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Effect of somatic cell co-culture on cleavage rate and embryo development of *in-vitro* fertilized goat oocytes

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ABSTRACT

The present study was undertaken to estimate effect of granulosa cell monolayer and oviductal epithelial cells co-culture on cleavage rate and embryo development of *in-vitro* matured and fertilized goat oocytes. The oocytes were matured in maturation media (TCM-199). After 27 h, matured oocytes were picked up and denuded and then transferred in fert-TALP medium. The capacitated sperm having concentration of 1×10^6 live sperm/ml were used for *in-vitro* fertilization. Oocytes were co-incubated with capacitated spermatozoa for 18 h at 38.5±1°C in an atmosphere of 5% CO_2 in humidified air. The fertilized oocytes culture in EDM over their respective cell culture i.e. granulosa cells monolayer and oviductal epithelial cells were obtained for further cleavage rate and embryo development (2, 4, 8, and > 8 celled) under steriozoom microscope at every 24 h with 50% replacement of pre-equilibrated EDM media up to 7 days. The cleavage rates with granulosa cells monolayer and oviductal epithelial cells co-culture, 48 h post insemination, were 43.52 and 45.27% respectively for goat oocytes. The present study indicated that both granulosa cell monolayer and oviductal epithelial cells co-culture behaved similarly for cleavage rate of *in-vitro* matured and fertilized goat oocytes, and oviductal epithelial cells co-culture significantly increased in embryonic development stage from 4 cell to > 8 cell stage (4 cell, 8 cell and > 8 cell) as compared to granulosa cell monolayer whereas granulosa cell monolayer co-culture significantly increased the embryonic development of 2 cell stage as compared to oviductal epithelial cells.

Key words: Embryo development, Granulosa cells monolayer, *In-vitro* maturation, *In-vitro* fertilization, Oviductal epithelial cells, Somatic cells.

Somatic cells are repeatedly used to improve *in-vitro* culture of early stage embryo. These include *in-vitro* co-culture with oviductal epithelial cells (Prichard *et al.* 1991, Crozet *et al*. 1995) and granulosa cell monolayers (Keskentepe *et al.* 1996, Mogas *et al.* 1997). It could provide embryonic growth factor or remove toxic substances from the culture medium or both. In goats, granulosa cells monolayer or oviductal epithelial cells have been used in IVM-IVF to improve embryonic development program (Singh *et al*. 2010). The oviductal cells are defined as crucial factor for better cleavage and further development of IVF embryos in cattle (Xu *et al.* 1987), goat (Watson *et al.* 1994) and buffaloes (Madan *et al.* 1994). The present study was aimed to evaluate the effect of granulosa cells monolayer and oviductal epithelial cells co-culture on cleavage rate and embryo development of *in-vitro* fertilized goat oocytes.

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MATERIALS AND METHODS

Collection and processing of abattoir ovaries: Goat ovaries collected from local abattoir in normal saline solution (NSS), having 100 IU/ml penicillin and 100 microgram /ml streptomycin sulphate at 37°C were brought to the laboratory and washed with physiological normal saline solution and DPBS (Dulbecco's phosphate buffer saline, P^H 7.3–7.4) before subjecting to the oocytes recovery.

Oocytes maturation: The oocytes were recovered by puncturing the visible surface of atretic follicle (1–5 mm diameter) with an 18-gauge needle into a 35 mm sterile glass petridish containing oocytes collection media (OCM). The oocytes were graded as excellent, good, fair and poor

depending upon the feature of cumulus layers and cytoplasm as per Kharche *et al.* (2008). Only excellent and good quality oocytes (grade A and B) were subjected to the process of *in-vitro* maturation.

The selected cumulus oocyte complexes (COCs) were washed 10 –20 times in 100 µl drop of oocyte handling media (OHM) followed by 8–10 washing in 100 µl drop of maturation medium (MM) which had been pre-equilibrated in a CO_2 incubator at 38.5±1ºC for 2 h. About 10–15 cumulus oocyte complexes (COCs) were placed in micro drop of 50 µl of the maturation medium (TCM-199, M-7528) containing either 20% concentration of EGS or 20% NCS or 10% NCS plus hormone (FSH @ 1 microgram / ml + LH @ 100 IU/ml + E_2 @ 1 microgram /ml) or 20% CFF which had been equilibrated at 38.5°C in a CO_2 incubator. The oocytes in the 50 µl droplets were covered with sterile mineral oil and cultured in humidified atmosphere of 5% CO_2 in air at 38.5±1°C for 27 h in a CO_2 incubator. The maturation of oocytes was confirmed by cumulus expansion and presence of first polar body after 27 h of incubation with maturation media.The *in-vitro* capacitation and *in-vitro* fertilization of *in-vitro* matured oocytes were done as per Singh *et al.* (2009).

