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Oocyte *in vitro* maturation with eugenol improves the medium antioxidant capacity and total cell number per blastocyst

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ABSTRACT

This study investigates the impact of eugenol (EU) supplementation on bovine oocyte *in vitro* maturation (IVM) and antioxidant capacity, as well as *in vitro* embryo production and quality after conventional *in vitro* fertilization (IVF). A total of 1077 cumulus oocyte complexes were cultured in TCM-199^{+} without EU supplementation (control treatment) or supplemented with EU at the concentrations of 10 μM (EU-10), 20 μM (EU-20), or 40 μM (EU-40). After IVM, the oocytes were subjected to IVF and embryo culture. The addition of EU at 40 μM to the IVM medium improved ($P < 0.05$) the antioxidant capacity and cleavage rate when compared to the control treatment. Moreover, a positive correlation ($r = 0.61$, $P < 0.03$) was observed between cleavage rate and EU concentration. The addition of EU at concentrations of 10 and 20 μM decreased ($P < 0.05$) the calreticulin (CALR) levels in expanded blastocysts when compared to the control treatment and EU-40 treatment. However, the EU-10 and EU-20 treatments had a greater ($P < 0.05$) mean total cell number (TCN) per expanded blastocyst when compared to the control treatment and EU-40 treatment. In conclusion, the addition of EU to the enriched culture medium during IVM of bovine oocytes improved the antioxidant capacity of the spent medium, as well as the cleavage rate and embryonic quality (i.e., TCN/expanded blastocyst), and reduced the endoplasmic reticulum stress (i.e., CALR levels) in the embryos. Thus, we recommend enriching the IVM medium with 10 μM EU for *in vitro* bovine embryo production.

1. Introduction

In recent decades, bovine *in vitro* embryo production (IVEP) has been used to optimize the use of gametes from genetically superior animals [1]. Despite all the research, IVEP efficiency in bovine species remained steady, with only 30–40% of the oocytes reaching the blastocyst stage when compared to an 80% rate of *in vivo*-produced embryos [2]. The negative outcomes of *in vitro*-produced embryos may be partially caused by variations in many exogenous factors like temperature, osmotic stress, shear stress, chemical exposure, and accumulation of reactive oxygen species (ROS) [3,4]. All these factors are believed to have implications on oxidative stress in the endoplasmic reticulum (ER; [5,6]) and reduction of embryo development potential through alterations in gene expression, epigenetic mechanisms, and metabolism [7].

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During oocyte *in vitro* maturation (IVM) and embryo development, various enzymes and metabolic pathways produce endogenous ROS [8]. The accumulation of ROS in cells leads to membrane lipids peroxidation, blockade of RNA transcription, damage of DNA, and decrease in the synthesis of proteins such as calreticulin (CALR). The CALR protein is a promiscuous chaperone of the ER with a high calcium-binding capacity and low affinity; moreover, the CALR protein is crucial for Ca^{2+} sequestering to the ER and different cellular processes, including signal transduction, gene expression, and protein trafficking [9–13]. Then, alteration in the CALR production may compromise oocyte maturation and development of *in vitro*-produced embryos [11,14].

The search for new potential antioxidants, such as eugenol (EU), represents a promising strategy to improve oocyte IVM conditions and embryo production and quality. EU (4-allyl-2-metoxyphenol) is a natural phenolic compound found in essential oils of some aromatic plants, such as *Myristica fragrans*, *Ocimum gratissimum*, and *Syzygium aromaticum* [15–17]. Compared to other antioxidants such as anethole [18] and ascorbic acid [19], EU is more chemically stable and has longer-term antioxidant activity during *in vitro* culture with direct and indirect effects against ROS and oxidative stress [18]. These EU characteristics are attributed to the presence of a hydroxyl group (-OH) on its aromatic ring [18,19]. In a recent study in which parthenogenetic activated bovine oocytes were used, EU at 83–120 μM concentration improved oxidative status, oocyte nuclear maturation rate, and *in vitro* embryo production [15]. In murine macrophages, 15 μg/mL EU was capable of reducing oxidative stress and increasing the activities of antioxidant enzymes [16]. In murine mesenchymal stem cell culture, EU at 10–12.5 μg/mL improved cell viability and the expression of genes related to cell survival and proliferation [20]. Moreover, in mononuclear human cell culture, EU (10, 20, and 40 μM) reduced inflammatory cytokines and oxidative stress markers [21].

