Stem cell-like outgrowths from \textit{in vitro} fertilized goat blastocysts

S S Pawar, D Malakar*, A K De & Y S Akshey

Animal Biotechnology Center, National Dairy Research Institute, Karnal 132 001, India

Received 27 August 2008; revised 18 March 2009

With an aim to isolate, culture and characterize goat embryonic stem cell-like cells derived from \textit{in vitro} fertilized goat blastocysts, slaughterhouse derived goat oocytes were \textit{in vitro} matured in maturation medium in 5\% CO\textsubscript{2} air at 38.5\textdegree{}C. Matured oocytes were fertilized \textit{in vitro} with fresh capacitated spermatozoa. Total 636 (36.5\%) cleaved embryos were obtained which were further co-cultured with goat oviductal epithelial cells (GOEC) for 7-10 days. GOEC culture system was better for formation of morula (150; 44.3\%) and hatched blastocyst (13; 3.8\%) than embryo development medium culture system, [morula (69; 23.1\%) and hatched blastocyst (5; 1.6\%)]. Out of total blastocysts (48) the primary colonies were formed in 23.3\% (7/30) blastocysts, and 66.6\% (12/18) of hatched blastocysts. The cells of the inner cell mass (ICM) derived primary colonies were small, aggregated and tightly packed in nature forming embryoid bodies on further subculture. The colonies were stained to see the expression of alkaline phosphatase and positive result was obtained. Goat embryonic stem cell like outgrowths were also characterized for Oct-4 expression and positive result was found. It could be concluded that ICM cells were isolated from \textit{in vitro} fertilized goat blastocysts and cultured for embryonic stem cell-like cells and expression of alkaline phosphatase and Oct-4 in these cells were positive.

**Keywords:** Blastocyst, Embryonic stem cells, Goat, Inner cell mass

Domestic animals are immunologically and physiologically more similar to human than rodent models and form better model for studying human pathology\textsuperscript{1}. Embryonic stem (ES) cells have 10-20 folds higher efficiency as somatic cell nucleus donor in cloning\textsuperscript{2} and transgenic animal production by nuclear transfer\textsuperscript{3}. ES cells are defined functionally as cells that have the capacity to self-renew and the ability to generate differentiated cells\textsuperscript{4,5}. ES cells were derived from the inner cell mass (ICM) of blastocyst and are pluripotent; these cells can differentiate into any cell type in the body, including gametes\textsuperscript{6,7}. These cells also provide an excellent tool for genetic engineering\textsuperscript{8} and this has been used extensively in investigations of functional genomics.

Murine ES cells have generated chimeric mice showing pluripotency in nature\textsuperscript{9} and cultured ICM on mouse embryonic fibroblast (MEF) in presence of leukemia inhibitory factor (LIF)\textsuperscript{10}. Blastocysts are either plated intact of feeder layer\textsuperscript{11,12} where they hatch and attach to feeder layer or the ICM is isolated from blastocyst either by immunosurgery\textsuperscript{12,13}, enzymatic digestion using trypsin\textsuperscript{11,12} or mechanical isolation\textsuperscript{11}. Except for intact blastocyst culture other methods require removal of zona pellucida, with 1\% pronase in PBS (w/v)\textsuperscript{14}.

Feeder layers of various types have been used for ES cell culturing; STO fibroblast in murine\textsuperscript{7}, bovine fetal fibroblast, bovine uterus epithelial cells, mouse embryonic fibroblast, human lung fibroblast for ES cell culturing in bovines\textsuperscript{13,15,16} and buffalo fetal fibroblast in buffalo\textsuperscript{11}. ES cell lines have been established in rodents\textsuperscript{7}, pigs\textsuperscript{17}, equine\textsuperscript{18}, sheep\textsuperscript{19}, bovines\textsuperscript{12}, primates\textsuperscript{20} and humans\textsuperscript{21,22}. The effect of oviductal epithelial cells (GOEC) co-culture system significantly enhanced the morula and blastocyst yield\textsuperscript{23}. The goat is a convenient domestic species for current biological investigation and application because it has diversified products of commercial value and relatively short gestation period. Considering these aspects attempts have been made for production of goat embryonic stem cells.

