Small Ruminant Research 100 (2011) 169–176

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Expression of growth and differentiation factor 9 (GDF-9) and its effect on the in vitro culture of caprine preantral ovarian follicles

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ARTICLE INFO

Article history:
Received 2 June 2010
Received in revised form 23 May 2011
Accepted 7 June 2011
Available online 1 July 2011

Keywords:
GDF-9
Oocyte
mRNA expression
Follicular growth and viability
Caprine

ABSTRACT

This study examined the expression of growth and differentiation factor 9 (GDF-9) in caprine ovarian follicles, and the effect of GDF-9 with or without FSH on the in vitro culture of preantral follicles. To evaluate the expression of GDF-9 in Experiment 1, follicles were recovered from 32 goat ovaries and the total RNA isolated and transcribed for real-time polymerase chain reaction (PCR). Experiments 2 and 3 each used a further 32 goat ovaries to provide preantral follicles of ≥150 μm. These follicles were isolated and cultured individually in 100 μL drops. In each experiment at least 45 follicles were used per treatment. Every 6 days, follicles were evaluated for viability, antrum formation and growth rate. At the end of the culture period, oocytes were submitted to in vitro maturation (IVM), viability tests and chromatin evaluation. In Experiment 2, follicles were cultured in a basal medium (control) or this medium supplemented with GDF-9 at a concentration of 100 ng/mL (GDF-9 100) or 200 ng/mL (GDF-9 200). The same media were used in Experiment 3, supplemented with recombinant FSH at a level of 100 ng/mL from day 0, 500 ng/mL from day 6 to 12 and 1000 ng/mL from day 12 to 18 of culture to form the three treatments: control FSH, GDF-9 (100) plus FSH and GDF-9 (200) plus FSH. Relative GDF-9 expression (Experiment 1) was greater in the secondary (18 units) than the primordial (1 unit) and the primary (1 unit) preantral follicles ($P < 0.05$). In the antral follicles, GDF-9 expression was significantly higher in the cumulus–oocyte complexes COC's < 3 mm (1.6 units) than those of >3 mm diameter (1 unit; $P < 0.05$), and in COC's < 3 mm and >3 mm (319.2 and 200.1 units, respectively), compared to their respective granulosa and theca cells (1 unit for each category, $P < 0.05$). In Experiment 2, GDF-9 supplementation significantly improved the survival of the follicles (60.8%, 66.0% and 77.4% for the control, GDF-9 100 and GDF-9 200, respectively; $P < 0.05$), follicular growth rate and antrum formation following 18 days of culture. Oocyte survival was approximately 100% in all treatments. More oocytes were submitted to IVM from GDF-9 100 (78.0%; $P < 0.05$), compared to GDF-9 200 (48.1%), but no suitable oocytes could be retrieved from the control (58.8%). The proportion of oocytes showing a resumption of meiosis, was not significantly different between treatments (41.4%, 35.9% and 36.0% for the control, GDF-9 100 and GDF-9 200, respectively). The addition of GDF-9 to the media supplemented with FSH (Experiment 3) did not significantly affect any of the variables studied. The proportion of oocytes submitted to IVM in Experiment 3 was 53.3%, 56.5% and 63.8% for the control FSH, GDF-9 100 plus FSH and GDF-9 200 plus FSH, respectively (no statistical differences). The resumption of meiosis was 75.0%, 60.9% and 60.7% for the control

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doi:10.1016/j.smallrumres.2011.06.001

FSH, GDF-9 100 plus FSH and GDF-9 200 plus FSH, respectively (NS). The occurrence of metaphase II was very low in both experiments. It was concluded that the supplementation of a basal medium with GDF-9 had a positive effect on the survival and development of caprine preantral follicles, but had no real effect in the presence of FSH.

1. Introduction

The study of folliculogenesis is of great importance in terms of the optimization of the female gametogenic potential, as 90–95% of the ovarian population resorts under the preantral category, and most follicles (99.9%) are eliminated through follicular atresia (Figueiredo et al., 2007). However, the initial development of folliculogenesis is very complex and involves the action of growth factors that could either be paracrine or autocrine, and may be exchanged between the oocyte, theca and granulosa cells (Eppig, 2001; Fortune, 2003; Van den Hurk and Zhao, 2005). Growth factors, as well as their receptors, are expressed in a stage-specific form and act synchronically to promote stimulatory and/or inhibitory effects. These are then responsible for the regulation of ovarian follicular development. Additionally, such substances act on the development of the oocyte competence, regarding fertilization and subsequent embryogenesis (Van den Hurk et al., 2000; Eppig, 2001; Matzuk et al., 2002). Certain studies have provided evidence regarding the role of several growth factors in folliculogenesis. Among the main factors involved in ovarian folliculogenesis, the Growth and Differentiation Factor 9 (GDF-9), must be highlighted.

