Comparative Study of Different Dilutional and Centrifugation Protocols for a Lower Density Gradient Separation Media in Isolation of Osteoprogenitors from Human Bone Marrow Aspirate

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Abstract

Introduction: Human bone marrow contains osteoprogenitors capable of differentiating into osteoblasts. Ficoll media is widely used in the isolation of human mesenchymal stem cells. Many centrifugation and centrifugation forces have been used; however, the relative isolation efficiencies of the different separation protocols have not been investigated.

Aim of the Study: The aim of the study was to compare the effectiveness of the two most commonly used centrifugation protocols for Ficoll-Paque PREMIUM 1.073, a lower density gradient centrifugation media in isolating osteoprogenitor cells from aspirated bone marrow.

Materials and Methods: Bone marrow was aspirated from anterior iliac crests, with a centrifugal force of 400 g and 1:1 dilution was used for protocol A, while a centrifugal force of 1000 g was used for protocol B after three dilution times with the buffer. Isolated mononuclear cells from each protocol were counted and compared. The number of Colony Forming Units-Fibroblasts (CFU-F) was counted and STRO-1 flow cytometry analysis was done in order to estimate the number of osteoprogenitors present in each protocol. Alkaline phosphatase activity in the cultures was determined histochemically as well as biochemically to determine and compare their osteoblastic differentiation capacity. Terminal differentiation of osteoprogenitors into mature osteoblasts and their ability to secrete calcium and phosphate minerals were detected by Alizalin Red S and von Kossa staining.

Results: The average numbers of isolated bone marrow mononuclear cells from the protocol A

and B were $6.87x10^7$±$4.84x10^7$ and $4.70x10^7$±$3.93x10^7$ respectively, which were statistically different. The mean±SD number of CFU-F in protocol A was 53±6/10^6 mononuclear cells, which was not significantly different from protocol B (51±8/10^6 mononuclear cells). Positive expression of STRO-1 (around 10%) was detected from both protocols. Alkaline phosphatase activity did not show any difference between protocols, however, significant increase in enzyme activity was detected on both protocols between day 7 and day 10. Formation of mineralized nodules in osteogenic culture was confirmed by positive alizarin red S and von Kossa staining in both protocols.

Conclusion: There was no detectable difference between the CFU-F forming capacity, STRO-1 positivity, osteogenic differentiation or mineralization abilities between protocols. Both protocols could isolate competent and functional osteoprogenitors, while Protocol A produced recovery of more mononuclear cells.

Keywords: Osteoprogenitor cells, Ficoll, CFU-F, STRO-1.

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Chapter 1

Introduction

Bone is a dynamic biological tissue that has the potential to repair itself throughout the life without leaving a scar [1, 2]. However, surgical treatments of trauma, congenital deformities or benign and malignant neoplasia may occasionally lead to formation of critical size defect which cannot be healed completely physiologically, and must be surgically reconstructed with bone grafting to prevent non-union defect and resultant aesthetic, functional, social and emotional complications.

Autogenous bone, transplanted from another part of the recipient body, is regarded as the "gold standard bone graft" and the most effective bone graft material. Grafted autogenous bone serves as a source of osteogenic cells (osteogenesis), a stimulator of mesenchymal cells (osteoinduction), and a mechanical or space filling agent (osteoconduction). However, there are many disadvantages and reported complications exists associated with autogenous bone grafting such as the need for a second operative site, limited and frequent insufficient quantity of bone graft, increased operation time and blood loss, the risk of donor site complications such as painful scar, hematoma, infection, fracture, gait and sensory disturbances [1, 3].

Bone substitutes such as Allografts and Alloplasts have been used as alternative grafting materials to avoid morbidity associated with donor site complication. Allografts, transplanted from genetically nonindentical members of the same species, have osteoconductive and weakly osteoinductive properties, but are not osteogenic. Therefore, longer time necessary to complete bone formation and vascular penetration, accelerated bone resorption, delayed or incomplete bone graft incorporation as well as potential risk of transmission of HIV infection and lack of histocompatibility can be encountered [1, 4-7]. Freeze-drying, demineralization and irradiation to reduce immunogenic potential can also reduce structural integrity, leading to graft fracture [8]. Bone alloplasts, composed of natural or synthetic materials, typically are

osteoconductive only, and not osteogenic, therefore posting similar problems seen in allografts except risk of infection and immunological reactions [9].

Induction of new tissue by growth factors requires large amounts of recombinant material, which may not be realistic in cases of massive defects [10]. Additionally, successful use of growth factors relies on the presence of a sufficient population of undifferentiated progenitor cells capable of responding to the inductive cues provided by the growth factor [11]. Such a population may not be available in aged or compromised patients [12].

Autogenous bone is the only type of bone graft to supply living, immunocompatible bone cells essential for osteogenesis, however, it has several disadvantages and is not applicable in every situation [13]. Bone allografts and alloplasts also have appropriate roles in bone repair; however lacks of osteogenic properties make them inferior to autogenous bone graft.

Since the effectiveness of these alternative bone grafting materials to generate bone healing or new bone formation totally depends on the presence of a sufficient number of competent osteoblast progenitor cells in the graft site, use of adult stem cells or osteoblast progenitor cells based tissue engineering technology has increased attention in recent years to potentiate the efficacy of these materials.

Tissue engineering approaches are based on the tissue engineering triad, which was derived from the three major components of tissues: cells, their extracellular matrix and a signaling system [12] (Figure. 1). Utilizing the principles of tissue engineering in a rational manner offers promise to regenerate or develop de-novo oral and craniofacial tissues.

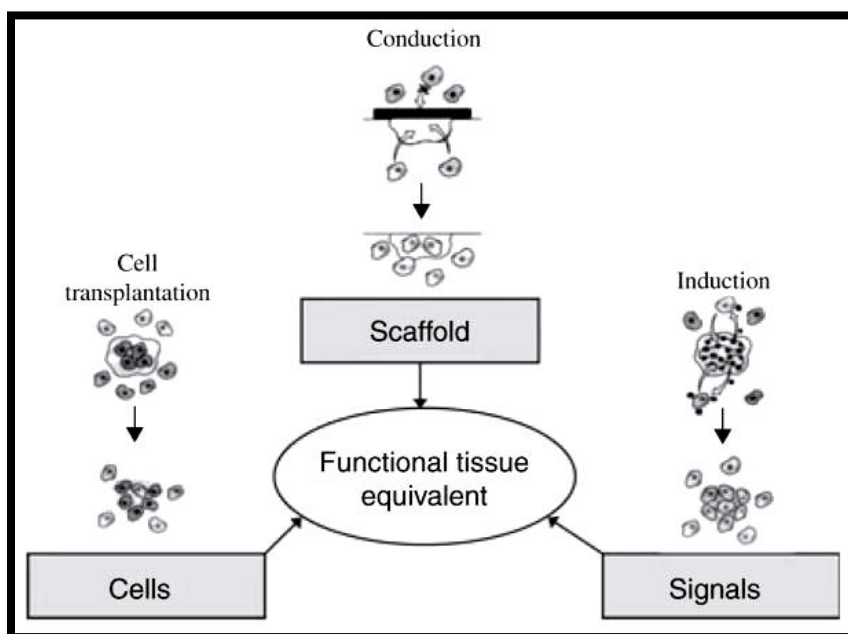


Figure 1. The Tissue Engineering Triad [14]. Cell transplantation, conduction and induction, can be used individually or in combination to optimize regeneration and engineering of a functional tissue

Osteoblast progenitor cells can be found in several locations throughout the body including bone marrow, trabecular bone, muscle, adipose tissue, periosteum, synovial memberane, articular cartilage, skin and periodontal ligament[15, 16].

Bone marrow contains a population of multipotent mesenchymal stem cells that generate the progenitors for osteogenic, chondrogenic, adiopogenic, and myogenic cells [17]. A number of studies have demonstrated the osteogenic property of marrow stromal or stem cells [3].

It is undeniable that even in the gold standard autologous bone graft, a large proportion of its osteogenic capacity resides in the bone marrow component of the graft because bone marrow contains the richest and most readily available repository of progenitor cells capable of differentiating into mature bone forming cells [18-20].

In the future, it is expected that utilization of osteoprogenitor cells isolated from bone marrow combined with osteoinductive and osteoconductive agents would possibly eliminate

the need for autogenous bone harvesting and reduce the problems associated morbidity and complications.

Chapter 2

Review of Literature

Bone Marrow

Bone marrow has two separate and distinct hematopoietic and the stromal systems which are not only coexist, but functionally cooperate [21]. The function of the bone marrow stroma is to provide structural and functional support for hematopoietic lineage cells. It is a unique connective tissue system composed of endothelial cells, adipocytes, smooth muscle cells, reticular cells, osteoblasts and stromal cells.

The osteogenic potential of bone marrow aspirate was first reported in 1869 by Goujon [22]. Since then, bone marrow has been used for long bone fracture repair, to treat nonunions and to potentiate the osteogenic effect of bone grafts or bone graft substitutes [15]. A number of authors have shown that bone marrow alone provides adequate bone formation and adequate defect healing [23]. Bone marrow can also be used to enrich grafting matrices such as xenograft, demineralized bone matrix, or ceramic materials especially in the larger defects and segmental defects [23]. Since bone marrow has a somewhat fluid consistency, their reduced ability to maintain at the defect site for an adequate duration to support osteogenesis can be compensated by using these scaffolds. When bone marrow is used with appropriate scaffold, it almost invariable produces faster and more consistent defect healing comparing with bone marrow or the carrier matrix alone and, in some cases, equivalent healing to autografts [24-27]. This synergistic effect most likely results from the physical containment of osteoprogenitors from marrow in the grafting matrix with minimal dispersal of cells from the defect site and a favorable surface or scaffold for bone mineral deposition [28].

Bone marrow stromal stem cells

A stem cell is a cell that has the ability of indefinite self-renewal and is capable of differentiationg into specialized cell types. Bone marrow stromal stem cells (BMSSC) are

multipotent cells capable of differentiating into several mesenchymal lineage pathways, to form bone, cartilage, muscle, marrow stroma, tendon, ligament, fat and other connective tissue types such as cardiomyocytes [16, 29, 30].

Friedenstein and colleagues isolated and cultured bone marrow fibroblasts in vitro and later established their osteogenic potential [31, 32]. Castro-Maloaspina et al [33] purified these cells populations, and Caplan [34] and Haynesworth et al [35] first identified them as progenitors by their ability to differentiate into osteoblasts, chondroblasts, myoblasts and other phenotypes. Owen discovered that when transplanted under the kidney capsule, these cells produced bone, cartilage, fat and other tissues [36]. Procktop first proposed the term stromal stem cell to describe its role as a multipotent precursor cell for nonhematopoietic tissues [37]. Manjumdar et al then culture expanded these cells and produced a variety of phenotypic progenitors [38], while Reyes et al determined that the differentiation capacity of osteogenic lineage cells cultured from bone marrow was maintained after as many as 40 doublings [39, 40].

