EFFECTS OF *QUERCUS INFECTORIA* SEMI-PURIFIED FRACTIONS ON MORPHOLOGICAL CHANGES, MINERALISATION AND ALKALINE PHOSPHATASE (ALP) ACTIVITY OF HUMAN FETAL OSTEOBLAST CELL LINE (hFOB 1.19)

by

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ANOVA	Analysis of variance
CO_2	Carbon dioxide
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphate
EC_{50}	Half maximal effective concentration/Median effective concentration
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
hFOB	Human fetal osteoblast
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NaOH	Sodium hydroxide
OD	Optical density
PBS	Phosphate buffered saline
QI	*Quercus infectoria*
SD	Standard deviation
TLC	Thin Layer Chromatography

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ABSTRACT

Osteoporosis is a serious disease of the bones. *Quercus infectoria* (QI) was reported to possess great medicinal values and possible to have an anabolic effect on bone metabolism due to antioxidant properties. Present study was performed to investigate the effect of semi-purified fractions of QI on the proliferation, alkaline phosphatase (ALP) activity, mineralisation and the morphology of hFOB 1.19. Semi-purified fractions of QI (Fraction A and Fraction B) were prepared by column chromatography methods. The hFOB 1.19 were cultured in Dulbecco's modified eagle F12 medium (DMEM F12) supplemented with a 10% fetal bovine serum (FBS), a 1% penicillin/streptomycin and then were treated with Fraction A and Fraction B at various concentration (0.1 to 99.0 μg/ml) for 72 hours. EC_{50} for positive control and fractions groups were measured by using MTT assay. Cell proliferation assay was performed to compare the number of cells viability after being treated from day 1 until day 14. The levels of ALP were measured at day 1, 3, 7, 10, and 14 by using ELISA and were compared among negative control, pamidronate, Fraction A and Fraction B. In addition, morphology of hFOB 1.19 was observed by using inverted microscope and mineralisation of hFOB 1.19 was determined by von Kossa staining for phosphate deposition and Alizarin red S staining for calcium deposition. The EC50 of hFOB 1.19 treated with Fraction A and Fraction B was 8.86 μg/ml and 9.92 μg/ml, respectively.

Both fractions were more effective compared to control drug, pamidronate (15.27 μg/ml). Viability of cells treated with both fractions was significantly higher compared to other two control groups from day 3 until day 14. ALP level of hFOB 1.19 treated with both fractions from day 3 to day 14, was significantly increased with time and concentration-dependant manner, also higher compared to other two control groups. The morphology of hFOB 1.19 treated with both fractions was uniformly spindle in shaped, higher in number and over-confluent. The hFOB 1.19 treated with Fraction B were significantly higher in number and ALP level compared to cells treated with Fraction A started from day 7 and onwards. In line with other findings, treatment with both fractions has improved the mineralisation activity by enhancing calcium and phosphate deposits of hFOB 1.19 cells better than the other two control groups. Greater intensity of Alizarin red content and von Kossa staining was observed in Fraction B as compared to Fraction A. Based on FTIR analysis, both fractions of QI were found to contain functional group of total phenolic content expressed mainly as tannin and gallic acid. In conclusion, semi-purified fractions of QI has enhanced cell proliferation, improved mineralisation and increased ALP level of hFOB 1.19 cells.

KESAN MANJAKANI (*QUERCUS INFECTORIA*) SEPARA TULEN KE ATAS MORFOLOGI, MINERSALISASI DAN AKTIVITI FOSFATASE ALKALI (ALP) OLEH SEL OSTEOBLAS MANUSIA (hFOB 1.19)

ABSTRAK

Osteoporosis adalah sejenis penyakit tulang yang serius. *Quercus infectoria* (QI) dilaporkan mempunyai nilai perubatan yang banyak dan mempunyai kesan anabolik terhadap metabolisme tulang disebabkan oleh ciri antioksidannya. Kajian ini dilaksanakan untuk menilai kesan separa tulen manjakani pada proliferasi, aktiviti fosfatase alkali (ALP), mineralisasi dan morfologi sel hFOB 1.19. QI separa tulen (Pecahan A dan Pecahan B) telah disediakan melalui kaedah kolum kromatografi. Sel hFOB 1.19 dikulturkan dalam medium *Dulbecco's modified eagle F12* (F12 DMEM) yang ditambah dengan 10% *serum fetal bovine* (FBS) dan penisilin 1% / streptomisin, dan kemudiannya telah dirawat dengan Pecahan A dan Pecahan B di pelbagai kepekatan (0.1- 99.0 µg/ml) selama 72 jam. EC_{50} untuk ubat kumpulan positif dan ubat kumpulan pecahan QI diukur dengan menggunakan ujian MTT. Ujian proliferasi sel telah dilaksanakan untuk perbandingan di antara bilangan sel selepas dirawat dari hari 1 hingga hari ke 14. Aras ALP telah dikira pada hari 1, 3, 7, 10 dan 14 dengan menggunakan ujian ELISA dan perbandingan antara kumpulan kawalan negatif, pamidronate, Pecahan A dan Pecahan B dilaksanakan. Di samping itu, morfologi hFOB 1.19 diperhatikan dengan menggunakan mikroskop songsang dan kandungan mineral hFOB 1.19 ditentukan oleh von Kossa bagi mineral fosfat dan pewarna Alizarin red S bagi mineral kalsium. Kepekatan berkesan manjakani separa tulen ke atas sel hFOB 1.19 adalah ml 8.86 µg / ml bagi sel yang dirawat dengan Pecahan A dan 9.92 µg /ml

