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Anethole Supplementation During Oocyte Maturation Improves In Vitro Production of Bovine Embryos

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Abstract

Oxidative stress is one of the most detrimental factors that affect oocyte developmental competence and embryo development in vitro. The impact of anethole supplementation to in vitro maturation (IVM) media on oocyte maturation and further bovine in vitro embryo production was investigated. Oocytes of slaughterhouse-derived bovine ovaries were placed in IVM with anethole at different concentrations of 30 (AN30), 300 (AN300), and 2000 μg/mL (AN2000), or without (control treatment). The oocytes were assessed for maturation rates, and for reactive oxygen species (ROS) and ferric reducing antioxidant power (FRAP) levels, and mitochondrial membrane potential. Embryo development was assessed by cleavage and blastocyst rates, and embryo cell number. The percentage of metaphase II oocytes were similar among the treatments (range, 77%-96%). Anethole at 300 μg/mL was the only treatment that yielded higher cleavage and embryo development (morula and blastocyst) rates compared to the control treatment. The ROS production in the oocytes after maturation did not differ among treatments. However, oocytes treated with anethole at 300 μg/mL had higher ($P < .05$) FRAP and mitochondrial membrane potential compared to the control treatment. Furthermore, AN300 treatment increased ($P < .05$) the average number of total cells in blastocysts compared to the control and AN30 treatments. The use of anethole at 300 μg/mL during IVM is suggested to improve the quantity and quality of bovine embryos produced in vitro. The beneficial effects of anethole on embryonic developmental competence in vitro seems to be related to its capacity to regulate the redox balance and improve mitochondrial function in oocytes and embryos.

Keywords

antioxidant, anethole, ROS, mitochondria, oocyte, embryo

Introduction

Although in vitro embryo production (IVP) protocols have progressed substantially in recent years, the IVP rates are still lower than their in vivo counterparts.[1] In a comprehensive recent review article on cattle,[2] the following general outcomes were reported for IVP: 70% to 90% maturation rate; 50% to 80% fertilization rate, including 1 to 2-cell stage; and blastocyst production rate of 20% to 40%. However, although promising alternatives to improve IVP in cattle have been recently reported, using an antioxidant substance (eg, lycopene), the blastocyst production rate was still low (eg, $\leq 29\%$).[3] For that reason, different approaches have been used to improve the efficiency of bovine IVP technology, enhancing, consequently, the production of metaphase II (MII) oocytes, and good-quality embryos. Improvement of culture media for both oocyte in

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vitro maturation (IVM) and in vitro embryo culture (IVC) are some of the main approaches nowadays used for several research groups.[4,5]

Several factors may impair oocyte and embryo development in vitro, such as nutritional imbalances, hormonal disturbances, and oxidative stress.[6,7] One of the main differences between the in vivo and in vitro environments is the high oxygen tension at which the oocytes are exposed in vitro,[8,9] leading to increasing the production of reactive oxygen species (ROS).[10] An excessive amount of ROS induces mitochondrial dysfunction and causes damage in DNA, RNA, and proteins,[11] as well as inhibits the sperm-oocyte fusion.[12] In vivo, the mitochondria are a major source of ROS, such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical; these radicals are produced endogenously by the proton electrochemical gradient during mitochondrial respiration.[13] However, ROS production is balanced by endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX).[14] Nevertheless, under in vitro conditions, oocytes and embryos have reduced antioxidant capacity, since in this system, they do not benefit from intrinsic antioxidant protection,[6] making it extremely necessary to supplement culture media with antioxidants.

