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Effect of α-tocopherol supplementation during *in vitro* maturation of oocytes on bovine embryonic development

Efecto de la suplementación de α-tocoferol en la maduración *in vitro* de ovocitos bovinos sobre el desarrollo embrionario

Acosta-Pérez, T.P. [1+], *Quezada-Casasola, A.* [1+], *Itzá-Ortiz, M.F.* [1], *Beristain-Ruiz, D.M.* [1], *Aréchiga-Flores, C.F.* [2], *Carrera-Chávez, J.M.* [1*]

[1] *Departamento de Ciencias Veterinarias. Universidad Autónoma de Ciudad Juárez, Av. Plutarco Elías Calles 1210, Fovissste Chamizal, C.P. 32310, Ciudad Juárez, Chihuahua, México.*
[2] *Unidad Académica de Medicina Veterinaria y Zootecnia, Universidad Autónoma de Zacatecas., Carretera Zacatecas-Fresnillo Km. 31.5, C.P. 98575, El Cordovel, General Enrique Estrada, Zacatecas, México.*

[+] *Estos autores contribuyeron igualmente a este trabajo*

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ABSTRACT

In vitro maturation of oocytes is a crucial process for *in vitro* production of embryos, a technology that still presents challenges to reach its full potential. The objective was to evaluate the addition of α-tocopherol during *in vitro* oocyte maturation and its subsequent effect on embryo development. A total of 1,019 bovine oocytes were used, classified as high and low quality, supplemented with ethanol and α-tocopherol at 100, 200, and 400 µM/mL during *in vitro* maturation, measuring maturation rates, cleavage, blastulation, and blastocyst expansion. Maturation was higher in high-quality oocytes ($p < 0.01$). Cleavage rate and percentage of expanded blastocysts were higher in embryos from high-quality oocytes (HQOE) supplemented with 400 µM/mL ($p < 0.05$). Blastulation rate was lower in HQOE supplemented with ethanol and 100 µM/mL ($p < 0.05$). In conclusion, high-quality oocytes exhibited higher maturation and embryo development rates compared to low-quality ones. α-tocopherol at 400 µM/mL increased cleavage and expanded blastocyst rates in HQOE. Ethanol reduced blastulation in HQOE, which was mitigated by the addition of 200 and 400 µM/mL of α-tocopherol.

KEY WORDS: Vitamin E, oxidative stress, cumulus expansion, embryo cleavage, blastocyst.

***Corresponding Author:**

José Maria Carrera-Chávez. Departamento de Ciencias Veterinarias. Universidad Autónoma de Ciudad Juárez, Av. Plutarco Elías Calles 1210, Fovissste Chamizal, C.P. 32310, Ciudad Juárez, Chihuahua, México. Telefono: (656) 688 1800.
E-mail: jose.carrera@uacj.mx

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RESUMEN

La maduración *in vitro* de ovocitos es un proceso crucial para la producción *in vitro* de embriones, tecnología que aún presenta desafíos para alcanzar su potencial, entre ellos el estrés oxidativo. El objetivo fue evaluar la adición de α-tocoferol (vitamina E) en la maduración *in vitro* de ovocitos y en el desarrollo embrionario subsecuente. Se utilizaron 1,019 ovocitos bovinos, clasificados como de alta y baja calidad, suplementados con etanol y α-tocoferol a 100, 200, y 400 µM/mL durante la maduración *in vitro*, midiendo las tasas de expansión de cúmulos de los ovocitos, escisión, blastulación y expansión del blastocito. La maduración fue mayor en ovocitos de alta calidad ($p < 0.01$). La tasa de escisión y el porcentaje de blastocitos expandidos fueron superiores en embriones de ovocitos de alta calidad (HQOE) adicionados con 400 µL ($p < 0.05$). La tasa de blastulación fue menor en HQOE adicionados con etanol y 100 µM/mL ($p < 0.05$). En conclusión, los ovocitos de alta calidad exhibieron mayores tasas de maduración y desarrollo embrionario en comparación a los de baja calidad. El α-tocoferol a 400 µM/mL incrementó la tasa de escisión y de blastocitos expandidos en HQOE. El etanol redujo la blastulación de HQOE, efecto que fue disminuido por la adición de 200 y 400 µM/mL de α-tocoferol.