bagi sel yang dirawat oleh Pecahan B. Kedua-dua pecahan bertindak lebih berkesan berbanding pamidronate (15.27 μg / ml). Bilangan sel yang dirawat oleh kedua-dua pecahan QI adalah lebih tinggi secara signifikan berbanding dengan dua kumpulan kawalan lain bermula dari hari ke 3 sehingga hari ke 14. Aras ALP yang dirembeskan daripada sel hFOB 1.19 yang telah dirawat dengan Pecahan A dan Pecahan B bermula daripada hari ke 3 hingga hari ke 14 meningkat secara signifikan mengikut masa dan tahap kepekatan, kedua-duanya juga lebih tinggi jika dibandingkan dengan dua kumpulan kawalan yang lain. Morfologi sel hFOB 1.19 yang dirawat dengan kedua-dua pecahan QI didapati berbentuk spindel (memanjang), mempunyai bilangan yang lebih tinggi dan penuh. Sel hFOB 1.19 yang dirawat dengan Pecahan B mempunyai bilangan dan aras enzim ALP yang lebih tinggi secara signifikan berbanding sel yang dirawat dengan Pecahan A bermula daripada hari ke 7 dan seterusnya. Selaras dengan penemuan lain, rawatan ke atas sel hFOB 1.19 dengan kedua-dua pecahan QI menambahkan aktiviti mineralisasi dengan meningkatkan mineral kalsium dan fosfat berbanding dengan dua kumpulan kawalan lain. Berdasarkan pemerhatian, kandungan keamatan pewarna Alizarin merah dan von Kossa lebih tinggi dalam sel yang dirawat oleh Pecahan B berbanding Pecahan A. Berdasarkan analisis FTIR, kedua-dua pecahan QI didapati mengandungi kandungan fenolik yang dirembeskan terutamanya tanin dan asid galik. Kesimpulannya, pecahan separa tulen QI telah menambahkan proliferasi sel, mineralisasi dan meningkatkan tahap ALP sel hFOB 1.19.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Bone which made up the human skeleton system is a connective tissue characterised by a mineralized extracellular matrix. Matrix mineralisation carried out by bone distinguishes them from other connective tissue where an extremely hard tissue that capable of providing support and protection is produced. Extracellular matrix produced consists of cells such as osteoblasts and osteoclasts (Ross and Pawlina, 2011). Homeostatic activity via bone remodelling process is carried out by these cells in order to maintain skeletal structural integrity. Bone resorption occurs by an equal degree of bone formation in a healthy adult from a balance process of osteoblast and osteoclast activity which indicated by the expression of various phenotypic markers such as alkaline phosphatase (ALP) and osteocalcin. Bone-specific ALP activity also associates with bone formation rate in metabolic disease of the bone other than normal physiological bone growth (Bolarin, 1996; Gundberg, 1993). Pathological conditions such as osteoporosis or osteopetrosis may occur from imbalance of this activity.

Osteoporosis means skeletal disorder characterised by progressive loss of normal bone density along with deterioration of its microarchitecture which predisposing a person to an increased risk of fracture and it is a major public health problem as it associates with age-related fractures (Yang *et al.*, 2006). Bone mass starts to decline around age of 35 and continually decreases thereafter in both men and women. Therefore, the occurrence of osteoporosis increases with age. In addition,

osteoporosis with hip fractures is two and a half times more common in women compared to men (Ammer, 2009).

Other additional factors that contribute to the risk of osteoporosis include low level of estrogen for a significant portion of a women's lifetime, lack of calcium in diet, inadequate exercise, family history with osteoporosis, excessive alcohol usage, and smoking (Scheiber and Torregrossa, 1998).

Currently available treatments for osteoporosis are designed to reduce bone loss or stimulate bone formation. The types of treatments include dietary, lifestyle changes and pharmacological intervention. Example of effective treatments prove to be effective in preventing osteoporosis are; bisphosphonates, calcitonin, and selective estrogen receptor modulators (SERMs) (Davidson, 2003). However, long-term usage of these treatments can induce unwanted adverse effects in most patients. For instance, hormone therapy treatment (HRT) has been revealed to induce patient with high risk of ovarian and breast cancer (Humphries and Sabrina Gill, 2003; Zhou *et al.*, 2008). This risk can be life-threatening if untreated properly.

Nowadays, most people searching for an alternative medicine in order to treat their health problems and this include osteoporosis. Alternative medicine has become an option due to the long-term side effects cause by synthetic drug or modern medicine although advantages of modern medicine in curing health problems far exceed the disadvantages.

Herbal remedy such as St. John's wort, gingko biloba, Echinacea, garlic, saw palmetto, ginseng, goldenseal, aloe, Siberian ginseng and valerian have been used among Americans for health maintenance (Mar and Bent, 1999). In Malaysia, Malay women prefer traditional modalities in health practices including the use of herbal

preparation for postpartum care. This practice is thought to revitalize and recover the reproductive functions (Soon and Hasni, 2005). Example of herbal used is gall of *Quercus infectoria Olivier* or "Manjakani" in Bahasa Malaysia (Grieve, 1971).

Quercus infectoria Olivier (Fagaceae) is a small tree widely distributed in Greece, Asia Minor, Syria and Iran. Attack from gall wasp, *Cypnis gallae-tincotoriae* (Samuelson, 1992) causing the tree bears galls to emerge on its young branches which has been used in combination with other herbs for remedy by postpartum women to restore the elasticity of the uterine wall (Muhamad and Mustafa, 1994). According to previous study, QI galls possesses pharmacological properties such as astringen (Dar *et al.*, 1976), anti-parkinsonian, anti-tremorine (Dar and Ikram, 1979), and anti-diabetic (Hwang *et al.*, 2000). It also beneficial for diseases due to biological agents by possessing antiviral (Hussein *et al.*, 2000), antibacterial (Fatima *et al.*, 2001), antifungal (Digraki *et al.*, 1999), larvicidal (Redwane *et al.*, 2002) properties. Furthermore, anti-inflammatory activity (Kaur *et al.*, 2004), and antioxidant activity as one of the its properties also have been proven.

