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In vitro maturation of nude caprine oocytes recovered during oocyte collection

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ABSTRACT

The present study was undertaken to investigate the effect of granulosa cell co-culture, granulosa monolayer and hormone on *in vitro* maturation of nude caprine oocytes. Nude oocytes (644) having evenly granulated cytoplasm were recovered by puncture technique. The oocytes were divided into 6 groups and matured in TCM-199 medium supplemented with 10% fetal bovine serum (FBS) and granulosa cell co-culture/ granulosa monolayer/ hormones. After maturation (27h), the oocytes were fixed for 15 min in 2.5% (w/v) glutaraldehyde in phosphate buffer saline, washed twice with PBS, stained with 0.1mg/ml DAPI in PBS and mounted on slides. The evaluation of nuclear status was done by epiflourescence microscope by observing either a polar body release or presence of two shiny chromatin spots. The maturation rate of oocytes in group 1, 2, 3, 4, 5 and 6 were 23.47, 32.40, 31.77, 26.00, 38.09 and 51.37%, respectively. The results of the study indicated that TCM-199 supplemented with hormones and co-culture granulosa cells significantly increases the maturation rate of *in vitro* matured nude caprine oocytes.

Key words: Caprine, *In vitro* maturation, Nude oocytes

Mammalian oocytes growth and development depends on the establishment of a fruitful molecular dialog between the oocyte and surrounding follicular (granulosa and cumulus) cells. Removal of cumulus cells before *in vitro* maturation (IVM) is detrimental to oocyte maturation in mice, rats, cattle and pigs (Ge *et al*. 2008). Co-culture with cumulus-oocyte complexes (COCs) or cumulus cells partially restore the development potential of denuded oocytes (DOs) in cattle (Ge *et al*. 2008) and mice (Ge *et al*. 2007). Granulosa cell monolayer or oviductal epithelial cells are used in *in vitro* maturation and *in vitro* fertilization (IVM-IVF) as crucial factor for better cleavage and further development of IVF embryos in goat (Watson *et al*. 1994, Singh *et al.* 2010). Workers have studied different aspects of IVM in goat oocytes (Pawshe *et al*. 1996, Kharche *et al.* 2006). The maturation medium and selection of supplements, hormones for IVM play an important role in *in vitro* development of oocytes. Also, the cumulus cells play an important role in developmental competence by promoting cytoplasmic and nuclear maturation through paracrine factors and gap junctions (Tanghe *et al*. 2002, Dey *et al*. 2012). Despite the importance of cumulus cells, the removal of them from oocytes or zygotes at various stages of development is inevitable for range of applications such as germinal vesicle (GV) transfer, somatic cell haploidization, cryopreservation of GV stage oocytes and pronuclear transfer (Dey *et al*. 2012). Denudation is also required for the injection of interfering RNA or mRNA into the oocyte to knockout or over express specific genes while evaluating their role in IVM or IVF processes (Xiong *et al*. 2008). A large number of oocytes are required for production of embryos through this technology. Utilization of nude oocytes recovered during oocyte recovery will increase the availability of matured oocytes required for *in vitro* embryo production. Therefore, the present investigation was undertaken to study the *in vitro* maturation of nude caprine oocytes.

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MATERIALS AND METHODS

Chemicals and media: Tissue culture media, TCM-199, mineral oil, antibiotics, D-PBS, BSA, EGS (Heat inactivated), follicle stimulating hormone, luteinizing hormone, estradiol–17ß and all other chemicals used for the *in vitro* maturation and *in vitro* culture media were purchased commercially. All the media used in the present study were filtered (0.22 µm) sterilized prior to use.

Source of ovaries and oocyte collection: Goat ovaries were collected from the local abattoir and transported to the laboratory within 2 to 3 h in 0.9% saline supplemented containing 100 IU penicillin-G and 100µg streptomycin sulphate per ml at 30 to 35°C. Oocytes were harvested by puncture method from large (>2mm diameter) follicles in oocyte collection medium. Recovered oocytes were graded as excellent (A), good (B), fair (C) and poor (D) quality as per method of Kharche *et al.* (2008).

