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Morphological comparison of bone marrow derived mesenchymal stem cell from albino rats and Indian chicken

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Summary

The bone marrow is a complex tissue containing stem cells with hematopoietic properties. These bone-marrow mesenchymal stem cells have been identified as the source of multipotent stem cells. Bone-marrow-derived mesenchymal stem cells (BM-MSCs) are also referred to as stromal progenitor cells which are self-renewing and expandable stem cells used for regenerative studies. Basically, the mesenchymal stem cell (MSC) has the unique property of plasticity and adherence. In this study we discuss the bone-marrow-MSC isolation and their cultural characterization based on plasticity, proliferation, and CD44 cell surface marker identification in Albino Rats and Indian Chicken. The results of comparative study in the two different species indicate that there are differences in the cell morphology and proliferation rate of MSC. This article provides general understanding of the cellular morphological difference of stem cells in the lower animal models, and paves the way for future research work into the selection of species.

Key words: BM-MSCs – Isolation – Morphology – Proliferation – Characterization

Introduction

Stem cells are defined as cellular entities with two main properties: self-renewal and the ability to differentiate along one or more lineages (Fuchs et al., 2004). Pittenger et al. (1999) reported that MSCs represent a very small fraction of the total population of nucleated cells in the marrow. Bone-marrow-derived mesenchymal stem cells (BM-MSCs) are also referred to as stromal progenitor cells, and they are self-renewing and expandable. MSCs constitute approximately 0.01-0.001% of the whole bone-marrow cells (Koc et al., 1999). Though MSCs occur in low quantity in bone-marrow aspirate, they can be separated from hematopoietic stem cells (HSC), because they adhere to plastic and glass (Colter et al., 2000). MSCs "gold standard" was defined in humans by Pittenger et al. (1999) as bone-marrow-derived fibroblasts cells able to differentiate under appropriate stimuli along three principal lineages: osteoblastic, adipocytic and chondrocytic lineages. This functional definition allows affirmation of the MSC nature of a cell population in the absence of strict specific markers. Phenotypically MSC has been defined as CD29, CD44, CD90, CD105 positive and negative for hematopoietic lineage markers such as CD45 and CD34 (Javazon et

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al., 2004). Nowadays BM-MSCs represent an ideal stem cell source for cell therapies and regeneration studies due to their multi-potent property. All the preliminary regenerative researches were carried out on animal models before being applied to humans for clinical practice. The results of our study reveal the properties of BM-MSCs in two different lower animal models. This comparison will help future research.

Materials and Methods

Isolation of chicken BM-MSC's

Chicken bone brought from an authorized slaughterhouse was washed twice in Phosphate Buffered Saline (PBS). Bone-marrow cells were flushed with maintenance of medium aseptically. The cells were separated by centrifugation (1500 rpm, 10 minutes) twice in fresh medium and once with Histopaque gradient solution centrifuged at 1500 rpm for 20 mins. The cells were counted before and after gradient separation. Then the counted cells were subjected for obtaining cell phenotype devoid of CD45 negative population using MACS (magnetic assorted cell separation). Primary antibodies: Sheep monoclonal anti CD-45 (Biological industries). Secondary antibodies: Goat anti-rabbit IgG conjugated with FITC- magnetic beads (Invitrogen). Single cell suspensions of MSCs were centrifuged at 1100 rpm for 10 minutes. Cell pellets were stained with anti CD45 antibodies and incubated in the dark for 10 minutes at 4-8°C. Cells were washed to remove unbound primary antibody by adding 1-2ml of buffer and centrifuged at 3000 rpm for 10 minutes. After washing, the cell pellet was re-suspended in 100 µl of anti CD45 FIT C antibodies and incubated for 15min at 4-8°C. Cells were washed to remove unbound antibodies. The cell pellet was re-suspended in 100 µl of anti-FITC micro beads/10^7, and incubated for 15 min at 4-8°C. Cells were washed to remove unbound antibodies. After washing, these cells were re-suspended in 500 µl of buffer. Then the cells were separated using magnetic assorted cell sorting (MACS) column, and the unlabelled cells were separated using a plunger. The cells were counted before and after MACS separation. The CD45- cells were plated in the density of $1x10^6$ nucleated cells/ml in 25 cm^2 flask (Nunc) in medium containing DMEM (Dulbecco's Modified Eagle's Medium) with glucose supplemented with 10% Platelet-rich plasma (PRP) and 2 mM L-Glutamine and simultaneously with 10% fetal bovine serum (FBS). The cultures were incubated at 37ºC in a humidified atmosphere containing 5% CO_2. The floating hematopoietic cells were removed at intervals during 24-48 hours of culture. The culture medium was changed after 2 days until confluence was reached.

