



Expression of growth and differentiation Factor 9 and cognate receptors during final follicular growth in cattle

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Abstract

Mutations in growth and differentiation factor 9 (GDF9) gene are associated to sterility or, paradoxically, increased ovulation rate in ewes. Despite its importance, the exact function of GDF9 in ovarian physiology is still poorly understood. This study aimed to investigate GDF9 function during dominant follicle growth and its regulation in follicular fluid. The regulation of GDF9 receptors in GnRH/LH-stimulated granulosa cells was also investigated. In a first experiment, a new follicular wave was induced and the intrafollicular GDF9 treatment into the largest growing follicle (8.5-9.5 mm) at both 100 (n = 3) and 1000ng/ml (n = 4) had no effect on follicular growth, estrus manifestation and ovulation compared to control (PBS-injected) follicles (n = 3). In a second experiment, follicles were obtained just after follicular deviation (day 4 after follicular emergence) and the abundance of GDF9 in follicular fluid did not differ between healthy dominant (n = 4) and atretic subordinate follicles (n = 4), as assessed by western blot analysis. Finally, mRNA expression of *BMPR2* and *TGFBR1* receptors was evaluated in granulosa cells obtained from preovulatory follicles (>12 mm diameter) obtained 0, 3, 6, 12 or 24 h after i.m. GnRH administration (n = 4-5 follicles/moment). Both receptors were significantly up regulated 12 h after GnRH treatment. Present results do not confirm the hypothesis that GDF9 inhibits dominant follicle growth and suggests a minor role in determining follicle fate. In the other hand, GDF9 receptors regulation in GnRH/LH-stimulated granulosa cells provides the first in vivo evidence of its involvement in the complex cascade of events that culminates in ovulation and luteinization in cattle.

Keywords: folliculogenesis, oocyte factors, ovulation.

Introduction

Folliculogenesis and follicle differentiation are processes that culminate in ovulation, being mainly coordinated by well-established endocrine mechanisms. In monovulatory species, from a cohort of growing follicles, only one is selected to continue growing (dominant follicle), whereas all the others undergo atresia (subordinate follicles; Ginther *et al.*, 1996).

Since all the follicles are under the same endocrine environment, ovarian-produced factors are also involved in dominant follicle selection, regulating important events such as proliferation of granulosa cells, differentiation and steroidogenesis. In this context, the involvement of oocyte-secreted factors such as growth and differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and their receptors, has been demonstrated in sheep (Knight and Glistler, 2006). Both proteins seem to interact to regulate important ovarian functions (Galloway *et al.*, 2000; Juengel, 2002; Hanrahan *et al.*, 2004).

Spontaneous heterozygous inactivating mutations in BMP15 and GDF9 genes induce superovulation, whereas homozygous mutation (absence of functional protein) causes sterility in ewes as a consequence of impaired preantral follicle development (Galloway *et al.*, 2000; Hanrahan *et al.*, 2004). These interesting findings were reproduced using passive and active immunization against these proteins. In both sheep and cattle, short-term immunization increased ovulation rate (Juengel *et al.*, 2004b, 2009), with no negative effects on fertilization, embryo development and pregnancy maintenance in ewes (Juengel *et al.*, 2004b).

On the other hand, long-term immunization blocked follicle development in sheep (McNatty *et al.*, 2007). Furthermore, passive immunization against GDF9 negatively affected ovulation and luteinization (Juengel, 2002), suggesting the involvement of this protein during the periovulatory period. In cattle, GDF9 stimulates theca cells proliferation but inhibits LH or IGF-stimulated steroidogenesis by downregulation of CYP11A1 enzyme and LH receptors (LHCGR) *in vitro*. A negative effect of GDF9 was also observed on estradiol secretion by granulosa cells treated with FSH and IGF *in vitro* (Spicer *et al.*, 2008). Taken together, these studies demonstrate that BMP15/GDF9 are essential during preantral follicle development, but are also involved in final follicular growth and ovulation (Juengel *et al.*, 2004a, 2009). However, the regulation and function of these proteins, especially during periovulatory period, is still poorly understood.

