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Steady-state levels of vasoactive intestinal peptide (VIP) mRNA in goat ovaries and the effect of VIP on the *in vitro* development of isolated preantral follicles

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Abstract

The aims of this study were to verify the steady-state level of vasoactive intestinal peptide (VIP) mRNA in goat follicles at various developmental stages and to investigate the influence of VIP on the survival, antrum formation and growth of secondary follicles cultured for 6 days. Primordial, primary and secondary goat follicles and small and large antral follicles were obtained to quantify *VIP* mRNA by real-time reverse transcription with the polymerase chain reaction. The influence of VIP and the presence or absence of follicle-stimulating hormone (FSH) on the development of secondary follicles and on mRNA expression for *VIP* and FSH receptor (*FSHR*) were determined after 6 days of culture. Survival, antrum formation and follicular diameter were evaluated every other day of culture. The levels of *VIP* mRNA in primary and secondary follicles were significantly higher than in primordial follicles. Cumulus–oocyte complexes (COCs) from both small and large antral follicles had significantly higher levels of *VIP* mRNA than their respective granulosa/theca cells. During culture, the addition of VIP and/or FSH had no effect on follicular development. However, the presence of FSH and/or VIP in the culture medium significantly reduced *VIP* mRNA levels, but did not alter *FSHR* mRNA levels. In conclusion, *VIP* mRNA was detected in all goat follicular categories and cellular types, VIP and/or FSH did not affect the development of secondary follicles and reduce the expression of *VIP* mRNA levels.

Keywords: caprine, culture, FSH, preantral follicles, VIP.

Introduction

Ovarian activity is regulated not only by gonadotropins and steroids but also by a number of neural inputs and paracrine regulatory mechanisms. The mammalian ovary is innervated by extrinsic nerves, which are both catecholaminergic and peptidergic in nature (Burden, 1985; Ojeda and Lara, 1989; Ojeda *et al*., 1989). Peptidergic innervation of the ovary was verified, among other ways, by the presence of vasoactive intestinal peptide (VIP; Ahmed *et al*., 1986).

Hulshof *et al.* (1994) demonstrated that the bovine ovary is innervated by VIP-positive nerve fibers beginning at the onset of follicular development and increasing with age. Furthermore, this neuropeptide is produced by nerve fibers innervating follicles at all stages of development in rodent (Ahmed *et al*., 1986) and avian (Johnson *et al*., 1994) ovaries. However, *VIP* mRNA levels in goat ovarian follicles have not yet been described.

Some findings suggest that VIP may play a vital role in several ovarian functions, such as the regulation of steroidogenesis (Ahmed *et al*., 1986; T**ö**rnell *et al*., 1988), cAMP accumulation (T**ö**rnell *et al*., 1988; Vaccari *et al*., 2006), plasminogen activator production (Johnson and Tilly, 1988) and oocyte maturation (T**ö**rnell *et al*., 1988). VIP has been shown to stimulate androgen and estradiol release *in vitro* (Davoren and Hsueh, 1985; Parra *et al*., 2007). Kowalewski *et al*. (2010) observed that VIP together with cAMP-analogs that activate type II protein kinase A increased phosphorylate steroidogenic acute regulatory protein and further increased steroidogenesis. Previous *in vitro* studies also demonstrated that VIP inhibits the apoptosis of rat and mice follicles (Flaws *et al*., 1995; Vaccari *et al*., 2006) and stimulates the development of isolated bovine primary and early secondary follicles (Hulshof, 1995). Moreover, we reported that the addition of VIP to the *in vitro* culture medium maintained the survival and increased the diameter of the early preantral follicles enclosed in caprine ovarian tissue (Bruno *et al*., 2010). Recently, Wang *et al*. (2012) found that exogenous VIP inhibited goose granulosa cells apoptosis and enhanced the expression of Bcl-2 gene, but did not affect cell proliferation.

