

# Effect of ovarian tissue storage in *Morus nigra* extract on the morphology and DNA fragmentation of ovine preantral follicles

## Efeito da conservação de tecido ovariano em extrato de *Morus nigra* sobre a morfologia e fragmentação de DNA de folículos pré-antrais ovinos

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### Abstract

This study demonstrated the effect of *Morus nigra* leaf extract during ovine ovarian tissue transportation on the survival and apoptosis of preantral follicles *in vitro*. High Performance Liquid Chromatography (HPLC) was used to determine the fingerprint chromatogram of the crude ethanolic extract. Four pairs of ovaries from four sheep were collected. The ovarian cortex was fragmented and one fragment was fixed in 10% buffered formaldehyde and processed for histological and TUNEL analyses (fresh control). The other fragments were placed in Minimal Essential Medium (MEM – control medium) or *M. nigra* extract (0.025; 0.05 or 0.1 mg/mL) and stored (simulating transport) at 4°C for 6, 12 or 24 h. Preserved fragments (6 h) were also destined to histological and TUNEL analyses. HPLC analysis confirmed the presence of antioxidant compounds (rutin, isoquercetin e kaempferitrin) in the extract. There was a decrease ( $P < 0.05$ ) in the percentage of morphologically normal preantral follicles after preservation in all treatments compared to the fresh control. The percentage of normal preantral follicles after preservation in *M. nigra* at 0.05 mg/mL for 6 h was higher ( $P < 0.05$ ) than in MEM or 0.025 mg/mL *M. nigra* and similar ( $P > 0.05$ ) to 0.1 mg/mL of the extract. Apoptosis increased ( $P < 0.05$ ) after conservation for 6 h in all treatments compared to the fresh control. Moreover, TUNEL positive cells decreased ( $P < 0.05$ ) after preservation in 0.05 or 0.1 mg/mL *M. nigra* compared to MEM or 0.025 mg/mL *M. nigra*. In conclusion, 0.05 mg/mL *M. nigra* extract can be used as a preservation medium for ovine ovarian tissue at 4°C for up to 6 h.

**Key words:** Ovine. Oocyte. Preantral. HPLC. Medicinal Plant.

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## Resumo

Este estudo demonstrou o efeito do extrato das folhas de *Morus nigra* durante o transporte na sobrevivência e apoptose de folículos pré-antrais *in vitro*. A CLAE (Cromatografia Líquida de Alta Eficiência) foi usada para determinar a impressão digital cromatográfica do extrato etanólico. Foram coletados 4 pares de ovários de 4 ovelhas. O córtex ovariano foi fragmentado e um dos fragmentos foi fixado em formaldeído tamponado a 10% e destinado às análises histológica e de TUNEL (controle fresco). Os demais fragmentos foram colocados em Meio Essencial Mínimo (MEM – meio controle) ou extrato de *M. nigra* (0,025, 0,05 ou 0,1 mg/mL) e conservados (simulando transporte) à 4 °C por 6, 12 ou 24 h. Os fragmentos conservados (6 h) também foram destinados às análises histológica e de TUNEL. A análise de CLAE confirmou a presença de compostos antioxidantes (rutina, isoquercetina e canferitrina) no extrato. Houve uma redução ( $P < 0,05$ ) na percentagem de folículos pré-antrais morfológicamente normais após conservação em todos os tratamentos em comparação com o controle fresco. A percentagem de folículos pré-antrais normais após conservação em *M. nigra* a 0,05 mg/mL por 6 h foi maior ( $P < 0,05$ ) do que em MEM ou *M. nigra* a 0,025 mg/mL e similar ( $P > 0,05$ ) ao extrato a 0,1 mg/mL. A apoptose aumentou ( $P < 0,05$ ) após conservação durante 6 h em todos os tratamentos em comparação com o controle fresco. Além disso, as células TUNEL positivas diminuíram ( $P < 0,05$ ), após conservação em 0,05 ou 0,1 mg/mL de *M. nigra* em comparação com MEM ou 0,025 mg/mL de *M. nigra*. Em conclusão, 0,05 mg/mL de *M. nigra* pode ser usado como meio de conservação de tecido ovariano ovino a 4 °C durante até 6 h.

**Palavras-chave:** Ovino. Oócito. Pré-antral. CLAE. Planta Medicinal.

## Introduction

Temporal storage of ovaries during transportation to the specialized laboratories can provide opportunities to rescue oocytes from ovaries of livestock or endangered animals that could be used for embryo production *in vitro*. Adequate medium, duration of storage and temperature at which ovaries are kept after death or excision may affect the potential of oocytes recovered from the ovaries (HIDEAKI et al., 2007).

