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Effect of Trolox addition as antioxidant in refrigeration medium in porcine spermatozoa

Efecto del agregado de trolox como antioxidante al medio de refrigeración del espermatozoide porcino

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ABSTRACT

The aim of this study was to analyze the effect of Trolox addition to semen diluents on porcine sperm refrigeration evaluated by functional tests in order to predict their potential fertilization capacity and to increase the knowledge regarding their quality parameters and refrigeration conditions to optimize porcine sperm conservation. A 200 µM Trolox concentration was added to a pool of boar semen samples diluted in Modena modified medium and stored at 17 °C. At days 1, 2 and 5 post-refrigeration, sperm motility, viability and acrosome integrity, pre-capacitation state, plasma membrane function and mitochondrial membrane potential were evaluated. In conclusion, the addition of 200 µM Trolox to seminal samples in the porcine species produced a significant increase in the preservation of plasma membrane functionality at day 1 and 5 compared with untreated samples. An increase in progressive motility evaluated by light microscopy was also observed on day 1 in treated samples compared to untreated samples. No significant differences were observed in vitality, mitochondrial membrane potential and pre-capacitation patterns between treated and untreated samples throughout the refrigeration time.

Keywords: (spermatozoa), (porcine), (refrigeration), (antioxidant), (trolox)

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RESUMEN

El objetivo del presente estudio fue analizar el efecto de la adición de Trolox al medio de refrigeración de semen porcino a través de pruebas funcionales, pudiendo así incrementar el conocimiento respecto a los parámetros de calidad y condiciones de refrigeración empleados para optimizar dicho proceso. Un pool de muestras de semen porcino fue diluido en medio Modena modificado, suplementando una parte con 200 µM de Trolox, y posteriormente refrigerado a 17 °C. Al día 1, 2 y 5 de refrigeración se evaluó la motilidad espermática, viabilidad e integridad acrosomal, estado de pre-capacitación, integridad en la función de membrana plasmática y potencial de membrana mitocondrial. En conclusión, la adición de Trolox 200 µM a muestras seminales en la especie porcina produjo un aumento significativo en el porcentaje de gametas con funcionalidad de membrana plasmática conservada en el día 1 y 5 en comparación con las muestras no tratadas. También se observó un incremento en la motilidad progresiva evaluada por microscopía óptica al día 1 en muestras tratadas con respecto a las no tratadas. No se observaron diferencias significativas en la vitalidad, potencial de membrana mitocondrial y patrones de pre-capacitación entre muestras tratadas y no tratadas a lo largo de ese tiempo de refrigeración.

Palabras clave: (espermatozoides), (porcino), (refrigeración), (antioxidante), (trolox)

INTRODUCTION

Gamete conservation is currently an essential biotechnology in animal production. To date, the most efficient method to store mammalian gametes for long periods of time is cryopreservation, which improves the biosecurity of stored samples, minimizes adverse effects related to infectious diseases and facilitates the constitution of a gene bank with an increased variety for each species, allowing to apply a higher selection pressure over the available males[13, 30]. However, in porcine production, gamete cryopreservation is not regularly used due to the damages which it generates in both sperm and oocytes.

During gamete preservation at low temperatures a series of changes take place in the plasma membrane, lipids suffer a reorganization which increases permeability and reduces enzyme activity[17]. This also increases reactive oxygen species (ROS) production, including superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2), which may derivate in oxidative stress and membrane lipid peroxidation[24]. Gamete aerobic metabolism, which is stimulated by their exposure to high oxygen concentrations during cryopreservation, results in higher ROS production by the mitochondrial respiratory chain and by NADPH oxidase, which is located in the plasma membrane[30].

It was reported that ROS have a dual effect in sperm function. At low concentrations ROS may induce capacitation by activating a cyclic AMP dependent cascade which stimulates tyrosine-kinases that phosphorylate tyrosine residues from proteins which participate in the capacitation process[24, 28]. These compounds also induce hyperactivation, help to maintain acrosome integrity, and promote sperm-oocyte fusion. At high concentrations, instead, they produce severe DNA damage, inhibit gamete fusion and reduce sperm motility[1].

