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Justicia insularis Improves the *in vitro* Survival and Development of Ovine Preantral Follicles Enclosed in Ovarian Tissue

Gildas Tetaping Mbemya[1], Denise Damasceno Guerreiro[1], Nathalie Jiatsa Donfack[1], Luciana Mascena Silva[1], Luis Alberto Vieira[1], Geovania Francisca Canafistula de Sousa[1], Benner Geraldo Alves[1], Aryele Pinto Izaguirry[2], Francielli Weber Santos[2], Phelix Bruno Telefo[3], Otília Deusdênia Loiola Pessoa[4], Johan Smitz[5], José Ricardo de Figueiredo[1] and Ana Paula Ribeiro Rodrigues[1]

1. Faculty of Veterinary (FAVET), State University of Ceará, Fortaleza 60714903, Brazil

2. Laboratory of Reproduction Biotechnology (Biotech), State of University of Pampa, Uruguaiana 97500970, Brazil

3. Laboratory of Biochemistry of Medicinal Plants, Food and Nutritional Sciences, Faculty of Science, University of Dschang, Dschang 3451381, Cameroon

4. Laboratory of Phytochemical Analysis of Medicinal Plants (LAFIPLAN), Federal University of Ceará, Fortaleza 60020181, Brazil

5. Follicle Biology Laboratory, Center for Reproductive Medicine, Brussels 1090, Belgium

Abstract: Objectives: Evaluating the addition effect of *J. insularis* extract and FSH on the survival, activation and ROS production after *in vitro* culture of ovine preantral follicles enclosed in ovarian tissue. Methods: In the first experiment, ovarian fragments were fixed (non-cultured control) or *in vitro* cultured in α-MEM$^+$ (cultured control), α-MEM$^+$ supplemented with FSH 50 ng/mL, or in α-MEM$^+$ supplemented with *J. insularis* (JUS0.3; 1.25 or 5 mg/mL) for 1 or 7 days, at 39˚C, 5% CO_2. In the second experiment, fragments were fixed or cultured in α-MEM$^+$ supplemented with anethole 300 µg/mL + FSH 50 ng/mL or in α-MEM$^+$ supplemented with anethole 300 µg/mL + 0.3 mg/mL JUS. Key findings: JUS0.3 was the only treatment that maintained the percentage of morphologically normal follicles similar to non-cultured control even after 7 days of culture. After 7 days of culture, a higher ($p < 0.05$) percentage of developing follicles was observed in JUS5 treatment compared with the other treatments except JUS1.25. In the second experiment, FSH maintained the percentage of normal follicles and promoted activation of primordial follicles. A reduction ($p < 0.05$) of stromal cell density was observed in MEM$^+$+ANE supplemented with JUS or FSH. Conclusions: *J. insularis* in a concentration-dependent manner maintained the levels of ROS and improved *in vitro* follicular survival and activation of ovine primordial follicles.

Key words: Medicinal plant, antioxidant, *in vitro* folliculogenesis, preantral follicles.

1. Introduction

The *in vitro* follicle culture studies have been performed either using primordial follicles enclosed in ovarian slices (*in situ* culture) or in the isolated form [1]. Such follicle culture systems have been used respectively to investigate *in vitro* early and late folliculogenesis at preantral follicle stage [2]. Culture systems for primordial follicles are important tools for studying the mechanism of oocyte development, and are a potential source of oocytes that can be used for *in vitro* embryo production. The factors that control primordial follicle activation and further growth of primary follicles are not well understood [2]. However, endocrine hormones, like FSH (follicle stimulating hormone), are known to regulate the production of several growth factors that play a critical role in primordial follicle activation and growth [3].

Corresponding author: Ana Paula Ribeiro Rodrigues, PhD., professor, reaearch fields: manipulation of oocyte and ovarian preantral follicles.

Some *in vitro* studies have demonstrated that the addition of FSH to the culture medium is important to maintain viability and to promote ovine follicular activation and further growth *in vitro* [3-5]. In addition, Magalhães et al. [6] showed that the addition of 50 ng/mL recombinant bovine FSH (rFSH) during *in vitro* culture of goat preantral follicles maintained viability, activation and follicular growth.

Despite the advances made in this field, the success of *in vitro* culture of preantral follicles is still very limited and the majority of reports are restricted to investigative studies to elucidate how early or preantral folliculogenesis works. As ARTs (assisted reproductive technique) to date, this tool has been limited to advance in mice, the only species to have been reported the birth of animals from embryos originated from completely developed preantral follicles *in vitro* [7, 8]. In large animals such as sheep [9] or even in human [10] the complete development *in vitro* of preantral follicles from a primordial to an ovulatory follicle has been hampered by growth arrested at the primary follicle stage [9, 10]. One reason for this shortcoming is that the requirements for *in vitro* growth are not well characterized due to lack of knowledge regarding activation of primordial and development of primary follicles compared to development of follicles in later stages [11]. In addition, it is known that during *in vitro* culture of preantral follicles, there is an increase production of reactive oxygen species: ROS [12], which can affect growth, survival and consequently can lead to cell death [13]. In this context, at the present time, there is an increase interest in natural products (medicinal plants) that prevent oxidative damages caused by the ROS and as a result may contribute to promote the activation, survival, growth of preantral follicles *in vitro* enclosed in ovarian tissue. Among these potential natural products, it is important to highlight *J. insularis* and anethole.

J. insularis T. Anders (family Acanthaceae), is an herbaceous and perennial plant, widely distributed in tropical area of Africa [14]. In ghomala'a (traditional language spoken in Western Cameroon), *J. insularis* is called "kwe mchie" [15]. Traditionally, in Senegal, the leaf decoction of *J. insularis* is given to women during the last month of pregnancy to reduce labour pains. In Cameroon and specifically in the Western region, their leaves are used in association with the leaves of three others medicinal plants (*Aloe buettneri, Hibiscus macranthus and Dicliptera verticillata*), to treat dysmenorrhoea and some cases of women infertility [15, 16]. The *in vivo* foliculogenic effect of their leaves has been related to their composition. Besides alkaloids, glycosides, polyphenols and triterpenoids, studies undertaken by by Telefo et al. [14] and Goka et al. [17] revealed the presence of flavonoids in their leaves which can act as a natural antioxidant. A mix of aqueous extract of *J. insularis* and others medicinal plants (*Aloe buettneri, Hibiscus macranthus* and *Dicliptera verticillata*) has also been proven, in a series of studies to induce ovarian steroidogenesis and folliculogenesis in female rats [17-19].

Anethole, other natural compound originated from *Croton zehntneri* Pax & K. Hoffm (family Euphorbiaceae), a plant locally known as "*canela* de *cunhã*" or "*canelinha*" in the Northeast of Brazil [20] has also showed antioxidant activity due its capacity to decrease the concentrations of ROS both *in vivo* [21] and *in vitro* [22]. Recently, Sá et al. [23] demonstrated that anethole reduced the levels of ROS, proving its antioxidant activity on goat isolated preantreal follicles (secondary stage) cultured *in vitro*.

