



Expression pattern of BMPR-2 and Fas and the regulatory role of BMP-2 and IGF-1 on granulosa cells function in buffalo

D VINODKUMAR¹, RAHILA GULZAR², S SELVARAJU³, S NAZAR⁴, S PARTHIPAN⁵, K V JAMUNA⁶, R V PRASAD⁷ and J P RAVINDRA⁸

ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bengaluru, Karnataka 560 030 India

Received: 6 April 2016; Accepted: 16 June 2016

ABSTRACT

The expression pattern of BMPR-2 and Fas was investigated in different developmental stages (1–3 mm, 3–5 mm, 5–8 mm and >8 mm) of follicles and the effect of bone morphogenetic protein-2 (BMP-2), insulin-like growth factor-1 (IGF-1) and Fas-ligand (Fas-L) was studied *in vitro* on granulosa cell function. BMPR-2 was mainly localized in the granulosa cells, oocytes and theca cells of healthy follicles from primordial to late antral stages and the expression pattern did not differ among different sizes of follicles. The Fas was predominantly expressed in granulosa cells of atretic follicles. In the granulosa cell culture, BMP-2 (30 ng/ml) and IGF-1 (100 ng/ml) significantly increased estradiol-17 β production when compared to control in all sizes i.e. small (3–5 mm), medium (5–8 mm) and large (>8 mm) follicles. This first study in buffalo revealed that BMP-2 rescues small size follicles, whereas IGF-1 rescues all size follicles from Fas mediated apoptosis in buffalo.

Key words: Buffalo, BMP-2, Estradiol-17 β , Fas-L, Follicles, IGF-1

Bone morphogenetic proteins (BMPs) are involved in ovarian folliculogenesis by regulating gonadotropin-induced steroidogenesis and mitosis in ovarian granulosa cells (Chakraborty and Roy 2015, Rossi *et al.* 2016). The BMPs act through BMP receptor II (BMPR-2) and BMPR type 1A or 1B, present in the granulosa cells, theca cells and the oocyte. The BMP-2 may play a role in ovarian follicular steroidogenesis in cattle (Selvaraju *et al.* 2013) and sheep (Vinod *et al.* 2014), but not reported in buffaloes. Further, elucidating the steroidogenic role of BMP-2, Fas-L and IGF-1 in different classes of follicles may help to understand the role of these molecules in follicle development and function during each cycle either towards ovulation or during superovulation. Hence, the objective of the present study was to localize BMPR-2 and Fas antigen during different stages of follicle development in buffalo and to study the effect of BMP-2, IGF-1 and Fas-L on granulosa cell steroidogenic function *in vitro*.

MATERIALS AND METHODS

Immunohistochemistry: The buffalo ovaries were

Present address: ¹Veterinary Surgeon (vinoddiva@rediffmail.com), Department of Veterinary Anatomy, Veterinary College, Bengaluru. ²Veterinary Assistant Surgeon (rahila905@gmail.com), Jammu and Kashmir. ³Senior Scientist (Selvaraju.S@icar.gov.in), ^{4,5}Ph D Scholar (nazarbt@gmail.com, shanmyli@gmail.com), ⁶Professor and Head (kolataluvj@yahoo.co.in), Department of Veterinary Anatomy. ⁷Dean (prashwin2002@yahoo.com), Veterinary College. ⁸Principal Scientist and Acting Head (jpravindra@yahoo.com).

obtained from the corporation slaughterhouse and transported to the laboratory in ice cold normal saline within 2 h of slaughter. The immunohistochemistry was carried out as described by Patil *et al.* (2012). The sections were overnight incubated in either rabbit polyclonal BMPR-2 (1:100 dilution) primary antibody or mouse monoclonal Fas (Fas-10; sc-8009 monoclonal antibody, 1:100 dilution) primary antibody at 4°C in a humidified chamber. The blocking buffer was added to negative control section instead of primary antibody. After overnight incubation, the slides were washed twice in PBS for 5 min each. For BMPR-2 expression studies, the slides were incubated with goat anti-rabbit biotin conjugated secondary antibody (1:400 dilution), and for Fas expression studies, the sections were incubated with rabbit anti-mouse biotin conjugated secondary antibody (1:200 dilution) for 1 h at room temperature. The sections were washed twice in PBS for 5 min. ABC (avidin-biotin complex) substrate was added to the section and incubated for 30 min at room temperature and sections were washed twice in PBS for 5 min each. Bound antibodies were visualized by incubating the sections in 3–3' diaminobenzidine, counterstained with Harris' haematoxylin, dehydrated through ascending grades of alcohol and observed under phase contrast microscope.

