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Expression of protein and mRNA encoding Insulin Growth Factor-I (IGF-I) in goat ovarian follicles and the influence of IGF-I on in vitro development and survival of caprine preantral follicles

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Abstract

The aim of this study was to investigate the protein and mRNA expression levels of IGF-I and the effects of IGF-I on preantral follicle survival and development, using an in vitro goat ovarian cortical culture system. The ovaries were used for immunohistochemical localization of IGF-I protein or used to demonstrate mRNA expression of IGF-I. For the latter goal, preantral and antral follicles, cumulus-oocyte complex, mural granulosa and theca cells were collected to study mRNA expression. For in vitro studies, ovarian cortex were cultured for 1 and 7 days with MEM supplemented IGF-I (0, 1, 10, 50, 100 or 200 ng/ml). Immunohistochemical results showed strong reactions for IGF-I in oocytes and granulosa cells of all follicular stages, except in granulosa cells of primordial and primary follicles. mRNA expression analysis demonstrated a discrete increase in the production of IGF-I during the transition from primordial to the primary and secondary follicle stages. After 7 days of culture, addition of 50 ng/ml of IGF-I to the medium showed the greatest percentage of normal follicles when compared with other concentrations. Furthermore, the highest percentage of primary follicles was observed after 7 days of culture in MEM^+ plus 10 and 50 ng/ml of IGF-I. Culture of ovarian tissue for 7 days in MEM^+ plus 50 ng/ml of IGF-I promoted the greatest increase ($P < 0.05$) in follicular diameter of all of the concentrations tested. In conclusion, IGF-I protein and mRNA were expressed in all follicular categories of goat. Furthermore, IGF-I maintained preantral follicle survival, promoted primordial follicle activation and stimulated the transition from intermediate to primary follicles.

Keywords: goat, insulin-like growth factor, ovary, preantral follicle.

Introduction

Several studies in reproductive biotechnology have focused on developing *in vitro* culture systems for immature oocytes enclosed in preantral follicles, since these follicles could be sources of fertilizable oocytes for *in vitro* embryo production in the future. Preantral follicle development can be divided into three stages: activation of primordial follicles, the primary to secondary follicle transition and further growth of secondary follicles (Fortune, 2003). Due to the protracted nature of preantral follicle development in large mammals, culture systems should be developed to accelerate follicular growth without inducing inappropriate follicular differentiation.

The actions of several local growth factors that are involved in the complex process of preantral follicular growth in mammals have been investigated *in vitro*. Many studies have highlighted the role of the insulin-like growth factor I (IGF-I) system in the control of follicular growth (Giudice, 1992; Armstrong and Webb, 1997; Armstrong *et al*., 1998; Woad *et al*., 2000). The bioactivity of IGF-I is controlled by its association with IGF-binding proteins (IGFBPs), which can either inhibit or facilitate IGF action (Monget and Bondy, 2000). The expression of mRNAs encoding IGF-I in ovarian follicles is developmentally regulated in a species-specific manner (Armstrong and Webb, 1997; Webb and Armstrong, 1998). Although it has been clearly established that IGF-I is expressed in the granulosa cells of rodents (Oliver *et al*., 1989) and pigs (Hatey *et al*., 1992), its expression in the ruminant remains controversial, with reports demonstrating both the presence (Spicer *et al*., 1993; Leeuwenberg *et al*., 1995; Spicer and Echternkamp, 1995; Yuan *et al*., 1998) and absence (Perks *et al*., 1995; 1999; Armstrong *et al*., 1998) of mRNA encoding IGF-I in both bovine and ovine granulosa cells.

The responsiveness of ovarian follicles to IGF-I has been demonstrated in numerous studies. When added during *in vitro* culture of preantral follicles, IGF-I has been shown to stimulate follicular growth in various species (rat: Zhou *et al*., 1991, Zhao *et al*., 2001; cow: Gutierrez *et al*., 2000; human: Louhio *et al*., 2000). In addition, IGF-I increased follicular diameter, increased DNA content, promoted functional integrity and stimulated steroidogenesis during rat preantral follicle culture (Demeestere *et al*., 2005). Moreover, IGF-I

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promotes proliferation of cultured human theca cells (Duleba *et al*., 1998). However, although IGF-I has a stimulatory effect on early follicle growth, McCaffery *et al*. (2000) highlighted the importance of its regulatory mechanism, showing that inappropriate exposure of bovine preantral follicles to IGF-I has a detrimental effect on oocyte development. However, much less is known about the involvement of IGF-I in the regulation of goat preantral follicular development.

In an attempt to address the lack of knowledge regarding the involvement of IGF-I on goat folliculogenesis, the present study was designed (1) to verify the patterns of expression of protein and mRNA of IGF-I in goat follicles at different stages of development and (2) to investigate a possible influence of different concentrations of IGF-I on the survival, activation and growth of goat preantral follicles cultured *in vitro*.

Materials and Methods

Messenger RNA and protein localization of IGF-I in goat follicles

To evaluate mRNA expression, ovaries (n = 30) from fifteen adult (1–3 years old) non-pregnant mixed-breed goats (*Capra hircus*) were collected at a local slaughterhouse. All animals were cyclic and in good body condition. The ovaries were stripped of surrounding fat tissue and ligaments and rinsed in saline (0.9% NaCl) containing antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin), after which 10 were used for isolation of primordial, primary and secondary follicles. The remaining ovaries were used for collection of COCs, mural granulosa and theca cells from small and large antral follicles. Primordial, primary and secondary follicles were isolated via a mechanical procedure described previously (Lucci *et al.*, 1999). After isolation, these follicles were first washed several times to completely remove the stromal cells and then placed by category into separate microtubes in groups of 10. This procedure was completed within 2 h, and all samples were stored at -80^{o}C until the RNA was extracted. From a second group of ovaries (n = 20), cumulus-oocyte complexes (COCs) were aspirated from small (1-3 mm) and large (3-6 mm) antral follicles. From the follicle content, compact COCs were selected as described by Van Tol and Bevers (1998). Thereafter, groups of 10 COCs were stored at -80°C until RNA extraction. To collect mural granulosa and theca cells, small (n = 10) and large antral follicles (n = 10) were isolated from ovaries (n = 5) and dissected free of stromal tissue with forceps, as described previously (Van Tol and Bevers, 1998). The follicles were then bisected and mural granulosa/theca were collected and stored at -80°C.