In porcine gametes the plasma membrane is rich in phosphatidylethanolamine and sphingomyelin and presents low phosphatidylcholine content[17] and also a low cholesterol concentration distributed asymmetrically, especially in the external layer, making the internal one more labile[17] and determining a low cholesterol/phospholipid relation. This makes the porcine sperm more susceptible to ROS-induced lipid peroxidation generated during their storage at low temperatures compared with other species[1, 22]. It has been reported that degenerative changes produced by storage also have effects on mitochondrial and acrosome membranes resulting in an increase in mitochondrial membrane potential and in sperm with

premature capacitation and acrosome reaction, respectively[18], having a negative impact on fertilization and pregnancy rates. For these reasons, to diminish the damage induced by thermal shock, porcine gametes are stored at temperatures above 15 °C[10, 12, 18, 23].

Recent studies have reported strategies to improve storage conditions using antioxidants to scavenge ROS or inhibit their generation. They tested refrigeration media supplemented with cysteine[2, 29], resveratrol[11], glutathione[13, 32], L-carnitine[32], or vitamin E and its analogues[2, 15, 32], among other compounds. A synthetic derivate from vitamin E called Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) scavenges ROS by means of a cromanol ring[21, 26]. Its effect has been proved in numerous cellular models like hepatocytes, miocytes, erithrocytes, timocytes and gametes[21, 31].

Our work aims at studying the effect of Trolox addition to semen diluents on porcine sperm refrigeration evaluated by functional tests in order to predict their potential fertilization capacity and to increase the knowledge regarding their quality parameters and refrigeration conditions to optimize porcine sperm conservation.

MATERIALS AND METHODS

Reagents and media

The materials used in these experiments were obtained from Sigma-Aldrich (St. Louis, Missouri), unless otherwise indicated.

Semen sample preparation for refrigeration

We worked with pools of donated semen samples with similar characteristics from three pigs in reproductive age obtained through manual pressure. Five pools from these three boars were used in this study, each being aliquoted for treatments with and without Trolox.

First, sperm concentration was determined and samples were incubated for 20 minutes at 37 ºC. Then, samples were separated in two equal aliquots diluted in Modena modified medium (52.61 mM glucose, 23.46 mM sodium citrate, 11.9 mM sodium bicarbonate, 6.99 mM EDTA, 46.66 mM Tris, 15.10 mM citric acid, 10 mg/L gentamicin, in tri-distilled water)[16]. One of the samples was treated with 200 µM Trolox[21]. Both aliquots were maintained at 25 ºC during 2 h and then stored at 17 °C.

To evaluate the conservation process, functional parameters were analyzed at days 0, 1, 2 and 5. Sperm motility was analyzed by optical microscopy, viability and acrosome integrity were evaluated using Trypan Blue stain by optical microscopy with Differential Interferential Contrast (DIC), pre-capacitation state was analyzed using chlortetracycline (CTC), plasma membrane functional integrity by Hyposmotic test (Hos Test) and mitochondrial membrane potential using 5,5,6,6-Tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) fluorochrome.

Vigor and motility evaluation

Vigor and motility were evaluated by optical microscopy using a thermal platinum at 37 °C, analysing progressive motility (%) and vigor (in a scale from 0 to 4)[19].

Sperm vitality and acrosome integrity evaluation

Each sperm suspension aliquot from the different treatments and their respective controls were incubated with an equal volume of Trypan Blue stain in TALP medium supplemented with bovine serum albumin (BSA) and calcium during 15 minutes at 37 °C. Then they were centrifuged at 600 g during 10 minutes and re-suspended in TALP + BSA and calcium to remove the stain excess. The samples were fixed with 5 % glutaraldehyde in TALP solution. Samples were observed by optical microscopy using DIC to determine the percentage of live sperm and acrosome integrity simultaneously[3]. The percentages of live spermatozoa with integer acrosome were determined as a difference between those obtained for each sample and those obtained at 0 h (control).

Sperm pre-capacitation evaluation

Sperm pre-capacitation and acrosome reaction percentages were determined using CTC[4], adding each sperm sample and chlortetracycline in equal volumes (500 µM). Glutaraldehyde at 0.1 % (p/v) final concentration was used to fix the

samples. Different sperm patterns were evaluated using a Carl Zeiss Jenamed epifluorescence microscope. Sperm pre-capacitation and acrosome reaction percentages were calculated as a difference between those obtained for each sample and those obtained at 0 h (control), leaving out those sperm which presented a pre-capacitation or acrosome reaction pattern attributed to refrigeration damage and incubation times.

