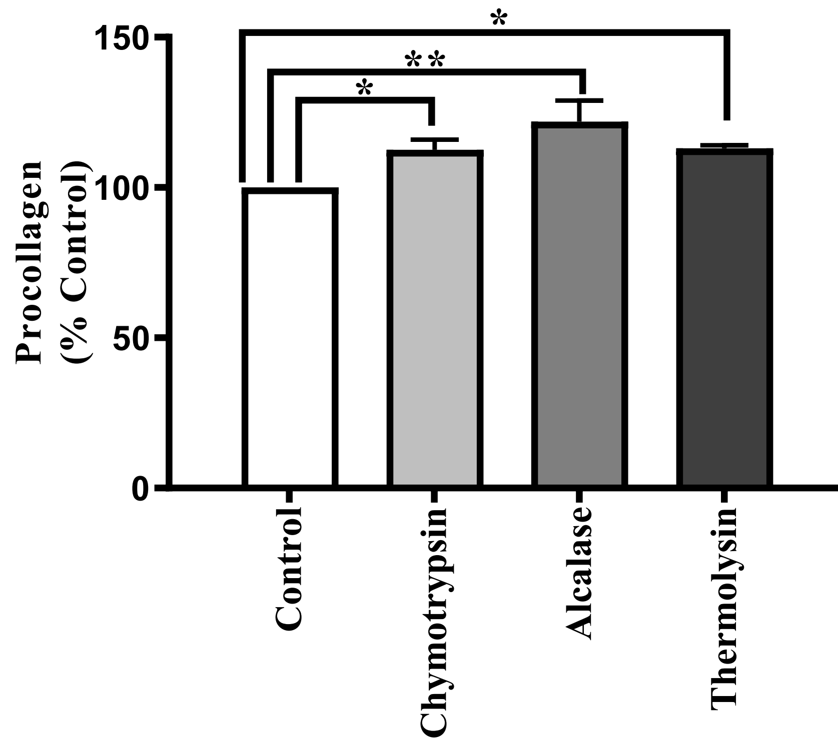
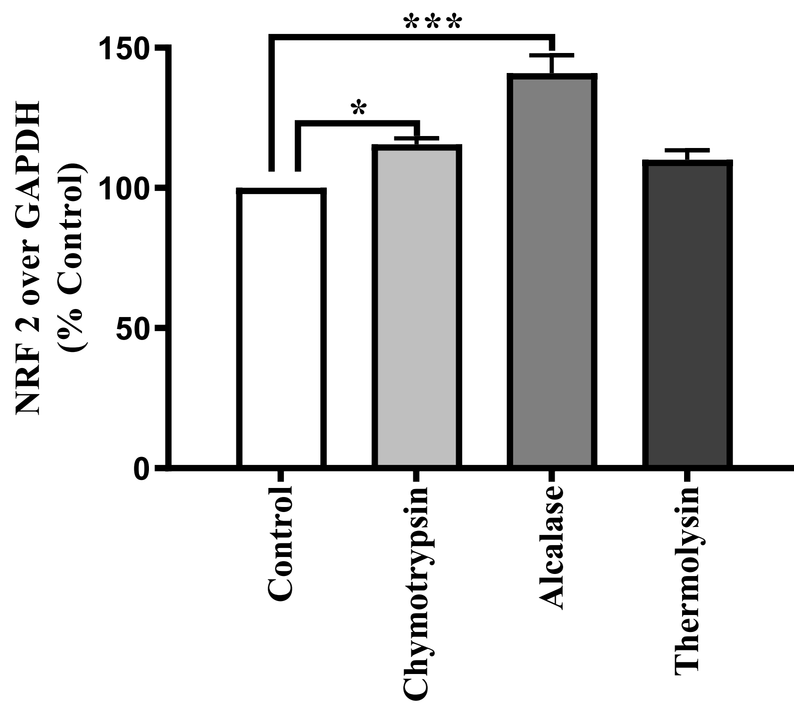


Figure 4.5- The *in vitro* effect of pea protein hydrolysates prepared with chymotrypsin [CT], alcalase [A] and thermolysin [Th] on NRF 2 expression in human osteoblast cell line U-2OS. The cells were treated with 1000µg/mL of hydrolysate. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further immunoblotted for NRF 2 and loading control (GAPDH) expressions. An increase in NRF 2 levels was observed. The data represented is mean ± SEM of 5 independent experiments. *, **, and * indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively as compared to control [C].**



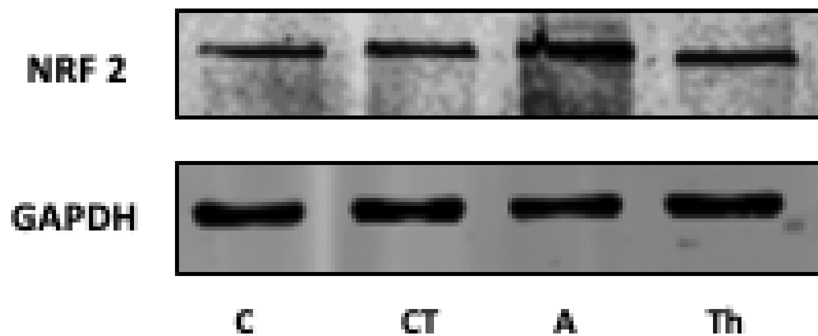
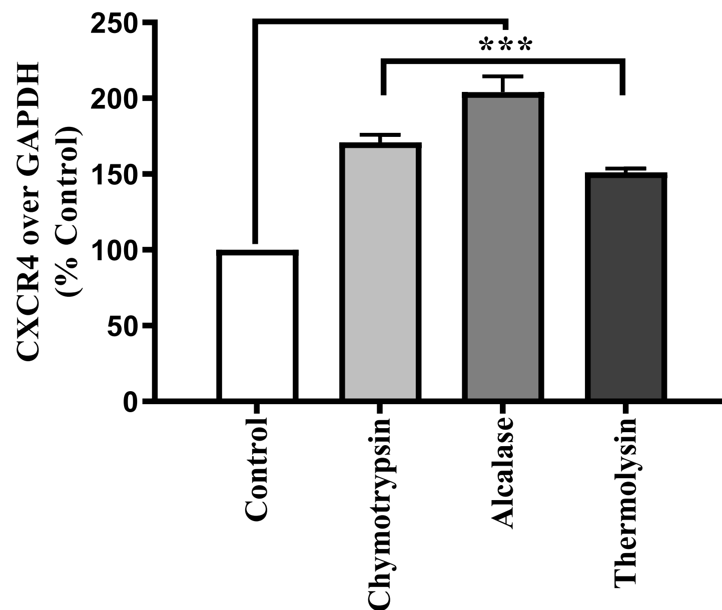


Figure 4.6- The *in vitro* effect of pea protein hydrolysates prepared with chymotrypsin [CT], alcalase [A] and thermolysin [Th] on CXCR4 expression in human osteoblast cell line U-2OS. The cells were treated with 1000µg/mL of hydrolysate. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further immunoblotted for CXCR4 and loading control (GAPDH) expressions. An increase in CXCR4 levels was observed. The data presented is mean ± SEM of 5 independent experiments. *, **, and * indicate p <0.05, p<0.01 and p<0.001 respectively as compared to control [C].**



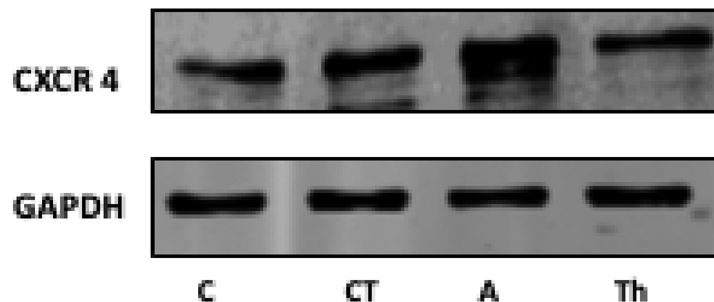
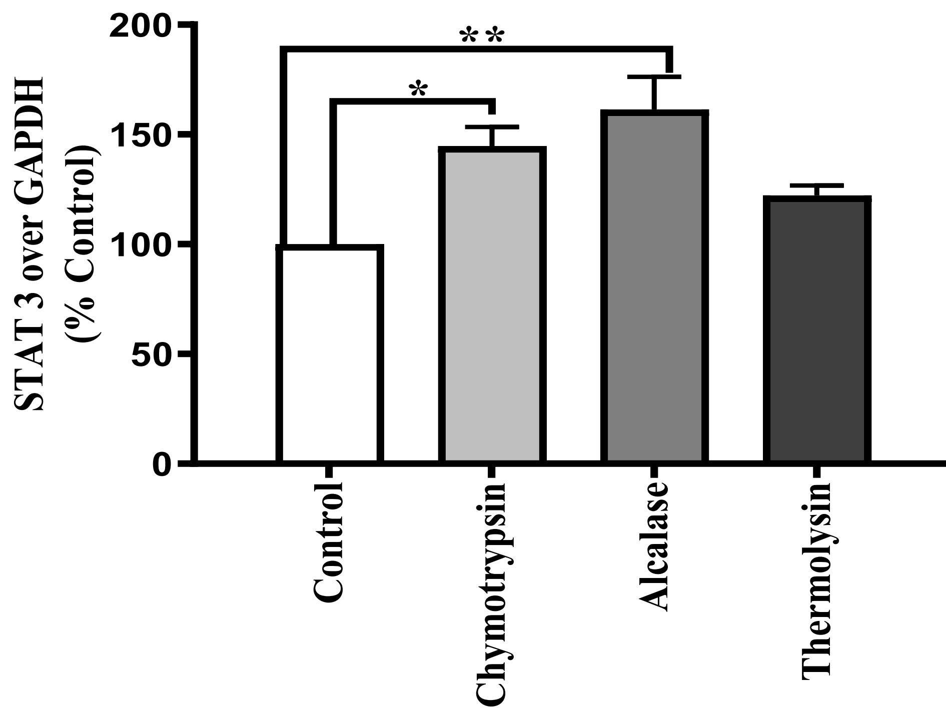


Figure 4.7- The *in vitro* effect of pea protein hydrolysates prepared with chymotrypsin [CT], alcalase [A] and thermolysin [Th] on STAT 3 expression in human osteoblast cell line U-2OS. The cells were treated with 1000µg /mL of hydrolysate. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further immunoblotted for STAT 3 and loading control (GAPDH) expressions. An increase in STAT 3 levels was observed. The data presented is mean ± SEM of 5 independent experiments. *, **, and * indicate p <0.05, p<0.01 and p<0.001 respectively as compared to control [C].**



