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—Original Article—

Resveratrol supplementation during *in vitro* maturation improves embryo development of prepubertal goat oocytes selected by brilliant cresyl blue staining

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Abstract. This study aimed to investigate the effect of resveratrol supplementation in maturation medium on the developmental ability and bioenergetic\oxidative status of prepubertal goat oocytes selected by brilliant cresyl blue (BCB). Oocytes collected from slaughterhouse-derived ovaries were selected by 13 μM BCB staining and classified as grown BCB+ and growing BCB- oocytes. All oocytes were matured *in vitro* in our conventional maturation medium and supplemented with 1 μM (BCB+R and BCB-R) and without (Control groups: BCB+C and BCB-C) resveratrol. After 24 h, IVM-oocytes were fertilized with fresh semen and presumptive zygotes were *in vitro* cultured for 8 days. Oocytes were assessed for blastocyst development and quality, mitochondrial activity and distribution, and levels of GSH, ROS, and ATP. BCB+R (28.3%) oocytes matured with resveratrol presented significantly higher blastocyst development than BCB+C (13.0%) and BCB- groups (BCB-R: 8.3% and BCB-C: 4.7%). Resveratrol improved blastocyst development of BCB-R oocytes at the same rate as BCB+C oocytes. No differences were observed in blastocyst quality among groups. GSH levels were significantly higher in resveratrol groups (BCB+R: 36554.6; BCB-R: 34946.7 pixels/oocyte) than in control groups (BCB+C: 27624.0; BCB-C: 27655.4 pixels/oocyte). No differences were found in mitochondrial activity, ROS level, and ATP content among the groups. Resveratrol-treated oocytes had a higher proportion of clustered active mitochondria in both BCB groups (BCB+R: 73.07%; BCB-R: 79.16%) than control groups (BCB+C: 19.35%; BCB-C: 40%). In conclusion, resveratrol increased blastocyst production from oocytes of prepubertal goats, particularly in better quality oocytes (BCB+).
Key words: Antioxidant, Blastocyst, *In vitro* fertilization (IVF), Juvenile ruminants

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Goat production in Mediterranean countries is economically and socially important. For these species, artificial insemination (AI) is the most used reproductive technology. However, *in vitro* embryo production (IVEP) using oocytes derived from prepubertal animals in conjunction with *in vitro* embryo transfer, termed as juvenile *in vitro* embryo transfer (JIVET), can accelerate genetic gain by shortening generation intervals. The addition of JIVET to artificial insemination yielded an extra 25 to 60% genetic gain in sheep programs [1]. The first births using JIVET were reported in the late 1970s [2] but the efficiency of this technology remains low, mainly due to the low competence of these oocytes compared to those from adult females. The reduced *in vitro* embryo development of these oocytes has been related to oocyte structural and molecular abnormalities [3–7], which are signs of their poor quality.

In order to improve *in vitro* embryo production, the selection of high-quality oocytes is crucial. Brilliant cresyl blue (BCB) staining is a non-invasive method used for the selection of immature oocytes [8]. BCB is a glucose-6-phosphate dehydrogenase (G6PD) substrate, from which it is reduced from blue to a colorless compound. G6PD activity gradually decreases as oocytes reach their maximum growth phase [9]. Thus, grown oocytes present a low G6PD activity and cannot reduce BCB, so they show a blue cytoplasm (BCB+), while growing oocytes with high G6PD activity reduce BCB and present an unstained cytoplasm (BCB-) [10]. Several studies in cattle [11, 12], sheep [13, 14], horses [15], goats [16], buffalos [17] and mice [18] showed that BCB+ oocytes presented higher embryo development competence compared to BCB- oocytes. However, in adult goats, 3.6% of morphologically good oocytes were found to show signs of degeneration following BCB staining [8].

An important factor contributing to the poor quality of *in vitro* matured oocytes could be their sensitivity to oxidative stress [19]. These oocytes are less able to maintain an appropriate redox homeostasis in response to oxidative stress generated by the *in vitro* condition compared to those from adult females [20]. This could be caused by an altered synthesis of endogenous antioxidants [20, 21]. The addition of antioxidants to the maturation medium has been proposed as a good strategy to overcome the effect of oxidative stress allowing an increase in oocyte embryo development [19, 22].

