The Pharma Innovation Journal 2019; 8(1): 170-173

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(1): 170-173

www.thepharmajournal.com
Received: 04-08-2018
Accepted: 06-09-2018

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Expression of growth differentiation factor 9 (GDF9) gene in ovine immature oocytes, *in vitro* matured oocytes and Parthenogenetic embryos

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Abstract
In vitro maturation (IVM) is essential technology under assisted reproductive technologies which enables oocytes to achieve maturation and acquire competence for subsequent embryonic division leading to blastocyst formation. This technique is valuable not only because it allows production of large numbers of oocytes, but also because they provide valuable *in vitro* model to study the gene expression at different maturational stages of oocyte. During maturation oocyte accumulates large amount of mRNA to dictate developmental competence of oocytes and further embryonic development. Using quantitative real-time qPCR assays, expression pattern of several classes of genes have been studied in Preimplantation stages of murine and bovine embryos.
In this study we investigate the expression of GDF9 gene at different developmental stages of sheep oocytes by using real-time qPCR technique. The relative expression of GDF9 was highest in immature oocytes than *in vitro* matured oocytes and parthenogenetic embryos.
In conclusion, GDF9 showed highest expression in immature oocytes, its expression decreased during maturation and embryo production.

Keywords: Oocyte, *In vitro* maturation, qPCR, gene expression, parthenogenesis

1. Introduction
Growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15(BMP15) are the members of transforming growth factor β (TGF-β) super family which are secreted from oocytes during folliculogenesis (Aaltonen *et al*., 1999) [1]. TGF-β superfamilyis a large family of structurally related proteins which controls various functions like proliferation, differentiation, and development etc. in the variety of cells or tissues in the body. They are essential for folliculogenesis and female fertility (Juengel *et al*., 2004a) [4]. *GDF9* is expressed in mammalian oocyte throughout follicular development (Aaltonen *et al* 1999) [1]. *GDF9* plays an important role in the development of primary follicles in the ovary (Juengal *et al*., 2004b) [5]. It has a critical role in granulosa cell and theca cell growth, as well as in differentiation and maturation of the oocyte (Hreinsson *et al*., 2002; Su *et al*., 2004) [3, 17].
GDF9 has been connected to differences in ovulation rate (McNatty *et al*., 2007) [12] and in premature cessation of ovary function (Kovanci *et al*., 2007) [8], therefore has a significant role in fertility. The cell surface receptor through which GDF9 generates a signal is the bone morphogenetic protein type II receptor (BMPR2) (Mazerbourg and Hsueh, 2006; Vitt *et al*., 2002) [11, 18]. A *GDF9* deletion results in decreased granulosa cell proliferation, abnormal oocyte growth and failure of follicles to develop past the primary stage (Dong *et al*., 1996) [2]. GDF9 is synthesized as pre-proteins which includes signal peptide, large proregion and small mature region. After removal of signal peptide, further intracellular processing results in secretion of biologically active mature region from proregion (McNatty *et al*., 2004) [13].

2. Materials and Methods
All chemicals and cultures were purchased from Sigma Chemicals Co. (USA) and all disposable plastic wares were purchased from Nunc, Denmark.

2.1 Collection of oocytes
The fresh sheep ovaries of mixed breeds were collected from slaughter house, in a tube containing warm isotonic saline (32–37°C) supplemented with 400IUmL^{-}1penicillin and

