

## Research Article

**Cite this article:** Silva AAA *et al.* (2018) Quercetin influences *in vitro* maturation, apoptosis and metabolically active mitochondria of goat oocytes. *Zygote* 26: 465–470. doi: 10.1017/S0967199418000485

Received: 20 March 2018  
Revised: 19 September 2018  
Accepted: 20 September 2018  
First published online: 15 February 2019

**Keywords:**  
Antioxidant; Flavonoid; GSH; Ovary

**Address for correspondence:**  
E.S. Lopes Júnior. Universidade Federal do Vale do São Francisco (UNIVASF). Colegiado de Medicina Veterinária - Laboratório de Fisiologia e Biotecnologia da Reprodução Animal. Rodovia BR 407 Km 12, Lote 543, Projeto de Irrigação Nilo Coelho, Petrolina, PE 56300-990, Brasil. Tel: +558721014816. Fax: +558721014845. E-mail: edilson.lopes@univasf.edu.br

# Quercetin influences *in vitro* maturation, apoptosis and metabolically active mitochondria of goat oocytes

A.A.A. Silva<sup>1</sup>, M.N.P. Silva<sup>1</sup>, L.B.F. Figueiredo<sup>1</sup>, J.D. Gonçalves<sup>1</sup>, M.J.S. Silva<sup>1</sup>, M.L.G. Loiola<sup>1</sup>, B.D.M. Bastos<sup>1</sup>, R.A. Oliveira<sup>1</sup>, L.G.M. Ribeiro<sup>1</sup>, R.S. Barberino<sup>2</sup>, B.B. Gouveia<sup>2</sup>, A.P.O. Monte<sup>2</sup>, D.M. Nogueira<sup>3</sup>, M.F. Cordeiro<sup>1</sup>, M.H.T. Matos<sup>2</sup> and E.S. Lopes Júnior<sup>1</sup>

<sup>1</sup>Laboratory of Physiology and Biotechnology of Animal Reproduction, Federal University of San Francisco Valley, Petrolina, Brazil; <sup>2</sup>Nucleus of Biotechnology Applied to Ovarian Follicle Development, Federal University of San Francisco Valley, Petrolina, Brazil; and <sup>3</sup>Brazilian Agricultural Research Corporation, Embrapa Tropical Semi-arid, Brazil

## Summary

The present study aimed to investigate the effect of quercetin as an alternative antioxidant to cysteamine on *in vitro* maturation. Oocytes were collected from goat ovaries, destined for *in vitro* maturation and distributed into three groups: CIS group, oocytes were immersed in MIV base medium; in Groups Q4 and Q8, oocytes were immersed in the medium of the CIS group, adding 4  $\mu\text{M}$  or 8  $\mu\text{M}$  of quercetin, respectively, and cultured for 24 h at 38.5°C with 5% CO<sub>2</sub>. The CIS and Q4 groups presented the same percentage of expanded cumulus cells, but the per cent in the Q8 group was significantly lower than that of the other groups ( $P < 0.05$ ). The oocyte retraction rate in the Q8 group was higher ( $P < 0.05$ ) than in the CIS and Q4 groups. Treatment with 8  $\mu\text{M}$  of quercetin presented a lower proportion of expanded oocytes than the CIS group and 4  $\mu\text{M}$  of quercetin ( $P < 0.05$ ). The percentage of MII oocytes was higher in the Q4 group than in the CIS group ( $P < 0.05$ ), but the percentages in the CIS and Q8 groups were similar. The rate of apoptosis was higher in the CIS group than in the other groups ( $P < 0.05$ ). In addition, oocytes matured with 4  $\mu\text{M}$  quercetin showed higher mitochondrial activity than matured oocytes in the CIS and Q8 groups ( $P < 0.05$ ). In conclusion, 4  $\mu\text{M}$  of quercetin can be used as an alternative to cysteamine in the *in vitro* maturation of goat oocytes.

## Introduction

*In vitro* embryo production (IVP) is a promising tool as it allows the optimized use of oocytes from donor females, as well as increasing the number of embryos produced under laboratory conditions (Choi *et al.*, 2013). *In vitro* maturation (IVM) of oocytes is the most important stage of IVP because it determines the successful fertilization, zygote formation, achievement of blastocyst stage and further pregnancy (Leoni *et al.*, 2009). However, currently, IVP in goats is marked by unsatisfactory rates when compared with results observed in other species such as human (Srivastava *et al.*, 2006) and bovine (Bueno and Beltran, 2008). Some studies have reported on goat oocyte physiology, as well as its metabolic requirements to achieve better IVM rates. This information may be the key to success for this biotechnology in such species (Zhou *et al.*, 2008; Souza-Fabjan *et al.*, 2016). One factor that culminates instability in IVM rates is the negative influence of high reactive oxygen species (ROS) production, which affects *in vitro* embryo development in conventional culture systems. This production occurs mainly in the IVM stage due to excessive oocyte manipulation, light exposure and high oxygen tension, causing an imbalance in the oxidation–reduction mechanism (Pu *et al.*, 2014). High ROS production can block or delay oocyte development and decrease the viability of these cells (Kang *et al.*, 2013). However, the equilibrium of ROS concentrations in ideal conditions has a positive effect on IVP because it promotes oocyte and embryo competency from energy generation during metabolism (Guemra *et al.*, 2013).

