

# Effects of resveratrol in bull semen extender on post-thaw sperm quality and capacity for fertilization and embryo development

Carolina Marinho Assunção<sup>a</sup>, Vivian Rachel Araujo Mendes<sup>b</sup>,  
Felipe Zandonadi Brandão<sup>c</sup>, Ribrio Ivan Tavares Pereira Batista<sup>c</sup>,  
Eliza Diniz Souza<sup>d</sup>, Bruno Campos de Carvalho<sup>d</sup>, Carolina Capobiango  
Romano Quintão<sup>d</sup>, Nadia Rezende Barbosa Raposo<sup>a</sup>, Luiz Sergio  
Almeida Camargo<sup>d,\*</sup>

<sup>a</sup> Federal University of Juiz de Fora, Rua José Lourenço Kelmer, s/n, Campus Universitário, CEP 36036-900, Juiz de Fora, MG, Brazil

<sup>b</sup> Federal University of Viçosa, Av. Peter Henry Rolfs, s/n - Campus Universitário, CEP 36570-900, Viçosa, MG, Brazil

<sup>c</sup> Fluminense Federal University, Rua Vital Brazil, 64, CEP 24320-340, RJ, Brazil

<sup>d</sup> Brazilian Agricultural Research Corporation - Embrapa Dairy Cattle, Rua Eugênio do Nascimento, 610, CEP 36038-330, Juiz de Fora, MG, Brazil

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## ABSTRACT

Resveratrol, a potent antioxidant, can be an alternative semen extender constituent to protect spermatozoa against reactive oxygen species (ROS); however, effects on sperm quality post-thawing and sperm function is not well understood. This study, therefore, was conducted to investigate effects of resveratrol supplementation to semen extender on sperm quality post-thawing. Bull semen was cryopreserved using extenders not supplemented or supplemented with 0.05, 0.1, or 1 mM resveratrol. Supplementation of extender with resveratrol at 0.05 mM resulted in greater ( $P < 0.05$ ) sperm progressive motility, average path velocity, straight linear velocity, linearity and straightness when compared with no or 1 mM supplementations. Furthermore, effects of 0.05 mM resveratrol supplementations on plasma membrane and acrosome integrity and sperm fertilization capacity using *in vitro* procedures were investigated. Supplementation of semen extender with resveratrol resulted in a greater ( $P < 0.05$ ) proportion of frozen-thawed spermatozoa with an intact acrosome and plasma membrane. Results from *in vitro* fertilization studies indicated there were no differences ( $P > 0.05$ ) when there was no supplementation or supplementation with 0.05 mM resveratrol on embryo development to the cleavage and blastocyst stages. In conclusion, addition of resveratrol to bull semen extender resulted in greater sperm quality post-thawing in a dose-dependent manner, with values for variables related to sperm quality being greater when there was resveratrol supplementation at the 0.05 mM concentration. Proportion of embryo developing to the cleavage and blastocyst stages after *in vitro* fertilization was not affected by resveratrol supplementation to semen extenders.

## 1. Introduction

Semen cryopreservation is an important factor for worldwide use of artificial insemination (AI) and consequently, cattle

\* Corresponding author at: Embrapa Dairy – Rua Eugênio do Nascimento, 610 - Dom Bosco, CEP: 36038-330, Juiz de Fora, MG, Brazil.  
E-mail address: [luiz.camargo@embrapa.br](mailto:luiz.camargo@embrapa.br) (L.S.A. Camargo).

production. Frozen-thawed semen of genetically elite bulls has been used to inseminate large numbers of cows, contributing to the greater global genetic improvement in cattle of most breeds. Furthermore, cryopreservation allowed for use of cattle genetic resources by making semen of genetically elite bulls available for farmers worldwide (Moore and Hasler, 2017).

Even though there is worldwide use of cryopreserved bull semen for AI, sperm cooling, freezing and thawing processes induce damage to bull spermatozoa and can compromise the viability of approximately half of sperm population, impairing spermatozoa quality and functions (Grötter et al., 2019; Hezavehei et al., 2018; Ugur et al., 2019; Watson, 1995). Among the damage that occurs is that resulting from lipid peroxidation and disruption of DNA integrity, both induced by a greater than optimal reactive oxygen species (ROS) release during cryopreservation (Chatterjee and Gagnon, 2001; Hezavehei et al., 2018; Ugur et al., 2019). Several extenders and cryoprotective compounds have been used to improve post-thaw sperm survival. Extenders based on egg yolk or milk and glycerol as cryoprotectants are commonly used for bull semen cryopreservation (Chen et al., 1993; Sieme et al., 2016), although animal protein-free extenders are also an alternative (Anzar et al., 2019).

