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Growth and differentiation factor – 9 (GDF-9) increases the in vitro growth rates of isolated goat early antral follicles

Fator de crescimento e diferenciação – 9 (GDF-9) aumenta a taxa de crescimento in vitro de folículos antrais iniciais caprinos isolados

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Abstract
This study aimed to investigate the effect of growth and differentiation factor 9 (GDF-9) during the *in vitro* culture of isolated caprine early antral follicles. The isolated and selected early antral follicles were individually cultured for 18 days, and the following treatments were tested: α-MEM$^+$ (control treatment) or α-MEM$^+$ supplemented with 200 ng/mL GDF-9. The following endpoints were evaluated: follicular growth and morphology, estradiol production, oocyte nuclear maturation, and relative expression of key genes related to steroidogenesis (*CYP19A1*, *CYP17*, and *insulin receptor*) and basement membrane remodeling (*MMP-9* and *TIMP-2*). In both treatments, a decrease was observed in the percentage of morphologically intact follicles with a concomitant increase in the rates of extruded and degenerated follicles ($P < 0.05$). The GDF-9 treatment showed higher rates of extruded follicles only on day 6 of culture ($P < 0.05$). Follicle diameter increased progressively throughout the culture period ($P < 0.05$) with similar diameters between treatments at all culture times ($P > 0.05$). Growth and differentiation factor 9 increased the daily growth rate from the first to the second third of culture, with higher values ($P < 0.05$) than control in the second third. Oocyte maturation rate as well as estradiol levels and relative mRNA expression for *CYP19A1*, *CYP17*, *MMP-9*, *TIMP-2*, and *insulin receptor* genes were similar between treatments ($P > 0.05$). This study shows for the first time that GDF-9 added to a culture medium increased the follicle growth rate of goat early antral follicles cultured *in vitro*.
Keywords: *in vitro* culture; antral follicle; goat; GDF-9

Resumo
Este estudo teve como objetivo investigar o efeito do GDF-9 durante o cultivo *in vitro* de folículos antrais iniciais caprinos isolados. Os folículos antrais iniciais isolados e selecionados foram cultivados individualmente por 18 dias, e os seguintes tratamentos foram testados: α MEM+ (tratamento controle) ou α-MEM+ suplementado com 200 ng/mL de GDF-9 (tratamento GDF-9). Os seguintes parâmetros foram avaliados: crescimento e morfologia folicular, produção de estradiol, maturação nuclear do oócito e expressão relativa de genes-chave relacionados a esteroidogênese (*CYP19A1*, *CYP17* e *receptor de insulina*) e remodelamento da membrana basal (*MMP-9* e *TIMP-2*). Em ambos os tratamentos, observou-se diminuição na porcentagem de folículos morfologicamente intactos com aumento concomitante nas taxas de folículos extrusos e degenerados ($P < 0,05$). O tratamento GDF-9 apresentou maiores taxas de folículos extrusos apenas no 6º dia de cultivo ($P < 0,05$). O diâmetro do folículo aumentou progressivamente ao longo do período de cultivo ($P < 0,05$) com diâmetros semelhantes entre os tratamentos em todos os tempos de cultivo ($P > 0,05$). O GDF-9 aumentou a taxa de crescimento diário do primeiro para o segundo terço de cultivo, sendo maior ($P < 0,05$) que o controle no segundo terço. A taxa de maturação oocitária assim como os níveis de estradiol e a expressão relativa de RNAm para os genes *CYP19A1*, *CYP17*, *MMP-9*, *TIMP-2* e *receptor de insulina* foram similares entre os tratamentos ($P > 0,05$). Em conclusão, este estudo mostra pela primeira vez que GDF-9 adicionado a um meio de cultivo aumentou a taxa de crescimento de folículos antrais iniciais caprinos cultivados *in vitro*.
Palavras-chave: cultivo *in vitro*; folículo antral; cabra; GDF-9

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1. Introduction

The *in vitro* follicle culture (IVFC) biotechnology aims to recover and culture *in vitro* ovarian follicles at different developmental stages to use them in assisted reproductive technologies. During IVFC several antioxidants and growth factors such as anethole and growth differentiation factor-9 (GDF-9), respectively, are known to have a key role in *in vitro* folliculogenesis when added to the culture media. Anethole or *trans*-anethole (1-Methoxy-4[1-propenyl] benzene) is a naturally occurring phenylpropanoid with several related properties, e.g. antioxidant, that can be obtained from various plant species[1]. Our group has recently reported promising results with the addition of anethole during the *in vitro* culture of caprine secondary follicles, which provided higher rates of oocyte meiotic resumption and lower reactive oxygen species (ROS) concentration when compared to other antioxidants, such as ascorbic acid[1]. Moreover, we described for the first time a pregnancy after the *in vitro* culture of caprine early antral follicles[2].