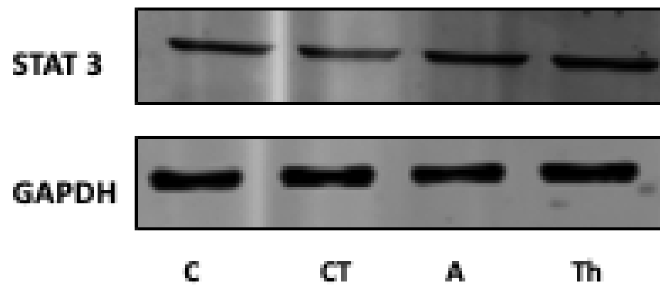


Figure 4.8- The *in vitro* effect of pea protein hydrolysates prepared with chymotrypsin, alcalase and thermolysin on CXCR4 qRT-PCR expression in human osteoblast cell line U-2OS. The cells were treated with 1000µg/mL of hydrolysate. After 24 h, the rna was extracted using Trizol and further precipitated in chloroform and isopropanol. The mRNA was further transcribed to cDNA. The qRT-PCR was performed using SYBR green master mix and data was expressed in terms of fold change. The data presented is mean ± SEM of 3 independent experiments. *, **, and * indicate p <0.05, p<0.01 and p<0.001 respectively as compared to control.**

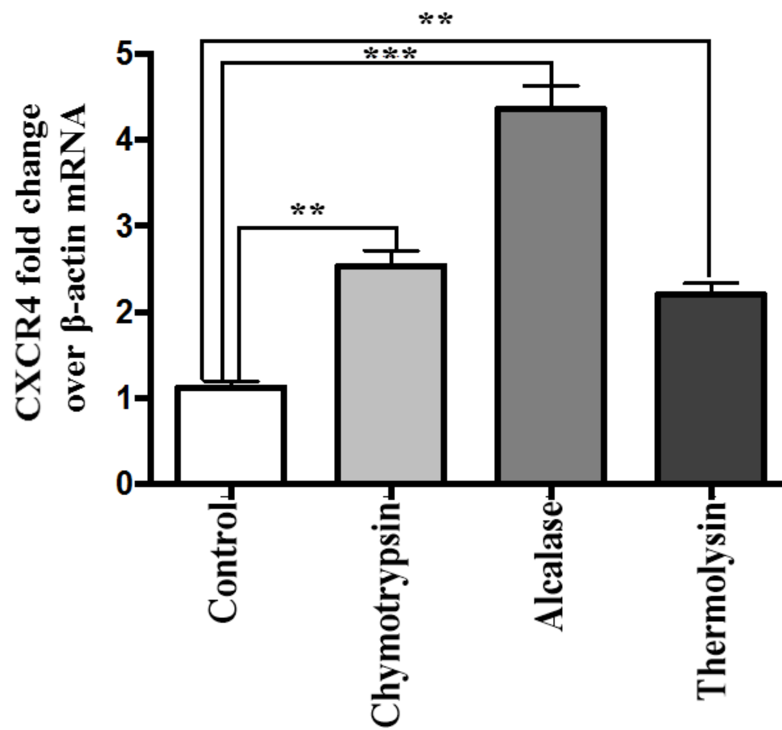


Figure 4.10- The *in vitro* effect of pea protein hydrolysates prepared with chymotrypsin, alcalase and thermolysin on MMP 9 expression in human osteoblast cell line U-2OS. The cells were treated with 1000µg/mL of hydrolysate. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further analyzed for MMP 9 expression. A decrease in MMP 9 levels was observed. The data presented is mean ± SEM of 3 independent experiments. *, **, and * indicate p <0.05, p<0.01 and p<0.001 respectively as compared to control.**

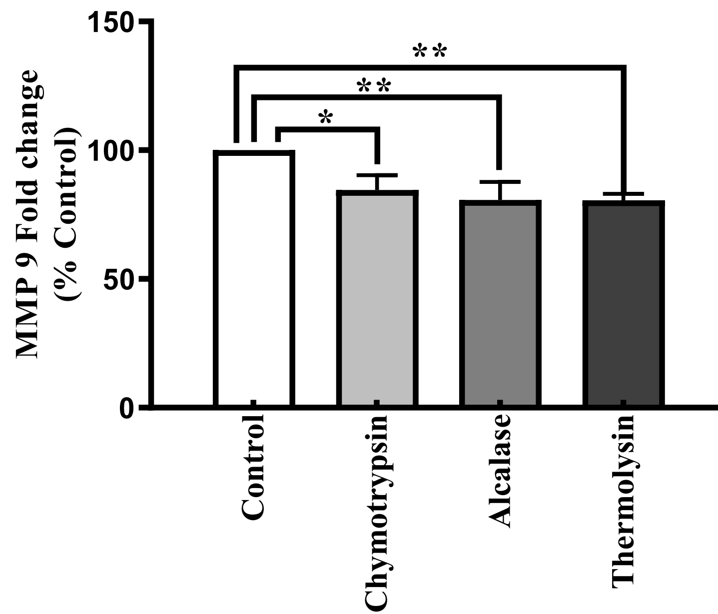


Figure 4.11- The *in vitro* effect of pea protein hydrolysates prepared with chymotrypsin, alcalase and thermolysin on MMP 1 expression in human osteoblast cell line U-2OS. The cells were treated with 1000µg/mL of hydrolysate. After 24 h, the cells were lysed, and protein was extracted. The extracted cell lysate was further analyzed for MMP 1 expression. A decrease in MMP 1 levels was observed. The data presented is mean ± SEM of 3 independent experiments. *, **, and * indicate p <0.05, p<0.01 and p<0.001 respectively as compared to control.**

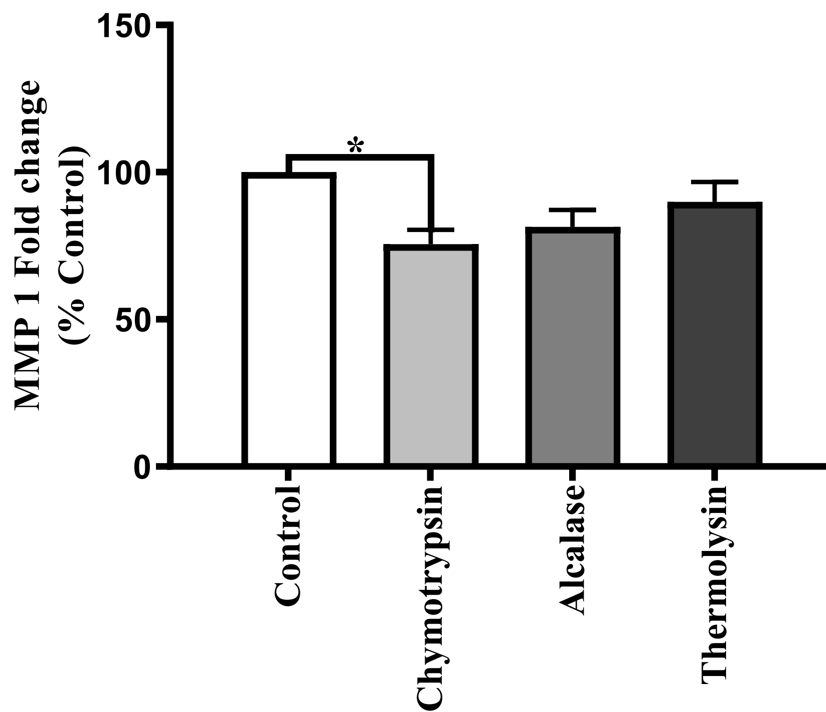
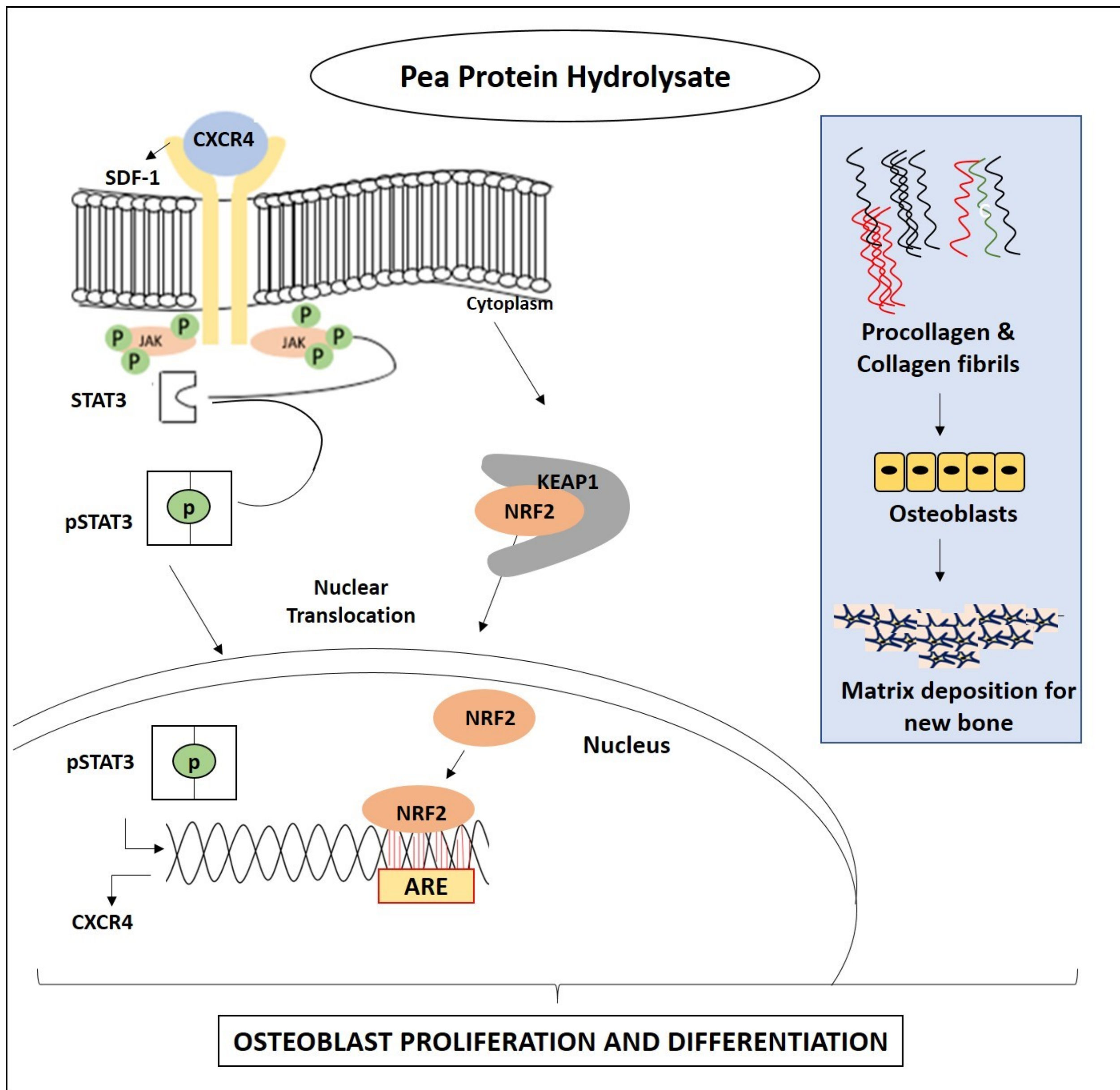