When these cells are plated at low density, bone marrow stromal stem cells (BMSSC) rapidly adhere and can be easily separated from the nonadherent hematopoietic cells by repeated washing. With appropriate culture conditions, distinct colonies are formed, each of which is derived from a single precursor cell, known as colony forming units-fibroblasts (CFU-F). These cells were later identified as progenitors by their ability to differentiate into osteoblasts, chondroblasts, and myoblasts [34, 35]. Each colony forming unit – fibroblasts (CFU-F) is derived from a single cell which represents the cell proliferation capacity [31].

Furthermore, if such cultures are allowed to develop for up to 20 days, phenotypic heterogeneity is also noted (Figure 2). Some colonies are highly positive for alkaline phosphatase (ALP), while others are negative, and a third type is positive in the central region, and negative in the periphery [41]. Some colonies form nodules (the initiation of matrix mineralization) which can be identified by alizarin red or von Kossa staining for calcium. Yet others accumulate fat, identified by oil red O staining [42], and occasionally, some colonies form cartilage as identified by alcian blue staining [23].

These findings demonstrated that marrow-derived, plastic adherent fibroblastic (stromal) cells were capable of differentiating mesenchymal lineage cells as well as supporting the growth and differentiation of various hematopoietic cell types.

These plastic adherent adult stem or progenitor cells from human bone marrow were originally referred to as fibroblastoid colony forming units, then in the hematological literature as marrow stromal cells, subsequently as mensenchymal stem cells, and most recently as multipotent mesenchymal stromal cells.

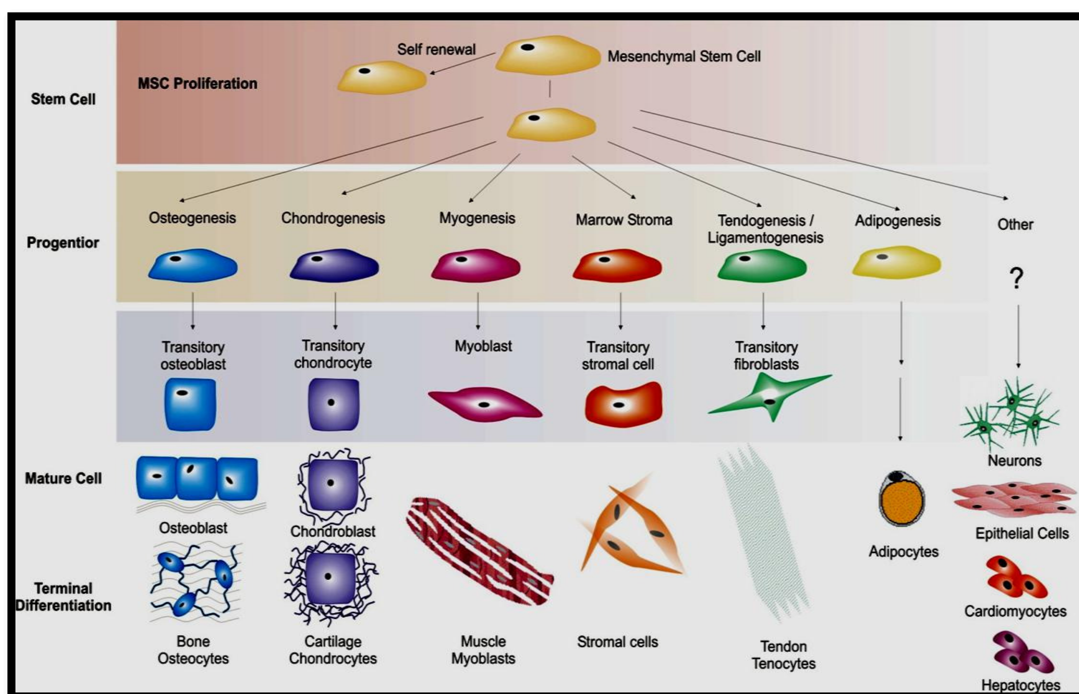


Figure 2. Differentiation of mesenchymal stem cells

The early preosteogenic stem and osteoprogenitor cells surface marker, monoclonal IgM antibody STRO-1 can be used to characterize the osteoprogenitor cells from the aspirates of human The STRO-1 antigen seems to be restricted to a subpopulation of cells that maintain immature, pre-osteoblastic phenotypes[43].

It is estimated that in every 1 million bone cells, only 2 to 5 stromal stem cells will be found and only 1 in 18,000 nucleated cells is estimated to be a stem cell [2, 3]. All stromal

stem cells do not perform the same function [44]. Only one third of all stromal stem cell are pluripotent and can be differentiated into osteoblasts, chondroblasts and adipoblasts [17]. Similar study revealed that one third of all bone marrow-derived cell clones are tripotent and they can differentiate into osteoblasts, chondroblasts, and adipoplasts. All the remaining cells are either bipotent (osteoblasts and chondroblasts) or unipotent (osteoblasts only) [45]. Therefore, the stromal stem cell population are comprised of subpopulations of undifferentiated stem cells and already differentiating progenitor cells [46].

Clinically, BMSSC can be applied in two main approaches. First approach is to use unexpanded BMSSC. After extracting from the bone marrow, it can be reintroduced into the recipient site with or without concentration. As an alternative, BMSSC can be expanded ex vivo to reach the desired number of stem cells before implantation. Both approaches are equally good in principle and the efficacy could possibly be the same [2].

Use of unexpanded stromal stem cells has several advantages over expanded cells. It is mostly used in clinical application because of the simplicity of the procedure. The technique is not only inexpensive, and it does not require extra instrumentation, but also favorable for ethical and regulatory reasons because it is minimally invasive and the patients need not to undergo two separate surgical operations and reduce the related risks of surgery and anesthesia. In addition to these advantages, the whole procedure can be safely performed within the surgical room, and the cells can be almost immediately reintroduced.

Since the cells are directly come from bone marrow, the risk of losing stem cells fraction in the cells extracted is minimal and more stem cells are delivered to the bone defect [44]. Jenson et al stated that the cultured cells may not function entirely like fresh bone marrow aspirate [44, 47, 48]. Moreover, there are evidences for hematologic stem cells cultured for hematologic transplantation showed reduced homing efficiency to both bone marrow and spleen. Similarly, there may be some altered function with expanded stem cells as well [44].

Although many studies have shown that the cultured mesenchymal stem cells implanted into critical-sized bone defects demonstrate profound osteogenesis, they also have

several disadvantages. Practical aspect of laboratory time and cost of cell culturing should be considered. The center needs to have specially trained technician and a research laboratory equipped with all the facilities necessary for cell culture.

According to the current estimates, the osteoprogenitor cells are limited in number, containing only less than 0.005% of the nucleated cells in the fresh marrow aspirates [20, 28, 49]. Therefore, the aspirated bone marrow should be reduced in volume to increase its osteoprogenitor cells content and to remove the polynuclear cells and non-nucleated cells so that the isolated mononuclear cell layer is obtained. The mononuclear cells layer contains stromal stem cells and osteoprogenitor cells but also contain other mononuclear cells such as leukocytes and monocytes and some of these cells may be a source of angiogenic or osteogenic cytokines with useful clinical synergistic effect [44].

Isolation of bone marrow mononuclear cells

The ability to isolate the marrow stromal cells with the most extensive replication and differentiation potential would naturally be of utmost importance for both theoretical and applicative reasons.

Bone marrow stromal stem cells can be isolated by different techniques by using Sepax processing system [50] (Figure. 3), COBE® 2991 cell processer [51] (Figure. 4), or automated cell capturing device such as Res-Q™ 60 BMC40 [52] (Figure. 5), as well as gravity filtration based technology known as Purecell™ Select System [53] (Figure. 6), or using Density Gradient Centrifugation reagents such as Ficoll [54] (Figure. 7) or Percoll [54] (Figure 8) etc.

Figure 3. Sepax® processing system

Figure 4. COBE® 2991 Cell Processor

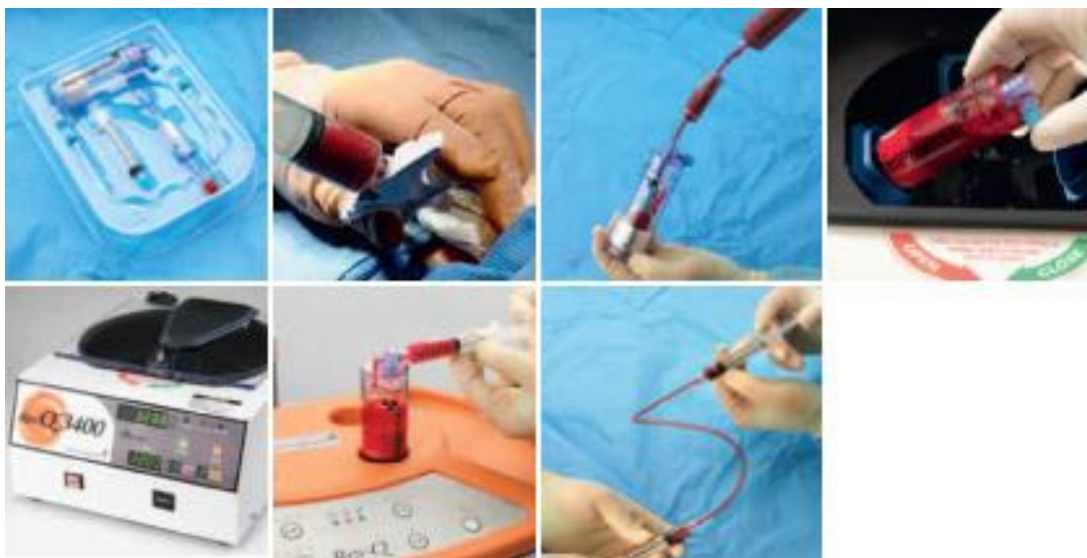


Figure 5. Res-Q™ 60 BMC Bone marrow concentration

Figure 6. Purecell™ Select System for whole blood mononuclear cells enrichment

Figure 7. Ficoll-Paque™ PREMIUM 1.073 Density Gradient Medium

Figure 8. Percoll™ Density Gradient Medium

Density gradient centrifugation

Density gradient centrifugation is a technique that allows the separation of cells depending on their density [55]. It is the most widely used method for isolating stromal osteoprogenitors cells from human bone marrow [54]. There are several advantages of Density Gradient Centrifugation reagents over other techniques requiring expensive devices and machine. Density Gradient Centrifugation medium are not expensive and mononuclear cells including stromal osteoprogenitor cells can be readily isolated by using a simple and rapid centrifugation procedure based on the method developed by Bøyum [55].

Ficoll Density Gradient Separation Media

Ficoll is a dentsity gradient separation media which can be used for in vitro isolation of mononuclear cells from peripheral blood, bone marrow and umbilical cord blood. Ficoll has been tranditionally used for separation of lymphocytes from blood by density gradient separation technique. Ficoll has also been applied in separation of mesenchymal stem cells from bone marrow [54].

