COLLAGEN SCAFFOLDS WITH *IN SITU* GROWN CALCIUM PHOSPHATE FOR OSTEOGENIC DIFFERENTIATION OF WHARTON'S JELLY AND MENSTRUAL BLOOD STEM CELLS

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ABSTRACT

COLLAGEN *IN SITU* GROWN CALCIUM PHOSPHATE SCAFFOLDS FOR OSTEOGENIC DIFFERENTIATION OF WHARTON'S JELLY AND MENSTRUAL BLOOD STEM CELLS

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The importance of developing new techniques for the treatment of bone and joint diseases is increasing continuosly together with the increase of human population and the average life span. Especially bone fractures as a result of osteoporosis are often seen in humans older than 50 years old. The expenses of bone and joint disease operations are very high and the duration of recovery is long. Because of these reasons World Health Organization, The United Nations and 37 countries announced that the years 2000-2010 is the Bone and Joint Decade. Tissue engineering is an alternative approach to clinically applied methods. In this study collagen scaffolds crosslinked with genipin, to improve the stability of foams in culture media, were prepared by lyophilization. To mimic the natural bone structure calcium phosphate mineral phase in the foam was formed by wet chemical precipitation. Collagen concentration (0.75% and 1%, w/v), freezing temperature (-20 ^{o}C and -80 ^{o}C) of the collagen solution before lyophilization and immersion duration (2x4 h and 2x48 h) of the foams in calcium and phosphate solutions for wet chemical precipitation were changed as process

parameters of foam production. Pore size distribution and porosity analysis as well as compression test were performed for characterization of the scaffolds. The foam with 1% w/v collagen concentration, frozen at -20 ^{o}C before lyophilization and immersed for 2x4 h in calcium and phosphate solution was chosen for *in vitro* cell culture studies. The defined foam had 70% porosity and pore sizes varying between 50 and 200 μm. The elastic modulus and compressive strength of the foam was calculated as 127.1 kPa and 234.5 kPa, respectively.

Stem cells isolated from Wharton's jelly (WJ) and menstrual blood (MB) were seeded to foams to compare their osteogenic differentiation. Both cells are isolated from discarded tissues and used in this study as an alternative to the commonly used cells which are isolated by invasive techniques such as bone marrow stem cells. Cells were seeded to collagen foams with and without calcium phosphate (CaP). It was observed that WJ cells proliferated during 21 days on collagen foams without CaP, but MB cell number decreased after day 14. Collagen foams with CaP supported the alkaline phosphate (ALP) activity compared to tissue culture polystyrene (TCPS) and foams without CaP. Contrarily lower cell numbers achieved on CaP containing collagen foams, possibly because of the calcium and phosphate concentration changes in the medium and as the result of osteogenic differentiation. ALP activity of both cell types increased almost 10 times and specific ALP activity (activity per cell) increased 40 times and 150 times for WJ and MB cells, respectively on the CaP containing foams compared to TCPS.

Therefore, in this study it was shown that *in situ* CaP formed collagen foams induce osteogenic differentiation of WJ and MB cells, and these cells isolated from discarded tissues can be used as alternative cell sources in bone tissue engineering applications.

Keywords: Bone tissue engineering, Genipin, Calcium Phosphate, Wharton's Jelly, Menstrual Blood

ÖZ

GÖBEK KORDONU VE MENSTRÜEL KANDAN İZOLE EDİLEN KÖK HÜCRELERİN KEMİĞE FARKLILAŞTIRILMASI İÇİN HAZIRLANMIŞ *IN SITU* KALSİYUM FOSFAT İÇEREN KOLLAJEN İSKELELER

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Kemik ve eklem hastalıklarının iyileştirilmesi için yeni tekniklerin geliştirilmesi artan nüfus ve ortalama yaşam süresinin artmasıyla birlikte önemini arttırmaktadır. Özellikle osteoporoz nedeniyle oluşan kemik kırıkları 50 yaş üstü insanlarda sıklıkla görülmektedir. Kemik ve eklem hastalıklarının tedavi masrafları fazla ve iyileşme süreleri uzundur. Bu sebeplerle Dünya Sağlık Örgütü, Birleşmiş Milletler ve 37 ülke 2000-2010 yıllarını Kemik ve Eklem Yılları olarak beyan etmişlerdir. Doku mühendisliği klinik olarak uygulanan yöntemlere bir alternatif olaral geliştirilmiş bir yaklaşımdır. Bu çalışmada hücre kültür ortamındaki stabilitesini arttırmak amacıyla genipinle çapraz bağlanmış kollajen iskeleler liyofilizasyonla üretilmiştir. İskeleler üzerinde kalsiyum fosfat (CaP) mineralleri çözelti presipitasyonu yöntemiyle oluşturulmuştur. İskelelerin üretimindeki değişkenler kollajen konsantrasyonu (% 0.75 ve % 1), kollajen çözeltisinin dondurma sıcaklığı (-20 ^{o}C ve -80 ^{o}C) ve iskelelerin kalsiyum ve fosfat çözeltileri içinde bekletilme süreleridir (2x4 sa ve 2x48 sa). İskelelerin karakterizasyonu için gözeneklilik ve gözenek dağılımı analizleriyle mekanik test

yapılmıştır. *In vitro* hücre kültürü çalışmaları için kollajen konsantrasyonu %1 olan, liyofilizasyondan önce -20 ^{o}C'de dondurulmuş ve kalsiyum ve fosfat çözeltilerinde 2x4 sa süre ile bekletilmiş iskeleler seçilmiştir. Bu iskelelerin gözenekliliği %70 olarak hesaplanmış, gözenek boyutlarının 50 ile 200 μm arasında değiştiği ölçülmüştür. İskelelerin elastikiyet modülü ve sıkıştırılma mukavemeti sırasıyla 127.1 kPa ve 234.5 kPa olarak hesaplanmıştır.

Göbek kordonu ve menstrüel kandan izole edilmiş kök hücreler kemik hücrelerine dönüşme potansiyellerinin karşılaştırılması amacıyla iskeleler üzerine ekilmiştir. Her iki hücre tipi de atık dokulardan elde edilmiştir ve kemik doku mühendisliğinde sıklıkla kullanılan fakat izolasyonu donöre acı veren kemik iliği hücreleri gibi hücrelere alternatif olarak bu çalışmada kullanılmıştır. Hücreler CaP içeren ve içermeyen iskelelere ekilmiştir. Göbek kordonu hücreleri CaP içermeyen iskeleler üzerinde 21 gün boyunca çoğalırken, menstrüel kandan izole edilen kök hücrelerin sayısında 14. günden sonra azalma gözlenmiştir. CaP içeren iskelelerin hücrelerin alkalen fosfataz (ALP) aktivitesini doku kültürü kabı ve CaP içermeyen iskelelere göre önemli ölçüde arttırdığı görülmüştür. Buna karşılık CaP içeren iskeleler üzerindeki hücre sayısında zaman içinde, ortamdaki kalsiyum ve fosfat iyon konsantrasyonunun değişmesi ve hücrelerin kemik hücrelerine farklılaşmasına bağlı olduğu düşünülen bir azalma gözlenmiştir. Hücre kültür kabına kıyasla CaP içeren iskeleler üzerinde her iki hücrenin de ALP aktiviteleri yaklaşık 10 kat, spesifik ALP aktiviteleri (hücre başına düşen ALP aktivitesi) de göbek kordonu ve menstrüel kan hücreleri için sırasıyla 40 ve 150 kat artmıştır.

Bu çalışmada *in situ* CaP oluşturulmuş kollajen iskelelerin kök hücrelerin kemik hücrelerine farklılaşmasını desteklediği ve atık dokulardan elde edilen göbek kordonu ve menstrüel kan hücrelerinin kemik doku mühendisliği için bir alternatif olabileceği gösterilmiştir.

Anahtar Kelimeler: Kemik Doku Mühendisliği, Genipin, Kalsiyum Fosfat, Göbek Kordonu, Menstrüel Kan

Dedicated to my family…

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3D	3 Dimensional
ALP	Alkaline Phosphatase
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
CaP	Calcium Phosphate
CSLM	Confocal Scanning Laser Microscopy
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
EDS	Electron Diffraction Spectra
EDTA	Ethylene Diamine Tetraacetic Acid
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan
GTA	Glutaraldehyde
HAp	Hydroxyapatite
HCA	Hydroxyl Carbonate Apatite
kDa	kilo Dalton
MB	Menstrual Blood
MSC	Mesenchymal Stem Cell
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium
MWCO	Molecular Weight Cut Off
OCP	Octa Calcium Phosphate
PBS	Phosphate Buffer Saline

PCL	Poly(ε-caprolacton)
PDS	Polydiaxonone
Pen/Strep	Penicillin/Streptomycin
PGA	Poly(glycolic acid)
PHA	Poly(hydroxyalkanoate)
PHB	Poly(3-hydroxybutyrate)
PHBV	Poly(hydroxybutyrate-*co*-valerate)
PLA	Poly(lactic acid)
PLGA	Poly(lactide-*co*-glycolic acid)
SDS-PAGE	Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
TCP	Tricalcium Phosphate
TCPS	Tissue Culture Poly Styrene
TEMED	Tetramethylethylenediamine
TGF-β	Tansforming Growth Factor β
WJ	Wharton's Jelly

CHAPTER 1

INTRODUCTION

The goal of this study was to design a scaffold which mimics the chemical composition and physical properties of natural bone with the stem cells isolated from discarded tissues (Wharton's jelly and menstrual blood) seeded on it and compare the bone forming efficiencies of these cells.

1.1. Structure and Function of Bone

Bone tissue is composed of organic and inorganic substances. Approximately 10% of wet bone mass consists of collagen, 65% calcium phosphate, 25% water and a very small amount of magnesium, sodium and bicarbonate, and because of this bone is considered a composite material (Weiner and Wagner, 1998). Collagen is a triple helical molecule. It is produced by osteoblasts and secreted into the extracellular protein medium. Each strand of the triple helix is composed of approximately 1000 amino acids of which roughly 1/3 proline and hydroxyproline, 1/3 is glycine and 1/3 charged amino acids. A triple helix is around 1.5 nm thick and 300 nm long and they lie vertically with 40 nm end-to-end gaps between two collagen molecules. There is 67 nm offset between two adjacent collagen molecules. These collagen molecules come together with a uniform pattern and form collagen fibrils which are approximately 80-100 nm thick (Figure 1.1.). Collagen fibrils congregate and form fibers (Weiner *et al*., 1998)

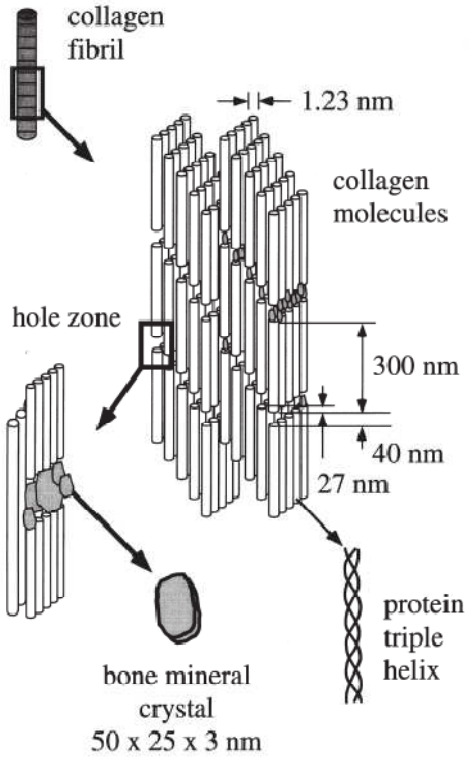

Figure 1.1. Organization and composition of collagen fibrils (Rho *et al*., 1998).

Calcium phosphate crystals are embedded in fibers. These hydroxyapatite-like crystals are carbonated and called dahllite (Weiner *et al*., 1998). The chemical formula for dahllite is $(Ca_5 (PO_4,CO_3)_3 (OH))$. Mature dahllite nanocrystals are plate-shaped (50 nm long, 25 nm wide) (Rho *et al*., 1998). Their thicknesses vary from 1.5 to 4 nm and they are the smallest biologically produced crystals known. There are also small amounts of hydrogen phosphate, sodium, magnesium, citrate, potassium and carbonate ions in bone mineral (Weiner *et al*., 1998, Rho *et al*., 1998). Other proteins in the bone structure such as bone sialoprotein, osteonection, osteocalcin and osteopontin arrange the alignment, size and crystal structure of minerals. These proteins contain the phosphorylated amino acids serine and threonine in their structure. These organically bound phosphate groups and carboxyl side chains of phosphorylated proteins are the binding sites for

calcium ions and thus regions for nucleation and crystal growth of minerals (Glimcher *et al*., 1984).

Water is another important component of bone tissue. It is found between the fibers and the triple helices and affects the mechanical properties significantly. Mechanical test results of dry and wet bone tissue give different results. The elastic modulus, ultimate tensile strength and hardness of dry bone are higher in dry state (Weiner *et al*., 1998).

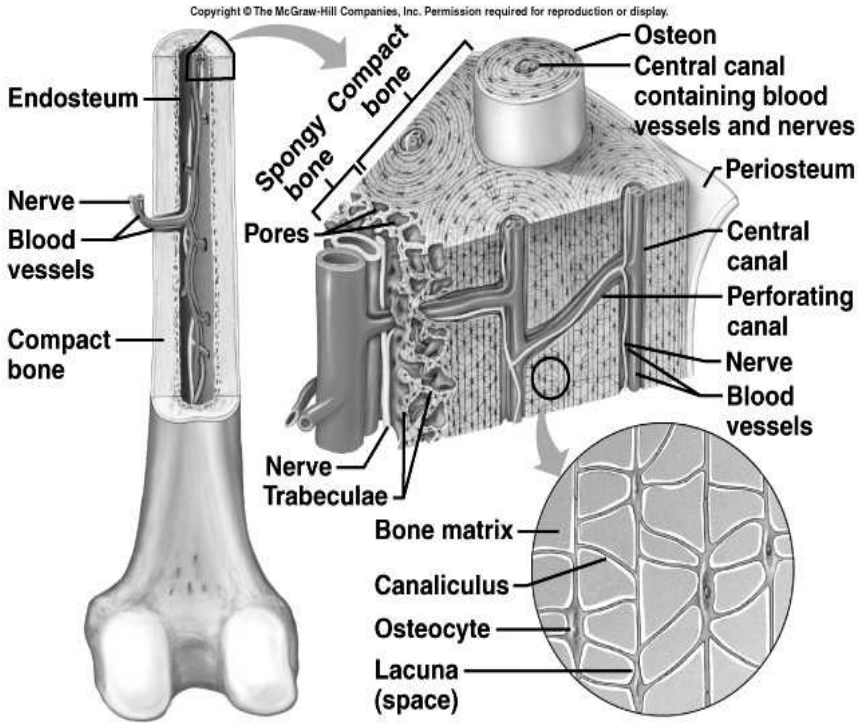

Figure 1.2. Typical structure of a long bone (http://biology.bangor.ac.uk/teaching/module/BSX1016/muscskel)

Bone is mainly organized as spongy (trabecular) and cortical (compact) bone (Figure 1.2.). Cortical bone is significantly more ordered than trabecular bone. In the cortical bone mineralized collagen fibers form flat lamellae that are 3-

7 μm wide and 3-8 lamellae are combined to form the hollow cylindrical osteons in which blood vessels are found. There is also another form of cortical bone which does not have particular patterns. This type of cortical bone is called woven bone and it is usually formed during fracture healing. Mechanical properties of cortical bone is related with its porosity, matrix organization and mineralization degree. The inner part of the cortical bone contains the bone marrow. Spongy bone is biologically more active than cortical bone. Bone remodeling occurs in the trabecular bone more often (Ott, 2002).

The diaphyseal ends of long bones and the interior parts of flat bones contain trabecular (spongy) bone. Trabecular bone is composed of collagen struts containing mineral and the marrow filling the trabeculae (Rho *et al.*, 1998). The pore size of trabecular bone varies between 400-500 μm (Ikada, 2006) and the porosity is around 50 and 90% (Karageorgiou *et al.*, 2005).

Bone tissue has very important functions such as supporting the body, protection of internal organs, providing a suitable surface for muscles to attach and moving of limbs. Bone deposits minerals and is a pH stabilizer. It also retains dangerous minerals such as lead. Almost 99% of calcium and 85% of phosphorus in the body are deposited in bone tissue. Excessiveness or deficiency of calcium and phosphate cause improper functioning of nerves and muscles (American Society for Bone and Mineral Research).

1.2. Bone Cells

Bone tissue is composed of mainly two different cell types. These are bone forming osteoblasts and bone resorbing osteoclasts. Osteoblasts are derived from pluripotent mesenchymal stem cells found in the bone marrow. These progenitor cells are capable of differentiating into a variety of cell types such as adipocytes, myocytes, chondrocytes in addition to osteoblasts and the type of differentiation is determined by tissue specific transcription factors. For example Runx2 (Cbfa1) and Sox 5/6/9 are the transcription factors which induces osteoblastic and chondrocytic differentiation, respectively.

Osteoblasts are uninuclear and have plenty of Golgi apparatus which is a main characteristic of secreting cells. Osteoblasts secrete a mixture of proteins, collagen type I being the most abundant. This non-calcified protein mixture is called osteoid. Osteoid is made up of collagen type I fibers and ground substance. Collagen type I is produced as tropocollagen inside the cell and transported outside of the osteoblasts as collagen fibrils. Ground substance is mainly composed of matrix proteins and proteoglycans such as osteocalcin, osteopontin and bone sialoprotein, and polysaccharides as chondroitin sulphate, and water. Osteoblasts synthesize the molecules such as membrane bound protein RANK and soluble factor macrophage colony stimulating factor (M-CSF) that communicate with other cells and agents that activate the osteoclasts. They have receptors for hormones such as parathyroid hormone and estrogen. Phosphate amount that the kidney excretes is also regulated by osteoblasts. Osteoblast cells have gap junctions that are responsible for the transportation of small molecules (smaller than 1 kDa) between cells. Gap junctions are very important because they carry mechanical, electrical and hormonal signals between cells. Some of the osteoblasts become flat shaped and form lining cells that cover the outer surface of the bone (periosteum), are locked in the extracellular matrix that they produced and become osteocytes, and some of them enter apoptosis and die.

Lining cells cover bone surface and are responsible for bone remodeling and mineralization. They secrete calcium ions when the calcium amount in the blood is decreased. They also protect the bone against the chemicals (like pyrophosphate) that destroy crystals.

Osteocytes are mature bone cells. They have extensions that maintain the communication of osteocytes with each other and with lining cells. They also secrete molecules like growth factors, prostaglandins and nitric oxide that activate the lining cells and the osteoblasts.

Osteoclasts are multinuclear cells and are derived from the same lineage as macrophages. Osteoclast precursors carrying RANK receptor on their surface circulate in the blood. Osteoblasts have RANK-L ligand and by the help of activating agents receptor and ligand interactions occur which cause the resorption of bone by osteoclasts.

1.3. Bone Remodelling and Critical Sized Defects

Some different hypotheses about bone remodeling exist (Cacchioli *et al*., 2006) but most of these hypotheses are the variations of Wolff's Law (1892). According to this law if the mechanical stimulus is sufficiently high bone formation occurs which results with the increase of bone mass and if the mechanical stress is too low bone is resorbed as a result of increased turnover. The general idea is that osteocytes serve as the mechanical sensors of bone. When the mechanical stress is in the normal range osteocytes secrete some inhibitory molecules such as MEPE (matrix extracellular phosphoglycoprotein) preventing the resorption of bone (Kulkarni *et.al*., 2010). If the mechanical stress on bone is too low in comparison to the biological load or too high that creates a micro crack in the bone, then the defect is sensed by osteocytes and the secretion of these inhibitory molecules is stopped. Osteocytes communicate with the osteoblasts and lining cells, and in return osteoblasts promote the remodeling of bone by activating pre-osteoclasts and the damage is repaired (bone regains its original mechanical strength) approximately in 4 months.

Bone formation and resorption occur continuously in a healthy bone tissue and some minor defects can be healed by bone itself. However, some accidents, some surgical treatments such as the resection of tumors, fractures that occur as a result of osteoporosis or tissue loss as a result of osteonecrosis may lead to nonunions (inability of the two sides of the broken bone cannot meet and fuse with each other) with bone loss. Such defects with sizes that can not be tolerated and healed by the tissue during a life time is defined as the 'critical sized defect' (Cacchioli *et al*., 2006).

1.4. Traditional Treatment Methods

The concept of replacing a damaged tissue with the healthy one has always been attempted with partial success. One of the first examples of tissue replacement is presented Tagliacozzi's work in the 16th century that he described the replacement of nose tissue with tissue from a forearm flap (Vacanti, 2007). Then in 19th century, the introduction of sterile techniques and application of anesthesia allowed the use of surgical treatments in repairing tissue defects (Vacanti, 2007).

1.4.1. Bone Grafts

Today, in most of the orthopedic and craniofacial surgeries grafts or implants are used for the therapy of bone defects. A graft is a transplant that carries live cells while an implant is a nonviable product that is applied to the wound side (Habal and Reddi, 1992). Bone grafts could be nonvascularized or vascularized. Nonvascularized grafts could be cortical or cancellous. There is also corticocancellous grafts and these are the most preferable ones because of better healing capabilities. Bone slurry, particulate bone and bone paste are the other graft choices available for surgeries.

Selection of the graft that to be used at the defect site depends on a variety of parameters. Cortical grafts are suitable for use in load bearing bones such as long bones but they are not suitable for maxillofacial bones which require trabecular bone. Cancellous grafts are commonly used in open fractures and oral cavity defects. If the vascularization capacity of the graft is high as in cancellous grafts this causes a reduction in the mechanical stress that the graft can withstand, so they are not suitable for load bearing defects. Corticocancellous grafts are the most promising ones because they both allow revascularization and have appropriate mechanical properties. Bone slurry is a type of graft that is composed of micro bone particles combined with blood and some other components such as collagen and growth factors. Bone paste is similar to bone slurry but is more viscous because it contains particulate bone in a gelatinous matrix.

An autograft is graft tissue that is obtained from another site on the patient's own body. Allograft, however, is taken from another person. Autografts are usually obtained from iliac crest, rib, tibial and cranial bones and they yield the best results because the donor and defect sites carry the same genetic material. Unavailability of sufficient donor tissue and donor site morbidity caused by healthy tissue removal are very important problems that must be overcome. In many operations donor site discomfort is more dominant than the original wound side. Allografts on the other hand have the risk of rejection by the patient's tissue. An immediate immune response is observed and this may result in the removal of the graft which necessitates another surgery which will then increase the healing duration and the cost of the process. In order to overcome these problems use of demineralized bone is preferred in certain applications. Use of demineralized bone is reported to give better results in children than in adults because the healing process is completely different in children and adults (Habal and Reddi, 1992).

