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Phytohemagglutinin improves the development and ultrastructure of *in vitro*-cultured goat (*Capra hircus*) preantral follicles

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Abstract

The objective this study was to determine the effect of phytohemagglutinin (PHA) on survival, growth and gene expression in caprine secondary follicles cultured *in vitro*. Secondary follicles (~0.2 mm) were isolated from the cortex of caprine ovaries and cultured individually for 6 days in α-MEM$^+$ supplemented with PHA (0, 1, 10, 50, 100, or 200 μg/mL). After 6 days of culture, follicle diameter and survival, antrum formation, ultrastructure and expression of mRNA for FSH receptors (FSH-R), proliferating cell nuclear antigen (PCNA), and neuronal nitric oxide synthase were determined. All treatments maintained follicular survival [α-MEM$^+$ (94.59%); 1 μg/mL PHA (96.43%); 10 μg/mL PHA (84.85%); 50 μg/mL PHA (85.29%); 100 μg/mL PHA (88.57%), and 200 μg/mL PHA (87.50)], but the presence of 10 μg/mL PHA in the culture medium increased the antrum formation rate (21.21%) when compared with control (5.41%, $P < 0.05$) and ensured the maintenance of oocyte and granulosa cell ultrastructures after 6 days of culture. The expression of mRNA for FSH-R (2.7 ± 0.1) and PCNA (4.4 ± 0.2) was also significantly increased in follicles cultured with 10 μg/mL PHA in relation to those cultured in α-MEM$^+$ (1.0 ± 0.1). In conclusion, supplementation of culture medium with 10 μg/mL PHA maintains the follicular viability and ultrastructure, and promotes the formation of antral cavity after 6 days of culture *in vitro*.

Key words: Caprine; Antrum formation; FSH-R; PCNA; Ultrastructure

Introduction

The development of efficient culture systems is extremely important both to understand the mechanisms that regulate follicular development and to assure the *in vitro* follicular growth up to the stage at which the oocytes are capable of being matured and fertilized *in vitro*. In different species, great advances have been made in culturing preantral follicles. In human, bovine and canine species, secondary follicles have grown *in vitro* until the stage of antral follicles (1-3). More satisfactory results were obtained in porcine (4), bubaline (5), ovine (6), and caprine (7) species, since a small number of embryos have been obtained after fertilization of oocytes from *in vitro*-grown secondary follicles. To improve this technique in domestic species it is necessary to study substances that may contribute to promote the oocyte growth and proliferation of granulosa cells. Several hormones and growth factors, such as follicle-stimulating hormone (FSH) (8,9), growth and differentiation factor-9 (10) and kit ligand (11), have been tested during the culture of caprine preantral follicles. Among them, FSH is the main regulator of ovarian function and its receptor has been demonstrated in goat preantral follicles (7). However, the effects of substances not produced by the ovaries, such as lectins, have not been described.

Lectins have been isolated from various sources and have a wide spectrum of biological activities (12). Most of them are proteins or glycoproteins that specifically bind to carbohydrates. In addition to recognizing sugars, some lectins promote mitogenic stimulation of lymphocytes (13), leukocytes (14) and fibroblasts (15). Phytohemagglutinin

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(PHA) is a lectin extracted from *Phaseolus vulgaris*, which bind to complex oligosaccharide containing N-acetylgalactosamine/galactose residues (15). Due to its ability to stimulate subpopulations of T cells, the PHA has been used as a mitogen to increase proliferation of various cell types *in vitro* (16). Fagbohun and Downs (17) demonstrated that mitogenic lectins, such as PHA, promote oocyte maturation and cumulus cell expansion in rats. On the other hand, Wang et al. (18) showed that the PHA has no apparent effect during maturation of bovine oocytes. Furthermore, PHA has been used to improve the efficiency of the nuclear transfer of somatic cells for oocytes from diverse species (19), because its capacity to induce closer contacts between adjacent cell membranes. In addition, PHA can stimulate the production of nitric oxide (NO) in different cells (20,21). Some studies have shown that neuronal NO synthase (nNOS) is expressed in ovarian follicles (22) and is involved in the control of folliculogenesis, steroidogenesis, oocyte maturation, and ovulation through the production of NO (23).