The present study has been undertaken to evaluate the effect of goat GOEC co-culture on the development of \textit{in vitro} fertilized (IVF) goat embryos. Culture of ICMs of blastocysts was carried out for production of embryonic stem cells and characterized with marker expressed as alkaline phosphatase and Oct-4.

---

*Correspondent author
Telephone:+91-184-9416741839
Fax:+91-184-2250042
E-mail:dhrubamalakar@gmail.com
Materials and Methods

**Chemicals and plasticware—** Unless otherwise mentioned, all chemicals and culture media used were cell culture grade/embryo tested and purchased from Sigma Chemical Co., St. Louis, Mo, USA. Plasticware was purchased from Nunk International, Denmark.

**Oocyte recovery and in vitro maturation—** Goat ovaries were obtained from a local slaughterhouse and washed thoroughly in pre-warmed normal saline solution (30°-35°C) supplemented with 50 μg/ml gentamycin sulphate. Oocytes were collected in oocyte collection medium (OCM) consisting of TCM-199 containing 25 mM HEPES, 200 mM L-glutamine, 3 mg/ml BSA, 50 μg/ml gentamycin and 10% fetal calf serum (FCS). Ovaries were picked up holding at the base of ovaries by a sterilized forceps and transferred to disposable 60 mm Petri dish containing 5 ml of oocyte collection medium. The ovary was sliced longitudinally with a scalpel blade by lateral mincing of the ovarian cortex. The oocytes were searched under stereozoom microscope (Nikon, Japan) and were picked up gently. All the oocytes were pooled in a 35 mm Petri dish with 3 ml oocyte collection medium. The collected oocytes were graded on the basis of the number of cumulus layers surrounding the oocytes, distribution of ooplasm and regularity in their shape. Only Grade ‘A’ and ‘B’ oocytes were selected for maturation. Selected oocytes (n=1744) were washed 5-6 times with maturation medium containing TCM-199 (HEPES modified), 0.5 μg/ml FSH, 10 μg/ml LH, 1 μg/ml estradiol 17β, 3 mg/ml BSA (fraction-V) and 10% inactivated estrus goat serum. Four drops each of 100 μl maturation medium were made in 35 mm dishes and covered with sterile mineral oil. These dishes were placed in the 5% CO2 in air prior to use for the equilibration. After washing the oocytes, 35 mm Petri dish from 5% CO2 incubator was taken out and 10 oocytes were placed in each drop of maturation medium. Then the Petri dishes with oocytes were incubated at 38.5°C under 5% CO2 in air with maximum humidity. Finally sperm pellet was resuspended in 3 ml of fertil-TALP medium containing 50 μg/ml heparin. Sperm suspension was incubated for 1 h at 38.5°C under 5% CO2 in air. A drop of sperm suspension was monitored at every step of processing to check the difference in the motility. Progressive movement, head-to-head attachment and glass surface attachment indicated the capacitation of spermatozoa. About 50 μl (2×10⁶ to 3×10⁶ sperms/ml) of sperm suspension was added to the 50 μl drop of fert-TALP medium and the Petri dish was covered with sterile mineral oil and incubated at 38.5°C under 5% CO2 in air for the equilibration of medium prior to in vitro fertilization. Matured oocytes after 27 h of maturation were taken out from maturation medium drops and maturation was accessed by examining the expansion of cumulus cells. The expanded cumulus cells were removed and oocytes were denuded by repeated pipetting in fert-TALP medium. The oocytes were then washed 5-6 times in fert-TALP medium. Ten denuded oocytes were placed in the sperm drop and co-incubated for 20 h at 38.5°C in 5% CO2 in air with maximum humidity.

**In vitro culture of embryos—** After 20 h of oocytes spermatozoa co-incubation, oocytes were taken out from fertilization drops and washed 5-6 times carefully in the embryo development medium (EDM) consisting of TCM-199 (HEPES modified), 0.5 mM sodium pyruvate, 100 μg/ml L-glutamine, 30 μg/ml gentamycin sulphate, 1% essential amino acids (EAA), 1% non-essential amino acids (NEAA), 10% FCS and 10 mg/ml BSA (Fraction-V). The attached spermatozoa were removed by repeated pipetting using Pasteur pipette without damaging the oocytes. The washed oocytes were put in 35 mm Petri dishes having 100 μl drop of Cleaved embryos were separated and transferred to four well culture dish containing EDM with goat oviductal epithelial cells (GOEC) for further development. Goat oviducts cells were prepared for co-culturing of embryos as described. Development of embryos was monitored for 7-10 days till the blastocyst formation.