Studies have demonstrated that storage of ovine and caprine ovarian fragments at 4°C results in proper rates of morphologically normal follicles compared to higher temperatures such as 20 or 35 °C (ANDRADE et al., 2001; MATOS et al., 2004; COSTA et al., 2005). Chaves et al. (2008) have shown that ovarian tissue transportation in Minimal Essential Medium (MEM) at 4°C for up to 4 h maintained the percentages of morphologically normal follicles similar to those observed in fresh tissues even after 7 days of *in vitro* culture. However, the use of synthetic media (e.g. MEM) could be expensive and less accessible. Therefore, the use of alternative media, such as medicinal

plant extracts, may be a promising and low-cost strategy for temporal storage of mammalian ovaries (GOUVEIA et al., 2015), especially due to the remarkable cytoprotective and antioxidant characteristics of these plants (TAG, 2015).

*Morus nigra* L. (mulberry) is a plant found from temperate to subtropical regions of the Northern hemisphere to the tropics of the Southern hemisphere and it can grow in a wide range of climatic, topographic and soil conditions (ERCISLI; ORHAN, 2007). In Northeastern Brazil, it is popularly known as “amora-miúra”, “amora-preta” or “amoreira”. The mulberry belongs to the genus *Morus* of the family Moraceae (ALMEIDA et al., 2011) and has been widely used in folk medicine as hepatoprotective, hypotensive, antipyretic, analgesic, diuretic, expectorant and antidiabetic (NOMURA, 1988; CHEN et al., 1995). Several compounds have been isolated from the roots, trunk bark and leaves of *M. nigra*, such as the piperidine alkaloid (moranolone), glycoproteins and phenolic compounds (flavonoids, stilbenes, coumarins) (NOMURA; HANO, 1994; KIM et al., 1999; TOSHIO et al., 2005; MAZIMBA et al., 2011). Some of these compounds (for example, flavonoids)

have antioxidant properties, reducing oxidative stress, reactive oxygen species (ROS) levels, lipid peroxidation and DNA damage in different cell types (MAZIMBA et al., 2011; VOLPATO et al., 2011; MATA-CAMPUZANO et al., 2012).

The leaves of *M. nigra* are commonly used by women in menopause as a substitute for the conventional hormonal replacement therapy, with a similar effect to that obtained after estrogen use (SILVA et al., 2003). Nevertheless, a recent study demonstrated that the administration of *M. nigra* did not exhibit any estrogenic activity nor did exert a toxic effect on the female reproductive system and on the embryonic development of rats (QUEIROZ et al., 2012). Moreover, no biochemical and macroscopic changes were observed after 30 days of *M. nigra* administration in rats, thus demonstrating its low toxicity (OLIVEIRA et al., 2013).

Thus, in view of its distribution throughout the world, *M. nigra* extract may be used as an alternative and a low-cost (it would cost about 10 or 40 times less than MEM or  $\alpha$ -MEM, respectively) preservation medium in the transportation of ovine ovarian tissue. However, there were no reports of the use of *M. nigra* extract as an *in vitro* preservation medium for sheep ovaries. The aim of the present study was to determine if the use of *M. nigra* extract as a preservation medium during ovine ovarian tissue transportation at 4°C would influence on the morphology and apoptosis of preantral follicles *in vitro*.

## Material and Methods

This experimental was approved and performed under the guidelines of Committee of Ethics and Deontology Studies and Research at the Federal University of San Francisco Valley (protocol n° 0007/040713). Unless indicated, chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### *Plant material and extract preparation*

Fresh leaves of *M. nigra* were collected in Petrolina-PE, (09° 23' 55" South and 38° – 40° 30' 03" West, Pernambuco, Brazil), from the Caatinga biome, and characterized by a semiarid climate, with average annual temperature of 26.4 °C. A voucher specimen (1764) is deposited at the Herbário Vale do São Francisco (HVASF) of the Federal University of San Francisco Valley. The leaves were dried in an oven at 40°C and pulverized and extracted at room temperature with 95% ethanol (Vetec, Duque de Caxias-RJ, Brazil) for 72 h. The extract was dried at 45°C for some hours under vacuum using rotavapor and the yield was approximately of 10% obtaining the crude ethanolic extract of the leaves of *Morus nigra*, which was dissolved in 0.9% saline solution, corresponding to concentrations of 0.025; 0.05 or 0.1 mg/mL.

### *Analysis of ethanolic extract by High Performance Liquid Chromatography (HPLC)*

Chromatographic equipment consisted of a Shimadzu® (Shimadzu, Kyoto, Japan) liquid chromatograph equipped with a diode array detector (DAD), with a quaternary system of pumps model LC-20ADVP, degasser model DGU-20A, detector PDA model SPD-20AVP, oven model CTO-20ASVP, auto sampler injector model SIL-20ADVP and controller SCL-20AVP. The data was acquired and processed using Shimadzu® LC solution1.0 software (Japan).

