PROLIFERATION EFFECTS OF HUMAN FETAL OSTEOBLAST CELL LINE (hFOB 1.19) TREATED WITH *QUERCUS INFECTORIA* GALLS EXTRACT

by

DALILA ROZELAN

Dissertation submitted in partial fulfillment of the requirements for the Degree of Bachelor of Health Sciences (Biomedicine)

May 2013

ACKNOWLEDGEMENT

Alhamdulillah, praise to Allah S.W.T upon His blessing I manage to complete my Biomedical Final Year Project Thesis in School of Health Sciences, Universiti Sains Malaysia. I am greatly indebted to my supervisor, Dr. Hermizi Hapidin, and co-supervisor, Dr. Hasmah Abdullah, for their guidance, great ideas and persistent help in regard to this research to complete my thesis. A great appreciation also to my course coordinator of Final Year Project, Dr. See Too Wei Chon who monitored, guided and gave a great motivation in finishing this research for two semesters.

I would also like to acknowledge Universiti Sains Malaysia for finding this research under the Short Term Grant 304/PPSK/61312042. I personally want to deliver a special thank to Wan Nurhidayah Wan Hanaffi, the postgraduate student who is also a research assistant under supervision of Dr. Hermizi Hapidin, for her continuous help and guidance. Her warmth cooperation and helping hand had eased me in facing the hardship in finishing my research project. Other than that, I also would like to thank to all my colleagues whose involve in teaching, giving ideas, and support me during my final year project.

Finally, I am very please to give my warming and heartiest thank to my family for their continuous support, motivation and inspiration. Last but not least, I would like to give my regard to all who, directly and indirectly, have lent their helping hand in this venture. May Allah bless all of you.

TABLE OF CONTENTS

CHAPTER 3: MATERIAL AND METHODOLOGY

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABC	Avidin-biotin-peroxidase complex
Abs	Absorbance
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
TMB	3,3',5,5'-tetramethylbenzidine

PROLIFERATION EFFECTS OF HUMAN FETAL OSTEOBLAST CELL LINE (hFOB 1.19) TREATED WITH *QUERCUS INFECTORIA* GALLS EXTRACT

ABSTRACT

The *Quercus infectoria* (QI) galls are traditionally reported to have great medicinal value such as astringent effect, anti-pyretic, anti-diabetic, anti-tremorine, anti-inflammatory, anti-bacterial, anti-viral and anti-oxidant activity. Those activities were postulated due to the presence of polyphenols which are proven to have an anabolic effect on the bone metabolism by modulating the proliferation, differentiation and mineralization of osteoblasts. Furthermore, QI galls also contain mineral compositions such as calcium, phosphorus, magnesium, iron, zinc, oxygen, potassium, aluminium, carbon, manganese, nickel and silica, which are important for bone metabolism. The present study was undertaken to evaluate the effect of QI galls extract on proliferation, bone formation markers such as alkaline phosphatase (ALP) and osteocalcin level, and morphology of hFOB 1.19 cells. The cells were cultured in Dulbecco's modified eagle medium F12 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and then were treated with QI galls extract at various concentrations ranging from 0.1 to 99.0 µg/ml for 72 hours. The proliferation activity of hFOB 1.19 treated with QI galls extract was measured by MTT assay with median effective concentration (EC_{50}) 10.30 µg/ml. This concentration was more effective compared to positive control drug, pamidronate which exert the EC_{50} at 16.09 µg/ml. In addition, the functional activity such as ALP and osteocalcin levels, were measured by enzyme-linked immunosorbent assay (ELISA) method at day 1, 3, 7, 10 and 14. The ALP activity for hFOB 1.19 treated with QI galls extract were increased in time dependent manner. The ALP highest activity in the QI galls

extracts treated cells recorded as 38.79 U/L. This trend were also observed in hFOB 1.19 treated with pamidronate. However, the cells treated with QI galls extract exerted higher ALP activity compared to cells treated with pamidronate (28.03 U/L). Meanwhile, osteocalcin level for hFOB 1.19 treated with QI galls extract also were increased in time dependent manner. The highest osteocalcin level in QI galls extract treated cells was 2.18 ng/ml. However, the osteocalcin level in hFOB 1.19 treated with pamidronate peaked at day 10 (1.54 ng/ml) and the level decreased afterward. Overall, the cells treated with QI galls extract still exerted higher osteocalcin level compared to cells treated with pamidronate. The morphology of hFOB 1.19 was observed by using inverted microscope from day 1 until day 14. The QI galls extract treated cells showed an increment in the cell number (percentage of raise in cell no. on day 1 = 81.48 % and day 14 = 96.18%). More interestingly, cells treated with QI galls extract remain uniformly elongated and over-confluent. Inversely, cells treated with pamidronate observed as rounded and less density (percentage of raise in cell no. on day 1 = 76.19 % and day 14 = 92.19 %). In conclusion, the result of functional activity and morphological changes of the cells were consistent and the effect of QI galls extract on proliferation of osteoblasts was much better than pamidronate. Thus, these suggest that QI galls extract might be a potent anabolic agent that able to enhance the proliferation, differentiation and mineralization of the osteoblast cells.

