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Effect of Trolox and resveratrol supplementation during the refrigeration of boar sperm

Efecto de la suplementación con Trolox y resveratrol durante la refrigeración de semen porcino

UNIVERSIDAD NACIONAL DE COLOMBIA

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Keywords:
Antioxidant
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Spermatozoa
Sperm preservation
Sperm viability

ABSTRACT

This research aimed to evaluate the possibility of improving the boar semen quality already refrigerated in commercial diluent by adding Trolox and/or resveratrol during the refrigeration process. Pools of refrigerated semen from boars of proven fertility were added with a) 200 µM Trolox, b) 50 µM resveratrol, c) 200 µM Trolox + 50 µM resveratrol, or d) no antioxidant supplementation (negative control) and conserved at 17 °C for 7 days. On days 1, 3 and 7 of refrigeration the following sperm parameters were evaluated: Motility, functions of the plasma membrane, pre-capacitated spermatozoa, viability and acrosome integrity, and mitochondrial membrane potential. Additionally, mitochondrial superoxide anion production was evaluated by flow cytometry. *In vitro* fertilization was performed using semen from day 3 of refrigeration. Data were analyzed using ANOVA (P<0.05). Evaluated parameters significantly decreased over refrigeration time (P<0.05), except the percentages of pre-capacitated spermatozoa. Samples supplemented only with Trolox maintained values similar to the control group, except for higher sperm motility on day 3 of preservation (P<0.05), whereas the addition of resveratrol caused a decrease in the studied parameters (P<0.05); the combination of both antioxidants showed intermediate values between each individual antioxidant treatment. No significant variations in mitochondrial superoxide anion production and cleavage rates were detected with each treatment with respect to the control. In conclusion, supplementing boar semen with Trolox and/or resveratrol does not cause a significant improvement in semen quality, once the refrigeration process has started, except for a higher motility with Trolox at day 3 of refrigeration, nor does it alter the mitochondrial production of reactive oxygen species and the fecundity capacity of semen *in vitro*.

Palabras clave:
Antioxidante
Estrés oxidativo
Espermatozoides
Conservación espermática
Viabilidad espermática

RESUMEN

El objetivo del presente trabajo fue evaluar la posibilidad de mejorar la calidad de semen porcino ya refrigerado en diluyente comercial por el agregado de Trolox y/o resveratrol durante el proceso de refrigeración. Mezclas de semen refrigerado provenientes de verracos de probada fertilidad fueron adicionadas con a) Trolox 200 µM, b) resveratrol 50 µM, c) Trolox 200 µM + resveratrol 50 µM, o d) sin suplementación antioxidante (control negativo) y conservados a 17 °C por 7 días. En los días 1, 3 y 7 de la refrigeración, se evaluaron los siguientes parámetros espermáticos: motilidad, funcionalidad de la membrana plasmática, espermatozoides precapacitados, viabilidad e integridad acrosomal y potencial de membrana mitocondrial. Además, se evaluó la producción de anión superóxido mitocondrial utilizando citometría de flujo. La fecundación *in vitro* se realizó usando semen del día 3 de refrigeración. Los datos fueron analizados por Análisis de Varianza (P<0,05). Los parámetros evaluados disminuyeron con el tiempo de refrigeración (P<0,05), excepto por los porcentajes de espermatozoides precapacitados. Las muestras suplementadas solo con Trolox mantuvieron valores similares a las del grupo control, excepto por una mayor motilidad espermática en el día 3 de conservación (P<0,05), mientras que la adición de resveratrol causó una disminución de los parámetros estudiados (P<0,05), la combinación de ambos antioxidantes mostró valores intermedios entre cada tratamiento antioxidante individual. No se observaron variaciones en los porcentajes de producción de anión superóxido mitocondrial y de clivaje embrionario en respuesta a la suplementación con cada antioxidante con respecto al control. En conclusión, la suplementación de semen porcino con Trolox y/o resveratrol, una vez que el proceso de refrigeración ha comenzado, no provoca un aumento significativo de la calidad seminal, excepto por una mayor motilidad con Trolox al día 3, ni altera la producción mitocondrial de especies reactivas del oxígeno y la capacidad fecundante del semen *in vitro*.

