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Leptin decreases apoptosis and promotes the activation of primordial follicles through the phosphatidylinositol-3-kinase/protein kinase B pathway in cultured ovine ovarian tissue

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Research Article

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Summary

This study evaluated the effects of leptin on primordial follicle survival and activation after *in vitro* culture of ovine ovarian tissue and if leptin acts through the phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) pathway. Ovarian fragments were fixed for histology (fresh control) or cultured for 7 days in control medium (α-MEM$^+$) alone or supplemented with leptin (1, 5, 10, 25 or 50 ng/ml). Follicle morphology, activation and apoptosis were analyzed. Next, the fragments were cultured in the medium that showed the best results in the absence or the presence of the PI3K inhibitor (LY294002), and immunohistostaining of p-Akt protein was assessed. After culture, the percentage of normal follicles decreased ($P < 0.05$) in all treatments compared with the fresh control. Moreover, control medium and 1 ng/ml leptin had similar ($P > 0.05$) percentages of normal follicles, which were significantly higher than those in other treatments. However, culture with 1 ng/ml leptin maintained apoptosis similarly ($P > 0.05$) to that of the fresh control and lower ($P < 0.05$) than that in α-MEM$^+$. Leptin did not influence follicle activation ($P > 0.05$) compared with the control medium (α-MEM$^+$). Culture in 1 ng/ml leptin with LY294002 decreased the normal follicles and increased apoptosis, inhibited follicle activation ($P < 0.05$), and reduced p-Akt immunostaining, compared with the medium containing 1 ng/ml leptin without PI3K inhibitor. In conclusion, leptin at 1 ng/ml reduces apoptosis and promotes the activation of primordial follicles compared with the fresh control after *in vitro* culture of ovine ovarian tissue possibly through the PI3K/Akt pathway.

Introduction

In vitro culture of ovarian cortical tissue has become an important tool to elucidate the unknown mechanisms of early folliculogenesis (Jones and Shikanov, 2019) and to support the development of the primordial follicle pool, to allow their future use in assisted reproductive techniques in humans and other mammalian species (Figueiredo *et al.*, 2019). Moreover, experiments using *in vitro* ovarian cultures allow faster screening of the follicular response to different substances such as growth factors, hormones, antioxidants and/or reproductive toxicants (Stefansdottir *et al.*, 2014; Guerreiro *et al.*, 2016, 2019; Santos *et al.*, 2019). Improvements in *in vitro* follicle culture will therefore enhance current fertility preservation strategies (Bus *et al.*, 2019). In this context, considerable research efforts have been undertaken to optimize the culture medium by supplying additional substances in order to reduce follicle apoptosis and to ensure optimum follicle growth. One potential candidate for medium supplementation is the hormone leptin.

Leptin, a 16 kDa adipokine primarily secreted by adipose tissue, was initially identified as a signal that regulates food intake and energy expenditure (Tsai *et al.*, 2012). In recent years, it has also been implicated in a wider range of physiological functions, including reproduction (Bilbao *et al.*, 2015; Taskin *et al.*, 2019; Zieba *et al.*, 2020). In mammals, *in vitro* studies have demonstrated that leptin enhances the development of isolated preantral (ovine 10 ng/ml leptin: Kamalamma *et al.*, 2016; Kumar *et al.*, 2019; and ovine 25 ng/ml: Macedo *et al.*, 2019) and early antral follicles (ovine 2 ng/ml: Menezes *et al.*, 2019), improves the developmental capacity of the oocytes and increases blastocyst rates (bovine 1 and 10 ng/ml: Boelhauve *et al.*, 2005; bubaline 10 and 50 ng/ml: Sheykhani *et al.*, 2016; 10 ng/ml: Panda *et al.*, 2017; and swine 10 and 100 ng/ml: Craig *et al.*, 2004).

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Some effects of leptin are mediated by its interaction with its receptor (LEPR) (Fruhbeck, 2006). Both leptin and LEPR proteins have already been demonstrated in follicular cells in caprine (Batista *et al.*, 2013) and ovine (Macedo *et al.*, 2019) species. The interaction between leptin and LEPR can activate the phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) pathway (Aragonès *et al.*, 2016; Seoane-Collazo *et al.*, 2020), which appears to be the main signalling pathway that regulates the growth and survival of primordial follicles (mice: Zhao *et al.*, 2014; swine: Moniruzzaman *et al.*, 2010; ovine: Santos *et al.*, 2019). In a human ovarian carcinoma cell line (OVCAR-3), leptin stimulates cell growth through the PI3K pathway (Chen *et al.*, 2013).

