The Pharma Innovation Journal 2021; SP-10(4): 102-107

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(4): 102-107

www.thepharmajournal.com
Received: 11-02-2021
Accepted: 13-03-2021

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# Characterization of embryonic stem cell like cells from pre-implantation goat (*Capra hircus*) embryos

**Ramkumaar RK and Ananthakumar KV**

**Abstract**
This study was carried out in goat (*Capra hircus*) pre-implantation embryos from which the stem cell like cells have been isolated and been characterized. Establishment of stable immortal ES cell lines using embryo as a source of isolation in domesticated farm animals, in particular for Goat, which are closer to human than mouse has not been reported. This information could contribute to the improvement of agricultural biotechnology, genetic modifications, developmental biology and regenerative medicine in humans, biotechnology and agriculture. Therefore, the discovery of effective protocols to derive and maintain ES cells and the induction of somatic cells from ES cells in goat is of importance.ES cells in their undifferentiated state are characterized by distinct morphology and expression of specific markers is an important criterion for pluripotent or undifferentiated state of cells. The expression of these markers is found to be exclusive to a particular species. *In vitro* derived embryos were subjected to enzymatic and mechanical method to derive the blastomeres. Blastomeres were cultured in knockout DMEM medium on mitotically inactivated goat fetal fibroblast feeder layer at 37 ºC until the primary outgrowth of ES-like cells were observed. The ES cell like cells was found positive for SOX2, OCT4 and NANOG pluripotent markers. They were morphologically dome shaped elevated in nature with compact cells and similar to the buffalo embryonic stem cells. In conclusion, the gES-like cells obtained in this study not only shows morphological similarity to authenticated ES cells but also had a positive for SOX2, OCT4 and NANOG markers confirmed by RT-PCR. Embryoid body formation by suspension culture was observed.

**Keywords:** *In vitro* fertilization, blastomere, embryonic stem cell, fetal fibroblast feeder layer, pluripotent markers

## Introduction
Goats are the most fertile animals among the domestic species with the conception rates in the range of 90 per cent. Assisted reproduction plays an increasingly important role in highly organized breeding programmes or utilizing goats as bioreactors in the context of gene pharming. Remarkable progress has been made in the field of assisted reproduction, including collection and handling of gametes, *in vitro* fertilization, oestrus control, embryo transfer, conservation and manipulation of gametes and embryos, transgenesis and pregnancy detection. The advances in assisted reproductive technology and the utilization of embryonic stem cell (Stice *et al*., 1994) [21] and primordial germ cell (Stokes *et al.,* 1994) [22] technology should enhance the efficiency of gene transfer in goats and other livestock.Embryonic stem (ES) cells are cells that have the capacity to self-renew as well as the ability to generate into differentiated cells. Embryonic stem cells are most frequently derived from the inner cell mass (ICM) of blastocysts. Embryonic stem cells (ES) are mainly used in cloning technology by introducing a enucleated zona (clones may differ in their cytoplasmic inheritance).The inner cell mass (ICM) gives rise to an ES cell line that is pluripotent, meaning the cells have the potential to develop into any cell type from all three germ layers-ectoderm, endoderm and mesoderm both *in vivo* and *in vitro.* Embryonic stem cell lines can be propagated *in vitro* indefinitely yet maintaining a normal karyotype. ES cells or ES cell-like cells have been produced in mice and other animal models, including chicken, bovine, equine, sheep, rabbit, hamster, pig and buffalo. In primates, ES cell lines have been produced in the rhesus monkey (Thomson *et al.*, 1995) [24] and the common marmoset (Thomson *et al.*, 1996) [25]. The first bovine ES cell line had remained pluripotent in culture for more than 150 passages (Mitalipova *et al.*, 2001) [16]. Applications of ES cells are wide, including application of therapeutic medicine in human, study of developmental biology, analysis of the characteristics of totipotent cells and gene targeting to produce genetically modified livestock. Goat embryonic stem cells are used as a powerful tool for studying early embryonic development,

gene targeting, cloning, chimera formation and transgenesis. Goat embryonic stem cells also help in overcoming the limitation on efficient gene transfer by providing abundant totipotent stem cells to be genetically manipulated by using conventional recombinant technologies. The use of ES cell technology in livestock will have enormous agricultural and pharmaceutical applications. Therefore the present study was carried out to isolate and characterize ES cells from goats using pluripotent markers.

