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Article

Resveratrol Reduces ROS by Increasing GSH in Vitrified Sheep Embryos

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Simple Summary: The in vitro production and cryopreservation of mammalian embryos generate reactive oxygen species (ROS) due to conditions of the system that can overcome their antioxidant protection. Resveratrol is an antioxidant used in in vitro systems improving blastocyst rates, but its effect on antioxidant enzymes such as glutathione (GSH) in embryos produced by in vitro fertilization (IVF) after vitrification has not been reported. The objective of this study was to evaluate the effect of resveratrol in the in vitro maturation medium (IVM) of sheep oocytes (Ovis aries) on the levels of ROS and GSH in embryos produced by IVF subjected to vitrification. Resveratrol was added at 0µM, 0.25µM, 0.5µM and 1µM during oocyte in vitro maturation (IVM). Matured oocytes were fertilized with thawed ram sperm. Embryos were cultured in sequential media until blastocysts, then were vitrified for 24 h and after heating they were stained with DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) to determine the presence of ROS and with Cell Tracker Blue® for the presence of GSH. The quantitative values of ROS and GSH were obtained through the Image J image processor. The results showed that resveratrol increases GSH and decreases ROS production ($p<0.05$). It is concluded that its use in sheep oocytes during IVM has a beneficial effect on embryos produced by IVF subjected to vitrification, protecting them from oxidative damage by promoting the synthesis of antioxidants.

Keywords: Resveratrol; ROS; GSH; vitrification

1. Introduction

The in vitro production of embryos is a biotechnology that impacts livestock systems by promoting genetic improvement. The cryopreservation of gametes and embryos has been widely developed worldwide in recent years [1], however, intrinsic processes can cause damage to cells and reduce embryo viability. One of the main factors detected is the increase in reactive oxygen species (ROS) [2], derived from an imbalance with the endogenous embryonic antioxidants.

To solve the damage, antioxidants such as resveratrol have been added to the in vitro culture media. Martínez et al. [3], observed that adding resveratrol at 0.25 and 0.5 µM to sheep oocytes (Ovis aries) during the in vitro maturation (IVM) improved their quality and promoted the compaction of morulae produced by somatic cell nuclear transfer (TNCS). Likewise, it is known that the supplementation of resveratrol (0.25 and 0.5 µM) in the IVM medium of mature cow and goat oocytes reduces intracellular ROS levels, increases glutathione (GSH) concentrations, stimulates embryonic development and gene expression [4]. In sheep, Zabihi et al. [5] did not find differences in the IVM

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rate but found significant differences in the blastocyst rate at concentrations of 0.25 and 0.5 µM of resveratrol, the quality of these being better at 0.5 µM.

In pigs, it is known that the addition of resveratrol during IVM and/or vitrification modulates the apoptotic process, improving the cryotolerance of vitrified porcine oocytes, while in bovine oocytes it reduces ROS levels, increases blastocyst rates and the number of embryonic cells. Particularly for bovine embryos, supplementation with low concentrations of resveratrol in the medium for in vitro development reduces ROS production and increases beta-oxidation levels, leading to higher survival rates after vitrification [6]. In the sow, Kwak et al. [7] used resveratrol at 0.1, 0.5 and 2.0 µM, during IVM of oocytes to evaluate its effect on ROS and GSH levels, on gene expression in mature oocytes, cumulus cells and blastocysts derived from the IVF. Observing that 2.0 µM of resveratrol improves the developmental potential of embryos, decreases ROS, and increases GSH But authors did not prove its effect on vitrified embryos.

Abdul Rahman et al. [8], say that "glutathione constitutes the major non-protein sulphydryl compound in mammalian cells, which confers protection against oxidative damage". Working with mice, authors directly added 0.01 mM of exogenous L-glutathione enzyme to the in vitro development culture media, showing that these improved intracellular GSH contents and vitrification outcomes in preimplantation embryos as observed through embryo morphology and preimplantation development. They also observed that this supplementation reduced the ROS levels in these embryos.

García-Martínez et al. [9], proved that glutathione ethyl ester supplemented to the IVM media protects bovine oocytes against oxidative stress induced by subsequent vitrification/warming, by preserving the mitochondrial distribution pattern, diminishing the cytoplasmic and mitochondrial ROS contents, and making embryo development like non-vitrified oocytes.