Despite the satisfactory results of leptin on isolated follicles, there has been no report of the effects of leptin on the *in vitro* culture of ovarian tissue in large mammals. Moreover, to optimize current culture systems for ovarian cortex, a better understanding of the signalling processes involved in follicle survival and activation is needed (Devos *et al.*, 2020). However, it is not known whether the PI3K/Akt pathway is involved in leptin effects on ovarian tissue. Therefore, the aims of this study were to analyse the effects of leptin on the survival, activation and *in vitro* growth of sheep primordial follicles cultured in ovarian cortical fragments and to verify if the PI3K/Akt signalling pathway is involved in leptin action.

Materials and methods

Unless noted otherwise, all chemicals used in this study were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

Source of ovarian tissue

Ovaries ($n = 20$) were collected at a local abattoir from 10 adult (1–3 years old) mixed-breed sheep for *in vitro* culture with different concentrations of leptin ($n = 10$ ovaries) or with the PI3K inhibitor ($n = 10$ ovaries). Immediately postmortem, the ovaries were washed once in 70% alcohol (Dinâmica, São Paulo, Brazil) followed by two washes in minimum essential medium buffered with HEPES (MEM–HEPES) and supplemented with antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin) and transported to the laboratory in MEM–HEPES at 4ºC (Chaves *et al.*, 2008).

In vitro *culture of ovarian tissue*

In vitro culture was performed according to Santos *et al.* (2019). In the laboratory, ovarian cortex samples from each ovarian pair ($n = 10$ ovaries from five sheep) were cut into slices approximately 3 mm × 3 mm (1-mm thick) in size using a needle and scalpel under sterile conditions. For each animal, one tissue slice was randomly selected and fixed for histological analysis (fresh control). The remaining slices of the ovarian cortex were cultured individually in 1 ml of culture medium in 24-well culture dishes for 7 days at 39°C in humidified air containing 5% CO_2. The base culture medium (control) consisted of α-MEM (pH 7.2–7.4) supplemented with 10 ng/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenium, 2 mM glutamine, 2 mM hypoxanthine, 50 µg/ml ascorbic acid and 1.25 mg/ml bovine serum albumin (BSA), which is referred to as α-MEM$^+$. To verify the influence of leptin on the *in vitro* culture of sheep ovarian cortex, fragments were cultured in α-MEM$^+$ (control) or in α-MEM$^+$ supplemented with different concentrations of human recombinant leptin (1, 5, 10, 25 or 50 ng/ml), which were chosen based on previous studies (Kammalama *et al.*, 2016; Macedo *et al.*, 2019). The culture medium was replenished every 2 days. Each treatment was repeated five times, therefore using the ovaries of five different animals.

Morphological analysis and evaluation of in vitro *follicular activation and growth*

For morphological examination, tissues from the fresh control and from all cultured treatments were fixed in 10% buffered paraformaldehyde (Dinâmica) for 18 h, dehydrated using graded ethanol (Dinâmica), clarified in xylene (Dinâmica), and embedded in paraffin (Dinâmica). Haematoxylin and eosin (Vetec, São Paulo, Brazil) was used to stain 5-µm thick serial sections for routine histological examination under a light microscope (Nikon, Tokyo, Japan; ×400 magnification). The preantral follicles were classified as histologically normal when an intact oocyte was present and surrounded by granulosa cells that were well organized in one or more layers and had no condensed nuclear chromatin. Atretic follicles were defined as those with a shrunken or vacuolated oocyte, condensed chromatin, disorganized granulosa cells detached from the basement membrane, and/or cell swelling (Santos *et al.*, 2019). Blind follicle counting was performed on every fifth section of the ovaries by one experienced investigator.

