

Impact of Antioxidant Supplementation on *In Vitro* Growth of Porcine Oocytes Derived from Early Antral Follicles

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ABSTRACT

Introduction: Oxidative stress (OS), a key factor impairing oocyte developmental competence, poses a major challenge in *in vitro* growth (IVG) systems. While antioxidant supplementation can mitigate OS-driven deficits, combining agents with complementary mechanisms—such as melatonin (Mela) and astaxanthin (Asta)—may offer synergistic benefits. This study evaluates the effects of Mela and Asta, individually and in combination, on oocyte quality, gene expression, and epigenetic markers during IVG. **Methods:** Growing oocytes from early antral follicles (EAFs; 1–1.5 mm) were cultured for 4 days in a 3D agarose system. Initial experiments tested Mela (1, 10, 100 $\mu\text{M}/\text{mL}$) to assess survival rates and expression of *BMP15*, *CAT*, *SOD1*, and *GPX4* via qPCR. Subsequently, the optimal Mela concentration was combined with Asta (0.05, 0.1, 0.25 $\mu\text{M}/\text{mL}$) to determine synergistic effects. Histone H3 methylation (Me-H3-K4) and global transcriptional activity (nascent mRNA accumulation) were analyzed to evaluate chromatin remodeling and transcriptional dynamics. **Results:** Mela supplementation significantly improved oocyte survival compared to controls, with 1 $\mu\text{M}/\text{mL}$ Mela yielding optimal results. Combining 1 $\mu\text{M}/\text{mL}$ Mela with 0.05 $\mu\text{M}/\text{mL}$ Asta further enhanced survival rates and upregulated antioxidant genes (*CAT*, *SOD1*, *GPX4*) and *BMP15*. This combination also elevated Me-H3-K4 levels and nascent mRNA accumulation, indicating enhanced transcriptional activity and chromatin remodeling. These changes correlated with reduced ROS impact and improved oocyte quality post-IVG. **Conclusion:** Co-supplementation with Mela (1 $\mu\text{M}/\text{mL}$) and Asta (0.05 $\mu\text{M}/\text{mL}$) synergistically mitigates OS during IVG by boosting antioxidant gene expression, enhancing epigenetic modifications (Me-H3-K4), and promoting global transcription. This strategy significantly improves oocyte developmental competence, offering a promising approach to optimize IVG systems.

Key words: IVG, porcine oocytes, antioxidant, gene expression, H3K4 methylation

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History

- Received: 09-12-2024
- Accepted: 07-4-2025
- Published Online: 31-5-2025

DOI : 10.15419/bmrat.v12i5.979



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INTRODUCTION

In vitro growth (IVG) is a methodological procedure that facilitates the development of oocytes from early-stage follicles by establishing a supportive environment with proper timing and essential nutrient supplementation. This method may have the potential to produce a large number of meiotically competent oocytes following *in vitro* maturation (IVM), thereby enhancing the implications for successful assisted reproductive technologies. Especially, ovarian tissue cryopreservation (OTC) offers fertility preservation and restoration of endocrine function for female cancer patients prior to undergoing gonadotoxic treatments, although it remains an experimental approach. Reimplantation of OTC is currently the only option to use preserved tissue. However, this procedure often carries the potential risk of re-inducing malignancy. Early antral follicles (EAFs)

serve as an intermediate stage between pre-antral and antral follicles, offering a promising source for obtaining mature oocytes because they are abundant and require a shorter culture duration compared with other pre-antral follicles (PAFs). However, challenges persist in culturing EAFs, and the efficiency of *in vitro* embryo production from EAF-derived oocytes remains substantially lower than that achieved with oocytes from small antral follicles (SAFs) and fully grown oocytes from large antral follicles (LAFs) in multiple mammalian species¹⁻⁵. This disparity underscores the need for further research and development in IVG of growing oocytes from EAFs, and various modifications have been suggested to overcome this problem^{6,7}.

Based on changes and additions to the substrate to support the culture of growing oocytes, we have successfully used an agarose gel 3D matrix for the culture of preantral follicles, which has improved

Cite this article : Chi Thien L, Do Truc Phuong L, Duy P, Lien Boi Linh N, Ba Anh My L, Dang Su Pham C, Ngoc Thao Vy N, Phan Van Anh T, Nhat Thinh N, Van Thuan N, Bui H-T. **Impact of Antioxidant Supplementation on *In Vitro* Growth of Porcine Oocytes Derived from Early Antral Follicles.** *Biomed. Res. Ther.* 2025; 12(5):7403-7417.

follicle growth and helped form fluid-filled cavities called antrum formation, marking the transition from preantral to antral follicles⁸. One of the main factors affecting reduced oocyte quality during culture is the production of reactive oxygen species (ROS), which cause DNA damage, protein misfolding, enzyme inactivation, cell apoptosis, and meiotic abnormalities^{8,9}. Supplementation of antioxidants is essential to reduce the harmful effects of ROS. We have successfully improved oocyte quality at the pre-IVM stage by supplementing the culture medium with ascorbic acid⁵.

Melatonin (N-acetyl-5-methoxy tryptamine; Mela) is produced by the pineal gland and plays an important role in regulating the circadian rhythm in mammals^{10,11}. This hormone is a powerful antioxidant, scavenging and neutralizing free radicals such as hydroxyl radicals, ROS, and reactive nitrogen species via its aromatic indole ring. As an antioxidant, it directly regulates the activity of several antioxidant enzymes, oxidative metabolism, and the electron transport chain¹². The addition of Mela has improved blastocyst quality; however, the quality of blastocysts derived from EAFs remains lower than those from LAFs. Based on the positive effects of Mela on oocytes during the IVM stage, we supplemented it during the IVG stage. Mela at 100 μM has been shown to significantly improve oocyte quality after the IVM stage¹³. After further testing with Mela at 1 μM , 10 μM , and 100 μM during the IVG stage, we observed an improved blastocyst rate compared to the non-supplemented group; nevertheless, the quality and overall blastocyst rate remained significantly lower than in the control LAFs group¹⁴. Therefore, we aim to conduct this study to gain deeper insights into the effects of Mela during IVG culture.

Astaxanthin (3,3'-dihydroxy- β , β' -carotene-4,4'-dione; Asta), a xanthophyll carotenoid found in various microorganisms and marine organisms¹⁵, has numerous biological functions, including blocking lipid peroxidation, anti-inflammatory activity, and scavenging ROS and hydroxyl radicals^{16,17}. Asta, which is a nonenzymatic antioxidant, neutralizes free radicals¹⁸. Its antioxidant capacity is more powerful than other antioxidants, such as vitamins C and E or other carotenoids¹⁹. Supplementation with Asta is beneficial for the maturation of porcine and bovine oocytes, helping to prevent heat shock^{20,21}. OCGCs cultured with 0.5 mg/L ($\approx$ 0.83 μM) Asta displayed improved quality compared to the non-treated group²². Asta also protects

granulosa cells from oxidative stress by upregulating phase II enzymes through NRF2 activation²³. Nevertheless, astaxanthin reduces granulosa cell growth and antrum formation²⁴. Therefore, the effects of Asta on oocytes after the IVG stage remain ambiguous. Consequently, concentrations of 0.05, 0.1, and 0.25 μM were selected for this study. Specific gene expressions during this stage need to be evaluated. It has been suggested that the simultaneous and exclusive presence of Mela and vitamin C antioxidants in the IVF medium reduces ROS, thus enhancing embryo development up to the blastocyst stage²⁵. Therefore, combining Mela and Asta antioxidants with different activities may have great potential to improve *in vitro* culture systems of growing oocytes from EAFs.

Porcine-growing oocytes isolated from EAFs (1–1.5 mm) predominantly exhibit uncondensed and dispersed chromatin in the germinal vesicle (GV, the oocyte nucleus), defined as filamentous/stringy chromatin (FC/SC) morphology²⁶. At this stage, oocytes remain transcriptionally active and functionally connected with the surrounding cumulus cells through gap junctions. However, these oocytes cannot spontaneously resume meiosis or support embryo development. With the transition to LAFs (4–6 mm), the porcine oocyte undergoes significant changes associated with the completion of the growth phase, such as the shift from FC/SC to higher degrees of chromatin compaction, namely the GV stage^{26,27}. We reported that histone H3 acetylation/methylation occurs in chromatin throughout the nucleoplasm of growing oocytes. In fully grown porcine oocytes, chromatin transitions from a decondensed state to a heterochromatin ring around the nucleolus. This acetylation/methylation is crucial for transcriptional activation and increases during oocyte growth. In addition, histone H3 methylation at lysine 4 (Me-H3-K4) is regulated by specific nuclear factors during folliculogenesis²⁶. Thus, assessing histone acetylation/methylation levels and chromatin structure can effectively predict the meiotic capacity of *in vitro*-grown oocytes.

