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Influence of Epidermal Growth Factor in the *In vitro* Development of Bovine Preimplantation Embryos

Sadekar Gautami Vinayak, Timiri Varadarajan. Meenambigai*, Angappan. Mangalagowri, Devarajan Reena and Kumanan. Vijayarani

Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Vepery, Chennai – 600 007

*Corresponding Author E-mail: meena_dharmaraj@yahoo.co.in

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ABSTRACT

Epidermal growth factor (EGF) plays a crucial role in early embryogenesis. We investigated whether in vitro culture medium supplementation with epidermal growth factor (EGF) has effects in the development of bovine oocytes and embryos. Cumulus–oocyte complexes (COCs) were matured in TCM 199 medium (control) and in TCM 199 medium with EGF at concentrations of 10 - 50 ng/ml. After maturation for 44–46 h, Metaphase II oocytes were assessed based on the degree of cumulus expansion and extrusion of first polar body. Homogenously expanded cumulus oocyte complexes (COCs) were fertilized, and the presumptive zygotes were cultured in the same groups. Then, 2, 4, 8 cells and morulae were collected for the analysis of quality. A concentration of 30 ng/ml of EGF in maturation medium increased MII oocyte numbers and did not affect oocyte maturation. Medium Supplementation with remaining concentration of EGF resulted in lower maturation of oocytes and in control embryos. EGF treatment also increased the cleavage rate and morula percentages respectively.

Key words: *EGF, In vitro, Oocyte, Maturation, Cleavage, Bovine.*

INTRODUCTION

Even though India has large population of cattle, the production performance is not that efficient. Biological constraints in cattle breeding is one of the reason behind it. By adopting assisted reproductive technology such as somatic cell nuclear transfer (SCNT), *in vitro* maturation (IVM), *in vitro* fertilization (IVF), *in vitro* culture and intracytoplasmic sperm injection (ICSI), it is possible to produce superior quality embryos from live or dead/slaughtered animals. Use of such good quality embryos will significantly help in increasing production potential.

Growth factors play important roles in the developmental capacity of oocytes and embryos in several species[7]. Preimplantation mammalian embryos express many growth factors and receptors, including epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1).

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EGF plays a key regulatory role in preimplantation embryonic development, and it stimulates both cellular proliferation and differentiation[8]. In particular, exogenous EGF enhances the developmental rate and mitogenesis of preimplantation bovine embryos[11].

Supplementation of IVM medium with EGF significantly increased (P<0.01) the nuclear maturation and cleavage rates compared to those with control medium and further concluded that EGF had a positive influence on cumulus expansion, nuclear maturation and post fertilization cleavage, but not on subsequent embryo development. EGF, the founding member of the EGF-family of ligands, was for many years believed to mediate the LH signal as it induced oocyte maturation and cumulus expansion in vitro in several mammalian species including human[6]. Epidermal growth factor (EGF) has important autocrine and/or paracrine effects during early embryonic development[5].

With these in back drop, the present study was carried out to see the influence of various concentrations of EGF on bovine preimplantation embryo development and to analyse its effects on maturation, fertilization and cleavage.

MATERIAL AND METHODS

Oocyte recovery and evaluation

Ovaries used for the present study were collected from Slaughter House. Ovaries were washed and rinsed thoroughly for five times in normal saline supplemented with gentamicin. The ovaries were then rinsed with Oocyte culture medium (OCM) and sliced with Bard Parker blade to release Cumulus Oocyte Complexes (COCs). The recovered oocytes were rinsed with TCM kept in 35mm petridishes and graded based on their cellular investment and homogenecity of ooplasm. Compact COCs with an unexpanded cumulus mass having more than four layers of cumulus cells and with homogenous, evenly granular ooplasm were taken for further maturation (Fig-1).

EGF supplementation and *In vitro* maturation of oocytes

TCM 199 medium was used for maturation of oocytes which was supplemented with 10 percent of Fetal bovine serum (FBS), one µl/ml penstrep, one µg/ml folltrophin (Bioniche, Canada), 0.021 IU/ml Lutenizing hormone (LH) and one µg/ml estradiol to prepare maturation medium for oocytes. In this media as per the trials, epidermal growth factor (10ng/ml, 20ng/ml, 30ng/ml, 40ng/ml and 50ng/ml) were added along with controlswithout EGF supplementation. After preparation of media, 6 dropltes, each containing 50 µl of media was laid in 35 mm petridish and were covered with sterile mineral oil (sigma 8410) and equilibrated under culture environment for 2 hours.

