Zygote

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Anethole improves blastocysts rates together with antioxidant capacity when added during bovine embryo culture rather than in the *in vitro* maturation medium

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Research Article

Cite this article: Anjos JC *et al*. (2019) Anethole improves blastocysts rates together with antioxidant capacity when added during bovine embryo culture rather than in the *in vitro* maturation medium. *Zygote* **27**: 382–385. doi: 10.1017/S0967199419000443

Received: 27 April 2019
Revised: 1 July 2019
Accepted: 19 July 2019
First published online: 27 August 2019

Keywords:
Anethole; Antioxidants; *In vitro* embryo production; Oxidative stress

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Summary

We performed the exposure of bovine oocytes to anethole during *in vitro* maturation (0 or 300 µg/ml), during *in vitro* embryo production (0, 30, 300 or 2000 µg/ml), or during both periods to determine the rates of 2–4 cells embryos, blastocysts rates and cells numbers, as well as the production of reactive oxygen species (ROS). Bovine ovaries ($n = 240$) were collected from a local abattoir after slaughter and cumulus–oocyte complexes (COCs) with homogeneous and non-dark cytoplasm, surrounded by two or more compact layers of cumulus cells, and an intact zona pellucida were selected for *in vitro* maturatuion (IVM). Mature oocytes were then submitted to *in vitro* fertilization (IVF) and *in vitro* embryo production (IVP) in culture medium supplemented or not with different concentrations of anethole, as described above. Although IVM medium supplementation with 300 µg/ml anethole improved the rates of bovine blastocysts formation, we demonstrated that IVP medium supplementation with 30 µg/ml anethole, regardless of IVM medium enrichment, considerably enhanced blastocysts rates. Furthermore, ROS levels were decreased only when anethole was added to the IVP medium without previous IVM medium supplementation.

Introduction

In vitro embryo production (IVP) is a consequence of successful steps of *in vitro* oocyte maturation (IVM), *in vitro* fertilization (IVF) and subsequent *in vitro* culture of presumptive zygotes. The IVP technique is routinely used to increase the proliferation of genetically selected animals, as well as a tool to understand physiological processes involved in early embryo development (Moore and Hasler, 2017). Although this technique is applied worldwide at different reproductive centres, optimization of results remains necessary (Wrenzycki, 2018).

Oxidative stress during IVM (Combelles *et al*., 2009; Sovernigo *et al*., 2017), IVF (Sapanidou *et al*., 2016), and IVP (Simões *et al*., 2013; Sá *et al*., 2019), is one of the factors limiting IVP success. Therefore, antioxidants are key compounds to improve *in vitro* embryo production (Torres *et al*., 2018, 2019).

Anethole appears as a promising alternative among the diverse antioxidant sources (Marinovi and Valcheva-Kuzmanova, 2015; Tavallali *et al*., 2017). Recently, we demonstrated that *in vitro* culture of preantral follicles in a medium supplemented with anethole resulted in improved follicular development and decreased levels of reactive oxygen species (ROS) (Sá *et al*., 2017). In a subsequent study, it was shown that IVM of bovine oocytes in the presence of 300 µg/ml anethole improved *in vitro* embryo production further (Sá *et al*., 2019). However, such strategy was not compared during the different steps of embryo production i.e. IVM, IVF and IVP.

The aim of the present study was to investigate the effects of anethole during IVM (300 µg/ml) and/or IVP (30, 300 or 2000 µg/ml) on bovine oocyte maturation and subsequent embryo development. To assess the effects, maturation, cleavage and blastocysts rates were evaluated, as well as blastocyst cell counting and ROS levels.

Material and Methods

Chemicals and medium

Unless otherwise mentioned, the culture media used in the present experiment were purchased from Vitrogen (Cravinhos, SP, Brazil). Anethole was purchased from Sigma (St. Louis, MO, USA).

Oocyte recovery

Bovine ovaries ($n = 240$) were collected from a local abattoir after slaughter and immediately transported to the laboratory in 0.9% (w/v) saline solution (NaCl) at 33–35°C within 1 h. Then, ovaries were washed twice with 0.9% NaCl, and cumulus–oocyte complexes (COCs) were collected from follicles (4–8 mm) using an 18-G needle connected to a 20 ml disposable syringe. COCs were selected under a stereomicroscope (SMZ 645 Nikon, Tokyo, Japan; ×100 magnification) in TCM-199. Only those COCs with homogeneous and non-dark cytoplasm, surrounded by two or more compact layers of cumulus cells, and an intact zona pellucida, were selected for IVM.

In vitro *maturation*

Selected COCs were washed three times in TCM-199 supplemented with 10% fetal bovine serum (FBS; Gibco) medium, previously equilibrate for at least 1 h at 38.5°C under 5% CO_2 in 100 % humidified air. The medium used for IVM was TCM-199 supplemented with 10% FBS, 5 μg/ml FSH, 50 μg/ml luteinizing hormone (LH) and 0.1 μg/ml estradiol (named control IVM medium) or control IVM medium supplemented with anethole at 300 μg/ml. Groups of 30 COCs were cultured in 100 μl of IVM medium on Nunc® Petri plates covered with mineral oil (60 mm) at 38.5°C, 5% CO_2 in air for 24 h.

