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Eugenol influences the expression of messenger RNAs for superoxide dismutase and glutathione peroxidase 1 in bovine secondary follicles cultured *in vitro*

E.M. Vasconcelos, F.C. Costa, A.V.N. Azevedo, P.A.A. Barroso, E.I.T. de Assis, L.R.F.M. Paulino, B.R. Silva, A.W.B. Silva, A.L.P. Souza and J.R.V. Silva

Postgraduate Programme in Biotechnology, Laboratory of Biotechnology and Physiology of Reproduction (LABIREP), Federal University of Ceará, Av. Comandante Mauricélio Rocha Ponte 100, CEP 62041-040, Sobral, CE, Brazil

Research Article

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Author for correspondence:
J. R. V. Silva. Biotechnology Nucleus of Sobral – NUBIS, Federal University of Ceará, Av. Comandante Maurocélio Rocha Ponte 100, CEP 62041-040, Sobral, CE, Brazil.
Tel:/Fax: +55 88 36118000.
E-mail jrvsilva@ufc.br

Summary

This study aimed to investigate the effects of eugenol on growth, viability, antrum formation and mRNA expression of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase 1 (GPX1) and peroxiredoxin 6 (PRDX6) in bovine secondary follicles cultured *in vitro*. To this end, bovine ovaries were collected from a local slaughterhouse and in the laboratory the follicles were isolated from the ovarian cortex. The follicles were then cultured in TCM-199^{+} alone or supplemented with different concentrations of eugenol (0.5, 5.0 and 50.0 μM). Follicular diameters and antrum formation were evaluated on days 0, 6, 12 and 18. Viability analysis was performed using calcein and ethidium homodimer. Real-time PCR was used to quantify mRNA levels for *SOD*, *CAT*, *GPX1* and *PRDX6* in cultured follicles. Follicular diameters and mRNA levels in follicles cultured *in vitro* were compared using analysis of variance and Kruskal–Wallis tests, while follicular survival and antrum formation were compared using the chi-squared test ($P < 0.05$). The results showed that secondary follicles cultured with eugenol maintained similar morphology and viability to follicles cultured in the control group. A progressive increase in follicular diameter was observed between days 0 and 12 for all treatments, except for follicles cultured with 50 μM eugenol. Eugenol (5.0 and 50.0 μM) increased mRNA levels for *GPX1* in cultured follicles, but 0.5 μM eugenol reduced mRNA levels for *SOD*. The addition of eugenol did not influence mRNA expression for *CAT* and *PRDX6*. In conclusion, eugenol supplementation reduces mRNA levels for SOD and increases mRNA levels of *GPX1* in bovine secondary follicles cultured *in vitro*.

Introduction

In mammals, preantral follicles represent approximately 90% of the ovarian follicular population, but most of them (around 99.0%) became atretic during their growth and maturation (Hsueh *et al*., 2015). Therefore, isolation and *in vitro* culture of these follicles open up a new possibility of maximizing the use of oocytes enclosed in preantral follicles and, consequently, have gained great scientific effect (Figueiredo *et al*., 2019). It is important to note that, in bovine species, primordial follicles are activated and begin to grow at least 100 days before ovulation (Britt, 1991), this means that, *in vitro*, these follicles need to be cultured for long periods. However, one of the main obstacles during *in vitro* culture of preantral follicles is the excessive production of reactive oxygen species (ROS), caused by factors such as manipulation, absence of physiological protection mechanisms and increased oxygen levels in the cellular environment (Sá *et al*., 2018). Such factors cause a redox imbalance, generating oxidative stress, which in turn compromises the quality of cultured follicles (Sies, 2015; Saeed-Zidane *et al*., 2017).

During *in vitro* culture, follicles are exposed to supraphysiological concentrations of oxygen (up to 20%) that can trigger oxidative stress and consequently reduce the ability of follicles to develop *in vitro* (Sá *et al*., 2018). Oxidative stress during *in vitro* culture can cause severe cellular damage such as lipid peroxidation, protein denaturation, alterations in nucleic acids and membrane disruption (Halliwell, 2014). Kashka *et al.* (2016) reported that lipid peroxidation of membranes, mitochondrial lesions and impaired cell integrity resulted in the low production of competent oocytes during *in vitro* culture. Additionally, lesions caused by ROS promote the inactivation of antioxidant enzymes, such as glutathione peroxidase 1 (GPX1), catalase (CAT), superoxide dismutase (SOD) and peroxiredoxins (PRDX) that act physiologically to balance cellular homeostasis (Sovernigo *et al*., 2017). To minimize the damage caused by oxidative stress during *in vitro* culture, substances with antioxidant activity were added to the culture medium (Barberino *et al*., 2015).

