



***In vitro* assessment of growth kinetics and differentiation potential of bone marrow derived mesenchymal and fetal fibroblast cells of caprine**

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ABSTRACT

The present study assessed comparative growth kinetics and differentiation potential of mesenchymal and fetal fibroblast cells of goats *in vitro*. The mesenchymal cells were isolated from bone marrow and fibroblasts were obtained from skin of 3–4 months old fetus. During culture, more than 99% viability of both cell groups indicated optimum culture conditions for *in vitro* experiments. The population doubling time (PDT) of BM-MSCs and FFCs were similar at third (18.25 ± 0.05 and 18.41 ± 0.07) and seventh (19.61 ± 0.06 and 19.68 ± 0.12) passages. But at 12th passage, FFCs cells had a significantly higher PDT (35.77 ± 0.68 vs 32.063 ± 1.0) as compared to BM-MSCs. Both groups of cells showed a characteristic sigmoid curve on plotting cell concentration verses days of culture. The stem cell like property was exhibited by BM-MSCs by differentiating into osteoblast, chondrocytes and adipocytes under specific media, which were further confirmed by specific staining of cells. Also, the BM-MSCs showed colonogenic property by forming different number of clones at third (60.5 ± 0.59), seventh (56.6 ± 0.5) and 12th (33.4 ± 0.45) passages. The fetal fibroblast cells did not exhibit colonogenic and differentiation potentials. This study concluded that although BM-MSCs and FFCs shared similar morphology and growth kinetics during initial passages but FFCs does not exhibit the stem cell like properties in terms of differentiation potency.

Key words: Differentiation, Fibroblast, Goat, Growth, Mesenchymal

In regenerative medicine, mesenchymal cells offer several advantages over embryonic or induced pluripotent stem cells, because of their easy isolation, *in vitro* culture, lower tumorigenic properties and intrinsic ability to self bio-preserve with minimal loss of potency. During *in vitro* culture, mesenchymal stem cells (MSCs) exhibit adhesion potential, retain non differentiating status for longer sub-cultures, and have potential to differentiate into chondrocytes, osteocytes and adipocytes *in vitro* (Phinney *et al.* 2007, Da Silva Meirelles *et al.* 2008, Augello *et al.* 2010).

At present no unique phenotype are identified that permits the reproducible isolation of MSC precursors with predictable developmental potential. The isolation and characterization of stromal cell function, therefore, still relies primarily on their ability to adhere to plastic and their expansion potential. Although MSCs appear as fibroblast like morphology but fibroblasts are widely considered to be terminally differentiated cells that are primarily responsible for the synthesis and remodeling of extracellular matrix in tissues. However, some reports have demonstrated

that fibroblasts were also able to differentiate into various lineages. In human, foreskin dermal fibroblasts could differentiate into adipocytes, osteocytes and chondrocytes (Chen *et al.* 2007) and fibroblast derived from bronchial and uterine tissues exhibited the differentiation potential into adipocytes and osteocytes (Kim *et al.* 2008, Strakova *et al.* 2008). These conflict reports addresses the necessity to investigate the comparative analysis of co-relations between mesenchymal and fibroblast cells.

MSCs were first described by Friedenstein *et al.* (1966) who reported isolation of cells from rat bone marrow tissue, using cell plastic-adherent property. Since then, the search for suitable biological models has become increasingly important as a way to produce results closer to possible application to human medicine as well as improving the quality of animal life. In most of the researches, small animals, such as rodents (Jeong *et al.* 2003, Da Rocha *et al.* 2012), swine (Gotterbarm *et al.* 2008) and canines (Sampaolesi *et al.* 2006, Alves *et al.* 2010) were used in experiments related to cell therapies and tissue regeneration for various diseases. However, MSCs from goat which are more similar to human than rodents has rarely gained attention. Goat, despite of consideration as an accepted model in stem cell therapy, viz. orthopedics surgery (Cunningham *et al.* 2010, Vonk *et al.* 2010, Lechner *et al.*

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1999), indeed remains to be fully investigated. In this context, the present study includes goat as a model to assess the growth and multilineage differentiation potentiality of bone marrow derived mesenchymal stem cells (BM-MSCs) *in vitro* as compared to fetal fibroblast cells (FFCs).