Other antioxidants have been proved in porcine, where Xiang et al. [10] found that when adding 1.5µM of astaxanthin to the culture media the developmental competence of parthenogenetic zygotes improved (51.7% blastocysts). This concentration also improved the blastocyst formation rate of vitrified cloned zygotes (23.3% blastocysts).

In bovine, Giraldo [11] determined the damage in vitrified embryos due to malondialdehyde (MDA) produced during the oxidation of phospholipids. By measuring MDA concentrations in media subjected to vitrification and post-vitrification with and without embryos, he found that in the first case it produced 35.26% more MDA compared to the second. He also observed that, at a higher concentration of cryoprotectants (DMSO-dimethyl sulfoxide + DMF-dimethylformin 20% each), the concentrations of MDA, ROS and thiobarbituric acid (TBAR) decreased.

Given that resveratrol shows an antioxidant effect that favors the IVM of oocytes, as well as the quality of embryos produced from them, in this study we evaluated its effect on the production of ROS, GSH and nuclei number of embryos produced by IVF after devitrification in sheep (Ovis aries).

2. Materials and Methods

Oocyte collection

Ovaries were collected from domestic sheep (Ovis aries) slaughtered at a local slaughterhouse and transported to the laboratory within 2 h at 30-35 °C in saline solution (0.9% NaCl) with 1% antibiotics (10,000 IU/mL of penicillin, 10 mg/mL of streptomycin sulfate and 25 µg/mL of amphotericin) (Antibac-Antifun 100x, In Vitro, SA). Once in the laboratory, the ovaries were washed and the cumulus-oocyte complexes (COC) were collected by puncturing the ovarian follicles (2 to 5 mm diameter) with a 10 mL syringe and a 20 G needle, containing TCM- 199® with HEPES (25 mM) with heparin (100 IU/mL) [3]. The COC were selected based on the ASEBIR [12] criteria.

In vitro maturation (IVM) of oocytes

IVM was carried out in TCM-199® supplemented with cysteine (0.57 mM), D-glucose (3.05 mM), polyvinyl alcohol (0.1%), sodium pyruvate (0.91 mM), 10% Fetal Bovine Serum (FBS, Microlab), 10 ng/mL of epidermal growth factor (EGF), 0.1 IU/mL-1 of recombinant follicle-stimulating hormone (FSH, Gonal-F Merck), 5 IU mL-1 of equine chorionic gonadotropin (eCG, Gonaforte Parfarm S.A.) and antibiotics (0.6%) (Antibac-Antifun 100x, In Vitro, SA). The IVM medium was added with the

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different concentrations of resveratrol. The COC were distributed in groups of 25 to 30 in cells of four-cell boxes (Nunc), in 400 µL of IVM medium from each treatment covered with mineral oil, culturing them for 24 h at 38.5°C, 5% CO2 and humidity at saturation. After IVM, 10% of the COC from each treatment were stripped from the cumulus cells (CC) in 0.5 mg/mL-1 of hyaluronidase, to determine the rate of in vitro maturation (IVM- rate of oocytes in Metaphase II or MII) [3] (Martínez et al., 2019) by observing the expansion of the CC and the presence of the first polar body [12], the rest was used for IVF.

In vitro fertilization (IVF) and in vitro development (IVD) of embryos

The COC showing expanded CC were washed in 50 µL of BO-IVF™ in vitro fertilization (IVF) medium (IVF Bioscience) covered with mineral oil and tempered at 38.5°C. Then 30 oocytes were distributed in four-cell culture dishes, in 100 µL of the IVF medium [13]. Straws with semen from fertile rams were thawed at 37°C before use. Post-thawing sperm motility was evaluated. "Swim up" was performed in Eppendorf tubes [2] in the IVM medium recovered from the oocytes that were matured from the control group [14]. Ten µL were taken from the upper part of the tubes with the motile sperm fraction (0.5 x 106 mL-1 sperm) and added to the IVF medium containing the oocytes from each treatment, incubating for 24 h under the same conditions [2]. Fertilized oocytes (zygotes) were washed twice in 50 µL of Cleavage medium (Cook Medical®) for the in vitro development (IVD) and transferred to a new culture dish by placing 25 zygotes per 50 µL of IVD medium covered with mineral oil. They were incubated for four days under the same conditions. On day 4 of culture, the embryos were transferred to another dish with 50 µL of Blastocyst medium (Cook Medical®), the IVD rate (early blastocysts) and embryonic morphology were evaluated in each treatment [2]. The embryos remained in Blastocyst medium (Cook Medical®) for an additional 24 h. The next day (day 5 of culture), early blastocysts were prepared for vitrification.

