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Femoral Head Bone vs Acetabular Subchondral Bone: Selecting the Optimal Anatomical Site to Obtain Mesenchymal Stromal Cells from Human Bone Marrow for Regenerative Medicine

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Abstract

Background: Mesenchymal Stromal Cells (MSC) have a great importance for the field of regenerative medicine. However, there is high variability in existing protocols for MSC *in vitro* expansion, which can lead to low reproducibility of pre-clinical studies and, even more critically, the reduced safety of patients undergoing clinical trials. Although bone marrow is one of the most important sources for the isolation and *in vitro* culture of MSC, the preferred anatomical location for obtaining bone marrow is often unclear, and this information is relevant for the interpretation of results obtained from preclinical and clinical trials.

Methods: In this study, we compared various biological characteristics of human MSC obtained from five total hip replacement surgery donors isolated from the bone marrow of two different anatomical sites: the femoral head bone (Fh) and the acetabular subchondral bone (Ac). Using the same surgical technique and collection volume, we compared the morphological characteristics, fibroblast colony forming unit (CFU-F) capacity, immunophenotype, capacity for differentiation (osteogenesis, condrogenesis, and adipogenesis) and population doubling time (PDT) of MSC isolated from these distinct anatomical locations.

Results: Here, we show that human MSC isolated from Fh have improved morphological and proliferative characteristics that are associated with higher *in vitro* efficiency for regenerative medicine protocols compared with cells obtained from Ac.

Conclusion: This report provides information regarding the importance of establishing a "standard" anatomical site for obtaining bone marrow as one of the most important requirements of the "pre-analytical" phase of MSC *in vitro* expansion for regenerative medicine.

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Introduction

For several years, Human Bone Marrow (hBM) has been an important source of Mesenchymal Stromal Cells (MSC) for use in tissue engineering and regenerative medicine [1-5]. Although hBM is one of the most important sources for these cells, recent studies have demonstrated that the MSC population is very heterogeneous, such that its capacity for proliferation and differentiation can vary significantly even if cells are isolated from the same tissue [1,6,7]. In addition to the intrinsic heterogenicity of this cell population, biological variability among different donors may affect the reproducibility of pre-clinical test results and patient safety in clinical trials. For these reasons, it is very important to define guidelines for the "pre-analytical" phase of the *in vitro* expansion of MSC as part of the process to guarantee the quality of cell expansion and validity of results related to regenerative medicine protocols. This short report compares the biological characteristics of MSC isolated from two different regions of hBM, namely, the Femoral Head Bone (Fh) and acetabular subchondral bone (Ac) of donor patients exposed to prosthetic hip replacement surgery, in order to contribute to the current knowledge regarding the pre-analytical conditions of MSC *in vitro* expansion.

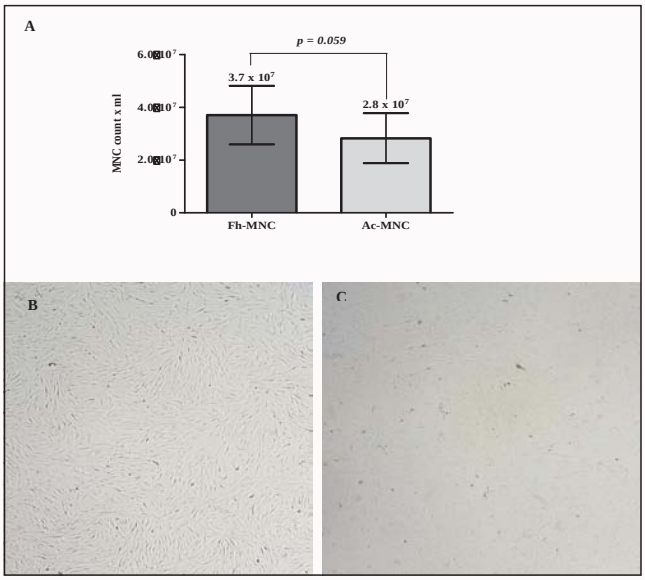

Figure 1: Mononuclear cells count (Fh vs. Ac). A) Higher CMN count was observed in the sample obtained from Fh. B) Cells with adherent fibroblastoid morphology isolated from Fh. C) Adherent fibroblastoid cells isolated from Ac.