PALABLAS CLAVE: Vitamin E, oxidative stress, cumulus expansion, embryo cleavage, blastocyst.

Introduction

The ability to generate large quantities of bovine embryos through *in vitro* production (IVP) at a relatively low cost, using slaughterhouse-derived ovaries, has brought significant benefits to modern livestock genetic improvement programs (Nogueira da Costa *et al.,* 2015). However, major challenges associated with this technology still exist, particularly concerning oocyte quality and the subsequent embryonic development. Although global *in vitro* embryo production has increased annually, a large proportion (up to 75 %) of oocytes retrieved from the follicular environment fail to achieve the necessary conditions for proper fertilization and high-quality embryos production (Krisher & Herrick, 2024). Technically, bovine IVP involves a three-step process: oocyte maturation, fertilization, and embryo culture up to blastocyst stage (Lonergan & Fair, 2014). In this context, *in vitro* oocyte maturation is a complex biochemical and structural process during which the female gamete acquires the competence for fertilization, an essential step for sustaining appropriate embryo development to the blastocyst stage and the potential birth of a live calf (Nogueira da Costa *et al.,* 2015).

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One of the main limitations of *in vitro* maturation (IVM) of oocytes is the lack or inadequate function of antioxidants, along with the presence of high concentrations of reactive oxygen species (ROS). Under normal physiological conditions, ROS participate in signaling pathways that support normal cellular development and function (Khan *et al.,* 2017). However, ROS are oxidizing molecules that can structurally damage various macromolecules, leading to cellular dysfunction and premature aging, and are considered partially responsible for the low efficiency of IVP systems (Chowdhury *et al.,* 2017; Khan *et al.,* 2017). In conventional IVP conditions, oocytes and embryos are exposed to oxidative stress due to light exposure, elevated oxygen concentrations, and altered levels of metabolic substrates (Remião *et al.,* 2016; Rocha-Frigoni *et al.,* 2016). If not rapidly and effectively neutralized, ROS can damage a wide array of cellular macromolecules, potentially leading to cell death. Thus, ROS must be continuously inactivated to maintain physiologically tolerable levels that contribute to normal cellular functions (Jeong *et al.,* 2006; Schoots *et al.,* 2018).

A viable approach to mitigate this issue is the addition of antioxidant compounds to the IVM medium. It has been reported that the inclusion of antioxidants in the maturation medium reduces ROS levels and increases blastocyst production (Remião *et al.,* 2016). Vitamin E, a non-protein, lipid-soluble antioxidant present in animal cells, is regarded as a key scavenger of ROS. Specifically, vitamin E has been shown to enhance the developmental rate of *in vitro*-produced embryos by mitigating cellular membrane damage caused by excessive ROS (Zhang *et al.,* 2012). Its active homolog, α-tocopherol, protects cell membranes from oxidation by reacting with ROS and lipid radicals generated during lipid peroxidation (Dalvit *et al.,* 2005). This is achieved through the hydroxyl group on its aromatic ring structure, which disrupts radical chain reactions and neutralizes free radicals (Engin, 2009). While the protective role of α-tocopherol during sperm cryopreservation is well established, acting as a membrane protector that inhibits lipid peroxidation and eliminates lipid-derived radicals (de Vasconcelos *et al.,* 2016; Jiménez-Aguilar *et al.,* 2021), its effect on oocyte fertilization competence following maturation and subsequent embryo development remains insufficiently understood (Vijayalakshmi *et al.,* 2020; Azam *et al.,* 2024). Based on the above, the objective of the present study was to evaluate the effect of α-tocopherol supplementation in the *in vitro* maturation medium on oocyte maturation rate and subsequent embryonic development.

Material and Methods

Ovary collection, recovery, and classification of cumulus-oocyte complexes

Ovaries were collected from non-pregnant Holstein Friesian cows with a calving history of ≥4 and a body condition score between ≥2.5 and ≤3 (Angel & Mahendran, 2024) slaughtered at the local abattoir in Ciudad Juárez, Mexico, under national guidelines for the humane handling of animals during transport (NOM-051-ZOO-1995) and for the humane slaughter of domestic and wild animals (NOM-033-SAG/ZOO-2014). Only ovaries without visible pathological structures were selected. The ovaries were rinsed twice, placed in 0.9 % saline solution at 37 °C, and transported to the laboratory within 2 hours of collection; 20 ovaries were processed per collection session.

