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Mitoquinone does not improve sperm cryo-resistance in bulls

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Abstract

Oxidative stress is associated with impaired post-thaw sperm quality. As mitochondria are the main source of reactive oxygen species (ROS) in sperm, the goal of this study was to evaluate effects of the mitochondria-targeting antioxidant Mitoquinone (MitoQ) during cryopreservation of bull sperm. Semen was collected from 11 Simmental bulls (two ejaculates per bull) and diluted in Triladyl® supplemented with various concentrations of MitoQ (0, 0.2, 2, and 20 nM) to a final concentration of 65×10^6 sperm/ml. After thawing (0 and 3 hr), we assessed the following sperm traits: sperm motility by computer-assisted sperm analysis (CASA), DNA fragmentation index by SCSA® and plasma and acrosome membrane integrity, intracellular calcium concentration, esterase activity, mitochondrial membrane potential and synthesis of ROS using two multicolour flow cytometric assays. After 3 hr of incubation, 20 nM MitoQ increased ($p < .05$) sperm ROS synthesis compared to Control, whereas none of the other quality parameters were altered ($p > .05$). Therefore, we concluded that addition of MitoQ to semen extender before cryopreservation of bull sperm was unable to improve post-thaw sperm quality. Furthermore, 20 nM of MitoQ increased frozen-thawed sperm ROS synthesis, without apparent negative effects on the evaluated sperm traits.

KEYWORDS

bovine, cryopreservation, MitoQ, oxidative stress, semen

1 | INTRODUCTION

Artificial insemination is a cattle breeding method implemented worldwide. Therefore, advances in bull sperm cryopreservation and subsequent fertility are a critical economic trait for breeding programmes (Grötter et al., 2019; Hitit et al., 2020). Increasing sperm cryosurvival persists as a major challenge, prompting ongoing research on new cryopreservation techniques and the addition of several constituents to semen extender (Chatterjee & Gagnon, 2001; Peris-Frau et al., 2020).

Production of reactive oxygen species (ROS) and the subsequent generation of controlled oxidative stress (OS) are important to several physiological pathways of sperm (Bollwein & Bittner, 2018; Moraes & Meyers, 2018). However, OS associated with sperm cryopreservation can reduce sperm quality (Gibb et al., 2020; Hitit et al., 2020). Sperm alterations induced by OS include membrane changes (fluidity, lipoperoxidation and ion channel dysfunction), reduced motility, DNA damage, mitochondrial electron transport disruption, and lower in-vitro and in-vivo fertility (Aitken et al., 2012,2016; Bahmyari et al., 2020; Bollwein & Bittner, 2018; De Castro et al., 2016).

Bull semen contains enzymatic antioxidants in both seminal plasma and sperm. Nevertheless, greater than 50% reduction in the enzymatic activity of both main sperm antioxidants (superoxide dismutase [SD] and reduced glutathione [GSH]) was observed after cryopreservation (Bilodeau et al., 2000), in addition to a decline in the antioxidant potential of seminal plasma caused by semen dilution prior to freezing (Gibb et al., 2020). Since both cooling and the freeze-thaw process increase sperm production of the superoxide radical ($O_2^{\bullet-}$) (Chatterjee & Gagnon, 2001), the aforementioned causes could contribute to excessive ROS production and subsequent OS.

Considering the deleterious effects of OS on sperm function, several studies were conducted to evaluate the influence of adding antioxidants to freezing media on post-thaw sperm quality (Amidi et al., 2016), but results have generally been inconsistent or controversial (Bahmyari et al., 2020). As sperm mitochondria are the main source of intracellular ROS, and $O_2^{\bullet-}$ is the primary ROS produced (Aitken et al., 2016; Bollwein & Bittner, 2018; Moraes & Meyers, 2018), mitochondria-targeting antioxidants could improve post-thaw sperm quality.

