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Effects of varying β-caryophyllene levels on nuclear and cytoplasmic maturation in bovine oocytes

Efeitos de níveis variáveis de β-cariofileno sobre a maturação nuclear e citoplasmática em oócitos bovinos

Antonia Beatriz Mendonça Pereira[1]; Lhara Ricarliany Medeiros de Oliveira[2]; Leonardo Vitorino Costa de Aquino[2]; Ana Lívia Rocha Rodrigues[3]; Vinicius Dantas da Silva[3]; Luciana Medeiros Bertini[4]; Alexsandra Fernandes Pereira[5]*

Highlights

- 5 µM β-caryophyllene promotes high rates of bovine oocytes with first polar body.
- 1 µM β-caryophyllene boosts cytoplasmic maturation rates in bovine oocytes.
- 1, 5 and 10 µM β-caryophyllene reduce ROS and increase GSH in IVM bovine oocytes.

Abstract

This study aimed to evaluate the effects of β-caryophyllene and its antioxidant potential at different concentrations during *in vitro* maturation (IVM) of bovine oocytes. Immature oocytes were divided into the groups: without antioxidant (control group); and groups supplemented with 100 µM cysteamine (CYS), 1 µM (β1), 5 µM (β5), and 10 µM (β10) β-caryophyllene. After 24h00 IVM, oocytes were analyzed for *cumulus* cell viability and expansion, presence of the first polar body (1PB), metaphase II (MII), cytoplasmic maturation, levels of reactive oxygen species (ROS), intracellular glutathione (GSH) and mitochondrial membrane potential (ΔΨm). *Cumulus* cell expansion rate was similar among all antioxidant groups (CYS [93.8%], β1 [91.8%], β5 [93.7%], and β10 [93.3%]), being higher than the control group (87.4%, $P < 0.05$). However, β-caryophyllene reduced *cumulus* cell viability compared to the control and CYS groups ($P < 0.05$). MII rates ranged from 79.6% to 90.0% ($P < 0.05$) with no difference between groups. β5 (93.8%) showed a higher 1PB rate compared to the other groups ($P < 0.05$). In terms of cytoplasmic maturation, β1 showed a higher number of oocytes with dispersed mitochondria (87.7%),

[1] Student of the Undergraduate Course in Biotechnology, Universidade Federal Rural do Semi-Árido, UFERSA, Mossoró, RN, Brazil. E-mail: antonia.pereira@alunos.ufersa.edu.br
[2] Doctoral Students of the Postgraduate Program in Animal Science, UFERSA, Mossoró, RN, Brazil. E-mail: lharagirs@hotmail.com; leonardovt@live.com
[3] Students of the Undergraduate Course in Biotechnology, UFERSA, RN, Brazil. E-mail: ana.rodrigues61941@alunos.ufersa.edu.br; viniciusd647@gmail.com
[4] Profª Drª, Instituto Federal de Educação, Ciência, e Tecnologia do Rio Grande do Norte, IFRN, Apodi, RN, Brazil. E-mail: luciana.bertini@ifrn.edu.br
[5] Profª Drª, UFERSA, Mossoró, RN, Brazil. E-mail: alexsandra.pereira@ufersa.edu.br
* Author for correspondence

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similar to group CYS (80.6%) and superior to other treatments (P < 0.05). Additionally, β1 ensured a greater heterogeneous mitochondria distribution pattern (84.2%) when compared to the other groups (P < 0.05). Upon the evaluation of oxidative stress in arbitrary fluorescence units (AFU), CYS, β1, β5 and β10 reduced ROS levels (0.37 ± 0.2 *vs.* 0.42 ± 0.1 vs. 0.55 ± 0.1 vs. 0.48 ± 0.1), respectively, when compared to the control (1.00 ± 0.5), (P < 0.05). A similar response occurred in the GSH analysis, where the groups with antioxidants (CYS, β1, β5 and β10) presented higher levels (1.00 ± 0.19 *vs.* 0.96 ± 0.22 vs. 0.95 ± 0.46 vs. 1.01 ± 0.26), respectively, compared to the control (0.50 ± 0.17), (P < 0.05). However, only β5 (0.73 ± 0.40) was able to decrease ΔΨm compared to the control (P < 0.05). In summary, lower concentrations (1 and 5 μM) of β-caryophyllene ensure a reduction in oxidative stress and promote better conditions for bovine IVM.
Key words: Antioxidants. Cysteamine. Oxidative stress. Reactive oxygen species.

