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Impact of post-thaw supplementation of semen extender with antioxidants on the quality and function variables of stallion spermatozoa

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A B S T R A C T

During cryopreservation procedures, the spermatozoa are exposed to physical and chemical stressors that generate an increase in the intracellular concentration of reactive oxygen species (ROS). If ROS concentrations are too great, this can lead to a state of oxidative stress that are detrimental to sperm quality. The aim of this study was to ascertain the profile the ROS production and assess the effects of post-thaw supplementation of a semen extender with different antioxidant compounds on the quality and function variables of frozen-thawed stallion spermatozoa incubated *in vitro*. Frozen-thawed stallion spermatozoa (2×10^6 cells/mL) were incubated with three different antioxidants (MnTBAP, NAC and FeTPPS) for 4 h at 38 °C. An untreated sperm suspension and a fresh sample were included as controls. Plasma membrane integrity (SYBR-14/PI), intracellular ROS concentration (DHE and ROS-ID™ total ROS/Superoxide Detection Kit), lipid peroxidation (BODIPY), DNA damage (TUNEL) and mitochondrial membrane potential (ΔΨm; TMRE/SYTOX) were evaluated by flow cytometry and fluorescence microscopy. In addition, sperm motility was evaluated using the ISAS system. Evaluations were performed at 0 and 4 h of incubation. The results indicate that superoxide anion is the main ROS produced by frozen-thawed stallion spermatozoa and that the use of MnTBAP improved sperm motility and viability, decreased the lipid peroxidation and DNA damage. In conclusion, this study provides relevant data to improve *in vitro* incubations conditions and to establish futures therapies using MnTBAP after thawing with the aim being to overcome the deleterious effects of semen cryopreservation and consequently preserve the stallion sperm quality through avoiding oxidative stress.

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1. Introduction

Cryopreservation of stallion spermatozoa allows long-term preservation of spermatozoa from particular stallions and facilitates its international semen trade, however, fertilizing capacity of frozen-thawed semen is still inferior to fresh or refrigerated semen (Loomis and Graham, 2008). Several factors have been reported to influence the cryosurvival of stallion sperm including osmotic stress, ice crystal formation, toxicity of the cryoprotectants (Ball, 2008; Pena et al., 2011), sample processing (Ellerbrock et al., 2018), and the variability among stallions (Neild et al., 2003; Ortega Ferrusola et al., 2009).

It is widely known that cryopreservation of sperm cells has deleterious effects in the plasma and acrosome membrane integrity. In addition, cryopreservation induces DNA fragmentation (Yeste et al., 2015), aberrant DNA methylation (Aurich et al., 2016), changes the pattern of RNA of epigenetic-related genes (Zeng et al., 2014), affects the Na-K^+ pump activity with ouabain-induced caspase 3 activation (Ortega Ferrusola et al., 2009), and mitochondrial functionality through induction of mitochondrial permeability transition (Treulen et al., 2018). This damage to the sperm cells is due mainly to three stressing events during the cryopreservation procedure, the thermic and osmotic shocks, and the oxidative stress (Reviewed by Ball, 2008).

Oxidative stress is the primary source of damage associated with semen cryopreservation (Yeste et al., 2015). The oxidative stress refers to the increase of intracellular concentrations of reactive oxygen species (ROS) (Agarwal et al., 2005). Because ROS are generated in spermatozoa when there are physiological conditions (de Lamirande and Gagnon, 1995), controlled ROS concentrations are needed for the important role of these factors in proper sperm function including fertilizing capacity (Du Plessis et al., 2015). During a pathophysiological imbalance, however, ROS accumulate and react with biomolecules (*i.e.*, membrane phospholipids, enzymes, chromatin), impairing structure and cellular functions (Agarwal et al., 2008; Aitken, 2017). There have been reports as a result of several studies that there are harmful effects of oxidative stress on sperm cells, including extensive damage by peroxidation of membrane lipids (Storey, 1997), oxidation of proteins (Morielli and O'Flaherty, 2015), DNA fragmentation and oxidation of bases (Barroso et al., 2000; Bui et al., 2018), sub-optimal mitochondrial membrane activity (Yeste et al., 2015; Treulen et al., 2018), and inactivation of enzymes associated with motility (de Lamirande and Gagnon, 1992). Nevertheless, there are reports that the addition of either enzyme scavengers or non-enzymatic antioxidants during *in vitro* manipulation of stallion spermatozoa protects against this oxidative stress (Baumber et al., 2005; Shojaeian et al., 2018). A valuable strategy to improve the quality of frozen-thawed spermatozoa, therefore, has been the identification and treatment of ROS generation through preventing its production or by antioxidant therapy by means of the addition of antioxidants to semen extenders before (Shojaeian et al., 2018), or after (Gadani et al., 2017) thawing.

Currently, there is a wide range of antioxidants used in semen extenders with different scavenger targets. The N-acetyl-L-cysteine (NAC) is an antioxidant that scavenges free radicals facilitating glutathione (GSH) biosynthesis and alleviating GSH depletion during oxidative stress (Roederer et al., 1992). Specifically, NAC reduces the production of intracellular hydrogen peroxide (H_2O_2) concentrations but reacts relatively slowly with the superoxide anion ($O_2^{\cdot-}$) (Owada et al., 2013).

Metalloporphyrins, a class of catalytic antioxidants, scavenge a wide range of ROS such as $O_2^{\cdot-}$, H_2O_2, peroxinitrite ($ONOO^-$), and lipid peroxyl radicals (Day, 2004) and are able to modulate RS-based redox signaling (Batinic-Haberle et al., 2010). The metalloporphyrin-5,10,15,20-Tetrakis (4-sulfonatophenyl) porphyrinate Iron (III) Chloride (FeTPPS), catalyzes the isomerization of peroxynitrite to nitrate, serving as a selective scavenger and catalyst for the decomposition of peroxynitrite (Misko et al., 1998). Manganese (III) tetrakis (4–69 benzoic acid) porphyrin (MnTBAP) is a synthetic metalloporphyrin that readily penetrates through cellular membranes. It possesses superoxide dismutase (SOD) and catalase-like activities (Cuzzocrea et al., 1999), and is also a potent inhibitor of membrane lipid peroxidation (Day et al., 1999). The MnTBAP compound may also inhibit the oxidative activity of peroxynitrite (Faulkner et al., 1994), but not the nitric oxide (Szabo et al., 1996).

Thus, regarding the valuable antioxidant capacities, the aim of the present study was to profile the *in vitro* ROS production and assess the effects of post-thawing supplementation of semen extender with the previously described compounds on the quality and function variables of frozen-thawed stallion spermatozoa.

2. Material and methods

2.1. Reagents and chemicals

All chemical reagents, as well as antioxidant (MnTBAP, NAC, FeTPPS) were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated.