Among the available mitochondria-targeting antioxidants, one of the best characterized is Mitoquinone (MitoQ) (Smith & Murphy, 2010). MitoQ is composed of tetraphenylphosphonium (TPP) and ubiquinone, an antioxidant that can insert itself in the inner mitochondrial membrane stimulated by the mitochondrial membrane potential (Gottwald et al., 2018; Ross et al., 2008). Since MitoQ has a positive charge, it readily passes through mitochondrial membranes and accumulates several hundred-fold within the mitochondria, where the respiratory chain reduces ubiquinone to ubiquinol, acting as a chain-brake antioxidant (Cochemé et al., 2007; James et al., 2004).

MitoQ improved post-thaw viability and decreased lipid peroxidation of yellow catfish sperm (Fang et al., 2014), prevented experimentally induced testicular degeneration in a mouse model (Zhang et al., 2019), and increased the blastocyst development rate of bovine oocytes subjected to OS (Marei et al., 2019). Better sperm quality and activity of enzymatic antioxidants (superoxide dismutase, catalase and glutathione peroxidase) were observed in thawed human sperm treated with MitoTEMPO, a mitochondria-targeting antioxidant also constituted by TPP (Lu et al., 2018). MitoTEMPO also improved sperm quality and the fertility rate of cooled-stored ram sperm (Zarei et al., 2021). However, to the best of our knowledge, there is no information about the use of MitoQ with bull sperm. Therefore, the aim of the present study was to determine effects of MitoQ added to the extender on the post-thaw quality of bull sperm.

2 | MATERIALS AND METHODS

At the time of the study, all bulls were on a routine semen collection schedule at an artificial insemination station. The biological samples were retrieved from ejaculates collected for commercial purposes. No additional handling of the animals was performed for this experiment; therefore, no approval from an ethical committee was considered necessary.

2.1 | Chemicals and reagents

Unless otherwise indicated, chemicals and reagents were obtained from Sigma-Aldrich Co. (Buchs, Switzerland). MitoQ was purchased from Biozol (MCE-HY 10016; Eching, Germany). The stock solution of Mito Q (20 mM) was prepared by diluting it in dimethyl sulfoxide. Prior to use, the stock solution was diluted (v:v) in Triladyl® (Minitube GmbH; Tiefenbach, Germany), supplemented with 20% egg yolk, to achieve the desired final concentration of MitoQ for each treatment. The TNE buffer was prepared with Tris-NaCl-EDTA buffer (0.01 M Tris, 0.15 M NaCl, 1 mM EDTA, pH 7.4). The Multicolour Assay I consisted of propidium iodide (PI; P4170), phycoerythrin conjugated agglutinin of *Arachis hypogaea* (PE-PNA; GeneTex GTX01509; Irvine, USA), and Calcein violet (C34858), Fluo-4 AM (F14201), and MitoProbe™ DiIC1 (M34151) purchased from Thermo Fisher (Whaltham, USA). The Multicolour Assay II consisted of MitoProbe™ DiIC1, Zombie Violet™ (423114; BioLegend; San Diego, USA), and 2',7' dichlorofluorescein diacetate (H_2DCFDA; D6883). For the Sperm Chromatin Structure Assay, acid detergent solution (0.15 M NaCl, 0.08 N HCl, 0.1% Triton-X 100, pH 1.2) and acridine orange staining buffer (0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl, 0.1 M citric acid, pH 6.0) were used.

2.2 | Preliminary evaluation of MitoQ toxicity on thawed bull sperm

To verify the potential toxicity of MitoQ, stored straws from four Simmental bulls (four straws/bull) were thawed in a water bath (38 ˚C, 30 s). Thawed semen samples from each bull were homogenized in Tyrode's solution (1:1, v:v) and aliquoted individually into five treatments supplemented with 40 µg/ml Hoechst 33342, for discrimination of sperm from non-sperm particles, and various concentrations of MitoQ: Control (no MitoQ), 0.2, 2, 20 and 200 nM.

Semen was examined by CASA, as reported (Ibanescu et al., 2020), using the same settings. Briefly, after 15 min of incubation at 38˚C, 6 µl of the sample mix was loaded into a pre-warmed (38°C) 4-chamber Leja slide (IMV Technologies), and the kinematic parameters from a minimum of 1000 sperm in no less than five randomly selected fields were assessed using an IVOS II CASA System (version 1.10.1; Hamilton Thorne Inc., Beverly, MA). Percentages of total and progressive motility were recorded for analysis.