Additionally, both Asta and Mela exert specific effects on gene expression. Mela indirectly reduces oxidative stress by stimulating the expression of many endogenous antioxidative enzymes (e.g., superoxide dismutase 1 [SOD1], glutathione peroxidase [GSH]) and has an inhibitory effect on pro-oxidative enzymes²⁸. Indeed, studies on the effect of Mela in IVG show an increase in the size of oocytes derived from the preantral follicle in mice²⁹,

sheep³⁰, and secondary oocytes of goats³¹. During the growth stage of oocytes, essential antioxidant genes such as *SOD1*, Catalase (*CAT*), and Glutathione Peroxidase 4 (*GPX4*) are expressed to protect oocytes from oxidative stress³². This study also analyzed the expression of Bone Morphogenetic Protein 15 (*BMP15*), which is critical for folliculogenesis and oogenesis support factors³³. Moreover, the accumulation of mRNA during oocyte growth plays a pivotal role in embryo quality at the preimplantation stage: mRNA synthesis increases during oocyte growth and is maintained for mature oocytes after fertilization³⁴. Besides that, we reported that paternal chromatin undergoes extensive remodeling following sperm entry into the oocyte, with maternally derived RNAs rapidly degraded after fertilization, before zygotic gene activation³⁵. Accordingly, protein synthesis relies predominantly on maternal mRNA that accumulates in the oocyte during the phase preceding zygotic gene activation. Hence, it is crucial to evaluate the expression of specific genes; global transcription is assessed by measuring nascent mRNA during *in vitro* growth of oocytes from EAFs.

In this study, we examine the effect of Mela and Asta supplementation in the IVG medium for growing oocytes at varying concentrations and in different combinations to explore the role of these antioxidants in oocyte growth. We evaluated their effects on porcine oocyte viability and the expression of specific genes using real-time PCR (RT-PCR), while also measuring nascent mRNA and Me-H3-K4 to assess global transcription. We found that supplementation with combined Mela and Asta at appropriate concentrations during IVG helps growing oocytes attain a quality nearly equivalent to fully grown oocytes isolated from LAFs.

METHODS

All the reagents and chemicals used in this research were ordered from Sigma Chemical Co. (St. Louis, Mo, USA), unless otherwise stated.

Ethics statement

The treatment of porcine ovaries used in this study followed the guidelines of the International University–Vietnam National University, Ho Chi Minh City, Viet Nam.

Oocyte cumulus granulosa cell complex (OCGC) collection

Porcine ovaries were collected from local slaughterhouses and transported to the laboratory within two

hours in phosphate-buffered saline (PBS; Dulbecco), supplemented with 100 IU penicillin and 100 mg/ml streptomycin. The SAFs (2–3 mm) and the LAFs (4–6 mm) were dissected from the ovaries. To collect EAFs, the ovarian cortex was sectioned into slices 1–2 mm thick using a surgical blade (No. 21). These slices were then placed under a microscope to dissect using a surgical blade (No. 11). After dissection, the follicles were washed in 25 mM HEPES-buffered TCM-199 containing 0.1% polyvinyl alcohol. The follicles were then placed in HEPES and opened using an 18-gauge needle under a microscope. Healthy OCGCs derived from these follicles, characterized by multilayered cumulus and granulosa cells with uniform ooplasm, were selected for the experiment.

Agarose gel preparation and coating dish for culturing

Agarose gel was used to mimic the *in vivo* microenvironment of the porcine ovarian matrix. To prepare a 1.5% agarose solution, mix low-gelling-temperature agarose (A9539) powder with PBS at a concentration of 1.5% (w/v), heat until fully dissolved, then transfer the hot solution to 1.5 mL centrifuge tubes and store at 4°C. For coating a 96-well dish, reheat the stored agarose gel in boiling water for 5 minutes to remelt it, then apply 30 μ L of the molten solution to the bottom of each well. The agarose hardens at room temperature for 10 minutes, and before use, the equilibrated medium is replaced with fresh medium⁸.

In-vitro growth (IVG) with antioxidant supplementation

The OCGCs from EAFs were transferred to an IVG medium and cultured under different IVG conditions for 4 days in an incubator with 5% CO₂ in humidified air at 38.5°C. Every well was filled with 120 μ L IVG medium and covered with 50 μ L mineral oil. Each well could culture 3–5 OCGCs.

The basic IVG medium contained α -MEM supplemented with 3% polyvinylpyrrolidone, 5% follicle fluid, 10% fetal bovine serum, 0.91 mM NaPy, 3 mM hypoxanthine, and 1 μ g/mL estradiol. This study was divided into two main experiments, with each IVG culture medium supplemented with different concentrations of antioxidants. In the first experiment, groups of Mela were divided into three different concentrations (1 μ M/mL, 10 μ M/mL, and 100 μ M/mL) to determine the optimal concentration for the next experiment. In the next experiment, Asta was combined at three different concentrations (0.05

$\mu\text{M/mL}$, 0.1 $\mu\text{M/mL}$, and 0.25 $\mu\text{M/mL}$) with the optimal concentration of Mela.

The OCGCs were cultured in a dish coated with agar. Half of the culture medium was replaced after 2 days. After IVG, the survival rate of OCGCs ($n = 54$) was evaluated by calculating the percentage of healthy cultured OCGCs. Oocyte diameter ($n = 25$) was measured after removing the surrounding cells, and both horizontal and vertical dimensions were recorded using a digital microscope.

mRNA extraction and real-time PCR

The mRNA extraction and RT-PCR were performed on the same day to prevent mRNA degradation. According to the manufacturer's instructions, total mRNA was extracted from samples (10–15 oocytes) using the Dynabeads™ mRNA DIRECT™ Kit (Invitrogen, Thermo Fisher Scientific). RT-PCR was performed using the Applied Biosystems™ Power SYBR™ Green RNA-to-CT™ 1-Step Kit (Invitrogen, Thermo Fisher Scientific), and the relative abundance of target mRNAs was examined with specific primers. Act- β was used as the reference gene. The primer sequences were listed in Table 1. Each RT-PCR reaction had a total volume of 10.0 μL , consisting of 3.0 pmol/ μL of each primer, 5 μL Master Mix, 0.08 μL Enzyme Mix, and 1 μL mRNA template. RT-PCR was processed using a thermocycler (Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, 96-well, 0.2 mL) over 45 cycles. The RT-PCR cycle conditions were performed as follows:

- Stage 1: Double-stranded DNA synthesis at 48.0°C for 30 minutes and lid heating at 95.0°C for 10 minutes.
- Stage 2: 45 cycles of denaturation at 95.0°C for 15 seconds, primer annealing, and extension at 60.0°C for 45 seconds.

Immunofluorescence staining and nascent mRNA detection

Each group of cultured oocytes was washed twice in an electro-permeabilization (EP) medium containing 0.25 M D-glucose, 100 μM CaCl_2 , 100 μM MgSO_2 and 0.1% PVP. They were then transferred into a drop of EP medium supplemented with 10 mM BrUTP for 2 minutes. The oocytes were placed in 20 μL of EP medium containing 10 mM BrUTP between two parallel stainless steel electrodes in a chamber. Electro-permeabilization was conducted with one direct current pulse at 30 V and 80 μs . Two minutes post-permeabilization, the oocytes were cultured in IVG

medium for 1 hour at 38.5°C with 5% CO_2 . Oocytes were fixed and immunostained, as described in a previous study³⁶. Indirect immunofluorescence detected newly synthesized transcripts incorporating BrUTP and histone methylation H3K4 (Me-H3-K4). The primary antibodies used include mouse monoclonal anti-bromodeoxyuridine (6 $\mu\text{g/mL}$, Roche-1117037600) and rabbit anti-methyl-histone H3K4. The secondary antibodies, Alexa-Fluor-568-labeled goat anti-mouse IgG and Alexa-Fluor-488-labeled goat anti-rabbit IgG, were used. DNA was counterstained with 2 $\mu\text{g/mL}$ 4,6-diamidino-2-phenylindole (DAPI) for 30 minutes. The levels of Me-H3-K4 expression and BrUTP were analyzed based on fluorescence intensity using the Nikon analysis program. Fluorescence intensity within each nucleus was measured across five different regions, and the cytoplasmic background value was subtracted. The resulting value was then multiplied by the nuclear volume to estimate the total fluorescence for the nucleus. Due to the limited sample size, no statistical analysis could be performed.

Statistical analysis

Each treatment was replicated at least 3 times. Descriptive statistics were performed to assess normality and homogeneity of variance prior to parametric analyses, using skewness values as part of the evaluation. One-way ANOVA was performed with the Statistical Package for Social Sciences (SPSS) version 20, with a significance level of $p < 0.05$. Data were reported as the mean $\pm$ standard error of the mean (SEM). RT-PCR data were analyzed using the Log2FC method, with statistically significant differences at $p < 0.05$. Based on the Log2FC method, the non-treated group was used as the reference. Therefore, the expression on the graph shows the increase or decrease relative to the non-treated group.

