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Effect of Different Growth Factors on *in vitro* Developmental Competence and Quality of Cattle Oocytes

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ABSTRACT

Background: The developmental competency of *in vitro* matured oocytes to the blastocyst stage is a big concern in laboratory conditions. This study aims in finding the best combination of growth factors for enhancement of cleavage as well as early embryonic developmental rates in cattle.

Methods: Fresh cattle ovaries were obtained from the nearest slaughterhouse in 0.15 M freshly prepared saline (35-38°C) supplemented with penicillin (400 IU/ml). A total of 924 COCs were aspirated from ovaries and transferred to maturation media for incubation (5% CO_2) at 38.5°C with maximum humidity for 24 h. After maturation, the matured oocytes were co-incubated with the capacitated sperms for *in vitro* fertilization in the FBO medium for 16 h. After fertilization, the cumulus cells were removed from the presumptive zygotes and incubated in the mCR2aa medium for 45-48 h. The cleavage rates of the zygotes were observed and further cultured in the blastocyst medium for 7-10 days. After every 24 h, 80% of culture media was replaced with fresh media. Three different combinations of growth factors (PDGF+IGF, EGF+FGF and T3+T4) used in this study.

Result: Supplementation of growth factors in culture media increased cleavage rate and blastocyst rate significantly ($p<0.05$). PDGF+IGF @ 10 ng/ml+75 ng/ml, EGF+FGF @ 20 ng/ml+10 ng/ml and T3+T4 @ 100 ng/ml+100 ng/ml showed the best results in terms of cleavage rates and blastocyst development.

Key words: Blastocyst, Cattle oocyte, Cleavage, Embryo, Growth factors.

INTRODUCTION

In vitro embryo production (IVEP) in cattle has been considered one of the most remarkable assisted reproductive technologies (ARTs) in the dairy sector to overcome/minimize infertility problems, failure in fertilization, chronic ovulation problem, heat and reproductive stress (Looney *et al.,* 1994; Block *et al.,* 2010; Gomez *et al.,* 2020), but also to obtain high genetic value embryos in fewer periods. In general, IVEP is focused on the production of 7-8 days old blastocyst that retains best birth and pregnancy rates to recipients for embryo transfer Randi *et al.* (2016). Despite improvised embryo production by IVEP, *in vitro* produced embryos still undergo stressful conditions like limited nutrient availability and assembly of toxic substrates and metabolic endproducts Ramos Ibeas *et al.* (2019). Previous studies showed that the use of growth factors has paracrine and autocrine effects in embryo development Ahumada *et al.* (2013). Several other factors like the use of bovine oviductal epithelial cells Scahmaltz Panneau *et al.* (2015) and reduced cell culture volume have enhanced blastocyst development Minasi *et al.* (2015). Some studies suggest that addition of EGF, IGF-1 or EGF + IGF along with PMSG and hCG gave optimum rates of maturation in porcine Mahanta *et al.* (2018). Activation of fibroblast growth factor (FGF) receptor and supplementation of FGF_2 at higher concentrations showed increased blastocyst development by activating interferon τ (*IFNT*) transcript profile Ozawa *et al.* (2013). Lee and Fukui (1995) reported that defined culture medium supplemented with EGF, FGF, or both synergistically enhanced blastocyst development. *In vitro* culture medium supplemented with thyroid hormones *i.e.* thyroxine (T4) and triiodothyronine (T3) showed improved cleavage rates and blastocyst development in bovine (Ashkar *et al.,* 2010). Piccirilli *et al.* (2018) also reported that thyroid hormones regulate protease expression and notch signal activation in embryo development. The present study aimed to improve *in vitro* embryo development by supplementing the culture medium with several growth factors and hormones.