2.3 | Effect of MitoQ during bull semen cryopreservation

2.3.1 | Bulls, semen processing and evaluation

Using an artificial vagina, semen samples were collected during June 2020 from Simmental bulls (n = 11; 1–6 years of age), housed at

an artificial insemination centre located in Germany (49°34′46″N, 10°36′40″E), under the same feeding and management conditions. At the time of collection, all bulls were under a routine semen collection schedule. For the experiment, two ejaculates (replicates) from each bull were used, collected within a 1-week interval. Ejaculates were evaluated in terms of volume, progressive sperm motility (phase-contract microscope) and sperm concentration (SDM 5 photometer, Minitube GmbH; Tiefenbach, Germany) immediately after collection. Ejaculates with a volume ≥1 ml, progressive sperm motility ≥65% and a sperm concentration ≥500 × 10^6 sperm/ml were further processed.

For each replicate, ejaculates were individually diluted in Triladyl® with 20% egg yolk to a final concentration of 65 × 10^6 sperm/ml. Subsequently, 20 ml of extended ejaculate from each bull were harvested and split into four equal aliquots supplemented with various concentrations of MitoQ (0.2, 2 and 20 nM) or unsupplemented (Control). Extended semen samples were equilibrated at 4°C for 24 hr (Bucher et al., 2019; Ibanescu et al., 2020), packaged in 0.25-ml straws (IMV Technologies; L'Aigle, France) and submitted to a standard freezing procedure. Frozen samples were stored in liquid nitrogen until analysed.

Sperm analysis was performed ~30 days after cryopreservation. For each analysis, four straws from each bull/replicate/treatment were thawed in a water bath (38°C, 30 s), pooled and homogenized in a pre-warmed (38°C) 1.5-ml laboratory tube. Pooled samples were assessed by means of Computer-Assisted Sperm Analysis (CASA) and flow cytometry, immediately after thawing and after 3 hr of incubation at 38˚C.

Semen examination by means of CASA was performed as described in Section 2.2. For each sample, percentages of motile, rapid and slow sperm were recorded.

2.3.2 | Flow cytometric analyses

Two multicolour assays were used to assess the functional characteristics of the sperm. A plot of forward scatter area and forward scatter height, followed by a plot of side scatter area and side scatter height, was designed to exclude doublets (Figure 1) in all flow cytometric analyses (Bucher et al., 2019) performed using a CytoFLEX flow cytometer V5-B5-R3 (Beckman Coulter Inc.; Fullerton, CA, USA) operated by the CytExpert Software (Version 2.4).

The Multicolour Assay I was performed to simultaneously assess plasma (PI_{neg}, intact plasma membrane; PI_{pos}, damaged plasma membrane) and acrosomal membrane integrity (PE-PNA; PNA_{neg}, intact acrosomal membrane; PNA_{pos}, damaged acrosomal membrane), intracellular calcium concentration (Fluo-4 AM; F_{neg}, low intracellular calcium; F_{pos} high intracellular calcium), esterase activity (Calcein Violet; C_{pos}, high esterase activity; C_{neg}, low esterase activity) and mitochondrial membrane potential (MitoProbe™ DiIC1; M_{pos}, high mitochondrial membrane potential; M_{neg}, low mitochondrial membrane potential). Final concentrations of the fluorescent probes, sample dilution prior to analysis, as well as the lasers and filters used during the assay, have been described (Arslan et al., 2019). A total of 10,000 sperm events/sample were measured and saved as an FCS file. Although several subpopulations can be identified with this approach, we focussed on the following sperm traits: $PI_{neg}PNA_{neg}$ (intact plasma and acrosomal membranes, relative to the total sperm population); $C_{pos}M_{pos}$ (high esterase activity and mitochondrial membrane potential, relative to total sperm population); $PI_{neg}PNA_{neg}F_{neg}C_{pos}M_{pos}$ (intact plasma and acrosomal membranes, low intracellular calcium, high esterase activity, and high mitochondrial membrane potential, relative to the total sperm population).