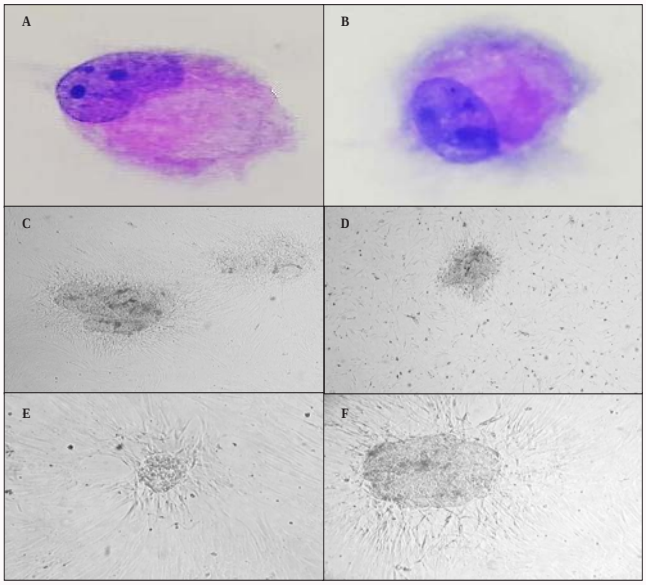

Figure 2: Morphological characteristics and colonies obtained from MSC. A) MSC isolated from Fh, prominent nucleoli are observed in the nucleus. B) MSC isolated from Ac. C-F) Colonies obtained from MSCs isolated from Fh.

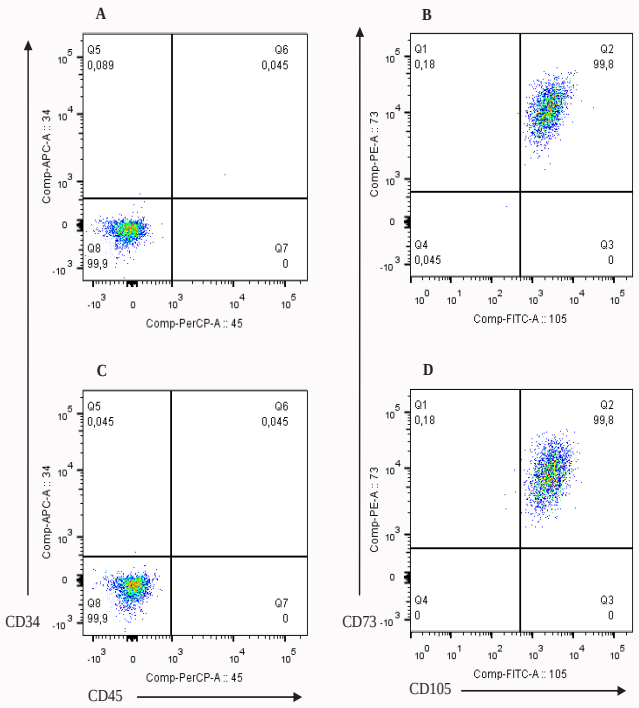

Figure 3: Determination of the MSC immunophenotype (Fh vs. Ac). (A-B) Fh MSC. (C-D) Ac MSC. No differences were observed in the expression of mesenchymal antigens (CD105, CD73) or hematopoietic antigens (CD34, CD45).

