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Enhancement of Developmental Competence of Immature Oocytes Supplemented with Growth Factors in Culture Media

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ABSTRACT

Background: *In vitro* embryo production is a valuable tool for understanding early mammalian development, therapeutic applications, excellent source for research in the field of developmental biology and production of valuable animals. The purpose of this study is to improve the production of *in vitro* cattle embryos using fibroblast and platelet derived growth factor as media supplement.

Methods: Ovaries were collected from local abattoir in 0.9% saline (30-35°C) supplemented with antibiotics. Cumulus oocyte complexes were aspirated, washed 5-6 times and placed in maturation media supplemented with growth factors and cultured in 5% CO_2 incubator at 38.5°C with maximum humidity. After 24 h oocytes were co-incubated with *in vitro* capacitated sperms for fertilization for 15-18 h and then presumptive zygotes were cultured for embryo development. Cleavage was observed after 40-42 h and embryos were co-cultured with oviductal cells for 7-9 days.

Result: The highest cleavage and blastocyst formation rates were 55.93±4.75, 57.06±4.78, 51.24±4.12 and 3.26±1.53, 2.42±1.02, 2.70±1.17 in FGF (1ng ml^{-1}), PDGF (10 ng ml^{-1}) and in combination of FGF and PDGF (1ng ml^{-1} each) respectively. It can be concluded that PDGF (10 ng ml^{-1}) enhanced cleavage rate and FGF (1ng ml^{-1}) enhanced blastocyst formation rate.

Key words: Cattle, Embryo, FGF, IVF, Oocyte, PDGF.

INTRODUCTION

In vitro embryo production (IVEP) is a valuable tool to provide an aid for the understanding of early mammalian development, having therapeutic applications, excellent source for research in the field of developmental biology and production of valuable animals. It's having advantages *viz*. reliability, reproducibility, use of prepubertal donors, slaughterhouse ovaries and senile or in post mortem cases (de Souza- fabjan *et al*. 2014).

This technique is necessary for cloning, transgenesis, conservation of endangered species, treatment of infertility and it contributes to stem cell biology to treat many diseases as well. IVEP coupled with cryo-conservation allow embryo marketing, easy transport of superior germplasms and pathogen-free genetic movement in commercial transactions (Paramio and Izquierdo, 2014). In spite of advances in designing media and culture systems, there are still prominent differences between embryos produced through *in vivo* and *in vitro* techniques. Culture conditions influence the quality and viability of *in vitro* produced embryos to a great extent. *In vitro* embryo development is strongly influenced by the events occurring during oocyte maturation, fertilization and the subsequent development of presumptive embryos. Type of breed, quality of oocytes, follicular size and micro-environment, fertilization atmosphere, and embryo culture milieu also influence the quality (Fiammetta, 2015). Production of embryos has been improved using different macromolecule supplementation in medium (Herrick *et al*. 2004; Kumar *et al*. 2020). To improve the culture system comparison of media supplements for oocytes maturation, fertilization and embryo culture have yielded varying results (Gasparini *et al*. 2006). 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 (Rajesh *et al.* 2020) with FCS (Totey *et al*. 1993) or estrus cow serum (Madan *et al*. 1994), follicular fluid (Das *et al*. 1996) has also been found to be necessary for achieving high maturation rates for cattle and buffalo oocytes. Supplementing the culture media with growth factors stimulated embryo development beyond the 8-cell stage (Thibodeaux *et al*. 1993; Yang *et al*. 1993; Borah and Biswas, 2020). The present study was conducted to enhance the *in vitro* development of Indian cattle embryo by supplementing culture media with platelet derived growth factor and fibroblast growth factor.

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MATERIALS AND METHODS

The present study was conducted in the Eastern Regional Station, ICAR-National Dairy Research Institute, Kalyani in the year of 2018-19. All chemicals/ biochemicals/ mineral oil from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA); disposable syringe filters (0.22 µm) were used from Millipore Corp., Bedford, MA, USA, plastic wares from Tarson Products Pvt. Ltd. (Kolkata, India). Disposable, nontoxic and non-pyrogenic plastic syringes and sterile disposable 19gauge hypodermic needles of Dispovan make, Kolkata, India unless otherwise mentioned.

