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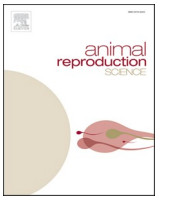

Effect of reduced water intake on ovarian reserve, leptin immunoexpression and impact of leptin on the *in vitro* culture of sheep secondary follicles

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ABSTRACT

This study evaluated the effect of reduced water intake on survival, apoptosis and immunoexpression of leptin in sheep preantral follicles, activation of primordial follicles, serum levels of leptin, estradiol (E2) and progesterone (P4), and *in vitro* maturation (IVM) of oocytes antral follicles, as well evaluated the effects of leptin on *in vitro* culture of secondary follicles isolated these animals. Ewes ($n = 32$) were divided into four groups: water *ad libitum* (Control – 100%), 80%; 60% and 40% of *ad libitum* intake. Blood was collected to determine, leptin, E2 and P4, before and after experiment. After the slaughter, ovarian cortex was used to histological and immunohistochemistry analysis and oocytes IVM. Moreover, isolated secondary follicles were cultured *in vitro* for 12 days in control medium (α-MEM+) or α-MEM+ with 10 or 25 ng/mL leptin. The reduction of water intake caused a linear decreasing effect on the percentages of normal preantral follicles, especially of primordial ($P < 0.05$), increased the apoptosis ($P < 0.05$) and decreased leptin expression in preantral follicles. The treatment with 60% of water intake showed greater total growth rate of isolated secondary follicles cultured with 25 ng/L leptin ($P < 0.05$), compared to those cultured in α-MEM+ . In conclusion, reduced water intake impaired the number of normal sheep preantral follicles, especially of primordial follicles, increased apoptosis and decreased leptin expression in preantral follicles. Moreover, secondary follicles from of ewes

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that receive 60% water intake increased follicular growth after *in vitro* culture with 25 ng/mL leptin.

1. Introduction

Small ruminants subjected to limited water intake were reported to exhibit reduction in reproductive performance (ovine: Barbour et al., 2005, Khnissi et al., 2019; caprine: Pacheco et al., 2018), and serum levels of estradiol and progesterone (ovine: Kumar et al., 2016; caprine: Pacheco et al., 2018). In addition, water-stressed animals often decrease their feed intake, which causes retardation of ovarian follicular growth (Blanc et al., 2004). Furthermore, water intake restriction decreases metabolic status and serum leptin levels in sheep (Houseknecht and Portocarrero, 1988; Chedid, 2009; Naqvi et al., 2015).

Leptin is a 16-kDa protein produced mainly by adipose tissue that plays an essential role in regulating energy homeostasis and interacts with the reproductive axis and the ovary in mammals (Dar et al., 2017; Heryani et al., 2019). The presence of leptin and its receptors in oocytes, granulosa and theca cells suggests a direct action of this hormone on folliculogenesis in bovine (Paula-Lopes et al., 2007; Martins et al., 2021), caprine (Batista et al., 2013; Gallelli et al., 2019) and ovine ovaries (Macedo et al., 2019, 2021). Results from previous studies have shown that leptin improves development and maturation of sheep oocytes from *in vitro* grown secondary and early antral follicles (Kamalamma et al., 2016; Macedo et al., 2019: Menezes et al., 2019), increases oocyte maturation and embryo production (bovine: Paula-Lopes et al., 2007; bubaline: Panda et al., 2017) and reduces apoptosis in cumulus cells (bubaline: Panda et al., 2017), preantral follicles (Macedo et al., 2021) and blastocysts (bovine: Boelhauve et al., 2005; caprine: Batista et al., 2014).

Thus, the present study aimed to answer at what level water intake reduction would adversely affect the ovarian follicular growth, reduce leptin and steroid hormone production in the ovaries, compromise ability of CCOs from antral follicles and whether such compromised ability could be restored by *in vitro* culture in leptin-supplemented medium.

2. Materials and methods

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

2.1. Experimental site and animals

The present experiment was approved and performed under the guidelines of the Ethics Committee on the Use of Animals at the Federal University of São Francisco Valley (UNIVASF), Petrolina, PE, Brazil (license number: 0002/241017). During the experimental period, the temperature, humidity, and temperature-humidity index (THI) of pens environment were measured, using Data Logger Thermo-hygrometer (HOBO U12–013, MicroDaq, Contoocook, NH, USA), coupled to a black globe thermometer. The average of minimum and maximum temperatures recorded were 24.56 °C and 33.83 °C, respectively, with relative air humidity of 63.90% and THI of 84.31.

Animal housing and experimental design were performed according to a previous study (Kumar et al., 2016) with some modifications. Thirty two clinically healthy, multiparous, non-pregnant Santa Inês crossbreed sheep, age of 2.3 ± 0.9 years with similar starting weight (32.2 ± 7.4 kg) were used for this experiment. Before starting the experiment, the animals were identified, weighed, treated against endo- and ectoparasites, and randomly allocated in pens of 1.00 × 1.20 m (one sheep per pen). Individual feeders and

Table 1
Chemical composition of the ingredients in the experimental diet.

