Expression of Growth Differentiation Factor-9 from buffalo follicular fluid – a marker gene for fecundity

K. Muralidharan[1], S. Eswari[2*] and K. Vijayarani[3]
Department of Veterinary Physiology
Madras Veterinary College
Tamil Nadu Veterinary and Animal Sciences University
Chennai- 600 007, Tamil Nadu, India

ABSTRACT

Growth factors synthesized from mammalian oocytes popularly known as Oocyte Secreted Factors (OSFs) play numerous role in ovarian folliculogenesis. Growth differentiation factor-9 (GDF-9) produced within the ovary plays an essential role during follicle maturation through their actions on granulosa cells, but their expression in follicular fluid has not been studied. The purpose of this study was to detect the temporal expression pattern of GDF-9 from the follicular fluid of buffalo ovary, which may be correlated with the oocytes and embryo quality. The sensitive messenger ribonucleic acid detection of GDF-9 from follicular fluid was determined by Reverse Transcriptase-Polymerase Chain Reaction using bovine oligonucleotide primers. Our finding showed the qualitative detection of GDF-9 mRNA transcripts from follicular fluid of buffalo ovary.

Key words: Buffalo, Follicular fluid, GDF-9 expression, RT-PCR

INTRODUCTION

Growth Differentiation Factor-9 (GDF-9), a prominent member of the Transforming Growth Factor-β(TGF-β) superfamily functions as paracrine factor in the regulation of granulosa cell proliferation and differentiation (Gilchrist *et al.,* 2006). Furthermore, GDF-9 is the first oocyte derived growth factor found to be indispensable in ovarian folliculogenesis and expressed throughout the development of the maturing follicle. Besides its activities in cumulus expansion, oocyte maturation and ovulation (Gui and Joyce, 2005; Yoshino *et al.*, 2006), italso play important roles during many steps of ovarian follicular development. GDF-9 involved in cell survival signaling (Orisaka *et al.*, 2006) and act as modulators of many other growth factors and endocrine hormones (Juengel *et al.*, 2004).

Given these results, GDF-9may be considered as a candidate gene for controlling ovulation rate in buffalo. Several studies have reported that supplementation of *in vitro* maturation media with FSH enhanced the cumulus

---

[1] MVSc scholar

[2] *Professor, Corresponding author email:drseswari@gmail.com

[3] Professor and Head, Bioinformatics and ARIS cell

expansion, nuclear maturation and cleavage rate of buffalo oocytes (Chauhan *et al*.,1996; Abdoon *et al*., 2001). Few other studies have reported the beneficial effects of including follicular fluid replacing hormones during *in vitro* maturation of buffalo oocytes (Chauhan *et al*., 1997).

In the ovaries, GDF-9 has been expressed in the oocytes of primordial follicles of sheep and cows (Bodensteiner *et al.,* 1999). The expression begins at the primary follicle stage in women (Aaltonen *et al.,* 1999), mice (Elvin *et al.,* 1999) and also in rats (Jaatinen *et al.,* 1999). GDF-9 mRNA expression has also been identified in extra-ovarian sites such as the pituitary, hypothalamus, placenta, testis and adrenal gland (Pennetier*et al*., 2004; Faure *et al*., 2005; Farnworth *et al*., 2006). Follicular fluid contains proteins, anticoagulants, enzymes, *etc*., thereby providing the oocytes with a microenvironment which contains the necessary regulatory factors (Fortune, 1994). Since GDF-9 affect oocyte development during folliculogenesis, follicular fluid level of this oocyte secreted paracrine factor may be important for oocyte function and subsequent embryo quality. Although GDF-9 and its functions in mammals have captured increased attention in recent years, much remains to be learnt about this molecule in buffalo. There is a lack of information on the availability of this growth factor from follicular fluid. Therefore, this study was designed to investigate the expression of GDF-9 from follicular fluid of buffalo ovary.