2.2. Semen collection and processing

Semen samples from four mature stallions (3 to 8 years old) with proven fertility were collected using an artificial vagina model Colorado twice a week during the breeding season of 2017 in compliance with the Universidad de La Frontera Scientific Ethics Committee (Act N° 057/2016). Horses were housed at a farm with similar feeding and activity management occurring for all animals. A mare with signs of behavioral estrus was used for stallions to mount for semen collection. Semen was collected using a pre-warmed (42 °C) and lubricated artificial vagina and filtered through gauze to remove the gel fraction. Immediately after collection, the ejaculates were immersed in a water bath at 37 °C until arrival at laboratory (approximately within 30 min). At least four ejaculates per stallion were collected, cryopreserved and analysed. Before cryopreservation, one aliquot of fresh semen was allocated for the assessment of the following sperm variables: plasma membrane integrity, mitochondrial membrane potential, lipid peroxidation, ROS production, DNA fragmentation and motility.

2.3. Semen cryopreservation

The ejaculates were separated into two aliquots, diluted 1:1 in stallion semen extender (BotuSemen, Nidacon, International AB, Mölndal, Sweden) and centrifuged at 600 *g* for 10 min at room temperature. The pellet was re-suspended in EquiPlus Freeze (Minitüb, Tiefenbach, Germany) and adjusted to 200×10^6 cells/mL. The samples were subsequently stored into 0.5 mL straws (Minitüb, Tiefenbach, Germany), heat sealed and balanced at 4 °C for 1 h. After equilibration, the straws were exposed to liquid nitrogen (LN_2) vapor for 15 min, plunged into and stored in LN_2 until thawed.

2.4. Semen evaluation after thawing

Straws were thawed individually in a water bath (37 °C), for 30 s and diluted in pre-warmed sperm TLP medium (spTLP: 100 mM NaCl, 3.10 mM KCl, 0.3 mM NaH_2PO_4, 21.6 mM sodium lactate, 2.0 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10.0 mM HEPES, and 1.0 mM pyruvate, pH 7.4 and 295 mOsm/Kg) (McPartlin et al., 2008), to a final concentration of 2×10^6 cells/mL. Aliquots of frozen-thawed sperm suspensions were incubated for 4 h at 37.5 °C. The same quality variables evaluated for fresh semen were assessed in frozen-thawed spermatozoa as described below. Sample analysis was performed on all semen samples immediately after thawing (0 h), as well as at the end of the incubation period (4 h).

2.5. Sperm membrane integrity

Spermatozoa with intact plasma membranes were detected using the SYBR-14 / PI (LIVE / DEAD Sperm Viability kit; Molecular Probes cat n° L-7011, Eugene, OR, USA) according to the manufacturer's instructions with some modifications. Two different fluorescent patterns were identified, red fluorescent spermatozoa considered to be cells that had lost the integrity of the plasma membrane (dead cells), whereas green fluorescent cells were considered as cells with an intact plasma membrane (live cells) (Fig. 1AII and III). Briefly, a volume of 2 μL SYBR-14 (100 nM) was added to 500 μL of sperm suspension (2×10^6 cells/mL). After 10 min of incubation at 38 °C, 2 μL propidium iodide (PI, 2.4 mM) was added and the suspension incubated for further 5 min at 38 °C. Technical controls were performed using fresh semen. The positive control was permeabilized with 0.2% triton x-100 plus 0.1% sodium citrate for 15 min at room temperature while the negative control was the untreated samples. The stained sperm samples were analyzed by flow cytometry and fluorescence microscopy. For each sample, the percentage of live cells was calculated.

2.6. Evaluation of mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi m$) was assessed using tetramethylrhodamine methyl ester perchlorate (TMRM). The TMRM is a cationic lipophilic, non-toxic and highly fluorescent compound which has been used to analyze the $\Delta\Psi m$ in somatic cells as well as in mammalian sperm. For each analysis, sample suspensions adjusted to 2×10^6 cells/mL were incubated for 15 min at 37.5 °C with 0.5 μM (final concentration) of TMRM. In addition, 0.5 μM SYTOX® Green (Molecular Probes, Life Technologies, Carlsbad, USA) was added to exclude dead cells. The stained sperm samples were analyzed by flow cytometry and fluorescence microscopy. For each sample, the fluorescence intensity (arbitrary unit; AU) and the percentage of spermatozoa with high $\Delta\Psi m$ were calculated.

2.7. Evaluation of ROS production

The measurement of ROS production was performed with dihydroethidium (DHE, Molecular probes, Life Technologies, Carlsbad, USA), which penetrates the cells and is oxidized by ROS (mainly $O_2^{\cdot -}$). Oxidized DHE binds to DNA and emits orange fluorescence; thus, changes in DHE fluorescence are proportional to ROS production (Carter et al., 1994). Sample suspensions adjusted to 2×10^6 cells/mL were incubated for 15 min at 38 °C with 2 μM DHE. The ROS production was also analyzed by fluorescence intensity (arbitrary unit; AU) of the probe. Again, 0.5 μM SYTOX® Green (Molecular Probes, Life Technologies, Carlsbad, USA) was added to exclude dead sperm. The stained sperm samples were analyzed by flow cytometry and fluorescence microscopy. For each sample, the fluorescence intensity (arbitrary unit; AU) was calculated. Technical controls were performed using fresh semen. In the case of TMRM and DHE assays, the positive control was done by incubating aliquots of sperm suspension with 5 μM ionomycin for 15 min at 37 °C, according to Treulen et., al (2015). An untreated control group was also included.

2.8. Profiling of ROS produced

To determine the profile of ROS generated, the ROS-ID™ total ROS/Superoxide Detection Kit (Enzo Life Sciences, Lausen, Switzerland) was used according to the manufacturer's recommendations. A positive control incubated with 100 μM pyocyanin to induce ROS production was included. Fresh sperm were used to establish the basal concentrations of ROS (negative control). This kit is designed to detect the production of reactive oxygen and/or superoxide species and reactive nitrogen species (RNS) in live cells using flow cytometry (Ponnala et al., 2012; Wang et al., 2015). The kit includes two fluorescent dye reagents as major components - Oxidative Stress Detection Reagent (Green) and Superoxide Detection Reagent (Orange). The non-fluorescent, cell-permeable ROS

