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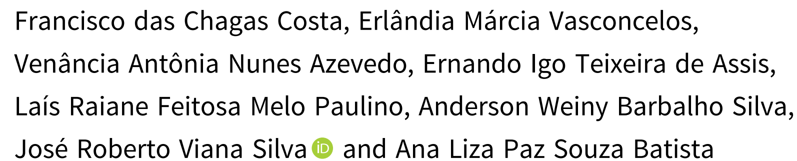
Aloe vera increases collagen fibres in extracellular matrix and mRNA expression of peroxiredoxin-6 in bovine ovarian cortical tissues cultured *in vitro*



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Research Article

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Summary

In vitro culture of ovarian tissue containing primordial follicles is an important tool to study the initiation of follicular populations and to develop efficient culture systems to support *in vitro* follicle growth. Considering that *in vitro* culture favours oxidative stress, it is very important to supplement culture medium with antioxidant substances such as *Aloe vera* extract. This study aims to evaluate the effects of different concentrations of *Aloe vera* on the distribution of collagen fibres in the extracellular matrix, follicular activation, development and survival in bovine ovarian cortical tissues cultured *in vitro*, as well as on expression of mRNAs for antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), peroxiredoxin 6 (PRDX6) and glutathione peroxidase 1 (GPX1)]. To this end, ovarian cortical tissues were cultured for 6 days in α-MEM alone or supplemented with different concentrations of *Aloe vera* extract (1.0, 5.0, 10.0 or 50.0%). After culture, fragments were fixed and processed histologically to evaluate follicular morphology and activation, as well as the extracellular matrix by staining with picrosirius red. The levels of mRNA for *SOD*, *CAT*, *PRDX6* and *GPX1* in cultured ovarian tissues were evaluated by real-time polymerase chain reaction (PCR). Ovarian tissues cultured with 10.0 or 50.0% *Aloe vera* had higher percentages of collagen fibres than tissues cultured in control medium. A significant increase in developing follicles was observed in ovarian tissues cultured in α-MEM alone or supplemented with 10% *Aloe vera* when compared with fresh control or tissues cultured with 1.0% *Aloe vera*. Presence of *Aloe vera* did not influence the percentage of morphologically normal follicles when compared with control medium. Ovarian tissues cultured with 50.0% *Aloe vera* had higher percentages of morphologically normal follicles than those cultured with 10.0% *Aloe vera*. Furthermore, 10% *Aloe vera* significantly increased mRNA levels for *PRDX6*. In conclusion, 10.0% *Aloe vera* improves extracellular matrix distribution in cultured tissues and increases the expression of mRNA for *PRDX6* after 6 days *in vitro*.

Introduction

Preantral follicles, i.e. primordial, primary and secondary follicles, represent approximately 90% of ovarian follicular population and are essential for the maintenance of fertility throughout the reproductive life of females (Sá *et al*., 2018). However, the vast majority of these follicles is lost due to the natural process of atresia (Figueiredo and Lima, 2017). Therefore, *in vitro* culture of ovarian tissue containing preantral follicles has been used in an attempt to avoid this huge follicular loss that occurs naturally *in vivo*. Isolation and *in vitro* culture of these follicles allow us to monitor follicular responses to hormones, growth factors, nutrients, vitamins, antioxidants and local factors during their development (Guerreiro *et al*., 2016). However, Magalhães *et al*. (2009) have shown that *in vitro* grown follicles have less developmental capacity than those grown *in vivo*. Although the birth of mice from primordial follicles grown, matured and fertilized *in vitro* has already been reported (O'Brien *et al*., 2003), in species such as caprine (Magalhães *et al*., 2011) and ovine (Arunakumari *et al*., 2010) only a few embryos have been produced *in vitro*. In bovine species, however, the results have been limited to the formation of early antral follicles *in vitro* (Mclaughlin *et al*., 2010). For these reasons, there is still a need to improve *in vitro* culture systems for preantral follicles in domestic species.

It is already know that during *in vitro* culture, preantral follicles are exposed to supraphysiological concentrations of oxygen (up to 20%; Sá *et al*., 2018), which can result in excessive formation of reactive oxygen species (ROS). Triggering oxidative stress can cause

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mitochondrial and DNA damage, peroxidation of lipid membranes, in addition to damage to cell proteins and consequent reduction in oocyte quality (Lins *et al.*, 2017). In addition, disturbing extracellular matrix (ECM) structure by oxidizing its compounds can have a negative effect on follicular dynamic. In fact, ECM is directly or indirectly involved in tissue remodelling, signalling, multiplication and cellular specialization during follicle development (Muncie and Weaver, 2018). As a consequence of increased production of ROS, ECM homeostasis can be severely disturbed, especially by changes in the activity of matrix metalloproteinases (MMPs). These proteases are responsible for remodelling and degradation of ECM components, including collagen (Verma and Hansch, 2007). As a consequence, tissues and cells can be severely damaged and negatively influence follicular development (Fu *et al.*, 2019).

Cellular defence mechanisms against oxidative stress involve enzymes such as superoxide dismutase (SOD), catalase, and peroxidases. SOD promotes a dismutation of superoxide radicals to H_2O_2, which is further detoxified to water and oxygen by catalase or glutathione peroxidase (GPx). Catalase is responsible for the conversion of H_2O_2 into water and oxygen, while GPx catalyzes the degradation of lipid peroxides, as well as H_2O_2 (Kala *et al.*, 2017). Peroxiredoxin 6 (Prdx6) is another enzyme with the ability to bind and reduce phospholipid hydroperoxides (Fisher *et al.*, 2018). In an attempt to improve *in vitro* culture systems, several non-enzymatic antioxidants extracted from plants have been highlighted due to their potential to combat ROS. Rutin (Lins *et al.*, 2017), extracts of *Morus nigra* (Gouveia *et al.*, 2019), *Amburana cearensis* (Gouveia *et al.*, 2016) and *Justicia insularis* (Mbemya *et al.*, 2018) have contributed to maintaining the balance between the production of ROS and the defence systems, favouring the maintenance of follicle growth and survival *in vitro*. In addition, it has been shown that *Aloe vera* extract has more than 75 different potentially active compounds including minerals, enzymes, vitamins, polysaccharides, phenolic compounds and organic acids that have several pharmacological properties, including antioxidant activity (Sumi *et al.*, 2019). *Aloe vera* can modulate oxidative stress due to the presence of antioxidant compounds such as α-tocopherol (vitamin E), carotenoids, ascorbic acid (vitamin C), flavonoids and tannins (Cesar *et al.*, 2018). Sumi *et al.* (2019) reported that *Aloe vera* increases the activity of antioxidant enzymes such as catalase, SOD and glutathione. The protective effect of *Aloe vera* on ECM integrity has also been reported (Saito *et al.*, 2016; Park *et al.*, 2017). Acemannan, a beta-(1,4)-acetylated mannan, is a bioactive polysaccharide present in *Aloe vera* gel that promotes skin wound healing through activating the AKT/mTOR-mediated protein translation mechanism in fibroblasts, resulting in increased secretion of collagen *in vivo* (Xing *et al.*, 2015). However, it is still not known if *Aloe vera* influences the collagen distribution in bovine ovarian tissues cultured *in vitro* and has a positive effect on primordial follicle survival and development. It is hypothesized that supplementation of culture medium with *Aloe vera* extract improves the integrity of collagen fibres in the ECM of cultured ovarian tissue, increases the expression of antioxidant enzymes and promotes primordial follicle development and survival.

