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Influence of follicular fluid and gonadotropin supplementation on the expression of germ cell marker genes during *in-vitro* maturation of caprine (*Capra hircus*) oocytes

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ABSTRACT

The present study aimed to investigate whether follicular fluid, gonadotropins (FSH, LH and estradiol-17β), and combination of both types of supplementation (follicular fluid + gonadotropins) in maturation medium influence the transcript abundance of germ cell marker genes (*MATER*, *ZAR1*, *GDF9* and *BMP15*) in goat oocytes. Grade I and II oocytes were collected from the goat ovaries and matured further under *in-vitro* conditions using four different maturation regimens *viz*, group B containing basal media (TCM 199 + BSA + serum), group C (basal media + 10% follicular fluid), group D (basal media + FSH + LH + estradiol-17β) and group E (basal media + 10% follicular fluid + FSH + LH + estradiol-17β). Group A consisted of immature oocytes. Expression profile of *MATER, ZAR1, GDF9,* and *BMP15* was analyzed in matured oocytes of different groups as well as immature oocytes using real-time PCR. The relative abundance of *MATER* gene in immature oocytes (group A) was found to be significantly higher ($p < 0.05$) compared to experimental groups. The relative expression of *ZAR1* was found to be significant different ($P < 0.05$) between group D and E. The relative expression of *GDF9* and *BMP15* was significantly higher ($P < 0.05$) in group E as compared to other experimental groups. These results indicated that the expression of *MATER* and *ZAR1* transcript were down regulated after maturation; however *BMP15* and *GDF9* transcripts were upregulated after maturation. For better *in vitro* embryo production (IVEP) out comes goat oocytes may be matured *in vitro* in TCM-199 supplemented with follicular fluid and gonadotropins.

1. Introduction

Innovative and efficient *in-vitro* techniques for oocyte maturation and fertilization in small ruminants like sheep and goats are more important than in large ruminants, due to their short generation interval, prolificacy, economical rearing, better visibility and large sized pronucleus for biotechnological experiments *viz*. during gene transfer, cloning and stem cell research (Kharche et al., 2009). Maturation of oocytes is the most important stage that determines the successful fertilization, zygote formation, attainment of blastocyst stage and subsequent implantation. Numerous studies have been focused to find better combinations of media supplements so as to further optimize the culture conditions. In some of mammalian species (porcine, murine, bovines, caprine and canines) the efficiency of *in-vitro* maturation is still quite low, hence supplements like EGF, BSA, progesterone and gonadotopin have been successfully used to improve the *in vitro* Culture (IVC) system (Kempisty et al., 2011). Among gonadotropin, LH was considered the primarily responsible factor for the resumption of meiosis *in-vivo*. However, FSH and not LH, was considered the hormone responsible for inducing oocytes maturation in isolated Cumulus Oocyte Complexes (COCs) *in-vitro* (Van Tol et al., 1996; Samartzi et al., 2008). Many studies have demonstrated the ability of FSH to stimulate mammalian oocyte maturation *in-vitro* (Schoevers et al., 2003; Ye et al., 2005). Among various genes expressed in germ cells, Maternal Antigen That Embryos Require (*MATER*), Zygote Arrest 1 (*ZAR1*), Growth Differentiation Factor 9 (*GDF9*), and Bone Morphogenetic Protein 15 (*BMP15*) transcripts were detected in the oocytes itself at a much higher level than in gonads and considered to be oocyte specific markers (Pennetier et al., 2004).

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For the production of good quality blastocysts the female germ cell (oocytes) must have high developmental competence, that is specifically characterized by the expression of some crucial oocytes marker genes *viz*; *BMP15*, *GDF9*, *c-kit*, *c-Mos*, *MATER, ZAR1, Estradiol*, *Leptin*, *NALP9*, *IGFBP1*, *p180* etc. during the period of growth, differentiation and oocytes maturation (Nath et al., 2013). However, there are meager studies on expression profile of these germ cell marker genes during *in-vitro* maturation in goat.

It is possible that a good quality oocyte may not reach to M-II (nuclear maturation) and henceforth may further lack the potential to develop upto blastocyst stage due to incomplete maturation medium supplements. Maturation media is critical in terms of providing correct milieu for oocytes. The maturation medium, selection of protein supplements, growth factors, antioxidants and hormones play an important role in *in-vitro* maturation (Yadav et al., 2013). Therefore, the present study was undertaken to evaluate the alteration in expression profile of germ cell marker genes following supplementation of gonadotropin during *in-vitro* maturation of goat oocytes.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and media used in this study were procured from Sigma Chemical Company, USA, unless otherwise indicated. Goat ovaries were obtained within 4 h of slaughter from a local abattoir located at Agra and were transported to the laboratory in a thermos flask containing sterile warm (35–37 °C) physiological normal saline solution (NSS) supplemented with antibiotics (100 IU/mL penicillin G and 100 μg/mL streptomycin sulphate).

2.2. Primers

The primers of *BMP-15, GDF-9 ZAR1* and *GAPDH* were designed by the Oligo Analyser software. The primers of *MATER* and ribosomal protein S15a (*RPS15A*) were taken from published sequence. The primers were procured from Biolink (Europhins) Pvt. Ltd., Bengaluru, India. The sequences and expected PCR product length are shown in Table 1.