KESAN EKSTRAK MANJAKANI (*QUERCUS INFECTORIA* GALLS) KE ATAS PROLIFERASI SEL OSTEOBLAS MANUSIA (hFOB 1.19)

ABSTRAK

Secara tradisional, manjakani dipercayai mempunyai nilai perubatan yang tinggi kerana kesan astringen, anti-piretik, anti-diabetik, anti-tremorin, anti-radang, anti-virus dan anti-oksidan, dan ini disebabkan oleh kandungan polifenol yang terdapat di dalamnya yang mana terbukti mempunyai kesan anabolik pada metabolisme tulang dengan memodulasikan proliferasi, pembezaan dan pemineralan sel osteoblas. Tambahan pula, manjakani juga mempunyai komposisi mineral seperti kalsium, fosforus, magnesium, zat besi, zink, oksigen, kalium, aluminium, karbon, mangan, nikel dan silika, yang penting untuk metabolisma tulang. Kajian ini telah dijalankan untuk menilai kesan ekstrak manjakani pada proliferasi, petanda pembentukan tulang seperti tahap alkaline phosphatase (ALP) dan osteocalcin, dan morfologi sel hFOB 1.19. Sel tersebut dikulturkan dalam medium Dulbecco's modified eagle F12 (DMEM/F12) yang ditambah dengan 10% serum fetal bovine dan 1% penisilin/streptomisin, dan kemudiannya dirawat dengan ekstrak manjakani pada kepekatan antara 0.1 to 99.0 µg/ml selama 72 jam. Proliferasi sel hFOB 1.19 yang dirawat dengan ekstrak manjakani diukur dengan ujian MTT dan kepekatan berkesan ekstrak manjakani (EC_{50}) ialah 10.30 µg/mL. Kepekatan ini adalah lebih berkesan berbanding dengan ubat kawalan positif, pamidronate dengan EC_{50} 16.09 µg/mL. Di samping itu, aktiviti fungsional sel seperti tahap enzim ALP dan osteocalcin, diuji dan diukur dengan kaedah 'enzyme-linked immunosorbent assay' (ELISA) pada hari 1, 3, 7, 10 dan 14. Aktiviti ALP bagi sel hFOB 1.19 yang dirawat dengan ekstrak manjakani adalah meningkat mengikut masa. Nilai tertinggi aktiviti ALP bagi sel yang dirawat dengan

ekstrak manjakani direkodkan sebagai 38.79 U/L. Keputusan ini juga diperhatikan sama dengan hFOB 1.19 yang dirawat dengan pamidronate. Walau bagaimanapun, sel yang dirawat dengan ekstrak manjakani memberikan nilai aktiviti ALP yang lebih tinggi berbanding dengan sel yang dirawat dengan pamidronate (28.03 U/L). Sementara itu, tahap osteocalcin bagi hFOB 1.19 yang dirawat dengan ekstrak manjakani juga meningkat mengikut perubahan masa. Nilai tertinggi osteocalcin bagi sel yang dirawat dengan ekstrak manjakani adalah 2.18 ng/ml. Namun begitu, tahap osteocalcin bagi sel yang dirawat dengan pamidronate memuncak pada hari ke-10 (1.54 ng/ml) dan kemudian menurun selepas itu. Secara keseluruhan, nilai tahap osteocalcin bagi sel yang dirawat dengan ekstrak manjakani adalah lebih tinggi berbanding dengan nilai tahap osteocalcin bagi sel yang dirawat dengan pamidronate. Selain daripada itu, morfologi sel hFOB 1.19 juga dicerap dari hari pertama hingga hari ke-14 dengan menggunakan mikroskop songsang. Sel yang dirawat dengan ekstrak manjakani menunjukkan peningkatan dalam bilangan sel (peratusan peningkatan bilangan sel pada hari pertama = 81.48 % dan hari ke-14 = 96.18 %). Lebih menarik lagi, morfologi sel-sel yang dirawat dengan ekstrak manjakani adalah memanjang secara keseluruhannya (seragam) dan penuh. Sebaliknya, morfologi sel-sel yang dirawat dengan pamidronate diperhatikan lebih membulat dan kurang tumpat (peratusan peningkatan bilangan sel pada hari pertama = 76.19 % dan hari ke-14 = 92.19 %). Kesimpulannya, keputusan aktiviti funsional serta perubahan pada morfologi sel osteoblas adalah konsisten dan dengan ini, kesan ekstrak manjakani ke atas sel tersebut adalah jauh lebih baik berbanding dengan kesan pamidronate. Oleh itu, ini menunjukkan bahawa ekstrak manjakani adalah ejen anabolik yang kuat yang mungkin dapat merangsang proliferasi, pembezaan dan pemineralan sel-sel osteoblas.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Human skeleton is an important structural support that has its own homeostatic activity to maintain its structural integrity, which is carried out by osteoblasts and osteoclasts, via a process termed as bone remodeling. In healthy adult, under normal circumstances, osteoblast as well as osteoclast activity is a balanced process where the bone resorption is always followed by an equal degree of bone formation, which is indicated by the expression of various phenotypic markers such as alkaline phosphatase (ALP) and osteocalcin. Other than normal physiological bone growth, bone-specific ALP activity also correlates with bone formation rate in metabolic disease of the bone (Bolarin, 1996; Grundberg, 1993). Any imbalance of the activity often leads to pathological situations such as osteoporosis or osteopetrosis.

Osteoporosis is a metabolic bone disease characterized by low bone mass and micro-architectural deterioration of bone tissue and it is a major public health problem because of its association with age-related fractures (Yang *et al.*, 2006). The occurrence of osteoporosis increases with age, where the bone mass starts to decrease at about age 35 and continually decreases thereafter in both men and women. Somehow, osteoporosis with hip fracture is two and a half times more common in women than in men (Ammer, 2009).

Generally, the white or Asian postmenopausal women with a thin body frame is considered at risk for developing osteoporosis, with additional factors include a lack of estrogen for a significant portion of a woman's lifetime, a sustained lack of calcium in the diet or poor absorption of calcium, inadequate exercise, a family history of osteoporosis, excessive alcohol use, and having engaged in smoking (Scheiber and Torregrosa, 1998). Treatments for osteoporosis are designed to reduce the bone loss or increase bone formation, or both. The treatment consists of dietary and lifestyle changes, along with pharmacologic intervention. Hormone replacement therapy (HRT) has been shown to be effective in preventing osteoporosis. The effective treatment options for women who have been diagnosed with the disease are: bisphosphonates, calcitonin, and selective estrogen receptor modulators (SERMs) (Davidson, 2003). However, the risks of long-term treatment of osteoporosis drugs are undesirable in most patients. For example, the hormone therapy treatment (HRT) for osteoporosis patient has exposed the patient at high risk for ovarian cancer (Zhou *et al.*, 2008), and breast cancer (Humphries and Gill, 2003). The other bone disease is osteosarcoma which is arises from mesenchymal cells and is pathologically characterized by spindle cells and aberrant osteoid formation (Gill *et al.*, 2013). This can be life-threatening if untreated properly.

