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Expression of cellular stress marker genes in caprine amniotic fluid derived mesenchymal stem cells

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Abstract
Stem cells are having potential applications in regenerative medicine due to their self- renewal and multilineage differentiation capability. *In vitro* cell culture induces environmental stress and leads to expression of cellular stress markers. In present study, we isolated and characterized caprine amniotic fluid derived mesenchymal stem cells (cAF- MSCs) to investigate the expression of cellular stress marker genes. Third passage cAF- MSCs were successfully differentiated in osteogenic and adipogenic lineages and it was confirmed by Alzarin red and Oil red O staining respectively. Total RNA was isolated from cAF-MSCs and cDNA was synthesized by M-MLV reverse transcriptase PCR to study the stress marker genes expression. Gene specific primers were synthesized and PCR conditions were standardized to study the expression of heat shock proteins 70 (HSP 70), heat shock proteins 90 (HSP 90) and Cu-Zn superoxide dismutase (Cu-Zn SOD) genes. Results revealed that the cAF-MSCs express all the three genes involved in cell stress defence mechanism including chaperone proteins (heat shock proteins HSP 70 and HSP 90) and regulation of oxidative stress (Cu-Zn superoxide dismutase). The expression of cellular stress marker genes in cAF-MSCs confirms that the cells are under environmental stress in *in vitro* culture conditions.

Keywords: Stem cell, amniotic fluid, stress markers, HSP 70, HSP 90, Cu-Zn SOD

Introduction
Progenitor cells well known as stem cells can differentiate into variety of cells with their unique ability of self-renewal (He *et al.*, 2009; Seita and Weissman, 2010) [9, 22]. Caprine bone marrow mesenchymal stem cells (cBM-MSC) have been identified and employed in different therapeutic models (Nair *et al.*, 2009; Quintavalla *et al.*, 2002) [15,20]. These multipotent stem cells can differentiate into various cell types including fibroblasts, osteoblasts, chondrocytes, adipocytes, myocardial cells, vascular endothelial cells, neurons,
hepatocytes, and epithelial cells (Jiang *et al.*, 2012; Li and Fu, 2012) [10, 13]. As a potential substitute for embryonic and adult stem cells, fetal adnexa-derived tissues stem cells have recently gained attention. They are a desirable choice in regenerative medicine and tissue engineering investigations due to their foetal characteristics, abundant developmental potential, and apparent absence of tumorigenicity (Marcus and Woodbury, 2008) [14]. Amniotic fluid stem (AFS) cells have been discovered within the diverse population of amniotic fluid cells, a class of multipotent cells. Mesenchymal stem cells (MSCs) can be obtained from amniotic fluid (AF) in an inexpensive and non-invasive manner. Because of their plasticity, low immunogenicity and great anti-inflammatory capacity, presumed stem cells produced from these tissues in the Veterinary industry are demonstrated as candidates for the therapeutics (Corradetti *et al.*, 2013) [3]. These cells are very appealing to practitioners and researchers in the field of regenerative medicine because of these characteristics. The amniotic fluid is a significant alternate source of foetal stem cells since it is extra-embryonic and offers relatively simple access for cell harvesting (De Coppi *et al.*, 2007b; Prusa *et al.*, 2003) [5, 18]. Recent developments have succeeded in mimicking *in vivo* culture conditions *in vitro* however it is not precisely the same. *In vitro* conditions are responsible for cellular stress and certain stress marker protiens are expressed in these conditions which protects cell. Heat shock proteins family is highly conserved proteins expressed in response to alcohol, metals, and heat shock (HS) can all activate HSF1, which then raises cellular HSPs (i.e., HSP 40, HSP 70, and HSP 90 (Quintana and Cohen, 2005; Ritossa, 1996) [19, 21]. It is well reported that HSPs act as molecular chaperones and have significant impact on stem cells (Fan, 2012) [6]. Certain HSPs which interact with transcription factors and are abundantly expressed in embryonic stem cells (ESCs) are crucial for healthy cell growth and function (Sreedhar *et al.*, 2004) [25].

