Indian Journal of Experimental Biology
Vol. 43, August 2005, pp. 693-701

# Isolation, identification and characterization of secretory proteins of IVMFC embryos and blood circulation of estrus and early pregnant goat

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*Received 13 January 2005; revised 27 April 2005*

The aim of the present study was to isolate, identify and characterize the secretory proteins of IVM oocytes and IVMFC embryos to evaluate its immunogenecity and identify of such proteins if any, in blood circulation of estrus and early pregnant goats. Oocytes were matured in TCM-199 with 1μg/ml, estradiol-17β; 0.5 μg/ml, FSH; 100 IU/ml, LH and 10% FCS on granulosa cell monolayer. After 18 hr of maturation, oocytes were further cultured in maturation medium containing 3 mg/ml polyvinyl alcohol (PVA) without serum and BSA for 12 hr and medium was collected. The IVF embryos of 4-8 cell stage were cultured in medium containing PVA without serum and BSA. Embryo culture medium was collected after 24 hr of culture and was pooled. The proteins were analyzed on SDS-PAGE (12.5%). Four secretory proteins of oocytes with approximately molecular weight of 45, 55, 65 and 95 kDa and three secretory proteins of embryos 45, 55 and 65 kDa were obtained on SDS-PAGE in silver staining. The protein profile of midluteal, estrus and early pregnant goat serum was similar and no variation was observed among the proteins on SDS-PAGE. Two secretory proteins of 55 and 65 kDa of both IVM oocytes and IVMFC embryos were observed on Western analysis. None of such proteins was observed in midluteal, estrus and early pregnant goat serum on western blotting. It can be concluded that IVM oocytes and IVMFC embryos secrete proteins in medium and two of them can develop antibody. The proteins secreted from embryos till morula stage was similar to that of oocytes. None of these oocyte/embryo released proteins were observed in blood circulation of estrus and early pregnant goats.

**Keywords**: Embryo, Goat, IVM, IVMFC, Oocyte, Secretory protein,

Earliest stages of embryogenesis in mammals are regulated by maternally inherited proteins, RNAs and proteins stored within the oocytes. Following fertilization the embryonic genome becomes transcriptionally active and embryonic transcripts and proteins of some of the maternal components required for early developmental process[1]. Timing of activation of embryonic genome varies among mammalian species[2,3].

Meng *et al.*[4] have observed no major changes in protein synthesis from prophase to prometaphase during meiotic maturation in rat oocyte. However, after entering into prometaphase-I, a polypeptide (24 kDa) disappeared and a polypeptide (34 kDa) expression becomes stronger unit metaphase-II. Liu *et al.*[5] have demonstrated the role of secreted proteins and gonadotropins in promoting maturation of porcine oocyte *in vitro*. The proteins of 30, 37, 45 and 46 kDa are secreted during the first 24 hr into the culture medium supplemented with follicular shell, which may facilitate full maturation of oocytes.

In xenopus and mouse oocytes a 39 kDa protein has been shown at a higher level at the metaphase II and it is suggested that it may stabilize the maturation[6]. Western blotting also detected the 39 kDa protein as a C-MOS protein in mature bovine oocyte[7,8]. Hue *et al.*[9] have analyzed the cyclin $B_1$ gene expression by Western blot, which reveals its presence in competent and incompetent goat oocytes. Simon *et al.*[10] have studied female infertility in mice lacking connexin-37 protein which is present in gap junctions between oocyte and granulosa cells and connexin-37 deficient mice lack mature Graafian follicles and fail to ovulate and develop numerous inappropriate corpora lutea.

