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Effect of resveratrol supplementation in conventional slow and ultra-rapid freezing media on the quality and fertility of bull sperm.

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ABSTRACT

The study investigated the impact of resveratrol (RES) on bull sperm cryopreservation employing conventional slow (CS) and ultra-rapid (UR) freezing methods on sperm quality and *in vitro* fertility. Twenty-four ejaculates from four bulls were divided into four groups based on the cryopreservation method and RES addition: CS-RES (n = 80), CS-Co (n = 80), UR-RES (n = 24), and UR-Co (n = 24). The CS freezing involved exposing sperm straws with 5% glycerol to liquid nitrogen (LN_2) vapors, while UR freezing submerged sperm drops with 100 mM sucrose directly into LN_2. Overall, sperm kinematic parameters and integrity of plasma and acrosome membranes significantly decreased ($P < 0.001$) after cryopreservation. Post-thaw values of motilities (total [TM] and progressive [PSM]), velocities (curvilinear and straight-line), beat cross frequency

(BCF), and sperm with intact plasma membrane/intact acrosome (PI-/PNA-) were higher ($P < 0.05$) with CS-RES and CS-Co treatments compared to UR-RES and UR-Co treatments. CS-RES treatment resulted in greater percentages ($P < 0.05$) of TM, PSM, PI-/PNA-, and fertility (blastocyst rate) than their control, CS-Co; while UR-RES showed higher BCF values ($P < 0.05$) than its control, UR-Co. Additionally, UR-RES treatment exhibited lower oxidative stress percentages than UR-Co ($P < 0.05$). This study presents the following conclusions: (1) the CS freezing resulted in better cryosurvival of bull sperm than UR freezing; (2) the RES supplementation to CS freezing medium improved sperm motility, membrane integrity, and fertility; and (3) despite low cryosurvival sperm and fertility, the RES addition to ultra-rapid freezing medium reduced oxidative stress.

Keywords: Resveratrol, bull sperm, conventional-slow freezing, ultra-rapid freezing

1. Introduction

Bull sperm cryopreservation and artificial insemination (AI) are crucial reproductive biotechnological procedures employed in bovine crossbreeding programs and genetic improvement. Despite its importance, the cryopreservation process carries the risk of sperm damage, compromising the functionality of a significant subpopulation of cells (Grötter et al., 2019). Various physical, chemical and biological factors, such as cold shock, oxidative stress, osmotic stress, and intracellular ice crystal formation, have been identified as determinants that affect the cryosurvival of bull sperm (Benson et al., 2012; Sharafi et al., 2022).

Conventional slow (SC) and ultra-rapid (UR) freezing methods have been utilized for cryopreserving bovine sperm, leading to varying outcomes in cryosurvival and cryoresistance (Grötter et al., 2019; Pérez-Marín et al., 2022). The UR freezing remains an important technique for sperm cryopreservation, providing a simple and cost-effective alternative to CS freezing. The UR freezing is achieved by directly submerging 30–50 µL drops of sperm samples (using disaccharides as cryoprotectant agent) into liquid nitrogen (LN_2) (Hidalgo et al., 2018). Sucrose, a disaccharide, is the most commonly used non-permeating cryoprotectant agent to safeguard sperm's physiological parameters (Isachenko et al., 2008). It is believed that this sugar induces cell dehydration by increasing the osmolality of the cryopreservation medium (Sieme et al., 2016). A previous report demonstrated that UR freezing produced higher cryoresistance rates of motility and viability of certain ungulate spermatozoa (e.g., gazelles, giraffes, and mouflon) than CS freezing (O'brien et al., 2019). However, evidence suggests that the UR freezing method may induce structural and molecular alterations in spermatozoa due to oxidative stress, similar to CS freezing, possibly attributed to the formation of extracellular ice crystals (Bóveda et al., 2020). Therefore, improvements are needed in CS or UR freezing media to enhance cryosurvival of bull sperm.

During cryopreservation, the cooling process can cause oxidative stress and cellular degeneration in bull sperm due to the excessive production of reactive oxygen species (ROS) (Desai et al., 2010) triggered by free radicals (i.e., H_2O_2, O_2^- and OH^-). The heightened generation of ROS and free radicals within cells can initiate lipid peroxidation (LPO), resulting in a deterioration of kinematic parameters like motility and membrane integrity in sperm (Lv et al., 2019). Consequently, these impacts can diminish fertilization potential by compromising lipids and proteins in the sperm membrane (Aitken and Krausz, 2001; Gao et al., 2017). Furthermore, the LPO induced by ROS during UR freezing is closely associated with mitochondrial damage (Aitken and Drevet, 2020). Therefore, in the effort to mitigate the detrimental effects of ROS, antioxidants have been shown to improve the quality of frozen-thawed bull spermatozoa (Amidi et al., 2016; Bucak et al., 2010).

Resveratrol (RES) is a stilbenoid well-known for its wide range of antioxidant properties and its effective role in cells (Cui et al., 2016). The RES can regulate the expression of antioxidant cofactors and enzymes (Li et al., 2018), eliminate free radicals (Garcez et al., 2010; Leonard et al., 2003), reduce ROS overproduction in mitochondria, and consequently, inhibit LPO (Seddiki et al., 2017).

Supplementing with RES to CS freezing media for cryopreserving spermatozoa in various species, including humans (Branco et al., 2010; Garcez et al., 2010), rams (Silva et al., 2012), goats (Al-Mutary et al., 2020; Lv et al., 2019), boars (Zhu et al., 2019), roosters (Najafi et al., 2019) and bulls (Assunção et al., 2021; Li et al., 2018), has demonstrated positive effects at different cellular and functional levels. Evidence indicates that the most suitable concentration of RES varies between species. In bulls, RES concentrations ranging 50–100 μM have been identified as more suitable for improving sperm quality after thawing (Assunção et al., 2021; Li et al., 2018). Some benefits provided by RES supplementation during cryopreservation include improvements in sperm motility, membrane integrity, and mitochondrial activity (Lv et al., 2019). Additionally, it helps prevent DNA damage, controls the ROS overproduction, and enhances the antioxidant defense system in sperm (Ahmed et al., 2020; Zhu et al., 2019). Furthermore, the addition of RES to washing and *in vitro* fertilization (IVF) media has increased the quality and percentage of bovine blastocysts (Li et al., 2018).