## Materials and Methods

### Chemicals

Dulbecco's modified eagle medium (DMEM), Fetal bovine serum (FBS), Fetal calf serum (FCS), HEPES buffer (2-[4-(2-hydroxyethyl) piperazin-1-1yl] ethane sulfonic acid), Tissue culture medium (TCM 199) were purchased from Gibco. cDNA synthesis kit was procured from Applied Bio system, β-mercaptoethanol, Jumpstart RED Taq ready mix, Ethanol, Ethylenediaminetetraacetic acid (EDTA), Follicle stimulating hormone (FSH), Lutenizing hormone (LH), Estradiol ($E_2$), glutamine, glycerol, hyaluronidase, methanol, Mitomycin C, paraformaldehyde, Phosphate Buffer Saline (PBS), Potassium chloride (KCL), pyruvate, Epidermal growth factor (EGF), Triton X-100, trypsin, Tween-20 were purchased from Sigma-Aldrich. All other chemicals used in this work were of analytical grade.

### Primers

Primer sequences of OCT4, NANOG and SOX2 were used in this study.

| | |
|---|---|
| NANOG | 5'-GCCCCTTAGTAAGCTGCTTTT-3' 5'-GGGGTGGTGGAAATCAGTAA-3' |
| SOX2 | 5'-AACCAAGACGCTCTCATGAAGAA-3' 5'-GTACTGCAGGGCGCTCAC-3' |
| OCT4 | 5'-CTTCAATCGCATATTCTTTAACCA-3'5'-GGAGGAAGCTGACAACCAACG-3' |

### Methods

Goat oocytes were used for *in vitro* embryo production. The ovaries were collected from slaughter house. The process of *in vitro* embryo production consisted of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC). The matured oocytes were inseminated with *in vitro* capacitated sperm. Derivation of blastomere from different stages of pre-implantation embryos was carried out by enzymatic and mechanical method. Blastomeres were cultured on mitotically inactivated goat fibroblast feeder layer (GFF).

### Goat ovary collection

Ovaries of goat were collected from corporation slaughter house, Chennai. Ovaries were washed three times by normal sterile containing 10µg/ml gentamicin sulphate and immersed in the same solution at 37 ºC during transportation until used. All processes were performed under aseptic conditions.

### *In vitro* maturation of oocytes

Cumulus oocyte complexes (COCs) were collected from ovaries by slicing. The cumulus oocyte complexes (COCs) were morphologically assessed and only oocytes that were fully surrounded with three to five layers of compact cumulus cells and homogenous cytoplasm were selected for IVM. All selected COCs (18 – 20 numbers) were cultured in 100µl drops of maturation medium (TCM 199) supplemented with 10 per cent fetal calf serum, 0.2mM pyruvate, 5µg/ml LH and 1µg/ml $E_2$at 38.0 ºC, 5 per cent $CO_2$ and 95 per cent relative humidity (RH) for 24 hrs. The droplets were covered with sterile mineral oil.

### *In vitro* fertilization (IVF) of goat oocytes

### Sperm preparation

Buck frozen semen straws were used for preparation of motile sperms. Two semen straws (0.25ml French mini) were thawed at 37 ºC for 30 sec in a water bath. Straws were then wiped with 70 per cent alcohol. 0.5 ml of semen was added to 4 ml of sperm Tyrode's albumin lactate pyruvate (TALP) medium in 15 ml centrifuge tube and centrifuged at 500g for 15 min. Supernatant was discarded and sperm pellet was collected. The sperm pellet was then layered slowly in the bottom of one ml fresh sperm TALP medium in three sterile 15 ml centrifuge tubes. Tubes were held at 45º angle and kept inside a $CO_2$ incubator for 30 min at 38 ºC under 5 per cent $CO_2$ concentration and 95 per cent RH for swim up. After incubation, supernatant from all the three tubes were collected, pooled, washed along with fresh 4ml of sperm TALP medium and centrifuged at 500g for 15min. The final pellet was collected and concentration of sperm was assessed by haemocytometer.