Beside pharmacological properties, *Quercus infectoria* (QI) galls also have various important mineral compositions such as calcium, phosphorus, portassium, magnesium, iron, manganes, zinc and nickel (Vermani *et al.*, 2010). Calcium and phosphorus are essential minerals for bone mineralization. Therefore, QI might beneficial in preventing bone disease such as osteoporosis. In addition, this herb also consists of phytochemicals which might possess anabolic effect on bone. The main constituent found in QI galls are tannin (50-70%), gallic acid (2-4%), ellagic acid, starch and sugar (Bruneton, 1999). Tannin is a phenolic compound that able to act directly on bone by modulating the osteoblast proliferation, differentiation, and mineralization (Habauzit and Horcajada, 2008; Trzeciakiewicz *et al.*, 2009). A review

paper has concluded that these phenolics or polyphenols can enhance the activity of osteoblast and mineralization, as well as osteoclastogenesis inhibition (Hermizi *et al.*, 2012).

Therefore, polyphenols and important minerals possess by QI which give anabolic effects on osteoblast has been the significant reasons of this study, as well as due to increase demand for alternative medications for osteoporosis treatment (Mitra *et al.*, 2001). Based on recent study, aqueous crude extract of QI was found to enhanced cell proliferation and increased bone formation markers (ALP and osteocalcin) (Hermizi *et al.*, 2015).

Hence, the semi-purified fractions of QI were prepared by column chromatography methods in this study. The extraction and purification techniques were applied in order to obtain the semi-purified polyphenol fractions from the crude extract of QI that may produce the most potent effect on bone. Then, the semi-purified fractions of QI was used to observe the proliferation, ALP activity, morphology characteristics and mineralisation activity on osteoblast through various techniques such as Enzyme-Linked Immunoabsorbent Assay (ELISA) and microscopy observation.

1.2 Problem statement

Bone is a mineralised connective tissue that provides protection to the organs of the body. Bone is formed before birth and continues to maintain its structural integrity until death, by a process known as bone remodelling. Balance process of bone resorption and formation occur through activity of osteoclasts and osteoblasts during bone remodelling. Osteoclasts and osteoblasts are responsible in bone resorption and bone formation process, respectively. These activities can be measure through expression of markers. These markers are divided into two types; bone formation markers (alkaline phosphatase, osteocalcin, type 1 collagen, osteopontin, and bone sialoprotein) and bone resorption markers (pyridinoline, hydroxyproline, free gamma carboxyglutamin acid, and tartarat-resistant acid phosphatase) (Seibel, 2005). However main focus in this study is bone formation markers especially alkaline phosphatase (ALP), which is the most specific biochemical marker of bone formation in detecting the osteoblastic activity.

Balance in bone resorption and formation is essential as imbalance in these activities can cause deterioration of micro-architectural of bone tissue that can lead to loss of bone mass and brittle bone. Individual suffered from this pathological situation usually diagnosed with osteoporosis. Osteoporosis is one of the major health problems among elderly especially when it is associated with bone fractures. Bone fracture especially hip bone fracture can lead to poor quality of life. Currently there are two pharmacological approaches used to treat osteoporosis. These include anti-resorptive agents such as bisphosphonates and calcitonin that used to inhibit osteoclastic bone resorption, and the anabolic agents that stimulate the osteoblastic bone formation. However, there are unwanted adverse effects from long-term usage of these treatments

for osteoporosis patient such as atypical fracture of bone and the risk of having esophageal, breast and ovarian cancer. Furthermore, these treatments also lack of anabolic agents to stimulate bone formation process.

In Malaysia, *Quercus infectoria* (QI) is one of the popular medicinal plants used traditionally in postpartum care and treatment of various ailments. Previous study has reported that the main phytochemical content of QI galls is phenolic compound or polyphenols. These compounds might have an anabolic effect on bone by modulating the proliferation, differentiation, and mineralisation of osteoblasts. In addition, the presence of mineral compositions such as calcium, phosphorus, magnesium, iron, and zinc are also important for bone metabolism. The presence of these beneficial compositions of QI galls turns this traditional preparation into a new natural and potential source for osteoporosis treatment, as well as overcome a wide range of adverse effects causing by current synthetic osteoporotic drugs.

1.3 Objectives of Study

1.3.1 General Objective

General objective of this study is to performed investigation on the effects of semi-purified fractions of *Quercus infectoria* (QI) on human fetal osteoblast (hFOB 1.19) cell line for its proliferative, alkaline phosphatase (ALP), morphology, as well as formation of mineralized deposits (calcium and phosphate).

1.3.2 Specific Objectives

These are the specific objectives of this study:

- To prepare semi-purified fractions from QI galls and determine its proliferative activity (EC_{50}) of hFOB 1.19 cell lines.
- To determine the ALP activity and formation of calcium and phosphate of the hFOB 1.19 cell lines treated with semi-purified fractions of QI.
- To access the morphological changes of the hFOB 1.19 cell lines treated with semi-purified fractions of QI by using inverted microscopy and image analyser.
- To detect the formation of mineralised deposits such as calcium and phosphate of the hFOB 1.19 cell lines treated with semi-purified fractions of QI after staining with von Kossa and Alizarin red S.

