EFFECT OF EPIDERMAL GROWTH FACTOR AND INSULIN ON *IN VITRO* MATURATION OF CAPRINE OOCYTES AND PRE IMPLANTATION EMBRYO DEVELOPMENT

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ABSTRACT

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Totally 1362 oocytes were collected from 358 goat ovaries obtained from abattoir, by slicing technique. The overall culturable oocytes recovery rate per ovary was 2.02.In experiment I(EGF @ 10 ng per ml), the number of inseminated oocytes cleaved day 1-2 were 145 embryos at 2-4 cell stage, 112 embryos at 8 cell stage on day 3, 75 embryos at 16-32 cell stage on day 4-5, 46 embryos at blastocyst stage on day 6-7.In experiment II(insulin @ 1 µg per ml), the number of inseminated oocytes cleaved at day 1-2 were 151 embryos at 2-4 cell stage, 123 embryos at 8 cell stage on day 3, 83 embryos at 16-32 cell stage on day 4-5 and 57 embryos at blastocyst stage on days 6-7.Inexperiment III (control) the number of inseminated oocytes cleaved at day 1-2 were 154 embryos at 2-3 cell stage, 98 embryos at 8 cell stage on day 3, 62 embryos at 16-32 cell stage on day 4-5 and 31 embryos at blastocyst stage on day 6-7. Thus supplementationof growth factors EGF and insulin to maturation and culture media can increase *in vitro* maturation rate and improve the embryo development rate in caprine.

Key words: Caprine, Epidermal growth factor, Insulin and Embryo development

INTRODUCTION

Goats play an important role in rural economy of India and thereby offer a means of livelihood to a large section of economically weaker population. Rapid progress has been made during last few decades in the development of advanced reproductive technologies for genetic improvement of farm animals. Large quantities of genetically superior embryos are needed for any embryo biotechnological research work. Technique of synchronization, superovulation and embryo transfer has significantly improved the supply of embryos. However, low efficiency and productive cost have reduced its extensive application. Hence, *in-vitro* maturation, fertilization and culture (IVMFC) of follicular oocytes has been considered as better alternative to embryo collection and transfer technique (Lonergan and Fair, 2008). This could surpass some of the set backs of embryo transfer technique and at the same time allows large scale and economical production of embryos for transfer and for biotechnological tools like sexing, splitting and cloning etc. IVMFC of oocytes of bovine and ovine have been studied extensively whereas fewer studies have been reported for goats. Realizing the importance of this technology in country like India, the present study was proposed using goat as experimental modelto study the effect of exogenous addition of epidermal growth factor (EGF) and insulin into the maturation and culture media for *in vitro* maturation and culture of caprine embryos.

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MATERIALS AND METHODS

A total of 358 goat ovaries were collected from the slaughter house and transported to the laboratory in 0.9 per cent saline containing penicillin (100 IU per ml) and streptomycin (50 mg per ml) at 30-35°C within 2 hrs of slaughter. Ovaries were trimmed to remove the extra ovarian tissues and washed 2-3 times with tap waterand 7-8 times with normal saline (at 38°C) supplemented with gentamicin.The washed ovaries were transferred in a sterile beaker containing warm

saline supplemented with gentamicin.The oocytes were harvested from ovaries by slicing.The collected follicular fluid along with oocytes collection medium was transferred in to a 50 ml sterile conical tube and kept at 37°C in a serological water bath (10 min) for the sedimentation of oocytes. The supernatant was discarded and the pellet was diluted with 50 ml of TL-HEPES medium and transferred into two-three petridishes (Falcon, 90 mm) for oocytes screening under zoom stereomicroscope. They were washed three to five times in oocytes collection medium and final washing was done in pre-incubated maturation medium.Oocytes were graded as A, B, C, D and E (Nandi *et al*, 1998). Only A and B graded oocytes were used for this experiment. Immature oocytes were matured *in-vitro* for 24-27 hrs in CO_2 incubator maintained at 38.3°C, 5 per cent CO_2 and 95 per cent relative humidity. A total of 234 oocytes were transferred to 100µl maturation droplets (@ 20 oocytes per droplet) supplemented with EGF @ 10 ng per ml (Experiment I), 241 oocytes were transferred to maturation droplets supplemented with insulin @ 1 µg per ml (Experiment II) and 247 oocytes were cultured in maturation medium without any supplement (control).

Sperm TALP and Fertilization TLAP media were freshly prepared every time. 50 µl fertilization droplets were made and covered with mineral oil and pre-incubated in CO_2incubator. The oocytes were then denuded from their cumulus attachment mechanically. The denuded oocytes were washed thrice in Sperm TALP and thrice in Fertilization TALP (pre-incubated) and transferred to fertilization droplets (pre-incubated).

Testes from adult buck collected from slaughter house, were washed thoroughly (after removing the tunica albuginea) with tap water and normal saline (37°C). Testes were sterilized with alcohol. The cauda epididymis was given deep cut and the semen was flushed into a petridish (90mm) with sperm TALP medium.Motile sperms were separated by swim up procedure (Parrish *et al.*, 1995).The oocytes were inseminated with 2 million of sperms/ml concentration and incubated for 18-24 hrs in CO_2 incubator.