Although a recent study reported the effect of EU addition during bovine oocyte maturation, it used only parthenogenetic activated oocytes [15]. The originality of the present study is based on the fact that we investigated the effect of the EU addition during IVM and its subsequent impact on IVEP, after conventional IVF rather than parthenogenetic activation. This aspect is extremely important considering the future commercial use of *in vitro*-produced embryos through IVF procedures. Moreover, we also investigated, for the first time, the impact of EU on ER stress of the *in vitro*-produced embryos. Therefore, the objective of the present study was to evaluate the antioxidant capacity, oocyte maturation, IVEP, and quality and ER stress of bovine embryos produced after supplementation of the IVM medium with different concentrations of EU.

2. Materials and methods

Unless indicated, the chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The study was approved by the Ethics Committee Board for the use and care of animals from the State University of Ceará (#05498214/2019). Procedures for oocyte recovery, maturation, and fertilization, as well as embryo culture, followed previously reported protocols [22], with slight modification. Ovaries (n = 300) were collected in a local slaughterhouse and transported to the laboratory. Cumulus-oocyte complexes (COCs) from antral follicles (4–8 mm in diameter) were aspirated, selected, and subjected to IVM.

2.1. In vitro maturation (IVM), expansion of cumulus cells, and nuclear maturation evaluation

The medium used for IVM was TCM-199 (Ref. 11150059; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 25 mg/mL sodium bicarbonate, 22 mg/mL sodium pyruvate, 50 mg/mL amikacin, 5 μg/mL FSH, 50 μg/mL LH, and 0.1 μg/mL estradiol. This medium was then named TCM-199^{+}. A total of 1077 COCs were shared in 4 treatments and cultured in TCM-199^{+} alone (control treatment) or supplemented with EU (Ref. E51791) at 10 μM (EU-10), 20 μM (EU-20), or 40 μM (EU-40). The EU used in the IVM medium of the present study was initially diluted in ethanol (1:20) to generate an EU stock solution (50 mg/mL). The stock solution was diluted in ultrapure water to make the following aliquots 100x concentrated: 1000 μM, 2000 μM, and 4000 μM. Therefore, 10 μL EU concentrated aliquots were diluted in each 1 mL of IVM medium to produce the final EU concentrations of 10, 20, and 40 μM, which contained 0.0031, 0.0062, and 0.0124% ethanol, respectively. Regarding our experimental design, similar studies [15,18,20,21] have not used ethanol as a positive control treatment, since ethanol at very low concentrations (e.g., ≤0.1%) had neutral effects on oocyte IVM and IVEP in cattle [23]. The EU concentrations used in the present study were based on a previous report that tested the effect of eugenol on human mononuclear cells [21], because at the time of the experimental planning and execution of the present study, the work of Oliveira et al. [15] had not been published. For the IVM process, COCs (n = 20/well/treatment) were incubated in 500 μL of maturation medium in five well dishes under mineral oil at 38.5°C in pre-mixed gas (6% CO_2 + 5% O_2 + 89% N_2) for 22–24 h. The experiment was repeated six times. After the IVM period, the degree of cumulus expansion from COCs (n = 193) was assessed under a stereomicroscope and classified as not expanded, partially expanded (the outer layer of cells was loosened), or fully expanded (all cumulus cells were loosened; [24]). Moreover, COCs (n = 368) were denuded for the evaluation of the percentage of first polar body (1 PB) extrusion in the perivitelline space under a stereomicroscope [25], and then, the oocytes were fixed and stained with Hoechst 33342 (10 mg/mL) for 15 min and further visualized with a fluorescent microscope. Only oocytes presenting 1 PB associated with the nucleus at the metaphase II stage (metaphase plate; MII) were considered matured according to Santos et al. [26].

2.2. Evaluation of antioxidant capacity by ABTS and DPPH free radicals' assays

Antioxidant activity was measured in 96-well flat-bottom plates using BIOTEK Elisa reader (model ELX 800, software "Gen5 V2.04.11"), according to the methodology described by Becker et al. [27], with some modifications. For the ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) method, $ABTS^{+}$ solution (7 mM, 5 mL) was mixed with 88 μL of potassium persulfate (140 mM). The mixture was stirred and kept in the dark at room temperature for 16 h. Then, 1 mL of this solution was added to 99 mL of ethanol. In 96-well plates, 300 μL of ABTS solution and 3 μL of the culture medium (sample) were used per well. For the DPPH (2,2-diphenyl-1-picrylhydrazyl) analysis, 180 μL of DPPH methanolic solution and 20 μL of culture medium were added to each well.