Ficoll is a synthetic high molecular weight polymer (MW 400 000) made by copolymerization of sucrose and epichlorohydrin and is manufactured especially for use in cell separation. Ficoll is non-toxic to cells. In 1968, Bøyum described a method using low viscosity Ficoll™ having the proper density and osmotic strength, to isolate mononuclear cells [55].

There are different types of Ficoll media available commercially. Ficoll-Paque PREMIUM 1.077 is sterile, standard density Ficoll PM400/sodium diatrizoate solutions of the proper density, viscosity and osmotic pressure. Ficoll-Paque PREMIUM 1.073 has a lower density (1.073 g/ml) compared to standard Ficoll having a density of 1.077 g/ml and was manufactured especially for isolation of lower density mononuclear cells, for example, mesenchymal stromal cells or monocytes. Unlike standard 1.077 g/ml Ficoll, the higher density lymphocytes and granulocytes will sediment through Ficoll-Paque PREMIUM 1.073 to the bottom of the tube, thereby enriching the lower density stromal cells at the interface.

Separation principle is as follows. Defibrinated or anticoagulant-treated blood was layered on the Ficoll-Paque PREMIUM solution and centrifuged for a short period of time by using swinging bucket rotor with brake off to allow better separation and to prevent disturbing of the pellet. Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer contains erythrocytes, which have been aggregated by Ficoll PM400 and therefore sediment completely in the Ficoll-Paque PREMIUM layer. The layer immediately above the erythrocyte layer contains mostly granulocytes, which at the osmotic pressure of the Ficoll-Paque PREMIUM solution, attains a density great enough to migrate through the Ficoll-Paque PREMIUM layer. At the interface between the plasma and the Ficoll-Paque PREMIUM layer, mononuclear cells are found together with other slowly sedimenting low density particles (e.g. platelets). Mononuclear cells are then recovered from the interface and subjected to short washing steps with a buffer solution to remove platelets, density gradient medium and plasma.

Ficoll has been used in clinical practice for several decades, and its efficiency in separating lymphocytes and its safety in clinical usage are well recognized [54]. The general manufacturer's protocol for using Ficoll to separate human mononuclear cells from human peripheral blood is as follows. Blood was diluted with the equal amount of buffer solution containing 2mM EDTA in sterile phosphate buffered saline (pH=7.2). The diluted bone marrow sample was carefully layer on Ficoll-Paque PREMIUM and care must be taken not to mix Ficoll-Paque PREMIUM and the diluted blood sample. Then the tube was centrifuged at 400xg for 40 min at 18 °C in a swing bucket rotor with brake off. After centrifugation, the upper layer containing plasma and platelets was removed using a sterile pipette, leaving the mononuclear layer undisturbed at the interface. The mononuclear cells layer was transferred to a sterile centrifuge tube using a sterile pipette and then diluted with buffer at least three volumes. The cells were suspended by gently drawing them in and out of a pipette. The cells were washed by centrifugation at 400 g for 10 min at 18 °C in a swing bucket rotor with brake off. After removing the supernatant, the mononuclear cells were resuspended in buffer and then centrifuged again at 100 g for 10 min at 18 °C in a swing bucket rotor (brake off) to remove platelets.

Although the method described by the manufacturer has been used with success to prepare mononuclear cells from blood, the optimum result may not be obtained due to the different in nature of bone marrow and blood such as cellularity, viscosity and weight. A number of studies using Ficoll to remove mononuclear cells from bone marrow used different centrifugation forces (from 350 g to 1800 g), as well as different ratio of dilution (1:1 to 1:3).

According the basic principles of centrifugation, differences in centrifugal force could affect the sedimentation rate and the viability of cells[56]. The centrifugation forces is directly proportional to the rate of sedimentation, i.e., a higher force can offer better separation, however, the viability of the cells will be reduced as the force increases. Accordingly, the ratio of dilution, in other words, the viscosity of the bone marrow could also affect the rate of sedimentation[56]. Therefore, we assumed that different in centrifugation forces and ratio of dilution might affect the enrichment efficacy of isolated bone marrow stromal cells and osteoprogenitor cells.

Currently, to our extent of knowledge, relative isolation efficiencies of these different separation protocols against the standard manufacturer's protocols have not been investigated. In this study, we hypothesized that increase in centrifugal force (1000 g) and degree of dilution (1:3) would improve enrichment efficacy of Ficoll 1.073 due to differences in the nature of the blood and the bone marrow.

Chapter 3

Objective of the study

The aim of the study was to compare the enrichment efficacy of the two different centrifugation protocols for the lower density gradient centrifugation media, Ficoll-Paque PREMIUM 1.073, in isolation of human bone marrow stromal osteoprogenitor cells.

Chapter 4

Materials and Methods

The study was approved by the Ethical Board of the Faculty of Dentistry, Prince of Songkla University. The study was performed in the Dental Hospital at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Prince of Songkla University, Hatyai, Thailand. Ten healthy volunteer patients without any systemic diseases having normal complete blood counts who would undergo secondary alveolar bone grafting surgery from anterior iliac crest bone grafts were included in the study.

Bone marrow Aspiration

Bone marrow was aspirated from the anterior iliac crests under general anesthesia. A rolled towel was placed beneath the buttocks of the patient to elevate and slightly rotate the anterior iliac crest. The surgical area was prepared in a routine fashion and draped with towels. The 2mm stab incision was made on the anterior iliac crest approximately 2cm posterior from the anterior superior iliac spine (ASIS) as shown in the Figure 9.

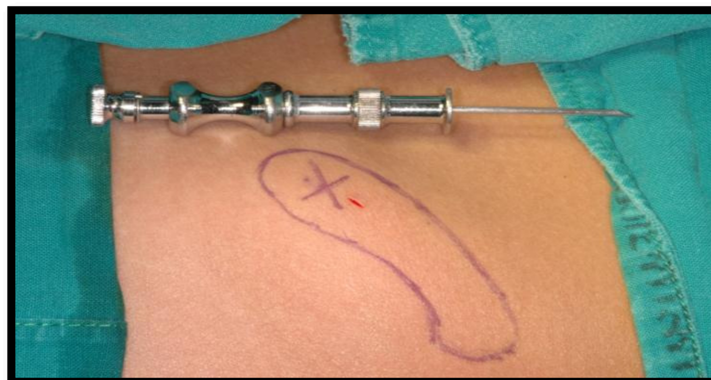


Figure 9. 2mm stab incision on anterior iliac crest about 1 cm posterior to the ASIS

A Klima-Rosegger bone marrow aspiration needle (Figure 10) (diameter 14 G, 1.5 inches long) was inserted about 2cm into the cancellous bone of the iliac crest between the inner and outer tables at a site approximately two centimeters directly posterior to the anterior superior iliac spine on the iliac crest.

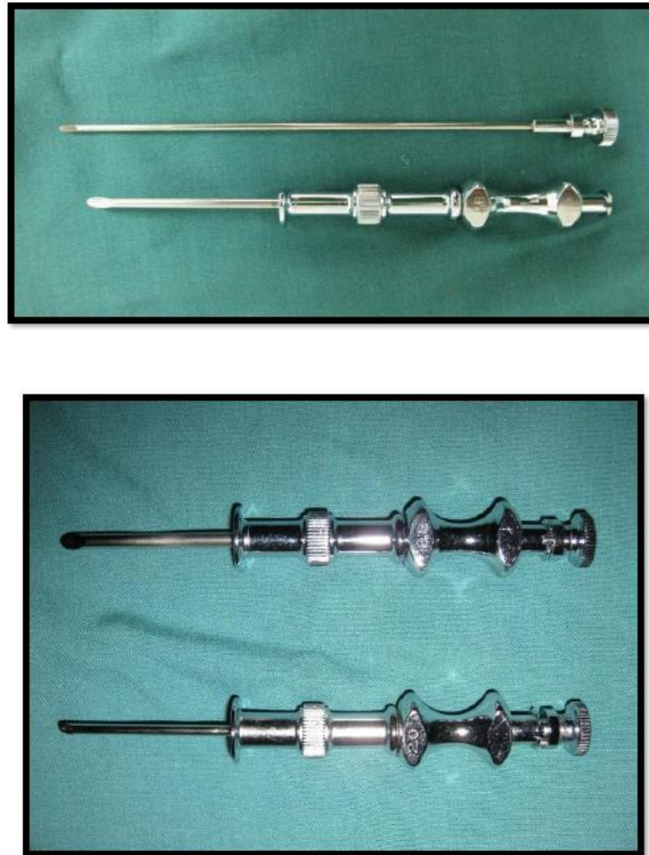


Figure 10. Klima-Rosegger bone marrow aspiration needle (diameter 14 G, 1.5 inches long), obturator removed (Above), and with obturator inside (Below).

After insertion of a beveled bone marrow aspiration needle into spongy bone, the obturator was removed, and a ten-milliliter plastic syringe was attached to the needle (Figure 11).

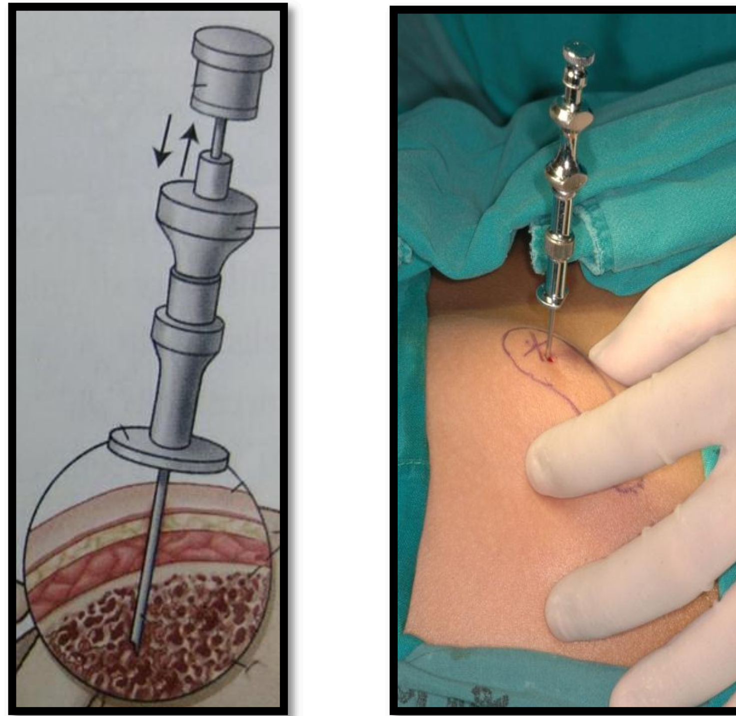


Figure 11. Insertion of bone marrow aspiration needle into the cancellous bone

Negative pressure was established by drawing the plunger back to approximately the six-milliliter marker until marrow began flowing into the syringe, the pressure was then reduced, and 2-4 ml of bone marrow was aspirated within three to six seconds at each time (Figure 12). If the marrow could not be obtained, the needle was rotated within the ilium so that the bevel of the needle could face the area of largest possible space to facilitate aspiration. Five successful aspirations were performed to get the 20ml bone marrow with a 1 cm distance between each bony insertion site to avoid dilution by aspiration from the previous area (Figure 13).

