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Sperm cryopreservation with supplementation of α-tocopherol and ascorbic acid in freezing media increase sperm function and fertility rate in Atlantic salmon (*Salmo salar*)

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ABSTRACT

The use of α-tocopherol and ascorbic acid in freezing medium prevents lipoperoxidation and damage by ROS (reactive oxygen species); however, the effects of these antioxidants in salmonid spermatozoa have not been studied. In this work the protective effects of antioxidants on the enzymatic activity and fertilization capacity of frozen Atlantic salmon spermatozoa were studied. Spermatozoa were frozen in Cortland® medium supplemented with 1.3 M DMSO, 0.3 M glucose, 2% BSA and different concentrations of α-tocopherol and ascorbic acid; spermatozoa frozen without antioxidants were used as control and fresh sperm. Samples loaded in 0.5 ml plastic straws were frozen at 68 °C/min (Freeze Control®). Thawing was carried out at 35 °C and post-thawing lipid peroxidation (LPO) was evaluated along with Glutathione (GSH/GSSG) levels; and Glutathione peroxidase (GPx) and Catalase (CAT) activity. Additionally, percentage of motility, mitochondrial membrane potential (ΔΨM), and fertilization rate were assessed. Results showed significantly lower LPO values (expressed as malondialdehyde concentration) in spermatozoa cryopreserved with α-tocopherol (0.1 mM and 0.5 mM) and with combinations of α-tocopherol/ascorbic acid (0.1 mM/1.0 mM and 0.5 mM/10 mM ratios) ($p < .05$). Moreover, spermatozoa frozen with α-tocopherol/ascorbic acid also exhibited significantly higher GPx and CAT activity as well as GSH/GSSG ratio compared to controls ($p < .05$). The production of O_{2i}^{-} also decreased with the combined treatment, however, no statistically significant difference was found compared to controls. Consistently with the improvement in antioxidant defences, the percentage of motility and ΔΨM were highest and significantly different in spermatozoa cryopreserved with α-tocopherol/ascorbic acid 0.1 mM/1.0 mM compared to controls, supporting the significantly higher fertilization rate exhibited by spermatozoa under the same treatment.

1. Introduction

Sperm cryopreservation can generate cellular damage that compromises the integrity of plasma membrane, motility and fertilization capacity of spermatozoa (Watson, 2000; Cabrita et al., 2010; Magnotti et al., 2016). These effects have been demonstrated in spermatozoa of various fish species: *Dicentrarchus labrax* (Zilli et al., 2003), *Sparus aurata* (Cabrita et al., 2005; Zilli et al., 2008), *Morone saxatilis* (He and Woods, 2004), *Oncorhynchus mykiss* (Merino et al., 2011) and *Salmo salar* (Figueroa et al., 2016). Oxidative stress has been considered one of the main causes of alterations in sperm function during cryopreservation. As such, an imbalance between ROS and the antioxidant system of spermatozoa leads to metabolic and functional alterations that increase the lipoperoxidation levels of membranes and oxidation of proteins (antioxidant enzymes), thus reducing the mitochondrial membrane potential (ΔΨM) and fertilization capacity of frozen spermatozoa (Ball,

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2008; Pérez-Cerezales et al., 2010; Figueroa et al., 2017).

Although semen antioxidant defense system is active in seminal plasma and within spermatozoa, its activity in cell cytoplasm is limited because of low availability in that compartment (Lahnsteiner and Mansour, 2010; Shiva et al., 2011). Additionally, diluting semen in freezing media causes reduction in the enzymatic and non-enzymatic antioxidant components of the seminal plasma; thus the spermatozoa are more vulnerable to oxidative stress during freezing (Figueroa et al., 2015). Various other studies have shown that in mammals and fish it is possible to reduce the damaging effects of ROS by supplementing the sperm freezing media with antioxidants (Zilli et al., 2005; Michael et al., 2007; Thuwanut et al., 2008a,b; Jeong et al., 2009; Cabrita et al., 2011; Figueroa et al., 2017). Despite this, the protective effects of these components may vary among species (Cabrita et al., 2010).

The vitamins E and C and the amino acids taurine and hipotaurine are powerful antioxidants naturally present in seminal plasma. Specifically, these antioxidants prevent the propagation of chain reactions induced by free radicals. Ascorbic acid and α-tocopherol are particularly important in these processes and both of them have the ability to prevent lipid peroxidation and reduce oxidative damage, hence improving sperm quality and reducing DNA fragmentation in thawed spermatozoa (Cabrita et al., 2010). There has been, however, little research to clarify the mechanisms by which oxidative damage is reduced in fish spermatozoa (Greco et al., 2005; Colagar and Marzony, 2009; Mendiola et al., 2010; Cabrita et al., 2011; Figueroa et al., 2017). Hence, the objective of this study was to evaluate the protective effects of α-tocopherol and ascorbic acid on lipid peroxidation and antioxidant enzyme activity in spermatozoa of Atlantic salom (*Salmo salar*) under cryopreservation conditions. We also measured the sperm motility rate, mitochondrial membrane potential, and fertilization rate of cryopreserved spermatozoa of Atlantic salmon, as key quality parameters strongly affected by freezing/thawing processes. Improvement of those parameters resulting from antioxidant supplementation during cryopreservation indicates good potential for optimization of this gamete preservation technique and its applications in the aquaculture industry.