MATERIALS AND METHODS

Isolation and *in vitro* culture of mesenchymal cells: Bone marrow was obtained surgically from the adult goats. The goats were anaesthetized by intramuscular injection of ketamine (22 mg/kg) and xylazine (0.2 mg/kg). About 5 ml of bone marrow were aspirated from epiphyseal region of tibia and collected into 15 ml tube containing 7,500 unit heparin. The contents were diluted with 5 ml of Dulbecco's phosphate buffer saline and immediately shipped on the ice to the laboratory. The bone marrow suspension was carefully layered on top of equal amount of histopaque solution. The suspension was centrifuged at 3,000 rpm for 20 min and thereafter, the intermediate buffy coat was carefully transferred into another tube containing 5 ml DPBS solution. The content was re-centrifuged at 3,000 rpm for 10 min, pellets were resuspended with DPBS. The diluted cell suspensions were added to 5 ml DMEM medium (Dulbecco's modified eagle medium), supplemented with 10% fetal bovine serum (FBS) and centrifuged at 1,500 rpm for 5 min. The cell pellet was resuspended in DMEM medium, plated at 5×10^6 cells in 25 cm² culture flasks and incubated under humidified atmosphere of 5% CO₂ and 38.5°C temperature. The media was replaced at every third day of incubation until the cultures became confluent.

Primary goat fetal fibroblast cell culture: Caprine fetal fibroblast cells were derived from skin pieces of approximately 3-month old goat fetus as per Freshney (2005). On 70–80% confluence, both primary flasks of mesenchymal and skin fibroblast cells were sub-cultured with 0.25% trypsin-EDTA. In both cell groups, the cells after third passage were used for experiments.

Cell viability, cell proliferation and population doubling time: Before plating, the viability of the cells was examined using 0.4% trypan blue stain. On microscopic examination, live and dead cells appeared as unstained and blue stained, respectively. On the basis of staining, the cell viability percentage was calculated as the ratio of viable and total cell count multiplied by 100. Primary cultures (24) comprising 12 each for BM-MSC and FFCs, were assessed for viability percentage at third passage.

Cell proliferation rate and population doubling time: To calculate cell proliferation rate (r), the total cell count at the time of seeding (N_1) and harvesting (N_2) between a specific time interval ($t_2 - t_1$) was recorded and the data were imposed to following formula:

$$\text{Proliferation rate } (r) = \frac{3.32 \times (\log N_2 - \log N_1)}{t_2 - t_1}$$

The population doubling time (PDT) was calculated as $1/r$. **Growth curve of proliferating cells:** Depending on the total cell count at the time of seeding and harvesting, a growth

curve of BM-MSC and FFCs at third, seventh and 12th passages was plotted. For each cell type, the harvested cells of previous passage (Second, Sixth and 11th) at 70–80% confluence were seeded in 8 tissue culture flasks (25 cm²) at cell density $3.8 - 4.0 \times 10^4$ cells/flask (approximately). The cells were maintained at 38.5°C and 5% CO₂ under humidified atmosphere of incubator. Every day, 1 flask of both type of cells were trypsinized to determine the cell numbers by haemocytometer count. Finally on eighth day, data derived from each tissue flask were used to plot the growth curve of BM-MSC and FFCs.

Colony forming unit (CFU) assay: At third, seventh and 12th passage, the colonogenic properties of cultured caprine mesenchymal cells were assessed by colony forming unit (CFU) assay. The cells were seeded at the rate of 100 cells/cm² in 24-well plate with DMEM fortified with 10% FBS. The cells were maintained at 38.5°C in 5% CO₂ incubator and the media were replaced twice a week. At day 14, the cells were rinsed with DPBS and fixed in formalin. To visualize the colonies, the cells were stained with 0.5% crystal violet solution. The cell colonies containing e^{30} cells were considered as clone and scored accordingly for different cell passages under inverted microscope.

***In vitro* multilineage differentiation:** At third passage, the MSCs were chemically induced to differentiate into different cell lineages, viz. osteoblast, chondroblast and adipocytes *in vitro*. In triplicates, each lineage was developed under the incubation of specific differentiation media for osteoblast, chondroblast and osteoblasts as per manufacturer's instructions. Fetal fibroblast cells were taken as negative control for each cell lineage. All cells were cultured for 21 days with replacement of media at 3 days intervals and finally stained for confirmation of differentiation. The osteoblast cells were stained by Von Kossa's method.