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All follicles measuring 2 to 8 mm in diameter were aspirated using a 10 mL syringe fitted with an 18-gauge needle. The follicular fluid was collected into pre-warmed 50 mL conical tubes containing 10 µL of heparin (5,000 IU/mL; Inhepar, PiSA Farmacéutica, Mexico). Cumulus–oocyte complexes (COCs) were allowed to settle in these tubes for 10 minutes. After sedimentation, COCs were collected and washed four times in a washing medium composed of 600 µL of TCM-199 (M4530, Sigma-Aldrich, Germany), 0.5 µL/mL of FSH (500 IU; Laboratorios Calier, Argentina), 5 % fetal bovine serum (FBS; F4135, Sigma-Aldrich, Germany), and 50 µL/mL of gentamicin (80 mg/mL; Rayere Pharmaceuticals, Mexico). A total of 1,019 COCs were recovered.

After washing, COCs were visually classified into two groups based on cytoplasmic homogeneity and the number of surrounding cumulus cell layers: a high-quality oocyte group, corresponding to COCs with score categories 1 and 2; and a low-quality oocyte group, corresponding to COCs with score categories 3 and 4 (Figure 1). The classification criteria were based on the system described by Stojkovic *et al.* (2001).

In vitro maturation

Maturation medium was equilibrated in a 5 % CO_2 atmosphere for 2 hours prior to the assignment of experimental treatments. COCs from both quality groups were placed in maturation medium at a density of 50 structures per 500 µL and randomly assigned to one of five experimental treatments: Control treatment (high-quality COCs, n = 105; low-quality COCs, n = 43), consisting of the commercial BO-IVM maturation medium (71001, IVF Bioscience, UK); Ethanol treatment (high-quality COCs, n = 100; low-quality COCs, n = 59), consisting of conventional maturation medium (TCM-199 supplemented with 5 % FBS, 10 % follicular fluid, 0.2 % sodium pyruvate [Gibco, USA], and 50 µg/mL gentamicin) supplemented with 0.1 % (v/v) of 90 % ethanol (Hycel, Mexico); 100 µM treatment (high-quality COCs, n = 185; low-quality COCs, n = 76), consisting of conventional maturation medium supplemented with 100 µM α-tocopherol (T3251, Sigma-Aldrich, Germany); 200 µM treatment (high-quality COCs, n = 154; low-quality COCs, n = 79), consisting of conventional maturation medium supplemented with 200 µM α-tocopherol; and 400 µM treatment (high-quality COCs, n = 161; low-quality COCs, n = 57), consisting of conventional maturation medium supplemented with 400 µM α-tocopherol. The α-tocopherol concentrations used in this study were selected based on the results reported in previous experiments (Naspinska *et al*., 2023; Tripathi *et al*., 2023).

COCs were matured at 38.5 °C in 5 % CO_2 under humidified atmospheric air (21 % O_2) for 22 hours. Oocyte maturation was assessed by observing cumulus cell expansion (Figure 1; Zhang *et al*., 2010; Caixeta *et al*., 2013).

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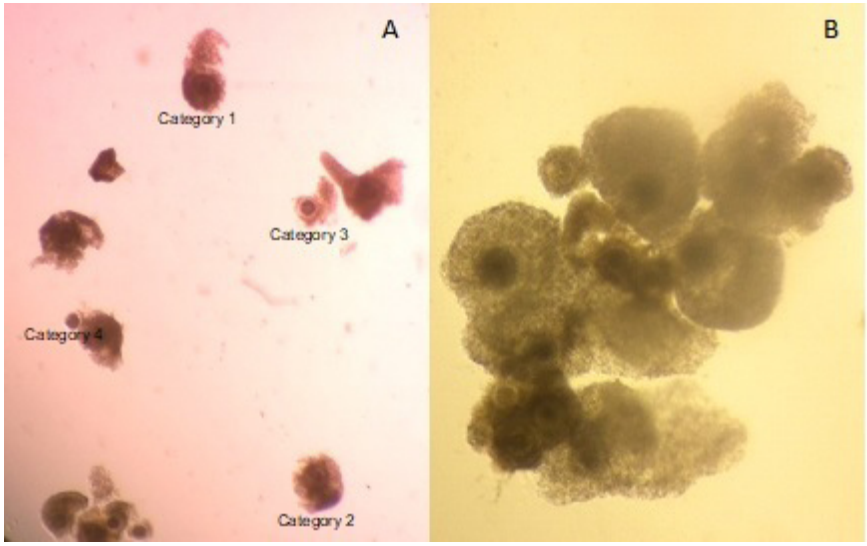