500µgmL¯1 streptomycin and transported to the laboratory within 2-4h. In the laboratory, the extra ovarian tissue was trimmed by using scissor and ovaries were washed 3-4 times with warm isotonic saline (32–37°C) containing 400IUmL¯1 penicillin and 500µgmL¯1streptomycin. Oocytes from follicles (>2mmindiameter) were harvested by puncturing withan 18-gauge needle. The collection medium consisted of HEPES buffered Tissue Culture Medium-199 (M-199) containing 0.3% bovine serum albumin (BSA).Oocyte searching was done under zoom stereo microscope and only grade A and grade B oocytes were collected by using Pasteur pipette. The aspirated oocytes were graded according to the criteriaal ready inusein the laboratory (Kharche and Birade, 2013) [7]. Grade A (excellent): Compact cumulus-oocyte-complexes (COCs) with an unexpanded cumulus mass having >4layersofcumuluscells, and with homogenous, evenly granulated ooplasm. Grade B (good): Compact cumulus-oocyte-complexes (COCs) with an unexpanded cumulus mass having 2-4 layers of cumulus cells, and with homogenous, evenly granulated ooplasm. Grade C (fair): Oocytes with only one layer of cumulus cell mass having evenly granulated ooplasm. Grade D (poor): Oocytes with no cumulus cell layer or incomplete cumulus cell layer and having unevenly granulated ooplasm. Oocytes were washed 3 to 4 times with the washing medium which consisted of Hepes buffered M-199 supplemented with 10% fetal bovine serum (FBS) (Hyclone, Canada), 0.68 mML-glutamine, 0.8m Msodium pyruvate and 50 µgmL¯1 gentamicin.

2.2 *In vitro* maturation of oocytes

Oocytes of usable quality (A & B grade) were selected and after washing thrice with IVM medium (bicarbonate buffered M-199 supplemented with 10% FBS, 5µgmL¯1 pFSH, 1µgmL¯1 estradiol-17β, 0.8m MS odium pyruvate and 50µgmL¯1gentamicin), groups of 15-20 COC's were cultured in 100µl droplets of IVM medium overlaid with mineral oil in 35 mm petri dishes and cultured for 24hin5% CO2 with maximum humidity at 38.5oC.

2.3 Parthenogenetic activation of *in vitro* matured oocytes

COC's after IVM, having expanded cumulus, were stripped of their cumulus using hyaluronidase (0.5mgmL−1) in T2 (where Tdenotes HEPES buffered M-199 supplemented with 2.0mMl-glutamine, 0.2mM sodium pyruvate, 50µgm L−1 gentamicin and the following number denotes the percentage of FBS, here2%). The 2ml eppondorf tube containing matured oocytes in hyaluronidase was incubated in an incubator at 37ºC for 2-3 minutes and thoroughly vibrated using vibrator for 1-2 minutes. All the denuded oocytes with compact and regular cytoplasm were used for parthenogenetic activation. Oocytes were washed in T20 (HEPES buffered M-199 supplemented with 20% FBS), here twice before activation by ionomycin/6-Dimethylamino purine (6-DMAP). Briefly oocytes were activated using 5µMionomycinin T2 and incubated at 37ºC for 5mins by using CO_2 incubator. After washing thrice in T20, the oocytes were incubated in 2mM DMAP for 3hours. Presumptive zygotes were washed thrice in culture medium and then cultured in 400µL of Research Vitro Cleave Media (K-RVCL50, Cook Australia Pvt. Ltd.) supplemented with 5% adult bovine serum (ABS, New Zealand), covered with 400µL mineral oil and kept undisturbed in CO2incubator for further development up today 7 of *in vitro* culture.

The immature oocytes, *in vitro* matured oocytes and parthenogenetic embryos (morula stage) were put into 2ml microfuge tubes containing 10-20µl DPBS and tube was immediately put in Liquid Nitrogen for freezing. The samples were stored at - 80ºC until RNA isolation process.

2.4 RNA isolation and cDNA preparation

Total RNA was extracted from pools of frozen immature oocytes (IM 50), *in vitro* matured oocytes(MO 50) and morulas (M 10) by kit method (RNA queous®-Micro Kit), and eluted in 10µl RNase free buffer.

The quality of RNA was checked by using micro volume UV-Vis bio-photometer (eppondorf bio-photometer) at optical density (OD) of 260 nm and 280 nm. The samples showing $OD_{260/280}$ ratio 1.8-2 were used for further experimental studies. The quantity of RNA was checked by using at OD of 260 nm. Equal concentration of RNA in all samples was used for synthesis of cDNA.