2. Materials and methods

All chemicals used in this study were purchased from Sigma (St. Louis MO, USA) unless otherwise indicated. All solutions were prepared using water from a Milli-Q Synthesis System (Millipore, Bedford, MA, USA). The mitochondrial permeability Detection Kit AK-116 (MiT-*E-Ψ*™, JC-1), the superoxide anion production detection Kit (DHE/SYTOX® Greed), the Total Glutathione Assay Kit and Catalase Activity Assay Kit (OxiSelect™) and the Glutathione Peroxidase Activity Assay Kit (GPx, K762-100) were purchased from Invitrogen (Oregon, USA), Roche Diagnostics GmbH (Mannheim, Germany), Biomol International LP (Pennsylvania, USA), Molecular Probes (Oregon, USA), Luxcel Biosciences Ltd. (Cork, Ireland), Promega Corporation (Madison, USA), Cell Biolabs, INC® (San Diego, USA) and BioVision® (Milpitas, USA) respectively.

2.1. Broodstock

This study was carried out in the Laboratory of Engineering, Biotechnology and Applied Biochemistry (LIBBA) at the Frontera University, Chile; Microbial Biomolecules Laboratory, Department of Biochemical-Pharmaceutical Technology at the University of Sao Paulo, Brazil and the Aquaculture Biotechnology Unit (BIOACUI) of the School of Aquaculture of the Catholic University of Temuco, Chile. The twenty male *S. salar* used for this study were two to three years old (sexually mature) with average mass of 8.1 ± 0.4 kg and a total length 83 ± 0.7 cm. They were provided by Hendrix Genetics Aquaculture S.A and Aquagen Chile S.A, a commercial farm in southern Chile. During the experimental period, the broodstock were kept in 3500 l fiberglass tanks with fresh water (550 l h^{-1}) at 8 °C and were maintained with a natural photoperiod.

2.2. Collection of gametes

Sperm collection was performed as described by Figueroa et al. (2016) with some modifications. Briefly, the twenty males were anesthetized in a 50 l tank with 125 mg l^{-1} MS-222 for 10 min. The urogenital pore was dried and semen was collected by gentle abdominal massage, directly into graduated, sterile, dry, disposable plastic containers, maintained at a temperature of 4 °C. Care was taken to not contaminate the samples with feces, mucus, or urine.

Immediately after collection, sperm motility and concentration were determined using a phase contrast microscope (Carl Zeiss Jena, Jena, Germany). Prior to motility analysis, the samples were diluted 1:3 (semen: medium) in Cortland non-activating medium (Trus-Cott et al., 1968). Sperm motility (pre-diluted sperm) was activated with Powermilt® and evaluated by subjective microscopic examination using a phase contrast microscope (Carl Zeiss, Jena, Germany) at 400× magnification. Sperm motility was assessed as described by Cosson (2008). Spermatozoa concentration in standard culture medium (Cortland) for fish spermatozoa (described by Figueroa et al., 2016) was determined with a Neubauer haemocytometer. Only 15 samples (different broodstock males) exhibited sufficiently high motility (> 80%) and average sperm concentration ($14 \times 10^9 \pm 2.7$ spermatozoa ml^{-1}) to be used independent in this study.

2.3. Freezing and thawing

Eight experimental groups were established according to the modified protocol of Martínez-Páramo et al. (2012): Group 1: Fresh sperm; Group 2: Frozen sperm without antioxidants; Group 3: Frozen sperm with α-tocopherol (0.1 mM); Group 4: Frozen sperm with α-tocopherol (0.5 mM); Group 5: Frozen sperm with ascorbic acid (1 mM); Group 6: Frozen sperm with ascorbic acid (10 mM); Group 7: Frozen sperm with α-tocopherol/ascorbic acid (0.1 mM/1 mM) and Group 8: Frozen sperm with α-tocopherol/ascorbic acid (0.5 mM/10 mM). The semen was frozen following the modified protocol of Figueroa et al. (2016). Frozen semen was diluted at 4 °C in Cortland medium supplemented with 1.3 M dimethyl sulphoxide (DMSO), 0.3 M glucose, and 2% bovine serum albumin (BSA). The dilution ratio was 1:3 (semen/cryoprotectant medium). For 7 to 10 min after dilution, the semen was stored in 0.5 ml plastic straws, which were sealed after filling. Subsequently, the straws were cryopreserved using the programmable freezing system *Freeze Control*® (Cryologic, Australia) at a rate of 62.3 °C/min from 4 °C to −120 °C in liquid nitrogen (N_2L) which is controlled by the software CryoGenesis 5.0 (Cryologic). After two months of frozen storage, the straws were removed from the cryotanks and thawed in a thermoregulated bath at 40 °C for 9 s. The thawed semen was immediately used for evaluation of oxidative stress level, antioxidant enzymatic activity, motility, ΔΨM, and fertilization rate.