For assessment of the follicular activation and growth (follicle and oocyte diameters), only histologically normal follicles with visible oocyte nucleus were recorded. The evaluation of follicular activation was performed by quantifying the follicles at different stages of development (Silva *et al.*, 2004), i.e. primordial (one layer of flattened granulosa cells around the oocyte) or growing follicles (intermediate: one layer of flattened to cuboidal granulosa cells; primary: one layer of cuboidal granulosa cells or secondary: two or more layers of cuboidal granulosa cells around the oocyte). The proportion of primordial and growing follicles was calculated at day 0 (fresh control) and after 7 days of culture. In addition, from the basement membrane, the major and minor axes of morphologically normal follicles and oocytes were measured using the Image-Pro Plus software (Media Cybernetics Inc.). The average of these two measurements was used to determine the diameters of the follicle and the oocyte, respectively.

Assessment of apoptosis by immunohistochemistry to active-caspase-3

After histological analysis, for a more in-depth evaluation of follicular quality, immunohistochemical analysis was performed in the fresh control and in treatments that showed the best results regarding follicular morphology. Immunohistochemistry was performed as described previously with some modifications. Briefly, sections (3-µm thick) from each block were cut using a microtome (EasyPath, São Paulo, Brazil) and mounted in Starfrost glass slides (Knittel, Braunschweig, Germany). The slides were incubated in citrate buffer (Dinâmica) at 95ºC in a decloaking chamber (Biocare, Concord, USA) for 40 min to retrieve antigenicity; endogenous peroxidase activity was prevented by incubation with 3% H_2O_2 (Easypath) for 10 min. Nonspecific binding sites were blocked using 1% normal goat serum (Easypath). Subsequently, the sections were incubated in a dark humidified chamber for 50 min at room temperature with rabbit polyclonal anti-activated caspase-3 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Thereafter, the sections were incubated for 20 min in EasyLink One polymer (Easypath). Protein localization was demonstrated with diaminobenzidine (DAB; Easypath), and the

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sections were counterstained with haematoxylin (Vetec) for 1 min. Negative controls (reaction control) were performed in the absence of the primary antibody. Follicles were analyzed using the above-mentioned light microscope connected to a computer equipped with Image-Pro Plus® software (Media Cybernetics), and if the oocyte and/or 70% of the surrounding granulosa cells were positively stained (brown staining), the follicle was considered apoptotic (adaptation of Langbeen *et al.*, 2016). The percentage apoptosis was calculated as the number of apoptotic follicles out of the total number of follicles (×100).

Pharmacologic inhibition of the PI3K pathway

The aim was to test the hypothesis that pharmacological inhibition of the PI3K pathway would inhibit the actions of leptin on primordial follicle survival and activation *in vitro*. For this *in vitro* culture, additional pairs of sheep ovaries ($n = 10$ ovaries from five animals) were collected, transported to the laboratory, and fragmented as described above. Ovarian fragments were cultured for 7 days in α-MEM$^+$ supplemented with 1 ng/ml leptin (concentration that showed the best results in the previous culture) in the absence or the presence of the PI3K inhibitor. For PI3K inhibition, the manufacturer recommended treating the cultured cells with LY294002 for 1 h before and for the duration of the stimulation. Therefore, 50 µM of the PI3K inhibitor LY294002 (Cell Signalling Technologies, Danvers, MA) was added to the α-MEM$^+$ for 1 h at 39°C under 5% CO_2. Next, the medium was supplemented with leptin. Total replacement of the medium was performed every 2 days for the culture in the absence or the presence of the PI3K inhibitor (the inhibitor was also replaced). The LY294002 concentration (50 µM) was chosen according to Adhikari *et al.* (2013). Each treatment was repeated five times. After this culture, the ovarian tissue was fixed, processed and used for histological (morphology and activation) and immunohistochemical (apoptosis) analyses as described above.

Evaluation of p-AKT immunostaining

To check the effectiveness of LY294002 in inhibiting the PI3K pathway and considering that Akt is one of the main downstream targets of PI3K (Cecconi *et al.*, 2012), Akt phosphorylation was evaluated after 7 days of culture with 1 ng/ml leptin in the absence or presence of LY294002. Immunohistochemistry was carried out as described above, but the slides were incubated with a rabbit polyclonal anti-p-Akt antibody (1:40; Santa Cruz Biotechnology) instead of the anti-activated caspase-3 antibody. Immunostaining was subjectively classified as absent, weak, moderate or strong using a microscope (Nikon) under ×400 magnification (Santos *et al.*, 2019).