Preparation of granulosa cells monolayer: Masses of goat cumulus and granulosa cells were isolated in a petri-dish following recovery of cumulus oocytes complexes (COCs) and washed 4–6 times in 100 µl drops of oocytes handling medium (OHM). Thereafter, 2–3 washings were given in 100 µl drops of maturation medium (MM) which were pre-equilibrated in a CO_2 incubator at 38.5±1°C for 2h. After washing, packed cumulus cells were placed in a micro-drops of 100 µl of the maturation medium (TCM-199, M-7528) containing 20% concentration of EGS, which had been equilibrated at 38.5±1°C in a CO_2 incubator. The packed cumulus cells in the 100 µl droplets were covered with sterile mineral oil and cultured in humidified atmosphere of 5% CO_2 in air at 38.5±1°C in a CO_2 incubator. After 48 h the expended cumulus masses forming monolayers were used for co-culture of presumptive zygote and early 2-cell-embryo. The preparation of granulosa cell monolayer was done as per Singh *et al.* (2010).

Preparation of oviductal epithelial cells: Goat oviducts were collected from slaughtered animal within 1h of their slaughter. Only those genitalia were selected where the ovaries contain either corpus haemorrhagicum or corpus luteum. The genitalia were brought to the laboratory in a thermos-flask containing physiological saline solution fortified with antibiotic (100 IU penicillin and 100 µg / ml streptomycin) kept at 4º C. In laboratory the oviducts were cleared from ligaments, connective tissue and adjoining blood vessels and 2 cm of the ampullary and isthmic region excised. The trimmed oviducts were then subjected to washing at least for 3 times with sterile physiological saline solution supplemented with antibiotics.

After washing with physiological saline solution, the oviducts were washed 3 times with Dulbecco, s phosphate buffer saline solution (DPBS) kept at 37º C. The oviduct was placed in a petri-dish and with the help of a pair of forceps its lumen was squeezed, which yield in thick fluid containing oviductal epithelial cells. The collected oviductal epithelial cells were mixed with oocyte collection media (OCM) and were subsequently passed through a syringe fitted with 26 gauge needle. The process resulted in collection of homogenized epithelial cells.

The homogenized oviductal epithelial cells were transferred to a centrifuge tube containing 5 ml of oocyte collection media (OCM) and were subjected to centrifugation at 200 rpm for 5 min. After centrifugation, the supernatant was discarded with the help of a pipette without disturbing the oviductal epithelial cells pellet. The process of washing of oviductal epithelium cell pellets was repeated, which was followed by washing in maturation media (100 µl) (pre-equilibrated at 38.5±1º C for 2 h in a CO_2 incubator) for 2–3 times.

The oviduct cell either in 100 or 200 µl droplets and covered with sterile mineral oil were cultured in a humidified atmosphere of 5% CO_2 in air at 38.5±1° C for 24 h in a CO_2 incubator. At the time of collection, oviductal cells were devoid of any cilliary (hair like projection on epithelium) movement but it appeared after 3–4 h of culture. After 20 h of culture, live cells were seen in rotatory motion. As expected in the middle of drop cells showed restricted movements while those on the periphery were highly motile. After 3 days of culture most of the cell formed vesicles attaining oval or round shape and become more transparent resembling blastocyst. The preparation of oviductal epithelial cell was done as per the procedures described by Singh *et al*. (2010).

In-vitro culture: Oocytes were washed in a culture media (embryo development medium, EDM) 8–10 times to separate adhering sperm cell, 18 h post-insemination. The oocytes were finally transferred either on monolayers (equilibrated with EDM under oil for 18 h) or 50 µl drop of EDM co-incubated with oviductal epithelial cells under sterile mineral oil (equilibrated for 2 h at 38.5±1° C in a CO_2 incubator) for 48 h in a drop. They were cultured at 38.5±1°C at 5% CO_2 in a CO_2 incubator as per Singh *et al*. (2010)

Assessment of cleavage rate and embryo development: Cleavage rate was evaluated 48h post-insemination, by counting the number of cleaved >2 cell embryos/number of oocytes used for fertilization. After evaluation of cleavage rate, embryo development was seen morphologically every 24 h under stereozoom microscope and classified as 2 cell, 4 cell, 8 cell and >8 cell embryos depending on a possible *in-vitro* cellular block. After every 24 h, 50% of the media was replaced with a fresh pre-equilibrated EDM media.

The difference between cleavage rates and embryo development stages were analyzed by the Chi Square analysis (Snedecor and Cochran 1989).

RESULTS AND DISCUSSION

Experiment 1: Statistical analysis did not reveal any significant differences between the cleavage rates of oocytes which were co-cultured with 2 different group of somatic cell co-culture i.e. granulosa cell monolayer and oviductal epithelial cells (Table 1).

Experiment 2: Statistical analysis between embryonic development stages (2 to >8 cell) of two different groups of cleaved oocytes revealed a significant difference ($\chi^2 = 10.35$, $P < 0.05$) which were co-cultured with 2 different co-culture system i.e. granulosa cell monolayer and oviductal epithelial cells (Table 1).

