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## Assessment of Mesenchymal Stem Cells in Bone Marrow Aspirate from Human Calcaneus

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### Abstract

Bone marrow aspirates (BMAs), due to their innate osteogenic potential, are well-documented supplements to osteoconductive and/or osteoinductive materials. The calcaneal body provides foot and ankle surgeons a convenient harvest site with low morbidity and minimal cost. In the current study, we attempt to identify and characterize multipotent mesenchymal stromal cells (MSCs) in BMAs harvested from human calcaneal body. Ten healthy patients between the age of 18 and 65 were enrolled in this study. BMAs were harvested from the patients without any reported post-operative complications related to the harvest. Cells isolated from all the aspirates were adherent to culture plates and expressed positive MSC surface markers CD105, CD90 and CD73 as well as low level of negative MSC markers CD34 and CD45. The cells maintained the ability to proliferate and differentiate into cells of mesenchymal lineages. The BMAs from the human calcaneal body offer a healthy source of multipotent MSCs.

### Keywords

foot and ankle surgery; orthobiologic materials; osteoconduction; osteoinduction; surface markers

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## Introduction

As podiatric surgery has continued to evolve and advance in complexity, so has the understanding of factors affecting healing - not only of soft tissue but also of bone. Many surgeons have looked towards orthobiologic approaches as a way of supplementing their surgical necessities. Recently the use of bone marrow aspirates (BMA) containing MSCs is becoming increasingly popular in foot and ankle surgery (1-3). In the continually evolving field, BMAs have arisen as a well-documented method to locally introduce endogenous growth factors such as Platelet Derived Growth Factor (PDGF) and Transforming Growth Factor  $\beta$  (TGF $\beta$ ) into an area to initiate healing of fusion sites, fresh fractures and areas of devascularization (4, 5). They have also been used to enhance healing at graft sites (6, 7). BMAs have been shown to have higher levels of both endogenous Bone Morphogenic Protein 2 (BMP2) and Vascular Endothelial Growth Factor  $\alpha$  (VEGF- $\alpha$ ), thus providing superior quality as compared to the harvest of peripheral blood (8).

Most orthobiologic materials, in comparison with the current “gold standard” of autologous bone graft, contain not only osteoconductive (scaffold) and osteoinductive (signal protein) properties, but also osteogenic qualities (MSCs) (1). The osteogenic qualities of BMAs (MSCs) have been well documented and used to supplement orthobiologic substances containing only osteoinductive and/or osteoconductive properties (9-11). Moreover, MSCs secrete a multitude of cytokines and growth factors, affecting surrounding cells and matrix to regulate tissue regeneration (12). Several studies have reported the successful aspiration of BMAs at sites with a high concentration of red marrow, such as vertebral body, the iliac crest, and the proximal tibia (13). Reproducible concentrations of hMSCs were also obtained from both the proximal humerus and the distal femur during arthroscopy (14, 15). However, many podiatric surgeons are limited with regard to being able to harvest aspirates from these sites due to factors such as state laws, hospital restrictions, or other inhibitory factors. Although many podiatric surgeons may choose to harvest autografts from the calcaneus or even tibia, this is not a benign procedure and may be associated with surgical morbidity (16). Replacement of red bone marrow by yellow bone marrow during the normal aging process further diminishes the options for the podiatric surgeons (18).

The current study is an attempt to identify the presence or absence of multipotent MSCs in the calcaneal body using a currently accepted method of sampling. As per the guidelines of the International Society for Cellular Therapy (ISCT) (19), minimum criteria for documenting the presence of hMSCs must include the following: a) Plastic adherence when maintained in standard culture conditions, b) Presence or enhanced expression (+) of cell surface markers such as CD105, CD73, and CD90 and absence or suppressed expression (-) of CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA class II, and c) Ability to differentiate into osteoblasts, adipocytes, and chondrocytes in vitro. To our knowledge, no such study exists in this regard. Our hypothesis is that the calcaneus contains a viable population of multipotent MSCs capable of differentiating into osteoblasts, chondrocytes, and adipocytes. As this study was an evaluation of presence of MSCs, patients were excluded for a variety of factors in an attempt to sample a “healthy” patient population.

