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Effect of Fibroblast Growth Factor 2 (FGF2) and Insulin Transferrin Selenium (ITS) on *In Vitro* Maturation, Fertilization and Embryo Development *in* Sheep

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ABSTRACT

The present study evaluated the effect of fibroblast growth factor 2 (FGF2) and insulin-transferrin-selenium (ITS) to the in vitro *maturation and embryo culture media on ovine oocyte maturation, cleavage and embryo development. Oocytes having more than five layers of unexpanded cumulus cells and granular homogenous ooplasm were cultured into 50 µL droplets of eight different culture systems: (i) TCM-199 (Tissue Culture Medium-199); (ii) TCM-199+10 ng/mL FGF2; (iii) TCM-199+20 ng/mL FGF2; (iv) TCM-199+30 ng/mL FGF2; (v) TCM-199+10 ng/mL ITS; (vi) TCM-199+20 ng/mL ITS; (vii) TCM-199+30 ng/mL ITS and (viii) TCM-199+20 ng/mL ITS+20 ng/mL FGF2 in a CO_2 incubator at 38.5^0C for 24 h. All the oocyte culture media were supplemented with 10% FBS, FSH (10 µg/mL) and gentamicin (50 µg/mL). The maturation rate was assessed based on the degree of expansion of cumulus cells and identifying first polar body extrusion into perivitelline space. The matured oocytes were inseminated with 1 to 2 million spermatozoa/mL in Brackett and Oliphant medium and the cleavage rate was checked after 42-48 h post insemination and further cultured for 6-7 days. Maturation and cleavage rates were significantly higher ($P<0.05$) in the oocytes cultured in TCM-199 +10% FBS+FSH (10 µg/mL) supplemented with both 20 ng/mL ITS and 20 ng/mL FGF2 as compared to the control. It was concluded that the supplementation of ITS and FGF2 in maturation medium was beneficial for improving maturation and cleavage rates of sheep oocytes. The addition of ITS and FGF2 in embryo culture medium did not improve the development of sheep embryos.*

Key words: fibroblast growth factor 2, insulin transferrin selenium, oocyte, embryo, sheep

INTRODUCTION

Oocyte maturation is the first and most critical step towards the successful *in vitro* embryo production. The intrinsic ability of the oocytes to resume meiosis, accept spermatozoa for fertilization, cleave after fertilization, and facilitate proper embryonic development that leads to the production of healthy offspring are dependent of various endocrine and intra-ovarian factors produced within the follicular niche (Binelli et al. 2010). Supplementing specific paracrine and endocrine components during *in vitro* maturation (IVM) of bovine cumulus-oocyte complexes (COCs) improved the success of *in vitro* embryo production and maximize embryonic competency to generate healthy offspring after embryo transfer. Various growth factors either endogenous and exogenous may be useful in oocyte maturation and growth. Previous studies have revealed that oocyte factors secreted in follicular fluid promoted the oocyte maturation (Gupta et al. 2001; 2005). The addition of ITS to *in vitro* maturation medium enhanced nuclear maturation (79% vs 54%),

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decreased polyspermy, improved embryo development and post-transfer survival (Hu et al. 2011). Another class of paracrine-acting factors that have received recent attention for their abilities to control follicular development and oocyte maturation are the fibroblast growth factors (FGFs). FGFs had been found to play crucial roles in folliculogenesis, including primordial follicle activation and regulation of granulosa and cumulus cell mitosis, apoptosis and glycolysis (Sugiura et al. 2007). Several other FGFs were found to be produced in oocytes and follicular somatic cells of cattle (Parrott and Skinner 1998), mice (Sugiura et al. 2007) and human (Ben-Haroush et al. 2005). In mice, FGF4 originated from inner cell mass inhibited the differentiation of trophectoderm (Feldman et al. 1995). Porcine FGF7 derived from endometrium stimulated the proliferation of trophoblast cells during the embryonic development (Ka et al. 2001). Increased bovine embryo development rates were also observed after supplementation with fibroblast growth factor 10 (FGF10), a theca derived factor (Zhang et al. 2010). A FGF of recent interest for a functional role during oocyte maturation is FGF2 (also known as basic FGF). bFGF (FGF2) acted as a mitogen, morphogen, and angiogenic factor to regulate early embryogenesis (Gospodarowicz and Bialecki 1979). The potential involvement of FGF2 in in-vitro maturation, fertilization and embryo development has not been examined in sheep. Hence, the present study investigated the effect of the addition of FGF2 and ITS to the maturation medium on *in vitro* maturation, fertilization and embryo development of sheep oocytes.