Vitrification and devitrification of embryos

Following Bhat et al. [15], the early blastocysts of each experimental group were vitrified using the Open Pulled Straw (OPS) technique [16]. Which consisted of washing the blastocysts in 800 µL of Holding medium (TCM-199 without HEPES supplemented with 10% FBS). They were then sequentially equilibrated first in 600 µL of Holding medium supplemented with 7.5% of ethylene glycol and 7.5% of DMSO for 3 min. Then in 600 µL of Holding medium with 0.342 g/mL of sucrose, 16.5% of ethylene glycol and 16.5% of DMSO for 20 sec. Immediately afterwards, 2 µL drops were formed with the embryos, collected by capillarity in an OPS, these were then placed inside straws (0.25 mL) that were heat sealed and subsequently immersed in the liquid nitrogen tank for 24 hours.

For devitrification, the OPS were thawed using the three-step technique [17]. First, each straw was removed from the nitrogen tank, the sealed end was cut, and the OPS was removed from the inside of the straw. The tip of the straw was introduced into 400 µL of Holding medium supplemented with 50% sucrose so that by gravity the embryos could go down, leaving them for 5 min. Embryos were then transferred to 1000 µL of Holding medium supplemented with 25% sucrose for 5 min. Finally, the embryos were washed in 400 µL of Holding medium without sucrose for 5 min. The devitrified early blastocysts were cultured again for 24 h in Blastocyst medium (Cook Medical®) under the same conditions until they reached the late blastocyst and hatched blastocyst stages [18].

Quantification of ROS and GSH in devitrified embryos

A sample of 10% of the devitrified early blastocysts from each group was taken, following the methodology of Martínez et al. [3]. At the end of post-vitrification IVD (day 7 of culture), 10 µM of DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) and 10 µM of the Cell Tracker Blue® stain were added to the medium containing the embryos, the culture dish was covered with aluminum foil and incubated for 30 minutes under the conditions described. Subsequently, the embryos were washed 3 times in 100 µL of DPBS (Dulbeco Phosphate Buffered Saline) and mounted on slides, covering them with coverslips and sealing them with nail polish. They were evaluated in an epifluorescence microscope with a 460 nm UV filter to evaluate ROS and 405 nm to evaluate GSH. Photographs were taken. The higher the fluorescence intensity, the presence of higher levels of ROS or GSH in the embryos was qualitatively considered. Fluorescence intensity was evaluated in an

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Image J image processor (version 1.53; Wayne Rasband, National Institute of Health, USA), to obtain semiquantitative data.

DAPI staining of nuclei

Prior to vitrification and after it, following the methodology of Vazquez-Avendaño et al. [19] with some modifications, on day 7 of culture the number of nuclei present in the blastocysts was determined. To do this, embryos of each group were washed for 2 min in 100 µL of DPBS (In vitro, S.A.), were fixed for 24 h in 400 µL of 4% paraformaldehyde, in the refrigerator. Subsequently, they were deposited on a slide in a 2 µL drop, removing the excess of the fixative. Ten 10 µL of a 2.5% solution of 4',6'-diamidino-2-phenylindole (DAPI) in DPBS was added to the slide and covered with a coverslip. A 405 nm UV filter was used to evaluate them. Once the images of each group were obtained, the nuclei of each embryo were counted.

Statistical analysis

The response variables analyzed were rate of in vitro maturation (IVM) of oocytes, embryonic segmentation, in vitro development (IVD), blastocysts with post-vitrification development capacity, presence of ROS, GSH and embryonic quality. These variables were analyzed considering the control group and the experimental groups (with the different concentrations of resveratrol) using Student's T test for paired samples. A one-way ANOVA was used considering the intragroup response variables, followed by the Duncan test, using the SAS Visual Analytical 2020.1 program, at a significance of $p<0.05$.

3. Results

3.1. Oocyte collection

Eight hundred and fifteen ovaries were collected from domestic sheep (O. aries), with an average aspiration rate of 3 oocytes/ovary. The IVM rate of oocytes treated with different concentrations of resveratrol was analyzed, IVF was verified by the embryonic segmentation rate and the IVD rate.