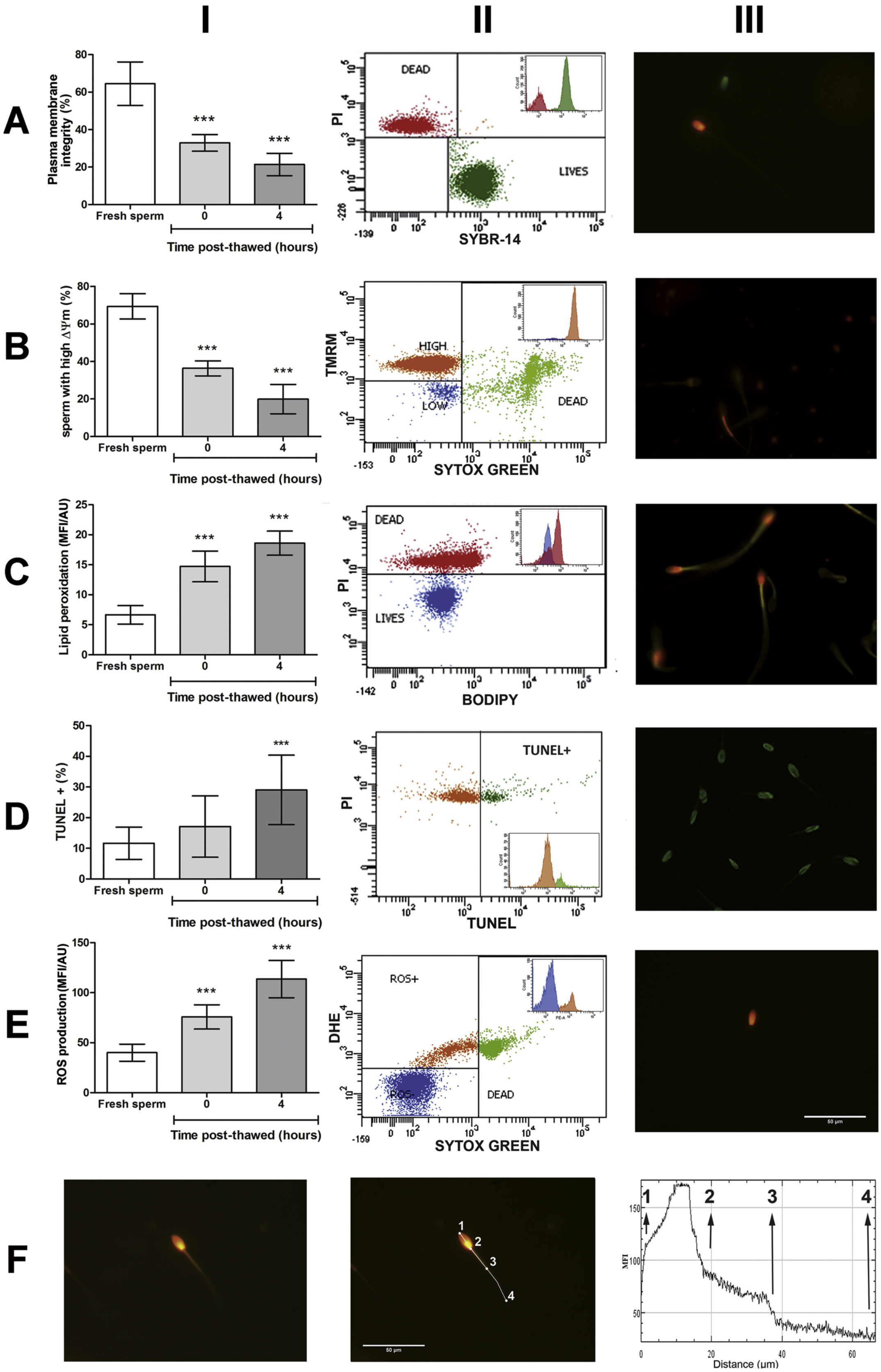

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Fig. 1. Analysis of stallion sperm quality variables by flow cytometry (A-E I and II) and fluorescence microscopy (A-E, III) in fresh, thawed and 4 h post-thawed sperm; (A) Plasma membrane integrity, (B) Sperm with high ΔΨm, (C) Lipid peroxidation, (D) TUNEL and (E) ROS production; Insets in cytometry scattergram (A-E II) show fluorescence histograms of each plots, respectively; Microscopy analysis of Superoxide Detection Reagent (F, I-III); (FI) Stained sperm, (FII) Area of sperm selected for fluorescence intensity analysis, (FIII) Fluorescence intensity plot of (1-2) sperm head, (2-3) midpiece, (3-4) sperm flagella; MFI: Medium fluorescence intensity, AU: Arbitrary units; Microscopy magnification of fluorescent images 100X; Data are presented as mean ± SD of eight experiments on different days (***$P < 0.001$).

detection dye (green probe) reacts directly with a wide range of reactive species, yielding a green fluorescent product indicative of cellular production of different ROS/RNS types. The green probe, however, has a relatively lesser sensitivity for $O_2^{\cdot-}$. The superoxide detection dye (orange probe) is a cell-permeable probe that reacts specifically with $O_2^{\cdot-}$, generating an orange fluorescent product. The stained sperm samples were analyzed by flow cytometry and fluorescence microscopy. For each sample, the percentage of spermatozoa with different production profiles of reactive species was calculated.

2.9. Evaluation of lipid membrane peroxidation

Lipid peroxidation was evaluated using BODIPY 581/591 C11, which once oxidized in sperm membranes, it changes its fluorescence spectral emission from red to green (Aitken et al., 2007). The BODIPY C11 was added so that there was a final concentration of 5 µM that was added to 0.5 mL of sperm suspension containing 2×10^6 cell/mL that was subsequently incubated for 30 min at 38 °C. 1 µL PI (2.4 mM) was added and the suspension incubated for further 5 min at 38 °C. An appropriate positive control was also prepared by incubating the samples with ferrous sulphate (80 µM), a membrane peroxidation promoter (Ortega Ferrusola et al., 2010). The stained sperm samples were analyzed by flow cytometry and fluorescence microscopy. For each sample, the fluorescence intensity (arbitrary unit; AU) was calculated.

2.10. Evaluation of DNA fragmentation

The percentage of cells with fragmented DNA was analyzed using the modified terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay with the *In-Situ* Cell Death Detection Kit, Fluorescein (Roche), according to the manufacturer's recommendations. Briefly, the samples were fixed for 1 h at 4 °C in PBS/BSA 1% (w/v) (pH 7.4) and permeabilized with 0.2% Triton X-100 + 0.1% sodium citrate for 1 h at room temperature. Then, permeabilized spermatozoa were incubated with the TUNEL reaction mixture in a dark environment at 38 °C for 1 h. Negative (omitting TdT from the reaction mixture) and positive (using DNAse I, 50 IU for 15 min at room temperature) controls were performed in each sample. To verify the successful cell permeabilization, samples were counterstained with PI (2.4 mM stock solution) for the last 5 min of incubation. The stained sperm samples were analyzed by flow cytometry and fluorescence microscopy. For each sample, the percentage of spermatozoa with fragmented DNA was calculated.

2.11. Evaluation of sperm motility

To evaluate sperm motility, computer-assisted sperm analysis was used through the Integrated Sperm Analysis System software (ISAS; Proiser, Valencia, Spain). Negative contrast was used and a minimum of 200 spermatozoa were examined for each evaluation using the adjustments for assessing stallion spermatozoa. Sperm suspensions were incubated for 4 h at 38 °C. Sperm motility was recorded at regular intervals of 1 h using 30×10^6 cells/mL as final cell concentration. Computer settings were according to Guimaraes et al., (2012), with some modifications: Number of frames 25/s; Maximum cell size 75 µm; Minimum cell size 12 µm; Velocity of rapid cells > 90 µm/seg; Straightness 35%; Temperature 37 ºC.

2.12. Analysis of supplementation of semen extender with antioxidants on quality sperm variables

Frozen-thawed stallion spermatozoa (2×10^6 cells/mL) were incubated at 37.5 °C for 4 h in SpTLP medium supplemented with different concentrations of MnTBAP (50 µM, 100 µM and 150 µM) used in equine sperm (Shojaeian et al., 2018) and macrophages (Tumurkhuu et al., 2007), NAC (5 mM, 10 mM and 15 mM) reported previously in bovine (Perez et al., 2015) and human sperm (Treulen et al., 2015), and FeTPPS (5 µM, 15 µM and 25 µM) reported for somatic cells (Lauzier et al., 2007; Liu et al., 2013). An untreated sperm suspension was included as control. The same sperm quality variables that were previously described (plasma membrane integrity, mitochondrial membrane potential, lipid peroxidation, ROS production, DNA fragmentation and motility) were evaluated. Sample analysis was performed immediately after thawing (0 h), as well as at the end of the incubation period (4 h).

