Resveratrol-supplemented holding or re-culture media improves viability of fresh or vitrified-warmed *in vitro*-derived bovine embryos

A suplementação dos meios de manutenção ou re-cultivo com resveratrol melhora a viabilidade de embriões bovinos produzidos *in vitro*

La suplementación de lós médios de mantenimiento o de recultivo con resveratrol mejora La viabilidad de lós embriones bovinos producidos *in vitro*

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Abstract
The effect of resveratrol supplementation on fresh (E1) or vitrified/warmed (E2) *in vitro* produced bovine embryos was investigated by evaluating the time-dependent response. After *in vitro* production, resveratrol (0.5 µM) was added to the incubation media and after two incubation periods with or without resveratrol, blastocysts were re-cultured for 24h. The rates of re-expansion, hatching, total cell number (TCN), apoptotic cells (ACN), reactive oxygen species (ROS) and intracellular glutathione (GSH) content were evaluated. For E1, the re-expansion rate differed at 6 and 10h within and between treatments (P<0.05), as did the re-expansion rate after 24h (P<0.01). The hatching rate increased after 10h with resveratrol (P<0.01) with differences within (P<0.05), but not between treatments after 24h of re-cultivation. At E2, hatching rate differed between treatments at 24h (P<0.01), with higher TCN in resveratrol-treated blastocysts after 10h (P<0.01). Resveratrol supplementation reduced ROS generation in E1 and E2 after 10h of incubation and increased GSH content (P<0.01). These results indicate that supplementation of holding re-cultivation medium with resveratrol for treatment of fresh or vitrified/warmed *in vitro* produced bovine embryos has a positive

and time-dependent effect. The reduction of ROS content, the increase of GSH and the anti-apoptotic ability of resveratrol are responsible for its protective effects, allowing an extension of embryo storage time before transfer to recipients.

Keywords: Antioxidant effect; Embryonic development; Holding; *In vitro* production; Warming.

Resumo

O efeito da suplementação com resveratrol sobre embriões bovinos produzidos *in vitro* frescos (E1) ou vitrificados/aquecidos (E2) foi investigado, avaliando a resposta tempo-dependente. Após a produção *in vitro*, o resveratrol (0.5 µM) foi adicionado aos meios de incubação e após dois períodos de incubação com ou sem resveratrol, os blastocistos foram re-cultivados por 24h. As taxas de reexpansão, eclosão, número total de células (TCN), células apoptóticas (ACN), espécies reativas de oxigênio (ROS) e conteúdo de glutationa intracelular (GSH) foram avaliados. Para E1, a taxa de reexpansão diferiu às 6 e 10h dentro e entre tratamentos (P<0,05), assim como a taxa de reexpansão após 24h (P<0,01). A taxa de eclosão aumentou após 10h com resveratrol (P<0,01) com diferenças dentro (P<0,05), mas não entre tratamentos após 24h de re-cultivo. No E2, a taxa de eclosão diferiu entre tratamentos às 24h (P<0,01), com maior TCN nos blastocistos tratados com resveratrol após 10h (P<0,01). A suplementação com resveratrol reduziu a geração de ROS em E1 e E2 após 10h de incubação e aumentou o conteúdo de GSH (P<0,01). Estes resultados indicam que a suplementação do meio de manutenção ou do meio para re-cultivo com resveratrol para tratamento de embriões bovinos produzidos in vitro frescos ou vitrificados e aquecidos tem um efeito positivo e dependente do tempo. A redução do conteúdo de ROS, o aumento de GSH e a capacidade anti-apoptótica do resveratrol são responsáveis por seus efeitos protetores, permitindo uma extensão do tempo de armazenamento embrionário antes da transferência para as receptoras.

Palavras-chave: Efeito antioxidante; Desenvolvimento embrionário; Manutenção; Produção *in vitro*; Aquecimento.

Resumen

Se investigó el efecto de la suplementación con resveratrol en embriones bovinos frescos (E1) o vitrificados/calentados (E2) producidos *in vitro*, evaluando la respuesta tiempo-dependiente. Tras la producción *in vitro*, se añadió resveratrol (0,5 µM) a los medios y se volvieron a cultivarlos durante 24hs. Se evaluaron las tasas de reexpansión, eclosión, número total de células (TCN), células apoptóticas (ACN), especies reactivas de oxígeno (ROS) y contenido de glutatión (GSH). En E1, la tasa de reexpansión difería a las 6 y 10hs dentro de los tratamientos y entre ellos (P<0,05), al igual que la tasa de reexpansión después de 24 hs (P<0,01). La tasa de eclosión aumentó después de 10h con el resveratrol (P<0,01) con diferencias dentro (P<0,05), pero no entre los tratamientos después de 24hs de recultivo. En E2, la tasa de eclosión difería entre los tratamientos a las 24hs (P<0,01), con mayor TCN en los blastocistos tratados con resveratrol después de 10hs (P<0,01). La suplementación con resveratrol redujo la generación de ROS en E1 y E2 tras 10hs de incubación y aumentó el contenido de GSH (P<0,01). Estos resultados indican que la suplementación con resveratrol para el tratamiento de embriones bovinos frescos o vitrificados/calentados tiene un efecto positivo y dependiente del tiempo. La reducción del contenido de ROS, el aumento del GSH y la capacidad antiapoptótica del resveratrol son los responsables de sus efectos protectores, permitiendo prolongar el tiempo de almacenamiento de los embriones antes de su transferencia a las receptoras.

