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Multipotent Differentiation Potential of Buffalo Adipose Tissue Derived Mesenchymal Stem Cells

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ABSTRACT

The present study has been undertaken for isolation, characterization and differentiation of Buffalo Adipose Derived Mesenchymal Stem Cells (bADMSCs). Cocktail enzymatic digestion method was used for isolation of bADMSCs which yielded optimum number of cells than other methods. Subcutaneous adipose tissue yielded comparatively higher number of Stem Cells (SC) than from omentum and bone regions. The growth and proliferation of SC were rapid when Mesencult medium was used, in comparison to Dulbecco's Modified Eagles Medium (DMEM). Expression of oct 4 gene by reverse transcriptase-polymerase chain reaction (RT-PCR) indicated the stemness of isolated bADMSCs. Magnetic cell sorting and immunostaining revealed that the isolated ADMSCs were cluster of differentiation 44 (CD44) positive. Buffalo ADMSCs were also induced to differentiate into adipogenic, osteogenic and chondrogenic lineages. Expression of specific marker genes-Peroxisome Proliferator Activated Receptor Gamma (PPAR γ) in adipogenic, Osteopontin in osteogenic and Collagen II in chondrogenic differentiated lineages were confirmed in buffalo Adipose Derived Stem Cells (ADSCs) by RT-PCR. The study clearly proves that Buffalo white adipose tissue is a source of multipotent SC and can be induced to differentiate into adipogenic, osteogenic and chondrogenic lineages. Further, this is the first study to report such finding in buffalo.

Key words: Mesencult, osteogenic, adipogenic, chondrogenic, CD44

INTRODUCTION

In recent years, interest has rapidly grown in the developmental plasticity and therapeutic potential of stromal cells isolated from adipose tissue, called Adipose-derived Stem Cells (ADSCs). This capacity is mediated by the presence of vascular and non-vascular cells that provide a pool of stem and progenitor cells with unique regenerative capacity (Prunet-Marcassus *et al*., 2006).

Adipose tissue is derived from the mesodermal germ layer and contains a supportive Stromal Vascular Fraction (SVF) that can be readily isolated (Gronthos *et al*., 2003). Subcutaneous adipose tissue is an attractive reservoir of progenitor cells, because it is easily accessible, abundant and self-replenishing. This SVF from adipose tissue consists of heterogeneous mixture of cells, including endothelial cells, smooth muscle cells, pericytes, leukocytes, mast cells and pre-adipocytes (Oedayrajsingh-Varma *et al*., 2006). In addition to these cells, the SVF contains an abundant population of multipotent ADSCs that possess the capacity to differentiate into cells of mesodermal

origin *in vitro*, e.g., adipocytes, chondrocytes, osteoblasts and cardiomyocytes (Guilak *et al*., 2004). These cells can be enzymatically digested out of adipose tissue (commonly from lipoaspirate) and separated from the buoyant adipocytes by centrifugation. A more homogeneous population emerges in culture under conditions supportive of MSC growth.

The propensity of stem cell research has acquired considerable attention in recent years due to the promising potential for future cell replacement based therapies (Meenambigai and Sejian, 2011). Remarkable improvement in livestock productions in the developed countries have achieved through research into how animals grow and how yield can be influenced and protected (Sejian *et al*., 2010). Regenerative medicine and cell replacement therapy is the most exciting cell based therapeutics in which scientist grow tissues and organs in the laboratory (Chhetri *et al*., 2010).

Application of Bioengineering technology to study the stem cell microenvironment can potentially revolutionize regenerative medicine by providing physicians with tools to regulate resident Adult Stem Cells (ASC) behavior (that is, self-renewal, differentiation, migration) in patients, cells for cell-based therapies, to replace defective tissues (Gilbert and Blau, 2011). The potential of Mesenchymal Stem Cells (MSCs) to diff erentiate into cells of the bone and cartilage lineages has led to a variety of experimental strategies to investigate whether MSCs can be used for tissue engineering approaches. Now, MSCs also has been known to display immunosuppressive properties which have prompted research on their capacity to suppress local inflammation and tissue damage in a variety of inflammatory autoimmune diseases and in particular, in rheumatoid arthritis (Maumus *et al*., 2011). The clinical use of stem cells in Veterinary medicine is clearly in its early stages. Applications for BM-MSC and AD-SVF cells in the treatment of musculoskeletal pathologies are currently in use in several species, although the differential efficacies of various approaches are still being investigated. Optimization of these stem cell-based therapies will focus on cellular origin, isolation, enrichment and processing as well as on the timing, route of administration, formulation and dosing of those therapies (Fortier and Travis, 2011). Oct 4 is highly expressed in pluripotent cells and becomes silenced upon differentiation. Oct 4 can re-establish pluripotency in somatic cells and proper reprogramming of Oct 4 expression is indispensable for deriving genuine induced pluripotent stem cell lines. Systemic and dynamic exploration of the protein complexes and target genes associated with Oct 4 will help to elucidate the role of Oct4 more comprehensively (Shi and Jin, 2010).

The population doubling no of human fetal bone marrow is about 2 times higher than BMMSC and their differentiation capacity superior (Abdel-Moneim and Said, 2007). Renal damage mediated by oxidative stress in mice was treated with aluminium chloride protective effects of taurine (Al-kahtani, 2010). The proliferative and developmental potential of stem cells promises an essentially unlimited supply of differentiated cells for basic research drug discovery and for transplantation therapeutics (Thomes and Yu, 2008). Production of red blood cells by bone marrow is stimulated by erythropoietin which is produced by the kidney when red blood cells are in short supply (Hulya Uz and Muberra, 2005). Impairment of kidney functions might lead to excessive circulation of toxins associated with damage of other tissues. Under such circumstances stem cell therapy comes handy (Al-Ankari, 2006).