Supplementation of extenders with antioxidants has been investigated in an attempt to protect the sperm against the oxidative stress and to improve sperm quality and functions post-thawing. Some antioxidants, such as amino acids and vitamins, can have positive effects on some values for sperm kinetic variables and plasma membrane and acrosome integrity post-thawing (Eidan, 2016; Sariözkan et al., 2009). There, however, is not always a positive effect of antioxidant supplements on sperm functions or fertility post-thawing.

Resveratrol (trans-3,5,4'-trihydroxy-trans-stilbene), a polyphenol found in several types of fruits, has potent antioxidant properties due to the radical scavenger activity of this compound, resulting in a reduction in ROS generation and lipid peroxidation (Fang et al., 2002; Leonard et al., 2003; Olas and Wachowicz, 2004; Rotondo et al., 1998; Stojanović et al., 2001). Supplementation of semen extender with resveratrol (10 mM) resulted in a reduction in DNA damage caused by the cryopreservation of sperm obtained from fertile and infertile men (Branco et al., 2010) and concentrations from 0.1–10 mM reduced lipid peroxidation post-thawing but there were negative effects on sperm motility of both fertile and infertile men (Garcez et al., 2010). Addition of 20 µg/mL resveratrol to cryopreservation media resulted in a reduction of mitochondria membrane activity, but there was no effect on motility and vigor of ram sperm after thawing of the semen (Silva et al., 2012). In contrast, supplementations of semen extender with 0.01 and 0.05 mM resveratrol resulted in a greater total and progressive motility, membrane and acrosome integrity and mitochondrial activity of goat sperm than supplementations with 0.1 and 0.25 mM resveratrol (Lv et al., 2019). Supplementations of bull semen extender with 1 mM resveratrol led to an increase in mitochondrial activity and motility subsequent to thawing of the semen, when there were evaluations using the computer-assisted sperm analysis (CASA), with there being no effects on values for other CASA variables (Bucak et al., 2015). It, however, is not known whether the effects of resveratrol on values for bull sperm variables are dose-dependent and whether resveratrol supplementations to semen extender can affect sperm function to the extent that it would be utilized in commercial bull semen extenders. With consideration of all of these previous findings, the aim of the present study was to determine the optimal resveratrol concentration for inclusion in bull semen extender that would result in spermatozoa having a greater motility and less plasma and acrosomal membrane damage as well as a retained capacity, after thawing, for *in vitro* fertilization leading to subsequent embryonic development.

## 2. Material and methods

All chemicals were from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless stated otherwise. Resveratrol was kindly provided by Nucleus of Identification and Analytical Quantification (NIQUA), Universidade Federal de Juiz de Fora, Brazil. Experimental procedures were consistent with the ethical guidelines for animal experimentation and were approved by local Committee (CEUA EGL 04/2015 protocol).

### 2.1. Experimental design

Semen from Holstein bulls was frozen using extenders containing no (0), or 0.05, 0.1, or 1 mM resveratrol dissolved in dimethyl sulfoxide (DMSO; final concentration of 0.01 % in semen extender). The DMSO (0.01 %) was also included in the control group (0 mM resveratrol). To ensure there were no effects of solvent on bull spermatozoa, the effect of 0.01 % DMSO in semen extender on sperm kinetic values was analyzed previously and there were no effects on frozen-thawed spermatozoa (Supplementary Tables S1 and S2). The first experiment was conducted to evaluate the effect of different concentrations of resveratrol in the extender on values for kinetic variables analyzed using CASA to identify a resveratrol concentration with positive effects on frozen-thawed semen. Semen contents post-thawing in straws from four ejaculates collected from eight bulls were evaluated in the first experiment, totaling 32 ejaculates being evaluated. The second experiment was conducted to evaluate the effect of inclusion of no (0) or 0.05 mM resveratrol to the semen extender on sperm acrosome and plasma membrane integrity and on sperm fertilization competence for inducing development of embryos after *in vitro* fertilization. Sperm acrosome and plasma membrane integrity assessment was performed by evaluating semen from 24 ejaculates after thawing that was collected from eight bulls, and *in vitro* fertilization was performed with sperm following thawing of semen from 16 ejaculates collected from four bulls.

### 2.2. Semen collection and processing

Eight Holstein bulls aged between 22 and 30 months were housed in individual sheltered pens and fed corn silage and balanced ration from a nutrient content perspective. Semen was collected in a pre-warmed disposable tube every 7–15 days using a rectal probe

for conducting the electroejaculation method. Sperm motility and vigor of fresh semen from at least four ejaculates of every bull were examined using optical microscopy procedures before initiating the experiments to ensure an average of sperm motility greater than 70 %.