Palabras clave: Efecto antioxidante; Desarrollo embrionario; Mantenimiento; Producción *in vitro*; Calentamiento.

1. Introduction

The increase in human population and the demand for food in the world has served as a stimulus for producers to seek alternatives to increase production over time, and in this context, the bovine *in vitro* embryo production (IVP) industry has grown considerably worldwide (Sanches et al., 2019). The IVP process involves oocyte maturation, oocyte fertilization, and culture of the *in vitro*-derived zygote to the blastocyst stage. Cell exposure to high atmospheric O_2 levels increases the production of reactive oxygen species (ROS). The manipulation, light temperature, maturation and culture media constituents, sperm, and O_2 stress during IVP increases ROS and may lead to a downregulated defense mechanism (Yu et al., 2014). During the entire IVP process, the amount of ROS might imbalance the availability of antioxidants as ROS are produced as a result of cellular metabolism; therefore, ROS levels play a critical role in the success of in vitro fertilization because the quality of the embryo could be impaired owing to harmful effects of the culture environment. The increase in cellular damage because of increased ROS production during the IVP process is well documented (Lee et al., 2012), as well the benefits of adding antioxidant molecules to supplement culture media and thus, decrease ROS production to improve the quality and to protect embryos against damage to DNA and other biomolecules, raising their developmental competence (Morado et al., 2009).

Pregnancy after the transfer of fresh IVP-derived bovine embryos is less likely than with fresh embryos produced *in vivo*, and cryopreservation decreases embryo survival (Ferraz et al., 2016). Currently, fresh embryo transfers are the most common choice in IVP; however, when the total embryo production is higher than the number of recipients, or because of the distance from laboratory to farm, an efficient protocol for embryo cryopreservation must be implemented (Sanches et al., 2019). Vitrification is the most efficient cryopreservation method for IVP embryos, which are more sensitive to cryoinjuries than those produced *in vivo* because, among other factors, they contain more intracellular lipid droplets. Antioxidant additives can improve embryo response to cryopreservation injuries and the benefits of resveratrol, a flavonoid with extra- and intracellular antioxidant properties has been demonstrated in different cellular models and it has the ability to reduce free radical production and to induce the expression of antioxidant genes (Sovernigo et al., 2017). Several studies have shown the beneficial effect of adding resveratrol to the media used for *in vitro* embryo production, allowing the embryos to recover from low oxidative metabolism (Madrid Gaviria et al., 2018) or their initial quality after cryopreservation (Madrid Gaviria et al., 2019) (Hara, Kim & Aoki, 2018). This improvement was also associated with improved embryo tolerance to cryopreservation by slow freezing (Hayashi et al., 2019) and by vitrification (Hayashi et al., 2018), including of the oocytes (Wang et al., 2014; Spricigo et al., 2017). Bovine embryos vitrified in the stage of 8-12 cells and and treated with resveratrol after warming showed improved development during the re-culture for 5 days (Hara, Kim & Aoki, 2018). However, owing to the growing demand for *in vitro*-derived bovine embryos, the addition of different products and molecules in the culture or holding media to improve post-culture viability of embryos easily and safely must be available to the field technician, because the conditions on the farm are not the same as in the IVP research or commercial production laboratory. To the best of our knowledge there are no studies showing the effect of adding resveratrol to the holding medium for fresh or vitrified-warmed *in vitro*-derived bovine embryos in the blastocyst phase. This study was designed based on the hypothesis that it is possible to improve the quality of fresh and vitrified-warmed IVP-derived bovine embryos in the pre-preincubation period, using the practical approach of adding resveratrol from a stock solution to the holding or *in vitro* culture (IVC) media.

2. Methodology

The experiments were developed as a quantitative laboratory research (Pereira et al. 2018). All experimental procedures were approved by the Ethics Committee on Animal Experimentation/Federal University of Goias, protocol #049/2015.

Unless otherwise indicated, all media for oocyte maturation, fertilization, embryo culture, and embryo cryopreservation were prepared by Progest® Animal Biotechnology (Botucatu, SP, Brazil).

Experimental design

The experimental design was completely randomized. Time-responsive effects of resveratrol on fresh (E1) and vitrified-warmed (E2) *in vitro*-derived blastocysts' developmental competencies were evaluated. Resveratrol was added to the incubation medium at a concentration of 0.5 µM. As control, a group of blastocysts were incubated in the absence of resveratrol. After the two incubation periods with or without resveratrol, blastocysts were re-cultured in the standard *in vitro* culture medium for up to 24 h. Blastocyst re-expansion and hatching rates were evaluated as developmental parameters and the number of total cells, apoptotic cells, ROS, and intracellular glutathione (GSH) content were assessed.