GDF-9 belongs to the super family of beta-transforming growth factors (TGF-β) and regulates the differentiation, proliferation and apoptosis of follicular cells. Such processes are essential for embryonic development, organogenesis, bone formation and reproduction (Chang et al., 2002). The activity of GDF-9 is mediated by the formation of a heterodimeric complex and its type I (ALK-5) (Mazerbourg et al., 2004) and II (BMPRII) receptors (Vitt et al., 2002). In the ovaries, GDF-9 has been expressed in the oocytes of primordial follicles of sheep and cows (Bodensteiner et al., 1999). In women (Aaltonen et al., 1999), mice (McGrath et al., 1995; Dube et al., 1998; Elvin et al., 1999) and rats (Hayashi et al., 1999; Jaatinen et al., 1999), the expression of GDF-9 begins at the primary follicle stage. In goats, the polymerase chain reaction (PCR) technique revealed the presence of GDF-9 mRNA in primordial, primary and secondary follicles, oocytes and granulosa cells of antral follicles and in the corpus luteum (Silva et al., 2004). In the ovine ovary, the expression of the BMPRII and ALK-5 receptors has been shown to increase with follicular growth (Qin Chen et al., 2009). These elements have previously been found in the granulosa cells of follicles from the primary to late antral stage (Souza et al., 2002). In goats, the BMPRII, BMPR1A (ALK-3) and BMPR1B (ALK-6) receptors have been identified in primordial, primary and secondary preantral follicles, oocytes, granulosa and theca cells of antral follicles and in CL's (Silva et al., 2004). The presence of GDF-9 receptors in the ovary demonstrates their importance in follicular development. Additionally, the elimination of the GDF-9 encoding gene in mice, has confirmed that GDF-9 is important for mouse follicular activation and growth (Barnett et al., 2006).

When used for in vitro culture of preantral follicles of rats, GDF-9 promoted the early transition of these follicles into the antral stage and suppressed follicular atresia (Orisaka et al., 2006). Martins et al. (2008) verified that in goats this factor maintains its viability and follicular ultrastructure and promotes the growth of preantral follicles that were cultured in ovarian tissue (in situ). Similarly, Hayashi et al. (1999) observed that the use of GDF-9, alone or in combination with FSH, favored the in vitro growth of secondary preantral follicles, isolated from mouse ovaries. Research has shown that FSH mediates the action of GDF-9 action, and may be mediated by FSH in cultures of oocyte–granulosa cell complexes in mice (Thomas et al., 2005) and granulosa cells isolated from sheep (Qin Chen et al., 2009).

Although GDF-9 is an essential factor for follicular development, there have been no studies focussed on the effect of this factor, with or without FSH, on the culture of preantral follicles isolated from caprine ovaries. In addition, even though GDF-9 expression has been recorded in the ovaries of goats, the quantification of specific mRNA in different follicular categories has not been performed. Thus, this trial was aimed at quantifying GDF-9 expression in different categories and compartments (*cumulus*–oocyte complexes – COC's – and mural cells) of the ovarian follicles, and evaluating the effect on in vitro culture of caprine preantral follicles, with or without FSH.

2. Materials and methods

This research was divided into three sequential experiments: the quantification of GDF-9 mRNA in caprine ovarian follicles (Experiment 1), and the in vitro culture of caprine preantral follicles in medium supplemented with GDF-9 (100 ng/mL or 200 ng/mL), without FSH (Experiment 2) and with FSH (Experiment 3).

2.1. Reagents and ovarian tissue

Unless otherwise specified, the culture medium and all other reagents used in this study were acquired from the Sigma Chemical Co. (St. Louis, MO, USA).

Ovaries were collected from adult mixed-breed goats (*Capra hircus*) from a local slaughterhouse. A total of 96 caprine ovaries, 32 per experiment, were used for the three experiments. Immediately after slaughter, the ovaries were washed in 70% alcohol and then twice with Minimum Essential Medium (MEM), buffered with HEPES (MEM-HEPES) and supplemented with penicillin (100 μg/mL) and streptomycin (100 μg/mL). Subsequently, all ovaries were transported to the laboratory within an hour (at 4 °C) (Chaves et al., 2008).

2.2. Experiment 1: GDF-9 messenger RNA expression in caprine ovaries

Sixteen caprine ovaries were used for the isolation of primordial, primary and secondary follicles. From the other 16 ovaries, COC's and mural cells (granulosa and theca cells) were collected from small (<3 mm) and large (>3 mm) antral follicles.

Primordial, primary and secondary ovarian follicles were isolated using a physical procedure (Lucci et al., 1999), performed within 2 h after collection. After isolation, the follicles were allocated according to their category, into groups of 10. From the second group of ovaries, selected from the follicular content of antral follicles, compact COC's were aspirated and grouped as smaller or larger than 3 mm in diameter (Van Tol and Bevers, 1998). To collect the mural theca and granulosa cell complexes, small and large antral follicles were isolated from the ovarian stroma, using 26 gauge needles, and then bisected. The COC's and mural theca and granulosa cell complexes were divided into groups of 10. All the samples were stored in microcentrifuge tubes at −80 °C, until RNA extraction.