RESULTS

Effects of melatonin on the quality of OCGCs after 4 days of IVG

After IVG culture, antrum formation in OCGCs did not show significant differences between the groups supplemented with Mela (Figure 1B). Nevertheless, Mela positively affected oocytes and granulosa cells, improving survival after 4 days of IVG. The OCGCs supplemented with Mela 10 μM had the highest survival rate, yet no significant differences were observed among the treatment groups (Figure 1C). Based on gene expression studies, Mela slightly promoted *BMP15* expression in growing oocytes

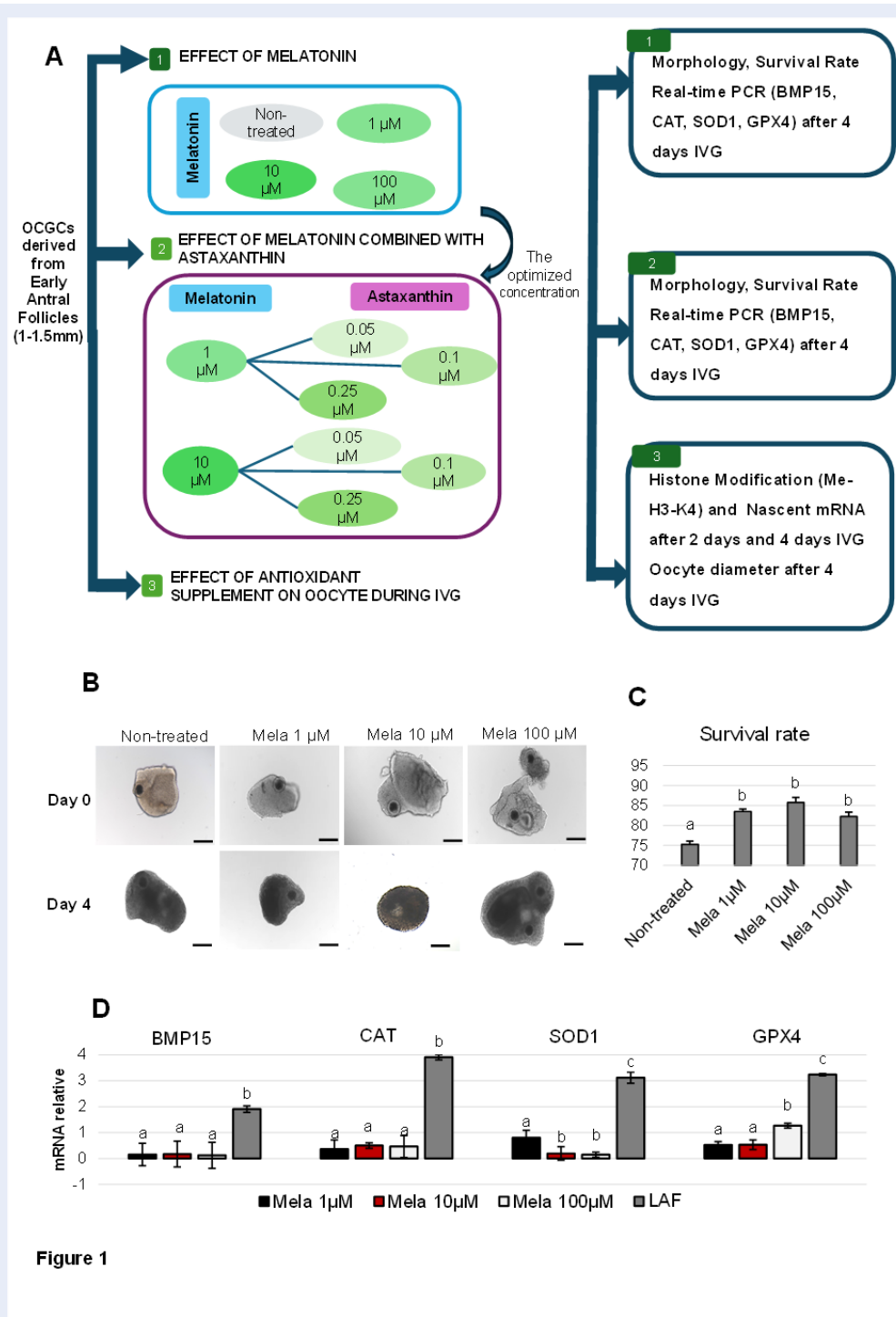


Figure 1

Figure 1: Experimental design and assessment of oocyte-cumulus-granulosa-complexes (OCGCs) supplemented with Mela after IVG. **A) Experimental design:** Experiment 1: OCGCs collected from EAF were cultured in different concentrations of Mela (1 μ M, 10 μ M, 100 μ M) supplementation; morphology, survival rate, and gene expression were assessed after 4 days. Experiment 2: OCGCs were cultured in different concentrations of combined Mela (1 μ M, 10 μ M) and Asta (0.05 μ M, 0.1 μ M, 0.25 μ M) supplementation; morphology, survival rate, and gene expression were assessed after 4 days. Experiment 3: Immunofluorescent staining of oocyte after IVG with different concentrations of melatonin groups and combined Mela (1 μ M, 10 μ M) and Asta (0.05 μ M, 0.1 μ M, 0.25 μ M) groups of Methylation Histone H3K4 (Me-H3-K4) and nascent mRNA on 2 and 4 days IVG and oocyte diameter were assessed after 4 days. Effect of Mela (1 μ M, 10 μ M, 100 μ M) on oocyte derived from EAF. **B) Morphology** assessment of the OCGCs was checked on days 0 and 4 in IVG. **C) The survival rate** of growth oocyte after 4 days of IVG. **D) RT-PCR results of marker gene** in OCGCs cultured with Mela (1 μ M, 10 μ M, 100 μ M) supplement. Data are presented as the mean $\pm$ S.E.M. a-c values without a common superscript differed significantly ($P < 0.05$). Bars = 150 μ m

Table 1: The sequence of PCR primers

Genes	Primer sequence (5'-3')	Product size
<i>Act-β</i>	Forward: ATCGTGCGGGACATCAAGGA	179bp
	Reverse: AGGAAGGAGGGCTGGAAGAG	
<i>BMP15</i>	Forward: ACCATGGTGAGGCTGGTGAG	178bp
	Reverse: CATGGCAGGAGAGGTGGAAG	
<i>CAT</i>	Forward: ACGTTGGAAGAGGACACCC	137bp
	Reverse: TCCAACGAGATCCCAATTACCA	
<i>SOD-1</i>	Forward: CACTGTGTACATCGAAGATTCTGTGA	97bp
	Reverse: GCCCAAGTCATCTGGTTTTTCA	
<i>GPX4</i>	Forward: ACGGGCACATGGTGAACCT	85bp
	Reverse: ACCTCCGTCTGCCTCATTG	

(**Figure 1D**). In addition, Mela enhanced the expression of the *CAT* and *GPX4* genes, thereby increasing antioxidant enzyme activity and reducing the effects of ROS. However, the differences between each treatment group were negligible, resulting in nearly identical oocyte qualities among the treatment groups.

Impact of the combined use of melatonin and astaxanthin on the quality of OCGCs following IVG after 4 days

The addition of combined Mela 1μM + Asta did not significantly affect antrum formation after 4 days of IVG (**Figure 2A**). However, supplementation with combined Mela 10 μM + Asta reduced the ability to form an antrum after the IVG stage (**Figure 2D**). The combined supplementation of Mela 1 μM + Asta significantly increased the oocyte survival rate after the IVG stage. Nevertheless, no significant differences were detected among the treatment groups (**Figure 2 B**). Similarly, the combined Mela 10 μM + Asta supplementation increased survival after 4 days of the IVG stage (**Figure 2E**). However, the quality of oocytes after 4 days of culture showed significant differences. In this case, supplementation with combined Mela 1 μM + Asta may enhance the expression of specific genes. In contrast, the combined Mela 10 μM + Asta groups generally decreased the expression of most of the genes studied (**Figure 2C, F**). OCGCs supplemented with combined Mela 1 μM + Asta 0.05 μM showed the highest quality after 4 days of IVG, yielding results comparable to fully grown oocytes (LAF). In contrast, groups supplemented with higher concentrations of Mela and Asta exhibited a significant reduction in quality. Both

combined Mela 1 μM + Asta and Mela 10 μM + Asta groups demonstrated an increase in *CAT* expression; however, it was still lower than LAF. OCGCs supplemented with combined Mela 1 μM + Asta showed increased expression of *SOD1* and *GPX4*, while a decrease was observed in the combined Mela 10 μM + Asta group. Among the treatment groups, the combined Mela 1 μM + Asta 0.05 μM group exhibited the highest expression for all genes studied. Therefore, supplementation with combined Mela 1 μM + Asta 0.05 μM significantly increased the oocyte survival rate and quality after IVG.