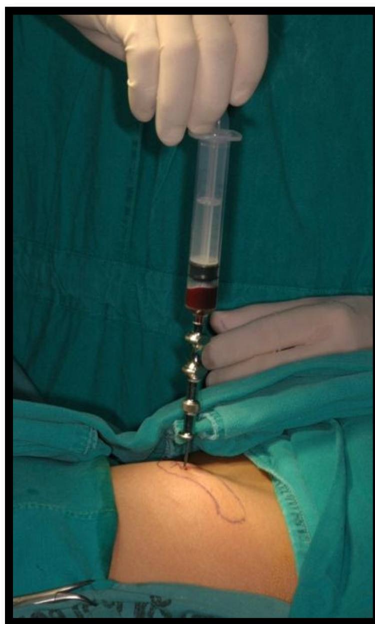


Figure 12. Bone marrow aspiration with ten-milliliter plastic syringe

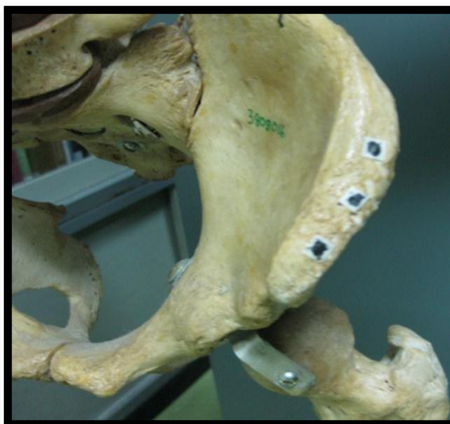


Figure 13. Demonstration of needle insertion sites (1 cm away from each other to prevent aspiration of peripheral blood oozed from previous needle injury site)

The marrow was aspirated in small fractions (< 4 mL) and continuous aspiration (more than 6 sec) was avoided to reduce the degree of dilution by peripheral blood. After successful aspiration, the syringe was detached and the sample was put into the test-tube containing 1 ml of heparinized normal saline containing 1000 units of heparin. The tube was inverted several times to ensure complete mixing to prevent clotting and then transferred to the laboratory.

Grouping

The experiment was performed to compare between 2 different centrifugation and dilution protocols as shown in Table 1.

A total of 20ml aspirated bone marrow was equally divided and put into two 50-ml plastic tubes. Each test tube contained 10 ml of bone marrow. Test tubes were named A and B randomly.

Table 1. Grouping

Protocol	Dilution	Force
A	1:1	400 g
B	1:3	1000 g

Bone Marrow Processing

Processing of the marrow samples was done in a class IIA biologic safety cabinet, and the samples were treated with universal precautions (Figure 14).

Figure 14. Class IIA Biosafety cabinet

Buffer was prepared by adding 2mM EDTA in sterile phosphate buffered saline (PBS) and adjusted to have a pH of 7.2 by pH meter (Figure 15), and was kept at 2-8 °C until used.

Figure 15. Measurement of pH of the buffer by pH meter

The heparinized bone marrow was first filtered through a 100μm cell strainer to remove bone fragments, cell clumps and fat. Wetting of the filter with buffer was carried out before use (Figure 16). The bone marrow in each tube was diluted with buffer in accordance with each protocol.

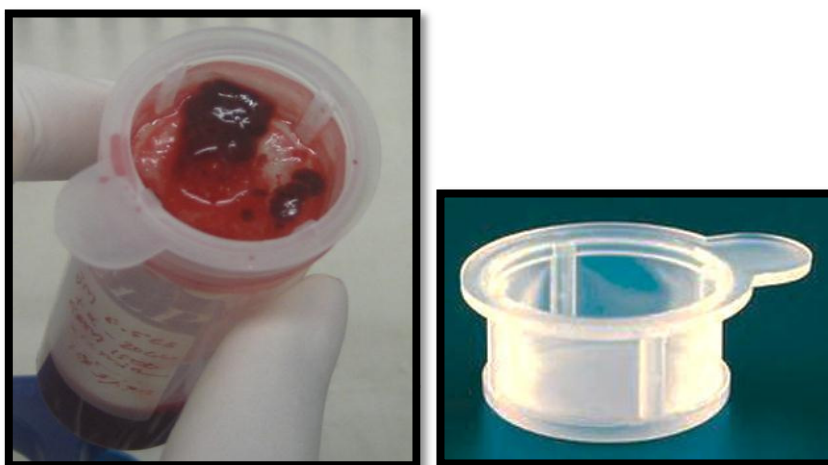


Figure 16. Filtering of aspirated bone marrow by 100μm cell strainer

Isolation of bone marrow mononuclear cells

Ten milliliters of Ficoll-Paque PREMIUM 1.073 density gradient centrifugation medium was added into two 50 ml centrifuge tubes which were labeled randomly as Protocol A and Protocol B. The diluted bone marrow samples were layered on Ficoll-Paque PREMIUM slowly and carefully to prevent mixing of the centrifugation medium and the diluted blood

samples as shown in the Figure 17. Then each tube was centrifuged at a given g force according to each protocol for 40 min at 18 °C in a swing bucket rotor with the brake off.

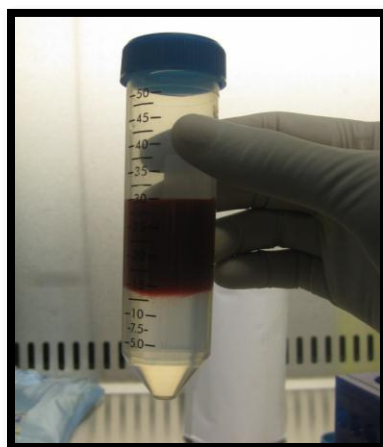


Figure 17. Careful layering of bone marrow over Ficoll-Paque PREMIUM 1.073 without mixing of bone marrow and density gradient media

After centrifugation, the upper layer containing plasma and platelets was removed using a sterile pipette, leaving the mononuclear layer undisturbed at the interface (Figure 18).

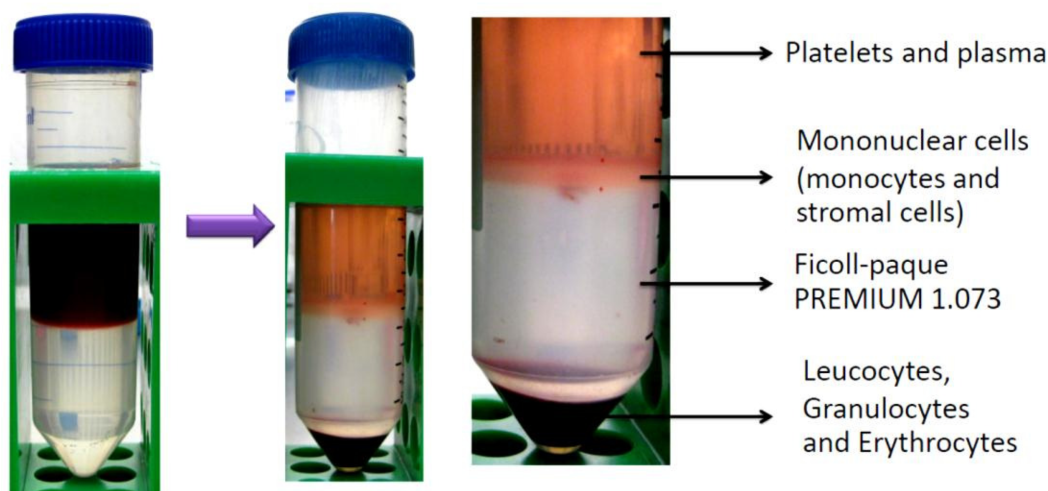


Figure 18. Mononuculear cell layer containing stromal cells

The mononuclear cells layer was transferred to a sterile centrifuge tube using a sterile pipette and then diluted with 20ml of buffer. The cells were suspended by gently drawing them in and out of a pipette. The cells were then washed by centrifugation at 400 g for 10 min at 18 °C in a swing bucket rotor with the brake off. After removing the supernatant, the mononuclear cells were resuspended in buffer and then recentrifuged at 100 g for 10 min at 18 °C to remove platelets in a swing bucket rotor with the brake off.

After removing the supernatant, the isolated mononuclear cells were suspended in the cell culture medium. Then by using 10ml sterile plastic syringes, isolated mononuclear cells were passed consecutively through 16, 20, 24 gauge needles to break up cell aggregates and to create single cell suspensions.

Counting the mononuclear cells

After centrifugal isolation of mononuclear cells from bone marrow aspirates by using Ficoll-Paque PREMIUM 1.073, 10 μL of bone marrow was mixed with 10 μL of 0.4% trypan blue stain. The mononuclear cells were counted and were checked for viability with standard Malassez hemocytometer (Figure 19).

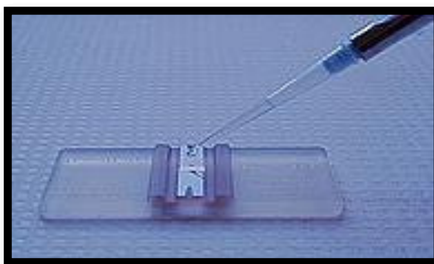


Figure 19. Malassez hemacytometer

Cell culture

The 10^6 mononuclear cells were plated in each well in the 6-well plates ($10cm^2$ in diameter for each well) in standard cell culture medium (α- MEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 1 % Fungizone) and incubated at 37˚C in a humidified 5% CO_2 environment. The cell growth was observed under inverted microscope every day. When plated cells became confluent around day 5-7, the culture medium was replaced with osteogenic medium containing α-MEM, 10% FBS, penicillin (100 units/ml), streptomycin (100 µg/ml), ascorbic acid (50 µg/ml),β-glycerophosphate (4 mM) and dexamethasone (100 nM). The media was changed every three days for the entire duration of culture.

Characterization of osteoprogenitors

1. Number of CFU-F colonies

After 10 days, cultured cells with high proliferative activity become colonies. Cell aggregate of more than 50 cells were counted as one alkaline phosphatase positive CFU-F colony by using a Nikon Eclipse Ti-S inverted microscope, and captured by the attached DS-Qi1 camera by using NIS-Elements Imaging Software. Results were expressed as the mean number of colony-forming units per 10^6 mononuclear cells.