Ejaculates collected for the experiments were diluted to  $160 \times 10^6$  sperm/mL with tris-egg yolk-based extender (Chen et al., 1993) and with there being 6% glycerol added that contained no resveratrol or that was supplemented with 0.05, 0.1 or 1 mM resveratrol. Extended semen was transferred in 0.25 mL straws ( $40 \times 10^6$  motile sperm/straws), sealed with polyvinyl alcohol powder and equilibrated at 4 °C for 4 h. Semen straws were placed horizontally in a nitrogen box at a distance of 5 cm above the surface of the liquid nitrogen for 15 min and then plunged into liquid nitrogen and stored for at least 2 weeks before being used in the experiments. Frozen semen in the straws from each ejaculate was thawed at 37 °C in water bath for 30 s and then processed using procedures consistent with those imposed for conducting of the experiments in the present study.

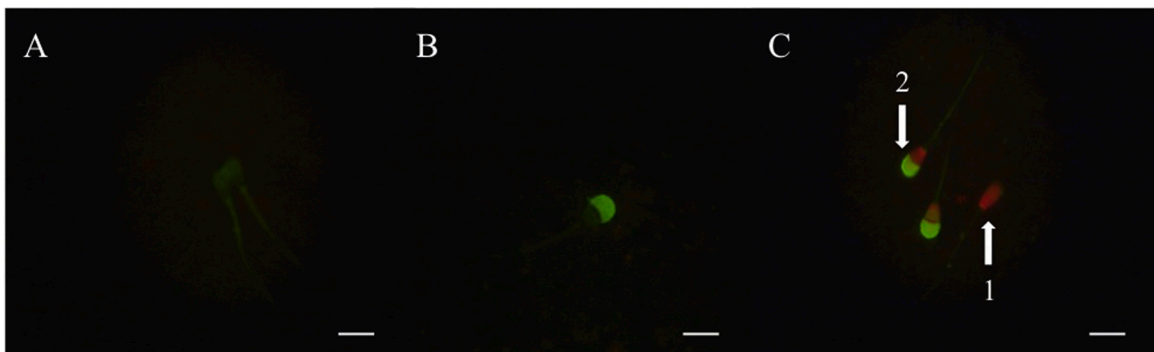
### 2.3. Computer-assisted sperm analysis assessment

Values for kinetic sperm variables of semen evaluated subsequent to thawing were determined using the Sperm Class Analyzer (SCA) CASA system (version 3.2.0, Microptic S.L., Barcelona, Spain), coupled to a phase-contrast and epifluorescence microscope (Nikon Instruments Inc.) equipped with a Basler Ace digital camera. Sperm aliquots of 10 µL were assessed on a pre-warmed (37 °C) counting chamber with a cover slip being applied before conducting the evaluations (Neubauer counting cell chamber). For each evaluation, five microscopic view fields including at least 200 cells were analyzed using the default configuration to bull sperm, with the dimensions of the sperm head detectable between 5 and 70 µm<sup>2</sup>. Spermatozoa were classified as being immobile, slow, medium or fast when there was a less than 10, between 10 and 25, between 25 and 50, or greater than 50 µm/s curvilinear velocity, respectively. Spermatozoa with a rectilinearity greater than 70 % were considered progressive.

The variables evaluated were: progressive motility (%), total motility (%), rapid motility (%), average path velocity (VAP, µm/s), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), linearity (LIN: VSL/VCL x 100, %), beat-cross frequency (BCF, Hz) and straightness (STR: VSL/VAP x 100, %). Assessments were performed in triplicate.

### 2.4. Acrosome and plasma membrane integrity assessment

Plasma membrane and acrosome integrity were assessed using a combination of propidium iodide (PI) with fluorescein isothiocyanate-conjugated *Pisum sativum agglutinin* (FITC-PSA) for staining. Briefly, for plasma membrane and acrosome integrity assessment, spermatozoa previously diluted in 150 µL HEPES-buffered TALP medium (125 mM NaCl, 3.1 mM KCl, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, H<sub>2</sub>O, 10 mM sodium lactate, 2 mM NaHCO<sub>3</sub>, 2 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.5 mM MgCl<sub>2</sub>·6H<sub>2</sub>O and 10 mM HEPES) were stained with 3 µL of PI (2 mg/mL) and 50 µL of FITC-PSA (100 µg/mL) for 8 min at 38.5 °C in a darkened area of the laboratory. Samples with stained spermatozoa were placed on separate slides with coverslips applied and evaluated immediately by epifluorescence microscopy (Zeiss, Jena, Germany) when there was a 400x and 1000x magnification using a filter set for excitation at 400–570 nm and emission at 460–625 nm. Plasma membrane and acrosome status were determined by evaluating the fluorescence emitted by each probe (PI and FITC-PSA): (1) PI-negative and FITC-PSA negative, intact plasma membrane and acrosome (IPMe/IAC Fig. 1A); (2) PI-negative and FITC-PSA-positive: intact plasma membrane/damaged acrosome (IPMe/DAC; Fig. 1B); (3) PI-positive and FITC-PSA-negative: damaged plasma membrane/intact acrosome (DPMe/IAC; Fig. 1C1); and (4) PI-positive and FITC-PSA-positive: damaged plasma membrane and acrosome (DPMe/Dac; Fig. 1C2). The same observer for each ejaculate examined at least 100 sperm cells per slide on duplicate slides.