The acquisition of adipose tissue is much less expensive than bone marrow with less invasive operation and is available in greater proportion. Clinically relevant stem cell (SC) numbers can be extracted from isolated adipose tissue since it possesses higher SC proliferation rate than BMSCs. Therefore, adipose tissue represents an abundant, practical and appealing source of donor tissue for autologous cell replacement (Lei *et al*., 2007). ASCs are under investigation for a variety of therapeutic applications. These cells are known to home to some tissues such as injured tissue.

During the past decade, numerous studies have provided preclinical data on the safety and efficacy of adipose-derived stem cells, supporting the use of these cells in future clinical applications. Various clinical trials have shown the regenerative capability of adipose-derived stem cells in subspecialties of medical fields such as plastic surgery, orthopedic surgery, oral and maxillofacial surgery and cardiac surgery. Although the mechanisms underlying the migration of ASCs remain to be determined, clarification of the roles of chemokine receptors and adhesion molecules on ASCs may lead to the development of therapeutic strategies to enhance the recruitment of cultured ASCs to injured or damaged tissue (Tobita *et al*., 2011).

Because of these favorable characteristics, interest has been growing in the application of ADSCs for cell-based therapies such as tissue engineering. With this prelude, the present study has been undertaken for isolation, characterization and differentiation of buffalo adipose tissue derived mesenchymal stem cells (bADMSCs) with the aim to use them as possible therapeutic cells.

MATERIALS AND METHODS

Collection of the sample: Buffalo white adipose tissue of either subcutaneous, omentum or bone origin was collected from the local abattoir and was transferred to the laboratory in sterile Dulbeccos phosphate buffered saline with antibiotics. Tissues were kept in room temperature and processed within 1 h. Storage of adipose tissues at 4°C was avoided as it reduced the digesting ability of the enzyme used. This study was carried out during 2010-11.

Isolation of stromal vascular fraction: Adipose tissue is a mesodermally derived organ that contains a stromal population containing microvascular endothelial cells, smooth muscle cells and SC. These cells should be enzymatically digested out of adipose tissue and separated from the buoyant adipocytes.

Approximately about 1 to 2 grams of adipose tissue was taken and was washed thrice with sterile Dulbeccos Phosphate Buffered Saline (DPBS) with antibiotics to remove external debris and blood. Then the tissue was minced well using sterile forceps and scissors and washed thrice with sterile DPBS with antibiotics. The above minced tissue was homogenized and subjected to three kinds of enzymatic digestion methods (Zhu *et al*., 2008) and were as follows: (1) Tissues were enzymatically digested in Dulbeccos Phosphate Buffered Saline (DPBS) supplemented with 0.5% Trypsin Versene Glucose (TVG); (2) 0.1% Collagenase with 1% Bovine Serum Albumin (BSA) and (3) Cocktail of enzymes, viz., 0.25% trypsin, 0.1% collagenase and 0.1% pronase with varying incubation periods of 1, 2 and 3 h accompanied by intermittent shaking every 15 min to ensure proper digestion of the tissue. The enzyme reaction was stopped by adding equal volume of medium with 10% serum. After digestion, 2 phases were formed-the floating digested adipose tissue in the upper phase and the lower phase contained stem cells, erythrocytes, leukocytes and smooth muscle cells. The lower phase was removed and centrifuged at 1200 rpm for 10 min. The supernatant containing mature adipocytes was discarded. The pellet was taken and washed twice with medium without serum by centrifuging at 1200 rpm for 5 min.

Establishment of primary cultures: Cells after washing was resuspended in Mesencult medium/Dulbeccos Modified Eagle Medium with high glucose (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1 X ABAM solution. Cells were cultured in 25 cm^2 tissue culture flasks with 8 mL of DMEM, 10% FBS, 1X ABAM solution under 5% CO_2 and 90% humidity at 39°C for 24 h. After 24 h, non-adherent cells were removed by washing with DPBS and fresh medium was added to the plate. This ensures the removal of erythrocytes and non-adhering cells without interfering with SCs in culture. Medium was changed every 72 h until they reached confluency.

Sub culturing adipose derived stem cells: The adipose cells were subcultured when they reached 75% confluency. The spent medium was removed and 1 ml trypsin (0.25%) was added and incubated for 5 min. Trypsin was removed and growth medium (mesencult with 10% FBS) was added. The cells were flushed and were distributed to culture flasks in 1:3 split ratios.

Characterization of undifferentiated ADMSCs

Separation of CD 44 positive ADSCs by MACS: Magnetic Cell Sorting (MACS) was done using magnetically tagged antibodies against CD44. Cells from a confluent plate were harvested using 0.25% trypsin and were pelted at 1200 rpm for 10 min. Primary antibodies (MACS Mouse anti-CD44 monoclonal antibody) in the ratio of 1: 500 were added and were incubated for 15 min. After incubation the cells were washed with Phosphate Buffer Saline (PBS) to remove unbound primary antibodies. To the pellet, Fluorescein Isothiocyanate (FITC) conjugated secondary antibodies in the ratio of 1:1000 dilution was added and incubated for 15 min. Cells were again washed with PBS to remove unbound antibodies. These cells were then incubated with MACS anti-FITC Micro beads for 15 min and washed with PBS. Then the cells were resuspended in the MACS buffer and were passed through the MACS Column placed in the magnetic field. The Magnetically labeled antibody bound cells alone gets bound to the column. These bound cells were eluted away from the magnetic field using elution buffer. The eluted CD 44 cells were cultured in a 25 cm^2 culture flask.