Follicle-stimulating hormone (FSH) acts through binding to the FSH receptor (FSHR), a G protein-coupled receptor superfamily member that is located exclusively in granulosa cells (Gudermann *et al*., 1995), from the primary follicle developmental stage onwards (Méduri *et al*., 2002). *FSHR* mRNA was detected in the preantral and antral follicles of goats(Saraiva *et al*., 2010a). FSH increased the *in vitro* survival and proliferation of granulosa cells, antrum

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formation and steroidogenesis in preantral follicles isolated from several species (mouse: Cortvrindt *et al*., 1997; sheep: Cecconi *et al*., 1999; buffalo: Sharma *et al*., 2009). In caprine species, FSH maintained the ultrastructural integrity, promoted the activation of primordial follicles and furthered the growth of preantral follicles enclosed in ovarian tissue (Matos *et al*., 2007; Magalhães *et al*., 2009). Moreover, the addition of FSH to the culture medium in a sequential way (day 0 to day 6 = 100 ng/ml; day 6 to day 12 = 500 ng/ml; day 12 to day 18 = 1000 ng/ml) maintained viability, stimulated antrum formation and reduced oocyte extrusion after 18 days of culture of isolated caprine secondary follicles (Saraiva *et al*., 2010a). In that same year, goat embryos were obtained from oocytes from isolated secondary follicles grown, matured and fertilized *in vitro* in the presence of FSH supplemented with growth hormone (Magalhães *et al*., 2011) or luteinizing hormone and epidermal growth factor (Saraiva *et al*., 2010b). FSH interacts with several intraovarian growth factors, which mediate its effect in regulating cellular interactions by autocrine and paracrine mechanisms, thus inducing follicular growth (Erickson and Shimasaki, 2001). Therefore, several growth and endocrine factors locally produced by the ovary are able to amplify or attenuate FSH action. However, among these factors, it is not known whether VIP, with or without an association with FSH, has an effect on the *in vitro* development of isolated caprine preantral follicles, as well as on *VIP* and *FSHR* mRNA levels.

Therefore, this study aimed to accomplish the following: (1) to determine the steady-state levels of *VIP* mRNA during different follicular stages in goat ovaries; (2) to investigate the influence of VIP and/or FSH on the development of isolated caprine preantral follicles after 6 days of *in vitro* culture; and (3) to verify the effects of VIP and/or FSH on *VIP* and *FSHR* mRNA levels in isolated caprine preantral follicles cultured for 6 days.

Material and Methods

Source of ovaries

Ovaries (n = 70) from 35 adult mixed-breed goats (*Capra hircus*, one to three years old) were collected at a local slaughterhouse, with 30 ovaries used for experiment 1 and 40 ovaries used for experiment 2. Immediately postmortem, the ovaries were washed in 70% alcohol followed by two washes in minimum essential medium (MEM). The ovaries were placed into tubes containing 15 ml of MEM plus HEPES (MEM HEPES) supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin, and they were transported to the laboratory at 4°C (Chaves *et al*., 2008) within 1 h. Unless mentioned otherwise, the culture media and other chemicals used in the present study were purchased from Sigma Chemical Co. (St Louis, USA).

Experiment 1: Steady-state levels of VIP mRNA in goat ovarian follicles

To evaluate steady-state mRNA levels, ovaries (n = 10) were used for the isolation of primordial, primary and secondary follicles using a mechanical procedure, as previously described (Lucci *et al*., 1999). After isolation, these follicles were washed twice to completely remove the stromal cells, and the follicles were then placed by category into separate Eppendorf tubes in groups of 10. This procedure was completed within 2 h, and all samples were stored at -80°C until the RNA was extracted.

The remaining ovaries were used for the collection of COCs, mural granulosa cells and thecal cells from small and large antral follicles. Compact COCs aspirated from small (1-3 mm) and large (3-6 mm) antral follicles were recovered from the ovaries (n = 15). Thereafter, groups of 10 COCs were stored at -80°C until RNA extraction. To collect mural granulosa and theca cell complexes, 10 small and large antral follicles were isolated from ovaries (n = 5) and dissected from stromal tissue as previously described (Van Tol and Bevers, 1998). The follicles were then bisected and granulosa and theca cell complexes were collected and stored at -80°C.