Statistical analysis

The percentages of morphologically normal, apoptotic, primordial and growing follicles were compared by chi-squared test. Data of follicle and oocyte diameters were submitted to Shapiro–Wilk test to verify normal distribution of residues and homogeneity of variances. Thereafter, data were submitted to the Kruskal–Wallis nonparametric test and the Student–Newman–Keuls test for comparisons among treatments. The results were expressed as the mean ± standard error of mean (SEM). Differences were considered to be statistically significant when $P < 0.05$. Data were analyzed using the BioEstat 5.0 procedure.

Table 1. Follicle and oocyte diameters (mean ± SEM) in the fresh control and after *in vitro* culture of ovine ovarian tissue in different concentrations of leptin

Treatments	Follicle diameter (µm)	Oocyte diameter (µm)
Fresh control	48.42 ± 5.77	34.73 ± 2.54
α-MEM$^+$	43.65 ± 5.34^A	31.83 ± 3.33
1 ng/ml leptin	45.68 ± 3.82^A	34.33 ± 3.48
5 ng/ml leptin	48.74 ± 5.57^A	36.62 ± 5.83
10 ng/ml leptin	41.40 ± 3.22^{*B}	31.44 ± 3.89
25 ng/ml leptin	36.39 ± 5.55^{*B}	32.36 ± 2.85
50 ng/ml leptin	35.54 ± 4.15^{*B}	29.52 ± 5.58

*Differs significantly from fresh control ($P < 0.05$).
^{A,B}Different letters denote significant differences among treatments ($P < 0.05$).

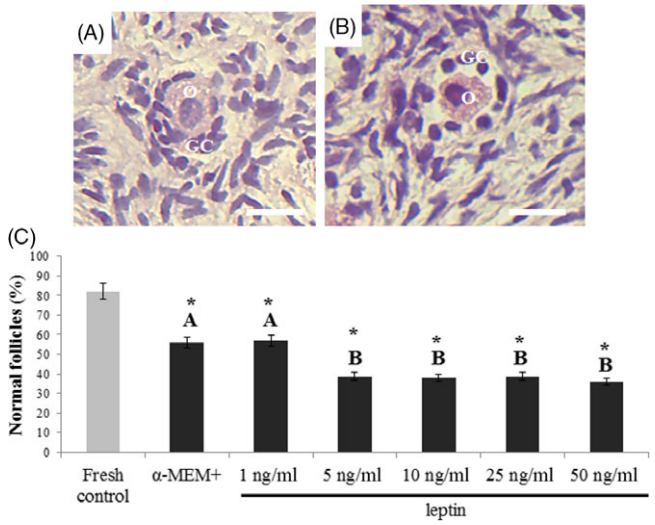

Figure 1. Histological sections of ovine ovarian fragments. (*A*, *B*) Normal follicle after *in vitro* culture in medium containing 1 ng/ ml leptin (*A*) and atretic follicle after culture in 50 ng/ ml leptin (*B*). GC, granulosa cells; O, oocyte; Scale bars: 30 µm (×400). (*C*) Percentages of ovine morphologically normal follicles in the fresh control and after 7 days of *in vitro* culture in α-MEM$^+$ or in different concentrations of leptin. *Differs significantly from the fresh control ($P < 0.05$). ^{A,B}Different letters denote significant differences among the treatments ($P < 0.05$).

Results

Follicular morphology and development after in vitro culture

Overall, normal (Fig. 1*A*) and atretic (Fig. 1*B*) follicles were observed in all treatments. After 7 days of culture, the percentage of morphologically normal follicles decreased significantly in all treatments compared with the fresh control (82%; Fig. 1*C*). However, the control medium (56%) and the medium containing 1 ng/ml leptin (57%) had similar ($P > 0.05$) percentages of normal follicles, which were significantly higher than those observed for other treatments (39, 38, 39 and 36% for 5, 10, 25 and 50 ng/ml, respectively).