1.4.2. Metallic Implants

In addition to bone grafts, metals and metal alloys are also used as implants and fixation devices. Stainless steel, titanium and Vitallium are most common metals and alloys used in the treatment of bone defects. Metals are usually preferred for load bearing bone defects because of their mechanical strength. One of the biggest problems in using metals for orthopedic implants is the mismatch of mechanical properties of bones and metals. The stiffness of metals is much higher than bones so they carry most of the load themselves. In addition to that use of metals and alloys has some other drawbacks. Metal surfaces are not suitable for the attachment of bone cells which causes a fibrous tissue formation around the implant and loosening of it. To overcome this challenge porosity of the metal surface is increased or the implant is coated with bioactive molecules such as hydroxyapatite (HAp) but still the release of some elements that evoke immune response and the risk of corrosion remain (Campbell *et al*., 1996).

1.4.3. Polymeric Implants

In addition to metallic implants, polymeric implants are also used in orthopedic surgeries. Especially bioabsorbable polymers are preferred over metallic implants in internal fixation operations. The first bioabsorbable polymeric implants for fracture fixation and osteotomies of extremities in human were introduced by a group from Helsinki University in 1984 (Rokkanen *et al.*, 1985).

Polyglycolic acid (PGA), polylactic acid (PLA) and polydiaxonone (PDS) are used to produce internal fixation devices in the shape of plates, screws and rods (Burns *et al*., 1998). These devices can be manufactured by moulding (compression and injection moulding) and sintering techniques (Ashammakhi and Rokkanen, 1997). These kind of polymeric devices are commonly used in fractures of shoulder, elbow, knee, femur, foot and toes (Rokkanen *et al*., 2000). They have some advantages such as the avoidance of a second surgery to remove the implant and this reduces the cost of operations. Additionally they prevent the mechanical shielding effect of metallic implants. But it was reported that in some of the surgical operations foreign body reactions may occur (Rokkanen *et al*., 2000).

Briefly, even satisfactory results are obtained with the traditional treatment methods there is still some drawbacks to overcome as mentioned above. In order to solve these problems new techniques are developing. Tissue engineering is one of the most promising alternatives to traditional methods which will be discussed in detail in the following section.

1.5. Bone Tissue Engineering

Tissue engineering is a new field of science which has been developing for the last 20 years. The term was first defined in 1988 by National Science Foundation of USA, as ‘use of engineering and life sciences principles to perceive structure and function relationship between normal and diseased tissues together

with the improvement of biological substitutes that can be used in tissue and organ function repair'(Cheung *et al.,* 2007). The first attempt of application was in the 1970s by a pediatric orthopedic surgeon, W. T. Green, who used bone spicules as a scaffold material and seeded chondrocytes for the treatment of a cartilage defect in mice (Vacanti, 2006). Although the result was not successful new attempts were made and in the following years research was continued in a multitude of applications leading to the first commercial product Apligraf®.

Tissue engineering concept is realized by the involvement of three constituents which are scaffold, cells and bioactive agents (e.g. growth factors) (Figure 1.3.). A tissue engineered construct need to satisfy not only the biological but also the physical and mechanical requirements of the target tissue. For example a tissue engineered construct for healing of bone defects should have the mechanical strength of the normal tissue until the regeneration of the site is completed. It should also integrate with the tissue, allow vascularization, and degrade gradually leaving its place to the newly formed tissue. For this reason a broad range of natural and synthetic materials are being investigated for use in scaffold production. Different cell sources, embryonic or adult, stem or fully differentiated are being sought, and the role of the biological cues is being investigated.

Tissue engineering is a serious alternative to the traditional bone defect treatment methods and has a number of drawbacks to overcome. Even though quite satisfactory *in vitro* results have been obtained, the problems such as the difficulty of having large amounts of cells on the scaffold and sustaining the viability and the phenotype of these cells until implantation while providing nourishment to the core of the 3D scaffolds exist.

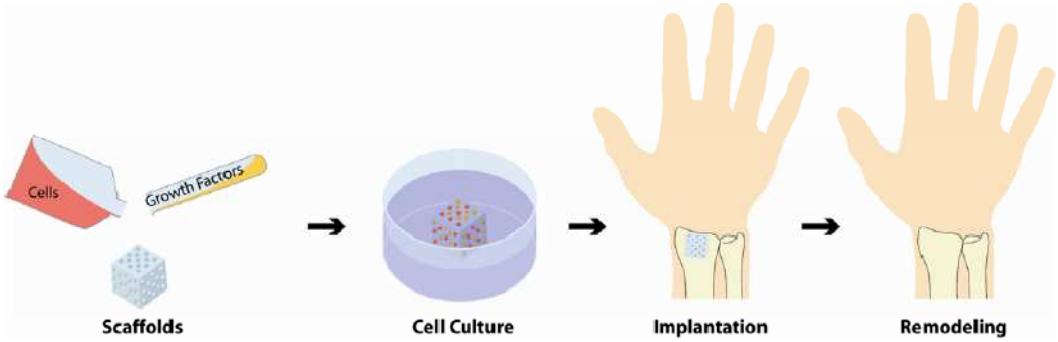

Figure 1.3. The concept of tissue engineering has three main components; scaffolds, cells and growth factors. Scaffolds provide the shape, a location for cells to adhere and grow in and the mechanical strengths. These three constituents, scaffolds, cells and growth factors are brought together *in vitro* and when the cell proliferation is reached to a satisfactory level, the construct is implanted to the defect. The construct is degraded in time and replaces its place with the newly formed tissue (Drosse *et al*., 2008).

1.5.1. Scaffolds

A wide variety of materials can be used in scaffold production such as natural or synthetic materials, composites or biologically modified materials. The scaffold materials should be biocompatible, with no toxic effect by them or by their degredation products. They should not cause an immune response when implanted into the body (Moroni *et al*., 2008). Scaffold surface is the interface with the body and affects protein adsorption and their tertiary structure, and thus influence cell-scaffold interactions (Hwang *et al*., 2008). For example, these properties of the scaffold influence the mineralization of cells and the morphology of mineral crystals formed by the cells (Hwang *et al*., 2008). Surface chemistry, topography, porosity, pore sizes, shapes and their connectivity and biodegradability are very important parameters to be considered when designing a scaffold (Chen *et al*., 2007).

1.5.1.1. Properties of an Ideal Scaffold

As has been stated until this point, scaffold design is very important in bone tissue engineering. Synthetic or natural materials chosen as scaffold materials should have some properties after a scaffold is formed using them. These are optimal fluid and bioactive molecule transport, structural integrity, controlled degradation to non-toxic products, appropriate surface chemistries that can be recognized and found attractive by the cells, the ability to induce signal transduction and matrix synthesis, and allowing organization and differentiation of cells (Leong *et al*., 2008).

The first parameter to be defined is the macro and microstructure of the scaffold (Drosse *et al*., 2008). In order to support the healing tissue, the scaffold should have a proper microstructure so that it can support vascularization, cell ingrowth, optimal fluid and oxygen flow (Drosse *et al*., 2008). This can be done via controlling the porosity of the scaffold. The attachment, proliferation and differentiation properties of anchorage dependent cells are affected by the surface roughness. Some studies demonstrated that osteogenesis can not be obtained on smooth surfaces *in vitro* (Karageorgiou *et al*., 2005). In a study, it was reported that higher porosity does not affect cell attachment but the proliferation of cells and oxygen and nutrient transport increases with increasing porosity. Contrarily the decrease in porosity increases ALP activity and osteocalcin expression, thus osteogenesis (Takahashi *et al*., 2004). Porosity also affects the mechanical properties of scaffolds. The increase in the porosity causes a decrease in the mechanical properties (Karageorgiou *et al*., 2005). For bone tissue engineering optimal porosity and the pore size should be 90% (Rezwan *et al*., 2006) and between 75 to 250 μm (Cheung *et al*., 2007), respectively. It was demonstrated that if the pores are smaller (around 10-75 μm), only fibrous tissue formed in the pores, and if the range is 75-100 μm then osteoid without a mineral phase filled the pores (Karageorgiou *et al*., 2005).

Osteoinductivity is one of the most important properties that a scaffold is expected to have. It means that the ability of the scaffold to not passively allow but actively induce bone formation and differentiation of preosteoblasts into

osteoblasts (Albrektsson *et al*., 2001). The scaffold should enhance the differentiation of osteoprogenitor and stem cells to achieve healing. Proliferation and differentiation of cells occur in a 3-D microenvironment where cell-cell communication is important, therefore the scaffold should allow this and to enhance differentiation and proliferation (Dawson *et al*., 2008a). Osteoconduction is the passive effect of the scaffold. An osteoconductive scaffold allows the bone cells to attach on its surface and to grow through the pores.

Biodegradability or bioresorbability are very important characteristics of scaffolds. The scaffold material should degrade or erode gradually in order to transfer the mechanical load to the newly forming tissue and thus eliminate the need for a second surgery to remove the implant. Degradation can be via simple hydrolysis or enzymatic. Additionally the scaffold must be easily sterilizable (Rezwan *et al*., 2006).

In order to fulfill these requirements scaffolds can be constructed by using both synthetic and natural materials.

1.5.1.2. Synthetic Materials

Synthetic materials used as scaffolds mainly include polymers such as polyglycolic acid (PGA), polylactic acid (PLA) and copolymer polylactide-co-glycolide (PLGA), ceramics such as calcium phosphates, bioactive glasses and metals like titanium. In addition to these materials some composites are used as tissue engineering scaffolds.

The use of synthetic polymers is very common because of their tunable properties such as mechanical properties and degradation rates, and features such as removal of degradation products by the body, easy processibility (Rezwan *et al*., 2006, Dawson *et al*., 2008a). Aliphatic polyesters such as PLA and PGA and their copolymer PLGA are among the most widely used. They are all approved by the US Food and Drug Administration (FDA) (Rezwan *et al*., 2006). Poly(ε-caprolactone) (PCL) is another polyester used as a scaffold material but according to the results it is reported that its degradation rate is too slow to be considered for most applications (Ikada, 2006). These polymers undergo bulk degradation and

their degradation products are acidic. If the tissue surrounding the scaffold has a poor metabolic activity and the vascularization is not adequate then these acidic degradation products may cause inflammation called the acidic sepsis (Hasirci, 2000).

Ceramics can be bioresorbable and bioactive or bioinert. Tricalcium phosphate (TCP) is a good example of bioresorbable ceramics. Its composition and crystal structure determines the degradation rate of the ceramic. HAp is the inorganic component of bone, and is used in many clinical studies but has a very low solubility. Bioactive ceramics such as calcium phosphates, low silica glasses and glass ceramics like the commercially available Bioglass® bond to the native bone tissue. They have molecules such as Na_2O and CaO in their structure which help the formation of hydroxyl carbonate apatite (HCA) on their surfaces that make them a suitable material for the cells to attach to (Roether *et al*., 2002; Carson *et al*., 2007). Bioactive glasses and glass ceramics are widely utilized as middle ear prosthesis and dental implants (Hench *et al*., 2002). In order to obtain a scaffold with the desirable physical and mechanical properties ceramics can be used together with polymers. The hydrolysis products of HAp and TCP can buffer the acidic byproducts of aliphatic polymers and therefore prevent immune responses (Hutmacher, 2000).

Another group of materials used in bone tissue engineering applications is metals. In some studies metals were used as scaffolds and seeded with stem cells. It was reported that mesenchymal stem cells could attach and differentiate into bone cells on titanium mesh scaffolds (Dawson *et al*., 2008a).

1.5.1.3. Natural Materials

Natural biomaterials have some advantageous properties such as being biocompatible, bioactive and having similar mechanical properties as natural tissues. They also provide a suitable surface for the attachment of the cells (Hwang *et al*., 2008). Despite their advantages natural biomaterials have also some disadvantages, like the difficulty of modifying their physicochemical properties, degradation rates, changing properties due to different sources, risk of

contamination with pathogens and viruses related with the source and difficulty in purification and sterilization (Dawson *et al*., 2008b). Usually they are not mechanically stable unless they are crosslinked (Meinel *et al*., 2005).

Natural materials used in scaffold construction are usually the components of extracellular matrix (ECM). Collagen is the most preferred ECM protein for scaffold production because collagen type I constitutes the 90% of bone ECM (Meinel *et al*., 2005) and is found in skin, tendons, artery walls, myofibrils, fibrocartilage and bone. Many cells including fibroblasts have a high affinity for type I collagen. Binding of fibroblasts to collagen is mediated by cell surface integrins. These integrins are activated by the help of soluble factors and by stimulating intracellular signalling cascades and transcription they regulate cell cycle and differentiation. Activation of type I collagen specific integrins mediate the osteogenic response of human bone marrow derived stromal cells. There are some commercially available collagen products, under the brand names of Collapat II®, Healos®, Biosite ® and Collagraft® (Dawson *et al*., 2008b).

Gelatin is another commonly used natural material in bone tissue engineering and is a derivative of collagen obtained by its hydrolysis. Gelatin also needs to be stabilized by crosslinking for bone tissue engineering applications. Stabilization of gelatin with common crosslinkers such as GTA, formaldehyde and epoxy resins prevent the enzymatic digestion of it. However, it was reported that the use of genipin which is a natural crosslinker extracted from gardenia fruits have a very low cytotoxic effect when compared with these most commonly used crosslinkers (Bigi *et al*., 2002).

Proteoglycans found in the extracellular matrix are also used in scaffold production. They are composed of a core protein and glycosaminoglycan (GAG) side chains, which are long unbranched polysaccharides, attached to the protein. Their properties vary according to the core protein or the GAG tails. The proteoglycans of the ECM are chondroitin sulphate, dermatan sulphate, keratin sulphate, heparan sulphate and hyaluronan (Ayad *et al*., 1998).

Not only the components but ECM as the whole tissue can be isolated and processed by decellularization, hydration or dehydration to prevent immunogenicity, to ease the handling and to prolong the shelf life but these

process stages alter some chemical and physical properties of the reconstituted tissue and affect cellular responses. The ECM contains the functional molecules of the tissue or organ that it is isolated from, so the components of ECM vary depending on the source tissue. (Badylak *et al.*, 2009).

Chitosan and silk are other frequently preferred natural materials used in bone defect healing research. Chitosan is a polysaccharide that can form insoluble complexes when combined with some connective tissue components such as collagen and glycosaminoglycans and was utilized as porous scaffolds, beads and films (Nettles *et al.*, 2002). Silk fibroin utilized in tissue engineering as a scaffold material. It has very desirable properties such as high strength, light weight, thoughness and elasticity (Wang *et al*., 2006).

Poly(hydroxyalkanoate)s (PHA) are another group of important materials of natural origin. They are produced by several different microorganisms from soil bacteria to blue-green algae and also by potatoes and grass as a result of genetic engineering. Poly(3-hydroxybutyrate) (PHB) and its copolymers containing various amounts of hydroxyvalerate (PHBV) are used most frequently. They are used as is as well as after surface modification. In one such study with osteoblasts it was reported that the oxygen plasma treated PHBV scaffolds have very suitable properties for use in bone tissue engineering (Torun Köse *et al*., 2003).

1.5.2. Cells

1.5.2.1. Cell Types

Many different types of cells have been used in bone tissue engineering applications until now. Primary cells with osteogenic capacity are isolated from mature tissues such as adult bone, periosteum, bone marrow and muscle cells (Hollinger *et al.*,2005, Marolt *et al*., 2010). However, discarded tissues obtained from dental therapies and fetal bones can be used as cell sources because of their stem cell content (Marolt *et al*., 2010). Stem cells are mostly preferred for tissue engineering studies because of their advantages over differentiated cells. Adult

stem cells such as mesenchymal stem cells and embryonic stem cells are very commonly used along with induced pluripotent stem cells (iPSCs), which are adult cells reprogrammed to show stem cell characteristics. Genetically engineered cells carrying osteogenic genes are new cell types used in recent tissue engineering studies (Marolt *et al*., 2010).

1.5.2.1.1. Stem Cells

Stem cells have certain characteristics that make them different from other, more specialized cells. The major property of stem cells is that they are able to divide and renew themselves for long periods unlike differentiated cells. When a stem cell divides, each newly formed cell has the capability to either remain as an undifferentiated stem cell or if it is induced by stimulatory factors it may become another type of cell with new special functions, such as brain cells, blood cells, etc (Bongso and Lee, 2005). Replication of cells for long periods is called proliferation. Tissue specific adult cells can replicate 50-60 times before senescence, which is called Hayflick limit, due to telomere shortening caused by cell division; in contrast, stem cells have high levels of telomerase that make them capable of renewing themselves for many more times without entering senescence (Roobrouck *et al*., 2008). If a parent stem cell proliferates, and the resulting daughter cells remain unspecialized, the parent cell is said to have the ability of long term self renewal (Lanza *et al*., 2006). Second, stem cells are unspecialized, they do not have any tissue specific markers that make them capable of performing specialized functions. However, unspecialized stem cells can become specialized cells carrying the characteristics of at least one kind of tissue specific differentiated cell.

Stem cells can be classified according to their differentiation potential. The terms totipotent, pluripotent, multipotent and unipotent are commonly used to define the developmental potential of stem cells (Bongso and Lee, 2005). After the fertilization of an egg by a sperm, a diploid cell which is called zygote is formed. Zygote is totipotent, which means that it has the potential to create an entire organism. Four days later in mouse and 7-10 days later in human, these

cells start to form a cell cluster called blastocyst. The inner cell mass of blastocyst starts to enter different lineages and form tissue specific cells (Roobrouck *et al*., 2008). These cells are pluripotent and have the potential to differentiate into any kind of cell including germ cells, but cannot give rise to a whole new organism on their own (Bongso and Lee, 2005). Multipotent stem cells are the cells which have the capability of forming several types of mature cells (Bongso and Lee, 2005). Some examples of multipotent stem cells are hematopoietic cells and brain cells which can form various neural cells and glia. Unipotent cells such as hepatocytes and some skin cells are also in undifferentiated state but they can only form one type of specialized cell. Because of this they are mostly considered as progenitor cells (Lanza *et al*., 2006).

Stem cells can also be classified based on their origin. Very broadly they are divided into two major groups: embryonic/fetal stem cells and adult stem cells (Bongso and Lee, 2005). Human embryonic stem cells are derived from four or five days old microscobic hollow ball shaped cell clumps, called blastocysts. The blastocyst is composed of three parts: the trophoblast, the surrounding layer of blastocyst; the blastocoels, the cavity inside the blastocyst, and the inner cell mass, the precursor of all body tissues (Bongso and Lee, 2005). Pluripotent human embryonic stem cells are isolated from the inner cell mass (Figure 1.4.). These cells have the potential to differentiate into a variety of specialized cells that belong to all three germ layers. Embryonic stem cells are also used for bone tissue engineering studies, however, the very complex culture conditions and the need for a feeder layer for their in vitro cultures are the main problems that still need to be overcome (Marolt *et al*., 2010). There are some ethical issues in their use and as a result despite their lower differentiation and self-renewal capacity adult stem cells are being studied more intensely (Verfaillie, 2002).

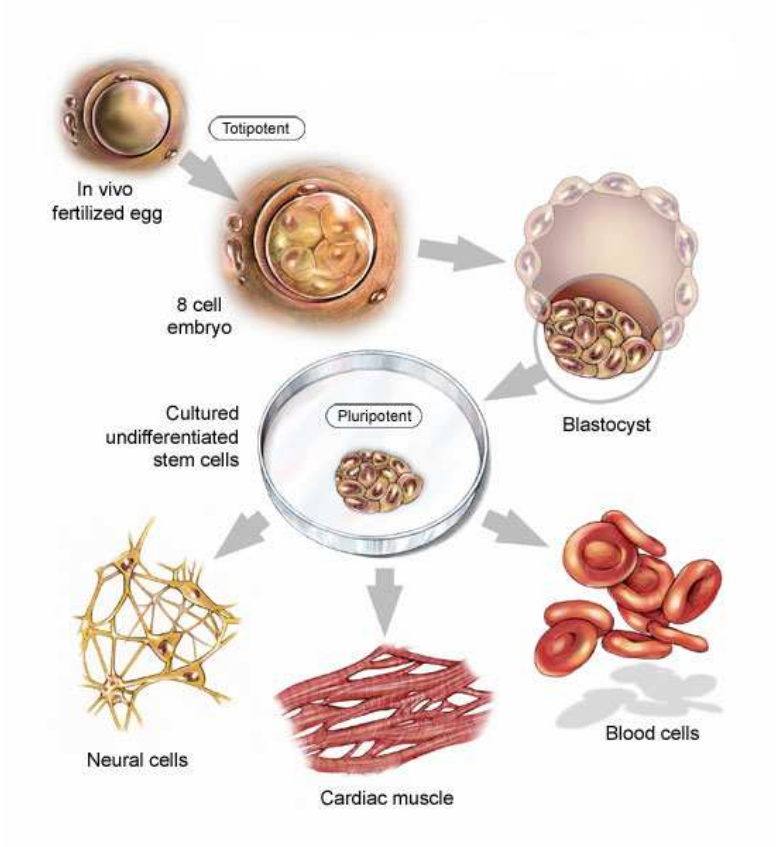

Figure 1.4. Isolation and culture of embryonic stem cells
http://www.amyshah.com/wp-content/uploads/2007/04/pluripotent-stem-cells.jpg

Adult stem cells or as sometimes referred somatic stem cells can be isolated from very different tissues. Hematopoietic stem cells are the most widely studied adult stem cells which can be isolated from bone marrow and peripheral blood. Types of other adult stem cells are mesenchymal, neural, epidermal, corneal, endothelial, gut, liver and pancreatic stem cells. Since they are less tumorogenic than embryonic stem cells and it is possible to obtain from different tissues, adult stem cells are gaining importance in regenerative medicine (Raghunath *et al*., 2005). Adult mesenchymal stem cells capable of forming bone specific cells are found in a variety of tissues such as bone marrow, adipose tissue, umbilical cord and cord blood, dental pulp, synovium, etc. Bone marrow is the most widely studied of all these tissues but the low number of cells obtained from

marrow aspirates, the decrease in the differentiation potential of bone marrow stem cells with the age of the patient and the invasiveness of the procedure of aspiration are some of its disadvantages (Marolt *et al*., 2010). Alternatively, stem cells isolated from adipose tissues such as abdominal fat are also used (Raghunath *et al*., 2005). It is known that some certain adult stem cells, especially bone marrow stem cells, can differentiate into cell types from all three germ layers (endoderm, ectoderm and mesoderm) (Meirelles *et al*., 2006). When they are transplanted into a damaged organ, they can engraft to the organ and repair the damaged site. This process is called plasticity or transdifferentiation (Alison *et al*., 2006). Recent studies showed that stem cells that are similar to the ones isolated from bone marrow stroma can be obtained from various regions of the umbilical cord (a tissue which is discarded after birth) such as blood, perivascular region, cord vein and connective tissue (Wharton's Jelly). It is very promising because of the cells having similar differentiation and renewal capacity as the bone marrow stromal cells and that they can be obtained easily without any invasive procedures (Hou *et al*., 2009).