Musculoskeletal problems can be effectively treated but many cannot be reversed so early treatment is important to get the best outcomes. Nowadays many people are looking for alternative medicine to treat the disease they have suffered from including the treatment for cancer and osteoporosis. This happens because people are more skeptical and worries of the long-term side effects of the synthetic drug or modern

medicine rather than the cure from the drug itself, even though the advantages far exceed the disadvantages.

About one in three Americans using some type of herbal remedy includes St. John's wort, gingko biloba, Echinacea, garlic, saw palmetto, ginseng, goldenseal, aloe, Siberian ginseng and valerian (Mar and Bent, 1999). In Malaysia especially the Malay women prefer traditional modalities in health practices including the use of herbal preparation for postpartum care for instance, which is thought able to revitalize and recover the reproductive functions (Soon *et al.*, 2007). The herbal used includes gall of *Quercus infectoria Olivier* or "Manjakani" in Bahasa Malaysia.

Quercus infectoria Olivier (Fagaceae) is a small tree widely distributed in Greece, Asia Minor and Iran. The tree bears galls that emerge on its young branches due to the attack of gall wasp, *Cypnis gallae-tincotoriae* (Samuelsson, 1992). The galls are used in combination with other herbs as drinking remedy by postpartum women to restore the elasticity of the uterine wall (Muhamad and Mustafa, 1994). Pharmacological evaluation of the galls has proven its properties as astringent (Dar *et al.*, 1976), anti-parkinsonian, anti-tremorine (Dar and Ikram, 1979), anti-diabetic (Hwang *et al.*, 2000), antiviral (Hussein *et al.*, 2000), antibacterial (Fatima *et al.*, 2001), antifungal (Digraki *et al.*, 1999), larvicidal (Redwane *et al.*, 2002), anti-inflammatory activity (Kaur *et al.*, 2004) and also the antioxidant activity of ethanolic extract of *Quercus infectoria* galls (Kaur *et al.*, 2008).

In addition, *Quercus infectoria galls* have various important mineral compositions such as calcium, phosphorus potassium, magnesium, iron, manganese,

zinc and nickel (Vermani *et al.*, 2010). These mineral compositions, especially calcium and phosphorus, are essential for bone mineralization, thus, may provide benefit to prevent bone disease like osteoporosis. Furthermore, *Quercus infectoria* galls also have plenty of important phytochemicals that possess an anabolic effect on the bone. The main constituents found in QI galls are tannin (50-70%), gallic acid (2-4%), ellagic acid, starch and sugar (Bruneton, 1999). Tannin, which is a phenolic compound, can act directly on the bone (Habauzit and Horcajada, 2008) by modulating the osteoblast proliferation, differentiation, and mineralization (Trzeciakiewicz *et al.*, 2009). Phenolics or polyphenols are phytochemicals which can enhance the activity of osteoblast and mineralization, as well as inhibit osteoclastogenesis (Hermizi *et al.*, 2012).

So, the presence of important minerals in *Quercus infectoria* galls, the anabolic effect of polyphenols on osteoblasts, as well as an increase demand for alternative medications that can prevent and cure osteoporosis (Mitra *et al.*, 2001), has been the significant reasons for this study. In this study, *Quercus infectoria* galls extract will be used to observe the proliferative activity on osteoblasts by Enzyme-Linked Immunosorbent Assay (ELISA) and microscopy observation.

1.2 Problem Statement

Bone is a supportive endoskeleton of vertebrates that provide protection to the organs of the body. The bone is formed before birth and continues to maintain its structural integrity until death, by a process known as bone remodeling. Bone remodeling usually involve a balance process of bone resorption and formation through the activity of osteoclasts and osteoblasts. Osteoclasts are responsible in bone resorption, whereas osteoblasts are bone cells that involve in the bone formation process. Both activities are

indicated by expression of markers which can be detected and measured. These markers are divided by two types; bone formation markers (alkaline phosphatase, osteocalcin, type I collagen, osteopontin, and bone sialoprotein) and bone resorption markers (pyridinoline, hydroxyproline, free gamma carboxyglutamin acid, and tartarat-resistant acid phosphatase). However, the focus of this study is only on the bone formation markers especially alkaline phosphatase (ALP) and osteocalcin, which are the most specific biochemical markers of bone formation in detecting the osteoblastic activity.

As mentioned before, bone resorption and formation are coupling process and must be balance. If the balance is disturbed, then the person may end up with deterioration of micro-architectural of bone tissue which may leads to loss of bone mass and brittle bone. The person who suffered with this pathological situation usually diagnosed as having osteoporosis. Osteoporosis has been recognized as the major health problem among elderly especially when it is associated with bone fractures. Bone fractures especially fracture of the hip bone has caused the person for having poor quality of life. Currently there are two pharmacological approaches used to treat osteoporosis; the anti-resorptive agents that used to inhibit the osteoclastic bone resorption, and the anabolic agents that stimulate the osteoblastic bone formation. The anti-resorptive agents, such as biphosphonates and calcitonin are currently available treatment for osteoporosis. However, these treatments have caused the unwanted long-term adverse effect to the patient such as atypical fracture of the bone and the risk of having esophageal, breast and ovarian cancer. In addition, these agents are also lack of anabolic agents to stimulate the bone formation.