## Patients and Methods

### Study Population

The study was conducted under approved protocol #10-51 at Memorial Hospital of Rhode Island. Ten patients were selected for this prospective, case controlled study. Patients were excluded in an attempt to sample a healthy patient population. Criteria for selection included several factors, such as age, surgical and disease history, smoking and substance abuse. Patients selected were between the ages of 18 and 65. All surgeries were elective in nature and no history of recent trauma to the lower extremity was present in any of the cases. Patients with a history of systemic diseases such as Diabetes Mellitus (DM), Rheumatoid Arthritis (RA), chronic obstructive pulmonary disease (COPD), chronic kidney disease (CKD), end stage renal disease (ESRD), and cardiovascular diseases were all excluded from the study. Patients with documented presence of peripheral artery disease (PAD) and/or peripheral neuropathy were also excluded from the study. Patients were required to have quit smoking at least a year to be eligible for the study as well. Chronic use of corticosteroids or other immunosuppressant therapy was also a criterion for exclusion from the study. Smoking, alcohol abuse, and substance abuse were also considered disqualifying factors from the study.

### Surgical Technique

Patients were placed in a supine position. An ipsilateral bump was placed under the hip to ensure adequate exposure of the lateral wall of the calcaneus. While carefully identifying and avoiding the sural nerve and peroneal tendons, 52 cc of BMA was collected along with 8 cc of heparin using an 8G Jamshidi needle inserted percutaneously through the lateral wall of the calcaneal body with continuous retrograde force. The skin wound was then closed with a single 4-0 nylon simple suture. Patients were followed for up to 10 months.

### Isolation of human mesenchymal stromal cells (hMSCs)

hMSCs were isolated by Percoll density centrifugation followed by plastic adherence as previously described (20, 21). The entire aspirate volume was mixed 1:1 with phosphate buffered saline (PBS). The diluted bone marrow was subsequently layered over a Ficoll-paque density gradient medium (1.073 g/mL) and centrifuged at 400x g for 30 min to isolate the human mononuclear cells (hMNCs). The mononuclear cells were then washed twice with PBS and resuspended in hMSC growth medium. The growth medium consisted of minimum essential medium alpha (MEM- $\alpha$ ) as basal medium supplemented with 10% fetal bovine serum (FBS), antibiotics-antimycotics (100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 0.25  $\mu$ g/mL fungizone), and 0.1 mM nonessential amino acids (Invitrogen). Cells were counted using a hemocytometer and trypan blue exclusion method and finally seeded at a density of  $10 \times 10^3$  cells/cm<sup>2</sup> on tissue culture treated plastic (TCP). The cells were kept in an incubator at 37 °C, 5% CO<sub>2</sub> and 95% humidity for at least 24 h before testing plastic adherence by observation of the culture specimens under a microscope. Observation of adherent cells deemed the specimen positive for the first minimal criterion of MSCs.

## Flow Cytometry

Cells seeded on TCP were washed with PBS to remove non-adherent cells present in the cell culture. The adherent cells were then detached from TCP using 0.25% trypsin-EDTA. Cells were resuspended in Pharmingen Stain Buffer (BD Biosciences) and subsequently stained with the respective fluorescently conjugated antibody. Flow cytometric analysis was performed on a FACSCalibur (Becton Dickinson) while a minimum of 20,000 events was recorded for each sample. Analysis was performed using FlowJo (Tree Star) on dot plots to obtain a more accurate quantification as compared to histogram analysis. Each specimen was tested for the presence of both positive (CD105 (SH2/Endoglin), CD73 (SH3/5' nucleotidase), and CD90 (Thymocyte Differentiation Antigen 1, Thy-1)) and negative (CD34, CD45) hMSC markers.

## Differentiation assays

The potential of isolated cells to differentiate into mesenchymal lineages was evaluated using StemPro Osteogenesis Differentiation Kit, StemPro Chondrogenesis Differentiation Kit, and StemPro Adipogenesis Differentiation Kit (Invitrogen) according to the manufacturer's instructions. Control cultures were run in parallel during all differentiation assays where cells were cultured in growth media without supplemental differentiation factors.