Ingredients			% in dry matter	
Elephant grass			46.0	
Ground corn			38.1	
Mineral mixture-sheep*			2.0	
Urea			0.7	
Soybean meal			13.2	
Total			100	
Specification	**Elephant grass**	**Ground corn**	**Soybean meal**	**Diet**
DM (g/Kg MN)	261.9	889.3	886.1	576.26
MM (g/Kg DM)	105.2	12.9	64.8	61.86
CP (g/Kg DM)	105.5	89.9	487.4	149.13
EE (g/Kg DM)	28.7	45.1	19.0	32.89
NDF (g/Kg DM)	708.7	111.6	15.46	370.56
ADF (g/Kg DM)	419.5	33.7	8.85	206.97
TCHO (g/Kg DM)	830.5	859.9	42.8	715.30
NFC (g/Kg DM)	174.0	642.0	27.85	328.31
TDN (g/Kg DM)	570.1	850.0	80.48	596.71

DM – Dry Matter; NM – Natural Matter; MM – Mineral Matter; CP – Crud Protein; EE – Ether Extract; NDF – Neutral Detergent Fiber; ADF – Acid Detergent Fiber; TCHO – Total Carbohydrates; CNF – Non-Fiber Carbohydrates; TDN – Total Digestible Nutrients. *Composition: P, 7.5%; Ca, 19%; Mg, 1%; S, 7%; Na, 14.3%; Cl, 21.8%; Fe, 500 ppm; Cu, 300 ppm; Zn, 4600 ppm; Mn,1100 ppm; I, 80 ppm; Co, 405 ppm; Se, 30 ppm.

water troughs were provided in each pen. The experimental period was 77 days, with the first 14 days for adaptation of the animals to the facilities, diet, and routine management. Thus, the animals were subjected to 63 days of experimentation (water intake), covering 3.7 estrous cycles. Estrus synchronization was not performed to avoid a possible alteration in the hormonal profile, thus masking the effects of reduced water intake on physiological follicular development.

The diet was composed of 70% fresh elephant grass (*Pennisetum purpureum*) and 30% concentrate, consisting of ground corn, soybean meal, mineral mixture-sheep and urea, formulated according to the requirements of the National Research Council (NRC, 2007) and calculated for a daily weight gain of 157 g. Table 1 describes the nutrient composition and percentage of dietary ingredients of the feed. During the experimental period, the diet was provided *ad libitum* twice a day.

2.2. Treatments

For the experimental conditions, sheep were randomly divided into four groups of eight animals each: *ad libitum* water (Control – 100%); 80%; 60% and 40% of water intake from the control group's water intake. The water was supplied in buckets and weighed before supplied and weighed again 24 h later (leftovers). The water lost through evaporation was also considered when calculating the water intake of the treatments. This variable was estimated using buckets randomly spread across the experimental shed, with the same amount of water available for each treatment. The difference in weight was determined over 24 h. Water was offered once a day at 09 h. The water intake was calculated daily according to the treatments. The water used for watering the animals came from the São Francisco River (Brazil). The water was transferred to the experiment site through pipes (Araújo et al., 2021; 2022).

2.3. Weight of animal body, uterus, and ovaries

At the end of the experimental period, ewes were slaughtered at a mean final body weight of 37.63 kg, after fasting for 16 h, according RIISPOA (2017). The animals were previously stunned and immediately slaughtered cutting the main blood vessels in the neck. After skinning, the animals were eviscerated, the uterus was removed and weighed. The average daily weight gain of the animals was calculated by the difference between the body weight at slaughter and the initial body weight dividing by the total number of experimental days.

The ovarian pairs ($n = 32$) of all animals were also collected and weighed. The ovaries were washed once in 70% alcohol (Dinâmica, São Paulo, Brazil) and twice in 0.9% saline solution supplemented with 100 μg/mL penicillin and 100 μg/mL streptomycin. Next, the ovaries were transported within one hour to the laboratory in tubes containing 0.9% saline solution and antibiotics at 4 °C (Soares et al., 2018) for morphological analysis of preantral follicles *in vivo*, *in vitro* maturation of oocytes from antral follicles, and *in vitro* culture of preantral follicles (secondary follicles).

2.4. Hormonal assay

Blood samples of each ewe were collected at the beginning and at the end of the experiment by jugular venipuncture using blood collection tubes with separator gel and clot activator (Cral Plast, São Paulo, Brazil). The serum was separated by centrifugation at 3500 rpm for 15 min, and then it was kept in 1.5 mL microtubes and stored at − 20 °C. Plasma progesterone (P4) and oestradiol (E2) concentrations were determined through the electrochemiluminescence method (ECLIA) in automated analytical equipment, Access 2® (Beckman Counter- USA) using commercial kits specific for the equipment. The intra-assay CV was 1.87% and 2.3%, and the assay sensitivity was 0.016 ng/mL and 0.004 pg/mL, respectively, for P4 and E2. Plasma leptin concentration was determined through the sheep leptin sandwich ELISA kit (FineTest®, Wuhan Fine Biotech Co., Ltd, China). The intra-assay CV was 4.5% and the assay sensitivity was 18.75 pg/mL.

2.5. Morphological evaluation of preantral follicles enclosed in the ovarian tissue

To verify if the reduction in water intake could influence *in vivo* preantral follicle morphology, *i.e.*, survival of primordial, intermediate, primary and secondary follicles, and primordial follicle activation, the ovaries were fragmented into small pieces and three ovarian cortical fragments (approximately 3 mm × 3 mm x 1 mm thick) were removed from each ovarian pair. The fragments were immediately fixed in 10% buffered formalin (Dinâmica, São Paulo, Brazil) for classical histology. After paraffin embedding (Dinâmica), five-μm thick serial sections were stained by Hematoxylin and eosin (Vetec, São Paulo, Brazil) and observed under a light microscope (Nikon, Tokyo, Japan; 400x magnification). Images were taken using a Nikon microscope with a camera. Preantral follicles ($n = 240$ from each experimental group) were classified in primordial (one layer of flattened pregranulosa cells around the oocyte) or growing follicles (intermediate: one layer of flattened to cuboidal granulosa cells; primary: one layer of cuboidal granulosa cells, and secondary: two or more layers of cuboidal granulosa cells around the oocyte and no sign of antrum formation, Silva et al., 2004). In addition, to evaluate the survival, the 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 those with a retracted or vacuolated oocyte, condensed chromatin, disorganized granulosa cells detached from the basement membrane, or cell swelling (Santos et al., 2019a; b). Follicles were evaluated using direct counts by one experienced investigator. Evaluation started from a random point and followed a clockwise direction until the cortical portion of that section had all been evaluated. Then, serial sections were analyzed until reached a total of 30 follicles per ovary. Only follicles in which the oocyte nuclei was visible were counted, and each follicle was examined in every section in which it appeared and matched with the same follicle on