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Eugenol (4-allyl-2-methoxyphenol) contains phenolic compounds in its composition that are responsible for protecting cells and tissues against damage caused by free radicals (Nagababu *et al.*, 2010). These authors reported that, *in vitro*, eugenol inhibits lipid peroxidation in liver cell mitochondria, while, *in vivo*, eugenol reduces cell necrosis in rat liver with induced hepatotoxicity. The phenol group found in eugenol is the aromatic phenylpropanoid that is present in several groups of plants including cloves (*Syzygium aromaticum*, Myrtaceae) and basil (*Ocimum basilicum*, Lamiaceae) (Alves *et al.*, 2017). Magalhães *et al.* (2018) showed that eugenol reduced lipopolysaccharide-induced protein oxidation *in vivo*, through both anti-inflammatory and anti-oxidative effects, avoiding damage to mice lung structure. Eugenol also demonstrated antioxidant activity on human cell lines cultured *in vitro* (Slamenová *et al.*, 2009). In addition, the ability of eugenol to inhibit ROS and lipid peroxidation was five times greater than that of α-tocopherol (Nagababu and Lakshmaiah, 1992; Nagababu and Lakshmaiah, 1994). Despite these studies, it is not known if eugenol influences mRNA expression of antioxidant enzymes such as *GPX1, CAT, SOD* and *PRDX6* and if it affects bovine preantral follicles growth and viability during *in vitro* culture.

The objectives of this study were to evaluate the effects of different concentrations of eugenol (0.5, 5.0 and 50.0 µM) on viability, growth, antrum formation and on the levels of mRNA for *SOD, CAT, PRDX6* and *GPX1* of bovine secondary follicles cultured *in vitro* for 18 days.

Materials and methods

Chemicals

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

Ovarian collection

Ovaries of multiparous cows ($n = 50$) were collected from a local slaughterhouse in Sobral, Ceará, Brazil. Immediately after removal, ovaries were washed in 70% ethanol for approximately 10 s, followed by two further washes in TCM-199 supplemented with penicillin (100 mg/ml), streptomycin (100 mg/ml) and HEPES. Then, the ovaries in TCM-199 at 4°C were transported within 1 h to a laboratory.

Follicle isolation and in vitro culture

In the laboratory, the ovarian cortex was fragmented (1–2 mm) with a sterile scalpel blade and the fragments were placed in HEPES-buffered TCM-199 supplemented with penicillin (100 mg/ml) and streptomycin (100 mg/ml). Secondary follicles measuring 150–250 µm in diameter were manually isolated with the aid of 26-gauge needles and using a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan). After isolation, follicles containing visible oocytes surrounded by granulosa cells and intact basement membrane, and without the presence of antral cavity, were selected for culture. Then, follicles were cultured individually in 100-µl droplets of culture medium in Petri dishes (60 × 15 mm; Corning, USA). The control medium, identified as TCM-199$^+$, consisted of TCM-199 (pH 7.2–7.4) supplemented with 3.0 mg/ml bovine serum albumin (BSA), 100 IU penicillin/streptomycin, 10 µg/ml insulin, 5.5 µg/ml transferrin and 5 ng/ml selenium (ITS), 50 µg/ml ascorbic, 100 ng/ml FSH, 2 mM glutamine and 2 mM hypoxanthine. The follicles were distributed randomly and cultured in TCM-199$^+$ alone or supplemented with 0.5, 5.0 or 50.0 µM of eugenol. The eugenol concentrations used were based on the study of Lopes *et al.* (2018). In each treatment, approximately 60 follicles were cultured. Follicles culture was performed at 38.5°C with 5% CO_2 in air for 18 days. Every 2 days of culture, 60 µl of medium was replaced with fresh medium. The percentage of follicles considered morphologically normal was evaluated on days 0, 6, 12 and 18 of culture. Follicles containing an opaque and/or extruded oocyte and having opaque granulosa cells were considered degenerated. For follicular diameter evaluation, two perpendicular measurements were performed in normal follicles using an inverted microscope with NIS-Elements 2.4 software (Nikon, Nikon Instruments Inc., Japan). To calculate daily growth rate, the difference in follicle diameter between days 0 and 18 was calculated and divided by 18 (days of culture).

