DOI: 10.21451/1984-3143-AR935

Anim. Reprod., v.14, n.4, p.1095-1102, Oct./Dec. 2017

Platelet-derived growth factor-BB (PDGF-BB) improves follicular survival, oocyte and follicular diameters, in a dose-dependent manner, after the *in vitro* culture of goat preantral follicles enclosed in ovarian tissue fragments

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Abstract

The aims of this study were to investigate the effects of different concentrations of Platelet-derived growth factor-BB **(**PDGF-BB) on the survival, activation, levels of ROS, and growth of goat preantral follicles enclosed in ovarian tissue. For this, ovarian fragments were cultured for 7 days in Alpha Minimum Essential Medium (α-MEM$^+$) with or without PDGF-BB (0, 25, 50 and 100 ng/ml). The results showed that both the 25 ng/ml PDGF and the 50 ng/ml PDGF treatments maintained the percentage of morphologically normal follicles from day 1 to day 7. In addition, the 25 ng/ml PDGF treatment showed a significantly higher percentage of morphologically normal follicles when compared to the other treatments. At day 7, greater ($P < 0.05$) follicular and oocyte diameters were observed in the 25 ng/ml PDGF and the 50 ng/ml PDGF treatments when compared to the cultured control treatment. On day 7 of culture, all the treatments tested had a significant increase in the percentage of developing follicles when compared to the non-cultured control. However, the percentage of follicle activation, as well as ROS production, were similar ($P < 0.05$) among the treatments, irrespective of culture time. In conclusion, PDGF-BB improved, in a concentration-dependent manner, follicular survival as well as oocyte and follicular diameter after *in vitro* culture of goat preantral follicle-enclosed in ovarian tissue fragments.

Keywords: goat, *in vitro* culture, PDGF-BB, preantral follicles.

Introduction

Folliculogenesis is defined as the formation of primordial follicles, their recruitment into the growing pool, and their further progression to the preovulatory stage in the ovary (Oktem and Oktay, 2008). In this process, proper metabolic bidirectional communication among the oocyte and somatic cells inside the follicle is crucial to control the follicular fate (Van Den Hurk and Zhao, 2005). Understanding the signals responsible for the initiation of folliculogenesis is an important step towards developing a successful *in vitro* culture system (Chaves *et al*., 2010). Thus, to achieve this goal, preantral follicles have been cultured in different *in vitro* culture systems, i.e., isolated form (Brito *et al*., 2014) or enclosed in ovarian tissue (*in situ* form; Bruno *et al*., 2009).

The *in vitro* culture of isolated follicles of farm animals aims to study mainly late preantral folliculogenesis, including the development of advanced secondary follicles to tertiary follicles (antral follicles). On the other hand, *in situ* culture of preantral follicles is an effective approach to study factors implicated in the regulation of early follicular development (primordial, intermediate, primary and early secondary stages). Among these factors, platelet-derived growth factor (PDGF) can be highlighted. PDGF is a dimeric glycoprotein composed of four different polypeptide chains, which can be linked by disulphide bonds in various combinations of two to five isoforms, as follows: PDGF-AA, -BB, -AB, -CC, and -DD (Rubin *et al*., 1988; Young *et al*., 1990; Berridge, 1993; Gaultier and Michel, 1999).

Several studies have shown that different PDGF isoforms, in a concentration-dependent manner, stimulate follicular activation (transition from primordial to developing follicles, when surrounding squamous pre-granulosa cells become cuboidal and begin to proliferate and grow; Fortune *et al*., 2000; Fortune, 2003; McLaughlin and Mclver, 2008; Kim, 2012) as well as the proliferation of granulosa and theca cells (rats: Duleba *et al*., 1999; Nilsson *et al*., 2006; humans: Hwu *et al*., 2009). In goats, our team demonstrated that PDGF-B increased follicular growth and antrum formation rates of isolated advanced secondary follicles in a concentration-dependent manner (Brito *et al*., 2012). Furthermore, it was verified in this species that there was the expression of PDGF-BB mRNA and protein in the early preantral and antral follicles (Brito *et al*., 2015). Despite the encouraging results achieved in goats using isolated secondary follicles in an advanced stage of development, the originality of the present work is based on the lack of information about the effect of PDGF-BB on early folliculogenesis, i.e., the culture of early preantral follicles (primordial, intermediate, and primary). This study is justified by the fact that follicular requirements vary according to the developmental stage. To date, the pattern of gene expression has been analyzed in goats in the transition from secondary to early tertiary follicles,

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Received: December 12, 2016
Accepted: July 12, 2017

and that analysis showed that there were ~2,466 genes specific only to this stage of development (Magalhães-Padilha *et al*., 2013).