Plasma membrane function evaluation

Plasma membrane functional integrity was evaluated using Hos Test, adding 50 µL of the sperm samples to 1 mL of a hyposmotic fructose-sodium citrate solution (100 mOsm)[27]. Samples were fixed using 15 µL glutaraldehyde and evaluated using optical microscopy, analysing the following patterns: 1) sperm with a flagellum swelling: positive reaction to the test, indicating a functional plasma membrane allowing liquid entrance; 2) sperm without swelling: negative reaction indicating a non-functional plasma membrane, not allowing liquid entrance.

Mitochondrial membrane potential evaluation

Mitochondrial membrane potential was analysed using JC-1 fluorochrome. The assay forms red fluorescent aggregates indicating an increased membrane potential and green fluorescent monomers indicating a decreased membrane potential[5]. Semen aliquots (500 µl) were incubated for 40 minutes at 37 °C with 0.5 µL JC-1 (3 mM) stock solution prepared with dimethyl sulfoxide[21] and then evaluated using an epifluorescence microscope using a 500 - 510 nm filter.

Statistical analysis

Results were expressed as mean ± standard deviation and were analyzed by two-way ANOVA, using post-hoc general contrasts for comparison among treatments (2 x 4 factorial analysis). Tukey Test was used as a post-ANOVA analysis to compare means (STATISTIX 7. 2000, Analytical Software for Windows, Version 7.0; Analytical Software, Tallahassee, Florida, USA). *P* values lower than 0.05 were considered significant.

RESULTS

Semen samples refrigerated without Trolox presented a (35.71 ± 6.47) %, (37.11 ± 4.26) %, (30.40 ± 5.90) % of viability and acrosomal integrity at day 1, 2 and 5 respectively. Semen samples refrigerated with Trolox, instead, presented a (44.20 ± 10.93) %, (36.14 ± 1.86) % and (37.66 ± 24.63) % of viability and acrosomal integrity at day 1, 2 and 5 respectively. It was observed a viability and acrosome integrity decrease compared with fresh semen [(68.09 ± 6.46) %], with no significant differences between Trolox treated and untreated samples. ($P > 0.05$, Figure 1).