The *Multicolour Assay II* mainly aimed to mainly assess ROS synthesis in sperm (H_2DCFDA). It was designed to avoid spectral overlap between H_2DCFDA and the Fluo-4 AM used in the first assay. However, it simultaneously evaluated plasma membrane integrity (Zombie Violet™; Z_{neg}, intact plasma membrane; Z_{pos}, damaged plasma membrane) and mitochondrial membrane potential (M_{pos} and M_{neg}). Synthesis of ROS was measured on the basis of median fluorescence intensity (MFI) of H_2DCFDA read from the histogram of each sample (Pool et al., 2020).

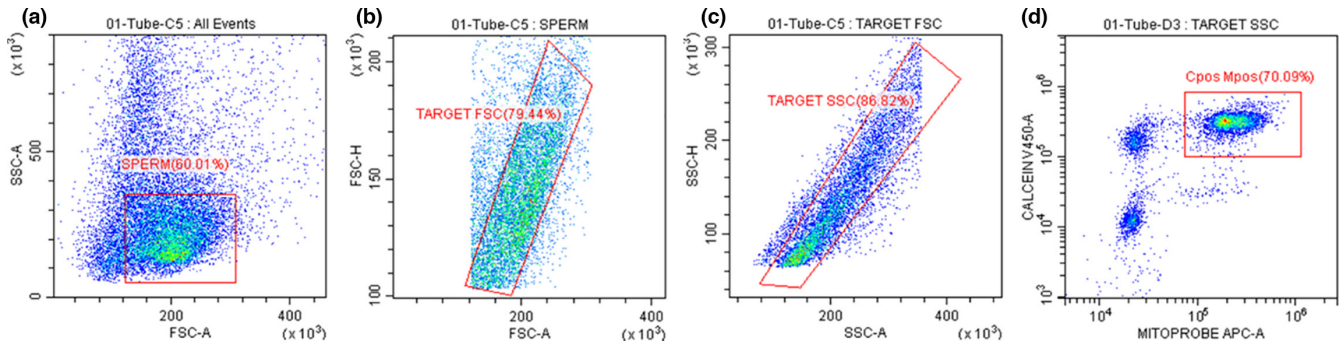

FIGURE 1 Sequence for gating sperm subpopulation with high esterase activity (Calcein Violet) and high mitochondrial membrane potential (Mitoprobe™ DiIC1). (a) Sperm population gating using FSC-A versus SSC-A. (b) Doublet exclusion using FSC-A versus FSC-H. (c) Doublet exclusion using SSC-A versus SSC-H. (d) Selected sperm subpopulation ($C_{pos}M_{pos}$). FSC-A, forward scatter area; SSC-A, side scatter area; FSC-H, forward scatter high; SSC-H side scatter high; $C_{pos}M_{pos}$, sperm subpopulation with high esterase activity and high mitochondrial membrane potential

In a reaction well of a 96-well plate maintained at 38°C, 5 μl of each sperm sample was diluted in 245 μl of Tyrode's solution containing MitoProbe™ DiIC1 (0.015 μM), Zombie Violet™ (1:100 dilution), and H_2DCFDA (100 μM). After 20 min of incubation in the dark (38°C), lasers with wavelengths of 405 nm (450 ± 45 nm V450 filter), 488 nm (525 ± 40 nm FITC filter), and 638 nm (660 ± 20 nm APC filter) were used to analyse Zombie Violet™, H_2DCFDA, and MitoProbe™ DiIC1, respectively. A total of 10,000 sperm events/sample were measured and saved as an FCS file. ROS synthesis was measured in the following sperm subpopulations: Z_{neg} only, M_{pos} only, and $Z_{neg}M_{pos}$.

2.3.3 | Sperm chromatin structure assay (SCSA)

Thawed sperm samples from each replicate/treatment were diluted in TNE buffer to a final concentration of 2×10^6 sperm/ml. Subsequently, 200 μl of the sperm suspension was diluted with 400 μl of acid detergent solution and homogenized for 30 s, followed by addition of 1.2 ml of acridine orange and incubation for 180 s at room temperature. After 3 min, samples were analysed after excitation with a laser at a wavelength of 488 nm. The DNA fragmentation index (%DFI) was calculated based on the histogram of sperm with single-stranded DNA (red fluorescence; 610 ± 20 nm ECD filter) versus the total sperm population (single-stranded DNA+ double-stranded DNA; green fluorescence; 525 ± 40 nm FITC filter), as reported (Bucher et al., 2019).