Substances that are inhibitory to ROS, i.e. antioxidants, have been to be considered significant components to supplement IVM medium. Some examples are cysteamine (Mudo *et al.*, 2015), cysteine (Pu *et al.*, 2014) and vitamin E (Silva, 2015). However, these substances have raised the cost of IVM medium, although they present satisfactory results. Therefore, the

addition of alternative substances with antioxidant action that could reduce the cost of maturation medium is necessary to improve oocyte IVM.

Quercetin (3,5,7,3'-4'-pentahydroxyflavone) is a natural flavonoid widely found in vegetables, grains, fruits, flowers, teas. It has therapeutic properties and antioxidant potential (Behling *et al.*, 2004). Positive effects of quercetin have been already observed on the IVM of bovine (Guemra *et al.*, 2013). The presence of quercetin promoted excellent oocyte maturation rates and blastocyst development in swine by reduction of ROS intracellular levels (Orlovski *et al.*, 2014). Furthermore, quercetin reduces the cost of IVM for oocytes because this flavonoid is 3.6 times less expensive than conventional antioxidant cysteamine (Guemra *et al.*, 2013). However, there have been no reports on effects of quercetin inclusion on oocyte IVM in goats.

Therefore, the aim of the present study was to evaluate the effect of two quercetin concentrations (4  $\mu$ M and 8  $\mu$ M) as an alternative oxidant to cysteamine for IVM of goat oocytes.

## Materials and methods

### Chemicals

Chemicals used in this study were purchased from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA) or from another company specified in the text.

### Aspiration of oocytes

Goat ovaries ( $n = 176$ ) were collected from a local slaughterhouse and transported to the laboratory within 3 h after collection in saline solution (NaCl 0.9%, Fresenius®, Dismed, Brazil) at 30°C (Souza-Fabjan *et al.*, 2016). Ovaries were washed three times in prewarmed fresh saline (30°C) and maintained in a water bath (MODEL 1102®, Fanem, Brazil). Oocytes were aspirated from all visible follicles between 2 and 6 mm in diameter with an 18-gauge short bevel needle (Solidor®, Lamedid Comercial & Serviços, Brazil) connected to a Falcon tube under controlled vacuum (30 mmHg or 6–8 ml/min). The collection tube was filled with 3–5 ml of tissue culture medium 199 (TCM199) supplemented with 50 IU/ml heparin, 50  $\mu$ g/ml gentamycin and 10% (v/v) of fetal calf serum (FCS).

### Evaluation of oocytes

After collection, cumulus–oocyte complexes (COCs) were transferred to 60 mm Petri dishes (Petri Dish60®, Nutricell, Brazil), analyzed under a stereozoom microscope (SMZ 645®, Nikon, Japan) and classified into different grades according to Avelar *et al.* (2012). Only COCs classified as Grade I (with more than three layers of compact cumulus cells) and Grade II (with one to three layers of cumulus cells finely granulated) were indicated for IVM.

### IVM of oocytes and experimental design

Selected oocytes were washed in collection medium until maturation procedures. Then, these COCs were allocated randomly into three maturation groups with 15–17 oocytes for the following groups:

- CIS group, with oocytes matured in TCM-199 supplemented with EGF (10  $\mu$ l/ml), follicle stimulating hormone (FSH)/LH

(10  $\mu$ l/ml), oestrus serum sheep (100  $\mu$ l/ml) and cysteamine (10  $\mu$ l/ml);

- Q4 and Q8 groups, with oocytes matured at medium used in the CIS group but without cysteamine and supplemented with 4  $\mu$ M or 8  $\mu$ M of quercetin, respectively.

The COCs were transferred to 5-well Petri dishes (Thermo Scientific®, Nutricell, Brazil) containing 500  $\mu$ l of maturation medium, under mineral oil, for 24 h at 38.5°C, in a humidified atmosphere of 5% CO<sub>2</sub> in air. Therefore, nine replicates were performed with 426 oocytes designated for IVM.

### In vitro maturation evaluation (IVM)

#### Morphological evaluation

In order to evaluate the expansion of cumulus cells, matured oocytes were evaluated under a stereozoom microscope (SMZ 645®, Nikon, Japan) to determine the presence or absence of cumulus cells expansion, according to published methodology (Avelar *et al.*, 2012). In addition, the cumulus cell expansion grade was evaluated and classified as Total (Grade I), Moderate (Grade II) or Light (Grade III), according Aghaz *et al.* (2015). Briefly, the scoring system spanned from 0 to +4. A score of 0 indicated no expansion; +1 indicated the minimum observable expansion in which cumulus cells had a glistening appearance; +2 indicated oocytes with partial expansion; +3 indicated oocytes with cumulus cells completely expanded, except corona radiata; and +4 indicated the maximum degree of expansion (Vanderhyden *et al.*, 1990).