Effect of antioxidant supplementation on histone modification of OCGCs and oocyte diameter after 2 and 4 days of IVG

SAF is the preceding stage of LAF; therefore, growing oocytes isolated from SAF must undergo a pre-IVM phase to attain competence equivalent to that of growing oocytes isolated from LAF⁵. Since the growing oocytes isolated from EAF are of a size comparable to those isolated from SAF, SAF is used for comparative evaluation after 2 days of IVG (**Figure 3C**). After 2 days of IVG, oocytes supplemented with Mela increased Me-H3-K4 expression compared to the non-treated group (**Figure 3B**). After 4 days of IVG, Me-H3-K4 levels decreased in all treatment groups as well as in the non-treated group. In **Figure 3D**, Me-H3-K4 is observed to be concentrated only in the heterochromatin ring, indicating the repression of transcriptional activities. This suggests that the addition of antioxidants does not lead to abnormalities in chromosome configuration²⁶. Therefore, the evaluation of Me-H3-K4 was only performed at IVG day 2. Due to the increase

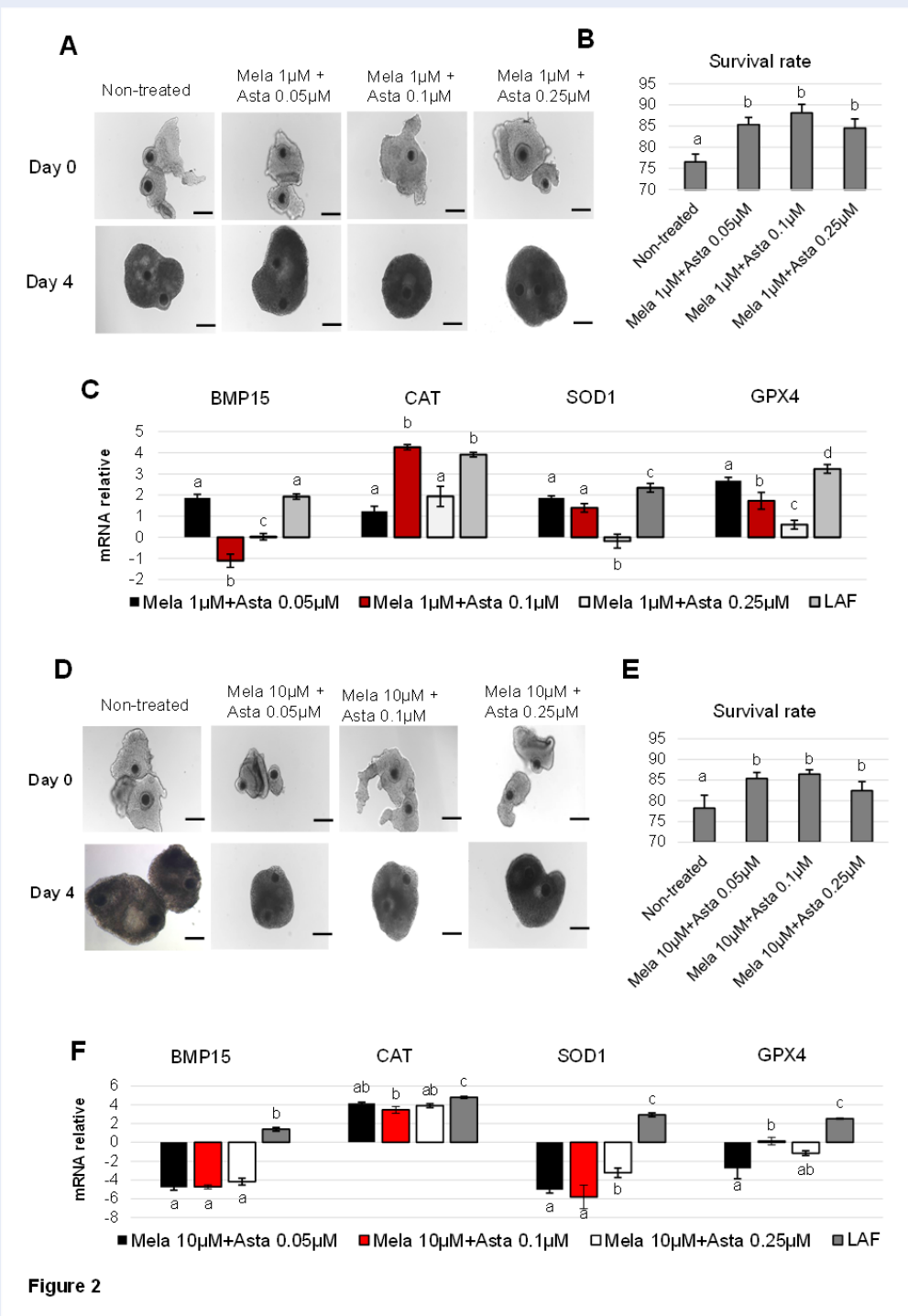


Figure 2: Impact of combined Mela and Asta supplementation on the quality of oocyte-cumulus-granulosa complexes (OCGCs) after IVG. Effect of Mela 1 mM + Asta on growing oocytes derived from EAFs. A) Morphology assessment of the OCGCs was checked on days 0 and 4 in IVG; B) The survival rate of growth oocyte after 4 days IVG; C) RT-PCR results of OCGCs cultured with Mela 1 μM + Asta supplement after 4 days IVG. Effect of Mela 10 μM + Asta on oocyte derived from EAF; D) Morphology assessment of the OCGCs was checked on days 0 and 4 in IVG; E) The survival rate of growth oocyte after 4 days IVG; F) RT-PCR results of OCGCs cultured with Mela 10 μM + Asta supplement after 4 days IVG. Data are presented as the mean ± S.E.M. ^{a-c} values without a common superscript differed significantly (P < 0.05), Bars = 150 μm.

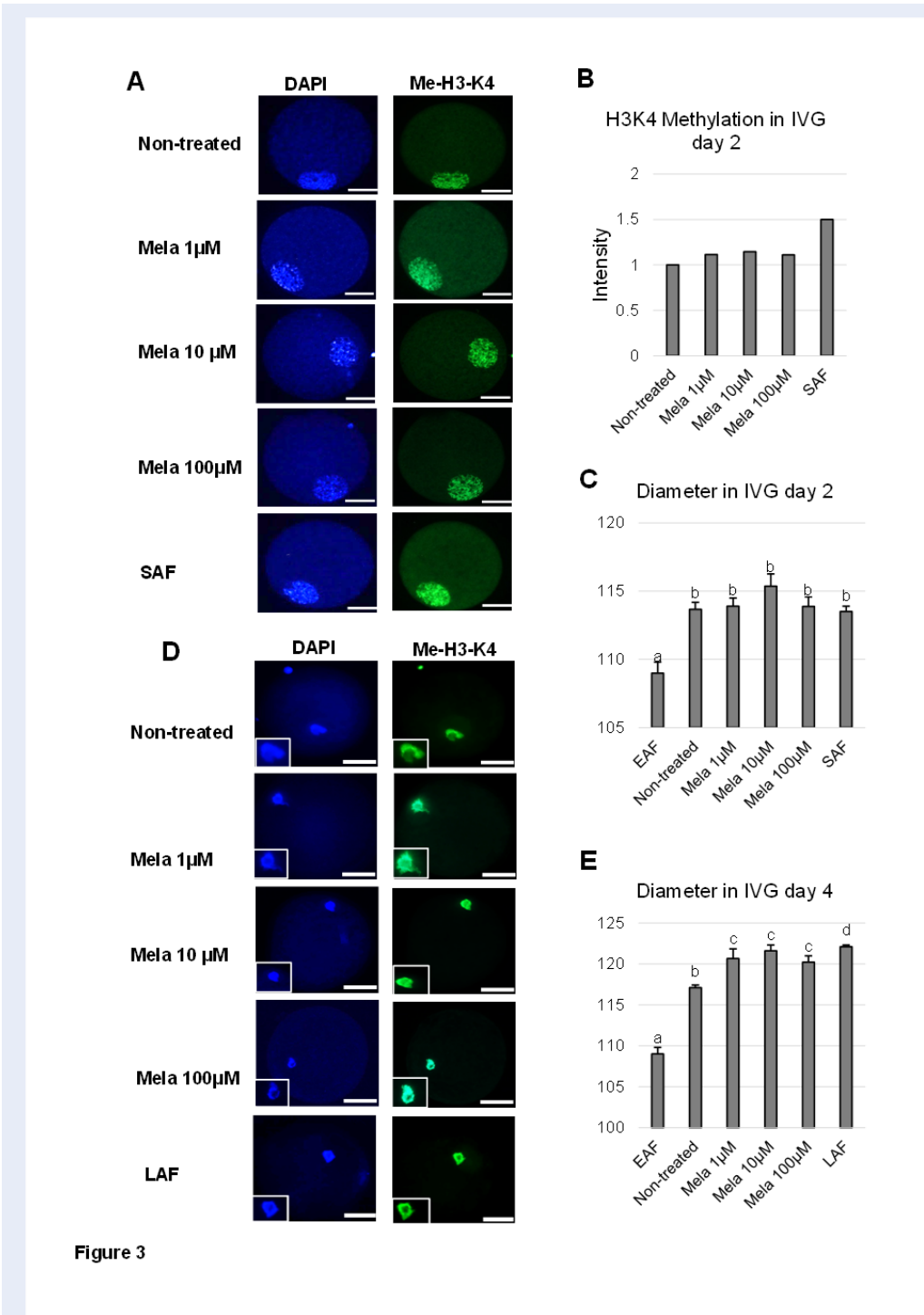


Figure 3: Effects of Mela supplementation on methylation of histone H3 and oocyte diameter after 2 and 4 days of IVG. **A)** Immunofluorescent staining of four groups of IVG porcine oocytes derived from early antral follicles (1–1.5 mm) supplemented with Mela (1 μM, 10 μM, 100 μM) after 2 days IVG. Methylation of Histone H3 at lysine 4 (Me–H3-K4) was expressed in green color. The nucleus and its membrane were stained with DAPI; **B)** The average Me–H3-K4 in corresponding oocytes in different concentrations of Mela (1μM, 10μM, 100μM) after 2 days IVG; **C)** Oocyte diameter supplemented with Mela after 2 days IVG; **D)** Immunofluorescent staining of four groups of IVG porcine oocytes derived from early antral follicles (1–1.5 mm) supplemented with Mela (1 μM, 10 μM, 100 μM) after 4 days IVG. Methylation of Histone H3 at lysine 4 (Me–H3-K4) was expressed in green color. The nucleus and its membrane were stained with DAPI; **E)** Oocyte diameter supplemented with Mela after 4 days IVG. Data are presented as the mean ± S.E.M. a-d values without a common superscript differed significantly ($P < 0.05$). Bars = 30 μm

in gene activity, a significant rise in oocyte size was also observed after 4 days of IVG (**Figure 3E**). Although oocytes exhibited a considerable increase in size following IVG, their diameter remained smaller than that of LAF. This suggests that Mela supplementation during IVG can enhance oocyte quality; however, it has not yet reached the level of LAF.