Cu-Zn superoxide dismutase also plays key role in regulation of oxidative stress. Common cellular stress markers include heat shock protein 70 (HSP 70), heat shock protein 90 (HSP 90) and Cu-Zn Superoxide dismutase (Cu-Zn SOD). Therefore, we have designed this study to investigate the expression of cell stress marker genes (HSP 70, HSP 90 and Cu-Zn SOD) in caprine amniotic fluid derived mesenchymal stem cells.

Material and Method

Present study was conducted in Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, Anjora, Durg, Chhattisgarh, India.

Collection and Processing of Amniotic Fluid

Gravid caprine uteri were collected immediately after slaughter from local abattoir aseptically in normal saline. Samples were transported to Stem Cell Culture Laboratory, Department of Veterinary Physiology and Biochemistry and processed within 1 hr. Gravid uteri were washed thrice with normal saline at 37 °C and caprine amniotic fluid was collected from the amniotic cavity aseptically with syringe. The amniotic fluid was centrifuged at 1200 rpm for 10 min and cell pellet was washed twice with PBS followed by final washing with serum fortified Dulbecco's Modified Eagle's Medium (DMEM).

Isolation and Culture of Caprine AF-MSCs

Cells were gently suspended and plated in tissue culture flasks with DMEM medium supplemented with fetal calf serum and incubated in 5% CO_2 incubator at 37 °C. Floating cells were discarded and media was replaced and replenished periodically. Adherent polygonal fibroid cells were observed and recorded periodically under inverted microscope. Culture media was changed after all 4th days and cells were passaged on day 14. Third passage caprine amniotic fluid derived MSCs (cAF-MSCs) were characterized and used for gene expression study.

Characterization of cAF-MSCs

Third passaged caprine cAF-MSCs were cultured in osteogenic differentiation media and after 14 days osteoblasts were stained with Alzarin red staining. Similarly cAF-MSCs were also differentiated in adipogenic lineage by incubation in adipogenic differentiation media (Himedia) and Oil red O staining at day 14 (Baghaban *et al*., 2008) [1].

Expression of Stress Marker Genes

Third passage caprine AF-MSCs were cultured and used evaluation of stress marker gene expression.

RNA extraction and cDNA synthesis

Total RNA extraction was performed using TRIZOL LS reagent (Invitrogen) according to manufacturer instructions. The RNA was treated with DNase to remove any possible DNA contamination. The purity and concentration of total RNA was checked using Nanodrop/DS-11 + Spectrophotometer (DeNovix). The isolated RNA samples were screened for the protein contamination by measuring ratio of OD 260/280. Total RNA was then reverse-transcribed and first strand cDNA was synthesized by using M-MLV Reverse Transcriptase (Lot. No- 28025013, Invitrogen) as per protocol.

Primers

Published primers were used for HSP70, HSP90, Cu-SOD and GAPDH. Details of the primers used, annealing temperature and accession no. are provided in Table 1.

Table 1: Details of primer sequence of genes with amplicon size and annealing temperature

Gene	Sequences 5'- 3'	Amplicon length (bp)	Annealing temperature (°C)	Accession No.
HSP70	F 5′AGCAAGCCAGCACAGCA 3′ R 5′GCGATGATTTCCACCTTC 3′	92	60 °C	AJ305315
HSP90	F 5′GGAGAGCAAAACCCTCACC 3′ R 5′ TGGCAATGGTTCCAAGGT 3′	83	60 °C	AJ431681
Cu-Zn SOD	F 5′AACCCCTTCAACAAAGAGCA 3′ R 5′ TTTGGCGACACCGTCTTC 3′	96	60 °C	AJ496219
GAPDH	F 5'GGGTCATCATCTCTGCACCT3 R 5'GGTCATAAGTCCCTCCACGA3'	176	60 °C	NM 001034034.2

Polymerase chain reaction

One microgram of total RNA was then reverse transcribed. PCR reactions were performed with a total volume of 25µl containing 12.5 µl PCR master mix (Dream Taq Hot Start Green PCR Master Mix, Lot No. 00520533, Thermo Scientific), 1 µl each of 0.5 mM gene-specific forward and reverse primer, 2 µl of template cDNA and 8.5 µl nuclease free water. PCR amplification was performed in thermocycler (T100 Thermal Cycler, BIORAD) at an initial 2 min denaturation step at 95˚C which was followed by 40 amplification cycles including denaturation at 94˚C for 30 sec, annealing at 60˚C temperature for 25 sec and extension at 72˚C for 12 sec followed by final extension step at 72˚C for 3 min. The PCR reaction was ended with hold at 4 ˚C and products were stored further at -20°C or visualized.