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The use of assisted reproductive biotechnologies has increased the need to develop reliable techniques for gamete preservation. The porcine species represents a greater challenge for the application of these biotechnologies, as boar sperm is particularly much more sensitive to cold damage due to its lipid composition (Giaretta et al. 2013). Thus, in pigs, frozen semen has a very low survival rate and 99% of artificial inseminations are carried out with refrigerated semen in liquid form between 15 and 17 °C (Yeste 2017). Furthermore, refrigerated boar semen quality can be altered after the refrigeration process due to conservation and transport conditions (Yeste 2017).

It is known that in both frozen and refrigerated semen, the conservation process increases oxidative stress; therefore, among the alternatives to improve the efficiency of these techniques, the supplementation of semen diluents with different types of antioxidants could be introduced. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a hydrophilic analog of α-tocopherol, the most common and biologically active form of tocopherols (vitamin E) (Giordano et al. 2020). The antioxidant mechanism of vitamin E and its analogues is mainly to inhibit lipid peroxidation of membrane phospholipids or lipoproteins, and to keep iron and other metal elements in a reduced state (Cao et al. 2022). On the other hand, resveratrol (3,5,4´-trihydroxystilbene) is a polyphenol, produced by more than 70 different types of plants in response to stressed conditions (Pasquariello et al. 2020). It has been reported in several cellular models that resveratrol has a reactive oxygen species (ROS) scavenging capacity and, like other phytoestrogen, acts via alpha and beta estrogen receptors activating the transcription of mitochondrial and nuclear target genes, controlling mitochondrial biogenesis and activating antioxidant enzymes (Gambini et al. 2015; Zhu et al. 2019; Pasquariello et al. 2020).

Some studies have reported the beneficial effect of the addition of Trolox or resveratrol to the frozen or refrigerated diluents before boar sperm preservation (Mendez et al. 2013; Varo-Ghiuru et al. 2015; He et al. 2020; Ribas-Maynou et al. 2021; Camporino and Córdoba 2022). However, there are no reports with respect to the addition of these antioxidants, nor their combination once the refrigeration procedure has been completed. Therefore, this research aimed to evaluate the possibility of improving the quality of boar semen already refrigerated in a commercial diluent by adding Trolox and/or resveratrol during the refrigeration process.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade and obtained from Sigma-Aldrich (Merk) unless otherwise stated. Fluorochromes were purchased from Sigma-Aldrich (Merk) or Life Technologies (Thermo Fisher).

Research Ethics Statement

No animals were used during this study. Ovaries were donated to our laboratory by a local slaughterhouse and semen was donated by a local farm. The research project was approved by the University of Buenos Aires (20020220300165BA, UBACyT 2023-2016).

Semen sample provision and experimental design

Refrigerated porcine semen samples obtained from fresh samples with a minimum of 70% motile and 80% viable spermatozoa were kindly donated by a local pig genetic company (Agroceres PIC Argentina, https://agroceresPIC.com.ar/). These boars belonged to a controlled program of a local artificial insemination centre, and they were kept under uniform feeding and handling conditions during the entire study. Five different pools from boars of proven fertility (three or four F1 Camborough/ line 337, Pietrain x Duroc Jersey males per pool) were refrigerated 1:1 in long-term commercial medium (Androstar Plus, Minitube; at least $31.4x10^6$ spermatozoa mL^{-1}) and transported to the laboratory at controlled temperature conditions (17 °C in an adiabatic container) within the first 24 hours.