Immunostaining of CD 44 positive ADSCs: MACS sorted ADMSCs were fixed with 4% paraformaldehyde for 15 min and washed thrice with PBS and incubated with 5% goat serum, 0.1% triton×100 for 1 h and washed thrice with PBS. Then ADMSCs were incubated with primary antibody (mouse anti CD 44) 1:500 overnight at 4°C and washed thrice with PBS to remove unbound primary antibodies. They were then incubated with Goat Anti Moure IgG (FITC conjugated) 1:1000 for 2 h and was washed thrice with PBS and observed for fluorescence.

Expression study of Oct4 gene in undifferentiated ADSCs by reverse transcriptase PCR: Total RNA was extracted from undifferentiated ADSC cell suspension (passage 3) using trizol as the cell denaturing agent. cDNA was synthesised briefly to 1.5 μg of RNA, 2 μL of random hexamers were added and incubated at 70°C for 5 min and kept in ice for 10 mins. Then 2 μL of 10×enzyme buffer, 2 μL of dNTP mix and 1 μL of R Nase inhibitor was added and maintained at 25°C for 5 min. To it 1 μL of reverse transcriptase enzyme was add to a total volume of 20 μL. Reaction was programmed at 25°C-10 min, 42°C-1 h and 75°C-10 min.

Differentiation of ADSCs: ADSCs were taken into 3 lineages differentiation viz. adipogenesis, osteogenesis and chondrogenesis at the third passage of ADMSC. Cells were seeded in 24 well plates and when confluency of approximately 75% was formed differentiation was induced. Adipogenesis was induced by culturing in Adipogenic Differentiation Medium (DMEM supplemented with 10% adipogenic supplement (Stem Cell Technologies, USA)), Osteogenesis was induced by culturing in osteogenic differentiation medium DMEM with 10% FBS, 1% ABAM (Antibiotic and Antimycotic solution), 10 mM β-glycerphosphate, 0.25 mM absorbic acid and 100 nM dexamethasone (Colleoni *et al*., 2005). Chondrogenesis was carried out by pellet culturing method (Bosnakovski *et al*., 2006). Two milliliter eppendorf tubes were seeded with approximately 50,000 cells and centrifuged at 1200 rpm for 5 min to form a cell pellet. Chondrogenic differentiation medium (DMEM with 10% FBS, 1% ABAM (Antibiotic and Antimycotic solution),

6.25 μg mL^{-1} insulin, 10 ng mL^{-1} transforming growth factor (TGF β1) and 100 nM dexamethasone. Francis *et al.* (2010) was added to the cell pellets. Culture media were changed every 3 to 4 days and was observed for 21 days. Differentiation profiles were recorded.

Histological staining of differentiated cells: Cells were washed with calcium and magnesium free DPBS, then fixed in 4% paraformaldehyde at 4°C for 2 h and again were washed four times with calcium and magnesium free DPBS prior to staining. Adipogenically differentiated buffalo ADSCs were stained with 1 mL of 2% Oil red O to detect intracellular lipid accumulation (Zhu *et al.*, 2008). Osteocyte induction was confirmed by 2% Alizarin Red S to verify mineralized calcium deposit (Williams *et al.*, 2008).Chondrogenically differentiated buffalo ADSCs were stained with 0.04% Toluidine blue stain to detect Proteoglycans (PGs) in the extracellular matrix (Kisiday *et al.*, 2008).

Characterization of differentiated lineages by reverse transcriptase PCR: RNA isolation, cDNA synthesis and amplification of specific genes were carried out as described earlier and the specific genes were amplified following a specific cycling condition. PPAR γ gene (389 bp) was amplified at an annealing temperature of 50°C (Ferneyhough *et al.*, 2007). Osteopontin gene (404 bp) at 60°C and Collagen II gene (147 bp) at 56°C (Li *et al.*, 2009).

RESULTS

Isolation of stromal vascular fraction: Adipose tissue was processed and subjected to enzymatic digestion for the isolation of stromal vascular fraction (Fig. 1). The stromal vascular fraction contained a mixture population of cells constituting hematopoietic cells, mature adipocytes,

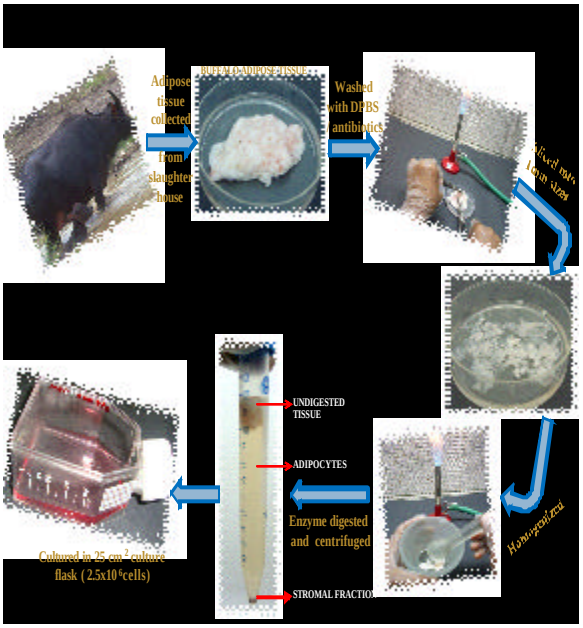

Fig. 1: Enzyme digestion of the subcutaneous adipose tissue

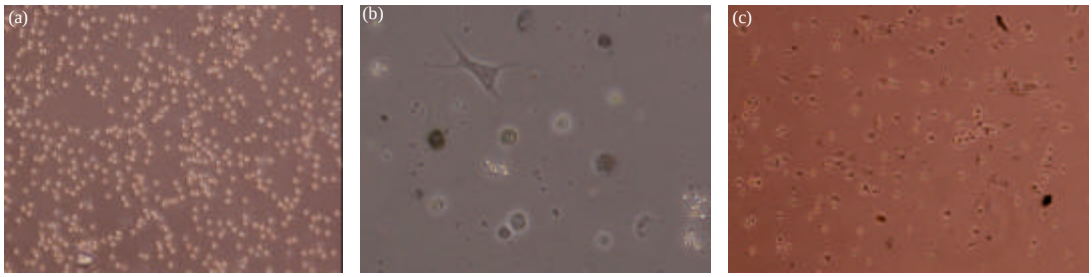

Fig. 2(a-c): Primary culture by trypsin digestion (b): ADMSC -24 h after Primary Culture (200 x) BCocktail Digestion (1 h) (c): ADMSC -24 h after primary culture (200 x) cocktail digestion (2 h)