The groups supplemented with the combined Mela 1 μM + Asta 0.05 μM and the combined Mela 10 μM + Asta 0.05 μM showed an increase in Me-H3-K4 expression compared to the other groups (**Figure 4B, C**). However, both the combined Mela 1 μM + Asta 0.05 μM and combined Mela 10 μM + Asta 0.05 μM groups showed lower intensity than SAF. Therefore, while the oocytes cultured for 2 days had a size similar to SAF, their quality was still lower than that of SAF.

The transcriptional activity of the oocytes supplemented with the combined Mela 1 μM + Asta 0.05 μM was higher, as indicated by the greater BrUTP intensity compared to the other two groups, although the combined Mela 10 μM + Asta 0.05 μM group showed a trend towards higher intensity than the non-treated group (**Figure 5A, B**). Therefore, supplementation with combined Mela 1 μM + Asta 0.05 μM enhances global transcription, improving oocyte quality through increased mRNA storage. Because global transcription only occurs during the FC/SC stage, by day 4 the oocytes have reached the GV stage, where transcription activity has ceased. Therefore, the assessment of global transcription cannot be performed on day 4. Corresponding to the increase in global transcription, the groups supplemented with appropriate antioxidants also showed a significant increase in size compared to the non-treated group. Among these, the combined Mela 1 μM + Asta 0.05 μM group resulted in a notable size increase compared to the other groups, although it was still smaller than LAF (**Figure 5C**). Therefore, antioxidant supplementation improved both oocyte quality and size, but it has not yet reached the level of LAF.

DISCUSSION

Our study reported that supplementation with the combination of Mela and Asta at appropriate concentrations improved oocyte quality by increasing Me-H3-K4 and global transcriptional activity of growing oocytes after 2 days of IVG. Finally, there was a significant increase in the expression of *BMP15*, an important folliculogenesis regulator gene, as well as antioxidant genes (*CAT*, *SOD1*, *GPX4*), thereby reducing the negative effects of ROS on

oocytes after IVG. Our culture system supported the growth of oocytes isolated from EAFs to achieve a size and quality equivalent to fully grown oocytes isolated from LAF.

Mela has been shown to mediate its effects on oocytes through MT1 and MT2 membrane receptors, binding to melatonin receptors in the plasma membrane and binding to intracellular proteins such as calmodulin and calreticulin, which helps increase the follicle survival rate¹³. It has proven effective in IVM culture by stimulating the resumption of meiosis³⁷. Additionally, Mela has been demonstrated to improve granulosa cell quality and mitochondrial activity and to reduce cathepsin B activities^{31,38,39}, thereby enhancing oocyte quality. This is consistent with the significant increase in oocyte diameter and marker gene activity (**Figure 1D**, **Figure 3E**). Although the differences in marker gene activity were not substantial compared to the non-treated group, a significant improvement in oocyte size was observed in the Mela-supplemented groups. However, OCGCs supplemented with 100 μM Mela exhibited a tendency toward reduced oocyte size compared to those treated with 1 μM and 10 μM Mela. This suggests that while 100 μM Mela still has some positive effects, its adverse impacts begin to emerge, leading to a reduced overall efficacy compared to lower concentrations.

Asta neutralizes reactive oxygen, nitrogen, sulfur, and carbon species by donating electrons or forming stable bonds with free radicals to inactivate them. Asta also induces antioxidant genes and suppresses apoptotic genes in bovine embryos⁴⁰. Asta may prevent glutamate-induced apoptosis by preserving redox balance and suppressing glutamate-triggered calcium influx and endoplasmic reticulum stress⁴¹. It is reported that Me-H3-K4 is highly enriched at transcription start sites, and its presence in enhancers has also been linked to increased transcriptional activity⁴². Thus, increased Me-H3-K4 was associated with increased gene expression. Moreover, combining both Mela and Asta in IVG is promising due to their different mechanisms of action. We found that combining Asta and Mela at optimal concentrations increased Me-H3-K4 levels and global transcription activity, resulting in improved oocyte quality after IVG. This also significantly improved the survival rate compared to the non-treated group.

Following a 4-day IVG culture, growing oocytes derived from EAFs exhibit chromatin morphology and oocyte diameter similar to those of fully grown oocytes derived from LAFs. Gene expression studies

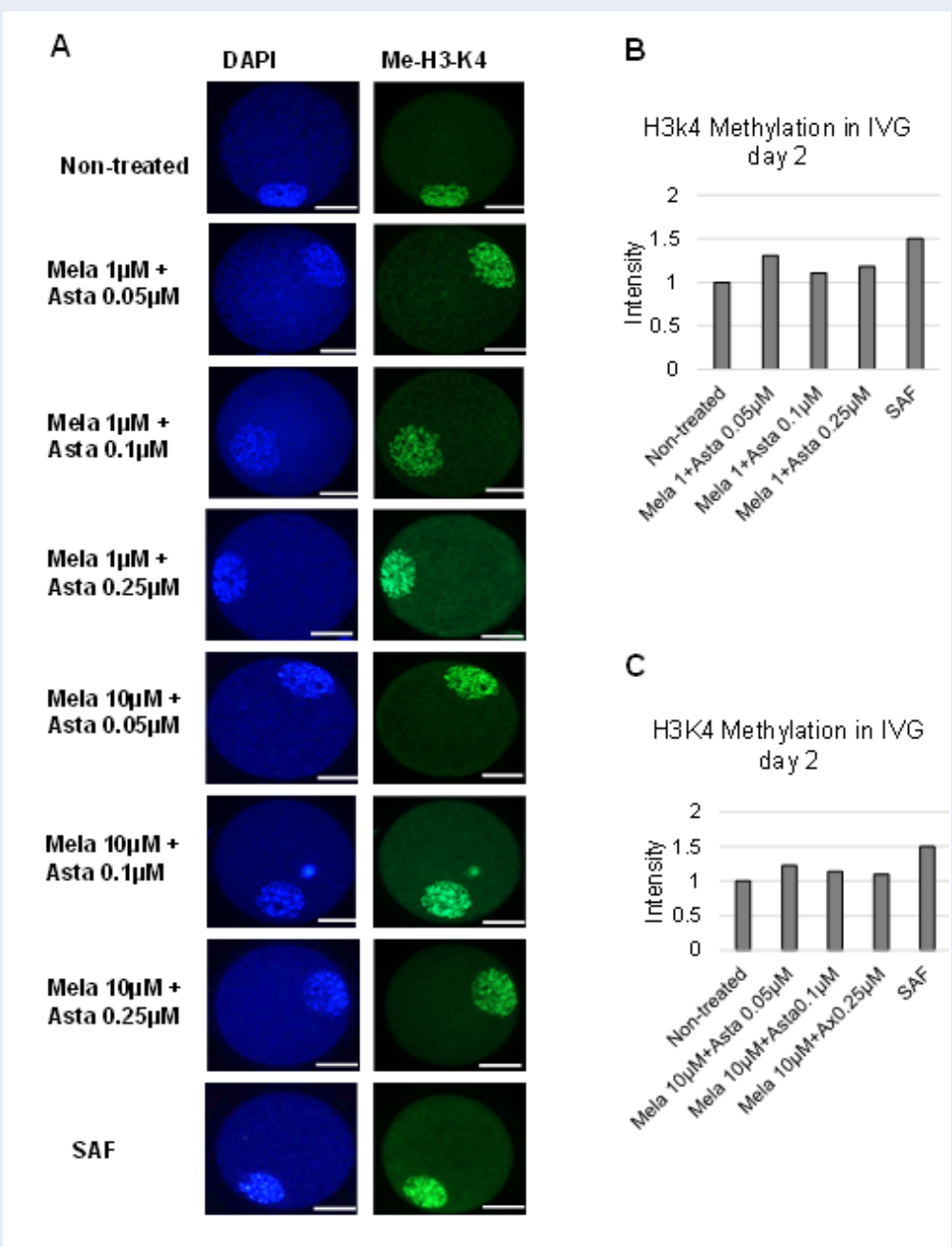


Figure 4: Effects of combined Mela and Asta on methylation of histone H3 after 2 and 4 days of IVG. **A)** Immunofluorescent staining of nine groups of IVG porcine oocytes derived from early antral follicles (1–1.5 mm) supplemented with Mela 1μM + Asta (0.05 μM, 0.1 μM, 0.25 μM), Mela 10 μM + Asta (0.05 μM, 0.1 μM, 0.25 μM) after 2 days IVG. Methylation of Histone H3 at lysine 4 (Me–H3–K4) was expressed in green color. The nucleus and its membrane were stained with DAPI; **B)** The average Me–H3–K4 in corresponding oocytes in different concentrations of combined Mela 1 μM + Asta (0.05 μM, 0.1 μM, 0.25 μM); **C)** The average Me–H3–K4 in corresponding oocytes in different concentrations of combined Mela 10 μM + Asta (0.05 μM, 0.1 μM, 0.25 μM). Bars = 30 μm