Assessment of preantral follicles viability using fluorescence microscopy

After 18 days of culture, follicles ($n = 20$/treatment) were incubated in 100-µl droplets of TCM-199 supplemented with 4 mM calcein-AM and 2 mM ethidium homodimer-1 (Molecular Probes, Invitrogen, Karlsruhe, Germany) at 37°C for a period of 15 min. The follicles were then washed three times in TCM-199 and then examined under a fluorescence microscope (Nikon, Eclipse, TS 100, Japan). Oocytes and granulosa cells were considered viable if the cytoplasm was positively stained with calcein-AM (green) and non-viable if the chromatin was positively labelled with ethidium homodimer (red) (Van Den Hurk *et al.*, 1998).

Expression of mRNA for SOD, CAT, PRDX6 and GPX1 in cultured follicles

Normal follicles that had been cultured for 18 days in each treatment were collected and then stored at −80°C until extraction of total RNA for further analysis of the levels of mRNA for *SOD*, *CAT*, *PRDX6* and *GPX1*. Total RNA extraction was performed 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. The primers were designed to perform amplification of *SOD*, *CAT*, *PRDX6*, *GPX1* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Table 1). GAPDH was used as the reference gene. The specificity of each primer pair was confirmed using 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 (Livak and Schmittgen, 2001).

Statistical analysis

Data on follicular diameters were submitted to statistical analysis using the Kolmogorov–Smirnov and Bartlett test to assess normal distribution and homoscedasticity, respectively. These data were then evaluated using ANOVA. For comparisons of follicular diameters and levels of mRNA between treatments, the Kruskal–Wallis

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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 ATGGCGCGTGGACAGTGGTCATAA	S AS	GI: 402744670
PRDX6	GCACCTCCTCTTACTTCCCG GATGCGGCCGATGGTAGTAT	S AS	GI: 59858298
GPX1	AACGTAGCATCGCTCTGAGG GATGCCCAAACTGGTTGCAG	S AS	GI: 156602645
SOD	GTGAACAACCTCAACGTCGC GGGTTCTCCACCACCGTTAG	S AS	GI: 31341527
CAT	AAGTTCTGCATCGCCACTCA GGGGCCCTACTGTCAGACTA	S AS	GI: 402693375

Table 2. Diameters and daily growth (mean ± standard error of the mean (SEM)) of bovine secondary follicles after 0, 6, 12 and 18 days of *in vitro* culture in TCM-199^{+} alone or supplemented with 0.5, 5.0 or 50.0 μM of eugenol (EUG)

	Day 0	Day 6	Day 12	Day 18	Daily growth
TCM-199^{+}	221.1 ± 5.5^{a}	260.0 ± 6.7^{b}	275.1 ± 8.4^{c}	278.2 ± 11.0^{c}	4.4 ± 3.5
EUG 0.5 μM	220.7 ± 6.3^{a}	273.7 ± 8.7^{b}	300.1 ± 10.7^{c}	307.1 ± 12.0^{c}	4.8 ± 4.1
EUG 5.0 μM	223.3 ± 5.5^{a}	269.2 ± 6.5^{b}	282.5 ± 9.6^{c}	290.6 ± 11.8^{c}	4.8 ± 3.8
EUG 50.0 μM	226.1 ± 5.1^{a}	300.4 ± 28.8^{b}	281.4 ± 8.4^{b}	284.5 ± 10.1^{b}	4.5 ± 2.9

Significantly different ($P < 0.05$).
^{a,b,c}Lowercase letters represent statistically significant differences between days of culture (between columns).

Table 3. Percentages of morphologically normal secondary follicles and of antrum formation after 18 days of *in vitro* culture in TCM-199^{+} alone or supplemented with 0.5, 5.0 or 50.0 μM of eugenol (EUG)

%	TCM-199^{+}	EUG 0.5 μM	EUG 5 μM	EUG 50 μM
Morphologically normal follicles	83.8% (52/62)^{a,b}	80.8% (55/68)^{b}	89.3% (67/75)^{a,b}	93.9% (62/66)^{a}
Antrum formation	23.1% (12/52)	23.6% (13/55)	23.9% (16/67)	27.4% (17/62)

Significantly different ($P < 0.05$).
^{a,b}Lowercase letters represent statistically significant differences between treatments (between columns).

test (GraphPad Prism software, version 5.0) was used. Percentages of follicles with different daily growth rate, follicular survival and antrum formation after *in vitro* culture within each treatment were compared using the chi-square test. Differences were considered significant when $P < 0.05$.