2.13. Flow cytometry and fluorescence microscopy

Flow cytometry measurements were developed in a FACSCanto II flow cytometer (Becton, Dickinson and Company). A total of 10,000 sperm events at 600 cells/sec were acquired from each measurement. Acquisition was performed with a sample aspiration speed of 60 µL/minute. Samples were acquired and analyzed with the software FACSDiva, version 6.1.3 (Becton, Dickinson and Company). Fluorophores were excited at 488 nm using an argon laser. Settings and compensation for each fluorescent analysis was made using the software FACSDiva, version 6.1.3. The green fluorescence staining (SYBR-14, BODIPY 581/591 C11, Oxidative Stress Detection Reagent, SYTOX Green, and TUNEL assay) was detected using a 530/30 nm bandwidth filter and the orange fluorescence (Propidium iodide, TMRM, Superoxide Detection Reagent and DHE) was detected using a 585/42 nm bandwidth filter. All assessments were conducted on logarithmic scales. Prior to the analysis, all stained samples were centrifuged at 300 x *g* for 5 min, and re-suspended in PBS (200 µl) (Aguila et al., 2015), at a final concentration of 2×10^6 cells/mL.

To avoid overestimating the sperm population (doublets and debris), the gating strategy with two dot plot analyses was used. A sperm gate was generated in the first dot plot analysis with forward scatter area (FSC-A) and side scatter area (SSC-A) on X and Y axes, respectively. In the second dot plot analysis of the first gate, another gate was generated with FSC-A and forward scatter width (FSC-W) on the X and Y axes, respectively. All subsequent fluorescence analyses were obtained from the second gate generated.

For fluorescence microscopy image acquisition, 10 µL of sperm aliquots stained with the different fluorophores were placed on separate glass slides. The images were acquired at 25 °C under 100x objective oil immersion (total magnification 1000x) with a light microscope equipped for epifluorescence (Axiolab drb, KT 450905, Zeiss, Oberkochen, Germany).

2.14. Statistical analysis

The experiments were conducted in duplicate and repeated at least eight times on different days. At least 2 straws per ejaculate of each stallion (n = 4) and at least two fresh semen samples per stallion were evaluated in each analysis. Results were expressed as mean ± standard deviation (SD) for cellular percentage for plasma membrane integrity, ΔΨm, sperm motility, DNA fragmentation, profile of ROS and mean fluorescence intensity (MFI) for the other assessments. The data analysis was performed using the Prism 6 software package (GraphPad, La Jolla, USA) applying D'Agostino's K2 test to assess the Gaussian distribution. The Brown-Forsythe and Bartlett`s tests were used to confirm homogeneity of variances. The stallion was considered as a random-effects factor. Statistical analyses occurred using a one-way ANOVA and Bonferroni's multiple comparison test. Statistical significance was established at $P < 0.05$.

3. Results

3.1. Sperm evaluation after thawing

3.1.1. Effect of cryopreservation on sperm membrane integrity

Evaluation of this variable was assessed to establish the magnitude of damage after the freezing-thawing process and how this damage changes after-thawing incubation. The results indicate that the frozen-thawed group had a lesser percentage of cells with intact plasma membranes compared with fresh samples (32.95 ± 4.42% and 64.48 ± 11.59%, respectively), and this difference increased at 4 h of incubation (21.40 ± 5.94% and 64.48 ± 11.59%, respectively; Fig. 1AI).

3.1.2. Effect of cryopreservation on mitochondrial membrane potential

The percentage of cells with high ΔΨm was less immediately after thawing compared to percentages for fresh semen, and this effect was greater at 4 h of the post-thaw incubation period (19.93 ± 7.86% and 69.38 ± 6.77%, respectively; Fig. 1BI). In addition, by using TMRM staining it was possible to differentiate sperm populations with a relatively greater and lesser ΔΨm (Fig. 1BII), and its fluorescence signal was mostly located in the midpiece of the spermatozoon (Fig. 1BIII), and cell region of mammalian sperm where mitochondria are located.

3.1.3. Effect of cryopreservation on peroxidation of membrane lipids and DNA fragmentation

Lipid peroxidation has been involved in loss of quality of cooled-stored or cryopreserved semen. Fig. 1C depicts the finding that frozen-thawed sperm undergo greater lipid peroxidation compared to sperm in fresh semen. Lipid peroxidation was also increased during post-thawing incubation (Fig. 1CI) and was associated primarily with dead spermatozoa positive for PI (Fig. 1CII). Interestingly, the fluorescent signal was located mainly in the midpiece of the spermatozoon (Fig. 1CIII).

To ascertain more completely the alterations that stallion spermatozoa undergo after thawing, there was also evaluation of the integrity of sperm DNA. The results indicate a non-significant increase in the percentage of cells with fragmented DNA immediately after thawing, however; at 4 h of post-thaw incubation, the percentage of cells with fragmented DNA was markedly greater compared to what occurred in the fresh sample ($P < 0.05$; Fig. 1DI-III).

3.1.4. Effect of cryopreservation on ROS production

Because cryopreservation can induce ROS production in spermatozoa (Wang et al., 1997), intracellular ROS concentrations were also analyzed. Immediately after thawing there was an increase of ROS production, with a further increase (greater than three times) at 4 h of post-thawing incubation compared to what occurred with fresh semen (Fig. 1EI and II). It, however, was observed that the fluorescent signal of both, DHE probe (Fig. 1EIII) and Superoxide Detection Reagent (Fig. 1 FI, II and III), were mainly located in the head region of the spermatozoa.

3.1.5. Profile of ROS in thawed stallion spermatozoa

To design an efficient strategy to prevent the oxidative stress, in addition to the evaluation of ROS concentrations, it is also important to profile ROS generation. Fig. 2 depicts that there were similar results as those depicted in Fig. 1D, frozen-thawed sperm