This study aims to evaluate the effect of different concentrations of *Aloe vera* extract (1.0, 5.0, 10.0 or 50.0%) on distribution of collagen fibres in the ECM, primordial follicles activation, development and follicular survival, as well as on expression of mRNA for antioxidant enzymes (*SOD*, *CAT*, *PRDX1* and *GPX6*) in bovine ovarian tissue cultured *in vitro* for 6 days.

Materials and methods

Chemicals

Unless otherwise indicated, culture medium and other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant material and extract preparation

The plant *Aloe vera* was cultured in a sandy clay soil type at Frecheirinha – Ce, northeast of Brazil (3°45′49.3′′S, 40° 50′14.0′′W), with a semi-arid tropical climate and temperatures ranging from 24ºC to 36ºC. To obtain the crude extract of *Aloe vera*, leaves at an intermediate stage of maturation were collected, properly disinfected, and transported to the laboratory. Then, the leaves were washed with distilled water and their surface parts were removed to expose a colourless gel that was extracted from the parenchyma of the leaves. The gel was filtered through a sieve and stored at 4ºC in sterile 50 ml Falcon tubes (Souza *et al.*, 2016). Before supplementation of culture medium, the extract was filtered again through 0.45-μm membranes.

Source of ovaries

Bovine ovaries ($n = 20$) from cycling mixed breed cows were collected at a local slaughterhouse. Immediately post mortem, the ovaries were washed once in 70% alcohol (Dinâmica, São Paulo, Brazil), and twice in minimum essential medium (α-MEM) buffered with HEPES (MEM-HEPES) and supplemented with antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin). After washing, each pair of ovaries was individually transported to the laboratory in Falcon tubes containing α-MEM supplemented with penicillin (100 μg/ml) and streptomycin (100 μg/ml) at 4ºC, within 1 h. This study was approved by the Committee of Ethics and Animal Welfare of the Federal University of Ceará (no. 04/2019).

In vitro *culture of ovarian tissue*

In the laboratory, using a sterile scalpel no. 22, ovarian cortical tissue from the same ovarian pair ($n = 10$ pairs) was cut into 22 slices (3 mm × 3 mm × 1 mm) in dissection medium composed of α-MEM supplemented with penicillin (100 μg/ml) and streptomycin (100 μg/ml). For each animal, two cortical slices were fixed in paraformaldehyde (4%) for 24 h at 4ºC for histological analysis (uncultured control) as well as for analysis of the ECM. The remaining fragments were cultured in 24-well culture dishes for 6 days (Passos *et al.*, 2016; Silva *et al.*, 2017). Culture was performed at 38.5°C in 5% CO_2 in an humidified incubator. The basic culture medium consisted of α-MEM (pH 7.2–7.4) supplemented with ITS (10 μg/ml insulin, 5.5 μg/ml transferrin, and 5 ng/ml selenium), 2 mM glutamine, 2 mM hypoxanthine, antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin) and 1.25 mg/ml of bovine serum albumin (α-MEM$^+$). The fragments were cultured in 1 ml of control medium (α-MEM$^+$) alone or in control medium (α-MEM$^+$) supplemented with different proportions of *Aloe vera* extract [1.0% (+99.0% α-MEM$^+$), 5.0% (+95.0% α-MEM$^+$), 10.0% (+90.0% α-MEM$^+$) or 50.0% (+50.0% α-MEM$^+$)]. The proportions of *Aloe vera* were chosen according to Souza *et al.* (2016). Every 2 days, 60% of the culture medium was replaced with fresh medium. Each treatment was repeated 10 times. After the end of the culture period, the fragments were taken for morphological analysis.

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Analysis of extracellular matrix

To evaluate collagen fibres distribution in the ECM, ovarian cortical tissues were stained with picrosirius red (Abcam Kit), according to the methodology described by Rittié (2017), with modifications. Briefly, 6.0-µm ovarian sections were dewaxed in xylol and incubated in Sirius Red solution (0.1%) for 1 h at room temperature. After removal of excess dye with acetic acid solution (0.5%), the sections were dehydrated and evaluated under an optical microscope (Nikon, Eclipse, TS 100, Japan) at ×400 magnification. For each treatment, the percentage of area occupied by collagen fibres in 10 different fields was measured with the aid of a DS Cooled Camera Head DS-Ri1 coupled to a microscope (Nikon, Eclipse, TS 100, Japan) and the images were analyzed using ImageJ software (v.1.51p, 2017). After staining, the collagen fibres were marked in red by picrosirius, while the follicles remained uncoloured. ImageJ software was used to quantify the percentage of collagen fibred in uncultured and cultured tissues. The staining intensity of collagen fibres was determined by measuring the average pixel intensity of the total area imaged after background subtraction.

Morphological analyses and assessment of in vitro *follicular growth*

Histological analysis was performed according to the methodology of Bizarro-Silva *et al.* (2018), with modifications. Fresh control (D0) and cultured ovarian fragments were fixed in paraformaldehyde (4%) for 24 h, dehydrated in increasing concentrations of ethanol, cleared with xylol and included in paraffin. Serial sections (6-µm thickness) were performed and, each fifth section was stained with haematoxylin and eosin. Only preantral follicles with oocyte nuclei in the section were analyzed to avoid double counting. Coded anonymized slides were examined under a microscope (Nikon, Tokyo, Japan) at ×100 and ×400 magnification. The developmental stages of follicles were classified as primordial (one layer of flattened or flattened and cuboidal granulosa cells around the oocyte) or growing follicles (primary: one layer of cuboidal granulosa cells, and secondary: two or more layers of cuboidal granulosa cells around the oocyte) (Figueiredo and Lima, 2017). These follicles were further classified as normal when an intact oocyte was present, surrounded by granulosa cells that were well organized into one or more layers, and had no pyknotic nucleus. Degenerated follicles were defined as those with a retracted oocyte, which had a pyknotic nucleus and/or was surrounded by disorganized granulosa cells, which are detached from the basement membrane (Telfer *et al.*, 2008). The percentages of healthy primordial and developing follicles were calculated before (uncultured control) and after culture in a particular medium.