Materials and Methods

Five hBM samples were collected from five patients during total hip replacement surgery, which originated from the Femoral Head (Fh) and acetabular subchondral bones (Ac), simultaneously in each donor. Samples were collected by orthopedic surgeons of Hospital Universitario San Ignacio (Bogotá, Colombia South América) using an acetabular reamer, in accordance with the recommendations of the ethics committees of Science Faculty of Javeriana University and Hospital Universitario San Ignacio. Each hBM sample was collected in a sterile tube containing ethylene diaminetetraacetic acid (EDTA) anticoagulant, and mononuclear cells were isolated by density gradient centrifugation (Histopaque d = 1.077 g/cm^3, Sigma-Aldrich, USA). Mononuclear cells were plated at a density of 1.6x10^5 cells/cm^2 in IMDM Glutamax-I (GIBCO, Invitrogen) according to previously published protocols [8,9]. MSC between 3 and 5 cell passages were used for allexperiments. According to ISCT recommendations for MSC characterization [10], morphological characteristics and the formation of fibroblastoid colonies (CFU-F)of Fh and Ac MSC were evaluated by cytospin (Hematoxylin & Eosin, CX21 Olympus® 100X) and inverted microscopy (CKX31 Olympus® 4X), respectively. For phenotypic analysis, adherent cells were trypsinized and labeled with combinations of monoclonal antibodies specific for CD34 (clone AC136, Miltenyi Biotec®), CD45 (clone 5B1, Miltenyi Biotec®), CD73 (clone AD2, Miltenyi Biotec®), and CD105 (clone 43A4E1, Miltenyi Biotec®). Data were acquired using a FACS ARIA-II flow cytometer (BD Biosciences®). DIVA and FlowJo software (BD Biosciences®) was used for data analysis. Osteogenic, adipogenic, and chondrogenic differentiation capacities were qualitatively determined according to previously published protocols [8,9]. Population doubling time (PDT) [11,12] was measured in hours, as calculated by the following equation: PDT =(T -T0) x $\log_2$ /(log N - log N0), where (T-T0) is the experimental incubation period (hours), N is the number of harvested cells, and N0 is the number of cells seeded. All assays were performed in triplicate. The following statistical tests were conducted using IBM SPSS v24 ™ software: Shapiro-Wilk test to compare data normality; Levene's test to analyze homogeneity of variance; T and Wilcoxon tests for comparison of means. A 95% confidence interval was defined for a significant p value ($p < 0.05$).

Results

A higher mononuclear cells (MNC) count was observed in the Fh sample compared to Ac (Figure 1A). In addition, during *in vitro* expansion a higher confluence was obtained from MSC isolated

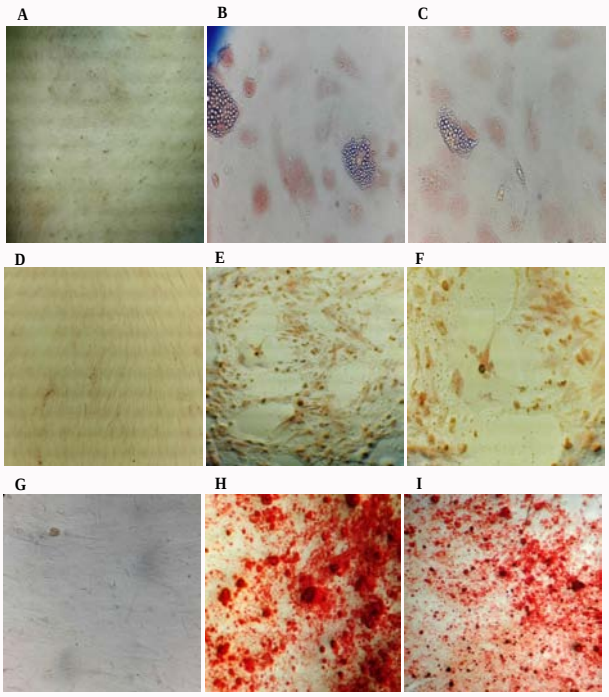

Figure 4: MSC differentiation capability (Fh vs. Ac). A) Negative control adipogenic differentiation of MSC (x10). B) Adipogenic differentiation from MSC of Fh (staining red oil) (x40). C) Adipogenic differentiation from MSC of Ac (staining red oil) (x40). D) Negative control osteogenic differentiation of MSC (x10). E) Osteogenic differentiation from MSC of Fh (von Kossa stain) (x10). F) Osteogenic differentiation from MSC of Ac (von Kossa stain) (x10). G) Negative control of chondrogenic differentiation (x10). H) Chondrogenic differentiation from MSC of Fh (Safranin stain) (x10). I) Chondrogenic differentiation from MSC of Ac (Safranin stain) (x10).