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MATERIALS AND METHODS

Chemicals, cell culture media and supplements

All chemicals and reagents were purchased from Sigma Chemicals Co. (St. Louis, MO, USA) and HIMEDIA Biosciences Pvt. Ltd., Mumbai, India unless otherwise

stated. All plastic wares used were purchased from Tarsons Products Pvt. Ltd. (Kolkata, India). The disposable syringe filters of 0.22 µm pore size were purchased from Milipore Corp., Bedford, MA, USA. Epidermal Growth Factor (from human recombinant, expressed in *E. coli*), Fibroblast Growth Factor (from bovine pituitary glands, the major mitogenic component is FGF_2), Insulin Growth Factor (from mouse recombinant, expressed in *E. coli*), Platelet Derived Growth Factor (from porcine platelets), 3,3',5-Triiodo-L-thyronine (≥95% HPLC) and L-Thyroxine (bioreagent) were purchased from Sigma-Aldrich, St. Louis, MO, USA. All the experimental works have been completed by 2022.

Collection of ovaries

Fresh cattle ovaries and oviducts were collected from the abattoir (Kolkata, India) in a thermos flask, containing saline (0.15 M NaCl) solution (35-38°C) with an addition of antibiotic (400 IU/ml penicillin) and transported to the laboratory (Animal Biotechnology Lab, ERS, ICAR-NDRI) within 2-3 hours. Ovaries were then trimmed for removal of adjacent tissues and washed 3-4 times in warm saline solution.

Collection of oocytes and *in vitro* maturation

The immature oocytes were collected from nonatretic surface follicles (3-8 mm diameter) of ovaries with the help of a 19 gauge hypodermic needle attached to a 5 ml disposable syringe filled with the aspiration medium (TCM-199 + DPBS + 0.3% BSA + 50 ug/ml gentamicin solution). Searching of oocytes was done under a zoom stereo-microscope at 40X magnification and was graded based on the cumulus layer. A and B grades of cumulus-oocyte complexes (COCs) were washed 4-5 times in washing medium (TCM-199 + 10% FBS + 0.81 mM Sodium Pyruvate + 50 ug/ml gentamicin solution) followed by twice in maturation medium (TCM-199 + 10 % FBS + 5 ug/ml FSH-P + 0.33 mM Sodium Pyruvate + 50 ug/ml gentamicin sulfate). Around 20-25 COCs were placed in 100 µl droplets of maturation medium overlaid with sterile mineral oil in 35 mm petri dishes and incubated for 24 h at 38.5°C in a 5% CO_2 incubator with maximum humidity.

Sperm preparation and *in vitro* fertilization

IVF-tested spermatozoa used in this study were from the same donor. The frozen cattle semen straws were thawed and suspended in 1.2 ml of working Brackett Oliphant (WBO) media each containing 10 µg/ml heparin, 0.57 mM caffeine sodium benzoate and 1.23 mM sodium pyruvate and kept for incubation at 38.5°C for 15 minutes. After the swim-up, capacitated sperms were taken by collecting supernatant (900 µl) from the Eppendorf tubes, leaving the seminal plasma at the bottom and allowing for centrifugation at 2000 rpm for 5 min. After the centrifugation, the pellet obtained was resuspended in 1.2 ml of WBO for washing and re-centrifuged at 2000 rpm for 5 min. Finally, the supernatant was decanted and the leftover pellet was suspended in 1 ml of Fertilization Brackett Oliphant (FBO) medium for the second wash and centrifuged at 2000 rpm for 5min. After decanting the supernatant, 50-100 µl of FBO is added to each tube and pipetted well before adding to the FBO drops (50 µl). The *in vitro* matured oocytes were inseminated with capacitated motile spermatozoa (1-2 million spermatozoa/ml) and placed in a 5% CO_2 incubator at 38.5°C for 15-18 h with maximum humidity.

Culturing of oviductal epithelial cells and embryo culture

The fresh oviducts were dissected using blunt-end scissors and washed in saline solution. The oviductal epithelial cells (OECs) were obtained by squeezing the oviduct with the help of a sterile glass slide and then washed 4-5 times in the washing medium. The cell chunks were then put into 100 µl drops of maturation media and incubated in a 5% CO_2 incubator at 38.5°C for 48 h with maximum humidity. After incubation, the healthy vibrant cells were picked up and washed 3-4 times in a washing medium. The cells were then added to the 100 µl drops of cleavage media (mCR2aa) overlaid with mineral oil.