### Oocyte preparation

Oocytes were observed for maturation status after 24 hrs. Matured oocytes were washed in pre-equilibrated fertilization TALP medium thrice in 35mm petridishes. Matured oocytes were transferred to pre-equilibrated 50µl droplets (8-10 oocytes/droplet) of IVF TALP medium supplemented with heparin (10µg/ml) with sterile mineral oil.

### Sperm oocyte co-incubation

Oocytes were inseminated with 2µl of sperm suspension which contained about two million actively motile sperms/ml and co-incubated for 18 hrs at 38 ºC in a humidified atmosphere of 5 per cent $CO_2$ in air and 95 per cent RH.

### *In vitro* culture of goat embryos (IVC)

After IVF, cumulus cells and excess spermatozoa surrounding the zona pellucida were removed manually by pipetting in TCM 199 supplemented with 10per cent FCS. Embryo culture medium (ECM) consisted of TCM 9 ml, 1 ml FCS, 20µl Gentamicin, 2 µl ITS, 10µl of EGF and 10µl of IGF-I. After 24 hrs, presumptive zygotes were washed in embryo culture medium and transferred to the ECM droplets. Presumptive zygotes were co-cultured with goat oviductal epithelial cells (GOEC) obtained from a metestural stage of oviduct in 100µl IVC droplets of embryo culture medium for 7 – 9 days under a humidified atmosphere of 5 per cent $CO_2$ at 38 ºC. The presumptive zygotes were observed for development at the 2 to 4 cell stage, 8 to 16 cell stage, morula and blastocyst stages.

### Preparation of goat fibroblast feeder layer

Goat fetuses (around 30 – 40days old) obtained from slaughtered animals were separated from uteri and then washed twice with sterile phosphate buffered saline (PBS). Skin biopsies were taken and washed six to eight times with Dulbecco's phosphate buffered saline (DPBS). Tissue pieces were transferred to cell culture flasks of different volumes. These tissue pieces were cultured in dulbecco's modified

eagle's medium (DMEM) supplemented with 10 percent FBS and 50 µg/ml gentamicin sulphate at 37 ºC and 5 per cent $CO_2$. The explants were removed after proliferation and establishment of fibroblasts. Monolayer fibroblasts were allowed to grow till confluency. Subsequent cell layers used as feeder layers were obtained by disaggregating the cell with 0.25 per cent trypsin-EDTA solution. Cells were harvested by trypsinisation and finally they were plated directly on to the surfaces of culture dishes and six well plates at the density of 2-2.5 x $10^4$ cells per $cm^2$ in a humidified atmosphere of 5 per cent $CO_2$ in air at 37 ºC until needed.

For preparation of a feeder layer, GEF were inactivated by treatment with 10µg/ml mitomycin-C for two hrs after which these were washed five times with DPBS. The medium of the flasks containing approximately 80-90 per cent confluent cultures of GEF was discarded and replaced with 10 ml medium containing 10 µg/ml mitomycin-C. The cells were then incubated with mitomycin - C at 37 ºC for two hrs in 5 per cent $CO_2$. The medium was then removed and the monolayers were washed twice with 10 ml sterile PBS.

**Blastomere culture**

Derivation of blastomere cell from two, four, eight, sixteen, morula and blastocyst of preimplantation embryos were collected by mechanical method. The resulting blastomeres were carefully transferred to another droplet. Individual blastomeres were seeded on mitomycin-C treated feeder layers and cultured in DMEM supplemented with 20 per cent FBS, 1,000 IU/ml murine leukemia inhibitory factor (mLIF), one per cent nonessential amino acids, 0.1 mm β-mercaptoehtanol and 2 mm L-glutamine. The culture medium was changed every 2nd day and further colonization of the cells were observed routinely under an inverted microscope. The cultured blastomeres were monitored for time of attachment, aggregation, colony formation, expansion and embryoid body formation.

