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Effect of ovarian tissue transportation in *Amburana cearensis* extract on the morphology and apoptosis of goat preantral follicles

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Abstract

The aim of the present study was to evaluate the effect of *Amburana cearensis* extract during caprine ovarian tissue transportation on the survival of preantral follicles *in vitro*. HPLC was used to determine the fingerprint chromatogram of the ethanolic extract. Five goat ovarian pairs were divided into fragments. One fragment was fixed for histology and TUNEL analysis (fresh control). The other fragments were placed in MEM or *A. cearensis* extract (0.1; 0.2 or 0.4 mg/ml) and stored at 4°C for 6, 12 or 24 h. Preserved fragments were also fixed for histology and TUNEL analysis. The presence of phenolic compounds (protocatechuic acid, epicatechin, *p*-coumaric acid, gallic acid and kaempferol) in the extract was confirmed using HPLC. The percentage of normal follicles preserved in 0.2 mg/ml *A. cearensis* for 6 h was similar to that observed in the fresh control. Moreover, the percentage of normal follicles was higher after preservation in 0.2 mg/ml *A. cearensis* for 6 h than the other *A. cearensis* treatments and similar to that found in MEM. There were no differences in the percentage of apoptotic cells between fresh control and those preserved for 6 h in MEM or 0.2 mg/ml *A. cearensis*. In conclusion, both 0.2 mg/ml *A. cearensis* or MEM can be used for the preservation of goat preantral follicles for up to 6 h. The use of *A. cearensis* is recommended due to the higher cost of MEM.

Keywords: caprine, HPLC, medicinal plant, oocyte, preservation, TUNEL.

Introduction

In vitro techniques are used to obtain mammalian embryos for research, genetic improvement or commercial purposes (Rajabi-Toustani *et al*., 2013). A common problem is often the large distances between the reproductive laboratories and farms. Successful embryo production depends on the maintenance of oocyte viability during transportation of the ovaries over long distances. In this context, preservation medium components are extremely important as well as the temperature and conservation period. In the caprine species, there is already a strategy available for the transport of fresh ovarian tissue. 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. These authors also demonstrated that chilling ovarian fragments at 4°C during transportation is better for maintaining the follicle viability than higher temperatures such as 20 or 35°C. However, besides being a rich source of nutrients, the use of MEM makes the technique more expensive. Therefore, other alternative media should be used.

At the present time, there is an increasing interest in natural antioxidants found in medicinal and dietary plants, which may contribute to prevent oxidative damages (Rajabi-Toustani *et al*., 2013). *A. cearensis* (Allemão) A.C. Smith (Fabaceae) is a tree commonly found in Northeastern Brazil, where it is popularly known as "cumaru" (Costa-Lotufo *et al*., 2003), "amburana" or "amburana-de-cheiro" (Leal *et al*., 2011). In traditional medicine, extracts of this plant have been used for the treatment of a wide range of diseases including respiratory problems in general, influenza, cough, expectorant, thrombosis, hypertension, inflammations and healing (Cartaxo *et al*., 2010). It is also claimed that *A. cearensis* exhibits antinociceptive, anti-inflammatory and bronchodilator activities (Leal *et al*., 1997, 2000; Canuto and Silveira, 2006; Oliveira *et al*., 2009).

Several compounds have been isolated from the trunk bark of *A. cearensis* (Canuto and Silveira, 1998; Bravo *et al*., 1999), such as coumarin, protocatechuic acid, isokaempferide, flavonoids, amburoside A and B. Some of these compounds are often related to the antifungal and antibacterial activity of this plant such as coumarin and amburoside A and B (Bravo *et al*., 1999). Moreover, some authors have reported that amburoside A can act as an antioxidant compound, presenting a neuroprotective effect on rat mesencephalic cell cultures (Leal *et al*., 2005).

