International Journal of Animal Biotechnology
ISSN: 2277 –4122

Available at http://inpressco.com/category/ijcsb

Original Research Article

Isolation, culturing and cryopreservation of putative amniotic fluid stem cells in sheep (*Ovis aries*)

Anil Khuttan[Á*], Kapil Dev[Á], Birbal Singh[B̂] and Sanjeev K. Gautam[Á*]

[Á]Department of Biotechnology, Kurukshetra University, Kurukshetra -136 119 Haryana India.
[B̂]Regional Station, Indian Veterinary Research Institute, Palampur-176 061 H.P. India.

Accepted Aug 14, 2012, Available online Oct 6, 2013, **Vol.3 (2013)**

Abstract

The current study was carried out to isolate, culture and cryopreserve the putative amniotic fluid stem (AFS) cells in sheep. AFS cells were cultured without feeder cells in DMEM containing 16% FBS, 1% penicillin/streptomycin and 1% L-glutamine in 5% CO2 in humidified air at 37±0.5˚C. After 6 days of culture different morphologies of cells were observed. Most of the cells started anchorage-dependent growth after day 7 of the culture. The putative AFS cells were cryopreserved using slow freezing method using DMSO as cryoprotectant. After freezing the cells for one week, it was observed that 70% of the cells were viable after trypan blue staining. This study shows that the putative amniotic fluid stem cells in sheep can be isolated, cultured and cryopreserved successfully under in vitro conditions.

Keywords: *Amniotic fluid, stem cells, cryopreservation, sheep.*

1. Introduction

Sheep is an important species of livestock for India which contributes greatly to the agrarian economy, especially in areas where crop and dairy farming are not economical, and plays an important role in the livelihood of a large proportion of small and marginal farmers and landless laborers (Malik et al, 1980). 40-43 available breeds of sheep have been classified on the basis of agro-ecological regions viz., a) Northern temperate region, b) North-Western arid and semi arid region c) Southern peninsular region and d) Eastern region (Arora and Bhatia, 2011) The production and reproduction of Indian Sheep is low, this may be due to the environmental conditions under which they are reared, it cannot be considered inefficient in low input system. Major reasons for this low productivity are inadequate grazing resources, disease problems and serious lack of organized efforts for genetic improvement. There is little selection of rams and bucks used for breeding, and much inter-mating among neighboring breeds takes place.

Therefore, researchers are attempting to improve sheep reproduction through innovative approaches using various reproductive technologies. Coordinated systems of reproductive management have been developed based on a thorough understanding of the endocrine, cellular and molecular factors controlling ovarian and uterine functions. Reproductive endocrinological interventions have contributed to improvements in sheep productivity, particularly through increased embryo production and the birth of the lambs through embryo transfer technology. Notable reports have presented a comprehensive account of the information on reproductive endocrinological advances, including ovarian follicular dynamics, knowledge of which may lead to better synchronization (as well as embryo production and transfer) in ewes.

The productive and reproductive efficiency of sheep is poor due to lower genetic potential which has not been studied in relation to functional genomics. Stem cells represent an ideal tool to study embryogenesis under in vitro conditions, particularly the genes involved in the functional development. A subset of cells found in amniotic fluid has been isolated and found to be capable of maintaining prolonged undifferentiated proliferation as well as able to differentiate into multiple tissue types encompassing the three germ layers. It has been found that amniotic fluid contains non-embryonic stem cells which can differentiate into adipogenic, osteogenic, myogenic, endothelial, hepatic and also neuronal cells lines. (Dev et al., 2012.) Such types of stem cells are very active and increase greatly without feeders. However, their properties have not been fully exploited, partially because unlike other embryonic sources such as embryonic stem (ES) cells, cell lines from amniocentesis samples have not been generated. Despite the wide and well established usage of human amniotic fluid cells in routine prenatal diagnosis, the current knowledge about origin and properties of these cells is limited. Availability of sheep stem cells could facilitate development of efficient methods for somatic cell cloning, transgenesis, and gene regulation in this species. If we have an AFS cell line in sheep, it could be used whenever required for

*Corresponding author: **Sanjeev K. Gautam**

any purpose like cloning, transgenic, stem cell genomics, screening of drugs and neurodegenerative disorders, etc. The properties of AFS cells have not been fully exploited, partially because unlike embryonic stem (ES) cells, cell lines from amniocentesis samples have not been generated. As for as the isolation and culturing of putative amniotic fluid stem (AFS) cells in sheep is concerned, to the best of our knowledge there is no report available in this species.

