

Secretory Proteins from Goat Oocytes Matured in Culture

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ABSTRACT : In this experiment, oocytes were collected from goat ovaries available in slaughterhouse by follicle puncture method. Morphologically culturable type of oocytes which having compact, multilayered cumulus granulosa cell complex and evenly granulated cytoplasm, was separated under a stereozoom microscope. Oocytes were washed thoroughly in maturation medium containing TCM-199, 1 µg/ml estradiol-17β, 0.5 µg/ml FSH, 100 µg/ml LH, 3 mg/ml BSA and 10% estrus goat serum. Washed oocytes were cultured into maturation medium on granulosa cell monolayer. Culture plate was then kept into CO₂ incubator at 38±1°C, maximum humidity and 5% CO₂ for 18 h. After maturation the oocytes were washed thoroughly with maturation medium containing polyvinyl alcohol (PVA) without serum and BSA and further cultured for 12 h for secretory proteins of oocytes. PVA medium was collected, pooled and concentrated by 5000 cut off centrifuge. Secretory proteins were separated on 12.5% SDS-PAGE. A total number of 3.41 oocytes per ovary were obtained and 2.17 culturable oocytes per ovary were cultured into maturation medium. After 18 h of maturation, 4,567 oocytes (1.82 oocytes per ovary) were further cultured into serum and BSA free PVA medium for its secretory proteins. Four secretory proteins of oocytes with approximately molecular weight of 45, 55, 65 and 95 kDa were obtained on SDS-PAGE in silver staining and three proteins with approximately molecular weight of 45, 55 and 65 kDa in Coomassie brilliant blue staining. In conclusion, four secretory proteins with approximately molecular weight of 45, 55, 65 and 95 kDa was obtained from *in vitro* cultured oocytes of goats. (*Asian-Aust. J. Anim. Sci.* 2002, Vol 15, No. 3 : 340-345)

Key Words : Goat Oocyte, Maturation, Granulosa Cell Monolayer, Polyvinyl Alcohol, Oocyte Secretory Proteins, IVM

INTRODUCTION

The mammalian oocytes maturation comprises of two steps (a) a period of oocyte growth and (b) a period of final nuclear and cytoplasmic preparation. The normal oocyte growth is related to its size and quality that continues with the process of folliculogenesis in first phase. The second phase is related to the process of meiosis. The growth of oocyte is essential for resumption of meiosis within the follicle that was arrested at the dictyate stage. Oocyte is not capable of resuming meiosis without further growth and development in most of the mammalian species. Nuclear as well as cytoplasmic maturation of oocyte is a prerequisite for successful fertilization leading to embryo development. During *in vivo* folliculogenesis, the oocyte and follicle undergo concurrent growth (Carnoll et al., 1991).

Not only oocytes are capable of resuming meiosis *in vivo* but also *in vitro* when they are released from their follicles and cultured in a suitable medium. Addition of gonadotropin (LH, FSH) and steroid (estradiol-17β) hasten the *in vitro* oocyte maturation. Nuclear progression of oocyte maturation is controlled by components of the cytoplasmic matrix (Eppig et al., 1994).

Culture media for *in vitro* maturation TCM-199 on Ham-F-12 are buffered with HEPES and/or bicarbonate

including hormones, various type of sera and amino acid supplements (Keskintepe et al., 1995). Generally oocytes from different species reach to metaphase II stage at different times of interval when culture *in vitro*. Bovine and ovine oocytes matured between 18 to 24 h (King et al., 1986; Cognie et al., 1992) but goat oocyte took little longer time (Crozet et al., 1995; Keskintepe and Brackett, 1996) in *in vitro* maturation.