However, there were no reports of the use of *A. cearensis* extract as an *in vitro* preservation medium in the transportation of ovarian tissue. The aim of the

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present study was to determine if the use of *A. cearensis* extract as a preservation medium during caprine ovarian tissue transportation at 4°C would influence the survival of preantral follicles *in vitro*.

Material and Methods

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 *A. cearensis* were collected in Petrolina, PE, Brazil. A voucher specimen (5545) 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 using rotavapor and the yield was approximately of 10% obtaining the crude ethanolic extract of the leaves of *A. cearensis*, which was dissolved in 0.9% saline solution, corresponding to concentrations of 0.1; 0.2 or 0.4 mg/ml.

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

Chromatographic equipment consisted of a Shimadzu® 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 andcontroller SCL-20AVP. The data was acquired and processed using Shimadzu® LC solution1.0 software (Japan).

The mobile phase consisted of solvents A-C using three pumps equipped with the chromatograph. Solvent A was 0.1% trifluoroacetic acid in acetonitrile, solvent B, 0.1% trifluoroacetic acid in HPLC grade water, and solvent C 100% methanol. A TSK-GEL Super-ODS (Supelco) column was used. The absorbance of the effluent was monitored at 250 and 330 nm. Flow rate was set at 1.0 ml/min, and column temperature was maintained at 37°C throughout the test. The initial solvent condition was 100% solvent B. A linear gradient was used to increase solvent A from 0 to 10% within 7 min. This solvent composition was maintained at an isocratic flow for 3 min. Solvent A was then increased from 10 to 40% using a linear gradient for 20 min. This composition was then maintained for 2 min and returned to the initial condition in 3 min. Sample sizes of 20 µl for the standard substances and crude ethanolic extract were injected during HPLC analysis (Cai *et al*., 2003). The concentrations of the standard substances (protocatechuic acid, epicatechin, *p*-coumaric acid, gallic acid and kaempferol) in *A. cearensis* samples were calculated from standard curves calibrated using the 50, 100, 125, 150 and 200 µg/ml.

Ovary collection and in vitro *preservation*

Ovarian cortical tissues (n = 10 ovaries) were collected at a local abattoir from five adult (1-3 years old) mixed-breed goats. 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 µg/ml penicillin and 100 µg/ml streptomycin).

Still in the slaughterhouse, the pair ovaries from each animal were divided into 13 fragments approximately 3 x 3 mm (1 mm thick). Then one ovarian fragment was taken randomly and fixed for histological and TUNEL analysis (fresh control). The other 12 fragments were randomly placed into tubes containing 5 ml MEM supplemented with antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin) or different concentrations of *A. cearensis* extract (0.1; 0.2 or 0.4 mg/ml) and stored at 4°C for 6, 12 or 24 h (Fig. 1). The temperature was maintained using thermoboxes with ice. Each treatment was repeated 5 times.

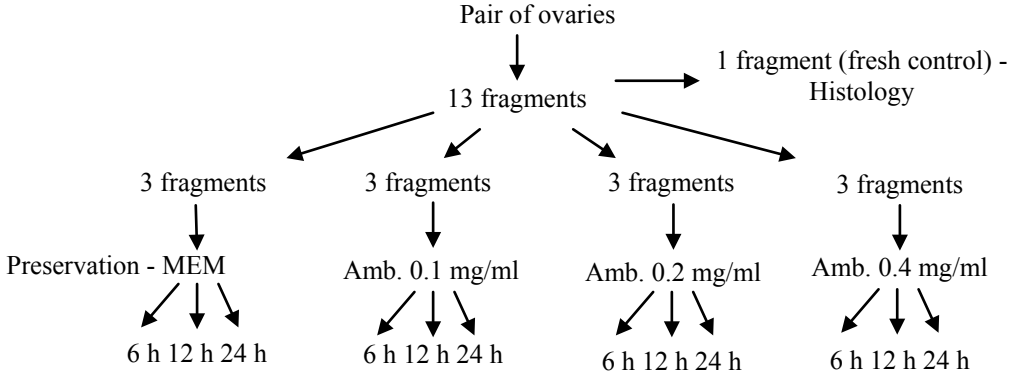

Figure 1. General experimental protocol for preservation of caprine preantral follicles in *A. cearensis* extract.