3.2. In vitro maturation (IVM) of oocytes, in vitro fertilization (IVF) and in vitro development (IVD) of embryos

The IVM rate was similar in oocytes treated with 0.5 and 1 µM of resveratrol and was higher compared to oocytes treated with 0 and 0.25 µM ($p\leq0.05$). The IVD, determined by the embryonic cleavage rate, was also similar for 0.5 and 1 µM of resveratrol and higher with respect to 0 and 0.25 µM ($p\leq0.05$). The blastocyst rate, although it did not show statistical differences between groups ($p\geq0.05$), it was observed that 0.25 µM of resveratrol reduces the rate of late blastocysts (Table 1).

Table 1. Rate of IVM, IVD and O. aries blastocysts from oocytes treated with resveratrol.

RESVERATROL	0 µM	0.25 µM	0.5 µM	1 µM
IVM	75 ± 6.5[a]	74 ± 8.5[a]	81 ± 6.1[b]	81 ± 6.5[b]
CLEAVAGE (IVD)	63.1 ± 5.4[a]	60 ± 4.6[a]	68.6 ± 4.7[b]	69.2 ± 4.7[b]
EARLY BLASTOCYSTS	8.4 ± 3.4[a]	13.5 ± 4.1[a]	16.2 ± 6[a]	16.8 ± 6.5[a]
LATE BLASTOCYSTS	30.2 ± 3.9[a]	25.6 ± 3.1[a]	30.2 ± 3.5[a]	31.4 ± 4.5[a]

Different literals a-b show significant differences in rows at $p\leq0.05$ (Mean ± SE). Considering an n in MIV=2,204 oocytes, DIV n=2,204 oocytes and embryos and blastocysts n=1,100 embryos.

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3.3. Vitrification and devitrification of embryos

No significant differences were found in the rate of late blastocysts after devitrification (p≥0.05). However, at 1 µM of resveratrol it looks higher (Figure 1 and Table 2).

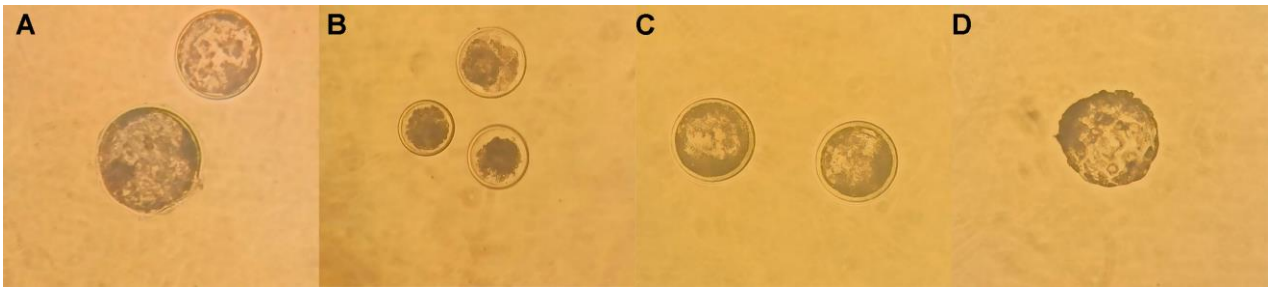

Figure 1. Late blastocysts produced by IVF from oocytes with resveratrol during IVM, after devitrification. A) 0 µM resveratrol (late expanded blastocysts); B) 0.25 µM resveratrol; C) 0.5 µM resveratrol (expanded blastocysts); D) 1 µM resveratrol (hatched blastocysts). A; B; D: Magnification 10X. C. Magnification 20X.

Table 2. Rate of late blastocysts produced by IVF from oocytes treated with resveratrol during IVM, after devitrification.

	Resveratrol			
Late Blastocysts	0 µM	0.25 µM	0.5 µM	1 µM
Mean ± SE	58 ± 20.9[a]	41 ± 23.6[a]	59 ± 7.3[a]	64 ± 13.8[a]

Same literal in a row shows no statistical differences (p≥0.05).

3.4. ROS and GSH levels in devitrified embryos

The qualitative evaluation of the presence of Reactive Oxygen Species (ROS) showed no differences in late blastocysts between groups, and glutathione (GSH) was present in all groups treated with resveratrol (Figure 2).

