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

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**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% CO₂. 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].
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 folliculogenic 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 °C. 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 follicles [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% CO₂ in air. These treatments were referred to as: MEM+(cultured control), FSH, JUS0.3,
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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 *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 µm². 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 $\leq 0.1$ 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 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).

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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.

<table>
<thead>
<tr>
<th>Follicular morphology (%)</th>
<th>Follicular development (%)</th>
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<tr>
<td></td>
<td>Primordial</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
<td>Non-cultured control</td>
<td>84.6 (127/150)</td>
</tr>
<tr>
<td>MEM+ (cultured control)</td>
<td>82.5 (109/132)?a 62.5 (70/112)?a 88.9 (97/109)?a 85.7 (60/70)?a 11.1 (12/109)?a 14.3 (10/70)?a</td>
</tr>
<tr>
<td>FSH</td>
<td>79.8 (99/124)?a 65.2 (62/95)?a 75.7 (75/99)?ab 87.1 (54/62)?a 24.3 (24/99)?ab 12.9 (8/62)?a</td>
</tr>
<tr>
<td>JUS0.3</td>
<td>91.3 (137/150)?ab 89.5 (111/124)?ab 77.4 (106/137)?ab 84.7 (94/111)?ab 22.6 (31/137)?ab 15.3 (17/111)?a</td>
</tr>
<tr>
<td>JUS1.25</td>
<td>58.1 (57/98)?c 47.3 (71/150)?c 85.9 (49/57)?AB 74.6 (53/71)?ABC 14.1 (8/57)?AB 25.4 (18/71)?ABC</td>
</tr>
<tr>
<td>JUS5</td>
<td>66.1 (52/111)?cd 21.9 (29/132)?cd 82.5 (66/80)?AB 65.5 (19/29)?AB 17.5 (14/80)?AB 34.5 (10/29)?AB</td>
</tr>
</tbody>
</table>

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

a,b Within a row and the same parameter evaluated, values without a common superscript differed (p < 0.05).

A,B,C,D Within a column, values without a common superscript differed (p < 0.05).

...follies were analyzed. The percentages of morphologically normal preantral and growing follicles 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+FISH significantly increased (p < 0.05) the percentage of developing follicles. In addition, at day 7 of culture, MEM++ANE+FISH 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+FSH 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\textsuperscript{+} anethole supplemented with FSH or J. insularis (experiment 2).

<table>
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<tr>
<td>Non-cultured control</td>
<td>95.5 (172/180)</td>
</tr>
<tr>
<td>MEM\textsuperscript{+} + ANE + FSH</td>
<td>91.4 (139/152)\textsuperscript{aB}</td>
</tr>
<tr>
<td>MEM\textsuperscript{+} + ANE + JUS</td>
<td>77.2 (139/180)\textsuperscript{aA}</td>
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\* Differs from non-cultured control (\(p < 0.05\)); \textsuperscript{a,b} Within a row and the same parameter evaluated, values without a common superscript differed (\(p < 0.05\)); \textsuperscript{A,B} Within a column, values without a common superscript differed (\(p < 0.05\)).

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

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

*Within treatment, values without a common superscript differed ($p < 0.05$); A,B Within day, values without a common 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*.
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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 common superscript differed \((p < 0.05)\); \(A, B\) Within day, values without a common superscript differed \((p < 0.05)\).

Fig. 6  Correlation between the follicular morphology and the reactive oxygen species. Each point of the graph represents 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 (experiment 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 believed 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 further 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**


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