The crude ethanolic extract (0.1 mg/mL) and chemicals reference (RUT – rutin, ISO – isoquercetin and KFT – kaempferitrin at concentrations of 15, 20, 30, 50 and 100 µg/mL) alone were subjected to analysis, under the following conditions: stationary phase consisted of a C18 column (250 x 4.6 mm, 5 µm), Supelco® (Supelco, Bellefonte, USA), at 37°C with a sample injection volume of 20 µl and the wavelength was 340 nm. Solvent A: water + 0.01% trifluoroacetic acid (v/v) and solution B: acetonitrile was used as mobile phase (0.8 mL/min) with a

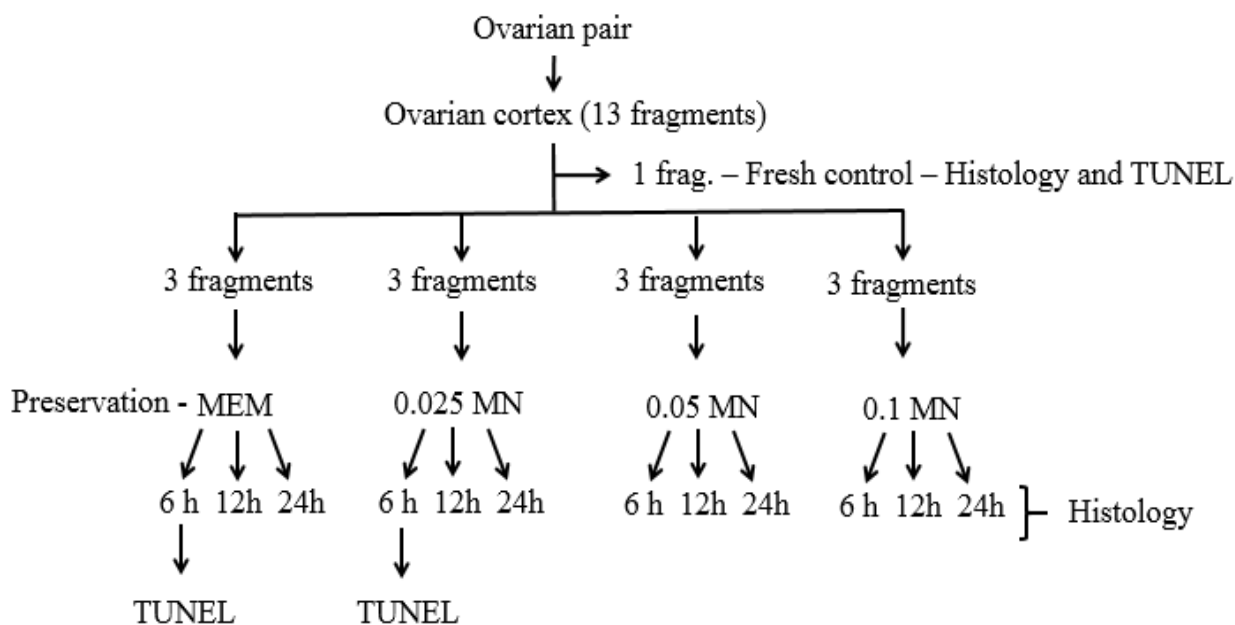
gradient program of: 35 to 65% B in 25 min, 65 to 85% B in 15 min, 85 to 100% B in 5 min, returning to 35% B after 10 min. All solutions were degassed and filtered through a membrane filter of 0.20  $\mu\text{m}$  pore (Millipore, Bedford, USA).

#### Ovaries collection and in vitro preservation

Ovarian cortical tissues (n=8 ovaries) were collected at a local abattoir from four adult (1–3 years old) mixed-breed sheep. Immediately postmortem, pairs of ovaries were washed once in 70% alcohol (Dinâmica) and then twice in MEM buffered with HEPES (MEM-HEPES) and supplemented with antibiotics (100  $\mu\text{g}/\text{mL}$  penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin).

Still in the slaughterhouse, the pair of ovaries from each animal were cut in half, after which the medulla, large antral follicles, and corpora lutea were removed. Following this, the ovarian cortex was divided into 13 fragments approximately  $3 \times 3 \text{ mm}$  (1 mm thick). Then, one ovarian fragment was taken randomly and fixed for histological and TUNEL analyses (fresh control). The other 12 fragments were randomly placed into tubes containing 5 mL MEM supplemented with antibiotics (100  $\mu\text{g}/\text{mL}$  penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin; control medium; CHAVES *et al.*, 2008) or different concentrations of *M. nigra* extract (0.025; 0.5 or 0.1 mg/mL), obtained from the dilution in 0.9% saline solution, and stored at 4°C for 6, 12 or 24 h (simulating transport) (Figure 1). The temperature was maintained using thermos boxes with ice. Each treatment was repeated 4 times.

**Figure 1.** General experimental protocol for the preservation of ovine preantral follicles in *Morus nigra* extract. MN: *M. nigra* leaf extract.