Experimental Procedures

In vitro embryo production (E1 and E2)

The IVP was performed as previously reported (Marques et al., 2018). Briefly, bovine ovaries were collected from local slaughterhouses, and cumulus-oocyte complexes (COCs; n = 1,500; 10 replicates) were aspirated from follicles between 3 and 8 mm in diameter. GI and GII oocytes (defined as more than three compact layers of cumulus cells and homogeneous cytoplasm and two compact layers of cumulus cells with less homogeneous cytoplasm in relation to the previous one, respectively) were subjected to the *in vitro* maturation process in groups of 30–35 COCs, matured in drops of IVC medium for 22–24 h at 38.5 °C in 5% CO_2 and saturated relative humidity without condensation. After maturation, COCs were incubated with conventional commercial frozen semen from a single Nellore bull with tested *in vitro* fertility and embryo production followed the previously reported routine (Marques et al., 2018). Probable zygotes remained for 7 days (D7) in drops of IVC medium at 38.5 °C in 5% CO_2. Morphological assessment of embryo quality was performed according to Bó and Mapletoft (2013). Development to the blastocyst stage was evaluated on D7 and fresh blastocysts were immediately subjected to the treatments (E1) or vitrified using open pulled straws (E2).

Resveratrol solution

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene, Sigma-Aldrich Chemical, St. Louis, MO, USA) 5.0 mM was prepared as a stock solution by dissolving resveratrol in ethanol (Sigma-Aldrich Chemical), stored in 10.0 µL aliquots, and kept frozen until use. The 0.5 µM concentration was obtained by dilution in the media according to the volume to be used. The final concentration was selected based on previously reported results showing an increase in the number of transferable bovine embryos using low concentrations of resveratrol during *in vitro* culture (Salzano et al., 2014).

Experiment 1- Growing in straw in holding medium

Six replicates were established with 307 D7-fresh Code 1 and 2 blastocysts. The blastocysts were placed in the holding medium with or without resveratrol 0.5 µM (Resv) and packed into 0.25 mL plastic straws. Straws were sealed and placed on a heating plate and kept at 36 °C for 6 and 10 h. At the end of the incubation period, embryos were evaluated for expansion rates, hatching, and quality. Embryos were then placed again in culture medium and re-cultured up to 24 h (38.5 °C in 5% CO_2). Incubation time was defined by the average time required to transport the fresh embryos from the laboratory to the farm. The two time points were selected based on a previous simulation performed in our laboratory.

Experiment 2 – Vitrification, warming, re-cultivation

Eight replicates were set up with 411 D7-fresh Code 1 and 2 blastocysts which were subjected to the vitrification protocol (Marques et al., 2018) using buffered HEPES with TCM-199 supplemented with 20% bovine fetal serum; 7.5% ethylene glycol and 7.5% dimethyl sulfoxide; and 15% ethylene glycol, 15% dimethyl sulfoxide, and 0.5 M sucrose. Embryos were loaded in open pulled straws (Vitri-Ingá® INGAMED, Maringá, PR), which were immersed in liquid nitrogen and stored in cryogenic cylinders for 60 days. Subsequently, the embryos were warmed on a heating plate at 38 °C and cultured in 200 µL drops of IVC medium (5–7 embryos/droplet; 38.5 °C in 5% CO_2) without (Control) and with resveratrol 0.5 µM (Resv). Embryo viability was recorded at 6 and 24 h after warming by evaluating the rate of embryos that re-expanded to the original size and developed to a hatching blastocyst. Incubation time for the cultivation after warming was based on previous studies showing the beneficial effect of using resveratrol during some steps of the embryo production on the viability of previously vitrified-warmed embryos after 24 h of post-warming culture (Salzano et al., 2014). The two time points were selected based on a previous transport simulation performed in our laboratory.

Detection of cellular apoptosis, ROS generation, and GSH levels

For the evaluations using fluorescent probes, 10 blastocysts from each treatment (Control and Resv) and incubation time (6 and 10 h) from E1 and 15 blastocysts from each treatment (Control and Resv) and incubation time (6 and 24 h) from E2 were analyzed.

The total cell number (TCN) and number of apoptotic cells (ACN) were evaluated with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. The nuclei of TUNEL-positive cells (fragmented DNA) were visualized at 450 nm (green), and all nuclei were visualized at 365 nm (blue). All cells labeled and visualized at both wavelengths were counted to determine the mean TCN and ACN. Intracellular ROS and GSH content were measured using dichlorohydrofluorescein diacetate (C400, Molecular Probes), and 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CellTracker Blue CMF2HC, Molecular Probes), respectively, as previously reported (Marques et al., 2021). ROS was detected as green (460 nm wavelength) and GSH as blue (370 nm wavelength) fluorescence, and to evaluate ROS levels and GSH content, the relative fluorescence intensity was considered directly proportional to intracellular ROS levels and GSH content.