Therefore, we hypothesized that the addition 50 µM RES (as per Assunção et al. [2021]) to CS and UR freezing media could enhance sperm quality by effectively reducing oxidative stress. Interestingly, there have been no reports on the utilization of RES in UR freezing. This study aimed to evaluated the impact of RES on bull sperm cryopreservation employing conventional slow or ultra-rapid freezing methods, with a focus on kinematic parameters, plasma and acrosome membrane integrity, oxidative stress, and *in vitro* fertility.

2. Materials and methods

All diluents and media were prepared in the Animal Reproduction Biotechnology Research Laboratory using reagent-grade chemicals purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and Merck KGaA (Darmstadt, Germany). Resveratrol and other reagents used in this study were purchased from Sigma-Aldrich Chemicals.

2.1 Animals, semen collection and initial evaluation

All animals were handled according to procedures approved by the Honorable Board of Directors of the Faculty of Agricultural Sciences from the University of Cuenca, and this research was carried out in accordance with the chapter 7.8 of the Terrestrial Animal Health Code-2019© OIE (07/8/2019), regarding the protection of animals used in scientific experiments.

Four adult and fertile bulls (two Holstein Friesian, one Brown Swiss, and one Red Angus), from 2–5 years of age, and clinically healthy were used in this study. All animals were housed at 'Irquis' farm (Cuenca, Ecuador, 3°04'48.1" S and 79°04'31.0" W) from University of Cuenca, using the same management system. The Irquis farms had a similar management system, based on grazing. Basal bulls' diet was exclusively grass-based (90% annual-perennial ryegrass (*Lolium perenne*) and 10% white clover (*Trifolium repens*), with occasional mineral supplementation. Water, vitamins, and mineral blocks were available ad libitum

Twenty-four semen ejaculates (six per bull, collected with a 7-day interval) were collected using an artificial vagina (42–43°C). The volume of each ejaculate was measured in a graduated conical glass tube in milliliters. Briefly, after collection, the semen was diluted 1:1 at 32°C with TCG-EY medium (313.7 mM Tris, 104.7 mM citric acid, 30.3 mM glucose, 0.54 mM Streptomycin, 2.14 mM Penicillin plus 6% egg yolk [v:v]); pH: 7.16 and osmolality: 354 mOsm/kg) and transported to the Animal Reproduction Biotechnology Research Laboratory located on the same Irquis farm. The percentage of motile sperm and the quality of mass motility scale 0–5 (scored on a scale from 0 [lowest] to 5 [highest]) were initially evaluated via phase contrast microscope (Nikon Eclipse, Nikon Instruments Inc., New York, USA). Sperm concentration was determined using a photometer (SDM 1, Minitube, Germany). Those ejaculates with a volume of 4–8 ml, a sperm motility value of >85%, a score of >3 on a mass motility scale, and a sperm concentration of > 800 × 10^6 sperm/ml were used in the subsequent experimental work.

2.2 Experimental design

Fresh-extended samples from each semen ejaculate was divided into four aliquots and then four treatments were conformed according to the cryopreservation method (conventional slow [CS] and ultra-rapid [UR] freezing) and the addition of 50 µM and 0 µM (control) of RES (Assunção et al., 2021): 1) CS-RES, 2) CS-Co, 3) UR-RES, and 4) UR-Co. The CS freezing treatments were frozen by exposing the sperm samples (packed in 0.5 ml straws) to LN_2 vapors according described by Tamay et al. (2022). The UR freezing treatments were frozen plunged drop-by-drop (30-µl/drop) of sperm samples directly into LN_2 following the procedure described by Pradieé et al. (2016) and O'Brien et al. (2019).

The pre-freezing and post-thawed samples from the four aforementioned treatments were evaluated for their sperm kinematics variables and membrane integrity. Oxidative stress and fertilizing capacity were analyzed only in post-thawed samples (See below).

2.3 Conventional slow (CS) freezing

Two aliquots from fresh-extended samples were diluted to initial concentration of 100×10^6 sperm/ml with TCG-EY medium (CPA free) at room temperature (22°C). Consequently, the equal volume of the same TCG-EY medium plus 10% glycerol, and plus 100- or 0-mM RES were added to the first aliquots for conforming CS-RES and CS-Co treatments, respectively. Therefore, the final volume of these samples prior freezing reached a final concentration of 50×10^6 sperm/ml, 5% glycerol, and 50 or 0 µM RES for CS-RES and CS-Co treatments, respectively (final osmolarity of freezing medium: 1116 mOsm/kg). Sperm samples were then placed in a refrigerator at 5°C for 3 hours (holding time for equilibration). Thereafter, sperm samples were manually filled in 0.50 ml straws (L'Aigle Cedex, France) and sealed with polyvinyl-alcohol (Sigma P8136, St. Lois, MO, USA). Straws were frozen using two ramps placed inside a Styrofoam cryo-box (Tamay et al., 2022) (30 × 29 × 31 cm of length, width and height respectively), that contained 3.4 liters of LN_2 (up to 4 cm of height). Straws were placed horizontally in the first ramp at 17 cm above the LN_2 surface and exposed to statics LN_2 vapors for 4 min, then placed in a second lower ramp at 7 cm above the LN_2 for 2 min more. Finally, the straws were plunged directly into liquid nitrogen (-196°C) and stored in a liquid nitrogen tank for 30 days before thawing.

All frozen straws from CS-RES (n = 80) and CS-Co (n = 80) were thawed by placing the straws in a water bath at 37°C for 30 seconds. The contents were poured into dry 1.5 ml Eppendorf tubes and incubated for 5 min at 37°C. Sperm quality parameters were subsequently evaluated (see below).