Isolation of total RNA from all categories preantral follicles (primordial, primary and secondary) and all components of small and large antral follicles (COCs, mural granulosa cells and theca cells) was performed using Trizol plus a purification kit (Invitrogen, São Paulo, Brazil). According to the manufacturer's instructions, 1 ml of Trizol solution was added to each frozen sample, and the lysate was aspirated through a 20-gauge needle before centrifugation at 10,000 g for 3 min at room temperature. All lysates were then diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding 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, which was comprised of 10 µl of sample RNA, 4 µl 5X reverse transcriptase buffer (Invitrogen), 8U RNAseout, 150U Superscript III reverse transcriptase, 0.036U random primers (Invitrogen), 10 mM DTT, and 0.5 mM of each dNTP. The mixture was incubated for 1 h at 42°C, for 5 min at 80°C, and then stored at -20°C. Negative controls were prepared under the same conditions but without the inclusion of the reverse transcriptase.

The quantification of *VIP* mRNA levels was performed using SYBR Green. PCR reactions were composed of 1 µl cDNA as a template in 7.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA), 5.5 µl of ultra-pure water, and 0.5 µm of each primer. The primers were designed to amplify *VIP*

mRNA. Glyceraldehyde-2-phosphate dehydrogenase (GAPDH) and beta-actin (Table 1) were used as endogenous controls for the normalization of the steady-state mRNA levels of the genes. 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 sec at 94°C, 30 sec at 60°C, and 45 sec at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a real time PCR Mastercycler (Eppendorf, Germany). The delta-delta-CT method was used to transform CT values into normalized relative steady-state mRNA levels.

Table 1. Primer pairs used for real-time PCR analyses.

Target gene	Primer sequence (5´→ 3´)	Sense	Position	GenBank accession nº
GAPDH	TGTTTGTGATGGGCGTGAACCA	S	287-309	GI:27525390
	ATGGCGTGGACAGTGGTCATAA	as	440-462	
Beta-actin	ACCACTGGCATTGTCATGGACTCT	s	187-211	GI:28628620
	TCCTTGATGTCACGGACGATTTCC	as	386-410	
UBQ	GAAGATGGCCGCACTCTTCTGAT	s	607-631	GI:57163956
	ATCCTGGATCTTGGCCTTCACGTT	as	756-780	
VIP	ACCAATCAAACGCCACTCAGATGC	s	360-384	GI:340253
	AGACTCTCCTTCACTGCTTCGCTT	as	483-507	
FSHR	AGGCAAATGTGTTCTCCAACCTGC	s	250-274	GI:95768228
	TGGAAGGCATCAGGGTCGATGTAT	as	316-340	

s= sense; as= antisense.

Experiment 2 - Effect of VIP and/or FSH on the follicular development and steady-state levels of VIP and FSHR mRNA

Isolation and selection of caprine preantral follicles

In the laboratory, the surrounding fat tissue and ligaments were stripped from the ovaries. Ovarian cortical slices (1-2 mm in diameter) were cut from the ovarian surface using a surgical blade under sterile conditions. Then, the ovarian cortex was placed in fragmentation medium consisting of MEM plus HEPES. Secondary follicles ≥200 µm in diameter were visualized under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) and manually dissected from the strips of ovarian cortex using 27.5 gauge (27.5 G) needles. After isolation, follicles were transferred to 100 µl drops containing fresh medium under mineral oil to further evaluate the follicular quality. Follicles with a visible oocyte, surrounded by granulosa cells, an intact basement membrane and with no antral cavity, were selected for culture.

Caprine preantral follicle culture

After selection, follicles were individually cultured in 100 µl drops of culture medium under mineral oil in Petri dishes (60 x 15 mm, Corning, USA). Control culture medium, called α-MEM$^+$, consisted of α-MEM (pH 7.2-7.4) supplemented with 3.0 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 hypoxantine and 50 µg/ml of ascorbic acid under mineral oil. Incubation was conducted at 39°C with 5% CO_2 in the air for 6 days. Fresh culture medium was prepared and incubated for 1 h prior to use. Preantral follicles obtained from each animal were randomly distributed in the following treatments: α-MEM$^+$ alone or supplemented with 100 ng/ml of recombinant FSH (rFSH: Tecnopec, Brazil), 10 ng/ml of VIP or both, constituting treatments α-MEM$^+$, FSH, VIP and VIP+FSH, respectively. Every other day, 60 µl of the culture media were replaced with fresh medium. The culture was replicated four times and a minimum of 35 follicles was used per treatment.