Another additive that has received growing attention for its use in IVFC is GDF-9, an oocyte-derived member of the transforming growth factor-beta (TGF-β) superfamily[3] that is present at all the stages of folliculogenesis *in vivo*[4]. *In vitro*, GDF-9 maintained survival, stimulated both oocyte and follicle growth, and promoted primordial follicle activation when the follicles were cultured *in situ*, i.e., within fragments of ovarian tissue of woman[5] and goat[6]. When preantral follicles were cultured in the isolated form, GDF-9 stimulated the growth of follicles and oocytes in mice[7]. Accordingly, our group reported the beneficial effects of adding 200 ng/mL GDF-9 to a medium containing ascorbic acid on the survival of goat isolated preantral follicles cultured *in vitro*[8]. Nevertheless, to the best of our knowledge, there is no information on the influence of GDF-9 on the *in vitro* culture of isolated early antral follicles in mammals.

Therefore, the present study aimed to investigate the effect of GDF-9 during the *in vitro* culture of isolated caprine early antral follicles. The following endpoints were evaluated: (1) follicular growth and morphology, (2) estradiol production, (3) oocyte nuclear maturation, and (4) relative expression of genes related to steroidogenesis and basement membrane remodeling.

2. Material and methods

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The study was previously approved by the Ethics Committee for Animal Use of the State University of Ceará, Fortaleza, CE, Brazil (approval no. 2422377/2016).

2.1 Chemicals and media

The reagents and chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.2 Collection of ovaries and selection of early antral follicles

Ovaries (n = 60) of 30 adult goats of mixed breed were collected at a local slaughterhouse. Immediately after slaughter, the ovaries were washed in alcohol (70%), followed by two washes in minimum essential medium (MEM) supplemented with penicillin (100 µg/mL), streptomycin (100 µg/mL), and HEPES (25 mM). The ovaries were transported to the laboratory in MEM within 1 h at 4 °C[9]. In the laboratory, fat, and connective tissue surrounding the ovaries were removed. Cortical slices (1 to 2 mm thick) were obtained with a surgical blade (under sterile conditions) and placed in a holding medium consisting of HEPES-buffered MEM supplemented with antibiotics (100 mg/mL penicillin and 100 mg/mL streptomycin). Early antral follicles approximately 300-350 µm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from strips of ovarian cortex using 26-gauge (26 G) needles. Only those morphologically normal were selected for *in vitro* culture[10].

2.3 In vitro culture conditions and media

The selected follicles were individually cultured in 100 µL drops of α-MEM (M5650, pH 7.2-7.4) supplemented with 3 mg/mL bovine serum albumin (BSA), 10 ng/mL insulin, 5.5 µg/ml transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine, 0.911 mM pyruvate, 50 ng/mL growth hormone (GH) and 300 µg/mL anethole, referred to as α-MEM^{+}[2]. The anethole concentration (300 µg/mL) was chosen based on a previous study performed by our group[1]. The following treatments were tested: α-MEM^{+} alone (Control treatment) or α-MEM^{+} supplemented with 200 ng/mL GDF-9 (GDF-9 treatment) for 18 days at 38.5 °C and 7.5% CO_2 under mineral oil. Fresh medium was prepared before use and pre-equilibrated overnight, with 60 µL medium being replaced in each drop every two days[2]. The experiment was replicated five times using an average of 12 ovaries per replicate, and approximately 65 follicles were used per treatment.

2.4 Assessment of follicular development

Follicle morphology and growth were evaluated every six days (Days 6, 12, and 18). During and after culture, follicles were classified into three types according to their morphological characteristics: extruded follicles were characterized by having a ruptured basement membrane; degenerated follicles exhibited an irregular contour, a darkened oocyte and/or granulosa cells, and a

reduced diameter; and intact follicles were characterized as translucent with an intact basement membrane and surrounded by homogeneous and bright granulosa cells with no signs of degeneration or extrusion[11]. The follicular growth rate in each culture interval (Days 0-6, 6-12, and 12-18) was calculated by considering the diameter of the period in which the follicles remained morphologically intact, that is, the final diameter minus the initial diameter of the considered period, divided by the number of culture days (six days). Follicular diameter was measured only in intact follicles and calculated from the basement membrane using the mean of two perpendicular measures of each follicle by an ocular micrometer (100X magnification) inserted into a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) every six days of culture (days 0, 6, 12, and 18 of culture).