In humans, deregulation of BMPs expression is associated with reproductive dysfunction such as premature ovarian failure (Dixit *et al.*, 2006). Reduced BMP15 and GDF9 expression was also reported in

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women with polycystic ovary syndrome (PCOS; Wei *et al.*, 2014). Furthermore, a mutation in GDF9 was identified in young women with reduced ovarian reserve, being associated to reduced production of mature protein, decreasing GDF9 effect on human granulosa cells proliferation *in vitro* (Wang *et al.*, 2013).

The growing evidence of the involvement of oocyte-secreted factors in regulating follicle development led to many *in vitro* studies to investigate their regulation and mechanisms of action. However, only a few studies have investigated the regulation of these proteins in different follicle classes and by gonadotrophins *in vivo*. Furthermore, to the best of our knowledge, there is no study manipulating GDF9 or BMP15 concentration in follicular environment. Thus, the present study aimed to determine the effect of GDF9 intrafollicular injection on follicular growth and compare its abundance in healthy and atretic follicles after follicular deviation. Furthermore, GDF9 receptors regulation during GnRH/LH-induced ovulation was also investigated.

Material and Methods

All procedures involving animals were reviewed and approved by the UFPEL's and/or UFSM Ethics for Animal Experimentation Committees.

Experiment 1: Follicular growth and ovulation after GDF9 intrafollicular injection

The effect of recombinant human GDF9 (rhGDF9; Sigma Aldrich) intrafollicular injection was investigated in non-lactating cyclic cows from Holstein and Jersey breeds. Cows were synchronized using a hormonal protocol (Zoetis, São Paulo, Brazil) consisting of 2 mg estradiol benzoate i.m. injection and insertion of an intravaginal device (IVD) containing 1 g progesterone on day 0 to induce follicular regression and a new follicular wave. Seven days later, prostaglandin F2 α (25 mg, i.m.) was administered to induce corpus luteum regression and daily follicular dynamics evaluation was initiated. IVDs were kept until growing follicles reached 8.5 to 9.5 mm diameter, when intrafollicular injections were performed. At least two transrectal ultrasound examinations using a 6MHz linear-array transducer (Pie Medical AquilaVet, Maastricht, The Netherlands) were performed before intrafollicular injection, to ensure that the follicles were growing. All structures larger than 6 mm were drawn according to their position in the ovaries. After identifying the healthy dominant follicle from each cow, treatments were performed followed by daily transrectal ultrasound evaluation. Intrafollicular injections were performed under caudal epidural anesthesia using 80 mg lidocaine chlorhydrate (4 ml lidocaine 2%) using a two-needles system attached to a 5MHz microconvex-array transducer (Pie Medical AquilaVet, Maastricht, The Netherlands) according to Ferreira *et al.* (2007). Injected follicles were daily monitored until follicular regression (atresia) or spontaneous ovulation.

Cows were randomly allocated to receive an intrafollicular injection of phosphate buffered saline (PBS, control group; n = 3) or recombinant human GDF9 (rhGDF9; Sigma Aldrich) at final concentrations of 100 (n = 3) or 1000 (n = 4) ng/ml. The doses of rhGDF9 were chosen based on *in vitro* studies (Mottershead *et al.*, 2011). The injected volume was determined according to the linear equation $V = -685.1 + 120.7D$, where "V" corresponds to the volume in microliters and "D" to the diameter of the follicle in millimeters (Ferreira *et al.*, 2007). The initial concentration of rhGDF9 was 10 times greater than the desired final intrafollicular concentration.

Experiment 2: Follicular fluid GDF9 abundance in dominant and subordinate follicles after deviation