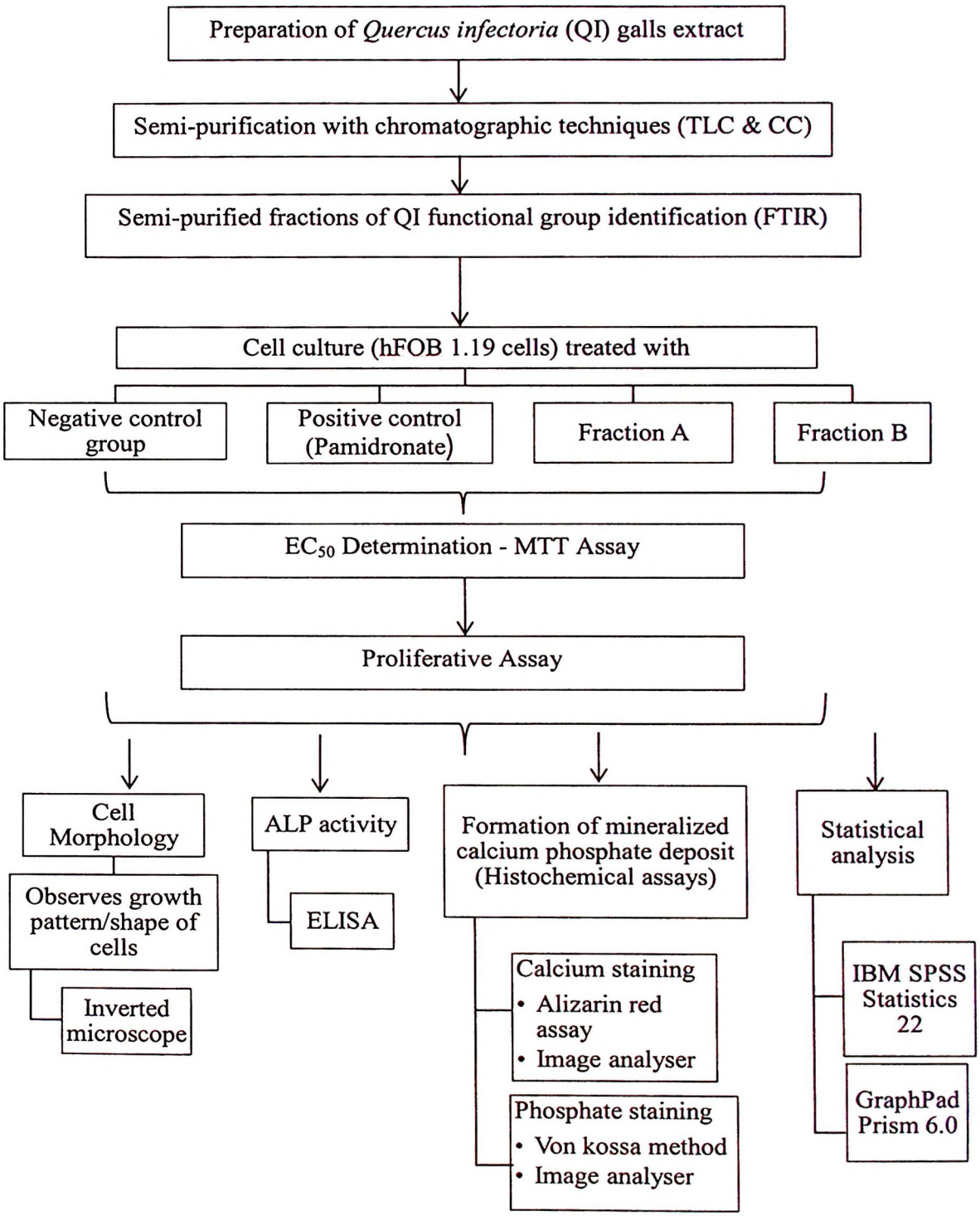
1.4 Hypotheses

These are the hypotheses of this study:

- EC_{50} of the hFOB 1.19 cell line treated with semi-purified fractions of QI is lower as compare to EC_{50} of the hFOB 1.19 cell line treated with pamidronate.
- Proliferation of hFOB 1.19 cell line treated with semi-purified fractions of QI is greater as compare to hFOB 1.19 cell line treated with pamidronate.

- hFOB 1.19 cell line treated with semi-purified fractions of QI has high level of bone formation markers (ALP) as compare to bone formation markers expressed by hFOB 1.19 cell line treated with pamidronate.
- Mineralisation of hFOB 1.19 cell treated with semi-purified fractions of QI is higher as compare to hFOB 1.19 cell line treated with pamidronate.

1.5 Experimental Design



CHAPTER 2

LITERATURE REVIEW

2.1 Bone

2.1.1 Structure and Function

Bone is a mineralised connective tissue which also composed of other several different tissues like cartilage, dense connective tissues, epithelium, adipose tissue, and nervous tissue. Together they work and makes up the skeletal system which contributes about 18% to human body weight (Tortora and Derrickson, 2011). Adult human skeleton system consist of 213 bones where 126 bones come from appendicular skeleton, 74 bones from axial skeleton and 6 bones of auditory ossicles (Clarke, 2008).

Bones can be categorized into four general types which are long bones, short bones, flat bones and irregular bones. Long bones which are formed by combination of endochondral and membranous bone formation consist of clavicles, humeri, radii, ulnae, femurs, tibiae, metacarpals, fibulae, phalanges, and metatarsals. Short bones include patellae, sesamoid bones, carpal and tarsal bones. Flat bones which form by membranous bone formation consist of skull, mandible, scapulae, sternum, and ribs. Irregular bones consist of various shape include vertebrae, sacrum, coccyx, and hyoid bone. Sesamoid bones develop in certain tendons and can be found at tendons where it passes over an angular structure such as hands and feet. It helps to protect the tendons from excessive wear and assist in movement by changing angle of tendon as they pass to their attachments. In addition, patella (knee cap) also is a kind of sesamoid bone (Clarke, 2008).

The skeletal system provides various functions for the body such as structural support and aid in movement and locomotion by providing levers for muscles. It also helps to protect important internal organs and structure. Apart from that, it performs the regulation of mineral homeostasis and acid-base balance. The skeleton also acts as a reservoir of growth factors and cytokines, and provide environment for haematopoiesis within marrow spaces (Clarke, 2008).

2.1.2 Histology of bone

Compact bone and spongy bone made up almost 80% and 20% of adult human skeleton overall, respectively (Eriksen *et al.*, 1994). Compact bone is the strongest form of all bones and makes up the bulk of the diaphysis of long bones which provide support and protection to the body. It is compact, dense, solid, and surrounds the marrow space. In contrast to compact bone, spongy bone which located in interior of bone (protected by a covering of compact bone) composed of honeycomb-like network of trabecula plates and rods dispersed in the bone marrow compartment and osteon can be found in both compact and spongy bones.

Compact osteons are called Haversian systems which are cylindrical in shape and form a branching network within compact bone. Each osteon consist of concentric lamellae arranged around a central (harversian) canal. The concentric lamellae which resembled the growth of tree are circular plates of mineralized extracellular matrix of increasing diameter surrounding by a small network of blood vessels, lymphatics and nerve. The Haversian systems interconnect with one another via Volkmann's canals that pierce the columns obliquely to the Haversian canals (Figure 2.1).