Isolation of total RNA was performed using Trizol (Invitrogen, São Paulo, Brasil). As per the manufacturer's instructions, 1 ml of Trizol solution was added to each frozen sample and the lysate was centrifuged at 10,000 x g for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and introduced onto a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNase-free DNase (340 Kunitz units/ml) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 µl RNase-free water.

Prior to reverse transcription, the eluted RNA samples were incubated for 5 min at 70°C, and then chilled on ice. Reverse transcription was then performed in a total volume of 20 µl made up of 10 µl sample RNA, 4 µl 5X reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNaseOUT, 150 units Superscript III reverse transcriptase, 0.036 U random primers (Invitrogen, São Paulo, Brazil), 10 mM DTT and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42°C followed by 5 min at 80°C and was then stored at -20°C. Minus RT blanks were prepared under the same conditions but without inclusion of reverse transcriptase.

Quantification of mRNA for IGF-I was performed using SYBR Green. PCR reactions were carried out using 1 µl cDNA as template in 7.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 µl of ultra-pure water and 0.5 µm of each primer. The primers were designed to perform amplification of mRNA for IGF-I. GAPDH and β-actin were used as endogenous controls for normalization of gene expression (Table 1). The thermal cycling profile for the first round of PCR 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. All reactions were performed in a Mastercycler real-time PCR machine (Eppendorf, Germany).

Localization of IGF protein was performed on serial 5-µm sections cut from the ovaries (n = 10) of five different goats. These sections were mounted on poly-L-lysine-coated slides, dried overnight at 37°C, deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase was blocked by incubating the deparaffinized sections in 3% hydrogen peroxide in methanol for 10 min. The sections were then washed with PBS, and the epitopes were activated by microwaving the sections for 7 min at 900 W in 0.01 M citrate buffer (pH 6.0). After microwaving, the sections were washed in PBS/0.05% Tween (PBS-T) before being incubated for 30 min with 5% normal goat serum in PBS to minimize non-specific binding. Then, the sections were incubated with polyclonal anti-IGF-I (Santa Cruz, CA, USA) diluted 1:50. After this incubation, sections were washed three times with PBS

and incubated for 45 min with biotinylated secondary antibody (anti-rabbit IgG from Santa Cruz, CA, USA) then diluted 1:200 in PBS containing 5% normal goat serum. Next, the sections were washed three times in PBS-T before being incubated for 45 min with an avidin-biotin complex (1:600-Vectastain Elite ABC kits; Vector Laboratories). The sections were then washed three times in PBS and stained with diaminobenzidine (0.05% DAB in Tris/HCl, pH 7.6, and 0.03% H_2O_2 - Sigma tablets) for a maximum of 10 min. The stained sections were rinsed in PBS and water then counterstained for 10 s in Mayer's hematoxylin. Finally, the sections were washed for 10 min in running tap water, and subsequently dehydrated in a graded ethanol series followed by xylene treatment and finally mounted for histological evaluation. The staining intensity was scored as follows: absent (-), weak (+), moderate (++) or strong (+++). Controls for non-specific staining were performed by (1) replacing the primary antibody with IgGs from the same species in which the specific antibody was raised, at the same concentration, and (2) incubating with diaminobenzidine reagent alone to exclude the possibility of non-suppressed endogenous peroxidase activity. For immunohistochemical analysis, the follicles were classified as described later for the culture.

Table 1. Oligonucleotide primers used for PCR analysis of goat cells and tissues.

Target gene	Primer sequence (5´→3´)	Sense	Position	GenBank accession no.
IGF-I	ATGCTCTCCAGTTCGTGTGT	s	182-202	GI: 217666 (2008) *Capra hircus* GHR
	TTGAGAGGCGCACAGTACATCT	as	341-363	
GAPDH	TGTTTGTGATGGGCGTGAACCA	s	287-309	GI: 27525390 (2005) *Capra hircus* GAPDH
	ATGGCGTGGACAGTGGTCATAA	as	440-462	
Beta-Actin	ACCACTGGCATTGTCATGGACTCT	s	187-211	GI: 28628620 (2003) *Capra hircus* Beta-Actin
	TCCTTGATGTCACGGACGATTTCC	as	386-410	

s: sense; as: antisense

In vitro culture of ovarian tissue and morphological evaluation

Ovaries (n = 10) from five adult (1-3 years old), non-pregnant mixed-breed goats (*Capra hircus*) were collected at a local slaughterhouse. The animals were cyclic and in good body condition. Immediately after the death of the animals, the ovaries were washed in 70% alcohol followed by two washes in 0.9% saline solution. The pair of ovaries from each animal was transported to the laboratory in 20 ml of Minimum Essential Medium (MEM) supplemented with penicillin (100 µg/ml) and streptomycin (0.25 µg/ml) within 1 h, at 33°C.

In the laboratory, the ovaries from each animal were stripped of the surrounding fat tissue and ligaments. Moreover, the medulla, visible growing follicles and corpora lutea were removed. The ovarian cortex of each pair of ovaries was then divided in 13 fragments (approximate size 3 x 3 mm, 1 mm thick) using a needle and scalpel under sterile conditions. One fragment was taken randomly and immediately fixed for histological examination (control). The 12 remaining fragments were placed individually in 24-well culture dishes, with each well containing 1 ml of culture medium. They were cultured for 1 or 7 days at 39°C in humidified air with 5% CO_2. The medium used was MEM (osmolarity: 300 mOsm/l, pH: 7.2) supplemented with ITS (insulin 6.25 µg/ml, transferrin 6.25 µg/ml and selenium 6.25 ng/ml), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine and 1.25 mg/ml BSA, which was called MEM^+ (all from Sigma Chemical Co. St Louis, MO, USA). The ovarian cortex fragments were cultured in MEM^+ alone or MEM^+ containing different concentrations of IGF-I (1, 10, 50, 100 or 200 ng/ml). Each treatment was repeated five times, and the culture medium was changed every two days.

