



An improved methodology for efficient isolation of mesenchymal stem cells from Caprine bone marrow

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Mesenchymal stem cells (MSCs), the multipotent stem cells reside in small number in many adult tissue and organ, and display the characteristics of self-renovation and trans-differentiation to a variety of other cell types such as cardiomyocytes, adipocytes, chondrocytes, osteoblasts, and neurons (Ullah *et al.* 2015). Bone marrow (BM) is the most common source of MSCs.

The density-gradient centrifugation is the commonly used method for isolation and enrichment of MSCs from BM (Pierini *et al.* 2012). However, due to the amount of histopaque used in the established protocol (3:4) and the cost of histopaque, the development of an easy and effective protocol for isolation of MSCs from large quantity of caprine BM is needed. Moreover, in farm animals, certain problems such as lack of standardized isolation methods and need to derive MSCs from large quantity of BM often result into longer duration and lower yield of the cells and thus hamper their clinical applications. However, a higher cell yield can be obtained by using a customized histopaque protocol for depletion of unwanted constituents such as erythrocytes and granulocytes. The concept behind modified method was to manage the large amount of BM with standard ratio of histopaque without affecting results. The ratio of bone marrow to histopaque required for isolation of MSCs is 3:4 ratios. Therefore, a large quantity of histopaque is needed to isolate MSCs from large animals as volume of BM collected varies from 20 to 40 ml. Besides this, the high cost of histopaque for isolation of MSCs from large animals encouraged us to develop a new modified method. Therefore, the aim of this study was to present the quick and efficient protocol for BM MSCs extraction using a modified density gradient centrifugation method.

Goat was deprived of food and water for 24 h before BM collection and put under deep sedation anesthesia

(Kharche *et al.* 2014) using xylazine (0.2 mg/kg body weight) and ketamine (4.4–6.6 mg/kg body weight).

[*Statement of ethics:* Ethical approval was taken for the present study for the IAEC and CPCSEA, New Delhi. The guidelines of the Institutional Ethical Committee/CPCSEA were followed for obtaining and handling of the biological samples.]

Bone marrow-derived MSCs were obtained from the iliac crest by biopsy from anesthetized goats. Once sedation developed, animals were restrained in lateral recumbency with surgical site on top; 2 mL of 2% lignocaine hydrochloride (Lox2%™) was infiltrated on the iliac crest in a fan like fashion to desensitize the portion from skin to periosteum. After that a stab incision of 1 cm was given on iliac crest, through which a sterile bone aspiration needle (16 G) was inserted. After achieving sufficient depth of the needle tip in the spongy/cancellous bone, the stylet was removed. Bone marrow was aspirated to a syringe filled with anticoagulant, ethylenediaminetetra-acetic acid (4 mg/ml EDTA, Sigma-Aldrich, Saint Louis MI/USA). At the culmination of collection procedure, the puncture area was disinfected with betadine (Nicodine™) followed by antibiotic for prevention and prophylactic action.

After collection of BM aspirate, isolation of caprine MSC was achieved by Histopaque-1077 (Sigma Aldrich, Saint Louis MI/USA) density gradient centrifugation. Based on the amount of blood and histopaque used, 7 groups were formed and compared.

Group 1 (control): In this group, 4 ml blood was carefully loaded on histopaque (3:4 ratios) in pre-sterilized 15 ml centrifuge tube followed by centrifugation at 2,500 rpm for 30 min at room temperature.

Group 2 (8 ml): In this group, 8 ml blood was centrifuged at 5,000 rpm for 10 min. Pellet obtained was dissolved in 4 ml PBS and re-suspended cells were carefully loaded on histopaque (3:4 ratios) in pre-sterilized 15 ml centrifuge tube followed by centrifugation at 2,500 rpm for 30 min at room temperature.

Group 3 (12 ml): In this group, 12 ml blood was centrifuged at 5,000 rpm for 10 min. Pellet obtained was

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dissolved in 4 ml PBS and re-suspended cells were carefully loaded on histopaque (3:4 ratios) in pre-sterilized 15 ml centrifuge tube followed by centrifugation at 2,500 rpm for 30 min at room temperature.

Group 4 (16 ml): In this group, 16 ml blood was centrifuged at 5,000 rpm for 10 min. Pellet obtained was dissolved in 4 ml PBS and re-suspended cells were carefully loaded on histopaque (3:4 ratios) in pre-sterilized 15 ml centrifuge tube followed by centrifugation at 2,500 rpm for 30 min at room temperature.