Resumo

Este estudo teve como objetivo avaliar os efeitos do β-cariofileno e seu potencial antioxidante em diferentes concentrações durante a maturação in vitro (MIV) de oócitos bovinos. Oócitos imaturos foram divididos nos grupos: sem antioxidante (grupo controle), suplementados com 100 μM de cisteamina (CIS), 1 μM (β1), 5 μM (β5) e 10 μM (β10) de β-cariofileno. Após 24h00 de MIV, oócitos foram analisados quanto à viabilidade e expansão das células do *cumulus,* presença do primeiro corpúsculo polar (1CP), metáfase II (MII), maturação citoplasmática, níveis de espécies reativas de oxigênio (EROs), glutationa intracelular (GSH) e potencial de membrana mitocondrial (ΔΨm). A taxa de expansão das células do cumulus foi semelhante entre todos os grupos antioxidantes [CIS (93,8%), β1 (91,8%), β5 (93,7%) e β10 (93,3%)], sendo maior que o grupo controle (87,4%, P < 0,05). Contudo, β-cariofileno reduziu a viabilidade das células do cumulus em comparação aos grupos controle e CIS (P < 0,05). As taxas de MII variaram de 79,6% a 90,0% sem diferença significativa (P < 0,05). β5 (93,8%) apresentou maior taxa de 1CP em comparação aos outros grupos (P < 0,05). Em termos de maturação citoplasmática, β1 apresentou maior número de oócitos com mitocôndrias dispersas (87,7%), semelhante ao grupo CIS (80,6%) e superior aos demais tratamentos (P < 0,05). Adicionalmente, β1 garantiu um maior padrão de distribuição heterogênea de mitocôndrias (84,2%) quando comparado aos demais (P < 0,05). Na avaliação do estresse oxidativo, em unidades de fluorescência arbitrárias (UFAs), CIS, β1, β5 e β10 reduziram os níveis de EROs (0,37 ± 0,2 vs. 0,42 ± 0,1 *vs.* 0,55 ± 0,1 vs. 0,48 ± 0,1), respectivamente, quando comparados ao controle (1,00 ± 0,5) (P < 0,05). Resposta semelhante ocorreu na análise de GSH, onde os grupos com antioxidantes (CIS, β1, β5 e β10) apresentaram níveis mais elevados (1,00 ± 0,19 *vs.* 0,96 ± 0,22 vs. 0,95 ± 0,46 vs. 1,01 ± 0,26), respectivamente, em comparação ao grupo controle (0,50 ± 0,17) (P < 0,05). Contudo, apenas β5 (0,73 ± 0,40) foi capaz de diminuir ΔΨm em relação ao controle (P < 0,05). Em síntese, concentrações menores (1 e 5 μM) de β-cariofileno garantem redução do estresse oxidativo, além de promover melhores condições para a MIV bovina.
Palavras-chave: Antioxidantes. Cisteamina. Espécies reativas de oxigênio. Estresse oxidativo.

Introduction

In vitro embryo production (IVEP) requires a set of steps to ensure embryo formation. Among these steps, *in vitro* maturation (IVM) stands out because it is the initial stage of embryonic development, responsible for promoting the maturation of immature oocytes (Lonergan & Fair, 2016). When oocytes are induced to IVM, conditions to which they are exposed in *in vitro* culture environments can result in changes in the oocyte quality, reducing the fertilization potential of the oocytes and preventing their development (García-Martínez et al., 2020). This phenomenon can be attributed to the insufficiency of intracellular antioxidants in controlling the increase in oxidative stress, resulting from the unbalanced production of reactive oxygen species (ROS), (Zhong & Zhou, 2013), which could lead to the interruption of oocyte IVM and reduce embryonic competence compared to *in vivo* embryos (Pioltine et al., 2021).

To minimize the consequences of oxidative stress, it is common to use synthetic antioxidants such as cysteamine. The use of 100 µM cysteamine during IVM of bovine oocytes promotes a nuclear maturation rate of 91.9%, while the group without antioxidant demonstrates a rate of 86.4% (Sovernigo et al., 2017). This also occurs at the levels of blastocyst formation, where the group treated with cysteamine presents a rate of 52.4% higher than the group without antioxidant, which presented 47.2% (Sovernigo et al., 2017). In addition to synthetic antioxidants, alternatives such as natural antioxidants derived from plants are currently being researched, as in the study by Santos et al. (2019), which tested the potential of *Syzygium aromaticum* L. essential oil in bovine oocytes and showed that a concentration of 20 µg/mL of the oil was able to reduce the mitochondrial membrane potential (ΔΨm) and improve embryonic development.

Subsequently, when evaluating the isolated bioactives from the essential oil of *Syzygium aromaticum* L. (eugenol, β-caryophyllene and eugenyl acetate), it was possible to observe rates above 82% of MII and 63% of blastocysts on D8, using a single concentration of these bioactives (Oliveira et al., 2022). Although β-caryophyllene was not highlighted as the best bioactive in this study, an improvement in the progression to MII (76.2%) was observed compared to the control group (62.1%) and there was also a reduction in ROS levels during IVM (Oliveira et al., 2022). However, the concentration used by the authors (19 µM β-caryophyllene) may have been above ideal for the bioactive to perform its function correctly, since its efficiency in reducing ROS has been demonstrated at low concentrations (1 µM) in rat C6 glioma cells and in human neuroblastoma SH-SY5Y cells (Assis et al., 2014; Ullah et al., 2021). Thus, lower concentrations of this bioactive can promote a reduction in oxidative stress and benefit the development of reproductive cells, such as oocytes.