Figure 4.13 The diagrammatic conclusion depicting the effect of pea protein hydrolysate prepared using different enzymes. The constituent bioactive peptides regulate the signalling of various phenotypic markers for osteoblast proliferation and differentiation. The activation of STAT3 by the action of cytokine (CXCR4) promote the activity of osteoblasts via JAK-STAT pathway. Moreover, NRF2 mediates bone formation through nuclear translocation at the ARE sites; thereby regulating bone remodelling. The increased expression of the most abundant component of bone, collagen, helps in depositing bone matrix.



CHAPTER 5- OVERALL SUMMARY AND DISCUSSION

5.1 Key Findings of the Present Research

The overall objectives of the current research study were to understand the regulatory roles of the pea protein derived bioactive peptides on the activity of osteoblasts and to explore its potential applications as a therapeutic treatment alternative for osteoporosis. The key findings of each study are listed below:

5.1.1 The first objective was to investigate the effect of pea protein derived tripeptide LRW on osteoblastic activity on mouse pre-osteoblast MC3T3-E1 cells (Chapter 3)

The diverse spectrum of bioactive peptides derived from either animal or plant source have now gained interest as nutraceuticals and functional foods because they exert beneficial positive effects on body functions [1]. Similarly, pea protein derived bioactive peptide LRW, is recognized as antioxidant, anti-inflammatory and ACE inhibitory peptide [2]. A similar study about IRW peptide derived from egg white protein have reported to stimulate osteoblast proliferation and differentiation [3].

In this study, pea protein derived tripeptide LRW directly stimulated osteoblast proliferation, differentiation and mineralization. On treatment with tripeptide LRW, osteoblasts exerted no toxic cellular response and exhibited increased proliferation. The treatment with different concentrations of LRW also increased the rate of cell migration. The tripeptide LRW also stimulated osteoblast proliferation and differentiation by increased expression of type 1 collagen, ALP and RUNX2. Furthermore, tripeptide LRW additionally promoted mineralization of calcium nodules for bone matrix formation. Moreover, tripeptide LRW also showed promoting effect on osteoblastogenesis by increasing OPG expressions.

Overall, pea protein derived tripeptide LRW reported increased activity of osteoblasts in stimulating bone formation markers.

5.1.1.1 The second objective was to determine the *in vitro* regulatory role of pea protein hydrolysate on osteoblast and osteogenesis (Chapter 4)

Protein hydrolysates are a mixture of oligo, tri and polypeptides which are generated after the enzymatic action on the parent protein [4]. Due to the presence of numerous peptides, hydrolysates exhibit structural functionalities and nutritional properties as compared to native proteins [5].

In the current research work, pea protein hydrolysates prepared with chymotrypsin, alcalase and theromlysin possessed no toxic cellular effect suggesting 100% viability. The

different pea protein hydrolysates up-regulated type 1 collagen and procollagen levels. The pea protein hydrolysates also activated JAK/STAT via upregulating CXCR4 expressions. Furthermore, pea protein hydrolysate also increased CXCR4 and STAT3 expressions at the molecular level. Metalloproteinase (MMPs) levels were also decreased suggesting inhibitory effect on bone resorption.

Largely, pea protein hydrolysate increased expression of bone matrix formation markers (COL1 A2 and procollagen) and inhibited levels of bone resorption markers (MMP-9 and MMP-1). Pea protein hydrolysate increased CXCR4, STAT3 and NRF2 expressions suggesting increased osteoblast proliferation and differentiation.

In conclusion, this thesis demonstrated the potential use of pea protein bioactive compounds as a potential therapeutic treatment alternative for osteoporosis through the management of bone remodeling via regulating osteoblast and osteoclast activity.

5.2 Significance of this research

In the current study, pea protein derived bioactive peptides has been widely studied for its bone health promoting effects. To our best knowledge, this is the first study reporting *in vitro* bone health promoting effects of pea protein bioactive peptides. This thesis has greatly contributed to the knowledge of pea protein bioactive compounds as a regulatory molecule on osteoblast activity paving a new way of value addition to the food industry. Thus, the study exhibits significance in both food industry and bone health management.

5.2.1 Significance to food industry

Food industry has shown emerging interest towards alternatives towards animal protein derived products. Therefore, the acceptability of pea protein derived bioactive peptides stands a strong chance at technological levels, as it contains a complete amino acid profile.

Worldwide, 143 products in food category and 36 products in drink category containing pea protein has been launched between 2011 and 2015 [6]. Global acceptability of pea protein is due to its nutritional importance in terms a complete protein, containing all the essential amino acids [7]. In addition to it, it also contains all branched chain amino acids which effectively increases muscle mass [8].

Therefore, the potential of pea protein in preventing osteoporosis and enhancing bone health will be of great interest to food, nutraceutical and pharmaceutical companies, which will

eventually bring health benefits to the food industry by developing valued added pea protein products.

5.2.2 Significance to Osteoporosis treatment

The number of osteoporosis-attributable fractures are reported to be 131,443 resulting in the estimated economic burden of osteoporosis in Canada to be $4.6 billion [9]. Even some of the pharmaceutical drugs used in the treatment of osteoporosis have reported to increase the risk of ovarian cancer and cardiovascular diseases [10]. Therefore, the transition towards natural alternatives using dietary interventions can provide safe opportunity for treating osteoporosis.

The finding of this research work provides a new approach for the treatment of osteoporosis with fewer side effects. Moreover, the regulatory role of certain effector molecules in the distinct signalling pathway have provided better insight for the development of new therapeutic approach in regulating bone remodeling.

5.3 Future research directions

It should be noted that this thesis also has some limitations in its research work. Firstly, we used murine cell line (MC3T3-E1) in the first *in vitro* study, validation of these effects of pea protein derived tripeptide LRW should be made with primary cells and human cell lines. Secondly, the metabolism of pea protein derived bioactive peptides after oral administration and its absorption in gastro-intestinal digestion is not known.

Based on the key findings and the limitations of the research work, the recommended future studies are outlined below-

1. The absorption mechanism for the pea protein bioactive peptides should be studied. The bioavailability of the pea protein derived bioactive peptides is necessary to estimate the efficacy of these bioactive peptides to produce its biological effect.
2. *In vitro* cell studies involve direct contact with bone cells. However, the transportation studies of the peptides in gastrointestinal tract to reach active site is not known. Therefore, *in vivo* evidences using protein labeling is required to track the transportation and circulation of bioactive peptides.
3. The human applicability of pea protein derived bioactive peptides can be will extrapolated using ovariectomized model of rats (OVX) to study the role of pea protein bioactive peptides on treatment of osteoporosis.

4. The regulatory role of pea protein bioactive peptides in case of diseased condition is remained to be established. Therefore, exploring more with diseased animal model can broaden the application of pea protein bioactive peptides in treatment of osteoporosis.
5. Pea protein bioactive peptides oral administration may also have affected immune functions, muscle synthesis, adipogenesis. The contributing effects of pea protein bioactive peptides can be further explored in these areas for better understanding the additional effects on human body.

5.4 References

[1] Bhat, Z.F., Kumar, S. and Bhat, H.F., 2015. Bioactive peptides of animal origin: a review. Journal of food science and technology, 52(9), pp.5377-5392.

[2] Wu, J., Yuan, L. and Aluko, R.E., 2006. Restriction of the *in vitro* formation of angiotensin II by leucinyl-arginyl-tryptophan, a novel peptide with potent angiotensin I-converting enzyme inhibitory activity. Bioscience, biotechnology, and biochemistry, 70(5), pp.1277-1280.

[3] Shang, N., Bhullar, K.S., Hubbard, B.P. and Wu, J., 2019. Tripeptide IRW initiates differentiation in osteoblasts differentiation via the RUNX2 pathway. Biochimica et Biophysica Acta (BBA)-General Subjects, 1863(6), pp.1138-1146.

[4] Korhonen, H. and Pihlanto, A., 2006. Bioactive peptides: production and functionality. International dairy journal, 16(9), pp.945-960.

[5] Brandelli, A., Daroit, D.J. and Corrêa, A.P.F., 2015. Whey as a source of peptides with remarkable biological activities. Food Research International, 73, pp.149-161.

[6] http://www.agr.gc.ca/eng/industry-markets-and-trade/international-agri-food-market-intelligence/pea-protein-isolate-in-food-and-beverage-products/? September 28, 2019.

[7] Tulbek, M.C., Lam, R.S.H., Asavajaru, P. and Lam, A., 2017. Pea: a sustainable vegetable protein crop. In Sustainable protein sources (pp. 145-164). Academic Press.

[8] Wolfe, R.R., 2017. Branched-chain amino acids and muscle protein synthesis in humans: myth or reality?. Journal of the International Society of Sports Nutrition, 14(1), p.30.

[9] Hopkins, R.B., Burke, N., Von Keyserlingk, C., Leslie, W.D., Morin, S.N., Adachi, J.D., Papaioannou, A., Bessette, L., Brown, J.P., Pericleous, L. and Tarride, J., 2016. The current economic burden of illness of osteoporosis in Canada. Osteoporosis International, 27(10), pp.3023-3032.

[10] Ottanelli, S., 2015. Prevention and treatment of bone fragility in cancer patient. Clinical Cases in Mineral and Bone Metabolism, 12(2), p.116.