2. Materials and Methods

2.1 Chemicals and media

All chemicals i.e. reagents, culture media and antibiotics used during the study were of cell culture grade, obtained from Hi Media Laboratories (Mumbai, India) unless otherwise indicated. Trizol was from Invitrogen (USA). Disposable 35 mmx10 mm cell culture Petri dishes, 6 well tissue culture plates, and centrifuge tubes were procured from Tarsons Products Pvt. Ltd. (Kolkata, India). Membrane filters were from Advanced Micro-devices (Ambala, India). The primers were got synthesized from GenxBio (India). The culture media were reconstituted freshly as per manufacturers' instructions and filter-sterilized (0.22µm) prior to use.

2.2 Transportation and collection of sample

Sheep amnion was obtained from a nearby abattoir, washed 2-3 times with isotonic saline fortified with 1% of penicillin/streptomycin and transported to the laboratory in a thermally insulated ice box within 5 hour. Uterine cut, fetus and membranes were located and AF was aspirated aseptically with the help of 20 ml syringe fitted with 16 gauge hypodermic needle. Twenty milliliters of AF was collected in Oakridge tubes. The appearance (fluid without cells, blood-cell-free, blood-cell, bloody or brown-coloured fluid), of fluid volume which was collected were observed and carefully documented.

2.3 Isolation and culture of AFS cells

AFS cells were isolated from sheep amniotic fluid after being centrifuged at 3000 rpm for 10 min and cells pellet was washed three times with Dulbecco's phosphate-buffered saline (DPBS) containing 1% antibiotics (penicillin/streptomycin) followed by seeded at density of 10^3 cells/ cm^2 in 6 well culture plates containing cell culture medium DMEM supplemented with 16% FBS, 1% L-Glutamine, 1% penicillin/streptomycin and incubated in humidified CO_2 incubator (Lark, China) at 37±0.5˚C in presence of 5% CO_2 in air (Dev *et al*., 2010). The cells were allowed to grow and sub cultured by passaging after achieving 80-90% of confluency without the use of feeder layer. Viability of the cells was determined by trypan blue dye method and cells were counted with a hemocytometer (ROHIM, India). Morphological features of the cells and their anchorage to culture plates were monitored and recorded regularly. After three to seven days of initial culturing, medium was replaced with fresh medium and cell attachment time, attachment rate, behavior with different medium were observed.

2.4 Cryopreservation and thawing of putative AFS cells

The putative AFS cells were cryopreserved using slow freezing method. The cells were collected and washed twice with PBS. Total two cryovials were used for freezing. Cryopreservation medium was prepared which contained 10% DMSO and 50% FBS and was named CP-M . CP-M was added drop wise in both the cryovials containing cells. The cryovials were put in minicooler and incubated at -8ºC for 12 hrs. After 12 hours the mincoolers were shifted to -20ºC and kept for 5 hour. After words the vials were transferred in LN_2 (-196ºC). The cryovials were kept in LN_2 for 7 days. For thawing, the vials were taken out from LN_2 container and put in water bath (37ºC) for 30 sec to 1 min. The cells were transferred to 6 well plates having fresh medium containing DMEM+10% FBS. The cells were washed 3-4 times in fresh medium.

2.5 Viability assay of putative AFS cells

For measuring the viability of the cells trypan blue assay was performed. The preparation of 1:1 dilution of the suspension using a 0.4% trypan blue solution was followed. Loaded the counting chambers of a hematocytometer with the dilution and observed the cells under compound microscope.

3. Results

3.1 Culturing of putative AFS cells

After collection, all the cells were spherical and variable in sizes. No anchorage was observed before 48–72 h of culturing the cells. After day 6, morphologically different cells viz., star shaped (62.7%), spherical with–out nucleus (1.9%), spherical with nucleus (26.4%), pentagonal (0.4%), and free floating and rounded cells (8.3%) were observed (Fig. 1). At day 7 of culturing, most of the cells converted into star shaped cells which subsequently started anchoring to surface (Fig. 2).

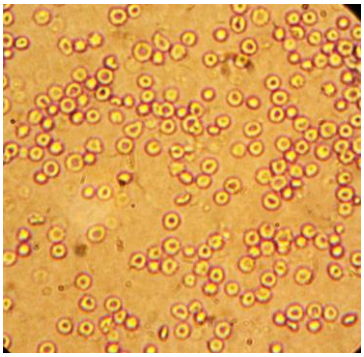

Fig1. Morphologically different cells observed after day 6

The anchorage–dependent cells subsequently gained typical fibroblast like shape and formed a confluent monolayer (Fig 3). A few spherical and freely floating cells were also visible (Fig 3). Instead of forming uniform cell monolayer, certain cell clumps were also observed. Initially, the cells reached 70–80% confluence after two weeks. However, the passaged cells exhibited higher growth rate, reaching a 90–100% confluence after day 6 of culturing.