After 15-18 hrs of incubation, the zygotes were obtained by removing the cumulus layers from the presumptive zygotes by gently repeated pipetting in a washing medium. The zygotes were further washed in mCR2aa medium and cultured in 100 µl of embryo culture media with added OECs. After 48 h of incubation, cleavage was observed and cleavage media was replaced by mCR2aa blastocyst medium (IVC media). The culture dish was kept in a 5% CO_2 incubator with maximum humidity at 38.5°C for 8 days and media was replaced within 48 h intervals.

Experimental design and statistical data analysis

The experiment was conducted for four replicates in three different concentrations (T_1, T_2 and T_3) under three different combinations (PDGF+IGF, EGF+FGF and T3+T4). Experimental data's were analyzed using simple One Way ANOVA. Means were compared using Duncan Multiple Range Test (IBM R Statistical Package for the social sciences R (SPSS version 16.0). Mean difference among groups were analyzed by Tukey's Multiple Comparison test (GraphPad Prisam 9.4.0). Graphs were made by using GraphPad Prisam 9.4.0. and the values were expressed as means±standard error.

RESULTS AND DISCUSSION

Effect of PDGF and IGF in different concentrations on cleavage and early embryonic development

PDGF and IGF were supplemented in the culture media (IVM, IVF and IVC) to assess the enhancement in IVEP concerning the control group. A total of 303 COCs were used in this experiment. All the matured COCs were fertilized *in vitro*. The experiment was conducted for four replicates and the mean percentage of their cleavage rates and the early embryonic development were presented in Table 1. The mean percentage of cleavage rate was 71.82±0.97 in the control group and 73.04±1.04, 75.70±1.29 and

87.49±1.31 in T_1, T_2 and T_3 groups respectively. This result demonstrated that there is no significant difference in cleavage rates between the control and the treatment groups, T_1 and T_2, but T_3 showed an increase in cleavage rate of about 87% ($p<0.001$) due to the enhanced proliferation property of PDGF that may have reduced the synthesis of the regulatory subunits of mitosis-promoting factors Jaskulski *et al*. (1988). The combination of PDGF+IGF used in this study had enhanced the potential development of the embryo by increasing its competency which also reported by Chandra *et al*. (2012). PDGF and IGF showed an increment in both cleavage and blastocyst rates when supplemented individually in the culture media (Umdor *et al*., 2021; Prasad *et al*., 2022). In this study, the mean percentages of the 4-cell, 8-cell and morula of the control group and three different treatment groups revealed no significant difference ($p>0.5$) due to the time/stage specific activity of IGF with PDGF Pathipati *et al*., (2021). The mean percentage of blastocyst development in control was 6.35±0.56 and 7.68±2.41, 7.16±1.10 and 16.30±1.33 in the T_1, T_2 and T_3 groups respectively. PDGF+IGF @ 10 ng/ml+75 ng/ml showed the best results in blastocyst development compared to the control, T_1 and T_2 groups ($p<0.05$) as it enhanced the survival competence of cleaved embryos Sirisathein *et al*. (2003).

Effect of EGF and FGF in different concentrations on cleavage and early embryonic development