For both assays (ABTS and DPPH), the dilutions of samples and positive standards used in the quantitative microplate evaluations started from a stock solution with a concentration of 20.000 to 0.78 μg/mL. The absorbances for the ABTS and DPPH assays were measured at 630 nm and 490 nm for a total of 10 min and 60 min of incubation, respectively. As a negative standard, all solutions (i.e., ABTS and DPPH diluted with its own solvent, as well as non-cultured IVM medium containing ABTS and DPPH solutions) were used. Finally, the antioxidant activity (AA) was expressed as the percentage of inhibition determined by the equation AA% = AC–AS/

AC x 100, where AA (%) stands for the percentage of antioxidant activity, AC stands for the absorbance of the negative control, and AS stands for the absorbance of the sample. The mean inhibitory concentration (IC50; μg/mL) was obtained using calibration curves, collected by plotting the different concentrations relative to AA%, and further analyzed by linear regression.

2.3. In vitro fertilization (IVF), embryo culture, and evaluation

After IVM, the COCs were placed in drops of 90 μL of TALP fertilization medium supplemented with 10 μg/mL heparin, 22 mg/mL sodium pyruvate, 50 mg/mL amikacin, 6 mg/mL bovine serum albumin (BSA; fatty acid-free), 2 μM penicillamine, 1 μM hypotaurine, and 0.25 μM epinephrine. The drops were inseminated with Percoll-purified sperm (1×10^6 sperm cells) and incubated in Petri dishes under mineral oil for 18–22 h at 38.5°C in 5% CO_2.

After conventional IVF, the presumptive zygotes were cultured in 500 μL drops of Charles Rosenkrans (CR2) modified medium supplemented with 50 mg/mL amikacin, 0.1 mM amino acids, 2.5% FBS, and 6 mg/mL BSA in five well dishes under mineral oil at 38.5°C under 6% CO_2 + 5% O_2 + 89% N_2. The cleavage rate was evaluated on day 3 of culture, and the blastocyst development kinetics was assessed and classified as: initial blastocyst (iB), blastocyst (B), expanded blastocyst (eB), and hatching or hatched blastocyst (hB) [28] after 7 and 10 days of culture. After that, the blastocyst (days 7 and 10) and hatching (day 10) rates were calculated. After 7 days of culture, only expanded blastocysts were fixed, and the total cell number (TCN) was quantified [8].

2.4. Calreticulin visualization in expanded blastocysts

After 7 days of culture, expanded blastocysts were fixed in 4% paraformaldehyde (PAF) for up to 1 h at room temperature. Embryos were further transferred to a fixation solution consisting of Phosphate-Buffered Saline (PBS) supplemented with 0.1% BSA and 0.1% Tween20 for 8–12 h at 4°C. Briefly, the embryos were transferred to a blocking solution composed of PBS plus 0.5% BSA, 0.2% sodium azide, 1% milk powder, 10% goat serum, 1% donkey serum, 0.1 M glycine, and 0.1% Triton X for 1 h under agitation and protection against light. Subsequently, these were incubated for 2 h at room temperature with CALR primary antibody (1:100; ab2907 - Abcam) under agitation in the dark as previously stated. After the incubation period, the embryos were submitted to three rinses in blocking solution for 5 min each and further subjected to an additional 2 h incubation with the labeled secondary IgG antibody (AlexaFluor® 488; 1:200; ab150077 - Abcam). Finally, the embryos were washed once more in blocking solution as described above, placed in 60-well slides (μ-Slide Angiogenesis IbiTreat, Ibidi GmbH, Germany) with DAPI (ab104139 - Abcam), and examined by confocal laser scanning microscopy (20 x magnification; Zeiss LSM 700META, Weimer, Germany) and fluorescence intensities were quantified by Image J software. The fluorescence intensities were quantified through analysis and measurement of color images in the Image J using the function 'Stack to RGB (red, green, blue)' to obtain particularly the average of the blue and green colors corresponding to the levels of DAPI and CALR, respectively. The negative control was set up by omitting the primary antibody. All evaluations were performed by a single operator.