2. Detection of Stro-1 surface marker

STRO-1 is a cell surface protein expressed by bone marrow stromal cells and is

known as an early osteogenic stem cell marker [43]. On day 10, the cultured bone marrow stromal cells were analyzed for expression of osteoprogenitor cells marker STRO-1 by flow cytometry. Adherent cells were released with trypsin/EDTA, and the action of trypsin was terminated by washing the cells with culture medium containing 10% FBS and centrifuged at 400g for 5 mins at 4˚C. The cells were washed twice with PBS. 10^6 cells were suspended in 90 µl of 10% PBS with 12 µl of mouse anti STRO-1 IgM antibody (Invitrogen, dilution 1:5) and incubated for 1 hour at 4˚C with occasional gentle mixing. The cells were then washed with PBS three times, resuspended in 90 µl of PBS with 10 µl of labeled secondary antibody, AlexaFluor 488 conjugate Goat anti-mouse IgM antibody (Invitrogen, concentration 1 µg), for 1 hr at 4˚C. The cells were then washed and resuspended in 500 µl cold PBS and fixed with equal volume of cold buffered 2% paraformaldehyde. For negative controls, each sample with omission of both antibodies, and omission of the STRO-1 antibody and omission of the secondary antibody were also analyzed by flow cytometry. Analysis of 10000 events was performed in a Cytomics FC 500 Flow Cytometer (Beckman Coulter, Inc).

Osteogenic differentiation

1. Characterization of osteoblastic phenotype

After being cultured in osteogenic induction medium for 14 days, cells were fixed with 10% neutral-buffered formalin for 5 mins, and then assayed for alkaline phosphatase (ALP) activity. Briefly, the substrate solution was prepared by dissolving 8mg of naphthol AS-TR phosphate in 0.3 ml of N,N'-dimethylformamide, while a separate solution of fast blue BB was prepared by dissolving 24 mg in 30 ml of 100 mM Tris (pH 9.6). The above solutions are mixed and then 10 mg of $MgCl_2$ was added and dissolved, and the pH was adjusted to 9.0 with 1N HCl. The cells were incubated with fresh substrate at 37˚C for 30 mins, then rinsed extensively with distilled water and photographed [54].

2. Alkaline Phosphatase quantitative assay

Alkaline Phosphatase (ALP) enzyme activity of the cell layer was measured in

triplicate manner at day 7,14 and 21. At the end of prescribed time periods, the cell layers were rinsed twice with PBS and osteoblasts were lysed by undergoing three freeze-thaw cycles (30 mins freeze at -20˚C then placed at room temperature for 15 mins for each cycle). 300µl of 0.2% Trition-X in PBS was added into each well, the cell layer was scraped off by using a cell scraper. The mixture was then transferred to the ependroff microcentrifuge tubes, vortexed vigorously for 1 mins and then incubated on ice for 90 mins. After incubation, the tube was vortexed again vigorously for 1 mins, centrifuged at 13000rpm for 5mins at 4˚C to remove insoluble cellular materials and debris, then a supernatant was collected for measurement of enzymatic activity.

The quantitative measurement of ALP activity was determined by formation of *p*-nitrophenol (pNP) (yellowish) , which is the end product of enzyme ALP and *p*-nitrophenolphosphate (colourless). 50 µl of supernatant was added to 50 µl *p*-nitrophenol-phosphate (4.34 mM) in 100mM glycine, pH 10.3, 1mM MgCl, mixed well and incubated at 25˚C for 60mins, and protected from light. The reaction was then stopped by adding 20µl of 1 M NaOH solution. After shaking the plate gently, the enzymatic activity was quantified by absorbance measurements at 405 nm in a micro plate reader and calculated according to a series of *p*-nitrophenol standards 0, 4, 8, 12, 16, 20 nmol/well pNPP standard. Background reading was corrected by subtracting the value derived from the zero standards from all standards, samples and the sample background control.

Enzymatic activity was normalized to total protein concentration by using bicinchoninic acid (BCA) from aliquots of the same samples with the Pierce® BCA Protein Assay Kit (Thermo Scientific). This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. The purple-coloured reaction product of this assay was formed by the chelation of two molecule of BCA with one cuprous ion. Bovine serum albumin standards were prepared in serial concentration of 25, 125, 250, 500, 750, 1000, 1500, 2000 µg/ml. 25µl of standard and unknown samples were mixed with 200µl of the working reagent (50 parts of reagent A contained sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide and mixed with 1 part of reagent B containing 4% cupric sulfate) in a 96 well

plate, and mixed thoroughly on a plate shaker for 30 seconds. The plate was incubated at 37°C for 60 mins, cooled to room temperature and then measured for absorbance at 562nm on a plate reader. Protein concentration of samples were calculated from a standard curve and ALP activity was standardized as nano-moles of *p*-nitrophenol liberated per milligram of total cellular protein [57].

Detection of Bone Mineralization

The presence of mineralized nodules (phosphate and calcium deposits) was confirmed cytochemically by using von Kossa and alizarin red S staining at Day 28.

1. Von Kossa (Phosphate staining)

Calcium phosphate deposits was detected by the Von Kossa technique in which phosphate deposits were stained black. At day 28, the monolayers were rinsed twice with ice cold PBS solution after removing the osteogenic medium and fixed with 10% formaldehyde for 15 mins and rinsed with distilled water. 5% silver nitrate solution was added and kept for 30 mins in a dark room and then the plate was exposed to bright sunlight until mineralized nodules were seen as dark brown to black spots [58].

2. Alizarin Red S staining (Calcium staining)

Alizarin Red S (sodium alizarin sulphonate) staining was used reveal the presence of calcium deposits. 2% alizarin red S solution was prepared in distilled water and the pH was adjusted to 4.1-4.3 using 0.5% ammonium hydroxide. Cultures were fixed with 10% formaldehyde for 15 mins, washed with distilled water and stained with alizarin red S for 15 mins. After removing excess incorporated dye with distilled water, red mineralized nodules became visible [59].

Chapter 5

Statistical Analysis

Statistical Analysis was carried out using SPSS 14.0 software (SPSS, Chicago, IL). The data were presented as the mean and standard deviation (SD). The paired t test was used to compare the differences between the two centrifugation protocols and $p<0.05$ was considered statistically significant.

Chapter 6

Results

Ten patients undergoing secondary alveolar bone grafting were volunteered to participate in this study. There was no intra operative and post operative morbidity or complications related to bone marrow aspiration and the surgery was uneventful.

The mean age of the patients was 9.5 years. The youngest patient was 4.6 years old and the oldest patient was 23 years old.

Figure 20 shows the normal bone marrow smear (left) and the isolated mononuclear cells after centrifugation (right).

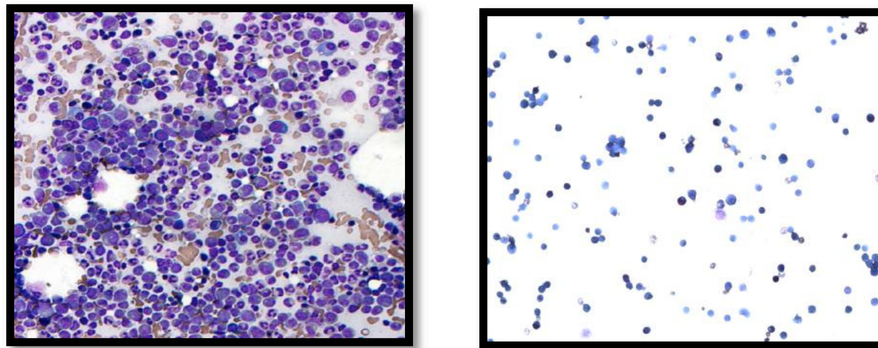


Figure 20. Bone marrow Weight's stain, before (left) and after centrifugal isolation (right)

Number of isolated mononuclear cells

The average numbers of isolated bone marrow mononuclear cells is shown in Figure 21.

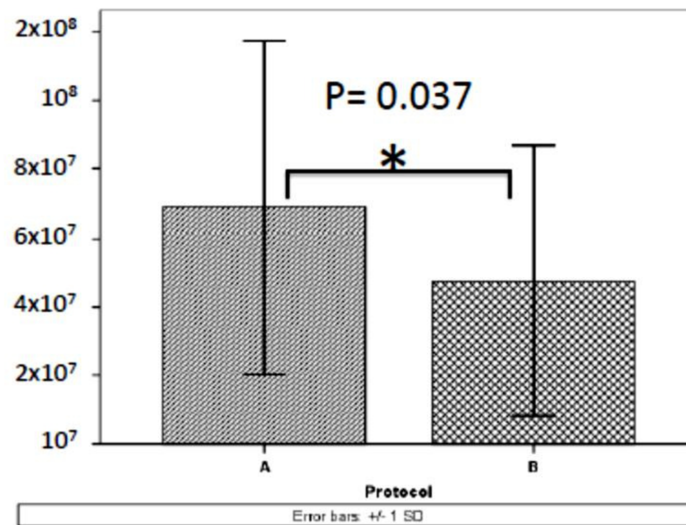
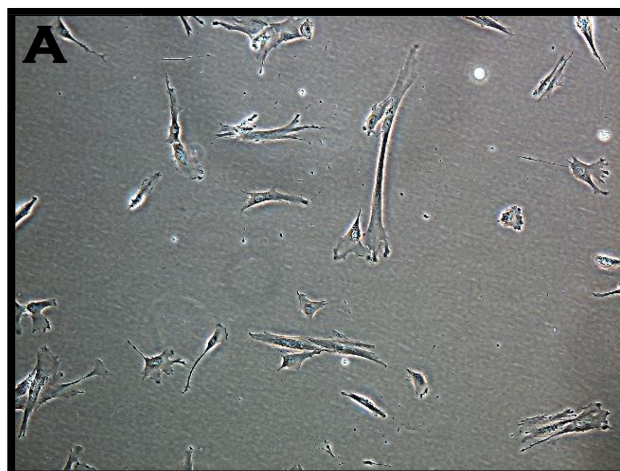


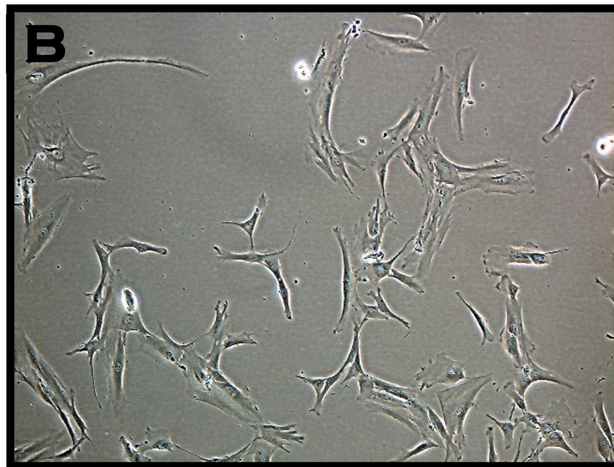
Figure 21. Isolated mononuclear cells count

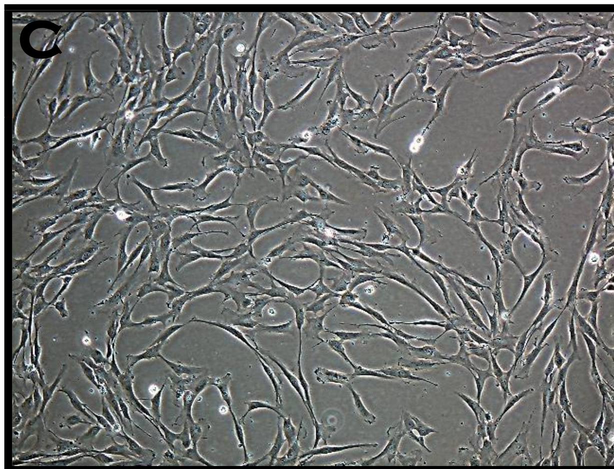
There was a significant difference here between protocols (p value 0.037*).* Thus, a lower centrifugal force produced recovery of more bone marrow derived mononuclear cells.

