

Effect of rutin on *in vitro* maturation of sheep oocytes from *in vitro* cultured preantral follicles

Efeito da rutina sobre a maturação *in vitro* de oócitos de ovelhas obtidos de folículos pré-antrais cultivados *in vitro*

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Highlights

Supplementation of the IVM medium with 10 µg.mL⁻¹ rutin improved meiotic resumption.

Supplementation of the IVM medium with rutin reduced oocyte DNA fragmentation.

The concentration of 0.1 µg.mL⁻¹ rutin is insufficient to prevent oxidative damage.

Abstract

The aims of this study were to determine the effect of rutin on *in vitro* maturation (IVM) of oocytes from *in vitro*-grown sheep secondary follicles and to analyze the possible involvement of the mammalian target of rapamycin (mTOR) pathway in IVM under the influence of rutin. Secondary follicles were cultured for 18 days in α -Minimum Essential Medium (α -MEM) supplemented with bovine serum albumin (BSA), insulin, glutamine, hypoxanthine, transferrin, selenium, ascorbic acid, and leptin (control medium: α -MEM⁺). Next, the follicles were evaluated for morphology, antrum formation, and follicular diameter, and the rate of fully grown oocytes (≥ 110 µm). Fully grown oocytes were submitted to IVM in Tissue Culture Medium 199 (TCM199) supplemented with fetal bovine serum (FBS), luteinizing hormone (LH), and recombinant

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follicle-stimulating hormone (rFSH) (IVM control medium), or in this medium with 0.1, 1, or 10 $\mu\text{g mL}^{-1}$ rutin. At the end of IVM, the oocytes were evaluated for mitochondrial activity, the reactive oxygen species (ROS) and glutathione (GSH) levels, the percentage of meiotic resumption, DNA fragmentation, and mTOR pathway involvement. After 18 days of *in vitro* culture, 77.5% of the follicles were normal and 77.7% became antral follicles with a diameter of 380.41 μm . Furthermore, almost 70% of the oocytes grew *in vitro*, reaching a diameter $\geq 110 \mu\text{m}$ and were submitted to IVM. Supplementation with 10 $\mu\text{g mL}^{-1}$ rutin significantly increased the percentage of oocytes that resumed meiosis (47.27%) compared with the control medium (30.43%). There was a significant increase in the ROS and GSH levels in oocytes matured with 0.1 $\mu\text{g mL}^{-1}$ rutin compared with the other rutin treatments ($p < 0.05$). Furthermore, oocytes matured with TCM199⁺ showed a higher ($p < 0.05$) percentage of DNA fragmentation (30%) than those that matured with 10 $\mu\text{g mL}^{-1}$ rutin (0%). After IVM, oocytes matured in the presence or absence of rapamycin showed a similar percentage of meiotic resumption (61.76% for TCM199⁺ 10 $\mu\text{g mL}^{-1}$ rutin and 70.73% for TCM + 10 $\mu\text{g mL}^{-1}$ rutin + rapamycin; $p > 0.05$). In conclusion, supplementation with 10 $\mu\text{g mL}^{-1}$ rutin increased meiosis resumption and reduced DNA damage.

Key words: Antioxidants. Flavonoids. Glutathione. Meiosis resumption. DNA damage.

Resumo

Os objetivos deste estudo foram verificar o efeito da rutina sobre a maturação *in vitro* (MIV) de oócitos provenientes de folículos secundários de ovelhas cultivados *in vitro* e analisar o possível envolvimento da via mTOR na MIV, sob influência da rutina. Os folículos secundários foram cultivados por 18 dias em meio α -Mínimo Essencial (α -MEM) suplementado com albumina sérica bovina (BSA), insulina, glutamina, hipoxantina, transferrina, selênio, ácido ascórbico e leptina (meio controle: α -MEM⁺). Em seguida, os folículos foram avaliados quanto à morfologia, formação do antro e diâmetro folicular e taxa de oócitos totalmente crescidos ($\geq 110 \mu\text{m}$). Oócitos totalmente crescidos foram submetidos à MIV em meio de cultivo de tecidos 199 (TCM199) suplementado com soro fetal bovino (FBS), hormônio luteinizante (LH), hormônio folículo estimulante recombinante (rFSH) (meio controle MIV) ou neste meio com 0,1, 1 ou 10 $\mu\text{g mL}^{-1}$ de rutina. Ao final da MIV, os oócitos foram avaliados quanto à atividade mitocondrial, concentração de espécies reativas de oxigênio (ERO) e glutathiona (GSH), porcentagem de retomada de meiose, fragmentação de DNA e envolvimento da via mTOR. Após 18 dias de cultivo *in vitro*, 77,5% dos folículos estavam normais e 77,7% tornaram-se folículos antrais, com 380,41 μm de diâmetro. Além disso, 70% dos oócitos que cresceram *in vitro* atingiram diâmetro $\geq 110 \mu\text{m}$ e foram submetidos à MIV. A concentração de 10 $\mu\text{g mL}^{-1}$ de rutina aumentou significativamente a porcentagem de oócitos que retomaram a meiose (47,27%) em comparação ao meio controle (30,43%). Houve um aumento significativo nas concentrações de ROS e GSH em oócitos maturados com 0.1 $\mu\text{g mL}^{-1}$ de rutina em comparação com os outros tratamentos com rutina ($p < 0,05$). Além disso, a maturação de oócitos em TCM199⁺ aumentou ($p < 0,05$) o percentual de fragmentação de DNA (30%) comparado ao tratamento com 10 $\mu\text{g mL}^{-1}$ de rutina (0%). Após MIV, ambos os tratamentos maturados na presença ou ausência de rapamicina apresentaram porcentagem semelhante de retomada meiótica (61,76% para TCM199⁺ 10 $\mu\text{g mL}^{-1}$ de rutina e 70,73% para TCM199⁺ 10 $\mu\text{g mL}^{-1}$ de rutina + rapamicina) ($p > 0,05$). Em conclusão, a concentração de 10 $\mu\text{g mL}^{-1}$ de rutina aumentou a retomada da meiose e reduziu os danos ao DNA.

