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Isolation and characterization of buffalo amniotic fluid stem cells

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ABSTRACT

Amniotic fluid (AF) present different cellular types in its composition and a specific group of these cells shows multipotent characteristics with high proliferation ratio and increased differentiation ability. In this study, we isolated and characterized adult stem cells from buffalo (*Bubalus bubalis*) to check their differentiation potential *in-vitro* and evaluated their plasticity. Single cell derived stem cell clones were isolated by limiting dilution. AF cells showed both fibroblast and epitheloid morphology. Amniotic fluid stem cells (AFSC) were differentiated into osteogenic, chondrogenic and adipogenic lineage by culturing in specific differentiation medium. Differentiation was confirmed by cell staining with alizarin red S, toluidine blue and oil red O specific for osteogenesis, chondrogenesis and adipogenesis respectively. Gene expression of stem cell markers were found to be positive for Oct- 4, Sox–2 and Nanog by RT- PCR.

Key words: Amniotic fluid cell, Buffalo, *In-vitro* differentiation, Stem cells

Stem cells are a special group of undifferentiated cells possessing two fundamental properties. They are capable of self-maintenance and differentiating into specialized tissue forming cells. Another important property of SC is their proliferative potential which allows them to divide many times and to be maintained as a cell population throughout the life of the multicellular body (Keller *et al.* 2005). Stem cells can be classified into 3 broad categories based on their time of isolation during ontogenesis; embryonic, fetal and adult. Every tissue or organ from brain to fat apparently contains a stem cell population (Kyba and Daley 2003). Given their multipotentiality, adult stem cells are considered as powerful tool for tissue repair and gene therapy. Large numbers of adult stem cells would be needed for regenerative medicine but the frequency and expansion capacity of these cells are limited and may decrease with age (Dippolito *et al*. 1999). Thus alternative source of adult stem cells need to be explored. Fetal tissues such as skin, liver, bone marrow, blood, kidney and spleen are rich sources of SC (Campagnoli *et al*. 2001, Anker *et al*. 2003). Up to now, some reports exist regarding the characterization of AFSC that should be considered as an interesting new source of prenatal stem cells devoid of ethical issues involved in embryonic stem cell research (Fauza 2004). These cells are routinely obtained utilizing minimal invasive techniques for prenatal diagnosis of fetal abnormalities but, despite their widespread and well-established use in prenatal genetic testing, current knowledge about their origin and properties is quite limited. The present study describes isolation and characterization of AFSC from buffalo *(Bubalus bubalis)*.

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MATERIALS AND METHODS

Specimen collection: Gravid buffalo uteri (55) were collected irrespective of age from Chennai Corporation abattoir and utilized in this study. The uterus was removed within 30 min of slaughter and washed in phosphate buffered saline (PBS) supplemented with antibiotics (penicillin 100 iu/ml and streptomycin 100 µg/ml) to remove blood and extraneous material and transported in a thermos flask to the laboratory.

Cell culture: Uterus was again rinsed with PBS supplemented with antibiotics and AF was aspirated from the uterine horn containing the fetus, using a 18G hypodermic needle and the fluid was centrifuged at 2500 rpm to get the cell pellet. This was repeated with PBS wash and finally with α-MEM. Cell pellet was suspended in fresh α-MEM with 20% fetal bovine serum. Cells were counted and seeded into suitable cell culture flask at the rate of 1×10^5 cells and incubated at 37°C in the CO_2 incubator. Cells were stained with hematoxylin and eosin stain to study cell morphology. The clonal AFSC were expanded serially with a spilt ratio of 1:3 and tested for the stem cell markers and differentiation potential.