Embryos were evaluated using epifluorescence microscopy (Olympus BX43, Tokyo, Japan), and images were captured using a camera (Olympus SC30, Tokyo, Japan) and saved in a TIFF format. The fluorescence intensities of the embryos were analyzed individually using Image J software (version 1.46; National Institutes of Health, Bethesda, MD) and normalized to those of the Control group.

Statistical analysis

All statistical analyses were performed using Sigma Plot 11.0 (Systat Software Inc, EUA). Data were first checked for normal distribution (Shapiro-Wilk test). Embryonic development and rates of re-expansion and hatching were compared using Fischer's chi-square test. The TCN and ACN were compared using the Kruskal-Wallis test. The Mann-Whitney test was used to analyze intracellular ROS and GSH levels. Data are presented as mean ($\pm$ SEM) and percentage. Differences ≤ 0.05 were considered significant.

3. Results

A total of 1,020 embryos were produced from 1,500 oocytes (68% success); 718 (70.4%) were morphologically classified as Code 1 and Code 2 (Bó & Mapletoft, 2013) and used for the two experiments.

Experiment 1

The results of resveratrol 0.5 μM treatment on fresh *in vitro*-derived bovine embryos are presented in Table 1. The re-expansion rate was calculated on the number of viable embryos and hatching rate was calculated on the number of embryos expanded within each moment. Incubation for 10 h improved embryo re-expansion and hatching in both treatments (Control, 28.9% and Resv, 23.3%, P< 0.05). After the re-cultivation period of 24 h, the highest re-expansion rate was achieved in the Resv 10 h embryos (36.6%, *P* < 0.05) and the hatched rate differed (P< 0.05) after 10 h (Control, 20.6% and Resv, 36.6%). Embryos incubated for 6 h before the re-cultivation of up to 24 h presented a higher hatched rate (Control, 43.3% and Resv, 48.4%) at the final time than those incubated for 10 h (Control, 25.0% and Resv, 27.2%; P < 0.05).

Table 1. Re-expansion and hatched rates of fresh *in vitro*-derived embryos after exposed or not to a holding medium with Resveratrol 0.5μM and re-cultured in IVC medium up to 24 h.

Treatment	n	Re-expansion (%)		Hatched (%)	
		6 hours	24 hours	6 hours	24 hours
Holding 6 h	81	28.9 (20/69)[a]	54.5 (30/55)[a]	-	43.3 (13/30)[a]
Resveratrol 6 h	86	23.3 (18/77)[a]	48.5 (33/68)[a]	-	48.4 (16/33)[a]
		10 hours	24 hours	10 hours	24 hours
Holding 10 h	88	43.2 (29/67)[b]	62.0 (36/58)[a]	20.6 (6/29)[a]	25.0 (9/36)[b]
Resveratrol 10 h	52	57.6 (30/52)[c]	80.4 (33/41)[b]	36.6 (11/30)[b]	27.2 (9/33)[b]

[a,b,c] Within a column, values without a common letter differed between and within treatments ($P < 0.05$).
Source: Authors.

There was an effect of treatment and incubation time on TCN (Figure 1, Figure 2) within the same incubation time. Control and Resv embryos differed regarding TCN [Control 6 h, 118.1 ± 3.2 versus Resv 6 h, 129.1 ± 2.5 (P< 0.001) and Control 10 h, 82.4 ± 2.5 versus Resv 10 h, 114.8 ± 1.9 ($P < 0.001$)]. Comparison between treatments showed a lower ACN in Resv 6 h (11.8 ± 1.0) and Resv 10 h embryos (11.0 ± 1.2) than in Control 6 h (15.0 ± 1.2) and Control 10 h (14.8 ± 1.2; P< 0.05).

Figure 1. Mean (± SEM) number of total cells (a) and apoptotic cells (b) recorded in *in vitro*-derived embryos exposed (6 or 10 hours) to medium with or without supplementation of Resveratrol (0.5 μM) and re-cultured in IVC medium up to 24 h.
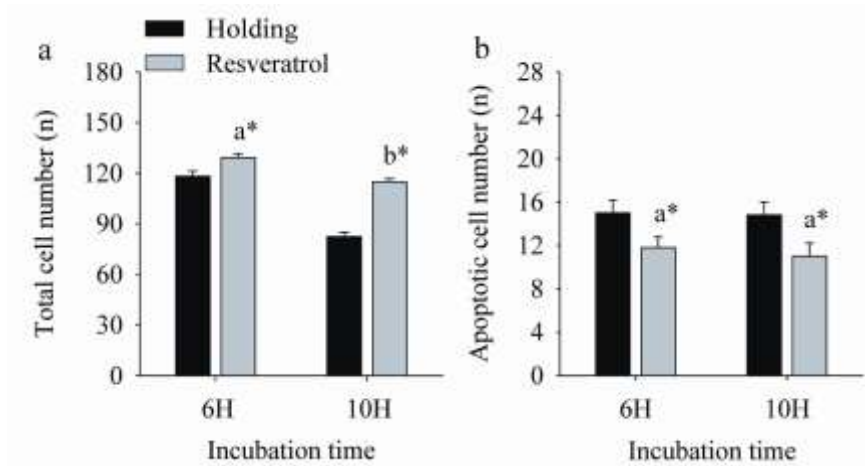

