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Effect of carvacrol antioxidant capacity on oocyte maturation and embryo production in cattle

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Research Article

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Summary

Carvacrol ($C_{10}H_{14}O$), an efficient phenolic antioxidant substance for several cell types, may become a useful antioxidant for female germ cells and embryo culture. This study investigates the effects of carvacrol supplementation on bovine oocytes in *in vitro* maturation (IVM) and embryo production. In total, 1222 cumulus–oocyte complexes were cultured in TCM-199^{+} alone (control treatment) or supplemented with carvacrol at the concentrations of 3 µM (Carv-3), 12.5 µM (Carv-12.5), or 25 µM (Carv-25). After IVM, the oocytes were subjected to *in vitro* fertilization and embryo production, and the spent medium post-IVM was used for evaluating the levels of reactive oxygen species and the antioxidant capacity (2,2-diphenyl-1-picryl-hydrazyl-hydrate and 2,2′-azinobis-3-ethyl-benzothiozoline-6-sulphonic acid quantification). A greater ($P < 0.05$) antioxidant potential was observed in the spent medium of all carvacrol-treated groups compared with the control medium. Moreover, the addition of carvacrol to the maturation medium did not affect ($P > 0.05$) blastocyst production on days 7 and 10 of culture; however, the total number of cells per blastocyst was reduced ($P < 0.05$) in two carvacrol-treated groups (Carv-3 and Carv-25). In conclusion, carvacrol demonstrated a high antioxidant capacity in the spent medium after oocyte maturation; however, although embryo production was not affected, in general, carvacrol addition to IVM medium reduced the total number of cells per blastocyst. Therefore, due to the high antioxidant capacity of carvacrol, new experiments are warranted to investigate the beneficial effects of lower concentrations of carvacrol on embryo production in cattle and other species.

Introduction

Although reproductive biotechniques have been used largely in the livestock industry, limited advances have been achieved regarding the increase in blastocyst rates (~30–40% over the last 30 years) in cattle (Seneda *et al*., 2020). Studies have suggested that inappropriate *in vitro* maturation (IVM) conditions account for the low blastocyst rate compared with its *in vivo* counterpart (De Vos *et al*., 2021). During *in vitro* embryo production (IVEP), excessive intracellular reactive oxygen species (ROS) production leads to detrimental effects on mitochondrial activity, meiotic spindle formation, DNA integrity, and chromatin configuration (He *et al*., 2016). Therefore, the use of antioxidants in *in vitro* culture systems has been shown to be an outstanding alternative to overcome or mitigate the detrimental effects of ROS (Liang *et al*., 2017; Tamura *et al*., 2020; Residiwati *et al*., 2021). Among ROS, hydrogen peroxide (H_2O_2) stands out from the other types of ROS due to its high reactivity in removing electrons from other molecules (Sies, 2017). Similar to ROS, reactive nitrogen species (RNS) are signalling molecules responsible for modulating the interactions between oocyte and sperm, as well as during early embryonic development (Pandey *et al*., 2010; Loren *et al*., 2017).

Several studies have shown some beneficial effects of antioxidants such as resveratrol (Silva *et al*., 2021), lycopene (Residiwati *et al*., 2021), and anethole (Anjos *et al*., 2019) on IVEP in cattle. A potential new antioxidant candidate is carvacrol ($C_{10}H_{14}O$), which has not been tested