BIBLIOGRAPHY

Reddi, S., Kumar, N., Vij, R., Mada, S.B., Kapila, S. and Kapila, R., 2016. Akt drives buffalo casein-derived novel peptide-mediated osteoblast differentiation. The Journal of nutritional biochemistry, 38, pp.134-144.

Abuajah, C.I., Ogbonna, A.C. and Osuji, C.M., 2015. Functional components and medicinal properties of food: a review. Journal of food Science and Technology, 52(5), pp.2522-2529.

Adebiyi, A.P. and Aluko, R.E., 2011. Functional properties of protein fractions obtained from commercial yellow field pea (Pisum sativum L.) seed protein isolate. Food Chemistry, 128(4), pp.902-908.

Ahn, C.B. and Je, J.Y., 2019. Bone health-promoting bioactive peptides. Journal of Food Biochemistry, 43(1), p.e12529.

Alberts, B., Bray, D., Hopkin, K., Johnson, A.D., Lewis, J., Raff, M., Roberts, K. and Walter, P., 2013. Essential Cell Biology. Garland Science.

Almeida, M.S., Cabral, K.M., Kurtenbach, E., Almeida, F.C. and Valente, A.P., 2002. Solution structure of Pisum sativum defensin 1 by high resolution NMR: plant defensins, identical backbone with different mechanisms of action. Journal of Molecular Biology, 315(4), pp.749-757.

Aluko, R.E., 2019. Food protein-derived renin-inhibitory peptides: *in vitro* and *in vivo* properties. Journal of Food Biochemistry, 43(1), p.e12648.

An, J., Yang, H., Zhang, Q., Liu, C., Zhao, J., Zhang, L. and Chen, B., 2016. Natural products for treatment of osteoporosis: The effects and mechanisms on promoting osteoblast-mediated bone formation. Life sciences, 147, pp.46-58.

Anderson, H.C., 2003. Matrix vesicles and calcification. Current Rheumatology Reports, 5(3), pp.222-226.

Arana-Chavez, V.E. and Bradaschia-Correa, V., 2009. Clastic cells: mineralized tissue resorption in health and disease. The International Journal of Biochemistry & Cell biology, 41(3), pp.446-450.

Arsenault, A.L., Frankland, B.W. and Ottensmeyer, F.P., 1991. Vectorial sequence of mineralization in the turkey leg tendon determined by electron microscopic imaging. Calcified Tissue International, 48(1), pp.46-55.

Awasthi, H., Mani, D., Singh, D. and Gupta, A., 2018. The underlying pathophysiology and therapeutic approaches for osteoporosis. Medicinal Research Reviews, 38(6), pp.2024-2057.

Azagra, R., Roca, G., Encabo, G., Aguyé, A., Zwart, M., Güell, S., Puchol, N., Gene, E., Casado, E., Sancho, P. and Solà, S., 2012. FRAX® tool, the WHO algorithm to predict osteoporotic fractures: the first analysis of its discriminative and predictive ability in the Spanish FRIDEX cohort. BMC Musculoskeletal Disorders, 13(1), p.204.

Bab, I. and Zimmer, A., 2008. Cannabinoid receptors and the regulation of bone mass. British Journal of Pharmacology, 153(2), pp.182-188.

Barać, M., Čabrilo, S., Pešić, M., Stanojević, S., Pavlićević, M., Maćej, O. and Ristić, N., 2011. Functional properties of pea (Pisum sativum, L.) protein isolates modified with chymosin. International Journal of Molecular Sciences, 12(12), pp.8372-8387.

Baron, R., 2008. Anatomy and ultrastructure of bone–histogenesis, growth and remodeling. MDText. com, Inc.

Behera, P., Kumar, R., Sandeep, I.V.R., Kapila, R., Dang, A.K. and Kapila, S., 2013. Casein hydrolysates enhance osteoblast proliferation and differentiation in mouse bone marrow culture. Food Bioscience, 2, pp.24-30.

Bensaha, T. and El Mjabber, H., 2016. Evaluation of new bone formation after sinus augmentation with two different methods. International Journal of Oral and Maxillofacial Surgery, 45(1), pp.93-98.

Beto, J.A., 2015. The role of calcium in human aging. Clinical Nutrition Research, 4(1), pp.1-8.

Bhat, Z.F., Kumar, S. and Bhat, H.F., 2015. Bioactive peptides of animal origin: a review. Journal of food science and technology, 52(9), pp.5377-5392.

Bhat, Z.F., Kumar, S. and Bhat, H.F., 2015. Bioactive peptides of animal origin: a review. Journal of Food Science and Technology, 52(9), pp.5377-5392.

Bhutani, G. and Gupta, M.C., 2013. Emerging therapies for the treatment of osteoporosis. Journal of Mid-life Health, 4(3), p.147..

Blair, H.C., Larrouture, Q.C., Li, Y., Lin, H., Beer-Stoltz, D., Liu, L., Tuan, R.S., Robinson, L.J., Schlesinger, P.H. and Nelson, D.J., 2017. Osteoblast differentiation and bone matrix formation *in vivo* and *in vitro*. Tissue Engineering Part B: Reviews, 23(3), pp.268-280.

Blair, H.C., Teitelbaum, S.L., Ghiselli, R. and Gluck, S., 1989. Osteoclastic bone resorption by a polarized vacuolar proton pump. Science, 245(4920), pp.855-857.

Bonewald, L.F. and Johnson, M.L., 2008. Osteocytes, mechanosensing and Wnt signaling. Bone, 42(4), pp.606-615.

Bonjour, J.P., 2011. Protein intake and bone health. International Journal for Vitamin and Nutrition Research, 81(2), p.134

Boskey, A.L., Spevak, L., Paschalis, E., Doty, S.B. and McKee, M.D., 2002. Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. Calcified Tissue International, 71(2), pp.145-154.

Boyce, B.F. and Xing, L., 2007. The Rankl/Rank/Opg pathway. Current Osteoporosis Reports, 5(3), pp.98-104.

Boyce, B.F. and Xing, L., 2008. Functions of RANKL/RANK/OPG in bone modeling and remodeling. Archives of Biochemistry and Biophysics, 473(2), pp.139-146.

Branca, C., Giudicianni, P. and Di Blasi, C., 2003. GC/MS characterization of liquids generated from low-temperature pyrolysis of wood. Industrial & Engineering Chemistry Research, 42(14), pp.3190-3202.

Brandelli, A., Daroit, D.J. and Corrêa, A.P.F., 2015. Whey as a source of peptides with remarkable biological activities. Food Research International, 73, pp.149-161.

Brot, C., Jørgensen, N., Madsen, O.R., Jensen, L.B. and Sørensen, O.H., 1999. Relationships between bone mineral density, serum vitamin D metabolites and calcium: phosphorus intake in healthy perimenopausal women. Journal of Internal Medicine, 245(5), pp.509-516.

Bruni, N., Capucchio, M., Biasibetti, E., Pessione, E., Cirrincione, S., Giraudo, L., Corona, A. and Dosio, F., 2016. Antimicrobial activity of lactoferrin-related peptides and applications in human and veterinary medicine. Molecules, 21(6), p.752.

Buckwalter, J.A. and Cooper, R.R., 1987. Bone structure and function. Instructional Course Lectures, 36, pp.27-48.

Caetano-Lopes, J., Canhao, H. and Eurico Fonseca, J., 2007. Osteoblasts and bone formation. Acta reumatológica portuguesa, 32(2).

Căpriţă, R., Căpriţă, A. and Creţescu, I., 2010. Protein solubility as quality index for processed soybean. Scientific Papers Animal Science and Biotechnologies, 43(1), pp.375-378.

Capulli, M., Paone, R. and Rucci, N., 2014. Osteoblast and osteocyte: games without frontiers. Archives of biochemistry and biophysics, 561, pp.3-12.

Carson, M. and Clarke, S., 2018. Bioactive compounds from marine organisms: Potential for bone growth and healing. Marine Drugs, 16(9), p.340.

Center, J. and Eisman, J., 1997. The epidemiology and pathogenesis of osteoporosis. Baillière's clinical endocrinology and metabolism, 11(1), pp.23-62.

Chakrabarti, S., Guha, S. and Majumder, K., 2018. Food-derived bioactive peptides in human health: Challenges and Opportunities. Nutrients, 10(11), p.1738.

Chakrabarti, S., Jahandideh, F. and Wu, J., 2014. Food-derived bioactive peptides on inflammation and oxidative stress. BioMed Research International, 2014.

Chapuy, M.C., Arlot, M.E., Duboeuf, F., Brun, J., Crouzet, B., Arnaud, S., Delmas, P.D. and Meunier, P.J., 1992. Vitamin D3 and calcium to prevent hip fractures in elderly women. New England Journal of Medicine, 327(23), pp.1637-1642...

Charles, J.F. and Aliprantis, A.O., 2014. Osteoclasts: more than 'bone eaters'. Trends in Molecular Medicine, 20(8), pp.449-459.

Chen, J.S. and Sambrook, P.N., 2012. Antiresorptive therapies for osteoporosis: a clinical overview. Nature Reviews Endocrinology, 8(2), p.81.

Chen, X., Wang, Z., Duan, N., Zhu, G., Schwarz, E.M. and Xie, C., 2018. Osteoblast–osteoclast interactions. Connective Tissue Research, 59(2), pp.99-107.

Cheng, S.L., Zhang, S.F., Nelson, T.L., Warlow, P.M. and Civitelli, R., 1994. Stimulation of human osteoblast differentiation and function by ipriflavone and its metabolites. Calcified Tissue International, 55(5), pp.356-362.

Chetram, M.A. and Hinton, C.V., 2013. ROS-mediated regulation of CXCR4 in cancer. Frontiers in biology, 8(3), pp.273-278.

Christoffersen, J. and Landis, W.J., 1991. A contribution with review to the description of mineralization of bone and other calcified tissues *in vivo*. The Anatomical Record, 230(4), pp.435-450.

Cintineo, H.P., Arent, M.A., Antonio, J. and Arent, S.M., 2018. Effects of Protein Supplementation on Performance and Recovery in Resistance and Endurance Training. Frontiers in nutrition, 5.

Clarke, B., 2008. Normal bone anatomy and physiology. Clinical Journal of the American Society of Nephrology, 3(Supplement 3), pp.S131-S139.

Clarke, B.L., 2009. New and emerging treatments for osteoporosis. Clinical Endocrinology, 71(3), pp.309-321...