In order to protect oocytes and embryos from oxidative stress during IVM and IVC, different antioxidants have been tested, such as ascorbic acid,[15] melatonin,[16] cortisol,[17] green tea,[18] and anethole.[19] The latter is the major component of the essential oil extracted from the plant *Croton zehntneri* (Euphorbiaceae), locally known as "canela de cunhã" or "canelinha," originating from northeastern Brazil.[20] The antioxidant activity of anethole has been confirmed in vitro in ML1-a cells[21] and caprine preantral follicles[19,22] and in vivo.[23] Anethole has been proposed to act as a synergistic antioxidant increasing the activity of primary antioxidants (eg, SOD, CAT, and GPX), and the levels of tripeptide glutathione (GSH).[24] Moreover, anethole has been shown to improve the survivability and development in vitro of isolated caprine secondary follicles.[19] Despite the beneficial effect of anethole on in vitro culture of preantral follicles, to the best of our knowledge, there are no studies regarding its impact on oocyte IVM and IVC. Therefore, the present study aimed to investigate the effect of anethole supplementation to IVM media by evaluating the following end points: (1) oocyte maturation, (2) levels of ROS, (3) total antioxidant potential, (4) mitochondrial membrane potential, (5) fertilization rate, and (6) embryo development in cattle.

Materials and Methods

Chemicals and Media

Unless otherwise mentioned, the culture media, anethole, and other chemicals used in the present experiment were purchased from Sigma (St. Louis, Missouri).

Collection of Oocytes

Bovine ovaries were collected from a local abattoir immediately after slaughter, transported to the laboratory in 0.9% saline solution (NaCl) supplemented with antibiotics (100 µg/mL kanamycin sulphate) at 33°C to 35°C within 2 hours and washed twice in the laboratory with saline solution.[25] The cumulus oocyte complexes (COCs) were collected from follicles (4-8 mm) using an 18-G needle connected to a 10-mL disposable syringe. The COCs were selected in Dulbecco phosphate buffered saline (DPBS) supplemented with 5.56-mM glucose, 1.25-mM sodium pyruvate, 15-µg/mL sodium heparin, 8-µg/mL phenol red, and 10-µg/mL gentamycin, under a stereomicroscope (X100 magnification; SMZ 645 Nikon, Tokyo, Japan;). Only COCs with homogeneous and nondark cytoplasm, surrounded by 2 or more compact layers of cumulus cells, and an intact zona pellucida, were selected for IVM.

In Vitro Maturation

The selected COCs were washed twice in DPBS and 3 times in IVM medium without hormone, previously equilibrated for at least 3 hours at 38.5°C under 5% CO_2. The medium used for IVM was Tissue Culture Medium (TCM-199) supplemented with 10% fetal bovine serum (FBS), 0.2-mM sodium pyruvate, 2-mM L-glutamine, 50-µg/mL gentamicin, 10-IU/mL equine chorionic gonadotrophin, and 10-IU/mL human chorionic gonadotrophin; this medium was referred to as TCM-199$^+$. For IVM, COCs were randomly allocated into 4 treatments as follows: TCM-199$^+$ (control) or TCM-199$^+$ supplemented with anethole at 30 (AN30), 300 (AN300), or 2000 µg/mL (AN2000). A total of 50 COCs per replicate were cultured in 4-well multidish (Nunc, Roskilde, Denmark) containing 500 µL of maturation medium, incubated for 24 hours at 38.5°C and 5% CO_2 in air. The experiment was replicated 6 times.

Assessment of Oocyte Viability and Chromatin Configuration

After IVM, COCs were mechanically denuded by repeated pipetting, and chromatin configuration was assessed by fluorescence microscopy (Nikon, Eclipse 80i, Tokyo, Japan). Oocytes were incubated for 30 minutes in 500 µL of PBS supplemented with 0.5% of glutaraldehyde and 10-µM hoechst 33342 (emission at 483 nm). According to the chromatin configuration, oocytes were classified as: germinal vesicle, germinal vesicle breakdown, metaphase I, MII, or degenerated when chromatin showed abnormal configuration.