Therefore, we aimed to evaluate lower concentrations of β-caryophyllene during bovine IVM, in order to analyze its effects on *cumulus* cells, nuclear maturation, cytoplasmic maturation and oxidative stress.

Material and Methods

The research was performed according to the Animal Ethics Committee of the Federal Rural University of Semi-Arid (nº 23.091.002360/2016-17). β-caryophyllene, along with other chemicals and reagents, was commercially obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. All experiments were performed at the Laboratory of Animal Biotechnology (LBA/UFERSA), in collaboration with the Mossoró Industrial Frigorific Slaughterhouse, where the ovaries were collected.

Experimental design

Immature oocytes were collected and divided into five experimental groups: without antioxidants (control group); and those supplemented with 100 µM cysteamine (CYS), 1 µM (β1), 5 µM (β5), and 10 µM (β10) β-caryophyllene. After 24 h IVM, oocytes were evaluated in two experiments. In the first experiment, oocyte maturation assessments were conducted with 15 replicates of *cumulus* cell expansion and viability, presence of first polar body (1PB), six replicates of metaphase II (MII) assessment, and three replicates of cytoplasmic maturation. In the second experiment, the antioxidant effect was evaluated according to: ROS, intracellular glutathione (GSH) and mitochondrial membrane potential (ΔΨm) levels with three replicates each.

Ovary collection, oocyte selection and IVM

Ovaries were collected from unidentified breeding cows at the slaughterhouse and transported to the laboratory in a thermo container immersed in saline (NaCl, 0.9%) plus antibiotic (penicillin, 0.05 mg/mL) solution at 37 °C. In the laboratory, *cumulus*-oocyte complexes (COCs) were obtained by follicular aspiration, in which follicles measuring 2 to 8 mm were aspirated using a needle and syringe (21 gauge, 5 mL) containing oocyte selection medium with TCM199-HEPES (Gibco-BRL, Carlsbad, CA, USA), supplemented with 0.2 mM sodium pyruvate, 10% fetal bovine serum (FBS, Gibco-BRL, Carlsbad, CA, USA), and 1% antibiotic and antifungal solution (Oliveira et al., 2022). With the aid of a stereomicroscope, oocytes that exhibited two or more layers of compacted *cumulus* cells and homogeneous cytoplasm were selected for IVM (Santos et al., 2019).

Subsequently, the oocytes considered viable were selected for IVM. The structures were grouped into 20–30 oocytes/drops (100 µL) of oocyte selection medium supplemented with 20 µg/mL FSH/LH (Pluset®, São Paulo, Brazil) and different concentrations of β-cariophylene according to the experimental groups. The droplets were covered with mineral oil and placed to mature for 24h00 in a controlled atmosphere (38.5 °C, 6.5% CO_2), (Oliveira et al., 2022).

Experiment 1: effect of different β-cariophylene concentrations on oocyte maturation

Nuclear maturation

After 24h00 IVM, COCs were evaluated for cumulus cell expansion, where structures that appeared expanded were

considered mature (Santos et al., 2019). The oocytes (1440 oocytes/15 replicates) were then denuded by successive pipetting with 0.1% hyaluronidase enzyme, and the cell suspension was stained with 0.2% trypan blue, in which the viable cells showed no staining, and the non-viable cells were stained blue (Santos et al., 2019).

The denuded oocytes were transferred to the selection medium to observe the first polar body (1PB) using a stereomicroscope, and oocytes showing 1PB were considered mature (Oliveira et al., 2022). Nuclear development was assessed using the Hoechst 33342 fluorescent probe, after which the oocytes were fixed in 4% paraformaldehyde for 30 min. The cells (496 oocytes/6 replicates) were labeled with 10 µg/mL of the probe for 15 min and collected for analysis under a fluorescence microscope (Olympus BX51TF, Tokyo, Japan). The cells that presented the nucleus in metaphase II (MII) were considered mature, and those in prophase I, metaphase I, anaphase I, and telophase I were considered immature (Oliveira et al., 2022).

Cytoplasmic maturation and mitochondrial organization

The denuded oocytes (337 oocytes/3 replicates) were incubated with 500 nM Mito Tracker Red® (CMXRos) for 30 min and taken to the fluorescence microscope for image acquisition and evaluation of different parameters. To assess cytoplasmic maturation, oocytes classified as peripherally distributed were considered immature and those with dispersed distribution were classified as mature (Oliveira et al., 2022). Moreover, patterns of mitochondrial aggregation were classified into pattern A: homogeneous, with small granulations distributed throughout the cytoplasm; and pattern B: heterogeneous, with clusters of large granulations distributed throughout the cytoplasm (Ambrogi et al., 2017). Therefore, the presence of pattern B was considered an indication of superior maturation rates.

