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Immunolocalization of leptin and its receptor in the sheep ovary and *in vitro* effect of leptin on follicular development and oocyte maturation

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ABSTRACT

The aims of the study were to characterize leptin and it is receptor (LEPR) proteins immunoexpression in ovine ovaries and to evaluate the effects of leptin on development of secondary follicles cultured *in vitro*. The ovaries were collected and fixed for immunohistochemical analysis. Additional pairs of ovaries were collected and secondary follicles were isolated and cultured, for 18 days, in α-MEM$^+$ alone or supplemented with 10 or 25 ng/mL of leptin. The antrum formation and fully grown oocytes rates were higher in 25 ng/mL leptin than all treatments. GSH levels and mitochondrial activity were higher in 10 or 25 ng/mL leptin than α-MEM$^+$. 25 ng/mL leptin showed a higher percentage of MII than the α-MEM$^+$. In conclusion, leptin and its receptor are expressed in ovine ovaries and 25 ng/mL leptin promoted higher *in vitro* maturation rates by improving follicular development, GSH levels and mitochondrial activity of ovine oocytes compared to control medium.

1. Introduction

The *in vitro* culture of isolated secondary follicles has been performed in sheep to obtain a larger number of meiotically competent oocytes (Kamalamma et al., 2016; Lunardi et al., 2016). However, the number of embryos produced from *in vitro* grown preantral follicles is very low (Arunakumari et al., 2010; Luz et al., 2012). One aspect that may be responsible is the generation of oxidative stress by the production of reactive oxygen species (ROS) under *in vitro* environmental conditions (Combelles et al., 2009; Yu et al., 2014). ROS can pass through cell membrane and induce numerous effects, including mitochondrial dysfunction (Guerin et al., 2001), which significantly decrease ATP synthesis in oocytes, resulting in decreased oocyte quality with adverse effects on maturation and fertilization (Van Blerkom, 2004; Ramalho-Santos et al., 2009; Chappel, 2013). As mitochondria are major sites of ROS production (Chen et al., 2003), maintenance of stable mitochondrial function in oocytes is important to prevent the direct production of ROS (Niu et al., 2017). Therefore, the use of substances with antioxidant properties during the *in vitro* culture of follicles may prevent mitochondrial dysfunction and oxidative damage or support the proper pro-/antioxidant balance by influencing, for example, intracellular glutathione (GSH) production, which protects oocytes against toxic injury due to oxidative stress (Ou et al., 2012).

Leptin, a 167–amino acid hormone (Zhang et al., 1994), is among the substances that have already demonstrated antioxidant activity (Bilbao et al., 2015) and beneficial action on follicular development, oocyte maturation and embryo production *in vitro* (Jin et al., 2009; Kamalamma et al., 2016; Keshrawani et al., 2016). Leptin is secreted mainly by adipocytes (Tsai et al., 2012) and is part of the adipokine family, which also includes resistin, adiponectin, visfatin, omentin and vaspin. It is involved in the regulation of food intake and energy homeostasis. Moreover, leptin is deeply involved in female reproductive system regulation by acting on the gonadotropic axis and on the ovaries (Catteau et al., 2015).

The hormone leptin exerts its biological effects by interacting with its receptor (LEPR) (Tartaglia et al., 1995). Expression of mRNA for

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leptin and LEPR have been reported in the oocyte, granulosa, cumulus and theca cells in antral follicles of different species (human: Cioffi et al., 1997; murine: Matsuoka et al., 1999; bovine: Paula-Lopes et al., 2007; caprine: Batista et al., 2013; ovine: Munõz-Gutiérrez et al., 2005; Taheri and Parham, 2016). In addition, the leptin and LEPR proteins have already been demonstrated in oocytes and granulosa cells from goat follicles at all stages of development (Batista et al., 2013).

Using the rat as a model, Bilbao et al. (2015) have demonstrated that a daily stimulus with low doses of leptin induces follicular growth, decreases ROS levels and Bax and active caspase-3 expression. *In vitro* studies have reported that supplementation of the maturation medium with leptin improved the developmental capacity of the oocytes, increased blastocyst rates (bovine: Boelhauve et al., 2005; bubaline: Khaki et al., 2014; Sheykhani et al., 2016; Panda et al., 2017; swine: Craig et al., 2004), and reduced apoptotic cells (bovine: Boelhauve et al., 2005; bubaline: Panda et al., 2017). Moreover, addition of leptin to the culture medium of ovine isolated secondary follicles increased antrum formation and follicular growth as well as the percentage of oocytes in metaphase II (Kamalamma et al., 2016). However, the mechanisms underlying the actions of leptin on sheep ovarian follicle survival and growth have not yet been clearly understood. Therefore, it is still necessary to evaluate whether the main effect of leptin involves oxidative stress response through GSH production and/or mitochondrial activity in follicular cells. Furthermore, additional immunohistochemistry to detect leptin and LEPR proteins would be important to evaluate if leptin could be involved in the ovarian function in sheep.