1.5.2.1.2. Mesenchymal Stem Cells (MSCs)

Up to date, it was reported in various studies that MSCs can be isolated from different tissues. Although the main resource of MSCs in most of these studies is the bone marrow, they were also isolated from other tissues such as the adipose tissue, trabecular bone, skeletal muscle, dental pulp and periodontal ligament, dermis and hair follicles, scalp subcutaneous tissue, synovial membrane, vascular wall and even peripheral blood, lung, thymus, spleen and liver. In addition to these adult tissues, MSCs are also found in fetal tissues such as fetal bone marrow, placenta, umbilical cord, cord blood and amniotic fluid (Roufosse *et al*., 2004, Covas *et al*., 2005, Krampera *et al*., 2006, Meirelles *et al*., 2006,Chen et al., 2008). MSCs can give rise to mesodermal tissue cells like osteoblasts, adipocytes, chondrocytes, myocytes, tenocytes and bone marrow stromal cells (Zipori, 2004, Covas *et al*., 2005, Krampera, 2006, Chen *et al*., 2008,). Besides,

MSCs are also able to form cells that have neuroectoderm and endoderm characteristic because of their plasticity (Chen *et al*., 2008).

These cells are mostly known for their adherence to plastic tissue culture surfaces and forming colonies (Zipori, 2004).

It is also known that MSCs play an important role in hematopoiesis, which is the proliferation, differentiation and maturation of blood cells, by both direct cell-cell contact or secretion of growth factors (Short *et al*., 2003, Roufosse *et al*., 2004). Another important function of MSCs is to suppress inflammatory responses by promoting proliferation and differentiation of progenitor cells reside in tissues by chemical cues they secrete and help the regeneration of injured tissues (Chen *et al*., 2008). This property of MSCs together with long term self renewal capacity makes them a promising tool for tissue engineering, regenerative medicine and gene therapy (Deans *et al*., 2000).

Bone marrow derived MSCs are the most widely used of all MSCs in tissue engineering and regenerative medicine applications but the invasiveness of the bone marrow aspiration and small amount of colony forming cells (1 in 10^4 or 10^5 cells) obtained from each aspiration creates the need to search for new sources of MSCs. Recently, waste tissues (such as umbilical cord) started to be considered for MSC isolation as an alternative to bone marrow. Further, recent studies demonstrated that MSC like cells exist in the menstrual blood, which is another waste tissue (Meng *et al*., 2007). These waste tissues will be discussed more deeply in following sections because in this study they were tested as new, bone tissue engineering cell types.

1.5.2.1.2.1. Wharton's Jelly MSCs (WJ MSCs)

Wharton's jelly (WJ) is the inner connective tissue of the umbilical cord. The umbilical cord is composed of two arteries helically wrapped around one vein, a porous mucoid tissue called Wharton's jelly that surrounds these vessels, allantois which is responsible from the removal of liquid waste and gas, and the outer amnion layer (Figure 1.5).

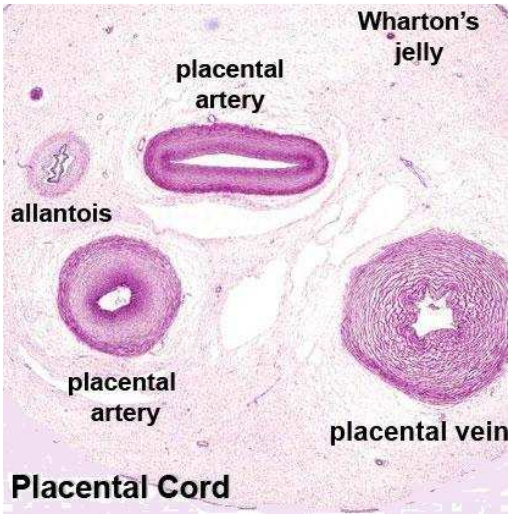

Figure 1.5. Structure of the umbilical cord
(http://embryology.med.unsw.edu.au/Science/ANAT2341lab04_4.htm#Whartonsjelly)

WJ is composed of collagen and elastin fibers, and in its pores proteoglycans and hyaluronic acid which form the viscous structure are found. It functions as the surrounding connective tissue like in cardiovascular vessels (Ferguson *et al*., 2009).

MSCs can be isolated from different parts of the umbilical cord and fetal tissues such as the umbilical cord blood, Wharton's jelly, amniotic fluid, placenta, fetal blood, subendothelial layer of cord vein and the perivasculature (Qiao *et al*., 2008, Hou *et al*., 2009, Hildebrandt *et al*., 2009, Ishige *et al*., 2009, Schneider *et al*., 2010). MSCs isolated from WJ can be induced to differentiate into adipocytes, osteoblasts, chondrocytes, neurons, cardiomyocytes and endothelial cells (Schneider *et al*., 2010, Chen *et al*., 2009, Ishige *et al*., 2009, Yucel *et al*., 2010, Kenar *et al*., 2010, Zhao *et al*., 2010). When induced for osteogenic differentiation, they produce osteoblastic markers such as ALP, collagen type I, bone sialoprotein, osteocalcin, osteonectin and osteopontin, and extracellular matrix in addition to calcium deposits (Schneider *et al*., 2010). They have surface markers similar to the bone marrow MSCs in addition to some embryonic markers. Because of being more primitive in comparison to the bone marrow

MSCs, their plasticity is higher and these MSCs are not rejected by the immune system when used *in vivo* (Zhao *et al*., 2010). The WJ cells are affected by some factors such as the age of the mother, genetic disorders of the fetus and pregnancy disorders (Ferguson *et al*., 2009). It was reported that the proliferation rate of MSCs are higher when fresher tissue is used (Qiao *et al*., 2008). The osteogenic differentiation potential of WJ MSCs is still a matter for discussion. In a recent study it was reported that even though WJ MSCs secreted more extracellular matrix, gene expression analysis showed that early and intermediate osteogenic differentiation markers such as osteopontin and alkaline phosphatase are expressed lesser than by bone marrow MSCs (Schneider *et al*., 2010). Similarly, when the osteogenic differentiation capacity of WJ, umbilical cord vein and artery cells were compared, WJ MSCs were found to have the lowest differentiation (Ishige *et al*., 2009). However, the advantageous properties of this cell source such as the noninvasive isolation and easy expansion make them an alternative source to be considered for bone tissue engineering applications.

1.5.2.1.2.2. Menstrual Blood MSCs (MB MSCs)

In humans and some other animals, the inner layer of uterus, endometrium, gets thicker during a defined period as a result of proliferation of cells that reside there and due to angiogenesis to prepare a suitable environment for a zygote to adhere during pregnancy. If the egg is not fertilized, the endometrium is shed from the body, and this process is known as menstruation. This is a 28 day period in humans. The thickness of endometrium increases by about 4-7 mm during the first half of the menstrual cycle (Gargett *et al*., 2008). The proliferation, differentiation and blood vessel formation capacities of these cells led the scientists to study their stem cell like characteristics.

It was reported that menstrual blood derived stem cell like cells have self renewal and multipotency properties (Zhang *et al*., 2009). These cells can undergo more than 68 doublings without any karyotypic anomalies, have a relatively short doubling time, approximately 19.4 h, and can differentiate into cells from all the three germ layers, such as osteocyte, adipocyte, myocyte, endothelial, neuronal

and pancreatic cells (Meng *et al*., 2007). Although these cells possess similar characteristics to stem cells, it is still contested whether they are stem cells or not. As a result of this they are labeled differently by researchers. Endometrial regenerative cells, menstrual blood stromal cells, menstrual blood derived stem cells and menstrual blood derived mesenchymal cells are the examples of the various terminology used to describe them (Zhang *et al*., 2009). They carry some but not all of embryonic stem cell markers (e.g. they are positive for Oct-4, but negative for SSEA-4 and Nanog), and this makes them resemble the human amniotic fluid stem cells (Meng *et al*., 2007).

These cells are very promising especially for females because of being autologous, non invasive and easily isolated multipotent cell source which can even serve as personal tissue banks (Meng *et al*., 2007). In addition, they can be used as an allogenic cell source because they are reported to be not rejected by the immune system (Zhang *et al*., 2009).

1.5.3. Growth Factors and Bioactive Agents

The third component of tissue engineering in addition to cells and scaffolds is the growth factors and bioactive agents. Growth factors are hormones or proteins that bind to the receptors on the cell surfaces and stimulate or inhibit cellular activities such as proliferation, differentiation, migration and apoptosis. Their concentration is important in regulating cellular activities and they are effective at pico or nanomolar concentrations (Hollinger *et al.*, 2005). The most commonly used growth factors in bone tissue engineering are the transforming growth factor β (TGF-β), bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF).

TGF-β is a superfamily of a group of proteins that take part in growth, differentiation and extracellular matrix production in bone. BMPs belong to a subgroup of TGF-β. BMP-2, BMP-4 and BMP-7 are the most important growth factors of this group used frequently in bone tissue engineering applications. However, in the literature it is reported that they have a species specific effect in

osteogenic differentiation and even though they have a positive effect on osteoblast differentiation in rodents *in vitro*, they are not good inducers for human bone formation (Kim *et al*., 2005; Vater *et al*., 2011). Additionally there is a potential risk of BMPs forming cancer (Kim *et al*., 2005).

FGFs and PDGF function in mitogenesis of mesenchymal cells. FGFs and VEGFs are also responsible for angiogenesis. They enhance bone formation and production of blood vessels.

IGFs are important in collagen type I production, and *in vitro* and *in vivo* proliferation and differentiation of bone cells (Hollinger *et al.*, 2005).

In addition to these growth factors, some bioactive agents are also used to trigger *in vitro* differentiation of MSCs into bone lineage. Dexamethasone, β-glycerophosphate and ascorbic acid are the most commonly used non growth factor differentiation agents for bone tissue engineering applications.

Dexamethasone is the most important component of an osteogenic medium and is a synthetic glucocorticoid. Glucocorticoids have an inhibitory effect on the proliferation of osteoblastic cells, and instead they induce their osteoblastic differentiation (Advani *et al*., 1997). The effect of dexamethasone on proliferation is known to be concentration dependent and at around physiological concentration (10 nM) it does not have an inhibitory effect, additionally when it is used in combination with ascorbic acid, its negative effect on collagen synthesis of cells is also overcome (Vater *et al*., 2011).

Ascorbic acid is another important constituent of the osteogenic medium. It is the reduced form of vitamin C, an essential vitamin for humans. Ascorbic acid is used as a cofactor in the hydroxylation of proline and lysine amino acids in collagen (Takamizawa *et al*., 2004, Le Nihouannen *et al*., 2010). Ascorbic acid stimulates the proliferation and ECM secretion of mesenchymal cells *in vitro* and it is suggested that this causes a higher mineralization and ALP activity (Choi *et al*., 2008; Coelho *et al*., 2000). However, ascorbic acid is unstable under cell culture conditions, so instead, ascorbic acid 2-phosphate, which is very stable and releases ascorbic acid under the action of ALP enzyme found at the plasma membrane, is mostly preferred (Takamizawa *et al*., 2004).

β-Glycerophosphate is used as the inorganic phosphate source in osteogenic medium for *in vitro* studies and it is also hydrolyzed by ALP (Vater *et al*., 2010). Phosphate ions released by hydrolysis stimulates the mineral formation by osteoblastic cells (Coelho *et al*., 2000).

1.6. The Aim and Approach of the Study

The aim of this study was to produce a tissue engineered bone construct that has a scaffold with both organic and inorganic compounds, and stem cells obtained from discarded tissues. The approach followed was to first produce collagen scaffolds carrying *in situ* prepared hyrdoxyapatite-like crystals to mimic the natural bone composition. The crystals were prepared by wet precipitation using an immersion method instead of adding commercial hydroxyapatite crystals. Collagen was crosslinked with genipin to increase the stability and mechanical properties of the foams. In most of the studies crosslinkers such as glutaraldehyde and formaldehyde are used. These crosslinkers have some cytotoxic effects so in this study a safer crosslinker, genipin, which is also used in food industry, was chosen. Scaffolds were physically and mechanically tested for their suitability for bone tissue engineering applications. The porous structure of foams was examined by scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM) and pore size distribution was studied by mercury porosimetry. Calcium to phosphate ratio of the crystals was determined with electron diffraction spectra (EDS).

In the literature there are numerous studies about bone tissue engineering. The critical sized defects in the bone can not recover without a suitable support material that fills the defect. In most of the studies the scaffold is seeded with bone marrow stem cells. Bone marrow is a reservoir for stem cells but the isolation technique of these cells is painful for the donor. In this study two different types of stem cells, both are isolated from discarded tissues (umbilical cord and menstrual blood) therefore which do not require invasive isolation techniques, were used. Menstrual blood stem cells have discovered recently and in the literature there are only studies about their differentiation potential on tissue

culture plates. But in this study the behavior of these cells are examined on three dimensional constructs. The approach was to compare the proliferation and differentiation efficiencies of these two cell types into osteoblastic cells in order to offer a new cell source obtained by non invasive approach for bone tissue engineering. Cell proliferation and differentiation on tissue culture polystyrene and scaffolds were examined with metabolic and enzymatic assays which allowed the assessment of the effect of the scaffold on these parameters. Furthermore, the behavior of cells was studied by microscopy.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Sprague-Dawley rat tails to extract collagen were kindly provided by Gazi University, Laboratory of Animal Breeding and Research Center (GÜDAM). Dialysis tubing (MWCO 10000) was purchased from Thermo Scientific (USA). Sodium phosphate monobasic and dibasic, sodium ammonium hydrogen phosphate and trisma base were obtained from Merck (Germany). Calcium chloride and sodium azide were from Riedel-de Haën (Germany). Genipin was obtained from Wako GmbH (Germany). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and Trypsin-EDTA (0.25 %) were purchased from HyClone (USA). HAM-F12 medium and penicillin/strepromycin (100 units/mL-100 μg/mL) were obtained from Biochrom AG (Germany). Alexa Fluor 488 Phalloidin and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Chemicon (USA). Dexamethasone, ascorbic acid, β-glycerophosphate and silver nitrate were from Sigma Chem Co (USA). ALP assay kit was obtained from Randox Laboratories (UK). CellTiter 96* AQ_{ueous} One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Germany). Sodium thiosulfate and dimethyl sulfoxide (DMSO) were obtained from Fluka and AppliChem, respectively.

2.2. Methods

2.2.1. Production of Collagen Scaffolds

2.2.1.1. Isolation of Collagen Type I from Rat Tails

The isolation process was carried out according to BIOMAT SOP 43, as reported earlier (Zorlutuna, 2009). Full length incisions were made on the Sprague Dawley rat tails and skins were stripped away. Tendons were removed from tails by forceps, placed in 0.5 M acetic acid and stirred at 4 ^{o}C until they were dissolved. The solution was then filtered through glass wool to remove undissolved tissue and dialyzed by using dialysis tubings of 10000 molecular weight cut off against dialysis buffer (5 L; 12.5 mM sodium phosphate dibasic, 11.5 mM sodium phosphate monobasic, pH 7.2) for several days with a change of buffer everyday. After the collagen precipitated out as a semi-solid gel, it was centrifuged (Sigma 3K30, UK) at 16,000 *g* for 10 min. The pellet was dissolved in 0.15 M acetic acid by stirring overnight. To reprecipitate the collagen, NaCl was added (5 % w/v) to the solution and it was stirred overnight, centrifuged once more for 10 min at 16,000 *g*. The pellet was dissolved again in 0.15 M acetic acid and dialyzed for 5 days against dialysis buffer by changing the buffer once a day to remove all traces of NaCl. Collagen precipitated in the dialysis tubings was centrifuged at 16,000 *g* for 10 min as before. Pellet was stirred in 70% ethanol for 48 h and after a final centrifugation step pellet was frozen at -80 ^{o}C (Sanyo MDF-U53865, Japan) overnight and lyophilized (FreeZone$^{6\ Plus}$ Freeze Dry System, Labconco Co., USA). The collagen powder was kept at 4 ^{o}C for long term storage.

2.2.1.2. SDS-PAGE of Isolated Collagen

SDS-PAGE was done to characterize the isolated protein from rat tails. In SDS-PAGE, proteins are seperated according to their molecular weights by applying electrical charge. SDS is an anionic detergent and is bonded to proteins and cover their surfaces with negative charge, resulting with the coverage of each

protein according to its mass. An electrical voltage is then applied and the negatively charged proteins migrate towards positive electrode. Smaller proteins move faster and reach the positive electrode first. Two gels with different acrylamide concentration is prepared, one is being the separating and the other is stacking gels. Stacking gel has lower acrylamide concentration and is more basic. This gel concentrates the proteins and provides a thin starting region. In order to prepare the separating and stacking gels, 30% acrylamide/bisacrylamide (37.5:1 w/w acrylamide/bisacrylamide) solution was made. First separating gel (12% v/v) was prepared and cast. For separating and stacking gels, the amounts given in Table 2.1. were used:

Separating gel was poured into the casting system and left at room temperature for polymerization and immediately 70% ethanol was added on it to prevent the oxidation of the gel. After the polymerization was complete, ethanol was removed and stacking gel was prepared and the combs were placed. After the stacking gel was polymerized sample loading solution (4% v/v β-mercaptoethanol, 0.025% v/v bromophenol blue, 14% v/v SDS, 46% v/v glycerol, 27% v/v H_2O and 9% v/v 0.5 M Tris-HCl, pH 6.8) was prepared, mixed with collagen samples (1:1 v/v) and the collagen samples (0.2% in 0.05 M acetic acid) of commercial source (BD Biosciences, USA) and isolated in the laboratory were denatured at 95 ^{o}C for 5 min. They were loaded to wells and the gel was run at 30 mA for 2.5 h (Bio-Rad Power Pac HC, Finland). The gel was stained with 0.2% w/v Coomasie Brillant Blue (in 12% v/v acetic acid, 50% v/v methanol and 38% v/v H_2O) overnight. The next day gel was destained with the solution containing 10% v/v acetic acid, 50% v/v methanol and 40% v/v H_2O.

Table 2.1. Separating and stacking gel compositions

	Separating Gel (12%)	**Stacking Gel (4%)**
Acrylamide/Bisacrylamide (30%)	4 mL	0.7 mL
Distilled water	3.4 mL	3 mL
1.5 M Tris-HCl (pH 8.8)	2.5 mL	-
0.5 M Tris-HCl (pH 6.8)	-	1.25 mL
10% SDS	100 μL	50 μL
10% Ammonium persulfate (APS)	50 μL	25 μL
TEMED	5 μL	5 μL
TOTAL	**10 mL**	**5 mL**

2.2.1.3. Preparation of Crosslinked Collagen Foams

Collagen isolated from the rat tails was dissolved in 0.05 M acetic acid (1.1 % w/v) on a stirrer avoiding the temperature to exceed 35 ^{o}C in order to prevent denaturation. Genipin was dissolved in 10 mM PBS (1 % w/v) and added to collagen solution (10 % v/v of final volume) to obtain 1% w/v collagen and 0.1% w/v genipin in the final solution and stirred rigorously for 2 min. Crosslinking was continued for 48 h at room temperature in a 24 well tissue culture plate (TCPS). Then the solution was frozen at -20 ^{o}C overnight, and lyophilized until complete dryness to obtain a porous structure. Afterwards, circular pieces with 7 mm diameter were cut by a biopsy puncher from the foams for use in further studies.

2.2.1.4. *In Situ* Formation of Calcium Phosphate Crystals on Collagen Foams

Lyophilized collagen foams were wetted with 10 mM PBS overnight, and then immersed in a sodium ammonium hydrogen phosphate solution (100 mM sodium ammonium hydrogen phosphate in 50 mM Tris, 1% NaN_3 pH 7.2) for 2 h, removed, blotted and then immersed in calcium chloride solution (100 mM calcium chloride in 50 mM tris, 1% NaN_3 pH 7.2) for 2 h at room temperature without washing between immersion steps (Total 4 h immersion=1 cycle). This cycle was repeated once more and then the foams were immersed in distilled water for 2 h to remove crystals not attached to the foam. Foams were then frozen at -20 ^oC overnight and lyophilized for 8 h. The same procedure was repeated by changing the immersion duration and sequence in order to study the effect of these changes on *in situ* crystal formation. In this case foams were immersed in each solution for 24 h instead of 2 h or the foams were first immersed in calcium solution instead of phosphate solution.

2.2.2. Characterization of Collagen Foams

2.2.2.1. Scanning Electron Microscopy (SEM)

Calcium phosphate treated, genipin crosslinked collagen foams were coated with 25 nm gold and examined under high vacuum with FEI QUANTA 200 scanning electron microscope (SEM) at Bilkent University UNAM for observation of calcium phosphate crystal formation, foam pore shape and sizes. Foams were also cut horizontally and vertically to study the pore size variation and crystal distribution along the thickness of the scaffold.

2.2.2.2. Electron Diffraction Spectra (EDS)

Calcium to phosphate ratio of calcium phosphate deposits on collagen foams were determined with EDS (Noran system SIX) attachment of JEOL (Japan, JSM 6400) SEM at the Metallurgical Engineering Department at METU.

Foams were coated with gold under vacuum and placed in the sample holder of electron microscope. The calcium to phosphate ratios were obtained from the peaks of graphs.

2.2.2.3. Pore Size Distribution Analysis

Pore size distribution of collagen foams with different collagen concentrations, freezing temperature and calcium phosphate treatment durations was analyzed by mercury porosimetry at METU Central Laboratory. Foams were examined at low pressure (0-50 psi) and the pore sizes of max 200 μm were measured.

2.2.2.4. Porosity Analysis

SEM micrographs of foams were converted to black and white format and the ratio of black area to total area was calculated by NIH Image J software the program which yields the porosity.

2.2.2.5. Compression Tests

Compression tests were performed on collagen foams by using a mechanical tester (Stable Micro Systems, MT-LQ, UK). Collagen foams made with 1% w/v collagen and 0.1% w/v genipin were selected for compression tests, because the foams with 0.75% collagen shrank when immersed in calcium phosphate solution because they were not strong enough. Three different groups of samples were tested. Each group consisted of 4 samples. These groups were: a) genipin crosslinked, b) genipin crosslinked and with calcium and phosphate formation for 4 h and 2 cycles, c) genipin crosslinked and with calcium phosphate formation for 48 h and 2 cycles. All the samples were fully dried by lyophilization, their thicknesses and diameters measured with a micrometer with a sensitivity of 0.1 mm before testing. The average diameter and thicknesses of the foams used in compression test were 12.0 mm and 3.4 mm, respectively.

Compression was applied at a displacement rate of 10 $mm.min^{-1}$. Young's modulus (E^*), elastic compressive strength (σ_{el}^*) and collapse elasticity (E_{col}) values were calculated according to Harley *et al*., 2007.

2.2.3. *In vitro* Studies

In the *in vitro* studies two types of human cells, menstrual blood and Wharton's Jelly derived mesenchymal stem cells, were used.

2.2.3.1. Isolation and Culture of Mesenchymal Stem Cells from Menstrual Blood (MB)

Menstrual blood from donors was collected in sterile Falcon tubes on the first day of menstruation and suspended in 5 mL expansion medium (DMEM high glucose complemented with 10% FBS and 100 units/mL-100 μg/mL Pen/Strep). Suspension was cultivated in a 25 cm^2 flask in humidified incubator (Sanyo, Model MCO-17AC, Japan) at 5% CO_2 and 37 ^oC overnight and the next day medium was changed. Medium was renewed every three days during cell culture.