Reactive oxygen species (ROS) are produced in metabolic and physiological cellular process. However, under certain conditions (i.e. *in vitro* culture), an imbalance in the ROS production may occur, which can jeopardize the survivability and development of these follicles *in vitro* (Talebi *et al*., 2012). Moreover, some studies have been investigating ROS production as an additional parameter of the *in vitro* culture's efficiency (Aguiar *et al*., 2016a, b; 2017; Paes *et al*., 2016). In fact, our research group has been measuring ROS levels in some articles (Carvalho *et al*., 2014; Castro *et al*., 2014; Sá *et al*., 2017) throughout the entire period of culture with the goal to demonstrate the efficacy of *in vitro* culture.

Therefore, to understand the effect of PDGF-BB on early folliculogenesis in goats better, the aim of this study was to investigate the effects of different concentrations of PDGF-BB on the survival, activation, growth, and levels of ROS of preantral follicles enclosed in ovarian tissue fragments.

Material and Methods

Chemicals

Recombinant human PDGF-BB, culture media, and other chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO, USA) unless mentioned otherwise.

Source of ovaries

Ovaries (n = 10) from 5 adult (1 to 3 years old), non-pregnant, mixed-breed goats (*Capra hircus*) were collected at a local slaughterhouse. Immediately postmortem, the ovaries were washed in 70% alcohol for 10 sec and then 2 times in minimum essential medium (MEM) supplemented with 100 µg/ml penicillin and 100 µg/ml streptomycin. Pairs of ovaries were transported within 1 h to the laboratory in MEM at 4°C (Chaves *et al*., 2008).

In vitro *culture of ovarian tissue*

In the laboratory, the ovaries from each animal were stripped of surrounding fat tissue and ligaments. The ovarian cortex of each pair of ovaries was then divided into 18 slices measuring approximately 3 x 3 mm and 1 mm thick using a needle and scalpel under sterile conditions. For each animal, two slices of tissue were randomly selected and immediately fixed for histological examination (non-cultured control: day 0). The remaining slices of ovarian cortex (17 slices) were placed individually in 24-well culture dishes, each well containing 1 ml of culture medium. The basic medium (cultured control), referred to as α-MEM$^+$, consisted of α-MEM supplemented with 10 µg/ml insulin, 5.5 µg/ml transferrin, and 5 ng/ml selenium, 2 mM glutamine, 2 mM hypoxanthine, and 1.25 mg/ml BSA. The ovarian cortex slices were cultured for 1 or 7 days at 39°C in humidified air with 5% CO_2.

Experimental design

For the experimental conditions, the base medium (α-MEM$^+$) was supplemented with PDGF-BB at different concentrations, as follow: PDGF 0 ng/ml (day 1 and day 7), PDGF 25 ng/ml (day 1 and day 7), PDGF 50 ng/ml (day 1 and day 7), and PDGF 100 ng/ml (day 1 and day 7). Each treatment was repeated five times (replicates), and the culture medium was changed every 2 days. The concentrations of PDGF-BB (50 and 100 ng/ml) used herein were chosen based on the previous study with the *in vitro* culture of caprine secondary follicles (Brito *et al*., 2012). In addition to these concentrations, we also included a lower concentration (25 ng/ml) of PDGF-BB to provide a concentration curve, which had not yet been established. At the end of each culture time (day 1 and day 7), all treatments were evaluated for morphology, development, and follicular and oocyte diameters. In addition, spent culture media at the end of 24 h of culture at day 1 and at day 7 of culture (after medium replacement at day 6) were stored at -80°C for ROS analysis.