adjacent sections to avoid double counting, thus ensuring that each follicle was only counted once (Barberino et al., 2022a). For assessment of primordial follicle activation (transition from primordial to growing follicles), only morphologically normal follicles were recorded and the proportion of primordial and growing follicles was calculated (Silva et al., 2004).

2.6. Immunohistochemistry for apoptosis and leptin expression in vivo

Immunohistochemistry for cleaved caspase-3 was carried out to measure the amount of apoptosis in follicular cells after treatments. In addition, cobarberinnsidering that water restriction can interfere with the production of leptin in the adipocytes (Houseknecht and Portocarrero, 1988; Chedid, 2009; Naqvi et al., 2015) and that leptin is expressed in sheep ovarian follicles (Macedo et al., 2019, 2021), we evaluated whether the reduction in water intake would influence the expression of leptin protein in the ovary. After histological analysis, immunohistochemistry was performed as described previously (Macedo et al., 2019, 2021), with some modifications, in the ovarian cortex of all the experimental groups. Briefly, Section (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, São Paulo, Brazil) 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, São Paulo, Brazil) for 10 min. Nonspecific binding sites were blocked using 1% normal goat serum (Easypath, São Paulo, Brazil). Subsequently, the sections were incubated in a dark humidified chamber for 50 min at room temperature with rabbit polyclonal anti-activated caspase-3 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-leptin [(H146): sc-9014] (1:40) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies. Thereafter, the sections were incubated for 20 min in EasyLink One polymer (Easypath, São Paulo, Brazil). Protein localization was demonstrated with diaminobenzidine (DAB; Easypath, São Paulo, Brazil), and the sections were counterstained with haematoxylin (Vetec, São Paulo, Brazil) for 1 min. For negative controls (reaction control), the tissues were incubated with blocking buffer, without the primary antibody included.

Images were taken using a Nikon microscope with a camera. Image analysis was performed using software Motic Images® Plus 3.0 (Motic China Group Co., Ltd.), with the assessor blind to treatment (Silva et al., 2023). The follicle was considered caspase-3-positive when it contained positively stained or apoptotic oocyte and/or 70% of the surrounding granulosa cells (Barberino et al., 2022b). The percentage of caspase-3-positive follicles was calculated as the number of caspase-3-positive follicles on the total number of evaluated follicles (×100). Approximately 26–31 preantral follicles were evaluated in each experimental group.

Immunostaining for leptin protein was analyzed in approximately 29–37 preantral follicles in each experimental group and subjectively classified as absent, weak, moderate or strong (Macedo et al., 2019). The slides were examined using a light microscope at 100 and/or 400x magnification.

2.7. In vitro maturation of sheep oocytes from antral follicles

To verify whether a reduction in water intake affects developmental competence of oocytes grown *in vivo*, *in vitro* maturation (IVM) of oocytes was carried out using cumulus-oocyte complexes (COCs) recovered from antral follicles enclosed in ovarian tissue fragments (1–2 mm thick). The recovery of the COCs was performed with sterile 26-G needles in tissue culture 199 medium supplemented with HEPES (TCM199-HEPES) and antibiotics. Only oocytes $\geq$ 110 μm in diameter with a homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells were selected for IVM (Cecconi et al., 1999; Luz et al., 2012). The COCs from the animals submitted to the different water intake levels (100%: $n = 46$; 80%: $n = 39$; 60%: $n = 39$% and 40%: $n = 21$) were transferred to drops of 100 μL of maturation medium composed of TCM 199 bicarbonate 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 at 39 ºC with 5% CO_2 in the air (Cecconi et al., 1999). Next, 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 was analyzed through observation of the intact germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) or metaphase II (MII; nuclear maturation).

2.8. In vitro culture of sheep secondary follicles in medium containing leptin

Finally, we evaluated whether the addition of leptin to the *in vitro* culture medium would mitigate the negative effects of water intake restriction and improve *in vitro* preantral follicle development. Isolation, culture, and follicle evaluation were performed according to a previous study (Menezes et al., 2017) with some modifications. At the same time of the recovery of COC described above, secondary follicles (approximately 180–302 μm in diameter) were visualized under a stereomicroscope (Nikon, Tokyo, Japan; 100x) and mechanically isolated by microdissection from the fragments of the ovarian cortex with 26-G needles. Next, the follicles were individually transferred to 100 μL droplets containing TCM199 for the quality evaluation. Follicles with a visible oocyte surrounded by two or more layers of granulosa cells, an intact basement membrane, and no antral cavity or cytoplasm darkness were selected for *in vitro* culture.