The first strand cDNA was synthesized by using Revert-Aid Kit (Fermentas, USA) asper the instructions of the manufacturer. Equal concentration of RNA was used for cDNA synthesis in all samples. Besides the negative RT control was also carried out in which all components were added except reverse transcriptase. The final volume of reaction mixture was 20µl. The cDNA synthesized were stored at -20˚C till further use.

2.5 Quantitative Real time PCR

Real time PCR was carried out for quantification of GDF9 transcripts in each experimental sample using SYBR Green master mix from Thermo Fisher, USA. RT-PCR was performed using forward primer 5ˈACAACACTGTTCGGCTCTTCACCC 3ˈ and reverse primer 5ˈ CACAAGAGTAACACGATCCAGGTT 3ˈhaving Gen Bank accession no. FJ529501.2 for GDF9, generating 129 bp size product and forward primer 5ˈCTTCCTGGGCATGGAATC 3ˈand reverse primer 5ˈ TCTTTCTGCATCCTGTCTGC 3ˈ having GenBank accession no. JXO46106 for β- actin, generating 150 bp size product. By using real-time thermal cycler each cDNA sample was analysed in duplicate. A non-template control (NTC) was prepared by nuclease free water without cDNA. Reactions were performed using final volume of 20 µland loaded into qPCR 96-well plate of 0.2ml volume. The plate was covered by adhesive type transparent film and centrifuged at 4000 rpm for a minute to ensure proper mixing of reagents and to remove air bubles. The plate was loaded in to the rmocycler.

The PCR cyclic conditions were 95˚Cfor10 min, then 40 cycles consisting of denaturation at 95˚C for 15 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec and melting curve analysis was performed to confirm the authenticity of amplified genes.

The comparative C_T method also known as $\Delta\Delta C_T$ method (Livack and Schmittgen, 2001) [10] was used to achieve the result for relative quantification. The threshold cycle value of reference gene (beta actin) was used to normalize the target gene (gdf9) signals. Fold change in the expression of target gene was calculated by using the formula $2^{-\Delta\Delta C_T}$ (Livack and Schmittgen, 2001) [10].

2.6 Statistical analysis

The data obtained in the current study was analyzed using a

computer-aided statistical software package SPSS 20.0. The differences between means were analyzed by unpaired Student's T-test and by one way ANOVA followed by Fisher's LSD test and significance was determined at $P<0.05$.

3. Results

3.1 *In vitro* maturation of oocytes and parthenogenesis

The results obtained during IVM and parthenogenesis are shown in table 1 below.

Table 1: *in vitro* development of parthenogenitically produced sheep embryos

S. No.	Total No. of oocytes	No. of oocytes put to IVM	Maturation %(n)#	Matured Oocytes used for embryo production	Embryo %(n)#	
					Cleaved %(n)# %(n)#	Morula %(n)# %(n)#
1	185	135	82.0±4.7 (109)	59	61.01±3.9 (36)	30.6±2.8 (11)
2	173	123	78.8±1.9 (97)	47	59.60±1.8 (28)	35.7±2.6 (10)
3	180	130	77.0±3.5 (100)	50	64.00±2.8 (32)	34.4±1.6 (11)
4	169	119	80.7±1.3 (96)	46	63.00±2.1 (29)	34.5±4.1 (10)
5	184	134	76.1±2.9 (102)	52	59.6±3.1 (31)	32.5±1.9 (10)
Total	891	641	78.92±2.5 (504)	254	61.4±1.9 (156)	33.5±2.0 (52)

#The values are presented as % mean ± S.E.M

3.2 Gene expression

In the present study, *GDF9* mRNA was detected and quantified in three developmental stages of oocytes *viz*, immature oocyte, *in vitro* matured oocyte and Parthenogenetic embryos (Morula stage).