Expression of mRNA for SOD, CAT, PRDX6 *and* GPX1 *in ovarian tissue*

For analysis of gene expression, ovarian tissues were stored at −80ºC immediately after the end of the culture period until the extraction of total RNA for further analysis of expression of *SOD*, *CAT*, *PRDX6* and *GPX1* mRNAs. Tissue cultured in control medium (α-MEM$^+$) and those cultured in medium supplemented with 10% *Aloe vera*, i.e. the treatment that improved ECM tissue distribution, were selected to investigate mRNA expression. For this, ovarian tissues were macerated using scalpels no. 22 under sterile conditions and then subjected to extraction of total RNA using a TRIzol® purification kit (Invitrogen, São Paulo, Brazil) in accordance with the manufacturer's instructions. Quantification of mRNA was performed using SYBR Green. PCR reactions were composed of 1 µl cDNA as a template in 7.5 µl of SYBR Green Master Mix (PE Applied Biosystems, Foster City, CA, USA), 5.5 µl of ultra-pure water, and 0.5 µM of each primer. Primers were designed to perform the amplification of *SOD*, *CAT*, *PRDX6*, *GPX1* and *GAPDH* (Table 1). The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was initial denaturation and activation of the polymerase for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C. The final extension was for 10 min at 72°C. All reactions were performed in a Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). The $\Delta\Delta C_t$ method was used to transform the C_t values into normalized relative expression levels.

Table 1. Primer pairs used for real-time PCR

Target gene	Primer sequence (5′→3′)	Sense (S), anti-sense (As)	GenBank accession no.
GAPDH	TGTTTGTGATGGGCGTGAACCA	S	GI: 402744670
	ATGGCGCGTGGACAGTGGTCATAA	As	
PRDX6	GCACCTCCTCTTACTTCCCG	S	GI: 59858298
	GATGCGGCCGATGGTAGTAT	As	
GPX1	AACGTAGCATCGCTCTGAGG	S	GI:156602645
	GATGCCCAAACTGGTTGCAG	As	
SOD	GTGAACAACCTCAACGTCGC	S	GI: 31341527
	GGGTTCTCCACCACCGTTAG	As	
CAT	AAGTTCTGCATCGCCACTCA	S	GI:402693375
	GGGGCCCTACTGTCAGACTA	As	

Statistical analysis

Statistical analysis was performed using GraphPad Prism (5.0) software. Data that did not show a normal distribution, according to Shapiro–Wilk test, underwent logarithmic transformation. The percentage of normal follicles and follicular activation in each treatment were evaluated using Tukey's test, and comparison between treatments using Fisher's exact test. Data for collagen fibres distribution were analyzed by Kruskal–Wallis test, followed by Dunn's comparison test. Levels of mRNA were analyzed by analysis of variance (ANOVA) and Student's t-test. The differences were statistically significant when the P-value was < 0.05.

Results

Assessment of ovarian extracellular matrix

Ovarian tissues cultured with 10.0 or 50.0% *Aloe vera* had higher percentages of collagen fibres than tissues cultured in control medium, but had similar percentages of collagen fibres when compared with uncultured tissues ($P < 0.05$) (Figure 1A,B).

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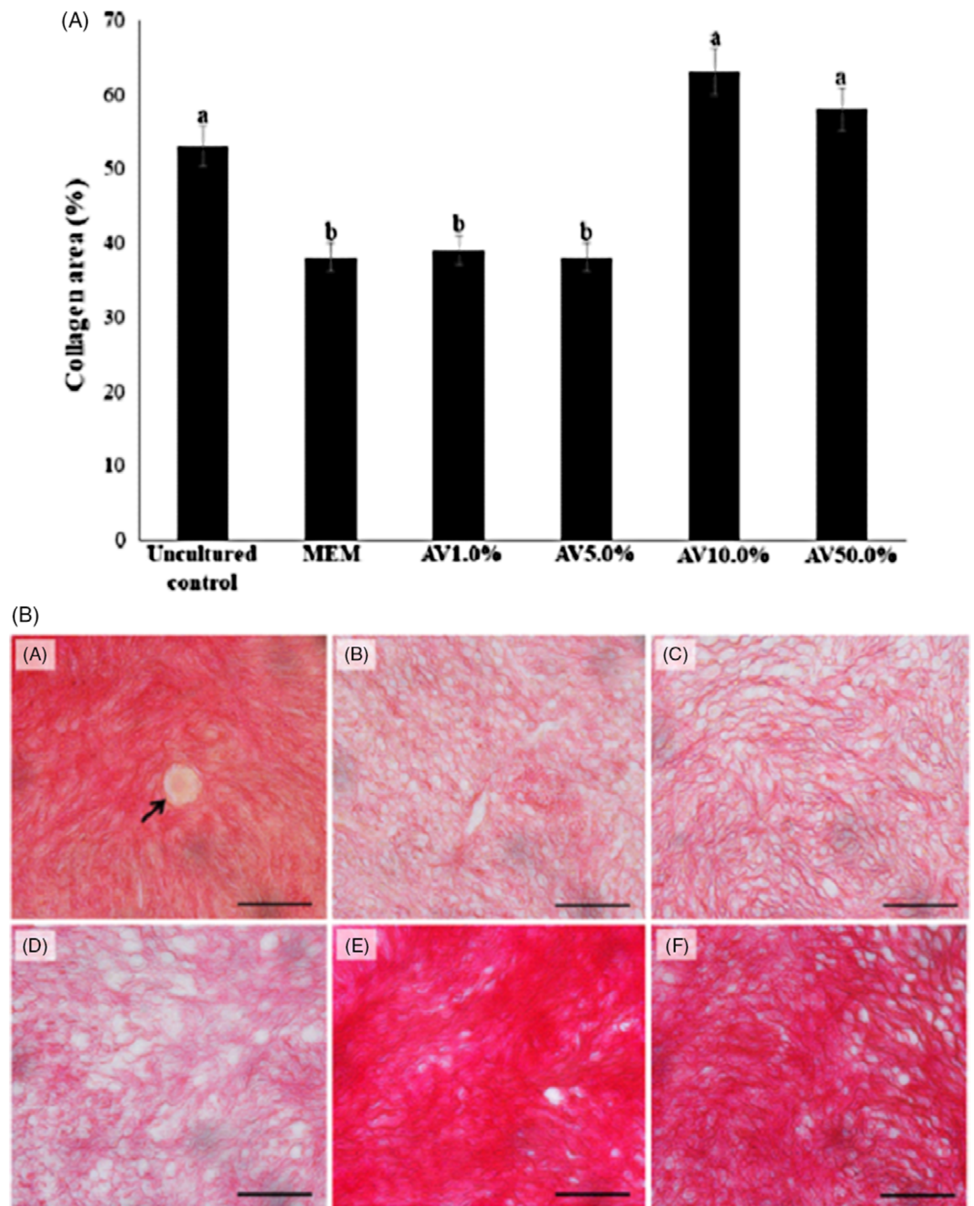