There is a traditional medicine in Malaysia, the *Quercus infectoria* galls or locally known as "Manjakani", is believed to have the potent anabolic agents which are beneficial in bone metabolism. The main phytochemical content of *Quercus infectoria* galls is phenolic compound or polyphenols, which have an anabolic effect on the bone by modulating the proliferation, differentiation and mineralization of osteoblasts. Furthermore, the presence of mineral compositions such as calcium, phosphorus, magnesium, iron, zinc, and many more, is also important for bone metabolism. So, the presence of these beneficial compositions of *Quercus infectoria* galls has made this traditional preparation a new natural and potential source for the treatment of osteoporosis, as well as overcome a wide range of adverse effect produced by current synthetic osteoporotic drugs.

1.3 Objectives of Study

1.3.1 General Objective

The general objective of this study is to observe the effect of gall extract of *Quercus infectoria* on the human fetal osteoblast (hFOB 1.19) cell line for its proliferative and functional activity (alkaline phosphatase and osteocalcin levels) as well as morphology.

1.3.2 Specific Objectives

The specific objectives for this study are:

- To determine the cell proliferative activity or effective dose (EC_{50}) of osteoblasts treated with QI galls extract.

- To observe the morphological changes of osteoblast cells treated with QI galls extract by using inverted microscopy.
- To determine the ALP activity and osteocalcin level expressed by osteoblast cells treated with QI gall extract.

1.4 Hypotheses

The hypotheses of this study are:

- EC_{50} of the hFOB 1.19 cell line treated with QI galls extract is lower as compare to EC_{50} of the hFOB 1.19 cell line treated with pamidronic acid.
- The proliferation of hFOB 1.19 cell line treated with QI galls extract is greater as compared to hFOB 1.19 cell line treated with pamidronic acid.
- hFOB 1.19 cell line treated with QI galls extract has high level of bone formation markers (ALP and osteocalcin) as compared to bone formation markers expressed by hFOB 1.19 cell hFOB 1.19 cell line treated with pamidronic acid.

CHAPTER 2

LITERATURE REVIEW

2.1 Bone

2.1.1 Structure and Function

Human skeleton is a complex system, well adapted to provide structural support, involves in movement and provide a protected environment for delicate internal organs. In addition, the bone houses the blood-forming (hematopoietic) elements within the marrow spaces and also serves as the major reservoir of calcium and a number of other vital minerals (i.e. phosphate, magnesium, and potassium).

Human adult skeleton consists of total of 213 bones, not including the sesamoid bones. The appendicular skeleton has 126 bones, axial skeleton 74 bones, and auditory ossicles six bones. Each bone constantly undergoes modeling throughout the human life to help it adapt to changing biomechanical forces, as well as remodeling to remove old or micro-damaged bone and replace it with new stronger bone to maintain its strength.

The five general classifications of bones according to their shape are long bones, short bones, flat bones, irregular bones, and sesamoid bones. Long bones are tubular bones which include clavicle, humerus, radius, ulna, metacarpal, femur, tibia, fibula, metatarsals, and phalanges. Short bones are cuboidal and can be found only in the wrist (carpus) and ankle (tarsus). Flat bones which are usually serve protective functions; include the skull, mandible, scapula, sternum, and ribs. Irregular bones are bones that have various shapes and this include the vertebrae, sacrum, coccyx, and hyoid bone.

Sesamoid bones are bones that develop in certain tendons and are found where tendons cross the ends of long bones in the limbs – they usually protect the tendons from excessive wear and often change the angle of the tendons as they pass to their attachments. Sesamoid bone include patella (knee cap).

2.1.2 Histology of Bone

The adult human skeleton is composed of 80% cortical bone and 20% trabecular bone overall (Eriksen *et al.*, 1994). Cortical bone is compact, dense and solid and it surrounds the marrow space, whereas trabecular or cancellous bone is composed of a honeycomb-like network of bony trabeculae separated by a labyrinth of interconnecting spaces containing bone marrow. Both cortical and trabecular bone are composed of osteons.

Compact bone is made up of parallel bony columns where in each column is composed of concentric bony layers or lamellae disposed around a central channel containing blood vessels, lymphatics and nerves. These neurovascular channels are known as Haversian canals, and with their concentric lamellae from the Haversian systems. The Haversian systems interconnect with one another via Volkmann's canals which pierce the columns obliquely to the Haversian canals (Figure 2.1).

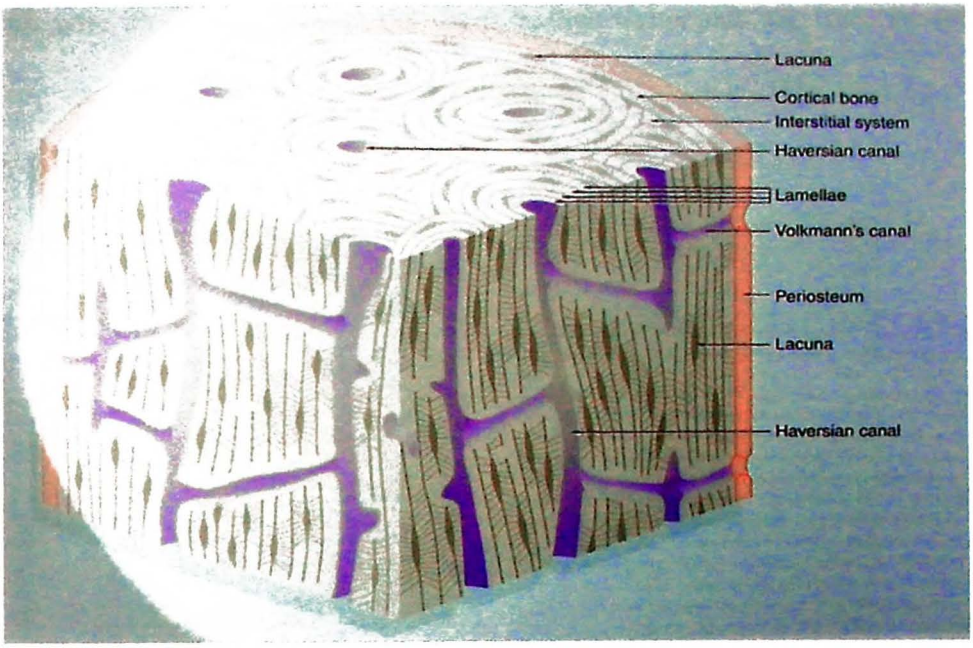

Figure 2.1: Schematic diagram of compact bone.
(Adapted from Wheater's Functional Histology, 1992)