**Sperm preparation and in vitro fertilization—** Fresh semen was collected from fertile healthy buck of the institute farm. Semen was collected in sterilized 15 ml plastic centrifuge tube using artificial vagina. A drop of neat semen was observed under microscope to check the semen quality and mass motility. Spermatozoa with more than 80% motility were preferred for the IVF. Semen (50 μl) was diluted with 3 ml of sp-TALP medium as per Parrish et al. The spermatozoa were allowed to swim-up during incubation for 15 min at 38.5°C under 5% CO2 in air. After incubation, the top 2 ml from the tube was collected and pooled in a sterile 15 ml centrifuge tube and centrifuged at 300 g for 10 min. Supernatant was discarded and sperm pellet was given one more washing in similar way using sp-TALP. It was monitored for 7-10 days till the blastocyst formation.
Preparation of goat fetal fibroblast feeder layer— Gravid uterus of goat containing fetus (age about 2 months) was collected from slaughterhouse. Fetus was dissected out using sterilized scissor and forceps. The fetus was then washed with Dulbecco phosphate buffered saline (DPBS) containing 50 μg/ml gentamycin sulphate. The skin of the fetus was exposed off using sterilized scissor and forceps. The collected skin explants were transferred to a dish containing 5 ml of 0.25% trypsin-EDTA and centrifuged at 33 g for 10 min. The supernatant was discarded and 4-5 washes were given in fetal fibroblast culture medium consisting of DMEM, 10% FCS, 1% NEAA and 50 μg/ml gentamycin sulphate. The cell suspension was plated in 4 well culture dishes and incubated at 38.5°C in 5% CO₂ in air. Medium was replaced after 48 h and the cells formed confluent monolayer after 6-7 days of plating. The feeder layer was subcultured by removing the cells from monolayer by mechanical streaking. The fibroblast cells were plated in fresh medium and incubated for 24 h. The medium was replaced after 24 h. The cells formed confluent monolayer after 3-4 days of plating.

Isolation of inner cell mass (ICM) from blastocysts— The inner cell masses (ICMs) were isolated from hatched blastocysts using mechanical and enzymatic isolation techniques. Mechanical isolation was performed using two fine glass needles under stereozoom microscope (Olympus, SZ61) as described by Verma et al.14. The zona pellucida of early and expanded blastocysts was removed by incubation in 1% pronase in DPBS (w/v) until zona pellucida dissolved completely. After the disappearance of zona pellucida the blastocysts were immediately given 4-5 washings in DPBS to inhibit further action of pronase. The zona free blastocysts were then transferred into 100 μl drop of DPBS. The inner cell masses were isolated from zona free blastocysts with the help of fine glass needles. For enzymatic isolation zona free blastocysts were transferred in to 0.25% trypsin-EDTA solution and observed under microscope until the trophectodermal cells became loose, and were shed from the ICM by pipetting as per Yadav et al.12. The isolated ICMs were then transferred in to DPBS.

Culturing of ICM derived cells— The confluent monolayer of goat fetal fibroblast feeder layer was washed twice with fresh fetal fibroblast culture medium. Then 1 ml of inactivation medium containing 10 μg/ml mitomycin-C in DPBS was added and incubated for 3 h. After 3 h, inactivation medium was discarded and again 5 washings were given with ES cell culture medium. The ICM cells were then seeded on mitomycin-C inactivated feeder layer. The ICM derived cells were cultured in ES cell culture medium which consisted of DMEM, 20% FCS, 1% NEAA, 0.1 mM β-mercaptoethanol, 1000 U/ml murine leukemia inhibitory factor (mLIF), 200 mM L-glutamine and 1% gentamycin sulphate. Culture medium was changed after every 48 h.

Characterization of goat embryonic stem cell-like cells by expression of alkaline phosphatase— The cells from primary colonies goat embryonic stem cell-like cells were subjected to stain to see the expression of alkaline phosphatase as it is robust marker for pluripotent embryonic stem cells. The staining procedure was carried out to see the expression of alkaline phosphatase14. Briefly, the cells were fixed in 4% paraformaldehyde in DPBS for 10 min. After fixation, the cells were washed thrice in DPBS, incubated for 30 min at room temperature in 25 mM Tris-maleate (pH 9.5) containing sodium alpha naphthyl phosphate (0.4 mg/ml), 8 mM MgCl₂ and Fast Red TR (1 mg/ml). The ICM derived cells took purple colour which is an indicator of pluripotent embryonic stem cells.