Palavras-chave: Antioxidante. Flavonóide. Glutathiona. Retomada de meiose. Apoptose.

Introduction

An *in vitro* culture system is a promising technique to obtain full *in vitro* development of preantral follicles in different domestic species ovine: (Kamamma et al., 2016); caprine: (Magalhães et al., 2011); bovine: (Antonino et al., 2019). Furthermore, *in vitro* maturation (IVM) of *in vitro*-grown oocytes remains an important possibility to obtain metaphase II oocytes, potentially increasing the number of embryos (Aguila et al., 2020). In sheep, the efficiency of these techniques still needs improvement because the number of mature oocytes and embryos produced from *in vitro*-grown oocytes is low (Arunakumari et al., 2010; Luz et al., 2012). One of the factors associated with low quality oocyte is the overproduction of reactive oxygen species (ROS) during culture and IVM, which leads to DNA damage, loss of membrane integrity, and mitochondrial dysfunction. Therefore, it remains necessary to use exogenous antioxidant supplementation in IVM medium to eliminate ROS and to prevent oxidative stress (Rakha et al., 2022).

Rutin is a polyphenolic bioflavonoid that is typically obtained from plants and fruits, especially apricots, cherries, grapes, grapefruit, plums, mulberry, and oranges (Ganeshpurkar & Saluja, 2017; Enogieru et al., 2018). Rutin is an antioxidant that can directly and indirectly neutralize ROS, activate antioxidant enzymes, remove harmful metal ions, inhibit oxidases, reduce oxidative stress caused by nitric oxide, and prevent lipid peroxidation and damage to mitochondrial DNA (Cai et al., 2019; Muhammad et al., 2019). Several *in vivo* studies have highlighted the antioxidant effects of rutin. It provided ovarian protection in mice exposed

to cisplatin (Lins et al., 2020); ameliorated metabolic, biochemical, and hormonal disturbances in rats with polycystic ovary syndrome (Jahan et al., 2016); and reduced ischemia-reperfusion ovarian injury in rats, decreasing malondialdehyde (MDA) and increasing glutathione (GSH) levels (Nayki et al., 2018). Supplementation of *in vitro* culture medium of sheep secondary follicles with rutin efficiently replaced three antioxidants (transferrin, selenium, and ascorbic acid), maintaining follicular viability and increasing GSH levels (Lins et al., 2017). Furthermore, rutin reduced apoptosis and increased activation of primordial follicles during *in vitro* culture of sheep ovarian tissue (Lins et al., 2021). Despite these satisfactory results, there are no reports on the effects of rutin on IVM of sheep oocytes from *in vitro*-grown secondary follicles.

The antioxidant protective effect of rutin on cardiomyocytes and cardiac function is related to activation of phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway (Fei et al., 2019). The mTOR pathway regulates the proliferative activity of cumulus cells during oocyte maturation (Kogasaka et al., 2013; Guo et al., 2018). mTOR is divided into two complexes, mTORC1 and mTORC2 (Tian et al., 2019), which act synergistically in oocytes, regulating cell activation and development (Chen et al., 2015; L. Li et al., 2021). Therefore, we hypothesize that rutin also acts in oocytes through the mTOR pathway. We aimed to determine the effect of rutin on IVM of oocytes from *in vitro*-grown sheep secondary follicles and to analyze the possible involvement of the mTOR pathway in IVM under the influence of rutin, evaluating the following endpoints: the percentage of

meiotic resumption, mitochondrial activity, glutathione and ROS levels, and DNA fragmentation.

Material and Methods

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Source of ovaries

Ovaries (n = 220) from 110 adult mixed-breed sheep (1-5 years old) were collected at a slaughterhouse. The pairs of ovaries were washed once in 70% alcohol and twice in HEPES-buffered Minimal Essential Medium (MEM-HEPES) supplemented with antibiotics (100 µg mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin). Then, the ovaries were transported within 1 h to the laboratory in tubes containing MEM-HEPES with antibiotics at 4°C (Chaves et al., 2008). The experiment was carried out at the Federal University of Vale do São Francisco, located in Petrolina, Pernambuco (09°23'55" S, 40°30'03" W) at an altitude of 376 m, with average annual precipitation around 400 mm.

Isolation, selection, and culture of secondary follicles

Isolation, selection, culture, and follicular evaluation were performed according to Barros et al. (2019). Ovarian cortical slices (1-2 mm thick) were cut from the ovarian surface using a surgical blade under sterile conditions and subsequently placed in fragmentation medium consisting

of MEM-HEPES with antibiotics (100 µg mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin). Secondary follicles, approximately 250-350 µm in diameter without antral cavities, were visualized under a stereo microscope (Nikon, Tokyo, Japan) and mechanically isolated by microdissection using 26-gauge (26 G) needles. Follicles selected for *in vitro* culture had an intact basement membrane, two or more layers of granulosa cells, and a visible and healthy oocyte that was round and centrally located within the follicle, without dark cytoplasm. After selection, isolated follicles (n = 876) were cultured individually in 100-µL droplets of culture medium under mineral oil in Petri dishes (60 × 15 mm, Corning, Sarstedt, Newton, NC, USA) at 39°C under 5% CO₂ for up to 18 days. The base control medium consisted of α-MEM (pH 7.2-7.4) supplemented with 3.0 mg mL⁻¹ bovine serum albumin (BSA), 10 ng mL⁻¹ insulin, 5.5 µg mL⁻¹ transferrin, 5.0 ng mL⁻¹ selenium, 2 mM glutamine, 2 mM hypoxanthine, 50 µg L⁻¹ ascorbic acid, and 25 ng mL⁻¹ human recombinant leptin (Macedo et al., 2019). Every 2 days, 60 µL of the culture medium in each droplet was replaced with fresh medium.