In Vitro Fertilization

Spermatozoa were obtained from frozen-thawed semen collected from one fertile bull. Thawed sperm were washed in a discontinuous gradient of 45/90% Percoll at 700*g* for 30 minutes at room temperature, and the pellet was initially resuspended with 5-mL Sperm-TALP.[26] The final pellet was resuspended in in vitro fertilization (IVF)-TALP medium[27] supplemented with 30-µg/mL

heparin, 30-μg/mL penicillinamine, 15-μM hypotaurine, and 1-μM epinephrine. After 24 hours of IVM, one half of the COCs were processed for IVF and were washed twice in IVF-TALP medium, then transferred in groups of 50 to 500 μL of IVF-TALP medium containing 2×10^6 spermatozoa/mL in 4 well Nunc multidishes. Spermatozoa and COCs were incubated for 18 hours at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

In Vitro Culture

Presumptive zygotes were removed from the IVF-TALP medium, mechanically denuded by repeated pipetting, and washed twice in synthetic oviductal fluid (SOF) medium supplemented with Basal Medium Eagle (BME) amino acids solution 50X, MEM amino acids solution 100X, and 5% FBS. Groups of 20 presumptive zygotes were transferred to 20-μL drops of SOF medium under mineral oil in a 30-mm Petri dish and cultured for 7 days at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Half of the medium was replaced every 2 days. Cleavage was evaluated on day 3 after IVF (day 0 = IVF), and blastocysts were evaluated on day 7.

Reactive Oxygen Species Levels

The levels of ROS in the culture medium were measured by a spectrofluorimetric method,[28] using 2′,7′-dihydrodichlorofluorescein diacetate (DCHF-DA) assay. Sample aliquot (50 μL) was incubated with 10 μL of DCHF-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) was measured for the detection of ROS. The DCF fluorescence intensity (FI) emission was recorded at 520 nm (with 480 nm excitation) 2 hours after the addition of DCHF-DA to the culture medium.

Evaluation of Total Antioxidant Potential

The total antioxidant potential was determined by the ferric reducing antioxidant power (FRAP) assay.[29] In the FRAP assay, the antioxidant potential in the culture medium was evaluated by measuring the conversion/reduction of Fe^{3+} to Fe^{2+}, which is chelated by 2,4,6-Tri (2-pyridyl) S-triazine (TPTZ) to form the Fe^{2+}-TPTZ complex, with a maximum absorbance at 593 nm. A curve with ascorbic acid was used as a positive control.

Oocyte Mitochondrial Membrane Potential and Mitochondrial ROS Production Assay

Oocytes from every treatment were assessed by fluorescent probes for mitochondrial membrane potential (MitoTracker® Orange, Molecular Probes®; Invitrogen, Ltd, Paisley United Kingdom),[30] and ROS (CM-H2DCFDA).[31] For mitochondrial membrane potential, oocytes were incubated in 2 μL/mL MitoTracker® Orange for 30 minutes at 37°C. For ROS assay, oocytes were incubated in 1 μL/mL H2DCFDA for 30 minutes. Six oocytes/treatment were used for each of the 2 assays described above.

After the incubation period of each fluorescent probe, oocytes were fixed in glutaraldehyde at room temperature for 15 minutes, and then transferred to PBS solution for up to 1 hour at room temperature protected from light for fluorescent image analysis. Oocytes were mounted on a slide for confocal laser scanning microscopy analysis (Zeiss LSM 710, Oberkochen, Germany). For each sample, images with 1024 × 1024 pixels were drawn to measure the FI. Parameters related to FI, such as laser energy, signal detection (gain), and pinhole size, were maintained at constant values for all measurements. Oocytes were observed at 40× objective magnification under oil immersion. A helium/neon laser ray at 543 nm (551-nm excitation and 576-nm emission) was used to identify the MitoTracker® Orange. An argon ion laser ray at 488 nm (495-nm excitation and 519-nm emission) was used to identify the H2DCFDA. Scanning was conducted with Z stack of 25 optical series from the top to the bottom of the oocyte with a step size of 0.08 mm to allow 3-dimensional distribution analysis.

Statistical Analyses

Statistical analyses were performed using Sigma Plot 11 (Systat Software Inc, San Jose, California). Comparison of means among treatments was analyzed by 1-way analysis of variance and Tukey test. The MII and cleavage and embryo development rates were analyzed by χ^2 test. Pearson correlation test was used to evaluate the association between MitoTracker levels and cleavage rates. Data are presented as a mean (± SEM) and percentage, and the statistical significance was set at $P < .05$.

