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*Original article*

# THE SYNERGISTIC ROLE OF CYSTEAMINE AND *CITRUS SINENSIS* PEEL ESSENTIAL OIL ON THE *IN VITRO* MATURATION OF PORCINE OOCYTE

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**Summary**

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The combined use of *Citrus sinensis* essential oil (EOCS) and cysteamine (CYS) can improve oxidative changes in porcine oocytes, boosting *in vitro* maturation (IVM) rates. This study evaluated the synergistic effect of EOCS and CYS on oocyte IVM using different EOCS concentrations: (i) 100 µM CYS (control), (ii) 10 µg/mL EOCS (10EOCS + CYS), (iii) 30 µg/mL EOCS (30EOCS + CYS), and (iv) 50 µg/mL EOCS (50EOCS + CYS). The optimal EOCS concentration was then compared to its synergistic or substitutive effect with CYS: (i) CYS (CYS group), (ii) the best EOCS concentration with CYS (EOCS + CYS), and (iii) the best EOCS concentration (EOCS group). The 50EOCS + CYS group performed better than the control with higher *cumulus* cell viability (92.4±0.6% vs. 75.1±4.9%), improved mitochondrial pattern (85.7±11.1% vs. 63.0±3.03%), and lower reactive oxygen species (ROS) levels (0.62±0.17 vs. 1.00±0.44). In the second experiment, while *cumulus* cell expansion, nuclear maturation, and ROS levels showed no differences, 50EOCS (92.4±0.6%) achieved better *cumulus* cell viability. It was concluded that 50EOCS did not exhibit a synergistic effect with CYS but was a viable alternative for porcine IVM.

**Key words**: essential oil, natural antioxidant, oocyte maturation, redox potential, synergism

## INTRODUCTION

Pigs make up about 40% of global meat consumption, reaching roughly 115.5 million tons in 2024, emphasising the importance of pork in the worldwide diet (Kim *et al.,* 2024; USDA, 2024). One way to boost productivity is through the rapid selection of animals using *in vitro* embryo production (IVEP), as this method can enhance productivity by increasing selection intensity and enabling the choice of

animals with high genetic value as gamete and embryo donors (Wu & Bazer, 2019; Currin *et al.,* 2022).

Although used as a reproductive biotechnique in pigs, IVEP has variable efficiency (Oller *et al.,* 2021), mainly due to differences in nuclear and cytoplasmic oocyte maturation rates (Gil *et al.,* 2010), *in vitro* fertilisation (Abeydeera, 2000), and inconsistent results with polyspermy (Suzuki *et al.,* 2003), leading to low embryonic development (Nguyen *et al.,* 2021). One way to improve IVEP rates is to optimise *in vitro* oocyte maturation (IVM) conditions (Zhao *et al.,* 2020b; Cánovas *et al.,* 2024). Abeydeera (2002) observed that many porcine oocytes did not reach proper *in vitro* nuclear and cytoplasmic maturation compared to *in vivo*. Using an appropriate culture medium can enhance these processes (Zhao *et al.,* 2020a; Chen *et al.,* 2021a).

In general, *in vitro* culture conditions can influence the excessive production of reactive oxygen species (ROS) in pig IVM (Xiang *et al.,* 2021). Antioxidants are a promising alternative to mitigate these effects (Oh *et al.,* 2023). Substances derived from natural sources, such as essential oils, have been added to IVM media to neutralise ROS levels, protect cells from oxidative stress, and enhance the efficiency of IVEP (Santos *et al.,* 2018). Recently, the essential oil from *Citrus sinensis* (EOCS), extracted from orange peels, has gained biotechnological interest due to its simple and sustainable extraction method, which uses industrial waste and converts it into a high-value product (Suri *et al.,* 2022; Lamine *et al.,* 2024). Moreover, oil bioactive compounds provide anti-inflammatory (Geraci *et al.,* 2017), antifungal (Sharma & Tripathi, 2008), and antioxidant (Farahmandfar *et al.,* 2020) benefits. Ferronatto & Rossi (2018) demonstrated that as an antioxidant, EOCS blocked free radicals by up to 81.4% in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays and 94.6% using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method.

Moreover, EOCS in cell cultures reduced superoxide radical generation and late apoptosis in HaCaT cell lines stimulated with $H_2O_2$ (Toscano-Garibay *et al.,* 2017). Tarhouni *et al.* (2024) observed that EOCS decreased ROS levels, preventing oxidative damage to testicular tissue and maintaining sperm quality in rats. Additionally, Aquino *et al.* (2023) evaluated the antioxidant effects of 10, 30, and 50 µg/mL EOCS on bovine oocytes, noting that at certain concentrations, it improved oocyte viability, promoted *cumulus* cell expansion, and reduced oxidative stress in a bovine model. The 10 µg/mL EOCS concentration notably increased oocyte maturation rates and embryo quality compared to the antioxidant cysteamine.

These results support the potential use of EOCS as a promising tool in IVEP, demonstrating antioxidant properties that aid gamete development during IVM. Unlike bovine oocytes, pig oocytes have a higher lipid content (McEvoy *et al.,* 2000; Mateo-Otero *et al.,* 2021), making them more vulnerable to oxidative reactions, which impact their viability and quality (Sun *et al.,* 2022a). Previous studies have shown that combined antioxidant supplementation promotes increased nuclear maturation of oocytes and enhances embryonic development (Li *et al.,* 2016; Silva & Silva, 2023). Using EOCS combined with CYS during IVM of porcine models could significantly boost meiotic efficiency and improve embryo quality *in vitro*, ultimately optimising pig production. Therefore, this study assessed the syner-

gistic effects of CYS and EOCS on porcine oocyte IVM.

## MATERIALS AND METHODS

All experiments were conducted in accordance with the Animal Ethics Committee of the Federal Rural University of Semi-Arid (No. 23091.002360/2016-17).

### *Chemicals and media*

Unless otherwise noted, all chemicals and reagents were acquired from Sigma Chemical Co. (St. Louis, MO, USA). The medium used for oocyte collection and manipulation (MCM) consisted of TCM-199 (Gibco-BRL, Carlsbad, CA, USA) supplemented with 0.3 mM sodium pyruvate, 5 µg/mL myo-inositol, 10% foetal bovine serum, and 1% antibiotic-antimycotic solution. For IVM, the medium included TCM-199 with 0.3 mM sodium pyruvate, 5 µg/mL myo-inositol, 10% follicular fluid, 5 ng/mL epidermal growth factor (EGF), 1% antibiotic-antimycotic solution, and 20 µg/mL FSH-LH (Pluset®, Hertape-Calier, Barcelona, Spain).