**Fig. 1.** Representative images of bull spermatozoa stained with fluorescent probes: propidium iodide (PI) and fluorescein isothiocyanate-conjugated *Pisum sativum agglutinin* (FITC-PSA); (A) PI-negative and FITC-PSA negative, spermatozoa with intact plasma membrane and acrosome (IPMe/IAC); (B) PI-negative and FITC-PSA-positive: spermatozoa with intact plasma membrane/damaged acrosome (IPMe/DAC); (C1) PI-positive and FITC-PSA-negative: spermatozoa with damaged plasma membrane/intact acrosome (DPMe/IAC); (C2) PI-positive and FITC-PSA-positive: spermatozoa with damaged plasma membrane and acrosome (DPMe/Dac); Magnification: 1000 x; Scale bars: 10 µm.

## 2.5. *In vitro* fertilization and embryo development assessment

Sperm fertilization capacity in inducing embryo development was assessed by evaluating embryo development to the cleavage and blastocyst stages after *in vitro* fertilization (IVF). Cumulus cells-oocyte complexes (COCs) were obtained from ovaries collected at commercial slaughterhouse and were *in vitro* matured for 24 h in TCM 199 (Gibco Life Technologies Inc., Grand Island, NY, USA) supplemented with 10 % estrous cow serum and 20 µg/mL FSH (Pluset, Calier, Barcelona, Spain) in a humidified atmosphere of 5% CO<sub>2</sub> and 38.5 °C in air. Frozen-thawed sperm from four bulls were centrifuged at 3600 g for 7 min in Percoll discontinuous density gradient (45 %–90 %) and the pellet was re-suspended in Fert-TALP medium (fertilization medium; Gordon, 2003) and centrifuged again at 520 g for 5 min. *In vitro*-matured COCs were co-incubated for 20–21 h with  $2 \times 10^6$  spermatozoa/mL in 100 µL drops of Fert-TALP medium supplemented with 20 µg/mL of heparin and 6 mg/mL of fatty acid free bovine serum albumin (BSA), in a humidified atmosphere of 5% CO<sub>2</sub> and 38.5 °C in air. Presumptive zygotes were completely denuded and cultured in a modified CR2aa medium with 2.5 % fetal bovine serum (FBS) when there was an atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90 % N<sub>2</sub> at 38.5 °C. Cleavage rate was evaluated 72 h post-fertilization and blastocyst rate was evaluated at day seven (D7) and day eight (D8). Analyses were performed with four replicates per bull, totaling 16 replicates per treatment.

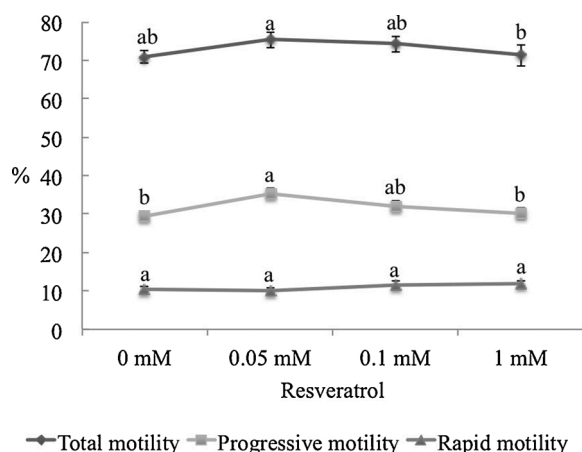
## 2.6. Statistical analysis

The distribution of variables was examined using the Shapiro-Wilk test and homogeneity was evaluated using the Levene's test. When data were not normally distributed, there was log transformations performed. Values for motility, vigor and CASA variables were analyzed using the mixed model repeated measures analysis (Mixed procedure of SAS, version 9.0, SAS Institute Inc, Cary, NC). Least square means comparisons were provided using the PDIF option of SAS. Resveratrol was treated as a fixed-effect factor and bull as a random effect. The effect of resveratrol on the proportion of sperm with intact or damaged plasma membrane and acrosome and on embryonic development was analyzed using binary logistic regression procedures (Logistic procedure of SAS). The 95 % confidence interval (CI) was used to estimate the precision of odds ratio (OR). Values are presented as means  $\pm$  standard error of means (SEM) and there were considered to be mean differences at the 95 % confidence level when there was a  $P < 0.05$ .