To compare GDF9 protein abundance between healthy dominant and regressing (atretic) follicles (day 4 of follicular wave), follicular fluid samples from a previous study were used (Gasperin *et al.*, 2014). Briefly, ovaries from four previously synchronized non-lactating cyclic *Bos taurus* cows (Red Angus, 400-500 kg body weight) were obtained on day 4 after follicular emergence (D0 was retrospectively identified as the last day when the largest follicle was smaller than 5 mm). Follicular fluid samples from the two largest follicles from each cows were obtained through aspiration. To quantify GDF9 protein abundance, western blots were run using 70 μ g protein samples. After boiling at 95 $^{\circ}$ C for 5 min, follicular fluid samples were subjected to 12% SDS gel and electrotransferred onto nitrocellulose membranes. After blocking for 2 h with 5% skimmed milk in PBS containing 0.1% tween-20 (PBS-T), blots were incubated overnight at 4 $^{\circ}$ C with 1:500 rabbit anti-human GDF9 (GTX108410; GeneTex., CA, USA) with agitation, followed by three washes (10 min each) with PBS-T. Then, blots were incubated with 1:5000 goat anti-rabbit IgG-HRP (ab6721; Abcam Inc., USA) for 2 h with agitation, followed by three washes (10 min each) with PBS-T. Finally, immunoreactivity was detected with Immun-Star WesternC Chemiluminescence Kit according to the manufacturer's instructions and visualized using Chemidoc analyser (BioRad, CA, USA). Quantification of GDF9 bands of the western blots was performed using Image Lab software (BioRad Laboratory).

Experiment 3: Granulosa cells mRNA expression of GDF9 receptors after induction of ovulation with GnRH

Thirty-one cyclic adult (3 to 8 years old) non-lactating *Bos taurus* cows (predominantly Red Angus and Hereford, 400-500 kg body weight) with body condition scores of 3 and 4 were submitted to a synchronization protocol. The hormonal protocol started on day 0 with the insertion of an intravaginal device (IVD) containing 1 g progesterone and i.m. injections of 2 mg estradiol benzoate and 250 μ g cloprostenol (two injections, 12 h apart). On day 9, IVDs were removed and only cows with follicles \geq 12 mm in diameter (n = 23 cows) received an i.m. injection of GnRH (100 μ g

gonadorelin acetate). Granulosa cells samples were obtained 0 (n = 4), 3 (n = 5), 6 (n = 4), 12 (n = 5) and 24 h (n = 5) after GnRH i.m. treatment (Santos *et al.*, 2012).

Granulosa cells total RNA was extracted using AllPrep DNA/RNA/protein kit (Qiagen, Mississauga, ON, Canada). Quantitation and estimation of RNA purity was performed using NanoDrop (Thermo Scientific - Waltham, USA; Abs 260/280 nm ratio) spectrophotometer (ratios above 1.8 were considered pure). To generate the cDNA, 200 ng RNA was first treated with 0.1 U DNase (Invitrogen; 37°C - 5 min). After DNase inactivation at 65°C for 10 min, samples were incubated in a final volume of 20 µl with iScript cDNA Synthesis Kit (BioRad) according to the manufacturer's instructions. To avoid cross-contamination, only granulosa cells samples that did not express *CYP17A1* mRNA after 30 PCR cycles were used in the study.

Real-time polymerase chain reactions (PCR) were conducted in a CFX384 real-time PCR detection system (BioRad, Hercules, CA) using SsoFast™ EvaGreen® supermix (BioRad, Hercules, CA) using the following primers sequences: *GAPDH*: F-GATTGTCAGCAATGCCTCCT and R-GGTCATAAGTCCCTCCACGA; *CYCLOPHILIN*: F-GGTCATCGGTCTCTTTGGAA and R-TCCTTGATCACACGATGGAA; *BMP2*: F-CCACTGGCCTCACTCCAAGT and R-CCCGACTGGCTGTGAAACAT; and *TGFBRI*: F-ACCACTGCAATAAAATAGAACTTCCA and R-TGACAGCTGCCAGTTCAACAG. Melting-curve analyses was performed to verify product identity. Samples were run in duplicate using 2 µl of diluted cDNA (1:20) in each well. The relative standard curve

method was used to assess the amount of a particular transcript in the samples, as previously described (Gasperin *et al.*, 2014). Results were expressed relative to the average of *GAPDH* and *cyclophilin* housekeeping genes.

Statistical analysis

Main effects of treatment group, day and their interaction on follicular growth were determined using the MIXED procedure (SAS Institute Inc., Cary, NC) with a repeated measure statement. Differences between follicular diameters at a specific time point were compared between groups using estimates. Protein and mRNA abundance data were submitted to ANOVA using JMP software (JMP version 7; SAS Institute Inc., Cary, NC). Comparison between groups was made by *least square means*. Results are presented as means ± standard error of the mean (s.e.m) and a P < 0.05 was considered statistically significant.