Culture and expansion of bone-marrow-derived MSCs were carried out as per the method described by Mangalagowri (2006). Briefly, upon reaching near confluence, the cells were detached from the culture flasks by treating with 0.25% trypsin containing 1mM EDTA for 5 minutes at 37ºC. The cells were washed with culture medium without Fetal Bovine Serum (FBS) to dilute trypsin, and the cell pellet containing MSCs was subjected to count using Neubauer chamber before subculturing.

Isolation of Wistar albino rat BM-MSC's

The eight-week-old Wistar albino rat was anesthetized by intra-peritoneal injection of ketamine (0.3 ml of diluted stock to 100 g young rat), followed by xylocaine (0.9 ml of diluted stock to 100 g young rats). Ten minutes later, the animal was laid down on its back, the skin over the forelimb and hind limb were aseptically cleaned. Two femurs and two tibias were dissected free of muscles and the adherent tissue. Both ends of the bone were cut, and the marrow cavity was flushed out with culture medium slowly injected at the end of the bone using a sterile 18-gauge needle. Bone-marrow-cells were subsequently suspended in minimal essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS).

The cells suspension was used for establishment of culture by plastic adherence, and was centrifuged on 400 g for 30 min. Mononuclear cells were removed from the gradient interface and washed with phosphate buffered saline (PBS). The suspension was then centrifuged at 2000 rpm for 5 min. The pellet thus obtained was dissolved in 1 ml of PBS; the cell count was done in a Neubauer chamber and tested for viability. The mononuclear cells were re-suspended in growth medium and plated in 25 cm^2 tissue-culture flasks made of polystyrene plastic at a density of $1x10^6$ cells/ml. Non-adherent cells were removed after 48 hours, replacing the media for every two to three days.

Characterization of mesenchymal stem cells

The chicken and albino rat MSCs were characterized in terms of their plasticity and adherence properties. Chicken BM-MSC was identified by CD44 marker analysis using RT-PCR as per the methodology and published primer of Khatri et al. (2010). Rat BM-MSC was identified by combining the density gradient centrifugation with plastic adherence as per the methodology of Polisetti et al. (2010).

Results

Chicken and rat bone-marrow cells were isolated and $1x10^6$ cells/ml of counted cells were cultured. Initially on the second day of cultured cells of both rat and chicken mesenchymal stem cells were seen with eccentric nucleus and process, along with mixed population of cells (Fig. 1a, b). The stem cells are distinct from other cells and can be viewed under microscope based on the morphology. On the fifth day the rats' MSC started growing elongated cells; and in chicken the cells were fusiform in shape with tapering ends (Fig. 2a, b). Cells were clustered as a colony forming units which formed thickly packed monolayer formation in in-vitro shown on the ninth day chicken MSC (Fig. 3b) and on eleventh day in rats MSC (Fig. 3a). This result indicated that the chicken's MSCs proliferation rate were faster than in rats. CD44 cell surface marker analyses were done in chicken by RT-PCR (reverse transcription polymerase chain reaction) and shown positive expression against negative control without reverse transcriptase (Fig. 4b), and in rats by immunocytochemistry for the confirmation shown in Fig. 4a.