Characterization of embryonic stem cells by Oct4— Goat embryonic stem cells like cells were characterized using ‘Cells-to-cDNA™ II’ kit (Ambion, USA). The kit is designed to produce cDNA from mammalian cells in culture without isolating RNA. The cDNA produced is specifically intended for use in polymerase chain reaction (PCR). Then the PCR product was run in 2% Agarose gel14.

Experimental studies
Experiment 1— Effect of goat oviductal epithelial cells (GOEC) co-culture system was evaluated on the development potential of in vitro fertilized goat embryos. The in vitro fertilized oocytes were
observed for 35 h and cleaved embryos were divided in two groups namely group ‘A’ and ‘B’. Group ‘A’ (298 cleaved embryos) was cultured in embryo development medium (EDM) consisting of TCM-199 (HEPES modified), 0.5 mM sodium pyruvate, 100 µg/ml L-glutamine, 50 µg/ml gentamycin sulphate, 1% EAA, 1% NEAA, 10% FCS and 10 mg/ml BSA (Fraction-V). Group ‘B’ (338 cleaved embryos) was co-cultured in EDM along with GOEC.

**Experiment 2** — Effect of stages of early and expanded blastocysts or hatched blastocysts were evaluated at the time of ICM derivation on the primary colony formation rate of embryonic stem cell-like cells. The ICM cells were isolated from early and expanded blastocysts mechanically after removal of zona pellucida using 1% pronase in PBS (w/v). ICM from hatched blastocysts were also removed mechanically. The isolated ICMs were plated on mitomycin-C inactivated goat fetal fibroblast feeder layer and primary colony formation rate of embryonic stem cell-like cells was monitored.

**Statistical analysis**— The data were analyzed using Chi-square test.

**Results**

**Experiment 1** — After *in vitro* maturation and fertilization of oocytes (Fig. 1), blastocysts were developed 7-8 days post fertilization (Fig. 2). Hatched blastocysts were formed 9-10 days post fertilization (Figs 3 and 4). Effect of co-culture system on number of blastocyst formed is shown in Table 1.

Results of Experiment-1 (Table 1) demonstrated that culturing of *in vitro* fertilized embryos in GOEC co-culture system was statistically significant ($P<0.01$) in morula formation than defined embryo development medium. The early and expanded blastocyst (Fig. 2) formation and number of hatched blastocysts (Fig. 3) were also statistically significant at $P<0.05$ in two culture systems in Chi-square test.

**Experiment 2** — For ICM isolation early and expanded blastocysts were made zona free by removal of zona pellucida using 1% pronase (Fig. 5). The ICM from hatched blastocyst and zona free blastocysts
were isolated using mechanical isolation technique and ICM cells plated on mitomycin-C treated inactivated goat fetal fibroblast feeder layer (Fig. 6). The primary colony formation of embryonic stem cell-like outgrowths (Figs 7, 8 and 9) were monitored in respect to source of ICM cells (early, expanded or hatched blastocyst). ICM cells derived from hatched blastocysts (Table 2) show statistically significant \((P<0.01)\) colony formation rate on goat fetal fibroblast feeder layer than the ICM cells isolated from early and expanded blastocysts. The cells from primary colonies were passaged and cells from fourth passage when subjected to alkaline phosphatase staining showed purple colour (Fig. 10) and Oct-4 expression (Fig. 11) is indicating the successful isolation of goat embryonic stem cell-like outgrowths. These cells began to lose their morphological features and began to differentiate after six passages.