Immediately after fragmentation, representative pieces of ovarian cortex were immersion-fixed for 12 h in 10% neutral buffered formaldehyde (pH 6.8-7.2) to serve as non-cultured control (fresh control). After 1 or 7 days of culture in each medium, the pieces of ovarian cortex were fixed as described above, dehydrated in a graded series of ethanol, clarified with xylene and embedded in paraffin wax. The samples were sectioned (7 µm thick) and mounted on charged slides, then allowed to dry overnight at 37°C before staining with Periodic Acid Schiff and hematoxylin. Follicle stage and survival were assessed microscopically on serial sections. Coded anonymized slides were examined on a microscope (Nikon, Japan) under 400X magnification.

The developmental stages of follicles have been defined previously (Silva *et al*., 2004) as primordial (oocyte surrounded by flattened granulosa cells) or developing follicles, i.e., intermediate (oocyte surrounded by flattened granulosa cells and at least one

cuboidal granulosa cell), primary (oocyte surrounded by a complete layer of cuboidal granulosa cells), or secondary (oocyte surrounded by two or more complete layers of cuboidal granulosa cells). Primordial and developing follicles were classified as morphologically normal (follicles containing an intact oocyte and granulosa cells well-organized in layers without pyknotic nucleus) and degenerated follicles (oocyte with pyknotic nucleus, retracted cytoplasm or disorganized granulosa cells detached from the basement membrane; Silva *et al*., 2000).

The percentages of primordial and developing follicles were calculated on day 0 (control) and after 1 or 7 days of culture in each medium. Overall, 150 follicles were evaluated for each treatment (30 follicles per treatment x 5 repetitions = 150 follicles). In addition, follicular diameter was taken using only normal follicles from day 0 and after culture. The follicle diameter was recorded from edge to edge of granulosa cell membrane, or from the outside edge of the theca cell layer when present. Oocyte diameter was recorded from edge to edge of the oocyte membrane and two perpendicular diameters were recorded for each oocyte. The average of these values was respectively reported as follicle and oocyte diameter. It is important to note that only follicles with a visible oocyte nucleus were evaluated to avoid double counting.

Assessment of preantral follicle viability by fluorescence

A viability study using an experimental protocol similar to that described in the above morphological investigation was performed with the aim of analyzing the effects of caprine ovarian tissue culture on the viability of preantral follicles. Based on the results of morphological analysis, the viability of follicles was further analyzed using a more accurate method of assessment based on fluorescent probes. Additional pairs of ovaries (n = 4) were cut into 18 fragments, of which six (n = 6) were immediately processed for follicle isolation. The remaining fragments were cultured for 7 days in the same conditions described above, in MEM^+ alone or MEM^+ containing 50 ng/ml of IGF-I.

After culture, goat preantral follicles were isolated from ovarian fragments using the mechanical method described previously by Lucci *et al*. (1999).

Isolated preantral follicles were analyzed using a two-color fluorescence cell viability assay based on the simultaneous determination of live and dead cells by calcein-AM and ethidium homodimer-1, respectively. While the first probe detected intracellular esterase activity of viable cells, the later labels nucleic acids of non-viable cells with plasma membrane disruption. The test was performed by adding 4 µm calcein-AM and 2 µm ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) to the suspension of isolated follicles, followed by incubation at 37°C for 15 min. After labeling, follicles were washed once by centrifugation at 100 x g for 5 min and resuspension in MEM, mounted on a glass microscope slide in 5 µl antifading medium (DABCO, Sigma, Deisenhofen, Germany) to prevent photobleaching, and finally examined using an a DMLB fluorescence microscope (Leica, Germany). The emitted fluorescent signals of calcein-AM and ethidium homodime-1 were collected at 488 and 568 nm, respectively. Oocytes and granulosa cells were considered live if the cytoplasm was stained positively with calcein-AM (green) and if the chromatin was not labeled with ethidium homodimer (red).

Statistical analyses

mRNA expression data in primordial, primary and secondary follicles were analyzed by ANOVA, and means were compared by Tukey's test, while *t*-tests were used for paired comparisons of mRNA expression in small and large antral follicles ($P < 0.05$). Culture data were analyzed statistically as follows. Kolmogorov-Smirnov and Bartlett's tests were applied to confirm normal distribution and homogeneity of variance, respectively. Analysis of variance was then performed using the GLM procedure of SAS (1999), and Dunnett's test was applied for comparison of control groups against each treatment tested (Steel *et al*., 1997), while the SNK test was used to compare different concentrations of IGF and days of culture. Differences among groups were considered significant if $P < 0.05$, and results are expressed as mean ± standard deviation.

Results

Messenger RNA and protein expression of IGF-I in goat ovarian follicles

Quantification of mRNA expression demonstrated a discrete increase in the production of IGF-I during the transition from primordial to primary and secondary follicle stages, but the differences were not statistically significant ($P > 0.05$, Fig. 1A). When the expression of IGF-I mRNA was compared between COCs as well as granulosa/theca cells collected from small and large antral follicles, no significant difference was observed ($P > 0.05$, Fig. 1B, C). Additionally, real-time PCR showed that granulosa and theca cells from small and large antral follicles produce significantly more IGF-I than their respective COCs ($P < 0.05$, Fig. 1D, E).

To confirm the presence of IGF-I protein in

follicular compartments, immunohistochemical studies showed a positive reaction for IGF-I in oocytes of primordial, primary, secondary and antral follicles (Fig. 2, Table 2). Granulosa cells of primordial and primary follicles had a moderate reaction for IGF-I, while a strong immunoreaction was observed in secondary and antral follicles (Fig. 2, Table 2). In addition, a weak immunoreaction for IGF-I was observed in theca cells from secondary follicles, but theca cells from small antral follicles had a moderate reaction. Moreover, a strong reaction for IGF-I was demonstrated in theca cells from large antral follicles (Fig. 2, Table 2).