Resveratrol (3,4,5-trihydroxy-trans-stilbene) is a small polyphenol synthesized by several plants, such as nuts, mulberry and grapes

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[23]. This phytoalexin is a potent antioxidant that, by activation of SIRT1, a NAD+ dependent deacetylase belonging to the sirtuin family, induces the upregulation of the endogen antioxidant system [24]. Recent studies highlighted that, SIRT1 acts as sensor of the redox state in oocytes and granulosa cells [24]. Furthermore, resveratrol is involved in the regulation of energy homeostasis [25], metabolism [26], estrogen levels [27], and genomic stability [28]. It has also been observed that resveratrol supplementation during *in vitro* maturation (IVM) positively affected oocyte quality, fertilization and embryo development outcomes in goats, cattle, and pigs [29].

This study investigated the effect of resveratrol supplementation of IVM media on the developmental ability of prepubertal goat oocytes selected by BCB. To this end the bioenergetic/oxidative status of *in vitro* matured oocytes, oocyte cleavage, and blastocyst formation and quality, following *in vitro* fertilization, were analyzed.

Materials and Methods

Materials

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, USA).

Oocytes collection and BCB staining

Oocytes were collected from ovaries of slaughtered juvenile (30 to 45-day-old) goats (*Capra hircus*). Oocytes with two or more complete layers of compact cumulus cells and with a uniform cytoplasm were selected for IVM. In the experiments two and three, morphologically selected oocytes were incubated with 13 µM BCB for 45 min under 5% CO_2 in air at 38.5°C. After BCB exposure, oocytes were classified based on cytoplasm coloration, BCB+ (blue) or BCB- (colorless), and were *in vitro* matured [13].

In vitro maturation, in vitro fertilization (IVF) and embryo culture (IVEC)

Groups of 25–30 oocytes were matured in TCM-199, supplemented with 5 µg/ml follicle-stimulating hormone, 5 µg/ml luteinizing hormone, 1 µg/ml 17 β estradiol, 10 ng/ml EGF, 10% fetal bovine serum, 5 µg/ml gentamycin, 1 mM L-glutamine, and 0.2 mM sodium pyruvate, for 24 h under 5% CO_2 in air at 38.5°C. After IVM, the oocytes were inseminated with fresh semen, obtained from three Murciano-Granadino bucks of proven fertility. Highly motile spermatozoa were selected using Bovipure density gradient kit (Nidacon EVB S.L., Barcelona, Spain). Oocytes were transferred into BO-IVF medium (IVF Bioscience; UK) for fertilization with 1×10^6 spermatozoa/mL for 20 h, under 5% CO_2, 5% O_2 and 90% N_2 atmosphere, at 38.5°C. After *in vitro* fertilization, presumptive zygotes were cultured in BO-IVC (IVF Bioscience; UK) for 8 days, under 5% CO_2, 5% O_2 and 90% N_2 atmosphere, at 38.5°C. The cleavage rate and blastocyst rate/blastocyst cell number were recorded at 48 h and on day 8.

Assessment of nuclear status

After IVM, denuded oocytes were fixed in ethanol and stained with 1 µM Hoechst 33342 solution (Invitrogen) for 1 h. The nuclear configurations were classified, using an epifluorescent microscope (Olympus BX50), as germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), or metaphase II (MII).

Blastocyst differential staining

Analysis of blastocyst cell numbers was performed by differential staining of the inner cell mass (ICM) and trophectoderm (TE) cell compartments [30]. Blastocysts were first incubated, briefly, for 15 sec in TCM199 with 1% Triton X-100 and 100 µg/ml propidium iodide, then transferred into an ethanol solution with Hoechst 33342 for 3 h. A digital image of each blastocyst was taken by epifluorescence microscopy and the numbers of TE (red) and ICM (blue) nuclei were counted using ImageJ software (ImageJ 1.5Oi).