Evaluation of follicular morphology and development after *in vitro* culture

Follicular morphology and development (n = 636 follicles) were analyzed every 6 days by using a pre-calibrated ocular micrometer and a stereo microscope (Nikon) at 100× magnification. Atretic follicles presented dark oocytes surrounded by granulosa cells, misshapen oocytes, a basement membrane that had ruptured, and/or oocyte extrusion. The

following characteristics were analyzed in the morphologically normal follicles: antral cavity formation, defined as the emergence of a visible translucent cavity within the granulosa cell layers; the diameter of follicles, measured from the basement membrane, which included two perpendicular measurements of each follicle; and the daily growth rate, calculated as the normal follicle final diameter minus its diameter on day 0, divided by the number of culture days (18 days).

After 18 days of culture, all healthy follicles were opened carefully with a 26 G needle under a stereo microscope (Nikon) for oocyte recovery. The percentage of fully grown oocytes ($\geq 110 \mu\text{m}$) was calculated as: (the number of acceptable quality oocytes [$\geq 110 \mu\text{m}$] recovered / the total number of cultured follicles) $\times 100$. These oocytes were submitted to IVM.

Maturation of ovine oocytes from in vitro-grown secondary follicles

IVM was performed in oocytes derived from *in vitro*-grown follicles. After culturing for 18 days, all oocytes enclosed in healthy follicles were collected carefully with 26-G needles under a stereo microscope (Nikon). Only oocytes $\geq 110 \mu\text{m}$ in diameter with a homogeneous cytoplasm and surrounded by at least one compact layer of cumulus cells ($n = 476$) were selected for IVM as described previously (Luz et al., 2012; Lunardi et al., 2016). The cumulus-oocyte complexes (COC) were transferred to 100- μL drops of maturation medium composed of control medium Tissue Culture Medium 199 (TCM 199) supplemented with 10% fetal calf serum (FCS), $1 \mu\text{g mL}^{-1}$ human recombinant follicle-stimulating hormone (r-FSH; Gonal-F; Serono

Laboratórios, São Paulo, Brazil), and $1 \mu\text{g mL}^{-1}$ luteinizing hormone (LH; ovine pituitary) under oil, and incubated for 24 h in 5% CO_2 in air (Ceconi et al., 1999). To evaluate the effects of rutin on IVM, the COC were matured in control medium alone or with 0.1, 1, or 10 $\mu\text{g mL}^{-1}$ rutin. The rutin concentrations were chosen based on a previous study (Lins et al., 2017). At the end of IVM, the oocytes ($n = 208$, approximately 52 per treatment) were incubated in phosphate-buffered saline (PBS) drops containing 10 mM Hoechst 33342 for 15 min at room temperature in the dark and visualized using an epifluorescence microscope (Nikon) with a UV filter (483 nm) at 100 \times magnification. The chromatin configuration (blue fluorescence) was analyzed by observing the intact germinal vesicle (GV), meiotic resumption (including germinal vesicle breakdown [GVBD] or metaphase I [MI]), or nuclear maturation (metaphase II [MII]).

Assessment of mitochondrial activity and ROS and GSH levels after IVM

After IVM, the oocytes ($n = 123$, approximately 30 per treatment) were recovered and the intracellular mitochondrial activity and ROS and GSH levels were measured using previously described methods (Bezerra et al., 2019). Briefly, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA; Invitrogen Corporation, Carlsbad, CA, USA), 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (CellTracker[®] Blue; CMF2HC; Invitrogen Corporation), and Mitotracker Red (Mitotracker[®] Red, CMXRos, Molecular Probes, Melbourne, Victoria, Australia) were used to detect ROS, GSH, and mitochondrial activity, which appeared

as green, blue, and red fluorescence, respectively. Oocytes (25-30 per treatment) were incubated in the dark for 30 min in PBS containing 10 mM H₂DCFDA, 10 mM Cell-Tracker Blue, and 100 nM Mitotracker Red. After incubation, the oocytes were washed with PBS for 30 min and fluorescence was observed under an epifluorescence microscope with UV filters (460 nm for ROS, 370 nm for GSH, and 579 nm for mitochondrial activity). The fluorescence intensities were analyzed with the ImageJ software (Version 1.41; National Institute of Health, Bethesda, MD, USA).

Detection of DNA fragmentation with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay after IVM

To assess DNA fragmentation after IVM, additional pairs of sheep ovaries (n = 20) were collected, transported to the laboratory, and fragmented as described above. The recovered oocytes were matured in TCM199+ and in the medium that presented the best meiotic resumption and that did not increase ROS production compared with the control medium (namely TCM199+ supplemented with 10 µg mL⁻¹ rutin). At the end of IVM, oocytes (n = 70, approximately 35 per treatment) from the treatment with the most desirable outcomes were subjected to the TUNEL assay as described previously (Macedo et al., 2017). Briefly, oocytes were fixed in 4% paraformaldehyde for 1 hour at room temperature. Thereafter, approximately 45 oocytes per treatment were washed three times in solution of PBS containing polyvinylpyrrolidone (PVP) and stored at