2.2.3.2. Isolation and Culture of Mesenchymal Stem Cells from Wharton's Jelly (WJ)

Wharton's Jelly mesenchymal stem cells used in this study were a gift of Drs. Halime Kenar and Deniz Yücel from METU BIOMAT. Briefly, umbilical cords were taken with the consent of mothers and isolated within 24 h. Umbilical cord tissues were cut into 2-3 cm pieces and washed with antibiotic (100 units/mL-100 μg/mL Pen/Strep) containing PBS. Arteries and vein were removed from tissue pieces manually with a forceps. Umbilical cord tissues were cut into smaller pieces, transferred to 6 well plates and cultivated in the expansion medium (DMEM low glucose:HAMF12 (1:1 v/v ratio) supplemented with 10% FBS, 100 units/mL-100 μg/mL Pen/Strep and 1 ng/mL bFGF) in a carbondioxide incubator with 5% CO_2 and at 37 ^oC. After two days, the medium was changed

and cells were incubated for a further two weeks with the renewal of medium every 3 days. At the end of two weeks tissues were removed from the 6 well plates and the cells were trypsinized and frozen in 90% FBS and 10% DMSO for use in further experiments. For *in vitro* experiments on TCPS and collagen foams, Wharton's Jelly MSCs were used from this stock. Cells were thawed in expansion medium, centrifuged at 3000 rpm for 5 min to remove DMSO and seeded to 75 cm^2 flasks in expansion medium. WJ MSCs were cultivated in medium in a humidified incubator with 5% CO_2 and at 37 ^oC with a change of medium every 3 days.

2.2.3.3. Morphology of WJ and MB MSCs

WJ and MB MSCs were stained with Alexa Fluor 488 labelled Phalloidin and DAPI for observation of the cytoskeleton and the nucleus, respectively, under a fluorescent microscope (Olympus IX70, Japan). For staining, the cells were washed with PBS (10 mM, pH 7.4) twice and fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS twice, permeabilized with 0.1% Triton X-100 (in 10 mM Tris-HCl buffer, pH 7.4) at room temperature for 5 min. After washing with PBS again, cells were incubated with 1% BSA (in PBS) at 37 ^oC for 30 min to block non-specific binding of the dye. Afterwards cells were stained with 0.5 μg/mL Phalloidin (in 0.1% BSA in PBS). Cells were then washed with 0.1% BSA in PBS 3 times. After that the nuclei of the cells were stained with DAPI (1/5000 dilution in PBS) for 10 min at room temperature. After washing with PBS cells were observed by a fluorescent microscope (Olympus IX70, Japan).

2.2.3.4. Behavior of WJ and MB MSCs on TCPS

2.2.3.4.1. Cell Proliferation on TCPS

Cell proliferation on TCPS was studied by using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation (MTS) assay. Cells in the wells of the TCPS were

washed with PBS (10 mM, pH 7.4) twice and incubated with 10% MTS containing DMEM low glucose completed with 10% FBS and 1% antibiotics for 2 h at 37 ^{o}C and 5% CO_2. MTS solution is composed of tetrazolium salt and an electron transfer reagent (PMS or PES) which are reduced by only living cells during cellular respiration. Tetrazolium salt is reduced to a colored formazan product (Figure 2.1) which is determined by measurement of the absorbance at 490 nm by an ELISA microplate reader (Molecular Devices, Inc. USA). These values are then converted to cell numbers by using a calibration curve.

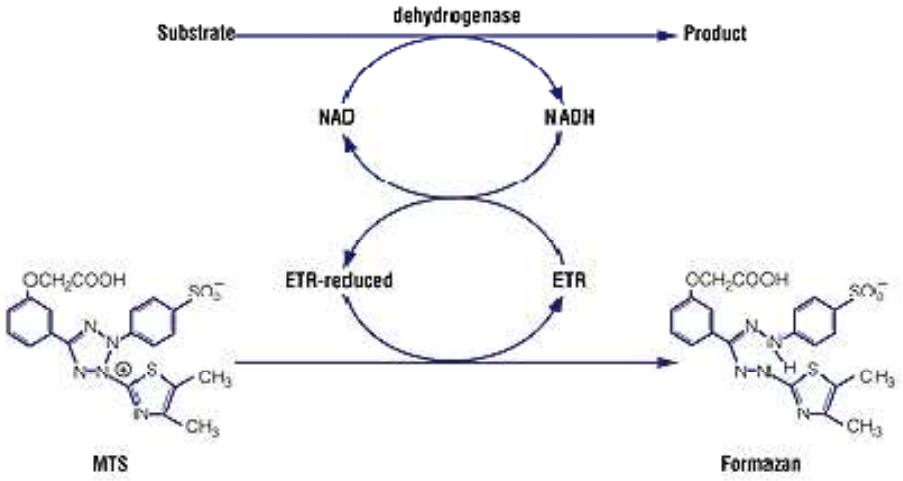

Figure 2.1. Schematic representation of MTS assay. Dehydrogenase enzymes found in living cells reduce NAD^{+}. NADH transfers its electrons to the electron transfer reagent (ETR). MTS tetrazolium salt is reduced by ETR and colored formazan product which is soluble in culture medium is formed.

2.2.3.4.2. Osteogenic Differentiation of WJ and MB MSCs

In order to perform differentiation studies of MB and WJ MSCs the cells were seeded at a density of $1.5x10^4$ cells/well in 24 well tissue culture plates (TCPS) and cultivated in control medium (DMEM high glucose complemented

with 10% FBS and 100 units/mL-100 μg/mL Pen/Strep) for 24 h. The next day the control medium was changed with osteogenic induction medium (DMEM high glucose supplemented with 10 nM dexamethasone, 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, 10% FBS and 100 units/mL-100 μg/mL Pen/Strep) and the cells were cultivated for 21 days in this osteogenic induction medium at 5% CO_2 and 37 ^oC in a carbondioxide incubator (Sanyo, Model MCO-17AC, Japan).

2.2.3.5. Evaluation of Osteoblastic Activity for WJ and MB MSCs on TCPS

The osteoblastic activity of MSCs was determined by Alkaline Phosphatase (ALP) enzyme assay and the mineralization was detected by von Kossa staining.

2.2.3.5.1. Alkaline Phosphatase (ALP) Enzyme Assay

For ALP assay, the cells in the 24 well plates were washed with 10 mM PBS and lysed with 0.1% Triton X-100 (in 10 mM Tris-HCl buffer, pH 7.4). Cell lysates were collected in sterile Falcon tubes and frozen at -20 ^oC. In order to burst the cells, cells were frozen and thawed 3 times and sonicated at 25 Watts for 5 min with a probe sonicator (Cole Parmer Instrument Co. 4710 Series, USA). After sonication, the lysate was centrifuged with a bench top centrifuge (Rotofix 32 Hettich, Germany) at 2000 rpm for 10 min. Aliquots (100 μL) from each sample were taken and 150 μL of 2-amino-2-methyl-1 propanol (AMP) buffer and p-nitrophenyl phosphate (substrate) were added, and incubated in 24 well plates in a humidified incubator (5% CO_2 and 37 ^oC) for 2 h in order to perform the reaction given in Equation 2.1. After incubation, 100 μL 0.2 M NaOH was added to each sample to stop the reaction. 200 μL from each sample was taken by a micropipette and transferred to a 96 well plate in order to measure the absorbance of samples with an Elisa plate reader (Molecular Devices, Inc. USA) at 405 nm. ALP enzyme activity was expressed as “μM substrate converted to product/min” by 100 μL cell lysate by using a calibration curve. 0.1% Triton X-100 (in 10 mM Tris-HCl buffer, pH 7.4) without cell lysate combined with 2-amino-2-methyl-1

propanol (AMP) buffer and p-nithophenyl phosphate substrate and 0.2 M NaOH served as the blank.

$$\text{p-nitrophenylphosphate} + H_2O \xrightarrow{\text{ALP}} PO_4^{-3} + \text{p-nitrophenol} \qquad \textbf{2.1.}$$

Specific ALP activities were calculated by dividing the activities calculated from 100 μL cell lysate to cell numbers determined by MTS assay.

2.2.3.5.2. von Kossa Staining

In order to detect mineral formation by the differentiated cells, von Kossa staining was performed. The cells in the TCPS were washed with 10 mM PBS twice, then fixed with 4% paraformaldehyde for 15 min at room temperature, washed with PBS and then with distilled water several times. Silver nitrate solution 1% (w/v) was prepared and the cells in each well of a 24-well plate were incubated with 500 μL of this solution for 30 min under UV light in the laminar hood. After incubation the cells were rinsed with distilled water and incubated with 5% (w/v) sodium thiosulfate for 5 min at room temperature to remove unreacted silver. In this staining protocol, calcium is reduced by ultraviolet light and silver replaces calcium and forms metallic silver, which can then be visualized by light microscopy. After incubation with sodium thiosulfate, cells were again washed with distilled water and observed under a light microscope (Olympus IX 70, Japan, with inverted phase contrast attachment) for detecting a brownish black stain.

2.2.3.6. Cell Seeding on Calcium Phosphate Containing Collagen Foams

Calcium phosphate containing collagen foams were sterilized by immersing in 70% ethanol in 6 well tissue culture plates for 3 h at room temperature in the laminar flow hood. Ethanol was then removed and 10 mM PBS was added onto the foams and incubated at room temperature for 3 h, with the exchange of the PBS every hour. After that PBS was replaced with the control

medium (DMEM High glucose supplemented with 10% and 1% antibiotics) and the foams were incubated at 37 ^{o}C, 5% CO_2 overnight. The medium was discarded and the foams were air dried in the laminar hood before cell seeding. WJ and MB MSCs at passage 2 were trypsinized with 0.05% and 0.1% trypsin/EDTA solutions, respectively. Cells counted with a hemocytometer were seeded to each scaffold ($5x10^4$ cells in 50 µL medium). Foams were maintained at 37 ^{o}C, 5% CO_2 for 4 h without addition of medium to improve cell attachment on the foams. Then 1 mL control medium was added onto each foam and maintained for 24 h before replacing the control medium with the osteogenic differentiation medium. Cells were incubated in osteogenic differentiation medium for 21 days. Cell attachment on foams was detected with MTS assay and Phalloidin-DAPI staining on first day of incubation. Cell proliferation and osteoblastic differentiation were determined by MTS and ALP assays, on days 14 and 21.

2.2.3.7. Behavior of WJ and MB MSCs on Collagen Foams

2.2.3.7.1. Cell Proliferation on Collagen Foams

Foams carrying the cells (from section 2.2.3.6.) were transferred to new 24-well plates 24 h after than seeding and cell proliferation on collagen foams was determined by MTS assay as described in section 2.2.3.4.1.

2.2.3.7.2. Evaluation of Osteoblastic Activity

Osteoblastic activity of cells on hydroxyapatite containing collagen scaffolds (from section 2.2.3.6.) was assessed by ALP enzyme assay on days 14 and 21 in osteogenic medium.

2.2.3.7.2.1. Alkaline Phosphatase (ALP) Enzyme Assay

Scaffolds seeded with MB and WJ MSCs (from section 2.2.3.6.) were washed with PBS twice. One mL 0.1% Triton X-100 (in 10 mM Tris-HCl buffer,

pH 7.4) was put in sterile Falcon tubes, scaffolds were transferred into tubes and frozen at -20 ^{o}C. Triton X-100 solution containing scaffolds with cells were thawed and frozen 3 times and sonicated at 25 Watts for 5 min with a probe sonicator without taking the scaffolds out of the medium. The rest of the procedure was applied as described in section 2.2.3.5.1.

2.2.3.7.2.3. von Kossa Staining

On the 21st day of osteogenic differentiation collagen foams were washed with PBS and the cells on foams were fixed with paraformaldehyde (4%, 15 min, room temperature). Scaffolds were then washed with PBS and PBS was discarded. Foams were frozen at -20 ^{o}C and ten μm thick sections were obtained on silane coated slides by using a cryomicrotome (Leica CM 1510 S, Germany) and the slides were kept at -20 ^{o}C until staining. The rest of the staining procedure was as described in section 2.2.3.5.2.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Characterization of Collagen Foams

3.1.1. SDS-PAGE of Isolated Collagen

The gel electrophoresis result of collagen type I isolated in our laboratory is shown in Figure 3.1. First lane in the figure demonstrates the protein ladder (Fermentas Spectra Multicolor Broad Range Protein Ladder, Canada), and the remaining lanes are loaded with commercial (C) and isolated (I) collagen. Collagen type I has doublets at 115 and 130 kDa, and at 215 and 235 kDa. Isolated collagen had bands only at these indicated places which shows the purity of the protein.

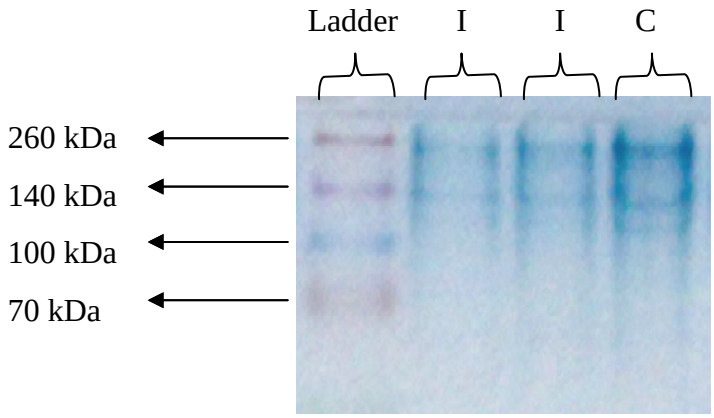

Figure 3.1. Gel electrophoresis result of isolated type I collagen. First lane is commercial ladder, I: isolated collagen, C: commercial collagen. Stacking gel concentration: 4%, seperating gel concentration: 12%

3.1.2. Determination of Pore Size and Distribution Using SEM and Mercury Porosimetry

Collagen foams that were crosslinked with genipin and those on which *in situ* calcium phosphate crystals were formed examined with SEM in order to determine the approximate pore sizes and their distribution along the thickness of collagen foams, and also the crystal structure of the newly formed calcium phosphate deposits. Two different collagen concentrations (0.75% and 1%, w/v), two freezing temperatures (-20^{o}C and -80 ^{o}C, before lyophilization) were used to test the effect of these variables; different immersion durations were also used (2x4 h and 2x48 h) as process parameters.

Pore size distribution of scaffolds was examined by mercury porosimeter. Since the maximum pore size that the device can measure is 200 μm, larger pores could not be examined. All the foams had pores varying from 5 to 200 μm except those with 0.75% collagen, frozen at -20 ^{o}C, untreated or treated with calcium phosphate for 2x4 h; and those of 1% collagen, frozen at -20 ^{o}C, untreated. These mentioned foams had pores starting from minimum 50 μm (Appendix A).

It was observed that increasing collagen concentration from 0.75% to 1% did not affect the average pore size of genipin crosslinked collagen foams significantly (Figure 3.2., Table 3.1.). However, for a given collagen concentration (1% w/v) pore sizes were more homogeneous and interconnected when they were processed at -20 ^{o}C rather than when processed at -80 ^{o}C (Figure 3.2.); and this was in accordance with the literature (Chen *et.al.*, 2007).

The porosity measurement was done by the NIH Image J program using the SEM micrographs. The average porosity was calculated to be 63%, but lower freezing temperature caused the porosity to decrease to as low as 16%. It was observed that the increasing collagen concentration from 0.75% to 1% did not affect the porosity very much, but the freezing temperature has a significant effect on porosity (Table 3.2.).

Table 3.1. Pore size distribution analysis results of collagen foams prepared under different conditions (porosimeter)

	Treatment Condition					
Freezing Temperature (°C)	**Collagen Concentration (%)**					
	0.75			**1**		
	U.T.	**2x4 h**	**2x48 h**	**U.T.**	**2x4 h**	**2x48 h**
-80	*20-30; 200 μm	30-50 μm	15-40 μm	30-50 μm	20-50 μm	20-30 μm
-20	200 μm	200 μm	*30-50; 200 μm	200 μm	*50-70; 200 μm	*15-30; 200 μm

U.T.: Untreated with calcium phosphate process
* These samples had 2 pore size peaks in the porosimeter

Table 3.2. Porosity of various foams prepared under different conditions (Image J).

	Treatment Condition					
Freezing Temperature (°C)	**Collagen Concentration (%)**					
	0.75			**1**		
	U.T.	**2x4 h**	**2x48 h**	**U.T.**	**2x4 h**	**2x48 h**
-80	77.70%	23.96%	16.02%	71.93%	68.37%	54.89%
-20	57.83%	51.59%	43.19%	75.43%	70.03%	56.69%

When the top surfaces of collagen foams were compared it was observed that the pores of the foams with the lower collagen concentration and freezing temperature were almost completely clogged, however the foam that had 1% collagen and frozen at -20 ^{o}C had pores large enough for cell seeding even after 2 cycles of calcium phosphate formation for both 4 h/cycle and 48 h/cycle (Figure 3.3., Table 3.1.).

Collagen foams with 0.75% w/v collagen concentration were mechanically weak and they had shrinkage problem after calcium phosphate treatment. Because of this foams with 1% w/v collagen concentration were chosen for tissue culture studies.

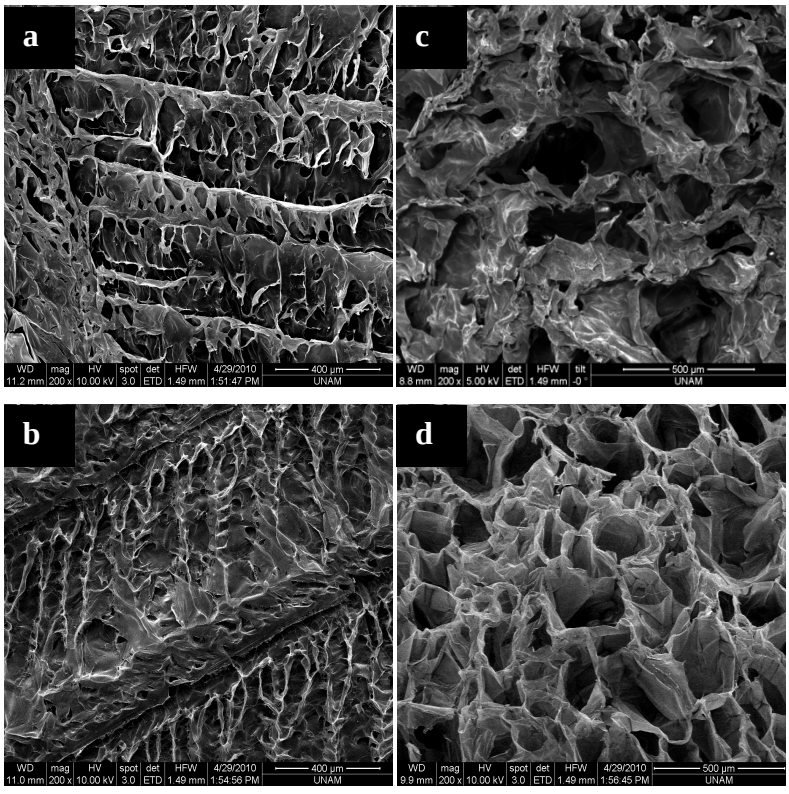

Figure 3.2. Scanning electron micrographs of untreated collagen foam top surfaces. a) 0.75% w/v collagen, frozen at -80 ^{o}C, b) 1% w/v collagen, frozen at -80 ^{o}C, c) 0.75% w/v collagen, frozen at -20 ^{o}C, d) 1% w/v collagen, frozen at -20 ^{o}C. (Scale bar=400 μm for a and b, scale bar=500 μm for c and d)

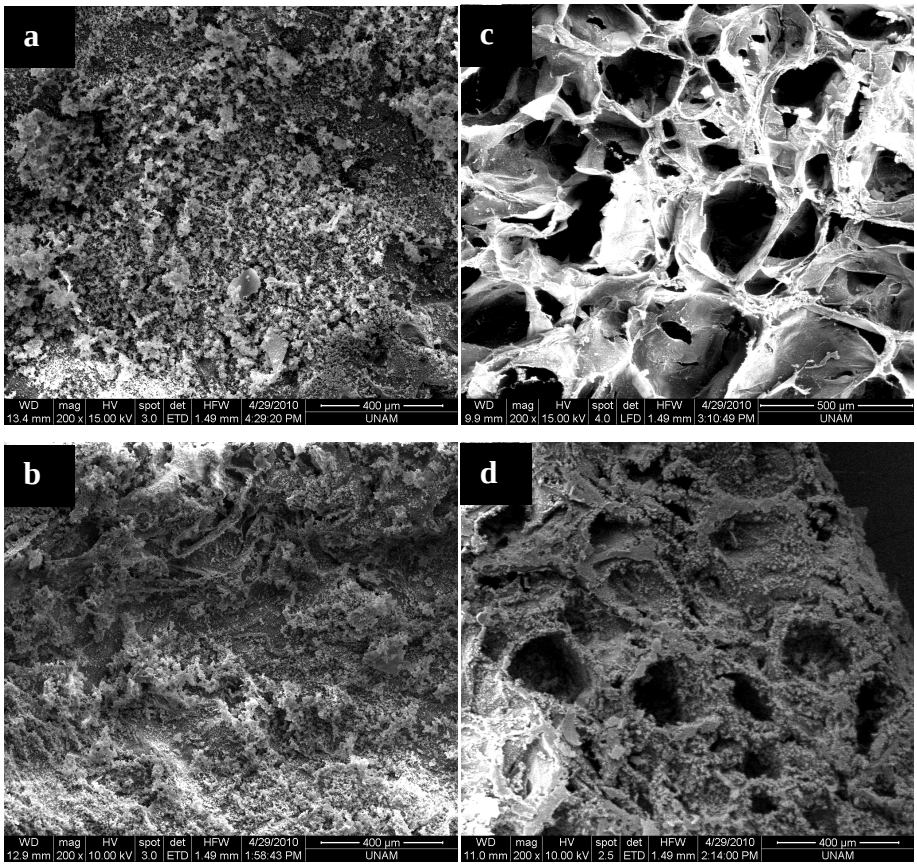

Figure 3.3. SEM images of collagen foams with *in situ* calcium phosphate formation under different conditions. a) Collagen foam with 0.75% w/v collagen concentration, frozen at -80 ^{o}C, 2x4 h/cycle CaP treatment b) 0.75% w/v collagen, frozen at -80 ^{o}C, 2x48 h/cycle CaP treatment, c) 1% w/v collagen, frozen at -20 ^{o}C, 2x4 h/cycle CaP treatment, d) 1% w/v collagen, frozen at -20 ^{o}C, 2x48 h/cycle CaP treatment

Horizontal and vertical cross-sections of the 1%, -20 ^{o}C foam showed that calcium phosphate treatment with 2x4 h/cycle did not lead to a significant decrease on pore sizes compared to untreated foams (Figure 3.4.).