Measurement of glutathione (GSH) and reactive oxygen species (ROS) levels

Denuded oocytes at the MII stage (presence of the first polar body) were incubated in the dark for 30 min with 10 µM 2'7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes, Eugene, OR, USA) or 10 µM 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (Cell Tracker Blue; CMF_2HC; Molecular Probes, Eugene, OR, USA) for reactive oxygen species or glutathione detection, respectively. An epifluorescent microscope with a UV filter (460 nm for ROS and 370 nm for GSH) was used to take digital images, and the fluorescence intensities of the oocytes were analyzed using ImageJ software [31].

Quantification of adenosine 5′-triphosphate (ATP) intracellular content

Groups of 6 MII oocytes were denuded by gentle pipetting and placed into Eppendorf tubes with 200 µl ultrapure water and stored at –80°C until their analysis. The ATP content of oocytes was measured using the adenosine 5-triphosphate bioluminescent somatic cell assay kit (FLASC) as described previously [32]. The bioluminescence generated by ATP-dependent luciferin-luciferase reaction was immediately measured using a luminometer.

Evaluation of mitochondrial distribution and activity

After IVM, MII oocytes were denuded and incubated in the dark for 30 min with 200 nM MitoTracker Orange CMTMRos (Molecular Probes, Eugene, OR, USA), under 5% CO_2 in air, at 38.5°C. After incubation, oocytes were fixed in 3% paraformaldehyde for 60 min at 38°C and stained with 1 µM Hoechst 33342 solution. Oocytes were stored at 4°C in the dark until their analysis [13].

Mitochondria analysis was performed using Leica TCS SP5 CLSM with LAS lite 170 Image software equipped with a 405 nm diode laser and a multiphoton laser. In each individual oocyte MitoTracker fluorescence intensities were measured at the equatorial plane as described previously [33]. Leica LAS AF Lite image analysis software package (Leica Microsystems GmbH, Wetzlar, Germany) was used for the quantitative analysis of fluorescence intensity. Mitochondrial distribution patterns were classified in two groups, as previously reported [34], with some modifications: 1) Pattern A: homogeneous fine, with small granulations spread throughout the cytoplasm; 2) Pattern B: heterogeneous clustered, with large granulations spread throughout the cytoplasm or located in specific cytoplasmic domains.

Experimental design

Experiment 1: Effect of resveratrol supplementation at different

Table 1. Effect of different resveratrol concentrations on embryo development and blastocyst cell numbers of prepubertal goat oocytes (Experiment 1)

Resveratrol concentration (μM)	No. oocytes	No. cleaved (% ± SE)	No. blastocysts/cleaved (% ± SE)	No. blastocysts/total (% ± SE)	Blastocyst cell number (mean ± SE)
0	164	100 (61.2 ± 2.0) [a]	11 (10.9 ± 2.5) [a]	11 (6.8 ± 1.6) [a]	127 ± 10.8
0.5	152	89 (56.7 ± 5.3) [a]	11 (14.5 ± 3.4) [a]	11 (7.9 ± 1.7) [a]	167.5 ± 23.8
1	163	137 (83.5 ± 2.8) [b]	32 (24.5 ± 2.0) [b]	32 (20.1 ± 1.3) [b]	156.5 ± 13.9

[a, b] Values with different superscript letters within a column differ significantly ($P < 0.05$). Three replicate trials were performed.

Table 2. Effect of 1 μM resveratrol, added to the IVM medium, on meiotic progression of prepubertal goat oocytes selected by brilliant cresyl blue (BCB) staining (Experiment 2)

Groups	No. oocytes	GV (% ± SE)	GVBD (% ± SE)	MI (% ± SE)	MII (% ± SE)
BCB+C	53	0	1 (2.2 ± 1.3)	4 (7.3 ± 2.3)	48 (90.4 ± 1.1)
BCB+R	46	0	0	3 (6.5 ± 0.1)	43 (93.5 ± 0.1)
BCB-C	47	0	1 (1.9 ± 1.1)	6 (12.9 ± 0.5)	40 (85.2 ± 0.6)
BCB-R	50	1 (1.9 ± 1.1)	1 (1.9 ± 1.1)	8 (16.4 ± 3.1)	40 (79.9 ± 1.0)

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II. Three replicate trials were performed.

concentrations on oocyte developmental competence.