Oocytes collection and *in vitro* maturation

Fresh cattle ovaries were collected at an abattoir immediate after slaughter and transported within 3 to 4 h to the laboratory in isotonic (0.9%) saline solution supplemented with penicillin (400 IU ml^{-1}) and streptomycin (50 µg ml^{-1}) maintained at 30-35°C. Follicular oocytes from apparently non-atretic surface follicles (>3 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 µg ml^{-1} gentamicin sulphate). All COCs with compact cumulus cell layer and homogenous, evenly granulated cytoplasm were washed 5-6 times in washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 50 µg ml^{-1} gentamicin sulphate), followed by 2-3 times in maturation medium (TCM-199 + 10% FBS + 5µg ml^{-1} FSH-P + 0.33mM sodium pyruvate + 5% Follicular fluid + 50µg ml^{-1} gentamicin sulfate + in treatment group FGF or PDGF or both). Then groups of 20-25 COCs were placed in 100 ml 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% CO_2 incubator with maximum humidity.

Sperm processing 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 Bracket Oliphant (WBO) medium (Bracket and Oliphant, 1975) with 10 µg/ml 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 200 ml of Fertilization Bracket Oliphant (FBO) media. The *in vitro* matured oocytes were washed twice with the FBO medium in the same maturation drop. The motile spermatozoa (1.5-2.0 million spermatozoa ml^{-1}) were inseminated into *in vitro* matured oocytes droplets and placed in 5% CO_2 incubator at 38.5°C for 15-18 h with maximum humidity.

Culture of oviductal epithelial cells and presumptive embryos

Fresh oviducts were dissected carefully with blunt end of 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 µl droplets of maturation media and incubated in 5% 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 for 5-6 times and were then cultured in 100 µl droplet in maturation media. 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 washed off from the oocytes by repeated gentle pipetting in washing medium. The zygotes were then washed 1-2 times with modified Charles Rosenkrans 2 amino acid (mCR2aa) medium and cultured in 100 µl of mCR2aa medium. After 48 h cleaved oocytes/embryos were shifted to 100 µl droplets of mCR2aa blastocyst medium and co-incubated with vibrant oviductal cells for 8 days.

Experimental design and statistical analysis

In this study the effect of fibroblast growth factor and platelet derived growth factor on *in vitro* embryo production was examined individually and in combination of different concentrations. Two groups were designed: (i) the control group and (ii) the treatment group. The maturation media was supplemented with three concentrations of FGF and PDGF (1, 5 and 10 ng ml^{-1}) and in combination of both FGF and PDGF (1, 5 and 10 ng ml^{-1} of each). The data of four replicates were analyzed by using simple one way ANOVA. Means were compared using Duncan Multiple Range Test (IBM® Statistical Package for the Social Sciences® (SPSS version 16.0). Graphs were made by using Microsoft office excel sheet.

RESULTS AND DISCUSSION

The present study was undertaken to examine the effects of growth factors (FGF and PDGF) on bovine early embryonic development when cultured *in vitro* with different concentrations. Total 1422 immature oocytes were used for the experiment in the present study. In the experiment 1 the effect of PDGF on *in vitro* cattle embryo development was significantly ($P<0.05$) improved (Fig 1) as compared to control group. The cleavage rate (57.06±4.78) in the treatment groups (@10 ng ml^{-1} is higher and having significant differences as compared to control group (37.74±3.53). From the present study it has been observed that 63.64% of 16-cell stage embryo reached to morula stage and 27.27% of 16-cell stage embryo reached to blastocyst stage. Similar results were reported in buffalo where cleavage rate was 30.60% when they used PDGF @ 1 ng