Upon arrival at the laboratory, four different sperm aliquots (6 mL) were added with a) 200 µM Trolox (Peña et al. 2003; Varo-Ghiuru et al. 2015), b) 50 µM resveratrol (Longobardi et al. 2017; Zhu et al. 2019), c) 200 µM Trolox + 50 µM resveratrol, or d) no antioxidant supplementation (negative control) and conserved at 17 °C for 7 days. On days 1, 3, and 7 of refrigeration, an aliquot (1 mL) from each treatment was collected, warmed at 37 °C for 10 minutes and then washed by centrifugation at 600x*g* for 5 minutes to remove the refrigeration diluent and to allow later evaluations. -

Sperm motility

Motility was visually determined using an Optic Microscope

(Jenamed 2, Carl Zeiss, Jena, Germany) under 400x magnification with a thermal stage at 37 °C, three times by the same observer (Peña et al. 2003).

Sperm viability and acrosome integrity

The percentage of intact acrosomes in live cells was determined by vital trypan blue stain using differential interference contrast (DIC) microscopy (Jenamed 2, Carl Zeiss, Jena, Germany) at 1000x magnification (Satorre et al. 2018). An aliquot of the sperm suspension was incubated with an equal volume of 0.25% Trypan blue in TALP at 37 °C for 15 minutes. The mixture was then centrifuged at 600×*g* for 10 minutes to remove excess stain and subsequently fixed with 5% formaldehyde in PBS. Live spermatozoa appeared unstained, while dead spermatozoa stained dark blue. Intact acrosomes presented a defined and integral membrane, whereas damaged acrosomes appeared blurred. For each treatment, 200 spermatozoa were analyzed under standardized conditions.

Pre-capacitated spermatozoa

Pre-capacitated spermatozoa percentages were determined through the modifications in fluorescence of chlortetracycline (CTC) patterns using an epifluorescence microscope (Jenamed 2, Carl Zeiss, Jena, Germany) at 400x magnification (Satorre et al. 2018). The CTC solution was freshly prepared daily by dissolving 500 µM CTC in a buffer containing 130 mM NaCl, 5 mM cysteine, and 20 mM Tris, pH=7.8. This solution was protected from light using foil. For the assay, 500 µL of the sperm suspension was mixed with an equal volume of the 500 µM CTC solution, and glutaraldehyde was added to achieve a final concentration of 0.1%. The prepared samples were then placed on a clean slide and sperm capacitation was examined using the epifluorescence microscope (Jenamed 2, Carl Zeiss, Jena, Germany) at 400× magnification. The three sperm patterns were as follows: a) intact non-capacitated sperm, with uniform fluorescence on the head, b) capacitated sperm, with a fluorescence-free band in the postacrosomal region, and c) acrosome-reacted sperm, with a fairly dull fluorescence on the head. The midpiece was fluorescent in all cells.

Sperm plasma membrane functionality

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the spermatozoa plasma membrane using an Optic Microscope (Jenamed 2, Carl Zeiss, Jena, Germany) under 400x magnification (Satorre et al. 2018). Aliquots (50 µL) of sperm solution were mixed with 500 µL of hypo-osmotic solution (sodium citrate 0.49%, fructose 0.9%, 100 mOsm kg^{-1}) and incubated at 38 °C for 60 min. Spermatozoa with swelling in the tail present a functional plasma membrane. Spermatozoa (n = 200) were counted under differential interference contrast (DIC) microscopy.

Sperm mitochondrial membrane potential

Mitochondrial membrane potential was evaluated using JC-1 (5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethyl benzimidazolyl carbocyanine iodide) fluorochrome. Aliquots (300 µL) of sperm solution from each treatment were mixed with 15 µL of 2 µM JC-1 (final concentration 0.1 µM) and incubated at 37 °C in the dark for 30 min. Glutaraldehyde (0.1%) was then added to the mixture (Camporino and Córdoba 2022). Spermatozoa were evaluated at 400x magnification using an epifluorescence microscope and 510-570 nm filters (Jenamed 2, Carl Zeiss, Jena, Germany) and classified as low or high membrane potential when showing green or red fluorescence in the middle piece, respectively (Wysokińska 2020). Two hundred spermatozoa were used for the evaluation.