Experiment 2: effect of different β-cariophylene concentrations on oocytes oxidative status

To assess the antioxidant activity and bioenergetic state of the oocyte, the levels of ROS, GSH and ΔΨm were measured. To evaluate ROS levels, denuded oocytes (284 oocytes/3 replicates) were washed in drops (100 µL) of phosphate buffer (PBS) and then incubated for 30 min at 38.5 °C and 6.5% CO_2 in 10 µL of fluorescent probe 2'-diacetate, 7'-dichlorodihydrofluorescein (H_2DCFDA) diluted in 500 µL of PBS. For quantification of GSH (321 oocytes/3 replicates), the same process was performed using 10 µM of 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (Cell Tracker Blue®). For the evaluation of ΔΨm (337 oocytes/3 replicates), the Mito Tracker Red® probe (CMXRos) was used at a concentration of 500 nM. Then, the samples were placed under the fluorescence microscope to obtain images, and the fluorescence intensity was measured by the arbitrary fluorescence unit (AFU) and analyzed using ImageJ software.

Statistical analysis

Data were expressed as mean ± standard error and analyzed using StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). Normality of the results was tested using the Shapiro-Wilk test, and homoscedasticity was tested using the Levene test. ROS, ΔΨm, and GSH were transformed by arcsine and analyzed by analysis of variance (ANOVA) followed by Tukey's test. All other data were analyzed by chi-squared test, and significance was set at $P < 0.05$.

Results and Discussion

After all repetitions of the oocyte recovery and selection process, a total of 386 ovaries were obtained and 1038 viable structures were recovered (2.7 viable oocytes/ovary). All viable oocytes were randomly distributed among the experimental groups. After IVM, it was possible to observe that the *cumulus* cells expansion rate was similar among the CYS, β1, β5, and β10 groups, all being higher than the control group (Table 1, $P < 0.05$). *Cumulus* cells are responsible for protecting the oocyte from external conditions, as they surround the oocyte, serving as a barrier between the nucleus and the in vitro environment (Hashimoto et al., 1998). Its expansion process occurs due to the increased production of hyaluronic acid, which is produced during oocyte maturation. Without adequate expansion, the quality of the oocyte may be compromised, affecting its development and preventing maturation (Allworth & Albertini, 1993).

Table 1
Effect of β-caryophyllene during IVM of bovine oocytes and its influence on cumulus cells and nuclear maturation

Groups	Characteristics of *cumulus* cells		Nuclear maturation	
	Expansion (% ± SE)	Viability (% ± SE)	MII (% ± SE)	1PB (% ± SE)
Control	87.4 ± 3.1[b] (250/286)	87.8 ± 2.0[a] (10914/12430)	90.0 ± 5.3[a] (54/60)	84.0 ± 3.2[b] (205/244)
CYS	93.8 ± 2.8[a] (273/291)	87.4 ± 2.2[a] (10375/11868)	90.0 ± 7.9[a] (44/49)	86.5 ± 6.0[b] (224/259)
β1	91.8 ± 2.4[a] (268/292)	82.1 ± 4.8[c] (10186/12399)	83.3 ± 11.1[a] (60/72)	88.3 ± 3.3[b] (234/265)
β5	93.7 ± 2.3[a] (269/287)	85.7 ± 3.0[b] (9848/11489)	79.6 ± 11.4[a] (43/54)	93.8 ± 1.6[a] (229/244)
β10	93.3 ± 2.0[a] (252/270)	85.3 ± 2.7[b] (8813/10337)	80.6 ± 12.5[a] (50/62)	82.9 ± 4.6[b] (219/264)

[a,b,c:] $P < 0.05$; SE: standard error.

However, all concentrations of β-caryophyllene reduced the viability of these cells compared to the control and CYS groups (Table 1, $P < 0.05$). These results may be corelated to the anticancer potential of β-caryophyllene, which was proven through its apoptotic properties in multiple myeloma cells by Mannino et al. (2021) using a concentration of 50 and 100 µM, reporting an 80% reduction in the viability of these cells. However, this same study revealed that, although β-caryophyllene has apoptotic potential, it did not affect cell proliferation. This corroborates the results observed in the present study, in which the groups treated with the bioactive showed expansion of *cumulus* cells, even with a reduction in their viability.