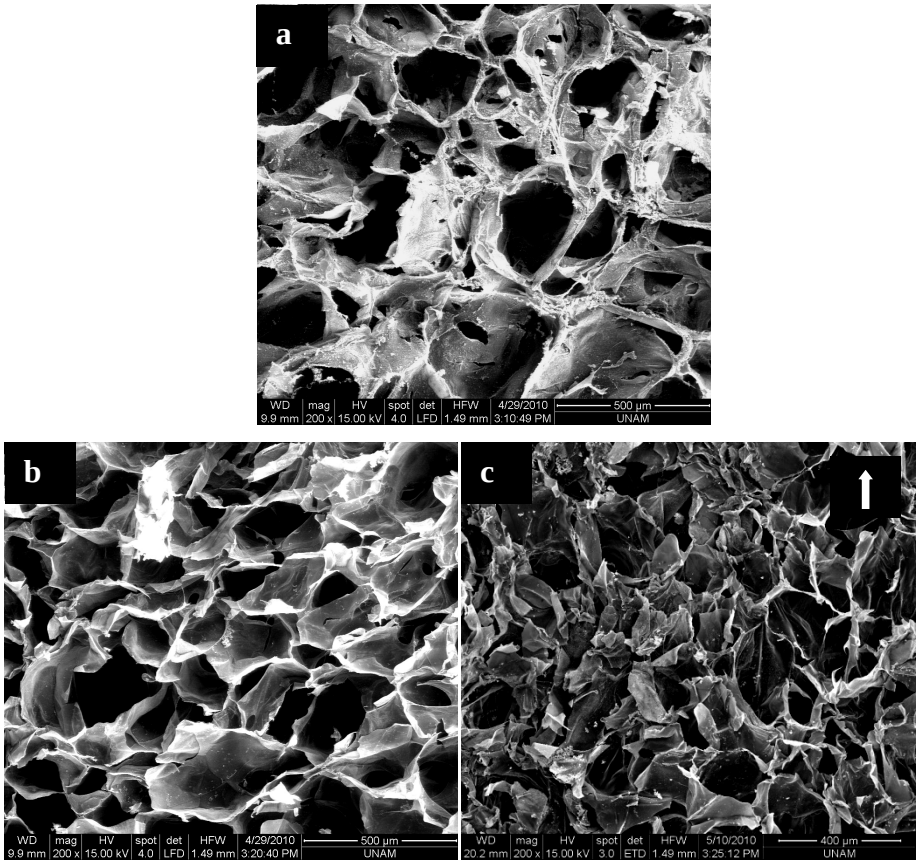

Figure 3.4. Scanning electron micrograph of a collagen foam prepared from 1% w/v collagen solution, frozen at -20 ^{o}C, treated *in situ* to form calcium phosphate with 2x4 h treatments. a) Top surface, b) horizontal cross-section, c) vertical cross-section, arrow: upper side of the vertical cross section (x200)

It was observed that when the foams were treated first with calcium and then phosphate solution, the crystals formed were needle-like, and when the order was reversed, the crystals had a rosette shape (Figure 3.5.).

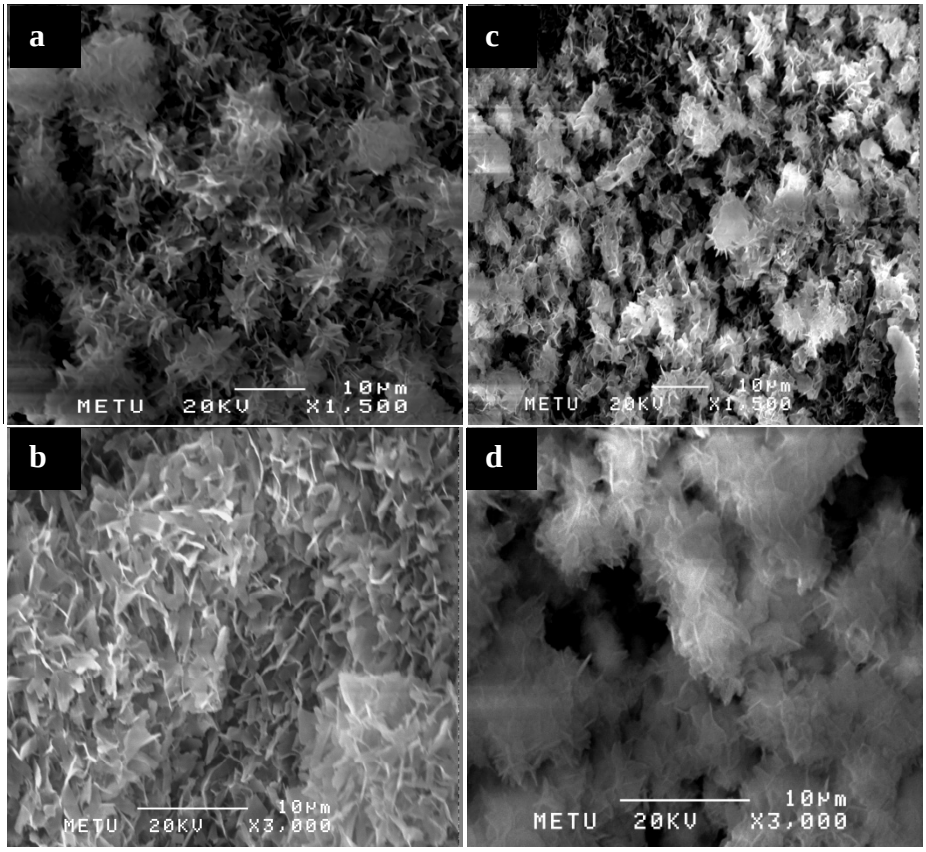

Figure 3.5. Scanning electron micrographs of crystals obtained by different immersion sequences (2x48 h). (a) Calcium first, (b) higher magnification of (a), (c) phosphate first, (d) higher magnification of (c)

Crystal morphologies vary depending on some parameters such as the pH, temperature, molarity of the solutions used and the existence of a support material. It was reported by Wang *et al.* in 2010 that solution pH defines the crystal shape for wet chemical production of HAp crystals as in this study. According to their results when the solution pH is 8 the crystals obtained are a mixture of needle-like and rectangular structures. In another study, Yaylaoğlu *et al*. reported in 1999 that if the precipitation of calcium phosphate crystals occurs in the solution without a support, they have needle-like structures but if a gelatin template is used the crystals are rosette-shaped.

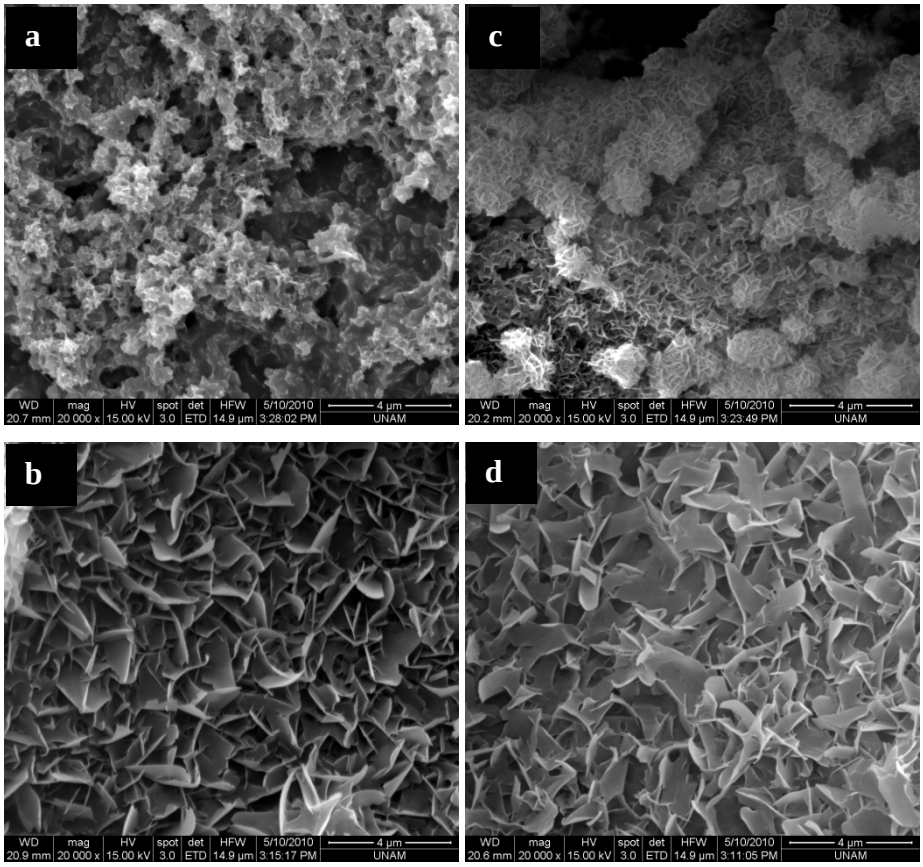

Figure 3.6. SEM images of calcium phosphate crystals formed *in situ* on collagen foams (1% w/v, frozen at -20 ^{o}C). a) Surface, 2x4 h/cycle, b) horizontal cross-section, 2x4 h/cycle c) surface, 2x48 h/cycle, d) horizontal cross-section, 2x48 h/cycle (x20000).

The crystal structures on the surface and horizontal cross-sections of foams were examined at a higher magnification (x20000) and it was observed that crystals formed on collagen scaffolds, both on the surface and the core of the scaffold. Rosette–shaped structure is a characteristic of octacalcium phosphate (OCP) ($Ca_8H_2(PO_4)_6.5H_2O$) crystals (Campbell *et.al.*, 1996). The crystals obtained in this study resemble the OCP which is known to mediate the formation of hydroxyapatite (HAp) (Figure 3.6). Needle-like structure is a characteristic of HAp crystals, but the bone apatite has a different composition than HAp which is carbonated and has less calcium and these crystals are plate shaped.

3.1.3. Electron Diffraction Spectra

The calcium phosphates formed on the foams using different immersion durations and sequences were examined with the EDS attachment of SEM. It was observed that for the foams as the immersion duration is increased, the atomic ratio of calcium to phosphate increases (Table 3.3.). This ratio was also higher when the foam was immersed first in phosphate solution rather than calcium. Since the natural HAp has a Ca to P ratio of 1.67, to mimic the mineral in the natural tissue, higher immersion durations and first phosphate approach is needed.Since the calcium to phosphate ratios were closer to natural compact bone for the phosphate first samples, for collagen foams with higher (1% w/v) collagen concentration, only the immersion duration was changed. According to the results obtained (Table 3.4.) calcium to phosphate ratio on the surface was 1.63 for the foams immersed for 2 cycles of 48 h. This value decreased to 1.43 when the horizontal cross-section was analyzed. With shorter immersion duration the values were lower as expected from Table 3.3 but also the core had a lower ratio than the surface.

Table 3.3. Calcium to phosphate atomic ratios of collagen foams with 0.75% w/v prepared under different immersion sequences and durations/cycle

	Phosphate first		**Calcium first**	
Duration (h)	**2x48**	**2x4**	**2x48**	**2x4**
Ca/P atomic ratio	1.55	1.30	1.31	1.03

Table 3.4. Calcium to phosphate atomic ratios of collagen foam prepared using 1% (w/v) collagen concentration with the phosphate first approach

Duration (h)	**2x48**		**2x4**	
	Surface	Horizontal	Surface	Horizontal
Ca/P atomic ratio	1.63	1.43	1.27	1.07

As Iijima *et al.* reported in 1997, biological apatites are formed by some precursors such as amorphous calcium phosphate (ACP) ($Ca_3(PO_4)_2.3H_2O$) and octacalcium phosphate (OCP) ($Ca_8H_2(PO_4)_6.5H_2O$). Crystals of OCP are plate-like and are thus similar to biological apatites. Hydroxyapatite (HAp) is thermodynamically more stable than OCP, so OCP can transform HAp under neutral pH.

Solution pH is another important parameter as the dissociation of acidic and alkali groups on collagen and the binding of ions on collagen molecule are affected by the pH. It was reported in the same study that a collagen molecule has approximately 230 acidic and 250 basic residues and the number of amide groups is larger than carboxyl groups. Because of this the pI of collagen is higher than neutral pH and this causes the collagen molecule to carry a positive charge under neutral conditions. Thus, the morphology and the Ca:P ratio of crystals formed might have been affected by the immersion sequence.

Molecular formula of OCP is $Ca_8H_2(PO_4)_6.5H_2O$ where the Ca:P is 1.33. According to the results obtained from EDS analysis the Ca:P atomic ratio was 1.30 for 0.75% collagen foams and 1.27 for 1% collagen foams treated with calcium and phosphate solutions with a 2x4 h/cycle. Thus, they are practically the same. When the immersion duration of foams was increased these values also increased to 1.55 and 1.63 for 0.75% and 1% collagen foams, respectively. For shorter duration Ca:P ratios were similar to OCP and longer duration they were

similar to HAp which can be concluded as OCP mediated HAp formation on these collagen foams.

3.1.4. Compression Test Results

The mechanical properties of scaffolds prepared to serve as tissue substitutes are very important and compression is the main load that bones are subjected to.

The stress-strain curves of elastic foams with open cell structures have three main parts (Figure 3.7.). These are the linear elastic region where the pore borders bend, the collapse plateau region where these borders are distorted and a densification region where all the pores collapse (Harley *et.al*., 2007).

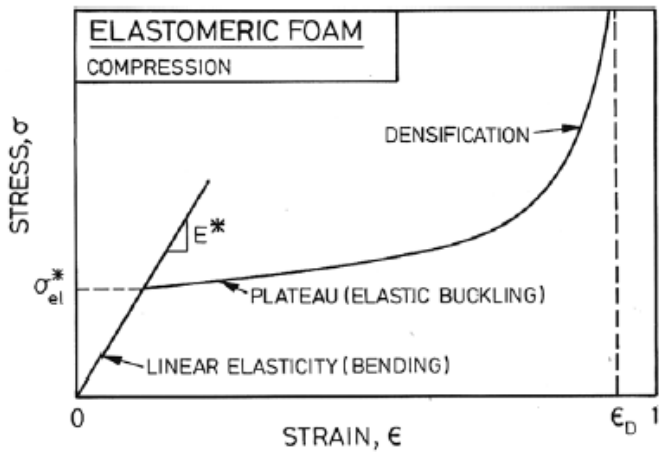

Figure 3.7. Schematic representation of stress-strain curve of an elastic foam with open cell structure obtained after compressive testing (Harley *et.al*.,2007)

Stress-strain curves of collagen foams with different CaP treatment durations or without treatment were obtained after carrying out compression tests. The Young's moduli (*E**) of foams were calculated from the slopes of the linear

region. Elastic compressive strength (σ_{el}*) are calculated as the stress at the intersection of the elastic region and the elastic buckling plateau, E_{col} was calculated from the slope of the linear regression of collapse plateau and the results are listed (Table 3.5.)

Table 3.5. Mechanical properties of collagen foam with *in situ* calcium phosphate calculated from stress-strain curves obtained by compression tests (1% collagen and phosphate first)

Treatment conditions	E^* (kPa)	E_{col} (kPa)	σ_{el}*(kPa)
Untreated foam	33.0±11.3	50.6±17.8	16.5±6.1
4 h/cycle CaP treatment for 2 cycles (2x4)	234.5±45.7	663.4±73.0	127.1±19.9
48 h/cycle CaP treatment for 2 cycles (2x48)	242.7±26.5	745.1±90.4	135.9±18.4

Here, it is observed that E^* has increased almost 8-fold after the formation of CaP on the foam. σ_{el} was improved almost to the same level. The E_{col} was affected the most; 13-15 fold increase was observed. However, these values were still much lower than the mechanical properties of natural cortical and spongy bone. Compressive strength of spongy bone is around 4-12 MPa, and elastic modulus of the same type of bone is 0.1-0.5 GPa (Rezwan *et.al*., 2006).

Kanungo *et.al*. reported in 2008, that they have formed calcium phosphate crystals on collagen-glycosaminoglycan foams by a triple co-precipitation method, and produced foams with different Ca:P ratios. They applied a uniaxial compression test to the foams and calculated the E^*, σ_{el}* and E_{col} values in dry state. Their results of E^* was 204-1000 kPa, somewhat higher than obtained in our

case. For σ_{el}*, 28.4-102 kPa and E_{col} 67.4-123 kPa, both of which are significantly lower then the values found in the present study.

In a study by Al-Munajjed *et.al*. in 2009, the same immersion method of CaP formation with this study was applied to collagen foams and the elastic modulus was increased to 10.3 kPa, almost 37 fold when compared with pure collagen foam, where pure collagen foam's elastic modulus was calculated as 0.28 kPa. Either case, the present study yielded better results.

In this study, the longer duration has a slight positive contribution which is so small that the improvement is not worth the long incubation durations. Because of this, shorter immersion duration was used for the formation of CaP crystals on the foams that were used in *in vitro* studies.

3.2. *In vitro* Studies

Cell proliferation and differentiation studies were carried out on TCPS and then on the collagen scaffolds. Menstrual blood contains a mixture of different cell types including fibroblasts. MSC-like cells also express fibroblastic surface markers (Gargett *et al*., 2008), and this makes it difficult to distinguish fibroblasts and MSC-like cells by flow cytometry. Therefore, characterization of MB MSCs was not performed by flow cytometry. MSC-like cells become dominant and highly purified at passage 3 and only the MSC-like cells have osteogenic differentiation capacity in this cell mixture; because of this, *in vitro* studies were performed without further isolation of the cells.

3.2.1. Cell Culture on TCPS

3.2.1.1. Cell Proliferation

After WJ and MB MSCs were seeded in 24-well TCPS, cells were incubated in their expansion media for 24 h and then the expansion medium was replaced with the osteogenic differentiation medium. MTS cell proliferation assay was done on the first day after the differentiation medium was added. Cell

proliferation assay results showed that for WJ MSCs the number of the cells attached to the tissue culture plate surface were around the seeding density for both control cells and differentiation samples (Figure 3.8.). WJ MSCs are incubated in a nutrient rich medium with bFGF which supports mitogenesis for MSCs before they are seeded to TCPS for osteogenic differentiation. The control medium which the cells are incubated in after detaching them from the flasks does not contain these nutrients and this may cause the WJ MSCs not to proliferate during the first day the MTS assay is applied. On the 14^{th} day of incubation the cell number of WJ MSCs were almost 8 fold higher in comparison to the seeded number of cells for both the control and differentiated cells. On the 21^{st} day of differentiation the cell numbers were almost the same with the 14^{th} day regardless of differentiation state.

For MB MSCs the behavior was significantly different. The cell number on the first day was higher by about 2.4 fold of the original seeding density, most probably because the control group is composed of a cell mixture containing other cell types such as fibroblasts and epithelial cells with MSC like cells. This high proliferation rate was maintained later. There was not a significant difference between the cell numbers in the control and differentiation media on the first day. The increase in the cell number during the first two weeks was very high; around 20 fold for control and 12 fold for the differentiated cells. The reason for such a high proliferation rate in comparison with WJ MSCs may be the shorter doubling time of these cells (19.4 h) (Meng *et al*.,2007) when compared with WJ MSCs (25.5 h) (Yücel, 2010). Unlike the WJ, with MB, there was a significant detrimental effect of the differentiation medium on cell proliferation. The number of the cells grown in osteogenic differentiation medium was almost half of the cells grown in the control medium for both 14^{th} and 21^{st} days of culture (Figure 3.9.).

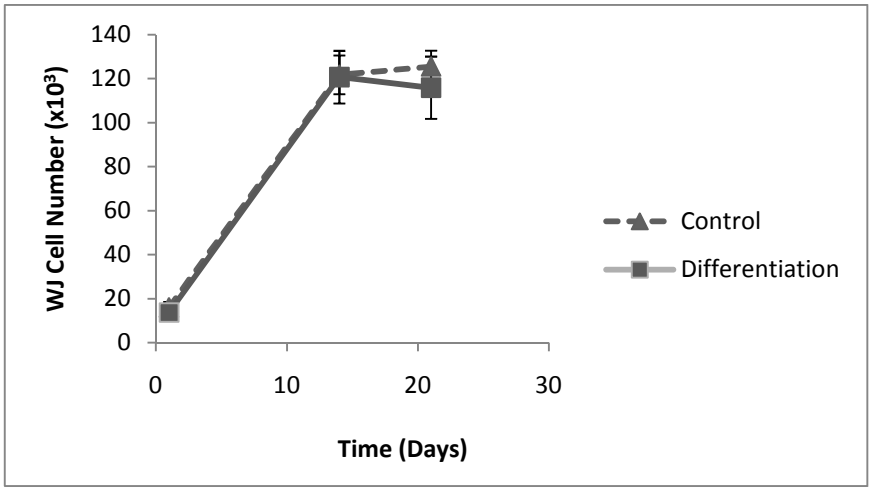

Figure 3.8. WJ MSC adhesion and proliferation in control and osteogenic differentiation media (MTS assay)

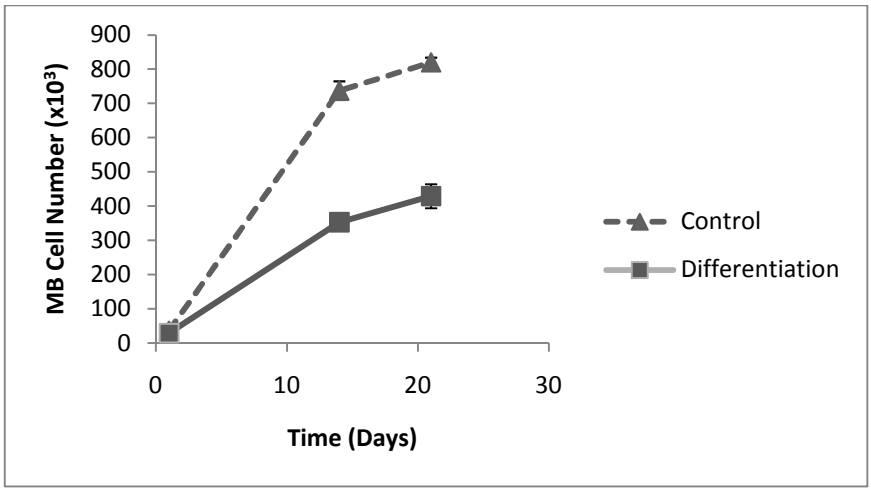

Figure 3.9. MB MSCs adhesion and proliferation in control and osteogenic differentiation media (MTS assay)

3.2.1.2. Determination of Osteogenic Differentiation

The osteogenic differentiation of WJ and MB MSCs was determined by ALP enzyme assay as mentioned in section 2.2.3.5.1. Alkaline phosphatase (ALP) is an enzyme found on the surface of osteoblast cell membrane and is responsible

for the removal of phosphate from many phosphate carrying molecules and ions, especially from calcium salts. Its optimal working media are alkaline environments. This enzyme is found in many tissues such as liver, bile duct, kidney and placenta in addition to bone. It is a very important marker of osteogenic differentiation for *in vitro* studies. WJ and MB MSCs were tested for their ALP enzyme activity and the results are presented as "μM substrate converted to product/min by 100 μL of cell lysate" (Figure 3.10). Since the osteogenic markers are produced during the first 21 days of induction and then the ALP activity reaches stability, the culture was stopped at 21st day of incubation and activities were determined. Specific ALP activities (ALP activity per cell), were also calculated. On day 14 of culture, ALP activities for both cell types grown in the differentiation medium were higher than those grown in the control media. The difference in ALP activity between the control and differentiated cells was almost 2 fold for WJ MSCs; for MB MSCs the difference was not as large (Figure 3.10.). On day 21, ALP activity profile of WJ MSCs was very similar to day 14 results, with a slight increase in activity for both cells grown in control and osteogenic media. On the other hand ALP activity of MB MSCs in both control and differentiation media on day 21 were lower when compared with day 14 results but the cells in the differentiation medium still had high values.

