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Impact of the antioxidant quercetin on morphological integrity and follicular development in the in vitro culture of *Bos indicus* female ovarian fragments

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Abstract

We evaluated the effect of quercetin on the in vitro culture of bovine ovarian fragments in relation to morphology, development, and oxidative stress. Ovaries ($n = 12$) from Nelore heifers ($n = 6$) were used. Each pair of ovaries was divided into nine fragments, and one fragment from each animal was fixed in Bouin solution for 24 h (histology control) or frozen (− 80°C; control for oxidative stress). Other ovarian fragments ($n = 8$) were distributed into concentrations of 0, 10, 25, and 50 µg/mL of quercetin added to the culture medium for 5 or 10 d. Data were analyzed by chi-square test or ANOVA followed by Tukey's test ($P < 0.05$). Treatment with 25 µg/mL quercetin resulted in the highest proportion of total intact follicles for 5 (67.3%) and 10 d (57.1%); the concentration of 25 µg/mL also presented the best proportion of developing follicles for 5 d (68.7%) and 10 d (62.8%). Treatment with 25 µg/mL quercetin resulted in significant ferric reduction for 10 d of culture, but not for 5 d. No difference ($P > 0.1$) was observed in the production of reactive oxygen species or in the oxidative degradation of lipids between treatments and non-cultivated controls. Treatment with 25 µg/mL quercetin preserved the morphological integrity of the developing follicles for 5 and 10 d of culture, in addition to promoting the best antioxidant potential after 10 d of culture in bovine ovarian fragments.

Keywords In situ culture · Folliculogenesis · Oxidative stress · Ovary · Bovine

Introduction

Culturing fragments of the ovarian cortex (in situ) is a biotechnology of assisted reproduction that allows the culture of preantral follicles (Gonçalves *et al*. 2008). This methodology has advantages relative to other follicular culture methods because of its ease of execution and shorter time to obtain fragments including follicles because it is not necessary to isolate individual follicles (Picton *et al*. 2008; Higuchi *et al*. 2015). In addition, this culture method provides adequate support for the follicle included in the tissue to maintain its three-dimensional structure, preventing damage to follicular organization and preserving the union of follicular cells with the ovarian stroma (Abir *et al*. 2006; Rossetto *et al*. 2011).

Several studies have evaluated the supplementation of culture medium in vitro with different concentrations of hormones (FSH) (Max *et al*. 2017; Bizarro-Silva *et al*. 2018), growth factors (EGF) (Max *et al*. 2018), and antioxidants (ascorbic acid (Gomes *et al*. 2015) and alpha-lipoic acid (Gomes *et al*. 2018) to understand the effects of these substances on the development and viability of ovarian follicles in cattle and horses. The in vitro environment, however, does not have similar amounts of antioxidants to those found in vivo and exposes gametes to an excess of reactive oxygen species (ROS) (Agarwal and Majzoub 2017). This increase in the production of ROS is due to the in vitro microenvironment, which has higher oxygen concentrations than

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those reported in vivo (Luvoni *et al*. 1996). Excess ROS can promote damage to cell structures (lipid membranes, DNA, proteins) (Agarwal *et al*. 2012), and decrease oocyte quality, inducing apoptosis (Tamura *et al*. 2008).

In this context, one form of protection for cells against damage caused by ROS is the addition of antioxidants, which have favorable effects on cell survival when prepared for in vitro culture systems (Silva *et al*. 2011). Antioxidants protect tissues from the action of the hydroxyl radical (OH) and the superoxide anion (O2-), which are highly reactive species involved at the beginning of lipids (Galati *et al*. 2002). Among antioxidants, the flavonoid group sequesters free radicals and can chelate metal ions (Kandaswami and Middleton 1994).

Quercetin (3,5,7,30,40-pentahydroxyflavone) is a flavonoid with a variety of biological activities (Mariani *et al*. 2008; Batiha *et al*. 2020). One study has suggested action of quercetin in ovarian cells related to proliferation, apoptosis, and hormone release, and this activity may be specific (Sirotkin *et al*. 2019). In addition, quercetin significantly decreased ROS levels compared to the control group in mature swine oocytes (Kang *et al* 2016). However, it is not yet possible to determine the action and adequate doses of quercetin in the culture of bovine ovarian follicles. Thus, the hypothesis of the present study is based on the supplementation of in vitro culture medium with quercetin, with the prediction that its antioxidant effect promotes growth and integrity of the follicles in bovine ovarian tissue. The objective of this study was to evaluate the effect of adding different concentrations of the antioxidant quercetin to the in vitro culture of follicles included in the ovarian tissue of *Bos indicus* females.