Results

In the first study, all injected follicles grew independently of treatment (moment effect). Cows from all groups demonstrated estrus and ovulated (Fig. 1). There was no significantly effect of group and no interaction of group and time after intrafollicular injection (P > 0.05).

No significant difference were observed in GDF9 protein abundance in follicular fluid from dominant compared to subordinate follicles collected after follicular deviation (P > 0.05; Fig. 2). Both *BMP2* and *TGFRBI* receptors were significant upregulated 12 h after GnRH treatment (Fig. 3).

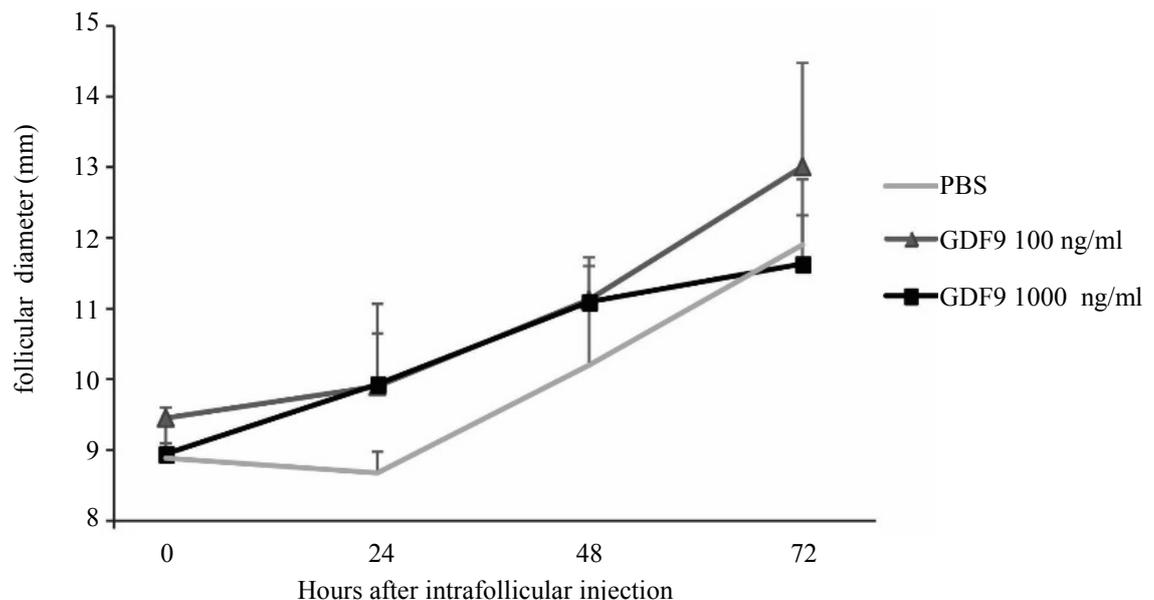


Figure 1. Effect of GDF9 intrafollicular injection on bovine follicular growth. Animals received intrafollicular injection of PBS (control; n = 3) or rhGDF9 at 100ng/ml (n = 3) or rhGDF9 at 1000ng/ml (n = 4).