2.5 In vitro maturation (IVM)

At the end of culture, all healthy follicles were carefully and mechanically opened. Cumulus-oocyte complexes (COC) containing oocytes with homogeneous cytoplasm surrounded by at least one compact layer of cumulus cells were selected for IVM[11]. The IVM medium consisted of tissue culture medium 199 (TCM 199) supplemented with 1 µg/mL 17β-estradiol, 5 µg/mL luteinizing hormone (LH), 0.5 µg/mL rFSH, 10 ng/mL epidermal growth factor (EGF), 1 mg/mL BSA, 22 µg/mL pyruvate, 50 ng/mL insulin-like growth factor 1 (IGF-I), and 100 µM cysteamine (2). The COC were matured individually at 38.5 °C in 7.5% CO_2 for 32 h[11]. Fresh medium and mineral oil were prepared before use and pre-equilibrated overnight.

2.6 Assessment of oocyte viability and chromatin configuration

After IVM, COC were mechanically denuded and incubated individually in 10 µL drops of PBS supplemented with 5.5 µg/mL Hoechst 33342 and 1% glutaraldehyde at room temperature for 30 min, and processed as described previously[10]. After incubation, oocytes were washed three times in PBS and mounted with mounting medium containing 5 µg/mL Hoechst 33342. Oocyte viability and chromatin configuration were evaluated under a fluorescence microscope (Nikon, Eclipse 80i, Tokyo, Japan). According to the chromatin configuration, oocytes were classified as germinal vesicle (GV), metaphase I (MI), metaphase II (MII), or degenerated (DEG).

2.7 Follicular wall RNA extraction and real-time PCR (RT-qPCR)

The samples were processed as described previously[12]. Three pools of 10 follicular walls (granulosa and theca cells) from early antral follicles were collected from each treatment after the culture period for total RNA isolation with the Trizol® reagent method (Invitrogen, Carlsbad, CA, USA). Purification of the total RNA was performed with PureLinkTM RNA Mini Kit (Ambion®, Carlsbad, CA, USA) according to the recommendations of the manufacturer. After the extraction, the RNA concentration was determined using the NanoDrop System (Thermo Scientific NanoDrop Products), performed with 2 µL of material. Before the cDNA synthesis, all samples were standardized with the same amount of RNA to minimize qPCR variability. For cDNA synthesis, the instructions of the Superscript III RT-PCR manual (Invitrogen, Carlsbad, CA, USA) were followed using random primers (Invitrogen, Carlsbad, CA, USA) from 1 ng of total RNA.

Primers were designed to perform the mRNA amplification of aromatase (*CYP19A1*), cytochrome P450 17α-hydroxylase/17,20-lyase (*CYP17*), matrix metalloproteinase-9 (*MMP-9*), tissue inhibitor of metalloproteinases-2 (*TIMP-2*), and *insulin receptor* (Table 1). As endogenous controls, two candidate reference genes, glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) and peptidylprolyl isomerase A (*PPIA*), were selected to study expression and stability and to normalize gene expression in all samples. Reactions were performed using an IQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Detection of PCR products was measured by monitoring the increase in fluorescence emitted by the marker Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). For all amplifications, one dissociation curve (melting curve) was constructed for the verification of unspecific amplifications arising from contamination. The qPCR thermal cycle was as follows: initial denaturation and activation of the polymerase 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. The final extension was for 10 min at 72 °C. Transcripts of target genes were quantified from the difference of the Cq values (threshold cycle PCR) in relation to transcripts of the endogenous gene, *PPIA*. First, the average Cqs of the three readings for each sample, both the target gene and the endogenous gene, was determined. From each sample, the subtraction of the mean value of the Cqgene-target to Cqgene-endogene provided ΔCq. Subsequently, one ΔCq corresponding to a calibrator was chosen, normalizing all values by subtracting the resulting ΔCq chosen, to obtain the ΔΔCq. Finally, the final value of relative quantification was given by $2^{\Delta\Delta Cq}$, where the calibrator or standard sample chosen was equal to one[13].

2.8 Estradiol assay

The potential relationship between follicular development and estradiol production in early antral follicles was investigated using the spent culture medium collected on day 18 of *in vitro* culture from each treatment and stored at –80 °C until assay. Estradiol levels were measured only in spent culture medium from where

follicles produced oocytes that resumed meiosis after *in vitro* maturation[14]. An enzyme-linked fluorescent assay (ELFA) (Vidas® Estradiol II – 30431) and an automatic analyzer (miniVIDAS®, bioMérieux SA, Lyon, France) were used to determine the estradiol concentrations. The intra-assay coefficient of variation was 5%.