MATERIALS AND METHODS

Oocyte collection and evaluation

Ovaries were collected from non-pregnant slaughtered sheep from a local slaughterhouse and transported to the laboratory in normal saline (0.9% NaCl). Ovaries having visible follicles with a diameter of 1 to 6 mm were aspirated using a 20-G hypodermic needle attached to a syringe containing 0.5-1.0 mL of aspiration media. The aspiration medium consisted of TCM-199, Dulbecco's phosphate-buffered saline (PBS), 0.3% bovine serum albumin, heparin (10 µg/mL) and gentamicin (10 µg/mL). Cumulus oocytes complexes were graded by the morphological appearance of the cumulus cells investments and homogeneity of ooplasm under a stereo zoom microscope (magnification 300X). Only oocytes having more than five layers of unexpanded cumulus cells and granular homogenous ooplasm were recovered and selected for *in vitro* maturation (IVM).

Maturation of oocytes *in vitro*

All the selected oocytes for IVM were washed once with aspiration medium and three times with IVM medium, then oocytes were cultured in the maturation medium, which consisted of TCM-199 (25 mM HEPES, with Earle's salt and L-glutamine, Sigma USA), supplemented with 10% FBS, FSH (10 µg/mL) and gentamicin (50 µg/mL) and finally covered with mineral oil (Sigma, USA) (oocytes cultured in groups 7-10 oocytes/50 µL droplet). Oocytes were cultured at 38.5ºC, 5% CO_2 and 95% humidity for 24 h. The degree of cumulus cell expansion was determined after 24 h of IVM and oocyte with expanded cumulus cell mass to at least two diameters away from the zona pellucida were considered as cumulus expanded. The maturation of oocytes was further evaluated by identifying the first polar body in the perivitelline space after denuding them.

Experimental design

Cumulus oocytes complexes, 7-10 in a group, were cultured into 50 µL droplets of seven different culture media in 35-mm Petri dishes: (i) TCM-199; (ii) TCM-199+10 ng/mL FGF2; (iii) TCM-199+20 ng/mL FGF2; (iv) TCM-199+30 ng/mL FGF2; (v) TCM-199+10 ng/mL ITS; (vi) TCM-199+20 ng/mL ITS and (vii) TCM-199+30 ng/mL ITS. FBS, FSH and gentamicin were added at the level of 10%, 10 µg/mL and 50 µg/mL, respectively in all the treatment groups. The concentration of FBS added was optimal for the IVM of sheep oocytes as found in an earlier study (Mondal et al. 2013). Since both FGF2 and ITS at 20 ng/mL level were found to be optimum for *in vitro* maturation of sheep oocytes when used individually, the combined effect of FGF2 and ITS in maturation medium was also investigated. Maturation was assessed and determined as described above.

In vitro fertilization and embryo development

Fresh semen was collected from a ram using electronic ejaculator and spermatozoa were capacitated using Brackett and Oliphant (BO) medium, supplemented with 10 µg/mL heparin

plus 2.2 mg/mL caffeine as capacitating agents. Before transfer to fertilization drops, the oocytes were washed four times in BO medium. After the matured oocytes had been denuded, they were co-cultured with capacitated spermatozoa. A 5.0 µL aliquot of sperm suspension, containing 1 - 2 $X10^6$ sperm/mL was added into the fertilization drop (7-10 oocytes per 40 µL fertilization drop). Fertilization was carried out by co-incubation of sperm and oocytes at 38.5ºC under 5% CO_2 in humidified air for 2-4 h. Thereafter, oocytes were transferred to embryo culture medium (same media as used in maturation medium except FSH) and incubated for 18-20 h.