[a,b] Values without a common letter differed between Resveratrol 6H and 10H ($P < 0.05$).
[*] Differ from the holding medium (control) within the same incubation time ($P < 0.05$).
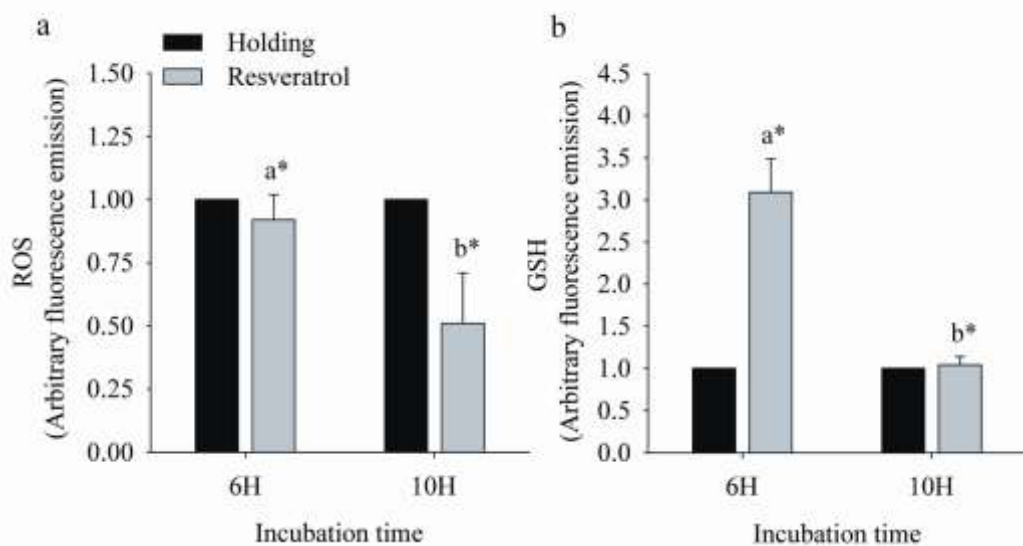Source: Authors.

Figure 2. Representative images of TUNEL staining recorded in in vitro-derived embryos exposed (6 or 10 hours) to Holding medium with or without supplementation of Resveratrol (0.5 μM) and re-cultured in IVC medium up to 24 h. The nuclei of TUNEL-positive cells (fragmented DNA, green), and all nuclei (blue), fluorescence microscopic imaging.
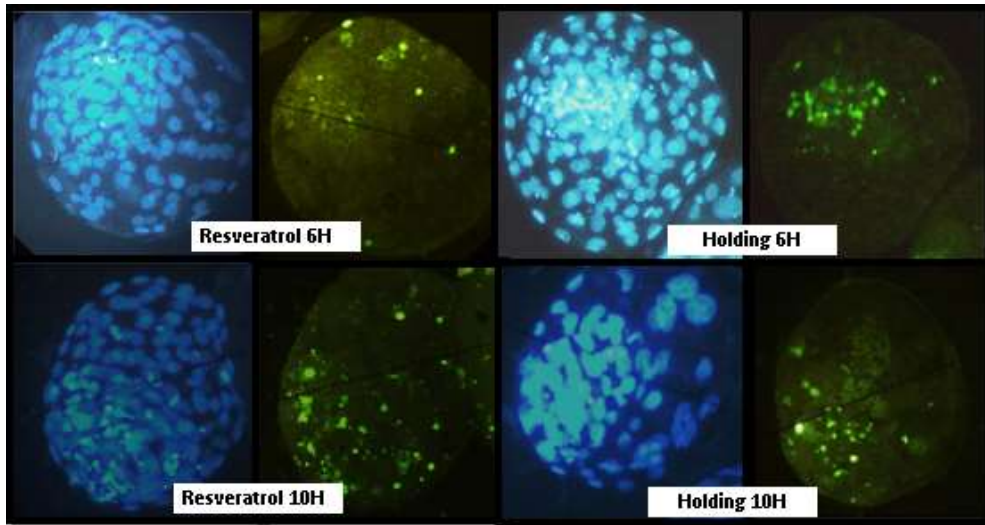
Source: Authors.