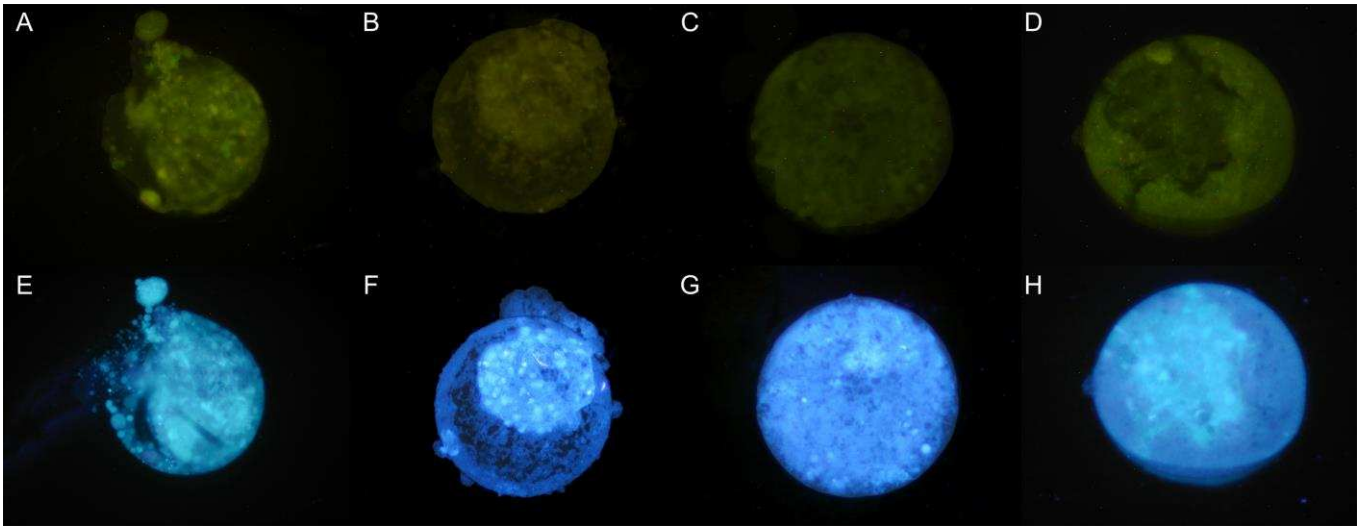

Figure 2. Qualitative evaluation of the presence of ROS and GSH in late blastocysts produced by IVF from oocytes treated with resveratrol during IVM, after devitrification. A. 0 µM; B: 0.25 µM; C: 0.5 µM and D: 1 µM resveratrol. Green images DCFH staining for ROS. Epifluorescence microscope (460 nm UV filter). Blue images Cell Tracker Blue® staining for GSH. Epifluorescence microscope (405nm UV filter). Magnification 10X.

The semiquantitative analysis (Image J) determined that ROS levels are reduced with the different treatments (0.25, 0.5 and 1 µM), particularly with 0.5 µM (p≤0.05) of resveratrol; while GSH

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levels increased at 0.25 and 0.5 µM of resveratrol with respect to the control group, being greater at 0.5 µM ($p \leq 0.05$) (Table 3 and Figure 3).

Table 3. Semiquantitative analysis (Image J) of fluorescence levels of ROS and GSH in late blastocysts produced by IVF from oocytes treated with resveratrol during IVM, after devitrification.

Variables	Concentración de resveratrol			
Fluorescence Intensity Units	0 µM	0.25 µM	0.5 µM	1 µM
ROS (Mean ± SE)	34.722 ± 768.4[a]	22.229 ± 4,314.2[bc]	12.929 ± 1,789.3[b]	24.447 ± 1,104.8[c]
GSH (Mean ± SE)	25.733 ± 9,637[a]	60.478 ± 10,586[b]	94.446 ± 9,589[b]	74.759 ± 14,800[ab]

a,b,c, Literals between lines show significant differences ($p \leq 0.05$).