Table 1. Oligonucleotide primers used for PCR analysis of goat follicles

Target gene	Primer sequence (5'→3')	Sense/anti-sense *	GenBank accession no.
Insulin receptor	ATGCCCTGGTGTCACTTTCCTTCT	S	XM_012177947.3 (*Ovis aries*)
	TTAGGTTCTGGTTGTCCAAGGCGT	AS	
CYP19A1	CGGCATGCATGAGAAAGGCATCAT	S	NM_001285747.1 (*Capra hircus*)
	ACACGTCCACATAGCCCAAGTCAT	AS	
CYP17	ACTGAATGCCTTTGCCCTGT	S	NM_001314145.1 (*Capra hircus*)
	CTGATTATGTTGGTGATCC	AS	
MMP9	TTTCCTCCTGGCTCAGGCATTCA	S	NM_001314269.1 (*Capra hircus*)
	GTTTCCGAAGTAGGTCGGGATCACA	AS	
TIMP2	AGAAGAAGAGCCTGAACCACAGGT	S	XM_018063674.1 (*Capra hircus*)
	TGATGTTCTTCTCCGTGACCCAGT	AS	
GAPDH	ATGCCTCCTGCACCACCA	S	XM_027541122.1 (*Ovis aries*)
	AGTCCCTCCACGATGCCAA	AS	
PPIA	TCATTTGCACTGCCAAGACTG	S	XM_018047035.1 (*Capra hircus*)
	TCATGCCCTCTTTCACTTTGC	AS	

S, Sense; AS, Anti-sense. Source: Data from the research itself.

2.9 Statistical analyses

Statistical analyses were carried out using Sigma Plot 11 (Systat Software Inc., USA). Normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) were previously evaluated. A comparison of means was analyzed using one-way ANOVA followed by the Student-Newman-Keuls posthoc test. The proportional variables (intact, degenerated, extruded, and meiotic resumption) were analyzed across treatments and days of culture by chi-square or G-test. Differences were considered significant at $P < 0.05$, and values are presented as mean (± standard error of the mean, SEM) or percentages.

3. Results and discussion

In both treatments, a decrease was observed in the percentage of morphologically intact follicles (Fig. 1A) with a concomitant increase in the rates of extruded (Fig. 1B) and degenerated follicles (Fig. 1C) ($P < 0.05$). Additionally, the treatment with GDF-9 did not affect the percentage of intact follicles compared to the control treatment at the different culture intervals evaluated ($P > 0.05$). In a recent study, the addition of GDF-9 at the same concentration as that used in this study also did not affect the viability of preantral follicles included in cultured ovarian fragments[15]. Moreover, supplementation of GDF-9 during *in vitro* culture of goat preantral follicles plus physiological concentrations of insulin (10 ng/mL) equally contained in the medium seems to have been unnecessary for follicle development[16]. The result obtained in this study is similar to those of previous works in which the addition of insulin (10 ng/mL) alone proved capable of maintaining the viability of isolated antral follicles[17,18]. This suggests that the maintenance of antral follicle viability during *in vitro* culture is not only dependent on specific factors such as GDF-9, but it is also related to the richness of the composition of the medium as a whole including insulin, transferrin, selenium, and anethole supplementation. In fact, anethole is a promising antioxidant, and recent studies show that anethole increased the percentage of meiotic resumption and mature oocytes after *in vitro* culture of isolated goat late preantral follicles[1]. Moreover, anethole was able to increase the *in vitro* embryo production after *in vitro* fertilization of caprine oocytes from *in vitro* culture of early antral follicles[2]. Compared to the control treatment, the GDF-9 treatment showed higher rates of extruded follicles only on day six of culture ($P < 0.05$).

The preservation of follicular wall during follicle growth depends on a fine balance between factors involved in basement membrane remodeling, e.g., matrix metalloproteinase-9 (*MMP-2* and *MMP-9*) and tissue inhibitor of metalloproteinases-2 (*TIMP-1* and *TIMP-2*)[19]. Even though the GDF-9 treatment showed higher rates of extruded follicles on day six of culture, at the end of the culture period (day 18), follicle extrusion rates were similar ($P > 0.05$) between the treatments. These results agree with the gene expression analysis performed on day 18 of culture that showed equivalent ($P > 0.05$) levels of mRNA for *MMP-9* and *TIMP-2* between the treatments (figure 2). Follicle diameter increased progressively throughout the culture period ($P < 0.05$), with similar diameters between treatments at all culture intervals ($P > 0.05$) as shown in Table 2.