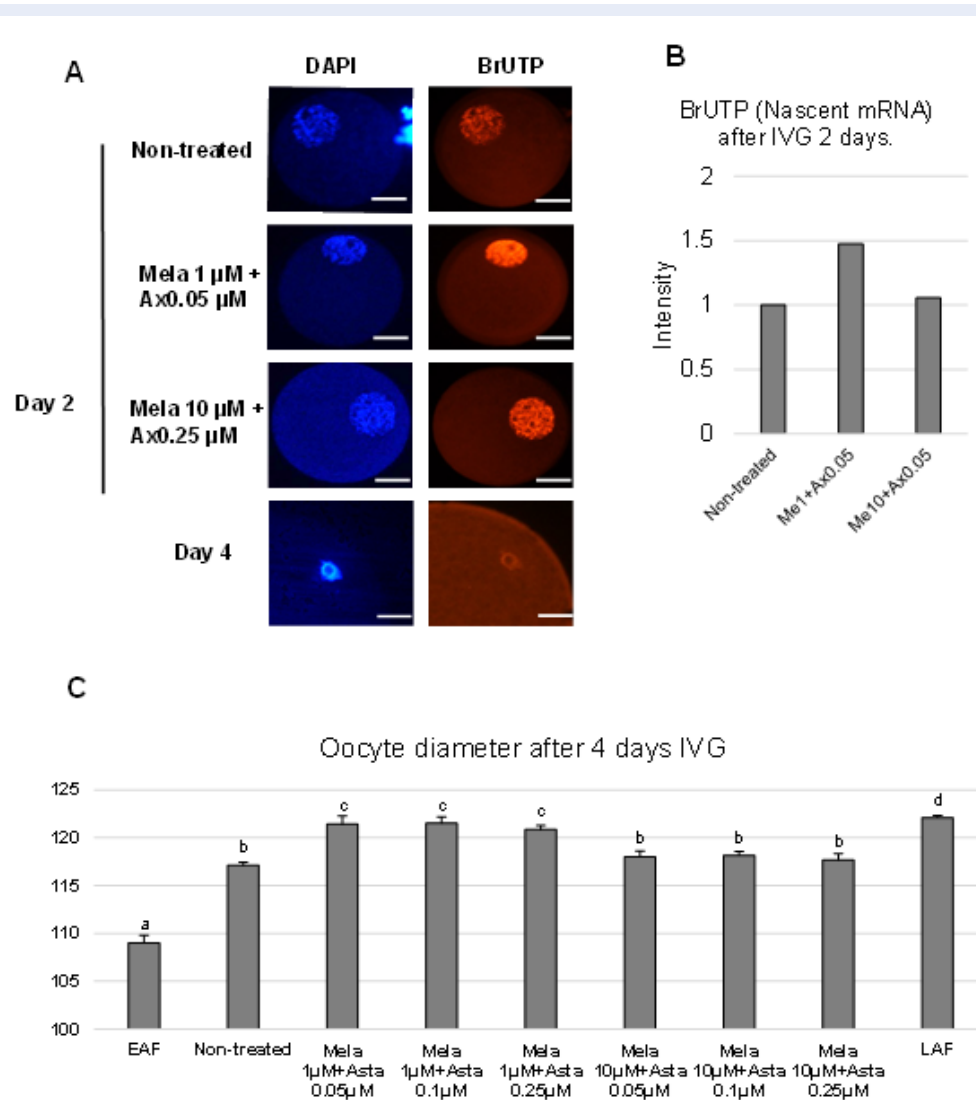


Figure 5: Effect of antioxidant supplementation on nascent mRNA synthesis in oocytes after IVG. A) Immunofluorescent staining of two groups of IVG porcine oocytes derived from early antral follicles (1–1.5 mm) supplemented with Mela 1 μ M + Asta 0.05 μ M, Mela 10 μ M + Asta 0.05 μ M. BrUTP (nascent mRNA) was expressed in red color. The nucleus and its membrane were stained with DAPI; B) The average nascent mRNA in corresponding oocytes in different supplemented with Mela 1 μ M + Ax 0.05 μ M group, Mela 10 μ M + Asta 0.05 μ M group, and non-treated group after 2 days IVG; C) Oocyte diameter after 4 days IVG. Data are presented as the mean $\pm$ S.E.M. a-d values without a common superscript differed significantly ($P < 0.05$). Bars = 30 μ m

on oocytes supplemented with Mela alone showed a minimal increase compared to the non-treated group. OCGCs cultured in IVG medium supplemented with Mela and Asta also exhibited a significant increase in CAT activity, similar to supplementation with both ascorbic acid and resveratrol⁴³ or coenzyme Q10, which increased antioxidant gene activities⁴⁴. In mammals, BMP15 is known to play a role in oocyte maturation and cholesterol biosynthesis, as well as enhancing oocyte competence and pro-

moting early embryo development⁴⁵. Interestingly, combinatory supplementation of astaxanthin and melatonin at lower concentrations demonstrated enhanced BMP15 activity levels in growing oocytes. Similarly, supplementation with Eugenol resulted in significantly higher BMP15 levels⁴⁶. Therefore, the significant increase in BMP15 and antioxidant gene activities contributes to a notable improvement in oocyte quality after IVG. Consequently, the supplementation of astaxanthin at a concentration of 0.05

and melatonin at a concentration of 1 during the IVG culture emerges as the most optimal culture condition.

However, supplementation with high concentrations of Mela led to a significant increase in ROS, consequently reducing oocyte quality^{47,48}. This is consistent with the significant reduction in the activity of antioxidant genes and BMP15 gene expression (Figure 2F). Moreover, a notable decrease in oocyte size was observed compared to the group supplemented with antioxidants at appropriate concentrations. While the supplementation of 10 μ M Mela and Asta did not directly induce oocyte death, it still exhibited negative effects on oocyte quality. This has been demonstrated, as supplementation with high concentrations of Kaempferol and ascorbic acid resulted in lower oocyte quality compared to supplementation with either compound alone⁴⁹. However, due to certain limitations of the study, it was not possible to clearly distinguish between healthy oocytes and those initiating the apoptosis pathway. As a result, it remains unclear whether the combination of Mela and Asta at inappropriate concentrations truly triggers apoptosis at IVG day 4.

This suggests that excessive supplementation of antioxidants may lead to the disruption of cellular metabolism. When Asta was supplemented with 1 μ M Mela, enhanced transcription and Me-H3-K4 levels were observed during 2 days of IVG. It can be concluded that the beneficial effects of Asta are obtained only when combined with lower concentrations of Mela, resulting in improved oocyte quality and overall outcomes in culture. Although OCGCs have been shown to improve quality through evaluations conducted after IVG, further in-depth assessment is necessary at the pre-implantation stage. This would provide a better understanding of mRNA accumulation throughout the IVG process, which could contribute to improving embryo quality. Additionally, this study has not yet evaluated the concentrations of ROS-related compounds such as superoxide, hydrogen peroxide, hydroxyl ions, peroxy radicals, and hypochlorite ions. Moreover, since the method for assessing nascent mRNA has not been fully optimized, the oocyte survival rate after BrUTP incorporation remains low, resulting in a limited sample size for evaluation. Therefore, additional methods should be implemented to further assess nascent mRNA synthesis capacity.

CONCLUSIONS

Growing oocytes isolated from EAFs can attain competence nearly equivalent to fully grown oocytes

isolated from LAFs when cultured in our optimal IVG culture systems. Supplementation of antioxidants at appropriate concentrations supports increases in oocyte size, survival rate, and quality by significantly enhancing Me-H3-K4 intensity, global transcription (nascent mRNA), BMP15 expression, as well as antioxidant gene expression (CAT, SOD1, GPX4).

ABBREVIATIONS

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 4',6-diamidino-2-phenylindole (DAPI), alpha minimum essential medium (α -MEM), analysis of variance (ANOVA), astaxanthin (Asta), beta-actin (ActB), bone morphogenetic protein 15 (BMP15), bromouridine triphosphate (BrUTP), carbon dioxide (CO₂), catalase (CAT), early antral follicles (EAFs), electro-permeabilization (EP), fetal bovine serum (FBS), filamentous/stringy chromatin (FC/SC), germinal vesicle (GV), glutathione (GSH), glutathione peroxidase 4 (GPX4), histone H3 lysine 4 methylation (Me-H3-K4), in vitro growth (IVG), in vitro maturation (IVM), large antral follicles (LAFs), log2 fold change (Log2FC), melatonin (Mela), melatonin receptor types 1 and 2 (MT1/MT2), nuclear factor erythroid 2-related factor 2 (NRF2), oocyte cumulus granulosa cell complexes (OCGCs), ovarian tissue cryopreservation (OTC), oxidative stress (OS), phosphate-buffered saline (PBS), polyvinylpyrrolidone (PVP), pre-antral follicles (PAFs), quantitative polymerase chain reaction (qPCR), reactive oxygen species (ROS), Real-time polymerase chain reaction (RT-PCR), small antral follicles (SAFs), sodium pyruvate (NaPy), standard error of the mean (SEM), Statistical Package for the Social Sciences (SPSS), superoxide dismutase 1 (SOD1), and tissue culture medium-199 (TCM-199).