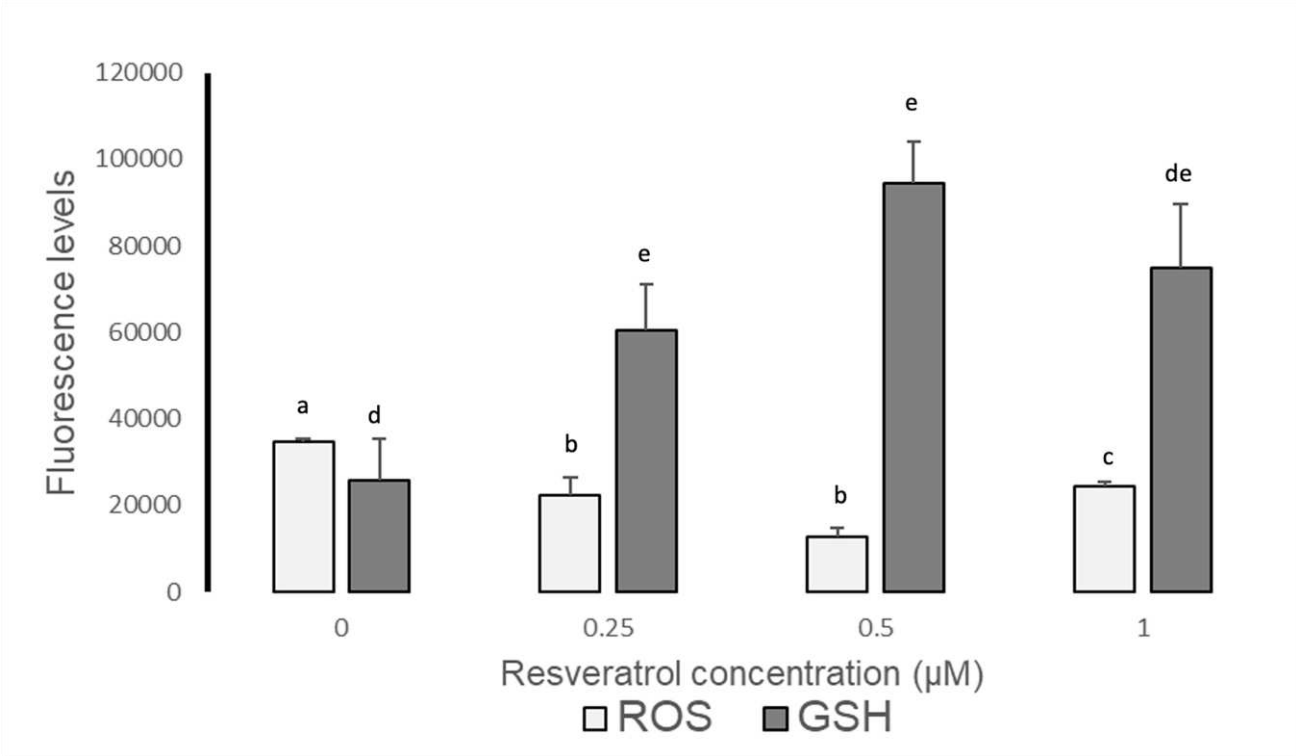

Figure 3. Fluorescence levels (Image J) of Reactive Oxygen Species (ROS) and glutathione (GSH) in sheep blastocysts produced by IVF from oocytes treated with resveratrol, after devitrification. Literals between bars show significant differences ($p \leq 0.05$).

3.5. DAPI staining of nuclei

3.5.1. Presence of nuclei in early blastocysts prior to vitrification

Prior to vitrification, blastocysts showed significant differences in the nuclei present between treatments, particularly for 0.5 and 1 µM of resveratrol, with respect to the control group ($p \leq 0.05$). Significant differences were also observed between 0.25 vs. 0.5 µM and between 0.5 vs. 1 µM ($p \leq 0.05$), with 1 µM being the treatment in which the embryos showed a greater number of nuclei, but not for 0.5 µM of resveratrol (Figure 4 and Table 4).

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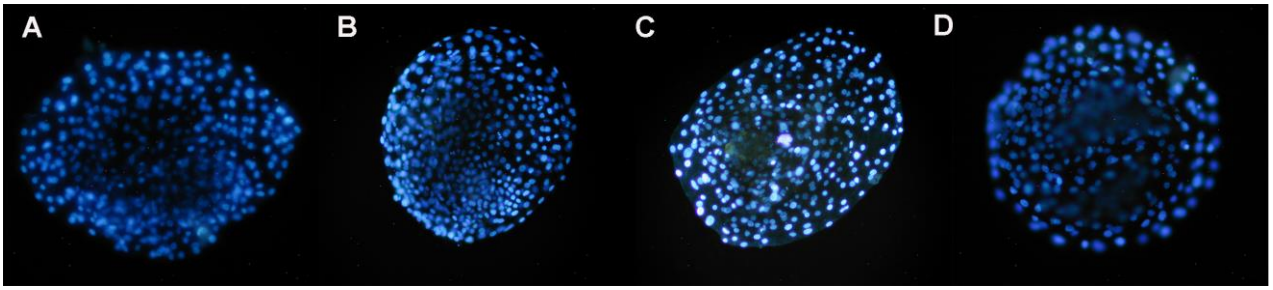

Figure 4. Presence of nuclei in early blastocysts prior to vitrification, produced by IVF from oocytes treated with resveratrol during IVM. Images A. 0 µM; B. 0.25 µM; C. 0.5 µM and D. 1 µM resveratrol. DAPI stain. Epifluorescence microscope (405 nm UV filter). 20X magnification.