The aim of this study was to evaluate the effect of different concentrations of PHA on survival, growth, antrum formation, and ultrastructure of caprine secondary follicles cultured *in vitro*. In addition, the effect of PHA on the expression of mRNA for proliferating cell nuclear antigen (PCNA), FSH, FSH receptors (FSH-R), and nNOS was analyzed.

Material and Methods

Chemicals

Unless otherwise stated, the culture media, the lectin PHA (Cat. No. L-1668) and other chemicals were purchased from Sigma-Aldrich Corp. (USA). The stock solution of the lectin PHA was prepared in phosphate-buffered saline, pH 7.2, and stored at -20°C.

Source of ovaries

Ovaries (n = 42) from 21 adult (1 to 3 years old) crossbreed goats (*Capra hircus*) were collected at a local slaughterhouse. Immediately postmortem, the surrounding fat tissue and ligaments were removed and the ovaries were washed in 70% alcohol followed by two washes in sterile saline solution. The ovaries were placed into tubes containing 20 mL alpha minimum essential medium (α-MEM), supplemented with 200 IU/mL penicillin and 150 μg/mL streptomycin and then transported to the laboratory at 4°C within 1 h.

Isolation and *in vitro* culture of preantral follicles

Ovarian cortical slices (1 mm) were cut from the ovarian surface, using a surgical blade under sterile conditions. The slices were subsequently placed in fragmentation medium, consisting of α-MEM supplemented with 150 IU/mL penicillin and 150 μg/mL streptomycin. Secondary follicles (~0.2 mm) were visualized under a stereomicroscope (SMZ 645 Nikon, Japan) and manually dissected from strips of ovarian cortex using 26 gauge (26 G) needles. Follicles with a visible oocyte, surrounded by two or more granulosa cell layers, an intact basement membrane and no antral cavity were selected for culture. After selection, follicles were cultured individually in 100 μL drops of culture medium under mineral oil on Petri dishes (60 × 15 mm, Corning, USA). The basic culture medium (α-MEM$^+$) consisted of α-MEM, pH 7.2-7.4, supplemented with 3.0 mg/mL bovine serum albumin, 1% ITS (10 μg/mL insulin, 5.5 μg/mL transferrin, and 5 ng/mL selenium), 2 mM glutamine, 2 mM hypoxanthine, 50 μg/mL ascorbic acid, 150 IU/mL penicillin, 150 IU/mL streptomycin, and 100 ng/mL FSH (from sheep pituitary, Sigma). Follicles were randomly distributed in each of the following treatments: α-MEM$^+$ alone (control) and α-MEM$^+$ associated with lectin PHA at concentrations of 1, 10, 50, 100, or 200 μg/mL. Incubation was carried out at 39°C and 5% CO_2 in air for 6 days. Previous studies have shown that goat preantral follicles begin their growth from 24 h of *in vitro* culture and antrum formation occurs after 6 days (24). Fresh media were prepared before use and incubated for 2 h prior to use. Every other day, 60 μL of the culture media was replaced with fresh medium. The culture was replicated four times, and at least 28 follicles were cultured per treatment.

Morphological evaluation of follicular development

After 6 days of culture, follicles were classified according to their morphology. A follicle was considered to be normal when presented a centrally located spherical and homogeneous oocyte, surrounded by compact layers of granulosa cells, and without apparent damage to the basement membrane in the beginning of culture. Those follicles that showed morphological signs of degeneration, such as darkness of oocytes and surrounding granulosa cells or those with misshapen oocytes, were considered to be degenerated. The follicular growth, survival and the presence/absence of the antral cavity were evaluated every 2 days of culture. Antral cavity formation was defined as a visible translucent cavity within the granulosa cell mass. Follicular diameter was measured only in healthy follicles by calculating two perpendicular diameters using Motic Images Plus 2.0. In order to better examine follicular morphology, transmission electron microscopy was performed to analyze the ultrastructure of caprine secondary follicles grown in control medium and in the treatment that provided the best.

