Author(s): Naung, Y.; Nuntanaranont, T.; Kamolmatyakul, S.; Suttapreyasri, S.

Title: Dilutional and centrifugation factors of lower density gradient separation media on osteoprogenitor cells separated from concentrated bone marrow aspirate

Conference: 23rd National Graduate Research Conference

Location: Rajamangala University of Technology, Isan, Thailand

Date: 23-24 December 2011

Year: 2011

Pages: 431 - 440

Abstract: ...

URLs:

FT: [REPLACE THIS with URL to Conference Paper (Click on 'FT' to EDIT)]

Abstract

Human bone marrow contains osteoprogenitors capable of differentiating into osteoblasts. Ficoll media is widely used in the isolation of human mesenchymal stem cells. The aim of the study was to compare the effectiveness of the two most commonly used centrifugation protocols for Ficoll-Paque PREMIUM 1.073 lower density gradient centrifugation media in isolating osteoprogenitor cells from aspirated bone marrow. Bone marrow was aspirated from anterior iliac crests, equally divided, 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. After isolation, 10⁶ cells were cultured in 6-well plates. The relative efficacies of each protocol were compared by their ability to produce alkaline phosphatase positive colony forming units-fibroblasts (CFU-F). The cultured monolayers were accessed for osteogenic differentiation and mineralization ability by cytochemical staining with alkaline phosphatase, von Kossa and alizarin red S staining as well as quantitative measurements of alkaline phosphatase activity were taken. The percentage of positive stromal osteoprogenitor cells marker STRO-1 expression was detected by Flow cytometry. The average numbers of isolated bone marrow mononuclear cells were 6.87x10⁷±4.84x10⁷ and 4.70x10⁷±3.93x10⁷ respectively, which were statistically different. The mean±SD number of alkaline phosphatase positive CFU-Fs in protocol A was 53±6/10⁶ mononuclear cells, which was not significantly different from protocol B (51±8/10⁶ mononuclear cells). Alkaline phosphatase activity did not show any difference between protocols, however, significant increase in enzyme activity was detected comparing 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. Assessment of matrix mineralization by optical density measurement revealed increased intensity of alizarin red S on each time interval, however, they did not differ significantly between the protocols. Positive expression of STRO-1 (around 10%) was detected from both protocols. There was no detectable difference between osteogenic differentiation and mineralization ability between protocols. Both protocols produced good results since they contained similar quantity of STRO-1 positive osteoprogenitors, however, using lower centrifugal force produced recovery of more mononuclear cells.

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

Introduction

Autogenous bone graft is the gold standard for grafting materials and their osteogenic capacity resides in the bone marrow and marrow stromal stem cells(1). Bone marrow contains two main cell systems; the hematopoietic and stromal systems. Bone marrow stromal system contains multipotent mesenchymal stem cells and other mesenchymal lineage progenitors including osteogenic, chondrogenic, adipogenic, and myogenic progenitor cells(2-5). Isolation of the mesenchymal stromal and osteoprogenitors cells from aspirated bone marrow can be a valuable alternative bone graft substitute to prevent grafting related morbidity and complications. The osteogenic potential of bone marrow aspirate was first reported in 1869 by Goujon(6). Later, Friedenstein and colleagues isolated and cultured bone marrow fibroblasts and demonstrated the development of fibroblastic colonies in vitro(7). These cells were later identified as progenitors by their ability to differentiate into osteoblasts, chondroblasts and myoblasts(8;9). Procktop proposed the term stromal stem cell to describe its role as a multipotent precursor cell for nonhematopoietic tissues(10).

The osteogenic potential of these cells can be confirmed by their ability of osteogenic
differentiation, mineralization and characterization by detecting surface markers of these osteoprogenitors. The early preosteogenic stem cell marker, monoclonal IgM antibody STRO-1 can identify all colony-forming osteogenic precursor cells from aspirates of human bone marrow(11).

Bone marrow stromal stem cells can be isolated by using density gradient centrifugation reagents such as Ficoll, a polymer of sucrose with a high synthetic molecular weight(12). Currently there are no clear indications on the appropriate dilution and centrifugation forces for using lower density gradient media Ficoll-Paque PREMIUM 1.073 in centrifugation of human bone marrow. Ficoll-Paque PREMIUM 1.073 has lower density compared to standard Ficoll having a density of 1.077 g/ml and can be used to isolate lower density mononuclear cells, for example, mesenchymal stromal cells or monocytes.