For chondrocytes, the cells were washed with DPBS and fixed with 4% formaldehyde solution for 30 min. After fixation, the cells were thoroughly washed with DPBS and stained with 1% alcian blue solution for 30 min. The stained cells were rinsed with 0.1 N HCl, added distilled water to neutralize the acidity and observed under light microscope.

The adipocytes were identified by staining of fat droplets inside the adipocytes. The differentiated cells were fixed by treatment with 4% formalin for 30 min at room temperature. The cells were washed with 70% ethanol and stained with Oil Red O solution in 99% isopropanol for 15 min. The cells were washed with PBS and visualized under microscope.

Statistical analysis: In the present study, 36 experimental trials comprising 18 trials each for 2 cell groups, viz. BM-MSC and FFCs, were conducted to evaluate the population doubling time. Under each cell group, 6 replicates of experiment were performed each for third, seventh and 12th passage. The data of different treatment groups were statistically analyzed by factorial analysis.

RESULTS AND DISCUSSION

Cell viability during *in vitro* culture: The fetal fibroblast

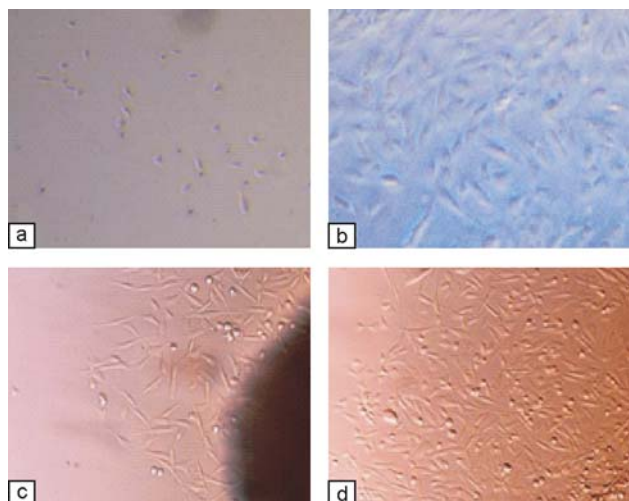


Fig. 1. Morphological features of BM-MSC and FFC culture. (a) fibroblast like appearance of BM-MSCs at day 3, (b) 70–80% confluency of BM-MSCs; (c) emergence of FFCs at third tissue explant, (d) 70–80% confluency of FFCs.

cells started to emerge 2 days after tissue explants. In BM- MSC, the fibroblasts like structure were seen on 3–4th days post-culture (Fig. 1a). In 12 trials of primary cell culture, the viability of BM- MSC and FFCs were observed as 99.42±0.15 and 99.25±0.19, respectively, on first passage indicating optimum *in vitro* culture condition for further experiments (Fig. 1b,c,d).

Cell proliferation rate and population doubling time: In this study, the population doubling time based on cell proliferation rates of BM- MSC and FFCs were assessed at third, seventh and 12th passages. The results revealed that PDT was not significantly different at third and seventh passages within and between the fibroblast (18.41±0.07 and 19.68±0.12) and mesenchymal (18.25±0.05 and 19.61±0.06) groups (Table 1). PDT of 12th passage was significantly higher ($P \geq 0.05$) than third and eighth passages within each cell group. Also, the PDT of 12th passage of

Table 1. Comparative analysis of population doubling time of BM- MSC and FFCs at different passages

Types of cells	Passages		
	3 rd	7 th	12 th
BM- MSCs	18.250±0.050 ^c	19.610±0.06 ^c	32.063±0.996 ^b
FFCs	18.407±0.072 ^c	19.677±0.119 ^c	35.77±0.679 ^a

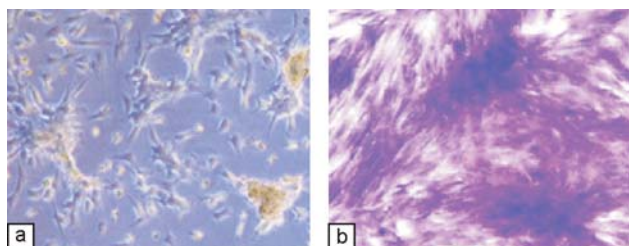


Fig. 2. Comparative growth curve of BM- MSC and FFCs at different passages representing characteristic lag, log and plateau phases.