Figure 1. A. Morphological characteristics of the oocyte-cumulus complexes used for classification into quality categories. B. Matured oocytes after 22 h of incubation. Based on Stojkovic et al. (2001).

Category 1, homogenous cytoplasm, compacted and multilayered cumulus; Category 2, cytoplasm with only a few irregular pigmentations, cumulus smaller than in category 1; Category 3, cytoplasm heterogeneous/ vacuolated, covered by a few layers of cumulus cells with small, denuded areas; and Category 4, cytoplasm heterogeneously pigmented, cumulus completely or in great part absent.

In vitro fertilization

After maturation, COCs from each group were washed once in warm commercial fertilization medium (BO-IVF, IVF Bioscience, UK) and transferred to 200 µL of BO-IVF (pre-equilibrated in a humidified atmosphere with 5 % CO_2 for 2 hours). Straws of frozen semen from two bulls with previously confirmed fertility were thawed at 37 °C for 30 to 40 seconds. Following thawing, sperm motility was assessed using a computer-assisted semen analysis system (AndroVision, Minitube, Germany). Thawed and evaluated semen was washed twice by centrifugation at 328 g for 5 minutes in 4 mL of commercial sperm preparation medium (BO-Semen Prep, IVF Bioscience, UK) at 37 °C. The supernatant was removed after each centrifugation, leaving approximately 250 µL of sperm suspension. Finally, the remaining supernatant was discarded, and the sperm pellet was resuspended by adding 100 µL of pre-warmed fertilization medium.

COCs from each treatment group were inseminated with the appropriate volume of the final sperm suspension to achieve a final sperm concentration of 2×10^6 cells/mL in each fertilization medium drop. Spermatozoa were co-incubated with the matured oocytes for 18 hours at 38.5 °C in 5 % CO_2 under a humidified atmosphere (21 % O_2).

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In vitro culture and embryo development assessment

Zygotes were transferred from the fertilization medium to pre-warmed washing medium (BO-Wash, IVF Bioscience, UK) and denuded using a vortex mixer (Barnstead International, USA) for 90 seconds. The denuded structures were then washed in washing medium, rinsed in culture medium (BO-IVC, IVF Bioscience, UK), and cultured in 200 µL of culture medium covered with a layer of mineral oil (BO-Oil, IVF Bioscience, UK) under a humidified atmosphere containing 88 % N_2, 6 % CO_2, and 6 % O_2 at 38.5 °C for 7 days. Embryos were considered to have reached the cleavage stage when at least two cells were observed within a single structure, approximately 48 hours after fertilization; the blastulation stage was identified by the presence of a clear cavity (blastocoel) within the zona pellucida, around day 6 post-fertilization; and the expanded blastocyst stage was characterized by an increase in diameter resulting in thinning of the zona pellucida (approximately 7 - 8 days after fertilization; Peippo *et al.,* 2011).

Statistical analysis

Proportions of categorical data, including matured oocytes (oocytes with expanded cumulus cells out of the total number of structures used per group), embryo cleavage (number of embryos with initial cell division out of the total number of fertilized structures per group), blastulation (number of blastocysts formed from cleaved embryos in each group), and blastocyst expansion (number of expanded blastocysts out of the total cleaved embryos per group), were analyzed using the LOGISTIC procedure with a two-factor model. The model included experimental treatments (α-tocopherol at 100, 200, and 400 µM/mL) and oocyte quality classifications (high and low) as main effects, as well as their interaction. Statistical analysis was performed using SAS software (version 9.0; Statistical Analysis System Institute Inc., USA). When no interaction between factors was observed ($p \geq 0.05$), the model was adjusted to include only the main effects. Differences were considered statistically significant when $p < 0.05$.