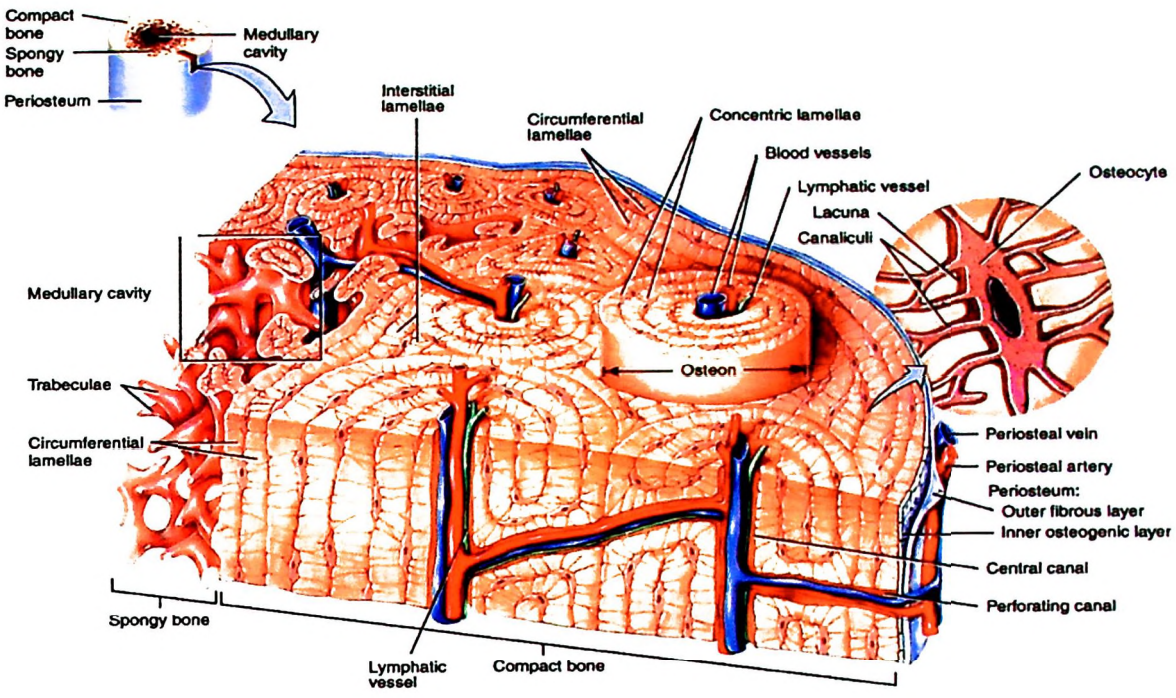


Figure 2.1: Schematic diagram of compact bone. Adapted from Tortora and Derrickson (2011)

Haversian systems remodelling involve continuous osteoclastic and osteoblastic activities which convert active cortical osteons to inactive cortical osteons. Osteocyte which performed bone resorption can be found in lacunae (small spaces between the concentric lamellae) while osteoblast that responsible for formation of new bone matrix lay down in the lamellae of the bone. During bone rebuilding, interstitial lamellae (fragments of older osteon) have been partially destroyed and replaced by deposition of new lamellae. The Haversian canal diameter decreases and osteoblasts are trapped as osteocytes in spaces called lacunae in the matrix through this activity. Thus, osteocytes are arranged in concentric rings within lamellae. Numerous minute interconnecting canals called canaliculi that radiates in all directions from lacunae. Inside canaliculi are slender finger-like processes of osteocytes (Tortora and Derrickson, 2011). Increased cortical remodelling causes an increase in cortical porosity and decrease in cortical bone

mass. Healthy aging adults normally experience thinning of the cortex and increased cortical porosity (Clarke, 2008).

Spongy bone, also referred as trabecular or cancellous bone is located in the interior of a bone which protected by a covering of compact bone. It consists of lamellae that are arranged in irregular pattern of thin columns called trabeculae. Spongy bone is different from compact bone in term of weight and function. Spongy bone is light which reduces the overall weight of bone. Reduction of weight allows bone to move readily when pulled by a skeletal muscle. It also helps to support and protect the red bone marrow. In contrast to compact bone, spongy bone does not have osteons. In other words, it does not contain Haversian systems and osteocytes exchange metabolites via canaliculi with blood sinusoids in the marrow. Spaces that are visible to the unaided eye between trabeculae are filled with red blood marrow in bones which produce the red blood cell and yellow bone marrow (adipose tissue) in other type of bones. The trabeculae also are lined by endosteum that consists of osteoprogenitor cells, osteoblasts, and osteoclasts (Figure 2.2).

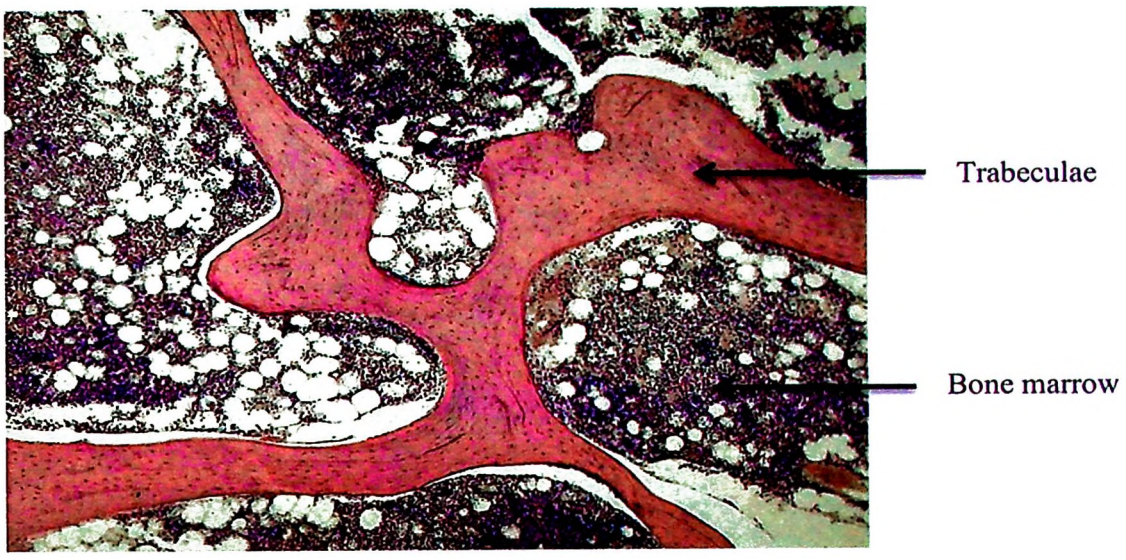


Figure 2.2: Spongy bone with x50 magnification of H&E stain. Adapted and edited from Department of Histology, Jagiellonian University Medical College (2006).