Morphological analysis and assessment of in vitro *follicular growth*

Tissues from all treatments (fresh control or cultured for 1 or 7 days) were fixed in 4% neutral buffered paraformaldehyde for 12 h and then dehydrated in increasing concentrations of ethanol. After being embedded in paraffin (Synth, São Paulo, Brazil), the caprine tissue pieces were cut into 7 µm sections, and every section was mounted on glass slides and stained by periodic acid-Schiff-hematoxylin. The follicle stage and survival were assessed microscopically on serial sections. Coded anonymized slides were then examined by microscopy (Nikon, Sendai, Japan) at 400X magnification.

The developmental stages of follicles have been defined as primordial (oocyte surrounded by a few flattened granulosa cells) or developing, i.e., intermediate (oocyte surrounded by flattened 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). These follicles were classified as morphologically normal (follicles containing an intact oocyte and granulosa cells well-organized in layers without a pyknotic nucleus) and degenerated follicles (oocyte with a pyknotic nucleus, retracted cytoplasm, or disorganized granulosa cells detached from the basement membrane), as described by Silva *et al*. (2004).

Overall, 150 follicles were evaluated for each treatment (30 follicles per treatment in one repetition x 5 repetitions). To calculate follicular activation and growth, only morphologically normal follicles with a visible oocyte nucleus are recorded, and the proportion of primordial and growing follicles is calculated at day 0 and after day 1 or day 7 of the culture in all treatments. In addition, from the basement membrane, major and minor axes of each normal oocyte and follicle were measured using a light microscope fitted with an eyepiece micrometer (Zeiss, Cologne, Germany) under400X magnification. The average of these two

measurements was used to determine the diameters of both the oocyte and the follicle.

Assessment of preantral follicle viability by fluorescence microscopy

Additional pairs of ovaries (n = 3) were collected from a slaughterhouse and then cut into fragments at the laboratory. Then, two fragments were immediately processed for follicle isolation (non-cultured tissue), and the remaining fragments were cultured for 7 days in basic culture medium (α-MEM^{+}) or in the treatment group (25 ng/ml PDGF-BB treatment) that provided the best outcome, i.e. a significantly higher percentage of morphologically normal follicles at day 7 of culture when compared with the other treatments. Goat preantral follicles were isolated from ovarian fragments using the mechanical method described by Lucci *et al*. (1999). Briefly, samples were cut into small pieces with a tissue chopper (The Mickle Laboratory Engineering Co., Gomshal, Surrey, UK) and adjusted to a sectioning interval of 75 µm. Therefore, samples were placed in MEM supplemented with 3 mg/ml BSA and were suspended 100 times with a large Pasteur pipette (inner diameter, 1600 µm) and 100 times with a smaller Pasteur pipette (inner diameter, 600 µm) to dissociate the preantral follicles from the stroma. The material obtained was then passed through a 200-µm nylon mesh filter. This procedure was performed within 10 min at room temperature. The viability of the preantral follicles was assessed by the trypan blue dye exclusion test. Briefly, 5 ml of 0.4% trypan blue (Sigma Chemical Co., St. Louis, MO, USA) was added to 100 ml of isolated and suspended preantral follicles, which were incubated for 1 min at room temperature. Subsequently, the follicles were examined with an inverted microscope (Nikon, Tokyo, Japan) and classified as nonviable or viable if they were positively or negatively stained with trypan blue, respectively.

Reactive oxygen species (ROS) analysis

The levels of reactive oxygen species were determined in spent culture media using a spectrofluorometric method (Loetchutinat *et al*., 2005). For this, culture media from all PDGF-BB-treated groups were incubated with 10 µl of 2',7'-dihidrodiclorofluorescein diacetate (DCHF-DA; 1 mM). The oxidation of DCHF-DA to dichlorofluorescein was measured for the detection of reactive species in the medium. The intensity of fluorescence emission was recorded at 520 nm (with 480-nm excitation) for 2 h after the addition of DCHF-DA to the medium.

Statistical analysis

Data for continuous variables (morphologically normal, primordial, and developing follicles) were initially submitted for Shapiro-Wilk and Bartlett tests to evaluate the normal distribution of the residues and homoscedasticity, respectively. Furthermore, confirming both requirements of the underlying analysis of variance (ANOVA), it was carried out using the GLM procedure of SAS (2002) according to a 4 × 2 factorial arrangement of groups, considering the PDGF-BB treatments and time of culture as the main effects. When any main effect or interactions were significant, means were compared accordingly by the Student-Newman-Keuls (SNK) test, whilst Dunnett's test was applied to compare the PDGF-BB-treated groups against the control, with the results expressed as means ± standard deviation (SD). Ordinarily, data for follicular viability were analyzed as the dispersion of frequency by a chi-square test, and the results were expressed as percentages. In all cases, differences were considered to be significant when $P < 0.05$.