Morphological evaluation of follicle development

Follicles were classified according to their morphology, and those showing morphological signs of degeneration, such as darkness of oocytes and surrounding cumulus cells or those with misshapen oocytes, were classified as degenerated. Follicular diameter was measured only in healthy follicles in the *x* and *y* dimensions (90º), by using an ocular micrometer (100X magnification) inserted into a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan) every other day of culture. To measure follicular growth, the mean increase in follicular diameter was calculated as follows: the diameter of viable follicles on day 6 minus the diameter of viable follicles on day 0 divided by the total number of viable follicles on day 6. In addition, the percentages of secondary follicles that reached the antrum formation *in vitro* were determined. Antral cavity formation was defined as a visible translucent cavity within the granulosa cell layers.

Steady-state levels of VIP and FSHR mRNA in goat ovarian follicles cultured in vitro

To evaluate the effect of VIP on the expression of *VIP* and *FSHR* mRNA after a 6 day culture period, groups of 10 follicles were collected at the end of the culture period and stored at -80°C until the extraction of total RNA. Quantification of mRNA was performed a

previously described, and the primers for *VIP* and *FSHR* are shown in Table 1. Beta-actin and Ubiquitin (*UBQ*) were used as endogenous controls to normalize gene expression.

Statistical analysis

For the cell culture experiments, follicles were considered to be the experimental unit, following the same approach as reported by Silva *et al*. (2010). Data from the follicular survival and antrum formation for each treatment were compared using the Chi-square test, with the results expressed as percentages. Follicular diameter data were submitted to the Kolmogorov-Smirnov and Bartlett tests to confirm normal distribution and homoscedasticity, respectively. Because of the heterogeneity of the variances, the follicular diameter, growth rate, and *VIP* and *FSHR* mRNA levels before and after culture were compared using the Kruskal-Wallis non-parametric test, SAS, 1999. The results were expressed as the mean ± standard error of the mean (SEM), and differences were considered to be significant when $P < 0.05$.

Results

Experiment 1 - Steady-state levels of VIP mRNA in goat ovarian follicles

Quantification of mRNA demonstrated that primary and secondary follicles had significantly higher levels of *VIP* mRNA compared to the primordial follicle ($P < 0.05$; Fig. 1 A). In addition, COCs and granulosa/theca cells from large antral follicles had higher levels of *VIP* mRNA than small antral follicles ($P < 0.05$; Fig. 1 B, C). Real time PCR showed that COCs from both small and large antral follicles had significantly higher levels of *VIP* mRNA than their respective granulosa/theca cells ($P < 0.05$; Fig. 1 D, E).