Activation of embryonic transcription represents the beginning of the transition from maternal to embryonic control of development. During this transition, the embryo begins to synthesize its own mRNA and then protein[11]. In the absence of the appropriate activation and subsequent embryonic gene expression, an embryo will simply fail to develop further beyond early cleavage division[12]. The

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maternal contribution to the successful development of the zygote begins during oogenesis, with the synthesis and accumulation of mRNAs and proteins. In addition to sustaining the embryo during early cleavage this maternal environment is also likely to contribute for development after onset of embryonic transcription[13]. In mammalian species, onset of embryonic transcription occurs at various times during early cleavage divisions, namely 2-cell stage in the hamster, 4-cell stage in human and pigs, 8-cell stage in cows and sheep and 16-cell stage in rabbits[14-18]. However, in comparison with other farm ruminants, there is probably no great difference in timing of shift in control of gene transcription from maternal to embryonic genome in goat[19]. The nutrient uptake of goat embryo is very similar to that of the sheep embryo[20].

Extensive studies of zygotic gene activation and its regulation in mammalian embryo have been done in mouse. Earlier studies have identified 2-cell stage embryo as the beginning of the transition from oogenesis to embryonic control of development[12]. Bovine embryo, produced *in vitro*, start gene expression at 2 to 4-cell stage. Importance of *in vitro* production of bovine embryo and development of the reverse transcriptase polymerase chain reaction made it feasible to analyze the transcription of developmentally important genes in early embryos[21-23].

The objectives of the present study were to isolate, identify and characterize the secretory proteins of IVM oocyte and IVMFC embryo and to find out of such proteins in blood circulation, if any, at estrus and early pregnant goat.

Permission was taken from CPCSEA (Institute ethic committee) during experiment on animals.

## Materials and Methods

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless stated otherwise.

### Recovery of the oocytes

Goat ovaries were obtained from the local abattoir to the laboratory within 1 hr. The cumulus-oocytes complexes (COCs) were collected after puncturing 2-8 mm follicle under sterile condition. Oocytes were matured *in vitro* in TCM-199 supplemented with 1 μg/ml, estradiol-17β; 0.5 μg/ml, FSH; 100 IU/ml, LH; and 10% estrus goat serum on granulosa cell monolayer[24] for 18 hr for secretory proteins of oocytes and 27 hr for IVMFC[24] embryos at 38° ±1°C under 5% $CO_2$ in air.

### Culture of oocytes for secretory proteins

The matured oocytes after 18 hr of culture were washed twice with 0.25% trypsin and 1 mg/ml hyaluronidase to remove the surrounding cumulus cells of oocytes. Finally, the oocytes were washed with medium containing 3 mg/ml polyvinyl alcohol (PVA)[24-26] without serum and BSA atleast 10 times to remove the serum and BSA of maturation medium. These oocytes were then transferred into a drop of 100 μl maturation medium containing 3 mg/ml PVA without serum and BSA under mineral oil in 35 mm plastic Petri dish and placed in 5% $CO_2$ incubator at 38°±1°C for 12 hr. Medium was collected into microfuge tube and preserved at -20°C.

### *In vitro* fertilization

*Collection of semen and processing of spermatozoa* — Semen was collected from 3 bucks with artificial vagina and pooled into a sterilized glass tube. Semen (50 μl) was taken into 3 ml quenched sperm-TALP[27] and centrifuged at 300 rpm for 5 min. The supernatant was removed and pellet was rewashed with 3 ml of sperm-TALP as above. Finally quenched 1 ml fertilization-TALP having 10 μg/ml heparin was added and kept into the $CO_2$ incubator for 2 hr.

*Preparation of matured oocytes* — After 27 hr of culture, cumulus expanded matured oocytes were collected and excess cumulus cells were removed by treating with 1 mg/ml hyaluronidase. They were then washed with oocyte culture medium (OCM)[24,28] and three times with quenched fertilization medium.

*Co-culture of processed spermatozoa and matured oocytes* — Processed spermatozoa were taken at the concentration of $2\times10^6$ spermatozoa/ml in fertilization-TALP containing 6 mg of BSA (fat free) and 10 μg/ml heparin in a 100 μl drop on 35 mm Petri dish and 50-100 matured oocytes were added into each drop. These fertilization drops were covered with mineral oil and kept into 5% $CO_2$ incubator at 38°±1 °C at maximum humidity for 6 hr.