Cojoc, M., Peitzsch, C., Trautmann, F., Polishchuk, L., Telegeev, G.D. and Dubrovska, A., 2013. Emerging targets in cancer management: role of the CXCL12/CXCR4 axis. OncoTargets and therapy, 6, p.1347.

Cooper, C., Campion, G. and Melton, L.3., 1992. Hip fractures in the elderly: a world-wide projection. Osteoporosis International, 2(6), pp.285-289.

Dahl, W.J., Foster, L.M. and Tyler, R.T., 2012. Review of the health benefits of peas (Pisum sativum L.). British Journal of Nutrition, 108(S1), pp. S3-S10.

Damoulis, P.D. and Hauschka, P.V., 1997. Nitric oxide acts in conjunction with proinflammatory cytokines to promote cell death in osteoblasts. Journal of Bone and Mineral Research, 12(3), pp.412-422.

Datta, H.K., Ng, W.F., Walker, J.A., Tuck, S.P. and Varanasi, S.S., 2008. The cell biology of bone metabolism. Journal of Clinical Pathology, 61(5), pp.577-587.

Dawson-Hughes, B., Tosteson, A.N.A., Melton, L.3., Baim, S., Favus, M.J., Khosla, S. and Lindsay, R.L., 2008. Implications of absolute fracture risk assessment for osteoporosis practice guidelines in the USA. Osteoporosis International, 19(4), pp.449-458.

De Laet, C., Kanis, J.A., Odén, A., Johanson, H., Johnell, O., Delmas, P., Eisman, J.A., Kroger, H., Fujiwara, S., Garnero, P. and McCloskey, E.V., 2005. Body mass index as a predictor of fracture risk: a meta-analysis. Osteoporosis International, 16(11), pp.1330-1338.

Depraetere, V., 2001. G-protein activation live. Nature cell biology, 3(5), pp.E107-E107.

Dong, X.Y., Guo, L.L., Wei, F., Li, J.F., Jiang, M.L., Li, G.M., Zhao, Y.D. and Chen, H., 2011. Some characteristics and functional properties of rapeseed protein prepared by ultrasonication, ultrafiltration and isoelectric precipitation. Journal of the Science of Food and Agriculture, 91(8), pp.1488-1498.

Drake, M.T., Clarke, B.L. and Khosla, S., 2008, September. Bisphosphonates: mechanism of action and role in clinical practice. In Mayo Clinic Proceedings (Vol. 83, No. 9, pp. 1032-1045). Elsevier.

Eming, S.A., Martin, P. and Tomic-Canic, M., 2014. Wound repair and regeneration: mechanisms, signaling, and translation. Science Translational Medicine, 6(265), pp.265sr6-265sr6.

Fakhry, M., Hamade, E., Badran, B., Buchet, R. and Magne, D., 2013. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. World Journal of Stem Cells, 5(4), p.136.

Feng, X. and McDonald, J.M., 2011. Disorders of bone remodeling. Annual Review of Pathology: Mechanisms of Disease, 6, pp.121-145.

Feng, X., 2009. Chemical and biochemical basis of cell-bone matrix interaction in health and disease. Current Chemical Biology, 3(2), pp.189-196.

Florencio-Silva, R., Sasso, G.R.D.S., Sasso-Cerri, E., Simões, M.J. and Cerri, P.S., 2015. Biology of bone tissue: structure, function, and factors that influence bone cells. BioMed research international, 2015.

Giangregorio, L.M., Papaioannou, A., Macintyre, N.J., Ashe, M.C., Heinonen, A., Shipp, K., Wark, J., McGill, S., Keller, H., Jain, R. and Laprade, J., 2014. Too fit to fracture: exercise recommendations for individuals with osteoporosis or osteoporotic vertebral fracture. Osteoporosis International, 25(3), pp.821-835.

Gillies, A.R. and Lieber, R.L., 2011. Structure and function of the skeletal muscle extracellular matrix. Muscle & Nerve, 44(3), pp.318-331.

Glimcher, M.J., 1998. The nature of the mineral phase in bone: biological and clinical implications. In Metabolic bone disease and clinically related disorders (pp. 23-52e). Academic Press.

Green, J., 1994. The physicochemical structure of bone: cellular and noncellular elements. Mineral and Electrolyte Metabolism, 20(1-2), pp.7-15.

Grigoriadis, A.E., Heersche, J.N. and Aubin, J.E., 1988. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. The Journal of Cell Biology, 106(6), pp.2139-2151.

Gullberg, B., Johnell, O. and Kanis, J.A., 1997. World-wide projections for hip fracture. Osteoporosis International, 7(5), pp.407-413.

Guo, S.A. and DiPietro, L.A., 2010. Factors affecting wound healing. Journal of Dental Research, 89(3), pp.219-229.

Hanley, D.A., Adachi, J.D., Bell, A. and Brown, V., 2012. Denosumab: mechanism of action and clinical outcomes. International Journal of Clinical Practice, 66(12), pp.1139-1146.

Hasegawa, T., Yamamoto, T., Tsuchiya, E., Hongo, H., Tsuboi, K., Kudo, A., Abe, M., Yoshida, T., Nagai, T., Khadiza, N. and Yokoyama, A., 2017. Ultrastructural and biochemical aspects of matrix vesicle-mediated mineralization. Japanese Dental Science Review, 53(2), pp.34-45.

Hauge, E.M., Qvesel, D., Eriksen, E.F., Mosekilde, L. and Melsen, F., 2001. Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers. Journal of Bone and Mineral Research, 16(9), pp.1575-1582.

Heaney, R.P. and Layman, D.K., 2008. Amount and type of protein influences bone health. The American Journal of Clinical Nutrition, 87(5), pp.1567S-1570S.

Hernlund, E., Svedbom, A., Ivergård, M., Compston, J., Cooper, C., Stenmark, J., McCloskey, E.V., Jönsson, B. and Kanis, J.A., 2013. Osteoporosis in the European Union: medical management, epidemiology and economic burden. Archives of osteoporosis, 8(1-2), p.136.

Hofbauer, L.C., Gori, F., Riggs, B.L., Lacey, D.L., Dunstan, C.R., Spelsberg, T.C. and Khosla, S., 1999. Stimulation of osteoprotegerin ligand and inhibition of osteoprotegerin production by glucocorticoids in human osteoblastic lineage cells: potential paracrine mechanisms of glucocorticoid-induced osteoporosis. Endocrinology, 140(10), pp.4382-4389.

Hoffman, J.R. and Falvo, M.J., 2004. Protein–which is best?. Journal of sports science & medicine, 3(3), p.118.

Holeček, M., 2018. Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. Nutrition & metabolism, 15(1), p.33.

Hood-Niefer, S.D., Warkentin, T.D., Chibbar, R.N., Vandenberg, A. and Tyler, R.T., 2012. Effect of genotype and environment on the concentrations of starch and protein in, and the physicochemical properties of starch from, field pea and fababean. Journal of the Science of Food and Agriculture, 92(1), pp.141-150.

Hopkins, R.B., Burke, N., Von Keyserlingk, C., Leslie, W.D., Morin, S.N., Adachi, J.D., Papaioannou, A., Bessette, L., Brown, J.P., Pericleous, L. and Tarride, J., 2016. The current economic burden of illness of osteoporosis in Canada. Osteoporosis International, 27(10), pp.3023-3032.

http://www.agr.gc.ca/eng/industry-markets-and-trade/international-agri-food-market-intelligence/pea-protein-isolate-in-food-and-beverage-products/? September 28, 2019

http://www.osteoporosis.ca/multimedia/pdf/publications/Drug_Treatments_EN.pdf

https://osteoporosis.ca/about-the-disease/, July 10, 2019

https://www.accessdata.fda.gov/scripts/InteractiveNutritionFactsLabel/protein.html, August 22, 2019.

https://www.bones.nih.gov/health-info/bone/osteoporosis/overview July 12, 2091

https://www.bones.nih.gov/health-info/bone/osteoporosis/overview, July 2, 2019

https://www.cysticfibrosis.ca/osteoporosis. July 10, 2019

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5335887/, July 12, 2019

https://www.nlm.nih.gov/nichsr/hta101/ta10107.html, July 13, 2019

Huang, Q.Y., Recker, R.R. and Deng, H.W., 2003. Searching for osteoporosis genes in the post-genome era: progress and challenges. Osteoporosis International, 14(9), pp.701-715.

Inan, S., 2019. The Potential Role of Nutraceuticals in Inflammation and Oxidative Stress. In Nutraceuticals-Past, Present and Future. IntechOpen.

Islam, M., Rahi, M., Jahangir, C.A., Rahman, M.H., Jerin, I., Amin, R., Hoque, K.M. and Reza, M.A., 2018. *In vivo* Anticancer Activity of Basella alba Leaf and Seed Extracts against Ehrlich's Ascites Carcinoma (EAC) Cell Line. Evidence-Based Complementary and Alternative Medicine, 2018.

Jia, M., Nie, Y., Cao, D.P., Xue, Y.Y., Wang, J.S., Zhao, L., Rahman, K., Zhang, Q.Y. and Qin, L.P., 2012. Potential antiosteoporotic agents from plants: a comprehensive review. Evidence-based Complementary and Alternative Medicine, 2012.

Jones, G., Winzenberg, T.M., Callisaya, M.L. and Laslett, L.L., 2014. Lifestyle modifications to improve musculoskeletal and bone health and reduce disability–A life-course approach. Best Practice & Research Clinical Rheumatology, 28(3), pp.461-478.

Kanis, J.A., McCloskey, E.V., Johansson, H., Oden, A., Ström, O. and Borgström, F., 2010. Development and use of FRAX® in osteoporosis. Osteoporosis International, 21(2), pp.407-413.

Kapinas, K. and Delany, A.M., 2011. MicroRNA biogenesis and regulation of bone remodeling. Arthritis research & therapy, 13(3), p.220.

Katikaneni, R., Ponnapakkam, A., Miller, E., Ponnapakkam, T. and Gensure, R.C., 2009. A new technique for precisely and accurately measuring lumbar spine bone mineral density in mice using clinical dual energy X-ray absorptiometry (DXA). Toxicology Mechanisms and Methods, 19(3), pp.225-231.

Kiewiet, M., Faas, M. and de Vos, P., 2018. Immunomodulatory protein hydrolysates and their application. Nutrients, 10(7), p.904.