Osteoprogenitor cells are resting cells that can differentiate into an osteoblast and secrete bone matrix. They are originated from mesenchymal stem cells in the bone marrow that have potential to differentiate into various different types of cells including fibroblast, adipocytes and muscle cells. These cells mainly found on the external and internal surfaces of bones and may also reside in the microvasculature supplying bone. Osteoprogenitor cells appear as flattened or squamous cells with lightly staining, elongate, or ovoid nuclei and inconspicuous acidophilic or slightly basophilic cytoplasm. Osteoblasts which derived from osteoprogenitor cells are differentiated bone-forming cell that secretes bone matrix during a process called osteogenesis (process of new bone formation). They are recognized by their cuboidal or polygonal shape and their aggregation into a single layer of cells lying in opposition to the forming bone. Osteoblasts then develop into osteocytes as they completely surrounded by osteoid or bone matrix (Ross and Pawlina, 2011).

Osteocytes are mature bone cell enclosed by bone matrix which previously secreted as an osteoblast. Each osteocyte resides within pockets in the matrix called lacunae that conforms to the shape of the cell. These cells extend cytoplasmic processes through canaliculi in the matrix to connect with processes of neighbouring osteocytes and bone-lining cells by means of gap junctions. The transport of nutrients throughout the bone is able to be performed through aid of these features. Osteocytes help to maintain and monitor the protein and mineral content of the matrix, as well as participate in the repair of damaged bone. Osteoclasts are large giant, multinucleate cells that can be found at sites where bone is being removed. They are not related to osteoblasts as they are derived from the fusion of mononuclear hemapoietic cells that give rise to granulocyte and monocyte cell lineage. Bone matrix is dissolve by osteoclasts through expression of acids and enzymes like matrix metalloproteinases (MMPs) and stored minerals are released through a process called osteolysis. Proper balance of osteoblasts and osteoclasts actions is essential to maintain proper strength of the bones (Ross and Pawlina, 2011).

2.1.3 Osteoblast proliferation

Osteoblasts are cuboidal cells which derived from mesenchymal stem cells (MSC) responsible in bone formation and mineralization. They are located along the bone surface comprising 4–6% of the total resident bone cells. These cells possess morphological characteristics of protein synthesizing cells, including abundant rough endoplasmic reticulum and prominent Golgi apparatus (Capulli *et al.*, 2014). They also secrete the osteoid toward the bone matrix (Damoulis and Hauschka, 1997).

Previous *in vitro* studies demonstrates the three differentiation stages of osteoprogenitor cells to osteoblasts. These stages include cell proliferation, matrix maturation, and matrix mineralization. During first stage (cell proliferation stage), osteoprogenitors undergo proliferation phase by dividing themselves to increase their numbers. According to Ross and Pawlina (2011), transcription factor called core binding factor alpha -1 (CBFA1) is the key factor that triggers the differentiation of osteoprogenitor cells. Mesenchymal stem cells differentiate into immature osteoblasts and it also help to synthesis and expressed bone morphogenetic proteins (BMPs) that plays role in the differentiation of osteoblast through the actions of Runx2, osterix, and b-catenin, where Runx2 is a master gene of osteoblast differentiation (Ducy *et al.*, 1997; Grigoriadis *et al.*, 1988; Komori, 2006).

Proliferation phase is when a pool of osteoblast progenitors expressing Runx2 and ColIA1 genes has been established during osteoblast differentiation. Osteoblast progenitors show alkaline phosphatase (ALP) activity, and are considered preosteoblasts (immature osteoblast) in this phase. High levels of osteopontin (OPN) expressed by immature osteoblast and then it differentiate into mature osteoblasts, which express high levels of osteocalcin (OCN). Finally, the mature osteoblasts are embedded in the bone matrix to become osteocytes. There is an increase in the expression of osteoblast-specific transcription factor osterix (Osx) and in the secretion of bone matrix proteins such as osteocalcin (OCN), bone sialoprotein (BSP) I/II, collagen type I (COLIa1) and parathyroid hormone receptor type I (PTH1R) during the transition of preosteoblasts to mature osteoblasts. In addition, the osteoblasts undergo morphological changes where they becoming large and cuboidal cells (Ducy *et al.*, 1997; Fakhry *et al.*, 2013; Komori, 2006).

All of these genes are upregulated and downregulated during proliferation, matrix maturation and mineralization phases asynchronously (Aubin, 1998). In general, during early phase ALP is upregulated and its expression decline once osteoblast matured. Expression of OPN then increases during progenitor proliferation and later during matrix maturation. This followed by the expression of OCN, BSP, COLIa1 and PTH1R during final stage which is matrix maturation and mineralisation. Generally, this is the heterogeneity in osteoblast gene expression.

2.1.4 Bone remodelling and mineral homeostasis

Bone remodelling is a physiological process that maintains the integrity of the skeleton by removing old bone and replacing it with a young matrix and it begins before birth and constantly continues until death (Sagalovsky, 2013). 90% of the bone surfaces are inactive when other 10% of bone surfaces in the skeleton are undergoing active remodelling. This remodelling process occurs over several weeks which involve a number of cellular functions directly towards to co-ordinated resorption and formation of new bone. Bone remodelling process rises in perimenopausal and early postmenopausal women however decline with further aging but the rate continues faster than in premenopausal women. As for men, bone remodelling is thought to increase mildly.