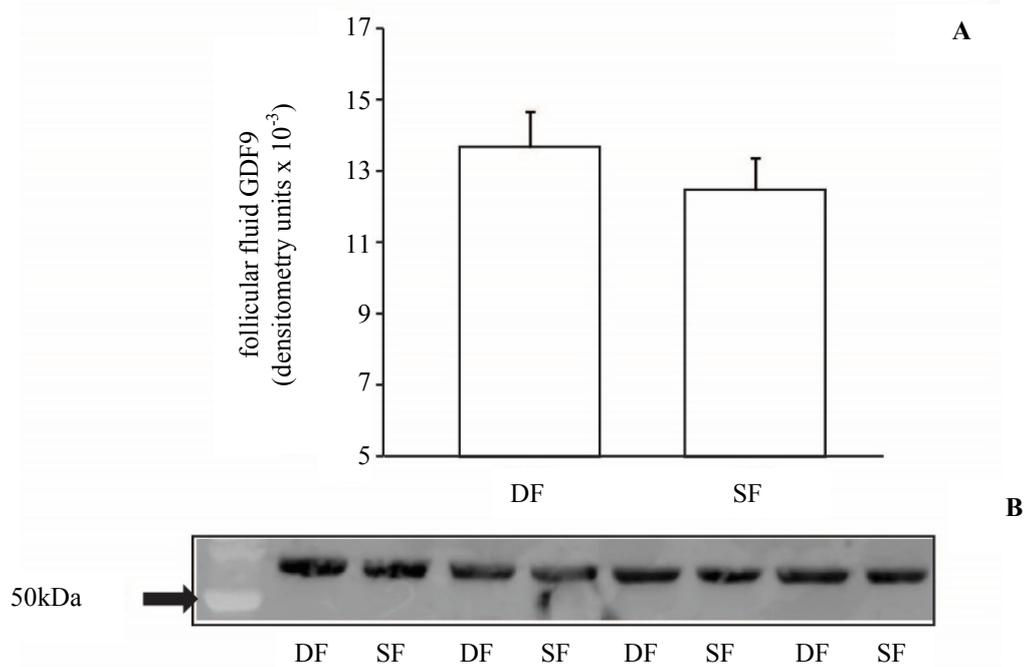


Figure 2. Abundance of GDF9 protein in follicular fluid from dominant (DF; n = 4) and subordinate follicles (SF; n = 4) obtained after follicular deviation (day 4 of follicular wave).

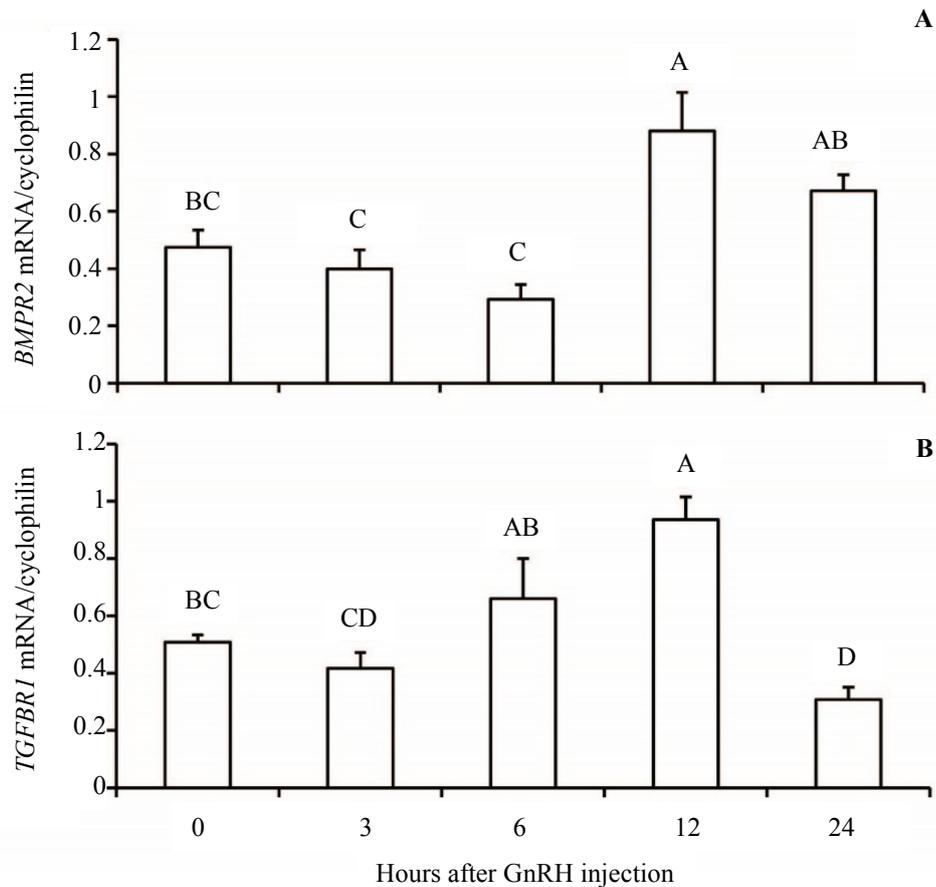


Figure 3. Abundance of *BMPR2* (A) and *TGFBR1* (B) mRNA in granulosa cells from follicles >12 mm, 0 (n = 4), 3 (n = 5), 6 (n = 4), 12 (n = 5) and 24 (n = 5) hours after GnRH administration. Different letters indicate significant difference (P < 0.05).