Table 1: Sources of buffalo adipose tissue

Fat source	Mesenchymal stem cell recovery
Subcutaneous fat	5×10^5
Omentum fat	3.8×10^5
Bone fat	4.2×10^5

Table 2: Enzyme digestion of the subcutaneous adipose tissue

Tissue type	Amount of tissue (g)	Enzyme	No. of trials	Cell types	ADMSCs recovery
Subcutaneous fat	1	0.5 % trypsin	5	Majority hematopoietic cells	Nil
Subcutaneous fat	1	0.1% collagenase	4	Mixed population of cells	1.5×10^5 cells
Subcutaneous fat	1	Cocktail (0.5% trypsin, 0.1% collagenase, 0.1% pronase)	40	Mixed population of cells	5×10^5 cells

stromal cells, vascular endothelial cells and vascular smooth muscle cells. Since adipose tissue is highly vascularised, higher proportion of hematopoietic cells were seen (Fig. 2a). Appearance of stellate and oval shaped mesenchymal stem cells 24 h post culture, subjected to 1 and 2 h cocktail enzyme digestion (Fig. 2b, c). Fourty nine trials were done and cultures were maintained till 6th passage level.

Sources of buffalo adipose tissue: Adipose tissues from subcutaneous, omentum and bone regions were processed for adipose stromal cell recovery. Subcutaneous adipose tissue yielded about 5×10^5 cells while omentum fat yielded about 3.8×10^5 and bone fat yielded about 4.2×10^5 (Table 1).

Enzyme digestion of the subcutaneous adipose tissue: Cocktail enzyme digestion yielded higher number of adipose derived stromal cells of about 5×10^5 cells while trypsin digestion did not yield stromal cells (Table 2).

Two hours incubation of buffalo adipose tissue in cocktail enzyme mixture yielded optimum number of cells -$4.940\pm65\times10^5$. Extension of the incubation time to three hours resulted in digestion of cell wall with less recovery of stromal cells ($0.90\pm15\times10^5$) leading to increase in non-viability percentage. On the other hand, 1 h incubation yielded $1.25\pm0.24\times10^5$ cells. The significance is that 2 h incubation yielded highest recovery of cells (Table 3).

Table 3: Optimization of cocktail enzyme digestion

	ADMSCs recovery		
Enzyme digestion (in hour)	Trypsin	Collagenase	Cocktail
1	Nil	$0.81\pm0.08\times10^5$	$1.25\pm0.24\times10^5$
2	Nil	$1.50\pm0.18\times10^5$	$4.94\pm0.65\times10^5$
3	Nil	$1.10\pm0.18\times10^5$	$0.90\pm0.15\times10^5$

Table 4: Culture media influencing growth and proliferation of stem cells

Culture media	ADSC expansion	Colony forming units (CFUS)
Mesencult	24 h	1st passage
DMEM	72 h	3rd passage

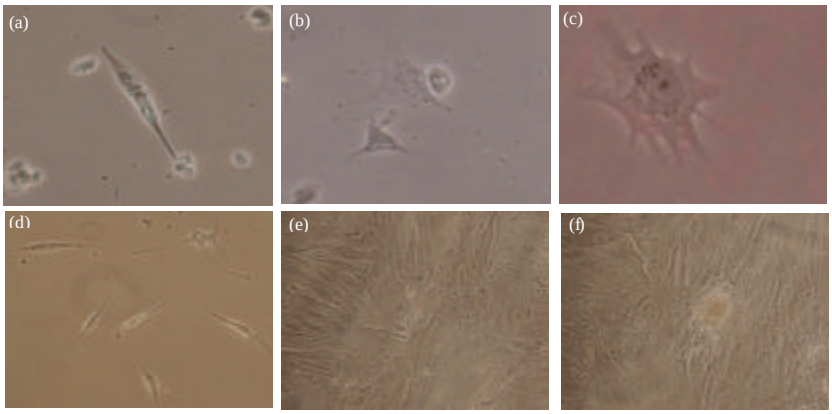

Fig. 3(a-f): Spindle shaped fibroblast (200×) (b): Spindle shaped fibroblast (c): Stellate shaped fibroblast (200×) (d): 40% confluency of ADMSCs on Day 7 (200×) (e): 90% confluency of ADMSCs on Day 25 (200×) (f):Colony forming unit (CFU) of ADMSC (200×)

Proliferation of adipose derived stromal cells: Adipose derived stromal cells became swollen and expanded to form fibroblast like structure in 24-48 h. Fibroblast with stellate and spindle shaped morphology was observed as reported earlier by Zhu *et al.* (2008) (Fig. 3, b and c). On 7th day 40% conflency of cells was detected (Fig. 3d). Adipose derived stromal cells reached 90% confluency (Fig. 3e) and formed colony forming units (CFUs) by 20 to 25 days (Fig. 3f).

Culture media influencing growth and proliferation of stem cells: Adipose stromal cells cultured in mesencult media showed rapid growth and Colony Forming Units (CFUs) in the first passage while in DMEM cells grew slowly and formed colony forming units only in the third passage (Table 4).