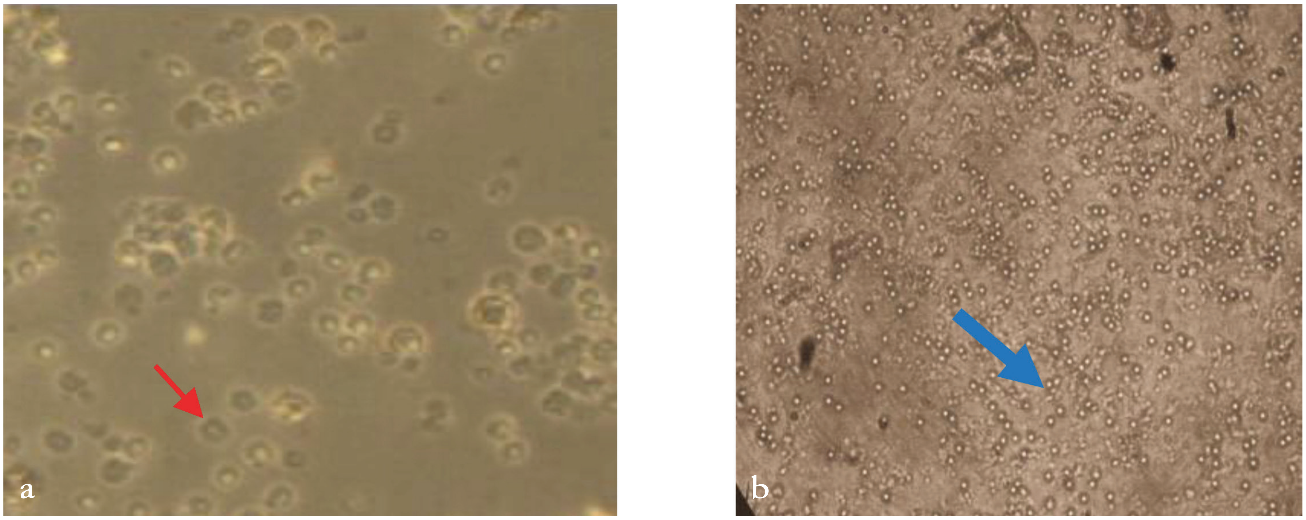

Fig. 1. Initial 2nd day MSC-*In vitro*. **a)** Albino rat. x200. **b)** Indian chicken - showed attaching cells on plate. x100.

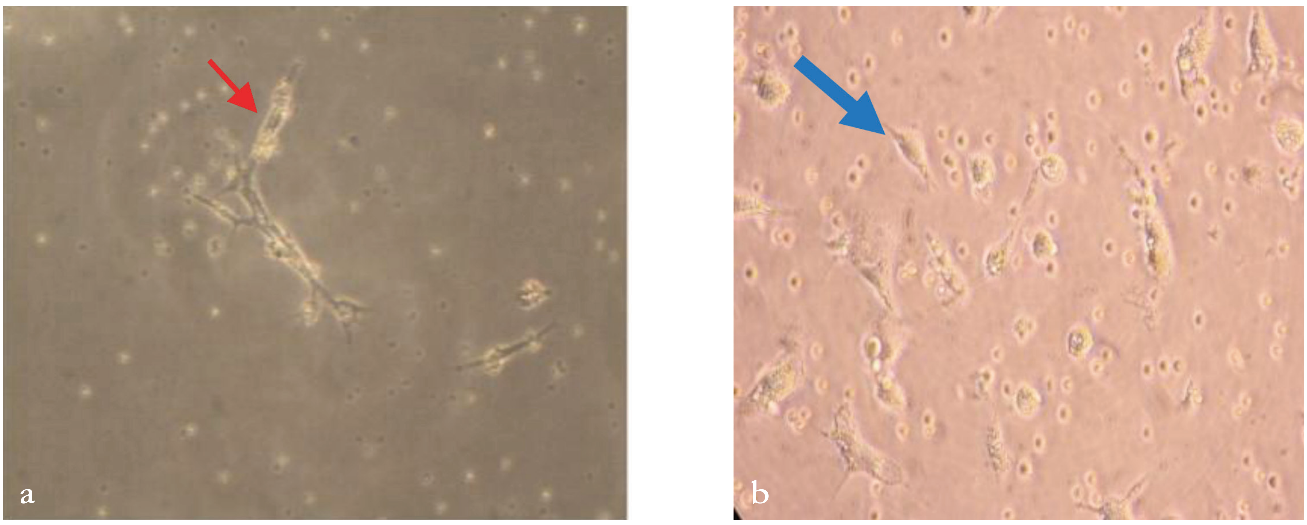

Fig. 2. 5th day MSC-*In vitro*. **a)** Albino rat showing elongated shaped cells. x200. **b)** Indian chicken showing fusiform with tapering ends. x200.