Cell culture

Within 24 hours of culture, the cells with adherence capacity from both protocols began attaching on plates in both protocols. Most of the cells that attached to the plastic surface exhibited a fibroblast-like spindle shape. Most cells attached to the surface within the first three days and non-adherent cells were reduced with subsequent medium change. The attached cells continued to proliferate and began differentiating into osteoblastic medium (Figure 22).







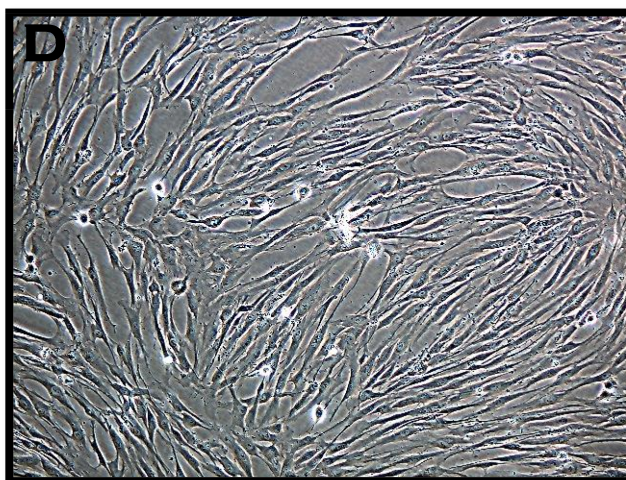


Figure 22. Proliferation of bone marrow mononuclear cells on different days

Characterization of osteoprogenitors

1. Formation of CFU-F

Fibroblastic colonies appeared and became clearer as the incubation period prolonged (Figure 23).

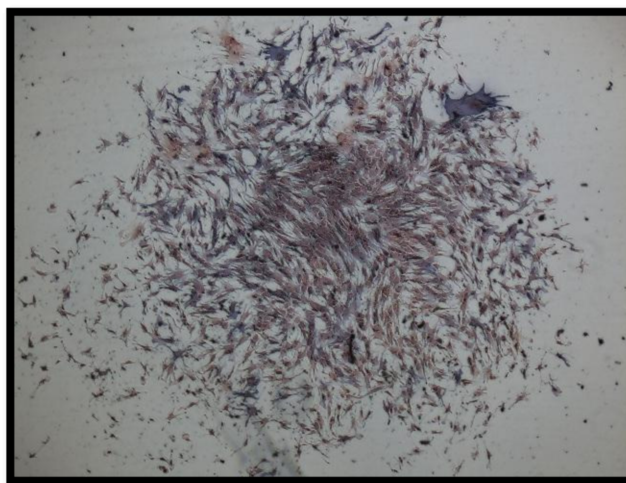


Figure 23. CFU-F colonies produced from single osteoprogenitor cells

2. Number of CFU-F colonies

Numbers of CFU-F colonies/ 10^6 cells produced from each protocol are shown in the Figure 24.

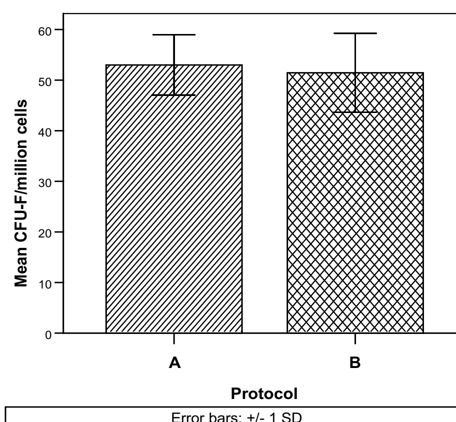


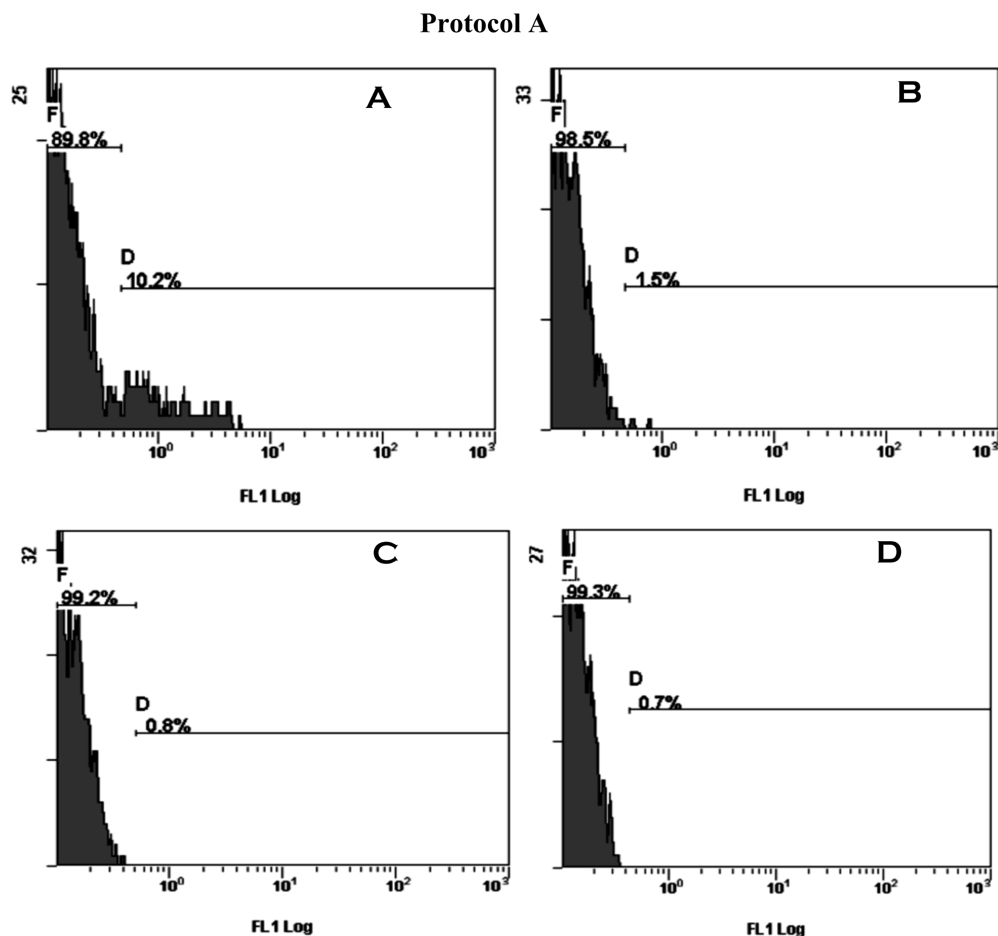
Figure 24. Number of CFU-F colonies from each protocol

Approximately 43-64 CFU-Fs were produced from 10^6 mononuclear cells in each well. The mean±SD number of alkaline phosphatase positive CFU-Fs in Protocol A was $53\pm6/10^6$ mononuclear cells, which was not significantly different from Protocol B ($51\pm8/10^6$ mononuclear cells) (Figure 24).

In the present study, there was no statistically different CFU-F forming capacity between each protocol.

3. STRO-1 Flow Cytometry Analysis

Cultured stromal mononuclear cells were analyzed to detect surface antigens by flow cytometry. STRO-1 is the novel monoclonal antibody which can identify stromal osteoprogenitor cells [60]. Positive expression of stromal osteoprogenitor cells marker STRO-1 was detected in both protocols by Flow cytometry analysis (Figure 25).



Protocol B

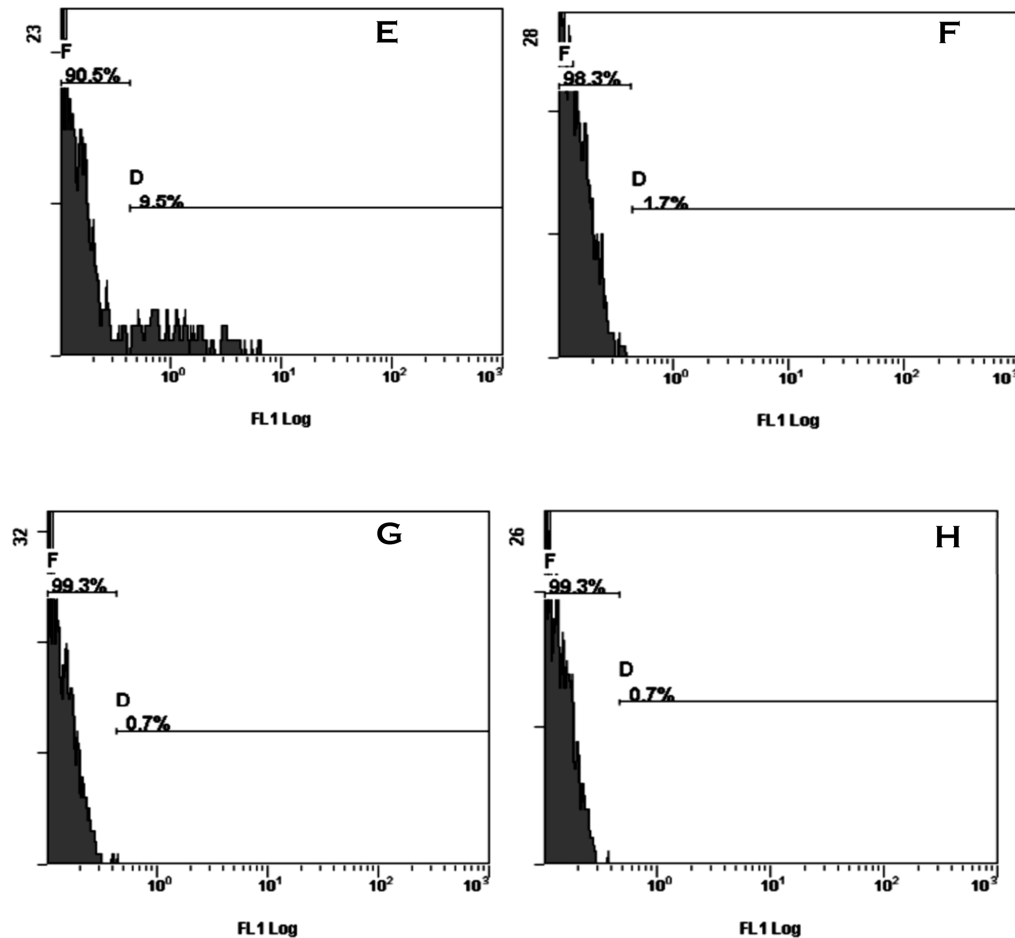


Figure 25. Fluorescence activated flow cytometry analysis of adherent cells 10 days after seeding showing STRO-1 positivity (No. A, E), compared with negative controls (B - D, F - H) : cells with AlexaFluor 488 conjugate Goat anti-mouse IgM antibody (B, F), cells with pure stro-1 (C, G), and cells only without any antibody (D, H)

Cells incubated with an unlabeled pure stro-1 antibody (C, G) showed no fluorescence expression which was similar to cells with no antibody staining (D, H). Small amounts of non-specific fluorescence expression was seen in the cells incubated with AlexaFlour 488 conjugated goat antimouse antibody (B, F) and their expression was added in the range of negative control cells in order to get truly positive stro-1 positive cells. No difference was detected statistically between both protocols, and both protocols were able to produce similar quantities of the STRO-1 positive osteoprogenitors.