The dose-responsive effects of resveratrol on oocyte developmental competence were evaluated. Resveratrol was added to the IVM medium at concentrations of 0.5 μM and 1 μM. A group of oocytes were cultured, in the absence of resveratrol, as control (C). After *in vitro* fertilization (IVF) cleavage rate, blastocyst formation and cell number were evaluated.

Experiment 2: Effect of 1 μM resveratrol supplementation on the developmental competence of oocytes selected by BCB staining.

Based on the results of experiment 1, we evaluated the effect of 1 μM resveratrol on the developmental competence of oocytes selected by BCB staining. Oocytes were matured *in vitro* with (BCB+R; BCB-R) or without (BCB+C; BCB-C) 1 μM resveratrol. After IVF and *in vitro* embryo culture, cleavage rate, blastocyst formation and cell number were assessed.

Experiment 3: Effect of 1 μM resveratrol supplementation on the bioenergetic/oxidative status of oocytes selected by BCB staining.

Oocytes at the metaphase II stage from the different groups were analyzed for: intracellular ROS and GSH levels (Experiment 3a), intracellular ATP content, mitochondrial activity, and organization (Experiment 3b).

Statistical analysis

For each experiment, at least three replicates were carried out. The oocytes used in each replicate were from the same group of abattoir-derived ovaries collected on the same day. After BCB selection, BCB+ and BCB- oocytes were randomly distributed across resveratrol groups.

Statistical analysis was performed using STATA\IC 11.0 software package. Data were first checked for normal distribution and were analyzed using the Shapiro-Wilk test. Data for maturation, cleavage, blastocyst rates and blastocyst cell number, expressed as mean values ± standard errors of mean (SE), were normally distributed and analyzed using one-way ANOVA, followed by Bonferroni's post-hoc test. Data for intracellular ROS and GSH levels, ATP content and mitochondrial activity, expressed as mean values ± standard errors of mean (SE), were not normally distributed and were analyzed with a non-parametric Kruskal-Wallis test. Active mitochondrial distribution was analyzed by Chi-square and Fischer's exact tests where appropriate. The overall chi-square was calculated and found to be significant before performing the Fischer's exact test to detect differences among experiment groups. Differences of 0.05 or less in the probability values were considered significant.

Results

Experiment 1: Effect of resveratrol supplementation at different concentrations on oocyte developmental competence and blastocyst cell number

Results on the effect of resveratrol supplementation, at different concentrations, on oocytes developmental competence after IVF are reported in Table 1. Cleavage and blastocyst rates were lower ($P < 0.05$) when 0 μM and 0.5 μM resveratrol was added to the IVM medium compared with the addition of 1 μM resveratrol. Total blastocyst cell numbers did not differ among groups (Table 1).

Experiment 2: Effect of 1 μM resveratrol supplementation on the developmental competence of oocytes selected by BCB staining

Supplementation with 1 μM resveratrol during IVM did not affect meiotic progression and the ratio of nuclear maturation within BCB+ or BCB- groups (Table 2). No differences were found in the cleavage rate among groups (Table 3). The blastocyst rate (blastocyst / total oocytes) was higher ($P < 0.05$) in the BCB+C group compared to that of BCB-C group. Resveratrol supplementation increased the blastocyst rate of BCB- oocytes at the same rate as BCB+C. BCB+R

Table 3. Effect of 1 μM resveratrol, added to the IVM medium, on embryo development and blastocyst cell number of prepubertal goat oocytes selected by brilliant cresyl blue (BCB) staining (Experiment 2)

Groups	No. oocytes	No. cleaved (% ± SE)	No. blastocysts/cleaved (% ± SE)	No. blastocysts/total (% ± SE)	Blastocyst cell number (mean ± SE)		
					Total	ICM	TE
BCB+C	110	87 (78.4 ± 3.6)	14 (16.0 ± 0.5) [a]	14 (13.0 ± 0.7) [a]	134.6 ± 7.4	30.2 ± 3.5	106.8 ± 21.8
BCB+R	116	103 (88.3 ± 2.6)	32 (32.1 ± 1.3) [b]	32 (28.3 ± 0.9) [b]	167 ± 12.6	43.4 ± 4	133.4 ± 12
BCB-C	95	67 (71.1 ± 6.9)	4 (8.3 ± 1.5) [a]	4 (4.7 ± 0.4) [c]	136 ± 4.9	32.5 ± 0.5	122.5 ± 28.5
BCB-R	88	67 (78.0 ± 8.9)	8 (11.1 ± 1.4) [a]	8 (8.3 ± 0.8) [ac]	120.5 ± 12.4	31.3 ± 5.5	89.2 ± 8.2