Materials and methods

The ovaries of animals from commercial slaughterhouses were used in this experiment. They were destined for slaughter following the rules established by Law No. 1283.

Collection and transportation of the ovaries For this study, two repetitions were performed: one intended for histological analysis, and the other for oxidative stress analysis. In each processing, six pairs of ovaries ($n = 12$) from Nelore heifers (*Bos taurus indicus*) with a mean age of 24 mo and body-condition score of 4 (on a scale of 1 to 5) (Lowman *et al*. 1976) were used in this study. The ovaries were washed with 0.9% sodium chloride solution, and the ovarian tissue was fragmented at the slaughterhouse. Fragments containing antral follicles and corpora lutea were excluded from the study. For histological analysis, a fragment was randomly selected and immediately fixed in Bouin solution (control group, not cultivated on day 0). For oxidative stress analysis, a fragment was separated into a 1-mL microtube, transported to the laboratory at 4°C, and frozen at − 80°C (ROS control group). The remaining ovarian fragments were deposited in 15-mL tubes with minimal essential medium (MEM; Gibco BRL, Rockville, MD; osmolarity 300 mOsm/L, pH 7.2), supplemented with 100 µg/mL of both penicillin and streptomycin, and transported to the laboratory under controlled temperature conditions (4°C; approximately 45 km; 40 min).

Elaboration of the agarose gel Agarose gel was created with a 1.5% agarose solution (Agarose Molecular Biology Grade; Kasvi, Brazil) in distilled water. Subsequently, this gel was sterilized in an autoclave for 15 min, deposited in a sterile Petri dish, and kept in an oven at 37°C for 24 h in order to provide sterility. The gel was divided, with the aid of a scalpel and sterile forceps, into supports of approximately 1 cm, which were packed in 24-well plates (Silva *et al.* 2017).

In vitro *culture of preantral follicles* The ovarian fragments were cultured in 1 mL of MEM supplemented with quercetin at concentrations of 0, 10, 25, and 50 µg/mL, and deposited in 24-well culture plates with agarose gel support. The culture medium was MEM with ITS supplementation (6.25 µg/mL insulin, 6.25 mg/mL transferrin, and 6.25 ng/mL selenium), 0.23 mM pyruvate, 2 mM glutamine, 2 mM hypoxanthine, 1.25 mg/mL of bovine serum albumin (BSA Gibco BRL), 20 UI/mL of penicillin, and 200 mg/mL of streptomycin. After the medium was prepared, plates were cultured for 5 or 10 d, with one fragment of ovarian tissue per treatment, under the same conditions of temperature (38.5°C), atmosphere (5% CO_2), and saturated humidity. The culture medium was prepared and balanced for 1 h before use in an oven, and complete medium change was carried out every 2 d.

Experimental design After ovarian fragmentation ($n = 9$) and removal of a fragment from the uncultivated control group (day 0), the remaining eight fragments were randomly distributed among the culture control group (MEM), and three different concentrations of quercetin supplementation were used: MEM supplemented with 10 µg/mL of quercetin (Q10), MEM supplemented with 25 µg/mL quercetin (Q25), and MEM supplemented with 50 µg/mL quercetin (Q50; Fig. 1), in 5- (day 5), or 10- (day 10) d cultures, as previously established (Kang *et al*. 2016). Two repetitions were performed to obtain samples of ovarian tissue for histological evaluation and oxidative stress analysis.

Histological processing To analyze ovarian follicle morphology, fragments of the ovarian cortex (uncultivated control and samples cultured for 5 or 10 d in vitro) were fixed by immersion in Bouin solution for 24 h, after which they were kept in 70% alcohol. After fixation, the tissues were

