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# Optimization of caprine embryo production in different media for generation of embryonic stem cell-like cells*

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ABSTRACT

The aim of the present study was to optimize the production of blastocyst for obtaining caprine embryonic stem cell-like cells. A total of 4372 cumulus oocyte complexes (COCs) were recovered by slicing the 1187 caprine ovaries and were matured in maturation media for 27 h in humidified atmosphere at 38.5°C with 5% $CO_2$ in $CO_2$ incubator. After 27 h of maturation, oocytes were denuded and were co-incubated with buck semen in fertilization medium (TALP medium + 8 mg/ml fatty acid free BSA and 50 µg/ml heparin) for 18 h. Good quality zygotes (2483) were selected and randomly divided into 2 groups (experiment 1), viz. Group 1 (1312) wherein the presumptive zygotes were cultured in RVCL while in Group 2 (1171) the presumptive zygotes were cultured in mCR2aa medium. The cleavage rate, blastocyst and hatched blastocyst production was significantly higher in Gr 1 (47.45±2.93, 10.13±1.31 and 3.90±1.13%) than Gr 2 (37.75±2.46, 4.20±0.93 and 1.66±0.72%). In experiment 2, after *in-vitro* fertilization, morula stage embryos and inner cell mass (ICM) from blastocyst and hatched blastocyst were used to isolate ES cell-like cells. Thus the results indicated that the RVCL medium is the best medium as far as the embryonic development up to blastocyst stage in comparison to mCR2aa media. Furthermore, the formation of putative embryonic stem cell colonies were higher from hatched blastocysts (91.6%) as compared to that of blastocysts (82.1%) and it was significantly higher than that from morulas (34.3%).

**Key words**: Caprine, Embryos, *In vitro* fertilization, Media, Putative embryonic stem cells

Over the past decade, the landscape for veterinary research in embryo technology and stem cell biology has reshaped dramatically. The initial focus of embryo technology in the domestic animals was to optimize breeding for improvement of production and health. In most parts of the world, however, the breeding-related use of such technologies are quantitatively limited. However, recently, renewed focus on domestic animal embryo technology and stem cell biology has emerged, due to the need for improved biomedical models for human diseases (Hall *et al.* 2013).

*In-vitro* fertilization is a well-established technology with a variety of applications in basic and applied sciences. The advances have allowed for progress in the study of early fertilization events so that the efficiency of embryo production *in vitro* can be improved. Experimentation in *in-vitro* maturation (IVM), *in-vitro* fertilization (IVF) and *in-vitro* culture (IVC) of goat oocytes has led to marked improvements in the production of embryos *in-vitro* and the birth of kids (Kharche *et al.* 2007, Kharche *et al.* 2011). In most mammalian species, the requirements for normal embryo development are not yet defined. The inadequacy of culture conditions leads either to a developmental block or to a loss of viability even though morphological aspects seem to be preserved. Despite the use of various methods, development of caprine embryos is frequently arrested at the morula stage. In an ideal scheme for *in vitro* embryo development, the culture medium supplements, additives and environment together would offer a milieu in which a matured and fertilized oocyte could cleave and develop up to the hatched blastocyst stage (Izquierdo *et al.* 1999).

The generation of ES cell lines in farm animal would be useful in the genetic engineering. Pluripotent stem cells were used for the production of chimeras or transgenic animals in bovine (Cibelli *et al.* 1998). Goat pluripotent stem cells might be a better material for developing new and to improve the yields of transgenic and cloned embryos or animals for developing nations.Therefore, the present study was undertaken to compare the two media for *in-vitro* embryo production so as to generate putative embryonic stem (pES) cell-like cells from morula and inner cell mass (ICM) from blastocysts and hatched blastocysts.

## MATERIALS AND METHODS

*Experiment 1: Optimization of blastocyst production—*

*Part of Ph.D. thesis.