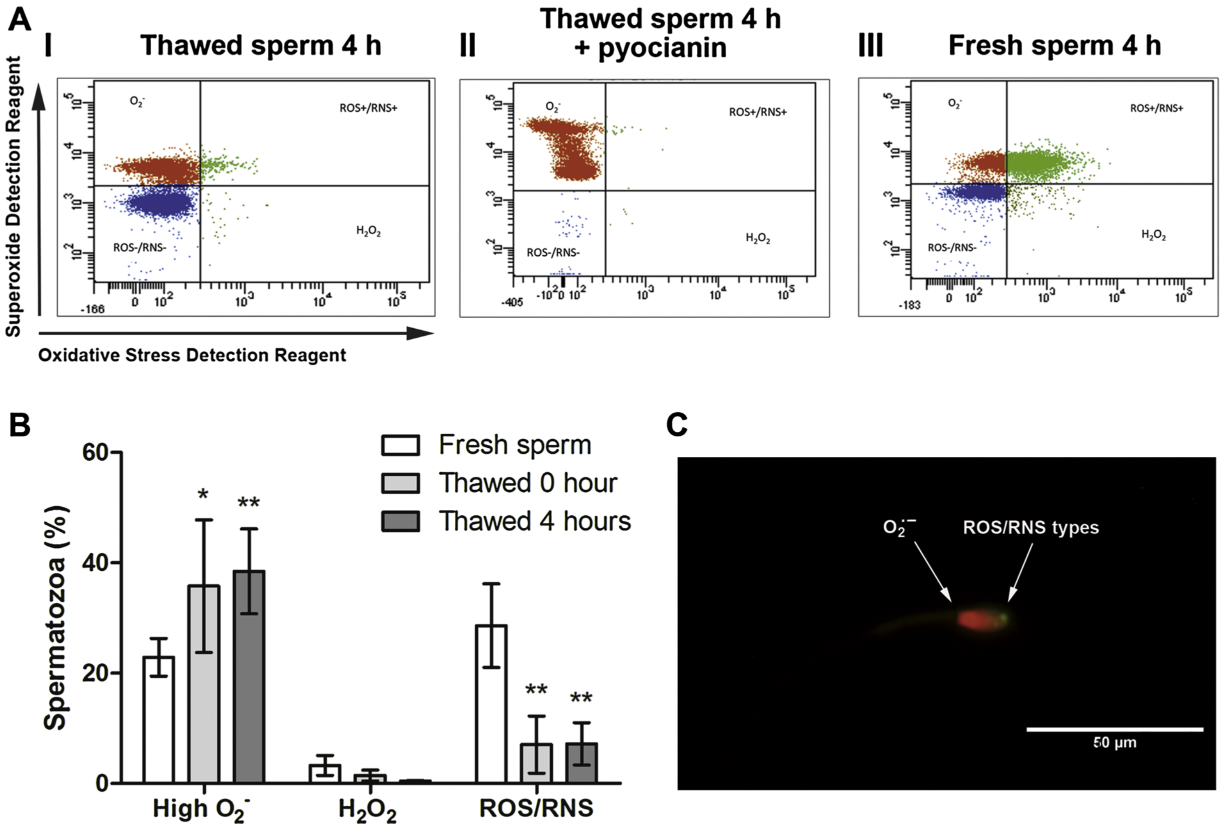

Fig. 2. Detection of comparative concentrations of ROS/RNS in fresh stallion spermatozoa (4 h of incubation), thawed and 4 h post-thawing; Values represent the mean ± SD of eight separate experiments (**P* < 0.05; ***P* < 0.01); (A) Representatives cytometry plot of ROS-ID™ total ROS/Superoxide Detection Kit staining (I) thawed sperm 4 h post-thawing, (II) thawed sperm co-incubated with pyocyanin and (III) fresh spermatozoa; (B) Prolife of ROS production of fresh and thawed sperm (C) Representative microscopy picture of stallion sperm stained with ROS-ID™ total ROS/Superoxide Detection Kit, at 4 h of post-thaw incubation; Magnification 100X.

had greater concentrations of ROS at both, 0 and 4 h post-thawing compared to fresh semen, and $O_2^{\cdot-}$ was the primary ROS produced after post-thawing incubation. The main ROS produced by thawed spermatozoa after 4 h of incubation was $O_2^{\cdot-}$ and a smaller percentage of cells produced RNS and other ROS (different to $O_2^{\cdot-}$) compounds (Fig. 2AI). Likewise, when thawed sperm were incubated with pyocyanin, a general ROS inducer, only one profile of $O_2^{\cdot-}$ was recorded (Fig. 2AII). Conversely, fresh sperm produced mainly RNS and other ROS types other than $O_2^{\cdot-}$ after 4 h of incubation (Fig. 2AIII and B). There were no changes in the production of H_2O_2 in both, fresh and thawed sperm during the incubation period (Fig. 2B). The fluorescence microscopy analysis revealed that the production of RNS and other ROS (indicated by green fluorescence signal) in stallion spermatozoa occurs mostly in the acrosomal region (Fig. 2C).

3.1.6. Evaluation of sperm motility after thawing

Considering that ΔΨm dissipation and overproduction of ROS have been associated with abnormal sperm motility (Agarwal et al., 2008), the effects of cryopreservation on sperm motility were subsequently analyzed. The results indicated that total and progressive motility of thawed sperm decreased markedly during the incubation time and this effect was greater compared with fresh sperm ($P < 0.05$; Fig. 3A and B, respectively).

3.2. Analysis of supplementation of semen extender with antioxidants on plasma membrane integrity and ROS production

The effect of different concentrations of MnTBAP (50, 100, 150 µM), NAC (5, 10 and 15 mM) and FeTPPS (5, 15 and 25 µM) on ROS production after thawing are depicted in Fig. 4. There was a decrease in the percentage of cells with relatively greater $O_2^{\cdot-}$ content when there was treatment with 150 µM MnTBAP at 4 h of incubation compared with the untreated control (17.4 ± 4.5% and 38.4 ± 7.6%, respectively) (Fig. 4A). The treatment with 25 µM of FeTPPS resulted in similar positive effects, decreasing the percentage of cells with relatively greater $O_2^{\cdot-}$ content after the onset of post-thaw incubation compared to the untreated control (7.2 ± 3.6% and 35.7 ± 6.1%). The treatment with 15 µM of FeTPPS was effective only at 4 h of post-thaw incubation compared with the untreated control (13.6 ± 3.3% and 38.4 ± 7.6%, respectively; Fig. 4B). None of the concentrations of NAC that were used decreased the percentage of cells with relatively greater $O_2^{\cdot-}$ content compared to the untreated control (Fig. 4C).

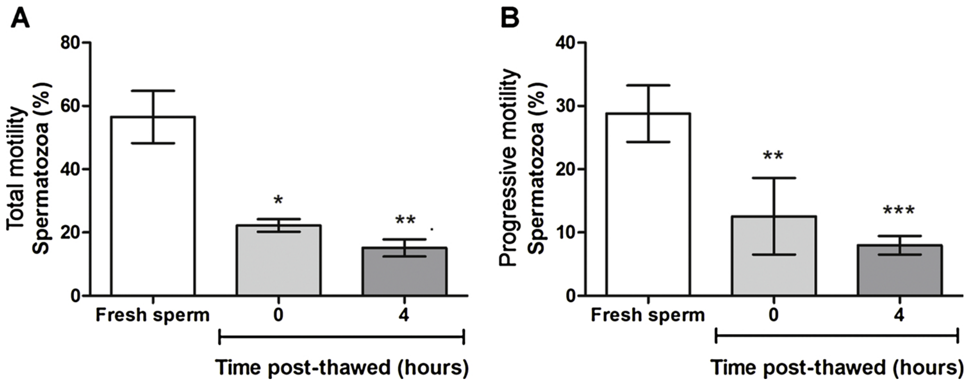

Fig. 3. Analysis of sperm motility in spermatozoa from fresh, thawed semen and 4 h after thawing; (A) Total motility, (B) Progressive motility; Data are presented as mean ± SD of at least eight experiments on different days (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