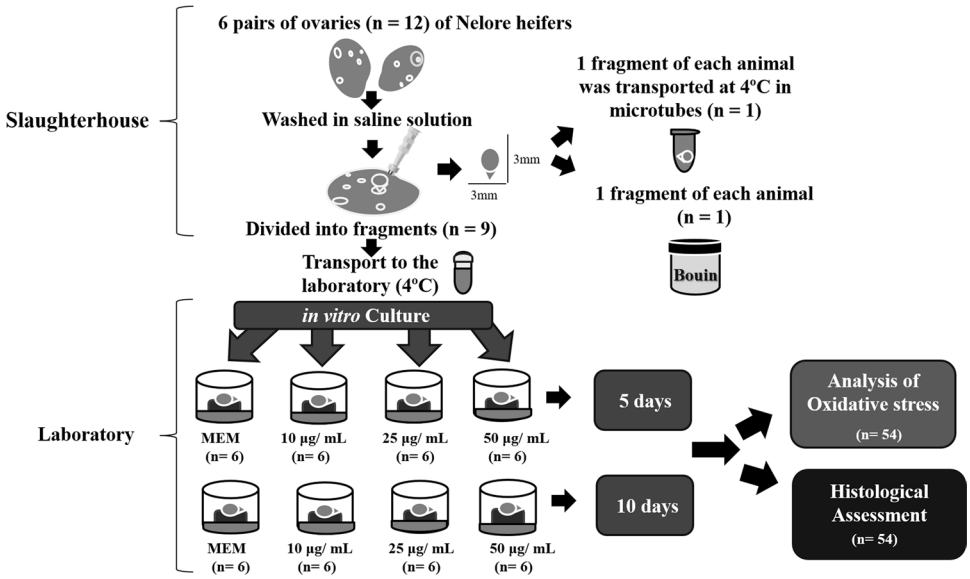

Figure 1. Experimental design used for in vitro culture of bovine preantral follicles in control medium (minimum essential medium—MEM) supplemented with different concentrations of quercetin (0, 10, 25, and 50 µg/mL)

dehydrated in a graded series of increasing solutions of ethanol, diaphanized in xylol, and embedded in paraffin for subsequent histological sections. Then, each fragment was sectioned to a thickness of 5 µm using a rotating microtome (Leica®, Wetzlar, Alemanha, Germany). For assembly of light microscope slides, an interval of 5 sections was used to avoid counting the same follicle more than once during reading. Slides were stained with periodic acid-Schiff (PAS) and hematoxylin, and analyzed by a single evaluator using optical microscopy (Nikon, Tokyo, Japan) at 40×magnification.

Analysis of follicular growth and integrity Preantral follicles were classified according to their stage of development into primordial (a single layer of flat granular cells around the oocyte; Fig. 2*A*), primary (a single layer of cuboid granulosa cells around the oocyte; Fig. 2*B*), or secondary (oocyte surrounded by more than one cell layer of the complete cuboidal granulosa; Fig. 2*C*) (Carámbula *et al.* 1999). Regarding integrity assessment, follicles were considered degenerate when they had at least one of the following characteristics: condensed oocyte nucleus, shrunken oocyte, pycnotic bodies in granulosa cells, low cell density, or rupture of the basement membrane (Silva-Santos *et al*. 2011). Only follicles with nuclei were counted.

Oxidative stress response analysis From collection to oxidative stress analysis, ovarian fragments were kept frozen in sterile microtubes at − 80°C. To measure antioxidant capacity, the ferric-reducing antioxidant power (FRAP) assay was performed as previously described (Katalinic *et al*. 2005; Borghi *et al*. 2021). Superoxide anion production was determined by the reduction of the redox dye nitroblue tetrazolium (NBT) (Rasquel-Oliveira *et al*. 2020), and lipid peroxidation was assessed by measuring levels of thiobarbituric acid reactive substances (TBARS) (Manchope *et al*. 2018; de Souza *et al*. 2020).