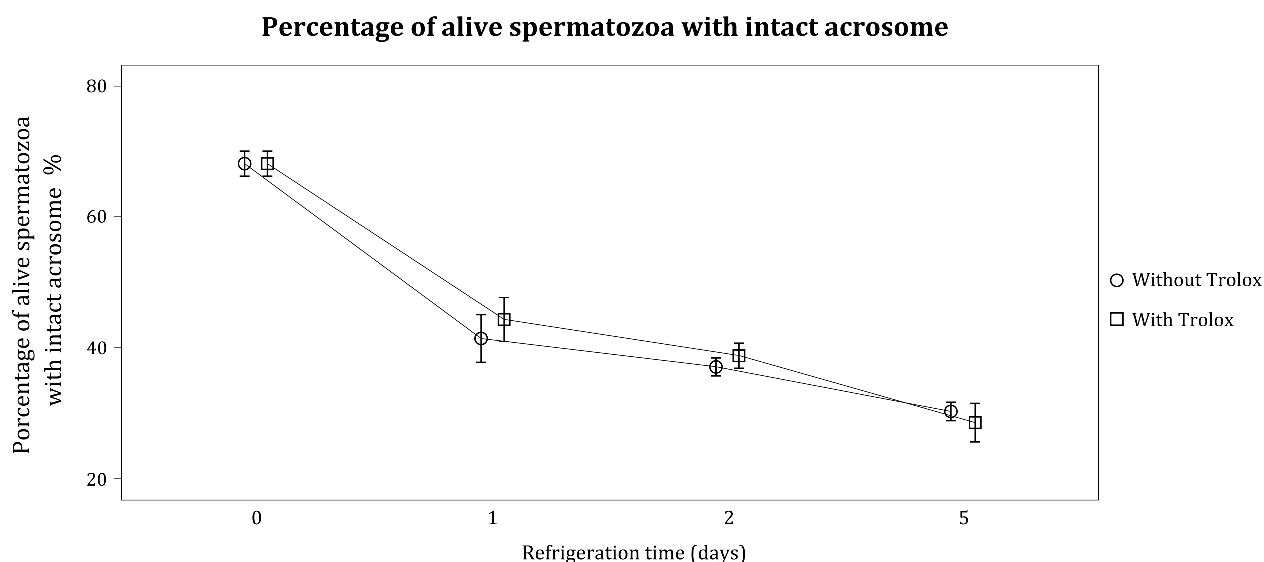

Figure 1. Percentage of alive spermatozoa with intact acrosome determined in fresh semen (day 0) and in samples treated and not treated with Trolox at days 1, 2 and 5 of refrigeration (n = 5). No significant differences were observed. ($P > 0.05$).

Plasma membrane function evaluated by Hos Test changed through the refrigeration process compared with fresh semen [(82.22 ± 4.73) %]. Furthermore, a significant increase in the gametes with conserved plasma membrane functionality was observed in Trolox treated samples at day 1 and 5 [(88.75 ± 3.20) % and (80.00 ± 3.46) % respectively] compared with untreated samples [(84.14 ± 1.46) % and (76.80 ± 8.70) % respectively] ($P < 0.05$, Figure 2).

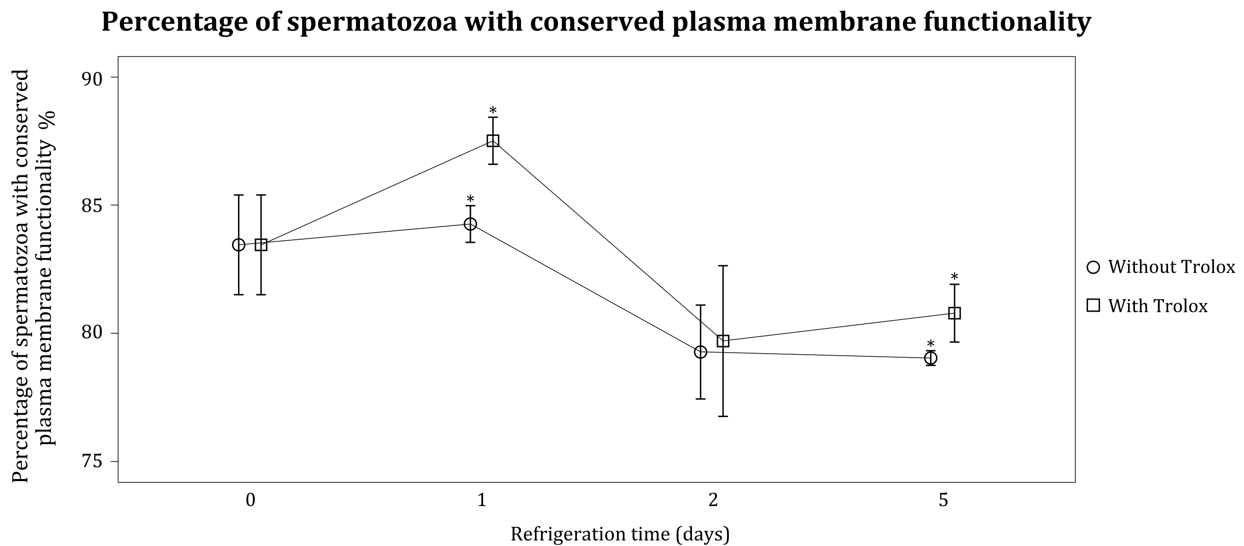

Figure 2. Percentage of sperm with conserved plasma membrane functionality determined in fresh semen (day 0) and Trolox treated and not treated samples at days 1, 2 and 5 of refrigeration (n = 5). Different symbols and numbers represent statistical differences ($P < 0.05$).