2.4 Ultra-rapid (UR) freezing

The other two aliquots were initially diluted at 100×10^6 sperm/ml with TCG-EY medium extender at room temperature (22°C). Similar to CS freezing, the same volume of TCG-EY extender plus 200 mM sucrose, and plus 100- or 0-mM RES were added to the first aliquots for conforming UR-RES and UR-Co treatments, respectively. Thus, the final volume of these samples prior UR freezing reached a final concentration of 50×10^6 sperm/ml, 100 mM sucrose, and 50 or 0 μM RES for UR-RES and UR-Co treatments, respectively (final osmolarity of freezing medium: 473 mOsm/kg). These samples were equilibrated for 30 min at 5°C (Martín et al., 2023), and then pipetted and plunged drop-by-drop (30 µl/drop), directly into LN_2 (-196ºC) from a height of about 15 cm. The spheres formed were stored in cryogenic vials (2 ml) (Wuxi NEST Biotechnology, Jiangsu, China) into LN_2 cryo-tank for 30 days before thawing.

Spheres from UR-RES (n = 48 cryotubes, 2 cryotubes per ejaculate) and UR-Co treatments (n = 48 cryotubes, 2 cryotubes per treatment and per ejaculated) were ultra-rapid thawed using a handmade warming device (STC-3008), which has aluminum plates warmed to 65°C, and which both converge towards its center. The spheres were slid onto the stages, and the rapidly thawed contents were recovered into 10 ml glass beakers. This thawing process lasted 3 seconds. Immediately, the content was transferred to 1.5 ml Eppendorf tubes and then centrifuged at 300 g for 5 minutes at room temperature. The supernatant was removed and then 500 µl of TCG-EY medium was added to the pellets; then, samples were incubated for 5 min at 37°C before sperm quality analysis (see below).

2.5 Sperm analysis

2.5.1. Kinematic parameters

Sperm kinematic parameters were objectively assessed using a CASA system (Sperm Class Analyzer, SCA Evolution 6.4.0.99-©2018 - software, Microptic S.L., Barcelona, Spain), coupled to a phase contrast microscope (Nikon Eclipse model 50i; Nikon Instruments Inc., New York, USA; negative phase contrast [Ph1] with green filter) with the following settings: 25 frames/s, head area 5–70 μm^2, velocity limit for slow sperm 10 µm/s, velocity limit for medium sperm 25 µm/s, velocity limit for fast sperm 70 µm/s, and minimal straightness for progressive spermatozoa 70%. An aliquot of 5 µl from each sample (pre-freezing and post-thawed samples) was placed on slides warmed at

37°C and covered with a coverslip. At least three fields and at least 200 sperm tracks per field (average: 600 spermatozoa per sample evaluated) were evaluated at 10 × magnification for each sample slide. Total motility (TM, %), progressive sperm motility (PSM, %), curvilinear (VCL, μm/s) and straight line (VSL, μm/s), linearity (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz) were assessed as described by Galarza et al. (2021).

2.5.2. Status of plasma and acrosome membranes

Sperm viability and acrosomal membrane status were determined by using a double association of fluorescent probes – propidium iodide (PI, Sigma P4170) and fluorescein isothiocyanate conjugated peanut (*Arachis hypogaea*) agglutinin (PNA-FITC, Sigma L7381) as described by Soler et al. (2005) and Santiago-Moreno et al. (2014). *Propidium iodide* is a DNA-specific stain that cannot enter the intact plasma membrane and, therefore, allows identification of viable cells that exclude the dye. The PNA-FITC is used to detect the integrity of the sperm acrosome. Sperm cells with an intact acrosome will have no fluorescence, and cells with a reacted or damaged acrosome will show green fluorescence. A total of 200 sperm cells per slide were examined using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc,. New York, NY, USA) with a triple-band pass filter (40 × magnification with an excitation: 450–490 nm, and emission: 520 nm) and classified into four categories (%): 1) intact plasma membrane/intact acrosome (PI-/PNA-); 2) intact plasma membrane/damaged acrosome (PI-/PNA+); 3) damaged plasma membrane/intact acrosome (PI+/PNA-); and 4) damaged plasma membrane/damaged acrosome (PI+/PNA+). In addition, the percentage of total integrity of plasma equivalent to viability (Total IP: [PI-/PNA-] + [PI-/PNA+]), and acrosome (Total IA: [PI-/PNA-] + [PI+/PNA-]) membranes were calculated (Galarza et al., 2021).

2.5.3. Oxidative stress

The oxidative stress (OS) of frozen-thawed sperm samples from four treatments was assessed using CellROX Deep Red Reagent® fluorescent probe (2.5 mM; CAT 10422 Life Technologies) diluted in DMSO for a final concentration of 2mM (working solution) and stored at -20°C in the dark as described by Tamay et al. (2022) with few modifications. This analysis was done using an epifluorescence microscopy (model Eclipse Ci. L 100 -240V, Nikon, Tokyo, Japan) at 40 × magnification using a three-filter combination: B-2A (excitement 450-490 nm and emission 520 nm), G-2A (excitement 510-560 nm and emission 590 nm), and UV-2E/C 2A (excitement 330-380 nm and emission 420 nm). An aliquot of 100 µl (20×10^6 sperm/ml) from each sperm sample was added to 2 µñ of CellROX (2 mM) and 2 ml of Hoescht 33342 (in PBS; 1 mg/ml) and incubated at 37°C for 30 minutes. After incubation, each sample was centrifuged for 5 min at 600 g, and the supernatant was removed; the pellet was resuspended in 50 µl of TALP-sperm medium (113.94 mM NaCl, 3.08 mM KCl, 0.30 mM NaH_2PO_4 H_2O, 1 mM Na-lactate, 1.97 mM CaCl $2H_2O$, 0.50 mM MgCl $6H_2O$, 10 mM HEPES sodium, and 25 mM $NaHCO_3$, 6 mg/ml BSA, 0.11 mg/ml Na pyruvate, and 5 µg/ml gentamycin; 326 mOsm/kg, pH 7.6). To evaluated sperm cells with presence of ROS, 5 µl aloquots of each sample was placed on a slide covered with a coverslip, and 200 cells were counted and categorized into three groups: 1) sperm with no OS (non-stained midpiece), 2) sperm with moderate OS (midpiece stained pale red), and 3) sperm with intense OS (midpiece stained strong red). Additionally, the percentage of the total OS (moderate OS + intense OS) was calculated.