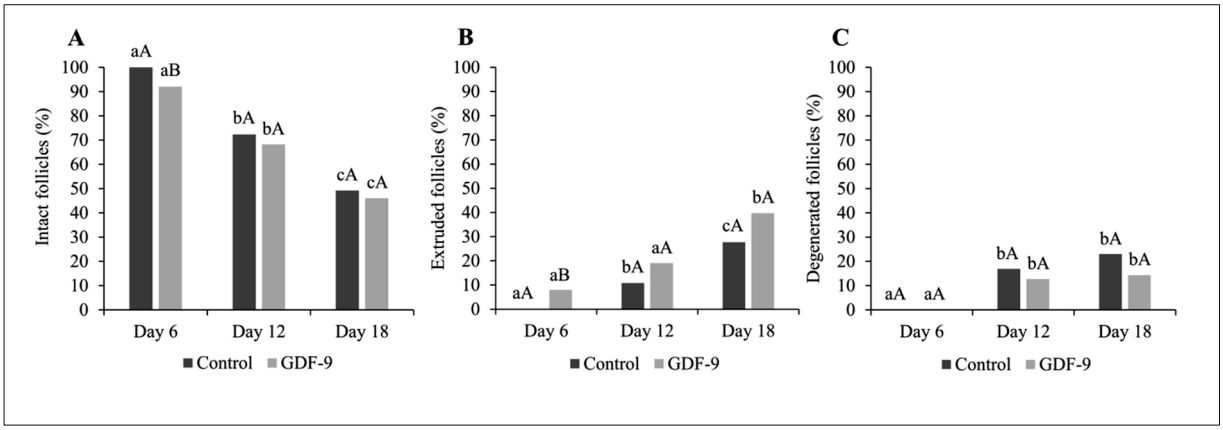

Figure 1. Percentage of morphologically (A) intact, (B) extruded, and (C) degenerated goat early antral follicles on days 6, 12, and 18 of culture in the absence (control treatment) or presence of GDF-9 (GDF-9 treatment). [a–c] Values without a common letter indicate a difference between days within the same treatment ($P < 0.05$). [A,B] Values without a common letter indicate a difference between treatments ($P < 0.05$).