from Fh (Figure 1B and 1C). Importantly, the same volume of bone marrow from each anatomical donor site was collected and processed. MSC from Fh showed prominent nucleoli and a greater generation of CFU-F (Figure 2A, Figure 2C-2F) in comparison to MSC obtained from Ac, which had less prominent nucleoli and weaker CFU-F capacity (Figure 2B). MSC phenotypic analysis revealed no significant differences in the expression of antigens (CD34, CD45, CD73, and CD105) between MSC isolated from Fh or Ac (Figure 3). The multiline age capacity (differentiation into chondrocytes, adipocytes, and osteocytes) of MSC isolated from the two anatomical sites was comparable under the same experimental conditions (Figure 4). With regard to population doubling time (PDT), MSC obtained from Fh had a shorter doubling time (9.97 h) than MSC isolated from Ac (13.83 h) ($p<0.05$) (Figure 5).

Discussion

There is a fundamental need for defining a particular anatomic site for the isolation of MSC in order to meet the needs and standards of regenerative medicine. This short report demonstrates that it is important to consider the place of extraction of human bone marrow to obtain MSC with favorable biological characteristics and establish standard protocols for cellular therapy. We observed particular differences between samples collected from Fhand Ac in terms of: i) the number of MNC obtained, ii) cellular morphology and CFU-F capacity and iii) PDT. Fh is located underneath highly vascularized spongy bone with intratrabecular spaces occupied by abundant bone marrow, whereas Ac samples were collected from subchondral

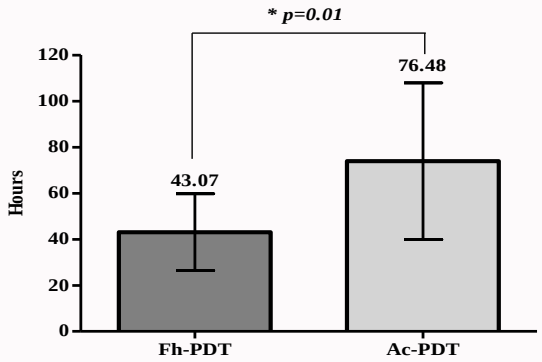

Figure 5: Population Doubling Time (Fh vs. Ac). MSCs isolated from Fh need less time to double their population compared to MSCs obtained from Ac ($p < 0,05$).

bone that has a laminar and poorly vascularized structure. These histological characteristics can influence the cellularity of the sample, which likely resulted in a greater number of MNC collected from Fh (Figure 1A).

Previous studies indicate that the ability to form fibroblast colonies is dependent on MSC *in vitro* expansion conditions [13-15] or external factors [16]. However, we observed a greater ability to generate CFU-F from MSC of Fh cultivated under the same experimental conditions as MSC from Ac, demonstrating that there are intrinsic MSC characteristics that result in a greater or lesser capacity to generate CFU-F (Figure 2C-2F). This finding is consistent with the results of Cox G et al., which demonstrate a greater ability to produce CFU-F from MSC isolated from long bone fatty bone marrow compared to those obtained from iliac crest aspirate. In addition, the same study revealed that this cell population contains a higher number of $CD45^{-/low}$ CD271+ MSC with greater capacity formultipotency [17].

Here, we show that MSC from Fh have prominent nucleoli, which are absent in MSC obtained from Ac. Importantly, this cellular structure is found in cells with high ribosomal activity, proliferation, and cell cycle regulation [18], so this result could be related to the shorter PDT observed in MSC obtained from Fh. Because a shorter PDT is associated with a greater proliferative capacity, MSC isolated from Fh can proliferate faster, which is an advantage for the development of cell therapy protocols (Figure 3).

In summary, in this report, we show that MSC isolated from Fh have biological characteristics that could favor their use for the efficient development of protocols for cell therapy or pre-clinical trials. In conclusion, in order to decrease the variability of human MSC *in vitro* expansion conditions, the femoral head bone should be considered as the anatomical site of choice for isolating these cells from human bone marrow.

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