It was therefore necessary to find out a customized protocol for this lower density gradient media for use in isolation of human mononuclear cells to produce the highest number of stromal stem cells as well as osteoprogenitors. 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 for isolation of human mesenchymal stromal osteoprogenitor cells.

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

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. 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.

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

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Dilution</th>
<th>Force, Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1:1</td>
<td>400 g x40 mins x18˚C</td>
</tr>
<tr>
<td>B</td>
<td>1:3</td>
<td>1000 g x40 mins x18˚C</td>
</tr>
</tbody>
</table>

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.

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

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. The bone marrow in each tube was diluted with buffer in accordance with each protocol.

Isolation of 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. 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. 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 20 ml 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 centrifuged 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 10 ml 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.

**Cell culture**

The 10° mononuclear cells were plated in each well in the 6-well plates (10 cm² in diameter for each well) in standard cell culture medium (a- 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% CO₂ 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.

**Osteoblastic Differentiation**

Osteoblastic differentiation of cultured cells from both protocols were determined by counting the number of alkaline phosphatase positive colony forming units-fibroblasts (ALP positive CFU-F) and by quantification of alkaline phosphatase activity.

**Cytostaining of CFU-F**

After being cultured in osteogenic induction medium for 14 days, cells were fixed with 10% neutral-buffered formalin for 5 mins, then assayed for alkaline phosphatase (ALP) activity. Briefly, the substrate solution was prepared by dissolving 8 mg 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 MgCl₂ 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.[13] Alkaline phosphatase-positive cell clusters of more than 50 cells were counted as one alkaline phosphatase positive CFU-F colony by using a Nikon Eclipse Ti-S inverted microscope, photographed and analyzed by Image-Pro Plus Software version 7.0. Results were expressed as the mean number of colony-forming units per 10° mononuclear cells.

**Alkaline Phosphatase Colorimetric 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). 300 µl of 0.2% Triton-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 eppendorf 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 5 mins 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 100 mM glycine, pH 10.3, 1 mM MgCl₂, mixed well and incubated at 25 °C for 60 mins, and protected from light. The reaction was then stopped by adding 20 µl 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 Cu²⁺ to Cu⁺ by protein in alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu⁺) 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
Detection of Stro-1 surface marker

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

Von Kossa (Phosphate staining)

Calcium phosphate deposits were 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(14).

Alizarin Red S staining (Calcium staining)

Alizarin Red S (sodium alizarin sulphonate) staining was used to 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(15).

To compare the amount of mineralization, Alizarin red-stained cultures were incubated with 100mmol/L cetylpyridinium chloride (CPC) for 1 hour to solubilize and release calcium bound Alizarin red into the solution(16). The released Alizarin red S were measured at 570nm using a microplate reader and read as Optical Density of Alizarin Red S released/well. The data were shown as mean±SD of triplicate wells(17).

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(18). 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. 106 cells were resuspended in 90 µl of 10% FBS 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).

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) and p<0.05 was considered statistically significant.

Results and Discussion

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.

The average numbers of isolated bone marrow mononuclear cells is shown in Table 2.

Table 2: The Average number of Isolated Mononuclear Cells

<table>
<thead>
<tr>
<th>Protoco</th>
<th>Range</th>
<th>mean±SD</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.59x10^7-1.75x10^7</td>
<td>6.87x10^7±4.84x10^7</td>
<td>0.037</td>
</tr>
<tr>
<td>B</td>
<td>7.20x10^6-1.22x10^7</td>
<td>4.70x10^7±3.93x10^7</td>
<td></td>
</tr>
</tbody>
</table>

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.

It was also possible that a higher centrifugations force (1000 g) might reduce the viability of cells. However, higher degree of dilution and relatively less viscosity of bone marrow cells might also increase the efficacy of Ficoll-Paque PREMIUM 1.073 density gradient medium to isolate more purified mononuclear cells, which may be reduced in quantity but it is high in osteogenesis capacity.

Therefore, the osteogenic capacities of mononuclear cells isolated by different protocols were...
compared by the number of alkaline phosphatase positive CFU-Fs produced.