MATERIALS AND METHODS

Buffalo ovaries were collected from a local abattoir at Perambur, Chennai within two hours of slaughter and washed five times in phosphate buffered saline (PBS) containing 100 IU/ml penicillin and 100 µg/ml streptomycin. The aspiration of non-atreticantral follicles (3-8 mm) was carried out using 18-gauge needle connected to a 10 ml disposable syringe (Fig. 1). The aspirated contents were centrifuged at 500g for 30 minutes in a cooling centrifuge at 4°C. After settling of follicular cells, supernatant part of follicular fluid was slowly aspirated and transferred into a fresh 1.5 ml microcentrifuge tube and then frozen at -80°C for RNA purification.

Total RNA was purified under RNase-free conditions at room temperature for each sample using Trizol Reagent (Invitrogen) according to the protocol of the manufacturer. In microcentrifuge tube, 250µl of cells free follicular fluid was lysed by the addition of 750µl Trizol followed by incubation at room temperature for 5 minutes. Then, 200µl of chloroform was added, vortexed for 5 seconds and incubated at room temperature for 5 minutes. The centrifugation at 12,000 rpm for 15 minutes at 4°C in cooling centrifuge separated the solution into an upper aqueous phase containing RNA and a lower phenol-containing organic phase. The upper aqueous phase was transferred to a clean, RNase-free eppendorf tube and again centrifuged at 12,000 rpm for 10 minutes at 4°C. Then, the pellet was washed with 1ml of 75% ice cold ethanol, subsequently centrifuged at 7500 rpm for 5 minutes at 4°C and ethanol was discarded. The microcentrifuge tube was air dried and suspended with 10µl of DEPC water. The purified total RNA was stored at 80°C. The quantity and purity of the RNA were determined by spectrophotometry.

The cDNA synthesis was carried out using Revert Aid M-MuLV Reverse Transcriptase Kit (Fermantas, USA)in a standard 20µl reaction mixture. One microgram oftotal RNA was used as template RNA for reverse transcription as per manufacturer's instructions. RT-PCR for GDF-9 was carried out by using the published (NCBI accession No: AB058416) forward (5' AGAAGCTGCTGAGGGTGTAAGATT3') and thereverse(5'AAGCAATTGAGCCATCAGGC3') primer sequences (Hosoe*et al.,* 2011) from bovine. These oligonucleotide primers amplified a fragment size of 401 base pair. The β-actin forward primer5'-GATGAGGCTCAGAGCAAGAGA-3'andreverse primer 5’-TCGTCCCAGTTGGTGACGAT-3’(amplicon size 596 was used as the internal control for Polymerase Chain Reaction). The PCR programme comprised an initial denaturation at 95°C for 5 minutes, followed by denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72 °C for 1 minutes and final extension at 72 °C for 5 minutes. Each PCR was performed for 30 cycles for each sample. Products of the RT-PCR were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

In this PCR study, the existence of GDF-9 mRNA expression in follicular fluid of buffalo ovary was observed. The presence of this fecundity gene from follicular fluid of buffalo was detected by using the reverse transcriptase PCR. Fig. 2 shows the PCR ampliconsof GDF-9 (NCBI accession No: AB058416) along with β-actin gene (NCBI accession No: BG689033) electrophoresed through 1.5% agarose gel.

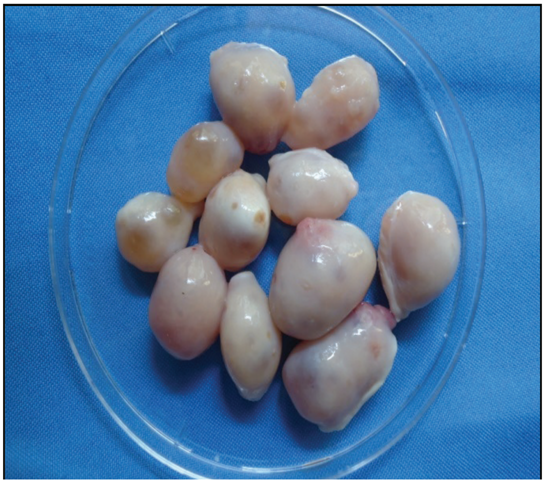

Fig.1. Picture showing ovaries of buffalo with follicles. Arrow mark showing fully grown good quality follicle from which the follicular fluid was collected.