For osteogenic differentiation, cells were seeded into 12-well plates at a density of 5,000 cells/cm<sup>2</sup> and incubated in growth medium for up to 4 days until cells reached confluency. The cells were then maintained in osteogenic medium for 21 days and medium was refreshed every 2 to 3 days. Cells that underwent osteogenesis were fixed with 4% formaldehyde and stained with 2% Alizarin red S solution (pH 4.2) for the detection of calcium accumulation.

Chondrogenic differentiation was induced in micromass culture. Micromass cultures were generated by seeding 5 µL droplets of cell suspension ( $1.6 \times 10^7$  cells/mL) in the center of 12-well plate wells. Cells were then cultivated for 2 hours and medium switched to chondrogenic medium. After 14 days of culture, cells were fixed with 4% formaldehyde and stained with 1% Alcian Blue for the detection of glycosaminoglycan in cartilage.

For adipogenic differentiation, cells were seeded into 12-well plates at a density of 10,000 cells/cm<sup>2</sup> and cultivated in growth medium until the cells reached confluency. The medium was then replaced with adipogenic medium and the cultures continued for 14 days. Cells that underwent adipogenesis were fixed with 4% formaldehyde and stained with oil red O to visualize lipid droplet formation.

## Results

Bone marrow aspirates were harvested from the calcaneus of the ten patients enrolled in the study. All patients were followed for an average of 4 months with a range of 2 months to 10 months of follow up. The suture was removed at approximately 2 weeks after bone marrow aspiration. All 10 patients remained non weight bearing (NWB) for at least 6 weeks with an average period of NWB being 12 weeks. A return to weight bearing (WB) status was

determined by healing factors in the primary procedure performed and was not influenced by the bone marrow aspirate. No evidence of any post-operative complications related to the harvest were noted including, but not limited to sural nerve injury, peroneal tendon injury, or calcaneal fracture. The described technique for harvesting BMAs from human calcaneus in the current study was safe, as demonstrated in other studies (17, 22, 23).

We next sought to identify the presence of hMSCs in BMAs from human calcaneus. We followed the guidelines set by the International Society for Cellular Therapy (ISCT) (19), to document the presence of hMSCs. To isolate hMSCs from calcaneus BMA, hMNCs were first isolated from BMA by density gradient centrifugation. The collected fraction containing hMNCs were then subjected to plastic adherence and attached cells grew into a confluent cell layer. All 10 specimens were observed to adhere to plastic under standard culture conditions. Figure 1 shows plastic adherence of a representative specimen 110211a at 16 days in culture. By this standard the requirement of plastic adherence was met, as all 10 were reported as positive.

Flow cytometric analysis was performed to study the expression of MSC surface markers. A subpopulation in the scatter plot was selected from the total population that was over 90% CD105 positive in all populations to exclude the dominant hematopoietic cell population and therefore also reducing variability between specimens (Fig. 2). This population was analyzed and percentages of cells were quantified using both the hMSC positive (CD73, CD90, CD105) as well as negative (CD34, and CD45) markers (Table 1). The exclusion marker CD34 was noted to be low in general and the CD45 marker substantially decreased over time. The inclusion markers CD73 and CD90, while initially only measured at about 10% of short-term cultures, were all found to be present in 100% of cells in long-term cultures. The positive marker CD105 was noted to be approximately 90% in all specimens taken, which was expected due to the subset being based on CD105<sup>+</sup> cells.

Finally, the potential of isolated hMSCs to differentiate into cells of mesenchymal lineages was evaluated. hMSCs isolated from all specimens had the ability to proliferate extensively and were capable of differentiating into osteoblasts, chondrocytes, and adipocytes with respective differentiation cues. Osteogenic differentiation was indicated by the alizarin red staining of calcium deposition at day 21 (Fig. 3A and D). Chondrogenic differentiation was indicated by the alcian blue staining of the sulfated proteoglycans deposits at day 14 (Fig. 3B and E). Oil red O staining at day 14 indicated the accumulation of lipid droplets during adipogenic differentiation (Fig. 3C and F). The multilineage differentiation potential varied among patients.