### *Experimental design*

This study was divided into two steps (Fig. 1). The first experiment aimed to select the best concentration to replace cysteamine (CYS) among the EOCS concentrations. Then, oocytes underwent IVM under the following groups: (i) 100 µM CYS (control group), (ii) 10 µg/mL EOCS (10EOCS + CYS group), (iii) 30

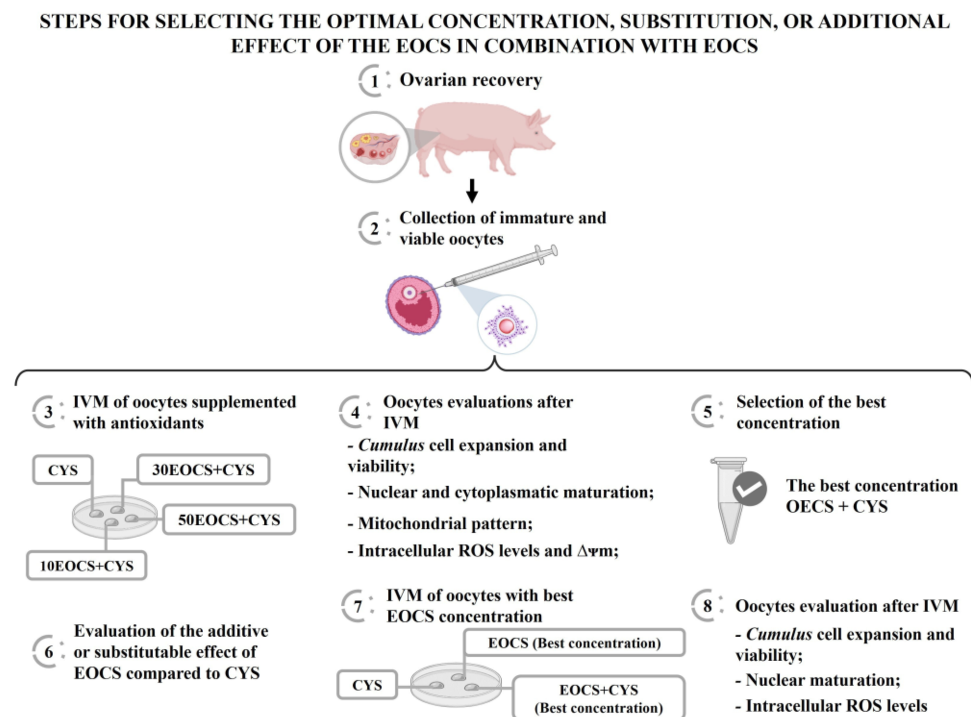


**Fig. 1.** Experimental design for establishing the optimal concentration of EOCS for use in the IVM of porcine oocytes.

µg/mL EOCS (30EOCS + CYS group), and (iv) 50 µg/mL EOCS (50EOCS + CYS group). The concentrations used were based on Aquino *et al.* (2023), where the authors found beneficial effects in controlling ROS and increasing the rate of expanded blastocysts compared to synthetic antioxidants. Ten replicates were performed to assess *cumulus* cell expansion and viability, the presence of the first polar body (1PB), nuclear and cytoplasmic maturation, ROS levels, and mitochondrial potential (ΔΨm).

The second experiment examined the synergistic or substitutive effects of the optimal concentration of EOCS relative to CYS on porcine oocytes. Accordingly, the following experimental groups were established: (i) 100 µM CYS (CYS group), (ii) the best EOCS concentration combined with CYS (EOCS + CYS group), and (iii) the best EOCS concentration alone (EOCS group). After three replicates, oocytes were evaluated for expansion, *cumulus* cell viability, 1PB, and ROS levels.

*Peel material, essential oil extraction, and chemical analysis*

Sweet and organic oranges were harvested in the Jaguaruana region, CE (4°50’02’’S, 37°46’51’’W), Northeastern Brazil, and their peels were processed as described by Aquino *et al.* (2023). *C. sinensis* peels were kept in a dry environment for seven days and then crushed. EOCS was extracted by hydrodistillation using a Clevenger-type apparatus connected to a 5 L round-bottom flask and a heating blanket (Caveiro *et al.,* 1976). Then, 371 g *of C. sinensis* peels were transferred to a flask with distilled water and heated to 100 °C. After 6 hours of extraction, the essential oil was recovered and dried through percolation with anhydrous $Na_2SO_4$. The samples were then stored at 4–8 °C and protected from light. Finally, the density and oil yield (% m/m) were determined at 0.84 g/mL and 2.9%, respectively.

Gas chromatography coupled with a mass spectrometer (GC/MS) (Shimadzu QP-2010 Plus Instrument, Kyoto, Japan) was used to analyse and identify the chemical constituents of EOCS. The analysis employed the following conditions: SH-Rtx-5 (5% diphenyl and 95% dimethylpolysiloxane) fused silica capillary column (30 m length × 0.25 mm diameter × 0.25 µm film thickness); carrier gas: He (1.78 mL/min, in constant linear velocity mode); injector temperature: 250 °C, in split mode (1:10); detector temperature: 250 °C; column temperature programming: 40–180 °C at 4 °C/min, 180–280 °C at 20 °C/min, followed by 280 °C for 10 minutes; mass spectra: electron impact at 70 eV. The injected sample volume was 1 µL (containing 1.0 mg of oil dissolved in 1000 µL of 99.9% pure dichloromethane).

The chemical constituents were identified by their gas chromatography retention times relative to known compounds. The retention times were expressed as Kovat’s indices (KI) and calculated using linear regression, comparing their mass spectra with published spectra (Geraci *et al.,* 2017). Only two compounds were identified: D-limonene (48.5%) and α-terpineol (40.2%), with 11.3% of components remaining unidentified.

*Recovery of pig oocytes and IVM*

Ovaries were collected at the slaughterhouse (Mossoró, Rio Grande do Norte, Brazil) and then transported to the lab in a heated saline solution (35–37 °C; NaCl, 0.9%) with 0.05 mg/mL penicillin. For follicular aspiration, oocytes were re-

trieved from follicles measuring 3 to 6 mm in diameter using a 21G needle and a 5 mL syringe containing MCM (Santos *et al.,* 2019).

After recovering immature and viable oocytes, structures were classified using a stereomicroscope (SZT-LED, Bel, Piracicaba, Brazil). Only oocytes with more than five *cumulus* cells and homogeneous cytoplasm layers were classified as grade I and subjected to IVM (Zhao *et al.,* 2022). For IVM, oocytes were divided according to the groups described in the experimental design. Twenty to thirty *cumulus*-oocyte complexes (COC) were maintained per drop (100 µL) covered with mineral oil and containing IVM medium, incubated for 44 hours at 38.5 °C with 6.5% $CO_2$ (Casillas *et al.,* 2020).

*Assessment of cumulus cell expansion and viability*

After IVM, COCs were examined with a stereomicroscope (SZT-LED; Bel, Piracicaba, Brazil) to evaluate *cumulus* cell expansion. Structures showing expansion were marked as mature. To assess cell viability, *cumulus* cells were removed through successive pipetting with 0.1% hyaluronidase. The cell suspension was stained with 0.2% trypan blue and observed for counting in a Neubauer chamber, considering viable (colorless) and non-viable cells (stained blue, Santos *et al.,* 2019).