Results

Effect of PDGF on follicular morphology

The percentages of morphologically normal preantral follicles (Fig. 1A) in non-cultured control and after 1 or 7 days of culture are shown (Table 1). After 1 day of culture, all treatments resulted in lower ($P < 0.05$) percentages of morphologically normal follicles than the non-cultured control group except when follicles were cultured in the absence of PDGF (0 ng/ml; $P > 0.05$). With the progression of the culture period from 1 to 7 days, no change in the percentage of morphologically normal follicles was observed in the 25 ng/ml PDGF and 50 ng/ml PDGF treatments ($P > 0.05$). In addition, at day 7 the 25 ng/ml PDGF treatment showed a significantly higher percentage of morphologically normal follicles when compared with the other treatments.

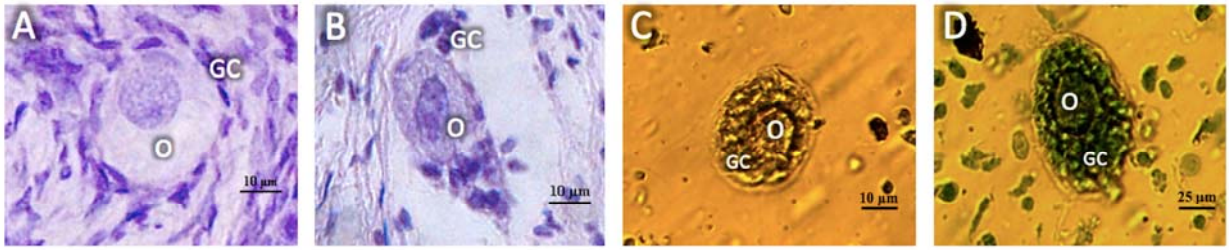

Figure 1. Photomicrographs of preantral follicles analyzed by optical microscopy. Histological sections after staining with periodic acid Schiff-hematoxylin, showing (A) normal preantral follicle and (B) degenerated follicle after culture in control medium (0 ng/ml PDGF-BB) for 7 days. Note the retracted oocyte (B). O: Oocyte; GC: Granulosa cells. Assessment of the viability of preantral follicles using trypan blue stain after culture for 7 days. Viable (C) or nonviable (D) isolated preantral follicle after *in vitro* culture in control medium (0 ng/ml PDGF-BB).

Table 1. Percentage of morphologically normal caprine preantral follicles in the fresh control (non-cultured) group and after *in vitro* culture for 1 or 7 days in the absence or presence of different concentrations of PDGF-BB.

Treatments	Normal follicles (%)	
Control (Day 0)	82.00 ± 1.82	
PDGF	Day 1	Day 7
0 ng/mL	76.67 ± 4.08 ^{Aa}	58.00 ± 3.80 ^{*Bb}
25 ng/mL	68.67 ± 1.82 ^{*Ab}	65.33 ± 2.98 ^{*Aa}
50 ng/mL	48.67 ± 5.05 ^{*Ad}	52.67 ± 4.35 ^{*Ac}
100 ng/mL	56.00 ± 4.35 ^{*Ac}	48.00 ± 5.05 ^{*Bc}

*Values differ significantly from fresh non-cultured controls (P < 0.05). ^{AB}Values with different uppercase superscript letters differ between columns (days) (P < 0.05). ^{abcd}Values with different lowercase superscript letters differ among rows (treatments) (P < 0.05).

Follicular development after in vitro *culture*

The percentages of primordial and developing follicles (intermediate, primary, and secondary) in fresh tissue or in tissues cultured for 1 or 7 days with different treatments are shown (Fig. 2A and 2B). Fresh ovarian tissues predominantly contained primordial follicles (69.10%). Under all culture conditions, after 7 days of culture, a significant increase in the percentage of developing follicles was observed compared with the non-cultured control, as well as when compared with day 1 of the culture (Fig. 2B). From day 1 to day 7, in all cultured treatments, a significant decrease was observed in the percentage of primordial follicles with a concomitant increase in the percentage of developing follicles. However, the percentage of developing follicles was similar (P > 0.05) among treatments regardless of the culture time.