Bone remodelling is mainly performed by clusters of bone-resorbing osteoclasts and bone-forming osteoblasts arranged within temporary anatomical structures known as "basic multicellular units" (BMUs). These cells sequentially carry out resorption of

old bone and formation of new bone. There are five phase in remodelling cycle which includes activation, reversal, formation, and termination (Raggatt and Partridge, 2010)

Activation Phase is the first stage of bone remodelling where the detection of an initiating remodelling signal occurs. There are several forms of signal such as mechanical strain on the bone that results in structural damage or hormone (e.g. estrogen or PTH) action on bone cells resulting to more systemic changes in homeostasis (Raggatt and Partridge, 2010). From these signals, the circulating mononucleated osteoclast precursors are recruited at the site of remodelling, followed by penetration into bone lining cell layer and fusion of the mononuclear cells to form multinucleated preosteoclasts (Figure 2.3).

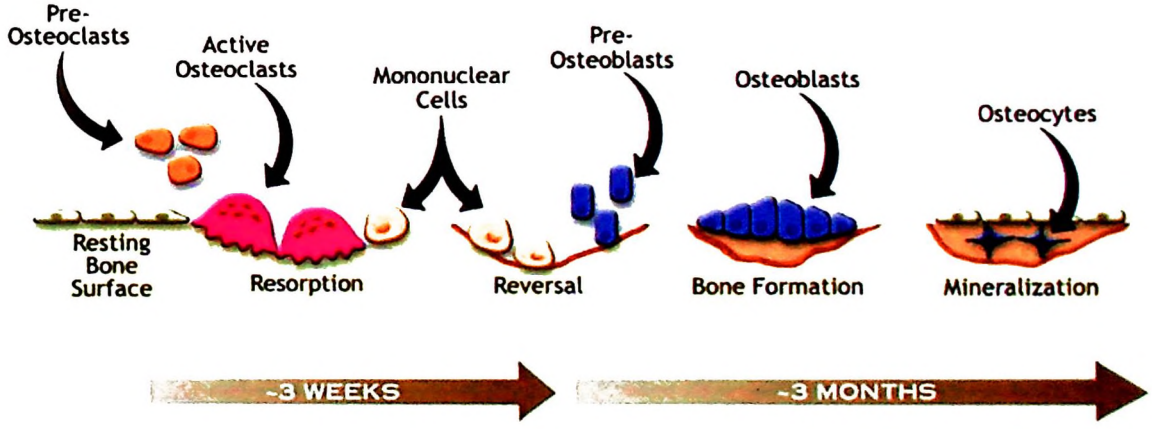


Figure 2.3: Schematic diagram of bone remodelling process. Consist of resting, resorption, reversal and formation phases. Adapted from Vaňhara (2015).

Resorption phase begins as osteoclastic resorption erodes a resorption lacuna that attach to the bone surface. Formation and activity of osteoclasts is controlled by cells of the osteoblast lineage that help to recruit osteoclast precursors to the remodelling site along with the modulation of the master osteoclastogenesis cytokines,

CSF-1, RANKL, and OPG expression in response to PTH (Tang *et al.*, 2009; Teti). CSF-1 responsible in stimulates proliferation and survival of osteoclast precursors, whereas RANKL and OPG stimulate and inhibit, respectively, osteoclast differentiation. In response to a variety of signals such as parathyroid hormone (PTH), tumor necrosis factor-α (TNF-α) and interleukin-1 (Il- 1), osteoblast and stromal cells produce RANKL membrane bound factor, promotes the binding of RANKL to the cytoplasmic membrane receptor RANK (receptor activator of NF-kB), which is a member of the tumor necrosis factor (TNF) receptor super family and subsequently induces both osteoclast differentiation and activation (Sagalovsky, 2013). In contrast, effect of RANKL is inhibited by OPG, a soluble decoy receptor for RANKL that prevents osteoclast development and subsequent bone resorption (Boyce, 2007). Resorption cavities then formed on the cancellous bone surface called Howship's lacunae resulting from osteoclastic bone matrix digestion together with the formation of cylindrical tunnels within the cortex (Dempster *et al.*, 2012). Early resorption process is carried out by multinucleated osteoclasts and then followed by mononucleated cells.

During reversal period, transition from osteoclast to osteoblast activity occurs where osteoclasts undergo apoptosis whilst osteoblasts are recruited and begin to differentiate. They are recruited to the site of remodelling to initiate formation phase. After withdrawal of the osteoclast from the resorption pit, bone-lining cells which include monocytes, osteoclasts and preosteoblasts, enter the Howship's lacuna and clean its bottom from bone matrix leftovers. Collagen remnants are removed and bone surface is prepared for subsequent osteoblast-mediated bone formation. Receiving or producing coupling signals for transition from bone resorption to bone formation within the BMU may be the final role of the reversal phase.

Formation phase begin through coordination of coupling signals. These signals are produced by osteoblast and osteoclasts where they coordinate this transition and direct bone formation precisely to sites of bone resorption. Previous studies proposed several candidate coupling mechanisms which include the soluble molecule sphingosine 1-phosphate and the cell-anchored EphB4-ephrin-B2 bidirectional signalling complex. Osteoclasts secrete sphingosine 1-phosphate to induce osteoblast precursor recruitment and promote mature osteoblast survival (Pederson *et al.*, 2008). Osteoclasts also expressed ligand ephrin-B2, whereas EphB4 receptors are expressed on osteoblasts. Forward signalling through EphB4 into osteoblasts enhances osteogenic differentiation and suppression of osteoclast differentiation are enhances by forward signalling through EphB4 and reverse signalling through ephrin-B2, respectively. In addition, osteoclasts differentiation is supressed by inhibiting the osteoclastogenic c-Fos/NFATc1 cascade (Zhao *et al.*, 2006). In general, EphB4-ephrin-B2 signalling complex possess ability to activate bone formation and inhibit bone resorption simultaneously at this critical transition point of the remodelling process.