in female germ cells and embryo culture. Carvacrol administered orally in different doses (10 and 20 mg/kg) to rats reduced ROS and increased antioxidant enzymes, such as catalase (CAT) and glutathione peroxidase (GPx), in testicular tissue (Güvenç *et al.*, 2018). *In vitro*, carvacrol has been successfully used as an antioxidant due to its relatively low toxicity demonstrated in *in vitro* culture of isolated cells (uterine carcinoma cells: Mastelić *et al.*, 2008; mesenchymal stem cells: Matluobi *et al.*, 2018; and smooth muscle cells: Lee *et al.*, 2015). In addition to its antioxidant properties, carvacrol has shown antibacterial, antifungal, anticancer, hepatoprotective, antispasmodic, vasorelaxant, immunomodulatory, and anti-inflammatory effects (Suntres *et al.*, 2015; Sharifi-Rad *et al.*, 2018; Ezz-Eldin *et al.*, 2020). Despite the encouraging results produced by the addition of carvacrol to *in vitro* cell culture, as mentioned above, to the best of our knowledge, the effects of carvacrol on IVEP in any species have not been studied. Therefore, the aim of this study was to investigate the effect of carvacrol supplementation at different concentrations (0, 3, 12.5, and 25 µM) during bovine oocyte IVM and its further impact on IVEP. Also, we compared among the treatments the levels of ROS, and the antioxidant capacity [2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) and 2,2′-azinobis-3-ethyl-benzothiozoline-6-sulphonic acid (ABTS) measurements] in spent medium after oocyte maturation.

Materials and methods

All procedures were approved by the Ethics Committee in Animal Experimentation of State University of Ceará (CEUA, UECE; #05498222/2019). Unless indicated, the chemicals used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Ovarian source and cumulus–oocyte complexes (COCs) collection

Ovaries ($n = 300$) were collected in a local slaughterhouse and transported to the laboratory, and cumulus–oocyte complexes (COCs) from antral follicles (4–8 mm in diameter) were aspirated and, after selection, destined for IVM. Harvesting of oocytes as well as IVM, fertilization, and embryo culture were performed as previously described (Rodrigues-Cunha *et al.*, 2016) with modifications.

Oocyte in vitro *maturation and experimental design*

TCM-199 medium supplemented with 10% fetal bovine serum (FBS), 25 mg/ml sodium bicarbonate, 22 mg/ml sodium pyruvate, 50 mg/ml amikacin, 5 µg/ml FSH, 50 µg/ml LH, and 0.1 µg/ml estradiol, here called TCM-199^{+}, was used for the IVM procedure. In total, 1222 COCs were cultured in TCM-199^{+} alone (control treatment) or supplemented with carvacrol at 3 µM (Carv-3), 12.5 µM (Carv-12.5), or 25 µM (Carv-25). Pure carvacrol (≥98% of purity, Ref. W224502) was diluted in ethanol (100%) to obtain a stock solution (1:20; 5%) that was further diluted in culture medium to produce the three working concentrations (3, 12.5, or 25 µM). Carvacrol (3 µM) was able to inhibit the increase in H_2O_2 production in vascular smooth muscle cells (Lee *et al.*, 2015). It was also reported that carvacrol (12.5 and 25 µM) increased levels of SOD enzyme activity in human mesenchymal stem cells (Matluobi *et al.*, 2018). COCs ($n = 20$/well/treatment) were incubated in 500 µl of maturation medium in five-well dishes under mineral oil. COCs were matured for 22–24 h at 38.5°C under pre-mixed gas (6% CO_2 + 5% O_2 + 89% N_2). The experiment was repeated six times.

Assessment of cumulus cell expansion

At the end of the maturation period, the degree of cumulus cell expansion in COCs ($n = 191$; 47–48 per group) was evaluated under a stereomicroscope and classified into low, partial, or total expansion (Marei *et al.*, 2009).

Evaluation of nuclear maturation

Oocyte maturation (MII) was evaluated by the extrusion of the first polar body ($n = 360$; 87–93 oocytes per group). As expected, the detection of the first polar body in the perivitelline space was associated with the visualization of the metaphase II plate using fluorescence microscopy after exposure of the oocytes ($n = 107$; 17–35 oocytes per group) to Hoechst 33342 (10 µg/ml) for 15 min (Santos *et al.*, 2019).

Levels of ROS (H_2O_2) in the culture medium

Frozen–thawed cultured medium (10 µl) was incubated with 0.9% phosphate-buffered saline (PBS), 100 U/ml superoxide dismutase (SOD), 0.5 U/ml horseradish peroxidase (HRP), and 50 µM Amplex Red as previously described (Paes *et al.*, 2020) with adaptations. For this analysis, the intensity of the fluorescence signal was measured using a microplate reader (Victor NIVO) under an excitation of 530 nm and emission of 595 nm, and the concentration of ROS was expressed considering the amount of H_2O_2 (µM).