One of the main ways to observe the maturation of an oocyte is through nuclear maturation (Figure 1), in which meiosis resumes and progresses to the MII stage. At this stage, the chromosomes are already aligned, forming the metaphase plate, which is essential to ensure the equitable division of genes (Sirard et al., 1998). In this context, no difference was observed between the groups regarding the MII percentages (Table 1, Figure 1C and D, $P > 0.05$), corroborating studies such as that of Sovernigo et al. (2017), which demonstrated that the use of antioxidants substances (vitamin C, carnitine and resveratrol) during the maturation process does not interfere with the progression of cell division. In addition, the formation of 1PB during the meiosis process is an indicator of maturation (Richard & Sirard, 1996). The present study demonstrated that, although concentrations of 1 and 10 µM do not affect the formation of said structure (Table 1), the concentration of 5 µM showed a significant increase compared to the other groups (Table 1, Figure 1A and 1B, $P < 0.05$). This information reinforces the hypothesis that lower concentrations may be more efficient, since in the study by Oliveira et al. (2022) the use of 19 µM of β-caryophyllene resulted in a rate of 69.8%, similar to the control group, which obtained 63.9%. In the present work, it was possible to achieve a rate of 93.8%, higher than that of the control and CYS groups (84.0% and 86.5%).

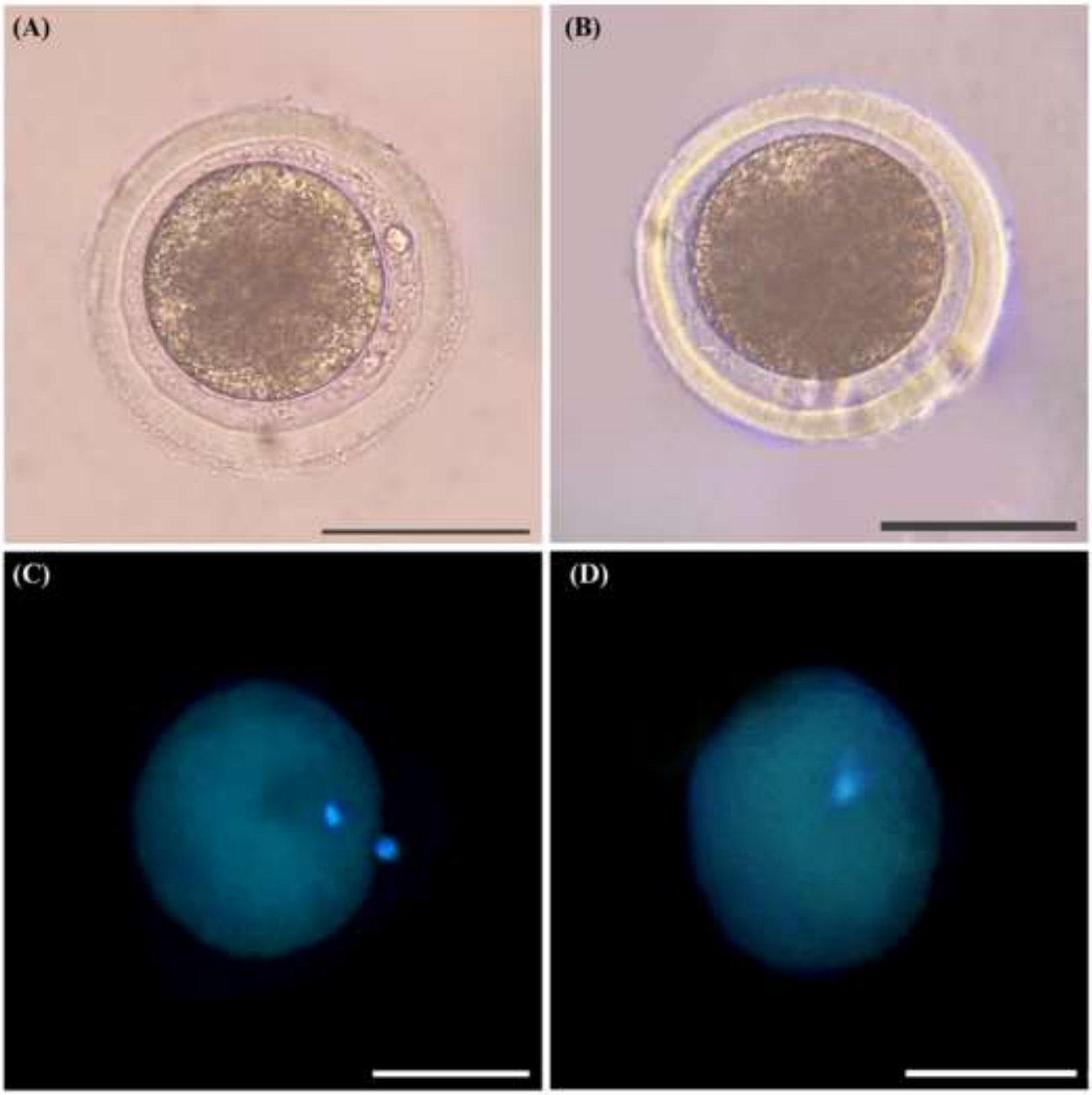

Figure 1. Representative images of nuclear maturation (presence of the first polar body and metaphase plate) of bovine oocytes matured with different concentrations of β-caryophyllene. **(A)** Oocyte with the presence of 1PB. **(B)** Oocyte without 1PB. **(C)** Immature oocyte at the germinal vesicle stage. **(D)** Matured oocyte in metaphase II. Scale bar 100 µm (20/40×).