#### Evaluation of chromatin configuration and oocyte DNA fragmentation

At the end of maturation, oocytes were submitted to terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay based on previous methodology (Gouveia *et al.*, 2016). Briefly, oocytes were denuded and fixed in 4% paraformaldehyde solution for 1 h at room temperature. Then, oocytes (approximately 10 oocytes per treatment group) were washed three times in solution of PBS/polyvinylpyrrolidone (PVP) and stored at 4°C in an Eppendorf tube (Eppendorf 1.5®, Eppendorf do Brasil, Brazil) with 400  $\mu$ l of PBS/PVP (Freezer Horizontal H400®, Electrolux, Sweden), prior to the TUNEL procedure. Oocytes were incubated in 100  $\mu$ l 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 100  $\mu$ l droplets containing DNase-free RNase (Invitrogen Corporation, Carlsbad, CA, USA) at room temperature for 1 h and washed three times in drops of PBS/PVP. TUNEL assay was prepared for 15 min as indicated by the manufacturer and at 4°C (In Situ Cell Detection Kit, Fluorescein: Boehringer Mannheim/Roche Diagnostics Ltd, Indianapolis, USA). For this purpose, 112.5  $\mu$ l terminal deoxynucleotidyl transferase enzyme and 112.5  $\mu$ l of marker solution of 2-deoxyuridine triphosphate 5-FITC were combined to give 225  $\mu$ l TUNEL mixture for the reaction. The experimental groups and the positive control were incubated with 15  $\mu$ l of this solution for 1 h at 37°C in a moist chamber (Equilam®, KEQ300UM, Brazil) in the dark. The negative control was incubated with 15  $\mu$ l of the marker solution. Oocytes were washed three times in 50  $\mu$ l drops of PBS/PVP and incubated in 50  $\mu$ l drops of PBS containing 10 mM Hoechst 33342 stain for 15 min at room temperature in the dark. Then, oocytes were washed three times in 50  $\mu$ l drops of PBS/PVP; slides (Coleman®, Roster, Brazil) were prepared for

evaluation using an epifluorescence microscope (Nikon E200®, Tokyo, Japan) at a magnification of  $\times 400$ . Oocytes were considered apoptotic when they exhibited marked chromatin green fluorescence. Finally, for oocytes, chromatin and the different meiosis stages: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII) were evaluated, following previously published methodology (Rajabi-Toustani *et al.*, 2013).

#### Assessment of oxidative stress markers (ROS and GSH intracellular levels) and metabolically active mitochondria

After IVM, 10 matured oocytes from each experimental group were denuded and intracellular reduced glutathione (GSH) and ROS levels and mitochondrial activity were measured as previously described by Lins *et al.* (2017), with minor modifications. Briefly, 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CellTracker® Blue; CMF2HC; Invitrogen Corporation), 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA; Invitrogen Corporation) and MitoTracker Red (MitoTracker® Red, CMXRos, Molecular Probes, Melbourne, Victoria, Australia) were used to detect intracellular GSH, ROS and mitochondrial activity levels as blue, green and red fluorescence, respectively. Oocytes (approximately 10 oocytes per treatment group) were incubated in the dark for 30 min in PBS supplemented with 10 mM of CellTracker® Blue, 10 mM of H<sub>2</sub>DCFDA and 100 nM MitoTracker® Red at 39°C. After incubation, the oocytes were washed with PBS and their fluorescence was observed under an epifluorescence microscope with UV filters (370 nm for GSH, 460 nm for ROS and 579–599 nm for active mitochondria). Fluorescence intensities of oocytes were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

#### Statistical analysis

Data on maturation rate, cumulus cell expansion and retraction rates, different grades of cumulus cell expansion rates and DNA fragmentation were expressed as percentages and compared using the chi-squared test using Epi Info software (Epi Info 7.1.5.0, Atlanta, GA, USA, 2015). Data for GSH levels and mitochondrial activity were submitted to the Shapiro–Wilk test to verify the normal distribution of residues and homogeneity of variances. Then, the Kruskal–Wallis non-parametric test was used for comparisons. When main effects or interactions were significant, means were compared using the Student–Newman–Keuls test. Data from apoptotic cells (TUNEL) were submitted to the least significant difference Fisher test and expressed as percentage. The results were expressed as the means  $\pm$  standard error of the mean, and differences were considered significant when *P*-values were  $< 0.05$ .

## Results

### Morphological evaluation after IVM

CIS group (control; Fig. 1A) presented the same percentage of expanded cumulus cells as the Q4 group (Fig. 1B; Table 1;  $P > 0.05$ ) but in the Q8 group the percentage was lower ( $P < 0.05$ ) than for the other groups (Fig. 1C). At 24 h from maturation onset, oocyte retraction rate in medium containing 8  $\mu$ M of quercetin was superior ( $P < 0.001$ ) to that of other treatments (Table 1).

For cumulus cell expansion grades, treatment containing 8  $\mu$ M of quercetin resulted in a lower proportion of expanded oocytes at the Grade I (Total) stage and a higher percentage of expanded oocytes at the Grade III (Light) stage compared with treatments containing cysteamine and 4  $\mu$ M of quercetin ( $P < 0.05$ ) (Table 2).