Group 5 (18 ml): In this group, 18 ml blood was centrifuged at 5,000 rpm for 10 min. Pellet obtained was dissolved in 4 ml PBS and re-suspended cells were carefully loaded on histopaque (3:4 ratios) in pre-sterilized 15 ml centrifuge tube followed by centrifugation at 2,500 rpm for 30 min at room temperature.

Group 6 (20 ml): In this group, 20 ml blood was centrifuged at 5,000 rpm for 10 min. Pellet obtained was dissolved in 4 ml PBS and re-suspended cells were carefully loaded on histopaque (3:4 ratios) in pre-sterilized 15 ml centrifuge tube followed by centrifugation at 2,500 rpm for 30 min at room temperature.

Group 7 (22 ml): In this group, 22 ml blood was centrifuged at 5,000 rpm for 10 min. Pellet obtained was dissolved in 4 ml PBS and re-suspended cells were carefully loaded on histopaque (3:4 ratios) in pre-sterilized 15 ml centrifuge tube followed by centrifugation at 2,500 rpm for 30 min at room temperature.

The modified methods (Gr 2 to 7) were compared with conventional method (Control Gr) so as to standardize the isolation method from large animals. For this, the cells were obtained by centrifugation over Histopaque-1077 (Sigma Aldrich, Saint Louis MI/USA). Mononuclear cells were recovered and washed twice with PBS. Finally, mononuclear cells were re-suspended in DMEM (Gibco, Thermo Fisher, USA) containing 15% FBS, 1% L-glutamine, 1% non-essential amino acids, 50 µg/ml gentamycin, 10 µg/ml antimycotic (Sigma Aldrich, Saint Louis MI/USA). Cells were seeded onto tissue culture flasks and expanded at 37°C and 5% CO₂. Following 48 h of culture the non-adherent cells were removed. Adhered cells were passaged at 80–90% confluence by trypsinization (0.25% trypsin solution) and seeded to a new culture at a density of 5,000 cells/cm². Culture medium was changed every 3–4 days.

Ten µl of the cells obtained in culture were diluted in 50 µl Trypan Blue (Sigma Aldrich, Saint Louis MI/USA). The solution was mixed properly by pipetting several time up and down and counted by automatic cell counter (Countess II FL Automated Cell Counter; Thermo Fisher Scientific) for total cell number.

For characterization of MSCs through ALP staining, the medium was removed from the MSC cultures, which were washed twice with DPBS. The monolayer was fixed with citrate-acetone-formaldehyde fixative solution for 1 min. After fixation, the monolayer was washed 3 times with deionized water for 1 min and incubated for 15 min at RT

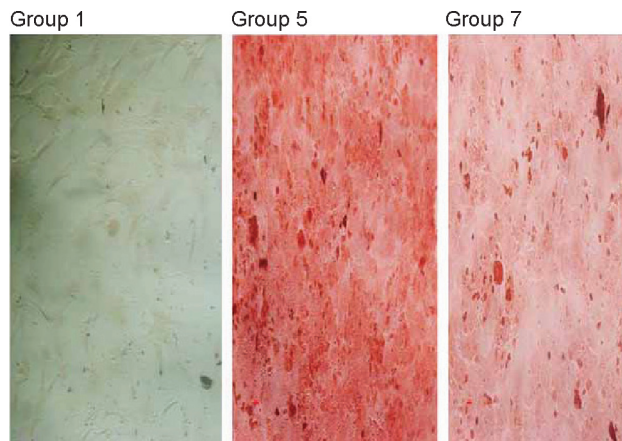


Fig. 1. Alkaline phosphatase staining image of group 1, group 5 and group 7.

with the alkaline dye. The monolayer was rinsed again 2–3 times with deionized water and counter stained with neutral red stain for 1–2 min. Finally, monolayer was washed several times to remove the extra neutral red stain and the response of the monolayer to ALP staining was observed (Fig. 1) under a bright field microscope (Nikon Instruments, Inc., Eclipse, TE 2000 U, Melville, NY, USA).