Despite the importance of the aforementioned natural products for ARTs, to the best of our knowledge, there is no study investigating the effect of the aqueous extract of *J. insularis* on *in vitro* folliculogenesis. In addition the effect of anethole on the *in vitro* culture of primordial follicle enclosed in ovarian tissue is not known. Therefore this study was conducted to (1) investigate the addition effect of FSH and *J. insularis* extract on the survival, activation, growth and ROS generation after *in vitro* culture of

ovine preantral follicles enclosed in ovarian tissue; (2) compare the efficiency of FSH and *J. insularis* on stromal cell density and all parameters above mentioned in culture medium containing anethole.

2. Materials and Methods

This study was approved and performed under the guidelines of the Ethics Committee for Animal Use of the State University of Ceará (N° 6004720/2015). Unless mentioned otherwise, the culture media, anethole and other chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, USA).

2.1 Source of Ovaries

Ovaries (n = 22) were collected at a local slaughterhouse from 11 adults (1-3 years old) mixed-breed sheep (*Ovis aries*). Immediately postmortem, ovaries were washed in 70% alcohol followed by two rinses in minimum essential medium (MEM) supplemented with 100 µg/mL penicillin and 100 µg/mL streptomycin plus 25 mM HEPES. Ovaries were transported within 1 h to the laboratory into tubes containing 15 mL of MEM-HEPES at 4 °C [5].

2.2 Plants Materials to Extracts Preparation and Culture Medium

The fresh leaves of *J. insularis* previously identified in the National Herbarium of Cameroon under voucher specimen code 34997 [17] were collected in Western Cameroon (Batoufam subdivision, Upper-Plateau division, 5°21′North 10°24′East, Altitude 1,515 m). The fresh leaves were then dried at room temperature in the shade. Subsequently, the plant extract decoction was prepared according to the protocol [16]. Finally, the plant decoction was lyophilized and kept in the freezer at -20 ºC. The lyophilized extract was then diluted in the distilled water to obtain the desired concentrations (0.3 mg/mL, 1.25 mg/mL and 5 mg/mL).

The basic culture medium consisted of α-MEM (pH 7.2-7.4) supplemented with 1.25 mg/mL bovine serum albumin, 10 µg/mL insulin, 5.5 mg/mL transferrin, 5 ng/mL selenium, 2 mM glutamine, 2 mM hypoxanthine and antibiotics (100 µg/mL penicillin-streptomycin) which was referred to as α-MEM$^+$ [24]. In a first experiment, to test the effect of *J. insularis* on the culture of ovine preantral follicles, the α-MEM$^+$ was supplemented with FSH or different concentrations of *J. insularis*, mentioned above. In a second experiment, the α-MEM$^+$ was supplemented with anethole + FSH or with anethole + *J. insularis*.

The concentrations of *J. insularis* were defined based on a concentration curve done according to *in vivo* [16] and *in vitro* [25]. Briefly, the best concentration *in vivo* (5 mg/mL) was divided per 4 to obtain 1.25 mg/mL which subsequently was divided by the same factor (4) to obtain 0.3 mg/mL. This latest concentration was close to the best concentration of *Amburana Cearensis*, and other medicinal plant was tested in *in vitro* culture of sheep preantral follicles [25]. The concentration of anethole used was chosen based on previous studies performed in our laboratory on *vitro* culture of goat preantral follilces [23].

2.3 Experimental Design

As briefly mentioned, this work was divided into two non-simultaneous experiments.

In the first one, sheep ovarian cortex from each ovarian pair (n = 5) was cut using a tissue slicer (Thomas Scientific, USA) into 22 fragments (approximately 3 × 3 × 0.5 mm). One fragment was taken randomly and immediately fixed for histological analysis and identified as non-cultured control, the remaining fragments were *in vitro* cultured in 1 mL of α-MEM$^+$; α-MEM$^+$ supplemented with FSH 50 ng/mL, or with different concentrations (0.3, 1.25 or 5 mg/mL) of lyophilized plant extract *J. insularis* for 1 or 7 days at 39 °C in 5% CO_2 in air. These treatments were referred to as: MEM$^+$ (cultured control), FSH, JUS0.3,

JUS1.25 and JUS5, respectively. The culture medium was equilibrated at least 3 h prior to use. Every two days, whole culture medium was replaced. Based on the histological analysis, the best treatments (higher percentage of morphological normal follicles) were selected to next experiment.

In the second experiment, ovarian fragments were obtained as previously described in experiment 1. The fragments from each ovarian pair (n = 6) were either fixed for histological analysis (non-cultured control) or *in vitro* cultured in 1 mL of α-MEM$^+$ + anethole 300 µg/mL + FSH 50 ng/mL or α-MEM$^+$ + anethole 300 µg/mL + *J. insularis* 0.3 mg/mL, corresponding to the following treatments: MEM$^+$+ANE+FSH and MEM$^+$+JUS+ANE, respectively.

2.4 Morphological Analysis and Evaluation of Follicular Growth in vitro

Before (non-cultured control) and after 1 or 7 days of culture, the ovarian fragments were fixed individually in 4 % buffered paraformaldehyde for 2 h. Subsequently, fragments were dehydrated in a graded concentrations of ethanol. After paraffin embedding (Synth, São Paulo, Brazil), the ovarian fragments were cut into 7 µm sections and mounted on glass slides and stained by periodic acid schiff-hematoxylin. Follicle stage and survival were assessed microscopically on serial sections.

The developmental stages of follicles are *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) [26]. These follicles were still classified individually as histologically normal when an intact oocyte was present, surrounded by granulosa cells which are well organized in one or more layers and that have no pyknotic nucleus. Atretic follicles were defined as those with a retracted oocyte, pyknotic nucleus, and/or disorganized granulosa cells detached from the basement membrane [26]. 150 follicles were evaluated for each treatment (30 follicles per each five repetitions) in both experiments.

To evaluate follicular activation, the percentages of healthy primordial and growing follicles were calculated before (non-cultured control) and after culture in each treatment. 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, regardless of its size [26].

2.5 Ovarian Stromal Cell Density

Ovarian stroma density was evaluated by calculating the stromal cell per 100 µm^2. For each treatment, ten fields per slide were assessed and the mean number of stromal cell per field was calculated in experiment 2 [27]. All evaluations and measurements were performed by a single operator.

2.6 Reactive Oxygen Species Levels

The ROS levels were determined by a spectrofluorimetric method [28], using 2', 7'-dihydrodichlorofluorescein diacetate (DCHF-DA) assay. Sample aliquot (50 µL) was incubated with 5 µL of DCHF-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein was measured for the detection of ROS. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 2 h after the addition of DCHF-DA to the medium. The correlation between the follicular viability and ROS levels was done to better understand the effect of ROS by the cells during *in vitro* culture of preantral follicles.