Table 1. Effect of granulosa cell co-culture, granulosa monolayer and hormone *on in vitro* maturation of nude caprine oocytes

Groups	Total no. of oocytes (n)	Total no. of oocytes matured	Maturation rate (%)
Group 1 (Maturation medium)	115	27 [a]	23.47
Group 2 (Maturation medium along with granulosa cell culture)	108	35 [b]	32.40
Group 3 (Maturation medium and over granulose cell monolayer)	107	34 [bc]	31.77
Group 4 (Maturation medium with FSH 5µg/ml+LH 5µg/ml+E2 1µg/ml)	100	26 [bcd]	26.00
Group 5 (Maturation medium with FSH 5µg/ml+LH 5µg/ml+E2 1µg/ml + insulin 50ng/ml)	105	40[bce]	38.09
Group 6 (Maturation medium with FSH 5µg/ml+LH 5µg/ml+E2 1µg/ml + insulin 50ng/ml and co-culture with granulose cell)	109	56[f]	51.37

Values in the same column with different superscripts differ significantly (P< 0.05).

Preparation of granulosa cell monolayer and granulosa cell co-culture: The present experiment was performed as per modified method of Sharma *et al*. (2001). Trimmed ovaries were extensively washed in warm sterile normal saline solution (NSS) and Dulbecco's phosphate buffered saline (DPBS). Each ovary was rinsed once in 70% ethyl alcohol (for 20 sec) and then transferred to a 100 mm culture dish containing DPBS. Follicular contents were aspirated from large (>4mm diameter) non-atretic surface follicles by a syringe and 22 gauge needle. Pooled aspirated cell in DPBS media were centrifuged at 50 g for 10 min. Supernatant was discarded and cells reconstituted with fresh DPBS and then centrifuged at 50 g for 10 min (process repeated for 8 times). Finally following the process, the cells were reconstituted in oocyte holding media (OHM) with 10% estrous goat serum (EGS). Seeding was done @ 0.5ml of cell suspension per well of a 24 well culture plate and cultured up to confluence (5–6 days) to establish monolayers in an incubator with 5% CO_2 under humidified air at 38.5+1°C, after every 48 h half of the culture media was replaced with fresh medium.

Moreover, some granulosa cell masses (collected from surface follicles, 2–5 mm diameter) of caprine ovaries were aspirated using a 5 ml syringe attached with 20 gauge needle. After thorough washing in oocyte holding media and then in maturation media these cells were used for co-culture in IVM.

In vitro *maturation of oocytes:* Selected nude oocytes (644) of granulated cytoplasm obtained during collection were used for *in vitro* maturation. Oocytes were washed for 10–14 times in oocyte holding medium (OHM) consisting of TCM-199, sodium pyruvate (0.25 mM), gentamicin (50 µg/ml), glutamine (100 µg/ml), BSA (3 mg/ml), finally 3–4 times in different maturation medium (Table 1). Nude oocytes (10–15) were transferred to a 50–µl drop, each of maturation medium under mineral oil in a polystyrene pre-warmed culture dish (35mm × 10mm). Oocytes were cultured for 27h at 38.5°C in 5% CO_2 of humidified air.

Evaluation of oocytes following in vitro *maturation:* Nude oocytes following *in vitro* maturation were kept in hypotonic solution of KCl for 5 min. After, oocytes were mounted on cover glass slide, fixed in 2.5% glutaraldehyde for 5 min at room temperature, and stained with 0.1µg/ml of 4, 6-diamidino-2-phenylindole (DAPI) (a fluorescent stain for nuclear material). The stained slides were observed for nuclear status evaluation and subsequent meiotic development in phase contrast microscope equipped with epifluorescent illumination and filters giving maximum transmittance at 405 nm (Fig. 1).