Figure 1. (A) Percentages of collagen in uncultured tissues and in tissues cultured for 6 days in control medium alone or supplemented with 1.0, 5.0, 10.0 or 50.0% *Aloe vera.* [a–c]Different lowercase letters indicate statistically significant differences between treatments. (B) Representative images of collagen fibres in uncultured tissues (a) and in tissues cultured for 6 days in control medium alone (b) or supplemented with 1.0, 5.0, 10.0 or 50.0% *Aloe vera* (c–f). The black arrow shows an unstained primordial follicle. Scale bars: 100 μm.

Effects of Aloe vera *on the activation and development of primordial follicles*

Ovarian tissues cultured with 10% *Aloe vera* showed a significant decrease in the percentage of primordial follicles and concomitant increase in developing follicles, when compared with uncultured control. Additionally, the percentages of developing follicles in tissues culture with 10% *Aloe vera* were higher than those seen in tissues cultured with 1.0, 5.0 or 50.0% of *Aloe vera*. (Figure 2A,B). The percentages of primordial follicles in ovarian tissues cultured with 1.0, 5.0 or 50.0% of *Aloe vera* did not differ from those observed in uncultured ovarian tissues ($P < 0.05$). Moreover, ovarian tissues cultured with 10% *Aloe vera* had similar rates of primordial/developing follicles when compared with those tissues cultured in control medium.

Effects of Aloe vera *on follicular morphology after* in vitro *culture*

In total, 932 preantral follicles were analyzed. Figure 3 shows the morphology of normal and degenerate follicles in uncultured and cultured ovarian cortical tissues. After a 6-day culture period, a significant decrease in the percentage of normal follicles in ovarian tissues cultured in all treatments was observed, compared with the uncultivated control ($P < 0.05$). The presence of *Aloe vera* (all concentrations) in the culture medium maintained the percentage of morphologically normal follicles when compared with tissues cultured in the control medium. Ovarian tissues cultured with 50.0% *Aloe vera* showed a higher percentage of morphologically normal follicles than those grown with 10.0% *Aloe vera* ($P < 0.05$) (Figure 4).

Levels of mRNA for SOD, CAT, PRDX6 *and* GPX1 *after* in vitro *culture*

Ovarian cortical tissues cultured in the presence of 10% *Aloe vera* had significantly higher mRNA levels for *PRDX6* ($P < 0.05$) than those tissues cultured with α-MEM^{+} alone (Figure 5). However, *Aloe vera* did not influence in the expression of mRNA for *SOD*, *CAT* and *GPX1* ($P > 0.05$).

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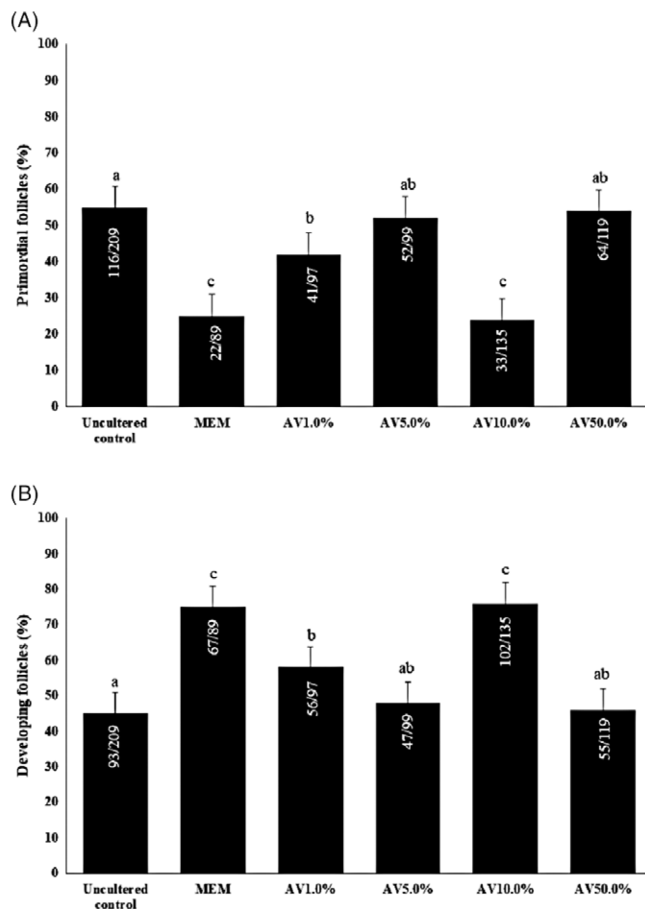

Figure 2. Percentages of primordial follicles (A), and developing follicles (B) in uncultured tissues and in tissues cultured for 6 days in control medium alone or supplemented with 1.0, 5.0, 10.0 or 50.0% *Aloe vera.* [a–c]Different lowercase letters indicate statistically significant differences between treatments.