2.7 Statistical Analysis

Statistical analyses were carried out using the Sigma Plot 11.0 software (Systat Software Inc, San Jose, California, USA). Data that were not normally distributed (Shapiro-Wilk test) were submitted to logarithmic transformation. The percentage of

morphologically normal and growing preantral follicles among treatments and days of culture were compared by Fisher's exact or chi-square tests. The Mann-Whitney test was performed to analyze the levels of reactive oxygen species and stromal cell density among treatments and days of culture. Spearman correlation test was used to assess the association between normal preantral follicles and reactive oxygen species. In addition, the association between stromal cell density and percentage of normal preantral follicles was evaluated by linear regression analysis. Data were presented as mean (± standard error of the mean) and percentage, unless otherwise indicated. Statistical significance was defined as $p < 0.05$ and probability values > 0.05 and ≤ 0.1 indicated that a difference approached significance.

3. Results

3.1 Sheep Preantral Follicles Morphology and Development

In the first experiment, a total of 1,374 preantral follicles were analyzed. Morphologically normal or degenerated follicles were observed in the non-cultured control as well as in the *in vitro* cultured ovarian tissues (Fig. 1).

The percentage of morphologically normal and follicular growth before and after *in vitro* culture is shown in Table 1 (experiment 1). JUS0.3 was the only treatment that maintained the percentage of morphologically normal follicles similar to non-cultured control even after 7 days of culture. In addition, this treatment showed a higher percentage ($p < 0.05$) of normal follicles than the other treatments regardless to the culture time. However, at day 7, *J. insularis* significantly reduced ($p < 0.05$) the percentage of morphologically normal follicles in a concentration-dependent manner. On the other hand, after 7 days of culture, a significantly higher percentage of developing follicles was observed in the JUS5 treatment compared to the other treatments except JUS1.25.

In the second experiment, a total of 1,286 preantral

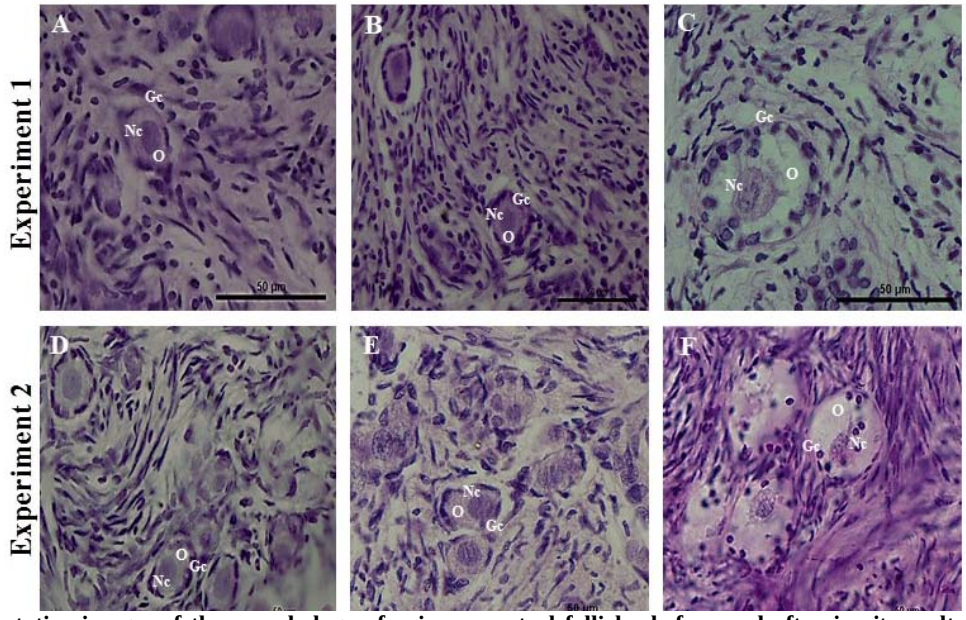

Fig. 1 Representative images of the morphology of ovine preantral follicles before and after *in vitro* culture. On the top panels we can see follicles from experiment 1 and on the bottom panels, follicles from experiment 2, after staining with periodic acid schiff-hematoxylin. Normal follicles are shown in non-cultured control (A), JUS0.3 (B), FSH (D) and FSH+ANE (E), while degenerated follicles are represented in JUS5 (C) and JUS+ANE (F) after 7 days of culture. Note the retracted oocyte with a pyknotic nucleus, disorganized granulosa cells (C and F). O: oocyte; Nc: oocyte nucleus; Gc: granulosa cells (400 x), bar 50 µm.

Table 1 Percentage of morphologically normal and growing preantral follicles before (non-cultured control) and after *in vitro* culture for 1 or 7 days in different treatments, in experiment 1.

	Follicular morphology (%)		Follicular development (%)			
			Primordial		Developing	
Non-cultured control	84.6 (127/150)		86.6 (110/127)		13.4 (17/127)	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
MEM^+ (cultured control)	82.5 (109/132)^{aA}	62.5 (70/112)^{*bA}	88.9 (97/109)^{aA}	85.7 (60/70)^{aA}	11.1 (12/109)^{aA}	14.3 (10/70)^{aA}
FSH	79.8 (99/124)^{aA}	65.2 (62/95)^{*bA}	75.7 (75/99)^{*aB}	87.1 (54/62)^{aA}	24.3 (24/99)^{*aB}	12.9 (8/62)^{aA}
JUS0.3	91.3 (137/150)^{aB}	89.5 (111/124)^{aB}	77.4 (106/137)^{aB}	84.7 (94/111)^{aA}	22.6 (31/137)^{aB}	15.3 (17/111)^{aA}
JUS1.25	58.1 (57/98)^{*aC}	47.3 (71/150)^{*aC}	85.9 (49/57)^{aAB}	74.6 (53/71)^{*aAB}	14.1 (8/57)^{aAB}	25.4 (18/71)^{*aAB}
JUS5	66.1 (52/111)^{*aC}	21.9 (29/132)^{*bD}	82.5 (66/80)^{aAB}	65.5 (19/29)^{*aB}	17.5 (14/80)^{aAB}	34.5 (10/29)^{*aB}

* Differs from non-cultured control ($p < 0.05$).

^{a,b} Within a row and the same parameter evaluated, values without a commom superscript differed ($p < 0.05$).

^{A,B,C,D} Within a column, values without a commom superscript differed ($p < 0.05$).

follicles were analyzed. The percentages of morphologically normal preantral and growing follilces in non-cultured control and after 1 or 7 days of culture in medium containing ANE supplemented with FSH or JUS are shown in Table 2. After *in vitro* culture, compared to non-cultured control, the percentage of normal follicles was reduced ($p < 0.05$), except in MEM^++ANE+ FSH treatment on day 1. Both cultured treated groups significantly reduced ($p < 0.05$) the percentage of normal follicles from day 1 to day 7 ($p < 0.05$).

After 1 and 7 days of culture, there was a significant reduction ($p < 0.05$) in the percentage of primordial follicles with concomitant increase ($p < 0.05$) in the percentage of developing follicles in both treatments compared to non-cultured control, indicating the follicular activation process. Furthermore, with the progression of the culture time, only MEM^++ANE+FSH significantly increased ($p < 0.05$) the percentage of developing follicles. In addition, at day 7 of culture, MEM^++ANE+FSH treatment showed a higher percentage of developing follicles than MEM^++ANE+JUS treatment.