## 3. Results

### 3.1. Sperm variable assessments using CASA

There was an effect of resveratrol concentration in semen extender on several CASA variables subsequent to thawing of the semen. Sperm progressive motility was greater ( $P < 0.01$ ) when there was supplementation with 0.05 mM than when there was no supplementation (0) or supplementation with 1 mM resveratrol whereas there was no difference in sperm progressive motility when there was supplementation with 0.1 mM as compared with when there was no supplementation or supplementations with resveratrol at the other concentrations ( $P > 0.05$ ; Fig. 2). There was no difference in sperm rapid motility when there was no supplementation or supplementations with resveratrol at the various concentrations, however, sperm total motility was less ( $P < 0.05$ ) when there was the 1 mM resveratrol supplementation compared with supplementation at 0.05 mM (Fig. 2). There was a greater ( $P < 0.05$ ) sperm VSL and VAP when there was a 0.05 mM resveratrol supplementation than with other resveratrol concentrations, whereas the VCL value was less ( $P < 0.01$ ) when there was supplementation with 1 mM resveratrol (Fig. 3A). There were the largest ( $P < 0.05$ ) percentages for sperm LIN and STR when there was supplementation with 0.05 mM resveratrol whereas there was a greater mean BCF when there was



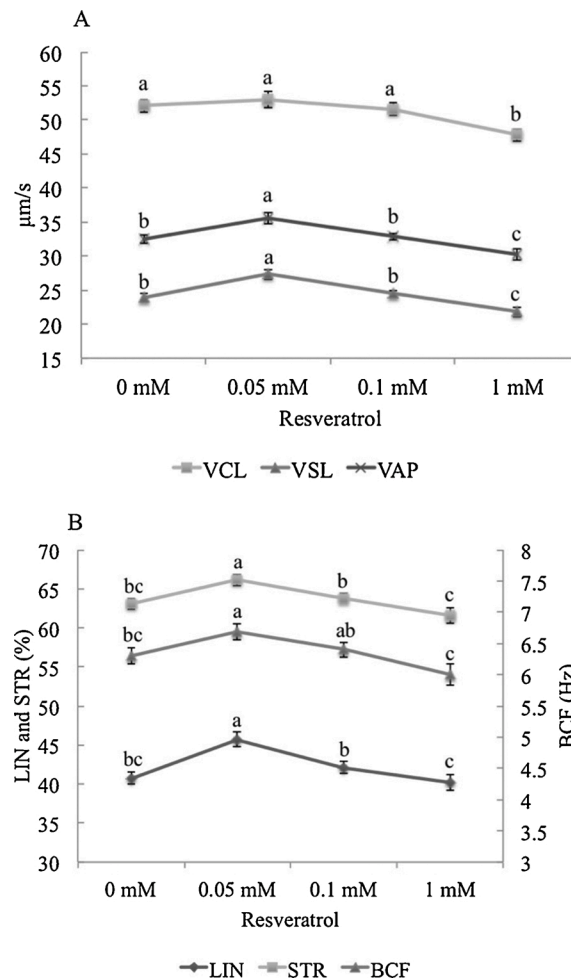
**Fig. 2.** Effect of resveratrol concentration in bull semen extender on motility of frozen-thawed sperm assessed evaluated using computer-assisted sperm analysis (CASA); a, b. Letters indicate difference among concentrations ( $P < 0.01$ ); Sample size: 32 ejaculates per treatment; Values are provided as means  $\pm$  SEM.

supplementation with 0.05 mM than when there was no (0) or 1 mM resveratrol supplementation (Fig. 3B). The sperm ALH was less ( $P < 0.05$ ) when there was supplementation with 1 mM resveratrol ( $2.2 \pm 0.04 \mu\text{m}$ ) than when there was no supplementation with resveratrol ( $2.36 \pm 0.03 \mu\text{m}$ ), with there being no difference ( $P > 0.05$ ) in ALH when there were supplementations with other concentrations of resveratrol ( $2.25 \pm 0.04$  and  $2.31 \pm 0.03 \mu\text{m}$  for 0.05 and 0.1 mM, respectively).

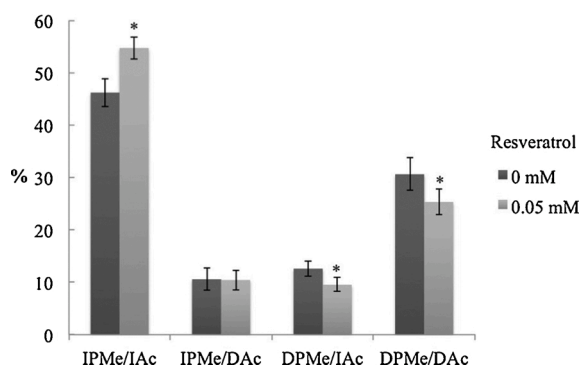
### 3.2. Plasma membrane and acrosome integrity and *in vitro* fertilization assessment