4°C in Eppendorf tubes with PBS/PVP until beginning the TUNEL procedure. Next, the oocytes were incubated in 50-µL droplets of permeabilizing solution (0.1% [v:v] Triton X-100 in 10 mM PBS) for 3 h at room temperature. Positive and negative controls were incubated in 50-µL drops containing DNase-free RNase (Invitrogen Corporation) at 37 °C for 1 h and washed three times in PBS/PVP. The TUNEL reaction mixture was prepared as indicated by the manufacturer (In Situ Cell Detection Kit, Fluorescein: Boehringer Mannheim/Roche Diagnostics Ltd., Indianapolis, IN, USA); 125 µL of this mixture contained 12.5 µL of terminal deoxynucleotidyl transferase enzyme and 112.5 µL of marker solution of 2-deoxyuridine triphosphate 5-FITC. The experimental oocytes and the positive control were incubated with 15 µL of this reaction mixture for 1 h at 37 °C in a moist chamber in the dark. The negative control was incubated with 15 µL of the marker solution only. After incubation, the oocytes were washed three times in PBS/PVP and then incubated in PBS drops containing 10 mM Hoechst 33342 for 15 min at room temperature in the dark. Thereafter, the oocytes were washed in PBS/PVP, and slides were prepared for evaluation using an epifluorescence microscope (Tokyo, Japan) at a magnification of 400×. TUNEL-positive oocytes were containing chromatin with green fluorescence.

Pharmacological inhibition of the mTOR pathway during IVM

The additional pairs of ovaries (n = 20) were also used to evaluate the effect of the pharmacological inhibition of the mTOR

pathway during IVM of oocytes to analyze whether the effects of rutin on IVM are linked to the mTOR signaling pathway. After culture, the oocytes ($n = 75$, approximately 35 per treatment) were submitted to IVM for 24 h at 39°C in 5% CO₂ in the standard medium supplemented with 10 µg mL⁻¹ rutin or in this medium in the presence of 100 nM rapamycin, an mTOR inhibitor. Next, the oocytes were incubated in PBS drops containing Hoechst 33342 and evaluated as described above. The inhibitor concentration was chosen according to Kordowitzki et al. (2020), who reported a reduction in the maturation rate of bovine oocytes.

Statistical analysis

The percentages of follicle survival, antrum formation, and retrieval of fully-grown oocytes after *in vitro* culture; the maturation rate; the percentage of TUNEL-positive oocytes; and the maturation rate after pharmacological inhibition of the mTOR signaling pathway were analyzed with the chi-squared test. The follicular diameter measurements were submitted to the D'Agostino-Pearson and Mann-Whitney test to determine whether they met the assumptions of a normal distribution of residues and homogeneity of variances. The mitochondrial activity and ROS and GSH levels were submitted to the Shapiro-Wilk test and ANOVA test to determine whether they met the assumptions of a normal distribution of residues and homogeneity of variances. The mitochondrial activity and ROS and GSH levels were assessed with the nonparametric Kruskal-Wallis test; when there was a significant difference,

the Student-Newman-Keuls test was used for pairwise comparisons. The results are expressed as the mean ± standard error of the mean (SEM) or percentage (%). Differences were considered significant when $p < 0.05$.

Results and Discussion

Secondary follicles were classified as morphologically normal when they showed centrally located oocytes and normal granulosa cells, which were enclosed by an intact basement membrane (Figure 1A). As early as 6 days of culture, most follicles displayed a small antral cavity (Figure 1B). After 18 days of culture, there were a few atretic follicles (Figure 1C). From day 0 to day 6, 12, and 18, there was a progressive reduction ($p < 0.05$) in the percentage of morphologically normal follicles (Figure 2A). Nevertheless, antral cavity formation (Figure 2B) and follicle diameter (Figure 2C) increased progressively ($p < 0.05$) throughout the 18-day culture. After 18 days, 77.5% of the follicles were normal and 77.7% became antral follicles, with an average diameter of 380.41 µm. Furthermore, almost 70% of the oocytes grew *in vitro* (diameter ≥110 µm) and were subjected to IVM. These results showed that the follicular culture system used, which was previously tested by our team (Macedo et al., 2019), maintained a high percentage of normal follicles (approximately 78%), and stimulated both antrum formation and follicular growth. It is noteworthy that 70% of the oocytes grew, reaching a diameter of 110 µm, demonstrating that *in vitro* culture of secondary follicles can be a promising technique to increase the number of competent oocytes.

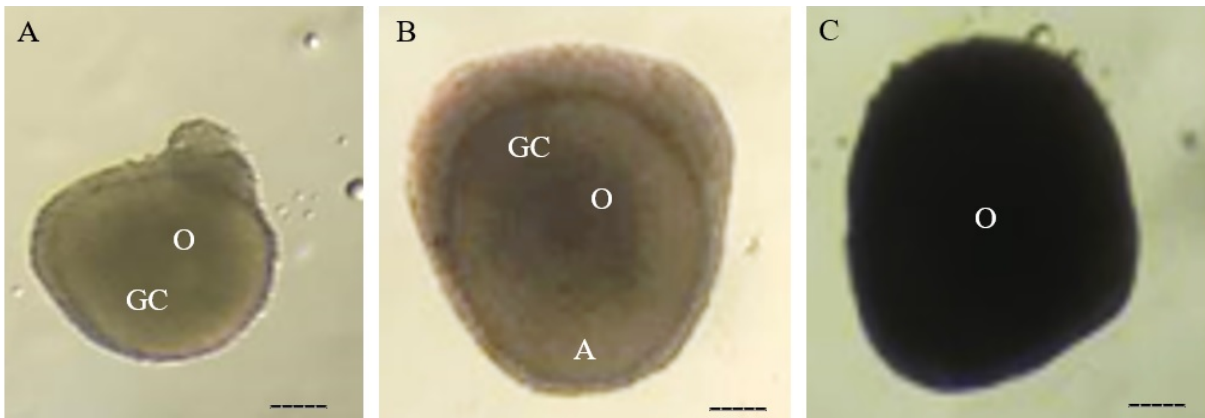


Figure 1. Morphologically normal secondary follicle at day 0 (A), antral follicle after 6 days of culture in 25 ng/mL leptin (B), atretic follicle (C). O: oocyte; GC: granulosa cell; A: antral cavity. Scale bars: 100 μm (100x).