**Subculture**

The primary colonies, obtained 5-7 days after seeding blastomeres on the feeder layers were disaggregated mechanically under microscope. Aggregates of 50 – 100 cells were individually reseeded onto a new feeder layer in 6-well cell culture plates. The colonies exhibiting typical morphological features of ES cell-like cells were sub cultured using mechanical dissociation until the cells remained in an undifferentiated state or when colony formation stopped.

**Characterization of embryonic cells**

Goat ES like cell colonies derived from different stage embryos and various methods were analysed for characterization. Reverse transcriptase-PCR for gene expressions were performed after fifth passage.

**Reverse transcriptase-Polymerase Chain Reaction amplification of OCT4, SOX2& NANOG**

**Preparation of ES colonies for RNA isolation and RT-PCR analysis**

Embryonic Stem like cell colonies from eight, sixteen, morula and blastocyst were collected and subjected for total RNA isolation by Trizol method, followed by RT-PCR analysis for OCT4, SOX2, NANOG gene expression. The product sizes of 314bp, 277bp, 317bp were confirmed by gel electrophoresis.

**RNA extraction**

RNA was extracted from ES colonies using RNA isolation reagent

1. ES colony samples were homogenized in 400µl of Trizol reagent per 200 µl of sample.
2. The homogenized samples were incubated for 5 min at 15 to 30 ºC and 80 µl of chloroform was added per 400µl of Trizol reagent.
3. The tube was vigorously shaken for 15 sec and incubated for 2 to 3 min at 15 to 30 ºC. Following centrifugation at 12,000g for 15 min at 4 ºC, the mixture was separated into a lower red, phenol-chloroform phase, an interface and colorless upper aqueous phase.
4. The top aqueous phase was transferred to a fresh tube and it was mixed with 200 µl of isopropyl alcohol to precipitate RNA. Then it was incubated for 10 min at 15 to 30 ºC and centrifuged at 12, 000g for 10 min at 2 to 8 ºC.
5. The supernatant was removed and the RNA pellet was washed with 400µl of 75 per cent ethanol. The sample was mixed gently by vortexing and centrifuged at 7000g for 5 min at 2 to 8 ºC.
6. After centrifugation, the supernatant was discarded and the RNA pellet was air dried partly but not completely. The resulting RNA was resuspended in 20µl of either in water of DNA/ RNase-free water. RNA was stored at – 70 ºC.

**cDNA synthesis**

cDNA was synthesised using Reverse Transcriptase enzymes per manufacturer's instructions (Applied Biosystem).

| | | |
|---|---|---|
| 10 x RT buffer | : | 2.0 µl |
| 25 x dNTP mix | : | 0.8 µl |
| 10 x RT random Primer | : | 2.0 µl |
| Multiscribe reverse transcriptase | : | 1.0 µl |
| RNAse inhibitor | : | 1.0 µl |
| NFW | : | 3.2 µl |
| RNA | : | 10 µl |
| Total reaction volume | : | 20 µl |

**Cyclic conditions**

For cDNA synthesis (Applied Biosystem)

| Temperature | Reaction time |
|---|---|
| 30ºC | 5 min |
| 50ºC | 60 min |
| 95ºC | 5 min |
| 4ºC | ∞ |

Reaction mixture

| Gene product | | | -Ve control | |
|---|---|---|---|---|
| 12.5µl | - | Master mix | - | 12.5µl |
| 1µl | - | Forward primer | - | 1µl |
| 1µl | - | Reverse primer | - | 1µl |
| 3µl | - | Template DNA | - | - |
| 7.5µl | - | NFW | - | 10.5µl |
| 25µl | - | Total reaction mixture | - | 25µl |

| Gene Product | Reaction condition annealing temperature/cycles | Product size(bp) |
|---|---|---|
| OCT4 | 58ºC/35 | 314 |
| SOX2 | 57ºC/35 | 277 |
| NANOG | 53ºC/35 | 317 |

| | | |
|---|---|---|
| Initial denaturation | 94ºC for 5 min | |
| Denaturation | 94ºC for 30 sec | |
| Annealing | 52ºC to 59ºC for 1 min | 35 cycles |
| Extension | 72ºC for 1 min | |
| Final extension | 72ºC for 10 min | |

After completion of the program, the tubes were held at 4ºC, till gel electrophoresis.