A total of 325 COCs were used for *in vitro* maturation and fertilization with supplementation of growth factors EGF and FGF in media. The combined effect of EGF and FGF is presented in Table 2. The control group showed a cleavage rate of (71.82±0.97). The treatment groups *i.e.* T_1, T_2 and T_3 showed cleavage rates of (66.11±0.55), (70.34±0.55) and (74.09±1.35) respectively. The three treatment groups showed a significant difference in cleavage rates ($p<0.001$) and T_3 showed the highest cleavage rate. The 4-cell stage, 8-cell stage and morula of treatment groups did not show any difference in level of significance but were significantly different from the control group ($p<0.005$). In the control group, the blastocyst development rate was (7.35±0.34) whereas T_1, T_2 and T_3 groups showed developmental rates of (7.98±1.09), (10.65±0.80) and (15.10±0.87) respectively. EGF+FGF @ 10 ng/ml+5 ng/ml showed significant differences from the control group ($p<0.005$) but the EGF+FGF @ 5 ng/ml+1 ng/ml did not show any significant difference with control and T_2 group respectively, showing minimal effect below 10 ng/ml. Park *et al*. (1997) reported that the exposure of bovine oocytes to the EGF (10 ng/ml to 50 ng/ml) during maturation in a chemically defined medium enhanced them to the blastocyst stage. The addition of growth factors EGF and FGF in their individual concentrations enhanced the early embryonic development in the IVC media respectively (Prasad *et al*., 2018; Umdor *et al*., 2021). Umdor *et al*. (2021) reported FGF @ 1 ng/ml as the best concentration for the development of cattle embryo and in combination with PDGF @ 10 ng/ml showed increase in cleavage and blastocyst development rate. According to Kumar *et al*. (2020), FGF @ 20 ng/ml enhanced the blastocyst development with higher total cell

Table 1: Effect of PDGF and IGF on early embryonic development.

Group	Control (Mean±SE)	Treatment 1 (T_1) (PDGF 1 ng/ml+ IGF 25 ng/ml) (Mean±SE)	Treatment 2 (T_2) (PDGF 5 ng/ml+ IGF 50 ng/ml) (Mean±SE)	Treatment 3 (T_3) (PDGF 10 ng/ml+ IGF 75 ng/ml) (Mean±SE)	Level of significance
Cleavage	71.82^{A}±0.97	73.04^{A}±1.04	75.70^{A}±1.29	87.49^{B}±1.31	0.000
4-cell	68.84 ±1.38	66.57±2.91	68.71±2.72	65.86±10.25	0.972 (NS)
8-cell	54.37±1.40	44.21±3.21	46.49±4.02	47.14±9.04	0.578 (NS)
Morula	26.09±5.39	31.34±2.08	28.89±1.59	34.31±8.02	0.700 (NS)
Blastocyst	6.35^{A}±0.56	7.68^{A}±2.41	7.16^{A}±1.10	16.30^{B}±1.33	0.005

^{A,B,C}Within a row, the same superscripts indicate non-significant difference statistically ($p>0.05$). NS: Non-significant.

Table 2: Effect of EGF and FGF on early embryonic development.

Group	Control (Mean±SE)	Treatment 1 (T_1) (EGF 5 ng/ml + FGF 1 ng/ml) (Mean±SE)	Treatment 2 (T_2) (EGF 10 ng/ml + FGF 5 ng/ml) (Mean±SE)	Treatment 3 (T_3) (EGF 20 ng/ml + FGF 10 ng/ml) (Mean±SE)	Level of significance
Cleavage	60.33^{A}±0.82	66.11^{B}±0.55	70.34^{C}±0.55	74.09^{D}±1.35	0.000
4-cell	43.76^{A}±2.65	53.36^{B}±±1.81	54.34^{B}±0.75	56.54^{B}±1.28	0.004 (S)
8-cell	29.39^{A}±1.36	38.65^{B}±0.72	36.84^{B}±1.06	39.92^{B}±0.58	0.000 (S)
Morula	16.92^{A}±1.54	30.67^{BC}±0.36	28.15^{B}±0.64	31.71^{C}±0.96	0.000 (S)
Blastocyst	$7.35^{A±}$0.34	7.98^{AB}±1.09	10.65^{B}±0.80	15.10^{C}±0.87	0.001

^{A,B,C} Within a row, the difference in superscripts indicate significant difference statistically ($p<0.05$).

S: Significant. Treatment groups show significant difference with the control group.

Table 3: Effect of T_3 and T_4 on early embryonic development.