### Analysis of chromatin configuration and DNA fragmentation

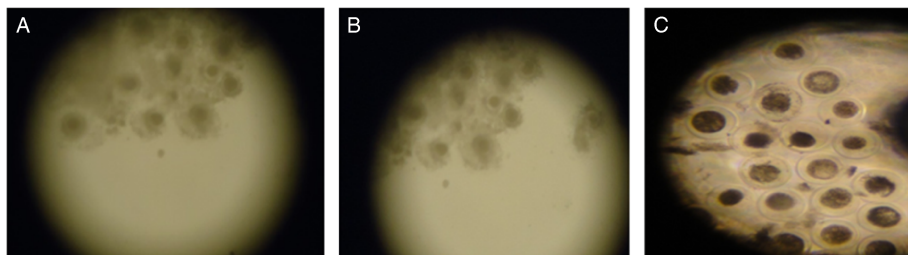
For nuclear maturation rate, the percentage of oocytes that reached the metaphase II stage was higher in medium supplemented with 4  $\mu$ M of quercetin than in medium containing cysteamine ( $P < 0.05$ ), but there was no significant difference between CIS and Q8 (Table 3; Fig. 2). There was a significant increase ( $P < 0.05$ ) in TUNEL-positive cells in the CIS group (28.2%) compared with the Q4 group (0%) or the Q8 (0%) group (Fig. 2).

### Quantification of reactive oxygen species levels (ROS), glutathione levels (GSH) and mitochondrial activity

Oocyte intracellular levels of ROS, GSH and mitochondrial activity are presented at Figs 3 and 4. Oocytes treated with 8  $\mu$ M of quercetin had lower ( $P < 0.05$ ) ROS and GSH levels compared with female gametes matured under CIS and Q4 group conditions; there was no significant difference between CIS and Q4 groups ( $P > 0.05$ ). Oocytes matured in the presence of 4  $\mu$ M of quercetin (Q4) presented higher ( $P < 0.05$ ) mitochondrial activity than female gametes treated under CIS and Q8 group conditions. Moreover, oocytes matured under Q8 group conditions presented lower ( $P < 0.05$ ) mitochondrial activity than female gametes treated under CIS group and Q4 group conditions.

## Discussion

This study is the first to report the effects of inclusion of quercetin as an alternative antioxidant to cysteamine on IVM of goat oocytes. IVM medium supplemented with 4  $\mu$ M of quercetin (Q4) or 100  $\mu$ M of cysteamine (CIS) presented similar expanded cumulus cells and oocyte retraction rates (they did not promote cell damage). Our results are similar to reports from Orlovski *et al.* (2014) that observed a high percentage of cumulus cells



**Figure 1.** Expanded goat oocytes matured *in vitro* in medium containing cysteamine (CIS group, (A), 4  $\mu$ M (Q4 group; (B) or 8  $\mu$ M (Q8 group; (C) of quercetin ( $\times 70$  magnification).

**Table 1.** Cumulus cells expansion and oocyte retraction rates (%) in goat cumulus–oocyte complexes (COCs) matured *in vitro* at medium containing cysteamine (CIS), 4  $\mu$ M (Q4) or 8  $\mu$ M (Q8) of quercetin

Treatments	Cumulus cells expansion rate (%)	Oocyte retraction rate (%)
CIS	67.81 $\pm$ 1.77 <sup>a</sup>	9.88 $\pm$ 1.16 <sup>b</sup>
Q4	71.86 $\pm$ 0.92 <sup>a</sup>	7.76 $\pm$ 0.73 <sup>b</sup>
Q8	46.86 $\pm$ 2.50 <sup>b</sup>	38.19 $\pm$ 3.20 <sup>a</sup>

Data are expressed as the mean  $\pm$  standard error of the mean (SEM) ( $n = 10$  replicates).  
<sup>a,b</sup>Within a column, values with different superscripts differ significantly ( $P < 0.05$ ).

**Table 2.** Effect of inclusion of quercetin on grade of expansion of goat cumulus–oocyte complexes (COCs) matured *in vitro* in medium containing cysteamine (CIS), 4  $\mu$ M (Q4) or 8  $\mu$ M (Q8) of quercetin

Treatments	Grades of expansion (%)			
	G1–Total	G2–Moderate	G3–Light	G1+G2
CIS	46.89 $\pm$ 1.59 <sup>a</sup>	36.67 $\pm$ 3.71 <sup>a</sup>	16.44 $\pm$ 3.39 <sup>b</sup>	83.56 $\pm$ 3.39 <sup>a</sup>
Q4	54.00 $\pm$ 3.13 <sup>a</sup>	34.27 $\pm$ 2.98 <sup>a</sup>	12.00 $\pm$ 1.33 <sup>b</sup>	89.09 $\pm$ 1.01 <sup>a</sup>
Q8	22.01 $\pm$ 3.80 <sup>b</sup>	18.27 $\pm$ 1.88 <sup>b</sup>	57.27 $\pm$ 3.62 <sup>a</sup>	42.73 $\pm$ 3.62 <sup>b</sup>

Data are expressed as the mean  $\pm$  standard error of the mean (SEM) ( $n = 10$  replicates).  
<sup>a,b</sup>Within a column, values with different superscripts differ significantly ( $P < 0.05$ ).