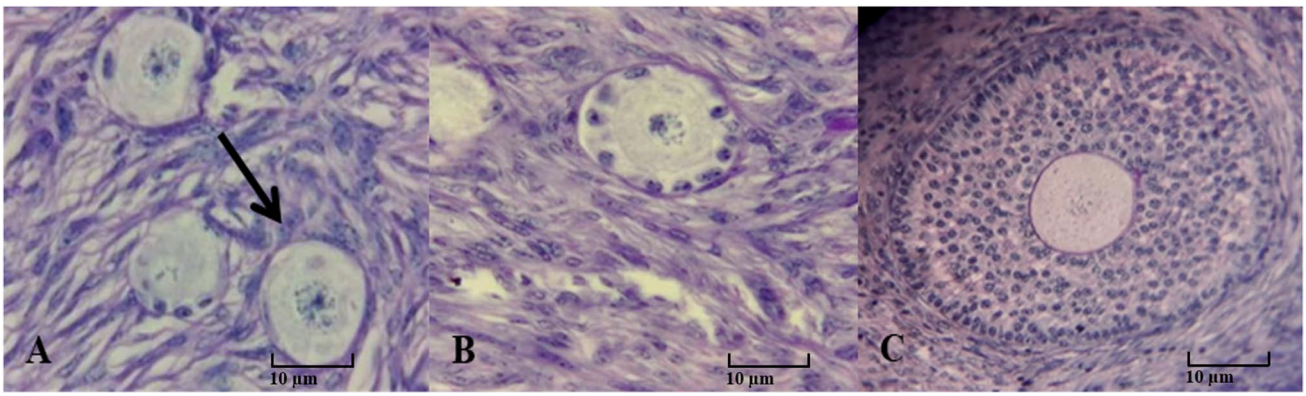

Figure 2. Morphological classification used for preantral follicles. (*A*) Intact primordial follicle (*arrow*), (*B*) intact primary follicle, and (*C*) intact secondary follicle. Sections were periodic acid-Schiff (PAS)- and hematoxylin-stained (100×magnification)

Ferric-reducing antioxidant power assay The FRAP assay was performed to verify ferric-reducing properties as an assessment of the capacity of samples to resist oxidative stress, as previously described. Frozen ovarian fragments were homogenized in ice-cold KCl buffer (500 µL, 1% weight/volume). Homogenates were centrifuged (200 $g \times 10 \times 4$°C), and the supernatants were used in the FRAP assay reagent solution (150 µL) and incubated at 37°C for 30 min. Absorbance was read at 595 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific; Vantaa, Finland) (Katalinic *et al*. 2005; Borghi *et al*. 2021). The results of ABTS and FRAP assays were equated against a standard Trolox curve (0.02–20 nmol).

Nitroblue tetrazolium assay Superoxide anion production was determined by the reduction of the redox dye NBT. Frozen ovarian fragments were homogenized with 500 µL of 1.5% KCl using an Ultra-Turrax homogenizor (Tissue-Tearor 985,370, BioSpec Products, Bartlesville, OK), and 50 µL of the homogenate was placed in a 96-well plate, followed by the addition of 100 µL of NBT solution (1 mg/mL, Sigma) and maintained at 37°C in a warm bath for 5 min. The supernatant was removed, and the formazan precipitate was solubilized by adding 120 µL of 2 M KOH and 120 µL of dimethyl sulfoxide (DMSO). The optical density was measured using a microplate spectrophotometer reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland) at 600 nm. The NBT reduction levels were corrected according to the total protein concentration, and the results were presented as NBT reduction (OD/mg of protein) (Manchope *et al*. 2018).

Thiobarbituric acid reactive substances assay Tissue lipid peroxidation was assessed by measuring TBARS levels. For this assay, 10% TCA was added to the homogenate to precipitate the proteins. The protein-free supernatant was then separated and mixed with TBA (0.67%). The mixture was kept in a water bath for 30 min at 100°C. Malondialdehyde (MDA), an intermediate product of lipid peroxidation, was determined by measuring the difference between absorbance at 535 and 572 nm using a microplate spectrophotometer reader. TBARS values were corrected for the total protein concentration, and the results were presented as TBARS (ΔOD A535–A572/mg of protein) (Manchope *et al*. 2018; de Souza *et al*. 2020).

Statistical analysis The proportion of intact follicles was determined from the total number of evaluated follicles (intact and degenerate). The proportion of primordial and developing follicles (primary and secondary) was determined from the total number of intact follicles in each studied group (uncultivated control on day 0, MEM control, Q10, Q25, and Q50) and at each culture time (days 5 and 10). Differences among follicular rates in each group and on each day were analyzed using the chi-square test. In the event of a significant effect, proportions were compared using a 2×2 proportion test to establish the ranking between treatments. Data from the oxidative stress analyses were subjected to ANOVA followed by the Bonferroni test. Results are presented as proportion or mean ± standard error of the mean (SEM). Analyses were performed using Minitab® 18.1.1 software. Differences were considered significant at $p < 0.05$.