The levels of intracellular ROS and GSH are shown in Figure 3. ROS differed between and within treatments (P< 0.01) irrespective of incubation time. Resv 6 h produced more ROS than Resv 10 h (P< 0.01). GSH content was higher in the Resv 6 h embryos, followed by that in Resv 10 h and Control 10 h (P< 0.01). After defining the fluorescence emission from Control embryos as "1," ROS and GSH of Resv embryos were 0.65 ± 0.1 and 1.3 ± 0.1, respectively.

Figure 3. Mean (± SEM) (a) reactive oxygen species (ROS) and (b) glutathione peroxidase (GSH) recorded in *in vitro*-derived embryos exposed (6 or 10 hours) to medium with or without supplementation of Resveratrol (0.5 μM) and re-cultured in IVC medium up to 24 h. Fluorescence intensity in the control group was defined as 1.0.

.[a,b] Values without a common letter differed between Resveratrol 6H and 10H ($P < 0.05$). *Differ from the holding medium (control) within the same incubation time ($P < 0.05$).
Source: Authors.

Experiment 2

The results of resveratrol 0.5 μM treatment on vitrified *in vitro*-derived bovine embryos after warming and re-cultivation are presented in Table 2 and Figure 4, 5 and 6. The hatching rate in the Resv embryos was higher (37.7%) than in the Control (19.1%) after 24 h of incubation (P< 0.01).

Table 2. Re-expansion and hatched rates of vitrified *in vitro*-derived bovine embryos warmed and re-cultured exposed or not to a medium with Resveratrol 0.5μM during 6 or 24 hs.

Treatment	n	Re-expansion (%)		Hatched (%)	
		6 hs	24 hs	6 hs	24 hs
Control	200	38.3 (41/107)[a]	56.6(47/83)[a]	14.6(6/41)[a]	19.1(9/47)[a]
Resveratrol	211	37.5(45/120)[a]	51.9(53/102)[a]	13.3(6/45)[a]	37.7(20/53)[b]

[a,b] Within a column, values without a common letter differed among treatments (P< 0.01).
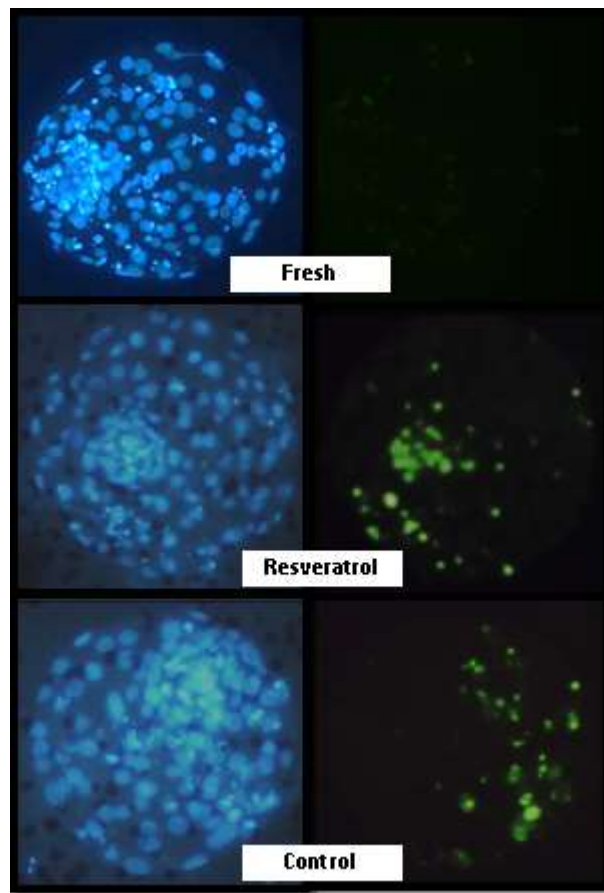Source: Authors.

For TCN and ACN evaluation, an additional group of 15 fresh embryos was included (Figure 4, Figure 5) to avoid errors in data interpretation. These variables were affected by vitrification, as evidenced by the differences regarding the above-mentioned parameters. Fresh embryos presented more TCN (131.6 ± 2.4) and fewer ACN (6.3 ± 0.8) than vitrified embryos (P< 0.01), irrespective of treatments. The TCN (102.7 ± 2.3) and ACN (11.5 ± 1.0) was affected by the addition of resveratrol in comparison to the Control (88.8 ± 2.5 and 23.6 ± 1.0, respectively; P< 0.01). Resveratrol decreased intracellular ROS levels and increased GSH content (Fig. 6) in comparison with that in the Control ($P < 0.01$). As the fluorescence emission was defined as "1," normalized ROS levels and GSH content were 0.65 ± 0.1 and 1.3 ± 0.1, respectively.