For modified method, bone marrow blood collected was randomly divided into 7 groups (Gr), viz. Gr 1 (control), Gr 2 (8 ml), Gr 3 (12 ml), Gr 4 (16 ml), Gr 5 (18 ml), Gr 6 (20 ml) and Gr 7 (22 ml). The numbers of cells isolated in these groups were 4.47×10^6 , 8.07×10^6 , 22.8×10^6 , 49.12×10^6 , 87.54×10^6 , 66.57×10^6 and 60.58×10^6 , respectively (Fig. 2). Therefore, it was observed that the maximum BM MSCs with minimum histopaque can be achieved in Gr 5 (18 ml) and thereafter the recovery of cells declined with increasing volume of blood. Similarly, the number of cells (cells/ml) attached in P0 was also observed and it was found that number of cells attached in Gr 1, Gr 2, Gr 3, Gr 4, Gr 5, Gr 6 and Gr 7 were 1.11×10^6 , 1.00×10^6 , 1.9×10^6 , 3.07×10^6 , 4.86×10^6 , 3.32×10^6 and 3.02×10^6 , respectively (Fig 2). Overall, 2.6% cells got attached to surface showing a less population of MSCs in bone marrow blood. Thus, this highest number of attached cells was observed in Gr 5, i.e. 4.4 folds higher compared with the Gr 1.

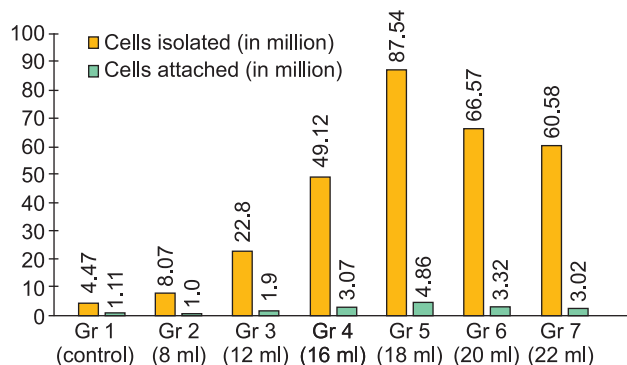


Fig. 2. The number of isolated and attached BMMSCs in different groups of experiment as expressed in millions.

The expression of ALP is one of the primary tests to check the actively proliferating cells undifferentiated cells. The goat MSCs established in present study, subjected to the ALP staining, stained red, indicating that a high expression of ALP (Fig. 1).

In the present study, we demonstrated a simplified method for efficient use of histopaque to isolate maximum number of MSCs from caprine bone marrow. For this, seven independent groups have been compared to obtain the minimum quantity of histopaque that can isolate MSCs from maximum amount of BM collected from the goats. For isolation and enrichment of the BM MSCs, the property of the MSCs to adhere plastic surfaces was explored. It is described that the adherence to the plastic surface is one of the well-known properties of MSC (Dominici *et al.* 2006) and thus BM-MSCs are usually purified through their physical adherence to the surface of cell culture plate (Nadri *et al.* 2007). Moreover, as per the guidelines of 'Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy', adherence to the plastic surface in standard culture conditions is one of the criteria to define MSCs. Therefore, in our study this property of the MSCs was used for screening of the MSCs from the pool of the cells isolated from the bone marrow of the goats.

Besides adherence to the plastic surfaces, expression of ALP is also an important characteristic of MSCs (Hanna *et al.* 2018). The BM MSCs we isolated from caprine BM aspirate and cultured showed high expression of ALP.

In conclusion, this study revealed that the total number of BM MSCs were 4.4 folds higher in Gr 5 than in control Gr, suggesting that 18 ml BM aspirate is preferable for recovery of highest number of MSCs using 3 ml of histopaque and modified protocol. Thus, we explained a simple and modified protocol for isolation of more number of BM MSCs from goats BM aspirate using minimal quantity of histopaque. This provides an efficient method that can be further applied in the field of cell biology and tissue engineering.

SUMMARY

The aim of this study was to investigate the effect of increasing volume of bone marrow (BM) aspirate of goats on yield of mesenchymal stem cells (MSCs). For this, yield of MSCs in seven groups (Gr; 1–7 Grs, including a control Gr) were compared. After isolation and enrichment of BM

MSCs with density gradient centrifugation using increasing volume of Histopaque-1077, the properties of MSCs to adhere the plastic surfaces and expression of alkaline phosphatase (ALP) were used for the identification of MSCs. The results indicated that 18 ml of BM aspirate (Gr 5) provided maximum number of MSCs using 3 ml of histopaque compared to the control and other Gr. The yield in Gr 5 was about 4.4 folds higher compared to the control Gr. In conclusion, these results demonstrated that in a single procedure the modified protocol yield significantly higher number of caprine MSCs from goat BM aspirate compared to the standard protocol. Thus, it can be used for isolation of more number of MSCs from a large quantity of bone marrow with least volume of consumables required for density-gradient centrifugation.

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