Discussion

The present study investigated for the first time the effect of *Aloe vera* on the maintenance of the ECM, follicular activation and levels of mRNA for *SOD*, *CAT*, *PRDX6* and *GPX1* in bovine ovarian cortical tissues cultured *in vitro* for 6 days. The presence *Aloe vera* extract in culture medium had no toxic effect on bovine ovarian tissues cultured *in vitro*. We showed that *Aloe vera* increased the number of collagen fibres in ECM of bovine ovarian tissue cultured *in vitro*, and kept ECM similar to uncultured tissues. ECM participates in the regulation of a variety of cellular functions in many tissues, inclusive the ovary. The formation, development and regression of ovarian follicles require considerable tissue remodelling, cell multiplication, and bidirectional communication among oocyte, granulosa cells, and theca cells, and the surrounding ECM (Berkholtz *et al.*, 2006). Therefore, in addition to providing mechanical support to cells, ECM is very important to cell–cell and tissue-cell communication, adhesion, migration, survival and proliferation (Muncie and Weaver, 2018). In addition, growth factors such as epidermal growth factor, fibroblast growth factor, transforming growth factor-β and amphiregulins can be stored in the ECM and released locally during the remodelling process through matrix cleavage (Gonella-Diaza *et al.*, 2018). Consequently, regulation of the mechanisms that control the integrity of ECM is necessary for the growth of follicles from primordial the ovulatory stages (Franchi *et al.*, 2020). Homeostasis of extracellular matrix is influenced by matrix metalloproteinases (MMPs) that are responsible for remodelling and degradation of ECM components, including collagens (Verma and Hansch, 2007). For these reasons, MMPs play an important role during follicular development (Kliem *et al.*, 2007). Although MMPs are regulated at the transcriptional level, a wide variety of stresses can result in excessive degradation of ECM. Among then, oxidative stress during *in vitro* culture of ovarian tissues can be a critical factor in this process (Quan *et al.*, 2013). In fact, Ali *et al.* (2019) demonstrated increased expression of MMP-2 and MMP-9 in endothelial cells under oxidative stress. In our study, supplementation of culture medium with 10 or 50% *Aloe vera* preserved the number of collagen fibre during *in vitro* culture. Previous studies have already reported that *Aloe vera* has a protective effect on ECM. Saito *et al.* (2016) reported a protective effect of *Aloe vera* on ECM of mice skin after irradiation by X-rays. These authors showed that *Aloe vera* prevents the increase of MMP-2 and MMP-9. A similar effect was reported by Park *et al.* (2017), i.e. *Aloe vera* was able to reduce the levels of mRNA and protein for MMP-9 in mouse tissues, which helped to maintain tissue structure.

In the present study, 10% *Aloe vera* treatment showed a similar rate of primordial follicles when compared with culture control treatment. Compared with ovarian tissue cultured with 10.0% *Aloe vera*, tissues cultured for 6 days in the presence of 1.0, 5.0 or 50.0% *Aloe vera* demonstrated a reduction in activation and follicular growth rates. These data show that 10% *Aloe vera* can be added to culture medium without influencing the maintenance of the primordial follicle reserve. Previous studies have shown that a 6-day culture period is sufficient to promote activation and development of primordial follicles. When fragments of ovarian cortex are cultured there is a significant shift of follicles from quiescent to the growing pool over short culture periods of 6–10 days (Telfer and Zelinski, 2013). It is known that the activation of primordial follicles is a very dynamic process and, despite enormous progress made in mapping their regulation, many molecular mechanisms are still not fully understood (Figueiredo and Lima, 2017). The reduction in follicular activation rates during culture in the presence of 1.0 or 5.0% *Aloe vera* can be associated with the reduction in collagen fibres described in this study. Woodruff and Shea (2007) reported that an appropriately rigid ovarian extracellular environment can be a necessary requirement for follicle survival and growth. Various growth factors, such as TGF-β superfamily members, are involved in primordial follicle activation and, considering that ECM components are capable of binding these factors (Smith *et al.*, 1999), reduction of ECM density can diminish the availability of these growth factors and downregulate primordial follicle activation and growth. To explain the reduction in activation in tissues cultured in 50.0% *Aloe vera*, Tseng *et al.* (2017) showed that high concentrations of bioactive components present in *Aloe vera* extract can block PI3K activation signalling. Additionally, Adhikari *et al.* (2009) reported that PI3K/Akt pathway activation in oocytes induces primordial follicle activation, while Santos *et al.* (2017) showed that blocking the PI3K pathway inhibits spontaneous activation of ovine primordial follicles *in vitro*.

A high percentage of normal follicles (70.0–80.0%) was observed in ovarian tissues cultured either in control medium alone or supplemented with *Aloe vera*. In general, the presence of *Aloe vera* in culture medium did not influence follicle viability, based on comparisons with tissues cultured in control medium. This could be due to the presence of antioxidants in control culture medium, such as transferrin, selenium and ascorbic acid. It is also

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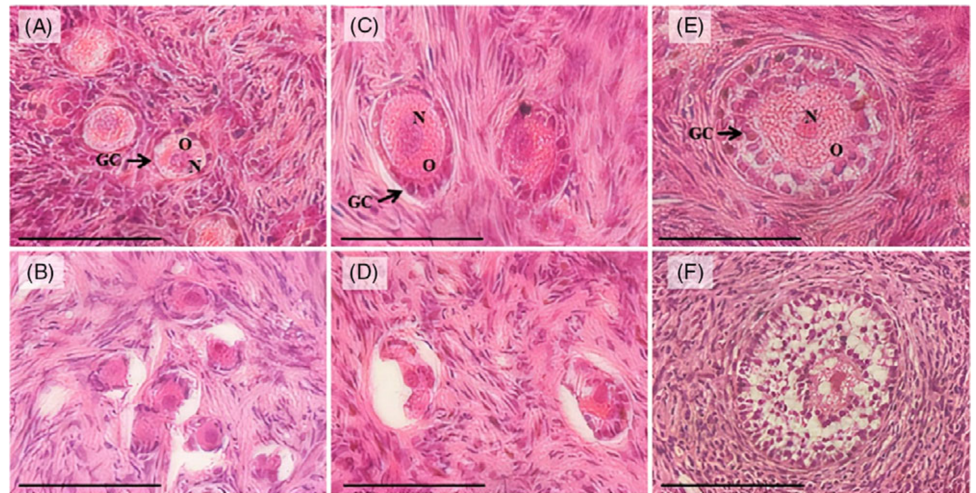

Figure 3. Representative images of sections of bovine ovarian tissue showing morphologically normal and atretic follicles. Normal primordial follicles (A) and atretic (B); normal (C) and atretic (D) primary follicle; normal (E) and atretic (F) secondary follicle. Granulosa cells (GC); oocyte (O); oocyte nucleus (N). Scale bars: 100 µm.

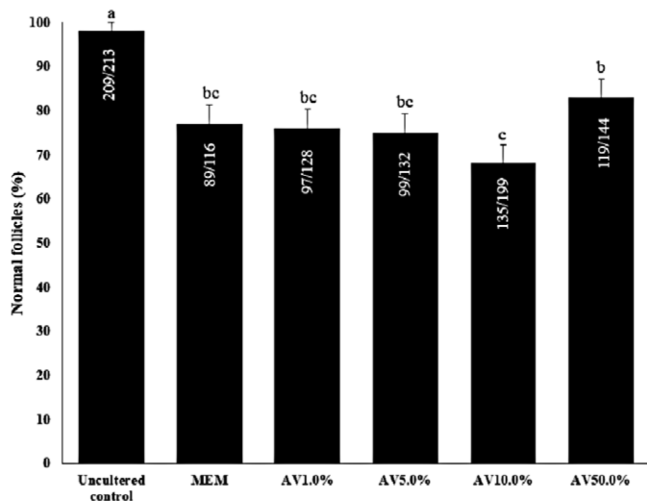

Figure 4. Percentages of morphologically normal follicles in uncultured tissues and in tissues cultured for 6 days in control medium alone or supplemented with 1.0, 5.0, 10.0 or 50.0% *Aloe vera.* [a–c]Different lowercase letters indicate statistically significant differences between treatments.