Results

Follicular growth and integrity In the present study, 1141 ovarian preantral follicles were evaluated. The uncultivated group was an internal control of the experiment to assess the initial conditions of the obtained ovarian tissue and presented 52.9% (92/174) intact follicles, 47.1% (82/174) degenerate follicles (92/174), 52.2% (24 intact/46 total primordial) primordial follicles, and 53.1% (68 intact/128 total development) follicles in development. Among the treatments (MEM, Q10, Q25, and Q50) for 5 or 10 d of culture, approximately 26% (295/1141) of the follicles were primordial (Fig. 2*A*), and 74% (846/1141) were developing follicles (Fig. 2*B* and *C*). Morphological evaluation revealed that 52.23% (596/1141) of the follicles were considered intact. Regarding the histological evaluation of the total intact follicles, supplementation with 25 µg/mL of quercetin resulted in the highest proportion of intact follicles (67.3%; 105/156; $P = 0.03$) compared to other treatments (MEM control and different concentrations of quercetin; Fig. 3*A*) cultured in vitro for 5 d. During in vitro culture for 10 d, supplementation with 25 µg/mL quercetin also resulted in the highest proportion of intact follicles (57.1%; 76/133; $P = 0.01$) compared to the other treatments (Fig. 3*B*).

Regarding the stage of follicular development, supplementation with different concentrations of quercetin resulted in similar ($P > 0.1$) proportions of intact primordial follicles regardless of whether the culture was conducted for 5 or 10 d (Table 1). However, when evaluating the percentage of developing follicles, the Q25 treatment at 5 d of in vitro culture was the best ($P = 0.05$) for maintaining the morphological integrity of the follicles in the histological analysis (68.7%), along with the Q50 treatment (57.5%) in the same period (Table 1). In the in vitro culture for 10 d, the Q25 treatment (62.8%) presented the highest proportion ($P = 0.008$) of developing follicles.

Analysis of oxidative stress-Ferric-reducing antioxidant power Analysis of FRAP for the 5-d cultures did not result in a difference between the control (uncultivated sample) and

other treatments ($P > 0.05$; Fig. 4*A*). However, 25 µg/mL of quercetin after 10 d of in vitro culture showed considerable antioxidant potential ($P = 0.03$), demonstrating the greatest capacity for reducing iron among the evaluated treatments (Fig. 4*B*).

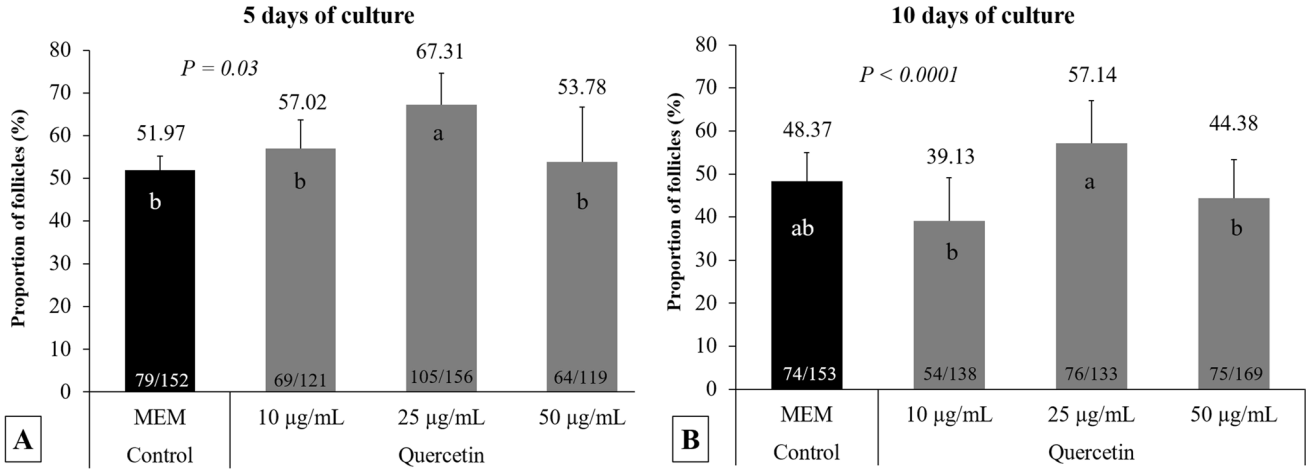

Figure 3. Total percentage of intact follicles evaluated during 5 (*A*) or 10 (*B*) d of in vitro culture of bovine ovarian fragments, treated with minimal essential medium (MEM) with different added concentrations of quercetin (10, 25, and 50 µg/mL). Different *lowercase letters* between *columns* (*a*, *b*), means statistical difference ($P < 0.05$) between treatments for each culture period