Assessment of 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 min in PBS supplemented with 0.5% of glutaraldehyde and 10 μM Hoechst 33342 stain (emission at 483 nm) with 10 μl/oocyte. From analysis of chromatin configuration, oocytes were classified according Cadenas *et al.* (2017) as: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), metaphase II (MII) or degenerated (DEG).

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 min at room temperature. The pellet was suspended with 5 ml sperm-Tyrode's albumin lactate pyruvate (TALP®) medium (Papa *et al.*, 2015). The final pellet was suspended in TALP medium, and COCs were washed twice with TALP medium, and then transferred in groups of 30 to 100 μl TALP medium supplemented with penicillamine, hypotaurine, and epinephrine (PHE) with 10 μg/ml of heparin. An aliquot containing 2×10^6 spermatozoa/ml was added to the Nunc Petri dishes under mineral oil. Spermatozoa and COCs were co-incubated for 18 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

In vitro *production of embryos*

The presumptive zygotes were removed from surrounding cells and sperm from successive pipetting and washed twice in CR2 modified medium both supplemented with 10% FBS and bovine serum albumin (BSA) (control IVP medium). Groups of 20 presumptive zygotes were transferred to 100 μl drops of the same wash medium under mineral oil in a 60-mm Petri dish and cultured for 7 days at 38.5°C in a humidified atmosphere of 5% CO_2 in air in the control IVP medium or control IVP medium supplemented with anethole at 30 μg/ml, 300 μg/ml and 2000 μg/ml cleavage was evaluated on day 3 after IVF and rates of blastocysts were evaluated on day 7. Half of the medium was replaced every 2 days.

Blastocysts cell counting

The quality of blastocysts was assessed by Hoechst 33342 staining for 10 min. After washed in 0.1% DPBS + BSA medium, blastocysts were mounted onto clean glass slides, then covered with a coverslip and examined under an inverted microscope equipped with epifluorescence (Nikon, Eclipse 80i, Tokyo, Japan) using NIS – Elements software version 3.00.

Assessment of ROS levels

ROS levels were determined using a spectrofluorometric method, using 2′,7′-dihydrodichlorofluorescein diacetate (DCHF-DA) assay (Loetchutinat *et al.*, 2005). Aliquots (50 μl) of *in vitro* embryo culture medium were organized in pools (from four different treatment combinations) and incubated in 10 μl of DCHF-DA (1 mM). The oxidation of DCHF-DA to fluorescent dichlorofluorescein (DCF) was measured to detect intracellular ROS levels. DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) 2 h after the addition of DCHF-DA to the medium.

Statistical analysis

Data analysis for this paper was performed using SAS software, SAS System version 9.1 (SAS Institute Inc., Cary, NC, USA). Data were compared by analysis of variance (ANOVA) calculated with the generalized linear mixed model (GLMM). Treatment means were compared according Fishers' least significant difference (LSD) test. Data are presented as mean ± standard error of the mean (SEM). The *P*-value of the statistical model is given per response parameter. Effects with *P*-values ≤ 0.05 were considered to be statistically significant.

Results

This study focused on early embryo development *in vitro*. Table 1 shows that presence of anethole in the IVP, or both IVM and IVP medium, did not affect the rates of 2–4-cell embryos. However, a significant positive effect of anethole was observed when evaluating blastocysts rates. When the IVP medium only was supplemented with the test compound, the highest rates of blastocysts were obtained with 30 μg/ml anethole, giving significantly higher blastocysts rate (35.7%) than that observed in the control group (25.4%). When both IVM and IVP media were supplemented with anethole, a significant increase in the blastocyst rate was observed if this compound was added at a concentration of 300 μg/ml in each medium (32.1%). However, such supplementation did not result in significantly higher blastocysts rate when compared with the control group. In summary, the period of anethole supplementation and not only its concentration significantly affected blastocyst rate. Furthermore, the blastocyst cell count did not show differences among treatments.

As anethole concentration did not affect blastocysts rates, the medium samples were pooled for ROS level analysis, considering the presence or absence of anethole in the IVM medium, IVP medium, or both. Figure 1 shows that the significantly lowest ROS levels were observed when anethole was added during IVP without previous IVM medium supplementation.

https://doi.org/10.1017/S0967199419000443 Published online by Cambridge University Press

Table 1. Mean (±standard error of the mean (SEM)) percentages of 2-cell embryos and blastocysts after exposure to different concentrations of anethole during IVM, IVP or both periods

Treatment	2–4-cell embryo (%)	Blastocysts (%)
IVM/IVP	87.4 ± 1.9	25.4 ± 2.8^{a,b}
IVM/IVP + 30 ANE	91.7 ± 5.5	35.7 ± 4.6^{c}
IVM/IVP + 300 ANE	86.7 ± 1.8	29.1 ± 5.4^{a,b,c}
IVM/IVP + 2000 ANE	80.9 ± 8.6	32.3 ± 2.5^{b,c}
IVM + 300 ANE /IVP	81.8 ± 5.3	23.7 ± 5.2^{a}
IVM + 300 ANE /IVP + 30 ANE	83.9 ± 2.9	24.0 ± 6.9^{a}
IVM + 300 ANE /IVP + 300 ANE	86.2 ± 5.8	32.1 ± 4.7^{b,c}
IVM + 300 ANE /IVP + 2000 ANE	82.7 ± 8.7	27.4 ± 7.5^{a,b}
LSD	8.28	8.02
P-value		
Treatment	0.197	0.026
Anethole concentration	0.147	0.067
Exposure period	0.424	0.032