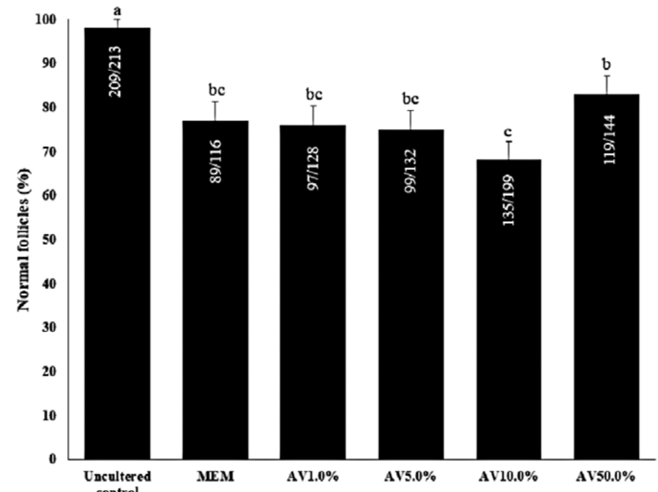

Figure 5. Levels of mRNA for *SOD* (A), *CAT* (B), *PRDX6* (C) and *GPX1* (D) in ovarian tissues cultured for 6 days in control medium alone or supplemented with 10.0% *Aloe vera*. [a–c]Different letters indicate significant difference between treatments ($P < 0.05$).

important to consider that *Aloe vera* has antioxidant activity. The maintenance of cell morphology of ovarian follicles in *in vitro* culture depends on the cellular and tissue redox balance (Sá *et al.*, 2018). Elevated levels of ROS can activate apoptotic clumps that result in follicular degeneration (Agarwal *et al.*, 2012). Cesar *et al.* (2018) reported the presence of antioxidants in *Aloe vera* leaves, such as ascorbic acid, carotenoids and soluble polyphenols. These authors also demonstrated that 10.0% *Aloe vera* increased the growth of HeLa cells in culture and reduced the cytotoxicity induced by H_2O_2. *Aloe vera* was also able to protect sperm cells against oxidative damage induced by bisphenol A (BPA) and X-rays, respectively (Bala *et al.*, 2017; Behmanesh *et al.*, 2018). In this sense, the protective effects of *Aloe vera* were associated with increased antioxidant defence that was enzymatic (glutathione reductase, glutathione peroxidase, catalase and SOD) and non-enzymatic (GSH) in nature. In addition, it has been shown that *Aloe vera* can reduce extensive damage to membrane lipids, therefore reducing apoptotic processes (Haritha *et al.*, 2014). In the present study, tissues cultured with 50% *Aloe vera* had higher percentages of normal follicles than those cultured with 10% *Aloe vera.* It is known that many locally produced growth factors regulate follicle survival. Probably, cytoprotective effects of nutrient constituents in *Aloe vera* such as acemannan, sugars, amino acids used in structural proteins, fats, and ions such as calcium, magnesium, potassium, and vitamins (Joseph and Raj, 2010) improve follicle survival when compared with tissues cultured in 10% *Aloe vera*, but not when compared with control medium. Sholehvar *et al.* (2016) showed that dental pulp stem cells cultured in medium supplemented with 50% *Aloe vera* had improved viability.

The presence of 10% *Aloe vera* in culture medium increased *PRDX6* mRNA levels in cultured tissues. Among mammalian peroxiredoxins, PRDX6 (Prdx6, 1-cys peroxiredoxin) is capable of reducing a wide range of substrates including H_2O_2, peroxynitrite, alkyl peroxides, and phospholipid peroxides (Perkins *et al.*, 2014; Fisher *et al.*, 2018). Due to its antioxidant properties, it plays an important role in maintaining the redox balance in mammalian cells including ovarian cells. For this reason, it is an important antioxidant defence in most cells exposed to oxygen (Sharapov *et al.*, 2019). Peroxideroxin 6 is the only peroxiredoxin capable of reducing phospholipid hydroperoxides through the activity of glutathione peroxidase (GPX). Animals with knockout of the *PRDX6*

gene, despite the normal expression of genes encoding other antioxidant enzymes, have a high sensitivity to oxidative stress, which is accompanied by a high level of oxidative damage to organs and tissues (Sharapov *et al*., 2019). Zha *et al.* (2015) demonstrated that PRDX6 can protect ARPE-19 cells, at least partially, through the PI3K/Akt signalling pathway. In the ovary, this pathway appears to play a regulating role in the growth and differentiation of ovarian follicles (Dupont and Scaramuzzi, 2016). The balance between PI3K/Akt substrates determines the acceleration, deceleration, survival and apoptosis of follicular growth (Zhou *et al*., 2017). In our study, increased levels of *PRDX6* mRNA can be associated with higher levels of collagen fibres in the ECM of cultured ovarian tissues.

In conclusion, 10.0% *Aloe vera* increases the number of collagen fibres in the ECM and the expression of *PRDX6* mRNA in bovine ovarian tissues cultured for 6 days. This demonstrates the potential for *Aloe vera* to be used as a supplement of culture medium of ovarian tissue.

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Statement of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Ethical standards. The authors declare that all procedures were performed according to national and institutional guides on the care and use of animals.

References

Adhikari D, Flohr G, Gorre N, Shen Y, Yang H, Lundin E, Lan Z, Gambello MJ and Liu K (2009). Disruption of Tsc2 in oocytes leads to overactivation of the entire pool of primordial follicles. *Mol Hum Reprod* **15**, 765–70.

Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A and Gupta S (2012). The effects of oxidative stress on female reproduction: a review. *Reprod Biol Endocrinol* **10**, 49.

Ali MM, Mahmoud AM, Le Master E, Levitan I and Phillips SA (2019). Role of matrix metalloproteinases and histone deacetylase in oxidative stress-induced degradation of the endothelial glycocalyx. *Am J Physiol Heart Circ Physiol* **316**, H647–63.

Arunakumari G, Shanmugasundaram N and Rao VH (2010). Development of morulae from the oocytes of cultured sheep preantral follicles. *Theriogenology* **74**, 884–94.