Sperm mitochondrial reactive oxygen species

The amount of mitochondrial superoxide anion produced in sperm cells was evaluated using MitoSOX Red fluorochrome. Aliquots of sperm solution ($1x10^6$ spermatozoa mL^{-1}) from each treatment were incubated with 2 µM MitoSOX Red at 37 °C for 15 min in the dark. Samples were then washed, and MitoSOX Red fluorescence was measured using a flow cytometer (BDFacsCanto II, Becton Dickinson, San Jose, USA). Argon laser excitation at 488 nm was coupled with emission measurements using 580 nm filters. Non-sperm-specific events were gated out and 50,000 cells were examined per independent sample (Zarzycka et al. 2014; He et al. 2017).

***In vitro* fertilizing capacity of Sperm**

Gilt cumulus-oocyte complexes *in vitro* maturation was performed following our laboratory protocol (Morado et al. 2023). Briefly, cumulus-oocyte complexes were obtained by aspiration of antral follicles (3-8 mm) from slaughterhouse ovaries and then matured in medium 199 (GIBCO, Grand Island, NY) supplemented with 50 µg mL^{-1} gentamicin sulfate, 10% (v/v) porcine follicular fluid, 2UI mL^{-1} equine chorionic gonadotropin, 10 ng mL^{-1} epidermal growth

factor, 0.57 mM cysteine, under mineral oil at 39 °C, 5% CO_2 in a humidified atmosphere for 44 h.

To evaluate the *in vitro* fertilizing capacity of refrigerated semen, sperm cells from each treatment at day 3 of refrigeration were washed in TBM (Tris Buffer Medium) by centrifugation and then co-incubated in the same medium at a final concentration of $5x10^5$ spermatozoa mL^{-1} with *in vitro* matured porcine oocytes for 3 h. Fertilized oocytes were washed in NCSU-23 medium (Yamanaka et al. 2009) and the remaining cumulus cells and spermatozoa were removed by gentle pipetting. Presumptive zygotes were incubated in NCSU-23 medium under mineral oil, at 39 °C in a 20% O_2 and 5% CO_2 atmosphere. Cleavage rate was evaluated 48 h post-fertilization. For this experiment, between 25 and 30 COCs were used for each treatment per trial and seven repetitions were performed.

Statistical analysis

Results for spermatic variables were expressed as mean ± standard deviation and were analyzed by two-way ANOVA followed by Tukey test and the comparison of means was carried out at each evaluation time. The normality of the data was evaluated using the modified Shapiro-Wilk test. Cleavage rates were expressed as mean ± standard deviation and were analyzed by one-way ANOVA followed by Tukey test. The normality of the data was evaluated using modified Shapiro-Wilk test. *P* values lower than 0.05 were considered significantly different. The statistical analysis was carried out using Infostat software.

RESULTS AND DISCUSSION

Some studies have attempted to evaluate the effect of Trolox and resveratrol on boar sperm quality, all of them performing the antioxidant supplementation before semen freezing or refrigeration procedures. This study analyzed the possibility of improving the quality of commercial semen by adding these antioxidants once the refrigeration procedure has started. Additionally, the combination of both antioxidants was evaluated considering that they have different antioxidant action mechanisms that could improve their protective capacity.

Five pools of refrigerated boar semen were analyzed in order to evaluate the effect of the addition of Trolox and/or resveratrol during 1, 3, and 7 days of preservation on sperm motility, viability and acrosome integrity, plasma membrane functionality, mitochondrial membrane potential, and pre-capacitated spermatozoa (Figure 1). As expected, except for the percentage of pre-capacitated spermatozoa

