STUDIES ON IN-VITRO CULTURING OF BUFFALO *(BUBALUS BUBALIS)* FETAL FIBROBLAST STEM CELLS

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ABSTRACT

The present study describes the isolation, in-vitro culturing and characterization of fetal stem cells from buffalo (Bubalus bubalis). During early stages of culture, buffalo fetal fibroblast (BFF) cells had heterogeneous morphology which developed into uniform fibroblast morphology later. Single cell derived BFF stem cell (BFFSC) clones were assessed for their osteogenic, chondrogenic and adipogenic potentials using specific differentiation medium. BFFSCs differentiated into osteogenic, chondrogenic and adipogenic lineages as observed by special staining. Total RNA was isolated from BFFSCs and checked for the presence of the three stem cell markers namely, Oct-4, Sox-2 and Nanog, and all three transcription factor genes were detected in differentiated BFFSCs.

Key words: Stem cells, Buffalo fetal fibroblast stem cells

INTRODUCTION

Stem Cells (SC) are a special group of undifferentiated cells capable of self-maintenance and differentiating into specialized tissue forming cells. Stem cells can be classified into adult, fetal and embryonic stem cells based on their time of isolation during ontogenesis. Adult stem cells do not differentiate spontaneously but can be induced to differentiate by applying appropriate growth conditions.

Adult stem cells have the disadvantage of not being immortal and most of them lose their pluripotency after a defined number of passages under in-vitro culture. This short life span may be a problem for clinical applications where a large amount of cells are needed (Musina *et al*., 2004). 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 have been found to be rich sources of mesenchymal stem cells (Campagnoli *et al*., 2001). Fetal tissues present different cellular types in its composition and a specific group of these cells shows multipotent characteristics as high proliferation ratio and increased differentiation ability. There is a paucity of information on the isolation and characterization of stem cells from domestic animals, especially from buffalo. In this study, we isolated and characterized fetal stem cells from buffalo (Bubalus bubalis) to check their differentiation potential in-vitro and evaluated their plasticity.

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MATERIALS AND METHODS

Specimen collection

Gravid uterus from buffaloes (Bubalus bubalis) was collected irrespective of age from the abattoir attached to the Chennai (India) corporation, within 30 min of slaughter and washed in phosphate buffered saline (PBS) supplemented with antibiotics (Penicillin 500 IU/ml and Streptomycin (500 mg/ml) to remove blood and extraneous material. The washed uterus was transported in PBS to the laboratory.

Culturing of fetal fibroblast cells

Primary fibroblasts were isolated from fetal tissues by trypsinization employing standard procedures. Cells were counted after trypan blue staining and cell density was adjusted to 106 cells /ml. Primary BFF cells were cultured in α-MEM with 20% fetal bovine serum. The cell suspension was distributed into suitable culture vessels at 106 cells /ml and incubated at 37°C. Cultures

were observed every 24 hr for their growth and stained with hematoxylin and eosin (HE) to study cell the morphology. Single cell clones were established by limiting dilution and subjected to serial passages.

In-vitro differentiation of single cell-derived clonal BFF stem cells (BFFSCs)

Single cell derived clonal BFFSCs were grown to 70%-90% confluence and shifted to osteogenic medium (RPMI1640 supplemented with 20% FBS, 0.1µg dexamethasone, 10mM glycerol phosphate, 0.2mM ascorbic acid), chondrogenic medium (RPMI1640 supplemeted with 20% FBS, 0.1µg dexamethasone, 50µg/ml ascorbic acid, 100µg/ml sodium pyruvate, 40µg/ml proline, 6.25µg/ml insulin transferring sulfate (ITS)), and adipogenic medium (RPMI1640 supplemented with 20% FBS, 0.5mM 3-isobutyl1-methylxanthine (IBMX), 1µg hydrocortisone, 0.1µg indomethacin) for three weeks. The differentiation potential for osteogenesis was assessed by the mineralization of calcium accumulation by alizarin red S staining. For adipogenic differentiation the cells were stained with oil red O stain for marked lipid droplet deposition. For chondrogenic differentiation, cells were subjected to toludine blue S staining.

Expression of marker genes

Total RNA was extracted from the clonal BFFSC using Trizol reagent (Invitrogen, USA). RT-PCR was performed by using the one step RT-PCR kit (Bangalore Genei, Bangalore) with gene specific primers for Nanog (272bp) Forward 5' GAATTCTGTACCACTGCCCC3', Reverse 5'AGCCTCCCTATCCCAGAAAA3', Sox-2 (260bp)Forward 5' TCAAGCCGTTTATCGAGAGG3', Reverse 5' ATCATGCTGTAGCTGCCGTT3', and Oct-4 (262bp) Forward 5' TGCTGCAGAAGTGGGTGGAG-GAAG3' Reverse 5' CCGAGCTGCTGGGCGATGTG3'. The reaction mixture consisted of 5µl of cDNA, 1µl each of forward and reverse primers and 13µl of prime Taq premix (Blue dye master mix, Bioscience) with the following cycle conditions for specific genes.