3.2 Evaluation of Stromal Cell Density and Correlation with Normal Follicular Morphology in MEM^+ +Anethole Supplemented with FSH or J. insularis

Stroma cells from non-cultured control, and after 1 or 7 days of culture in two treatments are shown (Fig. 2). Regardless the culture time, a significant reduction was observed ($p < 0.05$) in the percentage of stroma density compared to non-cultured control as well as from day 1 to day 7. When treatments were compared at day 1 of culture, the percentage of stroma density was significantly higher ($p < 0.05$) in MEM^++ANE+JUS treatment, while the opposite was observed at day 7 of culture.

As shown in Fig. 3, increase of the stromal cell density simultaneously results in the increase in the percentage of morphologically normal follicles.

3.3 Levels of Reactive Oxygen Species after in vitro Culture

The ROS levels were determined on days 1, 2, 4 and 6 of *in vitro* culture and is shown in Fig. 4 (experiment 1) and Fig. 5 (experiment 2).

In the experiment 1, regardless the culture time, addition of either FSH or JUS did not change the ROS levels compared to control treatment. However the control treatment showed a significant higher ($p < 0.05$) ROS levels on day 4 compared to day 1. In addition, only on day 2, the ROS levels were significant higher ($p < 0.05$) in JUS5 than JUS1.25 treatment. Finally, a negative correlation was observed between the percentage of normal follicle and the concentration of ROS.

With regard to experiment 2, from day 2 onwards,

Table 2 Percentage of morphologically normal and growing preantral follicles before (Non-cultured control) and after *in vitro* culture for 1 or 7 days in MEM$^+$+ anethole supplemented with FSH or J. *insularis* (experiment 2).

	Follicular morphology (%)		Follicular development (%)			
			Primordial		Developing	
Non-cultured control	95.5 (172/180)		69.1 (119/172)		30.9 (53/172)	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
MEM$^+$ + ANE + FSH	91.4 (139/152)^{aB}	61.1 (80/131)^{*bA}	58.3 (81/139)^{*aA}	18.7 (15/80)^{*bA}	41.7 (58/139)^{*aA}	81.3 (65/80)^{*bA}
MEM$^+$ + ANE + JUS	77.2 (139/180)^{*aA}	16.5 (16/97)^{*bB}	56.8 (79/139)^{*aA}	43.7 (7/16)^{*aB}	43.2 (60/139)^{*aA}	56.3 (9/16)^{*aB}

*Differs from non-cultured control ($p < 0.05$); ^{a,b} Within a row and the same parameter evaluated, values without a commom superscript differed ($p < 0.05$); ^{A,B} Within a column, values without a commom superscript differed ($p < 0.05$).

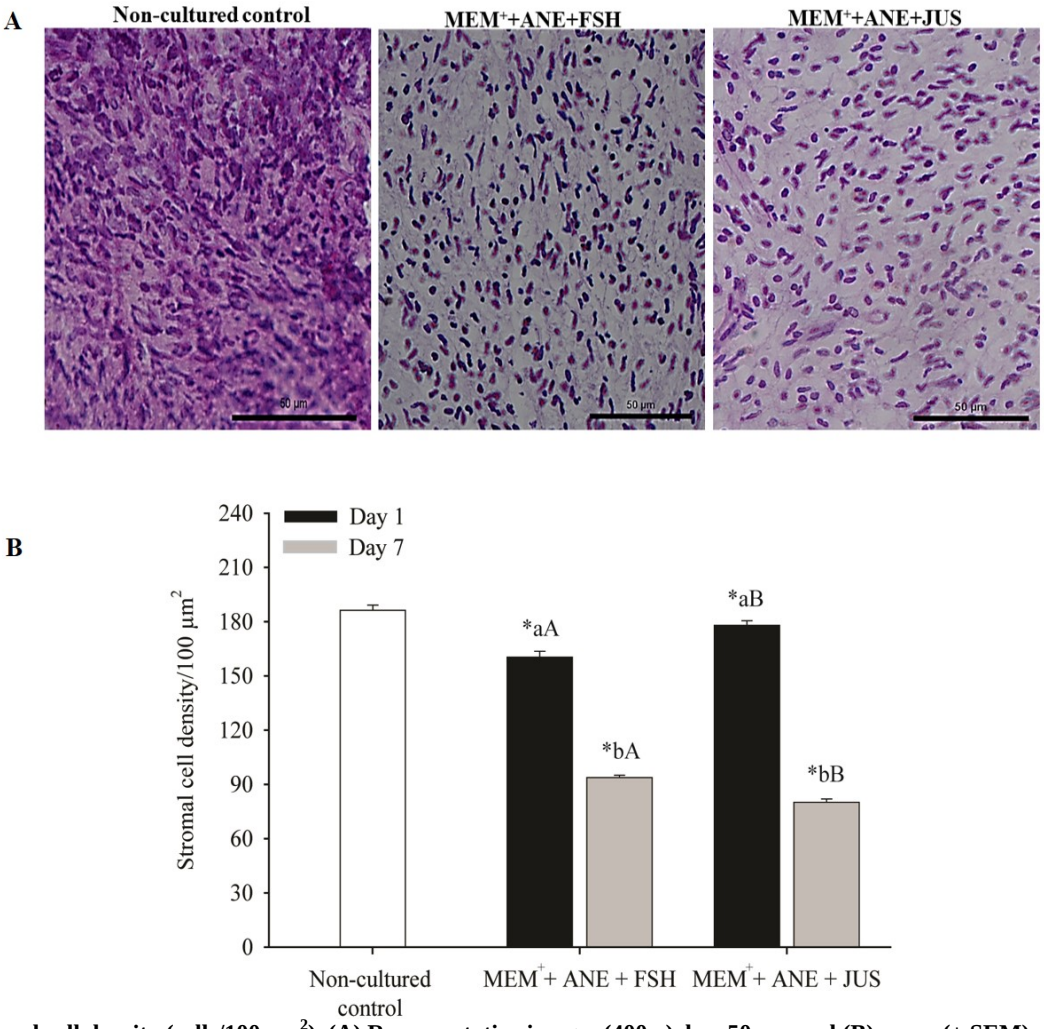

Fig. 2 Stromal cell density (cells/100 µm^2). (A) Representative images (400 x), bar 50 µm and (B) mean (± SEM) of ovarian stroma cell density in non-cultured control and after 7 days of *in vitro* culture in MEM$^+$+ANE+FSH and MEM$^+$+ANE+JUS treatments.

*Differs from non-cultured control ($p < 0.05$); ^{a,b} Within treatment, values without a commom superscript differed ($p < 0.05$); ^{A,B} Within day, values without a commom superscript differed ($p < 0.05$).