Evaluation of antioxidant activity using the DPPH method

The antioxidant activity of the culture medium after IVM was assessed using a previously described method (Becker *et al.*, 2019) with modifications. The stock solution was prepared by dissolving 13.8 mg DPPH in 100 ml methanol and then stored until needed. The culture medium (20 µl) was allowed to react with 200 µl of methanolic DPPH solution for 60 min in the dark at 25°C. Methanol (20 µl) was used for the blank control (100%), and the methanolic DPPH solution (220 µl) was added to the culture medium (20 µl) to avoid interference due to the sample's colour. The absorbance decrease was recorded at 490 nm. For all evaluated assays, absorbance measurements were performed in triplicate in a microplate reader. The dilutions of samples and positive standards used in quantitative microplate evaluations started from a stock solution with a concentration of 2000 to 0.78 µg/ml. The free radical scavenging activity or antioxidant activity (AA) was expressed as the percentage of inhibition determined by the equation AA% = AC − AS/AC × 100, in which, AA (%) stands for the percentage of antioxidant activity, AC stands for the absorbance of the negative control, and AS stands for the absorbance of the sample. The mean inhibitory concentration (IC_{50}; µg/ml) was obtained using calibration curves, collected by plotting the different concentrations in relation to AA%, and further analyzed by linear regression.

Antioxidant activity in the culture medium using the ABTS method

Antioxidant activity was assessed using the ABTS method as described by Re *et al.* (1999) with modifications. The ABTS solution (7 mM, 5 ml) was mixed with 88 µl of potassium persulfate

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Table 1. Influence of carvacrol during bovine oocyte IVM on the degree of cumulus cells expansion and oocyte nuclear maturation (presence of the 1st polar body)

	In vitro maturation			
	% Degree of cumulus cell expansion			% Nuclear maturation
Treatments	Low	Partial	Total	1st polar body
Control ($n = 141$)	0 (0/48)	8 (4/48)	92 (44/48)	78.5 (73/93)
Carv-3 ($n = 135$)	2 (1/48)	4 (2/48)	94 (45/48)	75.9 (66/87)
Carv-12.5 ($n = 139$)	2 (1/48)	15 (7/48)	83 (40/48)	77.1 (71/92)
Carv-25 ($n = 136$)	2 (1/47)	11 (5/47)	87 (41/47)	78.6 (69/88)

Within a column, no difference ($P > 0.05$) was observed among treatments.
Carv-3, 3 μM carvacrol; Carv-12.5, 12.5 μM carvacrol; Carv-25, 25 μM carvacrol.

(140 mM), agitated, and kept in the dark at room temperature for 16 h. Then, 1 ml of this solution was added to 99 ml of ethanol, and the absorbance was measured at 630 nm. A series of dilutions of the culture medium with decreasing concentrations of the stock solution, as used for the DPPH method, was prepared, and 3 ml of the ABTS solution was added to 30 μl of each of these solutions after 6 min. The AA% and the mean inhibitory concentration (IC_{50}; μg/ml) using ABTS were calculated as described above for DPPH.

In vitro *fertilization (IVF) and embryo culture*

After IVM, the COCs were placed in drops of 90 μl of Tyrode's albumin lactate pyruvate (TALP) fertilization medium supplemented with 10 μg/ml heparin, 22 mg/ml sodium pyruvate, 50 mg/ml amikacin, 6 mg/ml bovine serum albumin (BSA; fatty acid free), 2 μM penicillamine, 1 μM hypotaurine, and 0.25 μM epinephrine, inseminated with Percoll-purified sperm (1×10^6 sperm cells), and incubated in Petri dishes under mineral oil for 18–22 h at 38.5°C in 5% CO_2.

After IVF, the presumptive zygotes were cultured in 500-μl drops of CR2-modified medium supplemented with 50 mg/ml amikacin, 0.1 mM amino acids, 2.5% FBS, and 6 mg/ml BSA in 5-well dishes under mineral oil at 38.5°C under 6% CO_2 + 5% O_2 + 89% N_2.