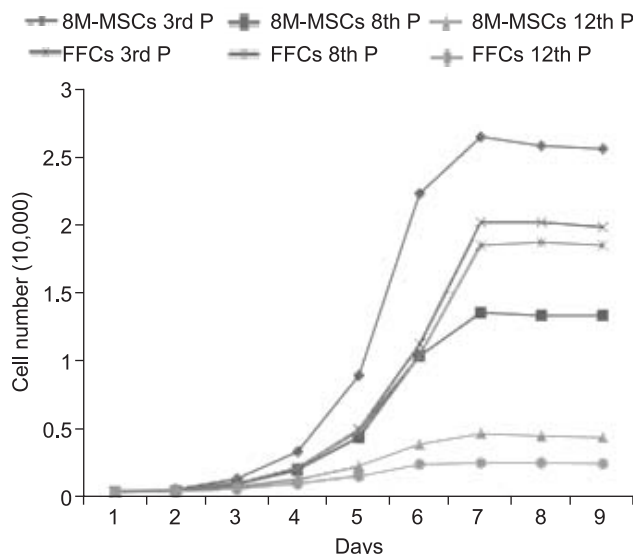


Fig. 3. Colonogenic property of BM- MSCs showing (A) different units of colonies (B) crystal violet stained CFU-F.

FFCs (35.77±0.68) was significantly higher ($P \geq 0.05$) as compared to BM- MSC (32.063±1.0) group.

Growth curve analysis: The present study compared the growth kinetics of MSCs and FFCs by assessing their population at harvesting period ranges from day 1 to 8. In all the cases, a characteristic sigmoid curve was obtained

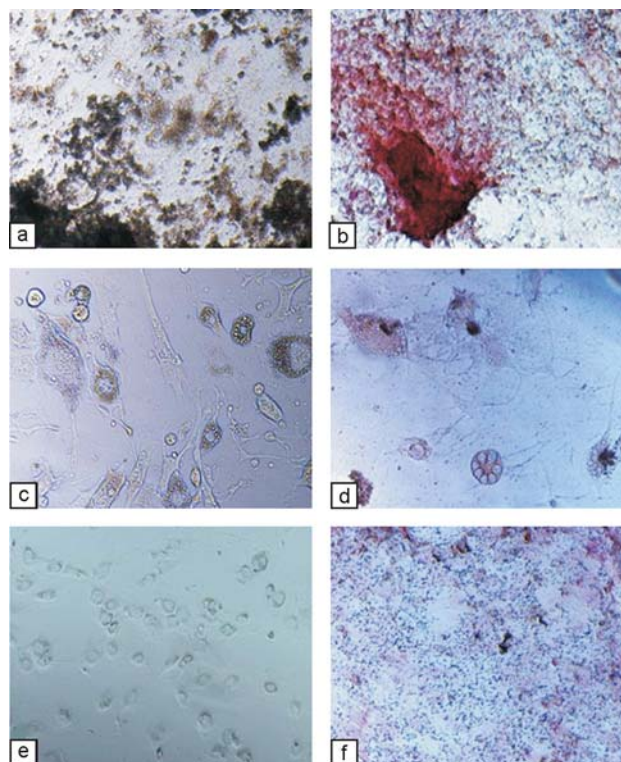


Fig. 4. *In vitro* multilineage differentiation of BM- MSCs. In Osteoblast, calcium deposits appeared as (a) black and (b) brownish black (von cossa's staining). Adipocytes were marked as (c) intracellular fat droplets, which appeared as (d) slight reddish on Oil Red 'O' staining. Chondrocytes were indicated as dark blue proteoglycans in the matrix (e) unstained (f) stained with Alcian blue.

(Fig. 2). However, it was noted that at 12th passages of both cells type, viz. BM-MSC and FFCs showed lower peak of curve as compared to earlier passages, probably due to higher population doubling time

CFU assay: On plating of 100 cells of BM-MSCs at third, seventh and 12th passages in 24 wells plate, almost 60.5 ± 0.59 , 56.6 ± 0.5 and 33.4 ± 0.45 clones, each consisting of 30–50 cells were formed, respectively on 12th day (Fig 3a,b). However, no such clones were observed in case of fibroblast cells at any passages.