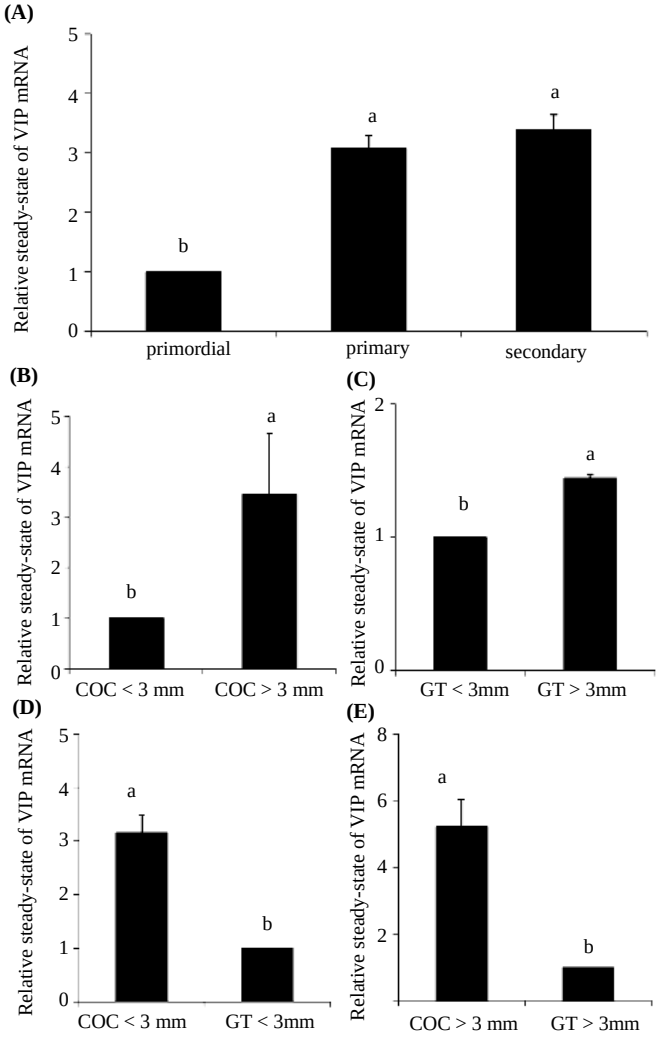

Figure 1. Steady-state levels of *VIP* mRNA in goat ovarian follicles (means ± SEM). A) Primordial, primary and secondary follicles. B) COCs from small and large antral follicles. C) Granulosa/theca cells from small and large antral follicles. D) COCs and granulosa/theca cells from small antral follicles. E) COCs and granulosa/theca cells from large antral follicles. [a,b]($P < 0.05$).

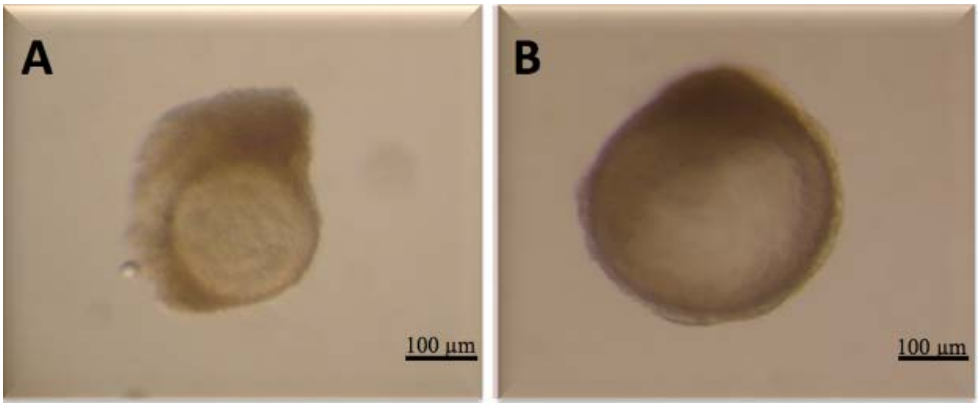

Figure 2. Preantral follicles from goats at day 0 (A) and antral follicles after 6 days of in vitro culture with 10 ng/ml VIP (B).

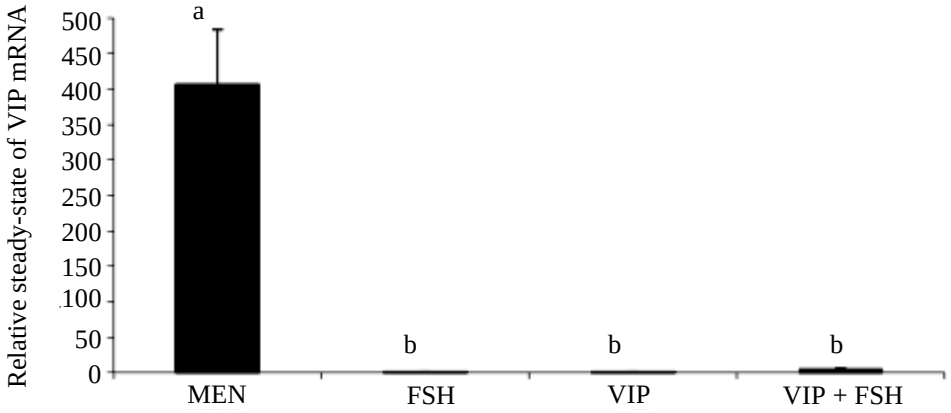

Figure 3. Steady state levels of *VIP* mRNA in goat preantral follicles cultured for 6 days in α-MEM^{+} supplemented with FSH, VIP or both. ^{a,b}($P < 0.05$).