Morphological analysis of preantral follicles preserved in situ

Ovarian fragments from each treatment, including the fresh control, were fixed individually in 4% buffered paraformaldehyde (Dinâmica) 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 µm and sections were stained using standard protocols with periodic acid-Schiff and haematoxylin (Vetec, Duque de Caxias-RJ, Brazil). Sections were examined by light microscopy (Nikon, Tokyo, Japan) at 400X magnification.

Preantral follicles were counted and evaluated in the section where the oocyte nucleus was visible. The developmental stages of 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). These follicles are 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, 150 follicles were evaluated for each treatment (30 follicles per treatment-replicate x 5 replicates = 150 follicles), totaling 1,950 preantral follicles.

Detection of apoptotic cells by TUNEL assay

Terminal deoxynucleotidyltransferase (TdT) mediated dUTP nick-end labeling (TUNEL) assay was used for a more in-depth evaluation of caprine preantral follicle quality before (fresh control) and after preservation in MEM or 0.2 mg/ml *A. cearensis*, which were the treatments that demonstrated the best results after 6 h of preservation according to previous histological analysis. 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). Antigen retrieval by microwave treatment was performed in sodium citrate buffer (pH 6.0; Dinâmica) for 6 min. Endogenous peroxidase activity was blocked by 3% H_2O_2 (Dinâmica) in methanol at room temperature 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 buffer, pH 7.6, 0.03% H_2O_2) during 1 min. Finally, sections were counterstained with Harry's haematoxylin in a dark chamber at room temperature for 1 min, dehydrated in ethanol, cleared in xylene, and mounted with balsam (Dinâmica). For negative controls, slides were incubated with label solution (without terminal deoxynucleotidyltransferase enzyme) instead of TUNEL reaction mixture.

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 random 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.

Statistical analysis

Percentages of morphologically normal follicles were submitted to ANOVA and the Tukey´s test was applied for comparison among treatments. Values of apoptotic cells were submitted to Qui-square and differences were considered to be statistically significant when $P < 0.05$. The results of follicular survival were expressed as the mean ± SD.

Results

HPLC Analysis

After analysis of the crude ethanolic extract through the HPLC method, it was possible to quantify five substances with different retention times: protocatechuic acid in 12.5 min (512.37 ± 5.05 µg/ml), epicatechin in 19.6 min (2.6 ± 0.02 µg/ml), *p*-coumaric acid in 22.7 min (1.146 ± 0.01 µg/ml), gallic acid in 24.7 min (3,566.24 ± 4346.77 µg/ml) and kaempferolin 35 min (1.01 ± 0.01 µg/ml; Fig. 2).

Effect of storage conditions on follicular morphology

Among the preantral follicles analyzed, 1,045 were primordial, 608 intermediate, 185 primary and 112 secondary follicles. The preantral follicles in the fresh control (Fig. 3A) and those preserved in control medium (MEM; Fig. 3B) or in 0.2 mg/ml *A. cearensis* (Fig. 3C) for 6 h showed centrally located oocytes and organized granulosa cells surrounded by normal intact basement membranes. After storage in 0.4 mg/ml *A. cearensis* for 24 h, atretic follicles with a retracted oocyte and pyknotic nucleus could be observed (Fig. 3D).