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*Collection of ovaries, recovery of oocytes and IVM:* Collection of ovaries, recovery of oocytes and IVM was done as per the protocols described by Kharche *et al.* (2016). Goat ovaries were obtained within 30–45 min of slaughter from a local abattoir located at Agra and transported to the laboratory in a Thermos flask containing sterile warm (37ºC) physiological normal saline solution (NSS) supplemented with antibiotics (100 IU/ml penicillin-G and 100 µg/ml streptomycin sulphate). A total of 4372 cumulus oocyte complexes (COCs) were recovered by slicing the 1187 caprine ovaries. Oocytes were matured in TCM199 supplemented with 10% fetal bovine serum (FBS) + 10% follicular fluid + FSH (5 µg/ml) + LH (10 µg/ml) + Estradiol (1 µg/ml) + EGF (10 ng/ml) + BSA (3 mg/ml) for 27 h in humidified atmosphere at 38.5°C with 5% $CO_2$ in $CO_2$ incubator. After 27 h of *in-vitro* maturation, matured oocytes were denuded with hyaluronidase enzyme (0.1%). After denudation, oocytes with evenly distributed granular cytoplasm with polar body extrusion cone or with polar body were selected and washed 3–4 times in medium for removal of residual cumulus cells. *In-vitro* fertilization was carried out as per the method described by Kharche *et al.* (2011) with slight modifications. Denuded oocytes from both groups were washed separately 10–20 times with Fert-TALP medium containing 10% FBS, 8 mg/ml fatty acid free BSA and 50 µg/ml heparin. Oocytes were co-incubated with 15–20 µl of the diluted buck semen so as to obtain a final sperm concentration of $1–2\times10^6$ sperm/ml for 18 h at 38.5°C with 5% $CO_2$ in humidified atmosphere. After 18 h of sperm-oocytes co-incubation, oocytes were washed in embryo development medium to remove sperm adhered to zona pellucida. Presumptive zygotes (2483) were selected and randomly divided into 2 groups, viz. Group 1 (1312) wherein the presumptive zygotes were cultured in research *in vitro* cleave (RVCL) medium while in Group 2 (1171) the presumptive zygotes were cultured in mCR2aa medium supplemented with amino acids. The cleavage rate was evaluated after 48 h under inverted phase contrast microscope (NIKON, Eclipse, TE 2000U) at 40×, 100×, and 200× magnification, using Hoffman Modulation Contrast (HMC) optics.

*Experiment 2: Production of putative embryonic stem cells colony from different stages of embryos:* A total of 1559 *in-vitro* oocytes were collected, matured and fertilized with capacitated sperms as per methods described above to obtain different stages of embryos for isolation of putative embryonic stem cell-like cells.

*Preparation of feeder layer, isolation and culture of embryos:* For the preparation of feeder layer, fibroblast cells were isolated from abdominal skin of goat foetus (40–60 days) and cultured. Embryonic fibroblast cells in log phase (80–90% confluent) were inactivated by adding 20 µg/ml Mitomycin-C for 4 h followed by washing with culture medium. Zona of embryos (morula) was dissolved with the 0.25% pronase and the ICM was mechanically isolated from expanded and hatched blastocysts produced by *in vitro* fertilization and was cultured on Mitomycin-C inactivated goat fetal fibroblasts feeder layer in stem cell culture medium (DMEM supplemented with FBS (20%), L-glutamine (1%), non essential amino acids (1%), gentamycin (50 µg/ml), LIF (40 ng/ml), SCF (20 ng/ml), IGF-1 (10 ng/ml) and β-FGF (4 ng/ml). Passage one was performed upon primary colony formation. Subsequent colonies were passaged mechanically using a microblade every 4–5 day and media was replaced after every 24 h.

*Passaging of embryonic stem cells:* Colonies were passaged when signs of differentiation were visible. Briefly, cells on the outer edge of the undifferentiated colony begin to differentiate first and remain on the perimeter of the colony to eventually form a band of differentiation. Strikingly, this band was of constant width in all colonies, independent of their size. As colonies grow and differentiate, they form a rounded colony shape with a fairly consistent band around the colony edge. For passaging, colonies were detached using microblade from feeder layer by making cuts all around the colonies followed by cutting the colony in 1:2 or 1:3 ratio depending upon its size. The small clumps of colonies were reseeded on fresh mitomycin-C treated feeder layers and cultured for 4–5 days.

*Characterization of goat ES-like cells colonies*

*Alkaline phosphatase staining:* Characterization of goat ES-like cells colonies were performed with alkaline phosphatase staining kit (Sigma Chemical Co., USA).

*Immunoflourescent detection of ES cell markers:* The expression of surface markers like SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, and intracellular marker (OCT4), was examined by carrying out immuno-flourescence staining with ES cells marker sample kit (Merck Life Science Pvt Ltd., India).

*RNA isolation from oocytes and cDNA synthesis:* RNA was isolated using tri-reagent as per manufacturer's instructions. The total RNA sample was treated with DNAase I to remove genomic DNA contamination. The RNA concentration and quality were assessed by Biophotometer. Reverse transcription was carried out using RevertAid$^{TM}$ cDNA synthesis kit (Thermo Scientific, USA) in a total 20 µl reaction volume following the manufacturer's instruction. One µg of total RNA was used in the reverse transcription as template.