Discussion

Oocyte-secreted factors have many different functions varying according to the species and experimental models (McNatty *et al.*, 2005; Spicer *et al.*, 2006). Based on previous results from bovine and ovine *in vivo* models (Juengel *et al.*, 2004b, 2009) suggesting that GDF9 acts as an inhibitor of granulosa cells differentiation, we hypothesized that its intrafollicular injection would inhibit dominant follicle growth and ovulation. However, using an *in vivo* model similar to the one used by Lv *et al.* (2009), GDF9 intrafollicular injection in follicles between 8.5 and 9.5 mm at final concentrations of 100 or 1000 ng/ml did not affect follicular growth and ovulation. Follicular differentiation in *Bos taurus taurus* cows occurs when the dominant follicles reach 8.5 mm and its hallmark is the expression of LH receptors (LHCGR) in granulosa cells, despite *LHCGR* expression increases in follicles larger than 9 mm (Gasperin *et al.*, 2015). Thus, a possible explanation for the lack of GDF9 effect is that injected follicles were already differentiated. GDF9 treatment in follicles around 7.5 and 8 mm should be performed in future studies to investigate a possible effect in inhibiting follicle differentiation (Gasperin *et al.*, 2012), since previous studies demonstrated that cells from small follicles (3 to 6 mm) are more susceptible to GDF9 effects on proliferation and steroidogenesis (Spicer *et al.*, 2008). Unfortunately, it is not possible to perform intrafollicular injections in follicles smaller than 6 mm.

Corroborating the aforementioned results, GDF9 abundance did not differ between follicular fluid collected from healthy dominant compared to atretic subordinate follicles. Based on previous studies suggesting an inhibitory role of GDF9 on antral follicle differentiation, we would expect decreased abundance in healthy growing follicles. However, in a previous study, GDF9 cognate receptors *BMPR2* and *TGFBR1* were not regulated at the expected moment of follicular deviation and after the establishment of follicular dominance in cattle (Gasperin *et al.*, 2014). GDF9 abundance was previously quantified in human follicular fluid, being mature protein levels positively correlated with nuclear maturation and embryo quality and negatively correlated to follicular fluid progesterone levels (Gode *et al.*, 2011), suggesting that GDF9 levels decrease during luteinization. However, in primates, GDF9 levels in follicular fluid were not altered after hCG treatment (Duffy, 2003). It is important to highlight that in the present study we only quantified the abundance of GDF9 pre-peptide and that post-translation modifications and interaction with other proteins may differentially regulate GDF9 function.

To investigate the previously-demonstrated association between GDF9 signaling and steroidogenesis (Juengel, 2002; Spicer *et al.*, 2008), the expression of *BMPR2* and *TGFBR1* mRNA was evaluated in granulosa cells from GnRH/LH-stimulated preovulatory follicles *in vivo* (Santos *et al.*, 2012). The significant increase in *BMPR2* and *TGFBR1* 12 h after GnRH treatment, suggests an involvement in

steroidogenesis regulation, since at this time-point estradiol levels sharply decrease (Santos *et al.*, 2012), preceding the increase in follicular fluid progesterone levels (Fortune *et al.*, 2009). Thus, beyond its role in granulosa cells steroidogenesis in small follicles (Spicer *et al.*, 2006, 2008), GDF9 may regulate steroidogenesis during luteinization in cattle.

Collectively, present results do not confirm the hypothesis that the oocyte secreted factor GDF9 inhibits dominant follicle growth in cattle. The fact that GDF9 protein abundance does not differ in healthy and atretic follicles suggests that this factor has a minor role in determining follicle fate. In the other hand, the regulation of GDF9 receptors in granulosa cells after GnRH/LH treatment provides the first *in vivo* evidence of its involvement in the complex cascade of events that culminates in ovulation and luteinization in cattle.

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