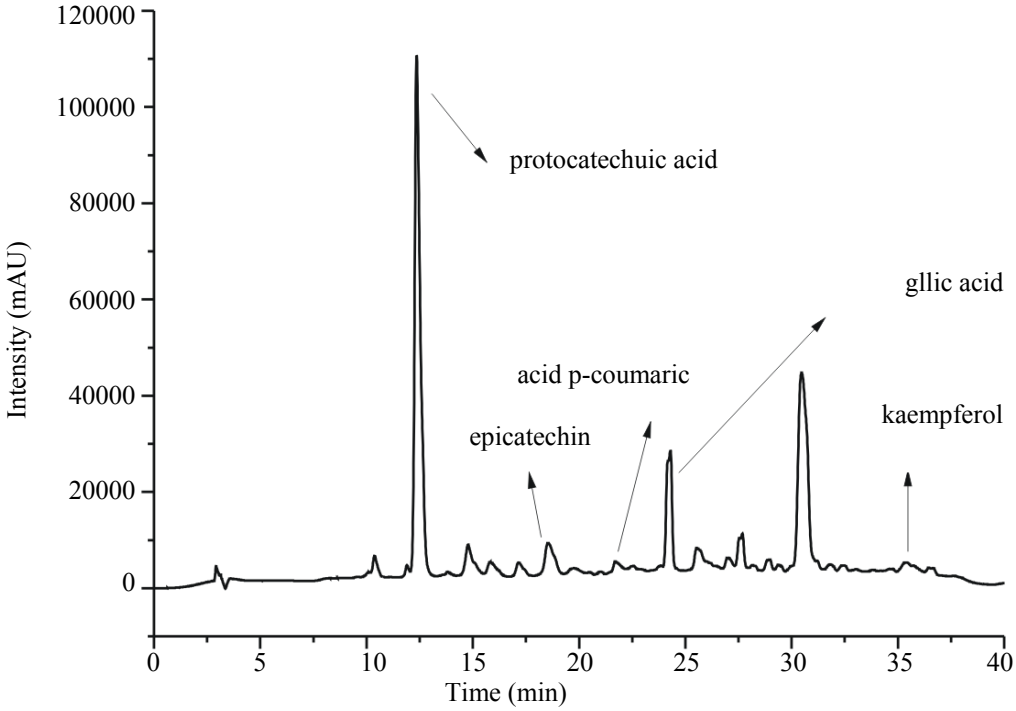

Figure 2. High performance liquid chromatography with diode array detector (HPLC-DAD) profiles of *A. cearensis* ethanolic extract.

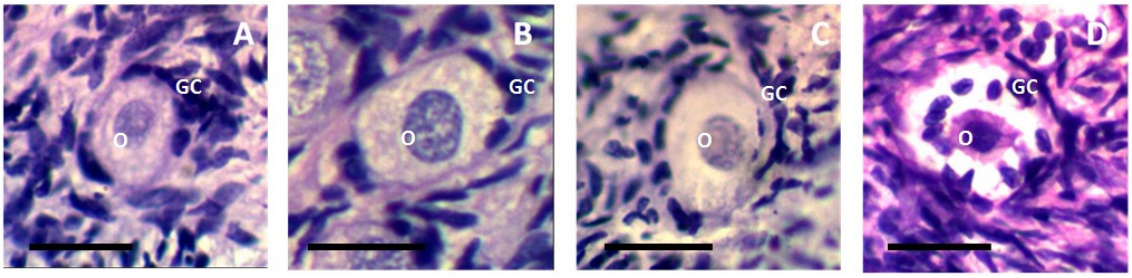

Figure 3. Histological sections of caprine ovarian fragments showing morphologically normal follicles in the fresh control (A) and after 6 h of preservation in MEM (B) or 0.2 mg/ml *A. cearensis* extract (C). Atretic follicle after 24 h of preservation in 0.4 mg/ml *A. cearensis* (D). O = oocyte; GC = granulosa cell. Scale bar: 30 µm (400X).