Characterization of undifferentiated ADMSCs Oct 4 gene: Expression of oct 4 gene in adipose derived stromal cells was confirmed by RT PCR which indicated the stemness of the isolated ADSCs (Fig. 4a).

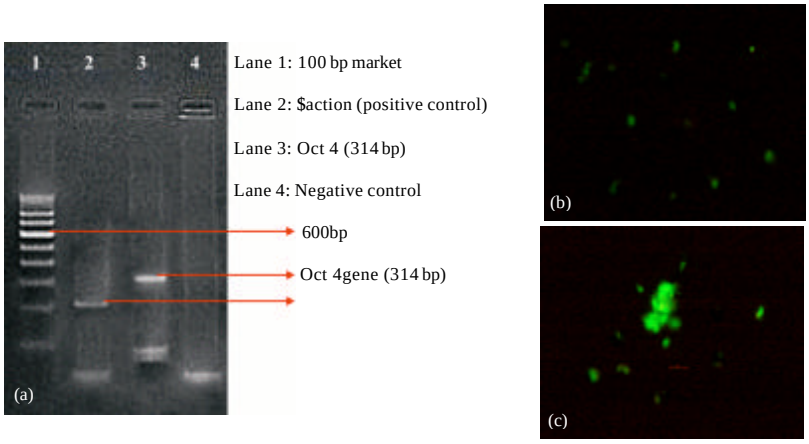

Fig. 4: Characterization of undifferentiated ADMSCS. (a) Expression of Oct 4 Gene in ADMSCs by RT PCR, Lane 1: 100 bp marker; Lane 2: β actin (positive control); Lane 3: Oct 4 (314 bp); Lane 4: negative control (b) Expression of CD 44 in ADMSCs in culture on Culture on Day 3 (200 x) and (c) Expression of CD 44 in ADMSCs in culture on Day 14 (200 x)

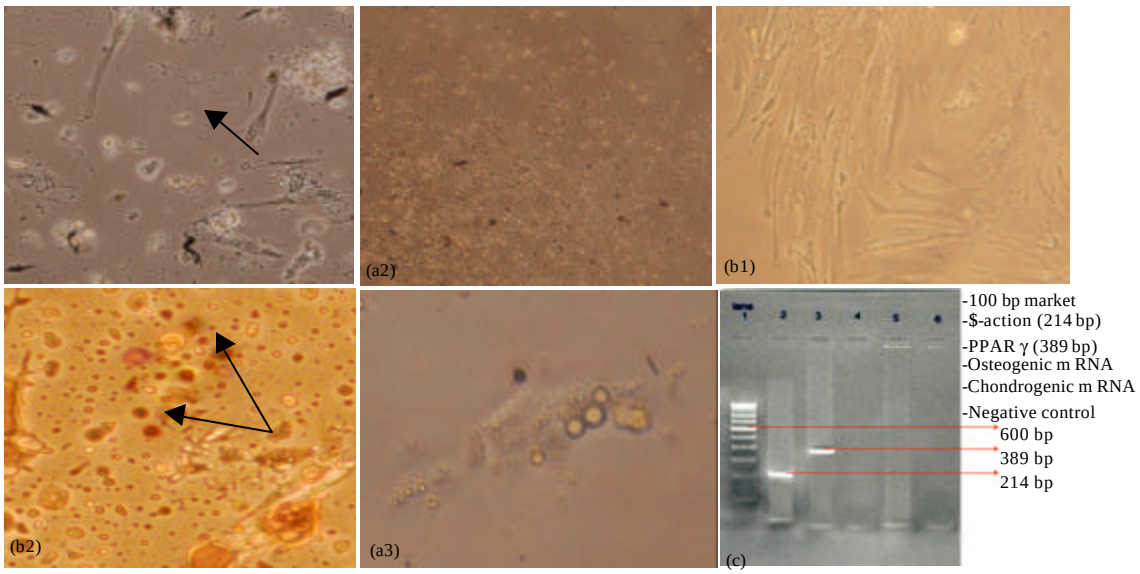

Fig. 5a: Lipid Vacuolation in adipogenically differentiating ADMSC (a) 1: On Day 7 (200 x) 5a 2: On Day 14 (100 x) 5a 3: Single differentiated ADMSC with lipid vacuoles on Day 21 (200 X); (b): Oil Red O Staining (200 x) 5b 1: Undifferentiated 5b 2: Differentiated (lipid vacuoles stained red) and (c): Expression of PPAR ? gene by RT PCR

CD 44: CD 44 positive adipose derived stromal cells of passage 3 stage were separated by Magnetic Cell Sorting (MACS). The sorted cells were confirmed by immunostaining using FITC conjugated antibodies (Fig. 4b, c).