*Assessment of nuclear and cytoplasmic maturation and mitochondrial distribution patterns*

Nuclear maturation was assessed by observing 1PB and identifying the nuclear stage (MII). Denuded oocytes were first examined under a stereomicroscope. Oocytes were deemed mature when 1PB extrusion was visible. To determine the nuclear stage, oocytes were fixed in 4% paraformaldehyde for 15 minutes and then stained with Hoechst 33342 (10 µg/mL) for another 15 minutes. Afterward, the oocytes were washed with phosphate-buffered saline (PBS), transferred onto glass slides, and examined using a fluorescence microscope (Olympus BX51TF, Tokyo, Japan, 370 nm). Oocytes with nuclei in MII were classified as mature, whereas those in other nuclear phases were considered immature (Lv *et al.,* 2010; Casillas *et al.,* 2020).

Additionally, oocytes were assessed for cytoplasmic maturation by labelling with MitoTracker Red® (CMXRos, Invitrogen, Carlsbad, CA, USA) at 500 nM. The oocytes were then incubated in 6.5% $CO_2$ for 30 minutes in the dark at 38.5 °C (Zhu *et al.,* 2024). After incubation, the cells were placed on glass slides for imaging with a fluorescence microscope (Olympus BX51TF, Tokyo, Japan, 560 nm). Oocytes were classified as peripherally distributed (immature), dispersedly distributed (mature), or with transitional mitochondria (between peripherally and dispersedly distributed, partially mature). Finally, mitochondrial organization was categorised into two patterns as described by Zhao *et al.* (2020b): Pattern A, homogeneous, with small granulations scattered throughout the cytoplasm; and Pattern B, heterogeneous clusters with large granulations spread throughout or located in specific cytoplasmic areas. The presence of Pattern B was associated with higher maturation rates.

*Assessment of oxidative status levels and mitochondrial membrane potential*

After IVM, matured oocytes were labelled with various fluorescent probes. The levels of ROS were measured using a 2',7'-dichlorodihydrofluorescein diacetate pro-

be ($H_2DCFDA$; Invitrogen, Carlsbad, CA, USA). Matured and denuded oocytes were washed in PBS and incubated for 30 minutes at 38.5 °C in 6.5% $CO_2$ in 500 µL PBS containing 10 µM $H_2DCFDA$ (Ryu *et al.,* 2023). After incubation, oocytes were rewashed in PBS and mounted on glass slides. Images were taken with a fluorescence microscope at 10× magnification (460 nm). The control group served as a calibrator, and the value for each treatment micrograph was divided by the calibrator's average to determine relative expression levels (arbitrary fluorescence units).

The MitoTracker Red® probe at 500 nM (CMXRos; Invitrogen) was used to analyse ΔΨm, reflecting bioenergetic activity. After IVM, the oocytes were washed in PBS and incubated with the probe in a controlled atmosphere containing 6.5% $CO_2$ for 30 minutes in the dark at 38.5 °C (Romek *et al.,* 2010). Subsequently, the cells were placed on glass slides, and images were captured using specific fluorescence at 560 nm. Image analysis to measure fluorescence intensity (in pixels) was performed as described for ROS quantification.

*Statistical analysis*

All data are expressed as mean ± standard error and analysed using StatView 5.0 (SAS Institute Inc., Cary, NC, USA). The Shapiro-Wilk test was performed to assess normality, and Levene's test was used to evaluate homoscedasticity. ROS, ΔΨm, and GSH analysis data were transformed using the arcsine function and analysed with analysis of variance (ANOVA) followed by Tukey's test. Other data were compared using a chi-square test. Differences were considered significant when $P<0.05$.

## RESULTS

*Effect of EOCS concentration on cumulus cells, nuclear and cytoplasmic maturation, mitochondrial distribution pattern, ROS levels, and ΔΨM*

In the first experiment, 282 ovaries were used to obtain 720 immature and viable oocytes, which were subjected to IVM, averaging 2.5 oocytes per ovary.

The results of cumulus cell expansion and viability are shown in Table 1. Initial-

**Table 1.** Antioxidant potential of different concentrations of EOCS combined with CYS during IVM of porcine oocytes and their impact on *cumulus* cells and nuclear maturation

| Groups | Evaluation of *cumulus* cells | | Assessment of nuclear maturation | |
|---|---|---|---|---|
| | Expansion (%) | Viability (%) | 1PB (%) | MII (%) |
| CYS | $64.4 \pm 5.0^{ab}$ (116/180) | $75.1 \pm 4.9^{c}$ (1862/2479) | $64.2 \pm 4.3^{a}$ (113/176) | $77.8 \pm 2.7^{a}$ (42/54) |
| 10EOCS+CYS | $70.8 \pm 4.4^{a}$ (126/178) | $88.0 \pm 2.6^{b}$ (3985/4528) | $66.5 \pm 2.9^{a}$ (121/182) | $70.2 \pm 2.4^{a}$ (40/57) |
| 30EOCS+CYS | $64.6 \pm 3.7^{ab}$ (128/195) | $87.6 \pm 2.9^{b}$ (2951/3369) | $70.1 \pm 4.5^{a}$ (122/174) | $69.0 \pm 6.3^{a}$ (40/58) |
| 50EOCS+CYS | $57.1 \pm 5.4^{b}$ (117/205) | $90.3 \pm 2.3^{a}$ (2623/2903) | $68.7 \pm 2.1^{a}$ (123/179) | $75.4 \pm 1.6^{a}$ (49/65) |

$^{a,b,c}$; Values with different superscript letters within columns are significantly different ($P<0.05$). 1PB, first polar body, MII, metaphase II.