Initial denaturation of 95°C for 5 min followed by 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) with 10 minutes at 72°C for final extension. The amplicons were visualized in 1.5% agarose gels.

RESULTS AND DISCUSSION

Culturing of cells

In this study, we explored fetal stem cell characterization and their multi lineage potential. Initially, the morphology of primary BFF cells was found to be heterogeneous which then developed into uniform fibroblast morphology. HE staining revealed spindle shaped cells (Fig 1) and single cell derived stem cell clones of BFF cells were obtained from 1st and 2nd passaged cells. The capacity of fetal explant tissues cells to differentiate into osteoblasts that produce mineralized matrices, chondrocytes that produce chondroitin sulfate and adipocytes that accumulate lipid vacuoles under in vitro conditions was assessed by specific staining and PCR.

In-vitro differentiation of osteocytes, chondrocytes and adipocytes from buffalo BFFSCs

Lineage differentiation potential of the BFFSC was assessed by culturing the cells in differentiation media for three weeks. When the cells were exposed to osteogenic differentiation medium, presence of extensive cellular calcium deposits were noticed in the cells when stained with alizarin red S which resulted in the spindle shaped BFFSC forming a mineralized matrix (Fig 2 A and B). The results of this study indicated that BFFSC differentiated into osteocytes when stimulated with specific medium.

When the cells were exposed to chondrogenic medium, the cells differentiated into chondrogenic lineage with the synthesis of chondrotin sulphate as evident from toludine blue staining (Fig 2 C and D). Close cell-to-cell contact and certain chondrogenic bioactive factors are the two main principles involved in enhancing chondrogenic differentiation. The best method to obtain strong cell-to-cell interaction is to culture the cells in a pellet or micro mass culture system (Johnstone *et al.*, 1998, Jones *et al.*, 2002). The bioactive factors that stimulate chondrogenesis include TGF-β1, bone morphogenic protein, fibroblast growth factor, and insulin-like growth factor (Mastrogiacomo *et al.*, 2001). In the present study, we used bioactive factors like TGF-β1, ITS, proline, dexamethsone and sodium pyruvate in culture medium for chondrogenic differentiation which gave good results.

Similarly, the cells on exposure to adipogenic medium differentiated into larger cells with the presence of lipid vacuoles and took orange colour when stained with Oil red O (Fig 2 E and F). Adipogenic differentiation was induced by using IBMX, indomethacin and hydrocortisone, a routinely used method to stimulate the differentiation of pre-adipocytes and stem cells. The mechanism of action of stimulants is not completely understood; however, certain studies indicate that the

effect is based on the mediation of cAMP metabolism (Gregoire *et al.*, 1998). 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.

Expression of marker genes

Total RNA was isolated from BFFSC and checked for the presence of the three stem cell markers namely, Oct-4, Sox-2, and Nanog. All three transcription factor genes were detected in differentiated BFFSC (Fig 3).

The importance of Oct-4, Sox -2 and Nanog genes for the maintainence of stem cells has been clearly established. Each established pluripotent stem cell line expresses Oct-4, Sox-2 and Nanog which reappears when cells start to differentiate (Pesce and Scholer *et al.*, 2001, Chambers *et al.*, 2003). Accordingly we have analyzed BFFSC for the expression of these markers. For RT-PCR amplification of Oct-4, Sox-2 and Nanog mRNA, three different sets of oligonucleotides were used. The results indicated that, differentiated BFFSC were found positive for Oct-4, Sox-2 and Nanog, characteristics of embryonic adult stem cells, thus suggesting their possible multipotentiality. The expression of Nanog, Oct-4 and Sox-2 was detected by RT-PCR. The co-expression of these stemness genes in this study confirmed that BFFSC included multipotent, committed and differentiated stem cells.

This is a preliminary study aimed at isolation and culturing of stem cells from buffalo fetal tissues. In conclusion, we have demonstrated that fetal stem cells 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 fetal fibroblast stem cells with good potential to differentiate into multiple lineages. With these techniques, the application of BFFSC 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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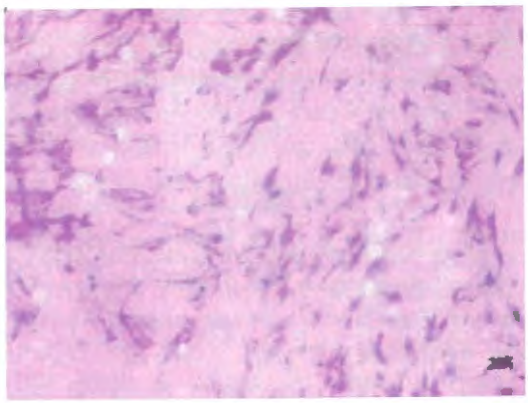

Fig 1. Undifferentiated clonal BFFSC stained with hematoxylin and eosin showing spindle shaped cells.