**Table 3.** Stages of nuclear maturation of goat oocytes matured *in vitro* in medium containing cysteamine (CIS), 4  $\mu$ M (Q4) or 8  $\mu$ M (Q8) of quercetin

Treatments	Stages of nuclear maturation, $n$ (%)			
	GV	GVBD	MI	MII
CIS	0 (0)	1 (6.67)	12 (80.0)	2 (13.33) <sup>a</sup>
Q4	0 (0)	1 (4.77)	8 (38.09)	12 (57.14) <sup>b</sup>
Q8	0 (0)	2 (11.76)	9 (52.94)	6 (35.30) <sup>a,b</sup>

<sup>a,b</sup>Within a column, values with different superscripts differ significantly ( $P < 0.05$ ).  
 GV, germinal vesicle; GVBD, germinal vesicle breakdown.

expansion rates in porcine oocytes matured at medium containing 25  $\mu$ g/ml of quercetin. These results suggested that supplementing with 4  $\mu$ M of quercetin or 100  $\mu$ M of cysteamine can influence the mucification process and quality of oocyte.

In contrast, supplementation with 8  $\mu$ M of quercetin at IVM medium promoted a lower cumulus cells expansion rate and a higher percentage of oocyte retraction than other treatments. A possible explanation is that the molecular structure of this flavonoid is similar to the structure of estrogens and therefore it probably interacts with estrogen receptors as well as a phytoestrogen. Quercetin would modulate oocyte activity (Orlovski *et al.*, 2014), promoting the decrease of synthesis and release of 17 $\beta$ -estradiol ( $E_2$ ) as well as the synthesis of growth factors from granulosa cells (Santini *et al.*, 2009). This flavonoid there affects the cellular environment, reducing the expansion of cumulus cells, as  $E_2$  added significant function to the mucification of intrafollicular cells (Kubo *et al.*, 2015).

In the present study, we observed a higher percentage of oocytes in the Q4 group that reached metaphase II stage than matured oocytes matured from the control group. By contrast,

Guemra *et al.* (2013) verified that there was no significant difference in the proportion of bovine oocytes that reached metaphase II stage after IVM in the presence of quercetin or cysteamine. It is important to emphasize that nuclear maturation does not depend directly on cumulus cells expansion (Crocomo *et al.*, 2015), as previously verified in cattle (Shirazi *et al.*, 2009) and goat (Hand *et al.*, 2006).

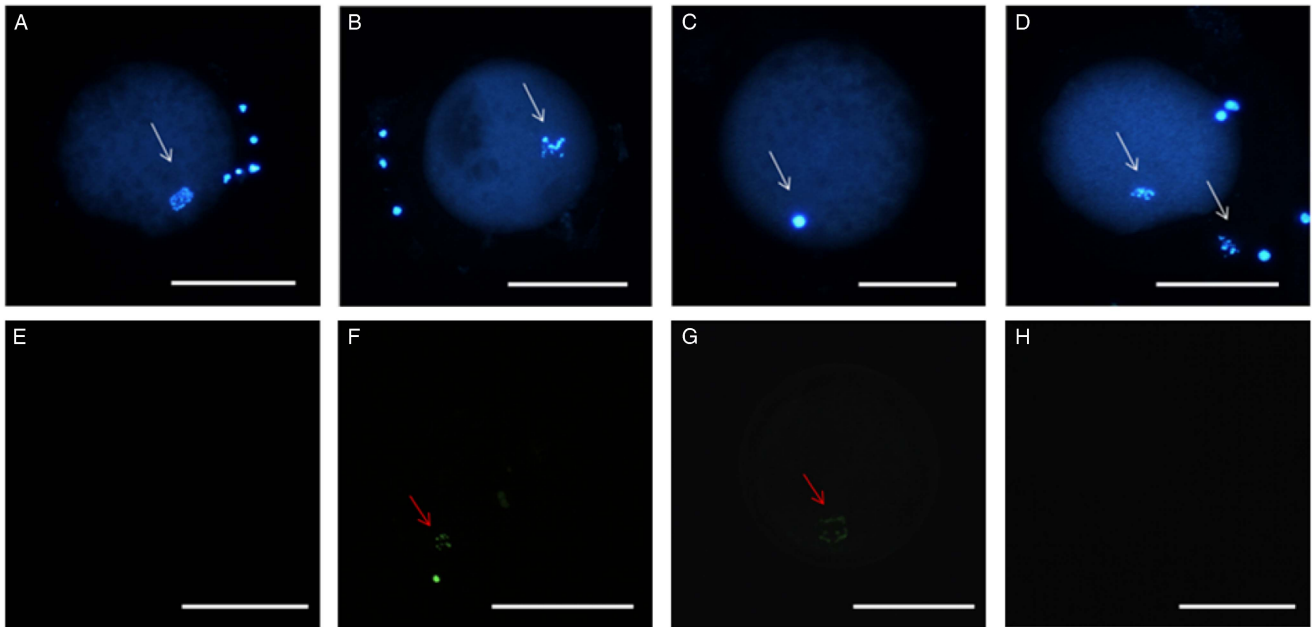
Although quercetin does not act directly on nuclear or cytoplasmic maturation (Kang *et al.*, 2013), the maturation rate observed for oocytes in medium containing 4  $\mu$ M of quercetin (Q4 group) can be explained by higher mitochondrial activity and lower DNA fragmentation, compared with female gametes matured with cysteamine (CIS group). Mitochondrial activity is considered to be an excellent marker of oocyte quality and viability. This is because active mitochondria ensure functional competence in oocytes mainly during cytoplasmic and nuclear maturation (Moussa *et al.*, 2015). Mitochondrial activity during nuclear and cytoplasmic maturation is higher in mature oocytes (Tarazona *et al.*, 2006). In addition, mitochondria have a significant function during programmed cell death, which starts with intracellular and extracellular signals propagated by mitochondrial that promote mitochondrial disruption and cellular death (Tanabe *et al.*, 2015).