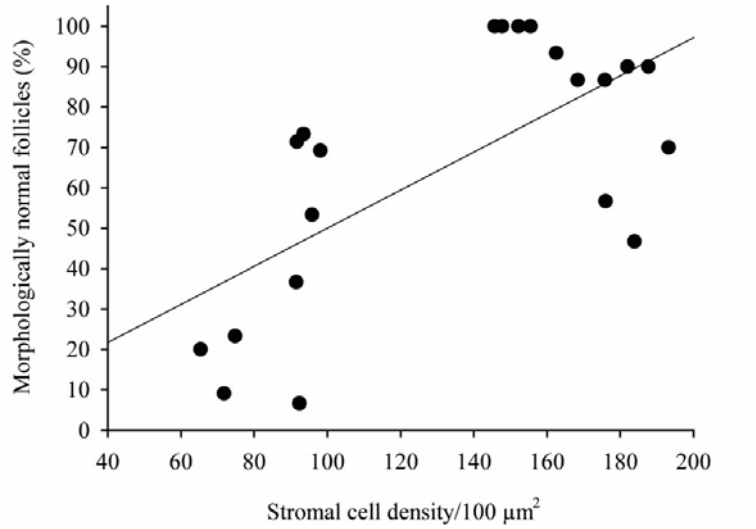

Fig. 3 Relationship of stromal cell density with percentage of normal preantral follicles. The association between variables (black line) was analyzed by linear regression [Normal preantral follicles = 2.887 + (0.471 × stromal cell density); r = 0.66; R^2 = 0.44; $p < 0.001$]. Each point on the chart represents one ovarian fragment evaluated.

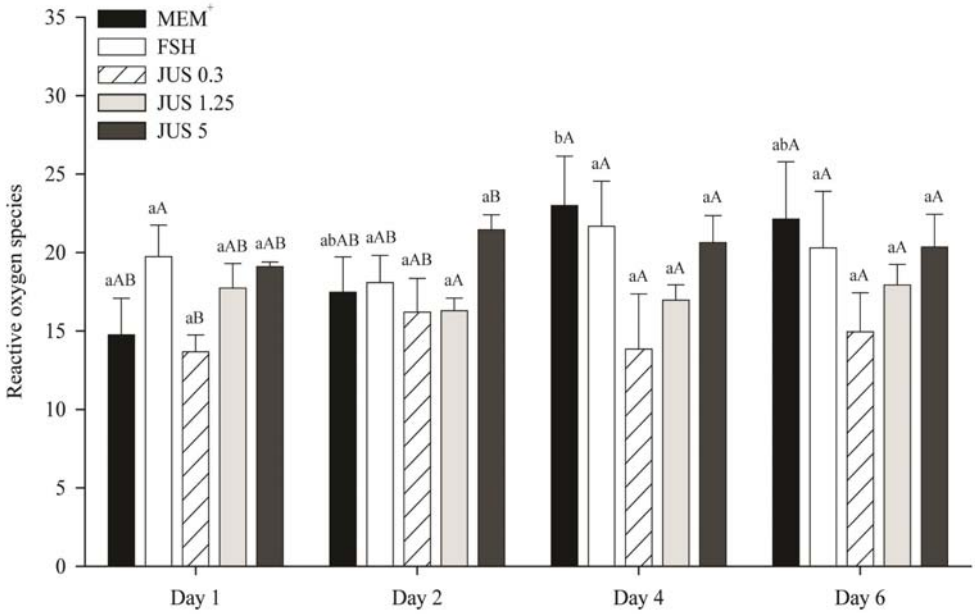

Fig. 4 ROS levels after 1 or 6 days in the absence or presence of FSH or different concentrations of *Justicia insularis*.
[a,b] Within treatment, values without a commom superscript differed ($p < 0.05$); [A,B] Within day, values without a commom superscript differed $p < 0.05$).

MEM+ANE+JUS treatment showed a significant higher ($p < 0.05$) ROS levels than MEM+ANE+FSH treatment. Contrary to MEM+ANE+JUS treatment, MEM+ANE+FSH significantly increased ($p < 0.05$) the ROS levels from day 1 to day 6.

As shown in Fig. 6, increase of the ROS levels simultaneously results in the decrease in the percentage of morphologically normal follicles.

4. Discussion

Although the action of FSH on the development of preantral follicles cultured *in vitro* within the ovarian cortex has already been investigated in different species (human [29], sheep [3], goat [6], bovine [30], buffalo [31], baboon [32]), this is the first study to describe the effects of the aqueous extract of *J. insularis*

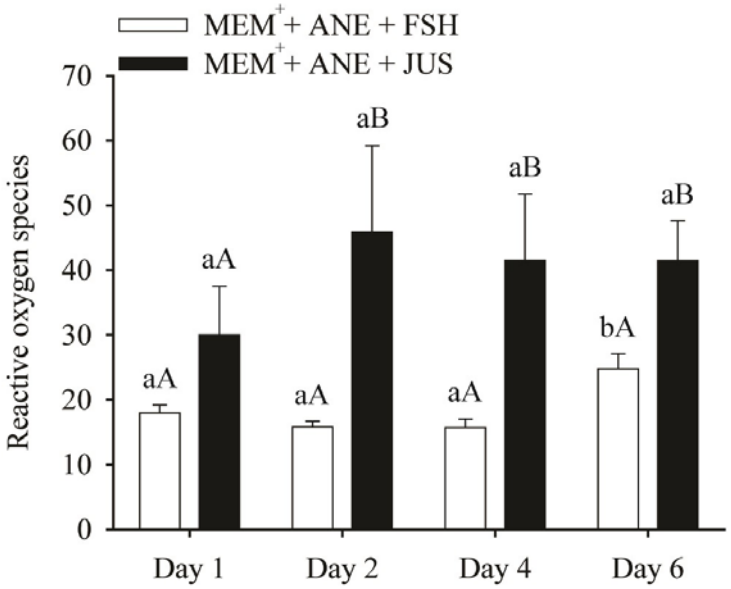

Fig. 5 ROS levels after 1 or 6 days in the presence of FSH + anethole and *Justicia insularis* + anethole.
[a,b] Within treatment, values without a commom superscript differed ($p < 0.05$); [A,B] Within day, values without a commom superscript differed ($p < 0.05$).