Each Haversian system forms as a result of continuous osteoclastic and osteoblastic activities. Osteoclasts are multinucleated giant cells which are responsible for the resorption of the bone, while osteoblasts are responsible for formation of new bone matrix. In Haversian system, osteoblasts lay down in the lamellae of the bone. With the deposition of successive lamellae, the diameter of the Haversian canal decreases and osteoblasts are trapped as osteocytes in spaces called lacunae in the matrix. The osteocytes are thus arranged in concentric rings within the lamellae. Between adjacent lacunae and the central canal are numerous minute interconnecting canals called canaliculi which contain fine cytoplasmic extensions of the osteocytes.

Cancellous bone is made up of a network of bony trabeculae that are thin and composed of irregular lamellae of bone with lacunae containing osteocytes. It usually does not contain Haversian systems and the osteocytes exchange metabolites via

canaliculi with blood sinusoids in the marrow. The trabeculae are lined by endosteum which contains osteoprogenitor cells, osteoblasts and osteoclasts (Figure 2.2).

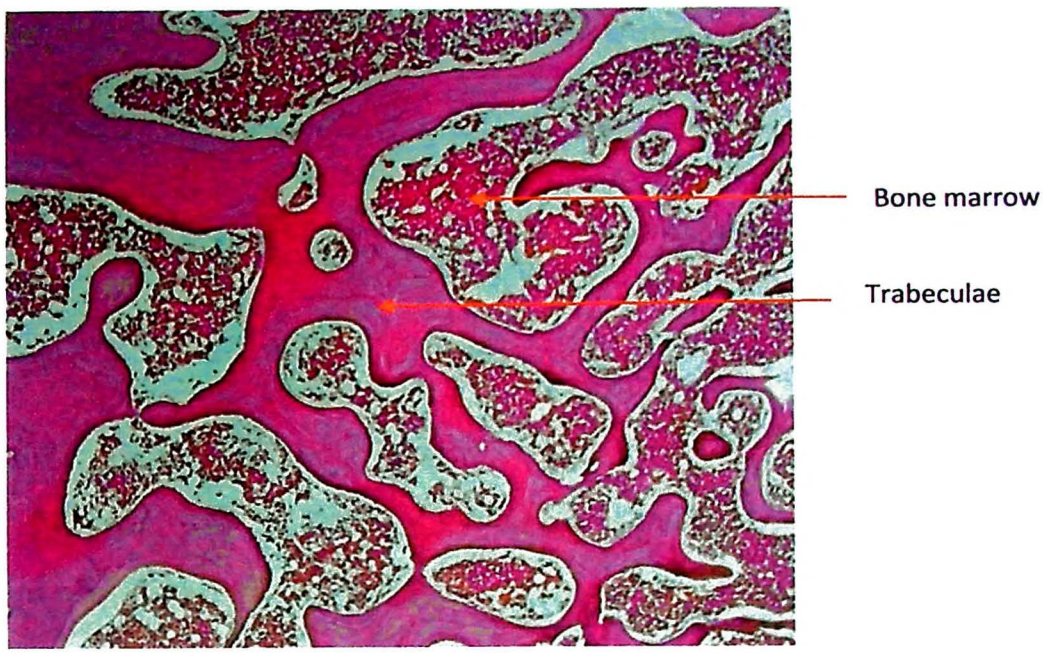

Figure 2.2: Cancellous bone with ×50 magnification of H & E stain. Cancellous or spongy bone is composed of a network of trabecular bone separated by a labyrinth of interconnecting spaces containing bone marrow.
(Adapted and edited from Wheater's Functional Histology, 1992)

The cells of bone are responsible for bone metabolism and repair. Osteoblasts are derived from a primitive mesenchymal cell called osteoprogenitor cells. Osteoblasts which are found lined up along the bone surface function in synthesizing osteoid and mediate mineralization. In normally developing bone, osteoid becomes calcified soon after deposition. Under conditions of calcium and phosphate ions deficiency (e.g. chronic renal failure, rickets), there is a lag in osteoid mineralization. Osteoclasts which are multinucleated phagocytic cells derived from the macropage-monocyte cell line, are capable of eroding bone. The resorption of bone performed by osteoclast contributes to

bone remodeling in response to growth or changing mechanical stresses on skeleton. Osteoclasts also involve in the long-term maintenance of blood calcium homeostasis through the response of parathyroid hormone and calcitonin. Parathyroid hormone stimulates osteoclastic resorption and release of calcium ions from bone, whereas calcitonin inhibits osteoclastic activity.

2.1.3 Osteoblast Proliferation

As mentioned before, osteoblast cells are important in bone formation and mineralization. Osteoblasts arise from osteoprogenitor cells which are located at periosteum and bone marrow. Based on in vitro culture studies, the differentiation of osteoprogenitor cells to osteoblasts process has three major stages: (i) cell proliferation, (ii) matrix maturation, and (iii) matrix mineralization. It has been a challenge to distinctly define the differentiation stages because the process is asynchronous where the progenitors are not going through the process at the same rate and they also do not behave in a similar way. However, the differentiation of osteoblast is associated with stage specific gene expression.

