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Comparative study on characterization and wound healing potential of goat (*Capra hircus*) mesenchymal stem cells derived from fetal origin amniotic fluid and adult bone marrow

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ABSTRACT

Caprine amniotic fluid (cAF) and bone marrow cells (cBM) were isolated, expanded and phenotypically characterized by mesenchymal stem cells (MSCs) specific cell surface markers. Both cell types were compared for multilineage differentiation potential by flow cytometry using specific antibodies against lineage specific markers. Furthermore, *in vitro* expanded cAF-MSCs showed higher expression of trophic factors *viz.* VEGF and TGF-β1 as compared to cBM-MSCs. Full-skin thickness excisional wounds created on either side of the dorsal midline (thoracolumbar) of New Zealand White rabbits were randomly assigned to subcutaneous injection of either fetal origin cAF-MSCs ($n = 4$) or adult cBM-MSCs ($n = 4$) or sterile PBS (control, $n = 4$). The rate of wound closure was found faster ($p < 0.05$) in cAF-MSCs treated wounds as compared with cBM-MSCs and PBS treated wounds especially on 21st day post-skin excision. Histomorphological examination of the healing tissue showed that wound healing was improved ($p < 0.05$) by greater epithelialization, neovascularization and collagen development in cAF-MSCs as compared to cBM-MSCs and PBS treated wounds.

1. Introduction

Stem cell therapy in veterinary regenerative medicine has evolved rapidly both experimentally and clinically. Caprine bone marrow mesenchymal stem cells (cBM-MSC) have been isolated, characterized and used in various therapy models, especially in bone repair and regeneration studies (Nair et al., 2009; Quintavalla et al., 2002). These multipotent stem cells are capable of differentiation into numerous 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). However, bone marrow collection requires invasive procedures, and yields comparatively a low number of stem cells upon processing than from fetal adnexa derived tissues (Pittenger et al., 1999; Pratheesh et al., 2014).

In recent years, stem cells from extra-embryonic/fetal adnexa derived tissues have emerged as a potential alternative to embryonic and adult stem cells. Their fetal properties, developmental potential and apparent lack of tumorigenicity make them an attractive option for regenerative medicine in cell therapy and tissue engineering studies (Marcus and Woodbury, 2008). Amniotic fluid cell population (AF-MSC) can successfully differentiate into connective tissue lineages and show favorable trophic support and immune-modulatory activities that could enhance self-regeneration (Antonucci et al., 2012; Joo et al., 2012; Soler et al., 2012), similar to MSCs derived from bone marrow(Pittenger et al., 1999) and adipose tissue (Mosna et al. 2010; Strioga et al. 2012). Since AF-MSCs are of fetal/extraembryonic origin, they lack or exhibit very low expression of highly polymorphic MHC class I molecules. Moreover, the AF-MSCs do not express surface MHC class II molecules and co-stimulatory molecules, such as CD40, CD40R, CD80, and CD86, thereby fail to induce an allogeneic or xenogeneic immune response in transplantation studies (Sarugaser et al., 2005; Kim et al., 2007). *In vivo* administration

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of amniotic fluid-derived cells had favorable effect in various injury models, including acute bladder injury (De Coppi et al., 2007), ischemic heart (Bollini et al., 2011), hyperoxic lung injury (Carraro et al., 2008) and acute tubular necrosis of the kidney (Perin et al., 2010). Apart from their extended *in vitro* life time (up to 250 passages), amniotic fluid stem cells demonstrate high trans-differentiation and angio-vasculogenic potential in organ tissues (Tsuji et al., 2010; Zhao et al., 2005; Kim et al., 2012). Paracrine factors released by the MSCs into the culture medium significantly enhanced the migration of macrophages, keratinocytes and endothelial cells and proliferation of keratinocytes and endothelial cells compared to fibroblast-conditioned medium(Chen et al., 2008).

In the recent past, bioengineered dressings and cell-based products have been utilized for various clinical applications in wound healing therapy. However, they could not produce significant improvement clinically and chronic wounds continued to be a serious medical problem. The role of MSCs in wound healing and the demonstration of 'MSC niche' within normal skin propose that the exogenous MSC administration is a hopeful solution to treat the non healing wounds (Kramperaa et al., 2006; Paquet-Fifield et al., 2009). Numerous reports demonstrate that, therapy using adult MSCs, both from bone marrow or adipose tissue, when applied either topically or systemically showed a convincing clinical advancement in the treatment of chronic wounds (Lu et al., 2011; Lee et al., 2012). Transcriptome analysis revealed that fetal origin stem cells in humans express more cell cycle genes in addition to genes involved in vasculogenesis, neurogenesis, Wnt, MAPKKK pathways compared to adult BM derived stem cells (Weng et al., 2011).

We have 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 (Pratheesh et al., 2013). These characteristics suggested that cAF-MSCs might be an ideal cell source and alternative to bone marrow derived MSCs (BM-MSCs) for stem cell therapy applications especially in veterinary regenerative medicine. Therefore, present study was designed to compare the wound healing potential of cAF-MSCs and cBM-MSCs upon xenogenic application over full skin thickness wounds induced in rabbit model.