2.3. In-vitro *maturation of oocytes*

Cumulus-oocyte complexes (COCs) were collected by using slicing method. Only excellent (Fig. 1A) and good (Fig. 1B) quality oocytes having homogenous cytoplasm with more than three layers of cumulus cells were used for *in vitro* maturation (Kharche et al., 2008). COCs were subjected to *in vitro* maturation (10–15 oocytes in 50 μL droplets) in tissue culture media-199 (TCM-199) supplemented with four different maturation regimens; group B (n = 223) containing basal media (TCM199 supplemented with 10% FBS + 3 mg/mL BSA), group C (n = 218, basal media + 10% follicular fluid), group D (n = 225, basal media + FSH – 5 μg/mL, LH – 10 μg/mL and estradiol 17β – 1 μg/mL) and group E (n = 285, basal media + 10% follicular fluid + FSH – 5 μg/mL, LH – 10 μg/mL and estradiol 17β – 1 μg/mL). Group A (n = 215) consisted of immature oocytes. Oocytes were cultured *in-vitro* for 27 h at 38.5 °C under humidified atmosphere of 5% CO_2 in air for maturation (Kharche et al., 2013). After maturation, the oocytes were denuded completely either by repeated pipetting or treatment with 0.1% hyaluronidase to remove COCs. The denuded oocytes (1166) thus obtained were subjected to RNA isolation and few oocytes (531) were used for staining with Hoechst dye (33342). Nuclear stages were distinguished by the morphology of chromatin materialas per Yadav et al. (2013). Oocytes with first polar body (Fig. 1C) and second metaphase plate (Fig. 1D) were classified as mature oocytes of second meiotic cell division (MII).

2.4. RNA isolation and cDNA synthesis

RNA was isolated using TriZol reagent (Ambion, Life Technologies) as per manufacturer's instructions. The total RNA sample was treated with DNAase I (Biolab DNA-free™) to remove genomic DNA contamination. Concentration and quality of isolated RNA were assessed by spectrophotometric analysis. Reverse Transcription was carried out using RevertAid™ cDNA synthesis kit (Thermo Scientific, USA) in a total 20 μL reaction volume following the manufacturer's instruction. One μg of total RNA was used in the RT as template. cDNA was synthesized according to manufacturing protocol. The quality of cDNA was assessed by an amplification reaction of housekeeping genes.

2.5. Real-time PCR

SYBR green-based real time was performed for *MATER, ZAR1, GDF9, BMP15* genes using *GAPDH* and *RPS15A* as housekeeping genes. The reaction was carried using Maxima SYBR Green qPCR Master Mix (Thermo Scientific, USA) in qPCR cycler (Step One plus, Applied Biosystems). The optimized reaction was carried out in a final reaction volume of 20 μL containing 1 μL (0.5 μM) of each forward and reverse primer, 4 μL of cDNA, 5.0 μL of nuclease-free water, and 10 μL SYBR Green qPCR master mix. Thermal profile used for amplification of all replicates consisted of an initial denaturation cycle of 10 min at 95 °C; 45 cycles of PCR (95 °C for 10 s, 60 °C for 10 s, 72 °C for 15 s) and melting curve profile (95 °C for 0.05 s followed by 70 °C for 1 min and 95 °C for 0.05 s) was set for fluorescence acquisition and reaction specificity. No Template Control (NTC) was placed with each reaction set up for checking any contamination in reaction components. At the end of the reaction, cycle threshold (C_t) values and amplification plot were acquired and relative expression of PCR product was determined by the equation suggested by Pfaffl (2001).

2.6. Statistical analysis

The percentages of M-II stage oocytes were calculated in different groups and data were analyzed by Chi square analysis. Expression pattern of genes were analyzed by one-way ANOVA (Tukey multiple comparison test) using graph-pad Prism V 5.0 software at 0.05% level of significance. Difference with $p < 0.05$ were considered statistically significant.

3. Results

From 361 ovaries, a total of 1793 oocytes were recovered by slicing technique, resulting in an average recovery of 4.9 oocytes per ovary. After 27 h, maturation rate was recorded on the basis of nuclear maturation of oocytes. The maturation rate was found to be significantly higher ($p < 0.05$) in all groups supplemented with either follicular fluid or gonadotropin as compared to non-gonadotropin (Group B, Table 2).

The expression pattern of *MATER*, *ZAR1*, *GDF9*, *BMP15* were analyzed between experimental groups A, B, C, D and E along with two housekeeping genes GAPDH and RPS15A. The relative expression pattern of *MATER* was found to be significantly down regulated ($p < 0.05$) in E groups than control group A (Fig. 2). Moreover, the expression pattern was differentiate between C and D groups. For *ZAR1* gene, the relative expression pattern of mRNA level was significant differences ($p < 0.05$) between group D and group E (Fig. 3).

The relative mRNA expression of BMP 15 gene was found to be significantly higher in group E ($p < 0.05$) than experimental groups