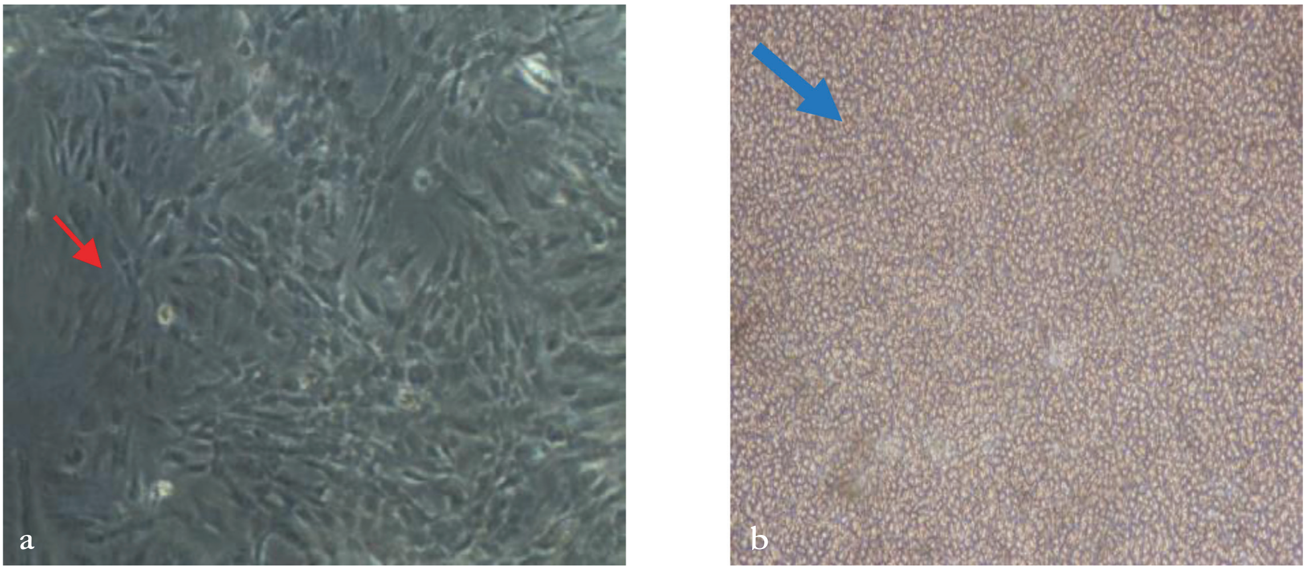

Fig. 3. Tightly packed monolayer of MSC-*Invitro*. **a)** Albino rat (11th day). x200. **b)** Indian chicken (9th day) showing monolayer. x100.