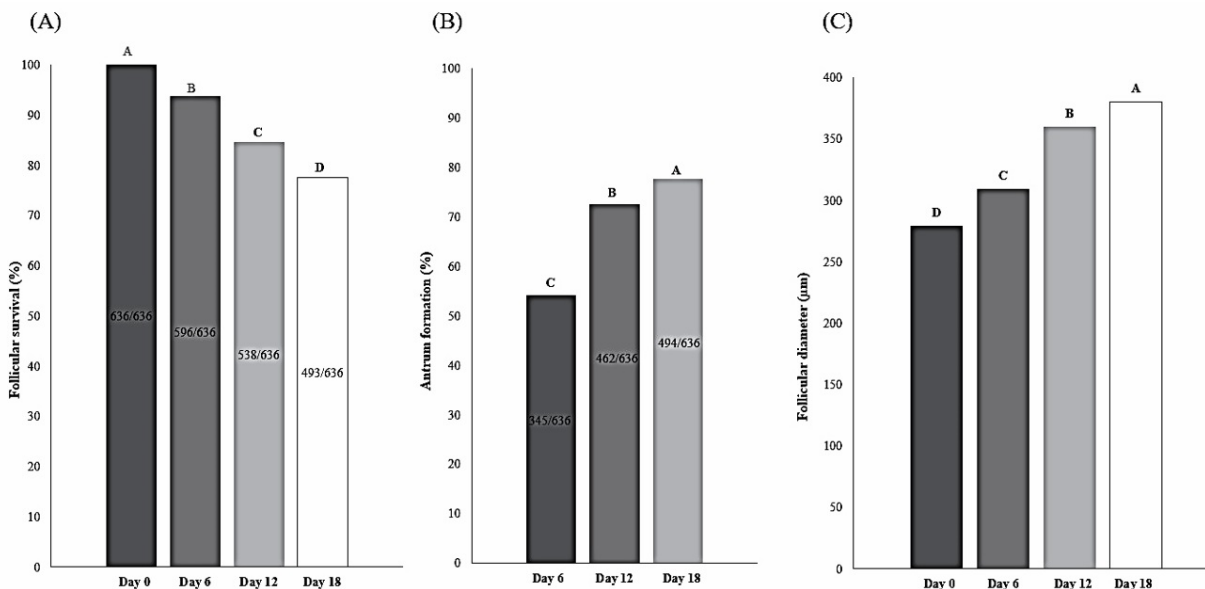


Figure 2. Percentages of normal follicles (A), antrum formation (B) and follicular diameter (C) during *in vitro* culture of secondary follicles. (A, B, C and D) Different letters denote significant differences among culture periods ($p < 0.05$).

After IVM of the recovered oocytes, we observed GV (Figure 3A), GVBD (Figure 3B), and MI (Figure 3C) oocytes in all treatments. Maturation in medium containing 0.1 or 1 $\mu\text{g mL}^{-1}$ rutin did not enhance ($p >$

0.05) meiotic resumption compared with the control medium (TCM199+: 30.43%), whereas medium containing 10 $\mu\text{g mL}^{-1}$ rutin significantly increased meiotic resumption (47.27%) (Table 1). There was no difference (p

> 0.05) in the mitochondrial activity among the treatments (Figure 4A-D and M). Furthermore, maturation in medium containing 0.1 $\mu\text{g mL}^{-1}$ rutin significantly increased the ROS levels in oocytes compared with the other treatments, whereas maturation in medium containing 1 or 10 $\mu\text{g mL}^{-1}$ rutin limited ROS production, with levels similar to the control (Figure 4E-H

and N). In addition, the GSH levels increased ($p < 0.05$) in oocytes matured in medium containing 0.1 $\mu\text{g mL}^{-1}$ rutin compared with the other rutin concentrations. However, there was no difference ($p > 0.05$) between 0.1 $\mu\text{g mL}^{-1}$ rutin and the control group (TCM 199⁺) regarding the GSH levels (Figure 4I-L and O).

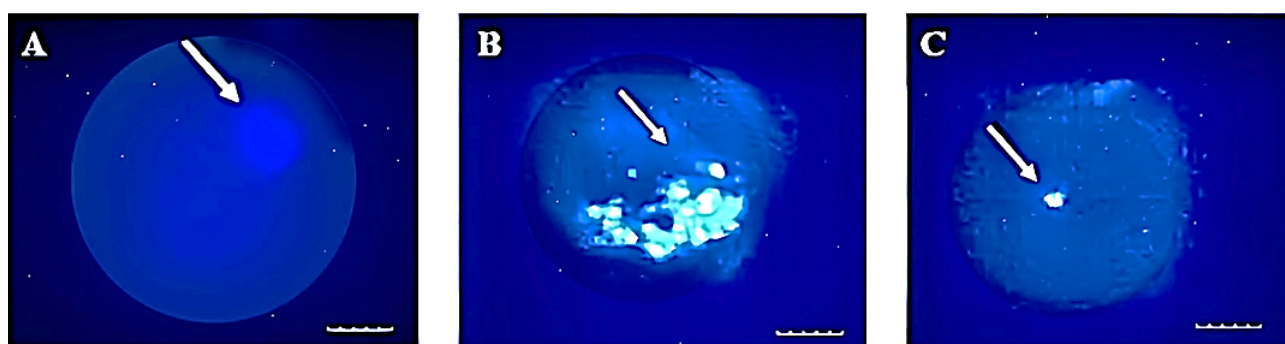


Figure 3. Epifluorescent photomicrographic images of ovine oocytes from *in vitro* grown secondary follicles stained with Hoeschst 33342 after IVM. Oocyte in GV (A), GVBD (B), and MI (C) in medium containing 0,1, 1 ou 10 $\mu\text{g.mL}^{-1}$ rutin, respectively. Arrow: nuclear chromatin. Scale bars: 50 μm (100x).