2.3.4 | Statistical analysis

The experiment was designed in randomized blocks, with two replicates constituted by eleven blocks (bulls) and four treatments (0, 0.2, 2 and 20 nM of MitoQ), eliminating the bull influence between treatments (St-Pierre, 2007). Sperm traits resulting from replicates and bulls were grouped, totalling 22 samples/treatment and are expressed as non-transformed means ± *SEM*. The variables used for comparison of sperm traits were the four treatments and post-thaw incubation time (0 and 3 hr). Differences between treatments within an incubation time and between incubation times within a treatment were assessed using the Friedman test, followed by a post hoc Wilcoxon test. For all analyses, results were considered significant at $p \leq .05$. All analyses were performed using SPSS Software (Version 24) and no data were transformed prior to analysis.

3 | RESULTS

No differences among treatments with 0.2, 2, and 20 nM of MitoQ during toxicity evaluation were observed ($p > .05$). However, progressive motility was higher in the Control than in the MitoQ 200 nM treatment after 15 min of incubation (31.20 ± 2.08 and 20.67 ± 1.27, respectively; $p < .05$). After 2 hr of incubation, both total and progressive motility were higher ($p < .01$) in the Control than in the MitoQ 200 nM treatment (43.50 ± 2.42 and 26.45 ± 1.18; 22.15 ± 1.93 and 1.85 ± 0.26, respectively).

There was no influence ($p > .05$) of the various MitoQ concentrations tested on kinematic parameters, sperm traits assessed using the Multicolour Assay I, and %DFI compared to the Control at both evaluation times (0 and 3 hr). Incubation time negatively influenced ($p < .05$) most of the evaluated sperm traits (Table 1). Furthermore, incubation time also increased the MFI of H_2DCFDA in all subpopulations, regardless of treatment. At 0 hr, MFI of H_2DCFDA in Mpos was higher ($p < .05$) in sperm incubated with MitoQ 20 nM compared to the Control. At 3 hr of incubation, higher MFI of H_2DCFDA was observed in all subpopulations (M_{pos} only, Z_{neg} only, $Z_{neg}M_{pos}$) of the MitoQ 20 nM treatment compared to the Control (Figure 2).

4 | DISCUSSION

To the best of our knowledge, this is the first report assessing effects of the addition of MitoQ to the extender on post-thaw bovine sperm quality. As recommended (Bahmyari et al., 2020), a preliminary simple dose-response test was performed to determine effects of MitoQ on thawed bull sperm. The reduction of total motility after 15 min of incubation, as well as that of both total and progressive motility after 2 hr of incubation, with progressive motility near to zero, indicated potential toxicity of MitoQ at 200 nM for thawed bull sperm. Although no sign of toxicity of MitoQ at 200 nM was reported on yellow catfish sperm, breast cancer cell lines displayed signs of toxicity when treated with MitoQ at 113 nM (Rao et al., 2010), implying differences among cell types and/or species. In other cell types, acute mitochondrial swelling was observed within 5 min after MitoQ treatment (Gottwald et al., 2018) and MitoQ can reach maximum mitochondrial absorption after 12 min (Ross et al., 2008), supporting our finding of a negative effect observed on sperm total motility after 15 min of incubation with MitoQ 200 nM. Therefore, the decision was made to exclude the MitoQ 200 nM sample from the cryopreservation experiment.