Corroborating our results, Jia *et al.* (2011) evaluated the effect of adding quercetin to chicken follicle cultures and cytotoxicity using cadmium. They observed that the inclusion of quercetin in the culture medium reduced the number of apoptotic cells. The antioxidant potential of quercetin successfully protected the cell from possible damage and ensured, consequently, a lower rate of apoptosis. Therefore, we suggested that the higher maturation rate, after supplementing with 4  $\mu$ M of quercetin, can be associated with higher oocyte quality that transferred to female gametes a greater ability to proceed to meiosis (Crocomo *et al.*, 2013).

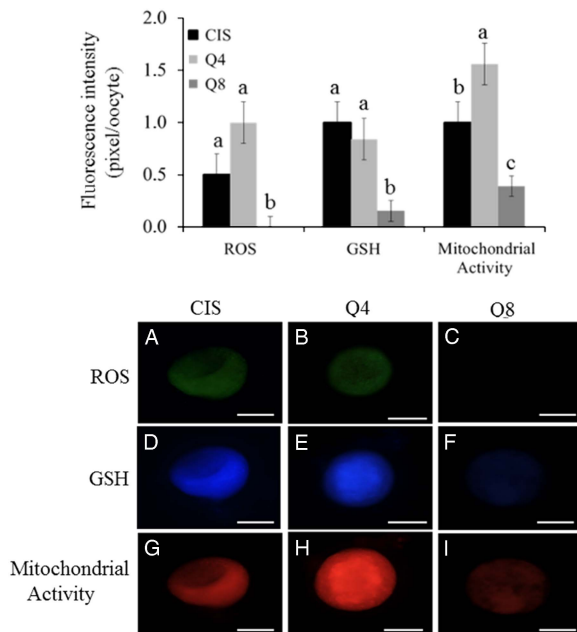
The positive effects of quercetin and cysteamine during IVM and oocyte culture are due to the antioxidant potential of these substances. In adequate concentrations, these antioxidants mediate the equilibrium during ROS production, resulting in cells of better quality and with higher viability (Orlovski *et al.*, 2014). There was no significant difference ( $P > 0.05$ ) between the CIS and Q4 groups regarding intracellular ROS and GSH levels. In contrast, Guemra *et al.* (2013) found lower GSH levels in oocytes matured in the presence of quercetin (2–10  $\mu$ M). Quercetin equilibrates ROS production because it interacts with superoxide ions, chelates iron ions and reduces lipid peroxidation, reducing cellular damage associated with oxidative stress (Johinke *et al.*, 2014). However, quercetin can increase or decrease GSH levels based on its concentration. In addition, oocytes have endogenous GSH, which it is found during maturation and metaphase II stage. Quercetin concentrations can be changed dependent on *in vitro* cellular culture systems adopted in the laboratory (Liu *et al.*, 2010).

An 8  $\mu$ M quercetin concentration reduced ROS, GSH and mitochondrial activity levels. Similar results were observed for bovine (Tvrdá *et al.*, 2016) and human (Khanduja *et al.*, 2002) sperm diluted in semen extender on the presence of quercetin. High concentrations of quercetin reduced the modulatory activity of  $Ca^{2+}$ -ATPase resulting in a higher influx of intracellular calcium, cell damage, mitochondrial disruption, reduction of mitochondria activity and lower production of ROS by mitochondria (Khanduja *et al.*, 2002; Tvrdá *et al.*, 2016).

In conclusion, 4  $\mu$ M of quercetin can be used as alternative to cysteamine for IVM of goat oocytes. However, more studies are



**Figure 2.** Stained chromatin after TUNEL reaction. (A) Germinal vesicle breakdown (GVBD) after treatment with 8  $\mu$ M of quercetin (Q8). (B) Oocyte in metaphase I after using cysteamine (CIS). (C) Oocyte in metaphase II after using 4  $\mu$ M of quercetin. Absence of TUNEL staining in the negative control (D) and Q4 group (E). Staining of fragmented DNA with TUNEL in the positive control (F) and after CIS treatment (G). Absence of labelling in the Q4 treatment group (H). White arrows indicate chromatin stained with Hoechst. Red arrows indicate chromatin stained using the TUNEL assay. Scale bar: 100  $\mu$ m.