#### Morphological analysis of preantral follicles preserved in situ

Ovarian fragments from each treatment, including the fresh control, were fixed individually in 10% buffered formaldehyde (Dinâmica, Diadema, São

Paulo, Brazil) for 18 h. Subsequently, fragments were dehydrated in a graded series of ethanol (Dinâmica), clarified with xylene (Dinâmica) and embedded in paraffin wax (Dinâmica). Tissues were sectioned serially at a thickness of 5  $\mu\text{m}$  and sections were

stained using standard protocols with hematoxylin eosin (Vetec, Duque de Caxias-RJ, Brazil). Sections were examined by light microscopy (Nikon, Tokyo, Japan) at 400× magnification. Preantral follicles were counted and evaluated in the section where the oocyte nucleus was visible.

The developmental stages of preantral follicles have been defined previously (SILVA et al., 2004) as 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, and secondary: two or more layers of cuboidal granulosa cells around the oocyte). Additionally, these follicles were classified individually as histologically normal when an intact oocyte was present and surrounded by granulosa cells that are well organized in one or more layers and have no pyknotic nuclei. Atretic follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane. Overall, 120 follicles were evaluated for each treatment (30 follicles per treatment x 4 replicates = 120 follicles), totaling 1,560 preantral follicles.

#### *Detection of apoptotic cells by TUNEL Assay*

Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick-end labeling (TUNEL) assay was used for a more in-depth evaluation of ovine preantral follicle quality before (fresh control) and after preservation in MEM or 0.025, 0.05 or 0,1 mg/mL of *M. nigra* for 6 h. TUNEL was performed using a commercial kit (In Situ Cell Death Detection Kit, Roche Diagnostics Ltd., Indianapolis, USA) following the manufacturer's protocol, with some modifications. Briefly, sections (5 µm) mounted on glass slides were deparaffinized and rehydrated through graded alcohols, then rinsed in PBS (pH 7.2). The slides were incubated in citrate buffer (Dinâmica) at 95°C in a decklocking chamber for 40 min to retrieve antigenicity, and endogenous

peroxidase activity was prevented by incubation with 3% H<sub>2</sub>O<sub>2</sub> (Dinâmica) and methyl ethanol (QEEL, São Paulo, Brazil) for 10 min. After rinsing in Tris buffer (Dinâmica), the sections were incubated with TUNEL reaction mixture at 37 °C for 1 h. Then, the specimens were incubated with Converter-POD in a humidified chamber at 37 °C for 30 min. The DNA fragmentation was revealed by incubation of the tissues with diaminobenzidine (DAB; 0.05% DAB in Tris/HCl pH 7.6, 0.03% H<sub>2</sub>O<sub>2</sub>) during 1 min. Finally, sections were counterstained with Harry's hematoxylin in a dark chamber at room temperature for 1 min, dehydrated in ethanol, cleared in xylene, and mounted with balsam (Dinâmica). For negative controls (reaction controls), slides were incubated with label solution (without terminal deoxynucleotidyl transferase enzyme) instead of TUNEL reaction mixture.

Preantral follicles apoptosis was evaluated as described previously (SANTOS et al., 2014). Only follicles that contained an oocyte nucleus were analyzed for apoptotic assay. The number of brown TUNEL positive cells (oocyte and granulosa cells) was counted in ten randomly fields per treatment using Image-Pro Plus® software. The percentage of TUNEL-positive or apoptotic cells was calculated as the number of apoptotic cells out of the total number of cells (x 100).

#### *Statistical analysis*

Percentages of morphologically normal were submitted to the Shapiro-Wilk test to verify normal distribution of residues and homogeneity of variances, followed by ANOVA and the Bonferroni's test was applied for comparison among treatments. Values of apoptotic cells were submitted to Qui-square test and differences were considered to be statistically significant when P<0.05. The results of follicular survival were expressed as the mean ± SD. The values have been verified using the software BioEstat 5.0.