When total and specific ALP activity of WJ MSCs grown in control and differentiation media are compared, it was observed that the trend was the same; ALP activity of differentiated samples was 2 fold of the control samples on days 14 and 21. For MB MSCs, the specific ALP activities in control and differentiation media were substantially different (ca. 2 fold) of the control group on days 14 and 21, and specific ALP activities on day 21 were also lower than day 14, like the total activities (Figures 3.10., 3.11.).

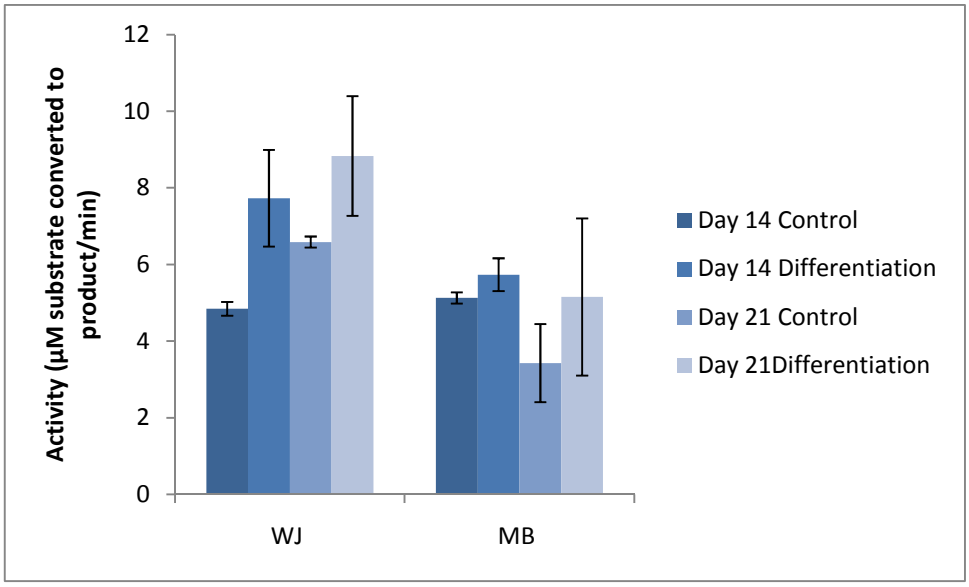

Figure 3.10. ALP activity of WJ and MB MSCs

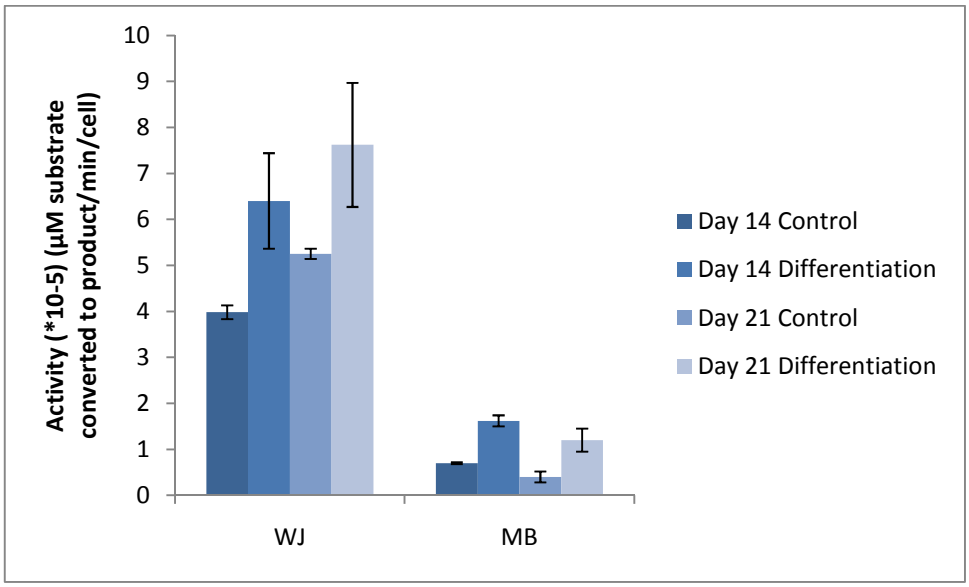

Figure 3.11. Specific ALP activities of WJ and MB MSCs on 14th and 21st days of osteogenic differentiation

When the total ALP activities of WJ and MB MSCs are compared it is observed that the activity of WJ MSCs is about 2 fold higher. When the specific activities of these cells are compared it is observed that their activity was 5-fold higher than that of MB MSCs.

The ALP activity of osteoblasts increases during the first 15 days of incubation and then starts to decrease (Hollinger *et al*., 2005). MB MSCs showed a similar differentiation profile to this, however, in this study, the ALP activity of WJ MSCs continued to increase during 21 days, but the total and specific activity of these cells were much lower than WJ MSCs. This indicates another difference between the WJ and MB cells.

3.2.1.3. Cell Morphology

Morphologies of MB and WJ MSCs in control and differentiation media were studied by staining with Phalloidin-FITC and DAPI for the cytoskeleton and nucleus, respectively. It was observed that both cells had fibroblastic structures, were attached to the tissue culture plate and during the 21 day incubation the cells continued to proliferate and reached confluency. MB MSCs are smaller than WJ MSCs and as was seen in the MTS assay, MB MSCs proliferate faster (doubling time of MB MSCs 19.4 h, and WJ MSCs 25.5 h) (Figure 3.12.).

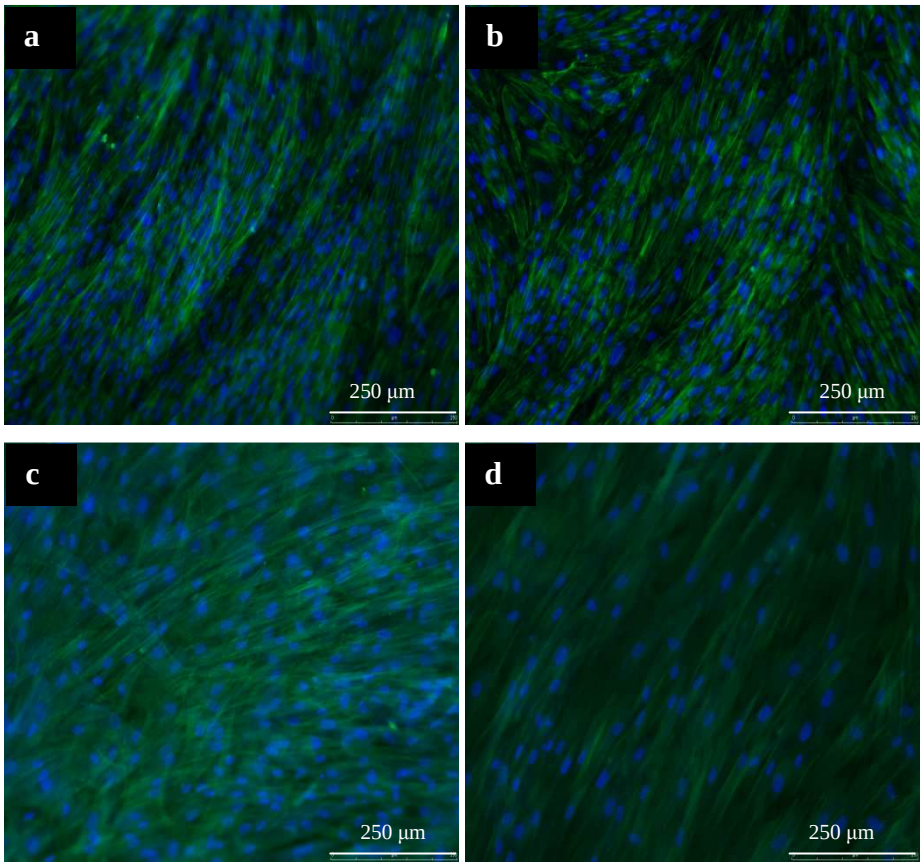

Figure 3.12. Fluorescence microscopy of MB and WJ MSCs on day 21. MB MSCs (a) in control medium, (b) in osteogenic differentiation medium. WJ MSCs in (c) control medium, d) in differentiation medium, (x10), Blue (DAPI): nuclei, Green (Phalloidin-FITC): cytoskeleton

3.2.1.4. von Kossa Staining

Bone forming cells secrete calcium phosphate into the extracellular environment and during osteogenic differentiation this mineral formation can be shown by von Kossa staining, which in this case was done on day 21 of osteogenic differentiation. Together with the increase of ALP expression matrix maturation occurs which is followed by the mineralization of ECM (Park, 2010).ALP activity of WJ MSCs was shown to be higher than MB MSCs with the

ALP assay, and as expected from these results, minerals formed by WJ MSCs were denser when compared with MB MSCs (Figure 3.13.).

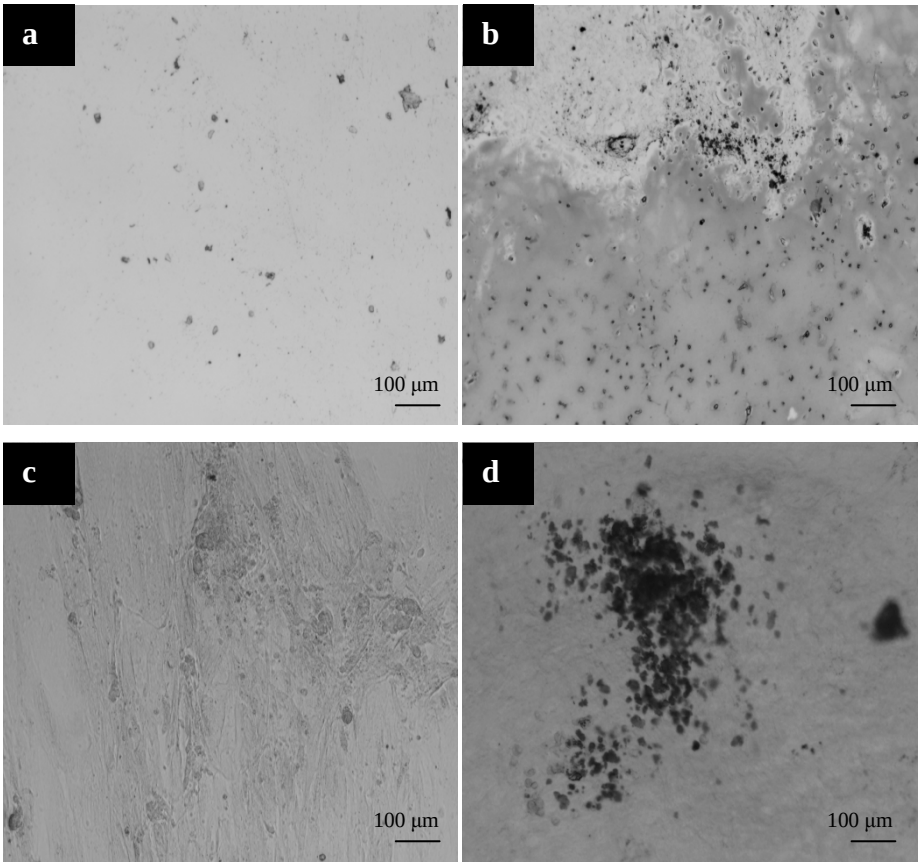

Figure 3.13. Light micrographs of von Kossa stained MB and WJ MSCs on day 21. MB MSCs grown in (a) control medium, (b) osteogenic medium. WJ MSCs grown in (c) control medium, (d) osteogenic medium (x10).

3.2.2. Cell Culture on Foams

3.2.2.1. Cell Attachment and Proliferation on Foams without Calcium Phosphate

The attachment and proliferation of cells were determined by MTS assay. On the day following cell seeding on collagen foams, MTS assay was done to determine the number of cells attached onto the foams. It was observed that the attachment efficiency was around 26% for WJ and 38% for MB MSCs, respectively. Pore sizes of the foams used for cell seeding in this study are around 200 μm, and Liu *et al*. reported in 2009 that large pores support the transport of nutrients and removal of wastes, but also cause low attachment efficiency and intracellular signalling. In addition, when the foams are incubated in the aqueous medium, they swell and the pore sizes increase making the situation worse.

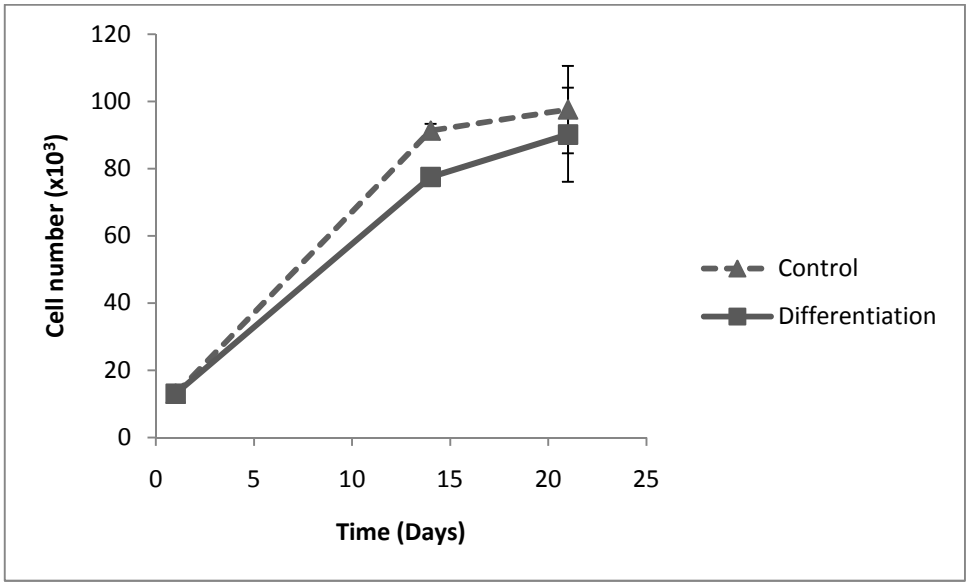

Figure 3.14. Proliferation of WJ MSCs on collagen foams without calcium phosphate and incubated in control and differentiation media (MTS assay).

On the 14th day of culture the cell number in both control and differentiation media for WJ MSCs increased around 7 fold of the first day. This increase in cell number continued during 21 days for the cells grown both in control and differentiation media, with a slightly higher cell number of control group (Figure 3.14).

The cell number of MB MSCs, however, increased during the first 14 days, but afterwards started to decrease (Figure 3.15). As it was seen in TCPS, cell number was lower in differentiation medium during 21 days.

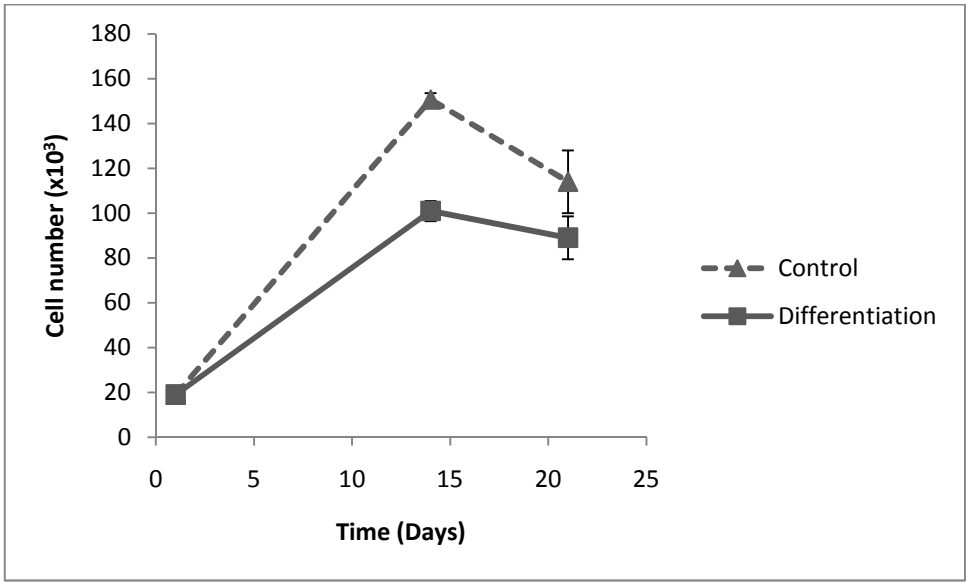

Figure 3.15. Proliferation of MB MSCs on collagen foams without calcium phosphate and incubated in control and differentiation media (MTS assay).

3.2.2.2. Cell Attachment and Proliferation on *in situ* Calcium Phosphate Produced Foams

The attachment and proliferation of cells were determined by MTS assay and Phalloidin-DAPI staining. On the day following cell seeding on collagen foams, MTS assay was done to determine the number of cells attached onto the foams. On the first day the cell number on the foams was low (Figure 3.16.). The average pore size of the foams decrease from 200 μm to 70 μm with the CaP crystal formation and this may prevent the adhesion and penetration of the cells into the pores and cause a decrease in cell number.

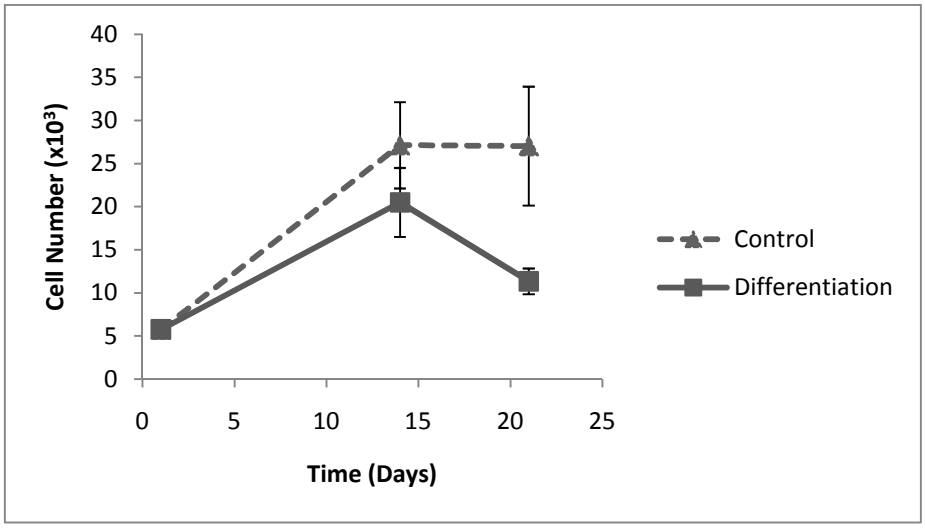

Figure 3.16. Proliferation of WJ MSCs on calcium phosphate containing collagen foams incubated in control and differentiation media (MTS assay).

On the 14th day in culture, the number of WJ MSCs incubated in the control medium increased 7 fold, like the same cells grown on TCPS. On the 21st day the cell number was the same with 14th day. In the differentiation medium a different trend than the control group and the cells incubated in TCPS was

observed. The number of cells on 14th day was 3 fold higher than the cells attached on first day. On 21st day this number decreased to half that of the 14th day. However, on TCPS in osteogenic medium, the cell number had increased around 8 fold of the seeding number between days 1 and 14 like the case for the control group as was mentioned in section 3.2.1.1.

On the other hand, MB MSCs displayed a totally different behavior on collagen foams than on TCPS. Cells on TCPS had proliferated during 21 days in both media. On collagen foams, however, cell numbers increased by only 2 fold in control media during 14 days but only 35% of these cells were viable on day 21 (Figure 3.17). In the differentiation media, cell viability was poorer; the number of cells decreased continuously from day 1.

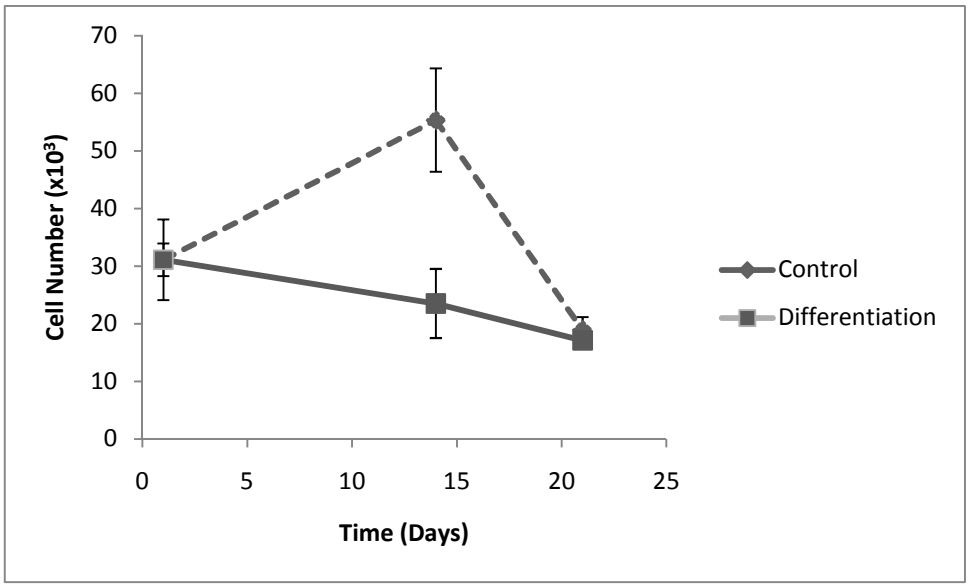

Figure 3.17. Proliferation of MB MSCs on calcium phosphate containing collagen foams in different media (MTS assay).

In a review published by Barrére *et al*. (2006) stem and osteoprogenitor cells are mobile and migrate on the scaffold materials in the early days of culture.

Later, they adhere to the substrate with strong focal points and start to differentiate. Additionally, the cells are affected by the calcium and phosphate concentration changes in the medium. The decending stability of calcium phosphates are as follows: HAp>TCP>OCP. The dissociation of calcium phosphates to release calcium and phosphate increases with decreasing stability and this may, as reported in the same review, cause cell death. Excessive Ca^{2+} in the extracellular environment may cause the uptake of it into the cell and storage of the ion in the endoplasmic reticulum (ER). The excess amount of Ca^{2+} may cause the ER stress and stimulate cell apoptosis (Orrenius *et al*., 2003)

In this study, as was discussed in section 3.1.3. Ca:P ratio of the foams were similar to OCP, which is thermodynamically less stable and possibly dissociates into its ions in the culture medium. Cells like to adhere HAp-like crystals and since the dissociation rate of crystals obtained in this study is higher than that of HAp, the cells attached to the crystals might lose contact and are removed by the renewal of medium during culture, leading to the decrease in the cell number.