Results

Effects of eugenol on follicular growth, antrum formation and viability

A significant and progressive increase in follicular diameter was observed from day 0 up to day 12 of culture in all treatments (Table 2). The only exception was secondary follicles cultured in medium supplemented with 50 μM eugenol, which had significant growth between days 0 and 6, but not after 6, 12 and 18 days. When comparisons were made between treatments, no significant difference was observed. No effect of eugenol on follicular daily growth rate was observed (Table 2).

Table 3 shows the percentage of normal follicles and antrum formation after 18 days of culture in control medium alone or supplemented with different concentrations of eugenol. The great majority of the follicles remained viable after culture in all treatments, and the percentage of normal follicles after culture in medium supplemented with 50 μM eugenol was higher than that seen in follicles cultured with 0.5 μM eugenol ($P < 0.05$). However, no significant differences were observed among the percentage of normal follicles after culture in control medium alone or supplemented with eugenol in the different concentrations. No differences were also observed in the percentage of antrum formation after follicle culture in the different treatments.

Effects of eugenol on follicular viability

Fluorescence microscopy analysis showed that, after 18 days of culture in control medium alone or supplemented with 0.5, 5.0 and 50.0 μM eugenol, all follicles previously considered morphologically normal by light microscopy were viable, as they stained positively for calcein-AM, but not for ethidium homodimer (Fig. 1). Conversely, some stromal cells organized around the follicles cultured in all treatments were positively stained with ethidium homodimer.

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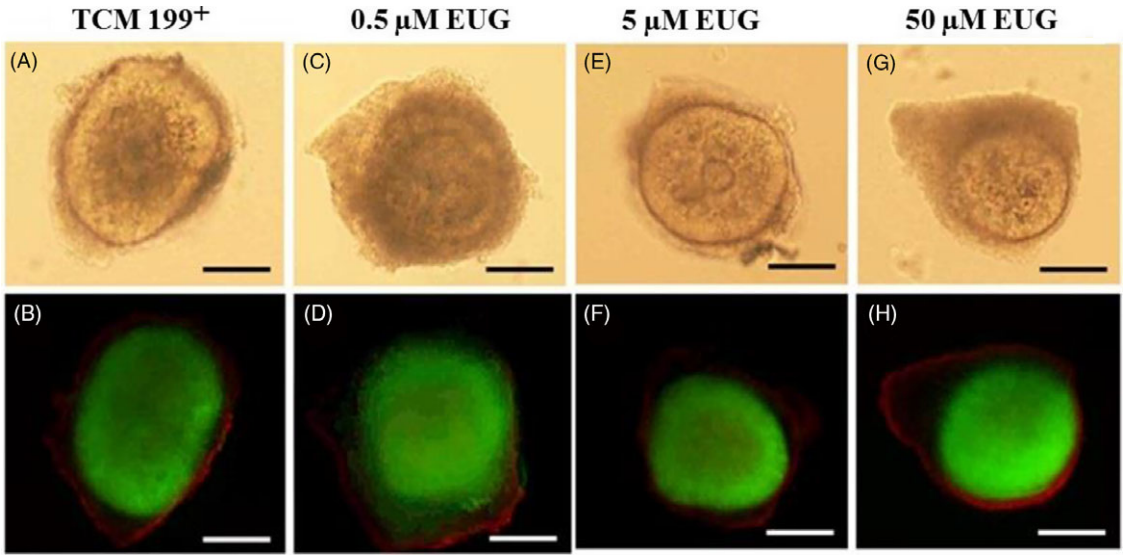

Figure 1. Viability of bovine secondary follicles cultured for 18 days after staining with calcein-AM (green) and ethidium homodimer-1 (red). Secondary follicles were cultured in TCM-199^{+} alone (A, B) or supplemented with 0.5 (C, D), 5.0 (E, F) or 50.0 (G, H) µM of eugenol (EUG). Scale bars represent 100 µm.