**Confirmation of Reverse Transcriptase-Polymerase Chain Reaction amplicons**

Amplified RT-PCR products specific for OCT4, SOX2 and NANOG, were confirmed by two per cent agarose gel electrophoresis. The results obtained were recorded in a gel documentation system used to check the size of the RT-PCR product.

**Embryoid body formation**

In the fifth passage goat ES cell-like cell colonies were removed from the feeder layer and disaggregated into small clumps using two fine needles. The dissociated single cells or small clumps were transferred to suspension culture dishes containing ES medium without mLIF and in the absence of feeder layer. The cells were fed every alternate day by tilting the plate, allowing the cells to settle and carefully removing the medium. The suspension culture was further examined for the formation of embryoid bodies

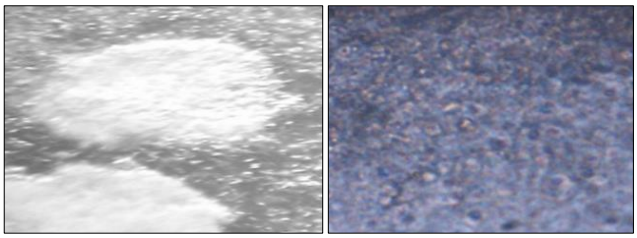
**Results and Discussion**

**Characterization of embryonic stem cell like cells**

Based on the above results, ES-like cells were isolated from embryos cultured in TCM-199 + GOEC medium. The blastomere cells were harvested by mechanical method. Primary colony formation was observed under inverted phase contrast microscope. ES like cells derived from morula and blastocyst were maintained upto fourth passage. ES cell like cells were collected and analysed for OCT4, SOX2 and NANOG. Embryoid body was developed using suspension culture method.

**Morphology of ES like cells**

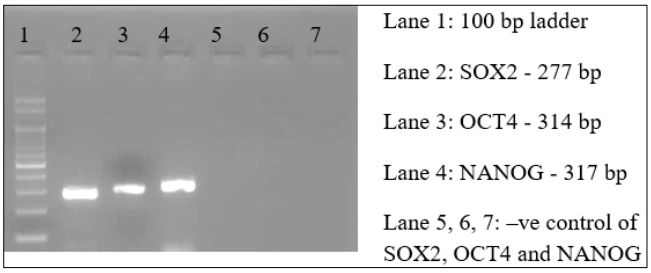
Goat ES like cell colonies were multicellular with distinct boundaries. The ES like cell colonies had a high nucleus to cytoplasm ratio and were densely packed with a clear border. Individual cells were large and round with indistinguishable cell to cell boundaries with a smooth surface. Morphologically, the goat embryonic stem cell like cells were dome shaped elevated in nature with compact cells and similar to the buffalo embryonic stem cells.



Characterization of goat embryonic stem cell-like cells colonies

**Stem cell specific marker analysis**

Pluripotency of gES like cells was demonstrated by expression of transcriptional markers OCT4, SOX2 and NANOG by RT-PCR



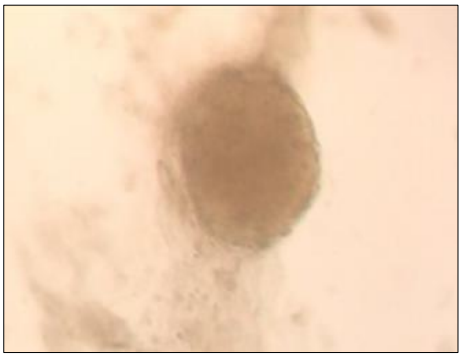

**Expression of transcriptional marker**

Reverse transcription of the extracted total RNA was carried out and the resulting cDNAs were amplified using gene specific primers. Amplicons of size specific for OCT4, SOX2 and NANOG were detected in ES like cells colonies. GEFF cells were not positive for OCT4, SOX2 and NANOG.