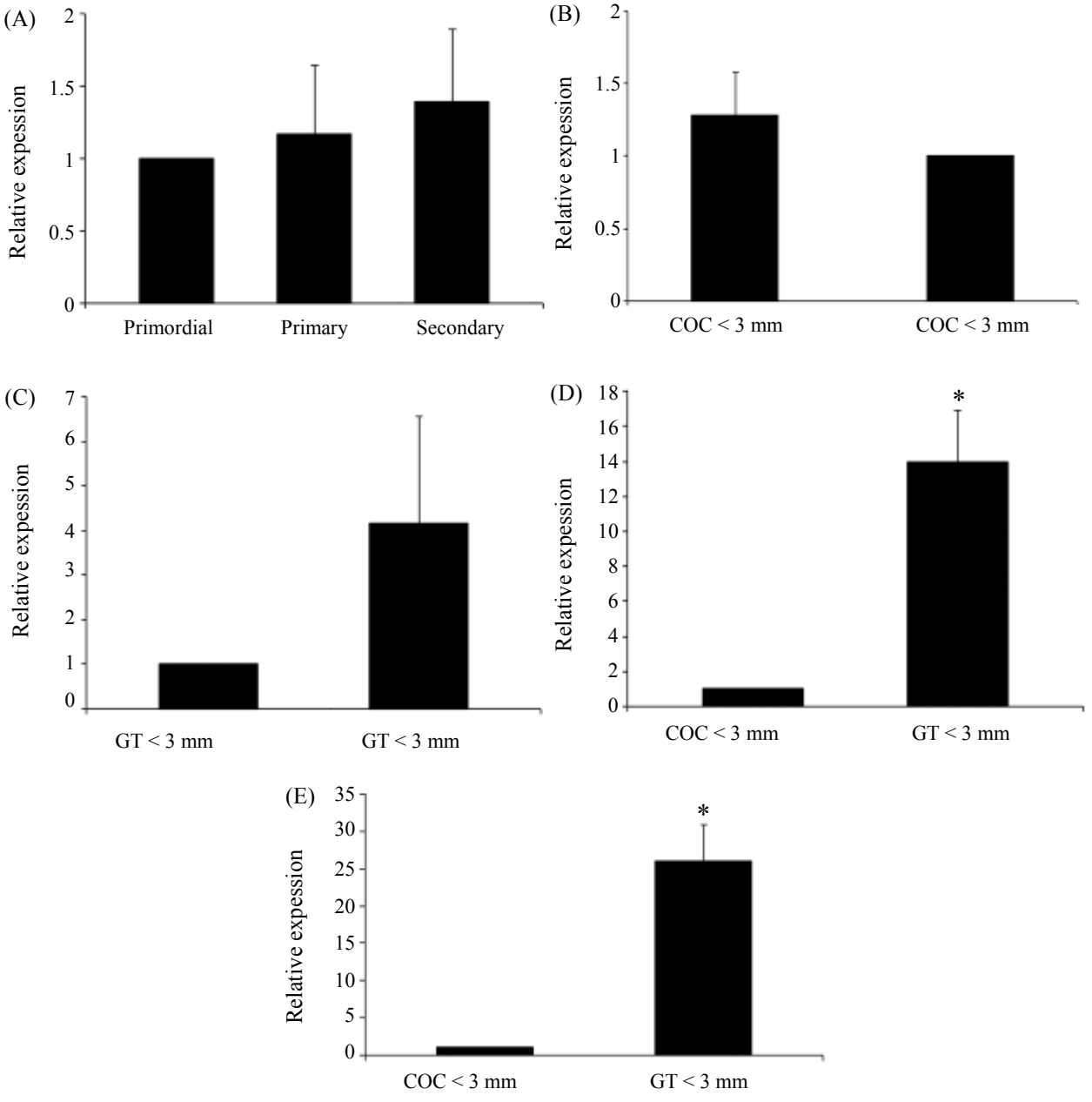

Figure 1. Expression of IGF-I mRNA (mean ± SEM) in goat ovarian follicles. (A) Primordial, primary and secondary follicles; (B) COCs from small and large antral follicles; (C) granulosa/theca (GT) cells from small and large antral follicles; (D) COCs and granulosa/theca cells from small antral follicles; (E) COCs and granulosa/theca cells from large antral follicles. *($P < 0.05$).