The percentage of spermatozoa with elevated mitochondrial membrane potential was reduced during storage compared with fresh semen [(65.11 ± 20.00) %]. Semen samples refrigerated without Trolox presented a (56.57 ± 7,81) %, (46.60 ± 7.86) %, (43.00 ± 7.81) % of spermatozoa with elevated mitochondrial membrane potential at day 1, 2 and 5 respectively. Semen samples refrigerated with Trolox, instead, presented a (60.80 ± 5.00) %, (60.20 ± 6.72) %, and (48.20 ± 22.52) % of spermatozoa with elevated mitochondrial membrane potential at day 1, 2 and 5 respectively. No significant differences were observed in the percentage of spermatozoa with elevated mitochondrial membrane potential between treated and untreated samples ($P > 0.05$, Figure 3)

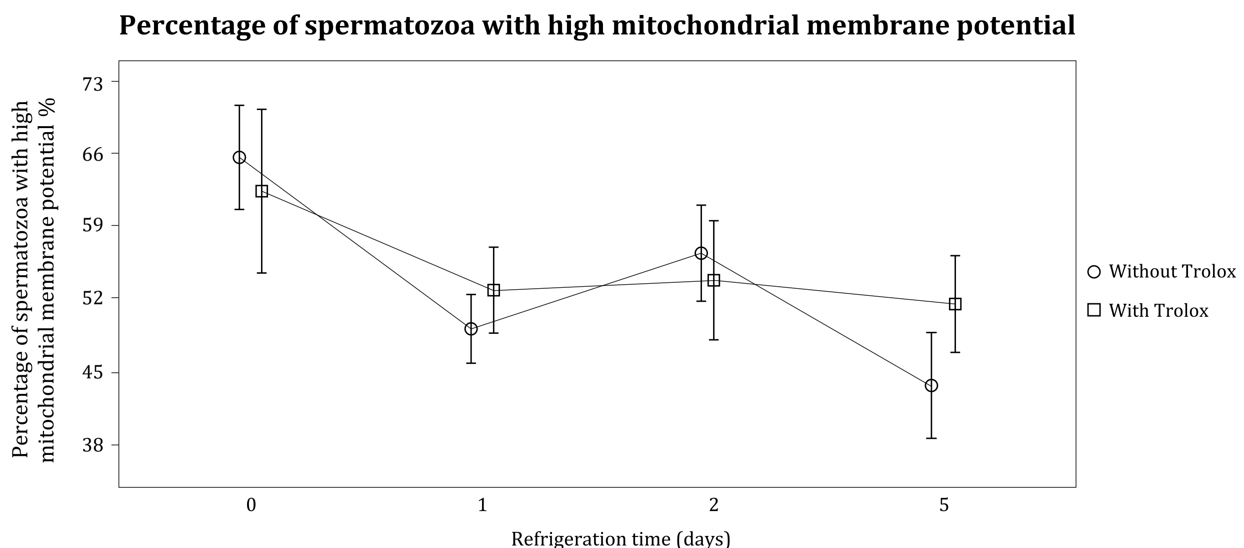

Figure 3. Percentage of spermatozoa with high mitochondrial membrane potential determined in the fresh sample (day 0) and in the samples treated and not treated with Trolox at days 1, 2 and 5 of refrigeration (n = 5). No significant differences were observed ($P > 0.05$).

The pre-capacitation percentage in stored samples increased compared to fresh semen [(14.91 ± 7.66) %]. Treated samples presented a (21.00 ± 5.66) %, (16.33 ± 3.67) %, and (23.80 ± 7.36) % of pre-capacitation at day 1, 2 and 5 respectively. Untreated samples presented a (14.50 ± 3.66) %, (18.14 ± 4.02) %, and (25.20 ± 9.96) % of pre-capacitation patterns at day 1, 2 and 5 respectively. No significant differences were observed between treated and untreated samples ($P > 0.05$, Figure 4).