**Embryoid body formation**

Embryoid body was formed using suspension culture method. Suspension culture of ES-cell-like cells in the absence of murine Leukemia Inhibiting Factor (mLIF) and feeder layer resulted in the formation of nearly round, three dimensional structure.

Expression of OCT4, SOX2 and NANOG by RT-PCR



Embryoid body formation (100x)

The parameters were optimized for further application for isolation of stem cells in our laboratory from goat embryos. ES like cells were isolated from embryos cultured in TCM-199 + 10 percent FBS medium with GOEC. The blastomere cells were harvested by mechanical method. ES like cells derived from morula and blastocyst were maintained up to fifth passage. ES like cell were characterized morphologically, expression of transcriptional markers OCT4, SOX2 and NANOG by RT-PCR. Embryoid body (EB) was developed using suspension culture method. The results of expression of the transcriptional markers were similar to Behboodi *et al.* (2011) [1] and De *et al.* (2011). The results observed from embryoid body formation by suspension culture method was similar to Vinoth (2011) [27].

**Morphological characterization**

Domeshaped embryonic stem cell colonies were reported in mouse (Evans and Kaufman, 1981; Martin, 1981) [7], porcine (Chen *et al*., 1999; Gerfen and Wheeler, 1995) [4] and buffalo (Verma *et al*., 2007) [26]. Primary colonies had a high nucleus to cytoplasm ratio and were densely packed with a clear border. Individual cells were large and round with indistinguishable cell to cell boundaries with smooth surface. The results were in agreement with De *et al.* (2011). In the present study, ES like colonies were observed as multicellular

with distinct boundaries. It was found that most of the colonies were flat shaped with clear boundaries which isin consistent with the morphology of embryonic stem cell colonies reported in human (Revazova *et al*., 2007) [20].

**Stem cell specific marker analysis**

Several stem cell transcriptional markers such as OCT4, SOX2 and NANOG, have been utilized for characterization. Expression of markers such as OCT4, SOX2 and NANOG were used for characterization of ES like cells in present study. Presence of the marker could be found by two different approaches. One approach was the analysis of mRNA, which would be useful identify the unique sequence of that gene; the second approach to analyse protein products in the cells.

**Expression of transcriptional markers**

OCT - 4 also known as POU5F1 (POU domain, class 5, transcription factor 1) is a protein that in humans is encoded by the POU5F1 gene OCT4 is a homeodomain transcription factor of the POU family. This protein was critically involved in the self-renewal of undifferentiated cells. OCT4 expression must be closely regulated; too much or too little would actually cause differentiation of the cells (Niwa *et al*., 2000) [8]. The expression of OCT4 in undifferentiated pluripotent cells has also been shown in various other species like canine (Hatoya *et al*., 2006) [10], goat (He *et al*., 2004) [13] and buffalo (Verma *et al*., 2007) [26].

NANOG is a home box-containing transcription factor with an essential function in maintaining the pluripotency of the ICM cells (Mitusi *et al*., 2003) [17]. Furthermore, over expression of NANOG is capable of maintaining the pluripotency and self-renewal characteristics of ES cells under the condition where normally the cells would be exposed to differentiation-including culture conditions (Chambers *et al*., 2003) [3]. NANOG transcripts appearing first in the inner cells of the morula prior to blastocyst formation (Mitusi *et al*., 2003; Chambers *et al*., 2003) [17, 3] are restricted to ICM in the blastocyst and are no longer detectable at implantation. Expression of NANOG reappears in the proximal epiblast at the development progress (Hart *et al*., 2004) [13]. NANOG seems to be one but out of the several factors that are expressed in pluripotent cells and are down regulated at the onset of differentiation. NANOG mRNA was detected in the ICM but not in the TE expanded goat blastocysts; a pattern that follows the expression observed in mice. The transcription factor SOX2 is a member of SRY sub-family of HMG box transcription factors that binds to the sequence CT/ATTG/T/AT/A and induces DNA bending that is helpful in regulation of transcription and chromatin architecture (Pevny *et al*., 1997). SOX2 participates in the regulation of the ICM and its progeny or derivative cells and is expressed in ES cells; but it is also expressed in neural stem cells. SOX2 expression is associated with uncommitted dividing stem and precursor cells of the developing central nervous system and indeed can be used to isolate such cells (Li *et al*., 1998; Zappone *et al*., 2000) [14]. SOX2 also marks the pluripotent lineage of the early mouse embryo, so that like OCT4, it is expressed in the ICM, epiblast and germ cells. It's down regulation correlates with a commitment to differentiate, such that it is no longer expressed in cell types with restricted developmental potential. Several markers other than OCT4, NANOG and SOX2 have now been realized to be exclusively expressed by ES cells (Kumar *et al*., 2009) [15]. One of the important features of embryonic stem cells is pluripotency. In this study RT-PCR results showed the expression of pluripotency related genes OCT4, SOX2 and NANOG.