Table 1. Total percentage of primordial or developing follicles (primary and secondary) evaluated during 5 or 10 d of in vitro culture of bovine ovarian fragments treated with supplemented minimum essential medium (MEM) or supplemented with different concentrations of quercetin (10, 25, or 50 µg/mL)

Treatment	5 d of culture			10 d of culture		
	Primordial % (*n*/*N*)	Developing % (*n*/*N*)	Intact total *N*	Primordial % (*n*/*N*)	Developing % (*n*/*N*)	Intact total *N*
MEM	47.7% (21/44)	53.7%^{b} (58/108)	79	47.0% (16/34)	48.7%^{b} (58/119)	74
Q 10	56.5% (13/23)	57.1%^{b} (56/98)	69	25.9% (14/54)	47.6%^{b} (40/84)	54
Q 25	50.0% (6/12)	68.7%^{a} (99/144)	105	43.6% (17/39)	62.8%^{a} (59/94)	76
Q 50	45.1% (18/39)	57.5%^{ab} (46/80)	64	36.0% (18/50)	47.9%^{b} (57/119)	75
P-value	0.87	0.05	317	0.16	0.008	279

Values followed by *superscript lowercase letters* (*a*, *b*) within the same *column* differ statistically ($P < 0.05$) between treatments

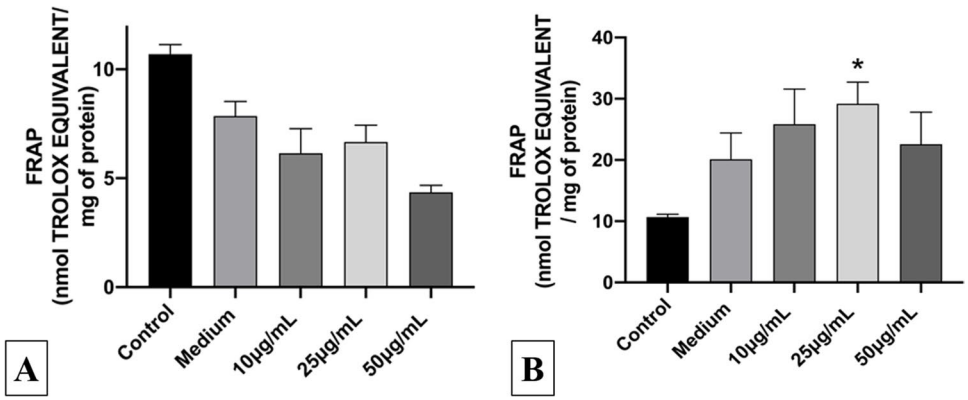

Figure 4. Effect of supplementation with quercetin (0, 10, 50 µg/mL MEM) on ferric-reducing ability of plasma (FRAP) after in vitro culture for 5 (*A*) or 10 d (*B*). *Differs from uncultivated control

Nitroblue tetrazolium The production of superoxide anions (free radicals), evaluated by the NBT assay, was not significantly different ($P > 0.05$) among treatments supplemented with different concentrations of quercetin and the control group, regardless of whether they were cultured for 5 or 10 d (Fig. 5).

Thiobarbituric acid reactive substances Oxidative degradation of lipids, as measured by the TBARS test, showed no significant difference ($P > 0.05$) among the treatments supplemented with different concentrations of quercetin and the control group at 5 or 10 d of in vitro culture (Fig. 6).

Discussion

This is the first study to evaluate the effects of adding quercetin to the in vitro culture of preantral follicles in the ovarian tissue of a bovine species. Additionally, we investigated the antioxidant potential of different concentrations of quercetin in cultured ovarian fragments, and the results obtained by the FRAP method were consistent with the main findings of the histological study. Therefore, the present study makes an important contribution to improving the in vitro culture system of preantral follicles in bovine ovarian fragments.

The present study reports a significant effect on the preservation of follicular integrity, as well as on the proportion of developing follicles when quercetin is used as an antioxidant supplemented into the minimum essential medium in the in vitro culture system of bovine ovarian fragments. The concentration of 25 µg/mL for 5 (67.3%) and 10 d (57.1%) of culture produced higher morphological integrity in follicles than did other concentrations. In addition, this concentration resulted in the highest proportion of developing follicles during the 5- and 10-d cultures. The ovarian fragmentation methodology used in the present study may have influenced the proportion of the total number of developing follicles described (74%). Hsueh and Kawamura (2020) reported that ovarian fragmentation used in the treatment of premature ovarian insufficiency interrupts the signaling of the Hippo pathway and promotes the polymerization of actin, resulting in follicular development and possibly triggering follicular activation. Thus, the ovarian fragmentation applied in the methodology of the present study may have contributed to the increase in the percentage of developing follicles.