Assessment of embryo development and blastocyst total cell number quantification

Embryo development was evaluated after 3, 7, and 10 days of culture, and the blastocysts were classified according to Stringfellow and Seidel (1998). The blastocyst (days 7 and 10) and hatching (day 10) rates were calculated. After 7 days of culture, only expanded blastocysts ($n = 118$; 26–33 expanded blastocysts per group) were fixed (0.5% glutaraldehyde) and stained with Hoechst 33342 (10 μg/ml) for 15 min. Embryos were visualized individually by fluorescence microscopy (emission of 370 nm, Olympus BX51TF, Tokyo, Japan), and the total cell number was quantified using ImageJ software (Oliveira *et al.*, 2021).

Statistical analyses

Statistical analysis was performed using Sigma Plot (version 11.0; Systat Software Inc., USA). The proportion variables were analyzed among treatments using Chi-square or Fisher's exact tests. One-way analysis of variance (ANOVA) followed using Tukey's post-hoc test was used to analyze the mean ROS, DPPH, ABTS, and the total number of blastomeres in the blastocysts. The association between carvacrol concentration and cleavage rates was evaluated using Pearson's correlation. Data were presented as percentage and mean ± standard error of the mean (SEM). Statistical significance was defined as $P < 0.05$ (two-sided).

Results

In vitro *maturation*

The influence of carvacrol during IVM of bovine oocytes is shown (Table 1) considering the degree of COC expansion (Figure 1A) and the evaluation of nuclear maturation rate (metaphase II and first polar body; Figure 1B). Overall, 89% of COCs had complete cell expansion after IVM, with no significant difference ($P > 0.05$) observed between treatments. Similarly, the maturation rate (range, 76–79%) did not differ ($P > 0.05$) between treatments.

ROS (H_2O_2) production and antioxidant capacity (DPPH and ABTS) levels in the culture medium

The ROS production and the antioxidant capacity levels were assessed in the spent medium after oocyte IVM (Figure 2). The levels of ROS, regardless of treatment, were similar ($P > 0.05$) to those of the control group. When carvacrol-treated groups were compared, high ($P < 0.05$) levels of ROS were observed in the Carv-25 μM treatment compared with the other two treatments (Figure 2A). However, when the carvacrol antioxidant capacity in neutralizing free synthetic radicals (DPPH and ABTS) was evaluated, carvacrol-treated groups led to greater antioxidant potential against those radicals compared with the control group. Moreover, a progressive antioxidant capacity was observed in a dose-dependent manner as the carvacrol concentration increased ($P < 0.05$) in the treated groups (Figure 2B,C).

In vitro *fertilization and embryo culture*

The impacts of carvacrol addition to the IVM medium on cleavage rate, percentage of blastocysts, blastocysts classes, hatched blastocysts, and the numbers of cells/blastocyst are shown (Figure 1 and Table 2). The addition of carvacrol at 25 μM to the IVM medium reduced ($P < 0.05$) the cleavage rate when compared with the control and Carv-3 treatments (Table 2); however, no significant correlation was observed between the cleavage rate and carvacrol concentrations ($r = -0.34$, $P > 0.05$). The total blastocyst and blastocyst hatching rates (Table 2; Figure 1C,D) as well as the rates of different blastocyst stages were similar among treatments (Figure 1E; $P > 0.05$). However, carvacrol reduced ($P < 0.05$) the mean total cell number per expanded blastocyst compared with