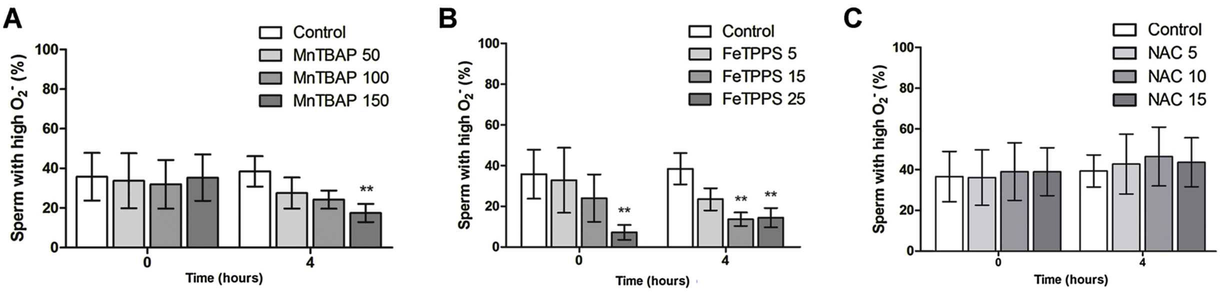

Fig. 4. Effect of extender supplementation with three antioxidants on $O_2^{\cdot-}$ production in thawed stallion spermatozoa; (A) Extender supplemented with MnTBAP, (B) Extender supplemented with FeTPPS, (C) Extender supplemented with NAC; Data are presented as mean ± SD of eight experiments on different days (**$P < 0.01$).

The two compounds (MnTBAP and FeTPPS) with the greater scavenger effects for $O_2^{\cdot-}$ were selected and there was evaluation of the effects on plasma membrane integrity. The results indicate that MnTBAP at all concentrations that were used had a protective effect on the plasma membrane at 4 h of post-thaw incubation compared to the untreated control (Fig. 5A). Conversely, although the supplementation with FeTPPS resulted in a decrease the $O_2^{\cdot-}$ production of a similar magnitude as MnTBAP, there was a deleterious effect on the sperm membrane (Fig. 5B). In addition, unlike MnTBAP, the FeTPPS induced a change in the ROS profile decreasing $O_2^{\cdot-}$ concentrations but increasing H_2O_2 concentrations at 0 and 4 h of post-thaw incubation (Fig. 5C to F).

3.3. Effects of supplementation of semen extender with MnTBAP on quality variables in frozen-thawed stallion sperm

After determination that post-thawed supplementation of the extender with MnTBAP was effectively inhibiting excessive production of $O_2^{\cdot-}$ without affecting the plasma membrane integrity, there was continuation in evaluating its antioxidant effects on other sperm quality variables such as ROS production, $\Delta\Psi m$, membrane lipid peroxidation, DNA fragmentation and motility. The results indicated that, although treatment with MnTBAP decreased ROS production as assessed by DHE staining (Fig. 6A), it did not prevent the $\Delta\Psi m$ dissipation (Fig. 6B). Treatments with all of the concentrations assessed, however, decreased lipid peroxidation (Fig. 6C). The treatment with MnTBAP also resulted in maintenance of the integrity of DNA similar to those basal data recorded after thawing (Fig. 6D). In addition, MnTBAP-treated thawed sperm had improvements in motility variables as evidenced by the greater percentages of total and progressive motility at 4 h of post-thaw incubation, compared to the untreated control (Fig. 6E and F).

4. Discussion

Stallion sperm are extremely sensitive to cell alterations generated by freezing, osmotic changes induced during the process and osmotic stress resulting from exposure to hypertonic media (Devireddy et al., 2002). In addition, the removal of seminal plasma, a source of natural antioxidants, and other factors, contribute to promotion of a state of imbalance known as oxidative stress (reviewed by Amidi et al., 2016). The effects of oxidative stress are particularly important in frozen-thawed stallion spermatozoa (Ball, 2008), not only during the *in vitro* storage procedure, but also after thawing process (de Andrade et al., 2012). In view of these data, the present study was conducted to assess the profile of ROS production and assess the effects of post-thawing supplementation of semen extender with different antioxidants on the quality and function variables of frozen-thawed stallion spermatozoa incubated *in vitro*.

Results of the present study are consistent with previous studies indicating that freeze-thawing procedures damage the plasma membrane of stallion spermatozoa (Ortega-Ferrusola et al., 2008; Hoffmann et al., 2011). Similar to plasma membrane damage, the