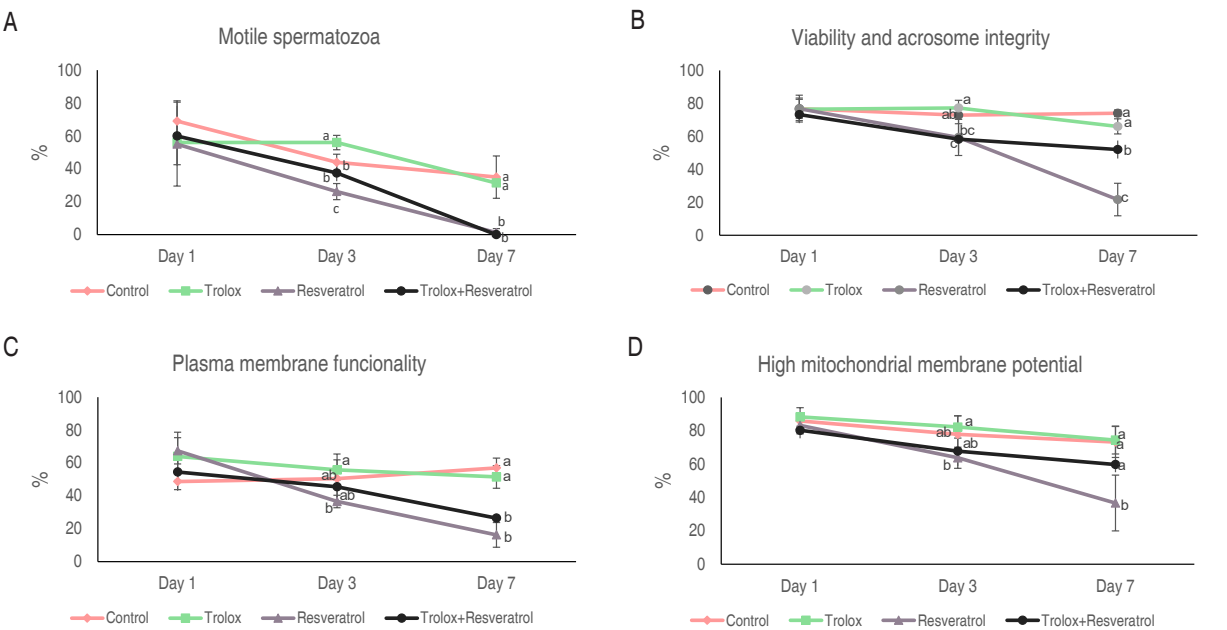

Figure 1. Evaluated sperm parameters. **A.** Percentage of motile spermatozoa. **B.** Percentage of viable spermatozoa with intact acrosome. **C.** Percentage of spermatozoa with functional plasma membrane. **D.** Percentage of spermatozoa with high mitochondrial membrane potential. Data are expressed in percentage ± standard deviation in 5 replicates. [a, b, c] Different letters indicate significant differences between treatments on the same evaluation day ($P<0.05$). All the evaluated parameters significantly decreased over refrigeration time ($P<0.05$).

(day 1=0.2±0.2% to day 7=2.3±0.3%), all the evaluated sperm parameters significantly decreased over the refrigeration time in the four studied groups (control, trolox, resveratrol, and trolox + resveratrol, $P<0.05$).

The addition of Trolox to already refrigerated sperm increased sperm motility at day 3 of preservation ($P<0.05$, Figure 1A), but it maintained similar values with respect to the control group for the other sperm parameters at all evaluated days (Figure 1A-D). The effect of Trolox supplementation on boar sperm is still controversial. It has been reported that the addition of Trolox (200 µM) during the freezing process improves frozen-thawed boar sperm viability, increases motility, mitochondrial activity and acrosome integrity (Peña et al. 2003; Varo-Ghiuru et al. 2015). The addition of Trolox (200 µM) to the refrigeration diluent increases motility and membrane functionality in boar sperm (Camporino and Córdoba 2022). Additionally, supplementation of Trolox (400 µg mL^{-1}) during the processing of pig semen doses to be stored at 15 °C for 72 h reduces malondialdehyde (MDA) production, indicating a reduction in lipid peroxidation (Mendez et al. 2013). On the other hand, it has been reported that the use of trolox (200 µM) in the diluent for boar semen refrigerated for 72 h causes a significant decrease in motility, tail membrane integrity and mitochondrial activity, and a significant increase in DNA fragmentation and lipid peroxidation (Zakošek Pipan et al. 2017). The findings in the present study suggest that the addition of Trolox to the sperm diluent once the refrigeration procedure has started exerts, in general, neither beneficial nor deleterious effect on sperm parameters.