Results and Discussion

In the present study, a total of 1,019 oocytes were obtained from 63 cows (an average of 16.1 oocytes per cow and 8.1 oocytes per ovary). No interaction effects were detected between the treatments and oocyte quality classification on the evaluated variables, including oocyte maturation, embryonic cleavage, blastulation, and blastocyst expansion (p = 0.29, 0.22, 0.26, and 0.78, respectively). Oocyte maturation rates across treatments and quality grades are summarized in Table 1. Specifically, in high-quality oocytes, the maturation rate showed no significant differences among treatments (p = 0.88), regardless of the level of α-tocopherol supplementation. It is known that α-tocopherol is present in animal cells, where it protects against oxidation by reacting with ROS and lipid radicals produced during lipid peroxidation, thereby interrupting radical chain reactions and exerting its antioxidant effect (Engin, 2009; Takahashi, 2012). However, it has been reported that during bovine embryo IVP, the amount of α-tocopherol present in the COCs is reduced by approximately 50 %, indicating a partial loss of antioxidant capacity during the process (Dalvit

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et al., 2005). In response, researchers have explored the addition of α-tocopherol as a ROS scavenger, and it has been used in various studies to evaluate its effects on bovine embryo IVP. Vásquez *et al.* (2014) added α-tocopherol at a concentration of 100 µM to the maturation medium and assessed its subsequent effect on ROS production in the culture medium, reporting that ROS levels were reduced in the α-tocopherol-treated group compared to the Control group (without α-tocopherol). In this regard, Vijayalakshmi *et al.* (2020) reported that the addition of 10 µL/mL (23 µM) increased the cytoplasmic and nuclear maturation rates of buffalo oocytes, although the addition of 20 µL/mL (46 µM) showed no difference compared to the control treatment; the authors attributed the beneficial effect on maturation to the protection of DNA and polyunsaturated fatty acids present in the plasma membrane. However, authors like Azam *et al.* (2024) reported that the addition of various concentrations of α-tocopherol (50 - 300 µM) in combination with α-linolenic acid (100 µM) during oocyte maturation and subsequent embryo development in buffaloes had no effect, suggesting that this may be due to the selected oocytes having sufficient quality to cope with oxidative stress. However, in the present study, low-quality oocytes treated with 400 µM of α-tocopherol showed a lower maturation rate compared to the 100 µM, 200 µM, Control, and Ethanol groups ($p = 0.02$). These results are consistent with previous studies (Wang *et al.,* 2002; Acosta-Pérez, 2020), which report that high concentrations of α-tocopherol may induce cytotoxicity in oocytes and reduce their maturation and development. However, this trend was not observed during cleavage, blastulation, or blastocyst expansion.

Table 1. Cumulus expansion of bovine oocytes of high (Categories 1 and 2) and low quality (Categories 3 and 4)* treated with different concentrations of α-tocopherol during *in vitro* maturation.

	Quality grades of oocytes	
Treatment	High quality (%)	Low quality (%)
100 µM	173/185 (93.5)^{Aa}	60/76 (78.9)^{Ab}
200 µM	145/154 (94.2)^{Aa}	52/79 (65.8)^{Ab}
400 µM	151/161 (93.8)^{Aa}	30/57 (52.6)^{Bb}
Control	101/105 (96.2)^{Aa}	32/43 (74.4)^{Ab}
Ethanol	93/100 (93.0)^{Aa}	40/59 (67.8)^{Ab}
Total	663/705 (94.0)	214/314 (68.1)

* Classification of categories according to Stojkovic *et al.* (2001).
$^{A, B}$ Different letters in the same column indicate a difference ($p < 0.05$)
$^{a, b}$ Different letters in the same row indicate a difference ($p < 0.01$)

When comparing oocytes based on their quality grade, it was observed that high-quality oocytes exhibited significantly higher maturation rates compared to low-quality oocytes across all experimental treatments ($p < 0.01$). In this regard, other authors (Krisher & Herrick, 2024)