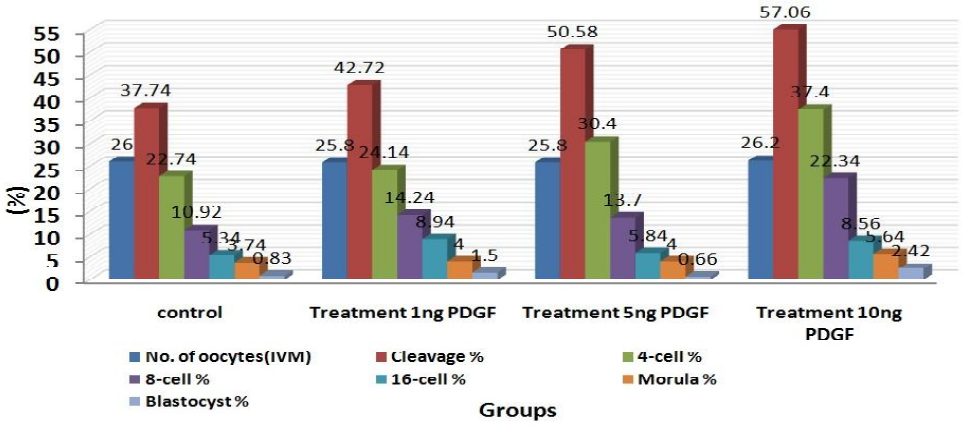

Fig 1: Effects of different concentration of PDGF supplementation on *in vitro* embryonic developmental stages.

ml^{-1} in culture media but the rate was increased significantly (42.00%) by adding EGF @ 20 ng ml^{-1} in culture media along with PDGF (Vikash *et al*. 2012). Lim and Hansel, 1996 explained that a higher proportion of 8-cell embryos (48.30%-50.80%) cultured singly developed beyond the 8-cell stage after addition of PDGF-AB or PDGF-AB + other growth factors than in basic medium alone where the rate was 30.30%. In another earlier study by Yang *et al*. 1993, it was observed that PDGF had the most pronounced beneficial effect with 70% of the 2 to 8 cell stage embryos cultured reaching the morula and blastocyst stages of development. It has also been suggested that PDGF may initiate gene expression of *in vitro* cultured bovine embryos during the fourth cell cycle (Yang *et al*. 1993). The present study revealed that supplementation of culture media with PDGF @ 10 ng ml^{-1} improves the early embryo development whereas lower concentration of has mild effects on embryogenesis.

In the experiment 2 results (Fig 2) show that FGF supplementation @ 1 ng ml^{-1} had significantly higher cleavage rate (55.93±4.75) as compared to control group and other two concentrations (5 ng ml^{-1} and 10 ng ml^{-1}). Although, there is not much significant difference in the mean percentage beyond the cleavage rate among control and treatment groups but results indicated that FGF with 1ng ml^{-1} concentration improved the development of 4-cell, 8-cell, 16-cell, morula and blastocyst (3.26±1.53) formation. It is also observed that there is a gradual decrease in the rate of developmental cell stages with increase in FGF concentration among treatments which might imply its non-beneficial effects at higher concentration. Diogenes *et al*. (2017) investigated the effect of fibroblast growth factor-10 during *in vitro* maturation on the developmental capacity of bovine oocytes. They reported that FGF-10 delayed the resumption of meiosis from 8 h onwards, not affected the percentage of oocytes reaching metaphase-II and not increased cumulus expansion at 22 h of maturation. They found no difference between treatments regarding embryo production, developmental speed and gene expression. Addition of 0.1 ng ml-1 FGF-10 to equine oocyte maturation media markedly improves their developmental competence to form blastocysts (20.91%) after fertilization as compare to control (7.28%) group (Reyes-Perea *et al.* 2019). In some other study reported that FGF receptor activation through signalling was important for optimal blastocyst formation and FGF-2 supplementation increased bovine blastocyst formation when provided at high concentrations. Using 20 µM SU5402 (inhibitor of FGF) on day 0 (Day of IVF) it was observed by Fields *et al*. (2011) that blastocyst formation rate was reduced on day 7 as compared to control (5.9±2.1 vs 16.9±2.4) which indicated that FGF has positive effect on embryo development.

In the third experiment total 499 cumulous oocyte complexes were used to study the combined effect of FGF and PDGF on *in vitro* embryo development which was significantly (P<0.05) improved (Fig 3). The results of this experiment revealed that there was significant differences between control and treatment group (FGF+PDGF) particularly in 4-cell stage and morula formation rate. The percentage of cleavage rate and subsequent embryonic cell stages were found to be higher in treatment group than the control group. The highest cleavage rate (51.24±4.12) and blastocyst formation rate (2.70±1.17) was observed in treatment group with FGF+PDGF (@1+1ng ml^{-1}. Similar results reported by Lim and Hansel, 1996 that embryo (beyond the 8-cell stage) development rate was higher (50.80%) when they used PDGF-AB with bFGF (1ng ml^{-1}) and transforming growth factor in the culture media as compare to basic medium alone where the rate was 30.30%. They also reported that a significantly higher percentage (62.60-65.80%) of 16-cell embryos developed to the morula stage when they used PDGF-AB with bFGF (1ng ml^{-1}) and transforming growth factor as compare to control group (30.20%). In another study Lim and Hansel, (2000) evaluated the effect of exogenous substances [platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and some other] on preimplantation bovine embryo development. They reported that embryo development to the blastocyst stage was regulated by exogenous substances including PDGF, FGF in a stage-specific manner. In buffalo Vikash *et al.* (2012)