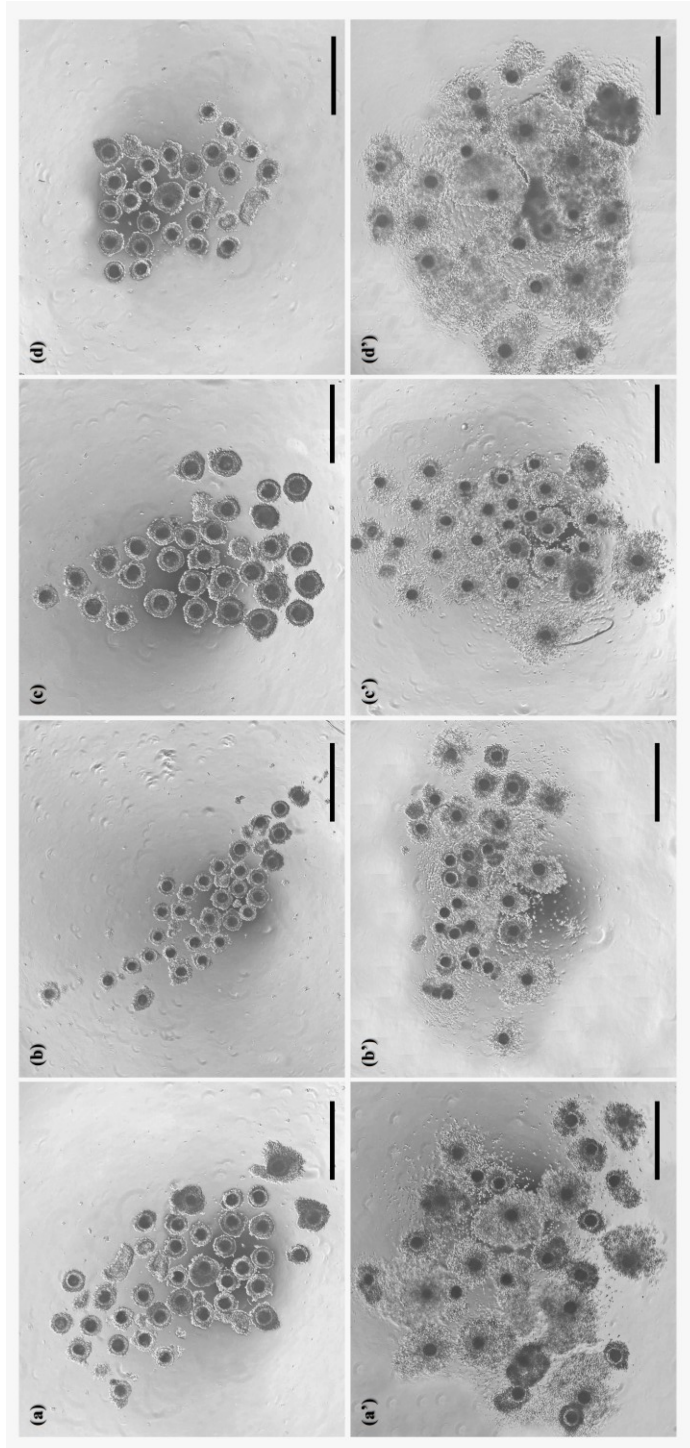


**Fig. 1.** Representation of porcine oocytes before and after *cumulus* cell expansion following 44 hours of IVM under different supplements. (a-a') Control group (100 µM cysteamine; CYS). (b-b') 10 µg/mL EOCS (10EOCS + CYS group). (c-c') 30 µg/mL EOCS (30EOCS + CYS group). (d-d') 50 µg/mL EOCS (50EOCS + CYS group). Scale bar: 50 µm, 4× magnification (a-a', b-b', c-c', d-d').

ly, all groups with *cumulus* cell expansion (Fig. 2A-D) were similar to CYS. Although this was observed, only the 50EOCS + CYS group maintained cell viability above 90% compared to the CYS, 10EOCS + CYS, and 30EOCS + CYS groups (Table 1). Moreover, no differences were found between groups after analysing 1PB extrusion and MII (Table 1 and Fig. 3A-B).

These findings were consistent after analysing cytoplasmic maturation (Fig. 3C-E). Finally, when examining the mitochondrial aggregation patterns of pig oocytes (pattern A or pattern B), the 50EOCS + CYS group outperformed the CYS group. However, it was similar to the 10EOCS + CYS and 30EOCS + CYS groups (Fig. 3F).

After evaluating intracellular ROS levels (Fig. 4A-E), the 50EOCS + CYS group significantly decreased the number of free radicals compared to the CYS group. However, it was similar to the 30EOCS + CYS group and better than the 10EOCS + CYS group. After assessing ΔΨm, no differences were observed among the groups (Fig. 4F-J).

*Synergistic or substitutive effect of EOCS to CYS on cumulus cell expansion and viability, nuclear maturation, and ROS levels*

To determine the presence, absence, and replacement of EOCS on the IVM of pig oocytes, 54 ovaries were used, resulting in the recovery of 141 immature and viable oocytes, averaging 2.6 oocytes per ovary.

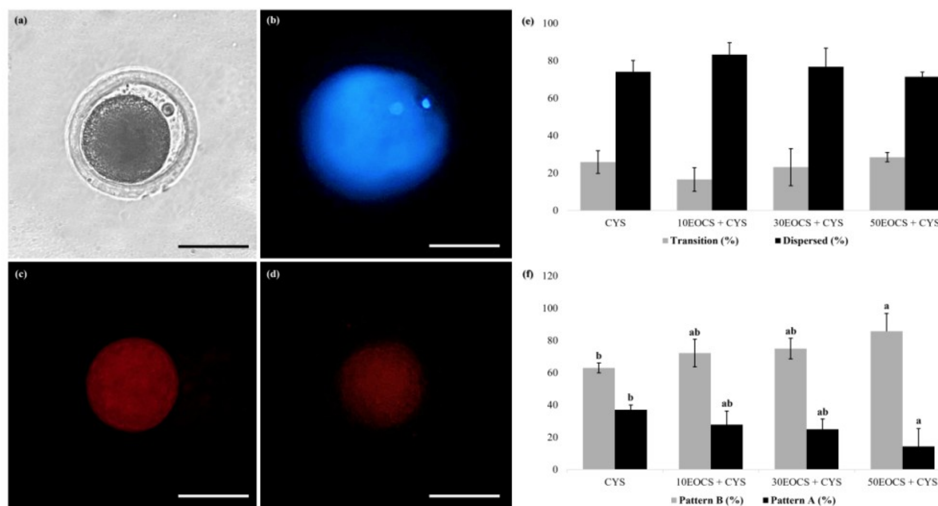


**Fig. 3.** Representation of the stages of nuclear and cytoplasmic maturation of porcine oocytes supplemented with 50EOCS + CYS after 44 hours of IVM. (A) Extrusion of the first polar body (1PB); (B) Porcine oocyte stained with Hoechst 33342; (C) Porcine oocyte with mitochondria dispersed throughout the cytoplasm, exhibiting type B mitochondrial aggregation patterns. (D) Porcine oocytes with mitochondria in transition throughout the cytoplasm, exhibiting type A mitochondrial aggregation patterns. Evaluation of cytoplasmic maturation and mitochondrial aggregation patterns of porcine oocytes after 44 hours of IVM. (E) Cytoplasmic maturation rate. (F) Mitochondrial aggregation pattern rate. Scale bar: 50 µm, 20× magnification (A, B); 100 µm, 40× magnification (C, D). [a,b]; Values with different superscript letters within the columns are significantly different (P<0.05).