Thus, the aims of this study were to 1) characterize protein expression for leptin and its receptor (LEPR) in ovine ovaries, and 2) to evaluate the effect of leptin addition to the base medium on the morphology (survival), growth, GSH and active mitochondria levels, as well as meiotic resumption of oocytes from ovine secondary follicles cultured *in vitro*.

2. Material and methods

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

2.1. Source of ovaries

For immunohistochemical studies, ovarian cortical tissues (n = 10 ovaries) were collected at a local abattoir from five adult (1–3 years old) non-pregnant mixed-breed sheep. The animals used in this study were non-pregnant and presumed to be undergoing normal oestrous cycles as judged by the presence of normal corpora lutea at slaughter. Immediately postmortem, the pairs of ovaries were washed once in 70% alcohol followed by twice washes in Minimum Essential Medium buffered with HEPES (MEM-HEPES) and antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin). Thereafter, the ovaries were fixed in 10% buffered formalin (Dinâmica, São Paulo, Brazil). For *in vitro* culture, additional pairs of ovine ovaries (n = 50 ovaries) were collected and transported within 1 h to the laboratory in tubes containing washing medium at 4 °C (Chaves et al., 2008).

2.2. Immunohistochemistry evaluation of LEP and LEPR expression

Immunohistochemistry was performed as described previously (Barberino et al., 2017). After 18 h of fixation, the ovarian tissue was dehydrated with increasing concentrations of ethanol (Dinâmica), clarified in xylene (Dinâmica), and embedded in paraffin (Dinâmica). Sections (5 μm thick) from each block were cut using a microtome (EasyPath, São Paulo, Brazil) and mounted in Starfrost glass slides (Knittel, Braunschweig, Germany). Thereafter, the slides were incubated in citrate buffer (pH 6.0) (Dinâmica) at 95 °C in a decloaking chamber (Biocare, Concord, USA) for 40 min to retrieve antigenicity, and endogenous peroxidase activity was prevented by incubation with 3% H_2O_2 (Dinâmica) and methyl ethanol (QEEL, São Paulo, Brazil) for 10 min. Non specific binding sites were blocked using 1% normal goat serum (Biocare) in phosphate-buffered saline (PBS, pH 7,2). Subsequently, the sections were incubated in a humidified chamber for 90 min at room temperature with rabbit polyclonal anti-leptin [(H146): sc-9014] (1:40) and anti-LEPR [(H – 300): sc–8325] (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Thereafter, the sections were incubated for 30 min with MACH4 Universal HRP-polymer (Biocare). Protein localization was demonstrated with diaminobenzidine (DAB; Biocare), and the sections were counterstained with hematoxylin (Vetec, São Paulo, Brazil) for 1 min. Negative controls (reaction control) underwent all steps except the primary antibody incubation.

Preantral follicles were classified as defined previously (Santos et al., 2014) in primordial (oocyte surrounded by as single layer of squamous or squamous and cuboidal granulosa cells), primary (oocyte surrounded by a single layer of cuboidal granulosa cells) or secondary (oocyte surrounded by two or more layers of cuboidal granulosa cells). Antral follicles were classified as early (< 3 mm presence of an antral cavity) or large (presence of a cavity filled with follicular liquid and well-developed theca layers). In the different follicular compartments (oocyte, and granulosa, cumulus, mural and theca cells), the immunostaining was classified as absent, weak, moderate or strong (Silva et al., 2004). The slides were examined using a light microscope (Nikon, Tokyo, Japan) at 100 and/or 400x magnification by two experienced researchers in a blinded manner to avoid variation.

2.3. Isolation and selection of secondary follicles

Isolation, selection, culture and follicular evaluation were performed according to Macedo et al. (2017). In the laboratory, the surrounding fatty tissues and ligaments were stripped from the pair of ovaries. Ovarian cortical slices (1–2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in fragmentation medium consisting of MEM-HEPES with antibiotics. Secondary follicles, approximately 240–270 μm in diameter without antral cavities, were visualized under a stereomicroscope (Nikon) and mechanically isolated by microdissection using 26-gauge (26 G) needles. These follicles were then transferred to 100 μL droplets containing base culture medium (α-MEM) for evaluation of quality. Follicles selected for *in vitro* culture had an intact basement membrane, two or more layers of granulosa cells and a visible and healthy oocyte that was round and centrally located within the follicle, without dark cytoplasm.

2.4. In vitro culture of ovine secondary follicles

After selection, isolated follicles were pooled and then randomly allocated to three treatments with approximately 45–50 follicles per treatment. Follicles were individually cultured (one follicle per droplet) in 100 μL droplets of culture medium under mineral oil in petri dishes (60 × 15 mm, Corning, Sarstedt, Newton, NC, USA) at 39 °C under 5% CO_2 for up to 18 days. The base control medium consisted of α-MEM (pH 7.2–7.4) supplemented with 3.0 mg/mL bovine serum albumin (BSA), 10 ng/mL insulin, 5.5 μg/mL transferrin, 5.0 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine and 50 μg/mL ascorbic acid, and then referred as α-MEM$^+$. For the experimental conditions, the control medium (α-MEM$^+$) was supplemented with 10 or 25 ng/mL human recombinant leptin (L4146; it has 92% molecular homology with ovine leptin). Every 2 days, 60 μL of the culture media was replaced with fresh media in each droplet.