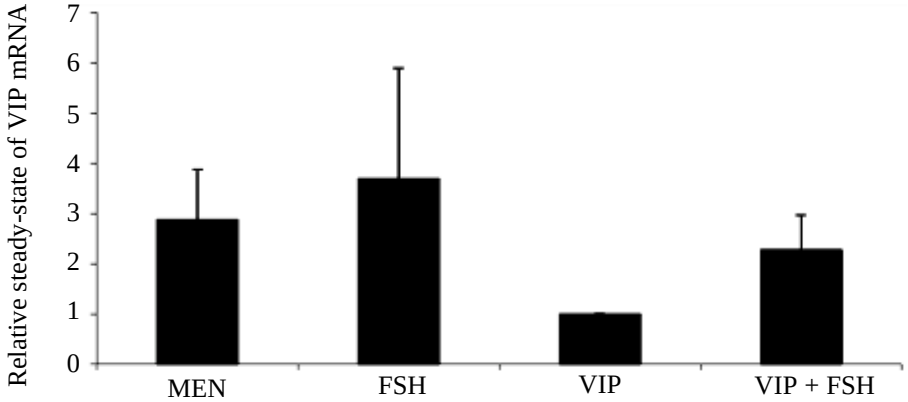

Figure 4. Steady state levels of *FSHR* mRNA in goat preantral follicles cultured for 6 days in α-MEM^{+} supplemented with FSH, VIP or both.

Experiment 2 - Effect of VIP and/or FSH on follicular development and steady-state levels of VIP and FSHR mRNA

Follicular survival and growth after in vitro *culture*

The rates of follicular survival were greater than 90% on day 6 of culture in all treatments tested. However, no significant differences were observed among the treatments (Table 2; $P > 0.05$). Figure 2 shows normal follicles before and after 6 days of *in vitro* culture. From as early as day 2 of culture, all treatments had follicles with an antral cavity. Furthermore, as the culture progressed from day 0 to day 6, a significant increase in the percentage of antral follicles was observed (data not shown; $P < 0.05$).

However, when the treatments were compared with each other, no significant differences were observed (P > 0.05).

The follicles in the *in vitro* culture showed an initial mean diameter of 221.59 ± 0.22, 237.73 ± 0.21, 223.94 ± 0.21 and 245.34 ± 0.22 µm for the α-MEM$^+$, FSH, VIP and VIP+FSH treatments, respectively (Table 2; P > 0.05). With the progression of the culture, an increase in follicular diameter was observed from day 0 to day 6 in all treatment groups (P < 0.05); however, no significant differences were observed among the treatments (P > 0.05). In addition, follicles cultured in α-MEM$^+$, FSH, VIP and VIP+FSH increased 16.94 ± 0.09, 19.22 ± 0.09, 18.89 ± 0.09 and 20.89 ± 0.08 µm/day, respectively. Similar to the diameter, no significant differences were observed when the treatments were compared to each other (P > 0.05).

Table 2. Survival (%), antrum (%) e diameter (µm) of goat preantral follicles cultured for 6 days in VIP and/or FSH.

Treatments	Survival (%) day 6	Antrum (%) day 6	Diameter ± SEM	
			day 0	day 6
α-MEM	100 (37/37)	72.97 (27/37)	221.59 ± 0.22^b	323.22 ± 0.27^a
FSH	91.89 (34/37)	72.97 (27/37)	237.73 ± 0.21^b	358.04 ± 0.30^a
VIP	97.30 (36/37)	75.68 (28/37)	223.94 ± 0.21^b	338.72 ± 0.27^a
VIP + FSH	100 (35/35)	85.71 (30/35)	245.34 ± 0.22^b	370.70 ± 0.27^a

Values with different letters between a column differ significantly (^{a,b}; P < 0.05).