In the present study 2 different co-culture systems i.e. granulosa cell monolayer and oviductal epithelial cells were used for enhancing cleavage rate and embryo development of *in-vitro* matured goat oocytes.

In this study, while comparing the effect of 2 monolayer of somatic cells on embryo development, the oviductal epithelial cells (OEC) showed significant increase in the embryonic development of 4 cell to > 8 cell stages as compared to granulosa cell monolayer (GCM), whereas granulosa cell monolayer found to show significant increased in embryonic development stage of 2 cell as compared to oviductal epithelial cells.

Several workers have used granulosa cell to induce nuclear and cytoplasmic maturation and suggest that these cell enhance the rate of fertilization and subsequent developmental capacity of the oocytes in bovine and caprine (Lu *et al*. 1988, Sharma *et al.* 2001, Teotia *et al.* 2001, Singh *et al*. 2010). Improvement in the *in-vitro* fertilization and culture in farm animals by incorporating granulosa cell monolayer co-culture could be due to the additional support in nutritional requirement or generation of specific signals to the developing oocytes (Staigmiller and Moor 1984). This support can be further enhanced by granulosa cell monolayer as it has been shown to produce growth factors (Mulheron and Schomberg 1992, Satoh *et al.* 1994).

Several investigators reported the superiority of co-culture with oviductal epithelial cells compared to culture without cells for goat (Sakkas *et al.* 1989, Prichard *et al*. 1991), cow (Eyestone and First 1989, Nagao *et al*.1994) and sheep (Gandolfi and Moor 1987) embryo development. It was also observed that cumulus and oviductal cells did not support embryonic development (Wiemer *et al.* 1991, Shamsuddin *et al.* 1993).

In goat, Keskintepe *et al*. (1994) obtained higher number of embryos reaching the morula stage with cumulus cells than with oviductal epithelial cells using an atmosphere of 90% of N_2 for embryo co-culture. However, Bavister (1988) opined that the role of oviductal tissue to supports early development *in-vitro* is not well known, but it seems likely that these cells produce material beneficial to the young embryos, remove substances with negative effects from the media and/or reduced the oxygen concentration or both.

Table 1. Effect of somatic cell co-culture on cleavage rate and embryo development of *in-vitro* fertilized goat oocytes

Somatic cell co-culture	Total number of replicate	Total number of ovaries	Recovered oocytes	Oocytes used for fertilization	Cleaved oocytes	Uncleaved oocytes	Cleavage rate (%)	Total number of embryos	2 cell (%)	4 cell (%)	8 cell (%)	> 8 cell (%)
Granulosa cell monolayer	38	457	803	648	282	366	43.52	282	182[a](64.54%)	71[a](25.18%)	19[a](6.74%)	10[a](3.55%)
Oviductal epithelial cell	30	349	617	497	225	272	45.27	225	117[b](52.00%)	67[b](29.78%)	24[b](10.67%)	17[b](7.55%)

* Value bearing different super script in a column differed significantly (P< 0.05); * Non Significant ($\chi^2 = 0.0013$) difference between the cleavage rate of 2 different group of somatic cell cultured oocytes; * Significant ($\chi^2 = 10.35$, $P < 0.05$) difference between the embryonic development stage (2 to > 8 cell stage) of 2 different group of somatic cell cultured cleaved oocytes.

Izquierdo *et al.* (1999) carried out study for cleavage and further embryo development of pre-pubertal goat oocytes. After 24 h of co-culture in TCM-199 with bovine and caprine oviduct epithelial and cumulus cells and following 48 h of post insemination the cleavage rates in different culture systems i.e. caprine cumulus cells, bovine cumulus cells, caprine oviductal epithelial cells, bovine oviductal epithelial cells and M-199 supplemented with 20% EGS were 42.6, 41.2, 41.5, 45.2 and 42.4%, respectively. In present study we have used caprine oviductal epithelial cells and granulosa cell monolayer (caprine) co-cultures for cleavage and further embryo development of goat oocytes. A cleavage rate of 45.27 and 43.52% for caprine oviductal epithelial cells and granulosa cell monolayer for goat oocytes were obtained. The present results are some what comparable with the above group of workers.

Various workers reported that the beneficial effect of the oviductal epithelial cells does not depend on the species from which the cells are taken since oviductal epithelial cells from cattle and buffalo are equally competent for development of goat embryo (Betteridge 1995, Yadav *et al.* 1998).

In conclusion, the present study indicated that both granulosa cell monolayer and oviductal epithelial cells co-culture behaved similarly for cleavage rate of *in-vitro* matured and fertilized goat oocytes and oviductal epithelial cell co-culture significantly ($P<0.05$) increased embryonic development stages from 4 cell to >8 cell stages (4 cell, 8 cell and > 8 cell stage) as compared to granulosa cell monolayer where as granulosa cell monolayer co-culture significantly ($P<0.05$) increased embryonic development of 2 cell stage as compared to oviductal epithelial cells.

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