2.5. Evaluation of follicle morphology and development

Analysis of follicular morphology was performed every six days of culture using a pre-calibrated ocular micrometer in a stereomicroscope

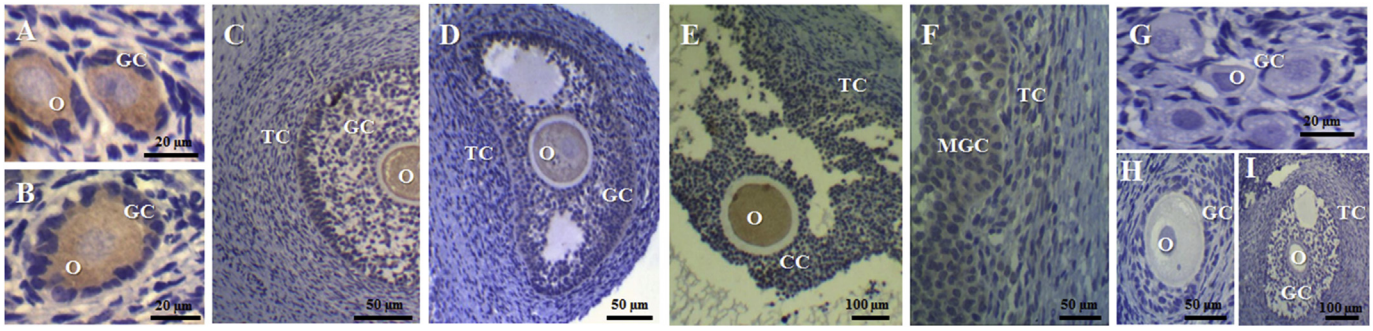


Fig. 1. Immunolocalization of leptin protein in ovine ovarian follicles. Primordial (A), primary (B), secondary (C), early antral (D) and large antral (E and F) follicles, and negative control (G, H and I). O: oocyte; GC: granulosa cells; CC: cumulus cells; MGC: mural granulosa cells; TC: theca cells (Figures A, B, E, F and G: 400x; Figures C, D, H and I: 100x).

(Nikon) at 100x magnification. Atretic follicles were those showing darkness of the oocytes and the surrounding granulosa cells, misshapen oocytes, rupture of the basement membrane and/or oocyte extrusion. The following characteristics were analyzed in the morphologically normal follicles: (i) antral cavity formation, defined as the emergence of a visible translucent cavity within the granulosa cell layers, (ii) the diameter of healthy follicles, measured from the basement membrane, which included two perpendicular measurements of each follicle, and (iii) the daily growth rate, calculated as the normal follicle final diameter subtracted from its diameter on day 0, divided by the number of culture days (18 days).

After 18 days of culture, all healthy follicles were carefully and mechanically opened with 26 G needles under a stereomicroscope (Nikon) for oocyte recovery. The percentage of fully grown oocytes, i.e., oocyte ≥110 µm, was calculated as the number of acceptable quality oocytes (≥110 µm) recovered out of the total number of cultured follicles (x 100). Furthermore, healthy oocytes were evaluated for intracellular glutathione (GSH) levels and mitochondrial activity.

2.6. Measurement of GSH levels and metabolically active mitochondria

After culture, the oocytes were recovered and intracellular GSH levels and mitochondrial activity were measured as previously described (Lins et al., 2017). Briefly, 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CellTracker® Blue; CMF_2HC; Invitrogen, Eugene, Oregon, USA) and MitoTracker Red (MitoTracker® Red, CMXRos, Molecular Probes, Melbourne, Victoria, Australia) were used to detect intracellular GSH and mitochondrial activity levels as blue and red fluorescence, respectively. Approximately 21 oocytes for each treatment were incubated in the dark for 30 min in PBS supplemented with 10 mM of CellTracker Blue® and 100 nM MitoTracker Red® at 39 °C. After incubation, the oocytes were washed with PBS and the fluorescence was observed under an epifluorescence microscope (Nikon) with UV filters (370 nm for GSH and 579 nm for active mitochondria) at a magnification of 100x. The oocytes were photographed and the digital images were analyzed by using the Image J software (National Institutes of Health, Bethesda, MD, USA). The fluorescence intensity was expressed as pixel/oocyte.