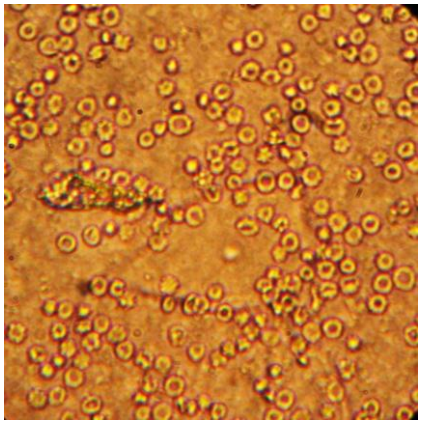

Fig.2 At day 7 of culturing conversion of cells into star shaped cells which subsequently started anchoring to surface

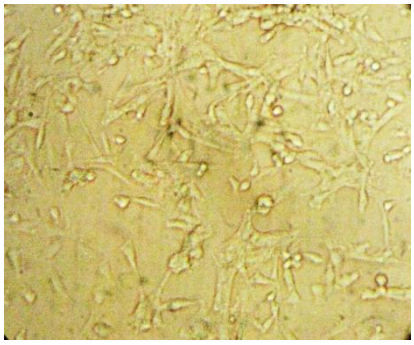

Fig. 3 The anchorage–dependent cells subsequently gained typical fibroblast like shape and formed a confluent monolayer

3.2 Cryopreservation and viability of putative AFS cells

After cryopreservation and thawing, the viability assay was performed to check the percentage of viable and dead cells. It was observed that 70% of the cells were viable after tryopan blue staining .

4. Discussion

Mammalian AF contains diverse cell types representative of three germ layers (Gosden, 1989; Fauza, 2004). Amniotic membrane and AF–derived cells have therefore, attracted a deal of attention globally as an alternative cell sources for transplantation and tissue engineering, and as a possible reserve of pluripotent stem cells that may be useful for clinical application in regenerative medicine (Delo et al, 2006; Parolini et al., 2009; Dobreva et al., 2010) and reproductive biotechnological applications (Zhao and Zheng, 2010). However, the potential of AF stem cells in livestock assisted reproduction and health applications are yet to be exploited. The establishment of pluripotent stem cell lines in domestic species could have great impact in the agricultural as well as in the bio–medical field (Yadav et al ., 2011). Accordingly, the study of the AF stem cells in live–stock species has become a new focus recently (Zhang and Chen, 2008). Efforts are being made to study various types of stem cells (Verma et al., 2007; Dev et al., 2012; Sritanaudomchai et al., 2007) in domestic animals. The present study is a preliminary effort to investigate whether the AFS cells in goat AF can be cultured and exhibit stem cell–like attributes. It has been observed that goat AF cells were able to grow without feeder cells. The choice of the culture medium and conditions chosen to grow goat AF cells are based on reports already established for human AF stem cells (De Coppi et al., 2007). However, the final selection was based on our preliminary observations on the growth of the cells in various combinations of culture media and supplements in buffalo species (Dev et al., 2012). After 3 to 5 days of incubation, the sheep AF cells had five different types of morphologically different cells (Fig. 1). The polygonal or star shaped cells were cultured for prolonged periods (at >10th passage). It was found that these cells transformed into fibroblast–like cells (Fig. 3). The cells had similar morphology to the AFS cells of buffalo (Dev et al., 2012) and as also reported by Mihu et al., 2009 where the authors observed the AF stem cells to show morphological features similar to fibroblasts. The sheep AF cells were found to have enlarged nuclei compared to adult skin fibroblasts, cumulus cells and granulose cells (data not shown). The AF cells, which were initially round in structure, started anchorage–dependent growth after day 3 to 5 of culture in vitro. After cryopreservation, the cells had around 70 % viability rate. Almost same viability rate has been observed in a study in our own lab by Sumita et al (Verma et al, 2012) which indicates that AFS cells of sheep can be cryopreserved successfully and can be used later on.

5. Conclusion

In summary, the present study is a preliminary attempt on isolation, culturing and cryopreservation of putative amniotic fluid stem cells in sheep. These putative AFS cells can be used in various purposes in future for various investigations. However, use of AFS cells in sheep therapeutic and assisted reproductive biotechnology needs further studies.

6. Acknowledgement

The authors are very thankful to Department of

Biotechnology, Kurukshetra University, Kurukshetra (Haryana) India for providing necessary facilities to carry out this work. We are greatly thankful to the Vice Chancellor, Kurukshetra University, Kurukshetra (Haryana) India.

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