*Gene expression:* The present study was carried out to find out the presence of the pluripotent stem cell marker in the pES cells in order for characterization. Oct 4, HNF-4, NF-68, SOX-2 and 18sRNA gene (Table 1) were amplified by polymerase chain reaction (PCR) and real-time PCR analysis. The RNA was isolated and PCR was standardized for gene expression. The SYBR green master mix was used for relative expression of Oct4, HNF-4, NF-68, SOX-2 using 18sRNA as housekeeping gene. The reaction was carried using Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA) in Roche 480 Light Cycler (Roche). The optimized reaction was carried out in a final reaction volume of 20 µl containing 1 µl (0.5 µM) of each forward and reverse primer, 4 µl of cDNA, 5.0 µl of

nuclease-free water, and 10 µl of SYBR Green qPCR master mix. Thermal profile used for amplification of all replicates consisted of an initial denaturation cycle of 10 min at 95°C; 45 cycles of PCR (95°C for 10 sec, 58°C for 10 sec, 72°C for 15 sec) and melting curve profile (95°C for 0.05 sec followed by 70°C for 1 min and 95°C for 0.05 sec) was set for fluorescence acquisition and reaction specificity.

*Statistical analysis:* The maturation rate of oocytes was calculated as a percentage. Cleavage rates between the different treatment groups were compared using the Chi-square test. The level of significance was recorded at the 5% level of confidence (Snedecor and Cochan 1989).The relative expression pattern of stem cell biomarker such as Oct4, HNF4, NF68, SOX2 along with housekeeping gene (18sRNA) in the stem cell was analyzed by ΔΔCt.

## RESULTS AND DISCUSSION

The oocytes used for embryo development after IVF were divided into two groups, viz. Group 1 (RVCL) and Group 2 (mCR2aa). The cleavage rate, blastocyst and hatched blastocyst production was significantly higher (Table 2) in Gr 1 (47.45±2.93, 10.13±1.31 and 3.90±1.13%) than Gr 2 (37.75±2.46, 4.20±0.93 and 1.66±0.72%) suggesting that RVCL might contain necessary growth factors required for embryo development which are lacking in mCR2aa medium.

The results demonstrated that the blastocyst production, in Gr 1 (RVCL) were higher than that reported by Izquierdo *et al.* (1999) in TCM 199 (9.1%), mSOF médium (6.2%), Ham's F-10 (4.2%), Chatot-Ziomek-Bavister (CZB) medium (3.4%) and Ongeri *et al.* (2000) in G1.2/G2.2 sequential culture medium (8.1%) in caprine oocytes while our results were lower than Lv *et al.* (2009) in modified synthetic oviduct fluid (mSOF) (16.6%), Charles Rosenkrans medium (16.2%), TCM 199 (15.1%) and De *et al.* (2011) in TCM 199 (32.89%) in caprine oocytes and Bevacqua *et al.* (2013) in synthetic oviductal fluid medium (38.7%) and Anchordoquy *et al.* (2017) in mSOF medium (44.6%) in bovine oocytes.

Furthermore, our results of blastocyst production, in Gr 2 (mCR2aa) were higher than that reported by Izquierdo *et al.* (1999) in Ham's F-10 (4.2%), in Chatot-Ziomek-Bavister (CZB) medium (3.4%) in caprine oocytes while our results were lower than Izquierdo *et al.* (1999) in TCM 199 (9.1%), in mSOF medium (6.2%), Ongeri *et al.* (2000) in G1.2/G2.2 sequential culture medium (38.1%), Lv *et al.* (2009) in mSOF (16.6%), Charles Rosenkrans medium (16.2%), TCM 199 (15.1%) and De *et al.* (2011) in TCM 199 (32.89%) in caprine oocytes. It is suggested that the difference in the blastocyst production might be due to difference in the species, culture media and culture conditions. Different culture media have been successfully used for small ruminant embryo development such as Chatot-Ziomek-Bavister (CZB) medium and Ham's F-10 (F-10) (Izquierdo *et al.* 1999), G1.2/G2.2 sequential culture medium (Ongeri *et al.* 2000), synthetic oviduct fluid (SOF) (Kharche *et al.* 2008, Bevacqua *et al.* 2013, Anchordoquy *et al.* 2017), SOF-AA medium (Oliveira *et al.* 2017), TCM 199 (Kharche *et al.* 2009, Pradeep *et al.* 2011), potassium simplex optimized medium (Pathak *et al.* 2013, Kouamo and Kharche 2015), modified Charles Rosenkrans medium with amino acids (mCR2aa) (Sharma *et al.* 2015) and Research Vitro Cleave-BSA (RVCL-BSA) (Kharche *et al.* 2016).