The cross sectional diameter of the follicle from the follicular basal lamina was measured using an ocular micrometer. Based on their size, the ovarian follicles were classified into 4 groups, viz. 1–3, 3–5, 5–8 and >8 mm diameter. Approximately, 30 follicles each of size, 1–3 mm, 3–5 mm, 5–8 mm and >8 mm were observed for the

immune-histochemical expression study. Follicles that were considered as healthy had an intact and well organised granulosa cell layers (>5 layers) with few pyknotic cells (<5%). The follicles that were considered as atretic had lesser granulosa cell layers (<5 layers) with more pyknotic/fragmented cells (>5%) and also had denuded granulosa cells in the antrum (Sreejalekshmi *et al.* 2011). The intensity of staining was graded by using a histograde system: -, negative; +/-, negligible staining; +, weak staining; ++, moderate staining; +++, strong staining.

Granulosa cell culture: The buffalo ovaries collected from corporation slaughterhouse were transported to the laboratory within 2 h of collection in ice cold Dulbecco's phosphate buffer saline (DPBS, pH-7.4) containing antibiotics and antimycotics. For culture, the granulosa cells were collected separately from 3 different size categories, viz. 3–5 mm (small), 5–8 mm (medium) and >8 mm (large) follicles and collected cells were pooled based on the size of the follicle and cultured. The granulosa cells culture was done in serum free culture media according to the procedure described by Selvaraju *et al.* (2013). The seeded cell culture plate was incubated in CO₂ incubator at 37°C, 5% CO₂ and 95% humidity for 6 days and every alternate day, 75% of the media was replaced with fresh media. On 6th day, the cells were treated with different combinations of BMP-2, Fas-L and IGF-1. Based on earlier studies, BMP-2 at 30 ng/ml (Vinod *et al.* 2014) and Fas-L at 10 ng/ml (Sunil *et al.* 2012) were added to granulosa cell culture. The treatment groups were as follows: control, BMP-2 (30 ng/ml), Fas-L (10 ng/ml), IGF-1 (100ng/ml), BMP-2 (30 ng/ml) + Fas-L (10ng/ml), Fas-L (10 ng/ml) + IGF-1 (100 ng/ml). On day 7, the media was removed and stored at –80°C for hormone estimation. The experiment was repeated thrice, and the average values from triplicates for each group were pooled for analysis.

Estradiol 17-β assay: Estradiol-17 β concentration in the cell culture media was estimated by radioimmunoassay technique by using commercially available RIA kit. The concentration of estradiol 17-β was expressed as pg/ml of the media. The intra- and inter-assay coefficients of variation were less than 10%.

Statistical analysis: The data were analyzed by using SPSS software. The average values of each group were pooled and analyzed using one-way ANOVA and post-hoc Duncan's test was used to detect statistically significant difference between groups. The values were expressed as mean±SEM and considered significant if P was < 0.05.

RESULTS AND DISCUSSION

Expression of BMPR-2 at different developmental stages of the follicle: The BMPR-2 was expressed in granulosa cells and theca cells in healthy buffalo follicles from the primary to the large antral stages (Fig. 1). The expression was stronger in granulosa cells than thecal cells and the oocytes expressed BMPR-2 at all developmental stages (Table 1) and the expression pattern did not differ among different sizes of tertiary follicles. Localization of BMPR-

Table 1. Cellular localization and relative intensity of BMPR-2 expression in buffalo ovarian follicles at different developmental stages

Classification of follicles	Relative intensity of BMPR-2	
	Granulosa cells	Theca cells
Non-atretic follicles		
Primordial follicles	++	-
Primary follicles	++	-
Secondary follicles	+++	+/-
Tertiary follicles	+++	+
Atretic follicles		
All classes	+ / ++	+

2 mainly in the granulosa cells suggests a potential role of BMPs in regulating follicle growth and steroidogenic function in buffalo ovary. A weak BMPR-2 expression was observed in the thecal layer of preantral and antral follicles of buffalo. Localization of BMPR-2 to theca cells suggests a probable role of BMPs in regulating androgen production, which will subsequently be used for estradiol-17β synthesis and follicular function.