Mitochondria are the main organelle responsible for promoting the formation of adenosine triphosphate (ATP), a molecule that serves as an energy source for cell development and functioning (Cummins, 2024). During the oocyte maturation, mitochondria move to the periphery due to high energy demand, moving to the center as the oocytes resume meiosis (Ambrogi et al., 2017). Thus, at the end of maturation, oocytes present a dispersed and homogeneous distribution. Hence, the β1 group presented a percentage of 87.7% ± 1.7 of dispersed mitochondria, higher than that of the control group (Figure 2C) and similar to the CYS group (80.6% ± 6.9, Figure 2C). Regarding the mitochondrial aggregation pattern (Figure 2D), oocytes classified in Pattern B were considered mature (Figure 2A), while Pattern A was considered immature (Figure 2B). Among the groups tested, β1 showed higher rates of oocytes found in Pattern B (84.2% ± 1.3), when compared to the other groups (control: 53.7% ± 11.8; CYS: 71.6% ± 12.7; β5: 57.6% ± 7.0; β10: 62.3% ± 7.7, Figure 2D, $P < 0.05$). These results emphasize that the reduction in the concentration of β-caryophyllene benefited the percentage of oocytes in Pattern B when compared with the findings of Oliveira et al. (2022), who achieve only 48.4%. These data are relevant since an inadequate distribution can impair the development of oocyte maturation (Zhang et al., 2022).

During maturation, it is important to evaluate the bioenergetic activity of the oocyte, since, in this context, the mitochondria synthesize ATP through β-oxidation via its electron transport chain (Ambrogi et al., 2017). This process results in a high release of O_2, which increases the production of ROS, impairing oocyte development. Therefore, oocytes must present a low level of ΔΨm (Al-Zubaidi et al., 2019). The use of β-caryophyllene at a concentration of 5 μM (0.73 ± 0.40 AFU) reduced ΔΨm levels compared to the other groups (Figure 3F, $P < 0.05$). However, β5 was similar to the β1 (0.88 ± 0.29 AFU) and CYS (0.95 ± 0.29 AFU) groups. Only the β10 group (1.00 ± 0.23 AFU) showed results similar to the control group (1.00 ± 0.29 AFU) (Figure 3C, 3F, $P > 0.05$).