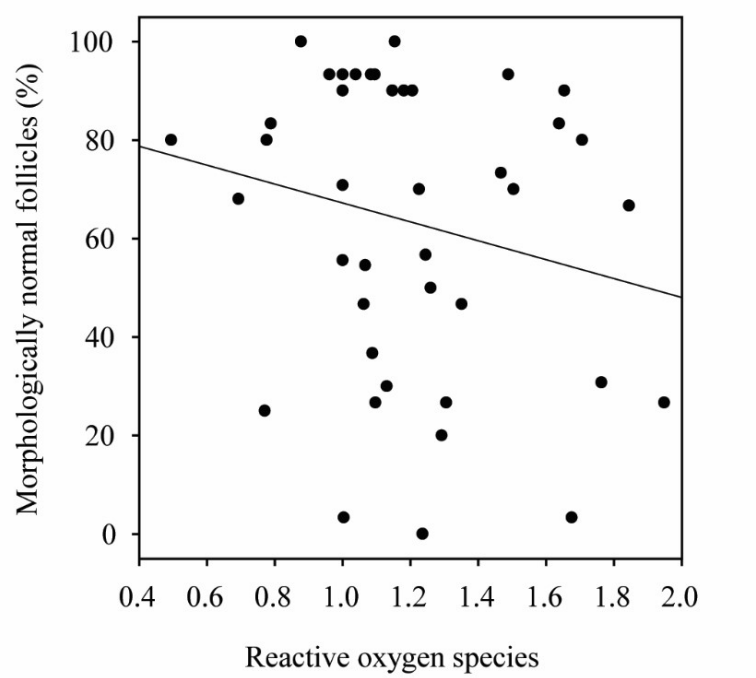

Fig. 6 Correlation between the follicular morphology and the reactive oxygen species. Each point of the graph represent a sample of the medium recovered during the *in vitro* culture (n = 42), r = - 0.25; *p* = 0.1.

on *in vitro* early folliculogenesis. *J. insularis* is a plant commonly used in the tropical area of Africa to ameliorate female reproductive disorders [16, 17] and it is known to have a FSH-like and antioxidant effects. Thus, this study demonstrated that adding *J. insularis* in a concentration-dependent manner maintained the levels of ROS and improved both *in vitro* follicular survival and activation of ovine primordial follicles enclosed in ovarian tissue when compared to the cultured control and FSH treatments. On the other

hand, addition of *J. insularis* or FSH in a culture medium containing anethole promotes beneficial effects on activation of ovine preantral follicles.

In the present study, JUS0.3 treatment maintained the percentage of normal follicles similar to the non-cultured control even after 7 days of culture and showed a significantly higher value than the other treatments. Such effect could be due to the action of FSH-like compounds of the extract of *J. insularis*. Phytochemical analysis of *J. insularis* has revealed the presence of alkaloids, glycosides, polyphenols, triterpenoids and flavonoids [14]. These metabolites are responsible for the induction of ovarian follicle growth and increase in the number of corpora lutea recorded during the fertility assay performed on immature female rats [16]. According to Andrade et al. [4], addition of FSH to the culture medium is important to the maintenance of morphology and activation of sheep primordial follicles, as well as for further follicular growth *in vitro* [33]. In addition, Thomas et al. [34] reported that FSH stimulates the expression of some growth factors, such as Kit Ligand, Bone Morphogenetic Protein-15 and Growth Differentiation Factor-9, which are important for the regulation of early folliculogenesis. The presence of FSH in the culture medium containing anethole also maintained follicular morphology.

J. insularis in high concentration (expeiriment 1) or in low concentration in the presence of anethole (experiment 2) have detrimental effects on the ovarian tissue. This could be due to an excess of antioxidant in the culture medium which can be harmful to preantral follicles. It is well known that oxidative metabolism is indispensable for energy production of follicles during *in vitro* culture [35], which in turn results in generation of ROS. Although a critical amount of ROS is essential for their physiological activities, excessive amount of them generates a contrary effect [12, 35]. Furthermore, some antioxidant substances act as pro-oxidants when used at high concentrations [36]. In this way, some studies have shown that high concentrations of ascorbic acid, an important antioxidant added to the culture medium, can inhibit important physiological processes in the ovary, resulting in follicular degeneration [37] besides promoting oxidative damage to cellular DNA [38].

This study also focused on the features of stroma tissue surrounding the follicles because any damage to this compartment may affect follicular morphology. After 1 and 7 days of culture, a reduction in the percentage of stromal cell density was observed in MEM^{+}+ANE supplemented either with *J. insularis* or with FSH compared to non-cultured control. The *in vitro* culture may have affected the bi-directional contact between germinal and somatic cells which are important to maintain the integrity of the cortex. In addition, in our opinion, cells can die during *in vitro* cultured by various factors such as nutrient deficiency in the medium, ischemia, generation of ROS, toxicity of the supplements. Our results are an in agreement with findings using sheep [39] and goat [27] ovarian tissues. Interestingly, a positive relationship between morphology and stromal cell density was observed. Previous reports have shown that ovarian stromal cells are responsible for the production of growth factors and peptides which are essential substances for follicular development [2].

Finally, we have evaluated the antioxidant effect of *J. insularis* and FSH in basic culture media in the absence (experiment 1) or presence (experiment 2) of anethole. In the first experiment (Fig. 4), addition of either FSH or JUS did not change the ROS levels compared to control treatment. The maintenance of ROS levels could be due to the capacity of *J. insularis* and FSH to scavenge ROS and their metal chelating properties. Similar results have been found during *in vitro* culture of ovine preantral follicles with *Amburana cearensis* [25]. According to Gouveia et al. [40], the extract of *A. cearensis* contains gallic acid, PCA (protocatechuic acid), epicatechin, *p*-coumaric acid and kaempferol. Gallic acid and PCA are endogenous plant phenols which stimulate the

production antioxidant enzymes such as catalase (CAT) and GPx (glutathione peroxidase) [41]. Reports from Telefo et al. [17] and Goka et al. [14] showed that *J. insularis* are constituted of several secondary metabolites among which the phenols and flavonoids, we thus belevied that those phenolic compounds act in a similar way as metabolic of *A. Cearensis* to maintain the ROS produced during *in vitro* culture.

In the present study, from day 1 to day 6, the presence of FSH in culture medium containing anethole increases the ROS levels. To date, it has been shown that FSH stimulates catalase activity in goat granulosa cells modulating intracellular ROS levels [42]. ROS inhibitors, in a concentration-dependent manner decreased oocyte maturation induced by FSH [43]. In our study, we suggest that an adequate FSH concentration (50 ng/mL) in a culture medium which contained anethole contributed to the maintenance of suitable levels of ROS after day 6 of culture, resulting in higher rates of follicle survival and activation. As above mentioned, we also observed that the combination between *J. insularis* and anethole increased the ROS level. This may be due to the high concentration of antioxidant in the culture medium. Indeed, antioxidant compounds in high concentration can be converted in pro-oxidant compounds. Some authors showed that some antioxidants could have oxidative action depending on the concentration [44]. Furthermore, during *in situ* culture, many cells such as granulosa, theca cells produce ROS. Those ROS with the time progression during *in vitro* culture caused injury to the follicular cells and thus affected the follicular morphology [12].

5. Conclusions

J. insularis in a concentration-dependent manner maintained the levels of ROS and improved both *in vitro* follicular survival and activation of ovine primordial follicles enclosed in ovarian tissue. Finally, as well as FSH 50 ng/mL, the addition of *J. insularis* 0.3 mg/mL in a culture medium containing anethole promotes beneficial effects on activation of ovine preantral follicles kept *in vitro* at least for a short-term. Although the mechanisms of interaction are unclear, these results open a vast field of research regarding interactions between plant extract and FSH in the development of primordial follicles until the antral stage. This study represents a hope for the use of medicinal plants in more complex experiments which aimed at the complete development of preantral follicles *in vitro*. *J. insularis* could be used for futher experiments to evaluate its beneficial effects on isolated preantral follicles development and oocyte maturation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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References

[1] Araújo, V. R., Gastal, M. O., Figueiredo, J. R., and Gastal, E. L. 2014. "*In vitro* Culture of Bovine Preantral Follicles: A Review." *Reprod Biol Endocrinol.* 12: 78.