Figure 4 details the effect of media and preservation period of ovarian fragments on the percentage of normal preantral follicles. Storage of ovarian fragments for 6 h in 0.2 mg/ml *A. cearensis* was the only treatment that maintained ($P > 0.05$) the percentage of morphologically normal follicles similar to that observed in the fresh control. Moreover, the percentage of normal follicles was higher ($P < 0.05$) after preservation of ovarian tissue in 0.2 mg/ml *A. cearensis* for 6 h than the other *A. cearensis* concentrations (0.1 or 0.4 mg/ml) and similar ($P > 0.05$) to that found in the control medium (MEM). After 12 h of preservation, the percentage of normal follicles decreased ($P < 0.05$) in fragments stored in 0.4 mg/ml *A. cearensis*, compared with the other treatments. There was no significant difference in the percentage of normal follicles in tissues preserved for 24 h ($P > 0.05$).The secondary follicles were the most affected by atresia in all the treatments.

Apoptotic cell detection

Figure 5A shows a normal follicle after TUNEL analysis, Fig. 5B shows that apoptosis occurred more frequently in the oocyte and Fig. 5C shows the negative control. Table 1 shows that there were no differences ($P > 0.05$) in the percentage of apoptotic cells (oocytes and granulosa cells) among fresh tissues (fresh control) and those preserved for 6 h in MEM (control medium) or 0.2 mg/ml *A. cearensis*. In all the treatments, no staining for TUNEL analysis was observed in granulosa cells.

Table 1. Percentage of apoptotic oocyte and granulosa cells before (fresh control) and after preservation of ovarian tissue in different treatments for 6 h.

Treatments	Oocyte (%)	Granulosa cells (%)	Total of apoptotic cells (%)
Fresh control	29.41	0	2.72
MEM / 6 h	15.67	0	1.47
A. cearensis 0.2 mg/ml/6 h	33.33	0	4.06

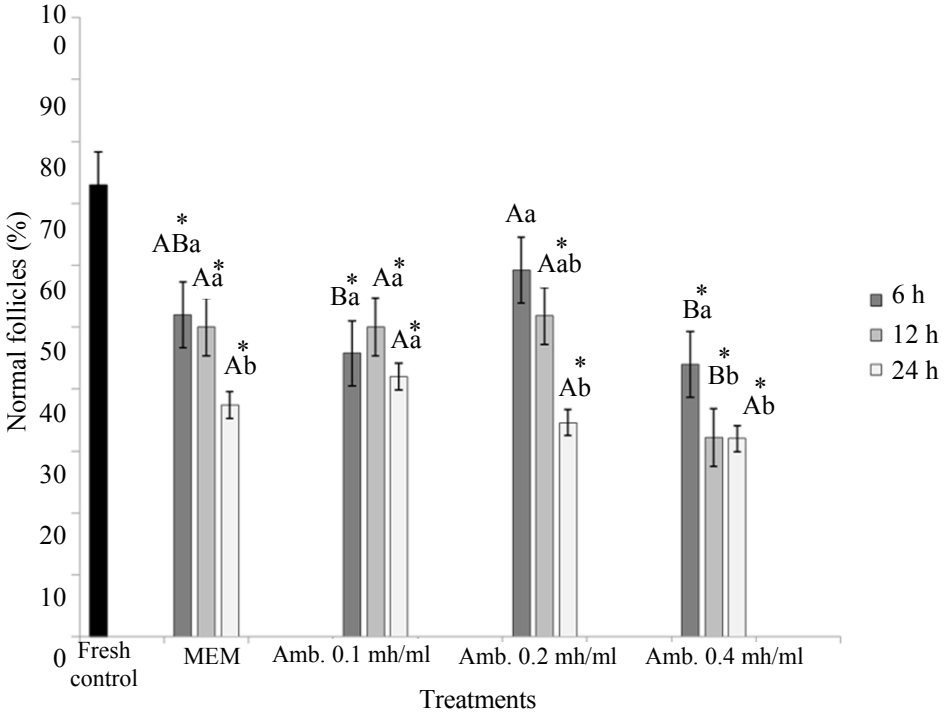

Figure 4. Percentages (mean ± SEM) of morphologically normal follicles in the fresh control and after preservation in MEM or *A. cearensis* extract. *Differs significantly from fresh control ($P < 0.05$). ^{A,B}Different letters denote significant differences among treatments (different media) in the same period ($P < 0.05$). ^{a,b}Different letters denote significant differences amongperiods in the same media ($P < 0.05$).