2.7. Maturation of ovine oocytes from in vitro cultured secondary follicles

In vitro maturation (IVM) was performed in oocytes derived from *in vitro* grown follicles after 18 days of culture in the control medium (α-MEM$^+$) and in the treatment that obtained the highest percentage of fully grown oocytes. For IVM, additional pairs of ovine ovaries (n = 10 ovaries) were collected, washed and transported to the laboratory as described above. After 18 days of culture, all oocytes enclosed in healthy follicles were carefully collected with 26-G needles under a stereomicroscope (Nikon). Only oocytes ≥110 µm in diameter with a homogeneous cytoplasm and surrounded by at least 1 compact layer of cumulus cells were selected for IVM as previously described (Luz et al., 2012; Lunardi et al., 2016). The cumulus–oocyte complexes (COCs) were transferred to drops of 100 µL of maturation medium composed of TCM 199 supplemented with 10% fetal calf serum (FCS), 1 µg/mL follicle stimulating hormone (human recombinant FSHr; Gonal-F; Serono Laboratórios, São Paulo, Brazil) and 1 µg/mL luteinizing hormone (LH; ovine pituitary) under oil, and incubated for 24 h under 5% CO_2 in the air (Cecconi et al., 1999). Thereafter, the oocytes were incubated in drops of PBS containing 10 mM Hoechst 33342 for 15 min at room temperature in the dark and visualized using an epifluorescence microscope (Nikon) with UV filter (483 nm) at a magnification of 100x. The chromatin configuration (blue fluorescence) was analyzed through observation of the intact germinal vesicle (GV), meiotic resumption (including germinal vesicle breakdown [GVBD] or metaphase I [MI]) or nuclear maturation (metaphase II [MII]).

2.8. Statistical analysis

Data from normal follicles, antrum formation, and retrieval of fully-grown oocytes after *in vitro* culture were expressed as percentages and compared by the Chi-squared test. Data from GSH, active mitochondria, diameter and daily growth rates were submitted to the Shapiro-Wilk test to verify normal distribution of residues and homogeneity of variances. Thereafter, Kruskal-Wallis non-parametric test was used for comparisons. When main effects or interactions were significant, means were compared by test Student Newman Keuls. Data from IVM were submitted to the Fischer exact. The results were expressed as the means ± standard error mean (SEM), and differences were considered significant when $p < 0.05$.

3. Results

3.1. Immunolocalization of leptin and its receptor (LEPR) in ovine ovaries

Oocytes of primordial (Fig. 1A) and primary (Fig. 1B) follicles showed a moderate reaction for leptin protein, while oocytes of secondary (Fig. 1C), early antral (Fig. 1D), and large antral follicles (Fig. 1E) showed a weak immunostaining. No reaction was observed for leptin protein in granulosa cells of primordial (Fig. 1A) and primary (Fig. 1B) follicles. However, a weak expression was observed in granulosa cells of secondary (Fig. 1C) and early antral (Fig. 1D) follicles, while theca cells did not show any positive immunoreaction in these follicular categories (secondary and early antral follicles). Cumulus, mural granulosa and theca cells of large antral follicles (Fig. 1E and F) showed a weak immunostaining for leptin protein. No staining was observed in the negative control (Fig. 1G, H and I).

Regarding to receptor, immunohistochemical analysis revealed a strong reaction for LEPR protein in oocytes of primordial (Fig. 2A),

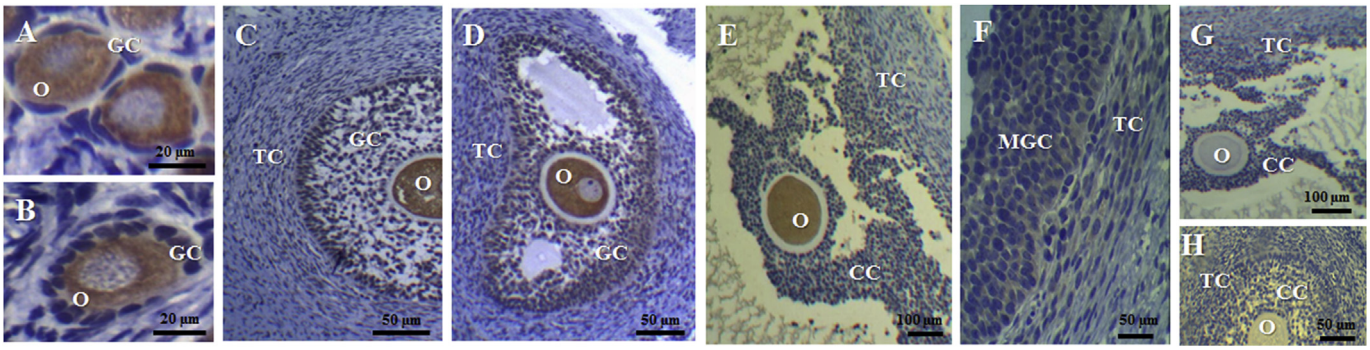


Fig. 2. Immunolocalization of leptin receptor (LEPR) protein in ovine ovarian follicles. Primordial (A), primary (B), secondary (C), early antral (D) and large antral (E and F) follicles, and negative control (G and H). O: oocyte; GC: granulosa cells; CC: cumulus cells; MGC: mural granulosa cells; TC: theca cells (Figures A, B, E, F and G: 400x; Figures C, D and H: 100x).

primary (Fig. 2B), secondary (Fig. 2C), and early antral (Fig. 2D) follicles, whereas a moderate reaction in oocytes of large antral follicles (Fig. 2E). Granulosa cells of primordial follicles (Fig. 2A) did not show a positive reaction for LEPR protein. However, granulosa cells of primary (Fig. 2B) and secondary (Fig. 2C) follicles showed a weak reaction, while granulosa cells of early antral follicles (Fig. 2D) showed a moderate reaction. In addition, a weak reaction was observed in theca cells of secondary and early antral follicles. Cumulus, mural granulosa and theca cells from large antral follicles (Fig. 2E and F) showed a weak reaction for LEPR protein. No staining was observed in the negative control (Fig. 2G and H).