In vitro *differentiation of single cell-derived clonal AFSC:* The clonal AFSC at passages 3,6,7 were grown to 70%–90% confluence and shifted to osteogenic (RPMI1640 supplemented with 20% FBS, 0.1µm dexamethasone, 10mM glycerol phosphate, 0.2mM ascorbic acid), chondrogenic (RPMI1640 supplemented with 20%FBS, 0.1µm dexamethasone, 50µg/ml ascorbic acid, 100µg/ml sodium pyruvate, 40µg/ml proline, 6.25µg/ml insulin transferring sulfate (ITS)), and adipogenic media (RPMI1640 supplemented with 20% FBS, 0.5mM 3-isobutyl1-methylxanthine (IBMX), 1µm hydrocortisone, 0.1mM indomethacin) for 3 weeks. The differentiation potential for osteogenesis was assessed by the mineralization of calcium accumulates by alizarin red S staining. For adipogenic differentiation, morphological changes were looked for after staining with oil red O and chondrogenic differentiation was assessed by staining the cells with toludine blue S.

Gene expression: Total RNA was isolated from the clonal AFSC using Trizol reagent. RT-PCR was performed with one step RT-PCR kit with gene specific primers for Nanog (Forward 5' GAATTCTGTACCACTGCCCC 3', reverse 5' AGCCTCCCTATCCCAGAAAA 3'- 272bp), *Sox*-2 (Forward 5' TCAAGCCGTTTATCGAGAGG 3' reverse 5' ATCATGCTGTAGCTGCCGTT 3'- 260bp), and *Oct*-4 (forward 5' TGCTGCAGAAGTGGGTGGAGGAAG 3', reverse 5' CCGAGCTGCTGGGCGATGTG 3'-262bp). The reaction mixture consisted of 5µl of cDNA, 1µl each of forward and reverse primers and 13µl of prime taq premix and the following cycle conditions were used for the specific genes. Initial denaturation at 95°C for 5 min, 25 cycles of 94°C for 30 sec (denaturation), 55°C for 30 sec *Oct-4*, 56°C for 30 sec *Sox-2*, 58°C for 30 sec *Nanog* (annealing), 72°C for 45 sec (extension), followed by 10 min at 72ºC for final extension.

RESULTS AND DISCUSSION

Isolation and culturing of AF cells: A major aim in stem cell research is the identification of new sources of stem cells. Although it is known that adult bone marrow derived stem cells can be rapidly expanded *in vitro*, there is little information about the characteristics and functional properties of stem cells in the AF. We report the isolation and characterization of adult stem cells from buffalo AF including their multi lineage potential. Primary culture cell morphology of AF cells was heterogeneous initially with the undifferentiated AF cells showed fibroblastic and epitheloid morphology during the early passages (Fig. 1). Single cell derived stem cell clones of AF cells were obtained from first and second passage cells. Undifferentiated clonal AFSC grew to 90% confluency during subsequent passages.

In-vitro *differentiation of osteocytes, chondrocytes and adipocytes from buffalo AFSC:* The multilineage differentiation capacity of the cultured AFSC was tested by culturing these cells under specific osteogenic, adipogenic and chondrogenic culture conditions. After the specific treatments, AFSC gave rise to adipocytes, osteoblasts and chondrocytes, when fibroblast were the predominant cell population. To investigate the osteogenic potential of the buffalo AFSC, third to seventh passaged cells were plated at a density of 3×10^3 cells/ml and cultured under conditions appropriate for differentiation. When induced to differentiate, the spindle shape of AFSC formed mineralized matrix as evidence by alizarin red S staining (Fig. 2). In this regard, it was reported that AFSC display a multilineage differentiation potential into fibroblast, adipocytes, and osteocytes (Anker *et al*. 2003). Our results indicated that the AFSC differentiated into osteocytes when stimulated with specific medium.

The chondrogenic potential of AFSC was evaluated by

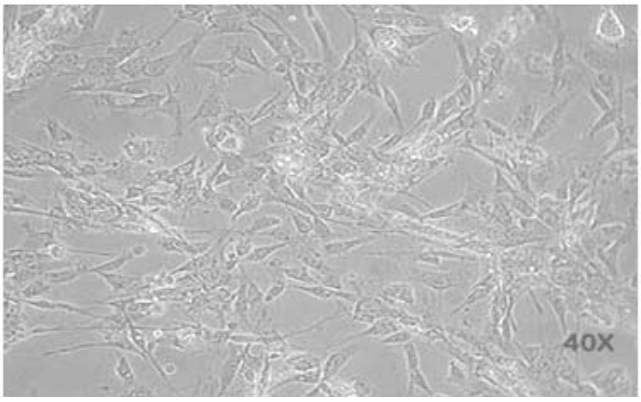

Fig. 1. Undifferentiated AF cells at second passage (unstained) showing fibroblastic and epitheloid morphology.