Total RNA extraction was performed using a Trizol purification kit, according to the manufacturer's instructions. Before reverse transcription, the RNA samples were incubated for 5 min (at 70 °C), and then cooled on ice. Reverse transcription was performed in a total volume of 20 μl, containing 10 μl of the RNA sample, 4 μl of reverse transcriptase 5× buffer, 8 units of RNAseout, 150 units of reverse transcriptase Superscript III, 0.036 U random primers, 10 mM DTT and 0.5 mM dNTP. The mix was incubated at 42 °C for 1 h, 80 °C for 5 min, and then stored at −20 °C. The negative controls were prepared under the same conditions, but reverse transcriptase was not included. The primers and all of the other products were acquired from Invitrogen (São Paulo, Brazil).

GDF-9 mRNA quantification was performed using SYBR Green. The PCR mix contained 1 μl of cDNA template, 7.5 μl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 μl ultrapure water and 0.5 μM of primer. The primers were designed to amplify mRNA from GDF-9 (forward 5′-ACAACACTGTTCGGCTCTTCACCC-3′ and reverse 5′-CCACAACAGTAACACGATCCAGGTT-3′) and two housekeeping genes: GAPDH (forward 5′-TGTTTGTGATGGGCGTGAACCA-3′ and reverse 5′-ATGGCGTGGACAGTGGTCATAA-3′) and β-actin (forward 5′-ACCACTGGCATTGTCATGGACTCT-3′ and reverse 5′-TCCTTGATGTCACGGACGATTTCC-3′) used as endogenous controls for the normalization of gene expression. The cycle profile for the first PCR step was an initial denaturation and polymerase activation for 15 min at 94 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C. Final extension was performed for 10 min at 72 °C. All of the reactions were performed using a Real Time PCR Mastercycler (Eppendorf, Germany). The delta-delta Ct (threshold cycle) method was used to transform the Ct values into normalized levels of relative expression (Livak and Schmittgen, 2001).

2.3. Preantral follicle isolation and selection

Fine fragments of the ovarian cortex (1–2 mm thick) were obtained with the aid of a sterile scalpel blade. The fragments were placed into the fragmentation medium, MEM-HEPES. The preantral follicles (≥150 μm) were then visualized under a stereo-microscope (SMZ 645 Nikon, Tokyo, Japan) and manually microdissected, using 26 gauge needles, attached to 1 mL syringes. Only follicles with visible oocytes surrounded by two or more layers of granulosa cells, with an intact basal membrane and without an antral cavity, were selected for culture. For Experiment 2 and 3, 8 ovaries were processed at a time, resulting in one replicate for each treatment, and 4 replicates being performed on different days.

2.4. Experiment 2: caprine preantral follicle culture with GDF-9 (basic medium without FSH)

After selection, follicles were individually cultured in 100 μL culture medium, in Petri dishes (60 mm × 15 mm, Corning, USA), under mineral oil for 18 days at 39 °C and 5% air CO_2. The basic culture medium consisted of α-MEM (pH 7.2–7.4), supplemented with 3 mg/mL bovine serum albumin (BSA), ITS (insulin 10 μg/mL, transferrin 5.5 μg/mL and selenium 5 ng/mL), 2 mM glutamine, 2 mM hypoxanthine and 50 μg/mL ascorbic acid without (control) or with GDF-9 at concentrations of 100 ng/mL (GDF-9 100) or 200 ng/mL (GDF-9 200). For each treatment, at least 50 follicles were used. Every 2 days, 60 μL of the α-MEM medium was replaced.

2.5. Experiment 3: caprine preantral follicle culture with GDF-9 (basic medium with FSH)

In this experiment, the basic culture medium was the same as that used in Experiment 2, except for the sequential addition of recombinant FSH: 100 ng/mL from day 0 (D0) to day 6 (D6), 500 ng/mL from D6 to day 12 (D12) and 1000 ng/mL from D12 to day 18 (D18) of culture. Total medium replacements were performed on days 6 and 12 of culture and partial replacements performed on the other days. The preantral follicles were randomly isolated and distributed between the treatments, i.e. the control FSH (only sequential FSH) or supplemented with 100 and 200 ng/mL of GDF-9 (GDF-9 (100) FSH and GDF-9 (200) FSH, respectively), and at least 45 follicles used in each treatment. The follicles were cultured for 18 days under the same conditions, as previously described for Experiment 2.

2.6. Morphological evaluation of follicular development

The ovarian follicles were classified as normal or degenerated, based on their morphological characteristics. The latter includes irregularities in the contour of the basal membrane, or the oocyte and darkening of the oocyte and the surrounding granulosa cells. On days 0, 6, 12 and 18, the normal follicles were measured using an ocular micrometer (100× magnification), attached to a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan). Two perpendicular measurements were performed. The daily increase in follicular diameter was calculated as follows: the final normal follicular diameter subtracted from the normal follicular diameter on day 0, divided by the number of culture days. Once a translucent cavity was visible between the granulosa cells, the antrum was considered to be formed.