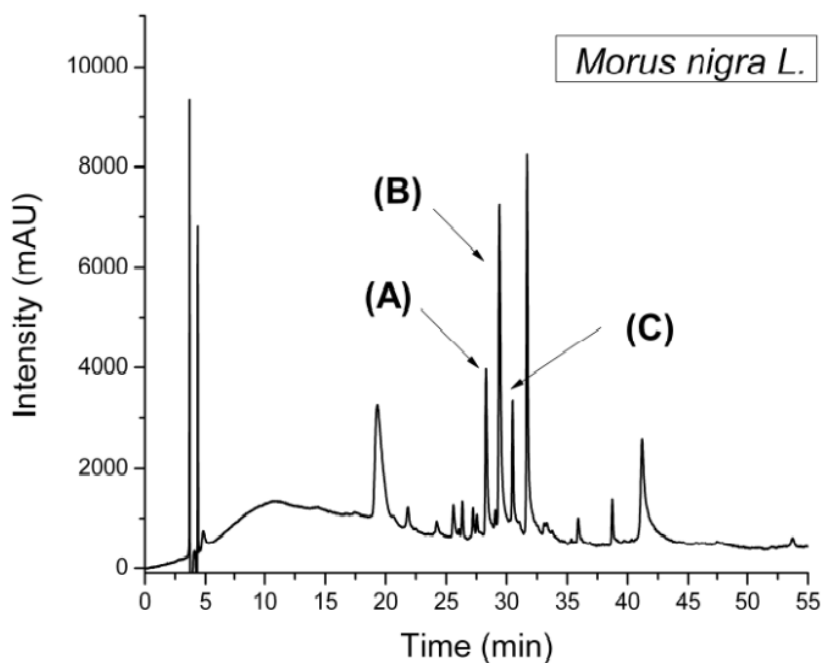
## Results

### HPLC analysis

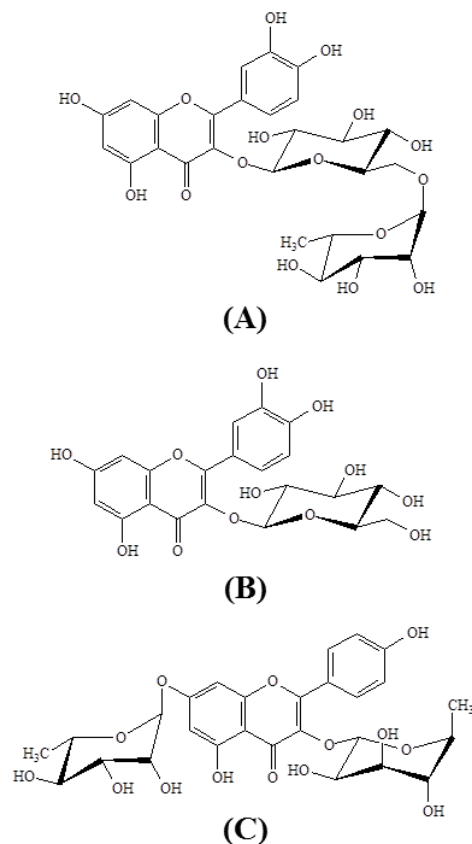
After analysis of the crude ethanolic extract through the HPLC method, it was possible to verify the presence of three substances with different retention times: rutin (A) in 28 min ( $\lambda$

= 252/354 nm), isoquercetin (B) in 29 min ( $\lambda$  = 253/354 nm) and kaempferitrin (C) in 31 min ( $\lambda$  = 247/354 nm; Figure 2). Among these substances, it was possible to accurately quantify rutin ( $5.05 \pm 1.3 \mu\text{g/mL}$ ) and isoquercetin ( $22.3 \pm 3.02 \mu\text{g/mL}$ ). The chemical structures of the compounds are shown in the figure 3.

**Figure 2.** High performance liquid chromatography with diode array detector (HPLC-DAD) profiles of *Morus nigra* ethanolic extract.



**Figure 3.** Chemical structures of the flavonoids rutin (A), isoquercetin (B) and kaempferitrin (C).



*Effect of storage conditions on follicular morphology*

Among the preantral follicles analyzed, 876 were primordial, 645 intermediate, 18 primary and 21 secondary follicles. The preantral follicles in the fresh control (Figure. 4A) and those preserved in 0.05 mg/mL de *M. nigra* (Figure 4B) for 6 h showed

centrally located oocytes and organized granulosa cells surrounded by normal intact basement membranes. After storage in 0.1 mg/mL *M. nigra* for 12 h, atretic follicles with a retracted oocyte and disorganized granulosa cells could be observed (Figure 4C).

**Figure 4.** Histological sections of ovine ovarian fragments showing morphologically normal follicles in the fresh control (A) and after 6 h of preservation in 0.05 mg/mL *M. nigra* (B); atretic follicle after 12 h of preservation in 0.1 mg/mL *M. nigra* (C). Hematoxylin-eosin stain. O = oocyte; GC = granulosa cell. Scale bar: Fig A: 80 µm (100x); B e C: 40 µm (400x).