Ultrastructural analysis of follicles cultured *in vitro*

Transmission electron microscopy was used to analyze the ultrastructure of preantral follicles cultured with α-MEM$^+$ alone or supplemented with 10 μg/mL PHA. Cultured follicles were fixed in Karnovsky solution (4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for at least 4 h at room temperature (approximately 25°C). After fixation, follicles

were embedded in drops of 4% low melting agarose, and kept in sodium cacodylate buffer. Specimens were post-fixed in 1% osmium tetroxide, 0.8% potassium ferricyanide and 5 mM calcium chloride in 0.1 M sodium cacodylate buffer for 1 h at room temperature, washed in sodium cacodylate buffer and counterstained with 5% uranyl acetate. The samples were then dehydrated through a gradient of acetone solutions and thereafter embedded in epoxy resin (Epoxy-Embedding Kit, Fluka Chemika-BioChemika, Switzerland). Afterwards, semi-thin sections (2 μm) were cut, stained with toluidine blue and analyzed by light microscopy at a 400X magnification. Ultra-thin sections (70 nm) were obtained from caprine preantral follicles classified as morphologically normal in semi-thin sections. Subsequently, ultra-thin sections were counterstained with uranyl acetate and lead citrate, and examined under a Morgani-FEI transmission electron microscope (FEI, The Netherlands).

Expression of mRNA for FSH-R, PCNA and nNOS in cultured follicles

To evaluate RNA expression, three groups of 8 follicles, from three different replicates, cultured either in control medium or in medium supplemented with 10 μg/mL PHA were collected and stored in microcentrifuge tubes at -80°C.

Total RNA was extracted using the TRIzol® reagent (Invitrogen, Brazil). According to manufacturer instructions, 1 mL Trizol solution was added to each frozen samples and the lysate was aspirated through a 20-gauge needle before centrifugation at 10,000 *g* for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 Kunitz U/mL) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 μL RNAse-free water. The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophotometer (Amersham Biosciences, England). Before the reverse transcription reaction, samples of RNA were incubated for 5 min at 70°C and then cooled in ice. From 2 μg of total RNA, the reverse transcription was performed in a total volume of 20 μL composed of 10 μL sample RNA, 4 μL reverse transcriptase buffer (Invitrogen), 8 U RNAsin, 150 U reverse transcriptase Superscript III, 0.036 U random primers, 10 mM DTT and 0.5 mM of each dNTP (Invitrogen). The mixture was incubated at 42°C for 1 h, subsequently at 80°C for 5 min, and finally stored at -20°C. The negative control was prepared under the same conditions, but without addition of reverse transcriptase.

Quantification of the mRNA for FSH-R, PCNA, and nNOS was performed by using SYBR Green. Each reaction in real-time (20 μL) containing 10 μL SYBR Green Master Mix® (Applied Biosystems, UK), 7.3 μL ultrapure water, 1 μL cDNA and 5 μM of each primer. Real-time PCR was performed in a thermocycler (Mastercycler® ep Realplex, Eppendorf, Germany). The primers designed to perform amplification of mRNA for FSH-R, PCNA and nNOS are shown in Table 1. This Table also shows the primers for β-actin and phosphoglycerate kinase, which were used as endogenous controls for normalization of gene expression. The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 10 min at 95°C, followed by 50 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C. The final extension was for 10 min at 72°C. The delta-delta-CT method was used to transform CT values into normalized relative expression levels (25).

Table 1. Primer pairs used in real-time PCR for quantification of FSH-R, PCNA and nNOS in cultured caprine follicles with 10 μg/mL phytohemagglutinin.