The differential expression of BMPR-2 was studied in different sizes of ovarian follicles of buffalo. The localization of BMPR-2 was observed in all the different sizes (1–3, 3–5, 5–8 and >8 mm diameter) of the ovarian follicles. The expression of BMPR-2 was strong in healthy follicles, but weaker in atretic follicles. The expression of BMPR-2 in theca cells was observed mainly in the healthy follicles. The early atretic follicles showed lesser expression than healthy follicles, whereas the late atretic follicles showed very less or no expression of BMPR-2 (Fig. 1). In the present study, expression pattern did not vary between different size classes, however, the relative BMPR-2 transcript levels increased with the growth of potential dominant follicle (Selvaraju *et al.* 2013).

Expression of Fas in different follicular groups: In the

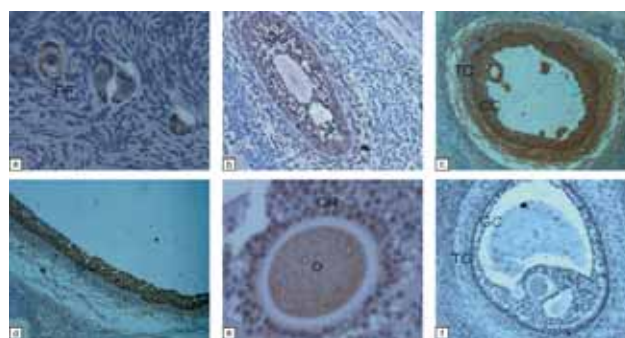


Fig. 1. Immunohistochemical staining of BMPR-2 in ovarian follicles of buffalo during different developmental stages. The paraffin embedded sections were treated either with BMPR-2 antibody or with normal goat serum (negative control) and detected using avidin-biotin complex and diaminobenzidine system. (a) Primordial follicle, (b) preantral follicle, (c,d) antral follicles, (e) oocyte with corona, (f, g) atretic antral follicle, (h) late atretic follicle and (i) negative control. (PF, primordial follicle; O, oocyte; DO, degenerated oocyte; GC, granulosa cells; DGC, denuded granulosa cells; TC, theca cells).

Table 2. Fas immunostaining and atretic pattern in different sizes of ovarian follicles of buffalo

Follicle size	Fas expression				Total positive (%)	Based on histological observation	
	Negative (%)	+	++	+++		Atretic (%)	Non-atretic (%)
1-3 mm	13	7	53	27	87	67	33
3-5 mm	17	10	33	36	83	71	29
5-8 mm	16	12	24	48	84	56	44
> 8 mm	25	-	75	-	75	75	25

+ , weak immunostaining; ++, moderate immunostaining; +++, strong immunostaining.

primordial follicles, Fas was detected in most of the atretic oocytes and also in few healthy oocytes apparently without any atretic sign (Fig. 2). In the atretic primary and secondary follicles, in addition to oocyte, granulosa cells also expressed Fas (Patil *et al.* 2012). This finding suggested that Fas-Fas-L system mediates oocyte apoptosis, which is the major mechanism of atresia in preantral follicles.

Among the 1–3 mm antral follicles, 30.3% were healthy and 66.7% were atretic, with the degree of atresia varying

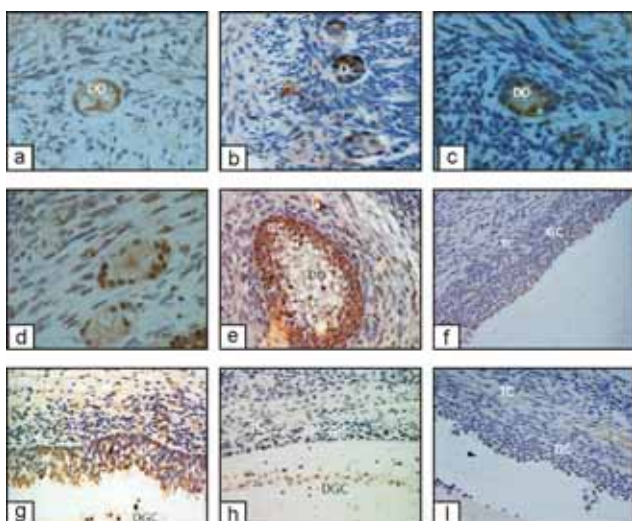


Fig. 2. Immunohistochemical staining of Fas in ovarian follicles of buffalo during different developmental stages. The paraffin embedded sections were treated either with Fas antibody or with normal goat serum (negative control) and detected using avidin-biotin complex and diaminobenzidine system. (a) Primordial follicle with completely degenerated oocyte showing strong Fas staining, (b) primordial follicle with healthy oocyte and intact nucleus showing positive staining for Fas in oocyte, (c) primary follicle showing Fas positive staining in oocyte, (d) primary follicle with degenerated oocyte showing Fas staining in oocyte as well as the granulosa cells, (e) secondary follicle with degenerated oocyte showing Fas staining in oocyte as well as the granulosa cells, (f) apparently healthy follicle with well organised and multilaminar granulosa cell layers showing mild Fas positive reaction in antral granulosa cells, (g) an early atretic follicle with Fas positive reaction in granulosa cells, (h) late atretic follicles showing positive staining for Fas in granulosa cells, (i) negative control. (O, oocyte; DO, degenerated oocyte; GC, granulosa cells; DGC, denuded granulosa cells; TC, theca cells).