Results

Effects of Anethole Supplementation on the Maturation Potential of Oocytes

To investigate the effects of anethole on oocytes in MII stage and after fertilization, oocytes were examined after IVM in the presence or absence of different anethole concentrations (Figure 1A and B). After IVM, all oocytes resumed meiosis. The percentages of MII oocytes were similar ($P > .05$) among the treatments and ranged from 77.3% to 95.7%. However, after IVF, the addition of anethole at 2000 μg/mL to the control medium increased ($P < .05$) the percentage of penetrated oocytes when compared to the control treatment only.

Levels of ROS and FRAP After IVM

The levels of ROS and FRAP measured in the culture medium after IVM are shown (Figure 2). Although ROS levels were similar among all treatments, the levels of FRAP were higher ($P < .05$) in the AN300 treatment compared to the control treatment.

Mitochondrial Membrane Potential and ROS Production Levels in the Oocyte

The levels of ROS (H2DCF-DA) and the mitochondrial membrane potential (MitoTracker® Orange) in the oocyte after IVM

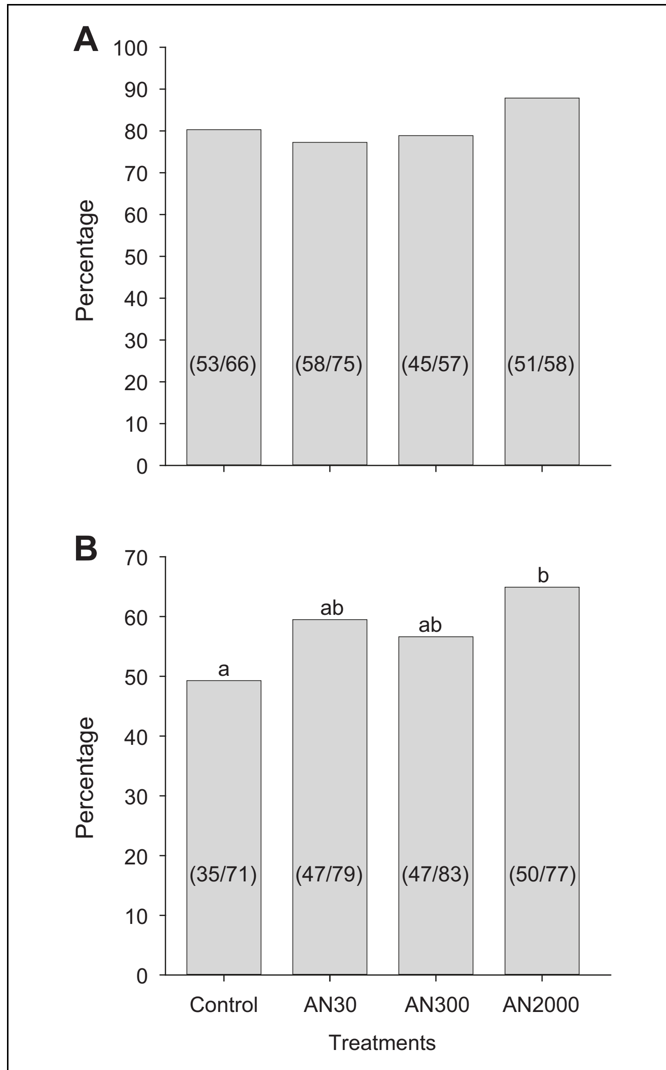

Figure 1. Nuclear maturation (A) and fertilization (B) rates in bovine oocytes after in vitro maturation in control medium with or without supplementation of anethole at 30 (AN30), 300 (AN300), or 2000 μg/mL (AN2000). [a,b]Different letters indicate significant differences among treatments ($P < .05$).