The results from the CASA assessment indicated that supplementation of semen extender with 0.05 mM resveratrol resulted in greater values for sperm kinetic variables. There was, therefore, an experiment conducted to evaluate the effect of supplementation of 0.05 mM resveratrol to semen extender on sperm plasma membrane and acrosome integrity and on the capacity of sperm to induce embryo development when there was *in vitro* fertilization. Supplementation of bull semen extender with resveratrol resulted in a greater ( $P < 0.01$ ) likelihood of preserving acrosome and sperm plasma membrane integrity after thawing (OR: 1.37; CI: 1.23, 1.53), resulting in a greater proportion of spermatozoa with IPMe/IAC subsequent to thawing than occurred when semen was not supplemented with resveratrol (i.e., control; Fig. 4). Consistent with these findings, supplementation with resveratrol resulted in a lesser ( $P < 0.01$ ) likelihood of damage to the acrosome and plasma membrane (OR: 0.76; CI: 0.67, 0.85), resulting in a lesser proportion of frozen-thawed spermatozoa with DPMe/DAC than in semen samples not supplemented with resveratrol (control). Supplementation with resveratrol also resulted in a lesser ( $P < 0.01$ ; OR: 0.73; CI: 0.62, 0.87) likelihood of the proportion of frozen-thawed spermatozoa with DPMe/IAC, and there was no effect ( $P > 0.05$ ) of resveratrol supplementation to bull semen extender on the proportion of spermatozoa with IPMe/DAC (Fig. 4).

There was evaluation of the effects of supplementation of semen extender with 0.05 mM resveratrol on the capacity of sperm to induce embryo cleavage and further embryo development after *in vitro* fertilization. There was no effect ( $P > 0.05$ ) of resveratrol



**Fig. 3.** Effect of resveratrol concentration in bull semen extender on values for CASA variables for frozen-thawed sperm; (A) curvilinear velocity (VCL), straight linear velocity (VSL) and average path velocity (VAP); (B) linearity (LIN), straightness (STR) and beat-cross frequency (BCF); a, b, c. Letters indicate difference among concentrations ( $P < 0.05$ ); Sample size: 32 ejaculates per treatment; Values are provided as means  $\pm$  SEM.



**Fig. 4.** Effect of resveratrol in bull semen extender on acrosome and plasma membrane integrity of frozen-thawed sperm; (\*) Asterisk indicates difference between treatments ( $P < 0.05$ ); IPMe/IAC: intact plasma membrane and acrosome, IPMe/DAC: intact plasma membrane/damaged acrosome, DPMe/IAC: damaged plasma membrane/intact acrosome, DPMe/DAC: damaged plasma membrane/damaged acrosome; Sample size: 24 ejaculates per treatment; Values are provided as means  $\pm$  SEM.

supplementation to semen extender on the capacity of sperm cryopreserved in this extender to induce cleavage of embryos and development to the blastocyst stage (Table 1).

#### 4. Discussion

Resveratrol has been investigated as an alternative to protect sperm from damage caused by cryopreservation processes in several species including humans (Branco et al., 2010; Garcez et al., 2010), sheep (Silva et al., 2012), goats (Lv et al., 2019), pigs (Zhu et al., 2019) and cattle (Bucak et al., 2015). In the present study, there was an effect of resveratrol on values for bull spermatozoa kinetic variables that was dose-dependent with there being positive effects with 0.05 mM supplementation to bull semen extender. In addition, spermatozoa cryopreserved with 0.05 mM resveratrol had greater plasma membrane and acrosome integrity. Resveratrol supplementation of the bull semen extender, however, did not have effects on capacity of sperm to induce embryo development after *in vitro* fertilization.

Percentage sperm motility and other values for kinetic variables are associated with the sperm capacity for transport to the fertilization site, and these variables are commonly used as criteria for evaluating semen quality (Chenoweth, 2002). Semen containing sperm with little or no motility is an indicator of there being sub-optimal fertility or infertility when there is use of this semen for AI or when bulls evaluated to have semen with these characteristics are used for natural mating. When there was supplementation with resveratrol at the 0.05 mM concentration, there was a greater sperm progressive motility when assessed using CASA procedures. In addition, values for VSL, VAP, LIN and STR variables were also greater when there was supplementation of semen extender with 0.05 mM resveratrol. These evaluations of sperm specific movements across the field of view are useful to determine sperm quality. The values for some of these variables such as those for VSL and VAP were positively correlated with estrous-non-return and pregnancy rates of cattle (Nagy et al., 2015), however, progressive motility is still considered to be one of the most important variables for assessing sperm quality (Seidel, 2012; Vincent et al., 2014).