Figure 4. Mean (± SEM) number of total cells (TCN) and apoptotic cells (ACN) recorded in vitrified *in vitro*-derived embryos warmed and re-cultured in a medium supplemented or not with Resveratrol.
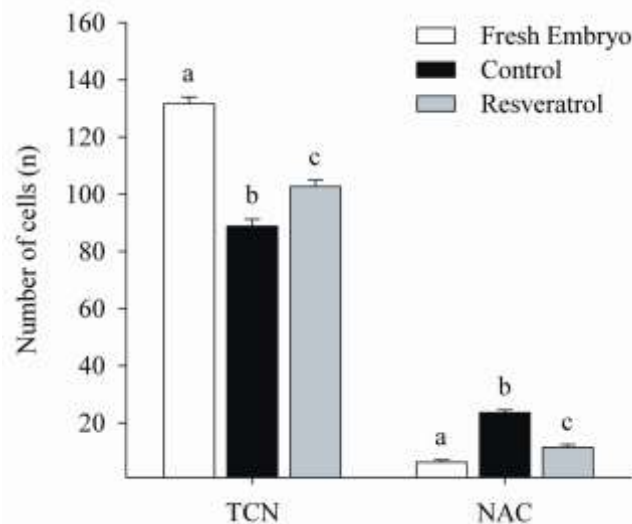

[a,b,c] Values without a common letter differed within the same parameter evaluated ($P < 0.05$).
Source: Authors.

Figure 5. Representative images of TUNEL staining recorded in vitrified in vitro-derived embryos warmed and re-cultured in a medium supplemented or not with Resveratrol (0.5 µM) up to 24 h. The nuclei of TUNEL-positive cells (fragmented DNA, green), and all nuclei (blue), fluorescence microscopic imaging.

Source: Authors.

Figure 6. Mean (± SEM) reactive oxygen species (ROS) and glutathione peroxidase (GSH) recorded in vitrified *in vitro*-derived embryos warmed and re-cultured in a medium supplemented or not with Resveratrol. Fluorescence intensity in the control group was defined as 1.0.
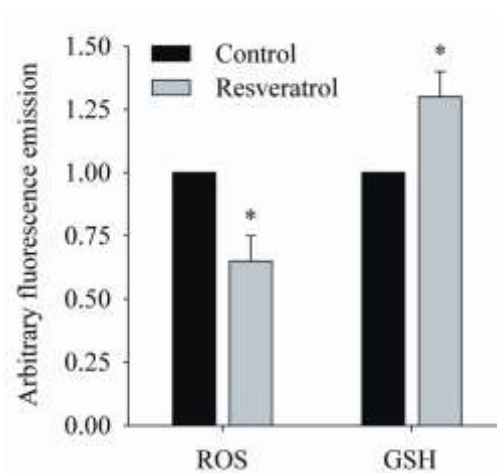
*Differ from the control within the same parameter evaluated ($P < 0.05$).
Source: Authors.

4. Discussion

Bovine IVP embryos present increased susceptibility with storage during transportation to the farm and to cryological damage and the result is reduced pregnancy rates after transfer (Hansen et al., 2010). Our main findings regarding bovine IVP embryos were that (i) the quality of fresh embryos incubated in a holding medium supplemented with resveratrol might be improved and (ii) post-warming viability can be enhanced by incubation of the embryos with resveratrol. The IVP bovine embryos are less developmentally competent and presented a poorer quality than *in vivo*-derived embryos [18]. Developmental ability and quality are associated with cryosurvival and pregnancy and can be explained by morphological, functional, cellular, and molecular competencies of the embryos [19]. *In vitro* survival of fresh IVP embryos plays an important role because pregnancy rates for cows receiving fresh embryos are higher than in those receiving cryopreserved embryos (Bó & Mapletoft, 2013). The transportation in portable incubators and maintenance of the embryos in a hypothermic medium at refrigeration temperature (Cavalieri et al., 2015; Ideta e al., 2013) have been suggested as alternatives to minimize the problem, as embryo viability and pregnancy rate may be influenced by the time spent in transportation Marinho et al., 2012). Embryonic stage at the time of packing (expanded or initial blastocyst), but not the time of transportation (6 or 9 h), was shown to influence hatching and degeneration rates Silveira et al., 2020). We proposed a short exposure period to a low concentration of resveratrol to improve developmental competence and quality of *in vitro*-derived embryos and to prevent oxidative stress during two important steps of embryo transfer – the transportation to the farm and post warming period.

Re-expansion and hatching rates

In E1, the evaluation at 10 h of incubation showed that Resv increased re-expansion by 14.4% and hatching rates by 16.0% (P< 0.05). Re-expansion rate is a good morphological indicator of embryo quality and the hatching rate is a biomarker of the quality and developmental capacity of blastocysts and has been used to determine the efficiency of embryo preservation methods (Do et al., 2018). In E2, Resv improved the re-expansion rate at 6 and 24 h of re-cultivation. The hatching rate was 18.6% higher (P< 0.01) for resveratrol than in the Control after re-cultivation. Hatching is a pre-requisite for uterine attachment and placentation of the pre-implantation embryo, and failures lead to pregnancy loss (Taniyama et al., 2011). Hatching rate may be impaired in cryopreserved embryos owing to the occurrence of zona pellucida hardening (Taniyama et al., 2011).