Secondary follicles were individually cultured in 100 μL droplets of culture medium under mineral oil in plastic Petri dishes (60 ×15 mm, Corning, Sarstedt, Newton, NC, USA) at 39 °C under 5% CO_2 for 12 days. Follicles isolated from animals of each group of water intake were divided in three *in vitro* culture treatments: control medium consisting 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^{+} (control) or α-MEM^{+} supplemented with 10 or 25 ng/mL human recombinant leptin (catalog number L4146). Every two days, 60 μL of the culture media was replaced with fresh media in each droplet. The concentrations of leptin were chosen according to a previous study (Macedo et al., 2019). Approximately 20–25 follicles were cultured per treatment, totaling 60–75 follicles for each experimental group of water intake.

Follicular morphology was assessed at day 12 of culture. Atretic follicles were those showing darkness of the oocytes and the surrounding granulosa cells, misshapen oocytes, rupture of the basement membrane or oocyte extrusion. The following endpoints were analyzed in the normal follicles: antral cavity formation; follicle diameter (measured from the basement membrane using a pre-calibrated ocular micrometer attached to a stereomicroscope at 100x magnification); total growth rate, calculated as the final diameter of the normal follicle (day 12) subtracted from its initial diameter (day 0). After 12 days of culture, all healthy follicles were carefully mechanically opened with 26 G needles under a stereomicroscope (Nikon) to recover the oocyte. The percentage of fully grown oocytes, *i.e.*, oocyte $\geq$ 110 μm, was calculated as the number of $\geq$ 110 μm oocytes recovered out of the total number of cultured follicles (x 100).

2.9. Statistical analyses

All analyses were performed using the SAS package (SAS. INSTITUTE. SAS/STAT. Version 9.1, Cary, NC, 2004). Data were tested for normal distribution using the Shapiro-Wilk test using PROC UNIVARIATE procedure and transformed when necessary (log10(x + 1). The *in vivo* experiment was performed in a completely randomized experimental design due to the homogeneity obtained in the selection of animals' body size and age. The body weight at slaughter, daily weight gain, uterus and ovary weight, Leptin, P4 and E2 concentrations (initial and final), *in vivo* survival of preantral follicles, percentage of primordial, intermediate, primary and secondary normal follicles, and follicular activation were submitted to ANOVA using PROC GLM and orthogonal polynomials with linear and quadratic effect. Data with time effect were analyzed using PROC MIXED procedure. The model included the effect of water intake (100 – 80 – 60% and 40%) in eight repetitions (32 animals). The apoptosis results were analysed by Chi-square procedure, considering as statistically significant when $P < 0.05$.

The *in vitro* experiment was also performed in a completely randomized experimental design, and PROC MIXED performed the ANOVA analysis. The model included the effect of water intake (100, 80, 60% and 40%), culture medium (α-MEM^{+}; 10 and 25 ng/mL leptin), culture time (0 and 12 days), and their interactions. The results were displayed as the average and standard error of the mean. Total follicular growth rate was analyzed by PROC GLM. However, due to significant heterogeneity of variance, the Kruskal-Wallis test was used by PROC NPAR1WAY. Results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effect of reduced water intake on body, uterus, and ovary weight

Body weight at slaughter, average daily weight gain, uterus and ovarian weights did not differ among different groups (Table 2).

3.2. Effect of reduced water intake on hormone levels

Serum levels of leptin, estradiol and progesterone did not differ among the groups neither at the beginning of the experiment nor at the end, except for the animals that were subjected to 40% water intake, which had a significant increase in the serum progesterone levels at the end of the experiment when compared to the beginning of the experiment (Table 3).

3.3. Effect of reduced water intake on preantral follicle survival and apoptosis in vivo

The ovaries from ewes that received *ad libitum* water (control group; Fig. 1A) and those from the animals that received 80% water

Table 2
Body weight at slaughter, average daily gain, uterus and ovaries weight and water and dry matter intake of Santa Inês crossbreed ewe submitted to different levels of water intake.

	Experimental groups*				MSE	Probability Effect	
Variable	100%	80%	60%	40%		Linear	Quadratic
Body weight at slaughter (Kg)	37.00 ± 0.70	38.43 ± 0.93	36.53 ± 0.73	38.56 ± 0.94	1.13	0.7919	0.8994
Average daily gain (kg)	0.078 ± 0.007	0.098 ± 0.007	0.065 ± 0.004	0.100 ± 0.003	0.01	0.7812	0.6114
Uterus (g)	65.60 ± 1.72	65.00 ± 1.86	60.00 ± 2.29	57.50 ± 1.80	2.66	0.2388	0.8648
Ovaries (g)	2.73 ± 0.10	2.55 ± 0.12	2.28 ± 0.08	2.21 ± 0.09	0.14	0.1647	0.8521
#Water intake (Kg/day)	1.79	1.41	1.11	0.73			
#Dry matter intake (kg/day)	1.156	1.113	1.170	1.180			

MSE: Mean Squared Error
* *Ad libitum* water (Control – 100%); 80%; 60%, and 40% water intake from the control group's water intake.
# Araujo et al. (2021).

Table 3
Serum levels (initial and final) of leptin, estradiol and progesterone of Santa Inês crossbreed ewe submitted to different levels of water intake.