The samples showed no differences in relation to the total antioxidant potential (as indicated by FRAP analysis) during culture for 5 d. However, the same concentration that was relevant for morphological integrity in histology (Q25 for both day 5 and day 10) resulted in a difference during in vitro culture for 10 d, in which an increase in

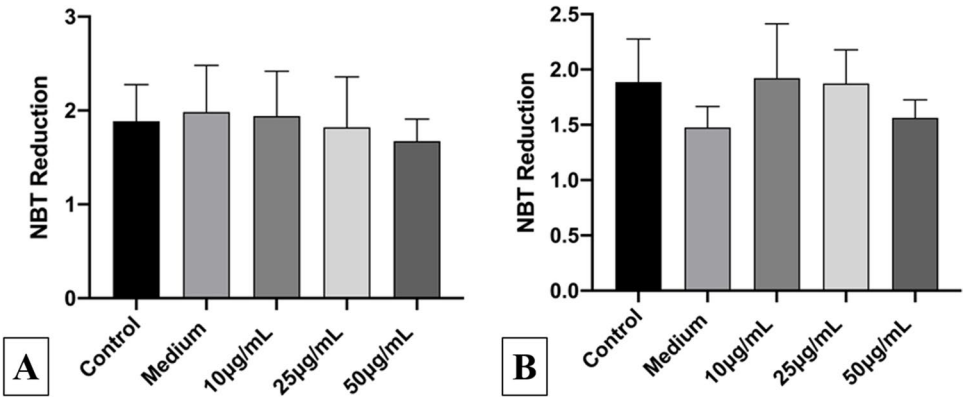

Figure 5. Effect of supplementation with quercetin (0, 10, 50 µg/mL MEM) on the production of superoxide anion (measured by NBT) after in vitro culture of 5 (*A*) or 10 d (*B*)

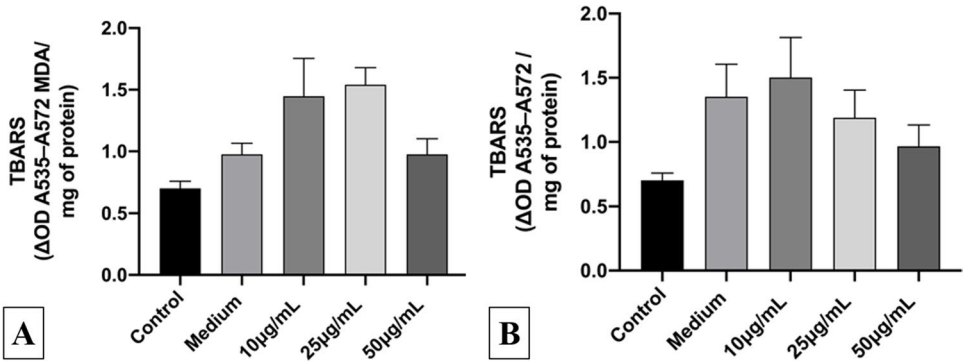

Figure 6. Effect of supplementation with quercetin (0, 10, 50 µg/mL MEM) on lipid peroxidation (measured by TBARS assay) after in vitro culture for 5 (*A*) or 10 d (*B*)

the antioxidant potential of the tissue was observed. The effect of quercetin may also be explained by the activation of Nrf2 (Ferraz *et al*. 2020; Albadrani *et al*. 2021). Nrf2 is involved in cellular adaptation and survival under oxidative challenges as well as promotes cell survival and plays a protective role against cell apoptosis (Bonay and Deramaudt 2015; Fan *et al*. 2017). Thus, it is reasonable to interpret that quercetin improved developing follicles early culture period (5 d) through activation of Nrf2, but did not change FRAP activity. However, additional study is needed to evaluate the participation of Nrf2 in the development of follicles in the early culture period with the supplementation of quercetin. Our data demonstrate that quercetin reverses the stress related to higher concentrations of oxygen in the in vitro environment after 10 d of culture. This result is interesting because the in vitro environment often provides gametes with conditions of greater oxidative stress and, consequently, greater formation of ROS (Agarwal *et al*. 2012; Agarwal and Majzoub 2017). On the other hand, at least at the concentrations tested in this study, quercetin did not affect superoxide anion production (NBT) or lipid peroxidation (TBARS) during in vitro culture for 5 or 10 d.