Kim, H.K., Cho, S.G., Kim, J.H., Doan, T.K.P., Hu, Q.S., Ulhaq, R., Song, E.K. and Yoon, T.R., 2009. Mevinolin enhances osteogenic genes (ALP, type I collagen and osteocalcin), CD44, CD47 and CD51 expression during osteogenic differentiation. Life Sciences, 84(9-10), pp.290-295.

Kiryushko, D., Bock, E. and Berezin, V., 2007. Pharmacology of cell adhesion molecules of the nervous system. Current Neuropharmacology, 5(4), pp.253-267.

Komori, T., 2006. Regulation of osteoblast differentiation by transcription factors. Journal of Cellular Biochemistry, 99(5), pp.1233-1239.

Komori, T., 2009. Regulation of osteoblast differentiation by Runx2. In Osteoimmunology (pp. 43-49). Springer, Boston, MA.

Kong, E., Hinds, P.W. and Agarwal, M., 2012. The Retinoblastoma protein in osteosarcomagenesis. In Osteosarcoma. INTECH Open Access Publisher.

Kong, Y.Y., Yoshida, H., Sarosi, I., Tan, H.L., Timms, E., Capparelli, C., Morony, S., Oliveira-dos-Santos, A.J., Van, G., Itie, A. and Khoo, W., 1999. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature, 397(6717), p.315.

Koopman, R., Crombach, N., Gijsen, A.P., Walrand, S., Fauquant, J., Kies, A.K., Lemosquet, S., Saris, W.H., Boirie, Y. and van Loon, L.J., 2009. Ingestion of a protein hydrolysate is accompanied by an accelerated *in vivo* digestion and absorption rate when compared with its intact protein. The American journal of clinical nutrition, 90(1), pp.106-115.

Korhonen, H. and Pihlanto, A., 2003. Food-derived bioactive peptides-opportunities for designing future foods. Current Pharmaceutical Design, 9(16), pp.1297-1308.

Korhonen, H. and Pihlanto, A., 2006. Bioactive peptides: production and functionality. International dairy journal, 16(9), pp.945-960.

Koutsopoulos, S., 2017. Peptide applications in biomedicine, biotechnology and bioengineering. Woodhead Publishing, p.387-408.

Kozielski, M., Buchwald, T., Szybowicz, M., Błaszczak, Z., Piotrowski, A. and Ciesielczyk, B., 2011. Determination of composition and structure of spongy bone tissue in human head of femur by Raman spectral mapping. Journal of Materials Science: Materials in Medicine, 22(7), pp.1653-1661.

Kristinsson, H.G. and Rasco, B.A., 2000. Fish protein hydrolysates: production, biochemical, and functional properties. Critical Reviews in Food Science and Nutrition, 40(1), pp.43-81.

Kukreja, P., Abdel-Mageed, A.B., Mondal, D., Liu, K. and Agrawal, K.C., 2005. Up-regulation of CXCR4 expression in PC-3 cells by stromal-derived factor-1α (CXCL12) increases endothelial adhesion and trans endothelial migration: role of MEK/ERK signaling pathway–dependent NF-κB activation. Cancer research, 65(21), pp.9891-9898.

La Manna, S., Di Natale, C., Florio, D. and Marasco, D., 2018. Peptides as Therapeutic Agents for Inflammatory-Related Diseases. International Journal of Molecular Sciences, 19(9), p.2714.

Lacey, D.L., Timms, E., Tan, H.L., Kelley, M.J., Dunstan, C.R., Burgess, T., Elliott, R., Colombero, A., Elliott, G., Scully, S. and Hsu, H., 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell, 93(2), pp.165-176.

Lane, N.E. and Kelman, A., 2003. A review of anabolic therapies for osteoporosis. Arthritis Res Ther, 5(5), p.214.

Larios-Cruz, R., Londoño-Hernández, L., Gómez-García, R., García-Galindo, I., Sepulveda, L., Rodríguez-Herrera, R. and Aguilar, C.N., 2019. Extraction of Bioactive Molecules through Fermentation and Enzymatic Assisted Technologies. High Value Fermentation Products: Human Health, 1, pp.27-59.

Lax, R., 2010. The future of peptide development in the pharmaceutical industry. PharManufacturing: The International Peptide Review, 2, pp.10-15.

Leali, P.T., Muresu, F., Melis, A., Ruggiu, A., Zachos, A. and Doria, C., 2011. Skeletal fragility definition. Clinical cases in Mineral and Bone Metabolism, 8(2), p.11.

Lee, K.S., Kim, H.J., Li, Q.L., Chi, X.Z., Ueta, C., Komori, T., Wozney, J.M., Kim, E.G., Choi, J.Y., Ryoo, H.M. and Bae, S.C., 2000. Runx2 is a common target of transforming growth factor β1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. Molecular and Cellular Biology, 20(23), pp.8783-8792.

Lerner, U.H., Kindstedt, E. and Lundberg, P., 2019. The critical interplay between bone resorbing and bone forming cells. Journal of Clinical Periodontology, 46, pp.33-51.

Li, J., Hao, L., Wu, J., Zhang, J. and Su, J., 2016. Linarin promotes osteogenic differentiation by activating the BMP-2/RUNX2 pathway via protein kinase A signaling. International Journal of Molecular Medicine, 37(4), pp.901-910.

Li, L., He, Y., Zhao, M. and Jiang, J., 2013. Collective cell migration: Implications for wound healing and cancer invasion. Burns & Trauma, 1(1), p.21.

Li, X., Qin, L., Bergenstock, M., Bevelock, L.M., Novack, D.V. and Partridge, N.C., 2007. Parathyroid hormone stimulates osteoblastic expression of MCP-1 to recruit and increase the fusion of pre/osteoclasts. Journal of Biological Chemistry, 282(45), pp.33098-33106.

Liao, W., Chakrabarti, S., Davidge, S.T. and Wu, J., 2016. Modulatory effects of egg white ovotransferrin-derived tripeptide IRW (Ile-Arg-Trp) on vascular smooth muscle cells against angiotensin II stimulation. Journal of Agricultural and Food Chemistry, 64(39), pp.7342-7347.

Lindsay, R., Hart, D.M., Forrest, C. and Baird, C., 1980. Prevention of spinal osteoporosis in oophorectomised women. The Lancet, 316(8205), pp.1151-1154.

Ma, Y.L., Zeng, Q., Donley, D.W., Ste-Marie, L.G., Gallagher, J.C., Dalsky, G.P., Marcus, R. and Eriksen, E.F., 2006. Teriparatide increases bone formation in modeling and remodeling osteons and enhances IGF-II immunoreactivity in postmenopausal women with osteoporosis. Journal of Bone and Mineral Research, 21(6), pp.855-864.

Mada, S.B., Reddi, S., Kumar, N., Kumar, R., Kapila, S., Kapila, R., Trivedi, R., Karvande, A. and Ahmad, N., 2017. Antioxidative peptide from milk exhibits antiosteopenic effects through inhibition of oxidative damage and bone-resorbing cytokines in ovariectomized rats. Nutrition, 43, pp.21-31.

Mangano, K.M., Sahni, S. and Kerstetter, J.E., 2014. Dietary protein is beneficial to bone health under conditions of adequate calcium intake: an update on clinical research. Current opinion in clinical nutrition and metabolic care, 17(1), p.69.

Manninen, A.H., 2009. Protein hydrolysates in sports nutrition. Nutrition & metabolism, 6(1), p.38.

Manolagas, S.C., 2006. Choreography from the tomb: an emerging role of dying osteocytes in the purposeful, and perhaps not so purposeful, targeting of bone remodeling. IBMS BoneKEy, 3, p.5.

Marcone, S., Belton, O. and Fitzgerald, D.J., 2017. Milk-derived bioactive peptides and their health promoting effects: a potential role in atherosclerosis. British Journal of Clinical Pharmacology, 83(1), pp.152-162.

Marie, P.J., 2006. Strontium ranelate: a dual mode of action rebalancing bone turnover in favour of bone formation. Current Opinion in Rheumatology, 18, pp.S11-S15.

Marks Jr, S.C. and Popoff, S.N., 1988. Bone cell biology: the regulation of development, structure, and function in the skeleton. American Journal of Anatomy, 183(1), pp.1-44.

Matsumoto, M., Kogawa, M., Wada, S., Takayanagi, H., Tsujimoto, M., Katayama, S., Hisatake, K. and Nogi, Y., 2004. Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU. 1. Journal of Biological Chemistry, 279(44), pp.45969-45979.

Meesters, D., Wijnands, K., Brink, P. and Poeze, M., 2018. Malnutrition and Fracture Healing: Are Specific Deficiencies in Amino Acids Important in Nonunion Development?. Nutrients, 10(11), p.1597.

Mellander, O.L.O.F., 1950. The physiological importance of the casein phosphopeptide calcium salts. II. Peroral calcium dosage of infants. Some aspects of the pathogenesis of rickets. Acta Societatis Botanicorum Poloniae, 55, pp.247-257.

Messina, M. and Messina, V., 2000. Soyfoods, soybean isoflavones, and bone health: a brief overview. Journal of Renal Nutrition, 10(2), pp.63-68.

Miller, S.C., Bowman, B.M. and Jee, W.S., 1989. Bone lining cells: structure and function. Scanning Microscopy, 3(3), pp.953-60.

Miller, S.C., Bowman, B.M., Smith, J.M. and Jee, W.S., 1980. Characterization of endosteal bone-lining cells from fatty marrow bone sites in adult beagles. The Anatomical Record, 198(2), pp.163-173.

Mitchell, D.G., Rao, V.M., Dalinka, M.K., Spritzer, C.E., Alavi, A., Steinberg, M.E., Fallon, M. and Kressel, H.Y., 1987. Femoral head avascular necrosis: correlation of MR imaging, radiographic staging, radionuclide imaging, and clinical findings. Radiology, 162(3), pp.709-715.

Mogensen, T.H., 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clinical Microbiology Reviews, 22(2), pp.240-273.

Mohanty, D.P., Mohapatra, S., Misra, S. and Sahu, P.S., 2016. Milk derived bioactive peptides and their impact on human health–A review. Saudi journal of biological sciences, 23(5), pp.577-583.

Morello, K.C., Wurz, G.T. and DeGregorio, M.W., 2002. SERMs: current status and future trends. Critical reviews in Oncology/Hematology, 43(1), pp.63-76.