[2] Picton, H. M., Harris, S. E., Muruvi, W., and Chambers, E. L. 2008. "The *in vitro* Growth and Maturation of Follicles." *Reproduction* 136: 703-15.

[3] Esmaielzadeh, F., Hosseini, S. M., Hajian, M., Nasiri, Z., Chamani, M., Afshar, A. M., et al. 2013. "Role of Follicle Stimulating Hormone in the Survival, Activation and Further Growth of *in vitro* Cultured Sheep Primordial Follicles." *Iran. J. Appl. Anim. Sci.* 3: 785-90.

[4] Andrade, E. R., Seneda, M. M., Alfieri, A. A., Oliveira, J.

A., Bracarense, A. P. F., Figueiredo, J. R., et al. 2005. "Interactions of Indole Acetic Acid with EGF and FSH in the Culture of Ovine Preantral Follicles." *Theriogenelogy* 64: 1104-13.

[5] Lima, L. F., Rocha, R. M. P., Alves, A. M. C. V., Saraiva, M. V. A., Araújo, V. R., Lima, I. M. T., et al. 2013. "Dynamized Follicle-Stimulating Hormone Affects the Development of Ovine Preantral Follicles Cultured *in vitro*." *Homeopathy* 102: 41-8.

[6] Magalhães, D. M., Araújo, V. R., Lima-Verde, I. B., Matos, M. H. T., Silva, R. C., Lucci, C. M., et al. 2009. "Impact of Pituitary FSH Purification on *in vitro* Early Folliculogenesis in Goats." *Biocell.* 33: 91-7.

[7] Eppig, J. J., and O'Brien, M. J. 1996. "Development *in vitro* of Mouse Oocytes from Primordial Follicles." *Biol. Reprod.* 54: 197-207.

[8] O'Brien, M. J., Pendola, J. K., and Eppig, J. J. 2003. "A Revised Protocol for *in vitro* Development of Mouse Oocytes from Primordial Follicles Dramatically Improves Their Developmental Competence." *Biol. Reprod.* 68: 1682-6.

[9] Cecconi, S., Barboni, B., Coccia, M., and Mattioli, M. 1999. "*In Vitro* Development of Sheep Preantral Follicles." *Biol. Reprod.* 60: 594-601.

[10] Mery, L., Lefevre, A., Benchaib, M., Demirci, B., Salle, B., Guerin, J. F., et al. 2007. "Follicular Growth *in vitro:* Detection of Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15) during *in vitro* Culture of Ovine Cortical Slices." *Mol. Reprod. Dev.* 74: 767-74.

[11] Muruvi, W., Picton, H. M., Rodway, R. G., and Joyce, I. M. 2009. "*In vitro* Growth and Differentiation of Primary Follicles Isolated from Cryopreserved Sheep Ovarian Tissue. Anim." *Reprod. Sci.* 112: 36-50.

[12] Costa, R. A. P., Romagna, C. D., Pereira, J. L., and Souza-pinto, N. C., 2011. "The Role of Mitochondrial DNA Damage in the Citotoxicity of Reactive Oxygen Species." *J. Bioenerg. Biomembr.* 43: 25-29.

[13] Lima-Verde, I. B., Rossetto, R., Matos, M. H. T., Celestino, J. J. H., Bruno, J. B., Silva, C. M. G., et al. 2009. "Androstenedione and Follicle Stimulating Hormone Involvement in the Viability and Development of Goat Preantral Follicles *in vitro*." *Anim. Reprod.* 7: 80-9.

[14] Goka, M. S. C., Telefo, P. B., Mbemya, G. T., Awouafack, M. D., Lienou, L. L., Yemele D. M., et al. 2016. "Potentialisation of Pregnant Mare Serum Gonadotropin Inducing Effect on Ovarian Follicles Growth by the Aqueous Extract of *Aloe buettneri, Dicliptera verticillata, Hibiscus macranthus* and *Justicia insularis* Leaves in Immature Rats." *Pharmacolology* 7: 328-36.

[15] Telefo, P. B., Lienou, L. L., Yemele, M. D., Lemfack, M. C., Mouokeu, C., Goka, C. S., et al. 2011. "Ethnopharmacological Survey of Plants Used for the Treatment of Female Infertility in Baham, Cameroon." *J. Ethnopharmacol.* 136: 178-87.

[16] Telefo, P. B., Tagne, S. R., Koona, O. E. S., Yemele, D. M., and Tchouanguep, F. M., 2012. "Effect of the Aqueous Extract of *Justicia Insularis* T. Anders (Acanthaceae) on Ovarian Folliculogenesis and Fertility of Female Rats." *Afr. J. Tradit. Complement. Altern. Med.* 9: 197-203.

[17] Telefo, P. B., Moundipa, P. F., Tchana, A. N., Tchouanguep, D., and Mbiapo, F. T. 1998. "Effects of an Aqueous Extract of *Aloe buettneri, Justicia insularis, Hibiscus macranthus, Dicliptera verticillata* on Some Physiological and Biochemical Parameters of Reproduction in Immature Female Rats." *J. Ethnopharmacol.* 63: 193-200.

[18] Telefo, P. B., Moundipa, F. P., and Tchouanguep, M. F., 2001. "Influence of the Aqueous Extract of *Aloe buettneri, Justicia insularis, Hibiscus macranthus* and *Dicliptera verticillata* on Fertility and Some Biochemical Parameters of Reproduction in Rats." *J. Cameroon Acad. Sci.* 3: 144-50.

[19] Telefo, P. B., Moundipa, P. F., and Tchouanguep, F. M. 2004. "Inductive Effects of the Leaf Mixture Extract of *Aloe buettneri, Justicia insularis, Dicliptera verticillata* and *Hibicus marcranthus* on *in vitro* Production of Oestradiol." *J. Ethnopharmacol.* 91: 225-30.

[20] Leal-Cardoso, J. H., and Fonteles, M. C. 1999. "Pharmacological Effects of Essential Oils Plants of the Northeast of Brazil." *An. Acad. Bra. Ciênc.* 71: 207-13.

[21] Freire, R. S., Morais, S. M., Catunda-Junior, F. E., and Pinheiro, D. C. 2005. "Synthesis and Antioxidant, Anti-Inflammatory and Gastroprotector Activities of Anethole and Related Compounds." *Bioorg. Med. Chem.* 13: 4353-8.