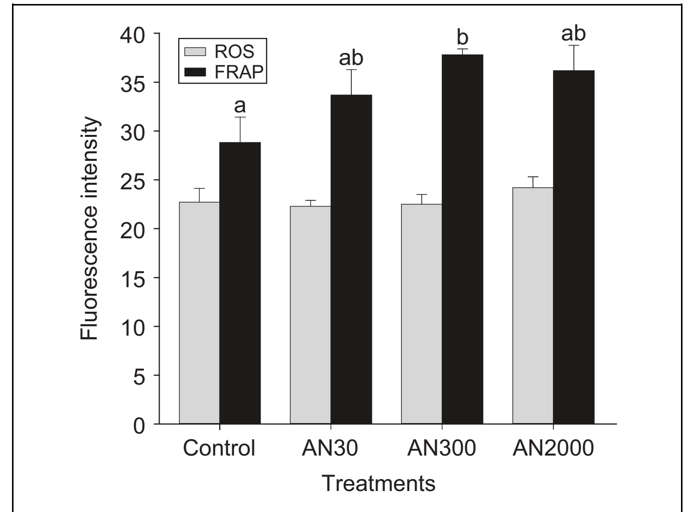

Figure 2. Fluorescence intensity of ROS and FRAP in the culture medium after in vitro maturation of bovine oocytes in control medium with or without supplementation of anethole at 30 (AN30), 300 (AN300), or 2000 μg/mL (AN2000). [a,b]Different letters indicate significant differences among treatments ($P < .05$). FRAP indicates ferric reducing antioxidant power; ROS, reactive oxygen species.

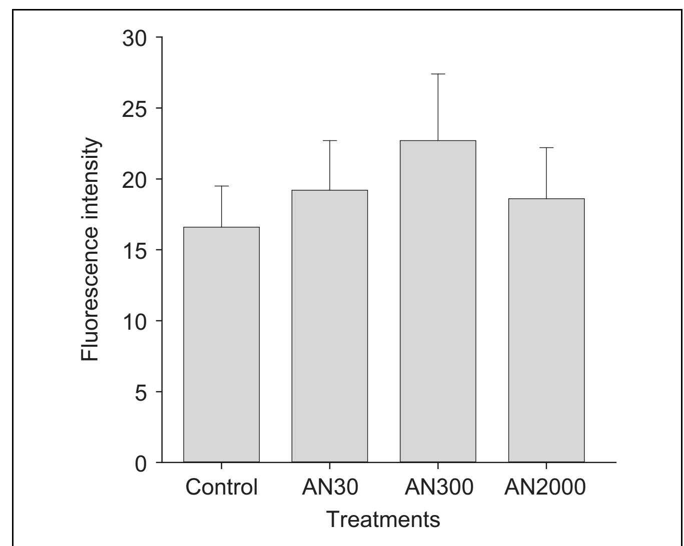

Figure 3. Mean (± SEM) fluorescence units of mitochondrial ROS after in vitro maturation of bovine oocytes in control medium with or without supplementation of anethole at 30 (AN30), 300 (AN300), or 2000 μg/mL (AN2000). No difference among treatments was observed. ROS indicates reactive oxygen species.

are shown (Figures 3 and 4, respectively). The ROS production in the oocytes after maturation did not differ ($P > .05$) among treatments. However, oocytes treated with anethole at 300 μg/mL had higher ($P < .05$) mitochondrial membrane potential when compared to the control treatment ± 0.2 vs 15.3 ± 2.9). In addition, a positive association between mitochondrial membrane potential and cleavage rate was observed by correlation analysis (Figure 5).

Effects of Anethole Addition to the IVM Medium on In Vitro Embryo Development

The impact of anethole addition to the IVM medium on embryo development is shown (Table 1). Anethole at 300 μg/mL was the only treatment that yielded higher ($P < .05$) cleavage rate and embryo development (morula + blastocyst) production compared to the control treatment. Furthermore, the AN300 treatment had greater ($P < .05$) average number of total cells in blastocysts compared to the control and AN30 treatments.