2.5.4. In vitro fertilization *(IVF)*

The fertilizing ability of sperm samples from four treatments was assessed by *in vitro* fertilization (IVF) assay according to Pradieé et al. (2018) with few modifications. Briefly, oocyte *in vitro* maturation was performed in 60 µl drops (15 oocytes per drop) of maturation medium (TCM-199-Earle's salt) supplemented with 10% FBS, 0.2 mM Sodium Pyruvate, 2 µg/ml Estradiol-17β, 5 µg/ml LH, 25 µg/ml FSH, 2 mM L-glutamine, 100 µM cysteamine, and 50 µg/ml gentamycin sulfate, at 38°C under an atmosphere of 6% CO_2 in air at maximum humidity. After 24 hours, the matured oocytes were washed twice in FERT medium (114 mM NaCl, 3.2 mM KCl, 0.3 mM NaH_2PO_4, 10 mM $C_3H_5NaO_3$ DL, 2 mM $CaCl_2$ $2H_2O$, 0.5 mM $MgCl_2$ $6H_2O$, 25 mM $NaHCO_3$, 6 mg/ml BSA, 0.2 mM sodium pyruvate, 20 µg/ml heparin, and 50 µg/ml gentamycin sulfate) and transferred to 60 ml drops of FERT medium (15 oocytes/drop).

For IVF, in each treatment, 4 straws were thawed (one straw per bull) and then a pool was formed. Sperm pooled samples from each treatment were maintained at 37°C for 5 min before sperm selection. Motile sperm from each treatment were selected by Percoll® density gradients centrifugation (Sigma-Aldrich, P1644) prior to IVF. For that, 200-µl sperm pooled sample of each treatment was placed on 45% Percoll top layer followed of 90% Percoll bottom layer; these gradients were prepared into 1.5 ml Eppendorf tube. The Percoll gradients were centrifuged for 15 min at 500 g and then pellet obtained was resuspended in 1 ml of TALP-sperm medium and recentrifuged at

300 g for 5 minutes. Finally, motile selected and capacitated sperm were resuspended in 60 µl of IVF medium and maintained for 5 min at 38.5°C prior to IVF. Sperm concentration was calculated using a Neubauer chamber (Marienfeld, Lauda-Königshofen, Germany) and an aliquot of this suspension was added to each fertilization 60 µl drop obtaining a final concentration of 1×10^6 sperm/ml. Gametes were co-incubated at 38.5 °C under an atmosphere of 6% CO_2 in air with maximum humidity. Eight IVF session were done and at least 200 *in vitro* matured zona-intact bovine oocytes were fertilized for each sperm treatment.

After 20 hours from IVF, the presumptive zygotes were transferred to 15 ml Falcon tubes containing 2 ml PBS at 38.5 °C, and vortexed for 3 min for removed cumulus cells. Groups of 10–15 presumptive zygotes from each treatment were places in 60-µl microdroplets of *in vitro* culture (IVC) medium (108 mM NaCl, 3 mM KCl, 25 mM $NaHCO_3$, 2.5 mM $C_6H_{10}CaO_6$ H_2O, 4 mM sodium pyruvate, BME, MEM, 0.02 mM glutamine, 100 UI/ml penicillin, 5 mg/ml BSA, 1 mM alanine, 1 mM glycine, and 3% (v:v) FBS), and cultured for 8 days in a triple gas incubator (6% CO_2, 90% nitrogen, and 5% O_2) at 38.5 °C and maximum humidity. The cleavage rate and blastocyst development rate from each post-thaw sperm freezing treatment were determined at 3 and 8 days after IVF, respectively.

2.6. Statistical analysis

Data were expressed as means ± S.E.M. Values for sperm variables that exhibited non-normal distributions, as determined by the Shapiro-Wilk test, were transformed to *arcsine* (for percentages values) or *Log10* (for numeric values) before analysis. The homogeneity of variances was checked using Levene's test. A Factorial ANOVA and Tukey post hoc multiple comparison test were employed to assess the effects of interactions among "*sperm sample type* (pre-freezing and post-thawing samples), *cryopreservation method* (CS and UR freezing), and *RES addition* (50 µM and 0 µM)" on sperm kinematic variables and status of plasma and acrosome membranes. Similarly, another factorial ANOVA and Tukey test were applied to explore the effects of interactions between the "*cryopreservation method* and *RES addition*" on oxidative stress and *in vitro* fertility, specifically focusing on post-thaw samples. Additionally, the "male factor" was included (except in *in vitro* fertility) as a covariable in all analysis due to variability among some bulls. Statistical significance was set at $P < 0.05$. All statistical analyses were conducted using Statistica software for windows v.12 (StatSoft Inc. Tulsa, OK, USA).

3. Results

3.1 kinematic variables assessment

The interaction *'sperm sample type × cryopreservation method × RES addition'* had a significant effect only on TM ($P < 0.01$). However, the interaction *'sperm sample type × RES addition'* had an effect on PSM, STR, and LIN ($P < 0.05$). In all the kinematic variables analyzed, the 'male' factor (bull) had a significant effect ($P < 0.05$) (Table 1).

Overall, sperm kinematic values significantly decreased ($P < 0.001$) after cryopreservation, irrespective of RES addition and cryopreservation method. In pre-freezing samples analysis, kinematic parameters (e.g., TM, PSM, VCL, VSL, STR, LIN, ALH, and BCF) after CS-RES and CS-Co freezing treatments were significantly greater

(P < 0.05) than after UR-RES and UR-Co freezing treatments. Supplementation with RES to the ultra-rapid freezing medium (i.e., UR-RES treatment) significantly improved TM percentage (P < 0.05) compared to its control treatment (i.e., UR-Co).