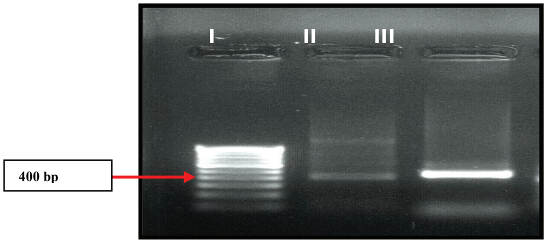

Fig. 2.Agarose gel showing PCR amplicons of GDF-9 (401 bp) and β-actin (endogenous control) from follicular fluid of buffalo ovary.Lane 1: 100bp Marker, Lane II: GDF-9 (401bp), Lane III: β-actin (596bp).

Folliculogenesis is a complex process dependent upon the intricate interplay of various growth factors and hormones. It is now well established that during ovarian folliculogenesis there is a dynamic interplay between the oocyte and the surrounding somatic cells that mutually influences growth and differentiation of the somatic granulosa cells and the oocyte (Gilchrist *et al.,* 2004). The members of the TGFβ superfamily influence a wide variety of growth and differentiation processes in a number of tissues and species. The newest member of this superfamily, GDF-9 appears to be no exception from this important function. Presumably, GDF-9 is the candidate gene for increasing the ovulation rate in sheep (McNatty *et al.,* 2003). The essential role of GDF-9 in fertility was demonstrated by targeted deletion of the GDF-9 gene in mice (Dong *et al.,* 1996). Long term immunization against GDF-9 in sheep disrupts early folliculogenesis and leads to the absence of normal follicles beyond the primary stage of development (Juengel *et al*., 2002). In addition, a naturally occurring mutation in this gene causes infertility in sheep (Hanrahan*et al.,* 2004). Positive staining for the GDF9 protein was identified in oocytes and granulosa cells in human ovarian samples (Oron*et al.,* 2010) and in buffalo oocytes (Kathirvel *et al.,* 2013).

In the present study, we have reported the expression of GDF-9 from the follicular fluid of buffalo ovary. There are no expression studies available to support the detection of GDF-9 gene from ovarian follicular fluid of buffalo. From previous ovarian expression studies, GDF-9 transcripts have been found to be localized in the oocytes from primordial follicles in cows and sheep (Bodensteiner *et al.,* 1999), whereas it is not found in primordial follicles of mice. These investigations suggest that expression pattern of GDF-9 differs between species. In goats, the polymerase chain reaction technique revealed the presence of GDF-9 mRNA in primordial, primary and secondary follicles, oocytes and granulosa cells of antral follicles (Silva *et al.,* 2004). Moreover it is reported earlier that the numbers of antral follicles per unit of ovarian mass and expression GDF9 serve as an important clue for higher prolificacy (Pramod*et al.,* 2013). Although results of our RT-PCR revealed the availability of GDF-9 expression from follicular fluid, the relative amount of mRNA transcripts of this gene need to be analysed through quantitative real time-PCR for further understanding the exact level of GDF-9 in follicular fluid of different category follicles. Additional studies on preantral follicles are needed to clarify the role of GDF9 as well as other growth factors.

In summary, the present study has demonstrated for the first time, GDF-9 expression from follicular fluid of buffalo ovary. This evidence of expression of GDF-9 in buffalo ovarian follicular fluid supports the effect of supplementation of buffalo follicular fluid during *in vitro* maturation of buffalo oocytes in improving the maturation rate. Moreover, the precise physiologic role of GDF-9 protein from follicular fluid in influencing oocyte needs further study.