In the present study, the assessed sperm traits were related to the main effects of cryopreservation and ROS on sperm function or capacitation-like changes, such as the percentage of rapid sperm, plasma and acrosomal membrane integrity, the intracellular calcium concentration, mitochondrial membrane potential, and DNA integrity (Alonso et al., 2017; Rahman et al., 2017; Rivlin et al., 2004; Ruiz-Díaz et al., 2020; Visconti et al., 2011). The reduction in sperm quality after incubation was an expected outcome and could be intensified by the concurrent increase in OS during incubation, as reported (Gürler et al., 2016; Murphy et al., 2013). However, the addition of various concentrations of MitoQ was not able to alleviate sperm cryodamage immediately post-thaw or prevent deleterious effects of incubation, in contrast

TABLE 1 Characteristics of frozen-thawed bovine sperm cryopreserved with Triladyl® without (Control) and with addition of various concentrations of Mitoquinone (MitoQ; 0.2, 2, and 20 nM) after 0 and 3 hr of incubation at 38°C

Parameter	Group	Duration of incubation (hr)	
		0	3
Motile sperm (%)	Control	44.85 ± 1.29[a]	41.59 ± 1.28[b]
	MitoQ 0.2 nM	43.29 ± 1.66[a]	42.98 ± 1.44[a]
	MitoQ 2 nM	44.48 ± 1.36[a]	40.90 ± 1.58[b]
	MitoQ 20 nM	45.93 ± 1.76[a]	41.71 ± 1.72[b]
Rapid sperm (%)	Control	38.60 ± 1.30[a]	36.85 ± 1.25[b]
	MitoQ 0.2 nM	37.27 ± 1.59[a]	38.41 ± 1.44[a]
	MitoQ 2 nM	38.10 ± 1.27[a]	36.44 ± 1.60[a]
	MitoQ 20 nM	39.72 ± 1.70[a]	37.30 ± 1.65[a]
Slow sperm (%)	Control	6.25 ± 0.22[a]	4.74 ± 0.31[b]
	MitoQ 0.2 nM	6.01 ± 0.20[a]	4.56 ± 0.25[b]
	MitoQ 2 nM	6.38 ± 0.29[a]	4.45 ± 0.40[b]
	MitoQ 20 nM	6.21 ± 0.25[a]	4.36 ± 0.37[b]
$PI_{neg}PNA_{neg}$ (%)	Control	56.46 ± 1.40[a]	53.25 ± 1.42[b]
	MitoQ 0.2 nM	55.89 ± 1.61[a]	52.70 ± 1.66[b]
	MitoQ 2 nM	56.77 ± 1.56[a]	53.16 ± 1.68[b]
	MitoQ 20 nM	55.75 ± 1.86[a]	52.63 ± 1.83[b]
$C_{pos}M_{pos}$ (%)	Control	58.86 ± 1.11[a]	53.98 ± 1.35[b]
	MitoQ 0.2 nM	57.92 ± 1.49[a]	53.50 ± 1.59[b]
	MitoQ 2 nM	58.93 ± 1.32[a]	54.06 ± 1.52[b]
	MitoQ 20 nM	57.83 ± 1.76[a]	53.56 ± 1.65[b]
$PI_{neg}PNA_{neg}F_{neg}C_{pos}M_{pos}$ (%)	Control	41.85 ± 1.76[a]	37.74 ± 2.13[b]
	MitoQ 0.2 nM	40.68 ± 1.82[a]	37.25 ± 2.04[b]
	MitoQ 2 nM	42.59 ± 1.78[a]	38.79 ± 2.12[b]
	MitoQ 20 nM	42.14 ± 2.05[a]	38.31 ± 2.20[b]
%DFI	Control	2.63 ± 0.13[a]	4.29 ± 0.31[b]
	MitoQ 0.2 nM	2.65 ± 0.15[a]	4.63 ± 0.45[b]
	MitoQ 2 nM	2.75 ± 0.12[a]	4.18 ± 0.35[b]
	MitoQ 20 nM	2.75 ± 0.12[a]	4.23 ± 0.39[b]

Note: Values are means ± *SEM* of two ejaculates from 11 bulls (total of 22 ejaculates).

For each sperm parameter, different lowercase letters indicate differences between times within treatment ($p < .05$). There was no difference among treatments ($p > .05$) within time.