Graded immature oocytes were rinsed in three 35 mm petridishes containing OCM followed by three prewashes in pre-incubated maturation medium. The washed oocytes were transferred into each droplet (10 COCs per droplet) and the oocytes were cultured for 27 hours in a humidified atmosphere with 5 percent CO2 in the air. Maturation rate was assessed based on the degree of cumulus expansion and extrusion of first polar body (Fig-2). Homogenously expanded cells were further taken for *in vitro* fertilization. Matured oocytes were washed in pre-equilibrated sperm TALP medium and then twice in IVF TALP medium and then were transferred to 75 microlitter of the droplets prepared using IVF TALP containing heparin (10 µg/ml) overlaid with sterile mineral oil and incubated.

In vitro fertilization and *In vitro* culture

Frozen bovine semen straws (0.25ml French mini) were thawed at 37 ^{o} C for 30 sec in a water bath. The tip was cut to release the semen from the straw into 5 ml of sperm TALP medium and then centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and 100 µl of pellet was distributed into three tubes with 1 ml of sperm TALP in each. These three tubes were kept in a slanting position for upward movement of sperms so as to get more motile spermatazoa in the supernatant fluid for which the tubes were

incubated for 45 minutes. The supernatant from each tube was collected without disturbing the lower part of fluid and then transferred together into a sterile 15 ml centrifuge tube. Centrifugation at1000 rpm for 5 minute was carried out and the supernatant was discarded. The final sperm pellet was checked for motility. Then prepared sperms were used for fertilization. To each 75 µl of droplets containing 15 oocytes, 4µl of sperm suspension was added and kept for incubation. This added sperm suspension contained about 3 million actively motile sperms/ml .The culture plate was incubated for 22 to 24 hours at 38.5^{o} C in a humidified atmosphere of 5 percent CO2 in air. (Fig-3).

Synthetic oviductal fluid medium (SOF) for IVC was prepared by addition of 9 ml SOF, 1ml 10 percent FBS, Essential amino acids (EAA) 200 µl, Non-essential amino acids (NEAA) 100 µl.. IVC droplets of 50 µl and SOF filtered in three 35mm petridishes were pre-equilibrated for 2 hours at 38.5^{o}C in a humidified atmosphere of 5 percent CO2 in the air. After 2 hours of incubation presumptive zygotes were washed in IVF-TALP medium and cumulus cells were denuded and then again washed twice in pre-equilibrated SOF and then transferred to the pre-equilibrated dropletsa and incubated. The cleaved embryos that were collected at 2 cell stage, 4 cell stage, 8 cell stage and morula stages were studied for their quality and development under the influence of EGF at varying concentrations (Fig-4).

RESULTS AND DISCUSSION

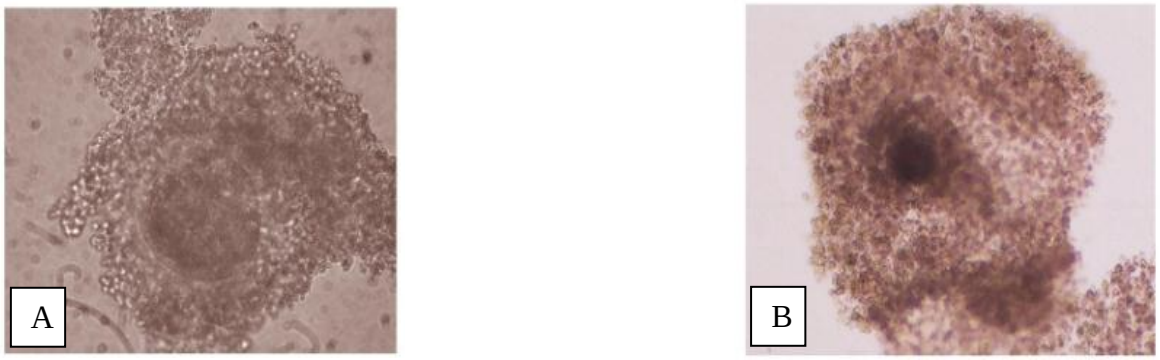
The effects of the influence of epidermal growth factor on maturation, fertilization and development of embryos are furnished in table 1. Out of 300 oocytes from five trials with EGF concentration of 10, 20, 30, 40, 50 ng/ml cultured under *in vitro* condition, showed homogenously expanded cumulus cells with the maturation per cent of 86 (258 oocytes), 89 (268 oocytes), 91 (273 oocytes), 82 (247 oocytes), and 77 (232 oocytes) respectively. For the control where no EGF was added showed maturation percentage of 84 (253 oocytes). Chi-square test revealed that there was highly significant difference between the maturation rates for different concentration of EGF. The percentage of cleavage rate was 80, 81, 85, 78, and 72 for EGF concentration of 10, 20, 30, 40, 50 ng/ml respectively. For the control where no EGF was added showed cleavage percentage of 78. Chi-square test revealed that there was highly significant difference between the cleavage rates for different concentration of EGF. The percentage of morula rate was 51, 53, 60, 50 and 46 for EGF concentration of 10, 20, 30, 40, 50 ng/ml respectively. For the control where no EGF was added showed morula percentage of 49. Chi-square test revealed that there was highly significant difference between the morula rates for different concentration of EGF.