2.7. In vitro maturation (IVM) of oocytes from preantral follicles grown in vitro

At the end of the culture period, the oocytes were recovered from the follicles, using 26 gauge needles. Oocytes were then washed twice in TCM199 medium, buffered with HEPES (TCM199-HEPES) and recorded to include the zona pellucida, as described for the follicles. It was important to note that only oocytes with a diameter of ≥110 μm were used for the maturation procedures. After that, the oocytes were cultured in TCM199, supplemented with 0.5 μg/mL FSH, 5 μg/mL LH, 1 μg/mL 17β-estradiol, 10 ng/mL recombinant Epidermal Growth Factor (EGF), 0.911 mmol/L pyruvate, 100 μmol/L cysteamine, 50 ng/mL recombinant Insulin-like Growth Factor I (IGF-I) and 1% BSA. The oocytes were grouped into the different treatment groups and cultured in 100 μL of medium on culture dishes (60 mm × 15 mm), under mineral oil for a period of 40 h in an incubator, at 39 °C, with 5% air CO_2.

2.8. Viability evaluation and oocyte chromatin configuration

A fluorescence microscopy was used to analyze the viability of the oocytes isolated from the caprine preantral follicles classified as morphologically normal under the stereomicroscope, after 18 days of culture. Briefly, the oocytes were incubated in 100 μL TCM-HEPES, supplemented with 4 μM calcein-AM, 2 μM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany), and 10 μM Hoechst 33342 (Sigma, Deisenhofen, Germany) at 37 °C, for 15 min. After incubation, the oocytes were washed three times with TCM-HEPES and evaluated under a fluorescence microscope (Nikon, Eclipse 80i, Tokyo, Japan). The oocytes with cytoplasm marked by calcein-AM (green) were considered to be viable and those that had chromatin marked with ethidium homodimer-1 (red) were considered to be degenerated (DEG). Hoechst was used to analyze the oocyte chromatin configuration by evaluation of the intact germinal vesicle (GV), germinal vesicle breakdown (GVBD) or metaphase II (MII) stage.

2.9. Statistical analysis

Data referring to the GDF-9 mRNA expression in primordial, primary and secondary follicles were analyzed using the Kruskal–Wallis test (SAS, 1999). The *t*-test was used for paired comparisons of RNA expression in the small and large antral follicles.

Data regarding the follicular survival, antrum formation and meiosis restart after in vitro culture were expressed as a percentage, and compared using the Chi-squared test. The data on the follicular diameter and growth rate after culture did not have the homogeneity of variance, and were thus compared using the non-parametric Kruskal–Wallis test (SAS, 1999). The results were expressed as mean values ± standard error of the mean (SEM). All differences were considered significant at a confidence level of $P < 0.05$.