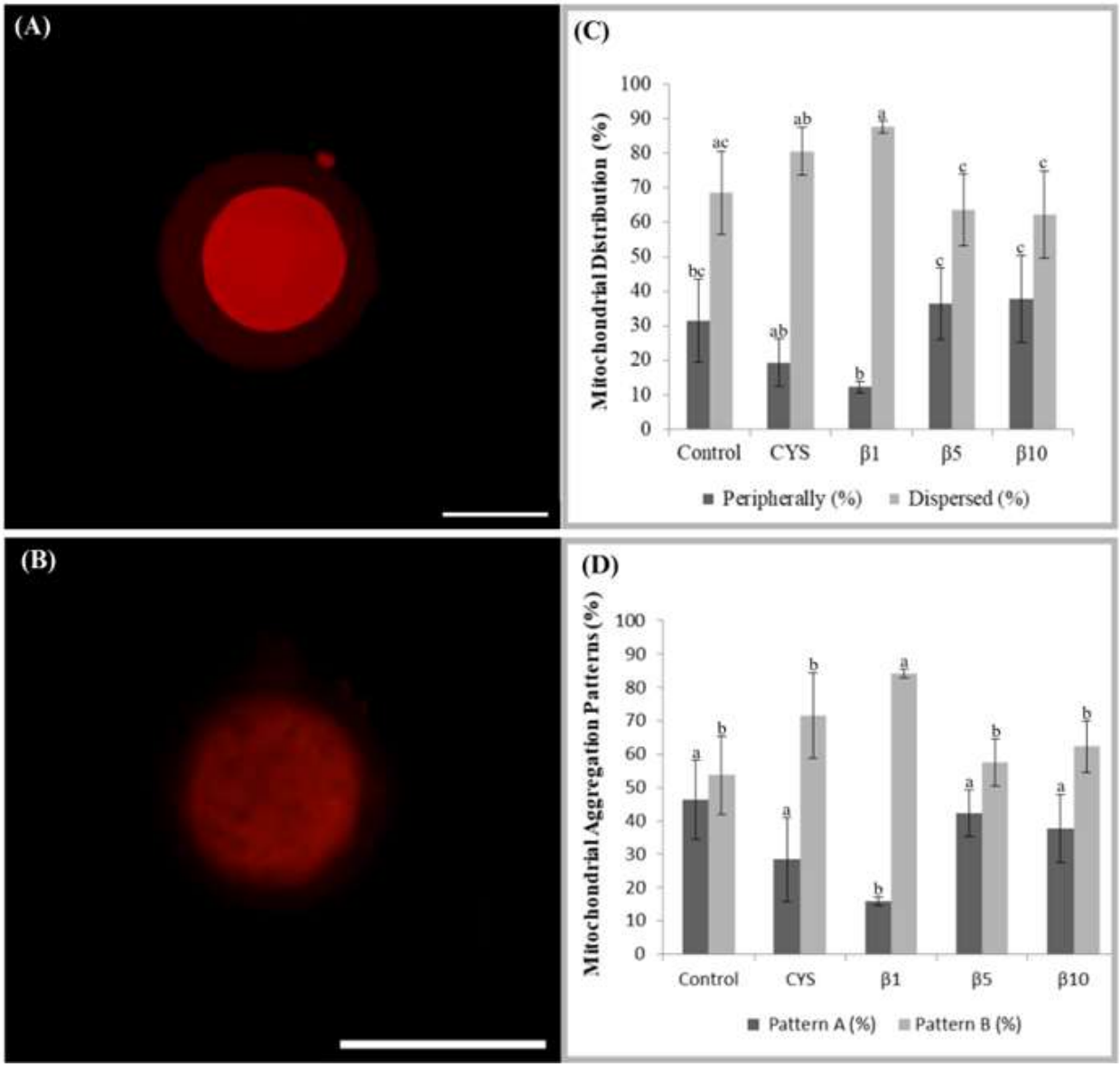

Figure 2. Cytoplasmic maturation of bovine oocytes matured with different concentrations of β-caryophyllene.
(A) Representative image of oocyte in Pattern A (immature). **(B)** Representative image of oocyte in Pattern B (mature). **(C)** Mitochondrial distribution. **(D)** Mitochondrial aggregation pattern. Different letters indicate statistical differences. Significance established at $P < 0.05$. Scale bar 100 µm (40×).