	Experimental groups*				MSE	Probability Effect	
Variable	100%	80%	60%	40%		Linear	Quadratic
Initial leptin (pg/mL)	635.7 ± 199.6	1086.5 ± 318.6	1225.2 ± 205.2	1424.3 ± 498.0			
Final leptin (pg/mL)	665.9 ± 203.8	756.7 ± 148.0	1160.7 ± 149.7	1455.3 ± 553.6			
Initial estradiol (pg/mL)	29.62 ^{A} ± 2.82	25.50^{A} ± 0.79	30.25^{A} ± 1.59	24.87^{A} ± 0.96	2.37	0.6686	0.8996
Final estradiol (pg/mL)	22.25 ^{A} ± 1.60	25.00^{A} ± 2.01	28.87^{A} ± 1.63	28.00^{A} ± 2.89	2.77	0.4129	0.7555
Initial progesterone (ng/mL)	1.84^{A} ± 0.20	1.72^{A} ± 0.17	1.63^{A} ± 0.13	1.03^{B} ± 0.12	0.17	0.1063	0.4792
Final progesterone (ng/mL)	2.28^{A} ± 0.18	1.35^{A} ± 0.18	1.21^{A} ± 0.13	1.82^{A} ± 0.27	0.22	0.4372	0.0869

Different letters in the column differ from each other ($p < 0.05$).
MSE: Mean Squared Error
* *Ad libitum* water (Control – 100%); 80%; 60%, and 40% water intake from the control group's water intake.

intake (Fig. 1B) showed morphologically normal preantral follicles. The ovaries from sheep that received 60% (Fig. 1C) or 40% (Fig. 1D) water intake displayed atretic preantral follicles with swollen and disorganized granulosa cells, retracted, vacuolated oocyte or condensed nuclear chromatin. The reduction in water intake promoted a decreasing linear effect in the percentage of morphologically normal preantral follicles (follicle survival), and more specifically, in the percentage of normal primordial follicles (Table 4). Furthermore, the reduction in water intake increased the number of caspase-3 positive follicles, *i.e.*, the treatments with 60% and 40% water intake had a greater percentage of apoptosis than control animals (100% water intake) (Fig. 2A-B). Reaction control did not show immunostaining (Fig. 2C).

3.4. Effect of reduced water intake on primordial follicle activation

The percentage of growing preantral follicles (intermediate, primary, and secondary follicles) did not differ among treatment groups, indicating that there was no follicle activation (Table 4).

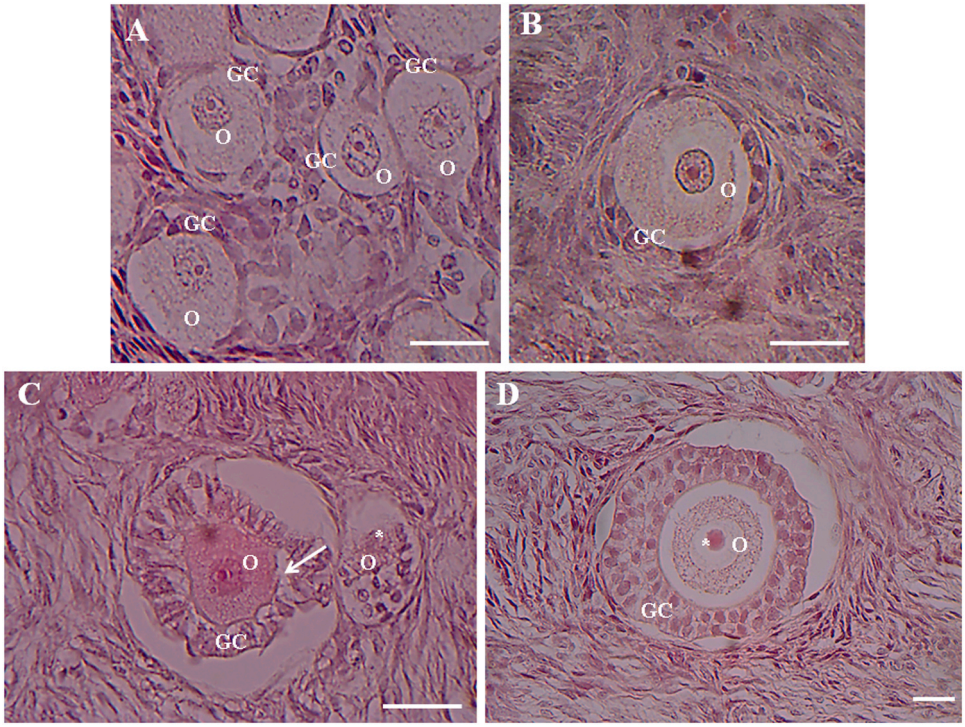

Fig. 1. Histological sections of ovine ovarian fragments (A–D): Normal primordial follicles in the control group (100% - *ad libitum* water; A) and normal intermediate follicle from sheep that received 80% water intake (B); atretic primary (C) and secondary (D) follicles from ewes that received 60% and 40% water intake, respectively. O: oocyte; GC: granulosa cells; Asterisk: pyknotic nucleus; Arrow: retracted oocyte. Scale bar: 20 μm (400 ×).

Table 4
Percentage of total morphologically normal preantral follicles, percentage of normal follicles in different stages (primordial, primary, and secondary follicles) and percentage of follicular activation of Santa Inês crossbreed ewes submitted to different levels of water intake.

	Experimental groups*				MSE	Probability Effect	
Variable	100%	80%	60%	40%		Linear	Quadratic
Preantral follicle survival (%)	56.25	49.17	45.00	32.08	3.03	[a]0.0053	0.5644
Normal follicles in each category (%)							
Primordial follicles	44.17	40.42	35.42	25.42	2.75	[b]0.0079	0.6579
Intermediate follicles	9.58	7.92	8.75	6.25	-	-	-
Primary follicles	0.83	0.42	0.42	0.00	-	-	-
Secondary follicles	1.67	0.42	0.42	0.42	-	-	-
Follicular activation (%)							
Primordial follicles	78.52	82.20	78.70	79.22	-	-	-
Intermediate follicles	17.04	16.10	19.44	19.48	-	-	-
Primary follicles	1.48	0.85	0.93	0.00	-	-	-
Secondary follicles	2.96	0.85	0.93	1.30	-	-	-
Growing follicles (intermediate, primary and secondary)	21.48	17.80	21.30	20.78	-	-	-