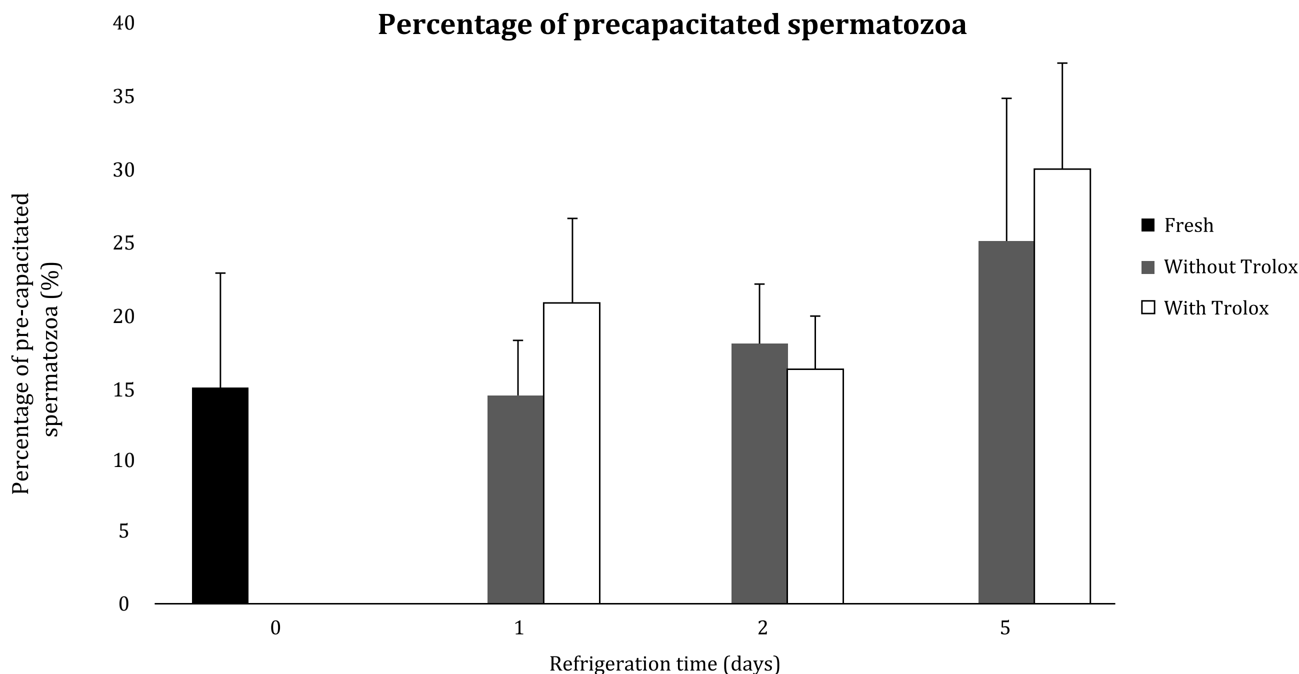

Figure 4. Percentage of pre-capacitation in spermatozoa determined in the fresh sample (day 0) and in the samples treated and not treated with Trolox at days 1, 2 and 5 of refrigeration (n = 5). No significant differences were observed ($P > 0.05$).

In refrigerated semen samples, a decrease in progressive motility was detected by optic microscopy with Trolox [(26.66 ± 13.91) %, (12.00 ± 2.73) %, and (5.00 ± 0.1) % at day 1, 2, and 5 respectively] and without Trolox [(16.12 ± 7.37) %, (5.00 ± 0.2) %, and (6.66 ± 2.88) % at day 1, 2, and 5 respectively] compared with fresh semen [(88.18 ± 5.6) %] during refrigeration time. A significant difference only was observed at day 1 between treated and untreated semen samples ($P < 0.05$).

DISCUSSION

It has been shown that during the storage process at low temperatures, seminal quality deteriorates due to ROS production. Although oxygen radicals have a positive effect at physiological concentrations in spermatozoa, being involved in intracellular signals that result in processes such as sperm capacitation, hyperactivation, and sperm-oocyte fusion[8,33,34], these cells are particularly vulnerable to oxidative stress due to their scarce cytoplasm, high polyunsaturated fatty acid concentrations and insufficient antioxidant mechanisms[25]. ROS such as superoxide anion, hydroxyl anion and hydrogen peroxide can affect the quality of stored spermatozoa by depleting antioxidant systems, causing lipid peroxidation, altering plasma and acrosomal membrane, reducing mitochondrial membrane potential and inducing protein denaturation with inactivation of glycolytic enzymes, DNA alterations and reduced motility[8, 9, 25].