The addition of resveratrol to already refrigerated semen diminished sperm motility ($P<0.05$, days 3 and 7, Figure 1A) and tended to decrease (day 3) or decreased ($P<0.05$, day 7) the other evaluated sperm parameters with respect to the control group (Figure 1A-D). The effect of resveratrol supplementation on boar sperm is also controversial. It was reported that the addition of resveratrol in boar sperm freezing diluent significantly improved post-thawing progressive motility, membrane and acrosome integrity, mitochondrial activity, glutathione level and antioxidant enzyme activity (Zhu et al. 2019). The addition of resveratrol (50 µM) during the refrigeration process improves visual motility, membrane and acrosome integrity, mitochondrial membrane potential, and decreased MDA content (Sun et al. 2020). On the other hand, Martín-Hidalgo et al. (2013) evaluated the addition of resveratrol to the extender of boar semen for its storage at 17 °C, indicating that this supplementation causes a lower response to capacitating stimuli, a decrease in the sperm ATP content and a reduction in mitochondrial membrane potential. Although there are no reports about the addition of resveratrol once the refrigeration process has started, Bucci et al. (2018) performed the antioxidant supplementation during the thawing process of frozen semen and found that the addition of resveratrol (2 mM) to thawing medium causes a negative effect on sperm motility and DNA integrity, while viability, acrosome integrity, mitochondrial function and lipid peroxidation were not influenced. These last studies are in part in line with the results of this study, which indicate that resveratrol addition during the refrigeration process of porcine semen tends to exert or exerts a deleterious effect on the sperm parameters with respect to the control. In this case, as resveratrol was added once the spermatozoa were already refrigerated and their metabolism was decreased, such incorporation in the sperm cells could not be as efficient as expected, increasing its concentration in the extender, which may have shifted their behavior to pro-oxidant activity. A similar idea has been proposed by Vongpralub et al. (2016), who mentioned that these extenders are suitable by themselves for long-term liquid preservation; having in mind that commercial farms retain the seminal plasma in the extended semen, and the plasma contains natural antioxidants, the subsequent addition of antioxidant molecules, may increase their content above the physiological values which may be detrimental to the cells. It was also observed that sperm samples preserved in the presence of resveratrol show the lowest mitochondrial membrane potential. This decrease could be associated with resveratrol ability to regulate mitochondrial permeability transition pore, increase mitochondrial superoxide anion production and to disrupt mitochondrial respiratory chain (Martín-Hidalgo et al. 2013). These events reduce the ATP content of the spermatozoa treated with resveratrol, which could explain the parallel decrease in sperm motility (Martín-Hidalgo et al. 2013); as it was also observed in sperm motility with the addition of resveratrol during refrigeration in the present study.

Interestingly, the percentages of sperm motility, viability and acrosome integrity, plasma membrane functionality and high mitochondrial membrane potential were higher in

samples added with Trolox with respect to those added with resveratrol at days 3 and 7 of preservation (P<0.05, Figure 1A-D). But these sperm parameters showed intermediate values when samples were supplemented with Trolox + resveratrol (Figure 1A-D). These results suggest that Trolox can protect sperm samples from the detrimental effects caused by the presence of resveratrol in the refrigerated diluent.