The results indicate that EGF enhances cumulus expansion in bovine COCs, in the same way as demonstrated by Downs (1989) for rodent oocytes. The present study demonstrates that exposure of cumulus enclosed bovine oocytes during *in vitro* maturation to EGF stimulated cumulus expansion (Kylie R. Dunning, 2015) and improved the percentage of oocytes undergoing nuclear maturation as well as the cleavage[5]. Study demonstrated that EGF enhanced oocyte fertilizability when cultured in defined medium[3, 10]. Present results are in agreement with previous reports of improvement in cleavage yields after culture with added EGF at the concentrations between 20ng to 30ng/ml of maturation media[4], where previous few studies demonstrated that COCs maturation and cleavage did not show much difference when treated with EGF at concentrations between 10ng to 100ng/ml of maturation media[2].

TABLE 1: Effect of Epidermal Growth Factor on maturation, fertilization and bovine embryo development under *in-vitro* conditions

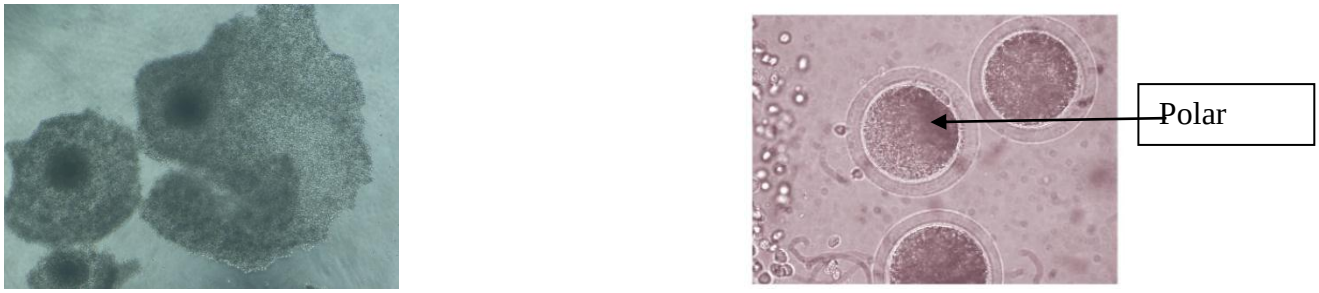
CONCENTRATION ng/ml	NO OF OOCYTES PER TRIAL	NO OF TRIALS	MATURATION %	CLEAVAGE %	MORULA %
10ng/ml	60	5	258 (86%)	245 (80%)	155 (51.66%)
20ng/ml	60	5	268 (89%)	255 (81%)	160 (53.33%)
30ng/ml	60	5	273 (91%)	285 (85%)	182 (60.66%)
40ng/ml	60	5	247 (82%)	237 (78%)	151 (50.33%)
50ng/ml	60	5	232 (77%)	224 (72%)	145 (48.33%)
control	60	5	253 (84%)	238 (78.33%)	148 (49.33%)
Chi square test				χ^2 = 27.40 **	χ^2 = 10.77 *