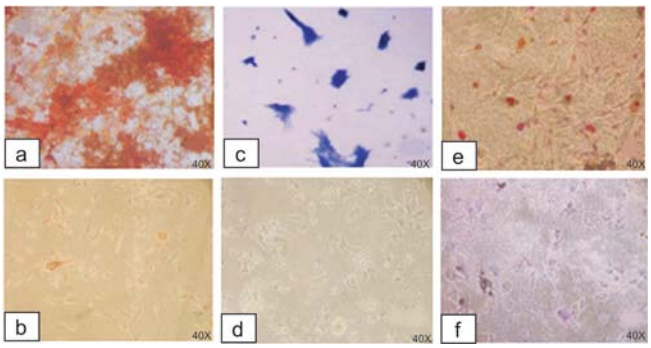

Fig.2. Differentiated AFSC into osteocytes, chondrocytes and adipocytes after 21 days of induction.**(a)** Osteogenic induction showed marked morphological changes and extensive extra cellular calcium deposition as demonstrated by positive Alizarin red S staining. **(b)** Osteogenic control in regular expansion medium maintained normal morphology and stained negative for Alizarin Red S. **(c)** Toludine blue stain of chondrogenic differentiated AFSC positive for chondritin sulfate. **(d)** Chondrogenic control in regular expansion medium maintained normal morphology and stained negative for Toludine blue **(e)** Positive adipogenic differentiation as demonstrated by morphological changes towards larger cells with marked lipid droplet deposition stained with Oil Red O. **(f)** Adipogenic control culture showing undifferentiated morphology and minor lipid droplet deposition.

culturing 1×10^6 cells/ml in chondrogenic medium. After 3 weeks of differentiation into chondroblast, toludine blue staining revealed the synthesis of chondroitin sulfate (Fig. 2). Close cell-to-cell contact and certain chondrogenic bioactive factors are widely accepted as being two main principles involved in enhancing chondrogenic differentiation. The bioactive factors that stimulate chondrogenesis include TGF-ß1, bone morphogenic protein, fibroblast growth factor, and insulin-like growth factor (Mastrogiacomo *et al*. 2001). In the present study, the potential of AFSC to undergo chondrogenesis has been analyzed after treatment with TGF-ß1, insulin transferrin sulphate (ITS), proline, dexamethsone and sodium pyruvate in monolayer culture

To assess the adipogenic potential, 3–7 passaged cells were plated at a density of 3×10^3 cell/ml and cultured in adipogenic medium. Morphological changes in cells as well as the formation of lipid vacuoles were noticeable as early as 1 week after differentiation and visualized by staining with oil red O (Fig. 2). Adipogenic differentiation was induced by using 3-isobutyl 1-methylxanthine (IBMX), indomethacin and hydrocortisone, a routinely used method to stimulate the differentiation of pre-adipocytes and stem cells. In our system, differentiation was apparent by the accumulation of lipid vacuoles within cells that developed, coalesced, and eventually filled the entire cell over a period of time. In this study, AFSC had limited differentiation potential due to the cell heterogeneity with high % of epithelial cells. However, the differentiation potential of AFSC could be improved in future studies by using additional cell selection procedures based on CD133 antigen expression.