The attachment of both cell types was also examined by Phalloidin-DAPI staining. Cells were seeded to scaffolds and fixed 24 h later. Confocal and fluorescence micrographs of stained cells on collagen foams were obtained. In the fluorescence microscopy studies UV filter and blue filter were used for observation of DAPI and phalloidin-FITC stained tissues, respectively. In the confocal microscopy studies, samples were excited with the laser at 488 nm and emission between 495 and 600 nm was collected for cells stained with phalloidin-FITC, and excited at 532 nm and emission was collected between 640 and 785 nm for collagen sponges. Despite the low adhesion efficiency of cells, the attachment of both cell types was good (Figures 3.18, 3.19). Besides, confocal micrographs of a 90 μm depth from the surface demonstrate that the cells had penetrated into the pores (Figure 3.20.).

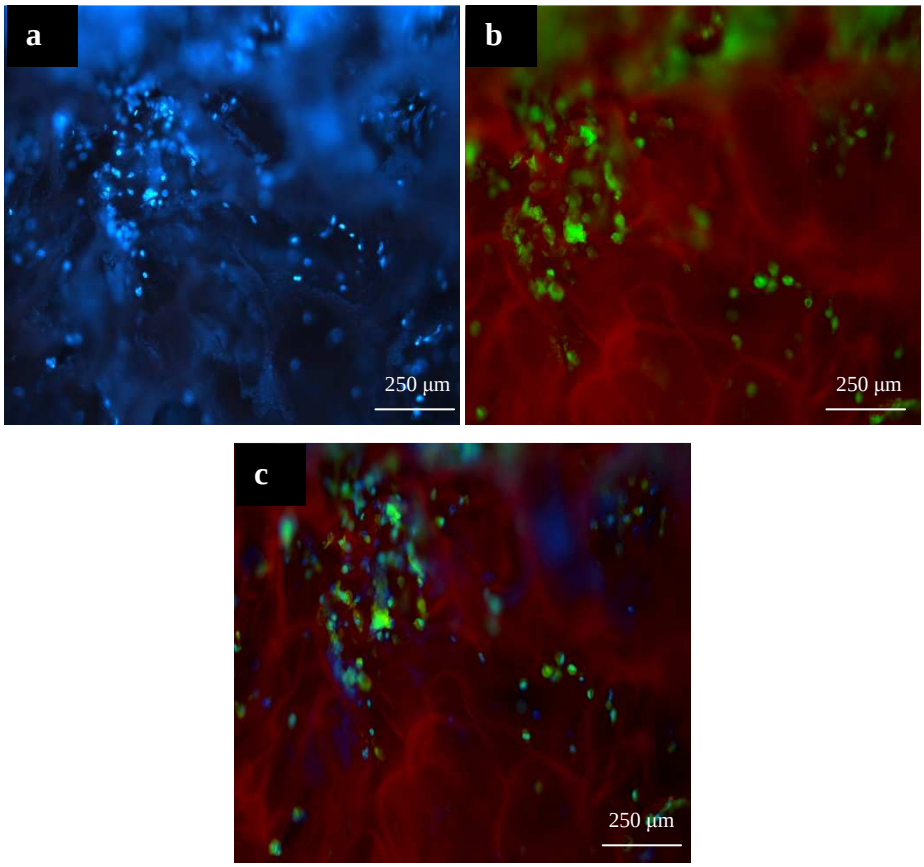

Figure 3.18. Fluorescence micrograph of MB MSCs on collagen foams 24 h after seeding. (a) Nuclei of cells stained by DAPI (blue), (b) ECM of cells stained with Phalloidin-FITC (green). Collagen scaffold had an autofluorescence (red), (c) overlay of (a) and (b) (x10)

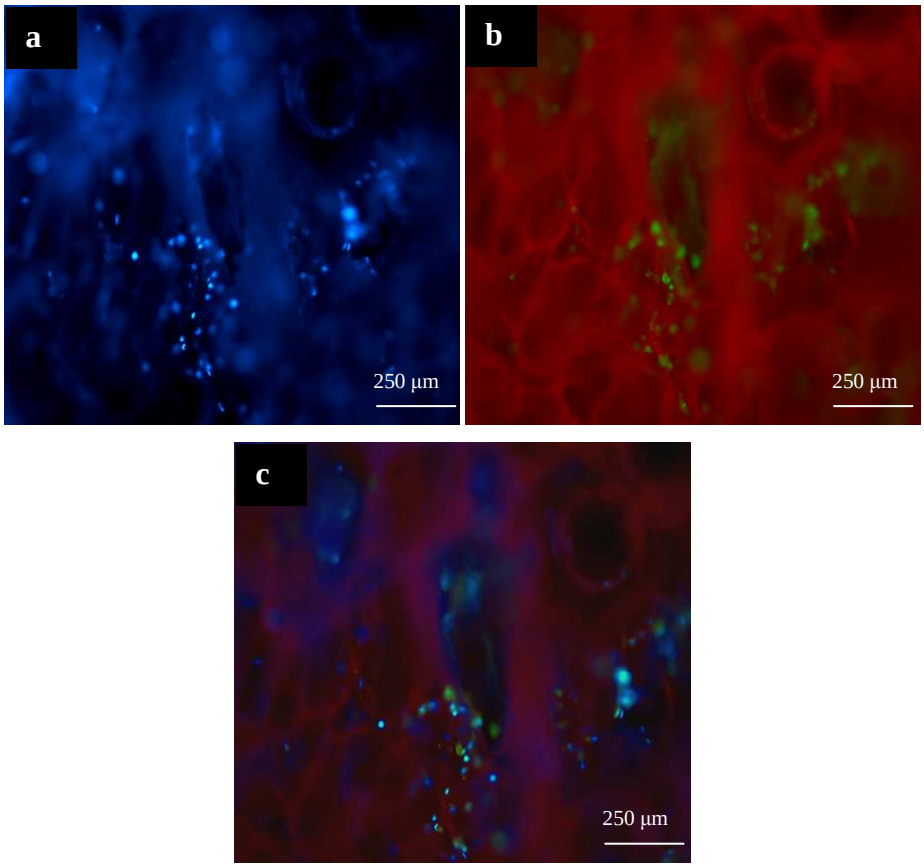

Figure 3.19. Fluorescence micrograph of WJ MSCs on collagen foams 24 h after seeding. (a) Nuclei of cells stained by DAPI (blue), (b) ECM of cells stained with Phalloidin-FITC (green). Collagen scaffold had an autofluorescence (red), (c) overlay of (a) and (b) (x10)

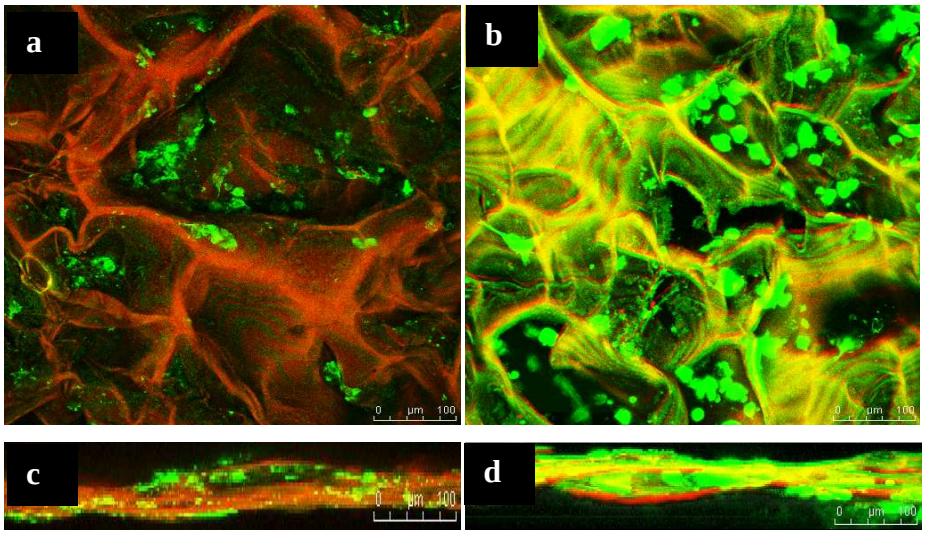

Figure 3.20. CLSM micrographs of WJ and MB MSCs on calcium phosphate carrying collagen foams. (a) WJ MSCs, (b) MB MSCs, (c) vertical cross-section of WJ MSCs, (d) cross-section of MB MSCs seeded foams. Stain: Phalloidin-FITC. Scale bar: 100 μm

3.2.2.3. SEM Analysis of WJ and MB MSCs on Collagen Foams

WJ and MB MSCs on collagen-CaP foams were fixed on 21st day of culture and images were obtained by SEM using the vertical cross sections of the foams. It was observed that these inner parts of the foams also have the cells and the cells grown in the osteogenic medium appear to have adhered to the foams and had fibroblastic extensions (Figure 3.21.).

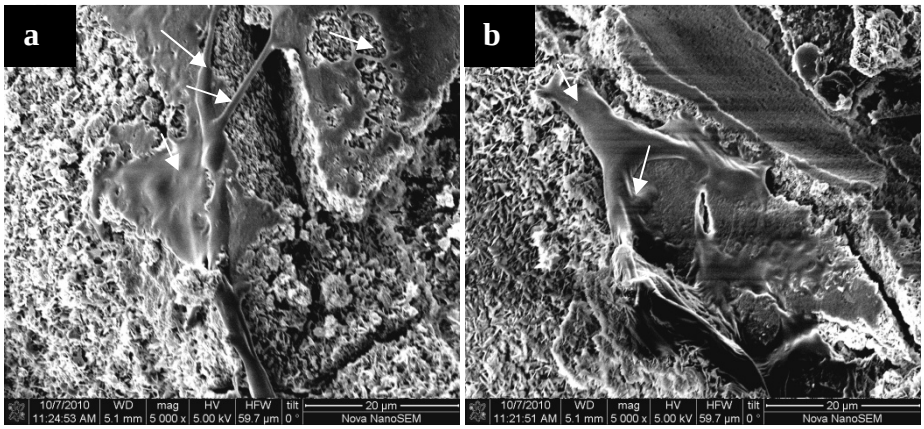

Figure 3.21. SEM micrographs of WJ and MB MSCs on collagen-CaP foams. (a) WJ MSCs, (b) MB MSCs. Day 21. Arrows indicate the extensions of cells

3.2.2.4. ALP Activity of WJ and MB MSCs on Collagen Foams without Calcium Phosphate

ALP activities of WJ and MB cells seeded onto collagen foams without calcium phosphate was determined to study the effect of the scaffold on their differentiation. It was observed that even the total values were higher than TCPS results (Figure 3.22), the ALP activity improvement upon incubation in differentiation medium for MB MSCs was almost the same with TCPS.The difference in total activities between the differentiation and control samples for WJ MSCs was not substantial and was similar to that on TCPS (Figure 3.22).

The specific ALP activity profiles of both cell types were quite similar to TCPS results. WJ MSCs had higher total and specific activity than MB MSCs, but the difference between WJ and MB MSCs was lower than that on TCPS (Figure 3.23).

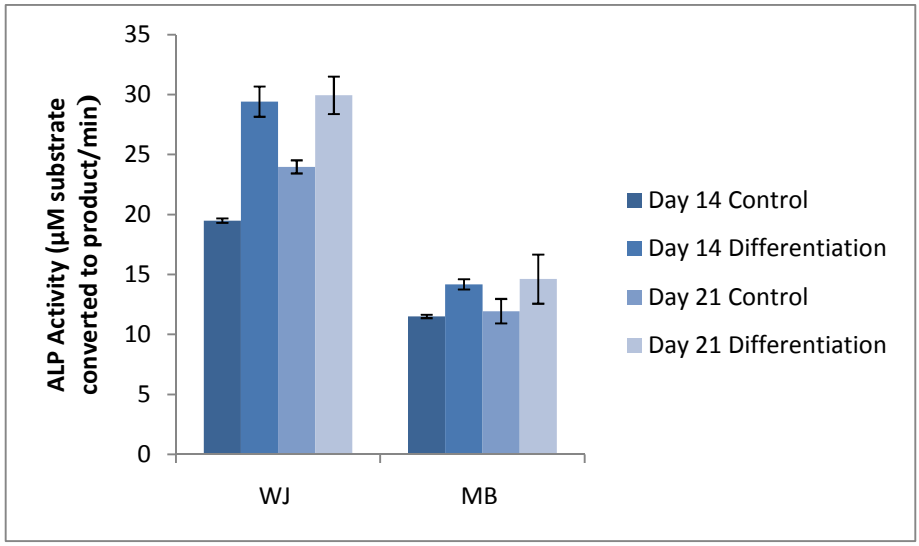

Figure 3.22. ALP activity of WJ and MB MSCs

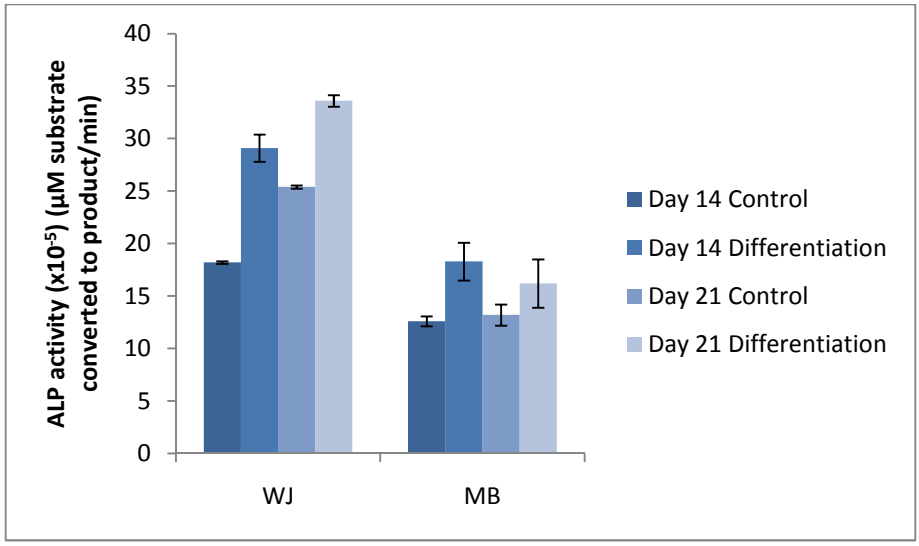

Figure 3.23. Specific ALP activities of WJ and MB MSCs on 14th and 21st days of osteogenic differentiation

3.2.2.5. ALP Activity of WJ and MB MSCs on *in situ* Calcium Phosphate Produced Collagen Foams

ALP activity of WJ and MB MSCs seeded on collagen foams were determined on days 14 and 21 in the control and osteogenic induction media. The results were much higher than the results on TCPS and on collagen foams without CaP for both cell types, which means that the scaffolds supported the osteogenic differentiation. On the 14th day of differentiation induction, for both cell types ALP activity of cells grown in osteogenic medium was twice as high as that in the control group and these values were 10 times and twice higher than the values obtained from cells on TCPS and foams without CaP, respectively (Figure 3.24.). In addition ALP activity of WJ MSCs was higher than those of MB MSCs as was also the case on TCPS on day 14.

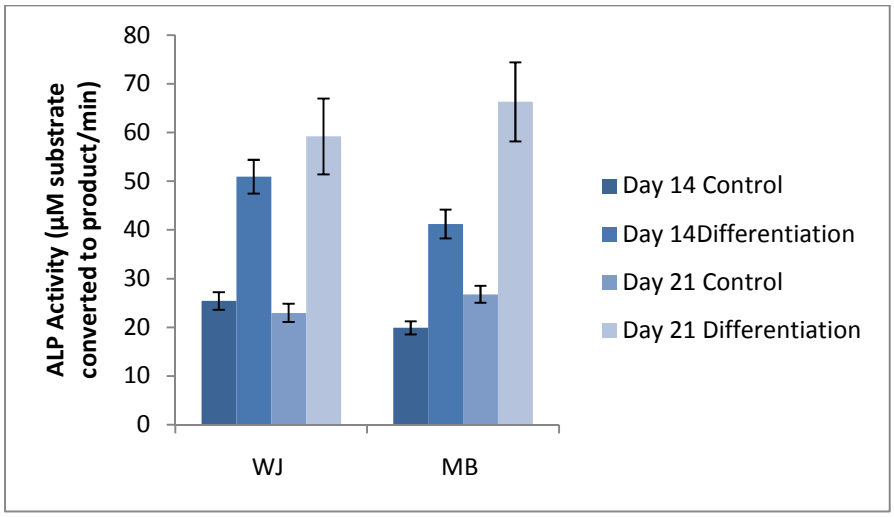

Figure 3.24. ALP activity of WJ and MB MSCs on collagen/CaP foams on 14th and 21st days of osteogenic differentiation. The ALP activity is expressed as µM substrate converted to product per minute for 100 µL cell lysate

On the 21st day of osteogenic induction ALP activity of both cell types were higher than on the 14th day. This result is similar for WJ MSCs on TCPS but for MB MSCs it was the opposite. Additionally MB MSCs had a higher activity on 21st day (Figure 3.24.).

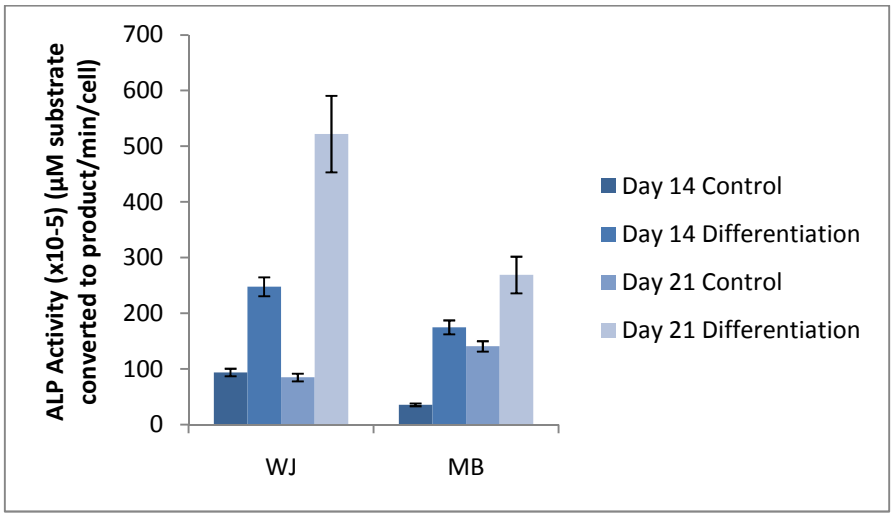

Figure 3.25. Specific ALP activity of WJ and MB MSCs on collagen/CaP foams on 14th and 21st days of osteogenic differentiation. The ALP activity is expressed as µM substrate converted to product per minute per cell

Specific ALP activities had a similar trend in the ALP activities of both cell types on day 14, but displayed a different profile on 21st day for MB MSCs (Figure 3.25.). The total ALP activity results of MB MSCs were slightly higher than WJ MSCs with osteogenic differentiation samples, but the activity of their control group was lower. But the situation was different for specific ALP activity. WJ MSCs had twice higher specific activity than MB MSCs, similar to the results obtained on TCPS; this value was 13 fold higher than on TCPS (Figure 3.25.). In addition, activity of MB MSC control group was almost twice higher than that of WJ MSC on day 2.

3.2.2.6. von Kossa Staining of WJ and MB MSCs on Collagen Foams

von Kossa staining was done for WJ and MB MSCs incubated on collagen-CaP foams after 21 days of incubation in control and osteogenic induction media. von Kossa staining is used to detect the calcium in a mineral. Because the scaffold itself here carries calcium, all the minerals attached to the scaffold and the mineral deposits produced by cells were stained. As a result it was not possible to distinguish the minerals produced by the cells (Figure 3.26.).

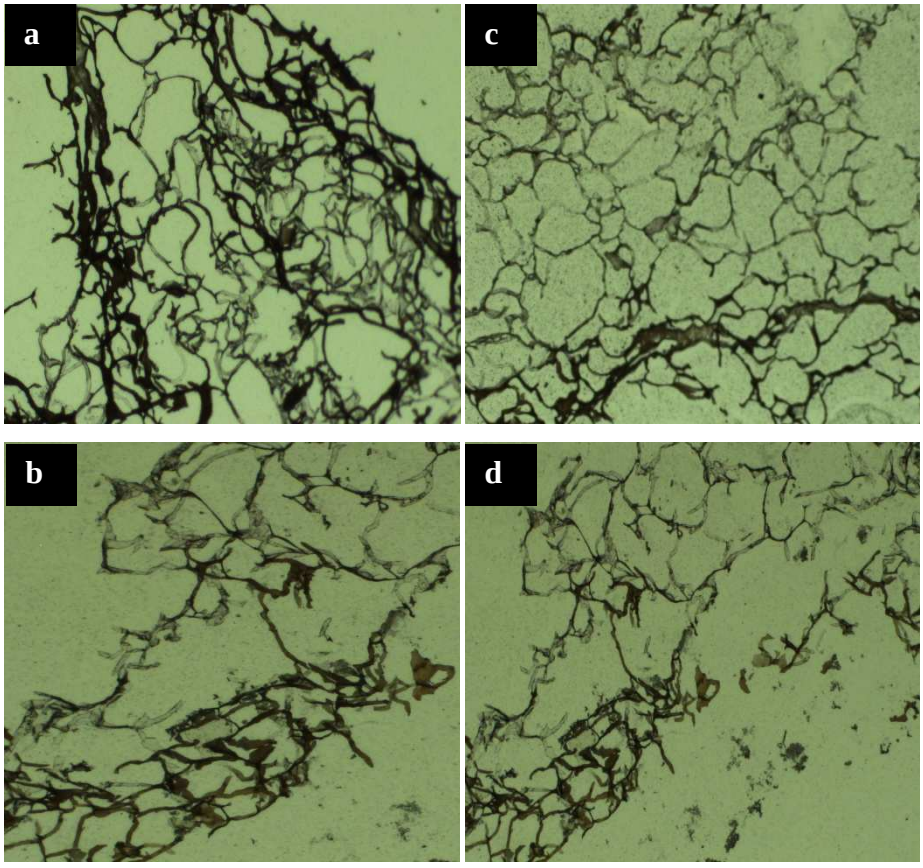

Figure 3.26. von Kossa staining of WJ and MB MSCs seeded on collagen-CaP foams and incubated for 21 days. (a) WJ MSCs grown in control, (b) WJ MSCs in differentiation, (c) MB MSCs in control, (d) MB MSCs in differentiation media

CHAPTER 4

CONCLUSION

Tissue engineering can be an alternative solution for the treatment of critical sized bone defects. In this study, collagen scaffolds with and without *in situ* grown calcium phosphate crystals were produced. Collagen was crosslinked with a natural crosslinker, genipin which is also used in the food industry, to overcome the cytotoxic effects of the most commonly used crosslinkers. Calcium phosphate crystals were formed by a double solution immersion method, introduced by our group previously. Porosity and pore size distributions were found to be suitable for cell seeding. Crystal morphology and the Ca:P ratio of crystals were similar to octacalcium phosphate (OCP) which is thought to be an intermediate phase in the biological apatite formation. Calcium phosphate crystals improved the elastic modulus and compressive strength of foams around 8 fold. These foams were also tested for *in vitro* cell culture studies. Two different types of stem cells (Wharton's jelly and menstrual blood), both of which were isolated from discarded tissues were seeded onto foams in order to see the effect of the foams on osteogenic differentiation and to compare the bone forming efficiencies of these cells. Cell proliferation on foams without calcium phosphate was good, but a decrease in the cell number was observed for foams with calcium phosphate especially after 14^{th} day in culture. Despite the decrease in cell number, the foams with calcium phosphate triggered osteogenic differentiation and the ALP activities of cells were found to be much higher compared to foams without calcium phosphate.