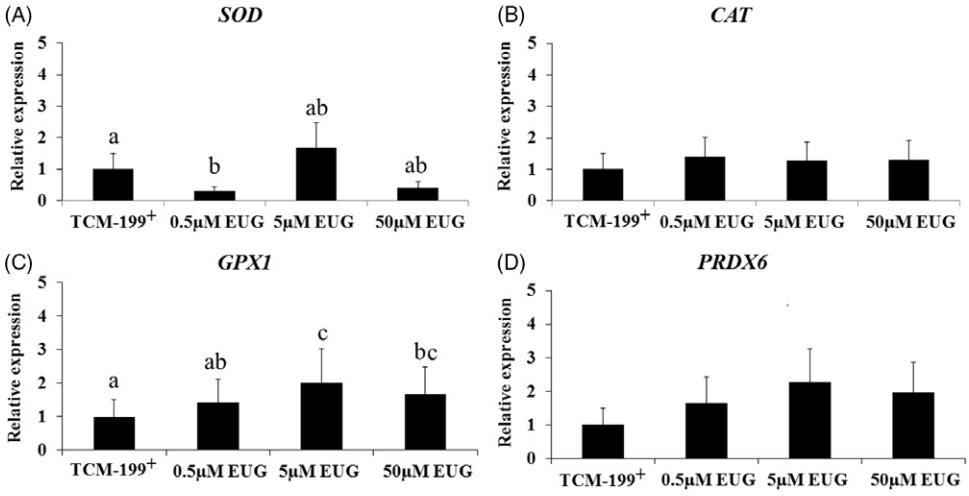

Figure 2. Levels of mRNA (means ± standard deviation (SD)) for (A) *SOD*, (B) *CAT*, (C) *GPX1* or *PRDX6* (D) in secondary follicles cultured *in vitro* for 18 days in TCM 199^{+} alone or supplemented with 0.5, 5.0 or 50.0 µM of eugenol (EUG). ^{a,b,c}Lowercase letters represent statistically significant differences between treatments ($P < 0.05$).

Expression of mRNA for SOD, CAT, PRDX6 and GPX1 in cultured follicles

Figure 2 shows the levels of mRNA for *SOD, CAT, PRDX6* and *GPX1* in bovine secondary follicle after 18 days of culture in TCM-199^{+} alone or supplemented with eugenol. The presence of 5.0 and 50.0 µM eugenol in culture medium significantly increased the levels of mRNA for *GPX1* in secondary follicles when compared with those cultured in control medium ($P < 0.05$). However, secondary follicles cultured in the presence of 0.5 µM eugenol had reduced levels of mRNA for *SOD*, when compared with control medium. Conversely, the presence of eugenol in culture medium did not influence the expression of mRNA for *CAT* and *PRDX6* in cultured follicles.

Discussion

This study shows that eugenol retains follicle viability and morphology, but it reduces follicle growth when used at high concentrations (50 µM). It has been reported previously that addition of 50 µM eugenol reduced the growth of human keratinocytes, but when nanoencapsulated it decreased its possible cytotoxic effect on this cell type and improved its anti-inflammatory effect (Lopes *et al.*, 2018). These authors showed that a controlled release device for this substance in the culture medium increased the positive action of eugenol. These results are in accordance with those obtained during culture of bovine secondary follicles in medium supplemented with higher concentrations of eugenol.

In bovine ovarian follicles cultured *in vitro*, we showed for the first time that 0.5 and 5.0 µM eugenol influences, respectively, the expression of mRNAs for *SOD* and *GPX1* in bovine secondary follicles cultured *in vitro*. Changes in the levels of antioxidant enzymes might be very important in the regulation of the response to oxidative stress (Salah *et al.*, 2019). SOD catalyzes the dismutation of the extremely reactive superoxide anion to H_2O_2, which can further be separated into water and oxygen by CAT or GPX. Reduction in the levels of mRNA for SOD in bovine secondary follicles cultured in the presence of 0.5 µM eugenol can be associated with diminished intracellular levels of reactive superoxide anion *in vitro*. Salah *et al.* (2019) reported that eugenol reduced ROS

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and O_2^- production in intestinal cell lines (HCT116) cultured *in vitro* and, consequently, these cells had reduced activities of SOD and CAT. It has been reported that phenolic antioxidants including eugenol have the capacity to enhance antioxidant enzyme activities (Tiku *et al.*, 2004; Zhao *et al.*, 2006). *In vivo*, eugenol also reduced peroxidation in mice liver and reduced oxidative stress (Tiku *et al.*, 2004). Reduction of lipid peroxidation in the oocyte is very important as the products of this process, such as malondialdehyde (MDA) and 4-hydroxynonenal (4HNE), have traditionally been viewed as toxic substances (Gallelli *et al.*, 2018). Eugenol possesses antioxidant properties due to its phenolic hydroxyl group that donates electrons to quench free radicals (Fouad and Yacoubi, 2011).