Mori-Okamoto, J., Otawara-Hamamoto, Y., Yamato, H. and Yoshimura, H., 2004. Pomegranate extract improves a depressive state and bone properties in menopausal syndrome model ovariectomized mice. Journal of Ethnopharmacology, 92(1), pp.93-101..

Moskowitz, R.W., 2000, October. Role of collagen hydrolysate in bone and joint disease. In Seminars in arthritis and rheumatism (Vol. 30, No. 2, pp. 87-99). WB Saunders.

Mühlbauer, M., Bosserhoff, A.K., Hartmann, A., Thasler, W.E., Weiss, T.S., Herfarth, H., Lock, G., Schölmerich, J. and Hellerbrand, C., 2003. A novel MCP-1 gene polymorphism is associated with hepatic MCP-1 expression and severity of HCV-related liver disease. Gastroenterology, 125(4), pp.1085-1093.

Mühlbauer, R.C., Lozano, A., Palacio, S., Reinli, A. and Felix, R., 2003. Common herbs, essential oils, and monoterpenes potently modulate bone metabolism. Bone, 32(4), pp.372-380.

Mukherjee, A. and Rotwein, P., 2009. Akt promotes BMP2-mediated osteoblast differentiation and bone development. J Cell Sci, 122(5), pp.716-726.

Muñoz-Torres, M., Alonso, G. and Raya, P.M., 2004. Calcitonin therapy in osteoporosis. Treatments in Endocrinology, 3(2), pp.117-132.

Narva, M., Collin, M., Lamberg-Allardt, C., Kärkkäinen, M., Poussa, T., Vapaatalo, H. and Korpela, R., 2004. Effects of long-term intervention with Lactobacillus helveticus-fermented milk

on bone mineral density and bone mineral content in growing rats. Annals of nutrition and metabolism, 48(4), pp.228-234.

Ndiaye, F., Vuong, T., Duarte, J., Aluko, R.E. and Matar, C., 2012. Anti-oxidant, anti-inflammatory and immunomodulating properties of an enzymatic protein hydrolysate from yellow field pea seeds. European Journal of Nutrition, 51(1), pp.29-37.

Neer, R.M., Arnaud, C.D., Zanchetta, J.R., Prince, R., Gaich, G.A., Reginster, J.Y., Hodsman, A.B., Eriksen, E.F., Ish-Shalom, S., Genant, H.K. and Wang, O., 2001. Effect of parathyroid hormone (1-34) on fractures and bone mineral density in postmenopausal women with osteoporosis. New England Journal of Medicine, 344(19), pp.1434-1441...

Niall Ferguson, 2004. Osteoporosis in focus. Pharmaceutical Press.

Noma, T., Takasugi, S., Shioyama, M., Yamaji, T., Itou, H., Suzuki, Y., Sakuraba, K. and Sawaki, K., 2017. Effects of dietary gelatin hydrolysates on bone mineral density in magnesium-deficient rats. BMC musculoskeletal disorders, 18(1), p.385.

Notelovitz, M., 1993. Osteoporosis: screening, prevention, and management. Fertility and Sterility, 59(4), pp.707-725.

Office of the Surgeon General (US, 2004. Bone health and osteoporosis: a report of the Surgeon General.

Office of the Surgeon General (US, 2004. Prevention and treatment for those who have bone diseases. In Bone Health and Osteoporosis: A Report of the Surgeon General. Office of the Surgeon General (US).

Office of the Surgeon General (US, 2004. The Basics of Bone in Health and Disease. In Bone Health and Osteoporosis: A Report of the Surgeon General. Office of the Surgeon General (US).

Ohara, H., Iida, H., Ito, K., Takeuchi, Y. and Nomura, Y., 2010. Effects of Pro-Hyp, a collagen hydrolysate-derived peptide, on hyaluronic acid synthesis using *in vitro* cultured synovium cells and oral ingestion of collagen hydrolysates in a guinea pig model of osteoarthritis. Bioscience, Biotechnology, and Biochemistry, 74(10), pp.2096-2099.

Ott, S.M., 2018. Cortical or trabecular bone: What's the difference? American Journal of Nephrology, 47(6), pp.373-375.

Ottanelli, S., 2015. Prevention and treatment of bone fragility in cancer patient. Clinical Cases in Mineral and Bone Metabolism, 12(2), p.116.

Pahwa, R. and Jialal, I., 2018. Chronic Inflammation. In StatPearls [Internet]. StatPearls Publishing.

Parfitt, A.M., 2002. Misconceptions (2): turnover is always higher in cancellous than in cortical bone. Bone (NY), 30, pp.807-809.

Parra-Torres, A.Y., Valdés-Flores, M., Orozco, L. and Velázquez-Cruz, R., 2013. Molecular Aspects of Bone Remodeling. In Topics in Osteoporosis. IntechOpen.

Patel, S., 2015. Emerging trends in nutraceutical applications of whey protein and its derivatives. Journal of Food Science and Technology, 52(11), pp.6847-6858.

Pavone, V., Testa, G., Giardina, S., Vescio, A., Restivo, D.A. and Sessa, G., 2017. Pharmacological therapy of osteoporosis: a systematic current review of literature. Frontiers in Pharmacology, 8, p.803.

Pawelec, K. and Planell, J.A. eds., 2018. Bone Repair Biomaterials: Regeneration and Clinical Applications. Woodhead Publishing.

Pellico, L.H. and Bautista, C., 2012. Focus on adult health medical-surgical nursing.

Perez, J.R., Kouroupis, D., Li, D.J., Best, T.M., Kaplan, L. and Correa, D., 2018. Tissue engineering and cell-based therapies for fractures and bone defects. Frontiers in Bioengineering and Biotechnology, 6, p.105.

Pergande, M. and Cologna, S., 2017. Isoelectric point separations of peptides and proteins. Proteomes, 5(1), p.4.

Pujiastuti, D.Y., Amin, G., Nur, M., Alamsjah, M.A. and Hsu, J.L., 2019. Marine Organisms as Potential Sources of Bioactive Peptides that Inhibit the Activity of Angiotensin I-Converting Enzyme: A Review. Molecules, 24(14), p.2541.

Putnam, S.E., Scutt, A.M., Bicknell, K., Priestley, C.M. and Williamson, E.M., 2007. Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 21(2), pp.99-112.4 3, Kumar and Clark, 2002.

Putnam, S.E., Scutt, A.M., Bicknell, K., Priestley, C.M. and Williamson, E.M., 2007. Natural products as alternative treatments for metabolic bone disorders and for maintenance of bone health. Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 21(2), pp.99-112.

Raggatt, L.J. and Partridge, N.C., 2010. Cellular and molecular mechanisms of bone remodeling. Journal of Biological Chemistry, 285(33), pp.25103-25108.

Ralston, S.H. and de Crombrugghe, B., 2006. Genetic regulation of bone mass and susceptibility to osteoporosis. Genes & Development, 20(18), pp.2492-2506.

Ramage, S.C., Urban, N.H., Jiranek, W.A., Maiti, A. and Beckman, M.J., 2007. Expression of RANKL in osteolytic membranes: association with fibroblastic cell markers. JBJS, 89(4), pp.841-848.

Rao, Q., Klaassen Kamdar, A. and Labuza, T.P., 2016. Storage stability of food protein hydrolysates—a review. Critical Reviews in Food Science and Nutrition, 56(7), pp.1169-1192.

Raveschot, C., Cudennec, B., Coutte, F., Flahaut, C., Fremont, M., Drider, D. and Dhulster, P., 2018. Production of bioactive peptides by Lactobacillus species: from gene to application. Frontiers in Microbiology, 9.

Reeve, J., Meunier, P.J., Parsons, J.A., Bernat, M., Bijvoet, O.L., Courpron, P., Edouard, C., Klenerman, L., Neer, R.M., Renier, J.C. and Slovik, D., 1980. Anabolic effect of human parathyroid hormone fragment on trabecular bone in involutional osteoporosis: a multicentre trial. Br Med J, 280(6228), pp.1340-1344.

Reginster, J.Y. and Burlet, N., 2006. Osteoporosis: a still increasing prevalence. Bone, 38(2), pp.4-9.

Rhee, C., Kim, J. and Tucker, H.O., 2017. Transcriptional Regulation of the First Cell Fate Decision. Journal of Developmental Biology & Regenerative Medicine, 1(1).

Ridiandries, A., Tan, J. and Bursill, C., 2018. The role of chemokines in wound healing. International journal of molecular sciences, 19(10), p.3217.

Rigamonti, E., Parolini, C., Marchesi, M., Diani, E., Brambilla, S., Sirtori, C.R. and Chiesa, G., 2010. Hypolipidemic effect of dietary pea proteins: Impact on genes regulating hepatic lipid metabolism. Molecular Nutrition & Food Research, 54(S1), pp.S24-S30..

Robinson, P.K., 2015. Enzymes: principles and biotechnological applications. Essays in Biochemistry, 59, pp.1-41.

Robinson, R.F. and Nahata, M.C., 2001. Prevention and treatment of osteoporosis in the cystic fibrosis population. Journal of Pediatric Health Care, 15(6), pp.308-315.

Rojas, A., Aguilar, R., Henriquez, B., Lian, J.B., Stein, J.L., Stein, G.S., Van Wijnen, A.J., Van Zundert, B., Allende, M.L. and Montecino, M., 2015. Epigenetic control of the bone-master Runx2 gene during osteoblast-lineage commitment by the histone demethylase. Journal of Biological Chemistry, 290(47), pp.28329-28342.

Rucci, N., 2008. Molecular biology of bone remodelling. Clinical Cases in Mineral and Bone Metabolism, 5(1), p.49.

Rucci, N., Rufo, A., Alamanou, M. and Teti, A., 2007. Modeled microgravity stimulates osteoclastogenesis and bone resorption by increasing osteoblast RANKL/OPG ratio. Journal of Cellular Biochemistry, 100(2), pp.464-473.

Rungruangmaitree, R. and Jiraungkoorskul, W., 2017. Pea, Pisum sativum, and its anticancer activity. Pharmacognosy Reviews, 11(21), p.39.

Sahni, S., Mangano, K.M., McLean, R.R., Hannan, M.T. and Kiel, D.P., 2015. Dietary approaches for bone health: lessons from the Framingham Osteoporosis Study. Current osteoporosis reports, 13(4), pp.245-255.

Salgado, P.C., Sathler, P.C., Castro, H.C., Alves, G.G., De Oliveira, A.M., De Oliveira, R.C., Maia, M.D., Rodrigues, C.R., Coelh, P.G., Fuly, A. and Cabral, L.M., 2011. Bone remodeling, biomaterials and technological applications: revisiting basic concepts. Journal of Biomaterials and Nanobiotechnology, 2(03), p.318.