**Figure 3.** (Upper) Intracellular levels of ROS, GSH and mitochondrial activity (pixel/oocyte) of oocytes matured under different concentrations of quercetin: 0  $\mu$ M (CIS); 4  $\mu$ M (Q4) e 8  $\mu$ M (Q8). Different small letters among columns, within of each parameter, indicate significant difference ( $P < 0.05$ ). (Lower) Epifluorescence photomicrograph of goat oocytes stained with dichlorofluorescein (A–C), cell tracker blue (D–F) and Mito Tracker Red (G–I) representing intracellular levels of ROS, GSH and mitochondrial activity (pixel/oocyte) respectively of oocytes matured using different concentrations of quercetin: 0  $\mu$ M (CIS); 4  $\mu$ M (Q4) and 8  $\mu$ M (Q8). Scale bar: 75  $\mu$ m.

necessary in order to find the ideal concentration of quercetin for IVM in goat oocytes and consequent *in vitro* fertilization and IVP.

**Financial support.** A.A.A. Silva was supported by CAPES and Federal University of San Francisco Valley. The authors wish to thank FACEPE for the financial support associated to project 'Effect of inclusion of quercetin and pentoxifylline on *in vitro* production of goat embryos at semiarid of North-eastern Brazil' (APQ10635.05/15).

**Conflicts of interest.** The authors have declared no conflict of interests.

**Ethical standards.** All protocols used in this study were approved by the Ethics and Deontology Committee in Studies and Researches of the Federal University of Saint Francisco Valley (Process 0004/120215/CEDEP/UNIVASF).

## References

- Aghaz F, Hajarian H, Shabankareh HK and Abdolmohammadi A (2015) Effect of sericin supplementation in maturation medium on cumulus cell expansion, oocyte nuclear maturation, and subsequent embryo development in Sanjabi ewes during the breeding season. *Theriogenology* **84**, 1631–5.
- Avelar SRG, Moura RR, Sousa FC, Pereira AF, Almeida KC, Melo CHS, Teles-Filho ACA, Baril G, Melo LM, Teixeira DIA and Freitas VJF (2012) Oocyte production and *in vitro* maturation in Canindé goats following hormonal ovarian stimulation. *Anim Reprod* **9**, 27–32.
- Behling EB, Senadão MC, Francescato HDC, Antunes LMG and Bianchi MLP (2004) Flavonoid quercetin: general aspects and biological actions. *Alim Nut* **15**, 285–92.
- Bueno AP and Beltran MP (2008) *In vitro* production of bovine embryos. *Electron J Vet Med* **6**, 1–7.
- Choi JY, Kang JT, Park SJ, Kim SJ, Moon JH, Saadeldin IM, Jang G and Lee BC (2013) Effect of 7, 8-dihydroxyflavone as an antioxidant on *in vitro* maturation of oocytes and development of parthenogenetic embryos in pigs. *J Reprod Dev* **59**, 450–6.
- Crocom LF, Marques Filho WC, Sudano MJ, Paschoal DM, Alvarenga FCL and Bicudo SD (2013) Effect of roscovitine and cycloheximide on ultrastructure of sheep oocytes. *Small Rum Res* **109**, 156–62.