During the first stage which is the cell proliferation stage, osteoprogenitors undergo proliferation phase by increasing their numbers. During this phase, histones and protooncogenes such as *c-fos* and *c-myc* are upregulated. Other genes like *cyclin B* and *E* are upregulated later postproliferatively (Smith *et al.*, 1995). A subset of CFU-ALP and CFU-F forming postproliferative progenitors are capable of forming colonies (CFU-O) that later become osteoblasts (Aubin, 1999).

The other most frequently studied osteoblast specific genes are alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN), type I collagen (COLIa1), bone sialoprotein (BSP) and parathyroid hormone receptor type I (PTH1R). All of these genes are upregulated and downregulated during proliferation, matrix maturation and mineralization phases asynchronously (Aubin, 1998). Generally, ALP is upregulated at early phase and its expression is decreased once the osteoblasts mature. This followed by the raise of OPN during progenitor proliferation and later during matrix maturation. Finally during matrix maturation and mineralization, OCN, BSP, COLIa1 and PTH1R are expressed. These generally explain the heterogeneity in osteoblast gene expression during its differentiation process.

2.1.4 Bone Remodeling and Mineral Homeostasis

Bone remodeling involves the removal of mineralized bone by osteoclasts followed by the new bone matrix formation that subsequently become mineralized by osteoblasts and it occurs in temporary anatomical structures known as basic multicellular units (BMUs). Bone remodeling is the process of which bone is renewed to maintain bone strength and mineral homeostasis. Remodeling begins before birth and constantly continues until death: at any one time, when about 10% of the bone surfaces in the adult skeleton are undergoing active remodeling, the remaining 90% are found to be quiescent. The duration of the remodeling cycle is about 6 months, most of this time being occupied by formation; about 10% of the skeleton is renewed by remodeling each year (Manolagas, 2000). Bone remodeling increases in perimenopausal and early postmenopausal women and then slows with further aging, but continues at a faster rate

than in premenopausal women. Bone remodeling is thought to increase mildly in aging men.

The bone remodeling unit involves a tightly coupled group of osteoclasts and osteoblasts that sequentially carry out resorption of old bone and formation of new bone. The remodeling cycle is composed of five distinct phases: quiescence/activation, resorption, reversal, formation and termination (Raggatt and Partridge, 2010) (Figure 2.3).

The first phase of bone remodeling is activation phase where the detection of an initiating remodeling signal occurs. The initiating signal can be either due to direct mechanical force on the bone that results in structural damage or hormone (e.g. parathyroid hormone or calcitonin) action on the cells of the bone. As the result, the circulating mononucleated osteoclast precursors are recruited to the site of the remodeling, bone lining cell layer is penetrated and fusion of the mononuclear cells to form multinucleated preosteoclasts.

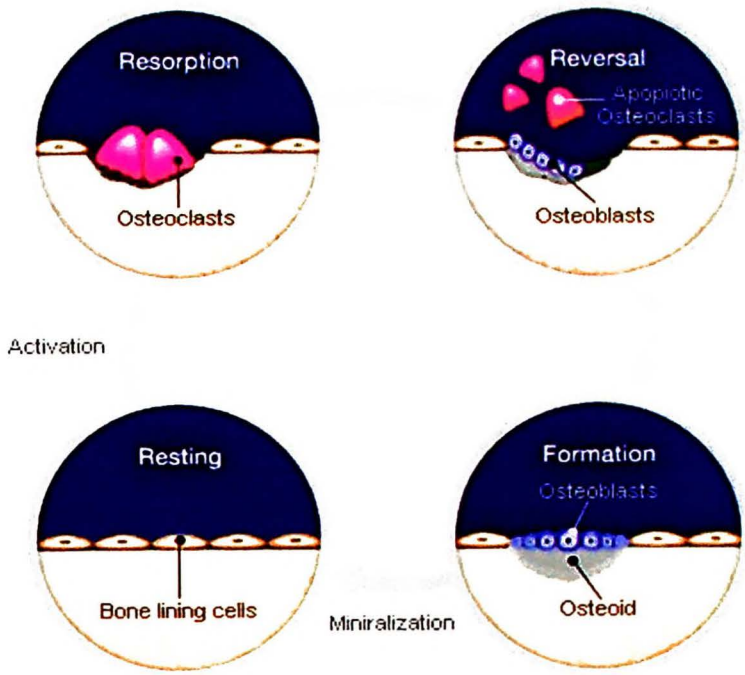

Figure 2.3: Schematic diagram of bone remodeling process of which consists of resting, resorption, reversal and formation phases.

Adapted from "The Bone Remodeling Cycle" http://www.sga-syndrom.de/Workshop/2010/Abstracts/Literatur/TOP3-Knochen/Bone/Bone-083.htm#refs. Accessed on 25th March 2013.

The resorption phase occurs when the osteoclasts start to erode the surface of the bone under regulation of local cytokines such as colony stimulation factor-1 (CSF-1) and receptor activator of NF-κB ligand (RANKL). CSF-1 stimulates proliferation and survival of osteoclast precursors (Insogna *et al.*, 1997), whereas RANKL promotes osteoclast precursors proliferation and in addition also coordinates the its differentiation and maturation, promotes resorption activity, and prolongs the life of mature osteoclasts (Burgess *et al.*, 1999). As the result of osteoclastic bone matrix digestion, resorption cavities are formed on the cancellous bone surface which is known as Howship's lacunae, and also the formation of cylindrical tunnels within the cortex (Dempster *et al.*,

2012). The resorption process is first taken place by multinucleated osteoclasts and later by mononucleated cells.

During the reversal phase, the Howship's lacuna is packed with mononuclear cells include monocytes, osteoclasts, and preosteoblasts. Preosteoblasts are recruited to the site of remodeling to initiate the formation phase. During this phase, the collagen remnants are removed and bone surface is prepared for subsequent osteoblast-mediated bone formation. The final role of the reversal phase may be to receive or produce coupling signals that ready for transition from bone resorption to bone formation within the BMU.