This study showed that 5 and 50 μM eugenol increased the mRNA levels of *GPX1* in bovine follicles cultured *in vitro*. Glutathione is the main nonenzymatic cellular defence mechanism against ROS, and GPX1 catalyzes the oxidation of glutathione into hydroperoxide, playing a role in repairing damage caused by lipid peroxidation (Kurutas, 2016). High expression of GPX1 in bovine granulosa cells is associated with follicle survival, as well as estradiol and progesterone secretion, as GPX1 is significantly upregulated in healthy follicles compared with small, healthy or atretic follicles (Ceko *et al.*, 2015). The absence of a positive effect of eugenol on the survival of bovine secondary follicles cultured *in vitro* could be due to differences in the cell types cultured (isolated granulosa cells versus secondary follicles) and stage of development, as granulosa cells were isolated from antral follicles. A previous study has shown that preantral follicles cultured in the presence of sodium selenite increases GPX activities and decreases ROS levels, therefore improving the development rate of mouse follicles *in vitro* (Abedelahi *et al.*, 2010). These authors also reported that low levels of GPX activity in cultured preantral follicles and high concentrations of ROS may be the major factors responsible for follicular regression *in vitro*. It is important to highlight that, during *in vitro* culture, cells are exposed to high concentrations of oxygen (up to 20%; Sá *et al.*, 2018). Salavati *et al.* (2012) showed that oocytes cultured in high oxygen concentrations showed increased GPX1 expression in response to high oxygen tension.

Expression of mRNA for *CAT* and *PRDX6* was not influenced by eugenol in *in vitro* cultured bovine secondary follicles. CAT has an important role during follicle development as it neutralizes H_2O_2 and maintains the balance in the production of ROS, and influences the production of steroidal hormones (Wang *et al.*, 2017). During meiotic maturation of mouse oocytes, it has been observed that catalase protects the genome from oxidative damage (Park *et al.*, 2016). CAT acts in the oocyte nucleus to avoid chromosomal defects, and inhibition of this enzyme is associated with chromosomal misalignment and DNA damage (Park *et al.*, 2016). PRDX6 is an enzyme with antioxidant activity that is widely present in the intracellular environment to promote the protection of cells against damage caused by exposure to high levels of oxygen (Wang *et al.*, 2006). Leyens *et al.* (2004) reported that *PRDX6* transcripts are stored in the oocyte for later use in early embryos. PRDX6 peroxide reductase activity controls the levels of hydrogen peroxides in cumulus–oocyte complexes and early embryos (Harvey *et al.*, 2002). PRDX6 can also use the glutathione accumulated both in the oocyte and cumulus cells during maturation to reduce its oxidized form during the peroxidation process (de Matos *et al.*, 1997). In addition to its peroxidase activity, PRDX6 expresses acidic calcium-independent phospholipase A2 and lysophosphatidylcholine acyl transferase activities in separate catalytic sites (Arevalo and Vázquez-Medina, 2018). Phospholipase A2 activity in cumulus cells is linked to the production of arachidonic acid for the production of prostaglandins, involved in the oocyte maturation process (Elvin *et al.*, 2000; Calder *et al.*, 2001). Fernandez *et al.* (2019) also reported that PRDX6 promoted the survival of human sperm *in vitro* through the activation of the PI3K-AKT pathway. Absence of changes in the levels of *CAT* and *PRDX6* can be associated with maintenance of the equilibrium between antioxidants and pro-oxidants during follicular culture.

In conclusion, eugenol reduces the levels of mRNAs for *SOD* and increases those of *GPX1* in bovine secondary follicles cultured *in vitro* for 18 days. Eugenol also kept follicle viability and morphology, but it reduced follicle growth when used at high concentrations (50 μM). This study showed the potential of eugenol to control oxidative stress during culture of bovine preantral follicles *in vitro*, as it increased the expression of mRNA for *GPX1,* an enzyme that catalyzes the oxidation of glutathione, and plays an important role in repairing damage caused by lipid peroxidation.

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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. This study was registered approved (Number: 03/2019) by the Committee of Ethics and Animal Welfare of the Federal University of Ceará.

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