ACKNOWLEDGMENTS

We would like to express our heartfelt gratitude to the International University – National University HCMC for providing an excellent research environment in the Cell Reprogramming Laboratory. We extend our thanks to the slaughterhouse Vissan, Tan Binh, and Ut Hao for supplying pig ovaries for research.

AUTHOR'S CONTRIBUTIONS

L.C.T and L.D.T.P designed the experiments, analyzed, investigate, writing – original draft. P.T.D and N.L.B.L and L.B.A.M and C.D.S.P and N.N.T.V and N.T.N: investigated, performed, and analyzed the experiments. N.V.T and H.T.B: project administration,

methodology, discussed the results, writing – review & editing. All authors read and approved the final manuscript.

FUNDING

This research is funded by Vietnam National University HoChiMinh City (VNU-HCM) under grant number B2022-28-01.

AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

- Kubo N, Cayo-Colca IS, Miyano T. Effect of estradiol-17 β during in vitro growth culture on the growth, maturation, cumulus expansion and development of porcine oocytes from early antral follicles. *Animal Science Journal*. 2015;86(3):251–9. PMID: 25410821. Available from: <https://doi.org/10.1111/asj.12283>.
- Ebrahimi M, Dattena M, Mara L, Pasciu V, Sotgiu FD, Chessa F. In vitro production of meiotically competent oocytes from early antral follicles in sheep. *Theriogenology*. 2024;226:253–62. PMID: 38950486. Available from: <https://doi.org/10.1016/j.theriogenology.2024.06.030>.
- de Sá NA, Ferreira AC, Sousa FG, Duarte AB, Paes VM, Cadenas J. First pregnancy after in vitro culture of early antral follicles in goats: positive effects of anethole on follicle development and steroidogenesis. *Molecular Reproduction and Development*. 2020;87(9):966–77. PMID: 32761832. Available from: <https://doi.org/10.1002/mrd.23410>.
- Barros RG, Lodde V, Franciosi F, Luciano AM. A refined culture system of oocytes from early antral follicles promotes oocyte maturation and embryo development in cattle. *Reproduction : The Official Journal of the Society for the Study of Fertility*. 2023;165(2):221–33. PMID: 36473031. Available from: <https://doi.org/10.1530/REP-22-0277>.
- Van NTT, My LBA, Thuan NV, Bui HT. Improve the developmental competence of porcine oocytes from small antral follicles by pre-maturation culture method. *Theriogenology*. 2020;149:139–48. PMID: 32272343. Available from: <https://doi.org/10.1016/j.theriogenology.2020.02.038>.
- Munakata Y, Sugimoto A, Shirasuna K, Kuwayama T, Iwata H. Xanthan gum and Locust bean gum gel supports in vitro development of porcine oocytes derived from early antral follicles. *The Journal of Reproduction and Development*. 2019;65(6):551–4. PMID: 31588063. Available from: <https://doi.org/10.1262/jrd.2019-074>.
- Munakata Y, Kawahara-Miki R, Shirasuna K, Kuwayama T, Iwata H. Polyacrylamide gel as a culture substrate improves in vitro oocyte growth from porcine early antral follicles. *Molecular Reproduction and Development*. 2017;84(1):44–54. PMID: 27864905. Available from: <https://doi.org/10.1002/mrd.22758>.
- Le BA, Nguyen LB, Lam DT, Lam CT, Nguyen NT, Nguyen VT. Agarose-based 3D culture improved the developmental competence of oocyte-granulosa complex isolated from porcine preantral follicle. *Theriogenology*. 2024;223:11–21. PMID: 38657435. Available from: <https://doi.org/10.1016/j.theriogenology.2024.04.010>.
- Shields HJ, Traa A, Van Raamsdonk JM. Beneficial and Detrimental Effects of Reactive Oxygen Species on Lifespan: A Comprehensive Review of Comparative and Experimental Studies. *Frontiers in Cell and Developmental Biology*. 2021;9:628157. PMID: 33644065. Available from: <https://doi.org/10.3389/fcell.2021.628157>.
- Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *Journal of Pineal Research*. 2008;44(3):280–7. PMID: 18339123. Available from: <https://doi.org/10.1111/j.1600-079X.2007.00524.x>.
- Kang JT, Koo OJ, Kwon DK, Park HJ, Jang G, Kang SK. Effects of melatonin on in vitro maturation of porcine oocyte and expression of melatonin receptor RNA in cumulus and granulosa cells. *Journal of Pineal Research*. 2009;46(1):22–8. PMID: 18494781. Available from: <https://doi.org/10.1111/j.1600-079X.2008.00602.x>.
- Khalil WA, Marei WF, Khalid M. Protective effects of antioxidants on linoleic acid-treated bovine oocytes during maturation and subsequent embryo development. *Theriogenology*. 2013;80(2):161–8. PMID: 23683689. Available from: <https://doi.org/10.1016/j.theriogenology.2013.04.008>.
- Cebrian-Serrano A, Salvador I, Raga E, Dinnyes A, Silvestre MA. Beneficial effect of melatonin on blastocyst in vitro production from heat-stressed bovine oocytes. *Reproduction in Domestic Animals = Zuchthygiene*. 2013;48(5):738–46. PMID: 23458773. Available from: <https://doi.org/10.1111/rda.12154>.
- Phuong LD, Thien LC, Pham CDS, Minh NU, Bao NTH, Truc LNT. Melatonin and cyclic adenosine monophosphate enhance the meiotic and developmental competence of porcine oocytes from early antral follicles during in vitro growth and pre-maturation culture. *Theriogenology*. 2025;237:129–42. PMID: 40015084. Available from: <https://doi.org/10.1016/j.theriogenology.2025.02.026>.
- Higuera-Ciapara I, Félix-Valenzuela L, Goycoolea FM. Astaxanthin: a review of its chemistry and applications. *Critical Reviews in Food Science and Nutrition*. 2006;46(2):185–96. PMID: 16431409. Available from: <https://doi.org/10.1080/10408690590957188>.
- Hama S, Uenishi S, Yamada A, Ohgita T, Tsuchiya H, Yamashita E, et al. Scavenging of hydroxyl radicals in aqueous solution by astaxanthin encapsulated in liposomes. *Biological & Pharmaceutical Bulletin*. 2012;35(12):2238–42. PMID: 23207776. Available from: <https://doi.org/10.1248/bpb.b12-00715>.
- Ambati RR, Phang SM, Ravi S, Aswathanarayana RG. Astaxanthin: sources, extraction, stability, biological activities and its commercial applications - a review. *Marine Drugs*. 2014;12(1):128–52. PMID: 24402174. Available from: <https://doi.org/10.3390/md12010128>.
- Sztretye M, Dienes B, Gönczi M, Cziráj T, Csernoch L, Dux L, et al. Astaxanthin: A Potential Mitochondrial-Targeted Antioxidant Treatment in Diseases and with Aging. *Oxidative Medicine and Cellular Longevity*. 2019;2019(1):3849692. PMID: 31814873. Available from: <https://doi.org/10.1155/2019/3849692>.
- Naguib YM. Antioxidant activities of astaxanthin and related carotenoids. *Journal of Agricultural and Food Chemistry*. 2000;48(4):1150–4. PMID: 10775364. Available from: <https://doi.org/10.1021/jf991106k>.