In vitro multilineage differentiation: In osteoinductive cultures, the cellular aggregates started to appear at 7–8th day of plating and gradually increased to form nodules like structure at the end of experiment (Fig. 4a). Following Vonkossa's staining, the calcium or minerals deposits appeared brownish-black (Fig. 4b).

Adipogenic differentiation: The presence of lipid droplets within the cytoplasm of differentiating cells was a marked indicator of adipose cells (Fig. 4c). The lipid droplets started to appear in the cytoplasm at 5–6 days of treatment and covered a wide area of cytoplasm by day 21. The lipid droplets appeared red with oil Red O staining which confirmed the adipogenic potential of BM-MSC cells (Fig 4d).

Chondrogenic differentiation: The chondrogenic potential of BM-MSC was confirmed by Alcian blue staining. After 21 days of culture, dark blue staining of chondrocyte indicates synthesis of proteoglycans by chondrocytes (Fig 4e,f).

In modern medicine, parallel to the great efforts for exploring the novel and alternative sources of stem cells in the body, the bone marrow appeared to be a promising reservoir of mesenchymal cells that could be used as multipotent stem cells. MSCs are defined by their 'fibroblast-like' morphology, characteristic plastic-adherence, specific marker expression profiles and differentiation into fat, bone and muscle (Dominici *et al.* 2006). Fibroblasts, on the other hand exist in virtually every organ in the body; defined as adherent cells, which are not endothelium, epithelium or hematopoietic in origin and which have the capacity to synthesize and remodel the extracellular matrix (Haniffa *et al.* 2009, Kuroda *et al.* 2010). The present study investigated that whether the fibroblast and mesenchymal cells exhibited only phenotypic similarities or shared growth kinetics and differential properties.

Our investigation found similar phenotypes for FFC and BM-MSCs as well as similar growth trends. The cell viability of both cell groups was more than 99%, indicating optimum cell growth under *in vitro* culture condition. The population doubling time of BM-MSC and FFCs were within 18–20 h up to seventh passage. After ninth passage, the morphology of cells appeared flattened as compared to earlier in case of both cell types. The changes in morphology were more evident at 12th passage where the PDT of both cells was significantly higher than earlier passages. It was noted that the PDT of fibroblast cells at later passage was

significantly higher than BM-MSC cells which might be indicative of its higher differentiated status and limited growth capacity. The growth kinetic of both cell groups, based on total number of cells against time showed a characteristic "S" shaped curve with characteristic lag, log, and stationary phases of a normal growth curve of mammalian cells (Freshney 2005).

In our study, despite sharing common phenotype and similar growth kinetics, the property of stemness was not evident in the fibroblast cells. The BM-MSCs showed colonogenic property as well as multilineage differentiation into osteoblast, chondrocyte and adipocytes, however FFCs failed to exhibit such characteristic features. The higher proliferation rates in earlier passages of BM-MSC were correlated with higher number of CFU and vice versa in 12th passage. However, this correlation was not observed in FFCs, where CFU was not observed at any passage, although proliferation rates in earlier passages were similar with BM-MSCs. The findings were in accordance to Alt *et al.* (2011), who reported that colony-forming capacity and differentiation potential are specific important properties that discriminate MSCs from fibroblasts. Interestingly, several studies showed that fibroblast cells of different origins not only express surface markers almost identical to MSCs, but could also differentiate into multilineage cells viz. adipocytes, chondrocytes and adipocytes (Sabatini *et al.* 2005, Lysy *et al.* 2007, Covas *et al.* 2008, Lorenz *et al.* 2008, Blasi *et al.* 2011).

The present study investigated the comparative growth and differentiation potential of BM-MSCs and FFCs of goats. The viability, PDT and growth curve of both cell types showed similar pattern during initial passages, but mesenchymal cells showed higher proliferation capacity as compared to FFCs at higher passage. The characteristic properties of colonogenicity and differentiation potential were evident of stem cells like feature of bone marrow derived fibroblast cells which were absent in fetal fibroblast cells.

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