2.4. Evaluation of oxidative stress and antioxidant enzyme activity

The sperm samples were centrifuged at 1800 rpm for 10 min at 4 °C, and then the supernatant was discarded and the pellet was resuspended with 1 ml lysis buffer (Tris 50 mM, NaCl 100 mM, EDTA 1 mM, EGTA 2.5 mM, Tween-20 0.1%, PMSF 100 μg/ml, pH 8.0) at a concentration of 2×10^9 sperm/ml. Cell disruption was performed with glass beads (1 mm diameter), and the samples were homogenized using a vortex at 40 Hz for 5 min. Afterwards, the samples were incubated in an ice bath for 2 min. Subsequently, the samples were centrifuged at 10,000 rpm for 30 min at 4 °C. The supernatant was aliquotted and stored at −80 °C for later evaluation of the following parameters:

2.4.1. Lipid peroxidation (LPO)

The protocol for substances reactive to thiobarbituric acid (Tbars)

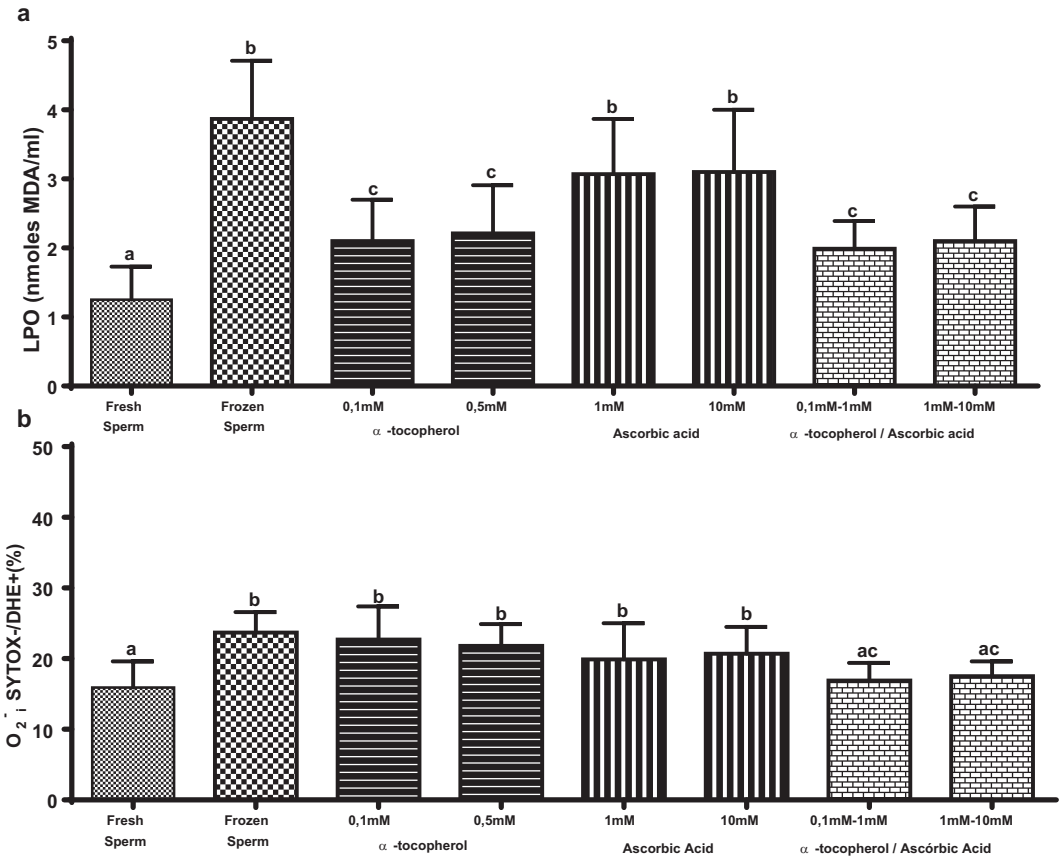

Fig. 1. Effect of cryopreservation in the incorporation of antioxidants in the freezing media of Atlantic salmon sperm. (a) Lipid peroxidation (LPO), and (b) Superoxide production (O_{2i}^{-}). Different letters show statistically significant differences ($p < .05$, $n = 10$).

described by Shaliutina-Kolesová et al. (2015) was adapted to evaluate lipid peroxidation in fish spermatozoa. A sample volume of 70 µl of each aliquotted treatment was incubated with 130 µl of thiobarbituric acid in a thermoregulated bath (98 °C) for 20 min and then cooled on ice for 5 min. The Tbars quantification was obtained by comparing the absorbance with a malondialdehyde (MDA) standard curve generated by hydrolysis catalyzed by 1,1,3,3-tetraethoxypropane acid. Absorbance was determined at 532 nm using a multimodal microplate reader (*SynergyTM HT*). The MDA values were expressed as nmol MDA/ml. The analysis in each trial was replicated three times.

2.4.2. Glutathione (GSH/GSSG)

The kit OxiSelect™ Total Glutathione Assay (Cell Biolabs, INC®) was used as a quantitative assay to measure the total glutathione content in the samples (GSH/GSSG). A sample volume of 100 µl of each aliquotted treatment was incubated with 25 µl glutathione reductase (1×) and 25 µl of nicotinamide adenine dinucleotide phosphate (1× NADPH) for 3 min. Afterwards, 50 µl of chromogen (1×) was added to start the reaction. Glutatione reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of NADPH. Subsequently, the chromogen reacts with the thiol group of GSH to produce a colored compound. The GSH/GSSG content was obtained by comparing the absorbance of the sample to a standard glutathione curve. Using a multimodal microplate reader (SynergyTM HT), the absorbance was determined at 405 nm during 10 min of incubation with 1 min reading intervals. The GSH/GSSG activity was expressed as µM/ml. The analysis in each trial was replicated three times.