ACKNOWLEDGMENT

The authors are thankful to the Dean, Madras Veterinary College and Director of Research, TANUVAS for the facilities provided to carry out the research work at Department of Veterinary Physiology, Madras Veterinary College, Chennai.

REFERENCES

Abdoon, A.S.S., Kandil, O.M., Otoi, T and Suzuki, T.(2001) Influence of oocyte quality, culture media and gonadotrophins on cleavage rate and development of *in vitro* fertilized buffalo embryos. *Animal Reproduction Science*, **65**:215–223.

Aaltonen, J., Laitinen, M.P.,Vuojolainen, K.,Jaatinen, R.,Horelli-Kuitunen, N.,Seppä, L.,Louhio, H.,Tuuri, T.,Sjöberg, J.,Bützow, R.,Hovata, O.,Dale,L and Ritvos,O.. (1999). Human growth differentiation factor 9 (GDF- 9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. *Journal ofClinicalEndocrinology&Metabolism,* **84**:2744–2750.

Bodensteiner, K.J., Clay, C.M.,Moeller,C.L and Sawyer,H.R. (1999). Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/ differentiation factor-9 in ovine and bovine ovaries.*Biology ofReproduction,* **60**:381–386.

Chauhan, M.S., Katiyar, P.K., Madan, M.L., Singla, S.K and Manik, R.S. (1996). Influence of follicle stimulating hormone on *in vitro* maturation and cleavage of buffalo (*Bubalusbubalis*) oocytes after *in vitro* fertilization. *Theriogenology,* **45**:243.

Chauhan, M.S., Palta, P., Das, S.K., Katiyar, P.K and Madan, M.L. (1997). Replacement of serum and hormone additives with follicular fluid in the IVM medium: effects of maturation, fertilization and subsequent development of buffalo oocytes *in vitro*. *Theriogenology*, **48**:461–469.

Dong, J., Albertini, D.F.,Nishimori, K., Kumar, T.R., Lu, N and Matzuk, M.M. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, **383**:531–535.

Elvin, J.A., Clark, A.T.,Wang, P.,Wolfman,N.M and Matzuk,M.M. (1999). Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *MolecularEndocrinology,***13**:1035–1048.

Farnworth, P.G., Wang, Y.,Leembruggen, P.,Ooi, G.T.,Harrison, C., Robertson D.M and Findlay,J.K. (2006). Rodent adrenocortical cells display high affinity binding sites and proteins for inhibin A, and express components required for autocrinesignallingby activins and bone morphogenetic proteins. *JournalofEndocrinology,* **188**:451–465.

Faure, M.O., Nicol, L.,Fabre, S.,Fontaine, J.,Mohoric, N.,McNeilly,A and Taragnat,C. (2005). BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary. *Journal ofEndocrinology,***186**:109–121.

Fortune, J.E. (1994).Ovarian follicular growth and development in mammals.*Biology of Reproduction.* **50**:225–232.

Gilchrist, R.B., Ritter,L.J and Armstrong,D.T. (2004). Oocyte-somatic cell interactions during follicle development in mammals. *Animal Reproduction Science,* **82–83**:431–446.

Gilchrist, R.B., Ritter, L.J.,Myllymaa, S.,Kaivo-Oja, N.,Dragovic, R.A.,Hickey, T.E.,Ritvos,O and Mottershead,D.G. (2006). Molecular basis of oocyte-paracrine signaling that promotes granulosa cell proliferation. *Journal ofCell Science,* **119:** 3811–3821.

Gui, L.M and Joyce I.M. (2005). RNA interference evidence that growth differentiation factor-9 mediates oocyte regulation of cumulus expansion in mice. *Biology of Reproduction,* **72**:195–199.

Hanrahan, J.P., Gregan, S.M., Mulsant, P., Mullen, M., Davis, G.H and Powell.R. (2004). Mutations in the genes for oocyte-derived growth factors GDF-9 and BMP-15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis Aries*). *Biology of Reproduction,* **70**:900–909.