After *in vitro* culture, there was a significant reduction in the percentage of primordial follicles (Fig. 2*A*) and an increase in the percentage of growing follicles (Fig. 2*B*) in all treatments compared with the fresh control. However, no difference ($P > 0.05$) in the follicular activation was observed between the control medium (α-MEM$^+$) and leptin-treated groups. As shown in Table 1, a significant reduction in the follicle diameter was observed when the

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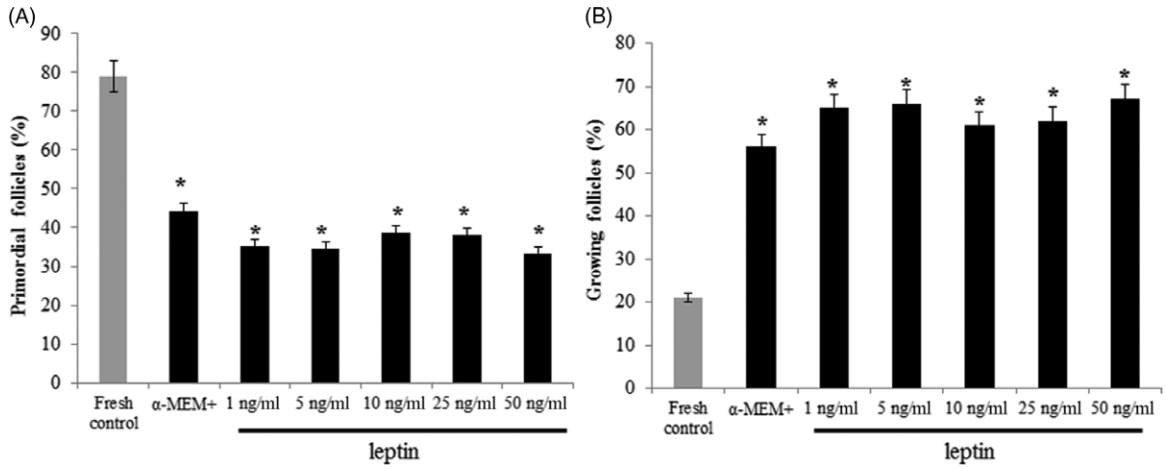

Figure 2. Percentages of primordial (*A*) and growing (*B*) follicles in the fresh control and after 7 days of *in vitro* culture in α-MEM^{+} or with different concentrations of leptin. *Differs from fresh control ($P < 0.05$).