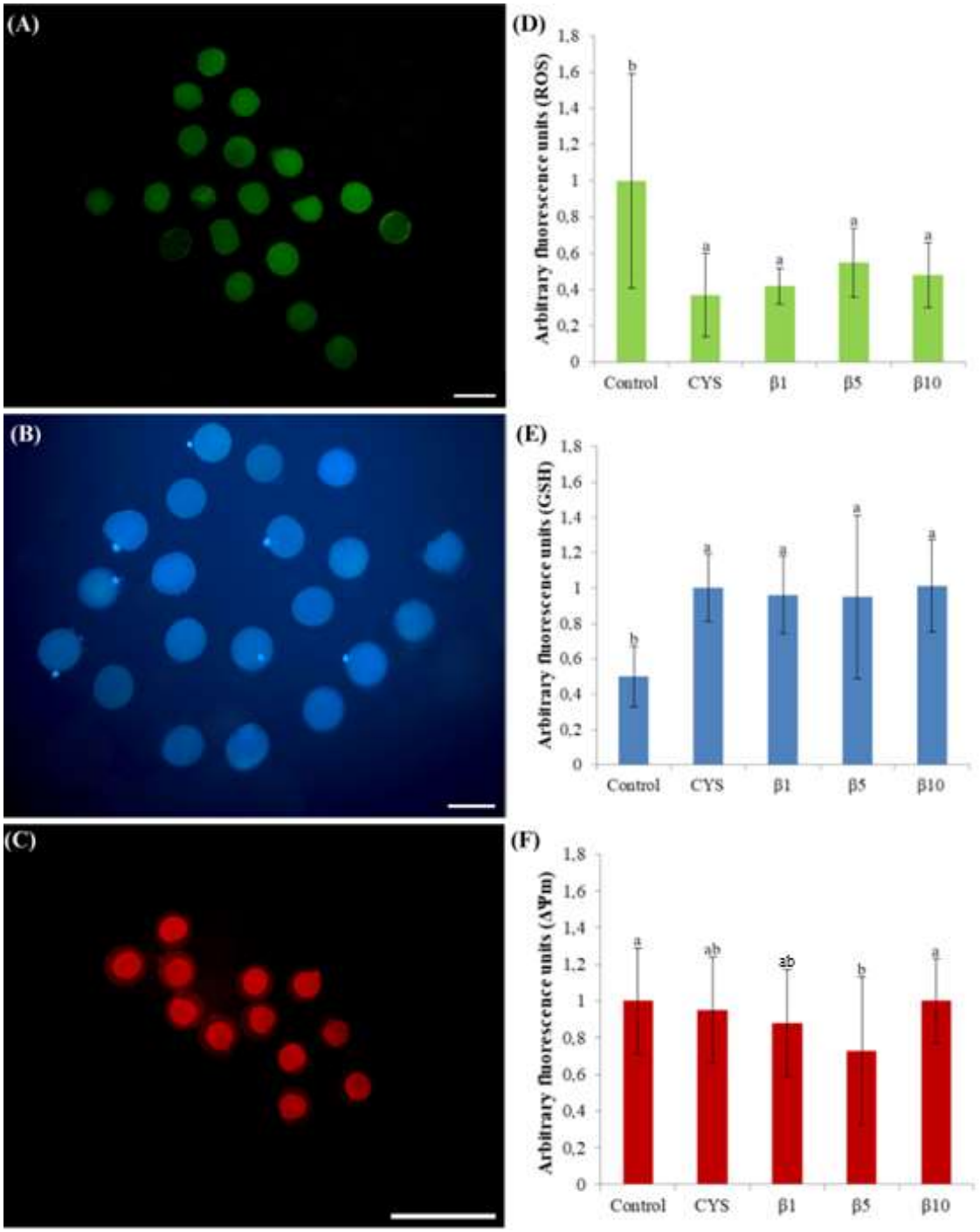

Figure 3. CEffect of different concentrations of β-caryophyllene on oxidative stress status during bovine oocytes maturation.
(A) Representative image of oocytes labeled with H_2DCFDA. **(B)** Representative image of oocytes labeled with CellTraker Blue. **(C)** Representative image of oocytes labeled with MitoTracker Red. **(D)** Quantification of ROS levels. **(E)** Quantification of GSH levels. **(F)** Quantification of ΔΨm levels. Different letters indicate statistical difference. Significance established at $P < 0.05$. A, C, E: Scale bar 100 µm (20/40×).

The animal organism has a redox control system that regulates the balance between the production of ROS and antioxidants (Davoodian et al., 2024). However, dysregulation of this system can lead to a significant increase in ROS, impairing oocyte development, causing changes in its mechanisms, structural problems, and even cell death (Alsalim et al., 2020). The present study demonstrated that β-caryophyllene is efficient concerning its antioxidant capacity, since all groups that used the bioactive as a supplement also achieved reduced ROS levels (β1: 0.42 ± 0.10 AFU; β5: 0.55 ± 0.19 AFU; β10: 0.48 ± 0.18 AFU) compared to the control group (1.00 ± 0.59 AFU), as well as presented similar results to the group supplemented with cysteamine (0.37 ± 0.23 AFU), (Figure 3A, 3D, $P > 0.05$), which is currently the most widely used chemical antioxidant for bovine IVM (Damayanti et al., 2022). Furthermore, all reduced concentrations demonstrated superior results to the use of 19 μM β-caryophyllene, which presented 0.84 AFU (Oliveira et al., 2022).

Following this principle, it is important to study this bioenergetic balance, since antioxidants can act through different mechanisms, such as acting directly in the reduction of ROS, capturing free O2 molecules, or increasing intracellular antioxidant levels (Zhou et al., 2010). Among these antioxidants, GSH stands out as an important antioxidant that can be synthesized from cysteine, glutamate, or glycine. GSH performs several intracellular functions, such as DNA and protein synthesis, in addition to being involved in the regulation of oxidative stress (Adeoye et al., 2018). In this context, the use of β-caryophyllene (β1: 0.96 ± 0.22 AFU; β5: 0.95 ± 0.46 AFU; β10: 1.01 ± 0.26 AFU) promoted a significant increase in GSH levels compared to the control group (0.50 ± 0.17 AFU), being similar to the result obtained with cysteamine (1.00 ± 0.19 AFU), (Figure 3B, 3E, $P < 0.05$), an antioxidant that has been shown to stimulate GSH synthesis, since it increases the intracellular supply of cysteine, which in turn stimulates GSH production (Zhou et al., 2010).

Conclusion

The present study demonstrated the antioxidant properties of β-caryophyllene since all concentrations tested reduced ROS levels and increased GSH. However, concentrations of 1 and 5 μM stood out as the most suitable for supplementation of the IVM medium. It was demonstrated that 1 μM concentration is able to promote efficient cytoplasmic maturation, while the 5 μM concentration ensures a higher percentage of 1PB. Furthermore, both concentrations reduced oxidative stress, decreasing ΔΨm levels. Thus, this study demonstrates that concentrations of 1 μM and 5 μM of β-caryophyllene were effective in promoting better oocyte development. The data are valuable for improving bovine IVM protocols, since they present an equally effective alternative to chemical antioxidants, capable of reducing oxidative stress and ensuring the development of suitable conditions for a possible IVEP.

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