After 18-24 hrs of sperm – oocytes co-incubation, the inseminated oocytes were washed in embryo culture medium to remove the excess sperm surrounding the zona. A total of 234 and 241 inseminated oocytes were transferred to embryo culture medium supplemented with EGF @ 10 ng per ml and insulin @ 1 µg per ml, respectively, and 247 fertilized oocytes were cultured in simple embryo culture medium for 7-8 days.

RESULTS AND DISCUSSION

Total 1362 oocytes were collected from 358 goat abattoir ovaries by slicing technique. The overall oocytes recovery rate per ovary was 3.8. Out of 1362 oocytes, 823 were good quality (A and B grade) oocytes. The overall culturableoocytes recovery rate per ovary was 2.02.In experiment I(EGF @ 10 ng per ml), the number of inseminated oocytes cleaved at day 1-2 were 145 embryos at 2-4 cell stage, 112 embryos at 8 cell stage on day 3, 75 embryos at 16-32 cell stage on day 4-5, 46 embryos at blastocyst stage on day 6-7. The percentage of cleavage was 61.9 (145/234) at 2-4 cell stage, 77.2(112/145) at 8 cell stage, 51.7 (75/145) at 16-32 cell stage and 31.7 (46/145) at blastocyst stage.

In experiment II(insulin @ 1 µg per ml), the number of inseminated oocytes cleaved at day 1-2 were 151 embryos at 2 cell stage, 148 embryos at 4 cell stage on day 2, 123 embryos at 8 cell stage on day 3, 83 embryos at 16-32 cell stage on day 4-5 and 57 embryos at blastocyst stage on days 6-7. The percentage of cleavage was 62.6 (151 / 241) at 2 cell stage, 98.0 (148 / 151) at 4 cell stage, 81.4 (123 / 151) at 8 cell stage, 54.96 (83 / 151) at 16-32 cell stage and 37.7 (57 / 151) at blastocyst stage.

Inexperiment III (control) the number of inseminated oocytes cleaved at day 1-2 were 154 embryos at 2 cell stage, 150 embryos at 3 cell stage on day 2, 98 embryos at 8 cell stage on day 3, 62 embryos at 16-32 cell stage on day 4-5 and

31 embryos at blastocyst stage on day 6-7. The percentage of cleavage was 62.3 (154 / 247) at 2 cell stage, 97.4 (150 / 154) at 4 cell stage, 63.6 (98/154) at 8 cell stage, 40.2 (62/154) at 16-32 cell stage and 20.1 (31 / 154) at blastocyst stage.

The percentage of blastocyst development (mean ± SE) was 7.66 ± 0.33, 9.50 ± 0.76 and 5.16 ± 0.47 in the EGF, insulin and control group, respectively. The percentage of blastocyst developed was significantly ($P< 0.01$) higher in treatment groups compared to the control group and no significant difference ($P >0.05$) was observed between treatment groups.

The results suggest that the supplementation of EGF and insulinin maturation medium significantly enhances the *in-vitro* maturation rate and embryo development competence of caprine oocytes. Goat cumulus cells express EGF receptors and EGF triggers signalling through the MAPK pathway during IVM in goat COCs (Gall *et al.*,2005).The intrinsic tyrosine kinase of EGF receptor (EGF-R) is activated by binding of EGF, resulting in EGF-R autophosphorylation and subsequent tyrosine phosphorylation of numerous substrates within the cell (Carpenter and Cohen, 1990).Epidermal growth factor improves not only nuclear maturation but also cytoplasmic maturation of cumulus enclosed caprine oocytes *in-vitro* (Cognie *et al.*,2004), cleavage rate as well as embryo development (Sirisathien *et al.*,2003).

Insulin is commonly used in cultured cells and tissues to increase cell viability due to its ability to remove pro-apoptotic molecules and phosphatidylinositol-3 kinase activation. In addition, insulin regulates important intracellular processes, such as amino acid transport, glucose and lipid metabolism, gene transcription and protein synthesis. Insulin stimulates granulosa and thecal cell proliferation and mitogenesis and synergizes with gonadotropins to stimulate granulosa and thecal cell steroidogenesis.

Addition of insulin to *in vitro* media promotes oocytes maturation and also affects their subsequent *in-vitro* development (Stefanello *et al.*, 2006). The results of experiment II demonstrated that insulin at a dose of 1 µg per ml stimulated nuclear maturation of cumulus-enclosed oocytes. The addition of insulin during oocyte maturation not only helped in achieving nuclear maturation but also influenced the cytoplasmic maturation and improved early embryonic development. These findings were consistent with previous reports (Pawshe *et al.*, 1998) showing that insulin enhanced embryonic cleavage rate and development when added to the oocyte maturation medium. The previous study suggested that insulin increased oestradiol production by the theca granulosa cells in serum free culture (Shores *et al.*, 2000). Furthermore, it was indicated that insulin acts as an anti-apoptotic factor during oocyte maturation (Wasielak & Bogacki, 2007). Supplementation of growth factors EGF and insulin to maturation and culture media can increase *in-vitro* maturation rate and improve the embryo development rate in caprine.

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