[a, b, c] Values with different superscript letters within a column differ significantly ($P < 0.05$). Four replicate trials were performed.

presented a higher ($P < 0.05$) blastocyst rate than the BCB+C, BCB-C and BCB-R groups (Table 3). The total, ICM and TE blastocyst cell numbers did not differ among groups (Table 3).

Experiment 3: Effect of 1 μM resveratrol supplementation on the bioenergetic/oxidative status of oocytes selected by BCB staining

Experiment 3a: Intracellular GSH levels were higher ($P < 0.05$) in both BCB+ (36554.6 ± 3049.25 pixels/oocyte) and BCB- (34946.8 ± 1877.8 pixels/oocyte) groups treated with resveratrol during IVM compared to their respective controls, BCB+C (27624.0 ± 1513.67 pixels/oocyte) and BCB-C (27655.42 ± 1489.8 pixels/oocyte) groups (Fig. 1). We did not find any significant differences in ROS levels among experimental groups (BCB+C: 32740.3 ± 3165.0; BCB+R: 26314.1 ± 2857.0; BCB-C: 59071.3 ± 14079.0; BCB- R: 30587.3 ± 3337.0 pixels/oocyte) (Fig. 1).

Experiment 3b: The intracellular ATP contents of the oocytes (Fig. 2) were not significantly different among groups (BCB+C: 3586.4 ± 203.6; BCB+R: 3219.0 ± 171.9; BCB-C: 3769.2 ± 267.6; 4083.1 ± 291.6 Fmol/oocyte).

We did not find any significant differences in the fluorescent image intensities of active mitochondria (BCB+C: 17.2 ± 2.6; BCB+R: 13.5 ± 1.4; BCB-C: 16.5 ± 2.5; BCB-R: 16.0 ± 2.4 AU) (Fig. 3).

Mitochondrial distribution patterns were different ($P < 0.05$) between control and resveratrol-treated oocytes in both BCB groups. Resveratrol-treated oocytes had a higher ($P < 0.05$) rate of pattern B mitochondria distribution (BCB+R: 73.07%; BCB-R: 79.16%) compared to controls (BCB+C, 19.35% and BCB-C, 40%; Fig. 4).

Discussion

In the present study, we investigated the potential beneficial effect of resveratrol supplementation of the maturation medium on the embryo developmental competence of prepubertal goat oocytes selected by BCB staining.

Increasing evidence proves that the addition of resveratrol during *in vitro* maturation has positive effects on *in vitro* embryo production in different species [29]. In adult goats, resveratrol improved the developmental potential of parthenogenetic-derived blastocysts and hand-made cloned blastocysts [35]. Resveratrol acts in a dose-dependent manner and the optimal concentration is species-specific [29]. We have demonstrated that, resveratrol at a concentration of 1 μM, significant increased blastocyst development of prepubertal goat oocytes compared to 0.5 μM and control groups. After selection with BCB, BCB+ oocytes, matured in the presence of resveratrol (BCB+R), developed to the blastocyst stage at significantly higher rates than those of the control group (BCB+C). Moreover, resveratrol positively affected BCB- oocytes, improving their competence to blastocyst development up to the rates of BCB+ oocytes matured in conventional medium.