* *Ad libitum* water (Control – 100%); 80%; 60%, and 40% water intake from the control group's water intake.
[a] Preantral follicle survival = 18.81 + 0.384x R^2 = 0.47
[b] Normal primordial follicles = 14.89 + 0.305x R^2 = 0.35

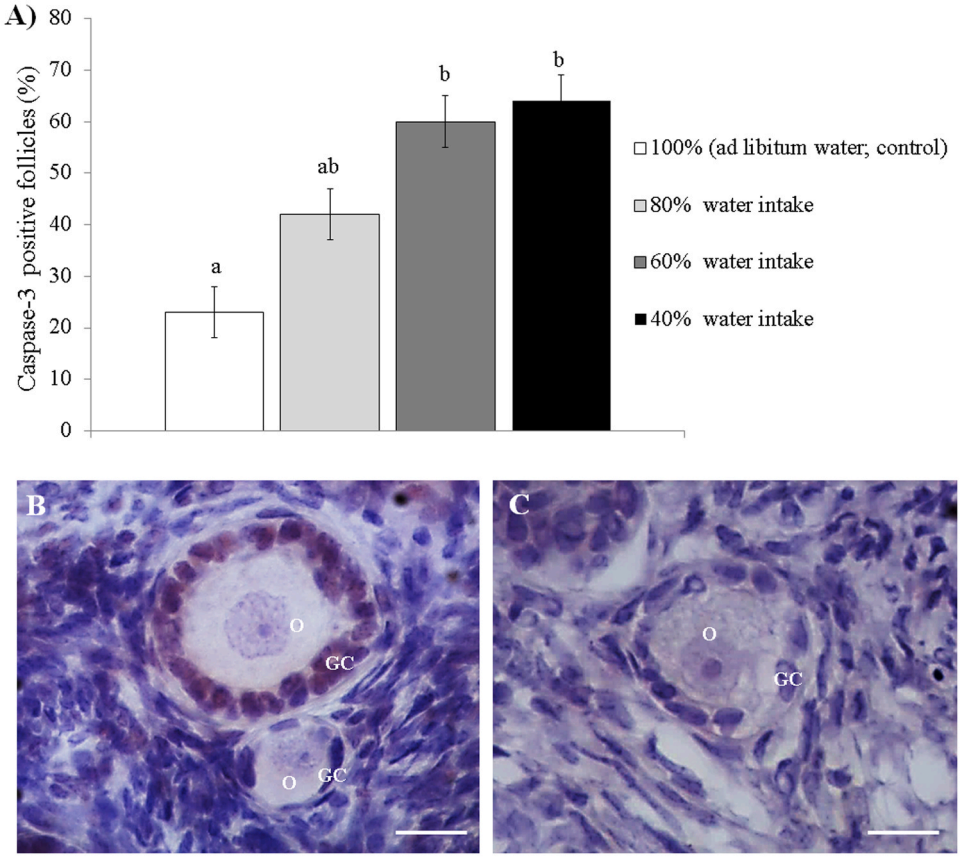

Fig. 2. Percentage of cleaved caspase-3 follicles of Santa Inês crossbreed ewes submitted to different levels of water intake (A). Immunohistochemical expression of cleaved caspase-3 in ovarian fragments (B-C): follicle with apoptotic cells in the top of Fig. 2B and normal follicle in the bottom of Fig. 2B; negative control (c). O: oocyte; GC: granulosa cells. Scale bar: 20 μm (400 ×).

3.5. Effect of reduced water intake on leptin immunolocalization

Oocytes from preantral follicles showed a moderate immunoexpression for leptin in ewes that received *ad libitum* water (control group - 100%; Fig. 3A) and a weak immunoexpression in animals that received lower levels of water intake (60% and 40%; Fig. 3B). No

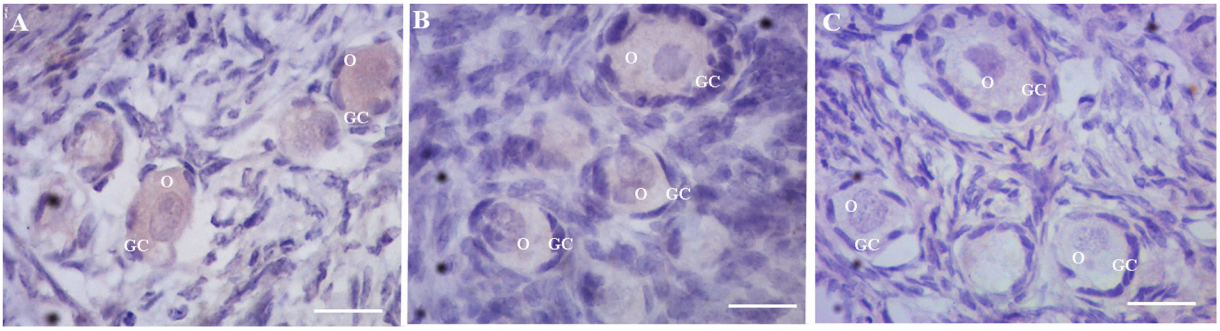

Fig. 3. Immunohistochemical analysis for the expression of leptin in sheep preantral follicles: follicles with moderate (A) and weak (B) immunostaining for leptin protein; negative control (C). GC: granulosa cells; O: oocyte. Scale bars: 25 μm (×400).

immunostaining for leptin protein was observed in the granulosa cells of any group. Reaction control did not show immunostaining (Fig. 3C).