*In vitro embryo development* — After 6 hr of co-incubation, oocytes were washed with OCM and embryo development medium (EDM)[24] containing medium 199 supplemented with 0.1 mg/ml, L-Glutamine; 0.027 mg/ml, sodium pyruvate; 0.05mg/ml, gentamycin; 3 mg/ml, fat free BSA; 3% FCS & 2% goat estrus serum) to remove the excess spermatozoa. The washed oocytes were then cultured on the granulosa cell monolayer, which was conditioned 24 hr with embryo development medium

(300 µl/well) covered under mineral oil. The culture plate was transferred into 5% $CO_2$ incubator as above.

*Separation of cleaved embryos for its secretory proteins* - Fertilized oocytes were observed under microscope for cleavage after 36, 40, 48, 60 and 72 hr. The cleaved oocytes were separated from uncleaved one and washed atleast 10 times with EDM containing 3 mg/ml PVA without serum and BSA. After washing, the embryos were cultured into 50 µl drop of EDM containing 3 mg/ml PVA without serum and BSA under mineral oil and kept at above 5% $CO_2$ incubator. After an interval of every 24 hr, half of the medium was collected into a microfuge tube and 25 µl PVA containing EDM was added to embryos. The medium was collected 4 times from 4 cell to morula stage of embryos and the collected medium was stored at -20°C for protein analysis and raising of antibodies.

**Raising of antibody into Rabbit**

*Raising of antibodies against oocytes generated proteins* - Equal volume (0.5 ml) of the oocyte culture medium from nearly 200 oocytes was added to 0.5 ml complete Freund's adjuvant and taken into a 2 ml glass syringe. The medium was thoroughly mixed with Freund's complete adjuvant with another 2 ml glass syringe connecting the two nasals with plastic tube. After half an hour of thorough mixing, the two medium emulsified and was taken into one syringe fitted with a 20 gauge needle. It was injected intradermally into a 6 months old adult male rabbit at 5 to 6 different sites. After 20 days another injection was prepared with 0.5 ml incomplete Freund's adjuvant and 0.5 ml medium and injected the same rabbit as above. Likewise another two injections were given at every 7 days interval to the above rabbit.

*Collection of hyperimmune serum from immunized rabbit* - Blood was collected from the heart of the immunized rabbit on day 7 after 4$^{th}$ injection. Serum was separated and centrifuged at 5000 rpm for 5 min. Supernatant was aliquotted into 0.5 ml in microfuge tube and stored at -20°C.

*Raising of antibodies against embryos generated proteins* - Proteins released by forty embryos during a period 24 hr in EDM containing PVA was taken and antibodies were raised against embryo generated proteins using same procedure as raising of antibodies against oocyte generated proteins.

*Collection of hyperimmune serum from immunized rabbit* - It was performed in the same method as in case of oocytes release proteins hyperimmuned serum collection.

**Concentration of oocytes and embryos generated proteins**

All the samples were thawed and pooled into 5000 cut off centrisart (Sartorius, Germany) and centrifuged at 5000 rpm for 30 min in 4°C. The supernatant in inner tube was discarded and distilled water was added upto the mark and centrifuged further as above. The process was repeated five times and volume was reduced to one ml out of 2.5 ml. All the salt solution of media containing PVA without serum and fat free BSA and below 5 kDa proteins was removed through the membrane of centrisart by the above process.

**Collection of blood from goat for oocytes and embryos released proteins**

Five ml blood was collected from goat expressed heat symptom for oocyte generated proteins on day estrus cycle and serum was separated immediately after clotting the blood. Serum was centrifuged at 5000 rpm for 5 min in 4°C, aliquot into microfuge tubes and stored at -20°C. The goat was serviced two times, 12 hr interval with proven bucks. Blood was collected on day 4, 6, 8, 10 and 12 from the day of service; serum was separated and stored as above. Palpation and birth of kid latter on confirmed the pregnancy. Midluteal phase blood was collected from goat and serum was separated and stored as above.