[22] Chainy, G. B., Manna, S. K., Chaturvedi, M. M., and Aggarwal, B. B. 2000. "Anethole Blocks both Early and Late Cellular Responses Transduced by Tumor Necrosis Factor: Effect on NF kappa B, AP-1, JNK, MAPKK and Apoptosis." *Oncogene* 19: 2943-50.

[23] Sá, N. A. R., Araújo, V. R., Correia, H. H. V., Ferreira, A. C. A., Guerreiro, D. D., Sampaio, A. M., et al. 2016. "Anethole Improves the *in vitro* Development of Isolated Caprine Secondary Follicles." *Theriogenology* 89: 226-34.

[24] Bandeira, F. T., Carvalho, A. A., Castro, S. V., Lima, L. F., Viana, D. A., Evangelista J. S. A. M., et al. 2015. "Two Methods of Vitrification Followed by *in vitro* Culture of the Ovine Ovary: Evaluation of the Follicular Development and Ovarian Extracellular Matrix." *Reprod.*

Domest. Anim. 50: 177-85.

[25] Barberino, R. S., Barros, V. R. P., Menezes, V. G., Santos, L. P., Araújo, V. R., Queiroz, M. A. A., et al. 2015. "*Amburana cearensis* Leaf Extract Maintains Survival and Promotes *in vitro* Development of Ovine Secondary Follicles." *Zygote* 1-9.

[26] Matos, M. H. T., Lima-verde, I. B., and Luque, M. C. A., 2007. "Essential Role of Follicle Stimulating Hormone in the Maintenance of Caprine Preantral Follicle Viability *in vitro*." *Zygote* 15: 173-82.

[27] Carvalho, A. A., Faustino, L. R., Castro, S. V., Silva, C. M. G., Campello, C. C., Figueiredo, J. R., et al. 2013. "Tissue Thickness May Influence the Outcome of Vitrification of Goat Ovarian Cortex." *Acta Sci. Vet.* 41: 1150.

[28] Loetchutinat, S., Kothan, S., Dechsupa, J., Meesungnoen, J., and Jay-Gerin, S. M. 2005. "Spectrofluorometric Determination of Intracellular Levels of Reactive Oxygen Species in Drug-Sensitive and Drug-Resistant Cancer Cells Using the 2',7'-Dichlorofluorescein Diacetate Assay." *Radiat. Phys. Chem.* 72: 323-31.

[29] Wright, C. S., Hovatta, O., Margara, R., Trew, G., Winston, R. M., Franks, S., et al. 1999. "Effects of Follicle-Stimulating Hormone and Serum Substitution on the *in vitro* Growth of Human Ovarian Follicles." *Hum. Reprod.* 14: 1555-62.

[30] McLaughlin, M., Bromfield, J. J., Albertini, D. F., and Telfer, E. E., 2010. "Activin Promotes Follicular Integrity and Oogenesis in Cultured Pre-antral Bovine Follicles." *Mol. Hum. Reprod.* 16: 644-53.

[31] Tamilmani, G., Varshney, P. K., Dubey, V. P., Pathak, M. C., and Sharma, G. T. 2013. "Influence of FSH on *in vitro* Growth, Steroidogenesis and DNA Synthesis of Buffalo (*Bubalus bubalis*) Ovarian Preantral Follicles." *Anim. Reprod.* 10: 32-40.

[32] Xu, M., Fazleabas, A. T., Shikanov, A., Jackson, E., Barrett, S. L., Hirshfeld-Cytron, J., et al. 2011. "*In vitro* Oocyte Maturation and Preantral Follicle Culture from the Luteal-Phase Baboon Ovary Produce Mature Oocytes." *Biol. Reprod.* 84: 689-97.

[33] Silva, J. R., Hurk, V. D., Matos, M. H., Santos, R. R., Pessoa, C., Moraes, M. O., et al. 2004. "Influences of FSH and EGF on Primordial Follicles during *in vitro* Culture of Caprine Ovarian Cortical Tissue." *Theriogenology* 61: 1691-704.

[34] Thomas, F. H., Ethier, J. F., Shimasaki, S., and Vanderhyden, B. C. 2005. "Follicle-Stimulating Hormone Regulates Oocyte Growth by Modulation of Expression of Oocyte and Granulosa Cell Factors." *Endocrinology* 146: 941-9.

[35] Engel, R. H., and Evens, A. M. 2006. "Oxidative Stress and Apoptosis: A New Treatment Paradigm in Cancer." *Fro. in Biosc.* 11: 300-12.

[36] Andrade, E. R., Maddox-Hyttel, P., Landim-Alvarenga, D. C., Silva, J. R. V., Alfieri, A. A., Seneda, M. M., et al. "Ultrastructure of Sheep Primordial Follicles Cultured in the Presence of Indol Acetic Acid, EGF, and FSH." *Vet. Med. Int.* 1-7.

[37] Murray, A. A., Molinek, M. D., Baker, S. J., Kojima, F. N., Smith, M. F., Hillier, S. G., et al. 2001. "Role of Ascorbic Acid in Promoting Follicle Integrity and Survival in Intact Mouse Ovarian Follicles *in vitro*." *Reproduction* 121: 89-96.

[38] Li, Y., and Schellhorn, H. E. 2007. "New Developments and Novel Therapeutic Perspectives for Vitamin C." *J. Nutr.* 137: 2171-84.

[39] Faustino, L. R., Santos, R. R., Silva, C. M. G., Pinto, L. C., Celestino, J. J. H., Campello, C. C., et al. 2010. "Goat and Sheep Ovarian Tissue Cryopreservation: Effects on the Morphology and Development of Primordial Follicles and Density of Stromal Cell." *Anim. Reprod. Sci.* 122: 90-7.

[40] Gouveia, B. B., Barros, V. R. P., Gonçalves, R. J. S., Barberino, R. S., Menezes, V. G., Lins, T. L. B., et al. 2015. "Effect of Ovarian Tissue Transportation in *Amburana cearensis* Extract on the Morphology and Apoptosis of Goat Preantral Follicles." *Anim. Reprod.* 12: 316-23.

[41] Mansouri, M. T., Farbood, Y., Sameri, M. J., Sarkaki, A., Naghizadeh, B., and Rafeirad, M. 2013. "Neuroprotective Effects of Oral Gallic Acid against Oxidative Stress Induced by 6-hydroxydopamine in Rats." *Food Chem.* 138: 1028-33.

[42] Behl, R., and Pandey, R. S. 2002. "FSH Induced Stimulation of Catalase Activity in Goat Granulosa Cells *in vitro*." *Anim. Reprod. Sci.*70: 215-21.

[43] Chen, Q., Zhang, W., Ran, H., Feng, L., Yan, H., and Um, X., 2014. "PKCδ and θ Possibly Mediate FSH-induced Mouse Oocyte Maturation via NOX-ROS-TACE Cascade Signaling Pathway." *PLoS One* 9: 1-8.

[44] Sadeghipour, M., Terreux, R., and Phipps, J. 2005. "Flavonoids and Tyrosine Nitration: Structure Activity Relationship Correlation with Enthalpy of Formation." *Toxicol.* 19: 155-65.