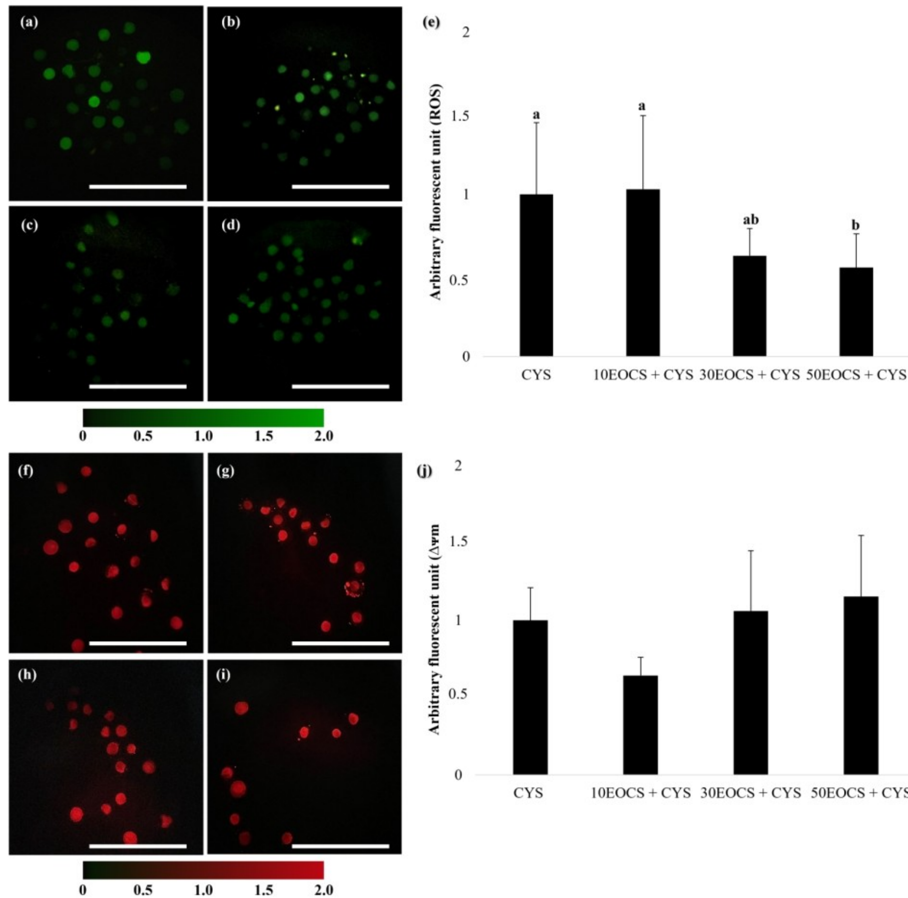


**Fig. 4.** Evaluation of intracellular ROS levels and ΔΨm in antioxidant-supplemented porcine oocytes after 44 h of IVM. (A-D) Representation of porcine oocytes after staining with the fluorescent probe $H_2DCFDA$; (E) Quantification of ROS fluorescence arbitrary units after IVM of porcine oocytes with different supplements. (F-I) Representation of porcine oocytes after staining with the fluorescent probe MitoTracker Red® (CMXRos); (J) Quantification of ΔΨm fluorescence arbitrary units after IVM of porcine oocytes with different supplements. Scale bar: 50 µm, 20× magnification (A, B, C, D, F, G, H, I). $^{a,b}$; Values with different superscript letters within columns are significantly different (P<0.05).

Additionally, the concentration of 50 µg/mL EOCS was optimal compared to 10 and 30 µg/mL, as this concentration maintained parameters related to nuclear maturation and bioenergetic value similar to the control group, while also predicting better characteristics of *cumulus* cells and reducing ROS levels.

Although no difference was observed between the groups after analysing *cumulus* cell expansion (Fig. 5A-C), only the EOCS50 group maintained rates greater than 92% cell viability compared to the CYS and 50EOCS + CYS groups (Fig. 5D). Furthermore, CYS resulted in higher viability of *cumulus* cells than the

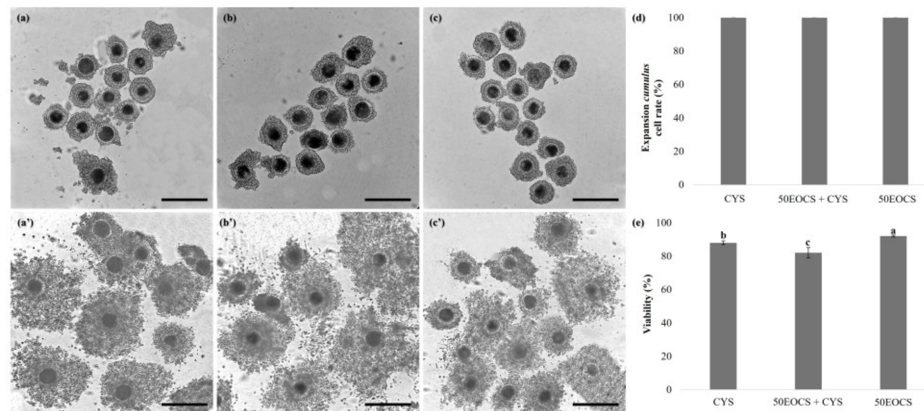


**Fig. 5.** Representation of porcine oocytes after 44 h of IVM supplemented with cysteamine, 50EOCS, or a combination of these antioxidants. (a-a') 100 µM cysteamine (CYS group). (b-b') 50EOCS concentration with CYS (EOCS + CYS group). (c-c') 50EOCS concentration (50EOCS group). (d) Expansion rate of *cumulus* cells. (e) Percentage of *cumulus* cells viability after trypan blue assay. Scale bar: 50 µm, 10× magnification (a-a', b-b', c-c'). [a,b,c]; Values with different superscript letters within the columns are significantly different (P<0.05).

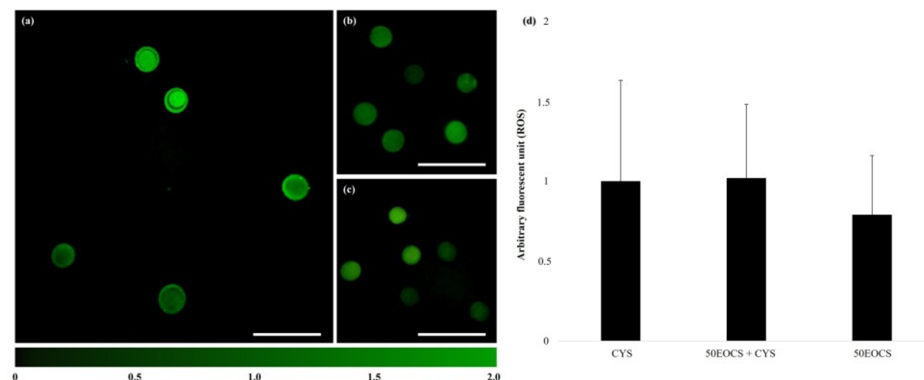


**Fig. 6.** Evaluation of intracellular ROS levels after 44 hours of IVM of porcine oocytes. (A-C) Representation of porcine oocytes stained with the fluorescent probe $H_2DCFDA$ under different supplements; (D) Quantification of ROS fluorescence arbitrary units after IVM of porcine oocytes with different antioxidant supplements. Scale bar: 50 µm, 20× magnification (A, B, C). [a,b]; Values with different superscript letters within the columns are significantly different (P<0.05).

50EOCS + CYS group (Fig. 5E). Despite this, no differences were seen among the groups for 1PB assessment (CYS: 74.4±1.6; EOCS50+CYS: 80.4±0.7; EOCS50: 78.0±1.1). Lastly, in terms of ROS levels (Fig. 6A-C), none of the groups differed significantly (Fig. 6D).

## DISCUSSION

This is the first study to assess the effects of EOCS supplementation during IVM in porcine oocytes, exploring its potential additive and/or substitutive effects on CYS. Different concentrations of EOCS

combined with CYS were tested and it was found that the 50EOCS + CYS group significantly increased *cumulus* cell viability, improved mitochondrial aggregation patterns, and decreased intracellular ROS levels.