Protein synthesis and phosphorylation patterns of bovine and ovine oocytes during their maturation have been reported previously (Kastrop et al., 1990b; Gall et al., 1993; Rehman et al., 2001). Qualitative and quantitative changes in protein synthesis by bovine follicular cells during preovulatory period have also been described by Rabahi et al. (1991). The newly synthesized mRNA and protein synthesis is required for both cumulus cell expansion and GVBD (Hunter and Moor, 1987). At least seven oocyte specific and five cumulus specific proteins that were stage dependent during maturation of bovine oocyte were reported (Wu et al., 1996). These result indicate that maturation of bovine oocyte is associated with synthesis of several distinct and temporarily expressed proteins which may play a role in the highly ordered sequence of events that culminates in oocyte maturation. For secretory proteins of oocytes, we cultured the oocytes for 18 h in maturation medium on granulosa cell monolayer, then transferred into serum and BSA free polyvinyl alcohol medium (Keskintepe and Brackett, 1996) and cultured 12 h for its secretory proteins and four proteins were obtained.

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MATERIALS AND METHODS

Preparation of granulosa cell monolayer

Collection of ovaries: Goat ovaries were collected from abattoir at unknown stage of cycle and brought to the laboratory in Dulbecco's phosphate buffer saline (DPBS) containing 200 IU/ml penicillin and 50 µg/ml streptomycin within 2 h of slaughter in a thermos (30-35°C). The surrounding tissues were trimmed and ovaries were washed one time with alcohol, five times with Normal Saline Solution and DPBS each. Washed ovaries were kept into a sterile glass beaker containing oocyte culture medium (OCM) at 37°C for granulosa cell culture and oocyte collection. Processing of granulosa cell for monolayer were performed as reported by Sharma et al. (2001).

Collection and maturation of oocytes

Processed ovaries were brought into laminar flowhood. Oocyte culture medium was poured into petridish and oocytes were released by follicle puncture method using 18-gauge needle under sterile condition. Morphologically culturable type of oocytes (having compact, multilayered cumulus granulosa cell complex and evenly granulated cytoplasm) was separated under microscope. These oocytes were washed at least 10 times in OCM to remove adhering bacteria.

Culture of the oocytes

Washed oocytes were further washed in maturation medium in a sterile petridish. RPMI-1640 medium of granulosa cell monolayer were replaced 24 h before putting the oocytes for culture with 300 µl/well maturation medium (TCM-199, 1 µg/ml estradiol-17β, 0.5 µg/ml FSH, 100 µg/ml LH, 3 mg/ml BSA and 10% estrus goat serum). Washed oocytes were put into this well in maturation medium under mineral oil. Culture plate was kept in the CO₂ incubator at 38±1°C, 5% CO₂ and maximum humidity for 18 h.

Oocytes cultured into PVA medium

The matured oocytes after 18 h of culture were washed two times with 0.25% Trypsin and 1 mg/ml hyaluronidase to remove the surrounding cumulus cells of oocytes. Finally, the oocytes were washed with PVA medium (Medium-199, 100 µg/ml L-glutamine, 27 µg/ml sodium pyruvate, 50 µg/ml gentamycin, hormone as above and 3 mg/ml PVA) at least 10 times to remove the serum of maturation medium. These oocytes were transferred into drop of 100 µl PVA medium under mineral oil in small 35 mm plastic petridish and placed in a CO₂ incubator for 12 h. PVA medium was collected into eppendorf tube and preserved at -20°C for secretory protein analysis.

Concentration of oocyte generated proteins

All the collected sample were thawed and pooled into 5000 cut-off centriscart (Sartorius, Germany) and centrifuged at 5,000 rpm for 30 minutes in 4°C. The supernatant in inner tube was discarded and distilled water was added up to the mark and centrifuged further at the above rate. All salt solution of PVA medium and below 5,000 Da proteins was removed through the membrane of centriscart by the above process.

Gel electrophoresis

Separation and determination of molecular weight of oocyte generated proteins were done on 12.5% SDS-PAGE using the method of Laemmli (1970) and modified by Okijama et al. (1993). A 12.5% separating and 4% stacking polyacrylamide gel containing 0.1% SDS was prepared. The electrophoresis was carried out in Tris-Glycine buffer on a vertical slab gel electrophoresis system (ATTO, Japan) using a constant current of 80v. Standard molecular weight sample markers were used to determine the molecular size of protein samples. Staining of SDS-PAGE with Coomassie brilliant blue and silver staining were done to detect the bands of proteins on gel after destaining according to the protocol of Sambrook et al. (1989).