Table 1

Meiotic stages (%) after *in vitro* maturation of oocytes from *in vitro* grown sheep secondary follicles. (A, B) Different letters denote significant differences ($p < 0.05$)

Treatments	GV (n)	Resumption of meiosis (n)
TCM199 ⁺	69.57 (32/46) ^B	30.43 (14/46) ^B
0,1 $\mu\text{g.mL}^{-1}$ rutin	64.29 (36/56) ^{AB}	35.71 (20/56) ^{AB}
1 $\mu\text{g.mL}^{-1}$ rutin	58.82 (30/51) ^{AB}	41.18 (21/51) ^{AB}
10 $\mu\text{g.mL}^{-1}$ rutin	52.73 (29/55) ^A	47.27 (26/55) ^A

Rutin is a natural, abundant flavonoid that has several beneficial pharmacological properties, including anti-inflammatory, anti-apoptotic, and antioxidant (Geçotek et al., 2017; Imani et al., 2021). Rutin can prevent

cell damage by neutralizing ROS, activating antioxidant enzymes, chelating negative metals, targeting oxidases, and reducing oxidative stress and damage to mitochondrial DNA (Seo et al., 2015; Yashavarddhan et

al., 2017; A. Singh et al., 2017; S. Singh et al., 2019). The secondary metabolites of rutin, including the flavonol quercetin and the disaccharide rutinose, are responsible for its antioxidant activity. They can remove or reduce ROS production, increasing the activity of endogenous antioxidants, such as GSH, the main intracellular component that prevents damage caused by oxidative stress in oocytes (Mukherjee et al., 2014). Supplementation of the vitrification

medium with quercetin reduced ROS accumulation; amplified antioxidant activity; and improved viability, the maturation rates, and developmental competence of sheep oocytes (Davoodian et al., 2021). In goat oocytes, quercetin could replace the antioxidant cysteamine in the IVM medium, increasing oocyte mitochondrial activity and ensuring greater cell viability and quality (A. A. Silva et al., 2018).