2.4.3. Glutathione peroxidase (GPx)

The kit Glutathione Peroxidase Activity Colorimetric Assay (BioVision®), which is an indirect quantitative assay, was used to measure GPx activity. GPx reduced the concentration of cumene hydroperoxide in the samples, oxidatively reducing glutathione (GSH) to oxidized glutathione (GSSG). The reduction of GSSG to GSH depends on NADPH consumption by glutathione reductase (GR). Therefore, the decrease in NADPH is proportional to the activity of GPx. A sample volume of 10 µl of each aliquotted treatment was incubated for 15 min with 40 µl of the following reaction mixture: 33 µl buffer; 3 µl 40 mM NADPH solution; 22 µl GR solution; 2 µl GSH solution to remove all GSSG from the samples. Subsequently, 10 µl of cumene hydroperoxide was added to start the GPx reaction. The GPx activity was obtained by comparing the absorbance with a standard NADPH curve for 10 min at 25 °C. Absorbance was determined at 340 nm using a multimodal microplate reader (SynergyTM HT). GPx activity was expressed as nmol/min/ml. The analysis in each trial was replicated three times.

2.4.4. Catalase (CAT)

The OxiSelect™ Catalase Activity Assay (Cell Biolabs, INC®) kit was used to quantify the CAT activity. The disintegration rate of hydrogen peroxide (H_2O_2) by CAT to water and oxygen is proportional to the concentration of catalase during 1 min of the reaction. The remaining hydrogen peroxide was combined with a reaction mixture (chromogen) that generates a coupling product called quinoneimine that is correlated with the amount of H_2O_2 left in the reaction mixture. A sample volume of 20 µl of each of the aliquotted treatments was incubated with 50 µl of H_2O_2 (12 mM) for 1 min. Subsequently, the reaction was stopped with 50 µl of Catalase Quencher®, and 5 µl of the suspension was transferred to a free well. Then 250 µl of chromogen was added to each well, and the mixture was further incubated for 60 min with continuous orbital movement. The CAT activity was obtained by comparing the absorbance with a standard catalase curve using a second-order polynomial equation. Absorbance was determined at 520 nm