Table 4. Number of nuclei present in early blastocysts prior to vitrification, produced by IVF from oocytes treated with resveratrol during IVM.

Variable	Resveratrol concentration			
Number of nuclei	0 µM	0.25 µM	0.5 µM	1 µM
Mean ± SE	267±28.8^{a}	260±33.2^{ac}	185±11.7^{bc}	354±21.5^{d}

Literals a-b-c-d: between lines show significant differences between treatments (p≤0.05). Day 7 of culture.

3.5.2. Presence of nuclei in late blastocysts after devitrification

At 48 h after devitrification, the quantification of nuclei in the blastocysts showed significant differences between 0.25 and 1 µM of resveratrol (p≤0.05), with the number of nuclei again being greater at 1 µM). However, in all treated groups the number of nuclei present in devitrified embryos was lower than that in non-vitrified embryos (Table 5).

Table 5. Number of nuclei present in blastocysts produced by IVF from oocytes treated with resveratrol during IVM, after devitrification.

Variable	Resveratrol concentration			
Number of nuclei	0 µM	0.25 µM	0.5 µM	1 µM
Mean ± SD	109±4.3^{a}	90±12.3^{a}	147±21^{b}	150±19.4^{b}

Literals a-b: between rows show significant differences between treatments (p≤0.05). Forty-eight hours after devitrification.

4. Discussion

Effect of resveratrol on IVM, DIV and blastocyst production

It is known that the maturation rate influences the success of IVF and subsequent embryonic development. Other factors that influence this are culture media, changes in pH, osmolarity, temperature, exposure to light, concentration of CO2 and oxygen, humidity, manipulation of cells and cryopreservation methods [20,21]. If the conditions are not adequate, they will generate stress to the oocyte by affecting the cytoplasm and cell membrane, causing changes in nucleic acids, lipids, and proteins [22,23].

This oxidative stress is caused by reactive oxygen species (ROS) produced by cellular metabolism that can damage and/or kill oocytes and embryos, through a decrease in ATP, blockage in embryonic development, alterations in methylation of DNA and histone modification [20,24,25].

Reactive oxygen species are produced naturally during cellular metabolism. However, during embryonic development, especially in the transition from morula to early blastocyst, an increase in ROS occurs and, consequently, at this stage the blastocyst is very susceptible to oxidative stress, especially when endogenous antioxidants (enzymatic and non-enzymatic) do not achieve balance. This is why there is a need to add antioxidants to the culture media [4]. As the embryo progresses in development, cellular metabolism increases and cells are more susceptible to oxidative stress,

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specifically in the mitochondrial respiratory chain. Complexes I and II produce superoxide and nitrile radicals that affect mitochondrial proteins and the function of different metabolic enzymes in the electron transport chain [26]. It has been reported that mitochondrial DNA (mtDNA) is more sensitive to oxidative stress than nuclear DNA [27], possibly because mtDNA lacks histones, which protect against such damage. Furthermore, it does not have an efficient repair system and is located near the inner mitochondrial membrane, the site of greatest ROS production [28,29]. Additionally, oxidative damage to mtDNA can induce mutations and alter mitochondrial function and integrity [30]. This oxidation-reduction imbalance, in humans, induces degenerative mitochondrial diseases such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis [31,32].

In an in vivo system, cells are protected from these effects by the enzymatic and non-enzymatic antioxidants present, for example, oocytes are protected by ovarian follicle fluid [33]. In contrast, in in vitro systems, antioxidants are added [34] such as α-tocopherol, vitamins, proteins, antioxidant enzymes, thiolic compounds, metal chelators, vitamin E and its derivatives, cysteamine, ascorbic acid and extracts of plants with antioxidant properties [35,34]. At physiological concentrations, ROS are essential for cell metabolism and signaling, while their overproduction will generate alterations [36,37].