**Sodium Dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

Separation and determination of molecular weight of oocytes and embryos generated secretory proteins were done on SDS-PAGE using the method of Lammli[29] and modified by Okijama *et al.*[30].

Staining of SDS-PAGE with Coomassie brilliant blue and silver staining was done to detect the bands of proteins on gel after distaining according to the protocol of Sambrook *et al.* [31].

**Western blotting of oocytes & embryos secretory proteins**

Western blotting was performed according to Towbin *et al.*[32] using kit (Sigma Chemical Co., USA) and protocol for Western blotting using biotinylated goat antirabbit IgG.

## Results

Oocytes were collected from the slaughterhouse goat ovaries by follicle puncture method. A total number of 8585 oocytes were obtained from 2515 ovaries and 5463 morphologically good quality oocytes were cultured for maturation. Among them 4567 oocytes were further selected and cultured in medium containing PVA without serum and fat free BSA for its secretory proteins.

*In vitro* matured oocytes number 1421 were selected for *in vitro* fertilization. These oocytes and capacitated spermatozoa were then co-incubated in drop of fertilization-TALP for 6 hr. Cleavage was observed after 40 hr and some of the oocytes showed cleavage even after 72 hr. A total numbers of 329 (23.15%) embryos in 2-8 cell stage were obtained and further cultured into EDM containing PVA without serum and BSA to obtain secretory proteins of embryos. All the embryos were cultured in PVA medium, on 3rd day reached to early morula stage and on 4th day reached to compact morula stage. Neither cell flattering or cell fusion was observed in those embryos.

Antisera were raised against the secretory proteins of IVM oocytes and IVMFC embryos in adult male (6 months) rabbits. Agar gel precipitation test (AGPT) was performed in secretory proteins of oocytes and its antisera (Fig. 1B), the titer was 1:8 and secretory proteins of embryos and its antisera (Fig. 1A), the titer was also 1:8. Both of the antisera were used against the medium containing PVA without serum and BSA in which oocytes and embryos were cultured for its secretory proteins. It was clearly showed the negative result (Fig. 1C,D). Similarly none of band was visible in mid-luteal goat serum in AGPT for antiserum of oocytes and embryos (Fig. 2C,D).

The secretory proteins of IVM oocytes and IVMFC embryos were analyzed and compared with midluteal, estrus and early pregnant goat serum in SDS-PAGE (12.5%). When gel was stained with Coomassie brilliant blue, three bands were appeared in oocytes proteins having approximate molecular weight of 45, 55 and 65 kDa (Fig. 3, Lane B) and in embryo proteins two bands were seen, having approximate molecular weight of 55 and 65 kDa (Fig. 3, Lane A). But in silver staining an extra band of 95 kDa protein appeared in oocytes (Fig.4, Lane ,G) and embryo proteins 45 kDa (Fig. 4, Lane H). The total bands of oocyte proteins were 45, 55, 65 and 95 kDa (Fig. 4, Lane G) and embryo proteins were 45, 55 and 65 kDa (Fig. 4, Lane H). Three bands of embryos generated proteins were similar to three bands of oocytes generated proteins having approximate molecular weight 45, 55 and 65 kDa.

The proteins bands of midluteal, estrus and early pregnant goat serum were similar in Coomassie brilliant blue (Fig. 3, Lane G, H; C, D; and E, F) and silver staining gel (Fig. 4, Lane A, B; E, F; and C, D) and no other extra band among the animals could be distinguished. The proteins bands of mid-luteal, estrus, and early pregnant goat serum were similar in

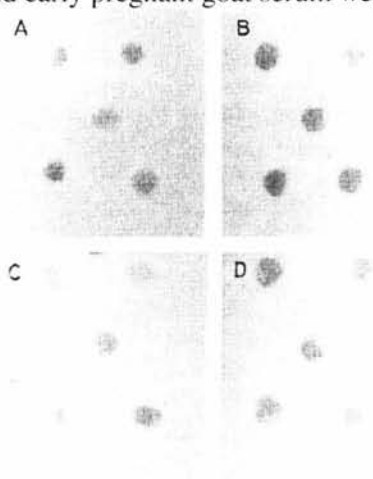


Fig. 1 — Illustrating the agar gel precipitation tests of secretory proteins of IVMFC embryos and its antisera showing white band (A), IVM oocytes and its antisera showing white band (B), EDM containing PVA medium and antisera of IVMFC embryo (C) and Maturation medium containing PVA and antisera of IVM oocytes (D) both showing no band at all.