Abbreviations: %DFI: high DNA fragmentation index, relative to the total sperm population; $C_{pos}M_{pos}$, high esterase activity and mitochondrial membrane potential, relative to the total sperm population; $PI_{neg}PNA_{neg}$, intact plasma and acrosomal membranes, relative to the total sperm population; $PI_{neg}PNA_{neg}F_{neg}C_{pos}M_{pos}$, intact plasma and acrosomal membranes, low intracellular calcium, high esterase activity, and high mitochondrial membrane potential, relative to the total sperm population.

with a positive influence of MitoQ on viability of yellow catfish sperm post-thaw (Fang et al., 2014) and positive effects of using a mitochondrial-targeted antioxidant during cryopreservation of human sperm on mitochondrial membrane potential, motility and membrane integrity (Lu et al., 2018).

Effects of MitoQ on oxygen consumption rate of other cell types are controversial, ranging from no influence (Gottwald et al., 2018) to impairment of oxidative phosphorylation (Pokrzywinski et al., 2016; Sun et al., 2017). The lack of influence of MitoQ on sperm kinematics observed in our study could be explained by the capacity of bovine sperm to produce ATP using both anaerobic (glycolytic) and aerobic (oxidative phosphorylation) pathways (Losano et al., 2017; Magdanz et al., 2019).

Unexpectedly, 20 nM MitoQ increased the MFI of H_2DCFDA in all assessed subpopulations. H_2DCFDA has been widely used to measure H_2O_2, although other compounds (OH, $ONOO^-$ and esterase) can also influence the H_2DCFDA signal (Gürler et al., 2016; Halliwell & Whiteman, 2004; Hitit et al., 2020; Zhang et al., 2018). MitoQ comprises a TPP molecule, and its absorption by cells depends on the mitochondrial membrane potential

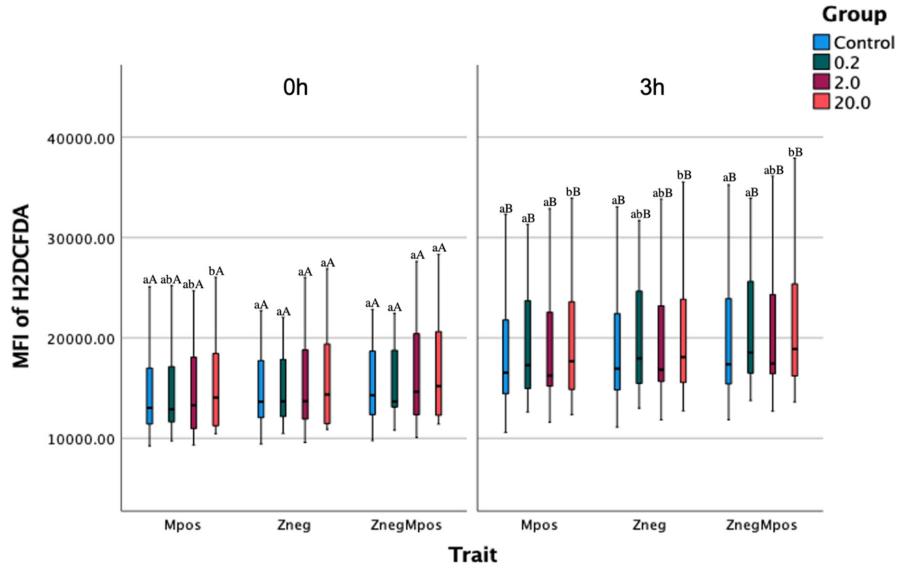

FIGURE 2 Boxplots containing traits of frozen-thawed bovine sperm cryopreserved with Triladyl® without (Control) and with addition of different concentrations of Mitoquinone (MitoQ; 0.2, 2, and 20 nM) after 0 and 3 hr of incubation at 38 ˚C. Each box contains data of two ejaculates from 11 bulls (in total 22 ejaculates). For each sperm parameter, different lowercase letters indicate differences among treatments within time, whereas different capital letters indicate differences between times within treatment ($p < .05$). MFI of H2DCFDA: median fluorescence intensity (MFI) of dichlorofluorescein diacetate (H_2DCFDA); Mpos: sperm with high mitochondrial membrane potential, relative to the total sperm population; Zneg: sperm with intact plasma membrane, relative to the total sperm population; ZegMpos: sperm with high mitochondrial membrane potential and intact plasma membrane, relative to the total sperm population