In vitro culture (IVC)

After 18 h, oocytes were washed in washing media {TCM199 with 10% FBS+gentamicin (50 µg/mL)} in order to remove the attached sperms. The embryos were then cultured in the same medium as used in maturation medium, except FSH for further development in CO_2 incubator at 38.5ºC, 5% CO_2 and 95% humidity and the cleavage was checked after 42-48 h post insemination. The embryos were then further cultured for 6-7 days in order to confirm the embryonic development.

Statistical analysis

The experiment was replicated seventeen times on different days. The percentage of maturation rate, cleavage rate and embryo development rate were analyzed by one-way ANOVA, followed by Tukey's test for comparisons of more than two groups using GraphPad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA). Differences between mean the values were considered significant when the probability values were < 0.05.

RESULTS

The effect of different concentrations of FGF2 and ITS, either alone or in combination of ITS (20 ng/mL) and FGF2 (20 ng/mL) on IVM, IVF and embryo development of sheep oocytes is shown in Table 1. Supplementation of ITS at 10, 20 and 30 ng/mL in maturation media significantly increased (P<0.05) the maturation rate of sheep oocytes as compared to the control. The maturation rate was significantly increased (P<0.05) relative to the control by supplementation of 20 ng/mL FGF2 in maturation medium. Significant increase (P<0.05) in cleavage rate was observed in the medium containing both 20 ng/mL ITS and 20 ng/mL FGF2 as compared to all the doses tested, except 20 ng/mL ITS. There was no significant difference in morula development rate in the media containing the different doses of ITS and FGF2 tested. Non-significant increase (P>0.05) in blastocyst development was observed in the medium containing 20 ng/mL ITS relative to the control. Significant decrease (P<0.05) in blastocyst yield was observed in the medium containing 10 ng/mL FGF2 as compared to those observed in the media containing 20 ng/mL FGF2, 30 ng/mL FGF2 and 20 ng/mL ITS+20 ng/mL FGF2.

Table 1 - Effect of supplementation of different doses of ITS (10, 20 and 30 ng/mL), FGF2 (10, 20 and 30 ng/mL) and combination of ITS (20 ng/mL)+ FGF2 (20 ng/mL) in maturation and embryo culture medium on maturation rate, cleavage rate and embryo development of sheep oocytes.

Treatments	Oocyte cultured	Maturation rate n (Mean ± SEM)	Cleavage rate n (Mean ± SEM)	Embryo development n (Mean ± SEM)	
				Morula -	Blastocyst
TCM-199+FBS (10%)+ FSH (10 µg/mL) - Control	158	101 (63.83±0.51)[a]	57 (58.29±4.12)[a]	6 (18.77±4.91)[a]	3 (11.50±1.51)[a]
Control+ITS (10 ng/mL)	133	101 (75.73±1.87)[b]	53 (53.23±4.54)[a]	6 (17.71±3.47)[a]	2 (11.11±0.0)[a]
Control+ITS (20 ng/mL)	124	101 (81.26±1.60)[b]	68 (69.68±4.96)[ae]	7 (20.00±4.91)[a]	13 (19.05±3.25)[a]
Control+ITS (30 ng/mL)	131	101 (76.89±1.42)[b]	56 (57.86±4.53)[a]	4 (23.61±1.39)[a]	3 (12.22±2.22)[a]
Control+FGF2 (10 ng/mL)	165	108 (65.72±1.26)[a]	50 (46.30±2.13)[acf]	9 (17.85±2.00)[a]	0.00[abcfh]
Control+FGF2 (20 ng/mL)	139	108 (77.31±2.88)[b]	58 (53.15±2.33)[a]	12 (20.75±3.10)[a]	7 (14.01±2.43)[ad]
Control+FGF2 (30 ng/mL)	167	108 (64.70±1.14)[a]	55 (51.11±2.67)[adf]	10 (17.46±4.05)[a]	5 (12.44±2.04)[ag]
Control + ITS (20 ng/mL) + FGF2 (20 ng/mL)	155	129 (82.43±1.78)[b]	106 (85.20±4.62)[be]	16 (15.96±1.94)[a]	15 (15.07±1.81)[ai]

[a-i] Different superscript in the same column differed significantly (P<0.05); SD standard deviation; Growth supplements (ITS and FGF2) were used both in oocyte maturation and embryo culture medium.