In post-thaw samples analysis, the values of TM, PSM, VCL, VSL, and BCF were higher (P < 0.05) after both CS freezing treatments than after both UR freezing treatments. Post-thaw TM and PSM values were greater (P < 0.05) after CS-RES treatment than their control treatment (i.e., CS-Co). Similarly, post-thaw BCF value was higher (P < 0.05) after UR-RES treatment than its control treatment (i.e., UR-Co) (Table 1).

3.2 Acrosome and plasma integrity analysis

The interaction *'sperm sample type* × *cryopreservation method'* had a significant effect on PI-/PNA- and PI-/PNA+ fluorescence categories (P < 0.01). However, the interaction *'sperm sample type* × *RES addition'* had an effect only on total IP variable (P < 0.05). The 'male' factor (bull) had a significant effect (P < 0.05) on the following fluorescence categories: PI-/PNA-, PI-/PNA+, total IP, and total IA (Table 2).

In both pre-freezing and post-thaw samples analysis, the percentage of sperm exhibiting an intact plasma membrane and acrosome (PI-/PNA-), as well as total intact plasma membrane (IP) and total intact acrosome (IA), were significantly higher (P < 0.05) in the CS freezing treatments compared to the UR freezing treatments. Similarly, pre-freezing and post-thaw percentages of sperm PI-/PNA-, total IP, and total IA were greater (P < 0.05) after CS-RES than after their control treatment (i.e., CS-Co). However, after UR-RES treatment produced a lower undesirable pre-freezing percentage (P < 0.05) of

PI+/PNA+ than after UR-Co treatment. In the same way, after CS-RES treatment produced a lower undesirable post-thaw percentage ($P < 0.05$) of PI+/PNA+ than after its control treatment (CS-Co) (Table 2).

3.3 Oxidative stress

The interaction of *'cryopreservation method × RES addition'* did not have a significant effect on any oxidative stress category examined. However, the 'male' factor (bull) had a significant effect in all oxidative stress categories ($P < 0.05$) (Figure 1).

After the freezing-thawing process, both CS-RES and CS-Co treatments resulted in a lower percentage of oxidative stress (total and moderated OE) than both UR-RES and UR-Co treatments ($P < 0.05$). Additionally, supplementation with RES to the ultra-rapid freezing medium efficiently reduced the percentage of oxidative stress after thawing, with a significantly lower percentage ($P < 0.05$) in UR-RES treatment compared to its control UR-Co treatment (16.9 ± 3.51% vs. 31.0 ± 5.38 %, respectively) (See Figure 1 and supplementary figure 1).

3.4 In vitro fertilization

The interaction between *cryopreservation method* × *RES addition* did not exhibit a significant effect on the cleavage and blastocyst rates. The cleavage rate was significantly higher ($P < 0.05$) after CS-RES treatment than after CS-Co, UR-RES, and CR-Co treatments (73.9 ± 3.12% vs. 59.8 ± 3.53%, 56.0 ± 4.99, and 52.7± 4.78%, respectively). Similarly, the blastocyst development rate was greater ($P < 0.05$) with CS-

RES treatment compared to CS-Co, UR-RES, and CR-Co treatments (22.5 ± 3.11% vs.10.2 ± 2.29%, 8.0 ± 3.88%, and 4.0 ± 2.8%, respectively) (See Figure 2 and supplementary figure 2).

4. Discussion

The results of this study demonstrated that both CS freezing treatments exhibited greater kinematics and membrane integrity before and after cryopreservation (*pre-freezing* and *post-thawed* samples, respectively) compared to both UR freezing treatments. Surprisingly, the addition of RES to the CS freezing medium increased the integrity of the plasma and acrosomal membranes before cryopreservation. After the freezing-thawing process, it was evident that UR freezing method caused major cryoinjuries to bull sperm than CS freezing due to the drastic reduction in motility and membrane integrity variables. Furthermore, RES supplementation to the CS freezing medium efficiently improved motility, membrane integrity, and *in vitro* fertility. On the other hand, despite the lower cryogenic response in UR freezing, the addition of RES to the ultra-rapid freezing medium reduced oxidative stress after thawing.

This superior cryosurvival results after CS freezing could be attributed to the freezing method used in this research, which included two freezing ramps within a cryogenic box that produced accelerating cooling rates (initial cooling rate 19°C/min, followed 47°C/min) (Tamay et al., 2022). In this sense, it has been previously shown that the use of freezing protocols with accelerating cooling rates yielded better cryogenic responses in sperm from bulls (Dias et al., 2018), rams (Galarza et al., 2019a; 2019b), and other species (Esteso et al., 2018). The UR freezing technique, despite being classified as an alternative method to CS freezing, has exhibited relatively low cryoresistance (i.e., low kinematics and/or mitochondrial activity) in bull sperm (Baiee et al., 2020; Hidalgo et al., 2018) and other species (O'Brien et al., 2019; Galarza et al., 2021; Caturla-Sánchez et al., 2018; Isachenko et al., 2008).

The reduction in motility and integrity of sperm membranes during UR freezing may be attributed to the cytotoxicity of the non-penetrating cryoprotective agent. This cytotoxicity likely occurs due to the extended exposure time (30 min) of sperm to high concentrations of sucrose (100 mM) before UR freezing. O'Brien et al. (2019) reported that sperm cryoresistance depends on the species. Their study showed that using 100 mM sucrose and 30 minutes of exposure prior to ultra-rapid freezing of sperm from wild ungulates (such as Iberian ibex, fallow deer, and aoudad) resulted in very low cryoresistance indices of motility and viability (10 to 20%). Conversely, Bóveda et al. (2020, 2018) utilized the same ultra-rapid freezing protocol for ungulates sperm and obtained variable results in motility and viability, ranging from 12.8% to 38.4%. Previous studies indicated that reducing sucrose exposure time improved kinematic variables in bovine (Baiee et al., 2020) and human (Isachenko et al., 2008) sperm. Our findings are consistent with the aforementioned studies.