RESULTS

Granulosa cells attachment was observed after 5-6 h of post-seeding and cell confluence was prominent within 4-5 days of culture. The oocytes were matured using this monolayer of granulosa cells.

Maturation of oocytes

A total number of 2,515 ovaries (3.41 oocyte per ovaries) were used for the study from which 8,585 oocytes were obtained and 5,463 (2.17 oocyte per ovary) morphologically good quality, healthy and equally distributed cumulus oocyte complex (figure 3) were selected from the total number of oocytes (table 1). Culturable oocytes (2.17 per ovary) were matured in maturation medium for 18 h and observed under microscope for their cumulus cell expansion (figure 4).

A total of 5,463 oocytes was cultured for 18 h in maturation medium and among them 4,567 good quality matured oocytes was further cultured in PVA medium without serum and BSA for study of secretory proteins of oocytes.

Secretory proteins of goat oocytes

The secretory proteins of IVM oocytes were analyzed in 12.5% SDS-PAGE. The gel was stained with Coomassie brilliant blue and silver staining procedures. When gel was stained with Coomassie brilliant blue three bands were

Table 1. Oocytes collected from ovaries, matured on granulosa cell monolayer and further cultured in PVA medium for its secretory proteins

	Number ovaries used for experiment	Number of oocytes collected from ovaries	Culturable oocytes obtained	Number of oocytes per ovaries	Number of culturable oocytes per ovaries	Oocytes cultured in PVA medium
	322	1,064	708	3.30	2.20	590
	356	1,218	763	3.42	2.14	632
	380	1,298	815	3.41	2.14	670
	310	1,065	668	3.43	2.15	552
	290	1,003	643	3.46	2.22	534
	265	911	578	3.44	2.18	491
	280	964	611	3.44	2.18	524
	312	1,062	677	3.40	2.17	574
Total	2,515	8,585	5,463	-	-	4,567
Mean±SE	-	-	-	3.41±0.017	2.17±0.010	-

observed in oocyte proteins having approximately molecular weight of 45, 55 and 65 kDa (figure 1 lane 1). But in silver staining technique an extra band appeared in oocyte proteins (95 kDa). The total bands of secretory proteins of oocytes were four and with approximately molecular weight of 45, 55, 65 and 95 kDa (figure 2 lane 1).

DISCUSSION

Results from the present study, 3.41 oocytes per ovary were obtained (table 1) which is slightly lower than 3.68 oocytes per ovary obtained Bonde (1995) and almost similar to Teotia (1998) who also obtained 3.38 oocytes per ovary. In this study direct maturation was not studied but

cumulus expansion was taken as the indication of the maturation under similar condition. Teotia (1998) observed 88.88% of maturation rate through direct manner in our study 83.5% were observed. Healthy, culturable oocytes with cumulus cell complexes were matured on granulosa cell monolayer that showed good cumulus expansion in most of the oocytes (figure 4). Xia et al. (1994) and Bagger et al. (1993) reported that cumulus might secrete a meiosis inducing substance that increased the maturation rate of oocytes.

Tyagi et al. (1997) observed 80.6% maturation of goat oocytes after 32 h on granulosa cell monolayer, however at 28 h 56.3% oocytes reached metaphase II stage in TCM-199 supplemented with FSH, estradiol-17 β and estrus