Sato, Y., Ren, X.S., Harada, K., Sasaki, M., Morikawa, H., Shiomi, S., Honda, M., Kaneko, S. and Nakanuma, Y., 2012. Induction of elastin expression in vascular endothelial cells relates to hepatoportal sclerosis in idiopathic portal hypertension: possible link to serum anti-endothelial cell antibodies. Clinical & Experimental Immunology, 167(3), pp.532-542.

Schaffler, M.B., Cheung, W.Y., Majeska, R. and Kennedy, O., 2014. Osteocytes: master orchestrators of bone. Calcified Tissue International, 94(1), pp.5-24.

Schultz, N.D., Giannini, A.V., Chang, T.T. and Wong, D.C., 1994. Food allergy. In the Best Guide to Allergy(pp. 111-135). Humana Press, Totowa, NJ.

Senevirathne, M. and Kim, S.K., 2012. Development of bioactive peptides from fish proteins and their health promoting ability. In Advances in food and nutrition research (Vol. 65, pp. 235-248). Academic Press.

Shaker, J.L. and Deftos, L., 2018. Calcium and phosphate homeostasis. In Endotext [Internet]. MDText. com, Inc..

Shang, N., Bhullar, K.S., Hubbard, B.P. and Wu, J., 2019. Tripeptide IRW initiates differentiation in osteoblasts differentiation via the RUNX2 pathway. Biochimica et Biophysica Acta (BBA)-General Subjects, 1863(6), pp.1138-1146.

Sharma, S., Singh, R. and Rana, S., 2011. Bioactive peptides: a review. Int J Bioautomation, 15(4), pp.223-250.

Shiozawa, Y., Havens, A.M., Pienta, K.J. and Taichman, R.S., 2008. The bone marrow niche: habitat to hematopoietic and mesenchymal stem cells, and unwitting host to molecular parasites. Leukemia, 22(5), p.941.

Small, E., 1999. New crops for Canadian agriculture. Perspectives on new crops and new uses. ASHS Press, Alexandria, VA. USA, pp.15-52.

Socorro López-Cortez, M., Rosales-Martínez, P., Arellano-Cárdenas, S. and Cornejo-Mazón, M., 2016. Antioxidants Properties and Effect of Processing Methods on Bioactive Compounds of Legumes. Grain Legumes, p.103.

Sözen, T., Özışık, L. and Başaran, N.Ç., 2017. An overview and management of osteoporosis. European Journal of Rheumatology, 4(1), p.46.

Stroup, J., Kane, M.P. and Abu-Baker, A.M., 2008. Teriparatide in the treatment of osteoporosis. American Journal of Health-System Pharmacy, 65(6), pp.532-539.

Sunyecz, J.A., 2008. The use of calcium and vitamin D in the management of osteoporosis. Therapeutics and clinical risk management, 4(4), p.827.

Surasani, V.K.R., 2018. Acid and alkaline solubilization (pH shift) process: a better approach for the utilization of fish processing waste and by-products. Environmental Science and Pollution Research, 25(19), pp.18345-18363.

Swarthout, J.T., D'Alonzo, R.C., Selvamurugan, N. and Partridge, N.C., 2002. Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. Gene, 282(1-2), pp.1-17.

Tabatabaei-Malazy, O., Salari, P., Khashayar, P. and Larijani, B., 2017. New horizons in treatment of osteoporosis. DARU Journal of Pharmaceutical Sciences, 25(1), p.2.

Tang, C.H., Chuang, J.Y., Fong, Y.C., Maa, M.C., Way, T.D. and Hung, C.H., 2008. Bone-derived SDF-1 stimulates IL-6 release via CXCR4, ERK and NF-κB pathways and promotes osteoclastogenesis in human oral cancer cells. Carcinogenesis, 29(8), pp.1483-1492.

Teitelbaum, S.L., 2000. Bone resorption by osteoclasts. Science, 289(5484), pp.1504-1508.

Thomford, N., Senthebane, D., Rowe, A., Munro, D., Seele, P., Maroyi, A. and Dzobo, K., 2018. Natural products for drug discovery in the 21st century: innovations for novel drug discovery. International Journal of Molecular Sciences, 19(6), p.1578.

Tian, G., Zhang, G. and Tan, Y.H., 2013. Calcitonin gene-related peptide stimulates BMP-2 expression and the differentiation of human osteoblast-like cells *in vitro*. Acta Pharmacologica Sinica, 34(11), p.1467.

Todd, Sanford, Davidsohn Clinical Diagnosis and Management by.

Tömösközi, S., Lasztity, R., Haraszi, R. and Baticz, O., 2001. Isolation and study of the functional properties of pea proteins. Food/Nahrung, 45(6), pp.399-401.

Torii, Y., Hitomi, K. And Tsukagoshi, N., 1994. L-ascorbic acid 2-phosphate promotes osteoblastic differentiation of MC3T3-E1 mediated by accumulation of type I collagen. Journal of Nutritional Science and Vitaminology, 40(3), pp.229-238.

Trouvin, A.P. and Goëb, V., 2010. Receptor activator of nuclear factor-κB ligand and osteoprotegerin: maintaining the balance to prevent bone loss. Clinical interventions in aging, 5, p.345.

Tsuboi, H., Matsui, Y., Hayashida, K., Yamane, S., Maeda-Tanimura, M., Nampei, A., Hashimoto, J., Suzuki, R., Yoshikawa, H. and Ochi, T., 2003. Tartrate resistant acid phosphatase (TRAP) positive cells in rheumatoid synovium may induce the destruction of articular cartilage. Annals of the rheumatic diseases, 62(3), pp.196-203.

Tsuchita, H., Goto, T., Shimizu, T., Yonehara, Y. and Kuwata, T., 1996. Dietary casein phosphopeptides prevent bone loss in aged ovariectomized rats. The Journal of nutrition, 126(1), pp.86-93.

Tuchendler, D. and Bolanowski, M., 2014. The influence of thyroid dysfunction on bone metabolism. Thyroid Research, 7(1), p.12.

Tulbek, M.C., Lam, R.S.H., Asavajaru, P. and Lam, A., 2017. Pea: a sustainable vegetable protein crop. In Sustainable protein sources (pp. 145-164). Academic Press.

Turner, R.M., Park, B.K. and Pirmohamed, M., 2015. Parsing interindividual drug variability: an emerging role for systems pharmacology. Wiley Interdisciplinary Reviews: Systems Biology and Medicine, 7(4), pp.221-241.

Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., Koga, T., Martin, T.J. and Suda, T., 1990. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proceedings of the National Academy of Sciences, 87(18), pp.7260-7264.

Ukon, Y., Makino, T., Kodama, J., Tsukazaki, H., Tateiwa, D., Yoshikawa, H. and Kaito, T., 2019. Molecular-Based Treatment Strategies for Osteoporosis: A Literature Review. International Journal of Molecular Sciences, 20(10), p.2557.

Väänänen, H.K. and Härkönen, P.L., 1996. Estrogen and Bone Metabolism. Maturitas, 23, pp.S65-S69.

Van Der Vorst, E.P., Peters, L.J., Müller, M., Gencer, S., Weber, C. and Döring, Y., 2019. G-Protein coupled receptor targeting on myeloid cells in atherosclerosis. Frontiers in pharmacology, 10, p.531.

Vashishth, D., 2005. Collagen glycation and its role in fracture properties of bone. Journal of Musculoskeletal and Neuronal Interactions, 5(4), p.316.

Wang, J., Su, Y., Jia, F. and Jin, H., 2013. Characterization of casein hydrolysates derived from enzymatic hydrolysis. Chemistry Central Journal, 7(1), p.62.

Weaver, C.M., Dwyer, J., Fulgoni III, V.L., King, J.C., Leveille, G.A., MacDonald, R.S., Ordovas, J. and Schnakenberg, D., 2014. Processed foods: contributions to nutrition. The American Journal of Clinical Nutrition, 99(6), pp.1525-1542.

Wojda, S.J. and Donahue, S.W., 2018. Parathyroid Hormone for Bone Regeneration. Journal of Orthopaedic Research®, 36(10), pp.2586-2594.

Wolfe, R.R., 2017. Branched-chain amino acids and muscle protein synthesis in humans: myth or reality?. Journal of the International Society of Sports Nutrition, 14(1), p.30.

World Health Organization, World Health Organization. Ageing and Life Course Unit, 2008. WHO global report on falls prevention in older age. World Health Organization.

Wu, J., Yuan, L. and Aluko, R.E., 2006. Restriction of the *in vitro* formation of angiotensin II by leucinyl-arginyl-tryptophan, a novel peptide with potent angiotensin I-converting enzyme inhibitory activity. Bioscience, biotechnology, and biochemistry, 70(5), pp.1277-1280.

Wu, L.N., Genge, B.R., Lloyd, G.C. and Wuthier, R.E., 1991. Collagen-binding proteins in collagenase-released matrix vesicles from cartilage. Interaction between matrix vesicle proteins and different types of collagen. Journal of Biological Chemistry, 266(2), pp.1195-1203.

Xie, W., Ji, L., Zhao, T. and Gao, P., 2015. Identification of transcriptional factors and key genes in primary osteoporosis by DNA microarray. Medical science monitor: International Medical Journal of Experimental and Clinical Research, 21, p.1333.

Xu, F. and Teitelbaum, S.L., 2013. Osteoclasts: new insights. Bone Research, 1, p.11.

Yoshiko, Y., Candeliere, G.A., Maeda, N. and Aubin, J.E., 2007. Osteoblast autonomous Pi regulation via Pit1 plays a role in bone mineralization. Molecular and Cellular Biology, 27(12), pp.4465-4474.

Zhang, C., Li, J., Hu, C., Wang, J., Zhang, J., Ren, Z., Song, X. and Jia, L., 2017. Antihyperglycemic and organic protective effects on pancreas, liver and kidney by polysaccharides from Hericium erinaceus SG-02 in streptozotocin-induced diabetic mice. Scientific reports, 7(1), p.10847.

Zhang, Y.J., Gan, R.Y., Li, S., Zhou, Y., Li, A.N., Xu, D.P. and Li, H.B., 2015. Antioxidant phytochemicals for the prevention and treatment of chronic diseases. Molecules, 20(12), pp.21138-21156.