Agarose gel electrophoresis

The confirmation of gene specific PCR amplicon was done by agarose gel electrophoresis. Amplified products were electrophoresed on 2.5% agarose gel stained with ethidium bromide in 1X TBE buffer initially at 100 V for 10 min followed by 80V for 30-45min. HSP70, HSP90, Cu-SOD and GAPDH gene amplified products were compared with 100bp DNA ladder and confirmed by expected amplicon size. Stained gene specific amplified product bands were visualized and recorded using a Gel Documentation System (BioRad).

Result and Discussion

Isolation and Characterization of cAF-MSCs

Mesenchymal Stem cells were successfully isolated and expanded *in vitro* and cAF-MSCs exhibited fibroblast-like morphology and displayed high nucleus-to-cytoplasm ratios. cAF- MSCs are plastic adherent and exhibited heterogenous

morphology however, mostly fibroblastoid like cells were observed and reached confluent stage on day 14 (Figure 1A). AF-MSCs were successfully isolated and cultured till third passage and phenotypically characterized by Alzarin red (figure 1B) and Oil red O staining (figure 1C) and confirmed their osteocytes and adipocytes differentiation capability. Observations in this study are in accordance with Pratheesh *et al*., 2017 [16], the amniotic fluid cells represent a heterogeneous population originated from the three germ layers. These cells share an epithelial origin and are derived from either the developing fetus or the inner surface of the amniotic membrane and resulted that fibroblastoid spindle shaped cell population could be isolated from the caprine amniotic fluid aspirates in their *in vitro* culture. Pratheesh *et al*., 2013 [17], previously reported that caprine AF-MSCs (cAF-MSCs) exhibit embryonic stem cell properties based on their pluripotency specific marker expression and also possess the potential to differentiate into osteo, adipo and chondrogenic lineages. Adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatogenic lineages are just a few of the many cell types that can develop from mesenchymal cells derived from amniotic fluid, which are also thought to be highly multipotent and capable of differentiating into all embryonic germ layers (De Coppi *et al*., 2007a) [4]. Osteogenesis was observed in induced AF-bMSCs by ALZ staining which is at par with previously reported findings in bovines (Jurek *et al*., 2020) [12]. Similarly, induced WJ-bMSCs were also confirmed for osteogenic lineage differentiation which has also been reported in adipose-derived bMSCs (Sreekumar *et al*., 2014) [26].

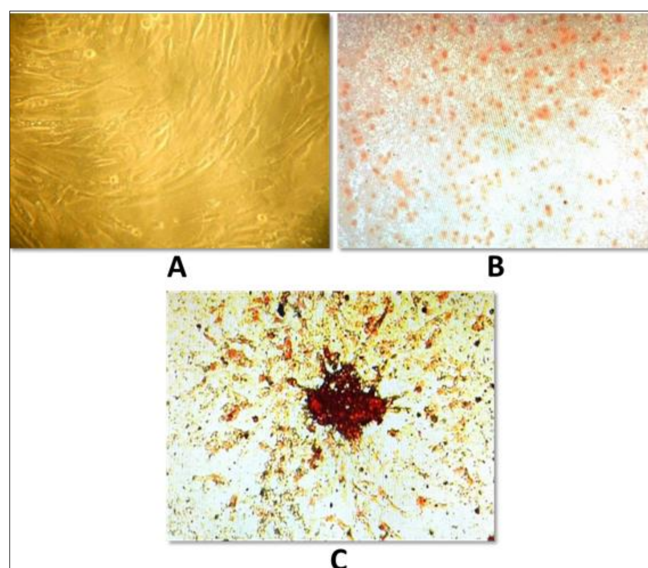

Fig 1: Isolation and Characterization of Caprine AF-MSCs (A. Confluent monolayer of cAF-MSCs on day 14, B. Alazirin Red Staining in Osteoblasts C. Oil red O Staining in Adipocytes