**Formation of CFU-F colonies**

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 to form a uniform confluent cell monolayer and became cubical, and some had the appearance of giant cells after changing to osteogenic media (Figure 1).

Fig 1. Proliferation of bone marrow mononuclear cells on different days: (A) Day 1, (B) Day 3, (C) Day 7, (D) Day 10.

Fibroblastic colonies appeared between day 6 or 7 and became clearer as the incubation period prolonged. Each human colony unit-fibroblasts (CFU-F) is derived from a single cell(19). CFU-F are considered colonies of stem and progenitor cells of mesenchymal lineage including osteoblasts(12). Expression of alkaline phosphatase activity is regarded as a marker of osteogenesis in bone-forming tissues(20). Therefore, alkaline phosphatase positive CFU-Fs produced by bone marrow stromal cells reflect the number of osteoprogenitor cells in each protocol (Figure 2).

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±6/$10^6$ mononuclear cells, which was not significantly different from Protocol B (51±8/$10^6$ mononuclear cells).

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

**Confirmation of osteogenic differentiation by alkaline phosphatase assay**

Osteoprogenitor cells cultured in osteogenic medium under the influence of dexamethasone, β-glycerophosphatase and ascorbic acid progressed to differentiation of osteogenic cells and in vitro nodule formation which was visualized by alkaline phosphatase cytochemical staining (Figure 4A) and quantified by colorimetric measurement (Figure 3).

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.

**Confirmation of osteogenic mineralization using von Kossa and alizarin red S staining**

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 4B,5B) and stained black when treated with silver nitrate in the von Kossa method (Figure 4C,5C).
When analyzed by spectrophotometry, osteoprogenitor cells cultured in osteogenic medium stained weakly on the 14th day, however the intensity increased on day 21 and 28 when exposed to alizarin red S, which was statistically significant for both protocols (p<0.05), demonstrating calcium deposition. However, assessment of matrix mineralization by quantitative measurement of the osteoprogenitor cells did not differ significantly between protocols (Figure 6), which means both protocols have similar osteogenic capacity.

**Characterization of stromal cells**

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(21). The STRO-1 antigen seems to be restricted to a subpopulation of cells that maintain immature, pre-osteoblastic phenotypes(22). Positive expression of stromal osteoprogenitor cells marker STRO-1 was detected in both protocols (Figure 7).

---

Fig 4. Osteogenic capacity of osteoprogenitor cells: (A) alkaline phosphatase positive osteoblastic cell, microscopic view, (B) Photograph showing presence of mineralized nodules confirmed by alizarin red S staining, (C) Photograph of Von Kossa stained mineralized nodules.

Fig 5. Phase contract Micrograph of 28-day-old mineralized nodules: (A) background control after fixing with formaldehyde without staining, (B) after Alizarin Red S staining, (C) after Von Kossa staining.

Fig 6. Osteogenic capacity of bone marrow stromal osteoprogenitor cells cultured in osteogenic medium: Detection of matrix mineralization by Alizarin Red S released per well on day 14, 21 and 28.
In this present study, to prevent false positive expression by nonspecific antigen-antibody binding and related fluorescence generation, double negative controls were included. Cells incubated with an unlabeled pure stro-1 antibody (7C, 7G) showed no fluorescence expression which was similar to cells with no antibody (7D, 7H). Small amounts of nonspecific fluorescence expression was seen in the cells incubated with AlexaFlour 488 conjugated goat antimouse antibody (7B, 7F) 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.

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 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, osteogenic differentiation or mineralization abilities between protocols and that both protocols produced good results and a similar percentage of STRO-1 positive osteoprogenitors.

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

Acknowledgements

Our sincere gratitude goes to Associate Professor Premjit Arpornmaeklong for her expert advice and help. We would like to thank Mr. Jakchai Jantaramano and Mrs. Siriwon Junrounnusit for technical assistance, Mrs. Somporn Stretrirutchai for assistance in flow cytometry and Mr. Mitch Atkins for proof-reading. We are also grateful for the financial support provided by Prince of Songkla University (Graduate School, Department of Oral and Maxillofacial Surgery, Faculty of Dentistry and Bone Research Center).

References