3.2. Follicular morphology and development after in vitro culture

Morphologically normal secondary follicles showed centrally located oocytes and normal granulosa cells, which were enclosed by an intact basement membrane (Fig. 3A). At day 6 of culture, antral (Fig. 3B), atretic (Fig. 3C) and extruded (Fig. 3D) follicles were observed. From day 0 to day 18, there was a reduction ($p < 0.05$) in the percentage of morphologically normal follicles in all treatments (Fig. 4). On day 12 of culture, the percentage of morphologically normal follicles was higher ($p < 0.05$) in 25 ng/mL leptin (71.7% than other treatments - 55.6% for α-MEM$^+$ and 61.1% for 10 ng/mL leptin). However, at the end of culture, no difference ($p > 0.05$) on the percentage of normal follicles was observed among treatments (33.3%, 42.6% and 39.1% for α-MEM$^+$, 10 and 25 ng/mL leptin, respectively) (Fig. 4).

The rates of antral cavity formation increased ($p < 0.05$) in α-MEM$^+$ and 25 ng/mL leptin treatments from day 0 to day 6 (Fig. 5). In addition, the medium containing 25 ng/mL leptin showed higher ($p < 0.05$) percentage of antral follicles than the other treatments on days 12 (7.4%, 1.9% and 26.1% for α-MEM$^+$, 10 and 25 ng/mL leptin, respectively) and 18 of culture (7.4%, 3.7% and 32.6% for α-MEM$^+$, 10 and 25 ng/mL leptin, respectively) (Fig. 5). There were no differences

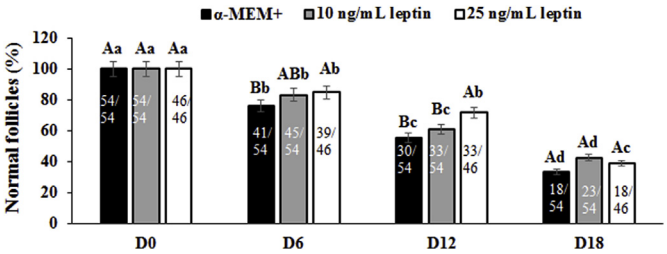


Fig. 4. Percentages of morphologically normal follicles during *in vitro* culture in control medium (α-MEM$^+$) or α-MEM$^+$ containing 10 or 25 ng/mL leptin. ($^{a, b}$) Different letters denote significant differences among culture periods in the same treatment ($p < 0.05$). ($^{A, B}$) Different letters denote significant differences among treatments in the same period ($p < 0.05$).

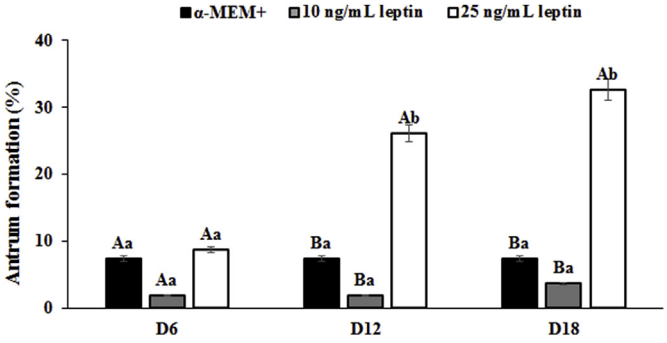


Fig. 5. Percentages of antrum formation during *in vitro* culture in control medium (α-MEM$^+$) or α-MEM$^+$ containing 10 or 25 ng/mL leptin. ($^{a, b}$) Different letters denote significant differences among culture periods in the same treatment ($p < 0.05$). ($^{A, B}$) Different letters denote significant differences among treatments in the same period ($p < 0.05$).

