

## Development of Cattle Embryo Through *In Vitro* Technique Using Epidermal Growth Factor as A Media Supplement

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### Abstract

The present study was conducted to produce cattle embryos through *in vitro* culture by supplementing media with epidermal growth factor. Immature cattle oocytes were collected from slaughterhouse ovaries, washed 5-6 times and cultured in maturation media (TCM-199+10% FBS+5  $\mu\text{g ml}^{-1}$  FSH-P+0.33 mM sodium pyruvate+50  $\mu\text{M}$   $\beta$ -Mercaptoethanol+50  $\mu\text{g ml}^{-1}$  gentamicin sulfate) supplemented with epidermal growth factor with three different concentrations (5, 10 and 20  $\text{ng ml}^{-1}$ ) for 24 h. in 5%  $\text{CO}_2$  incubator at 38.5  $^{\circ}\text{C}$  with maximum humidity. After 24 h matured oocytes were allowed for fertilization with capacitated sperms in Fert-BO media at 38.5  $^{\circ}\text{C}$  in  $\text{CO}_2$  incubator. After 15-18 h of sperm-oocyte co-incubation, the cumulus cells were washed off from the oocytes by gentle pipetting in washing medium. The oocytes were then washed 1-2 times with modified Charles Rosenkrans 2 amino acid (mCR2aa) media and cultured in 100  $\mu\text{l}$  droplet supplemented with epidermal growth factor, and cultured for cleavage. After 48 h cleavage was checked and further co-cultured with oviductal cells for development. In the present study the cleavage rate and morula formation rate were significantly higher in the treatment group as compared to control group. The mean percentage of cleavage rate was  $41.63 \pm 1.92$  in control group. The highest mean percentage of cleavage rate was  $55.13 \pm 1.45$  in 10  $\text{ng ml}^{-1}$  treatment groups. From the present study it could be concluded that epidermal growth factor may have induced the cleavage after fertilization.

**Keywords:** Cattle, oocyte, embryo, epidermal growth factor, IVF

### 1. Introduction

Production of large number of embryos through assisted reproductive techniques like *in vitro* maturation and *in vitro* fertilization have great potential for production of offspring and faster multiplication of superior germplasm or cryopreserve the embryos for future use (Cognie et al. 2003). Slaughterhouse derived ovaries provide a cheap and abundant source of large number of oocytes either for production of offspring or research purpose (Malakar et al., 2007). *In vitro* production of embryos involves oocytes recovery, maturation and fertilization with capacitated spermatozoa and culture of the produced embryos. Production of embryos from *in vitro* maturation and fertilization has been improved using different macromolecule supplementation in medium (Herrick et al., 2004). Improvement of developmental competence of mammalian oocytes by supplementation of *in vitro* maturation (IVM) media with hormones and serum supplements has been the subject of many investigations. Following the early report of Staigmiller and Moor, 1984, in which addition of granulosa cells, gonadotropins and estradiol to the culture media was

found to enable the sheep cumulus oocyte complexes (COCs) to mature outside follicles, supplementation of the IVM media with gonadotropins and estradiol has been found to be essential for acquisition of developmental capacity of oocytes in cattle (Brackett et al., 1989). Supplementation of the IVM media with fetal calf serum (FCS) (Staigmiller and Moor, 1984; Totey et al., 1993) or estrus cow serum (Brackett et al., 1989; Madan et al., 1994) has also been found to be necessary for achieving high maturation rates for cattle and buffalo oocytes. All these studies however, employed an IVM culture medium which was supplemented with gonadotropins, estradiol and a serum source. Supplementing the culture media with various growth factors such as insulin like growth factor-1 (IGF), epidermal growth factor (EGF) and other growth factors also boost up the developmental competence of *in vitro* cultured bovine oocytes (Neira et al., 2010; Ahumada et al., 2013) and embryo production. Various combinations of growth factors positively affect blastocyst formation rates in cultured bovine embryos (Sirisathien et al., 2003). The growth factor supplemented serum-free system was satisfactory for *in vitro* bovine embryo production (Dhali et al., 2011). EGF is shown



to have a positive effect during *in vitro* maturation and has been reported in follicular fluid at levels, which can stimulate meiosis. The present study was conducted to produce cattle embryos through *in vitro* maturation, fertilization and culture by supplementing culture media with epidermal growth factor.

## 2. Materials and Methods

All plastic wares were used from Tarson Products Pvt. Ltd. (Kolkata, India) and chemicals/ biochemicals/ paraffin oil from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA), the 0.22  $\mu\text{m}$  disposable syringe filters were used from Millipore Corp., Bedford, MA, USA. Disposable, nontoxic and non-pyrogenic plastic syringes and sterile disposable 19 gauge hypodermic needles of Dispovan, Kolkata, India unless otherwise mentioned.