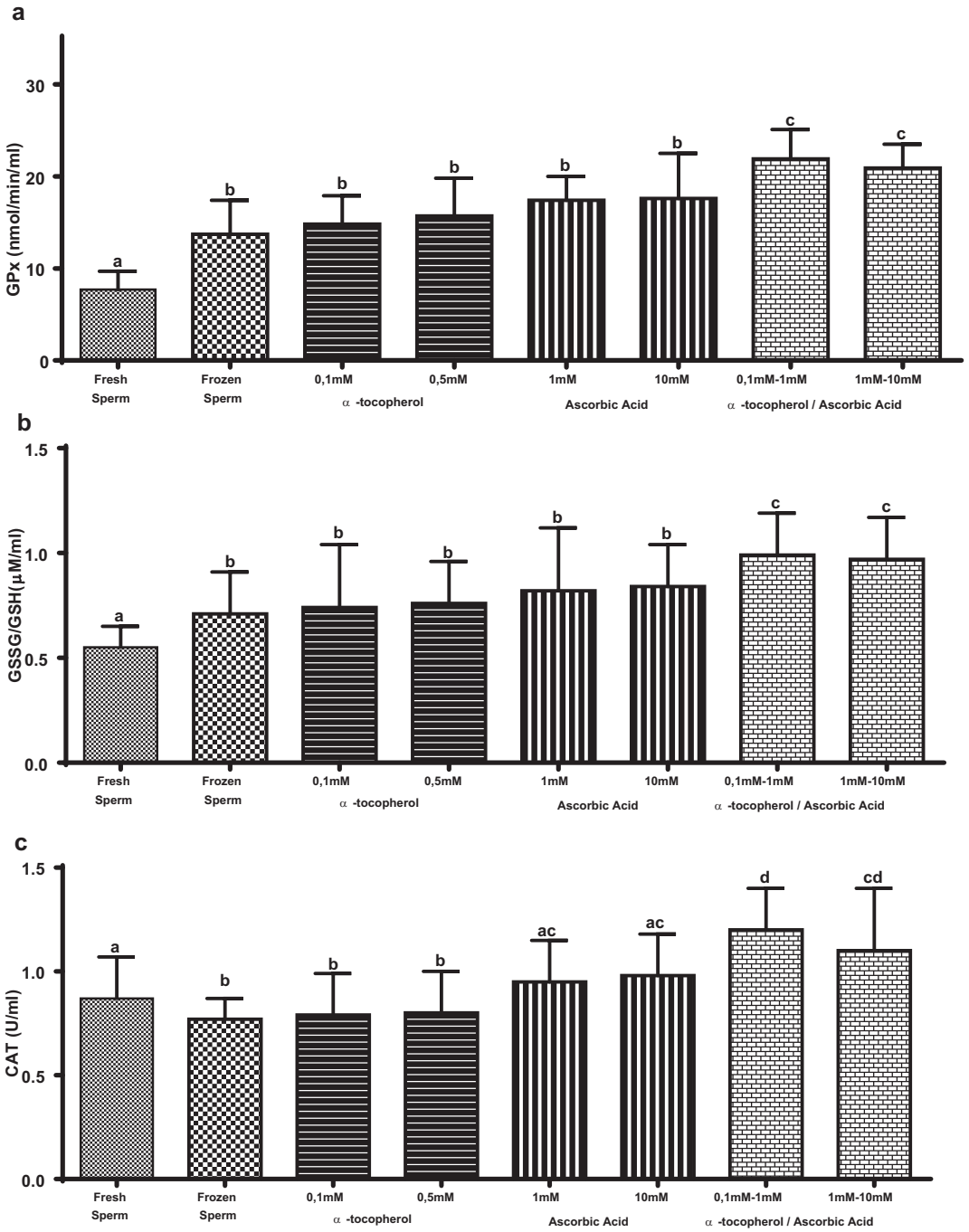

Fig. 2. Effect of cryopreservation in the incorporation of antioxidants in the freezing media of Atlantic salmon sperm. (a) Glutathione peroxidase (GPx), (b) Glutathione (GSH/GSSG) and (c) Catalase (CAT). Different letters present statistically significant differences ($p < .05$, $n = 10$).

using a multimodal microplate reader (SynergyTM HT). CAT activity was expressed as U/ml. The analysis in each trial was replicated three times.

2.5. Evaluation of sperm function

2.5.1. Motility

The percentage of motile spermatozoa (%) was determined using the modified protocol of Cosson (2004) and Li et al. (2012) for computer-assisted sperm analysis (CASA) using an optical microscope with stroboscopic light (Exposure Scope, CZK). Motility was determined using a phase contrast microscope (Olympus BX 41, Japan; with 200× magnification) after activation of motility. The samples were diluted in Powermilt® (280 mOs Kg-1 and pH 9.0). To prevent the spermatozoa from adhering to the slide, 0.25% (*w/v*) Pluronic (Sigma-Aldrich) was added to the activator. The spermatozoa were recorded with a digital video camera (SONY, SSC-G818) mounted on the microscope, filming at 25 frames per second at 50 Hz. The spermatozoa were analyzed using ImageJ CASA software (VirtualDub, WinDV) for image and video processing. The analysis was replicated three times for each trial.

2.5.2. Mitochondrial membrane potential

To evaluate changes in the mitochondrial membrane potential (ΔΨM), the AK-116 Mitochondrial Permeability Detection kit (MiTE-Ψ, BIOMOL International LP, Plymouth Meeting, PA, USA) was used. This kit includes a fluorescent cation JC-1 (dye, 5,50,6,60-tetrachloro-