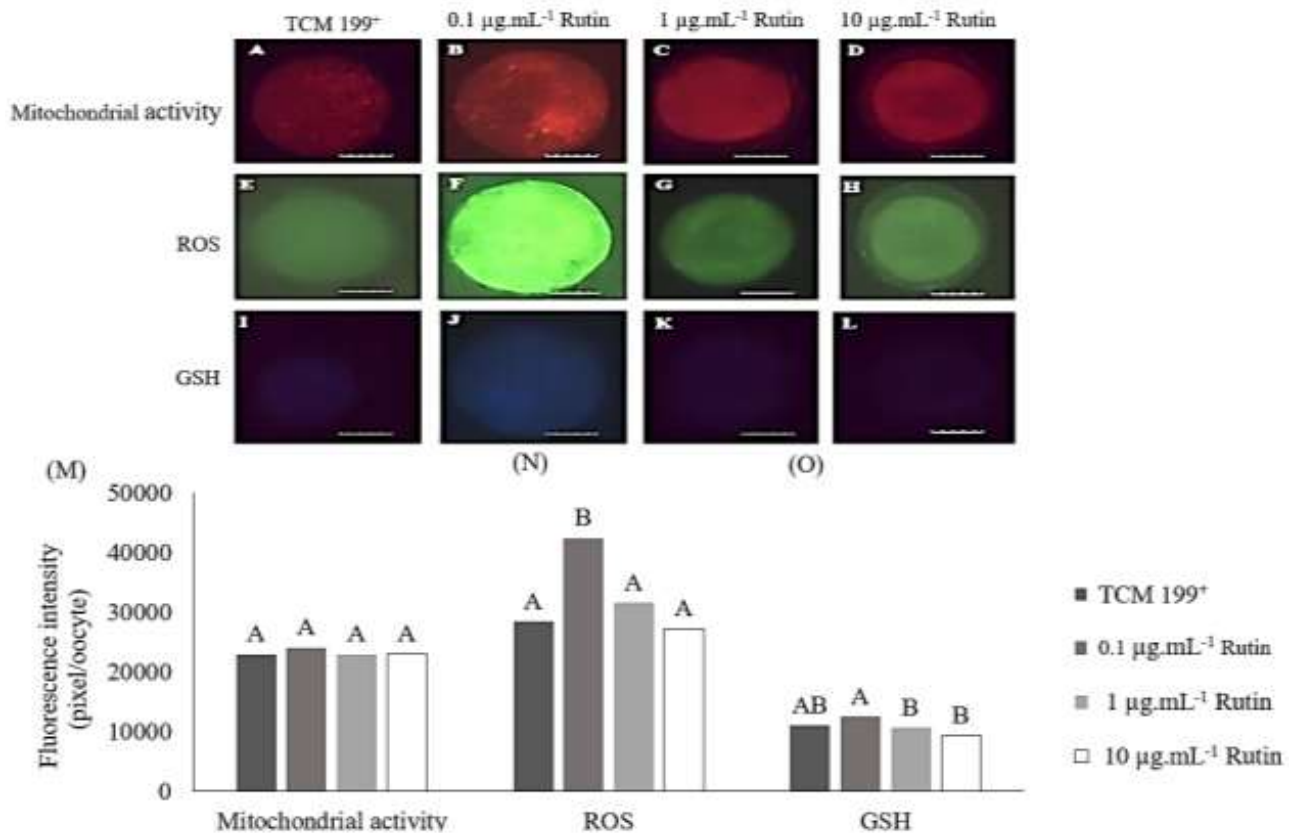


Figure 4. Detection of mitochondrial activity, ROS and GSH concentrations: oocytes cultured in TCM199⁺ (A, E and I) or control medium containing 0,1 (B, F and J), 1 (C, G and K) and 10 ug.mL⁻¹ (D, H and L) rutin. Scale bars: 50 µm (100x). Intracellular levels of mitochondrial activity, ROS and GSH in oocytes after in vitro maturation (M). (^{A, B}) Within each group, different letters denote significant differences among treatments (p < 0.05).

Mitochondrial activity and the ROS and GSH levels were similar in oocytes matured in the control medium and oocytes matured in medium containing $10 \mu\text{g mL}^{-1}$ rutin, suggesting that rutin modulated these markers during IVM. Similarly, rutin did not enhance energy metabolism of cultured hepatocytes (Fukaya et al., 2021). Furthermore, rutin showed cardioprotective action against ischemia-reperfusion injury in mice by increasing silent information regulator 1 (SIRT1), a histone deacetylase, which plays an important role in a variety of biological processes, regulating oxidative stress and antioxidant enzymes and promoting mitochondrial biogenesis and cell survival (Seo et al., 2015; Lin et al., 2018; H. Yang et al., 2019). ROS and GSH are important factors that affect oocyte maturation. The physiological ROS level plays an essential role in follicular rupture and acts by modulating the expression of genes that govern oocyte maturation processes (Kala et al., 2017). GSH, a ubiquitous intracellular free thiol compound, is involved in several cellular processes, including DNA synthesis and protein and amino acid transport (Nunes & Serpa, 2018). In sheep, oral rutin supplementation alleviated oxidative stress during the transition period, increasing the levels of superoxide dismutase, catalase, and GSH peroxidase (Ding et al., 2022). Therefore, considering that oxidative stress is one of the most detrimental factors affecting oocyte developmental competence and maturation, our results indicate that supplementation of IVM medium with $10 \mu\text{g mL}^{-1}$ rutin reduced ROS generation and maintained mitochondrial activity and GSH levels, protecting immature oocytes from damage caused by oxidative stress and thus improving meiotic resumption.

We found that $0.1 \mu\text{g mL}^{-1}$ rutin was insufficient to prevent oxidative damage in oocytes during IVM because it increased the ROS and GSH levels compared with the other rutin concentrations. During IVM, handling and lack of antioxidant defense mechanisms can generate large amounts of cellular oxygen and, consequently, increase ROS production (R. L. S. Silva et al., 2023). In the current study, we suggest that the high GSH levels observed in oocytes matured in medium containing $0.1 \mu\text{g mL}^{-1}$ rutin may represent an ineffective compensatory response of the oocytes to protect themselves against the *in vitro* oxidative stress.

After IVM, all oocytes showed chromatin that fluoresced blue due to Hoechst 33342 staining. The negative control did not show TUNEL staining (Figure 5A-E), while all oocytes showed DNA damage in the positive control (Figure 5B-F). Furthermore, we observed oocytes with DNA fragmentation in the control medium (Figure 5C-G) and healthy oocytes that matured in medium containing $10 \mu\text{g mL}^{-1}$ rutin (Figure 5D-H). Oocytes matured in TCM199+ showed a higher ($p < 0.05$) percentage of oocytes with DNA fragmentation (30%) than those matured in medium containing $10 \mu\text{g mL}^{-1}$ rutin, which did not show DNA fragmentation. A previous study demonstrated that oral rutin supplementation constrained apoptotic activity in the mammary glands of sheep, denoted by a reduction in caspase-9 and caspase-3 protein expression and the Bax/Bcl-2 ratio (Ding et al., 2022). In addition, in ovine species, rutin maintained follicular viability (Lins et al., 2017) and reduced apoptosis (Lins et al., 2021) during *in vitro* culture of isolated secondary follicles or ovarian tissue culture. These data indicate

the antiapoptotic effect of rutin, which is consistent with our findings. Therefore, maintenance of good-quality and healthy

oocytes may have contributed to increased meiotic resumption in medium containing rutin.