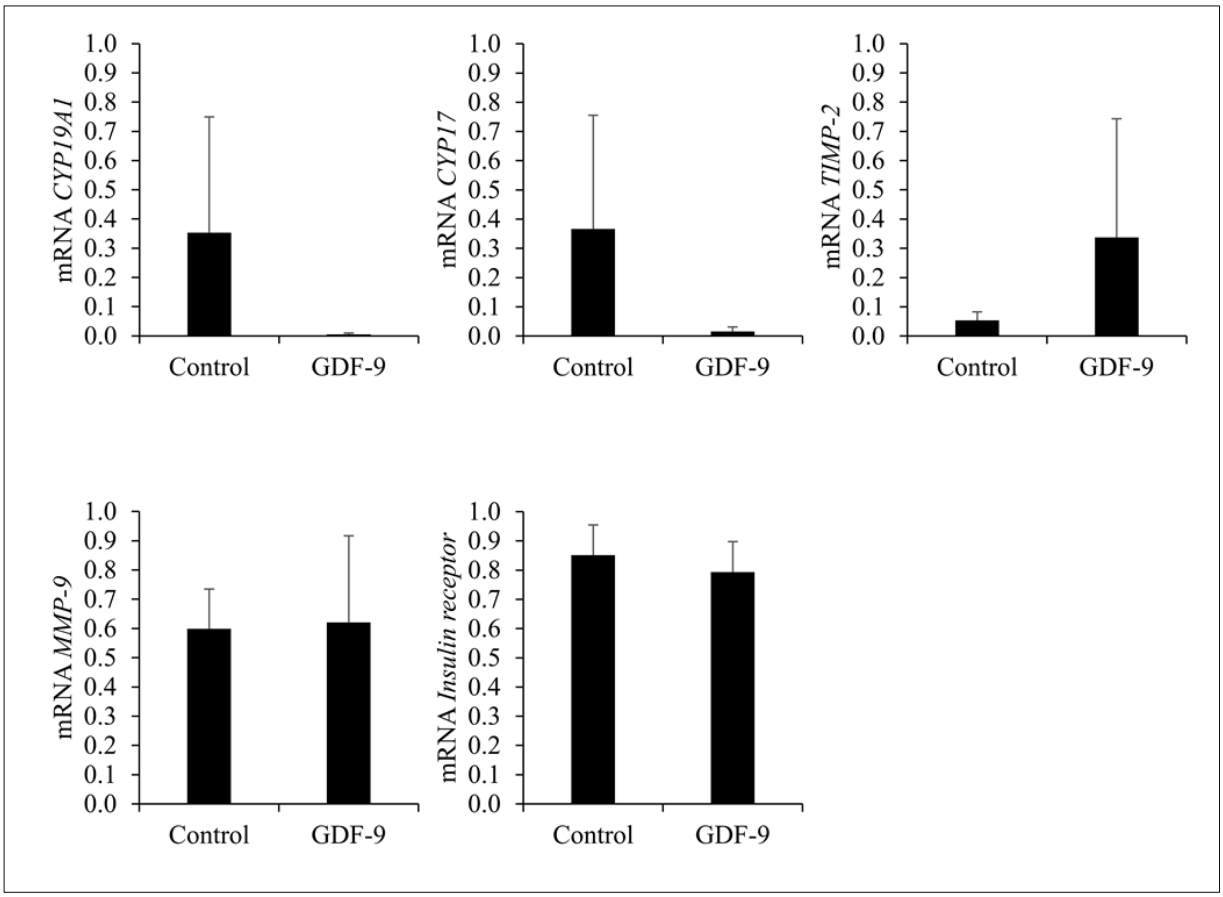

Figure 2. Mean (± SEM) relative mRNA expression of *CYP19A1*, *CYP17*, *TIMP-2*, *MMP-9*, and *insulin receptor* in goat early antral follicles cultured for 18 days in the absence (control treatment) or presence of GDF-9 (GDF-9 treatment). Between the treatments ($P > 0.05$).

Table 2. Mean (± SEM) follicular diameter (µm) of caprine early antral follicles cultured in the absence (control treatment) or presence of GDF-9 (GDF-9 treatment)

Treatment (n)	Follicular diameter (µm)			
	Day 0	Day 6	Day 12	Day 18
Control (63)	328.2 ± 3.7^{aA}	384.4 ± 8.3^{bA}	425.8 ± 11.6^{cA}	477.6 ± 16.8^{dA}
GDF-9 (65)	323.4 ± 2.8^{aA}	365.9 ± 5.9^{bA}	436.4 ± 10.1^{cA}	474.0 ± 15.4^{dA}

Abbreviations: SEM, standard error of the mean. $^{a-d}$ Different letters denote significant differences between culture periods within the same treatment ($P < 0.05$). Source: Data from the research itself.

On the other hand, GDF-9 increased the daily growth rate from the first to the second third of culture, with higher values ($P < 0.05$) in the second third compared to control (Table 3). However, this difference dissipated in the last third of culture (days 12-18). Unlike ours, a previous study showed that GDF-9 promoted the *in vitro* growth of human preantral follicles enclosed in ovarian tissue by stimulating the proliferation and differentiation of granulosa cells[5]. Differences between the culture system (isolated vs. *in situ*) and follicle stages (preantral vs. early antral) may explain these results. Additionally, GDF-9's effect of increasing the growth rate of antral follicles from the first to the second third of *in vitro* culture may be associated with the mitogenic effect of this substance on granulosa cells. A previous study showed that GDF-9 stimulated the progression from G0 and G1 phases to the S phase, and from the S phase to M phase of the cell cycle of human luteinized granulosa cells cultured *in vitro*[20]. A possible answer for the transient effect of GDF-9 for this parameter may be linked to the transcriptional profile of granulosa cells and oocyte. Although they exhibit BMPR-II receptors at all follicular stages[4], GDF-9 seems to be more required in the early phases of follicular development and during ovulation and oocyte maturation events, in goat species.

Table 3. Mean (± SEM) of follicular growth rates in each culture interval (µm/day) and estradiol concentration (pg/mL) of caprine early antral follicles cultured in the absence (control treatment) or presence of GDF-9 (GDF-9 treatment)

Treatment (n)	Mean follicular growth rates in each culture interval (µm)			E2 (pg/mL)
	Days 0-6	Days 6-12	Days 12-18	Day 18
Control (65)	9.4 ± 1.0^{aA}	7.0 ± 0.8^{aA}	6.7 ± 1.0^{aA}	146.4 ± 32.2^{A}
GDF-9 (63)	7.2 ± 0.7^{aA}	11.6 ± 1.0^{bB}	7.2 ± 1.1^{aA}	146.8 ± 54.8^{A}

Abbreviations: SEM, standard error of the mean. ^{A,B} Different letters within a column denote significant differences between treatments at the same time point ($P < 0.05$). ^{a,b} Different letters denote significant differences between culture periods within the same treatment ($P < 0.05$). Source: Data from the research itself.

All other evaluated parameters on day 18 were comparable between the two treatments, i.e., estradiol production (Table 3), mRNA levels for *Insulin receptor*, *CYP19A1*, *CYP17* (Figure 2), oocyte survival, growth, and maturation rates ($P > 0.05$) the last ones, shown in table 4. The mRNA expression for key genes relatated to steroidogenesis as well as the production of estradiol[21] showed that both control and GDF-9 treatments provided appropriate conditions for *in vitro* follicle development.