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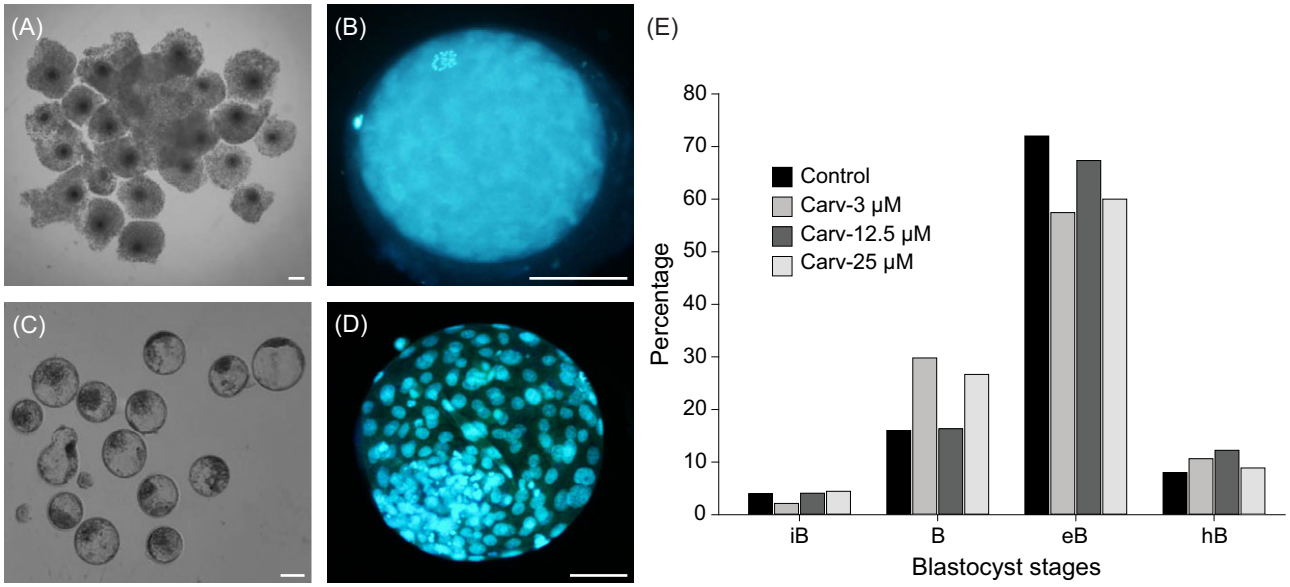

Figure 1. (A) Representative image of bovine oocytes with cumulus expanded after 22–24 h of IVM in the Carv-12.5 treatment. (B) Metaphase II oocyte, with the extrusion of the first polar body. (C) Blastocysts on day 7. (D) Expanded blastocyst stained with Hoechst 33342. Bars, 100 µm (A, C) and 50 µm (B, D). (E) Percentage of blastocyst stages on day 7 post-IVF: initial blastocyst (iB), blastocyst (B), expanded blastocyst (eB), and hatching or hatched blastocyst (hB) within the control and carvacrol treatments. No difference ($P > 0.05$) was observed among treatments.

the control, except for the Carv-12.5 treatment (Table 2; Figure 1D).

Discussion

This study reports, for the first time, the effect of carvacrol addition during oocyte maturation on *in vitro* embryo development in cattle. In the present study, the addition of carvacrol to the medium did not affect oocyte IVM but improved the antioxidant capacity (DPPH and ABTS) of the medium. Moreover, although carvacrol did not affect the levels of ROS in the medium, carvacrol supplementation in a dose-dependent manner reduced the cleavage rate and the number of cells per blastocyst.

The results of antioxidant supplementation on IVM in cattle are controversial. For instance, some studies in cattle have shown a positive effect of resveratrol (Wang *et al.*, 2014), lycopene (Chowdhury *et al.*, 2017), niacin (Kafi *et al.*, 2019), or dimethyl sulfoxide (DMSO; Ynsaurralde-Rivolta *et al.*, 2020), while others did not report any effect for the use of ascorbic acid, resveratrol, anethole (Sovernigo *et al.*, 2017; Sá *et al.*, 2019), and carvacrol (present study) on IVM. These discrepant effects of antioxidants on oocyte maturation might have been due to different *in vitro* culture conditions among studies. It is worth mentioning that, in our study, the results of IVM were obtained using low oxygen atmosphere (i.e. 5% O_2) conditions. Therefore, the use of carvacrol during IVM in other atmospheric conditions is warranted.