Somal *et al*. (2016) [23] isolated and *in vitro* expanded MSCs derived from caprine fetal adnexa {amniotic fluid (cAF), amniotic sac (cAS), Wharton's jelly (cWJ) and cord blood (cCB)} and studied *in vitro* growth kinetics, phenotypic properties and also their tri-lineage differentiation potential. Caprine fetal adnexa derived MSCs showed spindle shape resembled typical fibroblastoid morphology, stained positively to ALP, differentiated into osteogenic, chondrogenic and adipogenic lineages. Spitzhorn *et al*. (2017) [24] found that mesenchymal stem cells are present in heterogeneous populations in the amniotic fluid (AF), which is typically acquired during amniocentesis (MSCs). The cells had a spindle-shaped, fibroblast-like appearance, and their ability to differentiate into adipogenic, chondrogenic, and osteogenic differentiation was further studied. Oil Red O solution was used to stain developing fat droplets, which helped to reveal that the cells had successfully differentiated into adipocytes.

Expression of Stress Marker Genes in cAF-MSCs

The expression of stress marker genes in cAF-MSCs is shown in Figure 2, amplicon of 96 bp, 83 bp and 92 bp by PCR correspond to Cu- Zn SOD, HSP90 and HSP70 genes respectively. GAPDH was used as housekeeping gene and confirmation of gene specific PCR amplicon was done by agarose gel electrophoresis. It is observed that three stress marker genes HSP70, HSP90 and Cu- Zn SOD are expressed in caprine amniotic fluid derived mesenchymal stem cells. Similarly, Farcy *et al*. (2009) [7] investigated the transcriptional expression level of some genes involved in cell stress defence mechanisms, including chaperone proteins (heat shock proteins Hsp70, Hsp72 and Hsp90 (HSP)), regulation of oxidative stress (Cu-Zn superoxide dismutase, metallothionein (MT), in response to thermal stress. Gao *et al*. (2015) [8] determined the function of Hsp90 in controlling the migration of mesenchymal stem cells (MSCs), CXCR4 and VCAM-1 gene expression were elevated in MSC that had been treated with rhHsp90. Chen *et al*. (2015) [2] demonstrated that HSP70 (200 ng/ml) boosts hMSC mineralization and alkaline phosphatase activity. HSP70 dramatically increased the expression of osteo-specific genes, including the Runt family transcription factor Runx2 and osterix, under osteogenic induction conditions (OSX). Jin *et al*. (2020) [11] determined that MSCs overexpressing Hsp70 might increase their capacity and have a positive therapeutic effect on acute lung injury caused by phosgene (ALI). They used HSP70 to transduce MSCs, and after that, they examined how well the MSCs performed.

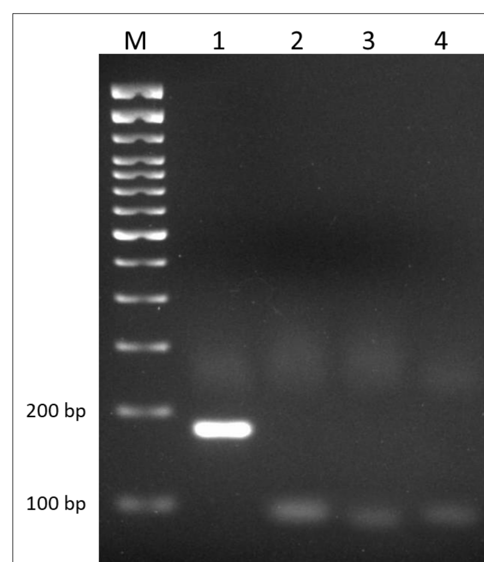

Fig 2: Agarose gel electrophoresis [M: 100bp ladder, 1: GAPDH gene (176 bp), 2: Cu-Zn SOD gene (96 bp), 3: HSP90 gene (83 bp) and 4: HSP70 gene (92 bp)]

Conclusion

Caprine AF-MSCs are plastic adherent fibroblast like cells that can differentiate into osteocytes and adipocytes. Cellular stress marker genes Cu-Zn SOD, HSP90 and HSP70 are expressed in caprine amniotic fluid derived mesenchymal stem cells. This reveals that *in vitro* culture conditions are stressful for cAF-MSCs and needs further modifications in microenvironment.

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Conflicts of interest

None declared.

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