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support these findings, concluding that grade 1 and 2 oocytes are more suitable for maturation and therefore have a higher fertilization potential, whereas grade 3 and 4 oocytes are considered unsuitable and not recommended for this process (Azam *et al.,* 2024). Several authors (Farghaly *et al.,* 2015; Kala *et al.,* 2017) have reported the presence of antioxidant enzyme activity in bovine oocytes and cumulus cells, suggesting that grade 1 and 2 oocytes are more likely to regulate ROS levels during *in vitro* maturation due to the greater number of surrounding cells. This may provide an additional antioxidant effect, as a relationship has been established between the concentration of antioxidant systems in cumulus cells and the maintenance and regulation of the cellular thiol redox state, thereby protecting the oocyte from oxidative damage by eliminating ROS (Deleuze & Goudet, 2010). On the other hand, an excess of free radicals, such as those generated by heat stress or the inherent handling associated with IVP, is associated with poor oocyte quality and meiotic arrest (Fabra *et al.,* 2020; Rakha *et al.,* 2022). This also helps explain why, in the present study, the addition of α-tocopherol to low-quality oocytes did not result in increased maturation rates. Although it is suggested that a large number of cumulus cells is required to achieve a high percentage of meiotic maturation, the metabolic activity of the oocyte itself is thought to play a significant role in the production and regulation ROS; therefore, cumulus cells are not the sole contributors to the protection against oxidative damage caused by external factors. While most antioxidant enzyme units within the oocyte and its surrounding cells depend on accumulation, denuded oocytes also possess intrinsic mechanisms to prevent deterioration due to ROS (Cetica *et al.,* 2001). This may explain why, in the present study, some denuded oocytes (grade 3 or even 4) were capable of reaching maturation, albeit to a lesser extent than grade 1 or 2 oocytes.

Based on the results reported by other authors (Remião *et al.,* 2016), it could be expected that, in addition to the intrinsic characteristics of grade 1 and 2 oocytes, which meet the morphological criteria for full *in vitro* development, the supplementation with any concentration of α-tocopherol would confer additional benefits, supporting oocyte development up to the blastocyst stage. This assumption is grounded in the notion that the antioxidant effect of α-tocopherol protects oocytes and embryos throughout the entire process by mitigating the harmful effects and reducing the production of ROS during handling, light exposure, and varying oxygen concentrations (Reis *et al.,* 2003).

Table 2 presents a comparison of embryonic development rates following fertilization, considering the different treatments and oocyte quality classifications. The results indicate that embryos derived from high-quality oocytes (HQOE) treated with 400 µM of α-tocopherol exhibited a significantly higher cleavage rate compared to those in the 100 µM, 200 µM, Control, and Ethanol groups ($p < 0.01$). These findings are consistent with those reported by Reis *et al.* (2003), who observed a positive effect of α-tocopherol supplementation in fetal bovine serum on blastocyst viability, and with the study by Vijayalakshmi *et al.* (2020), although the latter used a lower concentration in buffalo oocytes. Similarly, a study conducted on porcine embryos demonstrated that supplementation with 100 µM of α-tocopherol throughout the entire culture period or during the first 48 hours after the onset of culture resulted in a higher percentage of blastocysts compared to the control group and those supplemented at 96 or 120 hours after the beginning of culture (Hossein *et al.,* 2007). Another study involving porcine embryos supplemented with 0, 50, 100, and 200 µM of α-tocopherol reported a higher blastocyst rate in the 50 µM and 100 µM groups

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compared to the control and 200 µM groups (Jeong *et al.,* 2006). Additionally, these authors observed a significant increase in the number of inner cell mass cells, trophectoderm cells, and total cell count in embryos treated with 100 µM of α-tocopherol compared to the control group; they hypothesized that the beneficial effect of α-tocopherol supplementation was due to its anti-apoptotic properties, as it reduced the apoptosis index (Jeong *et al.,* 2006).

Table 2. Embryonic development of bovine oocytes with different quality grades treated with α-tocopherol during *in vitro* maturation.