In the present study, carvacrol concentrations (3, 12.5, and 25 µM) increased the antioxidant capacity (DPPH and ABTS) when compared with the control group. Among the antioxidant capacity assays that capture free radicals, DPPH and ABTS have commonly been used. Therefore, these two types of assays are able to measure the degree of absorption of free radicals attenuated by the presence of antioxidants in the solution (Re *et al.*, 1999; Morais *et al.*, 2021). Similarly to the findings of the present study, carvacrol has been reported to have a high capacity to scavenge free radicals through the DPPH and ABTS assays (Shahat *et al.*, 2002; Aristatile *et al.*, 2015), as well as some other types of ROS and RNS, due to the phenolic ring in its chemical structure (Jamali *et al.*, 2021). In this regard, an *in vivo* study has shown that carvacrol administered orally in rats using different doses (10 and 20 mg/kg) was able to reduce ROS and increase antioxidant enzymes in testicular tissue (Güvenç *et al.*, 2018). Considering that in this study, ROS levels (i.e. quantification of H_2O_2 levels) were similar between carvacrol-treated groups and the control group, we believe that the greater antioxidant capacity of carvacrol detected through the ABTS and DPPH assays might have been due to its effect in reducing RNS (Jamali *et al.*, 2021), such as nitric oxide (NO; Aristatile *et al.*, 2015), and other types of ROS, for example, the hydroxyl radical ($^{\bullet}OH$; Gavaric *et al.*, 2015) and the superoxide anion (O_2^{-}; Aristatile *et al.*, 2015).

Although in the present study, blastocyst production was not affected by the addition of carvacrol during IVM, after carvacrol supplementation, the cleavage rate and the number of cells per blastocyst were reduced in a dose-dependent manner despite the increased antioxidant capacity. In this regard, a cytotoxic effect of carvacrol on cancer cell membrane permeability has been demonstrated in a dose-dependent manner by an increase in apoptosis and a reduction in cell proliferation (Elbe *et al.*, 2020). Moreover, carvacrol at higher concentrations reduced the expression of proteins related to cell–cell adhesion, such as β-catenin (Elbe *et al.*, 2020), which has an important role in the fertilization process (Takezawa *et al.*, 2011). Considering that the balance between antioxidant capacity and production of free radicals is crucial for cell viability maintenance (He *et al.*, 2016), high concentrations of free radicals induce oxidative stress and cell damage, leading to problems in DNA molecules, proteins, and lipid membranes (Phaniendra *et al.*, 2015; Loren *et al.*, 2017). Studies have shown that the production and maintenance of ROS and RNS in the cell have beneficial effects on oocyte mitochondrial activity (Cajas *et al.*, 2020; McKeegan *et al.*, 2021) and embryo development (Pandey *et al.*, 2010; Loren *et al.*, 2017). In fact, after the use of high antioxidant concentrations in a preincubation period before IVM

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Table 2. Embryo development and quality after IVM of bovine oocytes exposed to different concentrations of carvacrol

	Embryo development and quality				
	% Cleavage	% Blastocysts		Quality of blastocysts	
Treatments	Day 3	Day 7	Day 10	TCN ± SEM[†]	Hatched[††]
Control ($n = 165$)	78.1 (129)[a]	44.8 (74)[a]	50.3 (83)[a]	172.8 ± 7.0 (33)[a]	88.2 (45/51)[a]
Carv-3 ($n = 168$)	77.9 (131)[a]	43.4 (73)[a]	50.0 (84)[a]	150.6 ± 7.2 (31)[b]	90.7 (49/54)[a]
Carv-12.5 ($n = 168$)	73.8 (124)[a,b]	42.2 (71)[a]	45.8 (77)[a]	165.7 ± 9.5 (26)[a,b]	85.1 (40/47)[a]
Carv-25 ($n = 170$)	65.8 (112)[b]	38.8 (66)[a]	42.9 (73)[a]	147.5 ± 7.3 (28)[b]	84.4 (38/45)[a]

[a,b]Within a column, values with different superscripts differ ($P < 0.05$).
Carv-3, 3 µM carvacrol; Carv-12.5, 12.5 µM carvacrol; Carv-25, 25 µM carvacrol.
[†]Total cell number (TCN) in each expanded blastocyst was quantified on day 7 of culture.
[††]Number of hatched embryos divided by the number of resulting embryos on day 10 of culture.