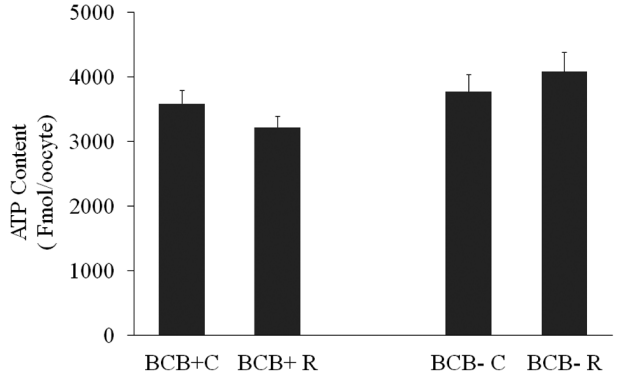


Fig. 2. ATP content (mean ± SE) of brilliant cresyl blue (BCB)-selected prepubertal goat oocytes matured with or without 1 μM resveratrol (Experiment 3b).

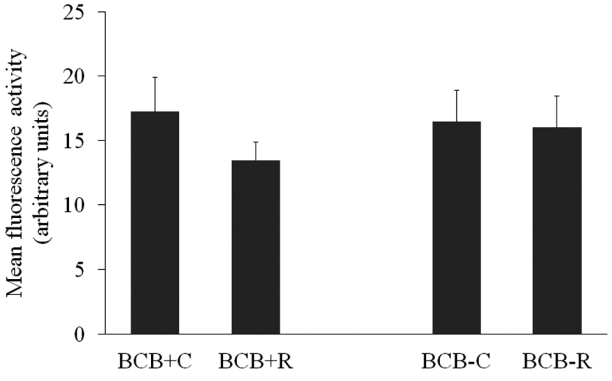


Fig. 3. Mitochondrial activity of brilliant cresyl blue (BCB)-selected prepubertal goat oocytes *in vitro* matured with or without 1 μM resveratrol (Experiment 3b). Fluorescence intensity was measured at the equatorial plane. Values are expressed as arbitrary units (Means ± SE).

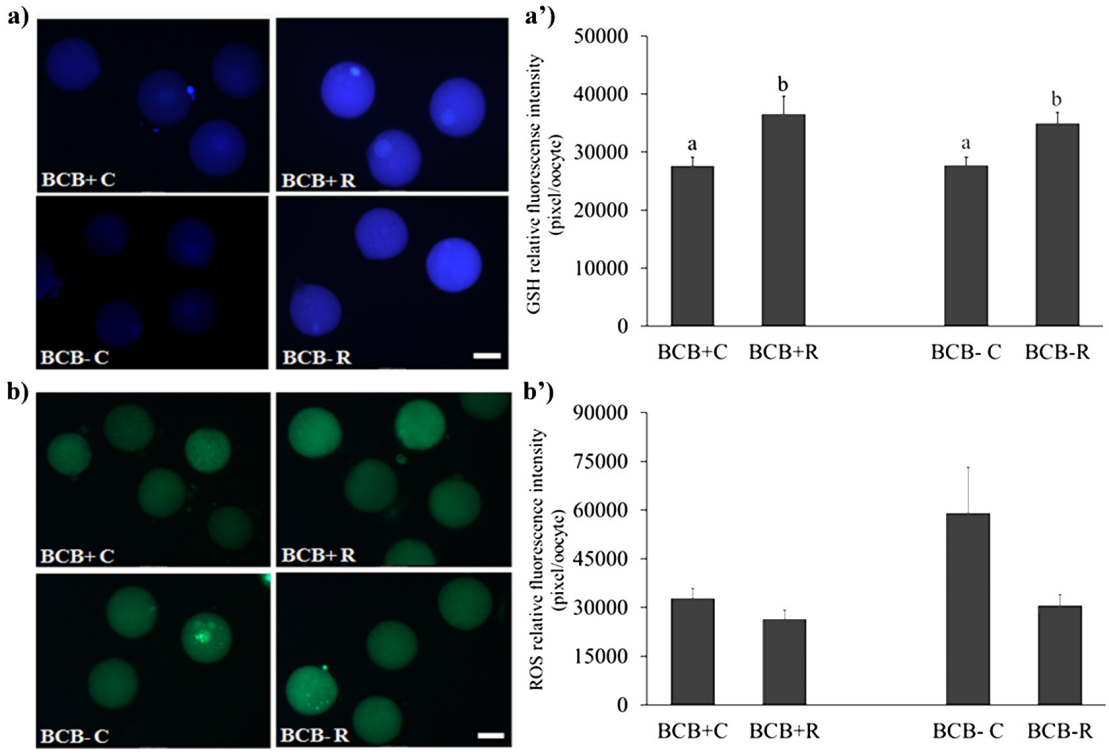
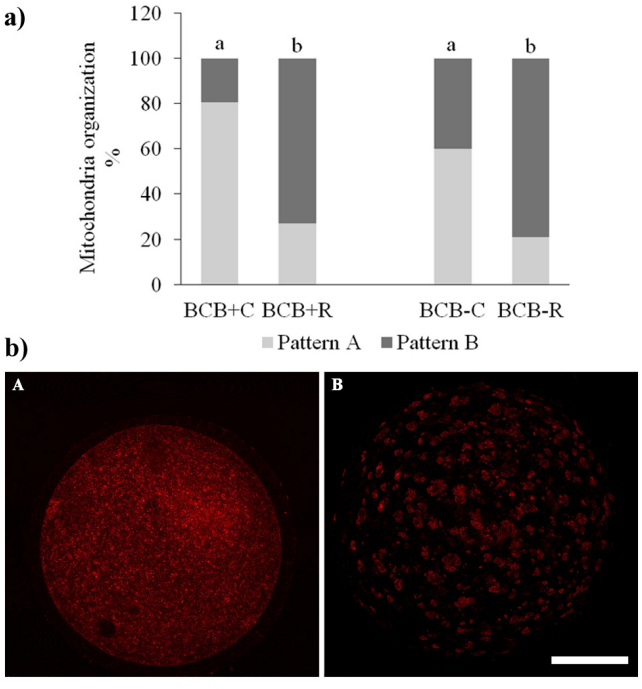