The RES supplementation to CS freezing medium enhanced some post-thaw kinematic variables and protected the integrity of plasma and acrosome membranes of bull sperm after cryopreservation. Our results on sperm motility and integrity of membranes are consistent with those reported by Assunção et al. (2021) and Bucak et al. (2015) in bull sperm. It has been previously shown that the addition of RES to CS freezing medium protected sperm against LPO and DNA damage caused by ROS (Silva et al., 2012). It has been suggested that the antioxidant properties of RES enhance mitochondrial biogenesis and removes free radicals (Ahmed et al., 2020), and in this way, could improve the cryogenic response of sperm by mitigating excessive ROS production (Bucak et al., 2015; Lv et al., 2019). Although the exact mechanism of action of RES is not completely clarified, studies have shown that RES can reduce ROS levels in human sperm, particularly the H_2O_2- and OH- (Leonard et al., 2003) and O_2- radicals (Gambini et al., 2015). Other authors have suggested that RES enhanced the antioxidant defense system (such as catalase, superoxide dismutase, and glutathione peroxidase) by promoting the phosphorylation of AMP-activated protein kinase (AMPK) (Zhu et al., 2019;Ahmed et al., 2020; Li et al., 2018).

Although conventional slow (CS) freezing demonstrated lower levels of oxidative stress compared to ultra-rapid (UR) freezing, our study revealed that the inclusion of RES in the UR freezing medium effectively mitigated oxidative stress. Sperm cells are particularly vulnerable to oxidative stress during UR freezing due to ROS-induced lipid peroxidation, leading to mitochondrial damage (Aitken and Drevet, 2020). Excessive ROS levels can result in mitochondrial dysfunction (Koppers et al., 2008). Hence, enhancing the antioxidant capacity of sperm mitochondria might reduce ROS production. It is proposed that RES exerted its antioxidant effects to counteract the oxidative stress induced by ultra-rapid freezing. Indeed, the reduction in the level of oxidative stress, coupled with the increase in BCF value after ultra-rapid freezing-thawing, allows us to define that RES plays an antioxidant role during the ultra-rapid freezing process of bull spermatozoa. This study represents the inaugural utilization of RES in the ultra-rapid freezing of bovine sperm.

The RES supplementation to the CS freezing medium improved *in vitro* fertility of bovine sperm despite the low percentages of cleavage and blastocyst rates. It has previously been shown that the addition of 50 μM RES preserved the fertilization potential of fresh bull sperm (Tvrdá et al., 2015). Contrary to that, it was shown that the addition of 50 µM RES to the CS freezing medium did not enhance *in vitro* fertility of bull sperm based on blastocyst rate (Assunção et al., 2021). In other species, such as the rooster (Najafi et al., 2019) and the ram (Al-Mutary et al., 2020), an enhancement in fertility and embryo hatching rate was noted when the sperm media were enriched with RES. Our results demonstrated that supplementing RES to the CS freezing medium improved *in vitro* fertility based on blastocyst formation rate. Nevertheless, the addition of RES to the UR freezing medium had no such effect.

4. Conclusions

This research demonstrated, firstly, that CS freezing resulted in better cryosurvival of bull sperm compared to UR freezing. Moreover, it was demonstrated that RES supplementation in the CS freezing medium improved sperm motility, membrane integrity, and fertility. Lastly, despite its limited effect on sperm quality and fertility, the addition of RES to ultra-rapid freezing medium reduced oxidative stress. Further investigation into the in vivo impact of resveratrol on fertility is recommended.

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Declaration of competing interest

None of the authors have any conflict of interest to declare.

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Figure captions

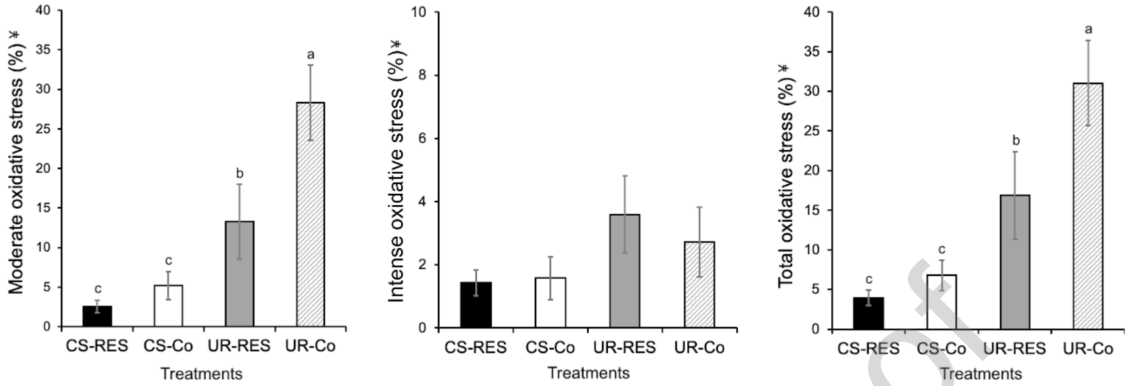

Figure 1. Post-thawed oxidative stress categories (%) from bull sperm samples previously diluted resveratrol (RES) and cryopreserved by conventional slow freezing (CS) or ultra-rapid (UR) freezing. CS-RES, conventional slow freezing with RES; CS-Co, conventional slow freezing control; UR-RES ultra-rapid freezing with RES; and UR-Co ultra-rapid freezing control. $^{¥}$ Differences between bulls (males) ($P < 0.05$). $^{a–c}$ Different superscripts within a same row differ significantly between treatments ($^{a–b–c}$ $P < 0.05$ and $^{a–c}$ $P < 0.01$).