3.6. *Effect of reduced water intake on in vitro maturation of oocytes from antral follicles*

The reduction in water intake did not affect meiosis resumption of *in vivo* grown oocytes in any treatment group (Table 5).

3.7. *Effect of leptin on the in vitro culture of secondary follicles from ewes submitted to water intake reduction*

After 12 days of culture, supplementation of the culture medium with leptin did not influence the percentage of normal follicles, antrum formation and fully-grown oocytes among groups (Table 6). There was no difference in total growth rate (Fig. 4) among different water intake groups, except for the animals of group of 60% that had the lowest result when the follicles were cultured in α-MEM^{+}. In addition, total growth rate (Fig. 4) were significantly greater when follicles from animals that received *ad libitum* water (control group) were cultured in α-MEM^{+} compared to those cultured in 10 ng/mL leptin but did not differ from 25 ng/mL leptin. Otherwise, the addition of 25 ng/mL leptin to the medium significantly increased the total growth rate (Fig. 4) compared to α-MEM^{+} (control medium) in those follicles from ewes that received 60% water intake. There were no interactions among water intake levels, media and culture time.

4. Discussion

The present study demonstrated for the first time the effects of the reduction in water intake on different phases of folliculogenesis and oocyte maturation in ewes, as well as in serum levels of leptin, estradiol and progesterone. The reduction of water intake, *i.e.*, 60% and 40% water intake decreased the percentage of normal preantral follicles, especially the primordial follicles, increased apoptosis and decreased leptin expression in sheep preantral follicles. Furthermore, supplementation of the culture medium with 25 ng/mL leptin mitigated the negative effects of 60% of water restriction and increased the follicular growth *in vitro*.

Considering that the ovarian preantral follicles (primordial, intermediate, primary, and secondary follicles) are the major source of oocytes, which can be used in assisted reproductive programs, and that the primordial follicles are considered the pool of ovarian reserve (Guo and Pankhurst, 2020), we investigated whether these follicles would be affected by a reduction in water intake. Interestingly, our results showed that the reduction of water intake (60% and 40%) decreased significantly the percentage of normal preantral follicles, specially primordial follicles, increased the percentage of apoptosis in follicle cells and decreased the leptin expression in preantral follicles. Small follicles do not have an independent capillary network and are dependent on the surrounding stromal vessels for their survival and early growth (Delgado-Rosas et al., 2009). Moreover, some studies claimed that a low flow of nutrients to the ovary for prolonged periods impairs the initial folliculogenesis (Britt, 1992; Nottle et al., 1997; Rondina et al., 2005). Therefore, probably the reduction of water intake may affect the cortical ovary vascularization, compromising the nutrients transport to the preantral follicles, as well as the production of hormones like leptin, which has an anti-apoptotic action for sheep preantral

Table 5
Percentage of meiotic stages after IVM of *in vivo* grown oocytes recovered from ewes submitted to different levels of water intake.

Treatments group of water intake*	% GV (n)	% GVBD (n)	% MI (n)	% MII (n)
100% (Control)	13.04 (06/46)	50.00 (23/46)	28.26 (13/46)	8.70 (04/46)
80%	23.08 (09/39)	33.33 (13/39)	35.90 (14/39)	7.69 (03/39)
60%	20.51 (08/39)	43.59 (17/39)	25.64 (10/39)	10.26 (04/39)
40%	23.81 (05/21)	52.38 (11/21)	19.05 (04/21)	4.76 (01/21)

($P > 0.05$).
* Ad *libitum* water (Control – 100%); 80%; 60%, and 40% water intake from the control group's water intake.

Table 6
Influence of leptin on the percentage of normal follicles, antrum formation and fully-grown oocytes after *in vitro* culture of isolated secondary follicles of Santa Inês crossbreed ewes submitted to different levels of water intake.

Treatments group of water intake*	Normal follicles (%)		
	α-MEM$^+$	10 ng/mL leptin	25 ng/mL leptin
100% (control)	62.5	72.5	50.7
80%	54.2	59.3	54.4
60%	59.6	59.6	63.6
40%	62.4	60.0	46.3
	Antrum formation (%)		
	α-MEM$^+$	10 ng/mL leptin	25 ng/mL leptin
100% (control)	25.0	32.1	15.3
80%	39.0	59.3	36.7
60%	18.2	40.0	27.1
40%	15.9	46.0	7.4
	Fully-grown oocytes (%)		
	α-MEM$^+$	10 ng/mL leptin	25 ng/mL leptin
100% (control)	17.0	49.9	17.4
80%	25.0	38.9	24.4
60%	22.6	41.0	32.3
40%	11.4	15.0	24.0

($P > 0.05$).
* Ad *libitum* water (control – 100%); 80%; 60%, and 40% water intake from the control group's water intake.

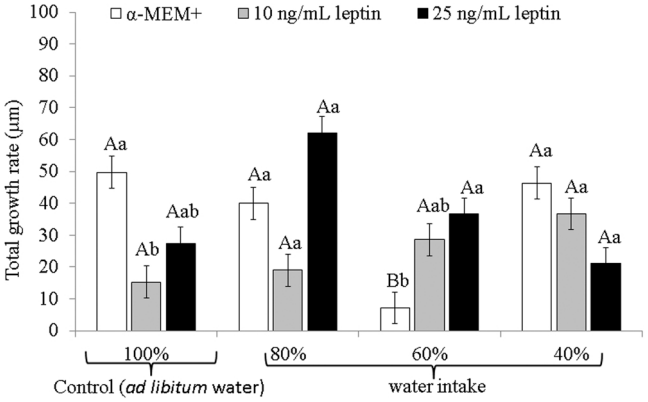

Fig. 4. Influence of leptin on the total growth rate after 12 days of *in vitro* culture of secondary follicles from ewes submitted to different levels of water intake. [A, B]Different letters denote significant differences among the different levels of water intake in the same culture medium ($P < 0.05$); [a, b]Different letters denote significant differences among different culture medium in the same level of water intake ($P < 0.05$).

follicles cultured *in vitro* (Macedo et al., 2021). Since the activation of the primordial follicles was not observed in any treatment, we suggest that a reduction in water intake can increase the loss of ovarian reserve due to death of primordial follicles. It is worth noting that this precocious depletion of the follicular pool may result in premature ovarian failure in the animals.