The formation phase is then taken place under coordination of coupling signals which are produced by osteoclasts and osteoblasts. The coupling factors or mechanisms involve during this phase including the soluble molecule sphingosine 1-phosphate and the cell-anchored EphB4-ephrin-B2 bidirectional signaling complex. Sphingosine 1-phosphate is produced and secreted by osteoclasts to induce osteoblast precursor recruitment, and promotes the survival of mature osteoblasts (Pederson *et al.*, 2008). EphB4 receptors are expressed on osteoblasts, whereas osteoclasts express the ligand ephrin-B2. Direct signaling through EphB4 to the osteoblasts enhances differentiation of osteoblasts. Reversely, ephrin-B2 signal to the osteoclast precursors suppresses osteoclast differentiation by inhibiting the osteoclastogenic c-Fos/NFATc1 cascade (Zhao *et al.*, 2006). Thus, EphB4-ephrin-B2 signaling complex is a unique remodeling mechanism which activates bone formation and inhibits bone resorption simultaneously at this critical transition point of the remodeling process.

Finally the termination phase occurs when an equal quantity of resorbed bone has been replaced. The termination factors that signal the remodeling cells to cease work are still unknown; however the return of sclerostin expression by the newly generated osteocytes, is one of the contributing factors that indicate the remodeling cycle has come to the end (Henriksen *et al.*, 2009). Following mineralization, the remaining mature osteoblasts undergo apoptosis. Some osteoblasts are trapped and buried in the matrix becoming osteocytes that maintain intimate contact with one another and with the cells on the bone surface. The remodeling cycle then return to the resting state when some of the remaining osteoblasts differentiate into flattened cells that lining and cover the bone surface.

The bone is the reservoir of calcium, and it serves a similar but not so unique role for phosphorus and magnesium. Skeletal calcium is controlled through the regulatory pathways of the gastrointestinal (GI) tract and the kidney, and this regulation is mediated in bone by the osteoblast and the osteoclast. Calcium reaches the bone by being absorbed from the diet in the GI tract. Unabsorbed calcium passes into the feces. In GI tract, dietary calcium is absorbed, enters the extracellular fluid (ECF) space and becomes incorporated into the bone through the process of mineralization of the organic matrix of bone, osteoid. The regulation of bone and bone mineral metabolism is under influence of three hormones; parathyroid hormone (PTH), calcitonin (CT), and vitamin D, to regulate three most important bone minerals; calcium, magnesium, and phosphorus.

2.1.5 Bone Formation Markers (ALP and Osteocalcin)

As previously discussed, bone is constantly undergoing remodeling process of which the formation and resorption of bone occurring in balance way. There are marker expressions that indicate the process of bone formation takes place or in other words, they prove the osteoblastic activity. The bone formation markers are alkaline phosphatase (ALP), osteocalcin, osteopontin, type I collagen (COLIa1), and bone sialoprotein (BSP). However, the serum osteocalcin and the serum activity of bone-specific ALP are considered specific biochemical indices of bone formation (Seibel and Woitge, 1999). The examples of bone formation markers commercially available are stated in Table 2.1.

Table 2.1: Commercially Available Bone Formation Markers.

Bone Formation Markers	Mechanism	Company
Bone-specific alkaline phosphatase (bALP)	Secreted by osteoblasts	Hybritech (Ostase), Metra (Alkphase-B)
Osteocalcin (bone Gla protein)	Secreted by osteoblasts	Nichols, Cis Biointernational
Procollagen type I C propeptide	Collagen-based	Orion, Metra (Prolagen)
Procollagen type I N propeptide	Collagen-based	Incstar

Adapted and edited from http://courses.washington.edu/bonephys/opmark.html. Accessed on 26th March 2013.

ALP is ubiquitously expressed and belongs to the category of molecules that are localized to cell membranes through a COOH-terminal glycan-phosphatidylinositol anchor. Isoforms produced by differential cleavage or preservation of the glycan-phosphatidylinositol anchor originate from different tissues, such as bone, liver, spleen, kidney, intestine, and placenta (Moss, 1992). Almost 95% of total ALP activity in the serum are accounts for bone and liver (bALP) isoforms. Bone ALP is produced by osteoblasts in extremely high amounts during bone formation phase of remodeling process and is an excellent indicator of total bone formation activity (Moss, 1992).

Osteocalcin or also known as bone Gla protein (BGP) is a non-collagenous protein synthesized by osteoblasts and released into the plasma also during bone formation. Most of circulating osteocalcin is a product of osteoblast activity and thus considered as a bone formation marker. The human osteocalcin gene is located at the distal long arm of chromosome 1. Various promoter elements contribute to basal expression and osteoblast specificity. The gene is then being modulated by vitamin D and glucocorticoid response elements (Sierra *et al.*, 2003). The function of osteocalcin has not clearly defined yet. However, it is assumed that the newly synthesized protein is incorporated into the bone matrix-binding calcium during mineralization process. Serum osteocalcin levels are significantly influenced by age, gender, and renal function (Young *et al.*, 1992).

2.2 Osteoporosis

2.2.1 Overview

Osteoporosis is an exaggerated loss in bone mass of mainly cancellous bone with impaired bone architecture which results in bone fragility and an increased risk of fracture. These changes result from the imbalance activity of osteoclasts and osteoblasts, where there are increased in osteoclastic activity and reduced in osteoblast function that characterizes postmenopausal osteoporosis.

The World Health Organization and International Osteoporosis Foundation define osteoporosis as "a systemic skeletal disease characterized by low-bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk."

In the United States, nearly 10 million people already have osteoporosis. Another 18 million people have low bone mass that places them at high risk for developing osteoporosis. More than 1.5 million Americans have fractures related to osteoporosis each year (Riggs and Melton, 1992). The prevalence will rise as the population ages. About 80% of people who have osteoporosis are women. Of people older than 50 years of age, one in two women and one in eight men are predicted to have an osteoporosis-related fracture in their lifetime (Melton *et al.*, 1992; Prelevic, 2001).