Target gene	Sequence (5′→3′)	Position	GenBank No.
β-actin	S: ACCACTGGCATTGTCATGGACTCT	188-211	GI: 28628620
	AS: TCCTTGATGTCACGGACGATTTCC	363-386	
PGK	S: AGCCTTCCGAGCTTCACTTT	444-466	GI: 77735550
	AS: AAACCTCCAGCCTTCTTTGGCA	541-563	
FSH-R	S: AGGCAAATGTGTTCTCCAACCTGC	250-274	GI: 95768228
	AS: TGGAAGGCATCAGGGTCGATGTAT	316-340	
PCNA	S: TGCCGAGATCTCAGTCACAT	566-586	GI: 77735938
	AS: TATGGCAACAGCTTCCTCCT	695-715	
nNOS	S: TGGAGGATGTGGCCAAGAAGATGA	1835-1859	GI: 358416368
	AS: TCTGGAAGCTGTACAGAACCGCAA	1942-1966	

S = sense; AS = antisense; PGK = phosphoglycerate kinase; FSH-R = follicle-stimulating hormone receptors; PCNA = proliferating cell nuclear antigen; nNOS = neuronal nitric oxide synthase.

Table 2. Follicular diameters of caprine secondary follicles cultured for 6 days in α-MEM$^+$ supplemented with different concentrations of PHA.

Period (days)	α-MEM$^+$	PHA (1 μg/mL)	PHA (10 μg/mL)	PHA (50 μg/mL)	PHA (100 μg/mL)	PHA (200 μg/mL)
0	181.30 ± 41.70^b	179.78 ± 47.66^b	173.98 ± 46.91^b	160.00 ± 49.39^b	181.49 ± 56.38^b	173.08 ± 48.15^b
2	199.98 ± 50.83^{ab}	197.67 ± 56.10^a	201.04 ± 58.11^{ab}	186.49 ± 58.81^{ab}	216.24 ± 69.20^a	206.50 ± 66.24^a
4	214.29 ± 59.30^a	205.64 ± 64.10^a	215.88 ± 66.40^a	194.30 ± 57.34^{ab}	228.08 ± 69.76^a	217.07 ± 62.14^a
6	225.96 ± 65.26^a	212.30 ± 60.01^a	228.34 ± 76.50^a	200.90 ± 54.27^a	230.76 ± 70.92^a	223.51 ± 64.25^a

Data are reported as means ± SD. α-MEM$^+$ = alpha minimum essential medium; PHA = phytohemagglutinin. Different superscript letters indicate statistically significant differences within a column ($P < 0.05$, paired *t*-test).

Statistical analyses

The percent of follicular survival and antrum formation after *in vitro* culture were compared by the Fisher exact test and the results are reported as percent. The data corresponding to the follicular diameter were subjected to the Shapiro-Wilk test and the Bartlett test for verification of normal distribution and homoscedasticity, respectively. Follicular diameters show homogeneity of variance and were compared by the paired *t*-test. The results are reported as means ± SD and differences were considered to be significant when $P < 0.05$. The levels of mRNA for FSH-R, PCNA, and nNOS in follicles cultured in the treatments were examined by the Mann-Whitney U-test. The results are reported as means ± SE and differences were considered to be significant when $P < 0.05$.

Results

Effect of PHA on survival and growth of goat secondary follicles

At the end of the period of culture, all treatments were capable of maintaining follicular survival and no significant difference ($P > 0.05$) among them were observed [α-MEM$^+$ (94.59%); 1 μg/mL PHA (96.43%); 10 μg/mL PHA (84.85%); 50 μg/mL PHA (85.29%); 100 μg/mL PHA (88.57%), and 200 μg/mL PHA (87.50%)]. Regarding the follicular diameter, from day 0 to 6 of culture, a significant increase in follicular diameter was observed in all treatments ($P < 0.05$), but no differences were found among them (Table 2).

Regarding the antrum formation, at the end of culture, follicles cultured in the presence of 10 μg/mL PHA showed a significant increase in antrum formation when compared to the control medium (α-MEM$^+$). On the other hand, the percent of antrum formation in follicles cultured with 1, 50, 100, and 200 μg/mL PHA did not differ either from the control treatment or among each other (Table 3).