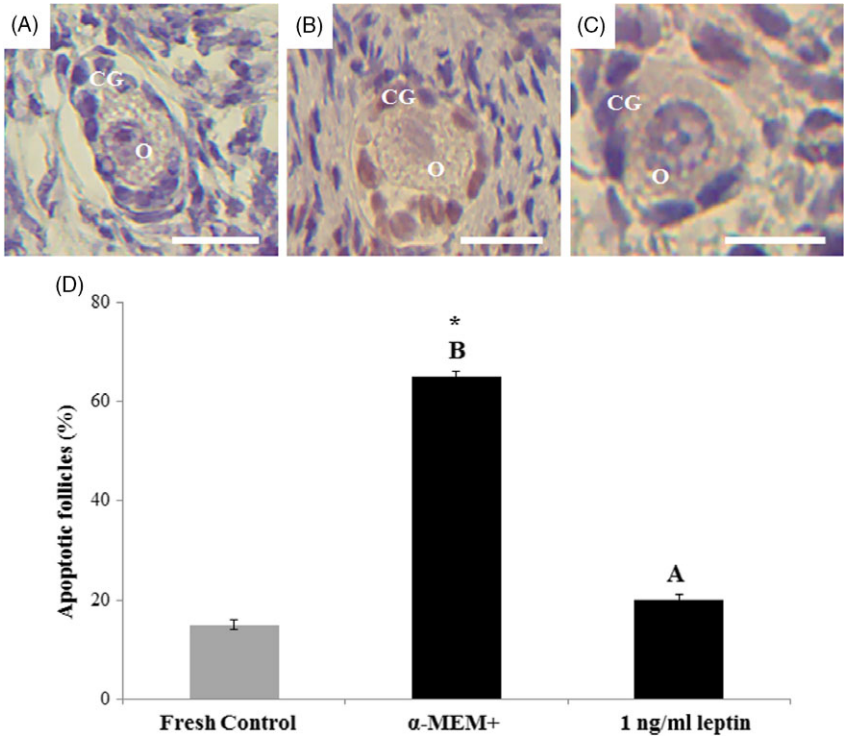

Figure 3. Immunohistochemical analysis for the expression of activated caspase-3 in ovine preantral follicles. (*A–C*) Normal follicle in the fresh control (*A*), apoptotic follicle (brown staining granulosa cells) cultured in α-MEM^{+} (*B*), and normal follicle cultured in medium containing 1 ng/ml leptin. GC: granulosa cells; O: oocyte. Scale bars: 25 µm (×400). Percentages of apoptotic follicles in the fresh control and after 7 days of culture in α-MEM^{+} or in 1 ng/ml of leptin. *Differs significantly from the fresh control ($P < 0.05$). ^{A,B}Different letters denote significant differences among the treatments ($P < 0.05$).

ovarian tissues were cultured in medium containing 10, 25 or 50 ng/ml leptin compared with the fresh control and other treatments. Additionally, there was no significant influence of leptin on the oocyte diameter.

Follicular apoptosis

Immunohistochemical analysis for apoptosis was performed in the fresh control (Fig. 3*A*) and in treatments that showed the best results for the percentage of morphological normal follicles, i.e. α-MEM^{+} (Fig. 3*B*) and medium containing 1 ng/ml leptin (Fig. 3*C*). The percentage of apoptotic follicles was similar ($P > 0.05$) between 1 ng/ml leptin (20%) and the fresh control (15%), and both groups had less apoptosis ($P < 0.05$) than that observed in the control medium (65%; Fig. 3*D*).

Follicular morphology, activation and apoptosis after inhibition of the PI3K pathway

The PI3K pathway inhibition was performed in follicles cultured with 1 ng/ml leptin because this treatment had less apoptosis than control medium (α-MEM^{+}). *In vitro* culture of ovine ovarian tissue in 1 ng/ml leptin with LY294002 decreased significantly the percentage of normal follicles (35.33%; Fig. 4*A*) and increased significantly the percentage of apoptosis (55%; Fig. 4*B*) compared with the medium containing 1 ng/ml leptin without PI3K inhibitor (56.67% and 20% for normal and apoptotic follicles, respectively). Moreover, the culture of ovine ovarian tissue with 1 ng/ml leptin and LY294002 significantly inhibited the *in vitro* activation of primordial follicles compared with medium without the inhibitor (Fig. 4*C*, *D*).

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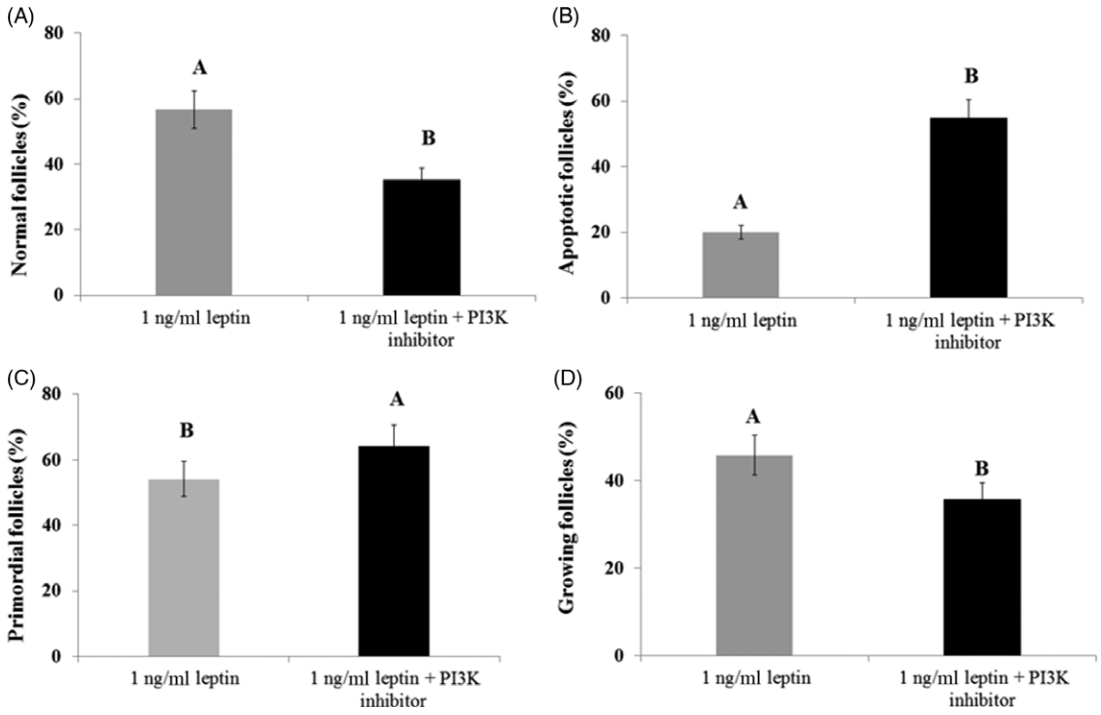