Osteogenic differentiation

1. Characterization of osteoblastic phenotype

Osteoprogenitor cells cultured in osteogenic medium under influence of dexamethasone, β-glycerophosphatase and ascorbic acid progressed to osteoblastic differentiation which is visualized by ALP cytochemical staining (Figure 26).

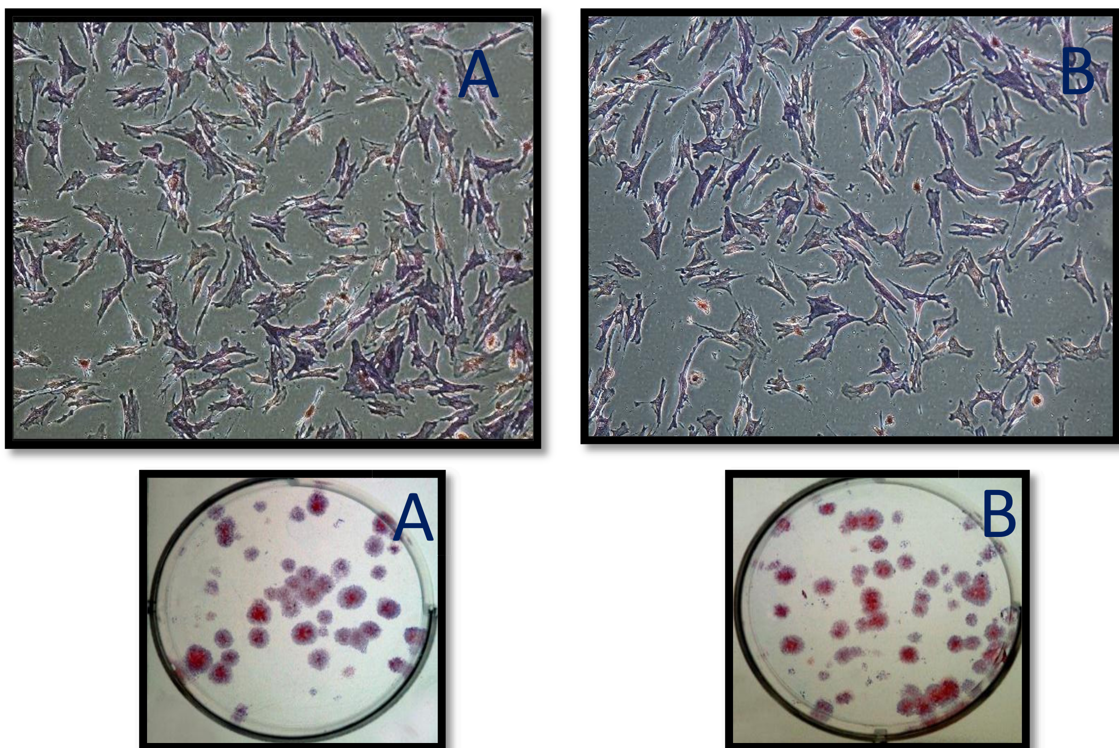


Figure 26. Alkaline phosphatase positive osteoblasts (Above) and CFU-F colonies produced from single osteoprogenitors in each protocol

2. Alkaline phosphatase quantitative assay

Alkaline phosphatase activities of the osteoblastic cells from each protocol were compared (Figure 27).

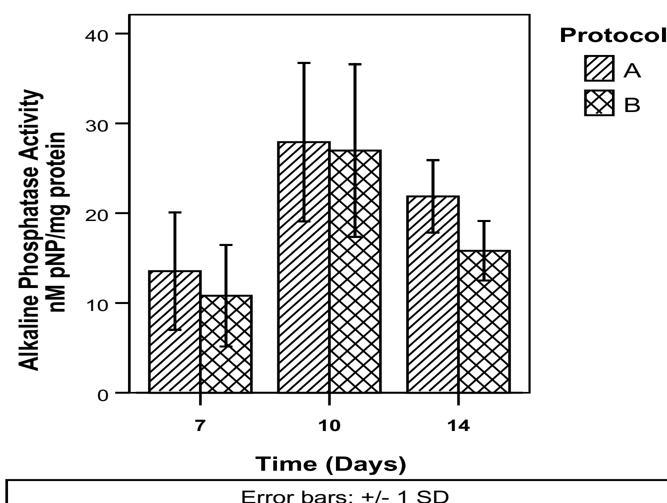


Figure 27. Osteogenic capacity of bone marrow stromal osteoprogenitor cells cultured in osteogenic medium: Alkaline phosphatase activity (nM pNP/mg protein) on day 7, 10 and 14.

The quantitative study of alkaline phosphatase revealed alkaline phosphatase activity by osteoblasts on day 7 and increased within a few days with its peak on day 10 and then it reduced in values. Alkaline phosphatase activity did not significantly differ between protocols on each time interval, however, a significant increase in enzyme activity was detected when compared between day 7 and 10 for both protocols.

Detection of in-vitro mineralization

Formation of mineralized nodules in osteogenic cell culture was confirmed by positive alizarin red S and von Kossa staining in both protocols.

The mineralized nodules appeared bright red when stained with alizarin red S (Figure 28).

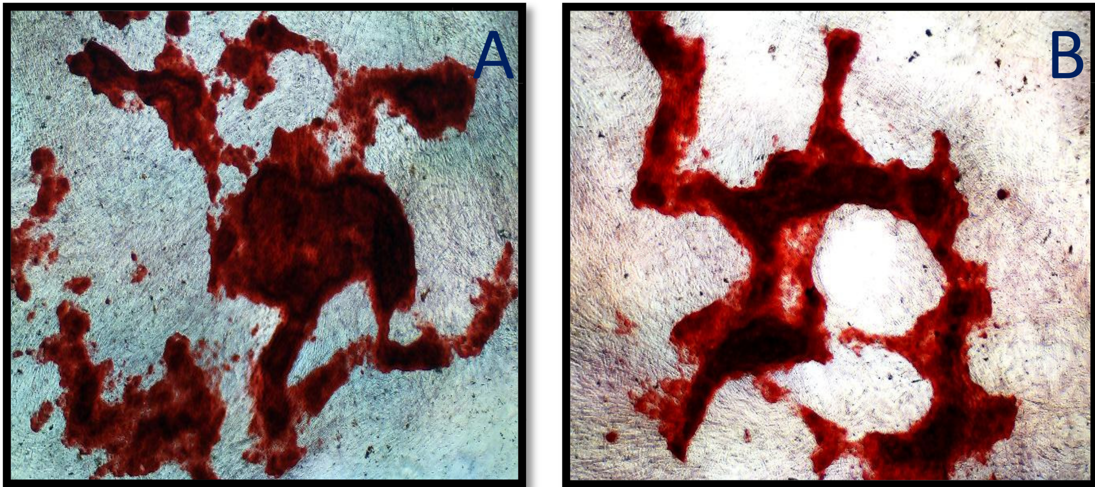


Figure 28. Phase contrast micrograph of 28-day-old mineralized nodules visualized by Alizarin Red S staining

In-vitro mineralized nodules stained black when treated with silver nitrate in the von Kossa method (Figure 29).

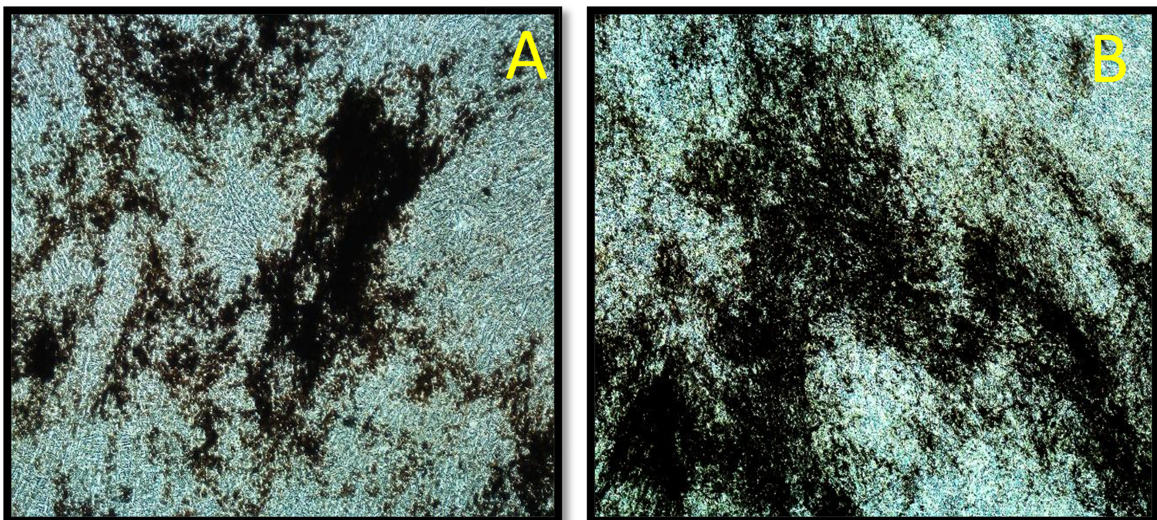


Figure 29. Phase contrast micrograph of 28-day-old mineralized nodules stained with von Kossa technique

Chapter 7

Discussion

A sufficient number of competent osteoprogenitor cells in the graft or healing site is essential for bone healing or regeneration. Recently, cell based (stem cells or osteoprogenitor cells) tissue engineering technology has increased in attention to potentiate bone healing or repair. Aspirated bone marrow serves as the richest supply of osteoprogenitor cells[3]. Isolation of the highest possible number of purified stromal stem and osteoprogenitor cells from the least possible amount of bone marrow is necessary.