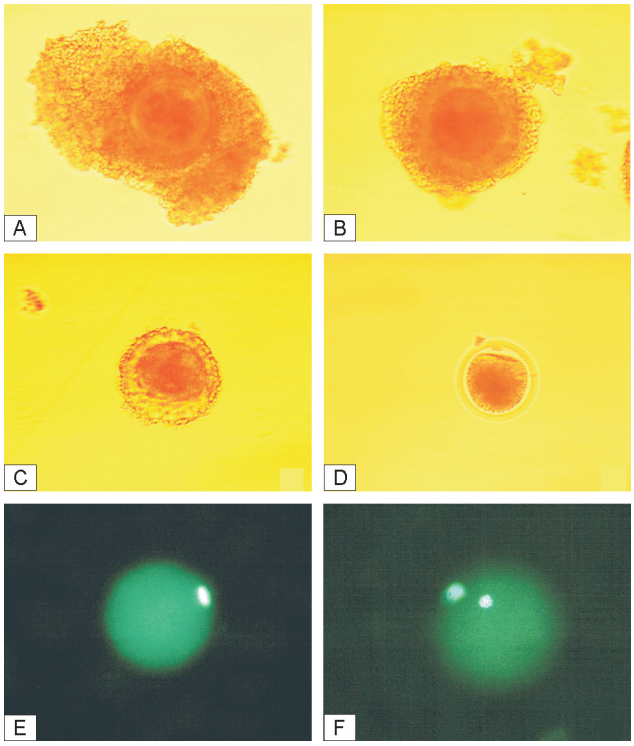

Fig. 1. (A-F) **A.** Excellent oocyte; **B.** Good oocyte; **C.** Fair oocyte; **D.** Poor oocyte; **E.** MII oocyte with polar body; **F.** MII oocyte with two shiny chromatin spot.

Statistical analysis: Maturation 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 Cochran 1989).

RESULTS AND DISCUSSION

The results of *in vitro* maturation in different culture and supplementations of nude oocytes are shown in Table 1. The percentage of maturation for denuded oocytes

obtained from non-atretic surface follicles (2–5 mm diameter) varied from 23.47 to 51.37. Sharma *et al*. (2001) reported a higher number of oocytes at M II stage (84.7%) when cultured over granulosa cell monolayer against 74.4% of M II stage oocytes co-cultured with granulosa cells. Also, Agrawal *et al*. (1995) found a maturation of 54.79% after 24h of co-culture with granulosa cells. In addition, Tyagi *et al.* (1997) reported a higher maturation rate (86.20%) of oocytes in goat over granulosa cell monolayer as compared to granulosa cell co-culture. The maturation rate observed in the present study was comparatively lower than that reported by the above workers due to the presence of nude oocytes. However, a numerical improvement in maturation rate with granulosa cell co-culture especially in nude oocytes was observed due to the existence of mRNA and protein (BMP15) in goat oocytes (Silva *et al*. 2005) it acts through an alternative signaling pathway and is able to regulate granulosa cell functions (Romaguera *et al*. 2010, McNatty *et al*. 2005a) and oocyte cytoplasmic maturation (Su *et al*. 2004, Romaguera *et al*. 2010).

In vitro maturation of caprine oocytes using TCM–199 supplemented with estrus goat serum (EGS)/FBS but without hormones revealed a maturation rate of 52.7% (Pawshe *et al*. 1996), 57.6% (Mogas *et al*. 1995), 61.9% (Kharche *et al*. 2005) and 83.12 % (Yadav *et al*. 2008). Mogas *et al*. (1995) reported that the addition of FSH, LH and estradiol-17ß as well as EGS in TCM-199 medium increased the maturation rate to 72.4% and 64.1% in adult and prepubertal goats, respectively. Similarly Pawshe *et al*. (1996) also reported a maturation rate of 62.6% following the supplementation of hormones and EGS. Rho *et al*. (2001) reported a 73% of maturation in oocytes cultured for 27 h matured in TCM-199 supplemented with 10% FCS and hormones (10 µg/ml FSH, LH and 1.0µg/ml estradiol-17ß). In present experiment the maturation rate in control group was 23.47%. Addition of hormones (FSH, LH and E_2) in medium supplemented with 10% FBS resulted in increased maturation rate (26.00%). However, these maturation rates are comparatively lower than that reported by other workers. Furthermore, in present study, addition of insulin along with hormones (38.09%) and its co culture with granulose cells (51.37%) in the TCM–199 media resulted a significant increase ($P<0.05$) in nuclear maturation rate. Insulin and insulin like growth factor effectively maintain the survival of oocyte and stimulated the growth. Combined effect of insulin-like growth factor (IGF–I) and hormones produces higher effect on survival and growth rate of oocytes. IGF-I is widely involved in the regulation of follicular development in different species. IGF-I and FSH synergistically enhanced the metabolic level of oocytes in pig (Adashi *et al*. 1985) and could maintain the growth and survival of most preantral follicles and culture in goats (Huanmin and Yong 2000). The result indicated that in *vitro* maturation of nude caprine oocytes in TCM-199 supplemented with hormones and co-culture with granulose cells significantly ($p<.05$) increases the maturation rate.

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