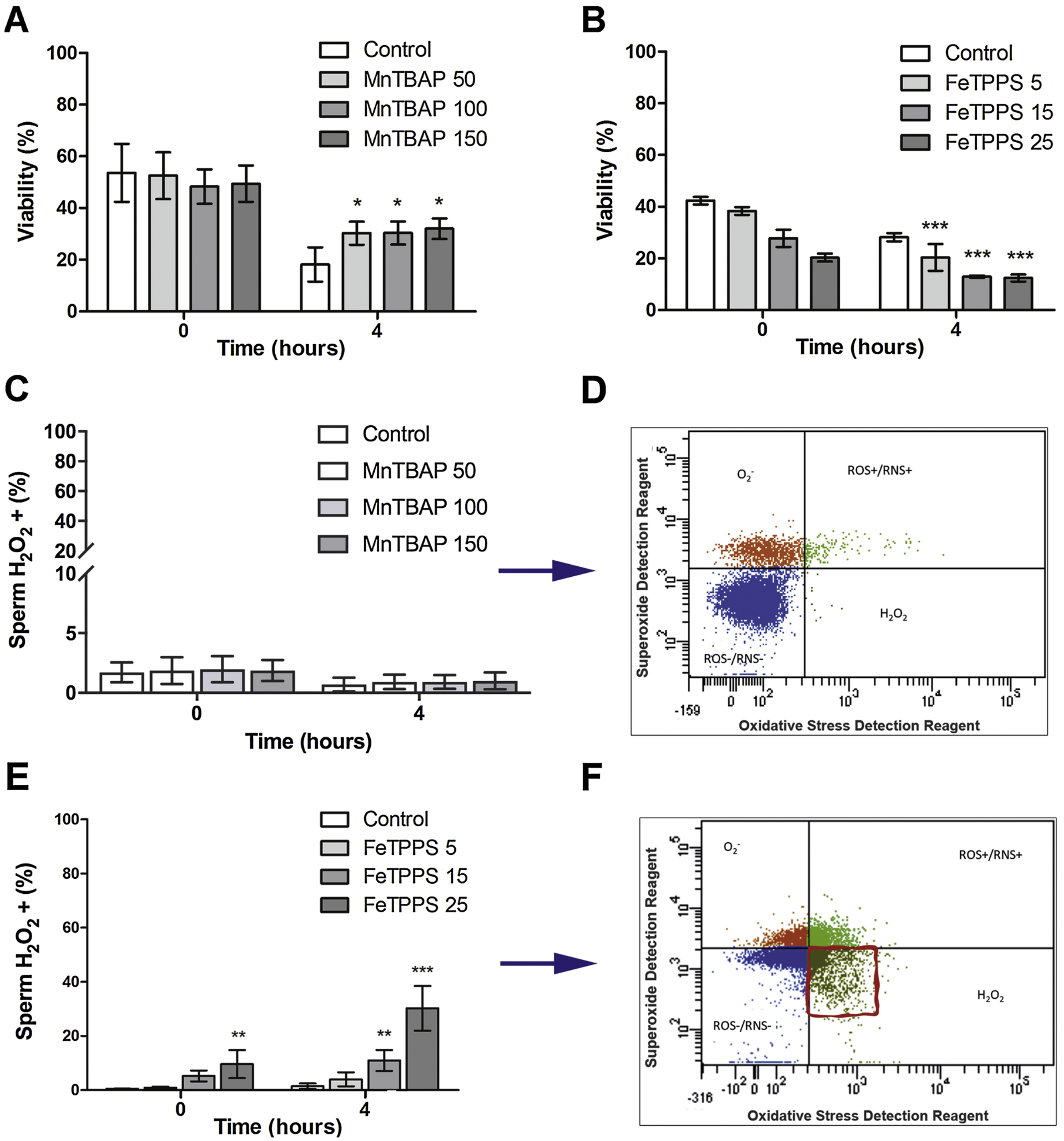

Fig. 5. Effect of extender supplementation with MnTBAP (A) and FeTPPS (B) on plasma membrane integrity in thawed stallion spermatozoa; (C) Effect of MnTBAP on intracellular H_2O_2 concentrations. (D) Representative example of one experiment by flow cytometry; (E) Effect of FeTPPS on intracellular H_2O_2 concentrations; (F) Representative example of one experiment by flow cytometry; Data are presented as mean ± SD of eight experiments on different days (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

mitochondrial membrane potential decreased immediately after thawing and this deterioration continued during the post-thaw incubation. Garcia et al. (2012) indicated that the changes in osmolarity of stallion sperm are more deleterious for the mitochondria than for the plasma membrane.

Similar to other studies, results of the present study indicate that cryopreservation was associated with increased in ROS production (Neild et al., 2003; Ball, 2008), and these ROS concentrations increased about three times at 4 h of post-thaw incubation compared to that of fresh sperm. This result suggests that it is important to supplement the medium with antioxidants not only prior to freezing but also after thawing. As expected, increased ROS concentrations were associated with increased lipid peroxidation. Cryopreservation can damage sperm cells *via* peroxidation of membrane lipids and production of toxic aldehydes leading to

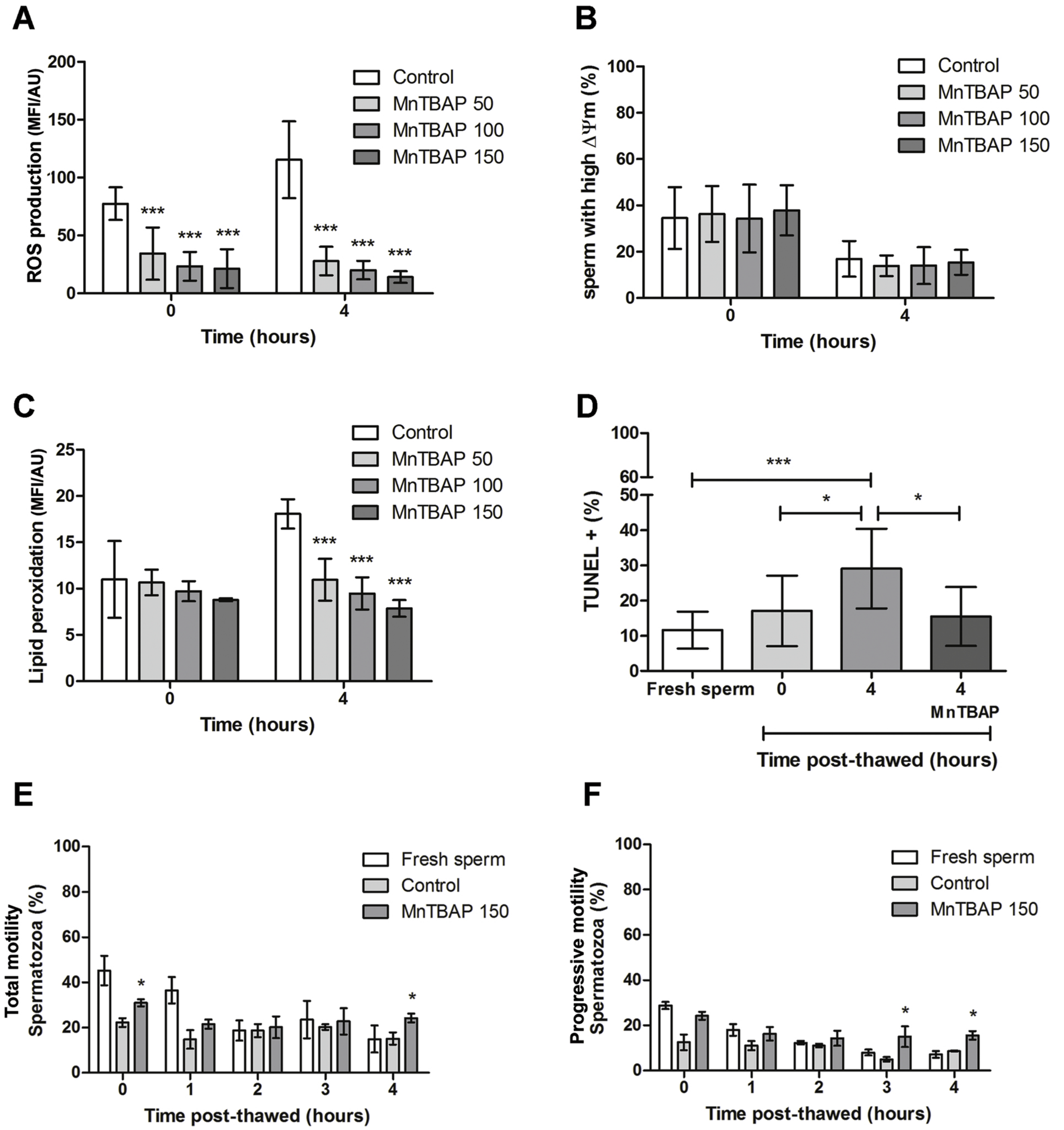

Fig. 6. Analysis of sperm quality variables in stallion spermatozoa at 0 and 4 h after thawing and supplemented with MnTBAP; (A) ROS production, (B) Sperm with high ΔΨm, (C) Lipid peroxidation, (D) DNA fragmentation, (E) Total motility, (F) Progressive motility; Data are presented as mean ± SD of eight experiments on different days (**P* < 0.05; ****P* < 0.001).

mitochondrial damage, reduction in membrane fluidity, loss of integrity of plasma membranes and damage of the sperm DNA (reviewed by Aurich et al., 2018), which is consistent with the deterioration of sperm DNA and motility observed in the present study.