Fig. 1. Effect of 1 µM resveratrol, added to the *in vitro* maturation medium, on GSH and ROS intracellular levels of prepubertal goat oocytes selected by brilliant cresyl blue (BCB) staining (Experiment 3a): intracellular GSH (a) and ROS levels (b) of *in vitro* matured prepubertal goat oocytes. Epifluorescence photomicrographs of MII oocytes stained with CellTracker Blue to determine GSH levels (a') and with 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) to detect ROS (b'). Values with different superscript letters (a *vs.* b) are significantly different ($P < 0.05$). Scale bar = 100 µm.



In our study oocyte nuclear maturation (MII stage) was not affected by resveratrol treatment, which is in line with results from other studies on bovines [36], goats [35] and pigs [37]. On the contrary, Wang *et al*. (2014) found that resveratrol promoted oocyte nuclear maturation due to its antioxidant properties and the induction of progesterone secretion [38]. We observed that oocytes with low G6PDH activity (BCB+) had higher developmental competence than those with high enzyme activity (BCB-). These findings have been previously shown in our laboratory in goats [16, 39], cattle [12] and sheep [13], and by other authors, in different species [11, 14, 15, 17, 18]. A recent study observed that in spite of similar mitochondrial distribution between both BCB groups, mtDNA content experienced a 1.9-fold increase in BCB+ cattle oocytes which confirmed their

Fig. 4. Mitochondrial organization of brilliant cresyl blue (BCB)-selected prepubertal goat oocytes *in vitro* matured with or without 1 µM resveratrol (Experiment 3b). a) Distribution of mitochondrial aggregation patterns in metaphase II prepubertal goat oocytes, different superscript letters (a *vs.* b) are significantly different ($P < 0.05$). b) Representative CLSM images of mitochondrial aggregation patterns in prepubertal goat oocytes after staining with MitoTracker orange CMTM Ros: A) Homogeneous small granulations spread throughout the cytoplasm (pattern A); B) Heterogeneous large granulations spread throughout the cytoplasm or are located in specific cytoplasmic domains (Pattern B). Scale bar = 50 µm.

higher competence compared to BCB- oocytes [40].