Figure 4. Percentages of morphologically normal (*A*), apoptotic (*B*), primordial (*C*) and growing follicles (*D*) after *in vitro* culture in 1 ng/ml leptin in the absence or presence of LY294002 (PI3K inhibitor). ^{A,B}Different letters denote significant differences among the treatments ($P < 0.05$).

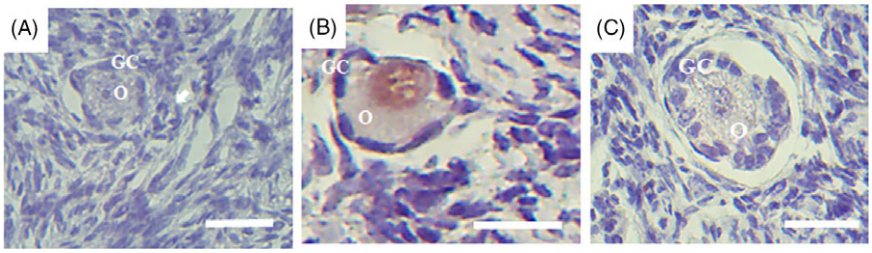

Figure 5. Immunohistochemical expression of p-AKT in the sheep ovary: follicles in 1 ng/ml leptin in the absence of LY294002 (*A*) or in the presence of LY294002 (PI3K inhibitor; *B*); Negative control (*C*). GC: granulosa cells; O: oocyte. Scale bars: 25 µm (×400).

Immunohistochemistry for Akt phosphorylation (p-Akt) after PI3K inhibition

The negative control did not show staining for phosphorylated Akt (Fig. 5*A*). After *in vitro* culture with 1 ng/ml leptin without LY294002, a moderate immunostaining for p-Akt was observed in the oocytes (Fig. 5*B*). Nevertheless, after PI3K inhibition, p-Akt immunostaining was absent (Fig. 5*C*).

Discussion

In recent years, some *in vitro* studies have demonstrated the beneficial effects of leptin on isolated ovarian follicle development, oocyte maturation and embryo production (Jin *et al.*, 2009; Kamalamma *et al.*, 2016; Keshrawani *et al.*, 2016; Macedo *et al.*, 2019; Menezes *et al.*, 2019). It is worth noting that *in vitro* culture of ovarian cortical tissue is an important tool to elucidate the regulatory mechanism and the signalling pathways involved in the activation of primordial follicles, which are the most predominant type of follicle in the ovary (Shea *et al.*, 2014; Telfer, 2019). Our study showed for the first time the effects of leptin on the follicles cultured within ovarian tissue slices.

In this study, ovarian tissue cultured in the control medium (α-MEM$^+$) or 1 ng/ml leptin resulted in greater percentages of histologically normal follicles than those found in other leptin concentrations. Although histological analysis remains the most classic method to determine the follicular atresia, the detection of early apoptotic changes may be beyond the scope of histological assessment (Bedaiwy and Hussein, 2004). Therefore, the use of a more sensitive method to evaluate cell quality is recommended. Using immunohistochemical analysis, we demonstrated that 1 ng/ml leptin was effective in preventing follicular apoptosis (only 20%, which was similar to that of the fresh control) compared with α-MEM$^+$ (65%). Previous studies have shown the anti-apoptotic activity of leptin on bubaline cumulus–oocyte complexes (Panda *et al.*, 2017), on bovine blastocysts from *in vitro*-fertilized oocytes (Boelhauve *et al.*, 2005) and human trophoblast cells triggered by high temperature (Pérez-Pérez *et al.*, 2016). It is likely that leptin is exerting its anti-apoptotic effects by interacting with its receptor (LEPR), which is expressed in sheep preantral follicles (Macedo *et al.*, 2019) and/or by enhancing the intracellular levels of antioxidant enzymes and decreasing the levels of inflammatory factors (Zwirska-Korczala *et al.* 2007; Bilbao *et al.*, 2015). In our study, as the ovaries from the fresh control had more morphologically