2.5. Statistical analysis

Statistical analyses were performed using Sigma Plot (version 11.0; Systat Software Inc., USA). The data normality and homogeneity of variance were previously evaluated using Shapiro-Wilk's and Levene's tests, respectively. The proportion variables were analyzed among treatments by chi-square or Fisher exact tests. One-way ANOVA followed by Fisher's post-hoc test was used to analyze the mean total number of blastomeres in the blastocysts. The association between EU concentration and cleavage rates was evaluated by the Pearson correlation test. Data are presented as percentage and mean ± standard error of the mean (SEM). Statistical significance was defined as $P < 0.05$ (two-sided).

3. Results

3.1. Effects of eugenol on cumulus cells' expansion and nuclear maturation

Representative images of oocytes with expanded cumulus cells (Fig. 1A) and MII plate associated with extrusion of the 1 PB (Fig. 1B) are shown after IVM. Regardless of treatment, all oocytes resumed meiosis, and the percentages of cumulus cells' expansion and nuclear maturation (1 PB) after IVM were similar ($P > 0.05$) among all treatments (Table 1).

3.2. Eugenol antioxidant capacity evaluated by the reduction of ABTS and DPPH free radicals

The EU antioxidant capacity was evaluated considering the reduction of free radical levels (ABTS and DPPH) in the spent medium after 24 h of oocyte IVM. Compared to the control treatments, all EU treatments (regardless of the EU concentration, i.e., 10, 20, and 40 μM) produced a greater antioxidant capacity, based on the lower ($P < 0.05$) levels of ABTS (Fig. 2A) and DPPH (Fig. 2B) radicals detected. The overall antioxidant capacity was proportional to the increase of EU concentrations in the medium ($P < 0.05$).

3.3. Effect of eugenol on the development and quality of in vitro-produced embryos

Representative images are shown for morphologically normal blastocysts (Fig. 1C) after *in vitro* embryo culture and blastocyst embryonic cells were stained with Hoechst 33342 (Fig. 1D). The addition of EU at 40 μM to the IVM medium improved ($P < 0.05$) the cleavage rate of the embryos when compared to the control treatment (Table 2). A positive correlation ($r = 0.61$, $P < 0.03$) was observed between the cleavage rate and EU concentration (Fig. 3A). The blastocyst rates on days 7 and 10 of culture and the hatching rates on day 10 (Table 2), as well as the rates of the different blastocyst stages, were similar among treatments (Fig. 3B; $P > 0.05$). However, the EU-10 and EU-20 treatments had greater ($P < 0.05$) mean TCN/expanded blastocyst when compared to the control and EU-40 treatments.

3.4. Effect of eugenol on calreticulin levels of in vitro expanded blastocysts

Representative images of expanded blastocysts after 7 days of culture are shown in the negative control, and CALR stained control treatment (TCM-199$^+$) and EU treatments (Fig. 4A–E). The EU-10 and EU-20 treatments decreased ($P < 0.05$) the CALR levels in expanded blastocysts when compared to the control and EU-40 treatments (Fig. 4F). Moreover, the EU-20 treatment had the lowest level ($P < 0.05$) of CALR from all treatments and differed ($P < 0.05$) as well from the EU-10 treatment.

4. Discussion

The present study demonstrated, for the first time, an effective EU antioxidant capacity at different concentrations in the IVM