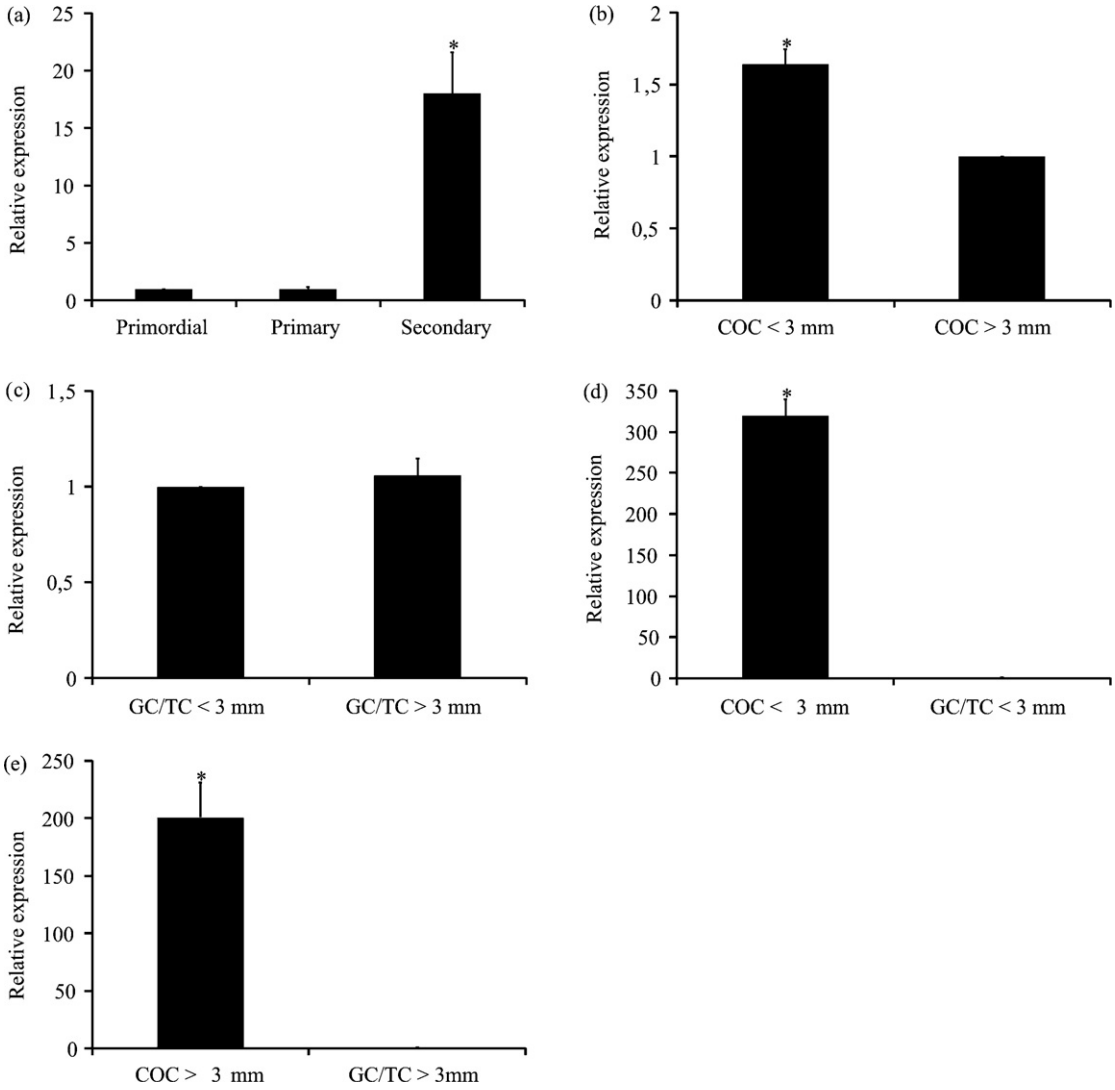

Fig. 1. Relative mRNA expression of GDF-9 in goat ovarian follicles (mean + SEM). (a) Primordial, primary and secondary follicles, (b) COC's from small and large antral follicles, (c) granulosa/theca cells of small and large antral follicles, (d) COC's and granulosa/theca cells of small antral follicles, and (e) COC's and granulosa/theca cells of large antral follicles. *Indicates that the differences between variants within a figure are significant, $P < 0.05$.

3. Results

3.1. Experiment 1: GDF-9 mRNA expression in caprine ovaries

Quantification of GDF-9 mRNA expression showed that the level of relative expression recorded in the secondary follicles, was significantly greater than those observed in the primordial and primary follicles ($P < 0.05$; Fig. 1a). GDF-9 mRNA expression in the COC's from small antral follicles was significantly greater than the expression observed in those recorded from large antral follicles ($P < 0.05$; Fig. 1b). The difference in expression of GDF-9 in the granulosa or theca cells was the small and not significant (Fig. 1c). The expression of mRNA in granulosa/theca cells from both small (Fig. 1d) and large (Fig. 1e) follicles was minimal and significantly lower ($P < 0.05$) than that occurring in the respective COC's.

3.2. Experiment 2: caprine preantral follicle culture with GDF-9 (basic medium without FSH)

3.2.1. Follicular and oocyte survival

Follicular viability declined progressively from day 0 and was significantly lower by day 12 for all treatments ($P < 0.05$; Fig. 2). Viability decreased at a slower rate for the GDF-9 (200) treatment than the control and GDF-9 (100), which recorded a significantly lower ($P < 0.05$) viability at day 0, compared to day 6 of culture. At day 18 the viability of follicles cultured on GDF-9 (200) was 80.8% and significantly higher than the control (60.8%) and GDF-9 (100) (66%).

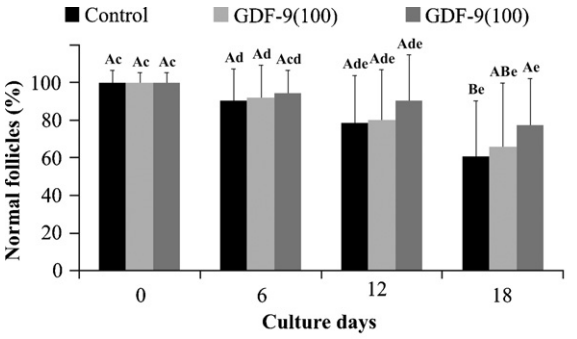

Fig. 2. Survival of goat isolated preantral follicles cultured in vitro (culture medium without FSH) for 18 days in the absence or presence of GDF-9 at concentrations of 100 ng/mL or 200 ng/mL.

Oocyte survival was similar for all treatments, with 98% of the control oocytes and 100% the GDF-9 (100) and the GDF-9 (200) oocytes marked with calcein-AM when visualized under a fluorescence microscope.

3.2.2. Antral cavity formation

The rate of antrum formation was significantly higher for GDF-9 (100) (78%) and GDF-9 (200) (62.3%) treatment than for the control (43.1%), on day 6 of culture. By day 12 of culture, the proportion of follicles achieving antrum formation were at or near the peak values recorded on day 18 (control 80.4%, GDF-9 (100) 92% and GDF-9 (200) 84.9% (NS; Table 1).