among different follicles (Fig. 2; Table 2). The healthy follicles (60%) showed insignificant and very weak positive reaction. The follicles in the late as compared to early atretic stage showed more intense positive reaction mainly in granulosa cells and a weak Fas positivity was observed in theca cells. Basal atresia was seen in some follicles with very slight degree or no atretic characteristics in granulosa cells on the antral side, but even then the intensity of Fas staining was more pronounced on the antral side. In the small (3–5 mm), medium and large size follicles, 82.8, 84 and 75% showed positive Fas staining (atretic follicle). The late atretic follicles with lesser granulosa cell layers and denuded cells showed strong staining. Though, the regulatory mechanisms of granulosa cell apoptosis might differ among species, in the present study, as similar to cattle, the Fas expression was observed in all atretic follicles and the intensity of expression was positively correlated with the degree of atresia.

All negatively stained follicles during histology were apparently non-atretic. A weak immunostaining was observed in some of those apparently healthy follicles. Since the histological staining is not efficient in detecting early stage of follicular atresia (Sreejalekshmi *et al.* 2011), these Fas expressed follicles even though apparently healthy based on histological findings, may be destined to undergo apoptosis soon. Hence, the Fas expression might occur in follicles during very early stage of apoptosis before other morphological and biochemical signs of degeneration or dysfunction.

Effect of BMP-2, IGF-1 and Fas-L on granulosa cell steroidogenic function: The cultured granulosa cells exposed to BMP-2 secreted higher estradiol-17 β , compared to control in all size classes (Fig. 3) indicating the beneficial effect of BMP-2 in improving ovarian function in buffalo. The BMP-2 secreted by the granulosa cells has both autocrine (acting on granulosa cells) and paracrine function. In cattle, a positive autocrine role of BMP-2 in dominant follicle development was reported (Selvaraju *et al.* 2013). It was also evident from the present study that the estradiol-17 β production by the granulosa cells cultured from medium and large size follicles was higher when compared to small size follicles. Since the intensity of expression of BMP-2 was stronger in granulosa cell when compared to theca cells, the granulosa cells might be the major target for the BMP-

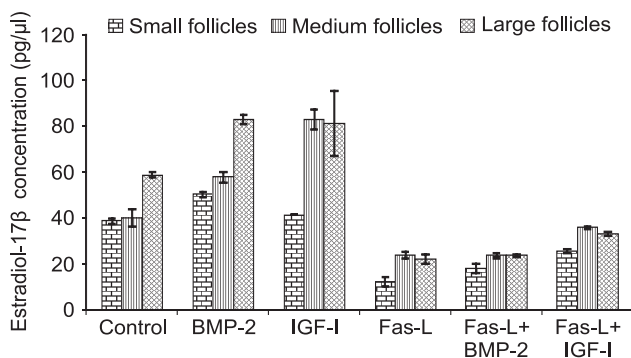


Fig. 3. The effect of bone morphogenetic protein-2 (BMP-2, 30 ng/ml), insulin-like growth factor (IGF-1, 100 ng/ml), Fas-L (10 ng/ml), FasL+BMP-2 and Fas-L+IGF-1 on estradiol-17 β (pg/ml) production from cultured granulosa cells collected from different sizes (small, 3–5 mm; medium, 5–8 mm and large, >8mm) of follicles. The cells were cultured in serum free minimum essential medium- α . The values (mean \pm SEM) were from 3 independent cultures, each treatment having four replicates.

2 in buffalo ovarian follicle development.

The addition of BMP-2 with Fas-L in the granulosa cells from the small follicles increased estradiol-17 β production in the culture and the levels were significantly ($P < 0.05$) higher than the Fas-L group (Fig. 3). However, addition of BMP-2 to the Fas-L did not increase the estradiol-17 β production from the granulosa cells of medium and larger size follicles, whereas addition of IGF-1 to the Fas-L exposed granulosa cells significantly ($P < 0.05$) increased estradiol 17- β production from all the 3 class of follicles. Though the addition of BMP-2 might overcome the effect of Fas-L by improving granulosa cell viability, BMP-2 failed to overcome the pro-apoptotic effect of Fas-L on large follicle granulosa cell function. This suggested that the biological functions of BMP-2 and Fas-L on granulosa cell functions in these classes of follicles may be mediated through different pathways.