(Gottwald et al., 2018; Lu et al., 2018; Pokrzywinski et al., 2016; Rao et al., 2010; Ross et al., 2008). However, in the present study, it is unlikely that differences in mitochondrial membrane potential or esterase activity influenced the MitoQ concentration within mitochondria or caused the higher MFI of H_2DCFDA in sperm treated with 20 nM of MitoQ, as no difference in the $C_{pos}M_{pos}$ subpopulation was detected among treatments using the Multicolour Assay I. Conversely, MitoQ can stimulate the release of one-electron oxidizing species, such as $O_2^{\bullet-}$ (Doughan & Dikalov, 2007; James et al., 2004; Pokrzywinski et al., 2016; Rao et al., 2010). As $O_2^{\bullet-}$ is converted by superoxide dismutase to hydrogen peroxide (H_2O_2) (Aitken, 2017; Sakamoto & Imai, 2017) considered one of the main ROS responsible for peroxidative damage (Gosalvez et al., 2017), this could explain the higher ROS synthesis in sperm treated with 20 nM of MitoQ in the present study.

Under aerobic metabolism, sperm mitochondria are an intracellular source of ROS (Aitken, 2017; Bollwein & Bittner, 2018; Moraes & Meyers, 2018) and, depending on oxidative balance, OS can reduce mitochondrial membrane potential, increase DNA damage and even stimulate an apoptotic cascade (Aitken et al., 2012; De Castro et al., 2016; Gibb et al., 2020; Gürler et al., 2016). Nonetheless, the higher ROS synthesis in sperm after MitoQ 20 nM treatment compared to Control had no effect on other sperm traits evaluated by CASA, Multicolour Assay I, and SCSA, indicating that under the ROS synthesis induced by MitoQ 20 nM, sperm were presumably able to maintain a balance between ROS generation and scavenging, as reported (Aitken, 2017; Bollwein & Bittner, 2018; Gibb et al., 2020; Moraes & Meyers, 2018). Additionally, bull sperm freezability was positively correlated with the percentage of thawed viable sperm producing H_2O_2 (Hitit et al., 2020), suggesting that cryo-resistant bull sperm are able to maintain plasma membrane integrity after freezing-thawing, even when exposed to H_2O_2. Furthermore, the same study reported the lack of a significant difference in mitochondrial oxidative stress among bulls with different freezability, leading the authors to infer that bull sperm maintained a balance between generation and removal of ROS by mitochondrial enzymatic antioxidants in the mitochondrial matrix. Perhaps optimal intercellular ROS production by mitochondria could be maintained while sperm undergo cryopreservation, as mitochondrial ROS are produced through metabolic substrates rather than through cryodamage. In our opinion, the presence of 20% egg yolk in the extender probably contributed to avoidance of deleterious effects of ROS on sperm as well, since egg yolk has antioxidative properties and is an effective ROS scavenger (Câmara et al., 2011; Chatterjee & Gagnon, 2001).

Our study confirmed that sperm cryosurvival results from a complex interaction of multiple factors related to sperm physiology and improvement of post-thaw sperm quality requires more than addition of supplementary antioxidants. In fact, the attempt to mitigate oxidative stress with a surplus of antioxidants targeting the main ROS source (i.e. mitochondria) can have the opposite effect. However, that increased ROS in the sperm had no apparent effects on any of the key physiological parameters determined during our experiment reinforced the need for further research on the fine balance between physiology and pathology of ROS concentrations.

5 | CONCLUSION

The addition of the mitochondria-targeting antioxidant MitoQ before cryopreservation of bovine sperm was unable to improve post-thaw sperm quality. Furthermore, 20 nM of MitoQ increased ROS synthesis in thawed sperm, without apparent negative effects on the evaluated sperm traits.

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CONFLICT OF INTEREST

There is no conflict of interest associated with this publication.

AUTHOR CONTRIBUTIONS

Diogo Ribeiro Câmara and Iulian Ibanescu: Conceptualization, methodology, validation, investigation, formal analysis, writing—original draft. **Mathias Siuda:** Investigation, data curation. **Heinrich Bollwein:** Resources, funding acquisition, supervision, writing—review and editing.

DATA AVAILABILITY

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

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