Vitamin E is an important component of the antioxidant system in sperm membranes, in which it appears to have a dose-dependent effect acting as a peroxyl lipid and alkoxyl radical scavenger, thus preventing lipid peroxidation. It has been shown by other authors that feeding boars with vitamin E supplements improved ejaculate volume and sperm concentration[25], while the addition of vitamin E to the semen extender/storage

solvent increased sperm resistance to lipid peroxidation. During cryopreservation, vitamin E supplementation had a positive effect on sperm motility, mitochondrial membrane potential and membrane integrity[8, 21, 26]. The addition of Trolox, a synthetic and water-soluble analog of vitamin E, proved to help reduce ROS levels in other cell models, allowing the normalization of the mitochondrial membrane potential and the restoration of its electrochemical proton gradient, acting as an efficient inhibitor of the mitochondrial proton leak that may occur under conditions of oxidative stress[7]. The aim of our study was to evaluate whether the addition of 200 µM Trolox to boar semen samples prior to refrigeration in Modified Modena medium[16] could help counteract the oxidative damage produced during refrigeration.

Trolox had no positive effect on the changes in the mitochondrial membrane potential experienced during refrigeration, being glucose the only oxidative metabolite that spermatozoa had available in the refrigeration medium[9, 33]. In accordance under these conditions, Trolox might not be enough to maintain mitochondrial membrane potential in fresh semen, which diminishes during the days in which it is refrigerated. This is in contrast with other studies which relate Trolox effect to Coenzyme Q-10 antioxidant action in the mitochondria. Coenzyme Q-10 or ubiquinone is a non-enzymatic antioxidant and energy promoter, membrane stabilizer and regulator of mitochondrial permeability transition pores. In spermatozoa, coenzyme Q-10 is concentrated in the intermediate part of the mitochondria, where it recycles vitamin E and prevents pro-oxidant activity[25]. In this way coenzyme Q-10 could preserve mitochondrial membrane potential, unlike what happened in our system.

Compared with other species, boar sperm are more vulnerable to peroxidative damage during cryopreservation due to their high polyunsaturated fatty acid content which serves as preferred substrates for ROS action in cell membranes[1,34]. The addition of 200 µM Trolox improved the plasma membrane function at days 1 and 5 post-refrigeration, suggesting that its action, consisting in breaking ROS bonds, would be helping to counteract the lipid peroxidation that occurs in membranes and therefore the change in their integrity during cooling, thus having a beneficial effect on sperm preservation.

It is important to mention that during the experiment, an increase in pre-capacitated spermatozoa percentage was observed with and without the addition of Trolox. This could be linked to changes in sperm redox state. It has been studied in species such as cattle, horses and humans that ROS at physiological concentrations may trigger various signaling cascades that result in the phosphorylation of proteins involved in the sperm capacitation process, such as protein kinase A (PKA), protein kinase C (PKC) and protein-tyrosine kinase (PTK), while at high concentrations there is an inhibition of these processes and marked cell damage[1, 6, 20], although these processes have not yet been fully elucidated in the porcine species.

Regarding viability and progressive motility, our results could indicate that the Trolox concentration used allowed us to obtain acceptable levels of sperm viability as a result of maintaining the plasma membrane function, but it was not enough to maintain adequate levels of progressive motility. It has been studied that ROS could produce the uncoupling of electron transport and oxidative phosphorylation in sperm mitochondria, with a coincident reduction in the number of sperm containing an internal transmembrane potential high enough to support mitochondrial ATP production and sperm motility[1]. It is also hypothesized that ROS may not alter mitochondrial function, but may exert a negative effect on the axoneme by disrupting ATP utilization or interfering with the contractile mechanism of the flagellum[14]. Some authors argue that boar sperm motility, acrosome integrity and lipid peroxidation are more sensitive indicators of oxidative stress than viability[1]. According to our data, progressive motility decreased compared with fresh semen throughout the refrigeration days. Although the maintained of sperm viability that was independent of Trolox effects and this also was not enough to maintain the mitochondrial potential that might provide energy for and adequate progressive motility.

CONCLUSION

Porcine spermatozoa are very susceptible to oxidative damage due to the characteristics of its membranes. The study of Trolox effect on them is extremely important to optimize seminal storage conditions at low temperatures. In our study, the addition of 200 µM Trolox in porcine semen diluent increased the preservation of plasma functionality and motility only at day 1, but did not modified another functional parameter. It should be noted that refrigeration is one of the most widely used methods in pig farms to preserve semen samples for artificial insemination, so the study of the effects of Trolox is important to improve sperm storage quality.

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