Gene expression

All 3 transcription factor genes (*Oct-4*, *Sox-2*, *Nanog*) were also detected in differentiated AFSC by RT-PCR. *Nanog* expressed only in differentiated cells. *Oct-4*, *Sox -2* and *Nanog* genes are reported to be essential for the maintenance of stem cells (Pesce and scholer 2001). We analyzed buffalo AFSC for the expression of these markers. It is noteworthy that differentiated AFSC were found positive for *Oct-4*, *Sox-2* and *Nanog,* characteristics of embryonic adult stem cells, thus suggesting their possible multipotentiality (Figs 3, 4). *Oct-4* upregulation was found to result in differentiation of ESC into extra embryonic mesoderm or endoderm while *Oct-4* knockout ESCs differentiated into trophoectoderm (Niwa *et al*. 2000, Nichols *et al*. 1998). Upregulation of *Nanog* allowed mouse ESC to remain pluripotent without the need for leukemia inhibitory factor (LIF) supplementation in the growth medium (Chambers *et al*. 2003, Mitsui *et al*. 2003). *Sox-2* is a regulatory transcription factor expressed in early development in many tissues including ESC (Avilion *et al*. 2003). The co-expression of these stemness genes in this study confirmed that AFSC included multipotent, committed and differentiated stem cells.

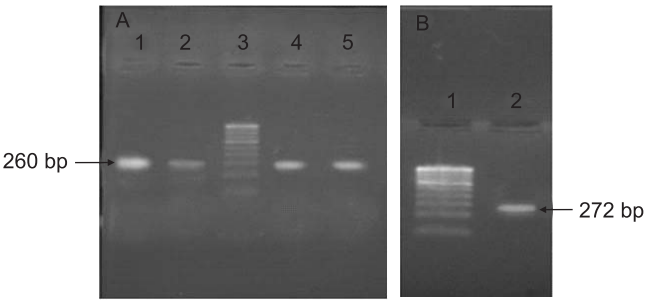

Fig.3. Gene expression in osteogenic differentiated and undifferentiated AFSC260bp. a) Lane 1 - 260bp *Sox-2* gene amplicon in osteogenic differentiated cells; Lane 2 - 260bp *Sox* 2 gene amplicon in undifferentiated cells; Lane 3 - 100bp DNA marker; Lane 4 - 262bp *Oct- 4* gene amplicon in osteogenic differentiated cells. Lane5 - 262bp *Oct- 4* gene amplicon in undifferentiated cells. b) Lane 1 - 100bp DNA marker; Lane 2 - 272bp *Nanog* gene amplicon in osteogenic differentiated cells.

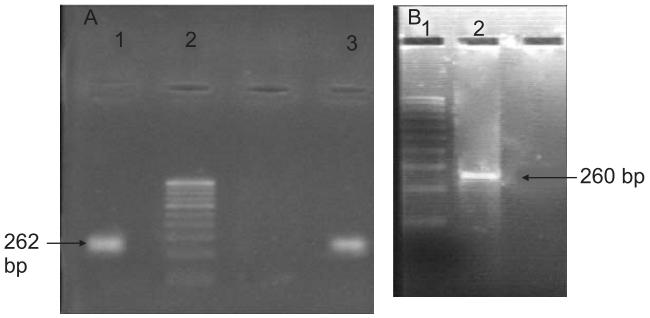

Fig.4. Gene expression in chondrogenic differentiated and undifferentiated AFSC. a) Lane 1, 3 - 262bp *Oct- 4* gene amplicon in chondrogenic differentiated cells; Lane 2 - 100bp DNA marker b) Lane 1–100bp DNA marker; Lane 2 - 260 bp *Sox-2* gene amplicon in chondrogenic differentiated cells.

In conclusion, we have demonstrated that adult stem cell from buffalo exhibited high proliferation rates and multilineage differentiation potential. With the approach reported in this study it could be possible to obtain single cell derived, clonally expanded amniotic fluid stem cells with remarkable potential to differentiate into multiple lineages. With these techniques, the application of AFSC can be further extended and can be used as an alternative source of adult stem cells to bone marrow and applicable to cell transplantation and regenerative medicine. In addition, these cells can be easily harvested and isolated, thereby making them attractive tools for further studies for physiological and for clinical applications.

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