When there was evaluation of several resveratrol concentrations for the cryopreservation of goat semen (Lv et al., 2019), there was a greater sperm motility when there was supplementation of the extender with 0.01 and 0.05 mM resveratrol, indicating a dose-dependent effect of resveratrol on values for sperm kinetic variables. For cryopreservation of bull semen, the optimal concentration of resveratrol for semen cryopreservation and retention of sperm viability has not been ascertained. In a previous study, there was a positive effect of 1 mM resveratrol after thawing of cryopreserved bull semen on values for total sperm motility and ALH; however, there was not evaluations of any other concentration of resveratrol (Bucak et al., 2015). Evaluations of fresh bull semen in a buffered-medium (Tvrdá et al., 2015) indicated there were positive effects of 5–50  $\mu$ M resveratrol on spermatozoa motility and protection against oxidative stress; however, investigation of the effects on frozen-thawed spermatozoa was not performed. Different from evaluations in previous studies, in the present study, there was evaluations for an optimal concentration of resveratrol for cryopreservation of bull semen and effects on *in vitro* fertilization results and resulting embryo development. The largest concentration of resveratrol (1 mM) had no effect on sperm progressive motility and had a detrimental effect on values for some kinetic variables such

**Table 1**

Effect of resveratrol in bull semen extender on embryo development after *in vitro* fertilization with frozen-thawed sperm.

Resveratrol	$n^1$	Cleavage (%)	Blastocyst D7 <sup>2</sup> (%)	Blastocyst D8 <sup>3</sup> (%)
0 mM	668	67.8 $\pm$ 3.5	27.5 $\pm$ 2.8	27.9 $\pm$ 3.2
0.05 mM	649	71.8 $\pm$ 3.1	28.6 $\pm$ 1.9	30.9 $\pm$ 2.2

<sup>1</sup>  $n$  = number of presumptive zygotes; <sup>2</sup>D7 = day seven post *in vitro* fertilization; <sup>3</sup>D8 = day eight post *in vitro* fertilization; 16 replicates (four bulls with four replicates per bull); Values are presented as means  $\pm$  SEM; No difference between groups ( $P > 0.05$ ).



as VSL, VCL and VAP. These findings are consistent with those from a study with human semen where there was a lesser sperm motility after cryopreservation and thawing of semen when there was supplementation with 0.1–10 mM resveratrol (Garcez et al., 2010). Supplementation of semen extender in the present study with 0.05 mM resveratrol resulted in greater values for kinetic variables indicating a dose-dependent effect, which is consistent with results after the cryopreservation of goat sperm (Lv et al., 2019).

The dose-dependent effect of resveratrol on sperm motility when there were evaluations of frozen-thawed spermatozoa in the present study is also consistent with results from other studies where there was supplementation of culture medium with resveratrol. The relatively smaller concentrations of resveratrol had protective effects (Wang et al., 2016) whereas the greater concentrations of 0.2 mM induced apoptosis and damage to the DNA (Kilic Eren et al., 2015) of human cells. Motility and viability of bull spermatozoa were impaired when sperm were cultured in medium containing large concentrations of resveratrol (Tvrdá et al., 2015). Supplementation of extenders with relatively larger concentrations of resveratrol also resulted in lesser values for kinetic variables during liquid storage of boar semen (Martín-Hidalgo et al., 2013). It, however, is not clear how the relatively larger concentrations of resveratrol affect the values for kinetic variables of frozen-thawed bull sperm.

The greater values for kinetic variables are not always associated with greater spermatozoa fertilization capacity and subsequent induction of embryo development because sperm capacitation, as well as acrosome and plasma membrane integrity are all important aspects of bull fertility (Aitken et al., 2006). After determining that 0.05 mM resveratrol supplementation of semen extender led to improvements in kinetics of frozen-thawed spermatozoa, there was, in the present study, also evaluation of whether this concentration of resveratrol could also contribute to preservation of sperm membranes during cryopreservation or affect the processes of fertilization. Resveratrol supplementation at 0.05 mM to bull semen extender resulted in a greater percentage of sperm with intact acrosome and plasma membrane, indicating there are positive effects on sperm quality after thawing of cryopreserved semen. There are similar findings when there was evaluation of buffalo sperm cryopreserved with 0.05 mM resveratrol (Longobardi et al., 2017). Because sperm cryopreservation processes lead to an increased generation of ROS (Aitken, 2020; Ugur et al., 2019), the mechanisms by which resveratrol enhances values for kinetic variables and membrane integrity subsequent to thawing of cryopreserved bull semen is likely related to the antioxidant properties of resveratrol. After evaluation of boar semen, Zhu et al. (2019) reported that the addition of resveratrol to the freezing extender activates 5'AMP-Activated Protein Kinase in sperm. This protein kinase is involved in regulating both cell metabolism and cellular redox status (Zhu et al., 2018), and once activated by resveratrol there is elimination of ROS and improvement of the antioxidant defense system of spermatozoa (Zhu et al., 2019).