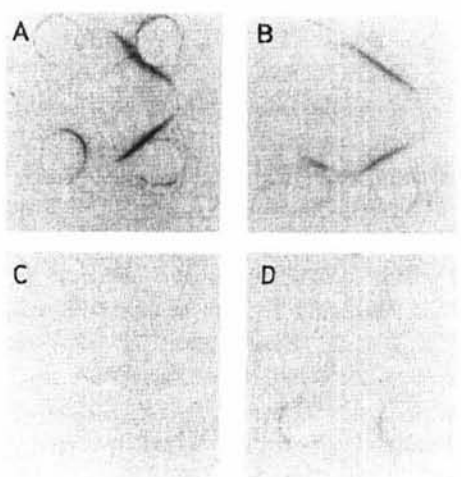


Fig. 2 — Illustrating the cross reactivity secretory proteins of IVMFC embryos and its antisera showing black band (A), secretory proteins of IVM oocytes and its antisera showing black band (B), arrow indicating band. Midluteal serum proteins and antisera of IVMFC embryo (C), and antisera of IVM oocyte (D) both are showing no band.

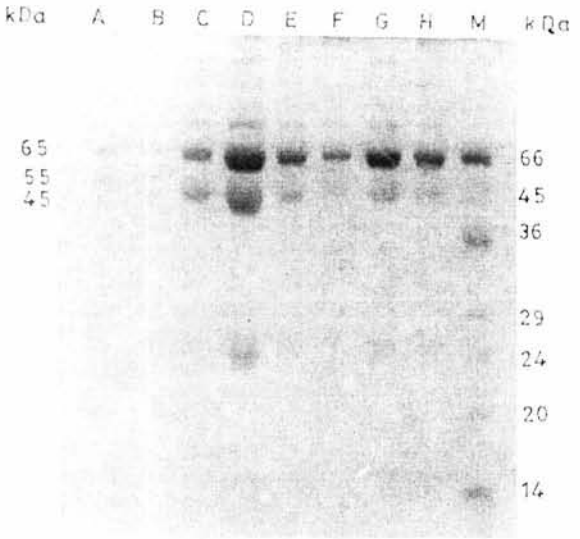


Fig. 3 — Illustrating the secretory proteins of IVMFC embryos showing 2 bands, 55 and 65 kDa (Lane: A), IVM oocytes showing 3 bands 45, 55 and 65 kDa (Lane: B), and midluteal (Lane: G and H), estrus (Lane: C and D), and early pregnant (Lane: E and F) serum proteins are similar bands, resolved on SDS-PAGE (12.5%) and stained with Coomassie brilliant blue.