Discussion

This study shows for the first time that anethole added to IVM medium, despite not affecting oocyte nuclear maturation, increased in a concentration-dependent manner the total

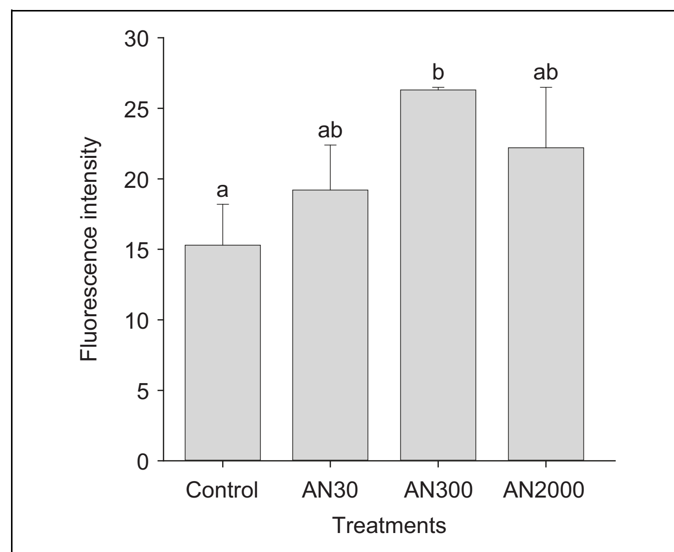

Figure 4. Mean (± SEM) fluorescence units of mitochondrial membrane potential after in vitro maturation of bovine oocytes in control medium with or without supplementation of anethole at 30 (AN30), 300 (AN300), or 2000 μg/mL (AN2000). [a,b]Different letters indicate significant differences among treatments ($P < .05$).

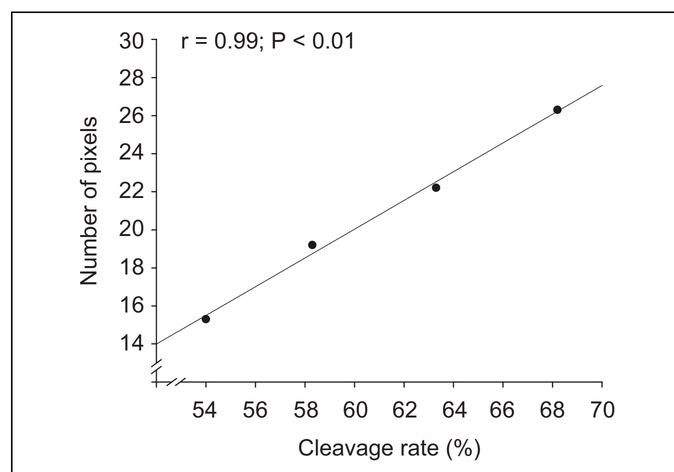

Figure 5. Correlation between oocyte mitochondrial membrane potential (number of pixels) and percentage of embryo cleavage rate. Each dot represents a treatment with their respective oocyte mitochondrial membrane potential versus its overall cleavage rate.

Table 1. In Vitro Embryo Development From Matured Bovine Oocytes in Control Medium With or Without Supplementation of Anethole at 30 (AN30), 300 (AN300), or AN2000.[a]

Treatment	n	Cleavage Rate (%)	Embryo Development (%)[b]	No. Embryo Cells
Control	100	54.0 (54)[c]	13.0 (13)[c]	119.9 ± 11.3[c]
AN30	108	58.3 (63)[c,d]	17.6 (19)[c,d]	124.5 ± 5.1[c]
AN300	107	68.2 (73)[d]	27.1 (29)[d]	144.1 ± 3.1[d]
AN2000	109	63.3 (69)[c,d]	18.3 (20)[c,d]	130.8 ± 4.1s[c,d]

[a]The experiments were replicated 6 times.
[b]Morula + blastocyst.
[c,d]Within a column, different letters indicate significant differences among treatments ($P < .05$).

antioxidant potential and mitochondria membrane potential of bovine oocytes and improved the quantity and quality(based on the number of embryonic cells) of in vitro produced embryos. Previous studies by our team demonstrated the protective effect of anethole against oxidative damage when added to culture medium of goat preantral follicles cultured either in situ or in the isolated form.[19,22]