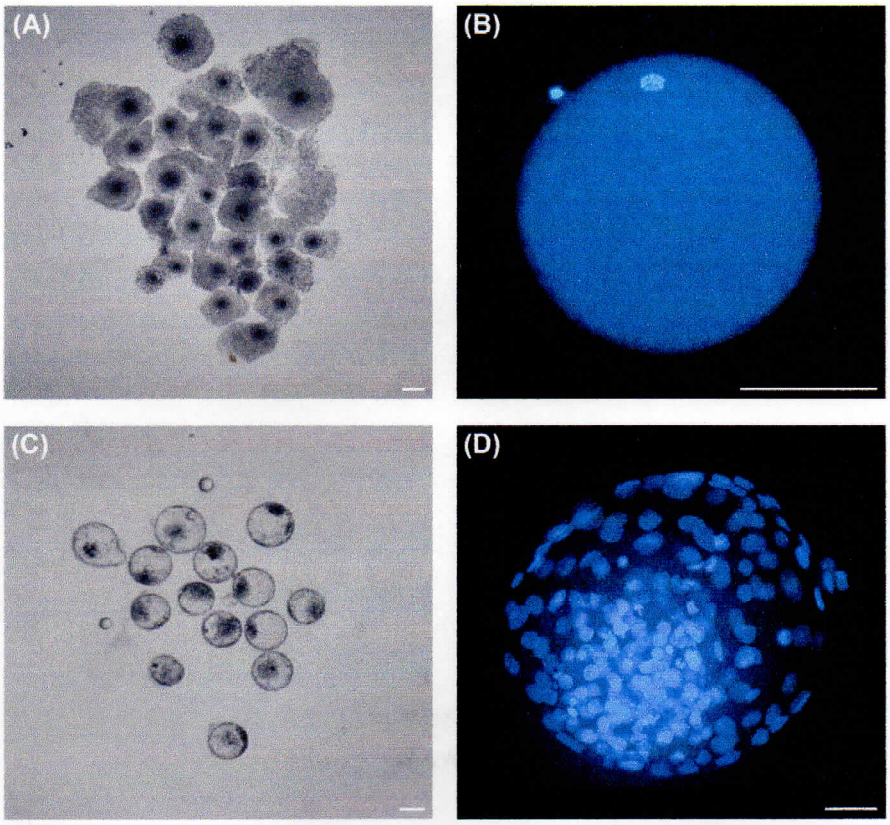

Fig. 1. Representative images obtained from (A) bovine oocytes with cumulus cells expanded after 22–24 h of IVM; (B) metaphase II oocyte, with the extrusion of the first polar body, after staining with Hoechst 33342; (C) blastocysts on day 7; and (D) expanded blastocyst stained with Hoechst 33342. All images were obtained from the EU-10 treatment. Scale bar = 100 μm (A and C) and 50 μm (B and D).

medium and showed that EU improved the development (cleavage rate) and quality (TCN and CALR production [ER stress levels] per expanded blastocyst) of bovine embryos produced by IVF using frozen semen.

Among numerous factors that may have contributed to low outcomes of IVM, the production of free radicals within the oocytes [15] and culture medium [29] lead to oxidative stress that affects IVM. For this reason, in the present study, the EU antioxidant capacity was evaluated, considering the levels of reduction of free radicals' reagents (i.e., ABTS and DPPH) in the spent medium after IVM, as a predictive marker of oocyte competence. Herein, the EU antioxidant activity increased with the increasing concentration of this compound in the tested samples. This finding agrees with recent reports that described the antioxidant activity of EU and its esters after analyzing the levels of ABTS and DPPH in the essential oil of an aromatic plant [30] and spent medium after *in vitro* follicle culture [18]. EU has a hydroxyl group (-OH) associated with an aromatic ring, which leads to high antioxidant activity over a longer period, when compared to the antioxidant capacity of other antioxidants, such as anethole [18] and ascorbic acid [19]. Concerning this aspect, the EU free radical scavenging activity can lead to the formation of phenolic radicals. These radicals are stable due to the resonance caused by the charge transfer and are not able to separate hydrogen from lipid or protein molecules, decreasing, therefore, the oxidation process [18,30]. In eucaryotic cells, EU is highly lipophilic, rapidly absorbed, and able to penetrate biological membranes, targeting intracellular organelles, such as mitochondria. In the mitochondria, the EU inhibits nicotinamide adenine

Table 1
Percentages of cumulus cells' expansion degrees and nuclear maturation in bovine oocytes *in vitro* matured in the different treatments.

Oocyte maturation				
	Degree of cumulus cells' expansion			Nuclear maturation
Treatments[†]	% Not expanded (n)	% Partially expanded (n)	% Fully expanded (n)	% 1 PB (n)
Control (n = 136)	0.0 (0/48)	4.2 (2/48)	95.8 (46/48)	81.8 (72/88)
EU-10 (n = 138)	2.0 (1/48)	12.5 (6/48)	85.5 (41/48)	73.3 (66/90)
EU-20 (n = 143)	2.0 (1/48)	10.4 (5/48)	87.5 (42/48)	80.0 (76/95)
EU-40 (n = 144)	0.0 (0/49)	4.0 (2/49)	96.0 (47/49)	75.7 (72/95)

No difference (P > 0.05) was observed among treatments. 1 PB, first polar body.
[†] TCM-199[+] alone (control treatment) or supplemented with eugenol at the concentrations of 10 μM (EU-10), 20 μM (EU-20), or 40 μM (EU-40). The total number of observations per treatment includes the number of COCs evaluated for cumulus cell expansion plus the number of COCs evaluated for nuclear maturation. Three replicates containing 21–32 oocytes in each replicate were performed.