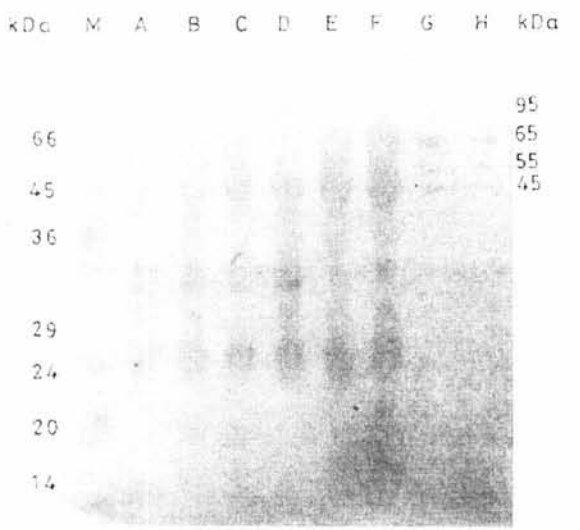


Fig. 4 — Illustrating the secretory proteins of IVM oocytes showing 4 bands 45, 55, 65 and 95 kDa (Lane: G), IVMFC embryos showing 3 bands 45, 55 and 65 kDa (Lane: H) and midluteal (Lane: A and B), estrus (Lane: E and F), & early pregnant serum proteins (Lane: C and D) are similar bands, resolved on SDS-PAGE (12.5%) and stained with silver nitrate staining. M- Molecular protein markers; A- Midluteal serum proteins of one goat; B- Midluteal serum proteins of other goat; C-Early pregnant serum proteins of one goat; D- Early pregnant serum proteins of other goat; E- Estrus serum proteins of one goat; F- Estrus serum proteins of other goat; G- Secretory proteins of IVM oocyte showing four bands, 45, 55, 65 and 95 kDa and H- Secretory proteins of IVMFC embryo showing three bands 45, 55 and 65 kDa.

Coomassie brilliant blue (Fig. 3, Lane G, H; C, D; and E, F) and silver staining gel (Fig. 4, Lane A, B; E, F; and C, D) and no other extra band among the animals could be distinguished.

Western analysis of midluleal, estrus and early pregnant goat serum with secretory proteins of IVM oocytes and IVMFC embryos was performed using antisera of oocytes generated proteins. Two protein bands of each lane of oocytes and embryos generated proteins were observed (Fig. 5a, Lane O and E) but none of the protein band appeared in the lane of midluteal, estrus and early pregnant goat serum proteins (Fig. 5a, Lane N, H and P). The molecular weight of two protein bands of oocytes and embryos were similar approximately molecular weight of 55 and 65 kDa (Fig. 5a, Lane O and E).

Western analysis was also performed using antisera of embryo generated proteins in midluteal, estrus and early pregnant goat serum proteins with secretory proteins of IVM oocytes and IVMFC embryos. Oocytes and embryos generated proteins lanes were having two protein bands in each lane and their molecular weight were similar i.e. 55 and 65 kDa (Fig. 5b, Lane O and E). None of the protein band of midluteal, estrus and early pregnant goat serum proteins was obtained in this immunoblot also (Fig. 5b, Lane N, H and P). Two protein bands of 45 and 95 kDa of oocytes and one protein band of 45 kDa of embryos secretory proteins were not observed in this Western.

## Discussion

In the present study 3.41 oocytes per ovary were obtained which was little less than 3.68 oocytes per ovary obtained[33] and almost similar[34] 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[34] has observed 88.88% of maturation rate through direct manner. Healthy, culturable oocytes with cumulus cell complex were matured on granulosa cell monolayer that showed optimum cumulus expansion in most of the oocytes obtained.

During oocyte growth and maturation, proteins are synthesized and zona pellucida glycoproteins[35, 36] that help in fertilization and embryo development until the embryonic genome becomes transcriptionally active[37]. Protein synthesis and phosphorylation occur during