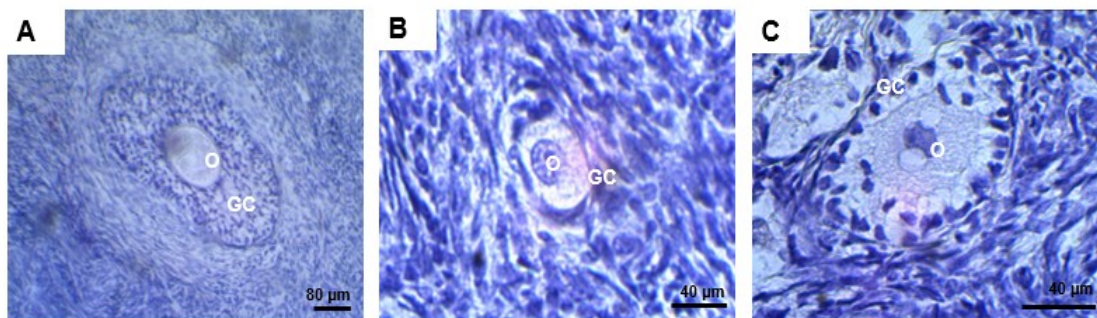
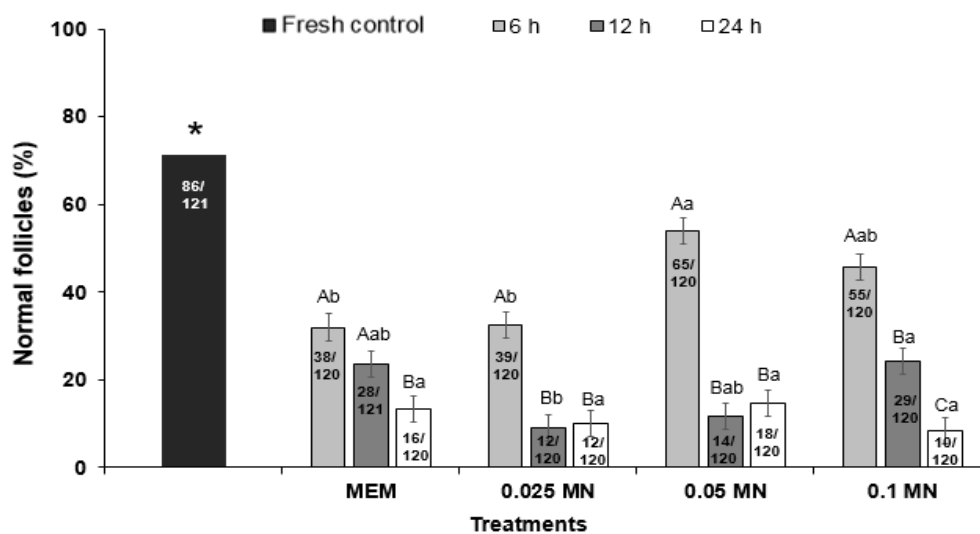


Figure 5 details the effect of media and preservation period of ovarian fragments on the percentage of normal preantral follicles. After histological analysis, there were 71.5% normal preantral follicles in the fresh control. There was a significant reduction in the percentage of morphologically normal follicles after preservation in all treatments ( $P < 0.05$ ) compared to the fresh control. Comparing the different media in the same

period, the percentage of normal preantral follicles after preservation in *M. nigra* at 0.05 mg/mL for 6 h (54%) was significantly higher ( $P < 0.05$ ) than in MEM (32%) or 0.025 mg/mL *M. nigra* (32.5%) and similar ( $P > 0.05$ ) to 0.1 mg/mL of the extract (45.75%). Comparing the control medium (MEM) with any concentration of *M. nigra* extract, no difference was observed after 12 or 24 h of storage ( $P > 0.05$ ).

**Figure 5.** Percentages (mean  $\pm$  SEM) of morphologically normal follicles in the fresh control and after preservation in MEM or *M. nigra* extract.



\*Differs significantly from fresh control ( $P < 0.05$ ). (a,b) Different letters denote significant differences among treatments (different media) in the same period ( $P < 0.05$ ). (A, B) Different letters denote significant differences among periods in the vsame media ( $P < 0.05$ ).

#### Apoptotic cell detection

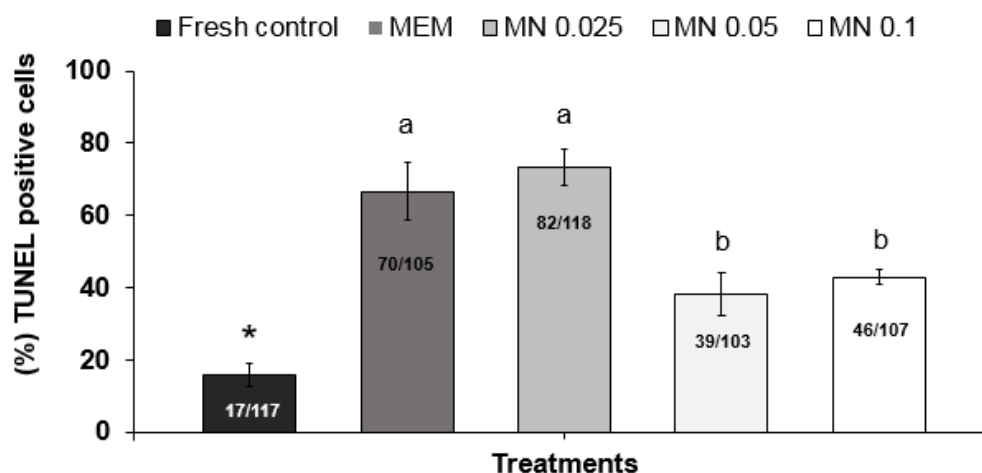
TUNEL analysis was performed only for the period of 6 h of preservation because at this period *M. nigra* extract (0.05 mg/mL) had higher percentages of normal follicles than the control medium. There was a significant increase ( $P < 0.05$ ) in the percentage of apoptotic cells (oocyte and granulosa cells) after preservation in all treatments compared with the fresh control (14.5%) (Figure 6). Ovarian tissue preserved at 0.05 mg/mL (38%) or 0.1 mg/mL (43%) *M. nigra* extract showed less apoptotic cells than storage in MEM (67%) or 0.025 mg/mL

(69.5%) *M. nigra* extract ( $P < 0.05$ ). No difference was observed in the percentage of TUNEL-positive cells after preservation in 0.05 mg/mL or 0.1 mg/mL *M. nigra* extract ( $P > 0.05$ ).