GDF9 mRNA expression during different developmental stages of oocyte shows significant differential changes. GDF9 mRNA showed decline in expression from immature oocytes to PA embryos significantly. *GDF9* mRNA expression was significantly higher in immature oocytes (1.0 fold) than *in vitro* matured oocytes (0.26 fold) and embryos (0.005 fold) ($P<0.05$) (fig.1).

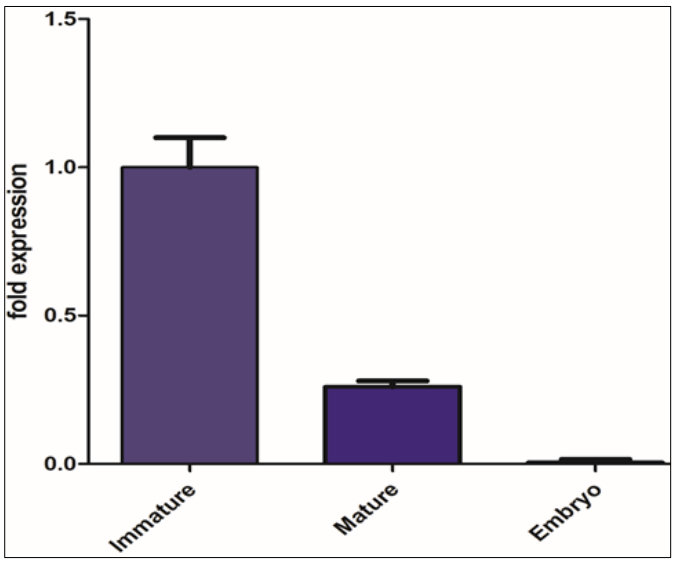

Fig 1: Fold expression in *GDF9 gene* at different developmental stages of sheep oocytes. *GDF9* mRNA expression is higher in immature oocytes than matured oocytes and embryos.

4. Discussion

In the present study, significant difference in the relative expression of *GDF9* transcripts showed a gradual decline during *in vitro* development of oocytes from immature to embryos stages.*GDF9* mRNA expression was significantly higher in immature oocytes than *in vitro* matured oocytes and embryos. This is in agreement with earlier findings that suggested the *GDF9* expression showed gradual decline during oocyte maturation. Porcine *GDF9* gene was found to be highly expressed in immature oocytes and declined slowly during oocyte maturation process (Li *et al*., 2000) [9]. Zhu *et al*., (2008) [20] studied the temporal and spatial expression patterns of *GDF9* gene in porcine COCs throughout *in vitro* maturation and reported that *GDF9* highly transcribed in oocytes from fresh COCs, whereas expression was gradually decreased during IVM. *GDF-9* was expressed throughout oocyte development and continued in early embryonic stages (Pennetier *et al*., 2004) [14]. Zeng and Schultz, (2003) [19] indicated that *GDF9* was not found in 8-cell embryo stage in mice model. Pennetier *et al*., (2004) [14] demonstrated that *GDF9* was expressed continuously in immature oocytes, *in vitro* cultured zygotes, two-, four-, five- to eight cell embryos but not detected after morula stage in bovine model. This suggested *GDF9* function is species specific until early embryo stage. It was also found that *GDF9* relative abundance in cumulus cells remained stable during IVM up to 12h of maturation and decreased significantly between 12h to 24h of maturation, in *Bubalus bubalis* (Kathirvel *et al*., 2013) [6]. Reyes *et al*., 2013 [16], found that *GDF9* was expressed in canine oocytes, mainly in earlier developmental stages, with low levels in mature oocytes *in vitro* and *in vivo*. But in one study in porcines, Prochazka *et al*., 2004 [10], found that there was no difference in expression of *GDF9* during oocyte maturation.

5. Acknowledgements

The authors are highly thankful to Division of Biotechnology, Faculty of Veterinary Science and Animal Husbandry, SKUAST Kashmir for providing all essential facilities in order to carry out this research.

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