## Discussion

The purpose of this study was to identify the presence of multipotent hMSCs in BMAs harvested from human calcaneus. In the continually evolving field of orthobiologics, the use of autologous BMA on its own or coupled with a carrier is emerging as a viable alternative to the use of autologous bone graft in the treatment of a variety of lower extremity pathologies (24). A variety of factors currently limit the podiatric foot and ankle surgeon in terms of ideal locations for harvest. This study was an attempt to present a reasonable

alternative to the previously documented harvest sites including the iliac crest, vertebral body, proximal tibia, and distal femur using a defined method of determining the presence of hMSCs. While there is no such thing as a “benign” surgical procedure, harvest of BMA from the calcaneus has been reported to have a relatively low risk of associated morbidity (17). Moreover, calcaneal bone marrow aspirate used in conjunction with carriers demonstrated enhanced success (25). Previous study identified the presence of osteoblastic progenitor cells in BMA from human calcaneus (26). However, definitive proof of the actual presence of multipotent hMSCs in BMA from human calcaneus has been lacking. To our knowledge, this study represents the first isolation, characterization and *in vitro* differentiation of hMSCs in BMAs harvested from human calcaneus. All 10 specimens collected from healthy donors were noted to be what is defined as “positive” for hMSCs according to the currently accepted standard of identification defined by the ISCT (19). Since the study was limited to two results (positive or negative for hMSCs), a score of 10/10 “positives” is statistically significant, indicating that BMA from calcaneus is a reliable source for hMSCs.

In the current study, we showed that hMSCs isolated from human calcaneus have high capacity of self-renewal and osteogenic differentiation, suggesting their potential use in bone regeneration. Due to the limited number of MSCs in unprocessed BMA, in order to gain the maximum potential the BMA can be further processed to concentrate MSCs or the isolated hMSCs be expanded *ex vivo* before implantation (7, 27). The *ex vivo* expanded MSCs and bone marrow aspirate concentrate (BMAC) can provide additional benefits as they both are rich sources of growth factors/cytokine/chemokines (28, 29). Further studies to evaluate the secretome of the *ex vivo* expanded calcaneal hMSCs and the growth factor/cytokine/chemokine profile in BMAC from human calcaneus will be needed.

Whether the BMA harvested from human calcaneus is as potent as that from the iliac crest and vertebral body is yet to be determined. Advanced analysis would be needed to identify the concentration of hMSCs in each aspirate and then compared to previously reported concentrations from other sites. One would expect a lower hMSC concentration in calcaneal BMA and this would require a higher volume for application. Previous reports suggest that BMA collected from the iliac crest had a higher mean concentration of osteoblastic progenitor cells compared with the calcaneus (26). Moreover, it is necessary to compare the osteogenic potential of BMAs from calcaneus to that of aspirates from other sites.

One limitation to this study is the relatively homogenous population studied. As a preliminary attempt to identify presence of hMSCs in BMAs from human calcaneus, patients with factors known to suppress regeneration were excluded. Future study is warranted to assess those factors as potential predictors of BMA quality.

In conclusion, the aspirates collected from the calcaneus contain a healthy population of cells which are able to adhere to plastic under standard culture conditions, showed the presence or enhanced expression (+) of cell surface markers such as CD105, CD73, and CD90 and absence or suppressed expression (–) of CD45, CD34; and showed the ability to differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro*. Based on the guidelines set by the ISCT, we infer that multipotent hMSCs were successfully isolated from bone

marrow aspirate collected from human calcaneus. They maintained the ability to proliferate and differentiate into cells of mesenchymal lineage. The bone marrow aspirate from the calcaneal body offers a reliable, healthy source of multipotent mesenchymal stromal cells.