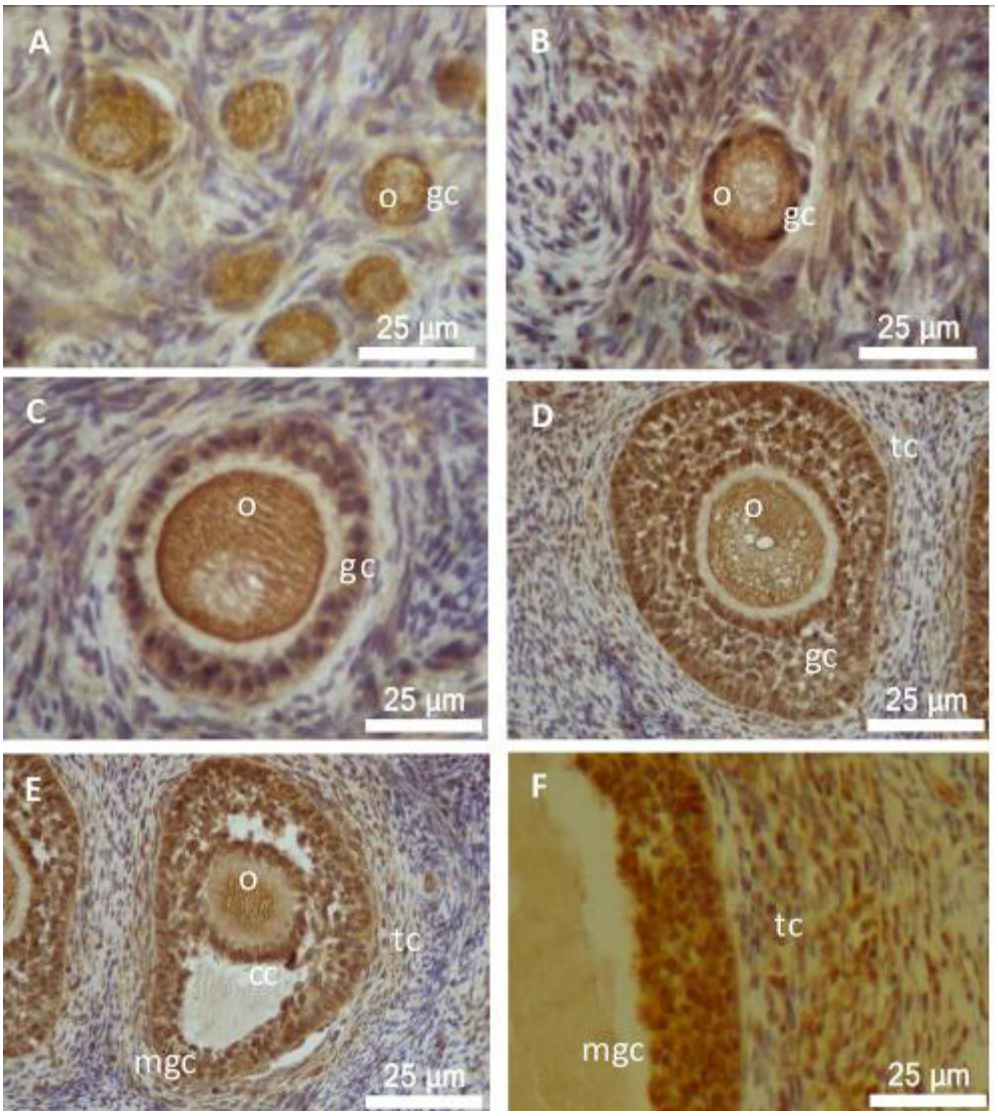

Figure 2. IGF ligand immunoreactivity in the different structures found within goat ovaries. (A) Primordial follicle; (B) intermediate follicles; (C) primary follicle; (D) secondary follicle; (E) antral follicle; (F) negative control reaction. o: oocyte; gc: granulosa cells; mgc: mural granulosa cells; cc: cumulus cells; tc: theca cells. (Original magnification ×400).

Table 2. Relative intensity of immunohistochemical staining for IGF-I ligand in goat ovarian follicles.

	Ovarian follicles			
Follicle compartment	Primordial	Primary	Secondary	Antral
Oocyte	+++	+++	+++	+++
Granulosa	++	++	+++	+++
Theca	NF	NF	+	+++

(-) absent; (+) weak; (++) moderate; (+++) strong immunoreaction. NF: not found.

Effect of IGF-I on preantral follicle survival

Histological analysis showed that degenerated (Fig. 3A) and normal (Fig. 3B) follicles were found in cultured ovarian cortical pieces. In degenerated follicles, a shrunken oocyte with a pyknotic nucleus or disorganized granulosa cells was observed. In total, 1,950 follicles were counted to evaluate follicular morphology, activation and growth. Figure 4 shows the percentage of normal follicles in non-cultured control (day 0) and after 1 or 7 days of culture of ovarian cortical pieces in different media. In all media tested, culture of ovarian tissue had significantly reduced ($P < 0.05$) percentages of normal follicles when compared to fresh tissue (87.5%), except when 100 and 200 ng/ml of IGF-I was added to the medium for 1 day of culture (90.1 and 83.9%,

respectively) and when 50 ng/ml of IGF-I was added for 7 days of culture (81.9%). When compared with control medium (MEM^+), after 1 or 7 days of culture, addition of 50, 100 and 200 ng/ml of IGF-I significantly increased ($P < 0.05$) the percentage of histologically normal follicles, except after 7 days with 200 ng/ml of IGF-I ($P > 0.05$). In addition, after 1 day of culture, the greatest percentage of normal follicles was observed with 100 and 200 ng/ml of IGF-I. After 7 days of culture, addition of 50 ng/ml of IGF-I to the medium showed the greatest percentage of normal follicles (81.9%) when compared with other concentrations ($P < 0.05$). Increasing the culture period from 1 to 7 days significantly reduced ($P < 0.05$) the percentage of intact follicles in all treatments, except in tissues cultured in MEM^+ plus 50 ng/ml of IGF-I ($P > 0.05$).

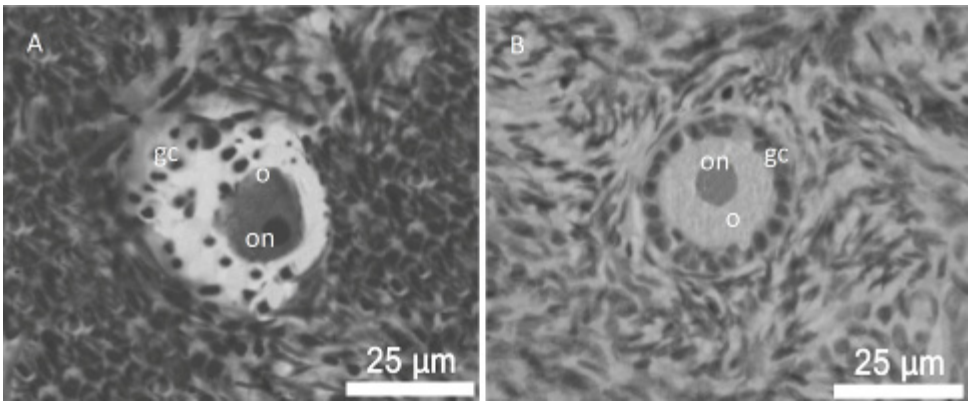

Figure 3. Histological section of non-cultured tissue after staining with periodic acid-Schiff-hematoxylin, showing degenerated primary follicle (A) and normal primary follicle (B). o: oocyte; on: oocyte nucleus; gc: granulosa cells. (Original magnification ×400.)