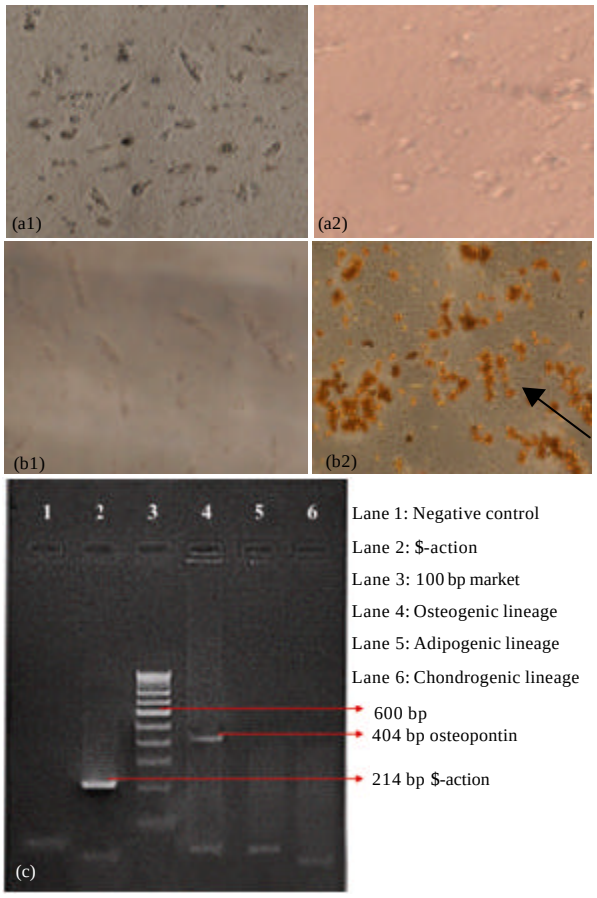

Fig. 6: Osteogenic differentiation of admscs from buffalo adipose tissue. (a): Cells attaining Cuboidal morphology 6a 1: On Day 7 6a 2: On Day 14 (b): Alizarin Red S staining on day 21 (200 x)(b) 1: Undifferentiated (b): Differentiated(calcium deposits were stained) (c): Expression of Osteopontin Gene by RT PCR

Differentiation of buffalo ADMSCs: After 3rd passage, ADMSCs of about 5×10^4 cell concentration was subjected to adipogenic, osteogenic and chondrogenic differentiation using specific differentiation media. In adipogenic differentiation, vacuolation of cells were observed from day 7 (Fig. 5a1-a3). On day 21 adipogenic differentiation was confirmed by Oil red O staining (Fig. 5b1, b2). Expression of PPARγ gene was confirmed by RT PCR on day 21 adipogenetically differentiated AD MSCs (Fig. 5c). Expression of osteopontin and collagen II genes were not observed in adipogenetically differentiated ADMSCs. In osteogenic differentiation, cells with ignited fibroblast morphology changed to cuboidal morphology from day 7 to day 21 (Fig. 6a). Osteogenic differentiation was confirmed by Alizarin red S staining (Fig. 6b1, b2). Expression of osteopontin gene was confirmed by RT PCR on day 21 osteogenetically differentiated ADMSCs (Fig. 6c). Expression of PPARγ and collagen II genes were not observed in osteogenetically differentiated ADMSCs. In chondrogenic differentiation, pellet culturing method was followed. Visible increase in the pellet size was observed on day 21 when compared with day 0 (Fig. 7a, b). On day 21

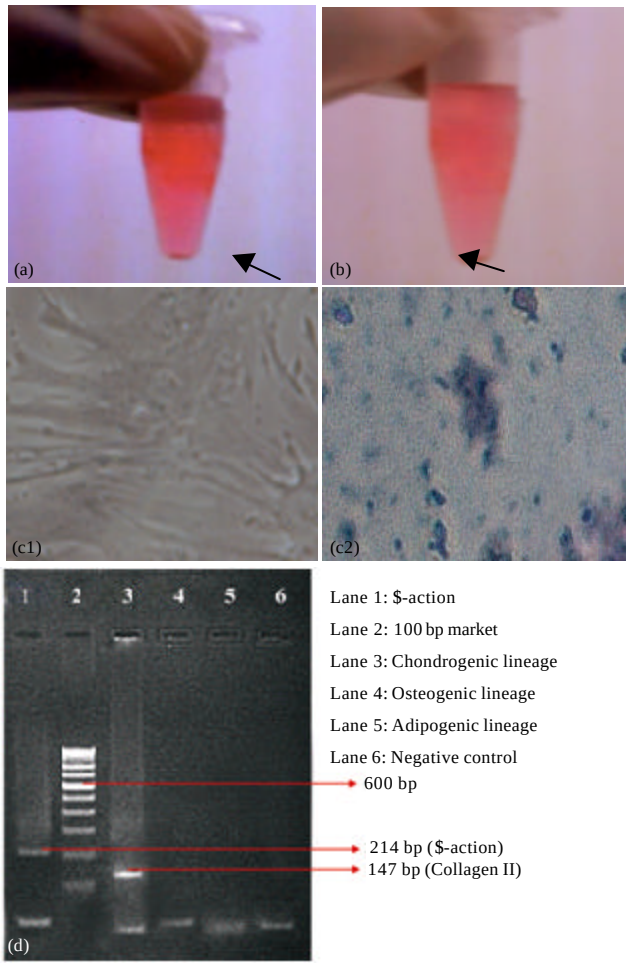

Fig. 7: Chondrogenic differentiation of admscs from buffalo adipose tissue (pellet culturing method (a): Pellet on Day 07 (b): Pellet on Day 21 (c1): Toluidine blue staining (200X) 7c1: Undifferentiated (c2): Differentiated (proteoglycans in ECM stained) (d): Expression of Collagen II gene by RT PCR

chondrogenic differentiation was confirmed by toluidine blue staining (Fig. 7c1, c2). Expression of collagen II gene was confirmed by RT PCR on day 21 in chondrogenetically differentiated ADMSCs. Expression of PPARγ and osteopontin genes were not observed in chondrogenetically differentiated ADMSCs (Fig. 7d).

DISCUSSION

Like marrow, adipose tissue is a mesodermally derived organ containing stromal population of cells (Zuk *et al.*, 2001). These cells can be enzymatically digested out of adipose tissue and separated from the buoyant adipocytes by centrifugation. Adipose tissue was washed, sliced and homogenized as a means of mechanical disruption. The minced fat becomes an excellent starting material for ADMSC isolation (Moore *et al.*, 1995), compared to a solid piece of fat that was utilized in this study. The stromal vascular fraction contained a mixture population of cells constituting

hematopoietic cells, mature adipocytes, stromal cells, vascular endothelial cells and vascular smooth muscle cells as reported earlier (Fraser *et al*., 2006; Strem *et al*., 2005). The stromal cells alone have the capacity to adhere to the substratum (i.e., culture flask) (Zhu *et al*., 2008). The results of the present study correlated with earlier workers.