Ovarian follicles preserved in 0.05 mg/mL *M. nigra* did not show or showed less apoptotic cells (Figure 7A). Nevertheless, TUNEL-positive cells (brown staining) were commonly found in ovarian tissue that had been preserved in MEM (Fig. 7B). Negative control did not show staining for TUNEL analysis (Figure 7C).



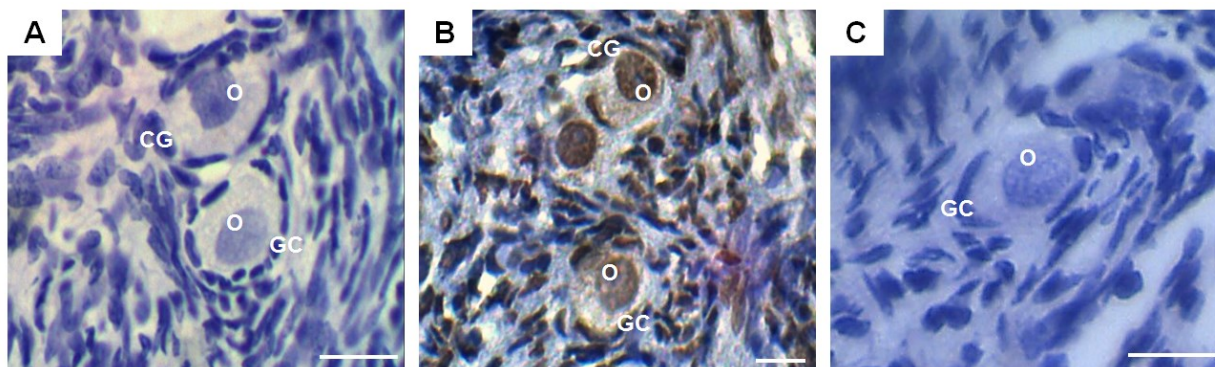
**Figure 6.** Percentages (mean  $\pm$  SEM) of TUNEL positive cells in the fresh control and after 6 hours of preservation in MEM; 0.025; 0.05 or 0.1 mg/mL *M. nigra*.



\*Differs significantly from fresh control ( $P < 0.05$ ).

(a, b) Different letters denote significant differences among treatments (different media) ( $P < 0.05$ ).

**Figure 7.** Apoptosis detection in ovine ovarian tissue after 6 h of preservation. Normal preantral follicle in 0.05 mg/mL *M. nigra* (A), apoptotic follicles in MEM (B), and negative control (C). Note the apoptosis in the oocyte and granulosa cells (brown) in figure B. O: oocyte; GC: granulosa cells. Scale bar: 30  $\mu$ m (400x).



## Discussion

This is the first report to demonstrate the beneficial effect of *M. nigra* extract on *in vitro* preservation of ovine ovarian tissue during transportation. One of the main challenges of the researches is to find the optimum composition for a storage solution, which might have less toxicity or damage for ovarian tissue. In this context, many studies have been performed to evaluate the efficiency of different solutions (MEM: CHAVES et al., 2008; saline solution: ANDRADE et al., 2002; Braun Collins solution: SILVA et al., 2000; coconut water solution:

ANDRADE et al., 2002; PBS: SANTOS et al., 2002) in the maintenance of follicle survival during the transport of the ovaries. Among these media, MEM has been one of the most commonly used for ovary storage in ovine and caprine species. MEM is rich in nutrients (glucose, vitamins and amino acids) and could be used alone (caprine: CHAVES et al., 2008) or enriched with others supplements (e.g. ITS, glutamine, hypoxanthine, and BSA; ovine: GONÇALVES et al., 2015) for maintaining follicle viability in ovarian tissue transportation. However, the use of commercial medium and/or

supplements makes the reproductive techniques more expensive. Therefore, other alternative media, such as *Amburana cearensis* extract, have also been successfully used to preserve caprine ovarian tissue at 4°C for 6 h (GOUVEIA et al., 2015), maintaining the percentages of normal follicles (approximately 60%) similar to that observed in the control medium (MEM).