### 2.1. Oocytes collection and maturation *in vitro*

Fresh cattle ovaries were collected immediately after slaughter at an abattoir, Kolkata, 2018 and transported within 3-4 h to the laboratory in isotonic saline solution supplemented with penicillin (100 IU  $\text{ml}^{-1}$ ) and streptomycin (50  $\mu\text{l}$   $\text{ml}^{-1}$ ) maintained at 30-35 °C. Follicular oocytes from apparently non-atretic surface follicles (3 to 8 mm in diameter) were aspirated with 19 gauge hypodermic needle to a 5 ml disposable plastic syringe containing oocyte aspiration medium (TCM-199+DPBS+0.3% BSA+50  $\mu\text{g}$   $\text{ml}^{-1}$  gentamicin sulfate) and categorized into A grade (>5 layer of cumulus cells), B grade (3-5 layer of cumulus cells), C grade (<3 layer of cumulus cells) and D grade (partial/without layer of cumulus cells). All A, B and C grade oocytes complexes with a compact cumulus cell layer and homogenous, evenly granulated cytoplasm were used for maturation. All the COCs were washed 4-6 times in washing medium (TCM-199+10% FBS+0.81 mM sodium pyruvate+50  $\mu\text{g}$   $\text{ml}^{-1}$  gentamicin sulfate), followed by 2-3 times in maturation medium (TCM-199+10% FBS+5  $\mu\text{g}$   $\text{ml}^{-1}$  FSH-P+0.33 mM sodium pyruvate+50  $\mu\text{M}$   $\beta$ -Mercaptoethanol+50  $\mu\text{g}$   $\text{ml}^{-1}$  gentamicin sulphate+epidermal growth factor). Then groups of 20-25 COCs were placed in 100  $\mu\text{l}$  droplets of maturation medium, covered with sterile mineral oil in a 35 mm petri dish and incubated for 24 h at 38.5 °C in a 5%  $\text{CO}_2$  incubator with maximum humidity.

### 2.2. Sperm preparation and *in vitro* fertilization

The spermatozoa used for IVF throughout the study were from the same donor that had been tested for IVF earlier. The spermatozoa were prepared for fertilization as described earlier (Das et al., 2013). Briefly, two straws of frozen-thawed cattle semen were suspended in 8 ml of Working Brackett Oliphant (WBO) medium (Brackett et al. 1975) with 10  $\mu\text{g}$   $\text{ml}^{-1}$  heparin and 0.57 mM caffeine sodium benzoate and 1.23 mM sodium pyruvate and incubated for swim-up at 38.5 °C. After 15 minutes of incubation progressively motile sperm cells were taken by collecting 4 ml of WBO medium from the top and centrifuged at 2000 rpm for 5 min. After that,

the supernatant was removed and the pellet was dissolved in 1.5 ml of BO medium and centrifuged at 2000 rpm for 5 min. Finally, the pellet was dissolved in 1 ml of Fertilization Brackett Oliphant (FBO). In the treatment group, the FBO was supplemented with different concentration of epidermal growth factor (5, 10 and 20  $\text{ng}$   $\text{ml}^{-1}$ ). The *in vitro* matured oocytes were washed twice with the FBO medium in the same maturation drop and inseminated with capacitated motile spermatozoa (2-4 million spermatozoa  $\text{ml}^{-1}$ ), and placed in 5%  $\text{CO}_2$  incubator at 38.5 °C for 15-18 h with maximum humidity.

### 2.3. Culture of oviductal epithelial cells and presumptive embryo

Fresh oviducts were dissected carefully with blunt end scissors and washed 3-4 times with washing media. Oviductal mucosal layer was carefully expelled by squeezing the oviduct with a sterile glass slide, and the cells were retrieved and transferred into petridish containing washing medium. Cell chunks were washed in washing medium for 5-6 times in washing medium. Cell chunks were then put into 100  $\mu\text{l}$  droplet of maturation media and incubated in 5%  $\text{CO}_2$  incubator at 38.5 °C for 24 h with maximum humidity. After 24 h of incubation cells were picked up and washed in washing media and were then cultured in 100  $\mu\text{l}$  droplet in maturation media covered with mineral oil. After every 48 h half of the medium used to replace with fresh medium. At the end of 15-18 h of sperm-oocyte co-incubation, the presumptive zygotes were separated from the drop and cumulus cells were removed by gentle repeated pipetting in washing medium. The oocytes were then washed 1-2 times with modified Charles Rosenkrans 2 amino acid (mCR2aa) medium and cultured in 100  $\mu\text{l}$  droplet of mCR2aa medium. The treatment group, the mCR2aa was supplemented with EGF with three different concentrations (5  $\text{ng}$   $\text{ml}^{-1}$ , 10  $\text{ng}$   $\text{ml}^{-1}$  and 20  $\text{ng}$   $\text{ml}^{-1}$ ). After 48 h cleaved oocytes/embryos were shifted to 10  $\mu\text{l}$  droplets of mCR2aa blastocyst medium and co-incubated with oviduct cells in 5%  $\text{CO}_2$  incubator with maximum humidity at 38.5 °C for 8 days.