	Cleavage		Blastulation		Blastocyst expansion	
	Quality grades of oocytes					
Treatment	1 and 2 (%)	3 and 4 (%)	1 and 2 (%)	3 and 4 (%)	1 and 2 (%)	3 and 4 (%)
100 µM	54/185 (29.2)^{Ba}	17/76 (22.4)^{Aa}	39/54 (72.2)^{Ba}	2/17 (11.8)^{Ab}	7/54 (13.0)^{Ba}	0/17 (0.0)^{Aa}
200 µM	48/154 (31.2)^{Ba}	22/79 (27.8)^{Aa}	42/48 (87.5)^{Aa}	6/22 (27.3)^{Ab}	4/48 (8.3)^{Ba}	0/22 (0.0)^{Aa}
400 µM	81/161 (50.3)^{Aa}	13/57 (22.8)^{Ab}	67/81 (82.7)^{Aa}	1/13 (7.7)^{Ab}	40/81 (49.4)^{Aa}	0/13 (0.0)^{Ab}
Control	37/105 (35.2)^{Ba}	11/43 (25.6)^{Aa}	35/37 (94.6)^{Aa}	6/11 (54.5)^{Ab}	3/37 (8.1)^{Ba}	0/11 (0.0)^{Aa}
Ethanol	38/100 (38.0)^{Ba}	16/59 (27.1)^{Aa}	27/38 (71.1)^{Ba}	5/16 (31.3)^{Ab}	4/38 (10.5)^{Ba}	0/16 (0.0)^{Aa}
Total	258/705 (36.5)	79/314 (25.1)	210/258 (81.3)	20/79 (25.3)	58/258 (22.4)	0/79 (0.0)

$^{A, B}$ Different letters in the same column indicate a difference ($p < 0.05$)

$^{a, b}$ Different letters in the same row under the same stage of development indicate a difference ($p < 0.01$)

Regarding embryos derived from low-quality oocytes (LQOE), these exhibited similar cleavage rates regardless of the concentration of α-tocopherol added ($p = 0.92$). Comparisons between oocyte quality grades within the same α-tocopherol supplementation level revealed significant differences only in the group supplemented with 400 µM, where HQOE showed higher cleavage rates than LQOE ($p < 0.01$). All other comparisons of cleavage rates between oocyte quality groups under the same treatment condition were not statistically different (100 µM group, $p = 0.26$; 200 µM group, $p = 0.6$; Control group, $p = 0.25$; and Ethanol group, $p = 0.16$). This suggests that the 400 µM α-tocopherol concentration may provide sufficient antioxidant activity to support the levels required during embryonic mitosis. However, this treatment effect was not observed during the blastocyst stage, likely because the antioxidant effect occurred primarily during maturation, and oocytes that failed to cleave were excluded from the experiment. Additionally, the ROS generated during *in vitro* maturation may exceed the antioxidant capacity of low-quality oocytes, compromising their developmental potential and quality (Azam *et al*., 2024).

In HQOE, blastocyst rates were higher in the 200 µM, 400 µM, and Control groups compared to the 100 µM and Ethanol groups ($p = 0.02$). In contrast, LQOE exhibited similar blastocyst rates across all treatment groups ($p = 0.06$), consistent with the findings of Tripathi *et al.* (2023), who

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also reported an increase in blastocyst cell number associated with a reduction in ROS following antioxidant supplementation.

Comparisons between oocyte quality grades under the same treatment revealed significantly higher blastocyst rates in HQOE compared to those derived from low-quality oocytes in all treatment groups ($p < 0.01$; Table 2). As previously noted, oocytes with quality scores of 3 and 4 (low quality) are less likely to achieve full embryonic development (Marei *et al.,* 2014). The findings of the present study indicate that supplementation with α-tocopherol at the evaluated concentrations during oocyte maturation did not exert cytotoxic effects on embryo formation and development.

In HQOE, the blastocyst expansion rate was significantly higher in the group treated with 400 µM α-tocopherol compared to the 100 µM, 200 µM, Control, and Ethanol groups ($p < 0.01$). Similar results were reported by Olson *et al.* (2000), who observed that a greater proportion of zygotes developed into expanded blastocysts when the culture medium was supplemented with 100 µM vitamin E (α-tocopherol being a specific form of vitamin E), compared to the control group. This stage of blastocyst development is characterized by high metabolic activity of transcription factors, whose synthesis and expression are known to be highly sensitive to oxidative stress in the embryo's environment (Kimura *et al.,* 2004). Although the present study did not include α-tocopherol concentrations higher than 400 µM (based on previous reports indicating that higher doses may induce cytotoxicity) it is plausible to suggest that the amount of α-tocopherol used in this experiment reduced oxidative stress up to the blastocyst expansion stage.