3.2.3. Follicular growth

Follicular diameter continued to increase until day 18 of culture reaching 483.9 μm for the control group, which was significantly lower ($P<0.05$) than the 564.3 μm recorded for the GDF-9 (100) and 542.7 μm for the GDF-9 (200) group (Table 2). These differences were reflected by the calculated daily growth rate which was significantly higher ($P<0.05$) at 18.4 ± 11.1 μm/d for the GDF-9 (100), compared to the control (14.3 ± 10.3 μm/d) and similar to that of the GDF-9 (200) group (17.1 ± 8.9 μm/d).

3.2.4. Ability to restart meiosis in oocytes grown in vitro with GDF-9

The proportion of COC's suitable for submission to IVM (≥110 μm) at day 18 of follicle culture was 78.0% for GDF-9 (100) and significantly higher ($P<0.05$) than for the control (58.8%) and GDF-9 (200) groups (48.1%). The rate of meiotic resumption (GVBD) in the control, GDF-9 (100) and GDF-9 (200) was, respectively, 41.4%, 35.9% and 36%, with the differences between treatments not being significant. The metaphase II stage was recorded in 3.3% of the control, 2.6% of the GDF-9 (100) and zero for the GDF-9 groups (200) (Fig. 3c and d).

Table 1
Percentage of antrum formation in goat preantral follicles goat strains cultured in vitro (culture medium without FSH), in the absence or presence of different concentrations of GDF-9 (100 ng/mL or 200 ng/mL) for 18 days.

Treatment	Day 0	Day 6	Day 12	Day 18
Control	0.0^{ae}	43.1^{bd}	78.4^{ac}	80.4^{ac}
GDF-9 (100)	0.0^{ad}	78.0^{ac}	92.0^{ac}	92.0^{ac}
GDF-9 (200)	0.0^{ae}	62.3^{ad}	83.0^{ac}	84.9^{ac}

Different superscripts (ab) indicate that differences between treatments within days are significantly different; $P<0.05$. Different superscripts (cde) indicate that differences between days within treatments are significantly different; $P<0.05$.

Table 2
Follicular diameter (μm) of preantral follicles isolated from goats with normal morphology after culture in vitro (culture medium without FSH), in the absence or presence of different concentrations of GDF-9 (100 ng/mL or 200 ng/mL) for 18 days.

Treatment	Day 0	Day 6	Day 12	Day 18
Control	205.2^{ae}	315.9^{ad}	442.4^{bc}	483.9^{bc}
GDF-9 (100)	224.7^{af}	333.9^{ae}	487.7^{ad}	564.3^{ac}
GDF-9 (200)	217.6^{ade}	335.8^{ad}	477.8^{adc}	542.7^{abc}

Different superscripts (ab) indicate that differences between treatments within days are significantly different; $P<0.05$. Different superscripts (cdef) indicate that differences between days within treatments are significantly different; $P<0.05$.

3.3. Experiment 3: caprine preantral follicle culture with GDF-9 (basic medium with FSH)

3.3.1. Follicular and oocyte survival

The proportion of normal follicles decreased continuously in all treatments until day 18 of culture, reaching values of 57.8% for the control FSH, 54.4% for the GDF-9 (100) FSH and 66% for the GDF-9 (200) FSH (NS) treatment groups (Fig. 4).

Oocyte survival, as indicated by calcein-AM staining, was 100% for the control, 88.5% for GDF-9 (100) FSH and 93.3% for GDF-9 (200) FSH (NS) treatments.

3.3.2. Antral cavity formation

Antrum formation was rapid up to day 6 of culture in all treatments (Table 3). Thereafter, development slowed and reached a peak, or near peak value by day 12 in all treatments. The values for antrum formation were 82.2% in the control, 74% for the GDF-9 (100) FSH and 74.5% for the GDF-9 (200) treatments at day 18 of culture. None of the differences between treatments within days of culture were significant.

3.3.3. Follicular growth

Follicular diameter increased steadily in all treatments, reaching a peak value of 534 μm for GDF-9 (100) FSH on day 12 of culture and peak values for the control FSH (478.6 μm) and GDF-9 (200) FSH (554,1 μm) on day 18 of culture

Table 3
Percentage of antrum formation in goat preantral follicles cultured in vitro (culture medium with FSH), in the absence or presence of different concentrations of GDF-9 (100 ng/mL or 200 ng/mL), for 18 days.

Treatment	Day 0	Day 6	Day 12	Day 18
Control	0.0^{c}	53.3^{b}	80.0^{a}	82.2^{a}
GDF-9 (100)	0.0^{c}	67.4^{a}	73.9^{a}	73.9^{a}
GDF-9 (200)	0.0^{c}	61.7^{a}	74.5^{a}	74.5^{a}

Different superscripts (abc) indicate that differences between days within treatments are significantly different; $P<0.05$.