In the present study, supplementing the IVM medium with anethole did not result in a higher proportion of oocytes that reached the MII stage. Likewise, other antioxidants have shown similar results when added to bovine IVM medium, that is, no effect on oocyte chromatin configuration.[32,33] In this regard, it has been suggested that intracellular antioxidant content at the end of IVM did not appear to affect oocyte nuclear maturation, but rather exerted a positive effect on cytoplasmic maturation and, consequently, on early embryonic development.[3]

After IVF, oocytes matured in vitro in the AN2000 treatment had a significant increase in the fertilization rate (ie, percentage of oocytes with 2 pronuclei formation, male and female). Gamete fusion induces the release of free calcium (Ca^{2+}) within the mammalian oocyte. Multiple Ca^{2+} oscillations trigger an orderly sequence of events that include the release of cortical granules, which prevent polyspermy, the completion of the second meiotic division, and the formation of the female pronucleus.[34] Anethole has been suggested as a trigger that may induce the opening of voltage-dependent Ca^{2+} channels.[35] Therefore, we believe that anethole at the concentration of 2000 μg/mL may have improved normal fertilization rates by opening Ca^{2+} channels, thus triggering the events necessary for pronuclei formation.

The addition of anethole during IVM altered neither the intra- nor extracellular ROS concentrations; nevertheless, the AN300 treatment increased the total antioxidant potential. The balance between ROS formation and elimination is known as the redox balance. In the oocyte, the main ROS scavenger system is GSH in the reduced state, and glutathione disulfide in the oxidized state. Glutathione uses a reducing power provided by oxidative metabolism, tied to redox couples such as $NADPH/NADP^{+}$, to counteract oxidation via ROS.[36] Any unbalance in this redox state may have deleterious effects on cells.[37] It has been previously reported that anethole may play an important role in the maintenance of the redox balance by either reducing ROS levels[22] or inducing the synthesis of cellular antioxidants such as CAT, SOD,[38] and GPX.[21] Accordingly, our results suggest that anethole improved the redox balance by increasing the total oocyte antioxidant capacity after maturation.

Interestingly, oocytes from the AN300 treatment had a higher mitochondria membrane potential. Mitochondria play a central role in energy production, cell metabolism, and cell death and are the main generator of free radicals in mammals.[39] For this reason, the mitochondrial pattern has been associated

with the quality and developmental capacity of mammalian oocytes and embryos.[40] Previous reports have shown that mitochondria membrane potential is lower in immature oocytes compared to the mature ones and that mitochondrial activity is related to oocyte competence.[41,42] Based on our findings of the mitochondria membrane potential and redox balance, we can suggest that anethole positively influenced oocyte cytoplasmic maturation in a concentration-dependent manner since higher rates of embryo production were obtained.

Regarding IVP, oocytes exposed to 300 μg/mL anethole during IVM showed higher rates of cleavage, embryonic development, and total cell number per blastocyst. It is well known that ROS levels increase,[43] and low adenosine triphosphate (ATP) production[44] can severely affect embryonic development in vitro. Variations in ROS concentration can affect the expression of several genes, including those for protein kinases, tyrosine kinases, and growth factors.[45] However, it has been suggested that redox balance rather than ROS ensures appropriate levels of gene expression related to cell cycle regulation.[46] In this regard, early embryo development and implantation potential have been correlated with mitochondrial function and activity.[47,48] The malfunction of the mitochondria generates a decrease in cellular ATP content that generally leads to cell apoptosis.[49] Here, we found a positive correlation between mitochondria membrane potential and cleavage rates. Therefore, the improvements in IVP in this study can be attributed to both the higher redox capacity and the higher mitochondrial membrane potential of the in vitro matured oocytes in the presence of anethole.

In conclusion, our results demonstrate that supplementing the IVM medium with 300 μg/mL anethole improves the quantity and quality of bovine embryos produced in vitro. Moreover, the beneficial effects of anethole on embryonic developmental competence may have been caused by its capacity to regulate the redox balance and improve mitochondrial function.

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