2. Materials and methods

All the chemicals used in this study were procured from Sigma (St Louis, MO, USA), unless otherwise indicated. The primary and secondary antibodies used for flow cytometry were purchased from Santa Cruz, Biotechnology (Santa Cruz, USA).

2.1. Isolation and culture of caprine amniotic fluid cells

Gravid uteri (n = 4) of goats at late gestation period (~2–3 months, based on fetal length and placentome size; Metodiev et al., 2012) were collected from local abattoir and transported in isotonic saline fortified with 1% of penicillin/streptomycin within 2 h to the Reproductive Physiology laboratory at the ICAR- Indian Veterinary Research Institute, Izatnagar India. Fetus and membranes were located and AF was aspirated aseptically with the help of 20 mL syringe fitted with 18G hypodermic needle. 30–40 mL of AF was collected *via* suction and centrifuged at 3000*g* for 20 min. The resulting pellet obtained was washed 2–3 times with phosphate buffered saline (PBS) with a final wash in Dulbecco's Modified Eagle's Medium (DMEM) fortified with serum.

2.2. Isolation and culture of caprine bone marrow cells

Adult healthy goats of either sex were used for bone marrow collection. Collection procedure was done under epidural anesthesia using 2% Lignocaine. All the procedures were in compliance with the guidelines of Institutional Animal Ethics Committee (IAEC). Bone marrow was aspirated with 16 G bone marrow biopsy needle from the aseptically prepared Iliac crest area. 5 mL of bone marrow aspirate was collected in a 10 mL heparinised syringe. The collected bone marrow samples were diluted with equal amount of sterile PBS and the mononuclear cells were isolated by density gradient method using histopaque 1077 as per manufacturer's instruction (Gade et al., 2012).

2.3. In vitro culture of caprine fetal and adult cells

The isolated cells from caprine amniotic fluid and bone marrow aspirates were seeded at a density of 10^3 cells/cm^2 in a 6 well culture plates containing DMEM supplemented with 15% FBS and 1% penicillin/streptomycin. Cultured plates were incubated in humidified CO_2 incubator at 37 °C in the presence of 5% CO_2 in humidified air. The cells were allowed to grow and were sub-cultured by passaging after achieving >80% confluency. For all the experiments cells were taken from the third passage.

2.4. Phenotypic characterization of MSCs

In vitro cultured cAF and cBM-cells were checked for their specific MSC markers by flow cytometry. Briefly, a single cell suspension of 1.0×10^6 cells/mL was placed in 100 μL of PBS supplemented with 2% FBS and was incubated with primary antibodies against CD-73, CD-105, Stro-1 and CD-34 (goat polyclonal, Santacruz Biotechnology, CA, USA) for 45 min. Thereafter the cells were washed with PBS, and were further incubated with FITC conjugated secondary antibodies (donkey anti-goat, Santacruz Biotechnology, CA, USA) for 40 min in the dark chamber. Finally stained cells were washed and re-suspended in PBS containing 2% FBS. Cell fluorescence was evaluated immediately in a FACS Calibur flow cytometer (Becton Dickinson, CA, USA), and the data was analyzed using Cell Quest software (Becton Dickinson, CA, USA). Isotype-identical antibodies served as controls.

2.5. Differentiation assay

2.5.1. Osteogenic differentiation assay

For osteogenic differentiation, the cells from either source were seeded at a density of 10,000 cells per well, and after 24 h, the expansion medium was replaced by the osteogenic differentiation medium (DMEM containing 15% FBS, 1% penicillin/streptomycin, 100 nM dexamethasone, 10 mM b-glycerophosphate and 0.05 mM L-ascorbic acid-2-phosphate). All cells were cultured for 21 days with medium changes every 3–4 day, and calcium deposition was evaluated by von Kossa staining. Cells maintained in regular growth medium were used as negative control.

2.5.2. Adipogenic differentiation assay

Adipogenesis differentiation kit (StemPro, Gibco) was used as per manufacturer's instructions for the adipogenic induction of cAF-MSCs and cBM-MSCs. MSCs from either source were cultured in specific induction media for 21 days, media were changed every third day, and differentiation was assessed by the presence of lipid droplets that were recorded after staining with Oil Red O stain.

2.5.3. Chondrogenic differentiation assay

Chondrogenic differentiation was induced in confluent monolayer cultures of cAF-MSCs and cBM-MSCs using a specific chondrogenesis differentiation kit (StemPro, Invitrogen). Differentiated cells were stained with Alcian Blue 8X (Sigma–Aldrich) after 18–21 days. Negative controls were prepared in a similar way except for the growth media in place of induction media.

2.6. Comparison of multilineage differentiation capacity by flow cytometry analysis of lineage specific markers

Cells of either source were *in vitro* expanded and differentiated in 75 cm^2 flasks with regular replacement of corresponding induction