It is well known that *cumulus* cell viability and expansion are linked to oocyte quality (Nevoral *et al.,* 2015; Lee *et al.,* 2018). Oocytes with greater *cumulus* cell expansion show higher nuclear and cytoplasmic maturation rates than COCs with incomplete expansion (Jin *et al.,* 2018). In this context, no difference was seen between the treatment groups and the CYS group regarding *cumulus* cell expansion, indicating that the combined use of EOCS and CYS did not negatively affect the *cumulus* cells, yielding results similar to the preferred antioxidant. Moreover, adding EOCS + CYS to the IVM medium increased *cumulus* cell viability compared to the CYS group, with 50EOCS + CYS showing even higher values than the other groups. Similar findings were reported by Aquino *et al.* (2023), where supplementation with EOCS resulted in higher viability rates compared to groups treated only with CYS.

The processes such as chromosomal condensation, meiotic spindle formation, and 1PB extrusion are essential for oocytes to reach nuclear maturation (Chen *et al.,* 2021b; Pei *et al.,* 2023). Although the different concentrations of EOCS combined with CYS did not differ between the treated groups and the CYS group after analysing 1PB and metaphase II, this result shows that the EOCS + CYS group did not negatively affect the progress of nuclear maturation, indicating that EOCS can be used as an additive in the IVM medium without hindering the oocyte's nuclear maturation process.

Cytoplasmic maturation, which is crucial for the developmental quality of oocytes, occurs alongside nuclear maturation (Abeydeera, 2002). This process includes the buildup of messenger RNA and proteins, cytoskeletal modifications, and redistribution of cytoplasmic organelles such as mitochondria (Choi *et al.,* 2021). In this context, our results showed no difference in the rate of cytoplasmic maturation between the groups. However, it was observed that the 50EOCS + CYS group demonstrated a better pattern of mitochondrial aggregation (pattern B). Similarly, studies indicate that using tannins as antioxidants during the *in vitro* maturation (IVM) of porcine oocytes promotes improved mitochondrial distribution, enhances cytoplasmic maturation, and reduces polyspermy during *in vitro* fertilisation (Sun *et al.,* 2022b). This improvement in mitochondrial distribution optimises the oocyte's energy efficiency by positioning mitochondria where they are most needed and facilitates the intracellular transport of vital substances for embryonic development (Yamochi *et al.,* 2016).

After evaluating ROS levels, the 50EOCS + CYS group differed from both the CYS and 10EOCS + CYS groups, though it remained similar to the 30EOCS + CYS group. This difference may be related to the concentration of chemical constituents in EOCS, such as D-limonene and α-terpineol, which have well-documented bioactive properties (Shah & Mehta, 2018; Ferronatto & Rossi, 2018). Additionally, α-terpineol, another component of EOCS, has shown significant antioxidant activity *in vitro* by eliminating hydroxyl and nitric oxide radicals, thereby preventing lipid peroxidation (Chopra *et al.,* 2021). Analysis of $\Delta\Psi m$ is an essential parameter for assessing the impact of EOCS + CYS on the functional integrity

of oocytes. Therefore, the various concentrations of EOCS combined with CYS did not differ from the results of the CYS group, as demonstrated in a bovine model (Aquino *et al.,* 2023). This indicates that EOCS was not toxic to the cells, allowing for high oocyte maturation rates.

The concentration of 50 µg/mL EOCS was tested in the second step for its synergistic potential with CYS, considering that it proved to be the most promising antioxidant additive to CYS in the first experiment. This study evaluated the effect of different antioxidant combinations on porcine oocyte maturation, focusing on *cumulus* cell expansion, viability, 1PB, and ROS levels. The results showed no difference in *cumulus* cell expansion among the groups. However, higher viability of *cumulus* cells was observed in the group treated only with 50EOCS, without CYS, indicating a positive effect of 50EOCS on maintaining cell viability. The lack of a synergistic effect between 50EOCS and CYS could be due to several factors. First, it is possible that combining these antioxidants does not produce an additive or synergistic interaction. Previous studies (Abeydeera *et al.,* 2000; Thongkittidilok *et al.*, 2022) indicated that combining antioxidants may not always yield additional benefits. These studies demonstrated that combining different antioxidants did not significantly enhance oocyte maturation or cell viability compared to using them individually.

In the current study, the 50EOCS + CYS group did not show a difference in the rate of 1PB compared to the 50EOCS and CYS groups alone. This indicates that both 50EOCS and CYS, when used separately, are equally effective at promoting nuclear maturation. However, their combination does not produce a greater effect. This lack of synergy might be because each antioxidant works through different pathways that do not enhance each other, or the combination could even hinder the effectiveness of each compound individually (Guerin *et al.,* 2001).

Although our study was specifically designed to investigate the potential synergistic or substitutive effect of EOCS in combination with CYS, a limitation of the present study is the absence of a control group cultured in a completely antioxidant-free maturation medium. Even though 100 µM cysteamine is routinely used as a reference condition in porcine IVM due to its well-established role in enhancing intracellular glutathione synthesis and cytoplasmic maturation (Matos & Furnus, 2000; Whitaker & Knight, 2020), this choice does not allow a full assessment of the isolated effects of EOCS or of the potential baseline oxidative status of oocytes in the standard medium. In addition, without physicochemical verification of the maturation medium after the addition of essential oil, such as pH, osmolarity, or potential micelle formation, uncontrolled interactions between oil constituents and culture components cannot be ruled out (Tian *et al.*, 2022). On the other hand, the maintenance of high *cumulus* cell viability and normal oocyte maturation rates in our results strongly supports that the culture environment remained stable and biocompatible throughout the experiment. These methodological constraints should be considered when interpreting the results, and future studies should incorporate both an antioxidant-free control and systematic medium characterisation to better elucidate the specific mechanisms and stability of oil-based supplements during oocyte maturation.

In summary, the results of this study show that although the combination of 50 µg/mL EOCS with CYS effectively con-

trolled free radicals, increased cell viability, and preserved cytoplasmic maturation without hindering nuclear maturation, 50EOCS alone is effective in maintaining *cumulus* cell viability and reducing ROS levels during porcine oocyte maturation. While further research is necessary to assess the effects of EOCS on *in vitro* fertilisation and embryonic development, these initial findings suggest that 50EOCS could be a promising and cost-effective additive for porcine oocyte IVM, offering new opportunities to enhance reproductive efficiency in pigs.

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## REFERENCES

Abeydeera, L. R., 2002. *In vitro* production of embryos in swine. *Theriogenology,.* **57**, 257–273.

Abeydeera, L. R., W. H. Wang, T. C. Cantley, A. Rieke, C. N. Murphy, R. S. Prather & B. N. Day, 2000. Development and viability of pig oocytes matured in a protein-free medium containing epidermal growth factor. *Theriogenology*, **57**, 777–785.

Aquino L. V. C., M. V. O. Santos, L. R. M. Oliveira, Y. B. F. Moura, T. L. Nascimento, L. M. Bertini & A. F. Pereira, 2023. Antioxidant effects of *Citrus sinensis* peel essential oil in a bovine oocyte model. *Livestock Science,* **276**, 105324.

Cánovas, M. G., I. Parrilla, C. Cuello, M. A. Gil & E. A. Martinez, 2024. Swine *in vitro* embryo production: Potential, challenges, and advances. *Animal Reproduction Science*, **270**, e107600.