Group	Control (Mean±SE)	Treatment 1(T_1) (T3 20 ng/ml + T4 20 ng/ml) (Mean±SE)	Treatment 2 (T_2) (T3 50 ng/ml + T4 50 ng/ml) (Mean±SE)	Treatment 3 (T_3) (T3 100 ng/ml + T4 100 ng/ml) (Mean±SE)	Level of significance
Cleavage	61.32^{A}± 1.43	64.58^{AB}±1.74	67.17^{B}±0.50	73.09^{C}±1.39	0.002
4-cell	62.77^{A}±0.80	66.72^{AB}± 2.12	68.47^{B}±0.97	70.53^{B}±1.93	0.041 (S)
8-cell	30.19^{A}±0.82	35.03^{B}±2.14	35.28^{B}±1.21	45.52^{C}±0.64	0.000 (S)
Morula	23.17±1.03	20.15±3.23	23.33±1.67	27.23±0.32	0.149 (NS)
Blastocyst	7.02^{A}±0.42	8.15^{A}±1.25	11.39^{AB}±2.47	14.54^{B}±1.06	0.029 (p<0.05)

Superscripts$^{AB,\ BC}$ within a row indicates non-significant difference statistically. S: Significant, NS: Non-significant.

number (TCN) but there was no significant differences observed in the total number of blastocysts in case of the other treatment groups. In the present study the addition of EGF @ 20 ng/ml to the FGF @ 10 ng/ml treated oocytes might have increased its competency to develop into blastocyst. The T_3 group, EGF+FGF @ 20 ng/ml+10 ng/ml showed the highest blastocyst formation rate and was significantly different from the control, T_1 and T_2 groups (p<0.005).

Effect of T_3 and T_4 in different concentrations on cleavage and early embryonic development

A total of 296 COCs were used in this experiment to determine the combined effects of T_3 and T_4 on IVEP presented in Table 3. All the *in vitro* matured COCs were fertilized and transferred to the culture media for early embryonic development. The mean percentage of cleavage rate shown by the control group was (61.32±1.43). The T_1, T_2 and T_3 groups showed the mean percentage of cleavage rates as (64.58±1.74), (67.17±0.50) and (73.09±1.39) respectively. The T_3 group (T_3@ 100 ng/ml +T_4 @100 ng/ml) showed a significant difference to the control, T_1 and T_2 groups (p<0.005). T_1 group, T_3+T_4 @ 20 ng/ml+20 ng/ml showed no significant difference in cleavage rates compared to the control and T_2 groups. The mean percentages of the 4-cell stage and 8-cell stage revealed significant differences between the control and treatment groups (p<0.05). Control and treatment groups showed no significant differences in the morula stage. The blastocyst development rate was higher in the T_3 group (p<0.05) but not in T_2 group (T_3+T_4 @ 50 ng/ml each)l. Singh *et al*. (2017; 2020) reported that T_3 @ 100 ng/ml and T_4 @ 100 ng/ml displayed an increase in cleavage rates. Costa *et al*. (2013), found that @ 50 ng/ml of T_3 enhanced higher rates of blastocyst development. Similar results were also demonstrated by Ashkar *et al*. (2010) that T_3 (50 ng/ml) and T_4 (50 ng/ml) provided better cleavage, blastocyst and hatching rates.

CONCLUSION

All combination of growth factors supplemented at various concentrations in the culture medium enhanced cleavage and blastocyst rates but PDGF+IGF @ 10 ng/ml+75 ng/ml, EGF+FGF @ 20 ng/ml+10 ng/ml and T_3+T_4 @ 100 ng/ml +100 ng/ml showed higher cleavage and blastocyst rates and these concentrations could be used for *in vitro* embryo production.

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Conflict of interest statement

- I as corresponding author wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest.
- I wish to confirm that there are no known conflicts of interest associated with this publication.
- I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.
- I further confirm that the order of authors listed in the manuscript has been approved by all of us.
- I confirm that I have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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