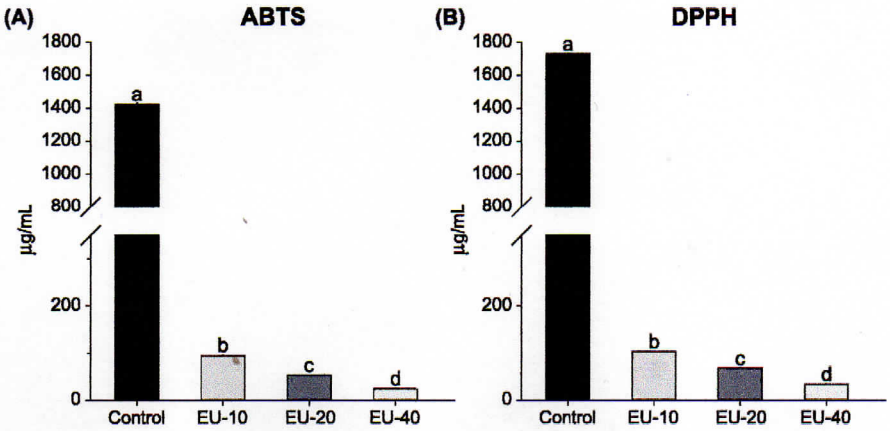

Fig. 2. Mean (±SEM) evaluation of antioxidant capacity by (A) ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) and (B) DPPH (2,2-diphenyl-1-picrylhydrazyl) free radicals' assays. The spent IVM medium without eugenol (control treatment) or supplemented with different concentrations of eugenol (10, 20, or 40 µM) were compared. Three samples from each treatment were analyzed in three replicates. [a–d] Values with different superscripts differ among treatments ($P < 0.05$).

Table 2
Embryo development and quality after *in vitro* maturation of bovine oocytes in the different treatments.

Embryo development and quality					
	% Cleavage (n)	% Blastocysts (n)		Quality of blastocysts	
Treatments[†]	Day 3	Day 7	Day 10	TCN/Expanded blastocyst on Day 7 (n)	% Hatched on Day 10 (n)
Control (n = 130)	64.6 (84)[a]	44.6 (58)[a]	46.1 (60)[a]	137.6 ± 4.6 (33)[a]	89.6 (26/29)[a]
EU-10 (n = 132)	69.7 (92)[ab]	38.6 (51)[a]	40.1 (53)[a]	164.4 ± 4.4 (29)[b]	80.0 (16/20)[a]
EU-20 (n = 129)	72.8 (94)[ab]	40.3 (52)[a]	42.6 (55)[a]	156.1 ± 3.7 (34)[b]	90.4 (19/21)[a]
EU-40 (n = 125)	79.2 (99)[b]	41.6 (52)[a]	44.8 (56)[a]	136.1 ± 5.1 (29)[a]	80.7 (21/26)[a]

[a,b] Different letters differ within the same column ($P < 0.05$). TCN, total cell number.
[†] TCM-199[+] alone (control treatment) or supplemented with eugenol at the concentrations of 10 µM (EU-10), 20 µM (EU-20), or 40 µM (EU-40). Three replicates containing 40–46 oocytes in each replicate were performed.

dinucleotide (NADH) oxidation and the reduction of adenosine triphosphate (ATP) levels [31]. Moreover, cell viability has been enhanced with the use of EU by inhibiting cell damage mediated by ROS and reactive nitrogen species (RNS; [21]). Furthermore, EU has been reported to have a potent modulating effect on intracellular concentrations of endogenous antioxidant substances, such as the reduced glutathione (GSH) and peroxidase enzymes (GPx), superoxide dismutase (SOD), and catalase (CAT; [16,21,32]).