Steady-state levels of VIP and FSHR mRNA in goat ovarian follicles cultured in vitro

Figure 3 shows *VIP* mRNA levels after 6 days of culture with the different treatments that were tested. Culture with FSH, VIP and VIP+FSH promoted a significant reduction in *VIP* mRNA levels in caprine preantral follicles after 6 days when compared with the control (P < 0.05). Regarding *FSHR* mRNA levels, no significant differences were observed among the various treatments (P > 0.05; Fig. 4).

Discussion

The current study demonstrates the presence of *VIP* mRNA in all follicular categories studied in goat ovaries, showing an increase in *VIP* mRNA levels during the transition from the primordial to the primary follicle. The presence of *VIP* mRNA (rat: Gozes and Tsafriri, 1986) and its receptors (mouse: Cecconi *et al*., 2004; Barberi *et al*., 2007; rat: Vaccari *et al*., 2006) were also detected in ovaries. VIP protein was also reported to increase with the appearance of secondary and antral follicles in bovine ovaries (Hulshof *et al*., 1994). These results suggest a role for this peptide in follicular growth.

COCs and granulosa/theca cells from large antral follicles had higher levels of *VIP* mRNA than small antral follicles, suggesting that VIP acts in a stage-dependent way because the mRNA expression levels increase with the progression of follicular development. In addition, COCs from either small or large antral follicles had higher *VIP* mRNA levels than their respective granulosa/theca cells. In rats, some studies have shown that VIP stimulates maturation in follicle-enclosed oocytes but could transiently inhibit, or not affect, the spontaneous maturation of cumulus-enclosed oocytes (T**ö**rnell *et al*., 1988; Apa *et al*., 1997). Furthermore, other studies have shown that VIP increases the levels of cAMP, which regulates oocyte maturation (Eppig and Downs, 1984) in preovulatory rat follicles (T**ö**rnell *et al*., 1988; Apa *et al*., 1997) and stimulates ovulation in perfused rat ovaries (Schmidt *et al*., 1990). In addition, VIP stimulates the production of plasminogen activator (Johnson and Tilly, 1988), which converts plasminogen into plasmin that acts in the follicular wall releasing the COCs.

In the current study, high rates of survival and antrum formation were observed after the *in vitro* culture period, and there was an increase in the follicular diameter after all treatments. These results occurred regardless of the addition of VIP and/or FSH to the culture medium. Previous *in vitro* studies have demonstrated that VIP inhibits apoptosis in rat preantral follicles (Flaws *et al*., 1995; Vaccari *et al*., 2006) and stimulates the development of isolated bovine primary and/or secondary follicles (Hulshof, 1995). Recently, a study performed by our research team has demonstrated that VIP is an important factor that promotes the growth of small preantral follicles enclosed in caprine ovarian tissue (Bruno *et al*., 2010). However, despite the fact that *VIP* mRNA was detected in caprine secondary follicles, no additional effect of this factor was observed after the culture of isolated follicles in this category when compared to the follicles cultured in α-MEM$^+$. This result may be due to the fact that α-MEM$^+$ is a medium that is rich in nutrients (amino acids, carbohydrates, B-vitamin complexes, vitamins C and D, inorganic salts and pyruvate) and DNA precursors, all of which promote cell division (Hartshorne, 1997). Another possible explanation for this finding is a different distribution of specific receptors in the different cell types surrounding the oocyte. Cecconi *et al*. (2004) demonstrated that VIP did not affect mouse follicular development and caused the inhibition of FSH stimulated follicular growth, antrum formation, granulosa cell proliferation and estradiol production. Moreover, other studies demonstrated that FSH inhibits follicle apoptosis (murine: Cortvrindt *et al*., 1998; bovine: Itoh *et al*., 2002), maintains the ultrastructural

integrity of goat preantral follicles after culturing cortical tissue (Saraiva *et al*., 2008), and promotes survival and antrum formation after an 18 day culture of isolated caprine secondary follicles (Saraiva *et al*., 2010a). However, in the present study, no additional influence of this hormone was observed after 6 days of culture when compared with the control medium. It is likely that the use of the different concentrations of FSH associated with a longer culture period of isolated follicles could improve the beneficial effects of FSH on follicular survival and viability.