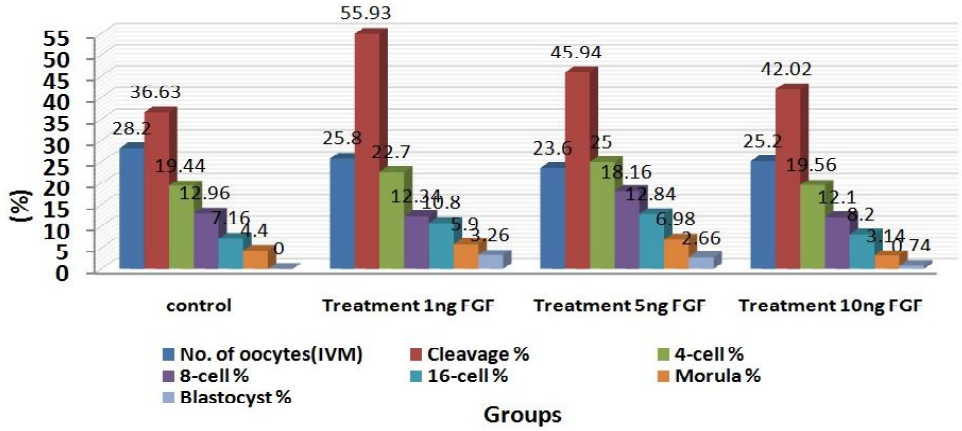

Fig 2: Effects of different concentration of FGF supplementation on *in vitro* embryonic developmental stages.

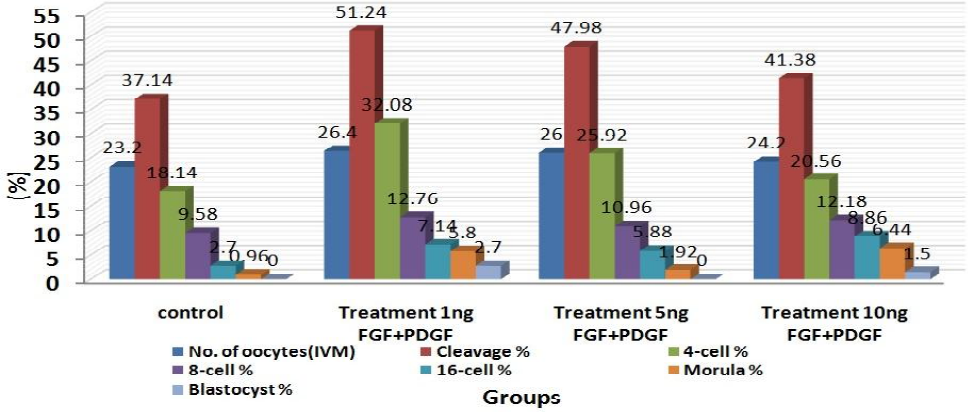

Fig 3: Effects of different concentration of FGF and PDGF supplementation on *in vitro* embryonic developmental stages.

reported that the cleavage rate was 30.6% with the supplementation of PDGF @ 1ng ml^{-1} in culture media. The cleavage rate was increased significantly *i.e.* 42.0% when they supplemented culture media with EGF @20ng ml^{-1} along with PDGF. The cleavage rate was increased further *i.e.* 45.0% with the supplementation of EGF @20 ng ml^{-1}) and IGF @ 100 ng ml^{-1} along with PDGF.

CONCLUSION

The results of the present study indicated that platelet derived growth factor and fibroblast growth factor individually and in combination significantly (P<0.05) affect *in vitro* cattle embryo development. Supplementation of PDGF @ 10 ng ml^{-1}, FGF @ 1 ng ml^{-1} and PDGF + FGF @ 1 + 1 ng ml^{-1} in *in vitro* maturation media enhanced cleavage and blastocyst formation rate.

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