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normal follicles than those cultured in 1 ng/ml leptin and similar percentages of apoptotic follicles, it could be speculated that follicles cultured in medium containing 1 ng/ml leptin are likely to undergo both necrosis and apoptosis, and that leptin is protecting the follicles from cell death by apoptosis. Considering that leptin is produced by oocytes from ovine primordial and primary follicles (Macedo *et al.*, 2019), we believe that the addition of a lower concentration of leptin (1 ng/ml) to the medium is sufficient to maintain satisfactory follicle survival and to prevent apoptosis in small follicles.

Interestingly, the decrease in the percentage of normal follicles after *in vitro* culture in 10, 25 or 50 ng/ml leptin was associated with a reduction in follicle diameter, which is indicative of follicle degeneration (Lunardi *et al.*, 2017). Another study has shown that exposure to high leptin concentrations inhibited human granulosa cell proliferation and increased caspase-3 mRNA levels (Lin *et al.*, 2017). Moreover, *in vitro* studies showed that rat ovarian tissue exposed to high concentrations of leptin (100 ng/ml) inhibited superoxide dismutase (SOD) activity compared with the control tissues (Bilbao *et al.*, 2015). Therefore, we suggest that leptin may have a concentration-dependent activity in follicular survival and development. At high concentrations, leptin seems to have a pro-oxidant effect and to be involved in cell death.

In the current study, all treatments promoted the activation of primordial follicles compared with the fresh control. The initiation of follicle growth starts with the differentiation of flattened granulosa cells into cuboidal, followed by their proliferation. Subsequently, the oocyte grows in size (Picton, 2001; Braw-Tal, 2002; Zhang *et al.*, 2014). Although our findings showed that leptin did not influence the percentage of growing follicles or the oocyte diameter compared with the control medium (α-MEM^{+}), we did not evaluate if leptin had a positive effect on the number of granulosa cells after *in vitro* culture, and this should be performed in future studies. It could be speculated that the period of culture of sheep ovarian tissue or the concentration of leptin used may not be adequate to promote an apparent increase in follicle activation and oocyte growth.

Considering that leptin maintains survival and promotes follicle growth by the PI3K/Akt pathway (Cirillo *et al.*, 2008; Ghasemi *et al.*, 2019), we hypothesized that the PI3K/Akt pathway would be involved in ovine follicular survival and activation in response to leptin. In addition, as Akt is one of the main targets of PI3K (Cecconi *et al.*, 2012), it is important to note the direct effect of LY294002 on Akt phosphorylation (Gharbi *et al.*, 2007). Our results showed that the expected inhibition of Akt phosphorylation after culture in 1 ng/ml leptin + LY294002 was associated with an increase in follicular apoptosis and inhibition of primordial follicle activation. Akt modulates granulosa cell apoptosis (Zhou *et al.*, 2019) and is a marker of primordial follicle activation (Reddy *et al.*, 2009; Santos *et al.*, 2019). One *in vitro* study has suggested that the PI3K/Akt pathway may be involved in the proliferative and anti-apoptotic effects of leptin on goose granulosa cells (Wen *et al.*, 2015). Therefore, although other pathways downstream may also come into play, we suggest that leptin reduces follicular apoptosis and stimulates *in vitro* activation of primordial follicles in sheep possibly through the activation of the PI3K/AKT pathway.

In conclusion, a 1 ng/ml leptin concentration reduced follicular apoptosis after *in vitro* culture of sheep ovarian cortical tissue. In addition, the PI3K/AKT pathway is possibly involved in the effects of leptin on survival and activation of sheep primordial follicles.

Declaration of interest. None of the authors have any conflict of interest to declare.

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Ethical standards. Not applicable.

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