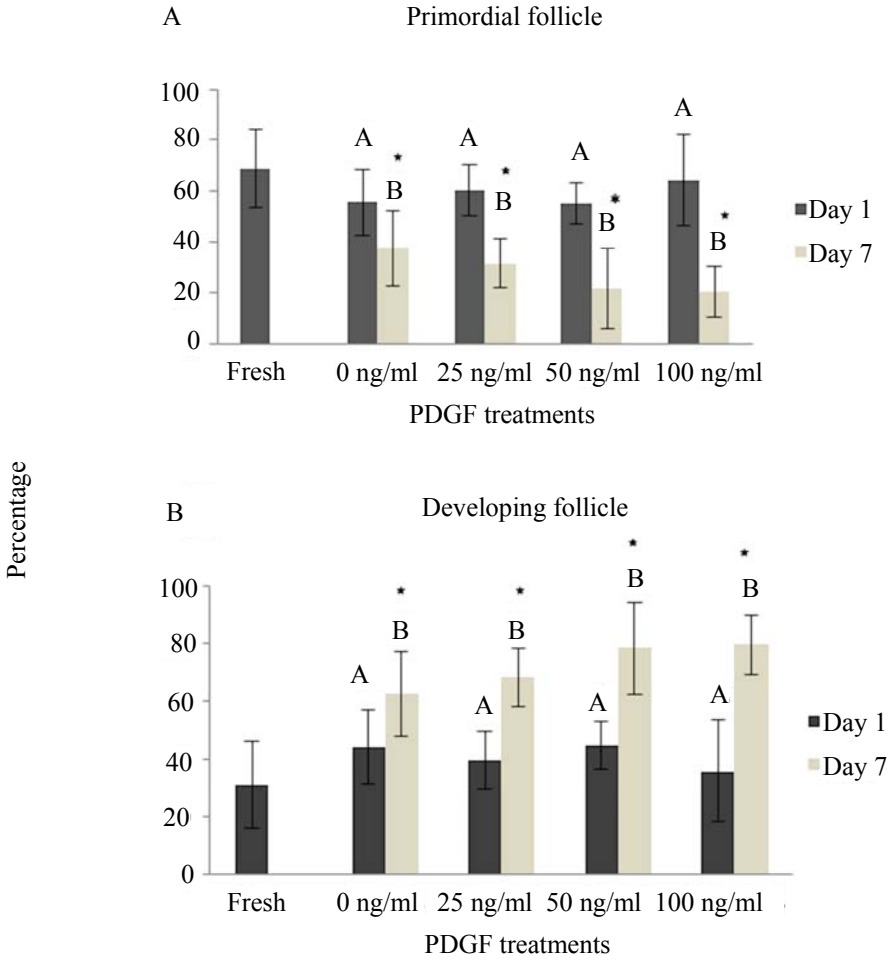

Figure 2. Percentage (mean ± SD) of (A) primordial and (B) growing follicles (intermediate, primary and secondary) in the fresh control (non-cultured tissue) and 1 or 7 days cultured tissue in the absence or presence of various concentrations of PDGF-BB. *Values differ significantly from fresh controls (P < 0.05). ^{AB}Values with different superscripts differ between days (P < 0.05) and among treatments within the same culture period (P > 0.05).

Follicle and oocyte diameters were measured, and the results are shown (Table 2). After 1 day of culture for all treatments, except for the 25 ng/ml PDGF treatment, mean oocyte and follicular diameters were higher than in non-cultured control (P < 0.05). However, after 7 days of culture no significant

differences were observed between the treatments and non-cultured control. Overall, oocyte and follicular diameters decreased significantly from day 1 to day 7, except for 25 ng/ml PDGF (follicle and oocyte) and 50 ng/ml PDGF (only for follicle) treatments. In addition, at day 7 of culture, the 25 ng/ml and 50 ng/ml PDGF treatments showed similar oocyte and follicular diameters, being both higher ($P < 0.05$) than 0 ng/ml PDGF alone (cultured control).