Sperm mitochondrial ROS production was evaluated with the fluorescent probe MitoSOX Red using flow cytometry. Two distinct subpopulations were observed; a subpopulation of sperm cells that did not bind to the probe (MS(-)), and a subpopulation that reacted to the probe (MS(+)) (representative histograms for days 3 and 7 are shown in Figure 2A). As shown in Figure 2B, mitochondrial ROS production for the MS(+) subpopulation was significantly higher on day 1 in comparison with days 3 and 7 of preservation (P<0.05). It is well known that the cryopreservation process affects sperm functionality, but Awda et al. (2009) demonstrated that intracellular levels of hydrogen peroxide are unchanged after the cryopreservation of boar sperm and that viable frozen-thawed sperm presents significantly less intracellular content of superoxide anion. The reported alterations could be caused by alterations in mitochondrial function and a decrease in oxidative phosphorylation, leading to lower ATP synthesis and ROS generation, causing both the reduction in superoxide anion content and sperm motility (Awda et al. 2009). On the same day of evaluation, no significant differences were observed in the production of mitochondrial superoxide anion between treatments. A possible explanation is presented by Guthrie and Welch (2006), who mentioned that boar sperm naturally presents low basal ROS concentrations, which do not differ between fresh and frozen-thawed sperm. Since physiological ROS production is not high, antioxidant supplementation can have detrimental effects on sperm cells (Bucci et al. 2018). *In vitro* sperm fertilizing capacity was evaluated by co-incubating *in vitro* matured porcine cumulus-oocyte

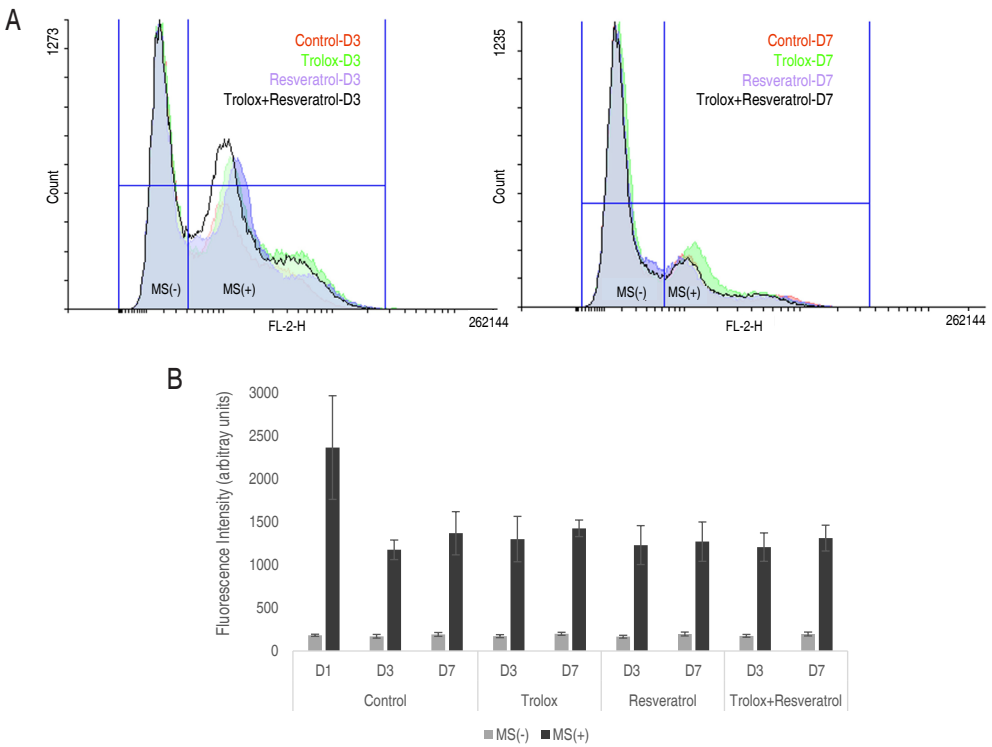

Figure 2. Mitochondrial superoxide anion production. **A**. Representative histograms of mitochondrial superoxide anion production evaluated with MitoSOX Red fluorescent probe using flow cytometry. Two different subpopulations are described; a subpopulation of sperm cells that did not bind to the probe (MS(-)), and a subpopulation that reacted to the probe (MS(+)). **B**. Effect of antioxidant treatment on mitochondrial ROS in refrigerated boar sperm. Results are expressed as arbitrary units of fluorescence intensity ± standard deviation in three replicates for negative (MS(-)) or positive (MS(+)) sperm cells to MitoSOX Red.