Fig. 1: Grades of bovine oocytes



A- Grade A B - Grade B

Fig. 2 : Influence of EGF on bovine oocyte maturation



Expansion of cumulus cells

Fig. 3: *In vitro* fertilization

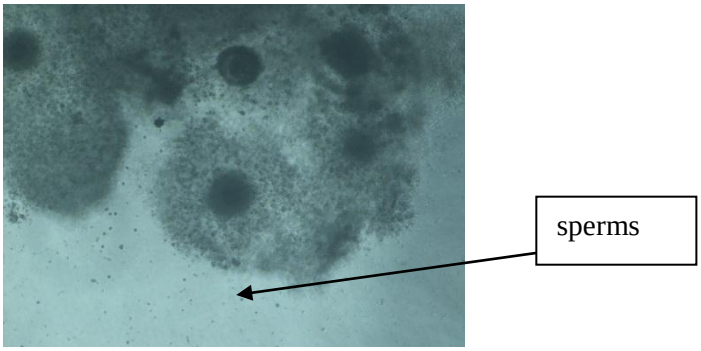
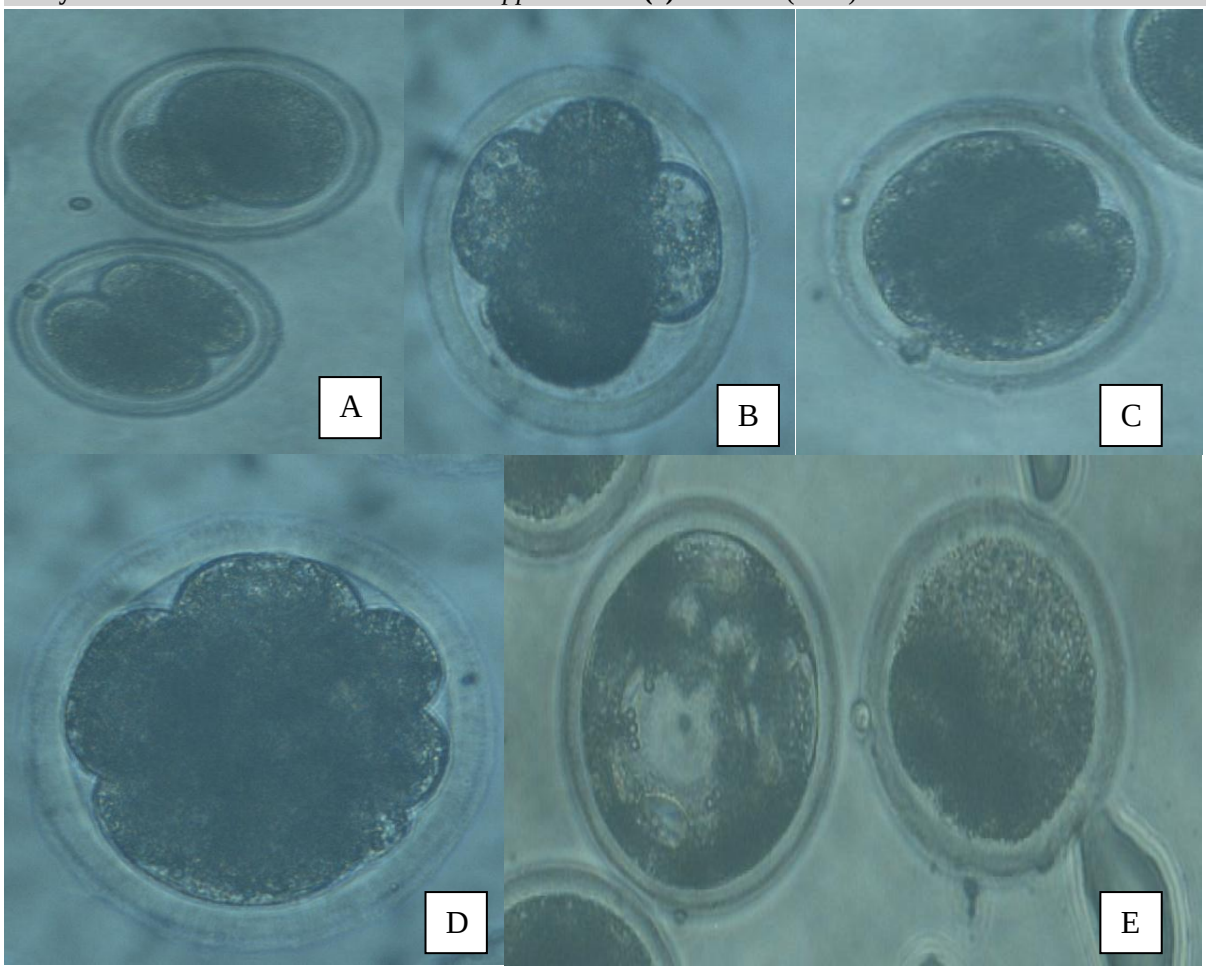


Fig. 4: Bovine Embryos developed under the influence of EGF



A- Two cell stage , B- Four cell stage , C- Eight cell stage , D- Morula, E- Blastocyst

CONCLUSION

In conclusion, supplementation of EGF in maturation media positively affected cumulus expansion and maturation to Metaphase II. However, 30ng/ml of maturation media was the optimum dose for the EGF supplementation. Epidermal growth factor supplementation increased embryo development rates and quality following IVF and IVC. Hence, the results suggest that EGF might be one of the major follicular factors responsible for stimulating oocyte cytoplasmic as well as nuclear maturation.

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