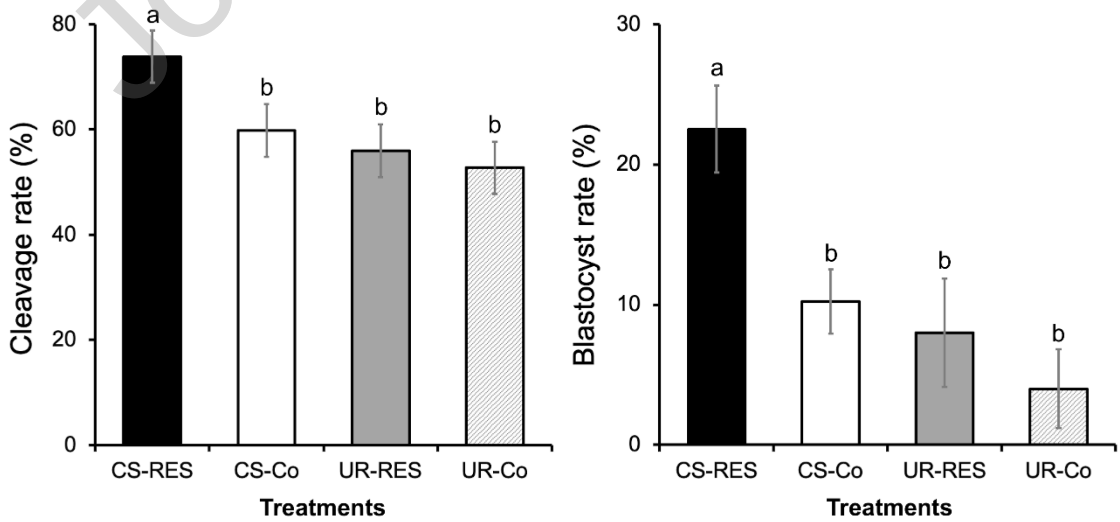

Figure 2. Post-thaw cleavage and blastocyst rates from bull sperm samples previously diluted resveratrol (RES) and cryopreserved by conventional slow freezing (CS) or ultra-rapid (UR) freezing. CS-RES, conventional slow freezing with RES; CS-Co, conventional slow freezing control; UR-RES ultra-rapid freezing with RES; and UR-Co ultra-rapid freezing control. [¥] Differences between bulls (males) ($P < 0.05$). [a–b] Different superscripts within a same row differ significantly between treatments ($P < 0.05$).

Supplementary figure 1. Assessment of oxidative stress of cryopreserved bull sperm stained with the CellROX deep red reagent® and Hoechst 33342 and observed under epifluorescence microscopy (40 × magnification). (A) Notice the spermatozoa without oxidative stress (green circles) and spermatozoa with oxidative stress based on the red staining of the midpiece: moderate (sperm with one yellow asterisk) and intense (sperm with two yellow asterisks). (B) Sperm observed at 60 × magnification

Supplementary figure 2. Zona-intact bovine oocytes in vitro matured (day 0, IVF), cleavage stage (day 3 post-IVF), and blastocyst development (day 8 post-IVF) of bull sperm samples previously diluted resveratrol (RES) and cryopreserved by conventional slow freezing (CS) or ultra-rapid (UR) freezing. CS-RES, conventional slow freezing with RES; CS-Co, conventional slow freezing control; UR-RES ultra-rapid freezing with RES; and UR-Co ultra-rapid freezing control

Table 1. Values of pre-freezing and post-thawed sperm kinematic (mean ± SEM) for bull sperm diluted with resveratrol (RES) and cryopreserved by conventional slow (CS) or ultra-rapid (UR) freezing.

Kinematic variable	Pre-freezing samples				Post-thawed samples			
	Conventional slow treatments		Ultra-rapid treatments		Conventional slow treatments		Ultra-rapid treatments	
	CS-RES (n = 24)	CS-Co (n = 24)	UR-RES (n = 24)	UR-Co (n = 24)	CS-RES (n = 80)	CS-Co (n = 80)	UR-RES (n = 80)	UR-Co (n = 80)
TM (%) $^{A\times B\times C,\ ¥}$	89.3 ± 1.84^{a}	90.3 ± 1.84^{a}	72.3 ± 4.33^{b}	54.3 ± 4.61^{cd}	59.6 ± 2.21^{c}	49.3 ± 1.97^{d}	8.0 ± 0.40^{e}	7.5 ± 0.54^{e}
PSM (%) $^{A\times C,\ ¥}$	57.1 ± 2.44^{a}	52.8 ± 2.77^{a}	23.8 ± 4.27^{cd}	17.1 ± 4.16^{d}	35.1 ± 1.99^{b}	28.0 ± 1.73^{c}	2.0 ± 0.15^{e}	1.8 ± 0.18^{e}
VCL (μm/s) $^{¥}$	91.7 ± 3.01^{a}	86.3 ± 2.80^{a}	60.4 ± 4.29^{bc}	54.5 ± 3.20^{cd}	69.1 ± 1.69^{b}	63.5 ± 1.64^{bc}	54.3 ± 2.37^{d}	49.3 ± 2.15^{d}
VSL (μm/s) $^{¥}$	40.2 ± 1.98^{a}	37.8 ± 2.20^{ab}	21.6 ± 1.75^{d}	18.1 ± 1.24^{d}	31.3 ± 1.10^{bc}	28.1 ± 1.04^{c}	22.3 ± 1.25^{d}	19.3 ± 1,31^{d}