Bala S, Chugh NA, Bansal SC, Garg ML and Koul A (2017). Protective role of *Aloe vera* against X-ray induced testicular dysfunction. *Andrologia* 49, 10.1111/and.12697.

Behmanesh MA, Najafzadehvarzi H and Poormoosavi SM (2018). Protective effect of *Aloe vera* extract against bisphenol A induced testicular toxicity in Wistar rats. *Cell J* **20**, 278–283.

Berkholtz CB, Shea LD and Woodruff TK (2006). Extracellular matrix functions in follicle maturation. *Semin Reprod Med* **24**, 262–9.

Bizarro-Silva C, Santos MM, Gerez JR, González SM, Lisboa LA and Seneda MM (2018). Influence of follicle-stimulating hormone concentrations on the integrity and development of bovine follicles cultured *in vitro*. *Zygote* **26**, 417–23.

Cesar V, Jozić I, Begović L, Vuković T, Mlinarić S, Lepeduš H, Borović Šunjić SB and Žarković N (2018). Cell-type-specific modulation of hydrogen peroxide cytotoxicity and 4-hydroxynonenal binding to human cellular proteins *in vitro* by antioxidant *Aloe vera* extract. *Antioxidants (Basel)* **7**, 125.

Dupont J and Scaramuzzi RJ (2016). Insulin signalling and glucose transport in the ovary and ovarian function during the ovarian cycle. *Biochem J* **473**, 1483–501.

Figueiredo JR and Lima LF (2017). Artificial ovary technology: applications, state of art, limitations and prospects. *Braz J Anim Reprod* **41**, 248–53.

Fisher AB, Vasquez-Medina JP, Dodia C, Sorokina EM, Tao JQ and Feinstein SI (2018). Peroxiredoxin 6 phospholipid hydroperoxidase activity in the repair of peroxidized cell membranes. *Redox Biol* **14**, 41–6.

Franchi FF, Hernandes MP, Coalho Ferreira AL, Vieira de Lima VA, de Oliveira Mendes L, Musa de Aquino A, Scarano WR and César de Souza Castilho A (2020). Fractal analysis and histomolecular phenotyping provides insights into extracellular matrix remodeling in the developing bovine fetal ovary. *Biochem Biophys Res Commun* **523**, 823–8.

Fu XH, Chen CZ, Wang Y, Peng YX, Wang WH, Yuan B, Gao Y, Jiang H and Zhang JB (2019). COL1A1 affects apoptosis by regulating oxidative stress and autophagy in bovine cumulus cells. *Theriogenology* **139**, 81–9.

Gonella-Diaza AM, Silveira Mesquita F, Lopes E, Ribeiro da Silva K, Cogliati B, De Francisco Strefezzi R and Binelli M (2018). Sex steroids drive the remodeling of oviductal extracellular matrix in cattle. *Biol Reprod* **99**, 590–9.

Gouveia BB, Macedo TJS, Santos JMS, Barberino RS, Menezes VG, Müller MC, Almeida JRGS, Figueiredo JR and Matos MHT (2016). Supplemented base medium containing *Amburana cearensis* associated with FSH improves *in vitro* development of isolated goat preantral follicles. *Theriogenology* **86**, 1275–84.

Gouveia BB, Barberino RS, Menezes VG, Macedo TJS, Cavalcante AYP, Gonçalves RJS, Almeida JRGS and Matos MHT (2019). DNA damage and primordial follicle activation after *in vitro* culture of sheep ovarian cortex in *Morus nigra* leaf extract. *Pesq Vet Bras* **39**, 85–92.

Guerreiro DD, Lima LFd, Rodrigues GQ, Carvalho AdA, Castro SV, Campello CC, Pessoa CdÓ, Gadelha CRF, Figueiredo JRd, Bordignon V and Rodrigues APR (2016). In situ cultured preantral follicles is a useful model to evaluate the effect of anticancer drugs on caprine folliculogenesis. *Microsc Res Tech* **79**, 773–81.

Haritha K, Ramesh B and Saralakumari D (2014). Effect of *Aloe vera* gel on antioxidant enzymes in streptozotocin-induced cataractogenesis in male and female Wistar rats. *J Acute Med* **4**, 38–44.

Joseph B and Raj SJ (2010). Pharmacognostic and phytochemical properties of *Aloe vera* Linn – an overview. *Int J Pharm Sci Rev Rev Res* **4**, 106–10.

Kala M, Shaikh MV and Nivsarkar M (2017). Equilibrium between anti-oxidants and reactive oxygen species: a requisite for oocyte development and maturation. *Reprod Med Biol* **16**, 28–35.

Kliem H, Welter H, Kraetzl WD, Steffl M, Meyer HHD, Schams D and Berisha B (2007). Expression and localisation of extracellular matrix degrading proteases and their inhibitors during the oestrous cycle and after induced luteolysis in the bovine corpus luteum. *Reproduction* **134**, 535–47.

Lins TLBG, Cavalcante AYP, Santos JMS, Menezes VG, Barros VRP, Barberino RS, Bezerra MÉS, Macedo TJS and Matos MHT (2017). Rutin can replace the use of three other antioxidants in the culture medium, maintaining the viability of sheep isolated secondary follicles. *Theriogenology* **89**, 263–70.

Magalhães DDM, Araujo VR, Verde IBL, Matos MHTd, Silva RC, Lucci CM, Báo SN, Campello CC and Figueiredo JRd (2009). Diferentes origens do Hormônio Folículo Estimulante (FSH) influenciam a viabilidade e o desenvolvimento de folículos pré-antrais caprinos. *Braz J Vet Res Anim Sci* **46**, 378–86.

Magalhães DM, Fernandes DD, Mororó MB, Silva CM, Rodrigues GQ, Bruno JB, Matos MH, Campello CC and Figueiredo JR (2011). Effect of the medium replacement interval on the viability, growth and *in vitro* maturation of isolated caprine and ovine pre-antral follicles. *Reprod Domest Anim* **46**, 134–40.

Mbemya GT, Cadenas J, Ribeiro de Sá NA, Damasceno Guerreiro D, Donfack NJ, Alberto Vieira L, Canafístula de Sousa FG, Geraldo Alves B, Lobo CH, Santos FW, Lima Pinto FDC, Loiola Pessoa OD, Smitz J, Comizzoli P, Figueiredo JR and Rodrigues APR (2018). Supplementation of *in vitro* culture medium with FSH to grow follicles and mature oocytes can be replaced by extracts of *Justicia insularis*. *PLoS ONE* **13**, e0208760.

https://doi.org/10.1017/S0967199421000824 Published online by Cambridge University Press

McLaughlin M, Bromfield JJ, Albertini DF and Telfer EE (2010). Activin promotes follicular integrity and oogenesis in cultured pre-antral bovine follicles. *Mol Hum Reprod* **16**, 644–53.