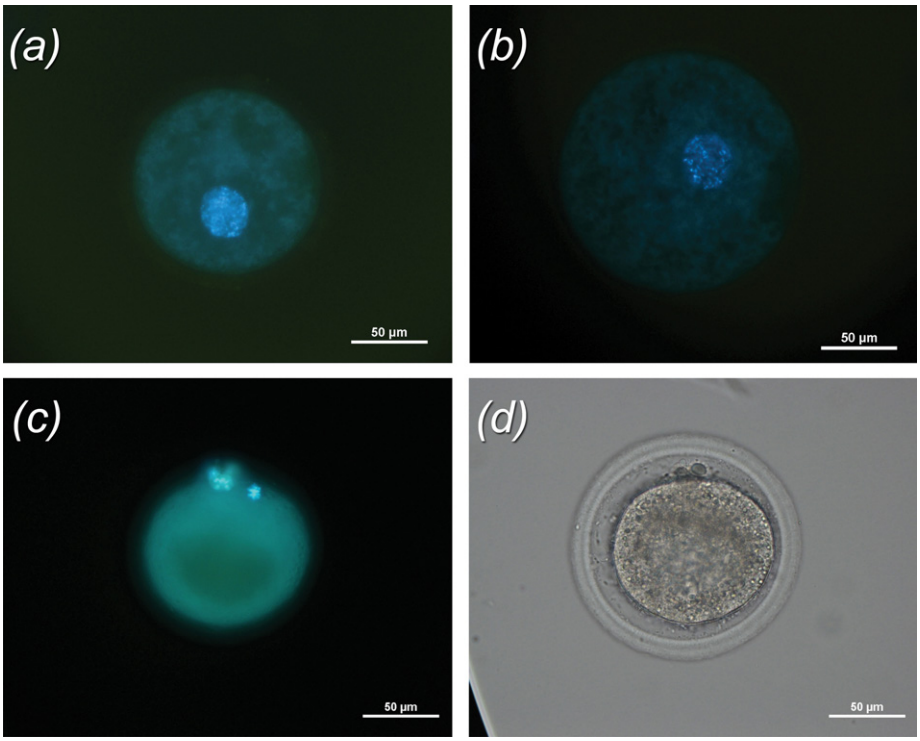

Fig. 3. Oocyte showing intact germinal vesicle (GV) (a), germinal vesicle breakdown (GVBD) (b) and metaphase II (MII) (c) after staining with Hoechst 33342, bright field image of MII (d) (400× magnification).

(Table 4). No significant differences were recorded between treatments within any day of culture. The daily growth rates achieved in the control (14.2 ± 8.2 μm/d) and GDF-9 (200) FSH (13.0 ± 9.7 μm/d) groups were significantly higher than for the GDF-9 (100) FSH (7.1 ± 10.6 μm/d) treatment.

3.3.4. *Ability to restart meiosis in oocytes grown in vitro with GDF-9*

The percentage of oocytes deemed suitable for IVM (≥110 μm) in the control FSH (53.3%), GDF-9 (100) FSH (56.5%) and GDF-9 (200) FSH (63.8%) did not differ among treatments. Similar results were observed for oocytes exhibiting GVBD (control FSH (75%), GDF-9 (100) FSH (60.9%) and GDF-9 (200) FSH (60.6%)).

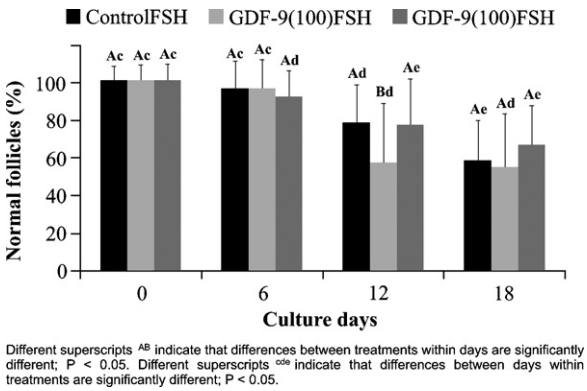

Fig. 4. Survival of goat isolated preantral follicles cultured in vitro (culture medium with FSH) for 18 days, in the absence or presence of GDF-9 at concentrations of 100 ng/mL or 200 ng/mL.

Table 4
Follicular diameter (μm) of preantral follicles isolated from goats with normal morphology after culture in vitro (culture medium with FSH), in the absence or presence of different concentrations of GDF-9 (100 ng/mL or 200 ng/mL) for 18 days.

Treatment	Day 0	Day 6	Day 12	Day 18
Control	215.8^{c}	327.5^{b}	427.7^{a}	478.6^{a}
GDF-9 (100)	232.2^{c}	361.0^{b}	534.0^{a}	528.6^{a}
GDF-9 (200)	225.5^{d}	330.8^{c}	474.9^{b}	554.1^{a}

Different superscripts (abcd) indicate that differences between days within treatments are significantly different; $P < 0.05$.