Table 4. Mean (± SEM) oocyte diameter and percentages of degenerated oocytes, germinal vesicle, meiotic resumption, metaphase I, and metaphase II of goat early antral follicles cultured during 18 days in the absence (control treatment) or presence of GDF-9 (GDF-9 treatment)

Treatment (n)	Oocyte diameter (µm)	DEG (%)	GV (%)	Meiotic resumption (%)	M I (%)	M II (%)
Control (65)	113.8 ± 2.3^{A}	49.23 (32/65) ^{A}	13.85 (9/65) ^{A}	36.92 (24/65) ^{A}	9.23 (6/65) ^{A}	23.08 (15/65) ^{A}
GDF-9 (63)	108.5 ± 2.2^{A}	38.10 (24/63) ^{A}	19.05 (12/63) ^{A}	42.86 (27/63) ^{A}	19.05 (12/63) ^{A}	19.05 (12/63) ^{A}

Abbreviations: SEM, standard error of the mean; GV, germinal vesicle; DEG, degenerate oocyte; MI, metaphase I; and MII, metaphase II. Source: Data from the research itself.

In the present study, the addition of GDF-9 stimulated follicle growth during culture but did not affect any other studied endpoints. A possible explanation for these results is the high quality of the control medium used in the present study, which contained anethole, among other supplements. In fact, anethole added during IVFC of caprine early antral follicles had previously been shown to increase follicle growth, estradiol production, oocyte growth, maturation, and *in vitro* embryo production[2].

4. Conclusion

This study shows for the first time that GDF-9 added to a culture medium containing anethole increased the follicle growth rate of goat isolated early follicles cultured *in vitro*. Nonetheless, it had no effect on either estradiol production or oocyte *in vitro* maturation. Considering the physiological importance of GDF-9 during activation and subsequent follicular development *in vivo*, future studies investigating the effect of GDF-9 addition during *in vitro* follicle culture on *in vitro* embryo production would be very important.

Conflict of interests

The authors declare no conflict of interest.

Author contributions

Conceptualization: A. L. C. Santos, A. C. A. Ferreira, J. R. Figueiredo. *Data curation*: A. L. C. Santos, A. C. A. Ferreira, J. J. H. Celestino and J. R. Figueiredo. *Formal analysis*: B. G. Alves. *Methodology*: A. L. C. Santos, A. C. A. Ferreira, N. A. R. Sá, R. F. Silva, G. J. Q. Palomino, J. Cadenas, E. P. F. Lopes. *Supervision*: A. P. R. Rodrigues and J. R. Figueiredo. *Writing, revision, and editing*: A. L. C. Santos, J. Cadenas, A. C. A. Ferreira, J. J. H. Celestino and J. R. Figueiredo.

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References

1. Sá NAR, Araújo VR, Correia HHV, Ferreira ACA, Guerreiro DD, Sampaio AM, et al. Anethole improves the *in vitro* development of isolated caprine secondary follicles. Theriogenology. 2017;1(89):226–34. https://doi.org/10.1016/j.theriogenology.2015.12.014

2. Sá NAR, Ferreira ACA, Sousa FGC, Duarte ABG, Paes VM, Cadenas J, et al. First pregnancy after *in vitro* culture of early antral follicles in goats: Positive effects of anethole on follicle development and steroidogenesis. Mol Reprod Dev. 2020;87(9):966–77. https://doi.org/10.1002/mrd.23410

3. Elvin JA, Clark AT, Wang P, Wolfman NM, Matzuk MM. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. Mol Endocrinol. 1999;13(6):1035–48. https://doi.org/10.1210/mend.13.6.0310

4. Silva JRV, Van Den Hurk R, Van Tol HTA, Roelen BAJ, Figueiredo JR. Expression of growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and BMP receptors in the ovaries of goats. Mol Reprod Dev. 2005;70(1):11–9. https://doi.org/10.1002/mrd.20127

5. Hreinsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJW, Hovatta O. Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. J Clin Endocrinol Metab. 2002;87(1):316–21. https://doi.org/10.1210/jcem.87.1.8185

6. Martins FS, Celestino JJH, Saraiva MVA, Matos MHT, Bruno JB, Rocha CMC, et al. Growth and differentiation factor-9 stimulates activation of goat primordial follicles *in vitro* and their progression to secondary follicles. Reprod Fertil Dev. 2008;20(8):916–24. https://doi.org/10.1071/RD08108

7. Cook-Andersen H, Curnow KJ, Su HI, Chang RJ, Shimasaki S. Growth and differentiation factor 9 promotes oocyte growth at the primary but not the early secondary stage in three-dimensional follicle culture. J Assist Reprod Genet. 2016;33(8):1067–77. https://doi.org/10.1007/s10815-016-0719-z

8. Almeida AP, Saraiva MVA, Araújo VR, Magalhães DM, Duarte ABG, Frota IMA, et al. Expression of growth and differentiation factor 9 (GDF-9) and its effect on the *in vitro* culture of caprine preantral ovarian follicles. Small Rumin Res. 2011;100(2–3):169–76. https://doi.org/10.1016/j.smallrumres.2011.06.001