In our study, after 6 h of preservation, an appropriate concentration of *M. nigra* extract (0.05 mg/mL) resulted in higher percentages of morphologically normal follicles (54.0%) and lower rates of DNA fragmentation (38.23%) than those observed in the control medium (MEM) or in 0.025 mg/mL *M. nigra*. Using the HPLC method, it was possible to identify three substances (rutin, isoquercetin, and kaempferitrin) in the crude ethanolic extract of *M. nigra*. We should take into account that environments in which the plant develops (temperature, relative humidity, solar radiation, soil) exert a direct influence on the chemical composition of the extracts (SUAREZ et al., 2003; FLOSS, 2004). Thus, knowledge of the chemical composition of *M. nigra* extract is essential for obtaining standardized extracts, which could be used as a commercial medium in the future.

In the current study, the substances identified in the *M. nigra* extract are flavonoids, a large group of polyphenolic compound found in many plant-based foods (MORETTI et al., 2012). The flavonoids exert antioxidant properties, which have been attributed to the ability of scavenging ROS. A relation between the chemical nature of flavonoids and their antioxidant activity has been established; in particular the number and the position of hydroxyl groups influence their antioxidant power (ÇELİK; ARINÇ, 2010). As they are amphipathic molecules, their permeability across membranes and their membrane affinity depend on the degree of hydroxylation, molecular configuration, and the length of the side chain (TAMMELA et al., 2004).

Our HPLC analysis showed that the compound

found in large amounts in the *M. nigra* extract was isoquercetin, which is a quercetin glycoside, a flavonoid present in plants (WANG et al., 2013). Isoquercetin has many biological properties, including antiviral (KIM et al., 2010) and antidiabetic (ZHANG et al., 2011). To date, there were no reports of the use of isoquercetin on folliculogenesis. However, its antioxidant effect was observed in other cells. Studies have demonstrated that isoquercetin protects neuroblastoma (SH-SY5Y) from the oxidative damage *in vitro* (SOUNDARARAJAN et al., 2008). In addition, isoquercetin is a potent neuroprotector in rats, being described as anti-inflammatory and antiapoptotic factor (WANG et al., 2013).

Rutin is also a glycoside of quercetin, in which the 3-hydroxyl group is replaced with rutinose, a disaccharide consisting of glucose and rhamnose (WEI et al., 2011). This flavonoid has antioxidant, anti-inflammatory and anti-cancer properties (SHEN et al., 2004). Rutin has been used to reduce the injury caused by ischemia/reperfusion in different organs, such as heart, brain and testes (JEONG et al., 2009; GUPTA et al., 2003; WEI et al., 2011). Recently, a study has shown that rutin decreases lipid peroxidation, ROS and DNA fragmentation in thawed deer spermatozoa after incubation at 37 °C (MATA-CAMPUZANO et al., 2012). Other authors demonstrated that rutin preserves the viability and motility of human spermatozoa when used in the sperm selection by swim-up procedure (MORETTI et al., 2012), besides being able to replace the addition of transferrin, selenium and ascorbic acid during 12-days *in vitro* culture of ovine secondary follicles, maintaining follicular viability and increasing GSH levels (LINS et al., 2017).

Another compound found in the *M. nigra* extract was the kaempferitrin, which is naturally occurring kaempferol glycoside, isolated from several plants (PEREIRA et al., 2011; ALONSO-CASTRO et al., 2013) and from fungus *Boverivar microspora* *Annulohypoxylon* (CHENG et al., 2011). Besides its antibacterial (DALMARCO et

al., 2010), antinociceptive and anti-inflammatory effects (DE MELO et al., 2009), kaempferitrin exerts antioxidant activity with similar potential to quercetin (DE SOUSA et al., 2004). Kaempferitrin also inhibits myeloperoxidase, a cytosolic enzyme, which participates in ROS production in neutrophils (REGASINI et al., 2008).

Therefore, in our study, it can be suggested that these three natural antioxidants may act isolated or in association to reduce the apoptosis of ovine preantral follicles preserved in *M. nigra* extract for 6 h. Moreover, the temperature (4 °C) used for preservation may also help to support the survival through a reduction in cell metabolism. However, the same concentrations of *M. nigra* extract were not sufficient for maintaining healthy follicles for 12 h or more. The follicles may need energetic substrates or other substances, like growth factors or hormones, to keep normal morphology for longer periods. Nevertheless, 0.025 mg/mL *M. nigra* seems to be insufficient to maintain follicular viability even for a short period (6 h).

It is important to note that the costs for purchasing the medium containing *M. nigra* extract may make *in vitro* preservation studies cheaper and accessible. These costs were limited to acquisition of 95% ethanol and 0.9% saline solution for extract dilution. Adversely, MEM is an expensive commercial medium that would cost about 10 times more than plant extract.

## Conclusions

Ovine preantral follicles can be successfully preserved in *M. nigra* extract at 4°C for up to 6 h. Therefore, due to the higher cost of MEM, the use of *M. nigra* extract (0.05 mg/mL) as a preservation medium is recommended. In future studies, we also suggest that the flavonoids identified in the *M. nigra* extract should be used alone to verify their antiapoptotic potential after ovaries storage.

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