Adipose tissues from subcutaneous (Fernyhough *et al*., 2008), omentum (Huang *et al*., 2007) and bone regions were processed for adipose stromal cell recovery. The yield of stromal cells from these three sources were more or less the same, yet subcutaneous adipose tissue comparatively yielded more number of cells than from omentum and bone fat. The abundancy of bone fat was comparatively less and omentum fat was highly vascularised (Dhanasekaran *et al*., 2010). Thereafter, subcutaneous tissue was used in the study, due to its abundancy, ease of collection *in vivo* for clinical applications (Nixon *et al*., 2008).

In the first few trials, trypsin digestion of adipose tissue was done but yield of stromal cells was nil, due to the presence of collagen fibres which are the dominant fibre type in most connective tissues. The primary function of collagen fibres is to add strength to the connective tissue . Hence collagenase was used to digest adipose tissue as described earlier by Zhu *et al*. (2008). According to Sakurada *et al*. (2007), enzymatic treatment for isolation of SCs may be carried out by digesting animal tissues with an enzyme such as collagenase, trypsin, pronase, dispase, elastase or hyaluronidase. SCs from brown adipose tissues were harvested by digesting with 0.1% collagenase, 0.1 dispase II and 0.05% trypsin in serum-free medium (Liu *et al*., 2010). However, in this study, subcutaneous adipose tissue was digested using cocktail of enzymes containing 0.25 trypsin, 0.1% collagenase and 0.01% pronase. Cocktail enzymatic digestion method yielded approximately 5×10^5 cells. The yield of ADSCs by cocktail enzyme method was higher than cells recovered when adipose tissue was digested with only collagenase. Cocktail enzyme digestion of buffalo adipose tissue at incubation periods of 1, 2 and 3 h were carried out and 2 h digestion yielded optimum number of cells of about $4.94\pm0.65\times10.5$ cells.

The stromal vascular fraction when seeded into culture flask, only the stromal cells adhered by 24 h and the non-adherent and suspension type cells were removed by a wash after 24 h. The adherent cells formed fibroblasts with spindle shaped and stellate shaped morphology. This was similar to earlier reports by Zhu *et al*. (2008) and Danisovic *et al*. (2007).

In this study, proliferative rate of buffalo ADSCs were higher when compared with DMEM. CFUs were higher in number in cells cultured with Mesencult medium. According to Jager *et al*. (2006), DMEM culture medium supported cell survival for bone substitutes that induced an alkaline reaction whereas MesenCult media promotes cell vitality in biomaterials which leads to an acidification of culture solution which was observed in the present study too.

Oct 4 is a mammalian pituitary-specific octamer a critical regulator of SC pluripotency and is highly expressed in the early stage of mammalian embryo and in the inner cell mass of the blastocyst. Recent studies have shown Oct 4 to be associated with the undifferentiated pluripotent state of SC populations derived from various adult tissues (Kim *et al*., 2009). Oct 4 is most frequently reported in hematopoietic and mesenchymal SCs of humans and mice as well as multipotent progenitors (Nayernia *et al*., 2006). Similar to the previous reports, in this study also Oct 4 gene expression was observed in buffalo adipose derived stromal cells. The expression of Oct 4 gene a 314 bp product was confirmed by reverse transcriptase-polymerase chain reaction. The expression of Oct 4 gene in MSCs ensures the pluripotent state of the isolated MSCs. Recently, the conservation of these proteins among mammalian species has become apparent and Oct-4, Sox-2 and Nanog are considered markers of cells that have pluripotent capabilities (Bernstein *et al*., 2006).

Expression of CD-44 in adipose derived stromal cells: MSCs are naturally heterogeneous. There are subpopulations of cells that have different shapes, proliferation and differentiation abilities. These subpopulations all express the known MSCs markers and no unique marker has yet been identified for the different subpopulations. Zhang and Chan (2010) used magnetic cell sorting to enrich the cells against two MSCs surface markers CD54 and CD90 among the isolated cells. Non-differentiated ADSCs contain different types of stromal cells with a large variety of CD marker expression (Trentz *et al*., 2009). Human adipose tissue expressed proteins include CD9, CD10, CD13, CD29, CD34, CD44, CD 49d, CD 49e, CD54, CD55,CD59, CD105, CD106, CD146 and CD166 (Gronthos *et al*., 2001). The cell surface phenotype of human ADSCs is quite similar to BM MSCs. When buffalo ADSCs were sorted by magnetically labeled CD44 antibodies using a magnetic cell sorter, about 0.005% cells were characterized as CD44 positive. CD44 expression on the surface of sorted ADSCs cells were also confirmed by immunostaining using FITC conjugated antibodies. CD44 a hyaluronate receptor, is crucial in the development of neo-extracellular matrix and plays a role in numerous pathologic and physiologic events. ADSCs also express high levels of CD54 when compared with BM-MSCs (De Ugarte *et al*., 2003). Different from reported values for human ASCs, porcine ASCs did not demonstrate positive expression for CD44 (Williams, 2009).

Differentiation of buffalo ADMSCs: ADSCs share many of the characteristics of its counterpart in marrow including extensive proliferative potential and the ability to undergo multilineage differentiation (De Ugarte *et al*., 2003). Adipose tissue-derived stem cells have the ability to undergo differentiation along classical mesenchymal lineages: Adipogenesis, osteogenesis and chondrogenesis,. In this study, the differentiation potential of buffalo derived ADSCs towards mesenchymal lineages have been proved.