On the other hand, some findings contradicted the utility of this test for selecting competent oocytes in bovines and pigs [8]. Moreover, the existence of a high caspase-3 activity in bovine blastocysts developed from BCB+ oocytes and a higher BCL-2 associated X protein (BAX) protein level in the BCB+ oocytes could imply a harmful effect of this staining [8]. In order to understand the reasons of the positive effect of resveratrol on embryo development of prepubertal goat oocytes, we evaluated the oxidative and bioenergetic status of oocytes. Our findings showed that resveratrol significantly increased intracellular GSH levels of *in vitro* matured oocytes in both BCB groups. In pigs [37], cattle [38] and goats [35], the beneficial effect of resveratrol on oocyte developmental competence has been associated with its antioxidant activity which increases intracellular GSH levels and decreases ROS levels. In our study, besides the increase in GSH content, resveratrol treatment did not affect ROS levels in both BCB+ and BCB- groups. Antioxidants do not always act in a univocal manner; indeed, increased GSH levels are not always associated with reduced ROS levels, as observed by other authors [22, 31]. Several reports suggest that IVM media supplementation with other antioxidants alleviated oxidative stress during *in vitro* maturation of poor-quality oocytes and improved early embryo development through a mechanism, including increase in GSH content [41–43]. GSH is the major non-protein sulfhydryl compound in mammalian cells and protects cells from oxidative damage [44]. The GSH level in oocytes increased as the oocyte resumed meiosis, and higher concentrations were found in mature oocytes than in immature [45]. The intra-oocyte GSH level can be considered as a marker of cytoplasmic maturity due to the close correlation with embryonic development [46]. In the adult goat, more competent oocytes (BCB+) presented higher intracellular GSH levels and the capacity to develop to the blastocyst stage after parthenogenetic activation [47]. Moreover, a reduction in GSH levels has been correlated with low developmental competence of oocytes derived from prepubertal mice and pigs [20, 21]. Several studies prove that GSH promotes decondensation of the sperm head and male pronucleus formation during fertilization [16,48], but also plays an important role in the development of parthenogenetic embryos [35, 37, 47].

Furthermore, GSH is involved in several biological processes, including DNA and protein synthesis, cell proliferation and protection of mitotic spindle from oxidizing agents [49, 50]. Another finding from our study was the effect of resveratrol on mitochondria organization. In fact, supplementation of the maturation medium with resveratrol induced a modification of active mitochondrial distribution in the cytoplasm of BCB+ and BCB- oocytes from a fine homogeneous pattern to a clustered distribution.

It has been shown that the activation of SIRT1 by resveratrol enhanced mitochondrial biosynthesis and degradation, thus, improving mitochondrial function and the developmental ability of oocytes [51]. In addition, resveratrol treatment could efficiently correct the defective phenotypes of mitochondrial organization in *in vitro* aged or methylglyoxal-treated mouse oocytes [52, 53]. Mitochondrial distribution and activity are considered good markers of oocyte quality. During *in vitro* maturation, changes in mitochondrial distribution and activity occur supporting oocytes maturation in cattle [54], dogs [55], goats [6], sheep [13], horses [56], pigs [57] and humans [58].

In a comparative study, Leoni *et al*. (2015) documented different active mitochondrial organizations in sheep MII-oocyte with high (adult) and low (prepubertal) developmental competence. A fine homogeneous dispersion of active mitochondria was observed at the GV stage in both oocyte types. This organization persisted in prepubertal MII-oocytes while adult MII-oocytes acquired a clustered distribution [34]. A clustered active mitochondrial organization was associated with maturation and high developmental competence in horse [56], dog [59], pig [60] and human [58] oocytes.

In our study, the presence of large clustered granules in resveratrol-treated MII-oocytes, which showed the highest GSH levels and developmental competence, suggests that the clustered mitochondrial phenotype may reflect the correct cytoplasmic maturity.

Quantitative analysis revealed that resveratrol neither affected ATP content nor the mitochondrial activity of prepubertal goat oocytes; indeed no significant difference was found among groups. In contrast, resveratrol treatment increased ATP content and the mitochondrial membrane potential in bovine *in vitro* matured oocytes [61].

In summary, the results of the present study show that supplementation of resveratrol during *in vitro* maturation improved embryo development to blastocyst stage, particularly in better quality oocytes (BCB+). Increased GSH levels and mitochondrial cluster distribution, could be some of the mechanisms underlying the positive effect of resveratrol supplementation on oocyte quality.

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