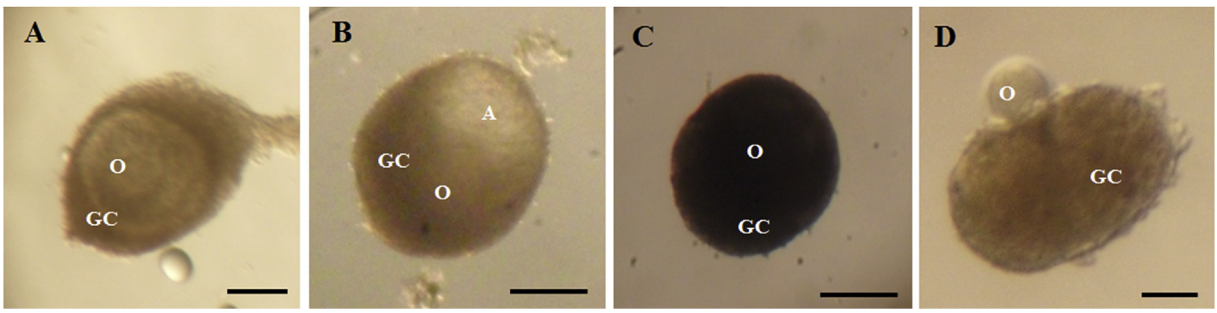


Fig. 3. Morphologically normal secondary follicle at day 0 (A), antral follicle after 6 days of culture in 25 ng/mL leptin (B), atretic (C), and extruded (D) follicles after 6 days of culture in control medium (α-MEM$^+$). O: oocyte; GC: granulosa cell; A: antral cavity. Scale bars: 100 μm (100x).

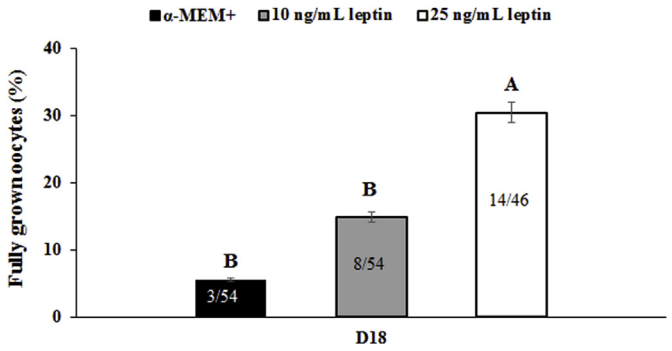


Fig. 6. Percentages of fully grown oocytes after *in vitro* culture of secondary follicles in control medium (α-MEM$^+$) or α-MEM$^+$ containing 10 or 25 ng/mL leptin. ($^{A, B}$) Different letters denote significant differences among treatments ($p < 0.05$).

($p > 0.05$) among treatments regarding to extrusion (no extrusion for α-MEM$^+$ and 10 ng/mL, and 8.7% for 25 ng/mL leptin), daily growth rate and follicular diameter (202.2 μm, 215.0 μm and 260.0 μm for α-MEM$^+$, 10 and 25 ng/mL leptin, respectively). At day 18, treatment containing 25 ng/mL leptin (30.4%) significantly increased the percentage of fully grown oocytes (i.e, ≥ 110 μm) compared to the control (5.6%) and 10 ng/mL leptin (14.8%) (Fig. 6).

3.3. Effects of leptin on intracellular GSH levels and mitochondrial activity

Regardless of the concentration, leptin significantly increased intracellular GSH levels and mitochondrial activity compared to the control medium (α-MEM$^+$) after 18 days of culture. Moreover, there was no difference ($p > 0.05$) between leptin concentrations (10 or 25 ng/mL) (Fig. 7).

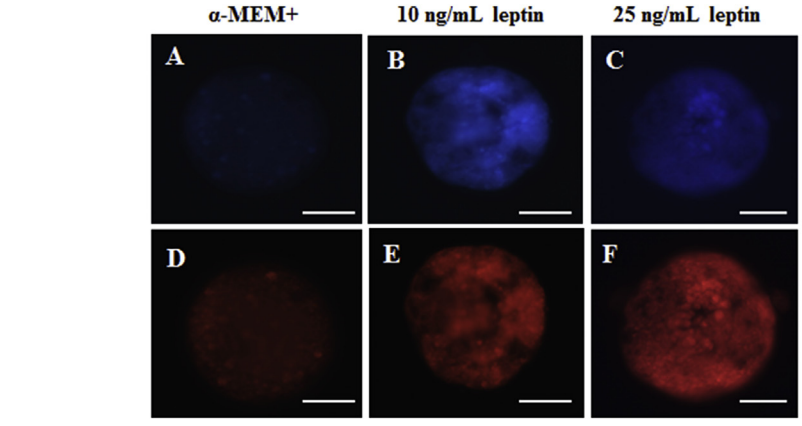
3.4. Chromatin configuration after IVM

Based on the best results of the percentage of fully grown oocytes, IVM was performed in the oocytes cultured in medium containing 25 ng/mL leptin, as well as in the control medium (α-MEM$^+$). After evaluation of the chromatin configuration, it was possible to observe oocytes in GV (Fig. 8A), GVBD (Fig. 8B), MI (Fig. 8C) and MII (Fig. 8D) in both treatments. However, the concentration of 25 ng/mL leptin showed a significantly higher percentage of oocytes in MII (33.33%) than the control medium (5.26%) (Table 1).

4. Discussion

To our knowledge, this study demonstrates for the first time a variable pattern of intensity and distribution of leptin and its receptor expression in sheep ovaries using immunohistochemical analysis. We have also demonstrated that 25 ng/mL leptin positively influences follicular and oocyte development (higher percentages of antrum formation and fully grown oocyte), increases GSH and active mitochondrial levels, as well as improves oocyte maturation compared to control medium.