Ultrastructural analysis of follicles cultured *in vitro*

The ultrastructural analysis showed that follicles cultured in α-MEM$^+$ had an abnormal profile, the oocyte cytoplasm was extremely vacuolated, having a greater open area and organelles were no longer recognizable in ooplasm (Figure 1A). Despite a regular zona pellucida and well-organized granulosa cells surrounding the oocyte, these follicles had reduced number of microvilli (Figure 1A). On the other hand, follicles cultured in medium supplemented with 10 μg/mL PHA (Figure 1B and C) presented a well-preserved oocyte, with visible organelles, such as Golgi complex, endoplasmic reticulum and lipid droplets. Mitochondria had some swollenness, but this feature is common in cultured cells. Granulosa cells were present with normal organelles and regular chromatin. Zona pellucida was preserved, and microvilli were evident.

Levels of mRNA for FSH-R, PCNA and nNOS in cultured follicles

Levels of mRNA for FSH-R, PCNA and nNOS in follicles cultured in α-MEM$^+$ alone or supplemented with

Table 3. Percentages of follicles showing signs of antrum formation after culture of secondary follicles in α-MEM$^+$ supplemented with different concentrations of PHA.

Period (days)	α-MEM$^+$	PHA (1 μg/mL)	PHA (10 μg/mL)	PHA (50 μg/mL)	PHA (100 μg/mL)	PHA (200 μg/mL)
0	0.00% (00/37)	0.00% (00/28)	0.00% (00/33)	0.00% (00/34)	0.00% (00/35)	0.00% (00/00)
2	0.00% (00/37)	0.00% (00/28)	6.06% (02/33)	0.00% (00/34)	0.00% (00/35)	0.00% (00/00)
4	0.00% (00/37)	3.57% (01/28)^A	12.12% (04/33)^A	0.00% (00/34)	5.71% (02/35)^A	0.00% (00/00)
6	5.41% (02/37)^B	7.14% (02/28)^{AB}	21.21% (07/33)^A	5.88% (02/34)^{AB}	5.71% (02/35)^{AB}	0.00% (00/00)

Data are reported as percent with number of antrum formations in parentheses. α-MEM$^+$ = alpha minimum essential medium; PHA = phytohemagglutinin. Different superscript letters indicate statistically significant differences within a row ($P < 0.05$, Fisher exact test).