Regarding oocyte IVM in cattle, the results of antioxidant supplementation on IVM medium are controversial. For instance, some studies in bovine have shown a positive effect of resveratrol [33], quercetin [34], melatonin [35], and anethole [8], while in other studies, the same antioxidants (resveratrol [36], quercetin [37], melatonin [22] and anethole [29]) had no effect on the IVM rates. These discrepant antioxidant effects on bovine oocyte IVM might have been due to different *in vitro* culture conditions among studies. In the present study, the addition of EU to the IVM medium did not affect the percentage of mature oocytes. Taken together, the results of this study and the above-described reports [22,29,36,37] suggest that the intracellular antioxidant content does not affect the oocyte nuclear maturation. However, it has been suggested that antioxidants are likely to improve oocyte cytoplasmic maturation and further embryonic development through mRNA storage and translation into proteins, such as enzymes with antioxidant activity [29]. Indeed, in the present study, EU supplementation during IVM increased the cleavage rate (EU-40 treatment) and the TCN/expanded blastocyst (EU-10 and EU-20 treatments). In this regard, low EU concentrations (10–12.5 µg/mL) increased cell viability and

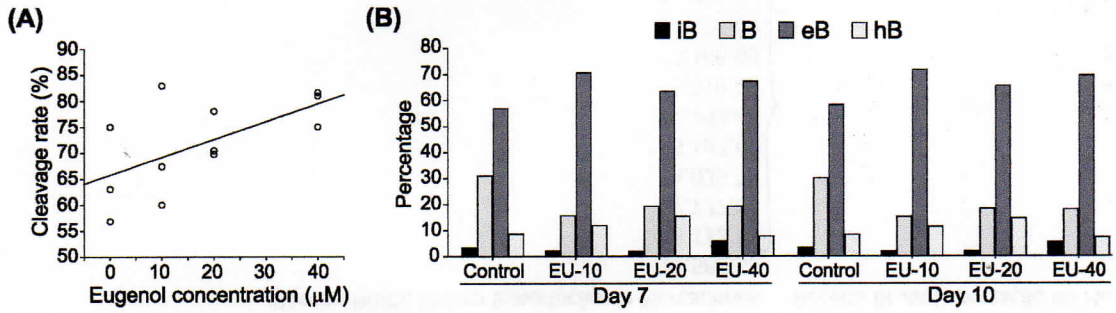

Fig. 3. (A) Positive correlation ($r = 0.61$; $P < 0.03$) observed between oocyte cleavage rate and eugenol concentration. Each dot represents a replicate within a treatment. (B) Percentage of blastocyst stages on day 7 and 10 post-IVF: initial blastocyst (iB), blastocyst (B), expanded blastocyst (eB), and hatching or hatched blastocyst (hB) within the control and eugenol treatments. No difference ($P > 0.05$) was observed among treatments.