The beneficial effect of IGF-1 on granulosa cells steroidogenic function is well established in cattle (Gutierrez *et al.* 1997) and addition of IGF-1 to the culture increased estradiol-17 β production in all the 3 follicle sizes from buffalo, but the magnitude of effect was highest in medium sized follicles compared to small and large follicles suggesting its importance in developing follicles for final growth in attaining dominance in bovine (Rovani *et al.* 2015). The study also suggested that the IGF-1 might overcome the pro-apoptotic effect of Fas leading to granulosa cell survival and maintaining its estradiol-17 β secretion in buffalo granulosa cells from medium size follicles. Hence, modulation of BMP-2 in small size follicles and IGF-1 expression in medium size follicle cells may help improving ovarian function and fertility in buffalo cow.

The presence of functional BMPR-2 in buffalo ovarian follicle implies that the BMP-2 may be involved in the processes of folliculogenesis and steroidogenesis. The study

also indicated that BMP-2 might play a key role in rescuing small size follicles, whereas IGF-1 is potentially protective in medium size follicles against Fas-Fas-L mediated apoptosis in buffalo. The rescuing effect of BMP-2 observed in small follicle could be used to enhance the number of follicles to grow from follicle pool for improving fertility in buffalo.

ACKNOWLEDGEMENT

The research work was carried out under the World Bank funded National Agricultural Innovation Project (NAIP)-C1056, ICAR. The authors thank the Director, National Institute of Animal Nutrition and Physiology, Bengaluru, for providing necessary facilities to carry out this work. The authors thank Carl-Henrik Heldin from the Ludwig Institute for Cancer Research, Uppsala, Sweden for the gift of the BMP receptor antibody.

REFERENCES

- Chakraborty P and Roy S K. 2015. Bone morphogenetic protein 2 promotes primordial follicle formation in the ovary. *Scientific Reports* 5: 12664. <http://doi.org/10.1038/srep12664>
- Gutierrez C G, Campbell B K and Webb R. 1997. Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. *Biology of Reproduction* 56: 608–16.
- Patil S K, Ravindra J P, Jamuna K V, Dhanya Joseph, Ravghavendra B S, Prasad R V and Selvaraju S. 2012. Assessment of immunolocalization of Fas antigen in the ovarian follicle and the role of IGF-1 in Fas-L induced granulosa cell apoptosis in water buffalo (*Bubalus Bubalis*). *Indian Journal of Animal Sciences* 82: 979–83.
- Rossi R O, da Cunha E V, Portela A M, Passos J R, Costa J J, Silva A W, Saraiva M V, Peixoto C A, Donato M A, van den Hurk R and Silva J R. 2016. Influence of BMP-2 on early follicular development and mRNA expression of oocyte specific genes in bovine preantral follicles cultured *in vitro*. *Histology and Histopathology* 31: 339–48.
- Rovani M T, Ilha G F, Haas C S, Rissi V B, Ferreira R, Bordignon V, Gonçalves P B and Gasperin B G. 2015. Regulation of TGF β family members around follicular deviation and final growth in bovine. *Animal Reproduction* 12: 599.
- Selvaraju S, Folger J K, Gupta P S P, Ireland J J and Smith G W. 2013. Stage specific expression and effect of bone morphogenetic protein 2 on bovine granulosa cell estradiol production: regulation by cocaine and amphetamine regulated transcript. *Domestic Animal Endocrinology* 44: 115–20.
- Sreejalekshmi P, Raghavendra B S, Sivasubramani T, Chandrashekaramurthy V, Jamuna K V, Prasad R V, Ravindra J P and Selvaraju S. 2009. Detection of follicular apoptosis in water buffalo (*Bubalus bubalis*) ovary by histology and nick end labelling technique. *Reproduction in Domestic Animals* 46: 59–65.
- Vinod Kumar D, Gulzar R, Selvaraju S, Nazar S, Parthipan S, Anand, Prasad R V, Jamuna K V and Ravindra J P. 2014. Effect of bone morphogenetic protein-2 (bmp-2) on sheep granulosa cell steroidogenic function. *Journal of Cell and Tissue Research* 14: 4233–36.