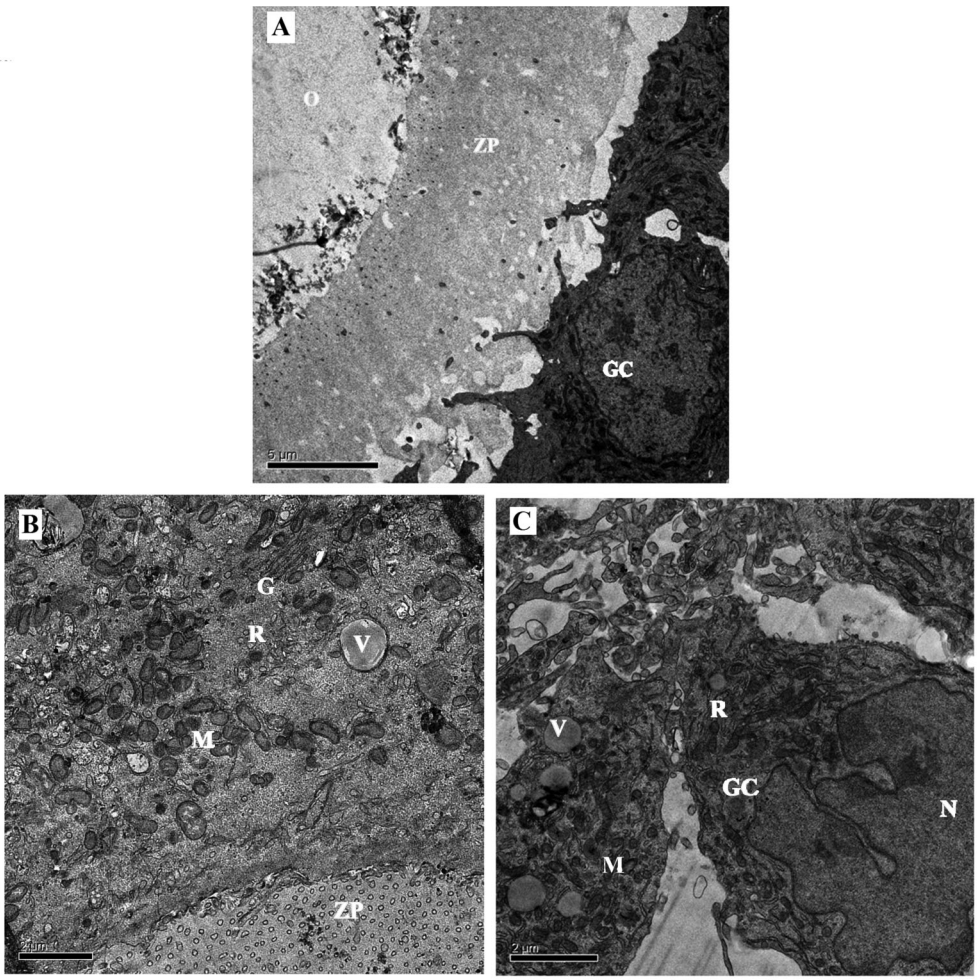

Figure 1. Transmission electron microscopy micrographs of (*A*) follicle cultured in α-MEM$^+$ showing abnormal ultrastructure, with no visible organelles and ooplasm; zona pellucida was present, with a paucity of microvilli. *Panels B* and *C* show follicles cultured with 10 µg/mL PHA with normal mitochondria, Golgi complex, endoplasmic reticulum, and zona pellucida. O = oocyte; N = nucleus; M = mitochondria; R = endoplasmic reticulum; ZP = zona pellucida; V = vesicles; GC = granulosa cells. Bars: *A* = 5 µm; *B* and *C* = 2 µm.

10 µg/mL PHA are shown in Figure 2. No significant difference in the levels of mRNA for nNOS between follicles cultured in control medium or medium supplemented with PHA was observed. However, the presence of PHA significantly increased the levels of mRNA for both PCNA and FSH-R, when compared with the control medium (Figure 2).

Discussion

The present study demonstrated that PHA (10 µg/mL) stimulates antrum formation, helps to keep ultrastructure integrity and increases the expression of FSH-R and PCNA in caprine preantral follicles cultured *in vitro*.

Morphological analysis showed that after 6 days of culture, high survival rates were observed in all the treatments. This is probably due to the use of a culture medium rich of nutrients such as vitamins, amino acids and minerals that have been successfully used to culture preantral follicles (26). FSH is known to support follicle viability by inhibiting the expression of anti-apoptotic proteins (27) and its presence in the culture medium certainly helped to maintain follicles viable. Other studies have shown that FSH is very important for preantral follicle survival in different species (2,28). On the other hand, despite the lack of a dose/response relationship, the presence of 10 µg/mL PHA increased the rate of formation of antral cavity. This may be associated with increased expression of the FSH receptors stimulated by PHA at this concentration. Previous *in vitro* studies have shown that this gonadotropin improves formation of antrum in cultured secondary follicles in caprine (9) and mouse (29) species.

The ultrastructural analysis confirmed the integrity of

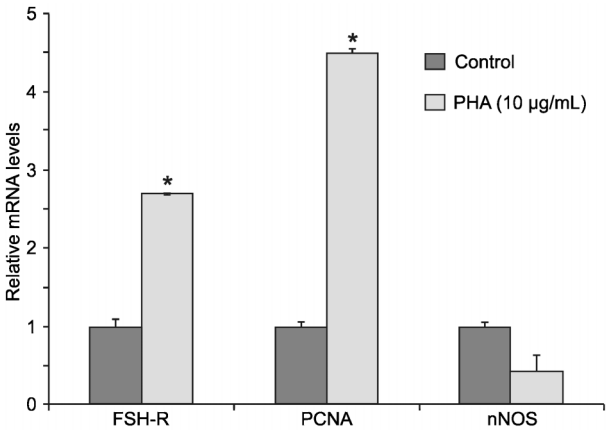

Figure 2. Relative expression of mRNA for FSH-R, PCNA and nNOS in caprine secondary follicles cultured for 6 days with 10 µg/mL PHA. FSH-R = follicle-stimulating hormone receptors; PCNA = proliferating cell nuclear antigen; nNOS = neuronal nitric oxide synthase; PHA = phytohemagglutinin. Data are reported as means ± SD. *$P < 0.05$ compared to control (Mann-Whitney U-test).