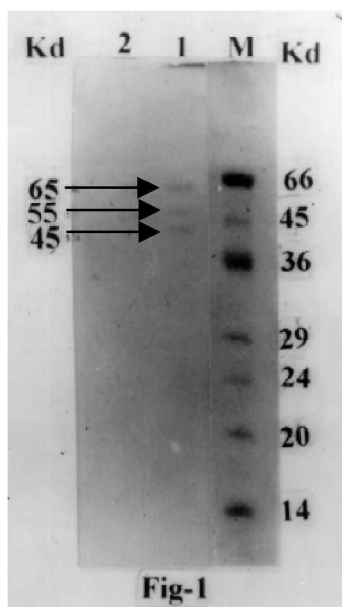


Figure 1. Lane-1- showed three proteins bands of goat oocyte when stained the SDS-PAGE gel with Coomassie brilliant blue

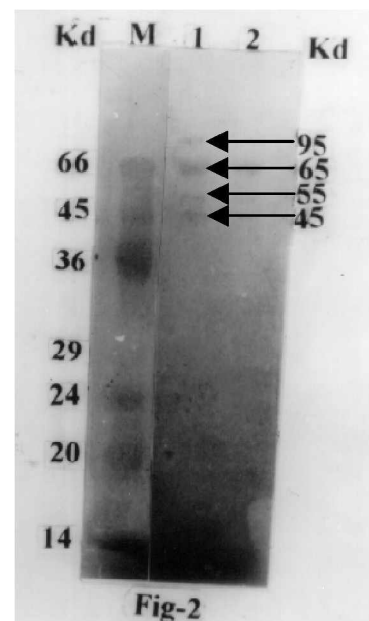


Figure 2. Lane-1- showed four proteins bands of goat oocyte when Stained the SDS-PAGE gel with silver staining

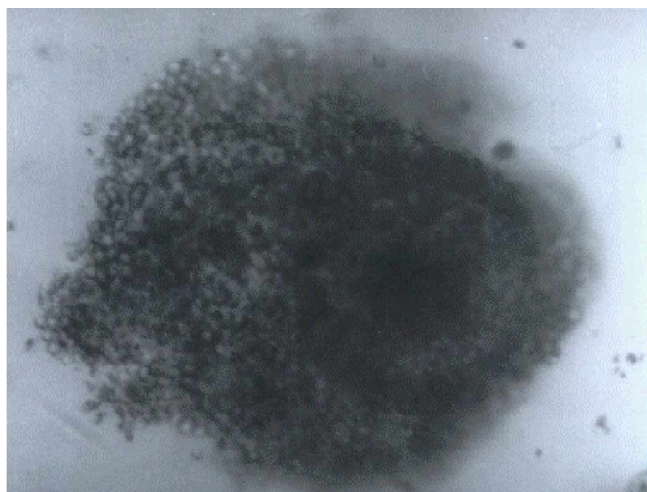


Figure 3. Morphologically good quality, healthy oocyte of goat showed before maturation under microscope (400x)

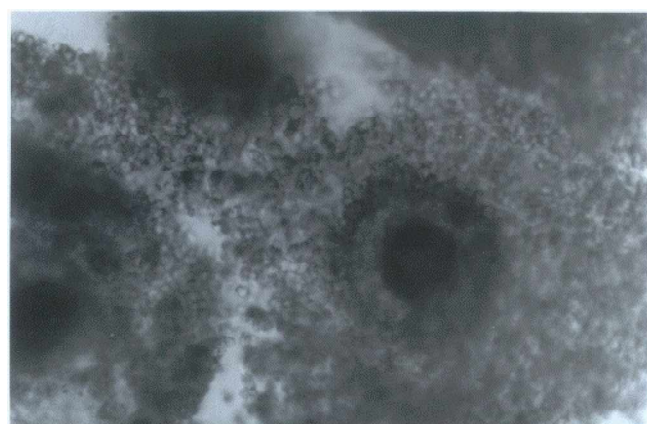


Figure 4. During *in vitro* maturation of goat oocyte showed cumulus cells expansion under microscope (400x)

serum. Teotia (1998) obtained 82.22% maturation in granulosa cell co-culture and 88.88% on granulosa cell monolayer at 27 h with the addition of LH in the media. Sharma et al. (1996) reported that goat oocytes began reaching metaphase II from 24 h onwards and 21.4% and 71.6% maturation rate were observed at 24 h and 32 h respectively in a media without LH but contained estrus serum. Maturation of goat oocytes was 72.9% in medium containing 5% estrus serum for 24-25 h culture as reported by Naqvi et al. (1992). Younis et al. (1991) reported that goat oocyte maturation range from 90 to 100% with addition of 10 $\mu\text{g/ml}$ LH and 39.5% cleavage rate were obtained. We also added 100 $\mu\text{g/ml}$ LH in our maturation medium and obtained 83.5% maturation.

