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# The aggregate nature of human mesenchymal stromal cells in native bone marrow

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**Abstract**

*Background aims.* The clinical application of mesenchymal stromal cells (MSC) faces several obstacles, such as the lack of a standard method for direct isolation as well as a low frequency and concern about the safety of their *in vitro* expansion. Although the density-gradient separation technique is used as the first step in most methods of MSC isolation to enrich mononuclear cells, the efficiency of this method has not so far been examined. This study was designed to address this issue. *Methods.* Human bone marrow (BM) samples were laid over Ficoll–Paque, and after centrifugation the upper and lower fractions were cultured separately. Surface markers, differentiation potential and the number of emerged cells were determined. *Results.* The isolated cells from both the upper and lower fractions were characteristic of MSC. Although it is commonly believed that MSC are single suspending mononuclear cells and so are enriched in the upper fraction of Ficoll–Paque after density-gradient separation, our data showed that considerable numbers of these cells were accumulated in the lower fraction. Further data indicated that MSC were actually present as cell aggregates in BM and they could be enriched effectively by a simple filtration method. *Conclusions.* The aggregate nature of MSC in BM is in agreement with the concept that they are one of the main elements of the hematopoietic stem cell niche. In addition, the simple filtration method proposed here to isolate cell aggregates may provide opportunities for instant stem cell therapy without the need for extensive *in vitro* expansion.

**Key Words:** *bone marrow, cell isolation, density-gradient separation, human mesenchymal stromal cells, stem cell therapy*

## Introduction

Mesenchymal stromal cells (MSC) are considered to be a promising tool for regenerative medicine (1,2) because of their unique characteristics such as easy isolation, high expansion potential and multi-lineage differentiation capacity (3). Although these cells have been shown to be of therapeutic value for several disorders in animal studies, their application to humans has not been widely standardized. The clinical use of MSC is hampered partly by the lack of a standard method for direct isolation, the low frequency of these cells and concerns about their expansion and safety for cell-based therapies (4,5). Therefore these problems have to be solved before their routine clinical use.

The isolation of MSC is usually conducted in two steps. First, density-gradient separation is used to reduce the volume of the samples and enrich mononuclear cells (MNC). Second, a purification step is used to isolate the MSC from the other MNC. Although different methods of purification, such as negative and positive selection, have been

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introduced (6–8), MSC are now routinely purified based on their plastic-adherence and proliferation capacity. Unfortunately, there are several concerns regarding the *in vitro* expansion of these cells, as several natural characteristics of these cells, such as the differentiation potential and chromosome composition, are modified during the process (9). Contamination with pathogens or other cell lines are among the other risks for the long-term culture of these cells (10). Thus the development of new methods for the direct isolation of a sufficient amount of MSC would certainly help in overcoming the obstacles currently faced by stem cell therapy.

Bone marrow (BM) is the most commonly used source of stem cells for clinical applications (5,11–13). To achieve the goal of isolation of a sufficient amount of MSC, the question of whether MSC actually represent a small fraction of BM, or the currently available methods are not efficient enough to isolate a significant proportion of these cells, needs be addressed. Although different studies have focused on increasing the efficiency of the purification step of MSC isolation (14,15) or increasing the proliferation rate of MSC through modification of the culture conditions (16–20), to the best of our knowledge no study so far has assessed the efficiency of the density-gradient separation step. The aim of this study was to examine the effectiveness of this method for the enrichment of MSC.

We report that a great proportion of MSC is lost during the density-gradient centrifugation step because of the aggregate nature of these cells. An alternative method for MSC isolation is suggested.

## Methods

### *Human samples*

BM samples were obtained from healthy male and female donors (15–55 years old) by aspiration from the posterior iliac crest. All BM samples were collected after informed consent was obtained. All procedures were carried out in accordance with the Stem Cell Technology Research Center (Tehran, Iran) guidelines. Ethical approval was obtained from the Ethics Committee of Stem Cell Technology Research Center.

### *Density-gradient separation technique*

Ten to twenty milliliters of heparinized human BM samples ($n = 10$) were diluted 1:1 (v/v) with phosphate-buffered saline plus Ethylenediaminetetraacetic acid (EDTA) (PBS/EDTA; GIBCO-BRL, Grand Island, NY, USA) and carefully laid over Ficoll–Paque (Healthcare Bio-sciences AB, Uppsala, Sweden) at a ratio of 10:3 (v/v) in 15-mL conical tubes. After centrifugation (20 min, 600 ***g***), the upper fraction containing the MNC layer was collected and washed with PBS. The lower fraction (cell pellet) was moved to a new tube and treated with red blood cell (RBC) lysis buffer (Sigma-Aldrich, St Louis, MO, USA). Both fractions were used for *in vitro* culture as described below.

### *Isolation of MSC with the filtration technique*

Ten to twenty milliliters of each BM sample ($n = 10$) were diluted 1:1 (v/v) with PBS/EDTA and filtered through a *c.* 20-μm strainer (a double-layered 40-μm nylon mesh; BD Biosciences, San Jose, CA, USA). The residue was cultured in complete medium as described below. In addition, the filtrate was treated with RBC lysis buffer and cultured.

### *Assessment of the amount of MSC-rich aggregates in BM collection bags*

BM collection bags ($n = 15$) that had been used for BM transplantation were received from the Shariati Hospital BM Transplantation Center (Tehran, Iran) and analyzed for the amount of residue that did not pass the 170-μm pore size filter of each bag. The volume of the residue was normalized to the total volume of the BM sample. Then the residue was cultured and assessed for the existence of MSC. To rule out the possibility that the residue was composed of cell aggregates and not bone fragments, after 24 h of culture colonies derived from the residue were stained with Alizarin Red. The number of MSC obtained after a 10-day culture was calculated for five bags.

### *Cell culture*

After isolation, the cells were suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin G (100 U/mL) and streptomycin (100 μg/mL), as well as 0.25 μg/mL amphotericin B (all from GIBCO-BRL), and cultured in 75-$cm^2$ culture flasks at 37°C and 5% $CO_2$. After 24 h, non-adherent cells were washed out and a fresh medium was added. The medium was changed every 3 days. The seeded cells were detached with trypsin and replated upon reaching 30–40% confluence in the first passage and 60–80% confluence in the following passages. The cells were observed immediately after seeding and then every day thereafter for 10 days using a light microscope. On day 10, the adherent layer was trypsinized, collected and the number of MSC determined using a Neubauer hemocytometer. The cells were also cultured for 2–3 months to assess the proliferation capacity.