Resveratrol has actions to reduce ROS generation (Fang et al., 2002; Olas and Wachowicz, 2004; Rotondo et al., 1998) and to stimulate activities of antioxidant enzymes such as superoxide dismutase and peroxidase (Fukui et al., 2010; Mokni et al., 2007) enhancing cell protection against oxidative stress. Greater than optimal concentrations of ROS can lead to damage of cell membranes by inducing lipid peroxidation (Su et al., 2019). In sperm such damage can affect the sperm movement and membrane integrity because when there is greater lipid peroxidation there is lesser sperm motility and there is a positive correlation with sperm membrane damage after thawing of cryopreserved semen (Trevizan et al., 2018). Because resveratrol can reduce lipid peroxidation in cells (Olas and Wachowicz, 2004), it is presumed that the positive effect of resveratrol on sperm motility and membrane integrity subsequent to thawing of cryopreserved sperm was due to its protective functions when there is greater than optimal ROS generation and lipid peroxidation. This hypothesis is corroborated by results from previous studies where there was lesser ROS generation after the cryopreservation of goat (Lv et al., 2019) and buffalo (Longobardi et al., 2017) sperm using 0.05 mM resveratrol.

Although sperm motility and acrosome integrity are prerequisites for and may have an effect on fertility (Harstine et al., 2018; Rodríguez-Martínez, 2013), assessments of these variables are not sufficient to predict fertilization capacity of sperm for induction of embryonic development. *In vitro* fertilization studies can provide information about the capacity of sperm to fertilize oocytes and induce embryonic development. In the present study, 0.05 mM resveratrol supplementation of semen extender had no effect on the capacity of sperm to induce embryonic development, to the cleavage and blastocyst developmental stages after *in vitro* fertilization. Results from a previous study with buffalo sperm indicated there was an improved normospermic penetration of oocytes after heterologous *in vitro* fertilization occurred using thawed semen that had been cryopreserved using 0.05 mM resveratrol (Longobardi et al., 2017). In this previous study, there was no effect on total penetration and proportions of embryos with cleavage as well as pregnancy rate after AI (Longobardi et al., 2017). Although 0.05 mM resveratrol supplementation of semen extenders can be effective for preservation of sperm kinetic patterns and membrane integrity after thawing of cryopreserved semen, there are no beneficial effects on the capacity of the sperm from these samples to induce embryonic development subsequent to IVF.

Supplementation of culture media with resveratrol when conducting *in vitro* oocyte maturation or embryo production can enhance oocyte and embryonic developmental capacity in pigs (Lee et al., 2010), cattle (Sovernigo et al., 2017), goats (Piras et al., 2019) and sheep (Zabihi et al., 2019). Supplementation of sperm washing and *in vitro* fertilization media with 0.1 mM resveratrol resulted in lesser sperm ROS concentrations and greater embryonic development to the cleavage and blastocyst stages when there was use of sex-sorted bull semen for conducting these procedures (Li et al., 2018), indicating that these supplementations with resveratrol can lead to improvement in fertility when this compound is added to the *in vitro* fertilization medium. In the present study, there was no resveratrol supplementations during *in vitro* fertilization because frozen-thawed semen was washed using Percoll discontinuous density gradient procedures to separate motile sperm and to remove the components in semen extender, including resveratrol. This may be the reason that there was no effects of resveratrol supplementations to semen on embryo development. It, therefore, is feasible that resveratrol supplementation to both semen extender and *in vitro* fertilization medium can contribute to enhancement of embryo development *in vitro*.

## 5. Conclusions

In conclusion, resveratrol in semen extender has a dose-dependent effect on values for sperm kinetics subsequent to thawing of cryopreserved semen. The concentration of resveratrol that was most effective in these regards was 0.05 mM because there was a greater acrosome and plasma membrane integrity, however, there were no effects of this supplementation on embryo development after *in vitro* fertilization. The resveratrol can be used at a lesser concentration in bull semen extenders to improve post-thaw sperm quality without compromising the fertilization capacity of sperm and inducing embryonic development *in vitro*. Further studies, however, are required to assess *in vivo* fertility by means of artificial insemination.

## Data statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## CRediT authorship contribution statement

**Carolina Marinho Assunção:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Vivian Rachel Araujo Mendes:** Methodology, Data curation, Investigation, Writing - review & editing. **Felipe Zandonadi Brandão:** Resources, Writing - review & editing. **Ribrio Ivan Tavares Pereira Batista:** Methodology, Data curation, Investigation, Writing - review & editing. **Eliza Diniz Souza:** Methodology, Data curation, Investigation, Writing - review & editing. **Bruno Campos de Carvalho:** Resources, Formal analysis, Writing - review & editing. **Carolina Capobianco Romano Quintão:** Methodology, Data curation, Investigation, Writing - review & editing. **Nadia Rezende Barbosa Raposo:** Conceptualization, Writing - review & editing. **Luiz Sergio Almeida Camargo:** Conceptualization, Supervision, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors report no declarations of interest.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.anireprosci.2021.106697>.

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