4. Discussion

This study quantified GDF-9 mRNA and its effect on the in vitro culture of caprine preantral follicles. The mRNA quantification revealed an increase in the GDF-9 mRNA expression, as follicular growth progressed, during the preantral phase. Silva et al. (2004) showed the presence of mRNA and the GDF-9 ligand and receptor proteins in all of the categories of the caprine ovarian follicles. However, it was not clear if the expression pattern of this factor varied during follicular development. In this study, secondary follicles showed an elevated GDF-9 mRNA expression level, when compared to the primordial and primary follicles.

Based on these findings, it was decided to test the effect of GDF-9 on secondary follicles, and the importance was confirmed via in vitro follicular development. It was observed that the COC's expressed significantly more mRNA, than did the granulosa/theca cells. This expression profile may be related to the oocyte contribution, which is the main source of GDF-9 in the COC's. Prochazka et al. (2004) observed that prepubertal gilt oocytes within growing follicles express four times more GDF-9 mRNA than the respective mural theca and granulosa cells. Using hybridization techniques, the presence of GDF-9 mRNA was exclusively confirmed in oocytes for all of the follicular stages in humans, except in the primordial follicles (McGrath et al., 1995).

Previous work performed by the group showed that GDF-9, at a concentration of 200 ng/mL, stimulated caprine primordial follicle activation and its progression to the secondary follicle stage, during in situ culture for 7 days (Martins et al., 2008). The current work here has demonstrated for the first time, the influence of GDF-9 on the in vitro development of isolated caprine secondary follicles during long-term culture (18 days). GDF-9, at a concentration of 100 ng/mL and without FSH, favored follicular survival, compared to the control group (Experiment 2). In humans, during the in vitro culture of preantral follicles enclosed in ovarian tissue, GDF-9 promoted follicular viability for 14 days and the progression from primordial and primary follicles, to the secondary stage (Hreinsson et al., 2002).

When FSH was not added to the basic culture medium (Experiment 2), the addition of GDF-9 (100 ng/mL) effectively stimulated follicular growth. However, although a positive effect of GDF-9 on follicular growth was recorded, it did not affect the percentage of oocytes that were $\geq$110 μm in diameter and destined for IVM. Thus it did not influence the oocyte growth rate. This fact suggests that GDF-9 predominantly promoted follicular growth, by stimulating granulosa cell proliferation. In fact, GDF-9 stimulates granulosa cell proliferation and the differentiation in preantral follicles and small antral follicles of immature rats (Vitt et al., 2000) and humans (Hreinsson et al., 2002) – which may explain the results achieved.

In addition, the increase in GDF-9 mRNA expression in the secondary follicles observed in this study, confirms the importance of this factor for caprine ovarian follicular development. Other research has highlighted the importance of GDF-9 regarding follicular growth, as this factor stimulated the growth of the primordial follicles in hamsters (Wang. and Roy, 2004), granulosa cell proliferation in immature rats (Vitt et al., 2000) and secondary follicular growth (130–150 μm) in rats (Hayashi et al., 1999).

In Experiment 2 (basic medium without FSH), no differences were recorded between the treatments in antrum formation. Similar results were recorded in the culture of caprine preantral follicles in a medium without FSH – which demonstrates that FSH is not essential for antrum formation during the in vitro culture of secondary follicles (Saraiva et al., 2010).

The GDF-9 positive effects (Experiment 2) on follicular survival and development were not recorded, when FSH was added (Experiment 3). Hayashi et al. (1999) recorded that GDF-9 and FSH, alone or in combination, favored isolated preantral follicles of immature rats. It has been confirmed that FSH may act on preantral follicular growth in goats (Matos et al., 2007; Magalhães et al., 2009), sheep (Campbell et al., 2004), cows (Gutierrez et al., 2000; Itoh et al., 2002) and mice (Cortvrindt et al., 1997). Thus, FSH is an important survival and follicular growth factor that stimulates the production of the different intra-ovarian growth factors (Thomas et al., 2005). Hence, a possible explanation for the absence of any significant effect of GDF-9 in the presence of FSH, is that this gonadotrophin may induce the expression of intra-ovarian factors – inhibiting any additional effects of GDF-9. However, FSH may also inhibit GDF-9, by regulating the expression of specific receptors (BMPRII and ALK-5) as was observed in ovine isolated granulosa cell culture (Qin Chen et al., 2009).

In caprine species, the production of fully grown oocytes from preantral follicle in vitro culture capable of restarting meiosis is a rare event (Silva et al., 2010). In the current research, oocytes capable of resuming meiosis and completing in vitro nuclear maturation (metaphase II), were obtained for the first time. Although this is progress in caprine preantral follicle culture, the IVM rates recorded were minimal and not affected by the treatments.

Based on these results, GDF-9 is essential for the regulation of caprine follicular development. Its respective mRNA is expressed during all the follicular stages and it also has a positive effect on follicular survival, precocity of antrum formation and production of oocytes, capable of restarting meiosis. Future studies are necessary to improve the maturation rates of oocytes from in vitro grown caprine preantral follicles.

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