Expression of OCT4 in ES cells was also reported in mouse (Evans and Kaufman, 1981; Martin, 1981) [8]. OCT4 plays a critical role in maintaining pluripotency and self-renewal of ES cells (Niwa *et al.*, 2000; Pesce and Scholer, 2001) [18], but its utility as a marker of pluripotency has been challenged recently by studies suggesting that it is expressed in a variety of differentiated cells, including peripheral blood mononuclear cells (PBMCs) (Tai *et al.*, 2005; Zangrossi *et al*., 2007) [23]. The expression of OCT4 and NANOG is strictly restricted to the ICM in mouse and human blastocysts (Boiani and Sholer, 2005; Cauffman *et al*., 2005) [2]. In case of goat, the expression of OCT4 was reported in both ICM and trophectoderm of blastocyst (He *et al*., 2006) [12]. In the present study, the expression of OCT4, SOX2 and NANOG were in concurrence with the reports of De *et al*., 2011 and 2013.

**Embryoid body formation**

Pluripotency was one of the defining features of ES cells. Perhaps the most definitive test of pluripotency was embryoid body formation. The cells under the suspension culture conditions were able to form simple EBs within a week. In the present study, embryoid body was developed using suspension culture method. Suspension culture of ES cell-like cells in the absence of mLIF and feeder layer resulted in the formation of embryoid bodies (EBs) within 7 days. The present results were in agreement with Verma *et al*. (2007) [26] [26], Haung *et al.* (2010) [11] and De *et al*. (2011) through suspension culture of ES cell-like cells in the absence of mLIF and feeder layer resulted in formation of embryoid bodies (EBs) within 2-3 days but it was contrary to Dang *et al* (2002) [5] and He *et al.* (2006) [12].

**Conclusion**

This study was designed to derive embryonic cells from *in vitro* produced goat (*Capra hircus*) pre implantation embryos to establish ESC colonies and to characterize them. There was no significant difference in the percentage of morula formation between goat oviductal epithelial co-culture medium and the medium without goat oviductal epithelial culture. The percent of primary colony formation was higher in mechanical method when compared to enzymatic method. The per cent of primary colony formation was higher in embryonic cell derived from compact morula followed by blastocyst, 16 cell embryo, 8 cell embryo and there was no ES colony formation from blastomere derived from 2 cell and 4 cell stage embryos. The primary colony formation was observed after 5-6 days of blastomere culture. The ES cell like colonies had a high nucleus to cytoplasm ratio and was densely packed with clear border. Individual cells were large and round with distinguishable cell to cell boundaries with smooth surface. Reverse transcriptase-PCR revealed expression of OCT4, SOX2 and NANOG genes in ESC confirming their undifferentiated stage and pluripotency of embryonic stem cells. Embryoid body was developed using suspension culture method. Suspension culture of ES cell-like cells in the absence of mLIF and feeder layer resulted in the formation of nearly round, three dimensional structure of resembling EB within 7 days. EB were composed to tight cell aggregates. This cell aggregates increased in size with time in culture.

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