9. Chaves RN, Martins FS, Saraiva MVA, Celestino JJH, Lopes CAP, Correia JC, et al. Chilling ovarian fragments during transportation improves viability and growth of goat preantral follicles cultured *in vitro*. Reprod Fertil Dev. 2008;20(5):640–7. https://doi.org/10.1071/RD07195

10. Cadenas J, Leiva-Revilla J, Vieira LA, Apolloni LB, Aguiar FLN, Alves BG, et al. Caprine ovarian follicle requirements differ between preantral and early antral stages after IVC in medium supplemented with GH and VEGF alone or in combination. Theriogenology. 2017;87:321–32. https://doi.org/10.1016/j.theriogenology.2016.09.008

11. Cadenas J, Maside C, Ferreira ACA, Vieira LA, Leiva-Revilla J, Paes VM, et al. Relationship between follicular dynamics and oocyte maturation during *in vitro* culture as a non-invasive sign of caprine oocyte meiotic competence. Theriogenology. 2018;107:95–103. https://doi.org/10.1016/j.theriogenology.2017.10.038

12. Jiatsa Donfack N, Alves KA, Alves BG, Pedrosa Rocha RM, Bruno JB, Lobo CH, et al. Xenotransplantation of goat ovary as an alternative to analyse follicles after vitrification. Reprod Domest Anim. 2019;54(2):216–24. https://doi.org/10.1111/rda.13340

13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001;25(4):402–8. https://doi.org/10.1006/meth.2001.1262

14. Ferreira ACA, Cadenas J, Sá NAR, Correia HHV, Guerreiro DD, Lobo CH, et al. *In vitro* culture of isolated preantral and antral follicles of goats using human recombinant FSH: Concentration-dependent and stage-specific effect. Anim Reprod Sci. 2018;196:120–9. https://doi.org/10.1016/j.anireprosci.2018.07.004

15. Campos LB, Silva AM, Praxedes ÉCG, Bezerra LGP, Freitas JLS, Melo LM, et al. Effect of growth differentiation factor 9 (GDF-9) on *in vitro* development of collared peccary preantral follicles in ovarian tissues. Anim Reprod Sci. 2021;226:106717. https://doi.org/10.1016/j.anireprosci.2021.106717

16. Dipaz-Berrocal DJ, Sá NAR, Guerreiro DD, Celestino JJH, Leiva-Revilla J, Alves BG, et al. Refining insulin concentrations in culture medium containing growth factors BMP15 and GDF9: An *in vitro* study of the effects on follicle development of goats. Anim Reprod Sci. 2017;185:118–27. https://doi.org/10.1016/j.anireprosci.2017.08.011

17. Chaves RN, Duarte ABG, Rodrigues GQ, Celestino JJH, Silva GM, Lopes CAP, et al. The Effects of Insulin and Follicle-

Simulating Hormone (FSH) During *In vitro* Development of Ovarian Goat Preantral Follicles and the Relative mRNA Expression for Insulin and FSH Receptors and Cytochrome P450 Aromatase in Cultured Follicles1. Biol Reprod. 2012;87(3):1–11. https://doi.org/10.1095/biolreprod.112.099010

18. Ferreira ACA, Maside C, Sá NAR, Guerreiro DD, Correia HHV, Leiva-Revilla J, et al. Balance of insulin and FSH concentrations improves the *in vitro* development of isolated goat preantral follicles in medium containing GH. Anim Reprod Sci. 2016;165:1–10. https://doi.org/10.1016/j.anireprosci.2015.10.010

19. Peralta MB, Baravalle ME, Belotti EM, Stassi AF, Salvetti NR, Ortega HH, et al. Involvement of Matrix Metalloproteinases and their Inhibitors in Bovine Cystic Ovarian Disease. J Comp Pathol. 2017;156(2–3):191–201. https://doi.org/10.1016/j.jcpa.2016.10.012

20. Huang Q, Cheung AP, Zhang Y, Huang HF, Auersperg N, Leung PCK. Effects of growth differentiation factor 9 on cell cycle regulators and ERK42/44 in human granulosa cell proliferation. Am J Physiol Endocrinol Metab. 2009;296(6):1344-53. https://doi.org/10.1152/ajpendo.90929.2008

21.Farkas S, Szabó A, Hegyi AE, Török B, Fazekas CL, Ernszt D, Kovács T, Zelena D. Estradiol and Estrogen-like Alternative Therapies in Use: The Importance of the Selective and Non-Classical Actions. Biomedicines. 2022;10(4):861. https://doi.org/10.3390/biomedicines10040861