**Discussion**

Goat embryonic stem cell-like cells were derived from *in vitro* produced goat blastocysts. The goat embryonic stem cell-like cells were subsequently cultured and characterized. Embryonic stem cells were derived from the inner cell mass (ICM) of blastocyst stage embryos\(^7\),\(^{20}\). Stage of blastocyst at ICM isolation was found to be a significant factor

---

**Table 1**— Effect of goat oviductal epithelial cells co-culture system on developmental potential of *in vitro* produced goat embryos  

<table>
<thead>
<tr>
<th>Embryo culture system</th>
<th>Total no. of cleaved oocytes</th>
<th>Morula Early and expanded</th>
<th>Blastocyst Hatched</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDM</td>
<td>298</td>
<td>69(23.1%)(^a)</td>
<td>11(3.6%) (^b)</td>
</tr>
<tr>
<td>EDM+ GOEC</td>
<td>338</td>
<td>150(44.3%)(^b)</td>
<td>37(10.9%) (^b)</td>
</tr>
</tbody>
</table>

Data are expressed as total number and percentage. Percentage with different superscripts \((a,b)\) within column are significantly different \((P<0.01)\) in Chi-square test.

EDM-Embryo development medium 
GOEC-Goat oviductal epithelial cells
in establishment of goat embryonic stem cell-like cells colonies (Figs 8 and 9). The ICM isolated from hatched blastocysts showed enhanced primary colony formation (66.6%) as compared to 23.3% primary colony formation in ICM obtained from early and expanded blastocysts. The mechanical isolation in goat was found comparatively easier for hatched blastocyst (Fig. 4) than the early and expanded blastocyst (Fig. 5).

Murine embryonic fibroblast (MEF) has been shown to produce the factors responsible for maintaining

<table>
<thead>
<tr>
<th>ICM source</th>
<th>Total no. of blastocyst processed</th>
<th>Primary colony formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early and expanded</td>
<td>30</td>
<td>7(23.3)a</td>
</tr>
<tr>
<td>Blastocysts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hatched blastocyst</td>
<td>18</td>
<td>12(66.6)b</td>
</tr>
</tbody>
</table>

Data are expressed as total number and percentage. Percentage with different superscripts (a,b) within column are significantly different ($P<0.01$) in Chi-square test
embryonic carcinoma cells in undifferentiated state by inhibiting their differentiation as reported by Martin and Evans. Feeder layer helps in maintaining embryonic stem cells undifferentiated state by virtue of secretion of various known and unknown factors such as leukemia inhibitory factor (LIF). LIF has been proved to inhibit differentiation of embryonic stem cells. Optimize murine LIF at 1000 IU/ml was used in ES cell culture medium as same concentration was reported earlier.

The initial primary colony formation took longer period than the subsequent colony formation after passaging. The goat embryonic stem cell-like cells colonies obtained from in vitro produced blastocysts were dome shaped, elevated in nature with compact cells, and similar morphologically to buffalo embryonic stem cells colonies as reported by Yadav et al.

The ES cell-like colonies were characterized by checking for the expression of alkaline phosphatase by staining and Oct-4 by PCR amplification. Alkaline phosphatase and Oct-4 expression has been used characteristically to identify pluripotent ES cells in many animal species. In the present investigation alkaline phosphatase and Oct-4 expression were observed in goat ES cell-like cells of 3rd and 4th passage. The alkaline phosphatase staining and Oct-4 expression results suggest that goat ES cell-like cells isolated in the present study were pluripotent in nature.

The oocyte recovery was 3.2 oocytes per ovary which was similar to that reported by Malakar and Majumdar. The effect of GOEC co-culture system significantly enhanced the morula and blastocyst yield. The culturing of goat embryos in GOEC co-culture system led to 10.9% fertilized oocytes reaching blastocyst stage as compared to 3.6% obtained in defined embryo development medium (EDM) alone. The enhancement of embryo development potential in GOEC co-culture system was attributed to several unknown growth factors secreted by GOEC which promote embryo development. Moreover GOEC exhibit ciliary movement which ensures the continuous rolling movement of embryos avoiding the embryo attachment to bottom of culture dish. The blastocyst yield in present study was more that reported by Malakar and Majumdar and Younis et al and less than the one reported by Katska et al.

Conclusion

It could be concluded that generation of goat embryonic stem cell-like outgrowths from in vitro produced goat blastocyst were established successfully and expression of alkaline phosphatase and Oct-4 was observed positive and higher percentage of blastocysts was produced when cleaved goat embryos were co-cultured with goat oviductal epithelial cells.

References


Notarianni E, Galli C, Laurie S, Moor R M & Evans M J, Derivation of pluripotent, embryonic cell lines from pig and sheep, J Reprod Fert, 43 (1991) 255.