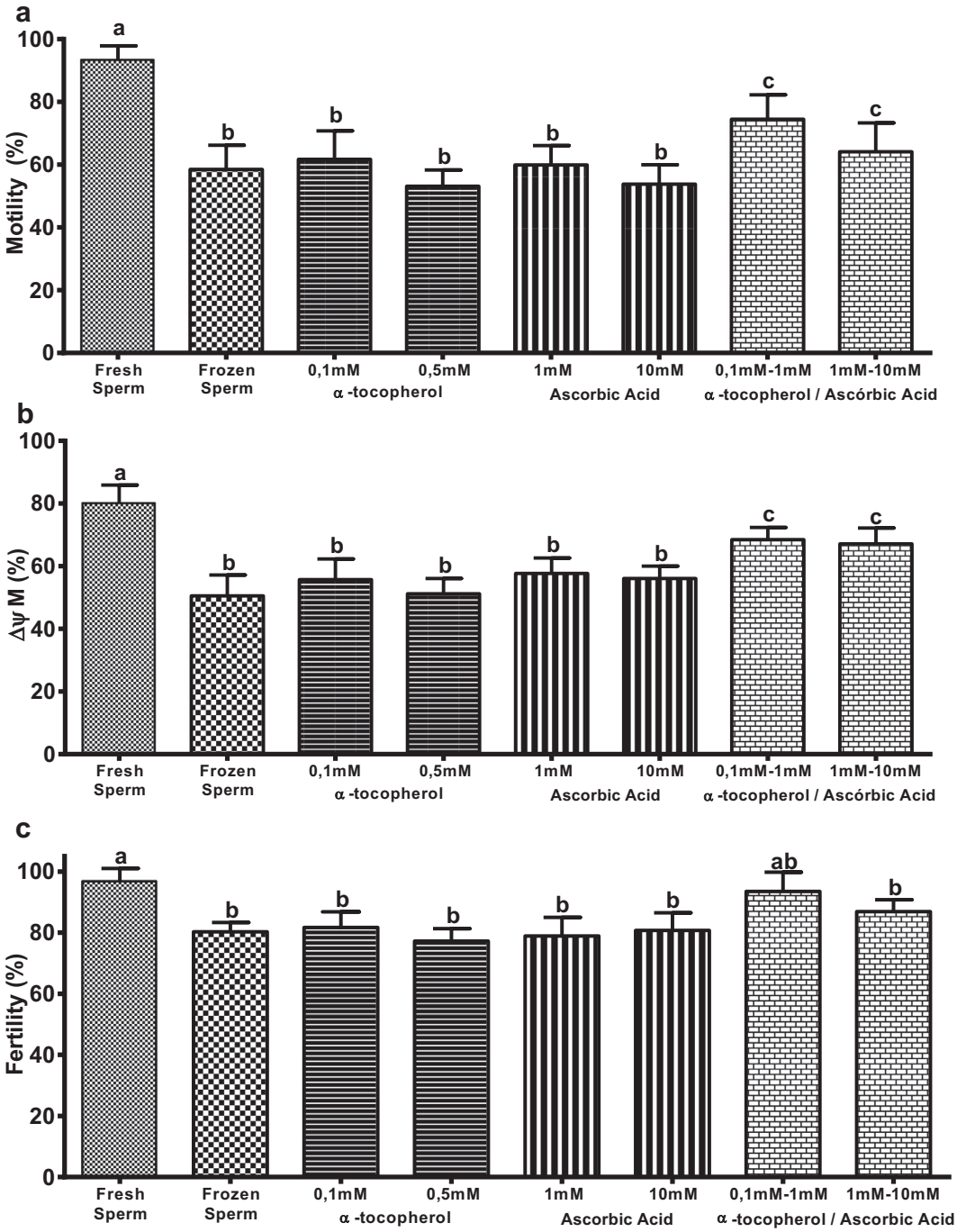

Fig. 3. Effect of cryopreservation in the incorporation of antioxidants in the freezing media of Atlantic salmon sperm. (a) Motility rate, (b) mitochondrial membrane potential (ΔΨM) and (c) fertility rate. Different letters present statistically significant differences ($p < .05$, $n = 10$).

1,10,3,30 tetraethylbenzymidazolyl carbocyanine iodide) which enters negatively charged mitochondria, forming dimers. The protocol described by Figueroa et al. (2016) was used with modifications. Briefly, 250 µl of spermatozoa suspension was centrifuged at 720 ×*g* for 5 min. The pellet was resuspended in 250 µl of JC-1 solution (3 mM JC-1 in DMSO) and was incubated for 15 min at 10 °C in the dark. Subsequently, the spermatic suspension was centrifuged for 5 min at 720 ×*g*, discarding the supernatant. The resulting pellet was resuspended in 400 µl of Cortland media for subsequent analysis by flow cytometry (BD FACS Canto II™). Sperm positive for JC-1 (intact potential) emitted red fluorescence. Measurements were taken in triplicated.

2.5.3. Intracellular superoxide (O_{2i}^{-})

The production of the superoxide anion (O_{2i}^{-}) was evaluated using the DHE (Dihydroethidium)/SYTOX® green (INVITROGEN®) kit following the protocol of Hagedorn et al. (2012) modified for fish spermatozoa. In general, the DHE probe is permeable and reacts with O_2^{-} to form 2-hydroxyethidium that is intercalated within ADN exhibiting red fluorescence. SYTOX is a green nucleic acid dye that only penetrates cells that have damaged membranes and does not penetrate the membranes of viable cells. To a sperm suspension of 250 µl (2.5×10^6 sperm/ml), 2.5 µl of DHE (5 mM) and 0.7 µl of SYTOX (5 mM) were added. These were then incubated for 10 min at 10 °C in the dark. Subsequently, the mixture was centrifuged at 1300 rpm for 5 min, discarding the supernatant and resuspending the pellet in 300 µl

of Cortland® medium. Detection was made using a flow cytometer (BD FACS Canto II™) considering live sperm that produced O_2^- (DHE +/SYTOX−). The production of O_2^- was expressed as a percentage in living cells. Measurements were taken in triplicated.

2.5.4. Fertilization rate

A pool of oocytes from 10 females (100 oocytes from each) was used for analyzing the fertilization of the fresh semen and cryopreserved treatments. The methods described by Figueroa et al. (2016) were used for the fertilization tests, and all of the tests were carried out in quintuplicate with 200 oocytes per replicate. The density of sperm used in the tests was 15×10^6 spermatozoa/oocyte. The eggs were incubated in an open flow chamber at 9 °C. After 16 h of incubation, fertilization was evaluated at 10 °C checking for the presence of the first cleavage stages (segmentation).

2.6. Multimodal reader

A *Synergy™ HTX Multi-Mode Microplate Reader* (BioTek®) was used to measure enzymatic activity. The samples were examined at a reading speed of 31 s for 96 wells. Fluorescence was measured using a *Xenon tungsten halogen flash* light source and emission (485/20 nm) and excitation (528/20 nm) filters together with a PMT detector (photomultiplier) to allow for high reading sensitivity.

2.7. Flow cytometry

A FACS Canto II (www.bdbiosciences.com BD Biosciences) flow cytometer was used to determine the mitochondrial membrane potential (JC-1). A minimum of 10,000 spermatozoa were examined in each assay at a flow rate of 100 cells s^{-1}. The excitation wavelength was 488 nm, and a solid-state laser (20 mW) and 633 nm He–Ne lamp (17 mW) were used. Green fluorescence was measured using FITC (533/30 nm), and red fluorescence was measured using PE (585/42 nm).