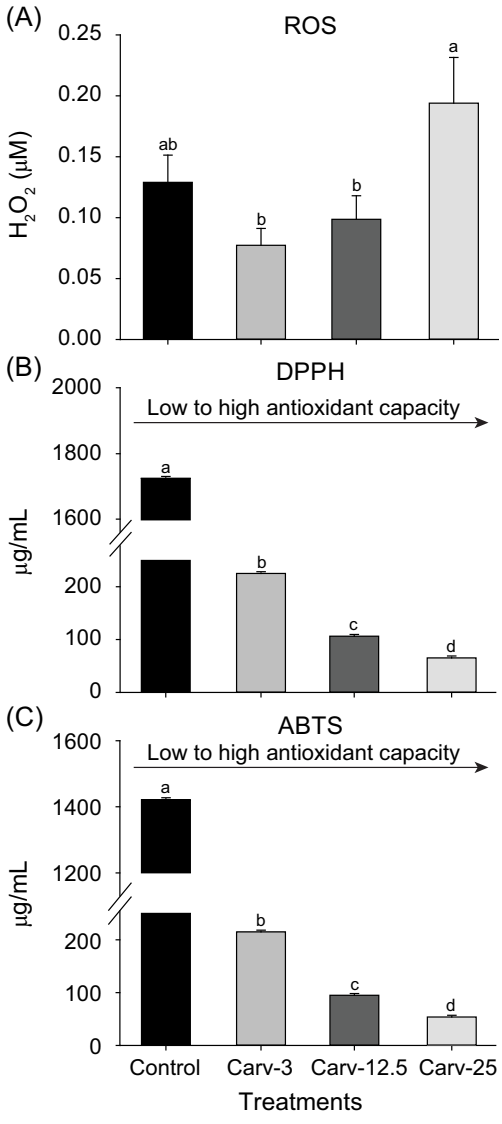

Figure 2. (A) Reactive oxygen species (ROS; quantification of H_2O_2) production and antioxidant capacity with (B) 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) or (C) 2,2′-azinobis-3-ethyl-benzothiozoline-6-sulphonic acid (ABTS) assays in the culture medium after bovine oocyte *in vitro* maturation (IVM) with different concentrations of carvacrol. Carv-3, 3 µM carvacrol; Carv-12.5, 12.5 µM carvacrol; Carv-25, 25 µM carvacrol. [a,b,c,d] Within columns, values with different superscripts differ ($P < 0.05$).

in mice, a reduction in free radicals reduced mitogen-activated protein kinase (MAPK) regulated by the activation of epidermal growth factor receptors (Shkolnik *et al.*, 2011). Moreover, a reduction in ROS during IVM reduced the intracellular calcium release and, consequently, the oocyte activation rate after IVM (Chaube *et al.*, 2008). In this regard, the MAPK pathway and the intracytoplasmic calcium release regulate *in vitro* fertilization and embryo development (Loren *et al.*, 2017). Therefore, we believe that the carvacrol concentrations of 3 and 25 µM used in the present study during the IVM process may have drastically reduced the levels of free radicals after fertilization. This result may have affected the signalling pathways mentioned above, therefore causing a reduction in the multiplication of trophoblast cells demonstrated by the lower number of blastocyst cells in the 3- and 25-µM carvacrol treatments. However, the numbers of cells per blastocyst in these treatments were compared with those reported for *in vivo*-produced embryos (Koo *et al.*, 2002).

In conclusion, a high antioxidant capacity of carvacrol was demonstrated in the spent medium after oocyte maturation. However, although the addition of carvacrol to the maturation medium did not affect blastocyst production, the total numbers of cells per blastocyst were reduced in two carvacrol-treated groups (Carv-3 and Carv-25). Considering the positive antioxidant capacity of carvacrol in the maturation medium, new experiments are warranted to investigate the effects of lower concentrations of carvacrol on embryo production in cattle as well as in other species.

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Conflict of interest. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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