Interestingly, in the present study there was a differential *in vitro* production of ROS between frozen-thawed and fresh sperm, $O_2^{\cdot -}$ was the primary ROS generated by stallion sperm after thawing which is consistent with the results of Baumer et al. (2000). Conversely, fresh sperm produced mainly RNS and other types of ROS different from $O_2^{\cdot -}$. There were indications in the results of a previous study by Aitken and Curry (2011) that because of cryopreservation the sperm mitochondria continues producing $O_2^{\cdot -}$, whereas nitric oxide generation is disrupted. Because of this imbalance in production of these substrates, the spermatozoa´s redox balance would shift from peroxynitrite to predominantly $O_2^{\cdot -}$, which is consistent with the findings in the present study. It appears as though the redox imbalance is related to the functional alterations of thawed stallion sperm, which result from a failure of sperm capacitation, which subsequently decreases the fertilizing capacity. These results allow for establishment of an effective supplement for sperm extenders with antioxidants. It, however, is important to determine previously the profile of ROS produced, which will probably depend on the storage and handling conditions and the species producing the spermatozoa.

The mechanism of sperm ROS production is not yet completely clear and there is a lack of knowledge regarding the sperm-metabolic mechanisms post-thawing. Stallion sperm produce ROS *in vitro via* NADPH oxidase and the membrane damage resulting from the freeze-thawing, increases the amount of ROS generated, possibly by increasing the permeability to NAPDH (Ball et al., 2001). The fluorescence microscopy analysis results from the present study indicate that ROS ($O_2^{\cdot-}$ and others different to $O_2^{\cdot-}$) generation was mainly located in the sperm head, which was probably the result of the enzymatic NADPH system. These findings, however, are in contrast with those reported by Ertme et al. (2017) where it was determined that ROS predominantly accumulated in the sperm midpiece. This inconsistency in results can be attributed to the fact that these authors induced oxidative stress artificially using the Xanthine-xanthine oxidase system, while in the present study stallion spermatozoa generated ROS naturally in response to freeze-thawing and *in vitro* incubation conditions. In addition, in the previous study there was monitoring of ROS concentrations using H_2DCFDA, a cell permeable probe that detects intracellular production of H_2O_2 (Yeste et al., 2015). On the contrary, in the present study was used for the first time in sperm cells, a commercial kit that allows for distinguishing between different reactive species as validated by fluorescence microscopy (Kato et al., 2013) and flow cytometry (Wang et al., 2015).

Furthermore, studies in somatic cells suggest that activation of membrane-associated phospholipase A2 (PLA2) in response to osmotic stress, promotes the activation of a NADPH oxidase, which would be the main enzyme responsible of $O_2^{\cdot-}$ increases in response to osmotic stress (Lambert et al., 2006). Aitken et al. (2006) speculated that the products generated by the PLA2 in human sperm would activate the NADPH oxidase with a concomitant increase in $O_2^{\cdot-}$ production. Thus, it is believed that the inhibition of both, NADPH oxidase and PLA2 could be an effective strategy to avoid the oxidative stress and to improve the quality variables of cryopreserved mammalian spermatozoa.

Results of previous studies indicate that addition of antioxidant molecules and/or enzymes to the freezing medium has a positive impact on the function and quality variables of spermatozoa (Bucak et al., 2010; Gadea et al., 2011; Kalthur et al., 2011). Results of the present study indicate that only MnTBAP and FeTPPS decreased the percentage of cells with relatively greater concentration of $O_2^{\cdot-}$. These results were expected because MnTBAP is a mimetic of SOD and although it has been described that FeTPPS has a primary role in the decomposition of $ONOO^-$, results of the present study indicate that FeTPPS has a basal superoxide dismutase effect, increasing the percentage of positive cells for H_2O_2, in agreement with previous data reported by Khan et al. (2011). The NAC, however, is an antioxidant that reduces H_2O_2 but reacts relatively slowly with $O_2^{\cdot-}$ (Owada et al., 2013), which may explain its poor antioxidant effect observed in the present study, because $O_2^{\cdot-}$ was the main ROS detected.

In the present study, after the observation that only MnTBAP and FeTPPS had a positive effect on $O_2^{\cdot-}$ decomposition, there was evaluation of the effects of these compounds on plasma membrane integrity. MnTBAP increased the percentage of cells with intact plasma membrane at all concentrations assessed. Even though there was a positive effect on $O_2^{\cdot-}$ decomposition, FeTPPS had no effect on plasma membrane integrity, because unlike MnTBAP, it does not contribute to breaking down the H_2O_2. Hydrogen peroxide is considered to be more toxic than $O_2^{\cdot-}$, because H_2O_2 is more stable and more readily crosses the plasma membranes (Halliwell, 1991). Thus, the increase of intracellular H_2O_2 would be responsible of the lesser sperm membrane integrity observed in the FeTPPS-treated group of the present study.

When there was evaluation of the effect of MnTBAP on other variables of cell quality and function in the present study, it was found that this antioxidant was also capable of suppressing lipid peroxidation and DNA fragmentation. In addition, there was a positive effect of this treatment on sperm motility, however, there continued to be mitochondrial membrane potential dissipation. Other mechanisms, in addition to oxidative stress, are involved in the deterioration of mitochondrial function, including mitochondrial permeability transition (Ortega Ferrusola et al., 2010; Treulen et al., 2018), release of hydrolytic enzymes from lysosomes (McGann et al., 1988) or damage to mitochondrial membranes produced by osmotic shock (De Oliveira et al., 2017).

MnTBAP has a broad array of antioxidant activities (Tumurkhuu et al., 2007), that have been investigated in different species including horses, sheeps, and rats. Shojaeian et al. (2018) reported that MnTBAP had beneficial effects on sperm quality, decreasing ROS concentrations and DNA fragmentation, and preserving plasma membrane integrity and viability when added to the cryopreservation medium. In most of studies relating the effects of antioxidant supplements there has been evaluations immediately or shortly after semen thawing (da Silva et al., 2011; Ertmer et al., 2017). In the present study, there was assessment during a longer post-thaw *in vitro* incubation period (4 h) which coincides with the interval for sperm placement in the uterus to reach the oviduct (Bader, 1982; de Andrade et al., 2012). This incubation time is also required for stallion sperm to have the "capacitation like" changes including tyrosine phosphorylation (McPartlin et al., 2008). The study of *in vitro* incubation conditions for stallion sperm is of increasing interest for the equine industry, owing to its implications for *in vitro* fertilization (IVF) technology, because efficient methods for standard IVF have not yet been identified (Reviewed by Leemans et al., 2016). In addition, the differential production of ROS between fresh and thawed sperm that occurred in the present study could be of interest to implement better strategies to preserve the fertilizing potential of raw and/or cryostored stallion sperm.

5. Conclusions

The results of the present study confirm for the first time a differential ROS production between frozen-thawed and fresh stallion spermatozoa incubated *in vitro* being superoxide anion the main ROS produced in frozen-thawed sperm. Supplementation of post-thawed semen extender with MnTBAP is an effective strategy to improve the quality of cryopreserved stallion sperm and preserve the fertilization potential. Future studies are still necessary to assess the effect of MnTBAP on the functionality of frozen-thawed stallion spermatozoa, in particular on *in vitro* sperm capacitation as well as on *in vitro* and/or *in vivo* fertilization potential.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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