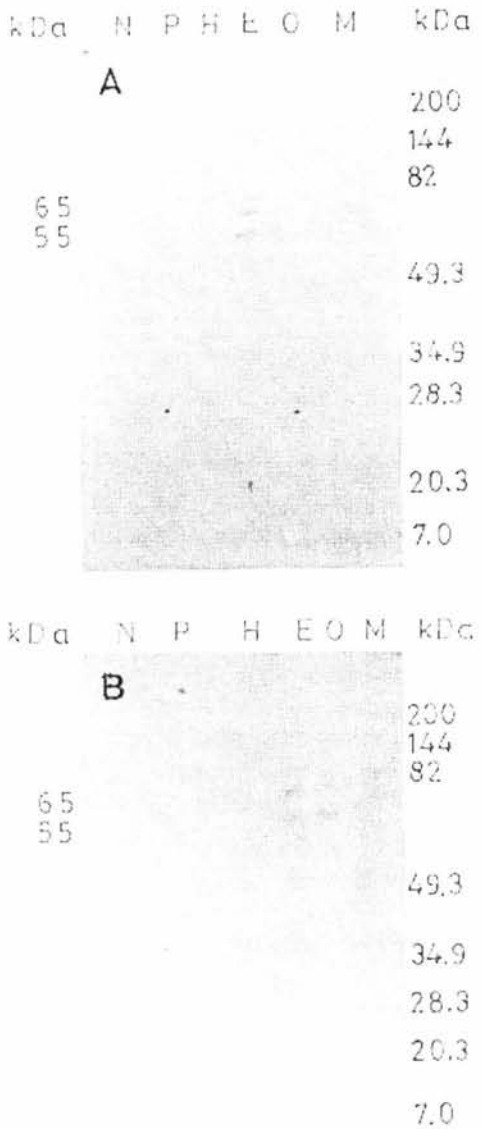


Fig. 5a — Illustrating in the Western blotting using antisera of oocytes secretory proteins of IVM showing 2 bands of 55 kDa and 65 kDa (Lane: O) and IVMFC embryos showing 2 bands of 55 kDa and 65 kDa (Lane-E) and midluteal (N), estrus (H), pregnant (P) goat serum showing no bands and M-molecular protein marker.

Fig. 5b — Illustrating in western blotting using antisera of IVMFC embryo released proteins, secretory proteins of IVM oocyte and IVMFC embryo with midluteal, estrus and early pregnant serum proteins. N-midluteal, P-early pregnant and H-estrus serum proteins showing no band; E-secretory proteins of IVMFC embryo showing two protein bands of approximate molecular weight of 55 and 65 kDa; O-secretory proteins of IVM oocyte showing two protein bands of approximate molecular weight of 55 and 65 kDa and M- Molecular protein marker.

maturation of bovine and ovine oocytes[38]. During the first 24 hr in maturation, porcine oocytes secreted 30, 37, 45 and 46 kDa proteins into culture medium[5]. At least seven oocyte-specific and five cumulus-specific proteins were synthesized during bovine oocyte maturation[8]. Rat oocyte contained seven polypeptides of molecular weight of 37, 56, 60, 66, 69, 80 and 97 kDa[4]. In Western blot a 65 kDa protein corresponding to cyclin $B_1$ protein was found in goat oocyte[9].

In the present study, 45, 55, 65 and 95 kDa proteins were obtained from *in vitro* matured goat oocytes after analyze on SDS-PAGE (12.5%) and silver staining (Fig.4 Lane D). These results were in agreement with those 45 kDa protein in porcine[5] 55 kDa protein in rat oocyte[4] and 65 kDa protein in goat oocyte[9]. Only 95 kDa proteins were observed in this study that has not been reported in earlier studies, though 97 kDa protein has been reported in rat oocyte[4]. With Coomassie brilliant blue staining of SDS-PAGE gel, proteins of 45, 55 and 65 kDa (Fig. 3, Lane B) were observed and 95 kDa proteins were not visible in stained gel in the present study. It suggested that probably very small quantities of these proteins were secreted from matured oocytes of goat.

Embryonic genome becomes transcriptionally active after fertilization and the timing of activation varies among different mammalian species[3]. In bovine embryo, the embryonic genome activated between 4 to 8-cells stage and results in synthesis of proteins that are required for cleavage. The timing of protein synthesis in early cleavage embryo has been reported in human and pig 4 cells stage[16] and in cow and sheep 8 cells stage[18]. The first major products of zygotic gene activity of mouse embryo occur as early at two cell stage that is a heat shock protein 70 kDa[39]. Two proteins of 17 and 24 kDa were isolated from *in vitro* culture medium of day 17 goat conceptus[40]. In the two cell stage of nuclear transfer embryo, 110 kDa proteins was obtained[41]. The total protein synthesis has been shown to be consistently high during first 2 cleavages, that reduces by 95% in the 3rd cell cycle and again increases at 5th cell cycle onward[15].