STR (%) [A×C, ¥]	70.6 ± 1.29[a]	69.5 ± 1.21[a]	62.2 ± 1.64[bc]	60.1 ± 1.12[c]	70.4 ± 0.70[a]	69.4 ± 0.71[a]	67.2 ± 1.09[ab]	64.0 ± 0.93[bc]
LIN (%) [A×C, ¥]	44.4 ± 1.94[a]	43.5 ± 1.74[a]	35.9 ± 1.46[bc]	33.6 ± 0.89[c]	42.4 ± 0.87[a]	41.5 ± 0.83[ab]	38.5 ± 1.10[bc]	36.7 ± 1.01[c]
WOB (%) [¥]	60.3 ± 1.63[a]	60.0 ± 1.51[a]	55.5 ± 1.10[ab]	54.2 ± 0.88[b]	57.5 ± 0.70[ab]	57.1 ± 0.65[ab]	58.7 ± 0.83[b]	54.9 ± 0.96[b]
ALH (μm) [¥]	3.7 ± 0.15[a]	3.5 ± 0.12[a]	2.9 ± 0.18[bc]	2.7 ± 0.12[cd]	2.8 ± 0.06[b]	2.7 ± 0.06[bc]	2.5 ± 0.08[cd]	2.4 ± 0.07[d]
BCF (Hz) [¥]	10.3 ± 0.26[a]	9.3 ± 0.34[ab]	6.3 ± 0.36[c]	5.7 ± 0.25[cd]	8.9 ± 0.23[ab]	8.3 ± 0.22[b]	6.2 ± 0.30[c]	5.0 ± 0.21[d]

CS-RES, conventional slow freezing with RES; CS-Co, conventional slow freezing control; ultra-rapid freezing with RES; and ultra-rapid freezing control. TM, total motility; PSM, progressive sperm motility; VCL, curvilinear velocity; VSL, straight line velocity; STR, straightness; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head displacement; and BCF, beat cross frequency. [A×B×C] Significant interaction between *sperm sample* × *cryopreservation method* × *RES addition* (P < 0,01). [A×B] Significant interaction between *sperm sample* × *cryopreservation method* (P < 0,05). [A×C] Significant interaction between *sperm sample* × *RES addition* (P < 0,05), [¥] Differences between bulls (males) (P < 0.05). [a–e] Different superscripts within a same row differ significantly between treatments ([a–b–c–d–e] P < 0.05 and [a–c, a–d, a–e, b–d, b–e, c–e] P < 0.01)

Table 2. Percentages (mean ± SEM) of status of plasma and acrosome membrane of pre-freezing and post-thawed sperm for bull sperm diluted with resveratrol (RES) and cryopreserved by conventional slow (CS) or ultra-rapid (UR) freezing.

Categories of fluorescence	Pre-freezing samples				Post-thawed samples			
	Conventional slow treatments		Ultra-rapid treatments		Conventional slow treatments		Ultra-rapid treatments	
	CS-RES (n = 24)	CS-Co (n = 24)	UR-RES (n = 24)	CS-RES (n = 24)	CS-RES (n = 80)	CS-Co (n = 80)	UR-RES (n = 80)	UR-Co (n = 80)
PI-/PNA- (%)$^{A\times B, ¥}$	80.1 ± 0.92^{a}	76.1 ± 1.12^{b}	68.0 ± 1.25^{c}	66.7 ± 1.45^{c}	53.2 ± 0.86^{d}	48.0 ± 0.89^{e}	15.2 ± 0.45^{f}	12.9 ± 0.40^{f}
PI-/PNA+ (%)$^{A\times B, ¥}$	1.1 ± 0.22^{ab}	1.0 ± 0.22^{ab}	1.7 ± 0.41^{a}	1.2 ± 0.24^{a}	0.9 ± 0.07^{b}	0.8 ± 0.10^{b}	1.0 ± 0.11^{b}	0.6 ± 0.08^{b}
PI+/PNA- (%)	8.1 ± 0.75^{cd}	10.0 ± 0.94^{d}	14.4 ± 1.51^{b}	13.9 ± 1.30^{b}	12.1 ± 0.47^{c}	12.3 ± 0.39^{c}	17.9 ± 0.60^{a}	17.8 ± 0.63^{a}

PI+/PNA+ (%)	10.8 ±0.95^{e}	12.9 ± 1.09^{e}	15.9 ± 1.43^{e}	18.2 ± 1.45^{d}	35.1 ± 0.88^{c}	38.9 ± 0.92^{b}	66.8 ± 0.85^{a}	68.5 ± 0.71^{a}
Total IP (%)$^{A\times C, ¥}$	81.1 ± 0.87^{a}	77.1 ± 1.09^{b}	69.7 ± 1.16^{c}	67.9 ± 1.41^{c}	54.1 ± 0.87^{d}	48.8 ± 0.91^{e}	16.2 ± 0.46^{f}	13.5 ± 0.42^{f}
Total IA (%)$^{¥}$	88.1 ± 0.99^{a}	86.1 ± 1.09^{b}	82.4 ± 1.31^{c}	80.6 ± 1.48^{c}	65.3 ± 0.83^{d}	60.3 ± 0.91^{e}	33.1 ± 0.77^{f}	30.7 ± 0.71^{f}

Treatments: CS-RES, conventional slow freezing with RES; CS-Co, conventional slow freezing control; ultra-rapid freezing with RES; and ultra-rapid freezing control. PI-/PNA-, intact plasma membrane/intact acrosome; PI-/PNA+, intact plasma membrane/damaged acrosome; PI+/PNA-, damaged plasma membrane/intact acrosome; and PI+/PNA+, damaged plasma membrane/damaged acrosome. $^{A\times B}$ Significant interaction between *sperm sample type* × *cryopreservation method* (P < 0,05). $^{A\times C}$ Significant interaction between *sperm sample type* × *RES addition* (P < 0,05). $^{¥}$ Differences between bulls (males) (P < 0.05). $^{a–e}$ Different superscripts within a same row differ significantly between treatments ($^{a–b–c–d–e–f}$ P < 0.05 and $^{a–c, a–d, a–e, a–f, b–d, b–e, c–e, c–f}$ P < 0.01).

Declaration of competing interest

None of the authors have any conflict of interest to declare.

Highlights

- Conventional slow freezing showed higher sperm cryosurvival than ultra-rapid freezing
- Resveratrol improved sperm motility and membrane integrity after slow freezing
- Resveratrol addition to slow freezing medium improved *in vitro* fertility
- Resveratrol reduced oxidative stress after ultra-rapid freezing