ROS are normally involved in reproductive events such as follicle development, ovulation, development of the corpus luteum, luteolysis, and early embryonic development (Rizzo *et al*. 2012; Wang *et al*. 2017). Nonetheless, an imbalance between free radicals and antioxidants can cause both failures in conception and in the rupture of the preovulatory follicle, which is eliminated in the formation of follicular cysts in cattle (Rizzo *et al*. 2009; Talukder *et al*. 2014). Although ROS are related to different stages of the reproductive cycle, an excess can lead to damaged cell structures, impairing the quality of oocytes (Tamura *et al*. 2008). In addition, excess ROS can affect mitochondrial function and lead to cell apoptosis in zygotes (Liu *et al*. 2000) and in oocytes subjected to follicular maturation in vitro (Tatemoto *et al*. 2000).

In this context, the addition of antioxidants to the culture medium is an alternative that is used to minimize the damage caused by excess ROS in vitro, and the antioxidant quercetin is one option. Sirotkin *et al*. (2019) reported that quercetin can modulate cell proliferation and apoptosis in ovarian cells, as well as the release of steroid and peptide hormones in the in vitro culture of ovarian cells, in addition to presenting activity that appears to be that of a specific chemical species. In the present study, quercetin was shown to promote antioxidant activity in ovarian tissues. This result corroborates the study by Wang *et al*. (2018), who reported that the addition of quercetin was efficient in regulating and increasing the antioxidant capacity of menopausal mouse ovaries both in vivo and in vitro.

The radical-scavenging activity of flavonoids is determined by their molecular structure and the hydroxyl group substitution pattern such as the availability of hydroxyl group in the phenolic rings of quercetin and the possibility of stabilizing the resulting phenoxyl radicals via hydrogen bonding or electron delocalization expansion (Cos *et al*. 2000; Ferraz *et al*. 2020). The most potent antioxidant flavonoids have a catechol substructure on their A- or B-ring, as well as a hydroxyl on C3 of the C-ring and an oxo-group on C4, which are required for successful iron chelation. The development of a planar molecule or an increase in the conjugation of double bonds in flavonoids is enhanced by the insertion of a double bond between C2 and C3 phenolic rings. The presence of hydroxyl at position 5 in conjunction with the oxo-group at position 4 can also contribute to antioxidative action (Ferraz *et al*. 2020). Quercetin has 5 hydroxyl groups at positions 3-, 3′-, 4′-, 5-, and 7. The presence of O-dihydroxy at B-ring allows stability after hydrogen donation, 2,3 double-bound bonds conjugated to 4-oxo-group on C-ring allows electron dislocation from phenoxyl radicals formed at B-ring, and combination of 2,3-double bond with 3-hydroxy and 5-hydroxy increase resonance stability is relevant to quercetin biological activity (Anand David *et al*. 2016). Thus, quercetin shows a higher antioxidant potential than other flavonoids due to its chemical structure.

The reported benefits of quercetin to other organs and systems have been well-established. In addition, it is already known that there is a specific and appropriate concentration of quercetin for cellular metabolism, since excessive amounts can be harmful to oocytes. For example, Kang *et al*. (2016) reported that a high concentration (100 µg/mL) of quercetin resulted in lower rates of oocyte maturation in pigs, possibly due to a toxic effect. These results corroborate ours, since the concentration of 50 µg/mL (the highest concentration in the present study) was not efficient in maintaining follicular integrity.

Emphasizing the importance of a balanced in vitro environment in relation to ROS, the purpose of the present study was to identify the best concentration of quercetin for the in vitro cultivation of bovine preantral follicles. In this context, at least based on our results, the concentration of 25 µg/mL of quercetin supplemented to MEM, for both 5 and 10 d of in vitro culture of ovarian fragments, was able to provide the best results for maintaining follicular integrity. In addition, we found that the same concentration in the 10-d cultivation resulted in the best antioxidant potential.

Conclusions

In conclusion, a concentration of 25 µg/mL was effective in maintaining the integrity and development of ovarian follicles in in vitro culture for 5 and 10 d. Additionally, we found that this same concentration increased the antioxidant potential after 10-d cultivation. The results of this study will

contribute to the improvement of the in vitro culture of preantral follicles in bovine species.

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