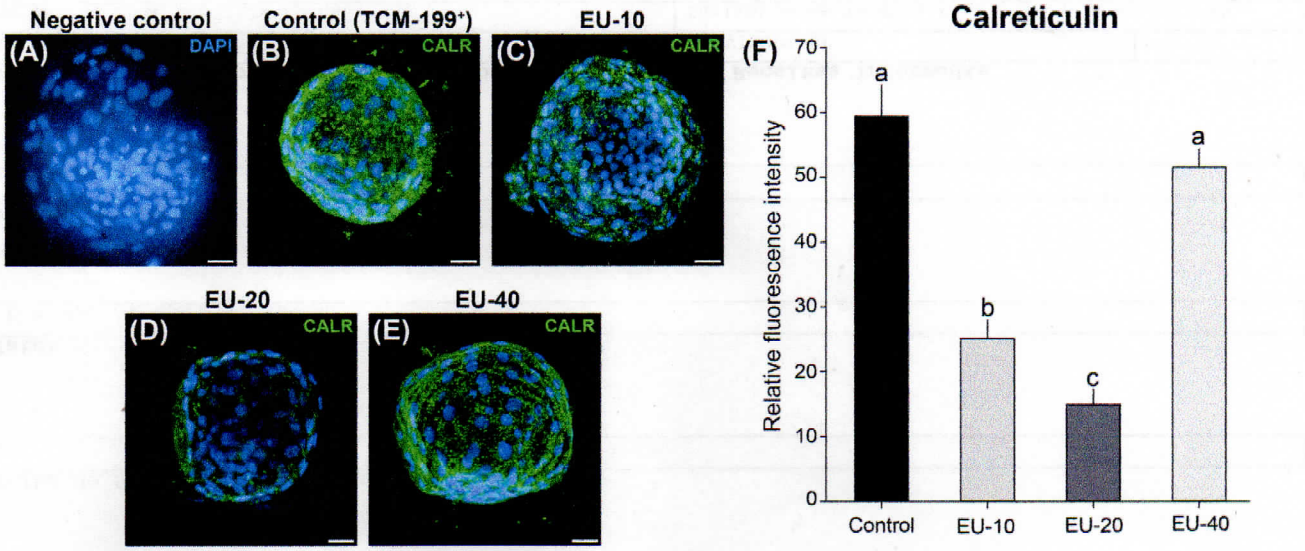

Fig. 4. Mean (±SEM) immunofluorescence signals (A–E) and (F) levels of calreticulin (CALR) in expanded blastocysts after 7 days of culture in the (A) negative control with DAPI only, (B) control treatment (cultured medium), and (C–E) eugenol treatments. Twenty embryos from each treatment were analyzed in three replicates. $^{a-c}$ Values with different superscripts differ among treatments ($P < 0.05$). Scale bar = 50 μm.

expression of genes related to cell survival and proliferation after *in vitro* culture of murine mesenchymal stem cells [17]. Moreover, in the present study, EU treatments produced a similar TCN/expanded blastocyst (range, 136.1 ± 5.1 to 164.4 ± 4.4 cells) when compared to *in vivo*-produced bovine embryos (122.5 ± 21.6 cells; [38]).

In the present study, although the use of EU at the highest concentration (40 μM) was able to increase the cleavage rate and the antioxidant capacity, at this concentration, EU did not improve embryo quality. A previous study has shown that EU is a competitive inhibitor of 17-β-estradiol α and β receptors [39]. Thus, we suggest that EU at the highest concentration (EU-40) during IVM may have reduced the efficiency of estradiol in improving oocyte maturation rate and embryo quality [40,41]. The addition of eugenol at 40 μM decreased the TNF-α and IL-6 cytokines levels during the *in vitro* culture of human mononuclear cells [21]. Previous studies showed that TNF-α promoted bovine oocyte growth and cytoplasmic maturation [42], while IL-6 improved embryonic quality in cattle [43]. Thus, in the present study, we suggest that EU at a 40 μM concentration may have reduced the TNF-α [42] and IL-6 [43] production during IVM, which could explain the lower TCN/expanded blastocyst (embryonic quality) observed in this treatment when compared to the other tested EU concentrations.

In the present study, CALR levels (ER stress) were higher in control and EU-40 treatments, which was associated with lower TCN/blastocysts (lower embryo quality). The ER helps to facilitate the proper folding and transport of proteins to other subcellular structures. During stress conditions, the chaperone proteins such as CALR hamper the binding of newly synthesized proteins and generate dysfunctional compounds. However, under heat shock and hypoxic and oxidative stress, the ER activates a complex set of signaling pathways known as the unfolded protein response (UPR), which includes increased production of chaperone molecules [44,45]. Previous reports have shown that EU at low concentrations promoted antioxidant and anti-inflammatory activities in preantral follicles, oocytes, and embryos [15,32,46]; however, at higher concentrations, EU was not beneficial to follicular development and embryonic quality [15,32,47]. These findings corroborate the results of our study, as the EU at the highest concentration (EU-40) did not improve embryonic quality.

In conclusion, the addition of EU to an enriched culture medium during IVM of bovine oocytes improved the antioxidant capacity of the spent medium (all EU treatments), enhanced the cleavage rate (EU-40) and embryonic quality (TCN/expanded blastocyst; EU-10 and EU-20), and decreased the CALR (ER stress) levels (EU-10 and EU-20) in expanded blastocysts. Thus, considering the comparable efficiency of 10 and 20 μM EU concentrations, we recommend enriching the IVM medium with 10 μM EU for bovine IVEP. Finally, the use of EU as a supplement represents a promising strategy to improve the quality of *in vitro*-produced embryos in cattle and potentially in other mammalian species.

Declaration of competing interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this work.

CRediT authorship contribution statement

A.F.B. Silva: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **L.F. Lima:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **A.N.P. Morais:** Methodology, Investigation. **L.L. Lienou:** Methodology, Investigation, Writing – original draft. **Y.F. Watanabe:** Methodology, Investigation. **D.C. Joaquim:** Methodology, Investigation. **S.M. Morais:** Methodology, Investigation. **D.R. Alves:** Methodology, Investigation. **A.F. Pereira:** Methodology, Investigation. **A.C. Santos:** Methodology, Investigation. **B.G. Alves:** Formal analysis. **D.M.M. Padilha:** Methodology, Investigation, Writing – original draft. **E.L. Gastal:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Visualization. **J.R. Figueiredo:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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