Adipogenic differentiation: Adipose derived stromal cells belonging to adipogenic origin easily differentiate into adipogenic lineage (adipocytes) either spontaneously or on induction with specific differentiation medium. The gene or factor responsible for signaling the multipotent SC to the adipocyte lineage *in vivo* is unknown (Otto and Lane, 2005). The most important stimulants, however, appear to be insulin and glucocorticoids. *in vitro*, the first step of adipogenesis is stimulated by insulin-like growth factor-1 (IGF-1). Growth hormone, glucocorticoids, insulin and fatty acids are stimulatory in both the early and late phases of adipocyte differentiation. Triiodothyronine (T_3) hormone stimulates the late phase of adipogenesis (Lee *et al*., 2003). In this study, a commercially available readymade adipogenesis inducing supplement was used (stem cell technologic Inc, USA). Vacuolation of cells as identified by the above reports were observed from day 7. ADSCs of passage 3 showed multiple intracellular lipid filled droplets in 80-90% of cells in adipogenic media. The cells containing lipid vesicles exhibited an expanded morphology with the majority of intracellular space occupied by droplets and lipid vesicles which is consistent with the phenotype of mature adipocytes. No lipid droplets were observed in undifferentiated ADSCs. Lipid vacuoles stained red when stained by Oil Red O stain. This result was in accordance with the report of Zhu *et al*. (2008). The expression of PPAR γ a marker of adipogenesis was confirmed by RT PCR. Fernyhough *et al*. (2008) stated that in numerous cases cytosolic vesicle formation prior to the cell beginning to accumulate lipid, in parallel with the expression of PPAR α a marker of adipogenic differentiation was observed. Non-lipid-filled intracellular vesicle walls possessed the structural protein perilipin. ADSCs express several adipocytic genes including lipoprotein lipase, aP2, PPAR (gamma) 2, leptin, Glut4 and develop lipidladen intracellular vacuoles, the definitive marker of adipogenesis (Wickham *et al*., 2003).

Osteogenic differentiation: The ability of MSCs to give rise to osteoblasts is well known. In the past decade, several groups have isolated cells from adipose tissue of humans and other species capable of differentiating into osteoblasts *in vitro* (Dragoo *et al*., 2003). Under osteogenic conditions, similar to those used for MSCs, ADSCs are observed to express genes and proteins associated with an osteoblasts phenotype, including alkaline phosphatase, type I collagen, osteopontin, osteonectin, steocalcin, bone sialo protein, RunX-1, BMP-2, BMP-4 BMP receptors I and II, PTH-receptor (Halvorsen *et al*., 2001). Dexamethasone is required for stimulation of osteogenesis *in vitro* (Lee *et al*., 2003). Ascorbic acid functions as a co-factor in the hydroxylation of proline and lysine residues in collagen and increases the synthesis of non-collagenous bone matrix proteins (Duplomb *et al*., 2007). β-glycerophosphate is essential for calcification and mineralization of the extracellular matrix (Knippenberg *et al*., 2006). In this study, buffalo ADSCs when exposed to osteogenic medium, the MSCs changed their morphology from spindle shaped to cuboidal as observed from day 7 which was similar to earlier reports by Colleoni *et al*. (2005). The mineralization of the extracellular matrix was detected by alizarin red S (Williams *et al*., 2008) which stains the calcium deposits. The expression of osteopontin gene (Colleoni *et al*., 2005) a marker of osteogenesis further confirmed the osteogenic differentiation of buffalo ADSCs. Osteopontin (OPN) is a highly acidic secreted phosphoprotein that binds to cells via an RGD (arginine-glycine-aspartic acid) cell adhesion sequence that recognizes the αVβ3 integrin, ALP activity, nodule formation and remodeling of bone are markedly enhanced by OPN overexpression (Kojima *et al*., 2004).

Chondrogenic differentiation: As reported earlier by Gimble and Guilak (2003) and Huang *et al*. (2004). ADMSCs can differentiate into various types of cells, including chondrocytes. MSCs undergo spontaneous *in vitro* chondrogenic differentiation (Bosnakovski *et al*., 2004) or differentiation in the presence of growth factors such as transforming growth factor-β (TGF-β), Bone Morphogenetic Proteins (BMPs) or IGF-1 (Indrawattana *et al*., 2004; Schmitt *et al*., 2003) in a pellet culture system. Chondrogenic differentiation is induced by the addition of insulin, TGF-b1 and ascorbate (with or without dexamethasone) to the medium (Winter *et al*., 2003). The most commonly used factor is TGF-β1 which acts through a multimeric complex involving two membrane serine/threonine kinase receptors and an intracellular signaling pathway involving a cascade of Smad proteins (Miyazono, 2000). In the present study, when buffalo ADSCs were induced towards chondrogenesis with specific induction medium with TGF-β in a pellet culture, there was continual increase in the size of the pellet. The cells mostly had round morphology in the pellet. This was similar to the one reported by Danisovic *et al*. (2007). The deposition of proteoglycans in the extracellular matrix was visualized by toluidine blue staining (Kisiday *et al*., 2008). Chondrogenesis was further confirmed by the expression of collagen II gene. Bosnakovski *et al*. (2006) observed significant 2-fold higher expression of the typical chondrogenic markers, collagen II (coll 2), biglycan (big) and cartilage oligomeric matrix protein (comp) in pellet than in the monolayer of chondrogenetically differentiated bovine bone marrow SCs. In this study, chondrogenic differentiation of buffalo adipose derived SCs was lower which was similar to the report of Danisovic *et al*. (2007).

CONCLUSION

The stromal cells of buffaloe adipose tissue have stemness characteristics with the ability to differentiate and express stem cell markers of adipogenic, osteogenic and chondrogenic lineages. This differentiation potential coupled with plasticity nature make these stromal adipose tissue derived mesenchymal stem cells valuable therapeutic candidates.

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