This study was done with the assumption that different centrifugation forces and different dilutions might have different enrichment efficacy of mononuclear cells including osteoprogenitor cells. In this study, by using Ficoll lower density gradient separation medium (1.073g/ml), relative enrichment efficacies of the two centrifugation protocols, manufacturer's protocol were compared.

The same amount of bone marrow was aspirated by using a standard proven technique for consistency in quality of aspirated bone marrow. This technique ensured that bone marrow was aspirated, not blood.

Bone marrow was aspirated from the anterior iliac crest because it was also the donor site for secondary alveolar bone grafting. Our aim was to avoid any added morbidity for the patients from bone marrow aspiration. Moreover, the area of iliac crest is broad and easily assessable, and the pain associated with needle aspiration is minimal, which provides considerable benefits for patients compared to open harvesting.

In our study, a lower centrifugal force (400 g in Protocol A) provided a higher number of isolated mononuclear cells. However, it was questionable whether the higher number came from an increase in number of osteoprogenitors or that of other unwanted cells. In contrast,

a higher centrifugal force (1000 g, Protocol B) isolated a less number of mononuclear cells. Again, whether or not the protocols could separate more purified osteoprogenitors and less number of other cells was also unknown.

The mean number ± SD of mononuclear cells isolated from both protocols was $6.87x10^7 ± 4.84x10^7$ and $4.70x10^7 ± 3.93x10^7$ respectively. Bone marrow cellularity and number of stem and progenitor cells greatly differ individually according to age, sex and physiological requirement of the body which leads to wide standard deviation when compared statistically. Therefore, a paired t test was used to compare the differences between the two protocols of individual patients separately.

In the present study, lower degree of dilution provided higher numbers of isolated mononuclear cells and verse versa. Higher viscosity of bone marrow (Protocol A) produced higher numbers of mononuclear cells; however, again, whether or not the protocols produced a higher number of osteoprogenitors still needs to be investigated further.

Therefore, CFU-F forming efficacy and STRO-1 positivity of isolated mononuclear cells were compared in order to estimate the numbers of osteoprogenitors presents in each protocol. Osteogenic differentiation and mineralization efficacy of these isolated osteoprogenitors were also compared.

It was found that both protocols could produce high numbers of CFU-F (no significant differences). In this study, 43-64 CFU-F per 1 million cells (around 0.0043% – 0.0064%) were isolated from bone marrow which was similar to the estimates of other studies (>0.005%) , therefore the techniques of aspiration, centrifugation and cell culture used in this study were comparable to others and thus reliable[20, 28, 49].

There were various agents in the literature used to isolated bone marrow stromal stem cells such as STRO-1, CD 18, CD 106, CD 146, etc. Among these, STRO-1 antibody has the highest affinity and efficiency for isolating all colony forming osteogenic precursor cells isolated from aspirates of adult bone marrow as a standalone agent. It has no reactivity to

hematopoietic stem and progenitors cells, and its specificity is strictly restricted to early preosteogenic stem cells[61-63].

Flow cytometry analysis confirmed the presence of equal number of osteoprogenitors by a STRO-1 osteoprogenitor cells marker (around 10±3%) in each protocol. Our results were also comparable to other studies done by Grothos (9.2%) and Simmons (14.7%), meaning both methods of separation could yield optimum number of STRO-1 positive osteoprogenitors [60, 64].

Isolated osteoprogenitors from both protocols showed alkaline phosphatase activity and no significant difference was detected in each protocol proving that competent and functional osteoprogenitors were able to recover from both protocols and the centrifugation process didn't alter their function.

In addition to this, mineralization was also detected in both protocols by Alizalin Red S and von Kossa staining as a proof of their ability to secrete calcium and phosphate minerals, reflecting the terminal differentiation of osteoprogenitors into osteoblasts.

Chapter 8

Conclusion

This study revealed that the total number of bone marrow derived mononuclear cells were significantly higher in Protocol A than in Protocol B, suggesting that a lower centrifugation force (400 g) was preferable in terms of recovery of more cells.

However, the present work showed that there was no detectable difference between CFU-F forming capacity, STRO-1 postivity, osteogenic differentiation or mineralization abilities between protocols.

Further studies with the higher number of patients are necessary to assess overall efficacy and function of these osteoprogenitor cells, and to find the optimum dilutional and centrifugation factors customized for isolation of stromal osteoprogenitor cells from human bone marrow aspirations.

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Appendices

Appendix 1

Preparation of Heparin

1. Draw 0.2 ml of heparin (5000 units/ml); add 0.8 ml of normal saline solution to get dilution of 1000 units/ml. Put into the 15 ml sterile plastic tube.
2. Attach 10 ml plastic syringe into bone marrow aspiration needle.
3. After taking 1 drop of bone marrow onto the side to do bone marrow smear, bone marrow will be ejected into 15 ml sterile plastic tube containing 1 ml of heparinized normal saline. (1000 units/ml for each 10 ml).

Appendix 2

Wright's Stain

Wright's stain is a neutral stain produced by the interaction of an acidic and a basic dye, producing a large salt molecule with a colored dye in both its parts. With the Wright's stain, blood cells exhibit four major staining properties that allow the cell types to be distinguished. Basophilia (affinity for methylene blue), azurophilia (affinity for the oxidation products of methylene blue called azures, which are reddish purple), acidophilia (affinity for eosin), and neutrophilia (affinity for a complex of dyes in the mixture, which are pale lilac). In a stained blood smear, erythrocytes bind eosin and appear orange to pink, nuclei purplish blue, basophilic granules very dark bluish purple, eosinophilic granules red to red-orange, neutrophilic granules reddish-brown to lilac, platelets violet to purple, and lymphocyte cytoplasm stains pale blue.

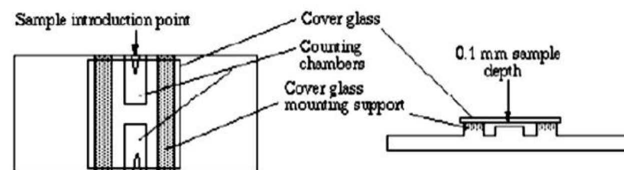
Staining Procedure for Blood Smears and Bone Marrow:

1. Prepare a film of blood or bone marrow on a microscope slide and allow to air dry.
2. Prepare three containers (e.g. coplin jars, or staining dishes). Fill one container with One Step Wrights Stain and the second and third containers with distilled or deionized water.
3. When stain volume in container 1 becomes insufficient, replace the stain. Do not replenish by adding new stain to the old.
4. To prevent evaporation, keep stain tightly covered when not in use.
5. Change the water in container 2 or 3 when an iridescent scum forms on the surface or when a dark blue discoloration occurs. It is very important to keep the rinse water clean.
6. Dip air dried slides in One Step Wrights Stain 60 seconds.
7. Dip slide in distilled or deionized water in container 2 for 90 seconds.
8. Dip slide in distilled or deionized water in container 3 for 50 seconds, using quick dips. As an alternative, the slide may be "swished" in distilled or deionized water for 50 seconds to remove the stain.

9. Wipe the back of slide.
10. Dry slides in vertical position, on absorbent surface. Do not blot the smear.
11. Apply oil and examine microscopically.

Appendix 3

Determining Cell Number and Viability with a Hemocytometer and Tryphan Blue Staining



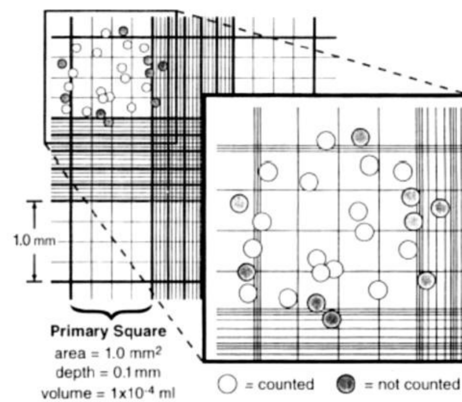
A hemocytometer is a thick glass slide with a central area designed as a counting chamber. The central portion of the slide is the counting platform which is bordered by a 1-mm groove. The central platform is divided into two counting chambers by a transverse groove. Each counting chamber consists of a silver footplate on which is etched a 3 x 3-mm grid. This grid is divided into nine secondary squares, each 1 x 1 mm. The four corner squares and the central square are used for determining the cell count. The corner squares are further divided into 16 tertiary squares and the central square into 25 tertiary squares to aid in cell counting. Accompanying the hemocytometer slide is a thick, even-surfaced cover slip. Cell suspension is applied to a defined area and counted so cell density can be calculated.

Preparing hemocytometer

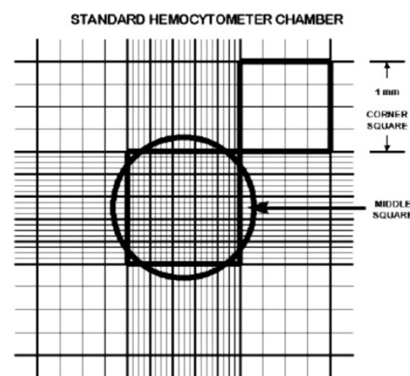
1. Clean surface of hemocytometer slide and cover slip with 70% alcohol.
2. Wet edge of cover slip slightly with water and press over grooves on hemocytometer. The cover slip should rest evenly over the silver counting area.

Preparing cell suspension

1. Dilute cells with 3 ml of α- MEM to obtain a uniform suspension. To determine number of viable cells by adding 0.4% tryphan blue according to the desired dilution factors. Mix thoroughly and let stand 1 min before loading hemocytometer. Non viable cells will take up the dye, while live cells will be impermeable to dye. Count only viable cells.



2. When using the hemocytometer, a maximum cell count of 50 to 200 cells per 1 x 1-mm square is recommended. Adjust dilution factor according to the desired cell count.



Loading hemocytometer

1. Use a sterile Pasteur pipette/ micropipette to transfer cell suspension to edge of hemocytometer counting chamber. Hold tip of pipette under the cover slip and dispense one drop of suspension.

Counting cells

1. Allow cells to settle for a few minutes before beginning to count. Blot off excess liquid.
2. View slide on microscope with 10x magnification.
3. Use a hand-held counter to count cells in each of the four corner and central squares. Five squares (four corners and one center) are counted. Count cells touching the middle line of the triple line on the top and left of the squares. Do not count cells touching the middle line of the triple lines on the bottom or right side of the square.

Calculating cell number

1. Determine the number of cells per millimeter by

$$\text{Cells/ml} = \frac{\text{number of cells counted}}{\text{number of squares counted}} \times 10^4 \times \text{dilution factor}$$

The number 10^4 is the volume correction factor for the hemocytometer: each square is 1 x 1 mm and the depth is 0.1 mm.

2. Determine the total number of cells

Total cells = Cells/ml x volume of original cell suspension

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Documentary Proof of Ethical Clearance

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The Project Entitled : Dilutional and Centrifugation factors of lower density gradient separation media on osteoprogenitor cells separated from concentrated bone marrow aspirate.

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