Assessment of follicle viability after culture

Follicles were classified as viable (Fig. 1C) or nonviable (Fig. 1D) if they were negatively or positively stained with trypan blue, respectively. After 7 days of culture, the percentage of viable follicles was similar between 0 ng/ml (68.33%) and 25 ng/ml (65.04%) PDGF-BB treatments, being both lower ($P < 0.05$) than the non-cultured control (72.73%) group.

ROS production

ROS production was evaluated after 1 and 7 days (Table 3) of culture in all tested treatments. The ROS production was not affected ($P > 0.05$) neither by the treatments nor by the culture time.

Table 2. Goat oocyte and follicle diameters (mean ± SD) in the non-cultured control and 1 or 7 days cultured tissue in the absence or presence of various concentrations of PDGF-BB.

Treatments	Follicular diameter (µm)		Oocyte diameter (µm)	
Control (Day 0)	24.78 ± 7.69		18.94 ± 1.39	
	Day 1	Day 7	Day 1	Day 7
0 ng/mL	$31.29 \pm 9.87^{*Aa}$	22.21 ± 6.48^{Bc}	$23.30 \pm 5.84^{*Ab}$	15.46 ± 3.39^{Bc}
25 ng/mL	26.31 ± 7.19^{Ab}	27.43 ± 5.40^{Aab}	18.34 ± 3.45^{Ac}	18.62 ± 1.99^{Aab}
50 ng/mL	$34.68 \pm 6.05^{*Aa}$	30.97 ± 9.68^{Aa}	$26.21 \pm 4.83^{*Aa}$	20.13 ± 4.32^{Ba}
100 ng/mL	$32.79 \pm 7.70^{*Aa}$	24.81 ± 5.15^{Bbc}	$24.13 \pm 5.70^{*Aab}$	16.08 ± 3.19^{Bbc}

*Values differ significantly from fresh non-cultured controls ($P < 0.05$). ^{AB}Values with different uppercase superscript letters differ between columns (days) ($P < 0.05$). ^{abc}Values with different lowercase superscript letters differ among rows (treatments) ($P < 0.05$).

Table 3. ROS production (mean ± SD) in the 1 or 7 days cultured tissue in the absence or presence of various concentrations of PDGF-BB.

Treatments	ROS production	
	Day 1	Day 7
0 ng/mL	20.11 ± 2.33	22.97 ± 1.97
25 ng/mL	20.38 ± 0.90	25.45 ± 4.73
50 ng/mL	21.05 ± 2.08	24.41 ± 4.20
100 ng/mL	22.98 ± 2.94	20.49 ± 1.59

There was no significant difference between treatments ($P > 0.05$).

Discussion

The present study demonstrated for the first time the effects of PDGF-BB on the *in vitro* survival, activation, and growth of early caprine preantral follicles (primordial, intermediate, and primary) enclosed in ovarian tissue.

In our study, at day 7 only, we observed a higher percentage of normal follicles in the 25 ng/ml PDGF treatment than in the other treatments, including the cultured control after histological analysis. In other words, PDGF affects early goat preantral follicle survival in a concentration and culture time-dependent manner. Some studies suggest that the role of PDGF in follicular survival occurs through the PI3K pathway. Taylor (2000) showed that PDGF increased phosphatidylinositol-3-kinase (PI3-kinase) expression in a porcine theca cell cultured *in vitro*. Moreover, PDGF has potent mitogenic, chemotactic, and antiapoptotic effects on different cell types (Vanhaesebroeck *et al*., 1997). In the present study, in addition to histological analyses, a cell viability test using trypan blue was performed in order to confirm follicle survival (Paes *et al*., 2016). We found that despite the treatment containing 25 ng/ml PDGF having a greater follicular survival compared to the cultured control group (PDGF 0 ng/ml), no difference was observed between these treatments after the evaluation of follicular viability when using trypan blue. This apparently contradictory result could be explained by the differences between the techniques (histology *vs*. viability test with trypan blue). Similar results after using the same techniques were reported in previous studies with canine (Lopes *et al*., 2008, 2016), equine (Haag *et al*., 2013) and ovine (Lunardi *et al*., 2012). It is known that in follicles with advanced stages of atresia, the basement membrane is discontinuous and in some cases full of holes, allowing for, as an example, the infiltration of leukocytes (Bagavant *et al*., 1999). Therefore, considering that atresia makes the basement membrane structure more fragile i.e., subject to rupture, the degenerate follicles detected within the ovarian tissue after histological analysis are more likely to be destroyed during the mechanical isolation of preantral follicles prior to the viability test using trypan blue and, consequently, no longer be detected by this test. As a matter of fact, follicle isolation involves a series of ovarian tissue cutting followed by the mechanical dissociation of the resulting fragments through pipetting (Lunardi *et al*., 2012).