In conclusion, Wharton's jelly and menstrual blood stem cells could be used as alternatives to common cells obtained by invasive techniques, such as bone marrow aspiration for bone tissue engineering applications. The ALP activity of Wharton's jelly cells was found to be higher than menstrual blood stem cells. However, osteogenic activities of these cells are lower than bone marrow stem cells.

A tissue engineered construct for bone tissue engineering should contain both the organic and inorganic phases to improve the osteogenic differentiation of stem cells. Collagen foams with calcium phosphate crystals have a similar chemical composition to native bone and these foams support osteogenic differentiation of Wharton's jelly and menstrual blood stem cells substantially when compared to collagen foams without calcium phosphate.

4.1. Future Prospects

Promising results for osteogenic activity were obtained using both the cell types, WJ and MB. Mechanical properties of the collagen foams were found to be lower than that of the natural bone, but the construct would serve perfectly well as a bone filler of high osteogenic capacity. However, conditions should be modified to improve the mechanical properties. Differentiation of cells into osteoblasts were detected and determined by mineralization and enzyme assays, but immunostaining could be performed to show the metabolic activity of the cells. As a result of the promising results obtained under *in vitro* conditions these constructs should also be tested *in vivo*.

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APPENDIX A

MERCURY POROSIMETRY RESULTS

Pore size distribution of collagen foams with different collagen concentrations, freezing temperature and calcium phosphate treatment durations were analyzed by mercury porosimetry. Production conditions of foams are given below figures.

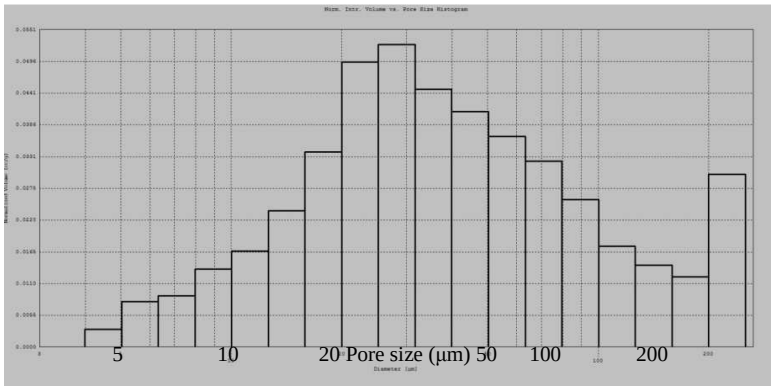


Figure A.1. 0.75% collagen, -80 ^{o}C, untreated foam

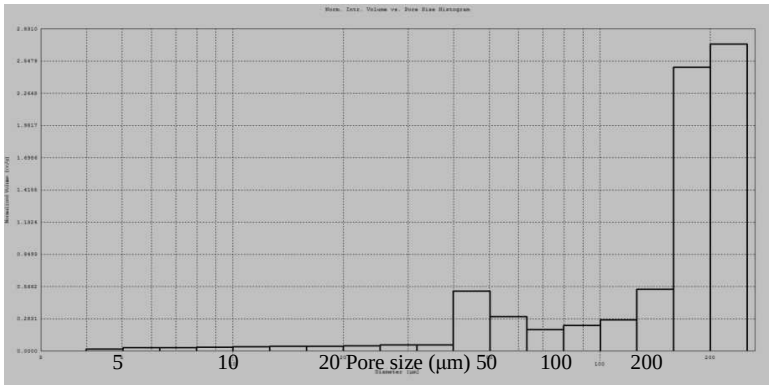


Figure A.2. 0.75% collagen, -20 ^{o}C, untreated foam

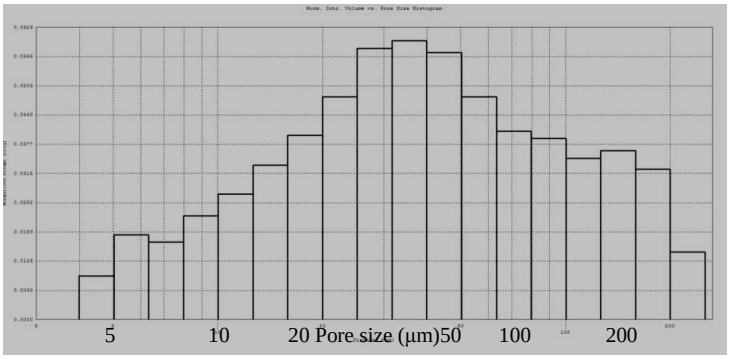


Figure A.3. 1% collagen, -80 ^{o}C, untreated foam

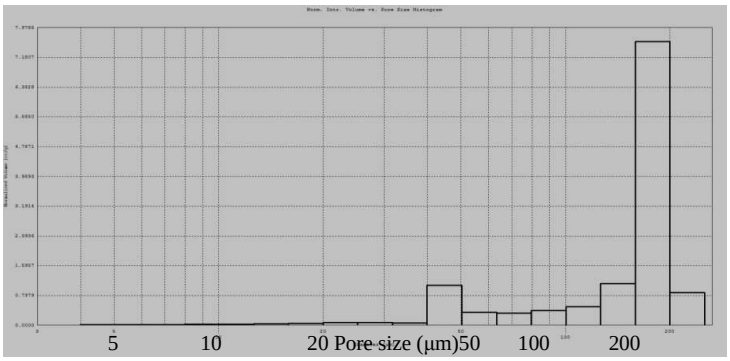


Figure A.4. 1% collagen, -20 ^{o}C, untreated foam

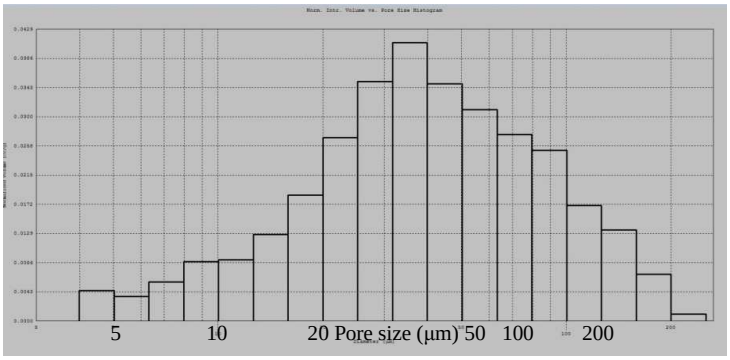


Figure A.5. 0.75% collagen, -80 ^{o}C, 2x4 h treatment

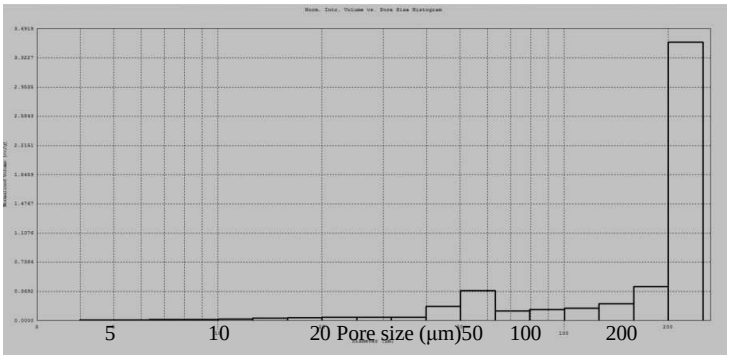


Figure A.6. 0.75% collagen, -20 ^{o}C, 2x4 h treatment

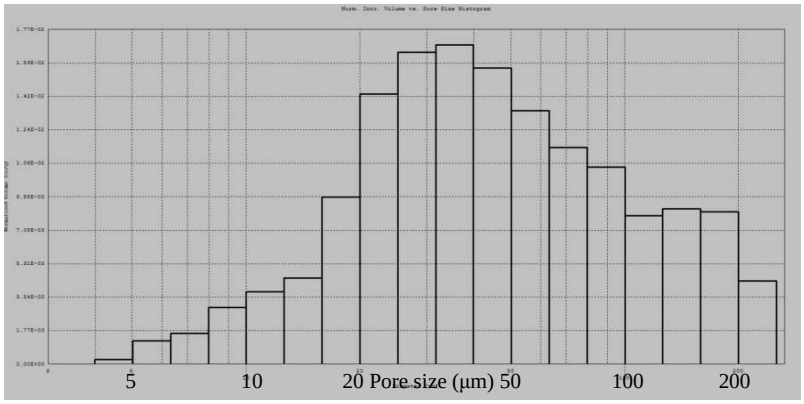


Figure A.7. 1% collagen, -80 ^{o}C, 2x4 h treatment

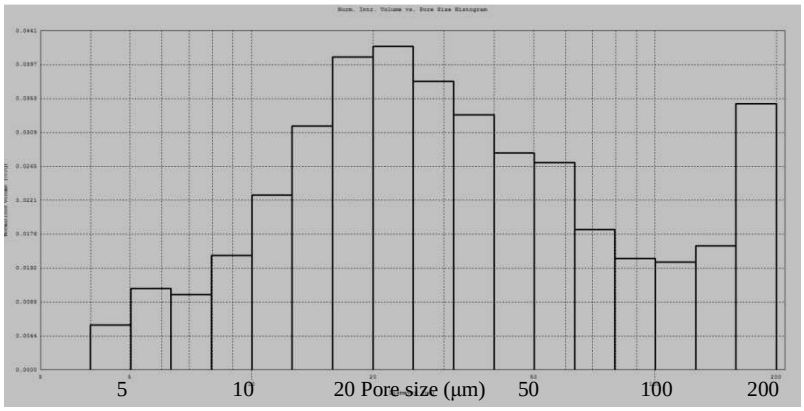


Figure A.8. 1% collagen, -20 ^{o}C, 2x4 h treatment

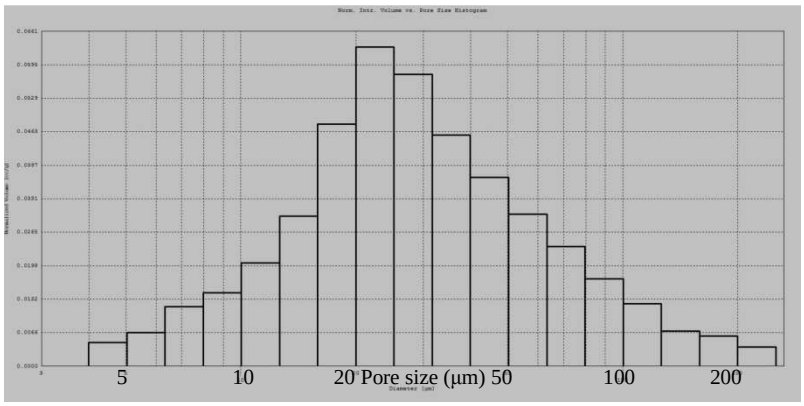


Figure A.9. 1% collagen, -80 ^{o}C, 2x48 h treatment

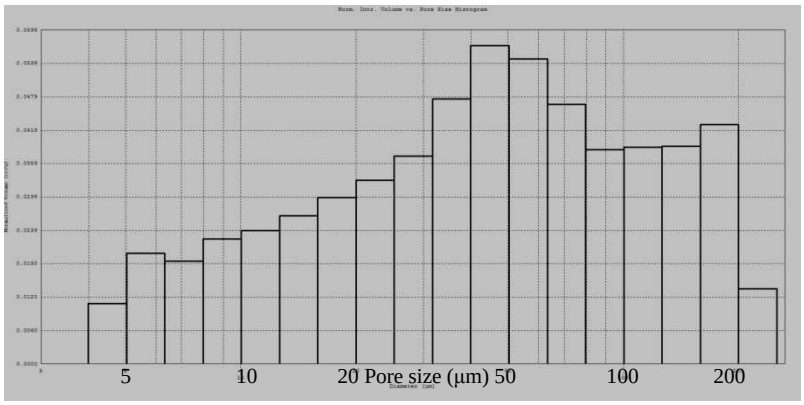


Figure A.10. 1% collagen, -20 ^{o}C, 2x48 h treatment

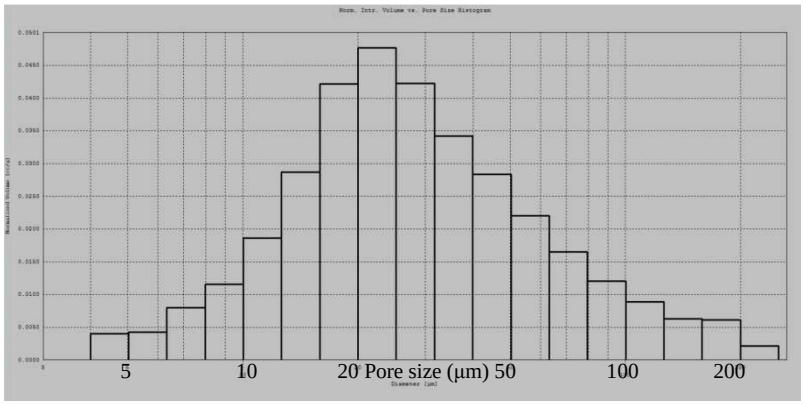


Figure A.11. 0.75% collagen, -80 ^{o}C, 2x48 h treatment

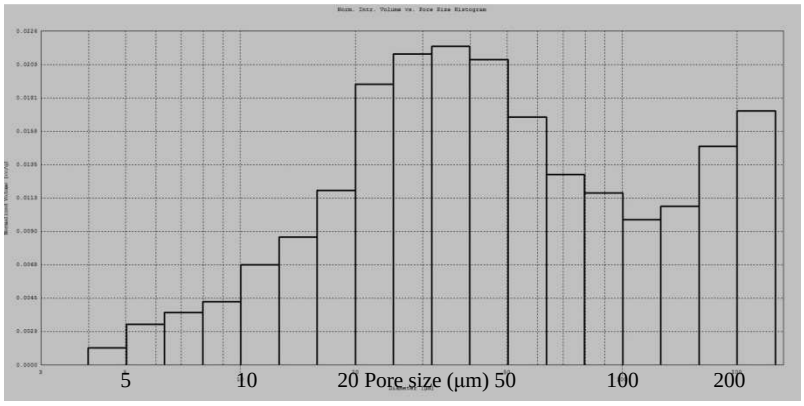


Figure A.12. 0.75% collagen, -20 ^{o}C, 2x48 h treatment

APPENDIX B

CALCULATION PROCEDURE USED AFTER COMPRESSION TESTS

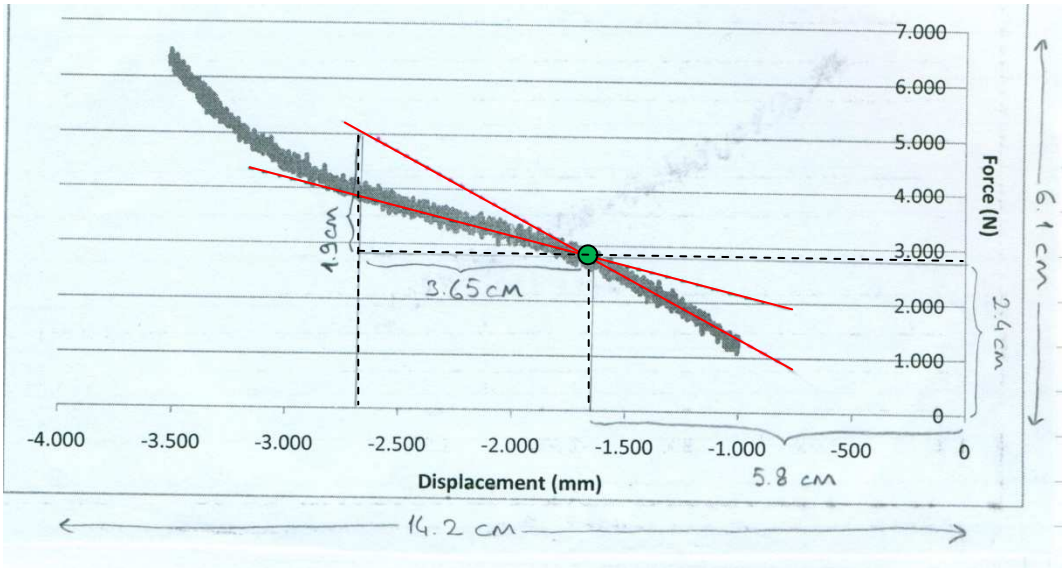
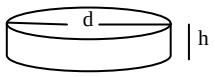


Figure B.1. Compression test results and data treatment

$h = 3.802\ mm$

$d = 1.5\ cm$

$A = 1.767 \times 10^{-4}\ m^2$



Schematic representation of a collagen foam

$$\varepsilon * = \frac{5.8\ \text{cm}}{14.2\ \text{cm}} \times 4\ \text{mm} = 1.634\ mm$$

$$A \times \sigma^* = \frac{2.4\ cm}{6.1\ cm} \times 7\ N = 2.754\ N \qquad \sigma^* = \frac{2.754\ N}{1.767 \times 10^{-4} m^2} = 15.6\ kPa$$

$$\Delta h = \frac{3.65\ cm}{14.2\ cm} \times 4\ mm = 1.028\ mm \qquad A \times \Delta\sigma = \frac{1.9\ cm}{6.1\ cm} \times 7\ N = 2.180\ N$$

$$E^* = 15.6\ kPa \times \frac{3.802\ mm}{1.634\ mm} = 36.3\ kPa$$

$$\frac{\Delta\sigma}{\Delta\varepsilon} = \frac{2.180\ N}{1.767 \times 10^{-4}\ m^2} \times \frac{3.802\ mm}{1.028\ mm} = 45.6\ kPa$$

E^*: Young's modulus

σ^*: Elastic compressive strength

ε^*: Strain

APPENDIX C

CALIBRATION CURVE FOR ALP ACTIVITY

The osteoblastic activity of MSCs was determined by Alkaline Phosphatase (ALP) enzyme assay. For calibration curve, absorbances of p-nitrophenol solutions with different concentrations were plotted.

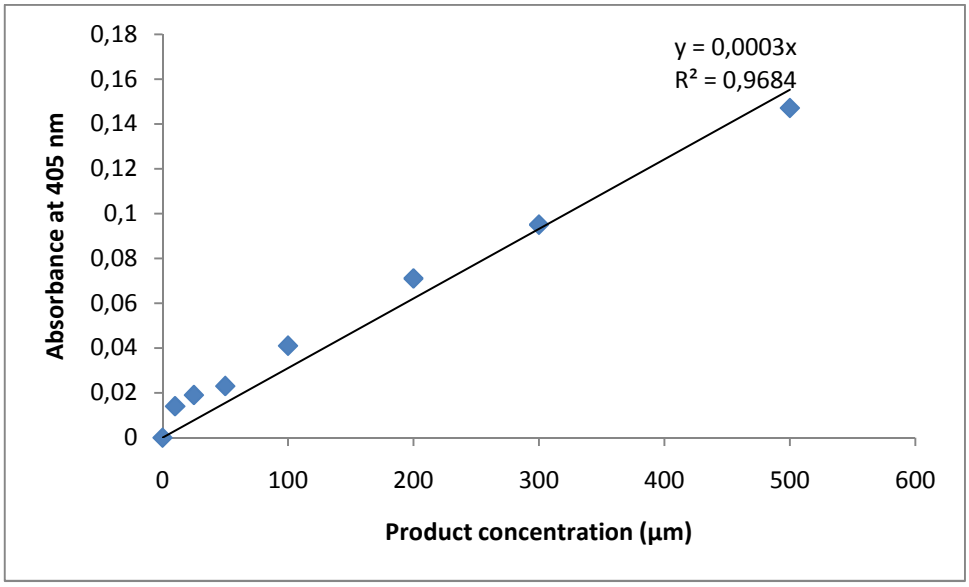


Figure C.1. The calibration curve for ALP activity, prepared by using p-nitrophenol at different concentrations

APPENDIX D

CALIBRATION CURVE FOR DETERMINATION OF CELL NUMBER

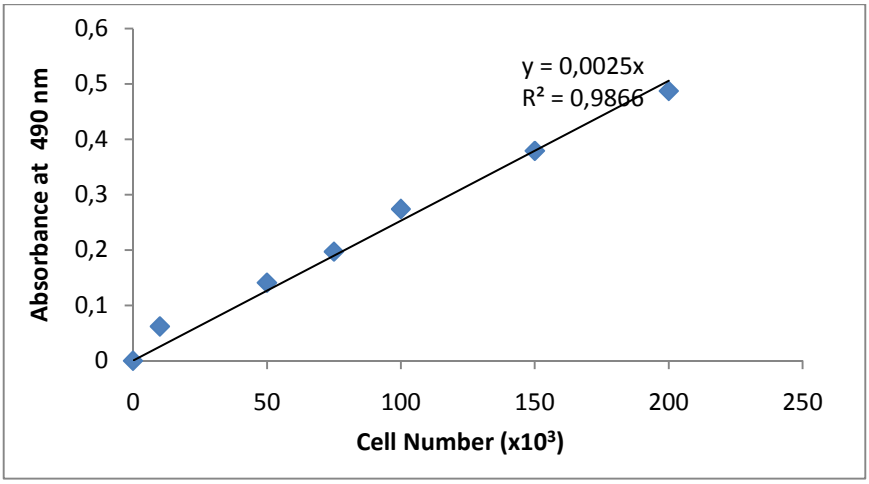


Figure D.1. Calibration curve for MB MSC obtained by using MTS assay

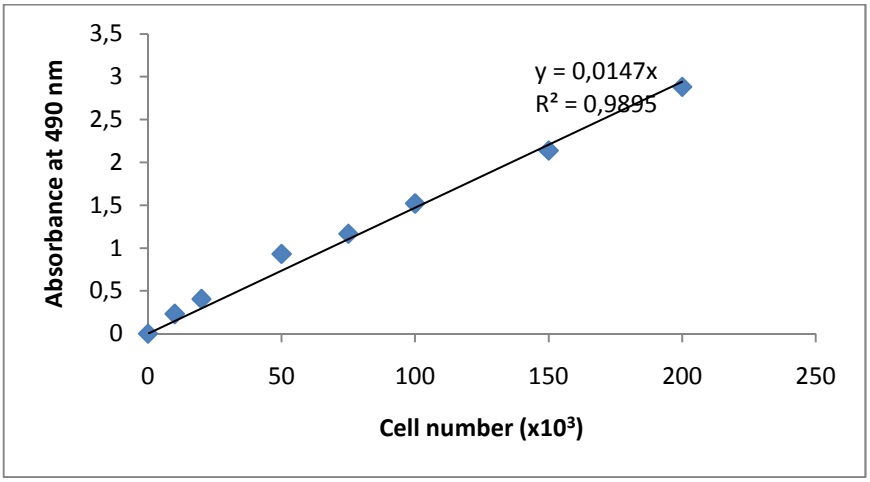


Figure D.2. Calibration curve for WJ MSC obtained by using MTS assay