Muncie JM and Weaver VM (2018). The physical and biochemical properties of the extracellular matrix regulate cell fate. *Curr Top Dev Biol* **130**, 1–37.

O'Brien MJ, Pendola JK and Eppig JJ (2003). A revised protocol for *in vitro* development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod* **68**, 1682–6.

Park CH, Son HU, Yoo CY and Lee SH (2017). Low molecular-weight gel fraction of *Aloe vera* exhibits gastroprotection by inducing matrix metalloproteinase-9 inhibitory activity in alcohol-induced acute gastric lesion tissues. *Pharm Biol* **55**, 2110–5.

Passos JR, Costa JJ, da Cunha EV, Silva AW, Ribeiro RP, de Souza GB, Barroso PA, Dau AM, Saraiva MV, Gonçalves PB, van den Hurk R and Silva JR (2016). Protein and messenger RNA expression of interleukin 1 system members in bovine ovarian follicles and effects of interleukin 1β on primordial follicle activation and survival *in vitro*. *Domest Anim Endocrinol* **54**, 48–59.

Perkins A, Poole LB and Karplus PA (2014). Tuning of peroxiredoxin catalysis for various physiological roles. *Biochemistry* **53**, 7693–705.

Quan J, Elhousiny M, Johnson NW and Gao J (2013). Transforming growth factor-β1 treatment of oral cancer induces epithelial–mesenchymal transition and promotes bone invasion via enhanced activity of osteoclasts. *Clin Exp Metastasis* **30**, 659–70.

Rittié L (2017). Method for picrosirius red-polarization detection of collagen fibers in tissue sections. *Methods Mol Biol* **1627**, 395–407.

Sá NAR, Bruno JB, Guerreiro DD, Cadenas J, Alves BG, Cibin FWS, Leal-Cardoso JH, Gastal EL and Figueiredo JR (2018). Anethole reduces oxidative stress and improves *in vitro* survival and activation of primordial follicles. *Braz J Med Biol Res* **51**, e7129.

Saito M, Tanaka M, Misawa E, Yao R, Nabeshima K, Yamauchi K, Abe F, Yamamoto Y and Furukawa F (2016). Oral administration of *Aloe vera* gel powder prevents UVB-induced decrease in skin elasticity via suppression of overexpression of MMPs in hairless mice. *Biosci Biotechnol Biochem* **80**, 1416–24.

Santos LdPd, Santos JMdS, Menezes VG, Barberino RdS, Gouveia BB, Cavalcante AYP, Lins TLBG, Gonçalves RJdS, Celestino JJdH and Matos MHTd (2017). Blocking the PI3K pathway or the presence of high concentrations of EGF inhibits the spontaneous activation of ovine primordial follicles in vitro. *Anim Reprod* **14**, 1298–306.

Sharapov MG, Novoselov VI and Gudkov SV (2019). Radioprotective role of peroxiredoxin 6. *Antioxidants (Basel)* **8**, 15.

Sholehvar F, Mehrabani D, Yaghmaei P and Vahdati A (2016). The effect of *Aloe vera* gel on viability of dental pulp stem cells. *Dent Traumatol* **32**, 390–6.

Silva AWB, Ribeiro RP, Menezes VG, Barberino RS, Passos JRS, Dau AMP, Costa JJN, Melo LRF, Bezerra FTG, Donato MAM, Peixoto CA, Matos MHT, Gonçalves PBD, Van den Hurk R and Silva JRV (2017). Expression of TNF-α system members in bovine ovarian follicles and the effects of TNF-α or dexamethasone on preantral follicle survival, development and ultrastructure *in vitro*. *Anim Reprod Sci* **182**, 56–68.

Smith JD, Bryant SR, Coupe LL, Vary CP, Gotwals PJ, Koteliansky VE and Lindner V (1999). Soluble transforming growth factor-beta type II receptor inhibits negative remodeling, fibroblast transdifferentiation, and intimal lesion formation but not endothelial growth. *Circ Res* **84**, 1212–22.

Souza ALP, Lima GL, Peixoto GCX, Silva AM, Oliveira MF and Silva AR (2016). Use of *Aloe vera*–based extender for chilling and freezing collared peccary (*Pecari tajacu*) semen. *Theriogenology* **85**, 1432–8.

Sumi FA, Sikder B, Rahman MM, Lubna SR, Ulla A, Hossain MH, Jahan IA, Alam MA and Subhan N (2019). Phenolic content analysis of *Aloe vera* gel and evaluation of the effect of *Aloe gel* supplementation on oxidative stress and fibrosis in isoprenaline-administered cardiac damage in rats. *Prev Nutr Food Sci* **24**, 254–64.

Telfer EE and Zelinski MB (2013). Ovarian follicle culture: advances and challenges for human and nonhuman primates. *Fertil Steril* **99**, 1523–33.

Telfer EE, McLaughlin M, Ding C and Thong KJ (2008). A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod* **23**, 1151–8.

Tseng HS, Wang YF, Tzeng YM, Chen DR, Liao YF, Chiu HY and Hsieh WT (2017). Aloe-emodin enhances tamoxifen cytotoxicity by suppressing Ras/ERK and PI3K/mTOR in breast cancer cells. *Am J Chin Med* **45**, 337–50.

Verma RP and Hansch C (2007). Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. *Bioorg Med Chem* **15**, 2223–68.

Woodruff TK and Shea LD (2007). The role of the extracellular matrix in ovarian follicle development. *Reprod Sci* **14** Supplement, 6–10.

Xing W, Guo W, Zou CH, Fu TT, Li XY, Zhu M, Qi JH, Song J, Dong CH, Li Z, Xiao Y, Yuan PS, Huang H and Xu X (2015). Acemannan accelerates cell proliferation and skin wound healing through AKT/mTOR signaling pathway. *J Dermatol Sci* **79**, 101–9.

Zha X, Wu G, Zhao X, Zhou L, Zhang H, Li J, Ma L and Zhang Y (2015). PRDX6 protects ARPE-19 Cells from Oxidative Damage via PI3K/AKT signaling. *Cell Physiol Biochem* **36**, 2217–28.

Zhou L, Xie Y, Li S, Liang Y, Qiu Q, Lin H and Zhang Q (2017). Rapamycin prevents cyclophosphamide-induced over-activation of primordial follicle pool through PI3K/Akt/mTOR signaling pathway *in vivo*. *J Ovarian Res* **10**, 56.

https://doi.org/10.1017/S0967199421000824 Published online by Cambridge University Press