follicles cultured in medium supplement with 10 µg/mL PHA. Previous dose response studies have shown that high concentrations of PHA (>80 µg/mL) have toxic effects on cultured fibroblasts, while low concentrations (0.1-10 µg/mL) have no positive effects (30). In agreement with these data, the present study has demonstrated that the optimal concentration of PHA to stimulate gene expression and antrum formation in cultured preantral follicles is 10 µg/mL. The increased expression of the FSH receptors stimulated by PHA may have contributed to maintain follicular ultrastructure. Previous *in vitro* studies have shown that FSH kept ultrastructural characteristics of caprine-cultured follicles (9,28). Studies using mice with deficiencies in FSH-R expression have allowed further elucidation of the role of FSH in ovarian follicles and it has been reported that mice lacking the FSH-R gene have structural alterations in the ovary (31). Furthermore, there is evidence to suggest that FSH-R deletion results in changes in oocyte structure and function, and disruption of oocyte-granulosa cell communication (32). In addition, PHA facilitates close cell contact by binding to the N-linked carbohydrate core structure (beta 1-6 branching) of glycoproteins on the cell membrane (33). In this study, this lectin may have recognized and mediated adhesion between carbohydrates present in granulosa cells and oocyte, maintaining the follicular ultrastructure. The communication between granulosa cells during the preantral and early antral stages is necessary to ensure subsequent oocyte developmental competence (34). The oocyte is the central regulator of follicular cell functions through the secretion of soluble growth factors such as BMP-15 and GDF-9, which act in the surrounding follicular cells to promote their proliferation and survival (35).

The addition of 10 µg/mL PHA to the culture medium increased the expression of mRNA for PCNA, indicating an influence of this lectin on granulosa cell proliferation, but a dose/response relationship was not demonstrated. PCNA performs the essential function of providing replicative polymerases with the high processivity required to duplicate the entire genome (36) and has been used as a marker of granulosa cell proliferation in various species (8,37,38). Although lectin PHA has a known role in the mitogenesis of various cell types (14-16), the increase in PCNA expression did not reflect an increase in follicular diameter during the culture, probably because of the short culture period. Although extensively studied, the mechanism of stimulation of mitosis by lectins is still not understood. It has been suggested that mitogenic lectins interact with components of cell membranes to stimulate cell proliferation (13).

The present study demonstrates the expression of nNOS in goat ovarian follicles, but no increase in the levels of mRNA for nNOS was observed in response to PHA stimulation during *in vitro* culture. The nNOS is an enzyme responsible for the synthesis of NO that was recently demonstrated to be expressed in bubaline granulosa cells and oocytes in different stages of development, indicating its role in the control of follicular growth (24). Nitric oxide can act as a pro- or anti-apoptotic agent in a variety of structures, including ovarian follicles (39). At low concentrations, NO has anti-apoptotic action, but with the increase of its production, it causes DNA damage and induces cell death by apoptosis (39). The role of NO in regulating ovarian function and reproductive systems has been described by Rosselli et al. (40), who observed correlations between ovarian hormones and NO production, since an increase in NO is correlated with increased steroidogenesis. Previous studies have shown that PHA can stimulate the production of NO in different cellular types (22,23), but this was not observed in cultured preantral follicles.

Addition of 10 µg/mL PHA during *in vitro* culture caprine secondary follicles stimulates antrum formation, increases the expression of FSH-R and PCNA, and helps to keep ultrastructural integrity of cultured follicles. These data may be useful for the development of an efficient culture system to promote oocyte growth and maturation *in vitro*.

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