Resveratrol is an antioxidant used in in vitro oocyte culture media, because promotes IVM rates. At 0.25 and 0.5 μM, resveratrol increases cleavage and blastocyst rates [4,7]. In the present study, oocytes treated with 0.5 and 1 μM resveratrol improved IVM, embryonic cleavage rate, and blastocyst production. It is known that the addition of this antioxidant during IVM favors the cytoplasmic and nuclear maturation of the oocyte. This effect could be related to its ability to increase the expression and nuclear translocation of transcription factors that prevent oxidative stress in the oocyte [3]. It could also be because it increases the concentration of intracellular reduced glutathione (GSH) and favors the concentrations of the enzyme glutathione peroxidase, essential for the embryonic oxidant balance [4]. This allows a decrease in ROS production, as has been observed in pigs, cattle, sheep, and goats.

Effect of resveratrol on embryo vitrification and devitrification

Vitrification subdues the cell to significant thermal and oxidative stress. In the present study, ethylene glycol was used as a cryoprotectant, since its efficiency in the vitrification of ovine and bovine embryos has been reported [16,38]. It has properties such as high penetration speed and low toxicity, in addition to avoiding removing the cryoprotectant prior to embryo transfer; that is, it does not interfere with embryo implantation in embryo recipient females [39].

On the other hand, the Open Pulled Straw (OPS) system allowed embryonic survival after vitrification (64%) at the highest concentration of resveratrol (1 μM), although it was lower than that reported by González [18], who obtained data greater than 70%.

Embryos are exposed to oxidative stress during vitrification, affecting rates of IVM, IVD and early blastocysts when compared to in vivo systems [4,5]. However, in the present study resveratrol supplemented during IVM reduced ROS levels in blastocysts after vitrification. Giaretta et al. [40] and Salzano et al. [41] reported that this antioxidant, when added to the culture medium has a positive effect on development and cryotolerance of blastocysts.

Effect of resveratrol on ROS and GSH levels in devitrified embryos

In the present study the vitrification and devitrification methods to which sheep embryos produced by IVF were subjected, allowed a reduction in ROS levels, which was minimal at 0.5μM, and an increase in GSH levels, reaching the highest levels at this concentration. Greater embryonic survival was achieved after devitrification, at 0.5 μM and 1 μM of resveratrol ($p<0.05$). This demonstrates that resveratrol, used in the IVM of oocytes, reduces embryonic oxidative stress by promoting the synthesis of endogenous antioxidants such as GSH.

Glutathione is the main non-protein sulfide component in mammalian cells and is recognized for protecting the cell from oxidative damage, regulating the intracellular redox balance [42]. It may also be important in biological processes such as DNA and protein synthesis, and cell proliferation during embryonic development [43]. In cattle, it has been considered an important marker of oocyte

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viability and quality [44]. Additionally, GSH synthesis during in vitro maturation is associated with the formation of the male pronucleus after fertilization [45,46] and development early embryonic [43].

During embryonic development, GSH is important in DNA and protein synthesis, and cell proliferation [44]. In cattle, it has been considered an important biochemical marker of oocyte viability and quality [46,43].

Effect of resveratrol on embryo quality

Hernández et al. [47], reported a low number of nuclei (44.2±9.9) present in parthenogenetic blastocysts of O. aries and in interspecies cloned blastocysts of O. aries and O. canadensis mexicana (46.7±8.1 nuclei). Lorenzo-Torres et al. [48], reported 122 nuclei in good quality blastocysts produced by IVF. These results are lower than those obtained in the present study, but they are similar to those reported by Agata et al. [49], Mastrorocco et al. [50] and Nadri et al. [51]. Before vitrification, resveratrol at 1μM showed a greater number of nuclei in early blastocysts. After devitrification, although the number of nuclei decreased, the highest value was also at 1μM of resveratrol. In both cases, 1 μM of resveratrol overcome the number of nuclei of control group and the other treatments.

It is shown that resveratrol not only favored the IVM rate of sheep oocytes but had a positive effect on the production of higher quality embryos, conferred cryotolerance capacity during the vitrification process.

5. Conclusions

Resveratrol supplemented at 0.5 μM and 1 μM during IVM of Ovis aries oocytes increased the rate of maturation, improved the development of sheep embryos to the early blastocyst stage from the fourth day of IVD, and favors embryonic cryotolerance to vitrification reducing ROS levels, an effect inversely proportional to GSH levels. But, at 0.25 μM it has a negative effect on IVM, IVD, blastocyst production rate and embryonic survival rate after vitrification. While at 1 μM it favors a greater number of nuclei present in sheep embryos produced by IVF, this indicates that its quality improves before and after vitrification.

6. Patents

Part of the methodology applied in this study was based on the patent No. 394003.

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