Water restriction can decrease feed intake, which is reported to cause retardation of ovarian follicular growth (Blanc et al., 2004), increase progesterone and decrease serum estradiol and leptin hormone levels in sheep (Naqvi et al., 2015; Kumar et al., 2016; El Khashab et al., 2018). Leptin is related to the reproductive axis and the ovary of mammals (Dar et al., 2017; Heryani, 2019) and to the *in vitro* development of sheep secondary follicles (Kamalamma et al., 2016; Macedo et al., 2019). Thus, we hypothesized that the addition of leptin to the *in vitro* culture medium would mitigate the negative effects of water intake and improve *in vitro* follicle development. Our results showed a decrease in growth rate of follicles cultured in α-MEM$^+$ from ewes that received 60% water intake compared to the other experimental groups. In this group (60% water intake), supplementation of the culture medium with 25 ng/mL leptin stimulated follicular growth compared to those follicles cultured in α-MEM$^+$ without leptin. Previous studies already pointed out that leptin increases the follicle diameter (Bilbao et al., 2015; Kamalamma et al., 2016), probably through stimulation of the granulosa cell proliferation (Wen et al., 2015). Therefore, considering our findings that the reduction of water intake decreased the leptin protein expression in oocytes, we propose that the addition of 25 ng/mL leptin to the culture medium was ideal to intake the low follicular production of this hormone, thus promoting *in vitro* growth of these follicles.

Regarding leptin serum level, our results demonstrated that the reduction in water intake did not affect the serum levels of this hormone. Leptin is produced by different organs, including ovaries (Macedo et al., 2019), but mainly by adipose tissue (Picó et al., 2022). The variations in the concentrations of this hormone are related to body fatness status (Delavaud et al., 2000). In the present

study, we believe that reduction in water intake did not affect the leptin serum levels because the body fat was not influenced by water intake (Araújo et al., 2021). However, the reduction in water intake decreased the leptin expression in preantral follicles. Thus, it appears that the preantral follicles have an autoregulative mechanism and can be relatively independent of the serum leptin levels.

Considering the importance of IVM for improvement of the fertilization and developmental capacity of ovine oocytes (Karami et al., 2011), this study also assessed the effect of water restriction on IVM of oocytes from *in vivo* grown antral follicles. The water intake reduction did not affect meiotic resumption. The similarity between the nutritional status of animals after water restriction might have contributed to the absence of significative difference in the meiotic stages because nutrition affects the number of oocytes that ovulate and their quality (Ashworth et al., 2009), confirming once again that animals adapting to semiarid conditions (Moura et al., 2016; Amorim et al., 2018) are resistant to water restriction (Santos et al., 2019a; b).

Finally, the present study demonstrated that the reduction in water intake did not affect serum levels of progesterone and estradiol. However, ewes submitted to 40% water intake had a significant increase in the serum level of progesterone at the end of the experiment when compared to the beginning. A previous study demonstrated that 50% water restriction did not change the levels of these hormones in goats (El Khashab et al., 2018). Nevertheless, another study highlighted that water-restricted groups of Malpura ewes either submitted to 40% continuous water restriction or submitted to a total water restriction alternated to *ad libitum* availability showed decreased plasma estradiol and increased progesterone levels (Kumar et al., 2016). These authors also observed that the groups submitted to water restriction showed the highest loss of body weight at the end of the experiment, the highest reduction of the average daily weight gain, and the lowest dry matter intake, as compared to the other experimental groups. These results highlight that water-restricted ewes suffered nutritional insufficiency. The serum levels of estradiol and progesterone in ewes are related to the nutritional status (Lamond et al., 1972; Kiyma et al., 2004; Kumar et al., 2016). Possibly, in the present study, serum levels of estradiol and progesterone did not differ among the experimental groups due to the absence of a significant difference in body weight at slaughter, average daily weight and dry matter intake. Thus, animals submitted to water restriction maintained their levels of estradiol and progesterone and nutritional needs, confirming their adaptability to tropical climate conditions as maintenance of carcass traits and meat quality when submitted to water restriction of at 24 h (Santos et al., 2019a; b) and their ingestion behavior when submitted to water saline levels (different levels of total dissolved solids; Moura et al., 2016).

5. Conclusions

In conclusion, the results indicated that the reduced water intake impaired the percentage of normal preantral follicles enclosed in ovarian tissue, especially the survival of primordial follicles, increased apoptosis and decreased leptin expression in preantral follicles from crossbred Santa Inês ewes. Furthermore, water intake reduction did not influence serum leptin, estradiol and progesterone levels, primordial follicle activation, as well as the IVM of oocytes from antral follicles. The addition of 25 ng/mL leptin to the culture medium reversed the detrimental effects of 60% of water restriction and increased follicle development *in vitro*.

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Declaration of Competing Interest

The authors declare no conflicts of interest with any other people or organizations in any financial or personal relationship.

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