In a previous study in mice, Nilsson *et al*. (2006) performed a short-term culture (2 days) of mouse ovaries and found that the addition of PDGF to a base medium (D-MEM Ham's F-12) significantly increased the primordial follicle activation rate and rose the mRNA expression of kit ligand (KL) in relation to the control treatment. However, in our study using a longer culture period, after 7 days of culture, all treatments increased the percentage of developing follicles compared to the non-cultured control. In addition, regardless of the treatment, the percentage of developing follicles increased significantly from day 1 to day 7 of the culture. However, during the culture, the proportion of developing follicles was similar among the treatments, demonstrating that PDGF did not affect primordial follicle activation. These findings are in agreement with previous studies (Fortune *et al*., 1998; Cushman *et al*., 2002) that demonstrated how the activation of primordial follicles can occur spontaneously, i.e., without the addition of growth factors or hormones.

Concerning follicle and oocyte diameters, contrary to 100 ng/ml of PDGF-BB, PDGF-BB at 25 and 50 ng/ml increased both parameters as compared to 0 ng/ml PDGF alone (cultured control) after 7 days of culture. This result shows the important role of PDGF in a concentration-dependent manner on *in vitro* follicular and oocyte growth. Brito *et al*. (2012) employed a different culture system from the one used in this work (culture of isolated large secondary follicles rather than primordial follicles enclosed in ovarian tissue) and reported that the addition of 50 ng/ml PDGF, in the presence of FSH, promoted *in vitro* follicular growth. Furthermore, this previous work showed a higher mRNA expression of PDGF receptors in caprine developing follicles than primordial follicles, suggesting an important role in the proliferation of follicular cells (Sleer and Taylor, 2007). Lee (2000) suggested that PDGF action on cell proliferation can occur via the binding to its receptor and can be followed by the activation of PI3K and Src signaling pathways resulting in the growth and differentiation of follicular cells.

However, the higher concentration of PDGF-BB (100 ng/ml) was not able to increase follicular and oocyte diameters. In fact, a previous study demonstrated that an overstimulation of PDGF-BB receptors makes insensible the PI3K or other pathways, jeopardizing the oocyte growth (Liu *et al*., 2014). In our study, we found that of regardless the PDGF-BB concentration used, ROS production was similar to the cultured control. This data shows that the best follicular survival rates observed in the presence of 25 ng/ml of PDGF, compared to the cultured control, were not associated with a reduced production of ROS. This fact indicates that the ROS levels were adequate for the functioning of cultured preantral follicles. Production of ROS has been used to assess *in vitro* oocyte quality (Ou *et al*., 2012; Martinho *et al*., 2014), and the balance between the production and degradation of ROS is an indicator of oxidative regulation (Rizzo *et al*., 2012; Winterbourn, 2014).

Previous studies have shown that ROS can act as second messengers and that adequate levels play an essential physiological and biochemical role, stimulating cell proliferation and differentiation (Talebi *et al*., 2012). However, in contrast to our study, Rigacci *et al*. (1997) found that the PDGF addition to the culture medium (D-MEM) increased glutathione and hydrogen peroxide levels in response to PDGF receptor activation in murine fibroblasts, contributing to the reduction of ROS levels. A possible reason for these differences might be the different cell types (ovarian tissue *vs*. fibroblasts) and culture conditions (5% CO_2/39°C using α-MEM *vs*. 8% CO_2/ 7°C using D-MEM).

In conclusion, PDGF-BB improved, in a concentration-dependent manner, follicular survival as well as oocyte and follicular diameter after *in vitro* culture of goat preantral follicles enclosed in ovarian tissue fragments.

Acknowledgments

This work was supported by this study was sponsored by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq grant number 407594/2013-2). R.F. Silva was supported by a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) Fellowship.

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