Hosoe, M., Kaneyama, K.,Ushizawa, K., Hayashi, K.G and Takahashi,T.(2011). Quantitative analysis of bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) gene expression in calf and adult bovine ovaries.*Reproductive Biology and Endocrinology,* **9**:33.

Jaatinen, R., Laitinen, M.P.,Vuojolainen, K.,Aaltonen, J.,Louhio, H.,Heikinheimo, K., Lehtonen,E and Ritvos,O. (1999). Localization of growth differentiation factor-9 (GDF-9) mRNA and protein in rat ovaries and cDNA cloning of rat GDF-9 and its novel homolog GDF-9B.*Molecular and Cellular Endocrinology,***156**:189–193.

Juengel, J.L., Hudson, N.L.,Heath, D.A.,Smith, P.,ReaderK.L and Lawrence,S.B. (2002). Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. *Biology of Reproduction,* **67**:1777– 1789.

Juengel, J.L., Bodensteiner, K.J.,Heath, D.A.,Hudson, N.L.,Moeller, C.L.,Smith, P.,Galloway, S.M.,Davis, G.H.,Sawyer, H.R and McNatty,K.P. (2004). Physiology of GDF9 and BMP15 signalling molecules. *AnimalReproduction Science,* **82–83**:447–460.

Kathirvel, M., Eswari, S., and Kumanan, V.(2013). Differential expression dynamics of Growth differentiation factor9 (GDF9) and Bone morphogenetic factor15 (BMP15) mRNA transcripts during in vitro maturation of buffalo (*Bubalusbubalis*) cumulus–oocyte complexes. *SpringerPlus,* **2:**206-211.

McNatty, K.P., Juengel, J.L.,Wilson, T.,Galloway, S.M.,Davis, G.H.,Hudson, N.L.,Moeller,C.L.,Cranfield, M.,Reader, K.L.,Laitinen, M.P.,Groome, N.P.,Sawyer,H.R and Ritvos,O. (2003). Oocyte- derived growth factors and ovulation rate in sheep. *Reproduction*, **61**(Suppl):339-351.

Orisaka, M., Orisaka, S.,Jiang, J.Y.,Craig, J.,Wang, Y.,Kotsuji,F And Tsang,B.K. (2006). Growth differentiation factor 9 is antiapoptotic during follicular development from preantral to early antral stage. *Molecular Endocrinolology,* **20**:2456–2468.

Oron, G., Fisch, B., Ao, A., Zhang, X.Y., Farhi, J., Haroush, A.B., Icekson,G.K and Abir,R. (2010). Expression of growth-differentiating factor 9 and its type 1 receptor in human ovaries. *ReproductiveBioMedicine Online,* **21**:109– 117.

Pennetier, S., Uzbekova, S.,Perreau, C.,Papillier, P., Mermillod,P and Dalbies-Tran, R. (2004). Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15, and VASA in adult bovine tissues, oocytes, and preimplantation embryos. *Biology of Reproduction,***71**:1359–1366.

Pramod, R.K., Sharma, S.K., Singhi, A., Pan, S and Mitra, A. (2013). Differential ovarian morphometry and follicular expression of BMP15, GDF9 and BMPR1B influence the prolificacy in goat. *Reproduction in Domestic Animimals,***48**: 803–809.

Silva, J.R.V., Van den Hurk, R.,VanTol, H.T.A.,Roelen,B.A.J. and Fiueiredo,J.R. (2004). Expression of growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and BMP receptors in the ovaries of goats.*Molecular Reproduction and Development,* **70**:11–19.

Yoshino, O., McMahon, H.E.,Sharma,S.and Shimasaki, S. (2006). A unique preovulatory expression pattern plays a key role in the physiological functions of BMP-15 in the mouse. *Proceedings of the National Academy of Sciences of the United States of America,***103**:10678–10683.