In this study, leptin and LEPR proteins were localized in oocytes of all stages of follicle development, which is in agreement to the immunoexpression found in goat oocytes (Batista et al., 2013). Leptin protein was expressed in the granulosa cells of sheep secondary and antral follicles, whereas LEPR was localized in the granulosa cells of primary, secondary and antral follicles. Moreover, leptin protein was found in theca cells of large antral follicles, while its receptor was demonstrated in secondary and antral follicles. Previously, leptin mRNA was expressed in theca cells and both leptin and LEPR mRNA were



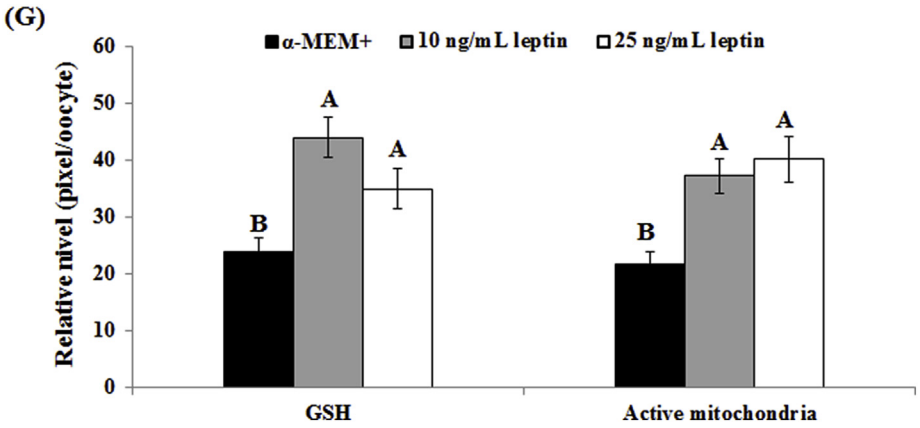


Fig. 7. Detection of intracellular levels of GSH and mitochondrial activity, respectively: oocytes cultured in α-MEM$^+$ (A and D) or control medium containing 10 (B and E) or 25 (C and F) ng/mL leptin. Scale bars: 50 μm (100x). Intracellular levels of GSH and active mitochondria in oocytes after *in vitro* culture (G). ($^{A, B}$) Within each group (GSH or active mitochondria levels), different letters denote significant differences among treatments ($p < 0.05$).

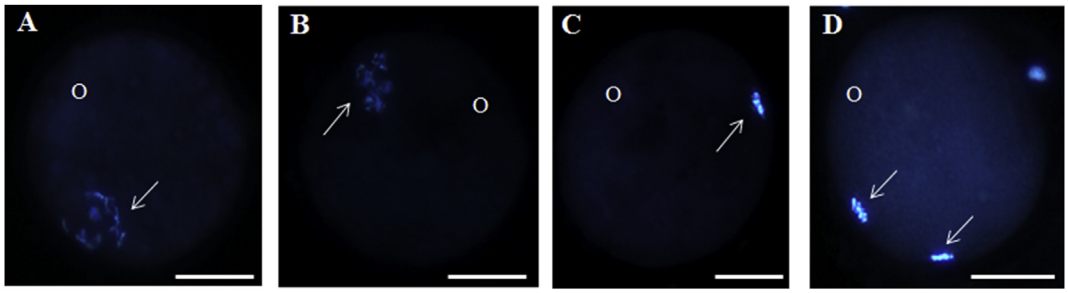


Fig. 8. Epifluorescent photomicrographic images of ovine oocytes from *in vitro* cultured secondary follicles stained with Hoeschst 33342 after IVM. Oocyte in GV (A), GVBD (B), MI (C) and MII (D) cultured in medium containing 25 ng/mL leptin. Arrow: nuclear chromatin. Scale bars: 50 μm (100x).

Table 1
Meiotic stages (%) after *in vitro* maturation of sheep oocytes from *in vitro* grown secondary follicles cultured in α-MEM^{+} or medium containing 25 ng/mL leptin.

Treatments	% GV (n)	% GVDB (n)	% MI (n)	% MII (n)
α-MEM^{+}	47.37 (9/19)	10.53 (2/19)	36.84 (7/19)	5.26 (1/19)^{B}
Leptin 25 ng/mL	23.8 (5/21)	9.52 (2/21)	33.33 (7/21)	33.33 (7/21)^{A}

($^{A, B}$) Different letter superscripts denote significant differences ($p < 0.05$) between treatments.

found in granulosa cells of antral follicles in sheep (Munõz-Gutiérrez et al., 2005) and goat (Batista et al., 2013). Using RT-qPCR, Pisani et al. (2008) detected the long form of LEPR in granulosa cells from ovine antral follicles greater than 2 mm in diameter. Moreover, leptin and LEPR proteins were reported in granulosa cells during all stages of goat follicular development (Batista et al., 2013). Additionally, leptin immunostaining was also found in granulosa cells, theca interna cells and luteal cells in swine (Phoophitphong et al., 2017). Therefore, the presence of a leptin signaling system in the ovary suggests that it could be involved in the ovarian function in sheep. Moreover, an evaluation of quantitative protein assay would be interesting to elucidate these mechanisms completely.