2.8. Statistical analysis

The data were expressed as means ± standard deviations. A non-parametric one-way ANOVA (Kruskal-Wallis test) and Dunn's multiple comparisons test were applied to evaluate the different treatments. The level of significance was set at $p < .05$. GraphPad Prism® (version 6.0) statistical software was used.

3. Results

3.1. Lipid peroxidation and production of O_{2i}^-

The concentration of MDA in spermatozoa cryopreserved with α-tocopherol (0.1 and 0.5 mM) or with a combination of α-tocopherol/ascorbic acid (0.1 mM/1 mM and 1 mM/10) (MDA values, respectively: 2.10 ± 0.6 nmol MDA/ml, 2.21 ± 0.7 nmol MDA/ml, 1.99 ± 0.4 nmol MDA/ml and 2.10 ± 0.5 nmol MDA/ml) was statistically lower than that found in spermatozoa frozen without antioxidants (3.87 ± 0.84 nmol MDA/ml; Fig. 1a; $p < .05$). Additionally, the production of O_{2i}^- (18.70 ± 2.5%) by sperm supplemented with α-tocopherol/ascorbic acid (0.1 mM/1 mM) was lower than that found for sperm frozen without antioxidantes (23.70 ± 2.9%; $p < .05$) and was lower than that of fresh spermatozoa (15.90 ± 3.7%; Fig. 1b).

3.2. Antioxidant defences

It was observed that GPx activity was significantly higher (21.90 ± 3.2 nmol/min/ml and 20.90 ± 2.6 nmol/min/ml) and the total GSH/GSSG content (0.99 ± 0.2 μM/ml and 0.97 ± 0.2 μM/ml) were found for spermatozoa cryopreserved with 0.1 mM/1 mM and 1 mM/10 α-tocopherol/ascorbic acid compared to that found for sperm frozen without antioxidants (GPx = 7.9 ± 1.1 nmol/min/ml; GSH/GSSG = 0.51 ± 0.1 μM/ml; $p < .05$, Fig. 2a,b). Similarly, significantly higher CAT activity was found for spermatozoa frozen with α-tocopherol/ascorbic acid (0.1 mM/1 mM) compared to sperm cryopreserved without antioxidants (0.79 ± 0.1 U/ml; $p < .05$; Fig. 2c).

3.3. Sperm function parameters

Spermatozoa frozen with 0.1 mM/1 mM and 1 mM/10 α-tocopherol/ascorbic acid had significantly greater motility (74.4 ± 7.8% and 64.1 ± 9.2%) than sperm frozen without antioxidants (58.5 ± 7.7%; $p < .05$; Fig. 3a). Similarly, sperm frozen with α-tocopherol/ascorbic acid had significantly higher ΔΨM (68.5 ± 3.9% and 67.1 ± 5.1%) than sperm frozen without antioxidants (50.5 ± 6.7%; Fig. 3b; $p < .05$). Lastly, a significantly higher fertilization rate was found for sperm frozen with 0.1 mM/1 mM α-tocopherol/ascorbic than samples frozen without antioxidants (80.3 ± 3.0%; $p < .05$) and the fertilization rate of sperm frozen with 0.1 mM/1 mM α-tocopherol/ascorbic did not differ from that of fresh sperm (96.9 ± 4.1%; Fig. 3c; $p > .05$).

4. Discussion

The obtained results show that supplementing freezing media with α-tocopherol and ascorbic acid leads to decreased membrane lipoperoxidation and O_{2i}^- production, which, in turn, increases the ΔΨM and fertilization capacity of Atlantic salmon spermatozoa. These results agree with those of Martínez-Páramo et al. (2012) and Liu et al. (2015) for cryopreserved seabass (*Dicentrarchus labrax*) and Japanese gilthead seabream (*Pagrus major*), showing a protective effect of these antioxidants on the integrity of the plasma membrane and mitochondrial function. Additionally, media supplemented with α-tocopherol (0.3 mM to 5 mM, respectively) has been shown to protect thawed canine and feline sperm from lipoperoxidation, protein oxidation, and sperm malfunction (Michael et al., 2007; Thuwanut et al., 2008a,b). Overall, the protective effect of α-tocopherol relies on its lipophilic characteristics, allowing this antioxidant to insert itself in the lipid bilayer of the cell membranes, thus transferring H^+ atoms to free radicals such as hydroxyl radical (HO·), superoxide anion ($O_2\cdot^-$) and peroxyl radical (ROO·), and eliminating them before they can interact with polyunsaturated fatty acids (PUFAs) on the cell membrane (Krishnamoorthy et al., 2007).

On the other hand, Mirzoyan et al. (2006) have shown that Russian sturgeon (*Acipenser gueldenstaedti*) sperm media supplemented with ascorbic acid confers increased cell integrity and sperm function compared to spermatozoa frozen without antioxidants. These results agree with those obtained here for Atlantic salmon spermatozoa frozen in media supplemented with ascorbic acid and combinations of ascorbic acid and α-tocopherol (0.1 mM to 1 mM). Ascorbic acid is a water-soluble vitamin, which effectively removes hydroxyl (HO·), superoxide ($O_2\cdot^-$) and hydrogen peroxide (H_2O_2) radicals. ROS can extract hydrogen from ascorbate, which then becomes monodehydroascorbate. Then, this antioxidant can gain another electron to become dehydroascorbate. In this process, ROS are reduced to H_2O, and the oxidized forms of ascorbate are relatively stable and do not cause cell damage. In addition, ascorbic acid plays an important role in the transformation of the tocopheryl radical (vitamin E-O·) to reduced α-tocopherol (vitamin E-OH), acting as a synergistic agent of the antioxidant activity of α-tocopherol (Kefer et al., 2009).