In LQOE, blastocyst expansion rates were similar across all experimental groups ($p = 1.0$), as no expansion was observed in any treatment. In the comparison between oocyte quality grades, a significantly higher proportion of expanded blastocysts was observed in HQOE compared to LQOE within the group treated with 400 µM α-tocopherol ($p < 0.01$). All other comparisons of blastocyst expansion rates between quality grades under the same treatment were not statistically significant (100 µM group, $p = 0.13$; 200 µM group, $p = 0.21$; Control group, $p = 0.44$; and Ethanol group, $p = 0.23$). In this context, given that low-quality oocytes possess fewer granulosa cells and considering that the *in vitro* culture process itself reduces the α-tocopherol content in cumulus-oocyte complexes (COCs) by approximately half (Dalvit *et al.,* 2005), the supplementation with exogenous α-tocopherol improved blastocyst quality. This finding is consistent with Báez *et al.* (2021), who reported that α-tocopherol supplementation affects embryonic development in a dose-dependent manner by reducing apoptosis and inducing the overexpression of genes involved in the oxidative stress response, such as *SOD2*. As observed in the present study, the number of embryos decreases as development progresses (Fabra *et al.,* 2020); therefore, future studies should consider increasing the number of oocytes analyzed to obtain more comprehensive data on advanced stages of embryonic development.

An interesting finding of the present study was the effect of ethanol on embryonic development in HQOE. Ethanol (90 %) was used as a solvent for α-tocopherol, following the supplier's recommendations, to allow for its addition to the corresponding experimental groups. Despite using a relatively low concentration of 0.1 % (v/v) of 90 % ethanol, a significant difference

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in blastocyst formation rates was observed between the Control and Ethanol groups in HQOE ($p < 0.05$). The use of ethanol as a solvent is common in reproductive technologies such as IVP when incorporating compounds with low or no water solubility; however, it is well recognized that high concentrations of ethanol can be detrimental to embryonic development (Avery & Greve, 2000). These authors reported that ethanol concentrations of 0.01% and 0.1% (the concentration used in the present study) do not affect COC maturation or subsequent embryonic development; however, concentrations of 0.3% and 1.0% harm embryonic development from the morula stage to day-9 blastocysts, suggesting that the threshold for detrimental effects is very narrow. This is likely due to ethanol's ability to alter the physicochemical properties of biological membranes, leading to changes in membrane activity. Since α-tocopherol is a lipophilic compound, it must be dissolved in solvents such as ethanol, chloroform, acetone, or dimethyl sulfoxide (DMSO). Therefore, it is recommended that future studies requiring ethanol as a solvent for antioxidant supplementation use concentrations of 0.01% or lower, and preferably dilute the antioxidant in DMSO, which has lower cytotoxicity than ethanol (Tsvetkov *et al.,* 2023; Van Nguyen *et al.,* 2023).

Conclusions

The maturation rate and embryonic development were higher in oocytes classified as high quality compared to those of low quality, regardless of α-tocopherol supplementation. However, cleavage rate and the percentage of expanded blastocysts were greater in high-quality oocytes supplemented with 400 µM α-tocopherol. Additionally, during the blastulation stage of embryos derived from high-quality oocytes, ethanol supplementation had a detrimental effect, which was counteracted when 200 or 400 µM α-tocopherol was added. Based on the results of the present study, further experiments are recommended to evaluate α-tocopherol at concentrations other than those tested here, as well as in combination with other molecules with demonstrated antioxidant capacity, in order to propose additional strategies to improve the efficiency of *in vitro* embryo production at a commercial scale. Moreover, due to its lower cytotoxicity, it is advisable to replace ethanol with dimethyl sulfoxide (DMSO) as the solvent for α-tocopherol.

Author contribution

Con APTP, QCA, and CCJM conceptualized the study, developed the methodology, and conducted experimental validation. All authors contributed to the writing, review, and editing of the final manuscript. All authors have read and approved the published version of the manuscript.

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Ethical declarations

The authors declare that all procedures were performed under the Mexican regulations for the humane treatment during animal handling and transportation (Official Mexican Standard NOM-051-ZOO-1995), and for the humane slaughter of domestic and wild animals (Official Mexican Standard NOM-033-SAG/ZOO-2014).

Informed consent statement

Not applicable.

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Conflict of interests

The authors declare no conflict of interest.

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