Casillas, F., Y. Ducolumb, A. López & M. Betancourt, 2020. Effect of porcine immature oocyte vitrification on oocyte-*cumulus* cell gap junctional intercellular communication. *Porcine Health Management,* **6**, 37.

Caveiro, A. A., F. J. A. Matos & J. W. Alencar, 1976. A simple and inexpensive steam generator for essential oils extraction. *Journal of Chemical Education*, **53**, 652.

Chen, P. R., B. K. Redel, K. C. Kerns, L. D. Spate & R. S. Prather, 2021a. Challenges and considerations during *in vitro* production of porcine embryos. *Cells*, **10**, 2770.

Chen, Y., C. Pan, Y. Lu, Y. Miao & B. Xiong, 2021b. HDAC8 drives spindle organization during meiotic maturation of porcine oocytes. *Cell Proliferation*, **54**, e13119.

Choi, H., J. Lee, J. D. Yoon, S. U. Hwang, L. Cai, M. Kim, G. Kim, D. Oh, E. Kim & S. H. Hyun, 2021. The effect of copper supplementation on *in vitro* maturation of porcine cumulus-oocyte complexes and subsequent developmental competence after parthenogenetic activation. *Theriogenology*, **164**, 84–92.

Chopra, B., A. K. Dhingra, K. L. Dhar & K. Nepali, 2021. Emerging role of terpenoids for the treatment of cancer: A review. *Mini-Reviews in Medicinal Chemistry,* **21**, 2300–2336.

Currin, L., W. Glanzner, K. Gutierrez, M. P. Macedo, V. Guay, H. Baldassarre & V. Bordignon, 2022. Optimizing swine *in vitro* embryo production with growth factor and antioxidant supplementation during oocyte maturation. *Theriogenology,* **194**, 133–143.

Farahmandfar, R., B. Tirgarian, B. Dehghan & A. Nemati, 2020. Changes in chemical composition and biological activity of essential oil from Thomson navel orange (*Citrus sinensis* L. Osbeck) peel under different drying methods. *Food Science & Nutrition,* **8**, 124–138.

Ferronatto, A.N. & R. C. Rossi, 2018. Extraction and application of orange peel essential oil as a natural ingredient. *Estudos Tecnológicos em Engenharia,* **12**, 78.

Geraci, A., V. Di Stefano, E. Di Martino, D. Schillaci & R. Schicchi, 2017. Essential oil components of orange peels and antimicrobial activity. *Natural Product Research,* **31**, 653–659.

Gil, M. A., C. Cuello, I. Parrilla, J. M. Vázquez, J. Roca & E. A. Martínez, 2010. Advances in technologies for the *in vitro* production of pig embryos. *Reproduction in Domestic Animals*, **45**, 40–48.

Guerin, P., S. El Mouatassim & Y. Menezo, 2001. Oxidative stress and protection against reactive oxygen species in the preimplantation embryo and its surroundings. *Human Reproduction,* **7**, 175–189.

Jin, J. X., S. Lee, E. M. N. Setyawan, A. Taweecheipaisankul, G. A. Kim, H. J. Han, C. Ahn & B. C. Lee, 2018. A potential role of knockout serum replacement as a porcine follicular fluid substitute for *in vitro* maturation: Lipid metabolism approach. *Journal of Cellular Physiology,* **233**, 6984–6995.

Kim, S. W., A. Gormley, K. B. Jang & M. E. Duarte, 2024. Current status of global pig production: An overview and research trends. *Animal Bioscience,* **37**, 719–729.

Lamine, M., Z. Hamdi, H. Zemni, F. Z. Rahali, I. Melki, A. Mliki & M. Gargouri, 2024. From residue to resource: The recovery of high-added values compounds through an integral green valorization of *Citrus* residual biomass. *Sustainable Chemistry & Pharmacy,* **37**, e101379.

Lee, S., J. X. Jin, A. Taweecheipaisankul, G. A. Kim & B. C. Lee, 2018. Synergistic effects of resveratrol and melatonin on in vitro maturation of porcine oocytes and subsequent embryo development. *Theriogenology,* **144**, 191–198.

Li, Y., J. Wang, Z. Zhang, J. Yi, C. He, F. Wang, X. Tian, M. Yang, Y. Song, P. He & G. Liu, 2016. Resveratrol compares with melatonin in improving *in vitro* porcine oocyte maturation under heat stress. *Journal of Animal Science and Biotechnology,* **7**, 1–10.

Lv, L., W. Yue, W. Liu, Y. Ren, F. Li, K.B. Lee & G. W. Smith, 2010. Effect of oocyte selection, estradiol and antioxidant treatment on *in vitro* maturation of oocytes collected from prepubertal Boer goats. *Italian Journal of Animal Science,* **9**, e11.

Mateo-Otero, Y., M. Yeste, A. Damato & E. Giaretta, 2021. Cryopreservation and oxidative stress in porcine oocytes. *Research in Veterinary Science*, **135**, 20–26.

Matos, D. G. & C. C. Furnus, 2000. The importance of having high glutathione (GSH) level after bovine *in vitro* maturation on embryo development: Effect of β-mercaptoethanol, cysteine and cystine. *Theriogenology*, **53**, 761–771.

McEvoy, T. G., G. D. Coull, P. J. Broadbent, J. S. Hutchinson & B. K. Speake, 2000. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. *Journal of Reproduction and Fertility,* **118**, 163–170.

Nevoral, J., M. Orsák, P. Klein, J. Petr, M. Dvořáková, I. Weingartová, A. Vyskočilová, K. Zámostná, T. Krejčová & F. Jílek, 2015. Cumulus cell expansion, its role in oocyte biology and perspectives of measurement: A review. *Scientia Agriculturae Bohemica*, **45**, 212–225.

Nguyen, H.T., T. Q. D. Nguyen, T. Somfai, N. T. Men, B. Beck-Woerner, N. V. Linh, B. X. Nguyen, J. Noguchi, H. Kaneko & K. Kikuchi, 2021. Excess polyspermy reduces the ability of porcine oocytes to promote male pronuclear formation after *in vitro* fertilization. *Animal Science Journal,* **92**, e13650.

Oh, S. H., S. E. Lee, D. H. Han, J. W. Yoon, S. H. Kim, E. S. Lim, H. B. Lee, E. Y. Kim & S. P. Park, 2023. Treatments of porcine nuclear recipient oocytes and somatic cell nuclear transfer-generated embryos with various reactive oxygen species scavengers lead to improvements of their quality parameters and developmental

competences by mitigating oxidative stress-related impacts. *Cell Reprogramming,* **25**, 73–81.

Oller, E. P., S. N. Serna, C. S. Ùbeda, J. S. Lopes, C. Matás, S. Ruiz, R. Latorre, A. O. López, R. Romar, S. Cánovas & P. Coy, 2021. Reproductive fluids, used for the in vitro production of pig embryos, result in healthy offspring and avoid aberrant placental expression of PEG3 and LUM. J. *Journal of Animal Science and Biotechnology*, **12**, 1–12.

Pei, Z., K. Deng, C. Xu & S. Zhang, 2023. The molecular regulatory mechanisms of meiotic arrest and resumption in oocyte development and maturation. *Reproductive Biology and Endocrinology,* **21**, 90.