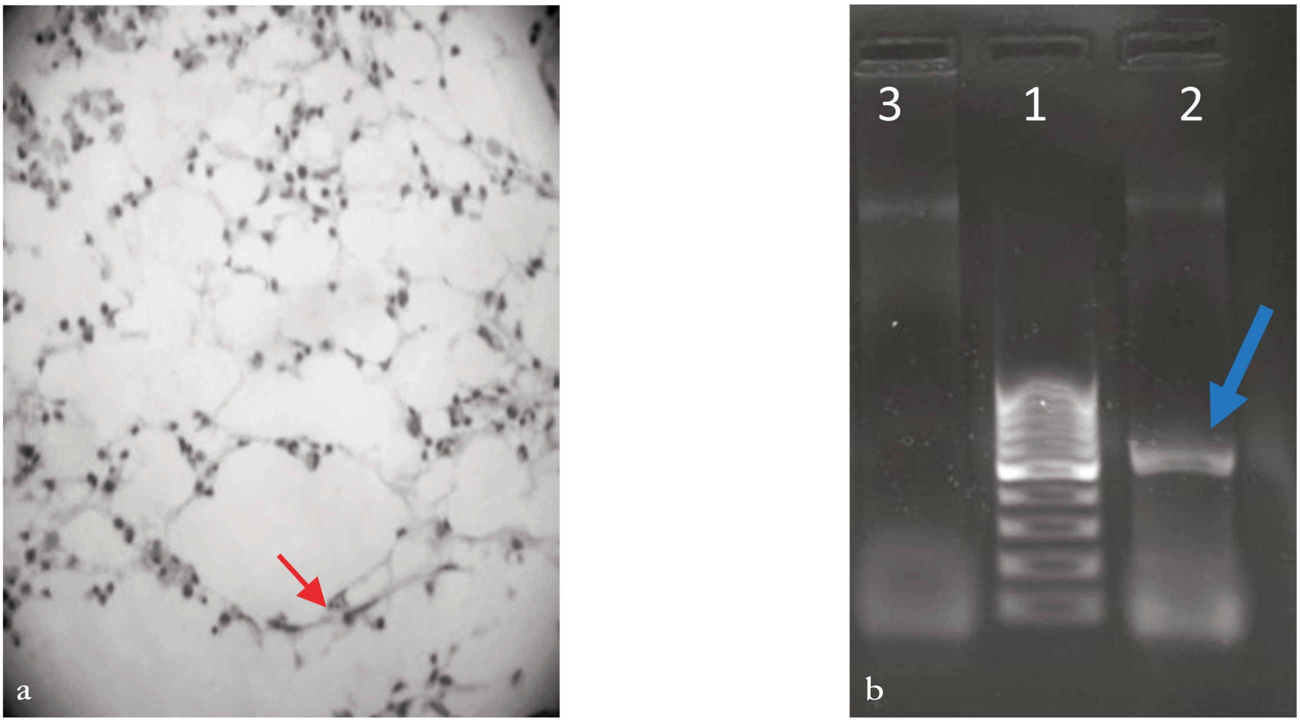

Fig. 4. CD44 confirmation in MSC-*In vitro*. **a:** Rat CD44 confirmation by immunocytochemistry. x200. **b:** Chicken CD44 confirmation by RT-PCR; lane 1 = 100 bp ladder, lane 2 = CD44 gene (701 bp), lane 3 = Negative control without reverse transcriptase.

Discussion

Bone marrows act as the mother source for mesenchymal stem cells, which play a vital role in modern regenerative medicine. Owen (1988) and Prockop (1997) reported that MSCs could be isolated in a relatively high number from cultures of bone marrow by selecting the cells that are adhered to tissue culture plastic, and which are proliferating rapidly. MSC population had been isolated via the methodology similar to that originally used by Friedenstein et al. (1970, 1976), and popularized by Caplan et al. (1991), which utilized the physical property of plastic adherence.

Isolation of MSCs were based initially on their ability to adhere to plastic, which apparently resulted in morphologically, phenotypically, and functionally heterogeneous populations of cells including reticular cells, fibroblasts, adiposites, and oestrogenic precursor cells (Minguell et al., 2001; Phinney, 2002; Baksh et al., 2004). Mesenchymal stem cells

(MSC) and Hematopoietic stem cells (HSC) can be isolated from mononuclear-cell fractions of bone-marrow aspirates and HSCs can be further enriched by immuno-magnetic isolation based on specific surface antigen like CD34+ and/or CD133. MSCs were found to lack unique surface antigen that could be used for positive selection and hence the general strategy for the enrichment of MSCs was formulated based on the adherence of cells to plastic plates in medium with low serum (Majumdar et al., 1998; Phinney, 2002).

Initially both rat and chicken mesenchymal cells were seen with eccentric nucleus and processes among the mixed population of cells. The mesenchymal cells are distinct from other cells and can be differentiated under microscope very clearly as described by Raimondo et al. (2005) and reported about the presence of mesenchymal stem cells with pseudopodia.

On the third day, when the culture medium was changed the mesenchymal stem cells were adherent to tissue culture plastic, as described by Prockop (1997) and Polisetti et al. (2010), and the other population of cells were removed due to lacking adherent property.

On the fifth day the rat mesenchymal stem cells started growing, and their long processes were seen as elongated cells. In chicken the cells were fusiform with tapering ends, as described by Raimondo et al. (2005). Kassis et al. (2006) discovered the isolation of the mesenchymal cells in mixed population, and reported that spindle shaped morphology of mesenchymal stem cells using fibrin micro bead.

On the ninth day chicken mesenchymal cells are clustered as a colony forming units which formed thickly-packed monolayer formation in in-vitro, and in rats MSCs form a monolayer on eleventh day, as described by Prockop (1997) and Polisetti et al. (2010). These results showed that the chicken MSCs proliferation rate were faster than in rats.

CD44 marker analysis was done in chicken by RT-PCR (Khatri et al., 2010), and in rats by immunocytochemistry. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed minimal criteria to define human MSC. The three criteria to define hMSC, such as plastic adherence with negative phenotype for CD45, CD14, and trilineage differentiation were suggested for animal MSCs, but the surface protein criteria for animal MSCs were not defined. MSCs lack hematopoietic markers such as CD14, CD34, and CD45 but expressed several surface proteins including SH2, SH3, CD29, CD44, CD71, CD90, CD106, and CD166 (Dominici et al., 2006).

Polisetti et al. (2010) reported that MSCs have been isolated from various species including mouse, rat, and rabbit; and human subjects have similar characteristic in part and some data suggested that variations occurred among them. The present study demonstrates that Albino rats and Indian chicken bone-marrow MSC were isolated, and their cultural characterization based on plasticity, proliferation, and CD44 cell surface marker identification was compared, which showed that there is a significant difference in the cell morphology and proliferation rate of MSC. This study was subjected to understand the cellular morphological difference of stem cells in animal models which will be helpful for future research.

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