The growth supplements, ITS and FGF2 in combination at the level of 20 ng/mL each were found to cause significant improvement ($P<0.05$) on the maturation and cleavage rates of sheep oocytes. However, the addition of ITS and FGF2, either alone or in combination in embryo culture medium did not improve the development of sheep embryos *in vitro.*

DISCUSSION

To the best of our knowledge, this is the first study to report the impact of FGF2 on IVM, IVF and embryo development of sheep oocytes. Here, ITS and FGF2, either alone or in combination were used as a supplement for the culture of sheep oocytes. Results suggested that the maturation and cleavage rates of sheep oocytes cultured in the presence of 20 ng/mL ITS and 20 ng/mL FGF2 combination was significantly higher in comparison to all the doses tested, except 20 ng/mL ITS. The fertilizing ability of sheep oocytes cultured in 20 ng/mL ITS was significantly higher as compared to those observed in 10 and 20 ng/mL FGF2 alone. This suggested that ITS had more beneficial effect on sheep embryo cleavage than FGF2. The transferrin and selenium has been reported to be essential for the catalytic activity of glutathione peroxidase and could serve as the antioxidant defense system in the oocyte (Cerri et al. 2009).

The present study showed that ITS supplementation in the maturation medium resulted higher oocyte maturation as compared to the control. Increment of oocyte growth in ITS supplement group could be due to increase in glutathione (GSH) concentration in oocytes. Glutathione, a thiol tripeptide component in all cell types has an important role in the transportation of amino acid, synthesis of the protein and DNA, and reduction of disulfide bonds (Meister and Anderson 1983). Insulin promoted glucose and amino acid uptake, lipogenesis, intracellular transport, and the synthesis of proteins and nucleic acids (Spicer and Echternkamp 1995). Transferrin being an iron carrier helped to reduce the toxic levels of oxygen radicals and peroxide. Selenium, as sodium selenite, has been reported as a co-factor for glutathione peroxidase and other proteins and used as an anti-oxidant in medium (Wu et al. 1973). In cell culture system, sodium selenite protected cell from oxidative damage, free radicals and obstructed lipid perioxide products (Ebert et al. 2006). Transferrin and Selenium played a role in the antioxidant defense system in the oocyte, which was essential for the catalytic activity of glutathione peroxidase (Cerri et al. 2009).

The exposure of sheep oocytes to the medium containing FGF2 (20 ng/mL), during the maturation, increased *in vitro* development to the morula and blastocyst stages. The similar results were observed for early blastocyst stage development but not at earlier stages (Sugiura et al. 2009). FGF2 as an oocyte competency factor was investigated because it has been produced by theca, granulosa and cumulus cells throughout folliculogenesis (Berisha et al. 2006). The transcripts for receptors for FGF2 and many other FGFs were evident in bovine and ovine blastocysts and peri-implantation bovine and ovine conceptuses (Cooke et al. 2009). Since FGF2 is reported to be endogenously produced by cumulous cells (Berisha et al. 2006), it was hypothesized that additional supplementation of FGF2 might improve oocyte development (Gupta et al. 2002). The supplementation of FGF2 (20 ng/mL) promoted oocyte maturation in buffalo. Zhang and Ealy (2012) reported that FGF2 increased blastocyst formation at day 7 because early blastocyst formation *in vitro* was associated with increased embryo competency after embryo transfer. It was reported that FGF2 did not affect the relative abundance of transcripts encoding factors that regulate cumulus expansion (EGFR, FSHR, HAS) (Assidi et al. 2008) and other cumulus competency markers (CTSB, SPRY2) (Sugiura et al. 2009) but improved the survival rates of cumulus cells. Present results showed that the addition of ITS and FGF2 in combination in maturation medium promoted the maturation and cleavage rates of sheep oocytes. No reports were available to compare results in this aspect.

In conclusion, the results of this study showed that the supplementation of ITS and FGF2 during *in vitro* maturation was beneficial for enhancing the maturation and cleavage rates of sheep oocytes.

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