According to the World Health Organization, the prevalence of osteoporosis among U.S. white women postmenopause is estimated to be 14% in those 50-59 years

of age, 22% in those 60-69 years of age, 39% in those 70-79 years of age, and 70% in those 80 years of age and older. Significant risk has been reported in people of all ethnic backgrounds. White and Asian racial groups, however, are at greatest risk.

In Malaysia, osteoporosis-related fractures have been recognized as a major health problem, particularly in the elderly. In 1997, the incidence of hip fracture among individuals above 50 years of age was 90 per 100 000. Hip fractures are associated with a high morbidity and mortality rate of up to 20% in the first year. The majority who survive are disabled and only 25% will resume normal activities. The direct hospitalization cost for hip fracture in 1997 is estimated conservatively at RM 22 million.

2.2.2 Types of Osteoporosis

Osteoporosis may be either a primary or a secondary form. Primary osteoporosis is the more common form and is due to the typical age-related bone loss from skeleton. It is classified as type 1 and type 2. Secondary osteoporosis results from the presence of other diseases or conditions that predispose to bone loss and is classified as type 3.

Type 1 osteoporosis is also known as postmenopausal osteoporosis that occurs with peak incidence in 60s and early 70s. The incidence in women is higher than men and it is believe that lack of estrogen hormone in the body is the culprit that causes this form of osteoporosis.

Type 2 or senile osteoporosis is usually associated with the decrease in bone formation along with the decrease of the ability of kidney to produce vitamin D, which

important in the calcium absorption that favours the bone anabolism. This type of osteoporosis occurs in women or men more than 70 years of age.

Type 3 or secondary osteoporosis is associated with a variety of conditions, including cancer, hormonal imbalance, gastrointestinal disorders, drug use, chronic renal failure, and poor nutrition. It is usually occurs equally in men and women of any age.

2.2.3 Treatment and Risks of the Treatment for Osteoporosis

The main aim of osteoporosis treatments is to reduce bone turnover and to increase in bone mass so that the incidence of bone fracture can be decreased. Most drugs currently available for the treatment of osteoporosis act by decreasing bone resorption. Antiresorptive drugs use include: selective estrogen modulators (SERMs), biphosphonate, calcitonin and many more.

Selective estrogen receptor modulators (SERMs) are compounds are designed to activate the estrogen receptors, but have different effects on different tissues. In other words, this drug act like estrogen in the desireable ways like stablizing the bone mass, improve lipid profile, and reduce hot flashes, but do not act like estrogen, for example, inhibit the estrogen receptor found in breast cells but activate the estrogen receptor present in uterine endometrial cells. Tamoxifen is a first SERM to reach market that is used to treat breast cancer. It has some beneficial effects on the bones, but it does stimulate the endometrium. Raloxifene is a newer SERM that has been approved by FDA for prevention and treatment of postmenopausal osteoporosis. Raloxifene

increases bone density at the spine, but its effect does not appear to be as powerful as the bisphosphonates, and it has been shown to reduce spine fractures (Ettinger *et al.*, 1999).

Bisphosphonates are potent selective osteoclastic bone resorption inhibitors. It is commonly used clinically for the treatment and prevention of postmenopausal osteoporosis. There are two groups of biphosphonate. The first group of biphosphonates are clodronate, etidronate, and pamidronate, while alendronate, risedronate, ibandronate, and zoledronate belong to the second group. The mechanisms of action of biphosphonate are inhibiting osteoclast adhesion to the mineralized matrix, reduce the osteoclast life span, and directly inhibit osteoclast activity through alteration of its cytoskeleton (Benford *et al.*, 2001).

Somehow, despite the benefits of these modern medicines in treating osteoporosis, the patients are exposed to the risk of other disease when under long term of treatment. SERMs for example, would inhibit cell proliferation in breast cells and prevent from breast cancer (Eriksen, 2012), but stimulate the proliferation of uterine endometrial cells and increase the risk of uterine cancer (Shang, 2006; DeMichele *et al.*, 2008). As for biphosphonates, patients are exposed to relative risks for incident invasive cancers of the oesophagus, stomach, and colorectum (Green *et al.*, 2010).

According to the survey, one in three adults is search out complementary and alternative medicine (CAM) approaches for their health conditions. Some CAM therapies that have been practiced in osteoporosis treatment are herbs, acupuncture, diet, and nutritional supplements. The herbal therapy, for instance, may suggest any herbal

preparations, soups and others that can affect calcium use by the body, and as a result improving bone density.

Some herbal preparations have been proven for their efficacy in treating osteoporosis. Er-Zhi-Wan (EZW), a traditional Chinese formulation, which has an antiosteoporotic effect without having hyperplastic effect on uterus (Cheng *et al.*, 2011). Another finding of herbal medicine has proven that *Withania somnifera* root extract contains estrogen-like withanolides which is important for anti-osteoporotic activity (Nagareddy and Lakshmana, 2006). An ayurvedic preparation, *Praval bhasma*, in a study indicates that this preparation is effective in the prevention of calcium and estrogen deficient bone loss and thus important for management of bone metabolic disorders such as osteoporosis (Reddy *et al.*, 2003).

So, with these evidence indicates that the alternative medicine is becoming popular nowadays. Thus, it is possible that in the future *Quercus infectoria* galls, with its positive role on osteoblastic activity, may become a potential agent in the osteoporosis treatment.

2.3 *Quercus infectoria* Galls Extract

2.3.1 Overview

Quercus infectoria Olivier (Fagaceae) is a small shrub mainly present in Greece, Asia Minor, Syria and Iran. This small tree can grow from four to six feet high, having crooked and shrubby-looking, with smooth and bright-green leaves. The galls arising in the branches of the tree are called as 'majuphal' in Sanskrit and 'machakai' in Kannada.