- Crocomio LF, Marques Filho WC, Ulian CM, Branchini NS, Silva DT, Ackermann CL, Landim-Alvarenga FC and Bicudo SD (2015) Effect of oil overlay on inhibition potential of roscovitine in sheep cumulus-oocyte complexes. *Reprod Dom Anim* **50**, 410–6.
- Gouveia BB, Macedo TJS, Santos JM, Barberino RS, Menezes VG, Müller MC, Almeida JR, Figueiredo JR and Matos MHT (2016) Supplemented base medium containing *Amburana cearensis* associated with FSH improves *in vitro* development of isolated goat preantral follicles. *Theriogenology* **86**, 1275–84.
- Guemra S, Monzani PS, Santos ES, Zanin R, Ohashi OM, Miranda MS and Adona PR (2013) *In vitro* maturation of bovine oocytes at medium supplemented with quercetin and its effect on embryo development. *Arq Bras Med Vet Zoot* **65**, 1616–24.
- Hand D, Lan GC, Wu YG, Han ZB, Wang HL and Tan JH (2006) Factors affecting the efficiency and reversibility of roscovitine (ROS) block on the meiotic resumption of goat oocytes. *Mol Reprod Dev* **73**, 238–46.
- Jia Y, Lin J, Mi Y and Zhang C (2011) Quercetin attenuates cadmium-induced oxidative damage and apoptosis in granulosa cells from chicken ovarian follicles. *Reprod Toxic* **31**, 477–85.
- Johinke D, De Graaf SP and Bathgate R (2014) Quercetin reduces the *in vitro* production of H<sub>2</sub>O<sub>2</sub> during chilled storage of rabbit spermatozoa. *Anim Reprod Sci* **151**, 208–19.
- Kang JT, Kwon DK, Park SJ, Kim SJ, Moon JH, Koo OJ, Jang G and Lee BC (2013) Quercetin improves the *in vitro* development of porcine oocytes by decreasing reactive oxygen species levels. *J Vet Sci* **14**, 15–20.
- Khanduja KL, Verma A and Bhardwaj A (2002) Impairment of human sperm motility and viability by quercetin is independent of lipid peroxidation. *Andrologia* **33**, 277–281.
- Kubo N, Cayo-Colca IS and Miyano T (2015) Effect of estradiol-17 $\beta$  during *in vitro* growth culture on the growth, maturation, cumulus expansion and development of porcine oocytes from early antral follicles. *Anim Sci J* **86**, 251–9.
- Leoni GG, Succu S, Satta V, Paolo M, Bogliolo L, Bebbere D, Spezzigu A, Madeddu M, Berlinguer F, Ledda S and Naitana S (2009) *In vitro* production and cryotolerance of prepubertal and adult goat blastocysts obtained from oocytes collected by laparoscopic oocyte-pick-up (LOPU) after FSH treatment. *Reprod Fertil Dev* **21**, 901–8.
- Lins TLBG, Cavalcante AYP, Santos JMS, Menezes VG, Barros VRP, Barberino RS, Bezerra MES, Macedo TJS and Matos MHT (2017) Rutine can replace the use of three other antioxidants in the culture medium maintaining the viability of sheep isolated secondary follicles. *Theriogenology* **89**, 263–70.
- Liu S, Hou W, Yao P, Zhanq B, Sun S, Nüssler AK and Liu L (2010) Quercetin protects against ethanol-induced oxidative damage in rat primary hepatocytes. *Toxic In Vitro* **24**, 516–22.
- Moussa M, Shu J, Zhanq XH and Zenq F (2015) Maternal control of oocyte quality in cattle: 'a review'. *Anim Reprod Sci* **155**, 11–27.
- Mudo GAM, Silva HAL, Bastos BDM, Silva AAA, Guimarães ASL, Medina FT, Cordeiro MF and Lopes Júnior ES (2015) Effect of different hormonal stimulation treatments on *in vitro* maturation of oocytes from pre-pubertal goats: preliminary results. In *XXV Congresso Brasileiro de Zootecnia, FORTALEZA*.
- Orlovski D, Miclea I, Zahan M, Miclea V and Pernes A (2014) Quercetin efficacy on *in vitro* maturation of porcine oocytes. *Anim Sci Biotech* **1**, 47–51.
- Pu Y, Wang Z, Bian Y, Zhang F, Yang P, Li Y, Zhang Y, Fang F, Cao H and Zhang X (2014) All-trans retinoic acid improves goat oocyte nuclear maturation and reduces apoptotic cumulus cells during *in vitro* maturation. *Anim Sci J* **85**, 833–9.
- Rajabi-Toustani R, Motamedi-Mojdehi R, Mehr MR and Motamedi-Mojdehi R (2013) Effect of *Papaver rhoeas* L. extract on *in vitro* maturation of sheep oocytes. *Small Rum Res* **114**, 146–51.
- Santini SE, Basini G, Bussolati S and Grasselli F (2009) The phytoestrogen quercetin impairs steroidogenesis and angiogenesis in swine granulosa cells *in vitro*. *J Biomed Biotechnol* **1**, 1–8.
- Shirazi A, Bahraee A, Ahmadi E, Nazari H, Heidari B and Borjian S (2009) The effect of the duration of *in vitro* maturation (IVM) on parthenogenetic development of ovine oocytes. *Avicenna J Med Biotechnol* **1**, 181–91.
- Silva AAA (2015) *In vitro* maturation of ovine oocytes on presence of vitamin E. Petrolina-PE. 48 pp. Monography (Work of Conclusion of Course) – Campus Agricultural Sciences/Federal University of San Francisco Valley.
- Souza-Fabjan JMG, Locatelli Y, Duffard N, Corbin E, Batista RITP, Freitas VJF, Beckers JF and Mermillod P (2016) Intrinsic quality of goat oocytes already found denuded at collection for *in vitro* embryo production. *Theriogenology* **86**, 1989–98.
- Srivastava S, Desai P, Coutinho E and Govil G (2006) Mechanism of action of L-arginine on the vitality of spermatozoa in primarily through increased biosynthesis of nitric oxide. *Biol Reprod* **74**, 954–8.
- Tanabe M, Tamura H, Taketani T, Okada M, Lee L, Tamura I, Maekawa R, Asada H, Yamagata Y and Sugino N (2015) Melatonin protects the integrity of granulosa cells by reducing oxidative stress in nuclei, mitochondria, and plasma membranes in mice. *J Reprod Dev* **61**, 35–41.
- Tarazona AM, Rodriguez JI, Restrepo LF and Olivera-Angel M (2006) Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced *in vitro*. *Reprod Dom Anim* **41**, 5–11.
- Tvrđá E, Tusimová E, Kováčik A, Paál D, Libová L and Lukác N (2016) Protective effects of quercetin on selected oxidative biomarkers in bovine spermatozoa subjected to ferrous ascorbate. *Reprod Dom Anim* **51**, 524–37.
- Zhou P, Wu YG, Li Q, Lan GC, Wang G and Gao D (2008) The interactions between cysteamine, cystine and cumulus cells increase the intracellular glutathione level and developmental capacity of goat cumulus-denuded oocytes. *Reproduction* **135**, 605–11.
- Vanderhyden BC, Caron PJ, Buccione R and Eppig JJ (1990) Developmental pattern of the secretion of cumulus expansion-enabling factor by mouse oocytes and the role of oocytes in promoting granulosa cell differentiation. *Dev Biol* **140**, 307–17.