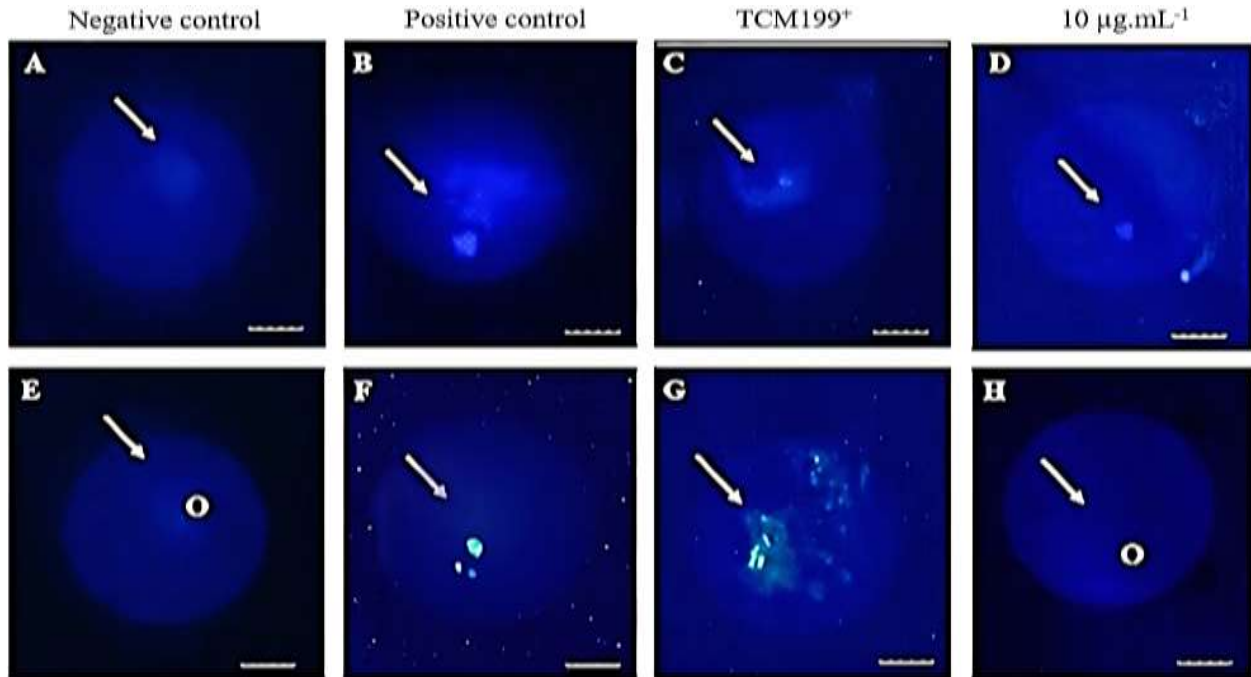


Figure 5. Representative DNA fragmentation of ovine oocytes after *in vitro* maturation. Normal oocyte in the negative control (A, E); oocyte with DNA damage in the positive control (B, F); TUNEL-positive oocytes in the control group (C, G); normal oocyte in the treatment 10 $\mu\text{g.mL}^{-1}$ rutin (D, H). Note the DNA fragmentation evidenced by green chromatin in F and G. Scale bars: 50 μm .

Previous studies have shown that the mTOR pathway is important in the formation of the meiotic spindle, distribution of cytoplasmic proteins, regulation of mitochondrial activity, cumulus cell expansion, autophagy, and blastocyst development murine: (Liu et al., 2018); bovine: (Kordowitzki et al., 2020; Alcaráz et al., 2022; Yang et al., 2022); swine: (Park et al., 2023). Considering that the antioxidant protective effects of rutin on cardiomyocytes are related to activation of the PI3K/Akt/mTOR signaling pathway (Fei et al., 2019), we tested the hypothesis that pharmacological inhibition

of the mTOR pathway with rapamycin inhibits meiotic resumption caused by rutin. We found that oocytes matured in the presence or absence of rapamycin showed a similar percentage of meiotic resumption (61.76% for TCM + 10 $\mu\text{g mL}^{-1}$ rutin and 70.73% for TCM + 10 $\mu\text{g mL}^{-1}$ rutin + rapamycin) ($p > 0.05$). To the best of our knowledge, this is the first study to test the effect of rapamycin on IVM of sheep oocytes. In a previous study, 100 nM rapamycin (Kordowitzki et al., 2020) was not toxic because the meiotic resumption rate was high, indicating good-quality oocytes. However, those authors

reported that 10 nM rapamycin reduced the maturation rate of bovine oocytes. Furthermore, supplementation of IVM medium with rapamycin inhibited the mTOR pathway and thereby induced autophagy (C. Yang et al., 2009) and decreased the rate of murine oocyte maturation (C. Yang et al., 2009; Lee et al., 2012). Our findings suggest that rutin does not act through the mTOR pathway to regulate meiotic resumption in sheep oocytes. Rutin might act through other signaling pathways, such as PI3K/AKT/FOXO3a (Lv et al., 2018; Fei et al., 2019; Y. Li et al., 2019), to improve oocyte developmental competence. However, further investigation is needed to evaluate the influence of other rapamycin concentrations on ovine oocyte IVM.

In the present study, only 19.5% of the oocytes matured in medium containing 10 $\mu\text{g mL}^{-1}$ rutin reached MI. At the end of the *in vitro* culture, the follicular and oocyte diameters were 380.41 and 110 μm , respectively. Researchers have reported a relationship between follicular size and oocyte developmental competence (Crozet et al., 1995; Ledda et al., 1999). After COC culture for 8 days, Crozet et al. (2000) observed that 42% of caprine oocytes (112 μm) resumed meiosis. However, in ovine species, almost 70% of oocytes with a diameter of 142 μm , from small antral follicles (<1 mm), reached MII (Ledda et al., 1999). Therefore, we suggest that an extended culture period would increase oocyte diameter (to at least 142 μm), which could be important to improve cytoplasmic and nuclear maturation and allow oocytes to reach MII. Moreover, the effects of higher rutin concentrations and/or longer mTOR pathway inhibition could be evaluated.

Conclusion

In conclusion, supplementation of the IVM medium with 10 $\mu\text{g mL}^{-1}$ rutin increased meiotic resumption; reduced DNA damage; and maintained mitochondrial activity and the ROS and GSH levels. Furthermore, inhibition of the mTOR pathway did not reduce the antioxidant effect of rutin. New studies focusing on the action of rutin on IVM of ovine oocytes from *in vitro*-grown preantral follicles are necessary to obtain more MII oocytes.

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Statement of conflict of interest

The authors have no conflict of interest to declare.

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