20. Namekawa T, Ikeda S, Sugimoto M, Kume S. Effects of astaxanthin-containing oil on development and stress-related gene expression of bovine embryos exposed to heat stress. *Reproduction in Domestic Animals = Zuchthygiene*. 2010;45(6):e387–91. PMID: 20210879. Available from: <https://doi.org/10.1111/j.1439-0531.2010.01584.x>.
21. Do LT, Luu VV, Morita Y, Taniguchi M, Nii M, Peter AT. Astaxanthin present in the maturation medium reduces negative effects of heat shock on the developmental competence of porcine oocytes. *Reproductive Biology*. 2015;15(2):86–93. PMID: 26051456. Available from: <https://doi.org/10.1016/j.repbio.2015.01.002>.
22. Li R, Wu H, Zhuo WW, Mao QF, Lan H, Zhang Y. Astaxanthin Normalizes Epigenetic Modifications of Bovine Somatic Cell Cloned Embryos and Decreases the Generation of Lipid Peroxidation. *Reproduction in Domestic Animals = Zuchthygiene*. 2015;50(5):793–9. PMID: 26280670. Available from: <https://doi.org/10.1111/rda.12589>.
23. Eslami M, Esfandyari S, Aghahosseini M, Rashidi Z, Hosseini SH, Brenjian S. Astaxanthin Protects Human Granulosa Cells against Oxidative Stress through Activation of NRF2/ARE Pathway and Its Downstream Phase II Enzymes. *Cell Journal*. 2021;23(3):319–28. PMID: 34308575.
24. Chelenga M, Sakaguchi K, Abdel-Ghani MA, Yanagawa Y, Katagiri S, Nagano M. Effect of increased oxygen availability and astaxanthin supplementation on the growth, maturation and developmental competence of bovine oocytes derived from early antral follicles. *Theriogenology*. 2020;157:341–9. PMID: 32853969. Available from: <https://doi.org/10.1016/j.theriogenology.2020.07.023>.
25. Navid S, Saadatian Z, Talebi A. Assessment of developmental rate of mouse embryos yielded from in vitro fertilization of the oocyte with treatment of melatonin and vitamin C simultaneously. *BMC Women's Health*. 2023;23(1):525. PMID: 37794412. Available from: <https://doi.org/10.1186/s12905-023-02673-w>.
26. Bui HT, Thuan NV, Kishigami S, Wakayama S, Hikichi T, Ohta H. Regulation of chromatin and chromosome morphology by histone H3 modifications in pig oocytes. *Reproduction : The Official Journal of the Society for the Study of Fertility*. 2007;133(2):371–82. PMID: 17307905. Available from: <https://doi.org/10.1530/REP-06-0099>.
27. Bui HT, Hwang KC, Kim JH, Thuan NV, Wakayama T, Miyano T. Effect of vanadate on the chromatin configuration in pig GV-oocytes. *The Journal of Reproduction and Development*. 2009;55(4):367–72. PMID: 19404002. Available from: <https://doi.org/10.1262/jrd.20173>.
28. Vázquez J, González B, Sempere V, Mas A, Torija MJ, Beltran G. Melatonin Reduces Oxidative Stress Damage Induced by Hydrogen Peroxide in *Saccharomyces cerevisiae*. *Frontiers in Microbiology*. 2017;8:1066. PMID: 28663741. Available from: <https://doi.org/10.3389/fmicb.2017.01066>.
29. Ganji R, Nabiuni M, Faraji R. Development of mouse preantral follicle after in vitro culture in a medium containing melatonin. *Cell Journal*. 2015;16(4):546–53. PMID: 25685745.
30. Rocha RM, Lima LF, Alves AM, Celestino JJ, Matos MH, Lima-Verde IB. Interaction between melatonin and follicle-stimulating hormone promotes in vitro development of caprine preantral follicles. *Domestic Animal Endocrinology*. 2013;44(1):1–9. PMID: 22920266. Available from: <https://doi.org/10.1016/j.domaniend.2012.07.001>.
31. Barros VR, Monte AP, Santos JM, Lins TL, Cavalcante AY, Gouveia BB. Melatonin improves development, mitochondrial function and promotes the meiotic resumption of sheep oocytes from in vitro grown secondary follicles. *Theriogenology*. 2020;144:67–73. PMID: 31918071. Available from: <https://doi.org/10.1016/j.theriogenology.2019.12.006>.
32. Petry A, Görlach A. Regulation of NADPH Oxidases by G Protein-Coupled Receptors. *Antioxidants & Redox Signaling*. 2019;30(1):74–94. PMID: 29466865. Available from: <https://doi.org/10.1089/ars.2018.7525>.
33. Su YQ, Sugiura K, Wigglesworth K, O'Brien MJ, Affourtit JP, Pangas SA. Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: BMP15 and GDF9 control cholesterol biosynthesis in cumulus cells. *Development (Cambridge, England)*. 2008;135(1):111–21. PMID: 18045843. Available from: <https://doi.org/10.1242/dev.009068>.
34. Bjerregaard B, Wrenzycki C, Philimonenko VV, Hozak P, Laurincik J, Niemann H. Regulation of ribosomal RNA synthesis during the final phases of porcine oocyte growth. *Biology of Reproduction*. 2004;70(4):925–35. PMID: 14627545. Available from: <https://doi.org/10.1095/biolreprod.103.020941>.
35. Bui HT, Wakayama S, Mizutani E, Park KK, Kim JH, Thuan NV. Essential role of paternal chromatin in the regulation of transcriptional activity during mouse preimplantation development. *Reproduction : The Official Journal of the Society for the Study of Fertility*. 2011;141(1):67–77. PMID: 20974742. Available from: <https://doi.org/10.1530/REP-10-0109>.
36. Bui HT, Yamaoka E, Miyano T. Involvement of histone H3 (Ser10) phosphorylation in chromosome condensation without Cdc2 kinase and mitogen-activated protein kinase activation in pig oocytes. *Biology of Reproduction*. 2004;70(6):1843–51. PMID: 14960481. Available from: <https://doi.org/10.1095/biolreprod.103.026070>.
37. Zhang J, Li F, Zhang X, Xie T, Qin H, Lv J. Melatonin Improves Turbot Oocyte Meiotic Maturation and Antioxidant Capacity, Inhibits Apoptosis-Related Genes mRNAs In Vitro. *Antioxidants*. 2023;12(7):1389. PMID: 37507927. Available from: <https://doi.org/10.3390/antiox12071389>.
38. Cao Z, Gao D, Tong X, Xu T, Zhang D, Wang Y. Melatonin improves developmental competence of oocyte-granulosa cell complexes from porcine preantral follicles. *Theriogenology*. 2019;133:149–58. PMID: 31100559. Available from: <https://doi.org/10.1016/j.theriogenology.2019.05.003>.
39. Gao Z, Luo K, Hu Y, Niu Y, Zhu X, Li S. Melatonin alleviates chronic stress-induced hippocampal microglia pyroptosis and subsequent depression-like behaviors by inhibiting Cathepsin B/NLRP3 signaling pathway in rats. *Translational Psychiatry*. 2024;14(1):166. PMID: 38538614. Available from: <https://doi.org/10.1038/s41398-024-02887-y>.
40. Jang HY, Ji SJ, Kim YH, Lee HY, Shin JS, Cheong HT. Antioxidative effects of astaxanthin against nitric oxide-induced oxidative stress on cell viability and gene expression in bovine oviduct epithelial cell and the developmental competence of bovine IVM/IVF embryos. *Reproduction in Domestic Animals = Zuchthygiene*. 2010;45(6):967–74. PMID: 19930137. Available from: <https://doi.org/10.1111/j.1439-0531.2009.01469.x>.
41. Lin X, Zhao Y, Li S. Astaxanthin attenuates glutamate-induced apoptosis via inhibition of calcium influx and endoplasmic reticulum stress. *European Journal of Pharmacology*. 2017;806:43–51. PMID: 28400209. Available from: <https://doi.org/10.1016/j.ejphar.2017.04.008>.
42. Wang H, Helin K. Roles of H3K4 methylation in biology and disease. *Trends in Cell Biology*. 2025;35(2):115–28. Available from: <https://doi.org/10.1016/j.tcb.2024.06.001>.
43. Costa FC, Silva BR, Filho FF, Bezerra VS, Azevedo VA, Silva AA. Ascorbic acid and resveratrol improve the structural integrity of the extracellular matrix and enhance follicular survival in cultured bovine ovarian tissue. *Theriogenology*. 2025;235:231–44. PMID: 39874799. Available from: <https://doi.org/10.1016/j.theriogenology.2025.01.020>.
44. Hosseinzadeh E, Zavareh S, Lashkarbolouki T. Antioxidant properties of coenzyme Q10-pretreated mouse pre-antral follicles derived from vitrified ovaries. *The Journal of Obstetrics and Gynaecology Research*. 2017;43(1):140–8. PMID: 27935208. Available from: <https://doi.org/10.1111/jog.13173>.
45. Caixeta ES, Sutton-McDowall ML, Gilchrist RB, Thompson JG, Price CA, Machado MF, et al. Bone morphogenetic protein 15 and fibroblast growth factor 10 enhance cumulus expansion, glucose uptake, and expression of genes in the ovulatory cascade during in vitro maturation of bovine cumulus-oocyte complexes. *Reproduction*. 2013;146(1):27–35. Available from: <https://doi.org/10.1530/REP-13-0079>.

46. Silva RF, Lima LF, Ferreira AC, Silva AF, Alves DR, Alves BG, et al. Eugenol Improves Follicular Survival and Development During in vitro Culture of Goat Ovarian Tissue. *Frontiers in Veterinary Science*. 2022;9:822367. PMID: 35573397. Available from: <https://doi.org/10.3389/fvets.2022.822367>.
47. Chen K, Zhu P, Chen W, Luo K, Shi XJ, Zhai W. Melatonin inhibits proliferation, migration, and invasion by inducing ROS-mediated apoptosis via suppression of the PI3K/Akt/mTOR signaling pathway in gallbladder cancer cells. *Aging*. 2021;13(18):22502–15. PMID: 34580235. Available from: <https://doi.org/10.18632/aging.203561>.
48. Moreira AJ, Ordoñez R, Cerski CT, Picada JN, García-Palomo A, Marroni NP, et al. Melatonin Activates Endoplasmic Reticulum Stress and Apoptosis in Rats with Diethylnitrosamine-Induced Hepatocarcinogenesis. *PLoS One*. 2015;10(12):e0144517. PMID: 26656265. Available from: <https://doi.org/10.1371/journal.pone.0144517>.
49. Santos JM, Monte AP, Lins TL, Barberino RS, Menezes VG, Gouveia BB. Kaempferol can be used as the single antioxidant in the in vitro culture medium, stimulating sheep secondary follicle development through the phosphatidylinositol 3-kinase signaling pathway. *Theriogenology*. 2019;136:86–94. PMID: 31254726. Available from: <https://doi.org/10.1016/j.theriogenology.2019.06.036>.