Cell number counting

In E1, TCN in the Control was lower than in Resv, irrespective of incubation time before the re-cultivation. The TCN of fresh D7 IVP-derived bovine embryo evaluated without any additional treatment ranged from 84.6 (Mori, Otoi & Suzuki, 2008) to 119 (Block, Bonilla & Hansen, 2010). Our results for Resv 10 h are similar to those of another study (114.8, Block, Bonilla & Hansen, 2010), representing an improvement in embryo quality, especially because these parameters were recorded after 24 h (10 h in the straw on the heating plate and 14 h of re-cultivation in the incubator). Embryo cell number indicates the viability of pre-implantation embryos (Papaioannou & Ebert, 1988). On day 14, the bovine embryos develop an embryonic disc, but approximately 25% of *in vitro* embryos fail to reach this stage (Block et al. ,2007); reduction in the apoptotic cells might improve the proportion of bovine embryos developing an embryonic disc (Block, Bonilla & Hansen, 2010). As differences in ACN were observed, results regarding cell counting for Resv embryos indicate an improvement in their quality. Vitrified IVP bovine embryos suffer additional cellular damage, presenting reduced cellular numbers. In E2, this was evident by the TCN in fresh (131.6) and vitrified embryos, disregarding the resveratrol IVC supplementation (Fig. 4, Fig. 5). The difference between TCN of the Control (88.8) and Resv (102.7) vitrified-warmed embryos was significant ($P < 0.05$), clearly owing to the reduction in apoptotic cells (ACN, 23.6 for Control and 11.5 for Resv), which probably contributed toward maintaining the inner cell mass number, increasing their viability. Apoptosis is a normal process during the pre-implantation

period to eliminate abnormal cells; the viability of vitrified embryos after warming is affected because of the cellular loss in the inner cell mass. Decreased inner cell mass and trophectodermal cell number in vitrified embryos probably cause placentary alterations, impacting pregnancy rates after *in vitro* embryo manipulation (Ufer et al.,2010).

ROS and GHS

In E1, ROS was similar between Control and Resv 6 h; however, a significant decrease (P< 0.01) was verified in the Resv 10 h embryos. Inversely, GSH intracellular content was higher in Resv 6 h ($P < 0.01$). Poor quality of embryos has been associated with ROS generation, leading to damage because of oxidative stress caused by the imbalance between pro- and antioxidants (Al-Gubory, Fowler & Garrel, 2010). ROS production increases up to the late morula stage during embryo *in vitro* culture and decreases in expanded blastocysts until values similar to those in oocytes are observed (Dalvit et al., 2005). The higher lipid content of IVP embryos induces higher ROS levels than in their *in vivo* counterparts, and culture media composition influences these factors (Romek et al., 2010). As our study was focused on the embryo response to extended incubation in the holding medium, the present results regarding ROS level and GSH content indicate advantages with the addition of resveratrol. Vitrification results in cellular and subcellular damage, which is gradually restored over a 24 h period. ROS modifies proteins and other macromolecules but are vital to signaling molecules within cells (Ufer et al., 2010). Intracellular content of ROS was 35% lower in the Resv embryos during the 24 h of the re-cultivation period than in the Control embryos (P< 0.01), confirming the protective effect of this antioxidant. Resveratrol maintains the cellular redox state, protecting the cell against oxidative injuries, and has been added to *in vitro* maturation culture media to produce bovine embryos with improved oocyte development and embryo competence and to improve cryotolerance of bovine IVP embryos (Silveira et al., 2020). ROS causes mitochondrial damage in oocyte and embryos; the mitochondria are vulnerable during early cleavage, and their maturation during *in vitro* culture may be incomplete, leading to inadequate coupling efficiency during blastocyst development. Resveratrol, as an activator of sirtuin deacetylases, which regulates proper mitochondrial function, enhances embryo quality through its action on mitochondrial maturation during embryonic development (Cagnone & Sirard, 2016). The GSH content was higher in the Resv embryos than in Control embryos ($P < 0.01$). GSH protects the cell from oxidative damage and regulates the intracellular redox balance, playing an important role in DNA repair, protein synthesis, and cell proliferation during embryo development and the level of GSH serves as a marker of embryo developmental competence. The re-cultivation of vitrified-thawed embryos during a period of 3 to 16 h could be an alternative to improve embryo recovery before the transfer to a recipient (Nedambale et al., 2006). We tested a re-cultivation period of 24 h after warming and verified a potential positive effect of this procedure using resveratrol as a practical alternative for field technicians, because embryos could be warmed and subjected to a re-culture in IVC media during transportation using portable incubators.

5. Final Considerations

Our findings suggest a time-dependent effect of a low concentration of resveratrol for both fresh and vitrified-warmed bovine embryos on the embryo quality regarding hatching rates, cell loss, and oxidative stress recovery, allowing the embryos to restore their developmental competence. In future, studies will be performed to investigate the phenotype of embryo developmental competence and quality and the pregnancy rate resulting from the use of these proposed strategies.

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