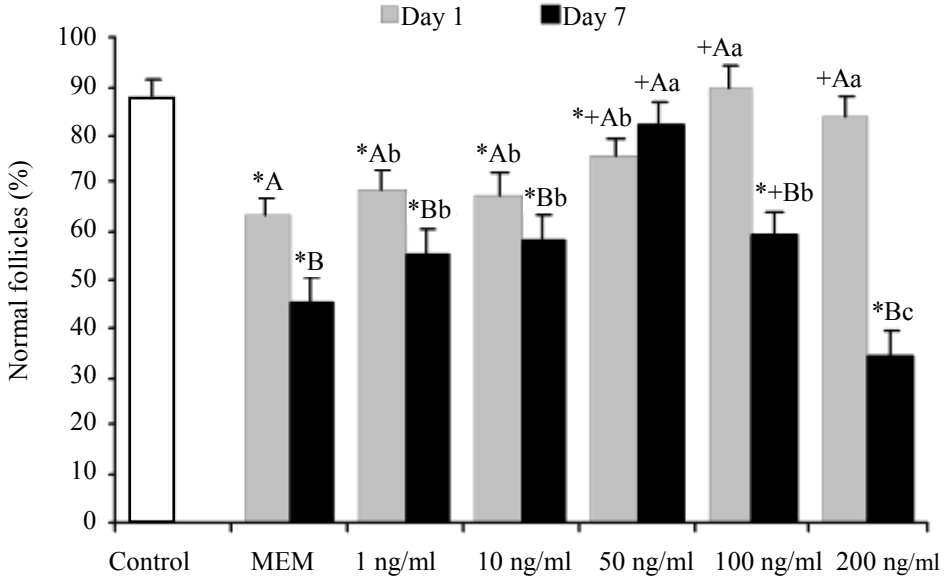

Figure 4. Percentage of normal follicles in non-cultured tissue and in tissue after culture for 1 or 7 days in MEM^+ and MEM^+ plus 1, 10, 50, 100, 200 ng/ml IGF-I.
*Denotes values that differ significantly from non-cultured ovarian cortex (control; $P < 0.05$).
+Denotes values that differ significantly from MEM^+ ($P < 0.05$).
^{AB}Values with different letters denote significant differences between cultured periods within a given medium ($P < 0.05$).
^{abc}Values with different letters denote significant differences among treatments ($P < 0.05$).

Viability assessment of follicles after culture

Based on the results of the morphological evaluation, goat preantral follicles were isolated from fresh control and from ovarian fragments cultured for 7 days with 50 ng/ml of IGF-I, and a viability trial using these treatments was performed. A fluorescence cell viability assay based on labeling of live and dead cells by calcein-AM and ethidium homodimer-1, respectively, was employed. Figure 5 shows viable preantral follicles after *in vitro* culture staining with calcein-AM and ethidium homodimer-1. The percentage of viable follicles after 7 days of culture was similar between MEM^+ (91.0%) and IGF-I (93.0%).

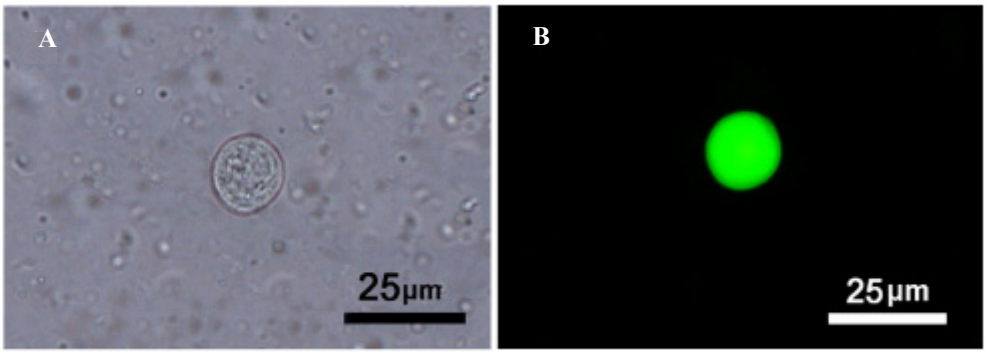

Figure 5. Mechanically isolated follicles visible under a fluorescence microscope after fluorescence staining for follicle viability (calcein-AM and ethidium homodimer-1). Preantral follicle in cultured ovarian tissue in MEM^+ containing 50 ng/ml of IGF-I for 7 days were visible by (A) light microscopy and (B) fluorescence microscopy. Scale bars represent 25 µm.

Goat primordial follicle activation and growth during in vitro culture

In non-cultured control, the percentages of primordial, intermediate, primary and secondary follicles were 74.5, 21.7, 2.4 and 1.4%, respectively. After 1 or 7 days of culture in all media tested, the percentage of primordial follicles ($P < 0.05$, Fig. 6A) was significantly reduced compared to fresh control. In contrast, there was a significant and concomitant increase in the percentage of intermediate and primary follicles in all treatments tested after 1 or 7 days of culture, when compared with fresh control ($P < 0.05$, Fig. 6B, C). Furthermore, cortical tissue cultured in MEM^+ plus 10 or 50 ng/ml of IGF-I for 7 days had a greater percentage of primary follicles than did tissue cultured with other concentrations of IGF-I ($P < 0.05$, Fig. 6C). When compared with MEM^+ alone, all IGF-I concentrations (except 1 ng/ml) significantly increased the percentage of primary follicles after 1 or 7 days. In addition, after 7 days of culture, the percentage of secondary follicles was significantly increased in medium containing 1, 10, 50 and 100 ng/ml of IGF-I, compared with fresh control ($P < 0.05$, Fig. 5C). Only ovarian cortex cultured for 7 days with 50 and 100 ng/ml had greater percentages of secondary follicles when compared to MEM^+ alone ($P < 0.05$, Fig. 6C).

Regarding the progression of the culture period (from day 1 to 7), a significant decrease in the percentage of primordial follicles was observed in all treatments except MEM^+ ($P < 0.05$, Fig. 6A). Moreover, a significant decrease in the percentage of intermediate follicles was also observed in 10, 50 and 100 ng/ml of IGF-I ($P < 0.05$, Fig. 6B), while all treatments resulted in a significant increase in the percentage of primary follicles ($P < 0.05$, Fig. 6C). All treatments promoted a significant increase in the percentage of secondary follicles with the progress of the culture period from 1 to 7 days, except when tissues were cultured with 200 ng/ml of IGF-I ($P < 0.05$, Fig. 6D).