IVM, *in vitro* maturation; IVP, *in vitro* embryo production; LSD, least significant difference.
[a–c]Different superscripts indicate significant difference among treatments ($P \leq 0.05$).
30 ANE: 30 µg/ml anethole; 300 ANE: 300 µg/ml anethole; 2000 ANE: 2000 µg/ml anethole.

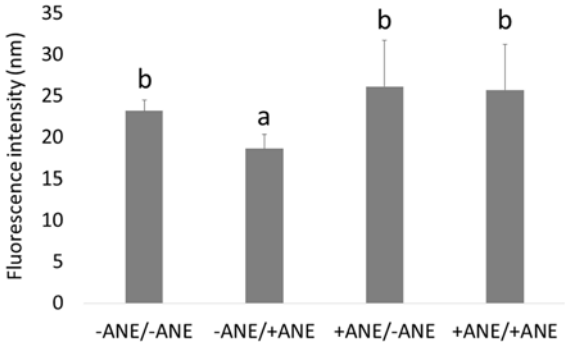

Figure 1. Effects of anethole supplementation during IVM and/or IVP on ROS levels in culture medium. ANE, anethole; IVM, *in vitro* maturation; IVP, *in vitro* embryo production; ROS, reactive oxygen species.[a,b] Different letters represent a significant difference among the treatments ($P < 0.05$).

Discussion

In this study we demonstrated the importance of anethole on early embryo development *in vitro*. IVM medium supplementation with 300 µg/ml anethole has been shown to improve the rates of bovine blastocysts formation (Sá *et al.*, 2019). Here, we demonstrated that anethole did not affect the rates of 2–4-cell embryos, but IVP medium supplementation with 30 µg/ml anethole, regardless of IVM medium enrichment, considerably enhanced blastocysts rates. In addition, ROS levels were decreased only when 300 µg/ml anethole was added to the IPV medium without previous IVM medium supplementation.

The period involved during cell preparation for IVM and IVP, e.g. handling, light, and other environment elements, are key factors that increase oxidative stress in the cells/embryos to be cultured (Yu *et al.*, 2014). Therefore, medium supplementation with antioxidants appeared as a possibility to decrease ROS production (Chowdhury *et al.*, 2017). Anethole is a phenylpropanoid derivative that exhibits antioxidant properties (Galicka *et al.*, 2014). It is known that this substance can directly scavenge ROS, as well as act as a chain-breaking peroxyl radical scavenger (Korkina, 2007), i.e. able to retard the chain oxidative processes (Roginsky, 2003). Furthermore, there are intrinsic factors, such as the oocyte donors, that will determine the risks of ROS production during embryo culture. For instance, Hidaka *et al.* (2018) showed that bovine IVP success depended on the glutathione concentration in COCs, and such levels will also depend on the donors. As it is not cost effective to check oocyte conditions from each donor at each collection, IVM medium supplementation with antioxidants appeared as the most practical alternative (Sovernigo *et al.*, 2017).

In the present study, we confirmed that IVM medium supplementation with anethole improved blastocysts rates. However, this success was linked to the presence of anethole in IVP medium rather than in IVM medium. When ROS levels were assessed in the media, we observed that oxidative stress decreased when IVP medium was supplemented with anethole. This compound supports glutathione (GSH) synthesis to counteract oxidative stress. However, at same time, anethole raises GSH, which is considered a lowering homocysteine agent (Giustarini *et al.*, 2014). With the knowledge that oocyte maturation is mediated by homocysteine (Liang *et al.*, 2007) and that successful embryo development will depend on the oocyte quality, it is not surprising that ROS levels were decreased when anethole was added into IVP medium only. Furthermore, under physiological levels, generated ROS are beneficial during oocyte maturation, as they will trigger meiotic resumption in mammalian species (Khazaei and Aghaz, 2017). Knowledge of the correct time to add antioxidants during *in vitro* culture is of great importance, especially for Nellore embryos that are more sensitive to oxidative stress (Souza *et al.*, 2018a,b).

In conclusion, when using anethole as an antioxidant, attention should be given to the time of medium supplementation. It was better to add this compound to IVP medium and not to IVM medium. These findings, however, cannot be translated to all different antioxidant sources and probably a combination of different antioxidants during the different periods of IVM and IVP would improve the rates of *in vitro* embryo production.

Financial support. This research received no specific grant from any funding agency, commercial or not-for-profit sectors

Competing interests. The authors declare that there is no conflict of interest that can be perceived as prejudicing the impartiality of the research reported.

Ethical standards. Not applicable.

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https://doi.org/10.1017/S0967199419000443 Published online by Cambridge University Press