In the present study, 45, 55 and 65 kDa proteins were obtained from IVMFC embryo culture medium (Fig. 4, Lane H). The protein profile was similar to

he secretory protein profile of oocytes with the exception of a 95 kDa additional protein from oocyte (Fig. 4, lane G). It seems that the genome activation ake place in goat embryos at late morula stage thus n the present study no new protein was observed.

In the present study, the secretory proteins of IVM ocytes and IVMFC embryos showed clear bands with its respective antisera raised in rabbit. (Fig. 1, A, 3) that the antibodies were actually formed against he oocytes and embryos release proteins. They cross-eacted with each other in both cases and the titer was 1:8. This showed that probably oocytes and early cell stage embryo released same type of proteins as has been reported earlier at embryos, maternal gene emain activated[37].

Secretory proteins of oocytes consist of 45, 55, 65 nd 95 kDa protein bands (Fig. 4, Lane G) and mbryo 45, 55 and 65 kDa protein (Fig. 4, Lane H). As reported earlier, of 45 kDa protein[5] 55 kDa protein[9] and 65 kDa protein[4] were identified from VM oocytes. In the present study, in addition of these rotein bands an additional band of 95 kDa protein vas observed. IVMFC embryos also had similar rotein of 45, 55 and 65 kDa. These results indicated hat genes turned on in oocyte remain ranscriptionally active during early embryo levelopment. Similar protein patterns were found in nidluteal, estrus and early pregnant goat serum. Fig. 4, Lane A, B; E, F; and C, D).

Western analysis of secretory proteins of IVM ocytes and IVMFC embryos with midluteal, estrus nd early pregnant goat serum proteins using antisera f oocytes generated proteins was carried out. In the resent study, two clear bands of 55 and 65 kDa were btained from oocyte (Fig.5a, Lane O) and 55 and 65 Da for embryo (Fig. 5a, Lane E) generated proteins. one of these bands were observed in midluteal, strus and early pregnant goat serum proteins in this Vestern blot (Fig. 5a, Lane N, H and P). A 65 kDa rotein of goat oocyte has been reported in Western nalysis[9]. In the present study, 65 kDa protein has lso been identified. Another protein of 55 kDa of ocyte was also found to be similar to 55 kDa of mbryo and they were immunologenic. But the roteins of 45 and 95 kDa of oocytes and 45 kDa of mbryos were not immunologenic. None of the roteins of midluteal, estrus and early pregnant goat erum proteins showed immunogenecity against the antisera of oocyte and embryo generated proteins. It seems that the amount of protein released by one or two oocytes in ovulation is very low and get further diluted when reaches in blood, if at all, it migrates from oviduct to blood. In case of early stage of embryo, a similar interpretation could be made as we cultured 10-20 embryos in 100 µl drop for 24 hr and when this medium was used for SDS-PAGE analysis without concentration we did not observed any band in those case.

Western blotting of secretory proteins of IVM oocyte and IVMFC embryo with midluteal, estrus and early pregnant goat serum proteins using antisera of embryo generated proteins. In the present study also two proteins band of 55 and 65 kDa were observed from secretory proteins of oocytes (Fig. 5b, Lane O) and embryo (Fig.5b, Lane E) as in earlier result using antisera of oocyte generated proteins. Only one report is available that reveals that 65 kDa protein of goat oocyte is immunogenic in nature[9]. None of the protein of midluteal, estrus and early pregnant goat serum proteins showed immunogenecity against the antisera of both embryo and oocyte generated proteins (Fig. 5b, Lane N, H and P).

It may be concluded that IVM oocytes released more proteins than IVMFC embryos till morula stage. Out of 45, 55, 65 and 95 kDa released proteins identified in oocyte, only 55 and 65 kDa proteins were found to be immunogenic and 45, 55, and 65 kDa embryo secreted proteins out of which 55 and 65 kDa proteins were immunogenic and similar to that of oocyte released proteins. None of the protein was found in blood circulation of midluteal, estrus and early pregnant goat.

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