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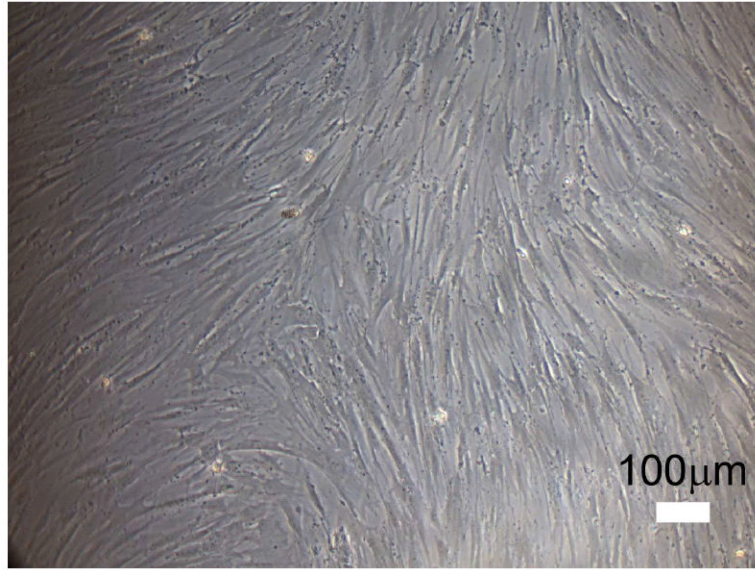
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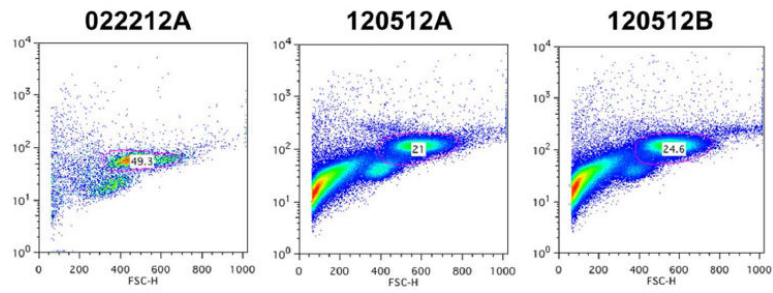
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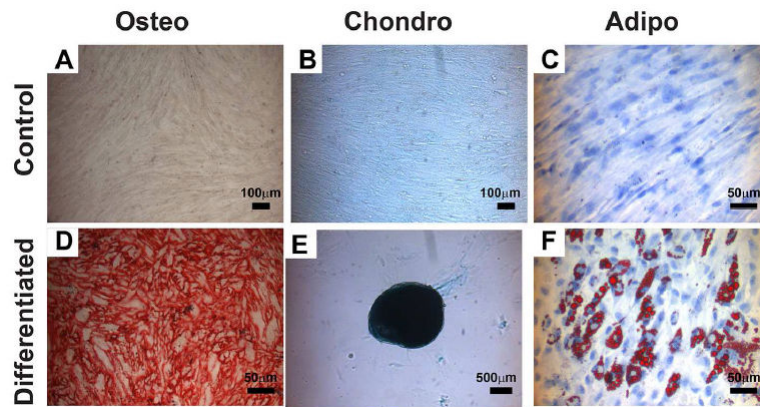




**Figure 1.** Stem cell morphology at day 16 (110211A). Cells attached to plastic and grew into a confluent cell layer.



**Figure 2.** Representative flow cytometry analysis of stem cell marker CD105. The gated subpopulations (pink circles, 49.3% in 022212A, 21% in 120512A, and 24.6% in 120512B) were selected for further evaluation of the expression of CD90, CD73, CD34 and CD45.



**Figure 3.** Differentiation of isolated hMSCs into mesenchymal lineages (032013A). Cells were shown to differentiate to osteogenic (Osteo), Chondro (chondrogenic) and adipo (adipogenic) lineages. Alizarin red staining indicated the calcium deposition during osteogenic differentiation (A and D). Alcian blue staining indicated the sulfated proteoglycans deposits during chondrogenic differentiation (B and E). Oil red-O staining indicated the accumulation of lipid droplets during adipogenic differentiation (C and F).

**Table**

Flow cytometric analysis of surface marker expression on isolated hMSCs

ID	Culture time (in days)	Flow Cytometry				
		CD34 (%)	CD45 (%)	CD73 (%)	CD90 (%)	CD105 (%)
110211A *	1	7.20	4.06	6.57	85.30	95.20
120512A	1	0.34	47.80	6.48	8.22	91.90
120512B	1	0.33	39.70	26.30	5.90	95.20
022212A	2	0.72	11.60	38.10	94.90	94.10
022212B	2	5.44	11.90	13.60	62.60	82.60
032112A	2	1.63	22.10	4.79	9.26	84.90
032013A	2	4.87	0.00	7.33	2.53	90.30
111711A	21	0.13	0.81	99.90	99.20	99.70
111711B	21	0.09	0.28	100.0	98.10	97.80
110211A *	28	1.02	0.40	99.60	100.00	99.60

\* Flow Cytometry performed before culturing (day 1) and on day 28 of culture