In present study, time of maturation was observed 18 h in maturation medium and 12 h for a further culture in PVA medium for secretory proteins of oocytes. Most of the worker reported 24 h or more than 24 h time of maturation,

24-25 h Naqvi et al. (1992), 27 h Teotia (1998), 32 h Sharma et al. (1996) and Tyagi et al. (1997). Furthermore, Sharma et al. (1996) reported that goat oocyte began reaching metaphase II from 24 h onwards. The difference in maturation time as reported by Sharma et al. (1996) and Tyagi et al. (1997) may be due to the absence of LH in their media.

Present study, demonstrated that granulosa cell monolayer provided favourable condition for goat oocyte maturation. It supports the earlier findings that granulosa cell monolayer may secretes a meiosis inducing substance (Xia et al., 1994) and growth factors (Sato et al., 1994) which helped in maturation of oocytes. On the other hand, granulosa cell co-culture systems may lack in building up required level of factors in the critical time which may explain the discrepancy between the observation of Teotia (1998) and the present study which resulted in 82.22% and 88.88% maturation rate, respectively. Tyagi et al. (1997) cultured goat oocytes on granulosa cell monolayer and obtained 56.3%, 74.4%, 80.6% and 86.2% M II stage oocytes at 28, 30, 32 and 34 h respectively. Granulosa cells in monolayers were viable and grew continuously that decrease the concentration of free radical and toxic substances result from production of more number of matured oocytes (Liu et al., 1995).

During oocyte growth and maturation, proteins are synthesized and stored that help in fertilization and embryo development until the embryonic genome becomes transcriptionally active (Park et al., 1999). Gall et al. (1993) reported that protein synthesis and phosphorylation occur during maturation of bovine and ovine oocytes. Liu et al. (1997) obtained 30, 37, 45 and 46 kDa secreted proteins during the first 24 h of maturation in culture medium supplemented with follicle shells. Wu et al. (1996) reported that at least seven oocyte-specific and five cumulus-specific proteins were synthesized during bovine oocyte maturation. Tatemoto and Terada (1995) also obtained a 39 kDa protein and indicated that it has an important role in oocyte maturation. Hue et al. (1997) found a 65 kDa polypeptide corresponding to cyclin B₁ protein in goat oocytes using Western blot. Meng et al. (1996) reported that rat oocytes contained seven proteins with molecular weight of 37, 56, 60, 66, 69, 80 and 97 kDa.

In the present study, 45, 55, 65 and 95 kDa proteins were obtained from goat oocytes culture medium after analyzing on 12.5% SDS-PAGE and staining with silver nitrate (figure 2 lane 1). These results were in agreement with 45 kDa protein in porcine oocyte of (Liu et al., 1997), 55 kDa protein in rat oocyte (Meng et al., 1996) and 65 kDa protein in goat oocyte of Hue et al. (1997). Only 95 kDa protein was observed in this study that has not been reported in earlier studies though Meng et al. (1996) had found a 97 kDa protein in rat oocyte.

In conclusion, three secretory proteins of goat oocytes were observed with approximately molecular weight of 45, 55 and 65 kDa when stained with Coomassie brilliant blue staining of 12.5% SDS-PAGE. But when the gel stained with silver staining four secretory proteins with approximately molecular weight of 45, 55, 65 and 95 kDa were obtained in this study. It may be assumed that small quantities of these proteins secreted from the matured goat oocytes.

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