Remodelling cycle concludes when an equal quantity of resorbed bone has been replaced. Termination phase occurs when terminating signal(s) expressed to inform the remodelling machinery to cease work however, this terminating signal is still unknown are largely unknown although a role for osteocytes is emerging. Previous research shows the expression of sclerostin that loss in order to initiate osteoblastic bone formation, likely returns toward the end of the remodelling cycle (Henriksen *et al.*, 2009). Following mineralisation, mature osteoblasts undergo apoptosis, revert back to a bone-lining phenotype or become embedded in the mineralized matrix. The cells then gradually flatten as they slow production and become quiescent lining cells. Some of the osteoblast differentiate into osteocytes and remain in the matrix. The resting bone

surface environment is re-established and maintained until the next wave of remodelling begin (Raggatt and Partridge, 2010).

Bone remodelling is mediated by a balance of osteoblast and osteoclast cell activity, which together, maintain bone mass and mineral homeostasis. Both decreased bone formation and increased bone resorption may result in bone loss. Therefore, the stimulation of bone formation may be another important factor for the prevention and treatment of bone loss. The amount of calcium absorbed in the intestine depends on habitual calcium intake.

Bone carry out important functions in the body, such as locomotion, support and protection of soft tissues, calcium and phosphate storage, and harbouring of bone marrow. It acts as reservoir of calcium, where this calcium homeostasis is controlled by regulatory pathways of gastrointestinal (GI) tract and the kidney. In addition, this regulation is mediated by osteoblast and osteoclasts in bone. Gastrointestinal tract absorbs calcium from diet, which then enters the extracellular fluid (ECF) space and diffuses into bone through the process of mineralisation of the organic matrix of bone, osteoid. As for unabsorbed calcium, it will be passed into the faeces. There are three hormones involve in regulation of bone and bone mineral metabolism including parathyroid hormone (PTH), calcitonin (CT), and vitamin D. These hormones help to regulate three most important bone minerals like calcium, magnesium and phosphorus. Despite its inert appearance, bone is a highly dynamic organ as it performs many important functions and continuously resorbed by osteoclasts and neoformed by osteoblasts.

2.1.5 Bone formation marker [Alkaline Phosphatase (ALP)]

Bone remodelling process constantly occurs in balance way which consists of bone formation and bone resorption activities, as previously discussed. These processes involve the expression of markers that indicate the remodelling process of bone; either bone formation or bone resorption takes place. Bone formation markers are products of active osteoblasts expressed during different phases of osteoblast development. Different aspects of osteoblast function and of bone formation are considered to be reflected by these markers. There are several markers expressed that related to the formation of bone process. These include an enzyme (alkaline phosphatase) and three byproducts of bone matrix synthesis (osteocalcin and amino- and carboxy-terminal procollagen I extension peptides. Among these bone formation markers, ALP enzyme and osteocalcin are the specific biochemical markers of bone formation where it also reflects osteoblastic activity (Duda *et al.*, 1988; Seibel and Woitge, 1999). All markers of bone formation are measured in serum or plasma and examples of cone formation markers commercially available are stated in Table 1.

Table 2.1: Commercially available bone formation markers

Bone formation markers	**Mechanism**	**Specimen type**	**Company**
Bone specific alkaline phosphate (bALP)	Secreted by osteoblast	Serum	Hybritech Metra Biosystems
Osteocalcin (bone Gla protein)	Secreted by osteoblast	Serum	CIS Bio. Osteometer Biotech
Procollagen type I C propeptide	Collagen-based	Urine/Serum	Osteometer Biotech
Procollagen type I N propeptide	Collagen-based	Urine/Serum	Ostex
Free deoxypyridinoline	Collagen-based	Urine	Metra Biosystems

Adapted and modified from Kress and Mizrahi (1999)

ALP is a membrane-bound tetrameric enzyme that attached to glycosyl-phosphatidylinositol moieties located on the outer cell surface and widely spread (Stinson and Hamilton, 1994). The precise function of the enzyme is yet unknown, but it obviously plays an important role in osteoid formation and mineralisation (Harris, 1990). The expression of alkaline phosphatase starts after cell proliferation of osteoblasts is ceased and reaches a maximum during matrix maturation, but declines during matrix mineralisation (Rosalki and Foo, 1984). Four gene loci which include three tissue-specific and one non-tissue-specific gene on chromosome code the physiological isoforms of ALP (Lian and Stein, 1999). Bone, liver, and kidney ALP are the latter that encodes for the most abundant isoforms. Post-translational modifications in the carbohydrate moiety help to differentiate these non-specifically encoded isoenzymes (Langlois *et al.*, 1994). Approximately 50% of the total ALP activity in

serum is expressed from the liver in adults with normal function liver and another 50% arises from bone. In contrast to children and adolescents, the bone-specific isoenzyme predominates almost up to 90% due to skeletal growth (Green *et al.*, 1971; Seibel, 2005).

Differentiation between the two main isoforms of circulating ALP is a challenge as both bone and liver expressed ALP marker. Results of bone ALP measurements may be high in subject with high liver disease (expression of high ALP activity), leading to false positive results. However, many techniques have been developed to differentiate between these two main isoforms of circulating ALP, including heat denaturation, electrophoresis, precipitation, selective inhibition and, more recently, immunoassays (Seibel, 2005). From these techniques, it can be concluded that B-ALP measurement draws a clearer difference between normal and pathological states with the upper reference range values, and thus increases diagnostic specificity in bone diseases. Among of these techniques, electrophoresis remains the gold standard for detecting decreased B-ALP concentration/activity and for confirmation of increased B-ALP in cases of severe liver diseases (Čepelak and Čvorišćec, 2009). Bone ALP is expressed in high amounts during bone formation phase of remodelling process, thus it has potential act as an excellent indicator for total bone formation activity (Moss, 1992). Although there is challenge exist in bone formation measurement, bone-specific ALP (BAP) isoenzyme is increasingly preferred as indicator to bone formation due to its higher specificity compared to other markers as previously mentioned.