Romek, M., B. Gajda, M. Rolka & Z. Smorąg, 2010. Mitochondrial activity and morphology in developing porcine oocytes and pre-implantation non-cultured and cultured embryos. *Reproduction in Domestic Animals,* **46**, 471–480.

Ryu, S. A., S. Baek, K.C. Kim, E.S. Lee & S.T. Lee, 2023. Effects of cumulus cells on the *in vitro* cytoplasmic maturation of immature oocytes in pigs. *Theriogenology,* **206**, 133–139.

Santos, M. V. O., A. A. Borges, L. B. Queiroz Neta, L. M. Bertini & A. F. Pereira, 2018. Use of natural antioxidants *in vitro* mammalian embryo production. *Semina: Ciências Agrárias,* **39**, 431–443.

Santos, M. V. O., L. E. Nascimento, E. A. Praxedes, A. A. Borges, A. R. Silva, L. M. Bertini & A. F. Pereira, 2019. *Syzygium aromaticum* essential oil supplementation during in vitro bovine oocyte maturation improves parthenogenetic embryonic development. *Theriogenology*, **128**, 74–80.

Shah, B. B. & A. A. Mehta, 2018. *In vitro* evaluation of antioxidant activity of D-Limonene. *Asian Journal of Pharmacy and Pharmacology,* **4**, 883–887.

Sharma, N. & A. Tripathi, 2008. Effects of *Citrus sinensis* (L.) Osbeck epicarp essential oil on growth and morphogenesis of *Aspergillus niger* (L.) Van Tieghem. *Microbiological Research,* **163**, 337–344.

Silva, B. R. & J. R. Silva, 2023. Mechanisms of action of non-enzymatic antioxidants to control oxidative stress during in vitro follicle growth, oocyte maturation, and embryo development. *Animal Reproduction Science*, **249**, 107186.

Sun, J. T., J. D. Yuan, Q. Zhang, X. Luo, X. Y. Qi, J. H. Liu, X. Q. Jiang, S. Lee, A. Taweecheipaisankul, Z. H. Liu & J. X. Jin, 2022a. Ramelteon reduces oxidative stress by maintenance of lipid homeostasis in porcine oocytes. *Antioxidants,* **11**, 1640.

Sun, J. T., J. H. Liu, X. Q. Jiang, X. Luo, J. D. Yuan, Q. Zhang, X. Y. Qi, S. Lee, Z. H. Liu & J. X. Jin, 2022b. Tannin reduces the incidence of polyspermic penetration in porcine oocytes. *Antioxidants.* **11**, 2027.

Suri, S., A. Singh & P. K. Nema, 2022. Current applications of *Citrus* fruit processing waste: A scientific outlook. *Applied Food Research,* **2**, e100050.

Suzuki, H., Y. Saito, N. Kagawa & X. Yang, 2003. *In vitro* fertilization and polyspermy in the pig: Factors affecting fertilization rates and cytoskeletal reorganization of the oocyte. *Microscopy Research and Technique,* **61**, 327–334.

Tarhouni, N., A. Bayoudh, A. B. Slima, S. Mekrazi, A. El Aguel, A. Khabir, I. Kallel & A. Soussi, 2024. Preventing bifenthrin-induced testicular toxicity in rats with *Citrus sinensis* ('Maltese Half-blood') essential oil: A multidisciplinary approach. *Journal of Essential Oil Bearing Plants*, **1**, 849–869.

Thongkittidilok, C., Q. A. Le, Q. Lin, K. Takebayashi, T. K. L. Do, Z. Namula, M. Hirata, F. Tanihara & T. Otoi, 2022. Effects of individual or in-combination antioxidant supplementation during in vitro maturation culture on the developmental competence and quality of porcine embryos. *Reproduction in Domestic Animals*, **57**, 314–320.

Tian, X., F. Wang, L. Zhang, Y. Lei & G. Liu, 2022. Essential oils as potential additives

in mammalian *in vitro* embryo production: Composition, mechanisms of action and safety considerations. *Animals*, **12**, 340.

Toscano-Garibay, J. D., M. Arriaga-Alba, J. Sánchez-Navarrete, M. Mendoza-García, J. J. Flores-Estrada, M. A. Moreno-Eutimio, J. J. Espinosa-Aguirre, M. González-Ávila & N. J. Ruiz-Pérez, 2017. Antimutagenic and antioxidant activity of the essential oils of *Citrus sinensis* and *Citrus latifolia*. *Scientific Reports,* **7**, 11479.

USDA, 2024. United States Department of Agriculture. Livestock and Poultry: World Markets and Trade. https://apps.fas.usda.gov/psdonline/app/index.html#/app/advQuery (17 April 2025, date last accessed).

Whitaker, B. D. & J. W. Knight, 2020. Mechanisms of cysteamine action during oocyte maturation and embryo development: A review. *Reproduction in Domestic Animals*, **55**, 893–902.

Wu, G. & F. W. Bazer, 2019. Application of new biotechnologies for improvements in swine nutrition and pork production. *Journal of Animal Science and Biotechnology,* **10**, 28.

Xiang, D. C., B. Y. Jia, X. W. Fu, J. X. Guo, Q. H. Hong, G. B. Quan & G. Q. Wu, 2021. Role of astaxanthin as an efficient antioxidant on the in vitro maturation and vitrification of porcine oocytes. *Theriogenology*, **167**, 13–23.

Yamochi, T., S. Hashimoto, A. Amo, H. Goto, M. Yamanaka, M. Inoue, Y. Nakaoka & Y. Morimoto, 2016. Mitochondrial dynamics and their intracellular traffic in porcine oocytes. *Zygote*, **24**, 517–528.

Zhao, H., Y. Dong, Y. Zhang, X. Wu, X. Zhang, Y. Liang, Y. Li, F. Zeng, J. Shi, R. Zhou, L. Hong, G. Cai, Z. Wu & Z. Li, 2022. Supplementation of SDF1 during pig oocyte in vitro maturation improves subsequent embryo development. *Molecules*, **27**, 6830.

Zhao, Y., Y. Xu, Y. Li, Q. Jin, J. Sun, E. Zhiqiang & Q. Gao, 2020a. Supplementation of kaempferol to *in vitro* maturation medium regulates oxidative stress and enhances subsequent embryonic development in vitro. *Zygote,* **28**, 59–64.

Zhao, Z., L. Yang, D. Zhang, Z. Zheng, N. Li, Q. Li & M. Cui, 2020b. Elevation of MPF and MAPK gene expression, GSH content and mitochondrial distribution quality induced by melatonin promotes porcine oocyte maturation and development in vitro. *PeerJ*, **8**, e9913.

Zhu, T., L. Yan, S. Deng, W. Ma, F. Xia, L. Wang, X. Ma, G. Li, Z. Shen, Y. Wang, Y. Fu, P. Ji, B. Wang, L. Zhang & G. Liu, 2024. Mitochondria of porcine oocytes synthesize melatonin, which improves their in vitro maturation and embryonic development. *Antioxidants,* **13**, 814.

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