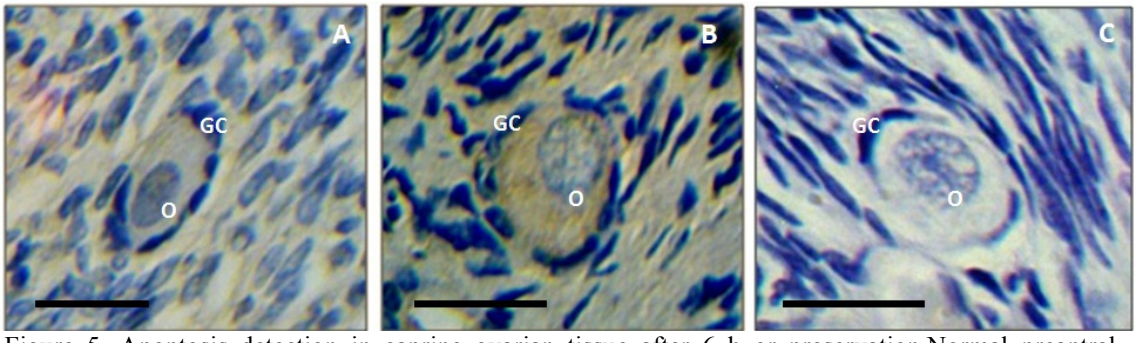

Figure 5. Apoptosis detection in caprine ovarian tissue after 6 h or preservation.Normal preantral follicle in 0.2 mg/ml *A. cearensis* (A), apoptotic follicle in MEM (B) and negative control (C). Note the apoptosis in the oocyte (brown) in Fig. B. O: oocyte; GC: granulosa cells. Scale bar: 30 µm (400X).

Discussion

To the best of our knowledge, this constitutes the first report to demonstrate the beneficial effect of *A. cearensis* extract on *in vitro* preservation of caprine ovarian tissue during transportation. Our results showed that appropriate concentrations of *A. cearensis* extract (0.2 mg/ml) maintain the percentages of morphologically normal follicles and the rates of follicular apoptosis similar to those observed in the

fresh control and the control medium (MEM). In the present study it was possible to identify and quantify five substances (protocatechuic acid, epicatechin, *p*-coumaric acid, gallic acid and kaempferol) in the crude ethanolic extract using the HPLC method. We should take into account that the seasonality of climatic elements such as temperature, relative humidity and solar radiation can alter the physiological behavior of plants and, consequently, their growth and development, as well as the chemical and biological composition of the soil (Inderjit and Dakshini, 1992; Floss, 2004). Thus, for a secondary metabolite, the variation of climatic elements can affect their concentrations in the plant (Suarez *et al*., 2003). Therefore, the environments in which the plant develops exert a direct influence on the chemical composition of the extracts.

In the current study, a compound found in large amounts in the *A. cearensis* extract was the gallic acid, which belongs to the group of antoxidant polyphenol (Tang *et al*., 2003). To date, there were no reports on the use of gallic acid on *in vitro* folliculogenesis. However, its antioxidant effect was observed in other cells. In rats, gallic acid has neuroprotective activity against 6-hydroxydopamine-induced oxidative stress via enhancement of glutathione peroxidase (GPx) levels (Mansouri *et al*., 2013). Moreover, gallic acid prevents DNA oxidative damage in human lymphocytes exposed to hydrogen peroxide treatment by an increase of the activities of antioxidant enzymes (superoxide dismutase, GPx and glutathion-S-transferase-π) and a decrease of intracellular (ROS) concentrations (Ferk *et al*., 2011).