Although the percentage of morphologically normal follicles was higher in 25 ng/mL leptin than other treatments at day 12, there was no difference among treatments regarding to follicle survival after 18 days. It is known that early antral follicles become dependent on FSH stimulation (Erickson and Shimasaki, 2001) and this hormone maintained the survival of ovine follicles cultured for 18 days (Barros et al., 2019). Therefore, in the present study, it is possible that the absence of FSH in the base culture medium may have influenced the similar percentage of normal follicles in the different treatments at the end of culture.

Nevertheless, after 18 days of *in vitro* culture, the concentration of 25 ng/mL of leptin effectively stimulated more antral cavity formation and oocyte development (higher percentages of fully grown oocytes). It is known that leptin upregulates the *in vitro* expression of the integral membrane protein aquaporin 9 (AQP9) in human placenta (Vilariño-García et al., 2018) and that AQP9 plays an important role in the antrum development of ovine follicles (Sales et al., 2015). Thus, a possible explanation for our results is that 25 ng/mL leptin may have stimulated the expression of AQP9, increasing antrum formation. Moreover, based on the present immunohistochemistry findings, it is likely that the strong immunoexpression for LEPR (more receptors) found in oocytes of secondary and early antral follicles (and moderate in theca cells of early antral follicles) may have increased follicular responsiveness to this hormone. An increase in concentration of ligand can induce a greater amount of the same type of response by activating more receptors, more cells, or by occupying receptor for a longer time (Gurdon et al., 1998). Thus, these data indicate that an increase in leptin concentration (25 ng/mL instead of 10 ng/mL) may be required to promote follicle development (antrum formation) and oocyte growth *in vitro* for up to 18 days. In contrast, 10 ng/mL leptin may not be sufficient to support this growth for a period greater than 6 days. These results were different from those reported previously in which 10 ng/mL leptin exhibited higher growth and antrum formation rates after *in vitro* culture of ovine secondary follicles (Kamalamma et al., 2016). These differences may be due to mean follicle diameter at the beginning of the culture (250–270 μm in our study x 250–400 μm), period (18 days in this study x 6 days), and base medium (α-MEM in this study x TCM 199).

Because mitochondria dysfunction is one of major reason that induces increase of ROS level and compromises oocyte quality (Igosheva et al., 2010; Ou et al., 2012), we further explored GSH level and mitochondrial activity. The GSH serves as an efficient scavenger and plays an important role in maintaining oocytes in a reduced environment, protecting them from oxidative stress. For example, decreased GSH indicated the progression of oxidative stress in mice with insulin resistance (Goud et al., 2008). Moreover, during oocyte maturation, mitochondrial membrane potential is highly important for oocyte quality (Qiang et al., 2007), and is an essential factor in the establishment of oocyte functional competence (Dumollard et al., 2007; Castaneda et al., 2013). In this context, the present study demonstrated that control medium without leptin (α-MEM+) had oocytes with less GSH and active mitochondria levels, which was associated with poor quality oocytes (smaller oocytes than 25 ng/mL leptin) and consequently affected oocyte maturation (5.26% in α-MEM + x 33.33% in 25 ng/mL leptin).

It is important to note that although there were no differences between both concentrations of leptin regarding to GSH or active mitochondria levels, the concentration of 25 ng/mL of leptin significantly increased the percentage of fully grown oocytes compared to 10 ng/mL. For this reason, IVM was performed only in oocytes that were cultured in medium containing 25 ng/mL leptin. Our rates of MII oocytes were higher than those previously obtained by Kamalamma et al. (2016) (33.33 % × 24.06 %, respectively). The beneficial effects of leptin on *in vitro* follicular development, oocyte maturation and embryo production have also been demonstrated in ovine (Kamalamma et al., 2016; Keshrawani et al., 2016) and bubaline (Panda et al., 2017) species. Some studies suggested that leptin and its receptor are directly involved in the regulation of oocyte maturation and embryo development (Kawamura et al., 2002; Craig et al., 2004; Kakisina, 2013). Additionally, it has been suggested that leptin may induce germinal vesicle breakdown *in vivo* via its action on the theca cells (Ryan et al., 2002) or *in vitro* by a direct action on the oocyte or indirect on cumulus cells (Paula-Lopes et al., 2007).

In conclusion, leptin and its receptor (LEPR) proteins are expressed in all ovarian follicle stages in sheep. Moreover, the concentration of 25 ng/mL of leptin improved antrum formation, oocyte growth, GSH levels, mitochondrial activity and oocyte maturation rates compared to control medium. Taken together, these results suggest that leptin has beneficial effects on the development of ovine oocytes by attenuating mitochondrial dysfunction and oxidative stress.

Conflicts of interest

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

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