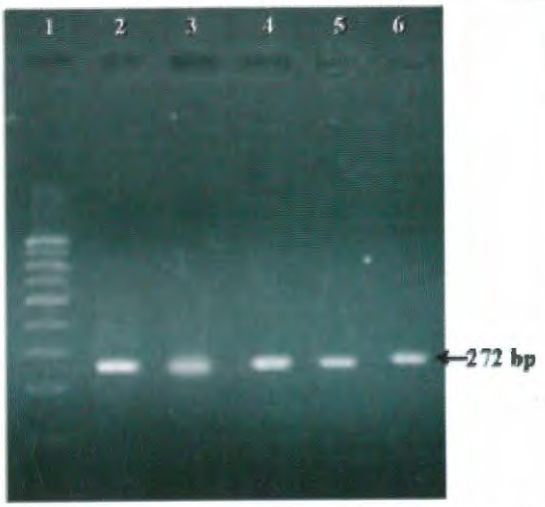

Fig 3. Gene expression in osteogenic differentiated and undifferentiated BFFSC. Lane 1 - 100bp DNA marker. Lane 2 - 260bp Sox-2 gene amplicon in osteogenic differentiated cells . Lane 3 - 260bp Sox-2 gene amplicon in undifferentiated cells. Lane 4- 262bp Oct-4gene amplicon in osteogenic differentiated cells. Lane 5 - 262bp Oct-4 gene amplicon in undifferentiated cells. Lane 6 - 272bp Nanog gene amplicon in osteogenic differentiated cells.

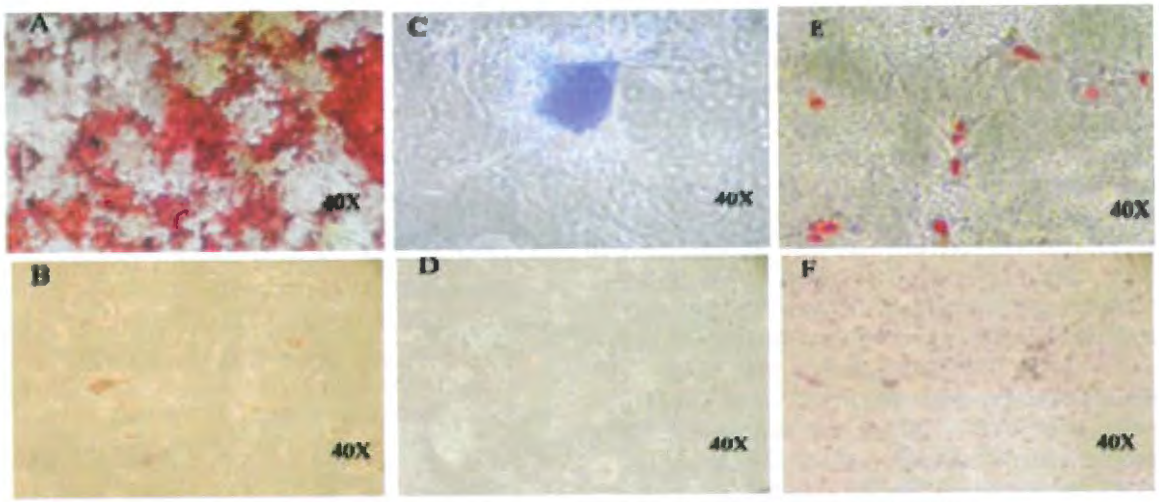

Fig 2 : Differentiated BFFSC into osteocytes, chondrocytes and adipocytes

(a) Osteogenic induction after 21 days showed marked morphological changes and extensive extra cellular calcium deposition as demonstrated by positive Alizarin red S staining. (b) Osteogenic control after 21 days in regular expansion media (MEM) maintained normal morphology and stained negative for Alizarin Red S. (c) Toludine blue stain of chondrogenic differentiated BFFSC positive for chondritin sulfate. (d) Chondrogenic control after 21 days in regular expansion media (MEM) maintained normal morphology and stained negative for Toludine blue (e) Positive adipogenic differentiation after culture in adipogenic media as demonstrated by morphological changes towards larger cells with marked lipid droplet deposition stained with Oil Red O. (e) Adipogenic control culture after exposure to regular expansion media showing maintenance of undifferentiated morphology and minor lipid droplet deposition.