Spermatozoa have a variety of antioxidant components including SOD (superoxide dismutase), CAT (catalase), GPx (glutathione peroxidase), GR (glutathione reductase), α-tocopherol, ascorbic acid, and glutathione (GSH), which can be altered during cryopreservation (Lahnsteiner and Mansour, 2010; Lahnsteiner et al., 2011; Osipova et al., 2016). Here we found lower CAT activity in Atlantic salmon

sperm frozen in media without antioxidant supplementation. This could be due to lower reduction rate of $O_2\cdot^-$ to H_2O_2 by SOD activity, thus increasing levels of intracellular $O_2\cdot^-$ that inhibits CAT activity (Lahnsteiner et al., 2011). According to Chen et al. (2010) and Lahnsteiner et al. (2011) the CAT and SOD activity of *Pagrus major*, *Salvelinus fontinalis* and *Oncorhynchus mykiss* sperm are affected during cryopreservation. However, these authors also found that, the activity of catalase is recovered by supplementing freezing media with α-tocopherol and ascorbic acid, favoring CAT activity in the regulation of intracellular ROS. On the other hand, Martínez-Páramo et al. (2012) have shown that addition of antioxidants such as α-tocopherol and ascorbic acid do not exert protective effects in motility and fertility during the cryopreservation of *Dicentrarchus labrax* sperm; however, these authors did find that additions of these antioxidants did neutralize the oxidative stress generated by cryopreservation.

Regarding GPx, it eliminates H_2O_2 generated by metabolic action and oxidative stress, while highly dependent on the concentration of GSH (glutathione). Our results showed that GPx activity and total glutathione concentration (GSSG/GSH) in Atlantic salmon spermatozoa supplemented with α-tocopherol and ascorbic acid were significantly higher than in controls. These results are similar to those found for frozen *Cyprinus carpio* and *Dicentrarchus labrax* spermatozoa (Li et al., 2010; Martínez-Páramo et al., 2012). Also, Krishnamoorthy et al. (2007) showed in mice that diets supplemented with α-tocopherol and ascorbic acid, lead to GPx activity and GSH concentration in spermatozoa, thereby decreasing levels of peroxynitrite ($ONOO^-$) and hydroxyl radicals ($HO\cdot$).

Reduced glutathione (GSH) is the main endogenous antioxidant produced by cells, and it participates directly in the neutralization of ROS and maintenance of exogenous antioxidants such as reduced (active) α-tocopherol and ascorbic acid. Stradaioli et al. (2007) and Gadea et al. (2011) have shown that GSH content in bovine and human sperm decreases after cryopreservation. Despite this, GSH can be regenerated from its oxidized form (GSSG) by glutathione reductase (GR) whose activity is induced by oxidative stress especially when the concentration of available glutathione (GSSG/GSH) in cells is low (Trenzado et al., 2007). Here, the concentration of glutathione (GSSG/GSH) in Atlantic salmon sperm did not decrease when sperm were supplemented with α-tocopherol and ascorbic acid rather the concentration increased significantly in the presence of these antioxidants. Similar results have been found for *Dicentrarchus labrax* spermatozoa supplemented with α-tocopherol and ascorbic acid, though no significant differences were found between sperm frozen with these antioxidants and those frozen without antioxidants (Martínez-Páramo et al., 2012).

In previous work (Figueroa et al., 2017) it is shown that greatest effects of cryopreservation are caused to the mitochondrial membrane potential, motility, and fertilizing capacity of frozen and vitrified fish spermatozoa. Also Kutluyer et al. (2014) reported that fertilization and hatching rates of frozen *Oncorhynchus mykiss* spermatozoa were not improved by supplementation with antioxidants. In this regard, lipid peroxidation of membranes, increased $O_2\cdot^-$ production, damage to midpiece, and ultrastructural alteration are believed to be relevant causes of cryodamage. Consistently, our results after supplementing the freezing media with antioxidants (α-tocopherol and ascorbic acid) showed increased ΔΨM, plasma membrane integrity, motility, and fertilization rate of Atlantic salmon spermermatozoa. Also, similar results were previously observed while cryopreserving *Oncorhynchus mykiss* and *Salmo salar* spermatozoa adding larger volumes of seminal plasma to the freezing media, which provide additional antioxidants defences (Figueroa et al., 2013, 2015). This is consistent with the results obtained here for the incorporation of α-tocopherol (0.1 mM) and ascorbic acid (1 mM)] in freezing media which allowed for increased motility (74%), mitochondrial integrity (68%) and fertilization capacity (93%).

Overall, the combined addition of α-tocopherol and acorbic acid to freezing media is advisable to optimize Atlantic salmon sperm cryopreservation. The use of these antioxidants can help to preserve sperm physiology and fertilization capacity during cryopreservation. The use of these antioxidants is a fundamental biotechnological tool for the genetic improvement and protection of species of aquaculture interest.

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