After 1 and 7 days of culture in MEM^+ containing 50 and 100 ng/ml of IGF-I, a significant increase in follicular and oocyte diameter was seen when compared with noncultured tissue and the other treatments ($P < 0.05$, Table 3). With the progression of the culture time from 1 to 7 days, a significant increase in follicular diameter was observed in all treatments except in MEM^+ and MEM^+ plus 200 ng/ml of IGF-I ($P < 0.05$). Nevertheless, ovarian fragments cultured in MEM^+ plus IGF-I at 50 ng/ml, after 7 days of culture, had greater follicular diameter when compared to the other treatments ($P < 0.05$).

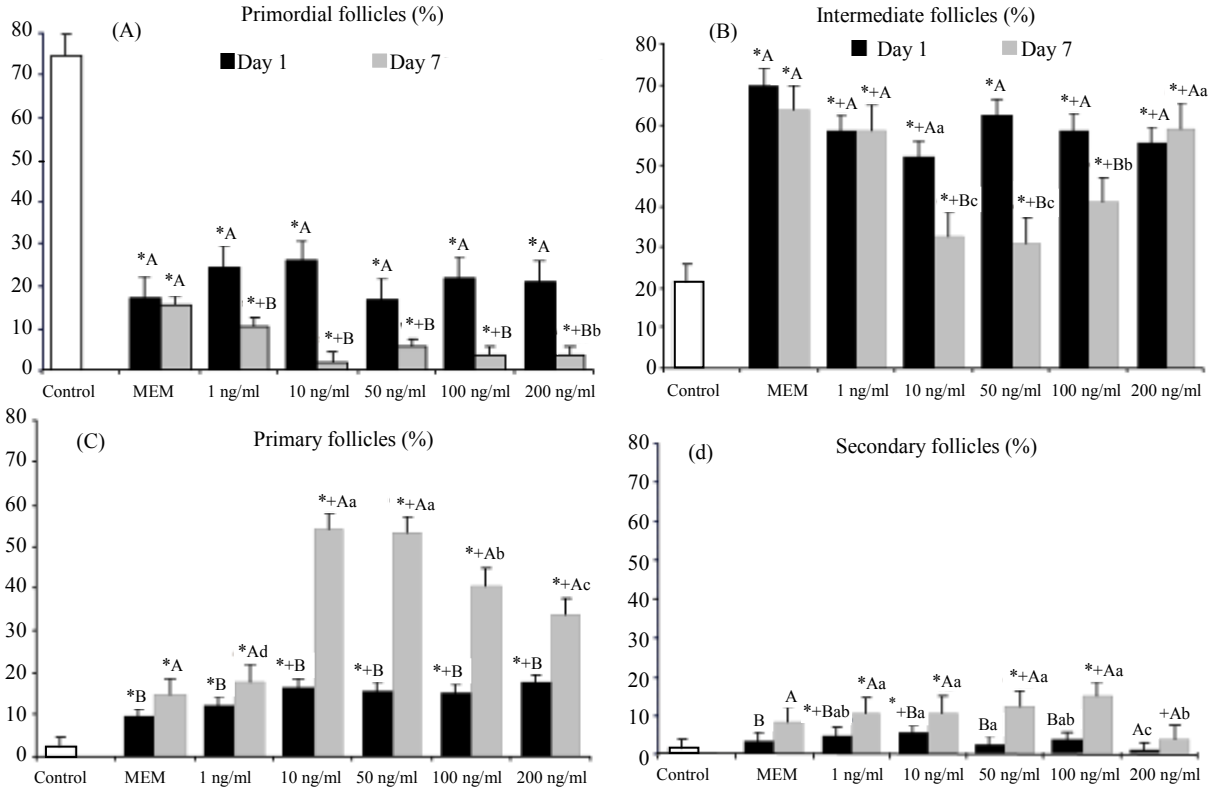

Figure 6. Percentages of primordial (A) and developing follicles (intermediate [B], primary [C] and secondary [D]) in non-cultured tissue (control) and in tissue cultured for 1 and 7 days in MEM^{+} medium and in MEM^{+} plus different concentrations of IGF-I.
*Denotes values that differ significantly from non-cultured ovarian cortex (control; $P < 0.05$).
+Denotes values that differ significantly from MEM ($P < 0.05$).
^{AB}Values with different letters denote significant differences between cultured periods within a given medium ($P < 0.05$).
^{abc}Values with different letters denote significant differences among treatments ($P < 0.05$).

Discussion

Analysis of the expression pattern of mRNA encoding IGF-I demonstrated a progressive increase in the production of IGF-I during the transition from primordial to primary and secondary follicle stages. The presence of IGF-I protein in these follicles, combined with the fact that IGF-I increases the percentage of primary follicles after 7 days of culture, indicates that IGF-I is involved in the control of preantral follicle development in goats. Wandji *et al*. (1998) showed that IGF-I mRNA levels were lower in mouse primary follicles than in large preantral and antral follicles. Moreover, in granulosa cells of mouse antral follicles, IGF-I mRNA was expressed in a gradient, with greater expression in the cumulus oophorus and in the vicinity of the antrum than in the mural compartment. In the present study, the greatest level of expression of IGF-I mRNA was observed in the granulosa/theca cells from large antral follicles of goats. In humans, it has been demonstrated that in small antral follicles (1-8 mm), the expression of IGF-I mRNA was restricted to theca cells (El-Roeiy *et al.*, 1993). In addition, IGF-I mRNA was also detected in the granulosa cells of rat antral follicles (Adashi *et al*., 1997). Conversely, Armstrong *et al*. (2000), using in situ hybridization, did not detect IGF-I mRNA in granulosa and theca cells in bovine follicles under different stages of development. Immunohistochemical localization of IGF-I shows that the thecal-interstitial cells of humans are the main sites of IGF-I biosynthesis (Hernandez *et al*., 1992).