In this study, the presence of VIP in the culture medium reduces the mRNA levels for itself. It is possible that the use of exogenous VIP, associated with its endogenous production during the culture, might have caused a down-regulation in the expression of *VIP* mRNA. Similarly, the addition of FSH to the culture medium reduces *VIP* mRNA levels. Flaws *et al*. (1995) verified that VIP can prevent follicular atresia in the absence of the influence of gonadotropin. Analysis of VIP receptor (VPAC1-R) mRNA levels in whole mouse ovaries showed that transcripts were present in untreated 22-day-old immature animals and were significantly down-regulated after gonadotropin stimulation (Barberi *et al*., 2007). These findings raise the possibility that VIP can promote the survival of follicles that have not yet acquired dependence upon gonadotropins for continued development. This proposal would be consistent with the fact that VIP can induce aromatase activity in immature follicles that are not yet responsive to FSH (George and Ojeda, 1987). Many of the effects of VIP on ovarian granulosa cells are similar to the action of gonadotropins (Ahmed *et al*., 1986; Johnson and Tilly, 1988; Ojeda *et al*., 1989; Johnson *et al*., 1994), suggesting that VIP may be important for regulating gonadotropin-independent development and ovarian follicle survival.

Although VIP stimulates the synthesis of cAMP, a key intracellular messenger involved in the formation of FSHR (Knecht *et al*., 1983; Tano *et al*., 1997), in the present study, the presence of VIP had no influence on *FSHR* mRNA levels in caprine preantral follicles cultured in vitro. Evidence also suggests the existence of cAMP-independent mechanisms, such as activin, that regulate the formation of FSHR in immature granulosa cells (Xiao *et al*., 1992; Nakamura *et al*., 1995; Tano *et al*., 1997). In contrast to our findings, VIP stimulation induced aromatase and FSHR expression at both the transcript and protein levels in rat neonatal ovaries several days before the ovaries became responsive to gonadotropins (Mayerhofer *et al*., 1997). In addition, there was no increase in *FSHR* mRNA expression when the medium was supplemented with FSH. It is possible that gonadotropins are not required for their own receptor expression during initial folliculogenesis, as shown in hpg/hpg mice, in which the development of *FSHR* mRNA levels was normal (O'Shaughnessy *et al*., 1997). Moreover, Saraiva *et al*. (2010a) used a sequential culture system with FSH and showed no increase in the expression of *FSHR* after 6 and 12 days of isolated caprine secondary preantral follicle culture. However, most studies show that gonadotropins stimulate the expression of their own receptors (Smith and Ojeda, 1986; Minegishi *et al*., 1997; Guglielmo *et al*., 2011). A second possible explanation for the limited response could be that FSH may have promoted a transcriptional down-regulation or decreased the stability of receptor mRNA induced by high FSH concentrations (Tilly *et al*., 1992; Tisdall *et al*., 1995; Xu *et al*., 1995).

In conclusion, the present study demonstrated that *VIP* mRNA was detected in all follicular categories and cellular types in caprine species, but levels of *VIP* mRNA in primary follicles were higher than the primordial follicle and COCs of both small and large antral follicles showed higher levels of *VIP* mRNA than their respective granulosa/theca cells. In the culture conditions used here, the presence of VIP and/or FSH did not affect follicular survival and development after 6 days of *in vitro* culture, in addition to reduces the mRNA levels for *VIP*, however without influences the mRNA levels for *FSHR*. More detailed studies on the action mechanisms of VIP on follicular development *in vivo* and *in vitro* are essential to better understand folliculogenesis. Furthermore, future applications of *in vitro* follicle culture systems would improve fertility preservation for humans and rare animal species.

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