*Production of embryonic stem cell-like cells:* In experiment 2, morula (32) and ICM from expanded

Table 1. Primer sequence of stem cell biomarkers used for expression in the putative embryonic stem cells.

| Gene | Primer 3'→5' | Size (bp) | Accession no. |
|---|---|---|---|
| OCT4 | F-GATATACCCAGGCCGATGTG<br>R- TCGATACTCGTCCGCTTTCT | 90 | EU926737 |
| SOX2 | F- TCCACATTCGAGATCAGCAA<br>R- CATGAGCGTCTTGGTTTTCC | 162 | DQ487021 |
| NF68 | F- AGGAAGATGCTGAGGAAGCA<br>R- GTTGACCTGATTTCGGGAGA | 183 | NM174121 |
| HNF4 | F- ACATCCCAGCCTTCTGTGAG<br>R- TCGTCAATCTGCAGCTCTTG | 110 | AF250028 |
| 18S rRNA | F- GAGAAACGGCTACCACATCC<br>R- GGACACTCAGCTAAGAGCATCG | 337 | AM711869 |

Table 2. Effect of culture medium on developmental potential of *in vitro* fertilized caprine oocytes

| Group | Presumptive zygotes | Cleaved | 2 cell | 4 cell | 8–16 cell | Morula | Blastocyst | Hatched blastocyst |
|---|---|---|---|---|---|---|---|---|
| Group 1 (RVCL) | 1312 | 551[a] (47.45±2.93%) | 71[a] (13.53±1.90%) | 104[a] (20.08±2.31%) | 134[a] (26.68±2.26%) | 155[a] (25.66±4.18%) | 58[a] (10.13±1.31%) | 29[a] (3.90±1.13%) |
| Group 2 (mcr2aa) | 1171 | 400[b] (37.75±2.46%) | 68[b] (18.43±3.08%) | 70[a] (17.90±2.45%) | 132[b] (34.60±3.53%) | 103[a] (23.18±4.1%) | 19[b] (4.20±0.93%) | 8[b] (1.66±0.72%) |

The values bearing different superscript within a column differed significantly (P<0.05).

Table 3. Relative expression pattern of stem cell biomarker such as Oct4, HNF4, NF68, SOX2 along with housekeeping gene (18sRNA) in the embryonic stem cells.

| | Mean cp of target | Mean cp of reference | Fold change |
|---|---|---|---|
| Control (18sRNA) | 31.01 | 25.55 | 1.000 |
| Oct4 | 32.49 | 31.01 | 15.780 |
| HNF4 | 36.41 | 31.01 | 1.042 |
| NF68 | 37.94 | 31.01 | 0.361 |
| SOX2 | 36.07 | 31.01 | 1.320 |

blastocyst (ExBs) (28) and hatched blastocyst (HB) (24) were used to produce ES cell-like cells. Statistically, the percentage of hatched blastocysts that attached to the feeder layer (91.6%) was higher than expanded blastocysts (82.1%) and significantly higher ($P<0.05$) than morula (34.3%) indicating more prominent ICM in case of hatched blastocysts than early or expanded blastocysts.

Our observation suggests that the source of ICM is an important criterion for ES cell derivation in goat where HBs had higher (91.6%) attachment rate and primary colony formation rate, obtained in relatively shorter time period in comparison to the ExBs (82.1%). This observation prompts towards better ICM compaction, presence of more viable ICM or higher ICM cell number leading to an increased efficiency of ES cell-like cell derivation from HBs and ExBs compared to morula. Further, superiority of HBs and ExBs over morula could be because of 2 more reasons. Firstly, since the HBs and ExBs were seeded intact on the feeder layers without being exposed to any proteolytic enzyme, the damage to the ICM cells could be expected to be minimal. Secondly, since HBs and ExBs are at a stage of development, which is more advanced than morula, that could have given them an edge over the morula. In earlier studies too, the isolation of ICMs either from morula, early stage of blastocyst gave an unsatisfactory result as compared to HB in various species (Verma *et al*. 2007).

*Characterization of goat ES-like cells colonies:* The morphology of ES cell-like cells derived either from morula, ExBs and HBs was same. The ES cell-like cell colonies were flat shaped with clear boundaries which was in consistent with the morphology of embryonic stem cell colonies reported in human (Revazova *et al.* 2007). During later passages, the cells colonies lost their integrity, compactness and floated in culture medium indicating sign of degeneration. In present study, morula, ExBs and HBs formed pluripotent stem cell like colonies as they expressed stem cell specific pluripotent markers. Putative ES cells initially characterized by alkaline phosphatase staining were positive. The primary colonies developed were immune-positive for ESC specific markers, viz. Oct4, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. The putative ES cell colonies expressed the pluripotency genes Oct4, Sox2, Nanog and FoxD (Mahapatra *et al.* 2014). Our result demonstrated that at mRNA level all four stem cell biomarkers were expressed in the embryonic stem cells**.** The Oct4 marker exhibited higher expressions relative to other markers while other markers showed low expression (Table 3). The HNF4, SOX2 had similar expression as a control and expression of NF68 in the stem cell was less than control.

It is concluded that RVCL induced significantly higher cleavage rate and blastocyst production as compared to mCR2aa media by providing better culture condition for embryonic development. Also HBs had higher attachment rate and primary colony formation rate, obtained in relatively shorter time period in comparison to the ExBs and morula.

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