The present study shows new evidence that IGF-I promotes goat ovarian follicle survival and development during 7 days of *in vitro* culture. Maintenance of follicular viability is a prerequisite for the culture of preantral follicles. Our results showed that the addition of IGF-I at 50 ng/ml promoted follicle survival in tissue slices cultured for 7 days, confirmed by histological and fluorescence analyses. These results suggest that IGF-I acts to prevent atresia. In goats, Zhou and Zhang (2005) demonstrated the stimulatory effect of 100 ng/ml of IGF-I during 9 days of culture on the maintenance of follicular viability. This would be consistent with previous studies that have shown that

IGF-I is able to suppress apoptotic DNA fragmentation in cultured rat follicles, acting as an antiapoptotic factor (Chun *et al*., 1994). In contrast, in our study, MEM^+ alone or with IGF-I at concentrations of 1, 10, 100 or 200 ng/ml decreased follicular viability after 7 days of culture, compared with follicular viability in MEM^+ with 50 ng/ml of IGF-I. We conclude that lower concentrations of IGF-I may be not sufficient for promoting the maintenance of goat follicular survival and that greater concentrations may show a detrimental effect on follicular health.

This study shows an increase in follicular activation during *in vitro* culture of goat ovarian cortical tissue for 7 days independently of growth factor addition, compared with fresh control. Similar results were obtained in previous studies in goat (Martins *et al*., 2005, 2008; Silva *et al*., 2006; Matos *et al*., 2007), cow (Wandji *et al*., 1996; Braw-Tal and Youssefi, 1997) and baboon (Fortune *et al*., 1998), in which the number of primordial follicles was dramatically reduced with a concomitant increase in the number of developing follicles after *in vitro* culture of ovarian tissue. Culture of newborn mouse ovaries in serum-containing media shows that the transition from primordial to subsequent stages can occur spontaneously *in vitro* (Eppig and O´Brien, 1996). It is possible that *in vitro* conditions improve follicle development by providing release of stimulatory factors or cessation of the production of inhibitory factors by stromal, granulosa, or pre-thecal cells. Thus, the culture of ovarian cortical tissue triggered the *in vitro* spontaneous activation of goat primordial follicles. Yang and Fortune (2002) suggested that the highest doses of insulin in ITS (3.12 and 6.25 µg/ml), a component of our culture medium, best supported the activation and maintenance of health of bovine primordial follicles. Other studies have shown that the IGF-I knockout mouse has normal follicle development, including the primordial to primary transition, up to the late preantral stage (Baker *et al*., 1996).

To our knowledge, this is the first study to report an increase in the percentage of goat primary follicles after stimulation with IGF-I (at 10 and 50 ng/ml) during 7 days of *in vitro* culture. A counterpoint to this fundamental role of IGF in goat preantral follicle development is the inhibitory effect of exogenous IGF-I on follicular activation and survival of bovine primordial and primary follicles *in vitro* (Fortune *et al.,* 2004). Our data suggests that a large amount of intermediate follicles is stimulated to progress to later developmental stages when treated with IGF-I. Another possibility is the fact that the primordial follicle pool is indirectly stimulated to grow into intermediate follicles and from intermediate to primary follicles. In addition, it has been reported that paracrine factors secreted by follicular somatic cells regulate many important aspects of follicular development in mammals (cat: Jewgenow and Pitra, 1993; Albertini *et al*., 2001; rat: Eppig, 1991; Eppig *et al*., 2002; pig and cow: Miyano, 2003). Thus IGF-I may increase the expression of other growth factors and hormone receptors in primary follicles and hence make them more receptive to those paracrine and autocrine factors, increasing their number. Alternatively, IGF-I may have improved survival of the pre-existing primary follicles. Previous studies have outlined the involvement of IGFBPs in the regulation of IGF-I bioavailability (Perks and Wathes, 1996; Armstrong *et al*., 1998, 2002; Yuan *et al*., 1998; Monget *et al*., 2002). In our study, we supposed that preantral follicles cultured in medium with 10 or 50 ng/ml of IGF-I were expected to be exposed to the actions of IGF-I, since at these concentrations, the regulation of IGF-I by its binding proteins (IGFBPs) would be bypassed, and IGF-I would have the potential to act via its receptors.

In the present study, after 7 days of culture, the greatest follicular diameter was observed after culture with 50 ng/ml of IGF-I, compared with fresh control and other concentrations of IGF-I. This effect may be due to an increased rate of mitosis in granulosa cells and an increase in oocyte size. Similarly, Itoh *et al*. (2002) reported that the addition of 20 ng/ml of IGF-I to the culture medium increases bovine preantral follicular and oocyte diameter *in vitro*. Stimulation of follicular growth may be due primarily to the greater number of granulosa cells. The physiological roles of IGFs have been well characterized in large antral follicles, but fewer studies have shown the effects of IGFs during earlier stages of follicular development. IGF-I may be involved in the growth and survival of rapidly growing, large preantral follicles and early antral follicles in mice (Adashi *et al*., 1997; Wandji, *et al*., 1998). Moreover, Zhao *et al*. (2001) demonstrated that rat preantral follicles (140-160 µm in diameter) cultured for 6 days in the presence of IGF-I (at concentrations of 1 and 10 ng/ml) led to a larger size while maintaining normal morphology (Zhao *et al*., 2001). In a recent study, Thomas *et al*. (2007) showed that follicular diameter was increased over control levels by the addition of 50 ng/ml of IGF-I during 6 days of culture. These findings are in agreement with the data presented in the current study.

In conclusion, our findings indicate that both mRNA and protein for IGF-I are expressed in different follicular compartments at various stages of follicle development, indicating that IGF-I is involved in the control of folliculogenesis in goats. In support of these data, *in vitro* studies have demonstrated that culture of goat ovarian cortical slices in medium supplemented with 50 ng/ml of IGF-I promote follicular survival, follicular growth and primordial to primary follicle transition. These results suggest that IGF-I plays an important role in goat preantral follicle development.

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