complexes with sperm samples treated with antioxidants on day 3 of preservation, because these samples still preserved a good motility. Seven repetitions were performed. As shown in Table 1, no significant differences were observed on cleavage rates between the four groups studied. Surprisingly, despite the differences found for sperm parameters, especially between Trolox and resveratrol, cleavage rates do not differ between treatments. Probably, there are still enough viable spermatozoa to maintain *in vitro* fertilizing capacity in the different sperm samples. These results coincide with several authors (Peña et al. 2003; Pech-Sansores et al. 2011; Maside et al. 2023) who indicated that subjective motility is not a reliable indicator of porcine sperm quality after thawing, and Martín-Hidalgo et al. (2013) who mentioned that conventional boar sperm parameters used to evaluate semen doses are insensitive to assess the fertilization potential of refrigerated semen. Although some studies have evaluated the effect of these antioxidants on classic parameters of boar sperm, to the best of current knowledge, only one has assessed the potential effect of resveratrol on *in vitro* fertilizing capacity. It has been reported that the addition of resveratrol in a frozen medium may increase sperm penetration rate and diminish monospermic rate in comparison with the control (Bucci et al. 2018). This study is the first one that evaluates the effect of Trolox supplementation, alone or combined with resveratrol, on *in vitro* fertilizing capacity of boar refrigerated sperm. Additionally, it would also be interesting to evaluate *in vivo* fertilizing capacity of these sperm samples, considering the effect of female genital tract conditions.

Table 1. Cleavage rate obtained from *in vitro* matured cumulus-oocyte complexes fertilized with refrigerated sperm treated with different antioxidants (Trolox and/or resveratrol).

Sperm treatments	Total number of matured COCs	Total number of cleavage embryos	Mean cleavage rate (%)*
Control	197	102	51.00±4.41
Trolox	201	97	46.41±2.95
Resveratrol	193	100	46.53±9.14
Trolox + Resveratrol	179	92	49.31±8.61

* Percentages are expressed as mean ± standard deviation in seven replicates (n= 25-30 COCs for each treatment per trial; $P<0.05$).

CONCLUSION

Previous studies have evaluated the addition of antioxidants, among them Trolox and resveratrol, to refrigerate diluents before the cooling process to avoid damage related to cold shock. However, there is not enough evidence whether the addition of these antioxidants to the semen extender collaborates to prolong seminal viability once the refrigeration process has been completed, considering that commercial farms sell semen already refrigerated. In this study, it was observed that supplementing refrigerated boar semen with Trolox and resveratrol does not cause a significant improvement in semen quality. When only Trolox is added, viability and acrosome integrity, plasma membrane functionality and mitochondrial membrane potential maintain a similar behavior to the control, except for an increased motility at day 3 of preservation with this antioxidant. Particularly, the addition of resveratrol generates a reduction in the sperm parameters studied over refrigeration time. The presence of Trolox prevents in part, the detrimental effect caused by resveratrol. It is important to highlight that the addition of these antioxidants did not generate changes in mitochondrial superoxide anion production, suggesting that although conservation processes usually increase ROS levels, in refrigerated porcine semen these levels would not exceed the cell physiological control capacity; so, the addition of antioxidant supplements after refrigeration had no beneficial effects. This idea is supported because no significant differences were observed in cleavage rates between treatments, which also was evidenced that commonly evaluated sperm parameters do not necessarily reflect the fertilizing capacity of sperm, at least with respect to *in vitro* embryo production system. Further studies that investigate the endogenous antioxidant sperm capacity could help to develop the proper antioxidant protocol supplementation to improve refrigerated boar sperm performance.

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CONFLICT OF INTERESTS

The authors have no conflict of interest.

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