### 2.4. Experimental design and statistical analysis

In the experiment, the effect of epidermal growth factor on *in vitro* fertilized embryo production was examined. The culture media were supplemented with three different concentrations of epidermal growth factor *i.e.* 1) 5  $\text{ng}$   $\text{ml}^{-1}$ , 2) 10  $\text{ng}$   $\text{ml}^{-1}$ , 3) 20  $\text{ng}$   $\text{ml}^{-1}$ . The control group was not supplemented with the growth factor. The data analysis was done by IBM® Statistical Package for the Social Sciences® (SPSS version 20). Descriptive statistics were performed to calculate mean and standard errors. Post hoc analysis or significant differences between means were determined by LSD/TUKEY HSD test.

## 3. Results and Discussion

In the present study all three different media like IVM, IVF and IVC have been supplemented with epidermal growth factor with three different concentrations. In control group there is no supplementation of epidermal growth factor.

The result showed that there exists significant differences ( $p < 0.05$ ) in cleavage rate, 4-cell stage, 8-cell stage, 16-cell stage and morula formation rate in treatment group. The Table 1 indicates that the cleavage rate and morula rate were significantly higher in the two treatment of EGF (5 and 10 ng ml<sup>-1</sup>) groups, as compared to control group. The mean percentage of cleavage rate, 4-cell stage, 8-cell stage, 16-cell stage and morula were 41.63±1.92, 25.29±1.43, 19.11±1.87, 9.71±1.70 and 7.26±0.98 respectively, in control group. In the treatment group, EGF (5 ng ml<sup>-1</sup>), the mean percentage of cleavage rate, 4-cell, 8-cell, 16-cell and morula stage were 49.11±1.22, 33.52±1.89, 24.68±1.82, 14.96±1.30 and 7.82±0.85, in the EGF (10 ng ml<sup>-1</sup>) were 55.13±1.45, 40.55±1.71, 30.98±2.25, 19.27±1.87 and 13.95±1.02 respectively and in the

EGF (20 ng ml<sup>-1</sup>) were 39.99±1.81, 25.95±2.23, 18.31±2.10, 11.39±1.15 and 5.29±0.50 respectively. The mean percentage of cleavage rate and morula is significantly higher in EGF (10 ng ml<sup>-1</sup>) i.e. 55.13±1.45 and 13.95±1.02 than the control group. The cleavage rate and morula formation rate is showing the significant difference between the EGF with (5 ng ml<sup>-1</sup>) 49.11±1.22 and 7.82±0.85 and EGF with (10 ng ml<sup>-1</sup>) 55.13±1.45 and 13.95±1.02, whereas, the treatment of EGF (20 ng ml<sup>-1</sup>) is showing the negative effect in both the cleavage as well as morula formation as compared to control. This shows that, if we increase the concentration of EGF beyond an optimum amount, it may become toxic and will have negative effect on both the cleavage and morula formation rate compared to control. The differences between control

Table 1: Effect of Epidermal growth factor on different embryo developmental stages in cattle

Group	No. of oocytes	Cleavage (Mean±SEM)	4-cell stage (Mean±SEM)	8-cell stage (Mean±SEM)	16-cell stage (Mean±SEM)	Morula stage (Mean±SEM)
Control	169	41.63±1.92 <sup>c</sup>	25.29±1.43 <sup>b</sup>	19.11±1.87 <sup>b</sup>	9.71±1.70 <sup>c</sup>	7.26±0.98 <sup>b</sup>
T <sub>1</sub> (5 ng ml <sup>-1</sup> )	210	49.11±1.22 <sup>a</sup>	33.52±1.89 <sup>a</sup>	24.68±1.82 <sup>ab</sup>	14.96±1.30 <sup>b</sup>	7.82±0.85 <sup>b</sup>
T <sub>2</sub> (10 ng ml <sup>-1</sup> )	207	55.13±1.45 <sup>a</sup>	40.55±1.71 <sup>a</sup>	30.98±2.25 <sup>a</sup>	19.27±1.87 <sup>a</sup>	13.95±1.02 <sup>a</sup>
T <sub>3</sub> (20 ng ml <sup>-1</sup> )	179	39.99±1.81 <sup>b</sup>	25.95±2.23 <sup>b</sup>	18.31±2.10 <sup>ab</sup>	11.39±1.15 <sup>b</sup>	5.29±0.50 <sup>b</sup>

Values (Mean±SEM) in the same column with different subscripts differ significantly ( $p < 0.05$ )

and treatment group is due to the presence of epidermal growth factor receptor (EGF-R) in the immature oocytes and cumulus cells. Epidermal Growth factor has a role in embryo growth and ovarian metabolism, as it plays a great role in the oocytes maturation and it stimulates the pattern of proteins neo-synthesised during oocytes maturation. The EGF-R (EGF-receptor) is involved in the cell proliferation and differentiation. Pat Lonergan et al., 1996, reported that epidermal growth factor have a positive effect during in vitro maturation (IVM) and reported in follicular fluid at levels capable of stimulating meiosis in a variety of species.

#### 4. Conclusion

The growth factor supplementation was satisfactory for in vitro embryo production. Nevertheless, the system was not efficient when embryos were produced from <3 mm follicles and cultured in vitro. The highest mean percentage of cleavage rate (55.13±1.45) and morula formation rate (13.95±1.02) were achieved with the supplementation of epidermal growth factor @ 10 ng ml<sup>-1</sup> in treatment group.

#### 5. Acknowledgement

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