Another compound found in the *A. cearensis* extract was the protocatechuic acid (PCA), which is one of the major benzoic acid derivatives from vegetables and fruits (Guan *et al*., 2009). PCA was highly effective in inhibiting the neurotoxicity in cultured rat adrenal gland pheochromocytoma cell line (PC12 cells) and augmented the activities of antioxidant enzymes such as superoxide dismutase, scavenging ROS or inhibiting their formation, thus reducing oxidative stress damage (An *et al*., 2006; Shi *et al*., 2006; Guan *et al*., 2009). Moreover, PCA inhibited the rotenone-induced apoptotic cell death in PC12 cells via ameliorating the mitochondrial dysfunction that is associated with oxidative stress damage (Liu *et al*., 2008).

The term flavonoid is a collective noun for plant pigments, mostly derived from benzo-*g*-pyrone (Hässig *et al*., 1999). The flavonoids are phenolic compounds (Havsteen, 2002) including isokaempferide, kaempferol, afrormosin and quercetin (Mann, 1987). One of the prominent and most useful properties of the flavonoids is their ability to scavenge ROS (Wang and Zheng, 1992). They are considered more efficient antioxidants than vitamins C and E (Gao *et al*., 2001). In the present study, it was possible to quantify two flavonoids, kaempferol and epicatechin. Choi (2011) has demonstrated that pretreatment with kaempferol prior to antimycin A exposure significantly reduced cell damage by preventing mitochondrial membrane potential dissipation and ROS production. Other authors showed that epicatechin has important cytoprotective effects, inhibiting human fibroblast death induced by hydrogen peroxide by a mechanism involving suppression of caspase-3 activity (Spencer *et al*., 2001). Moreover, coumarins (1,2-benzopyrone) comprise a class of natural antioxidant compounds distributed widely in plants (Egan *et al*., 1990; Lake, 1999). Studies have reported that coumarin inhibit lipoxygenase activity, lipid peroxidation, decrease the injury caused by oxidative stress and decrease the levels of ROS in different types of cells (Neichi *et al*., 1983; Martín-Aragón *et al*., 1998; Kaneko *et al*., 2003). Therefore, in our study, it can be suggested that these natural antioxidants, specially gallic acid and PCA, may act isolated or in association to support the survival of caprine preantral follicles preserved in 0.2 mg/ml *A. cearensis* for 6 h.

In the present study, the percentage of normal preantral follicles was higher when the ovarian tissue was preserved in 0.2 mg/ml *A. cearensis* for 6 h, compared to other plant concentrations. It is possible that 0.1 mg/ml *A. cearensis* may not be sufficient for the maintenance of follicular survival. A recent study showed that *p*-coumaric acid has cytotoxic effect on neuroblastoma after 72 h of treatment, promoting an increase in ROS levels (Shailasree *et al*., 2014). It is possible that a higher concentration (0.4 mg/ml) of *A. cearensis* potentiated the cytotoxic effect of *p*-coumaric acid, increasing the rates of follicular atresia.

Some *in vitro* studies have satisfactory results after conservation of ovarian fragments at 4ºC in MEM for up to 4 h (caprine: Chaves *et al*., 2008) or 12 h (canine: Lopes *et al*., 2009). MEM has many substances that help in the maintenance of follicular survival such as glucose, vitamin and amino acids (Hartshorne, 1997). However, the costs for purchasing this medium make the researches more expensive. Therefore, our findings encourage future studies of follicle preservation in *A. cearensis* because this medicinal plant is cheaper than MEM.

In conclusion, 0.2 mg/ml *A. cearensis* or MEM can be used with the same effectiveness for the preservation of goat preantral follicles at 4ºC for up to 6 h. However, due to the higher cost of MEM, the use of *A. cearensis* extract as a preservation medium is recommended. More studies should be performed to investigate the effect of the isolated compounds of *A. cearensis* on the oxidative stress of our *in vitro* model.

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