Journal of Equine Veterinary Science 141 (2024) 105168

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.elsevier.com/locate/jevs

Effect of Mitoquinone on sperm quality of cryopreserved stallion semen

Ahmed R. Elkhawagah [a], Gian Guido Donato [b,*], Mariagrazia Poletto [b], Nicola A. Martino [c], Leila Vincenti [b], Laura Conti [d], Denis Necchi [e], Tiziana Nervo [b]

[a] Theriogenology Department, Faculty of Veterinary Medicine, Benha University, Egypt
[b] Department of Veterinary Science, University of Torino, Grugliasco, Italy
[c] Department of Biosciences, Biotechnologies, and Environment, University of Bari Aldo Moro, Bari, Italy
[d] Department of Molecular Biotechnology and Health Sciences, University of Torino, Grugliasco, Italy
[e] Keros Insemination and Embryo Transfer Center, Passendale, Belgium

ARTICLE INFO

Keywords:
Cryopreservation
Flow cytometry
Mitoquinone
Sperm quality
Stallion semen

ABSTRACT

This study aimed to investigate the effect of mitochondria-targeted antioxidants (Mitoquinone, MitoQ) on the quality of frozen-thawed stallion semen. Semen samples collected from three fertile stallions aged 10 - 13 years, were filtered, centrifuged in a skimmed milk-based extender, and diluted to a final concentration of 50 × 106 sperm/mL in freezing medium. Diluted semen was divided into five experimental groups supplemented with MitoQ at concentrations of 0 (control), 25, 50, 100, and 200 nM and then subjected to freezing after cooling and equilibration. After thawing, semen was evaluated for motility and kinetics at different time points. Sperm viability, plasma membrane, acrosome, DNA integrity, mitochondrial membrane potential, apoptosis, and intracellular reactive oxygen species (ROS) concentrations were evaluated. The results revealed that MitoQ at concentrations of 25, 50, and 100 nM improved ($P< 0.01$) the total sperm motility after 30 minutes of incubation. In addition, 25 nM MitoQ improved the sperm amplitude of lateral head displacement values ($P< 0.01$) after 30 minutes of incubation. Conversely, negative effects on sperm motility, kinetics, and viability were observed with the highest tested concentration of MitoQ (200 nM). The various concentrations of MitoQ did not affect the plasma membrane, acrosome, and DNA integrity, or the mitochondrial membrane potential and intracellular ROS concentrations. In conclusion, supplementation of MitoQ during cryopreservation, had a mild positive effect on sperm motility and kinetics especially at a concentration of 25 nM, while the highest concentration (200nM) has a detrimental effect on motility and viability parameters of frozen-thawed stallion sperm.

1. Introduction

Equine artificial insemination is one of the most widely used assisted reproductive techniques worldwide [1]. The cryopreservation of stallion sperm improved the horse industry's genetic foundation by increasing sperm life span, disseminating superior genetic resources globally, and avoiding the risks of transportation and natural mating [2–4]. The quality of frozen-thawed stallion semen is lower than that of fresh or cooled semen [3,5] due to the adverse effects of cryopreservation on stallion semen compared to other species such as cattle [6]. Improving post-thaw sperm quality and longevity would result in a longer functional life post-insemination, making the timing of AI relative to ovulation less critical than at present and increasing pregnancy rate [7]. During freeze-thawing procedures, the sperm is subjected to a variety of conditions that reduce its fertility potential [8]. Cryopreservation causes osmotic and oxidative stress, as well as the formation of ice crystals, which causes changes to the plasma membrane, increased release of reactive oxygen species (ROS), lipid peroxidation, early tyrosine phosphorylation, DNA fragmentation, and decreased sperm motility and fertility [2,9–11]. Due to the high level of polyunsaturated fatty acids in stallion spermatozoa, reactive oxygen species (ROS) as superoxide anions ($O_2\bullet-$), hydroxy radical (OH), and hydrogen peroxide (H_2O_2) induce membrane lipid peroxidation, resulting in oxidation of sperm protein and subsequent carbonylation [12–15].

The increased level of ROS in stallions during cryopreservation is attributed to the insufficient antioxidant enzymes in the sperm cell as a result of the elimination of seminal plasma during cryopreservation semen processing [16] as well as the restricted distribution and limited volume of sperm cytoplasm which contains antioxidant enzymes [17].

The mitochondrion is the primary source of sperm intracellular ROS

* Corresponding author.
E-mail address: gianguido.donato@unito.it (G.G. Donato).

https://doi.org/10.1016/j.jevs.2024.105168
Received 9 April 2024; Received in revised form 27 June 2024; Accepted 14 August 2024
Available online 14 August 2024

and the most vulnerable organelle to cryopreservation-induced oxidative stress [18,19]. During oxidative phosphorylation, the mitochondrial membrane potential is the most important factor in ATP synthesis [20]. As a result of mitochondrial damage and depolarization, stallion sperm motility is reduced because it is primarily dependent on ATP production from oxidative phosphorylation [21,22]. Furthermore, sperm mitochondrial function is linked to sperm vitality, morphology, and fertility [23,24]. Therefore, the incorporation of mitochondria-targeting antioxidants in semen extender could improve the quality of frozen-thawed semen.

Mitoquinone (MitoQ) is a mitochondrial antioxidant consisting of triphenylphosphonium (TPP, a lipophilic cation) and ubiquinone (coenzyme Q10; [25]). It can penetrate mitochondrial membranes and accumulate within the mitochondria [26,27]. Ubiquinone is reduced within the mitochondria to ubiquinol, which protects the mitochondria from oxidative damage due to its powerful antioxidant activity [28]. Recent studies have shown that the incorporation of MitoQ into a semen extender exerted a positive stimulating effect on post-thawing semen characteristics in various species, including fish [29], humans [30], ram [31], buffalo [32], rooster [33], and goat [34]. In addition, MitoQ has been recorded to ameliorate the quality and fertility rate of liquid-preserved ram semen [35].

To the best of our knowledge, the effects of MitoQ supplementation on cryopreserved stallion sperm have never been investigated to date. Therefore, due to the promising effects observed on other species and the need for improved protocols for semen cryopreservation in the equine breeding industry, the present study aimed to determine the effects of MitoQ added to the extender on the post-thaw quality of stallion sperm.

2. Material and methods

2.1. Animal ethics

All procedures in this study were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) and in compliance with European Community Regulation 86/609. No experimental animals were used since semen collection procedures were performed for commercial purposes and only the sperm unnecessary to prepare the commercial semen doses was used in this experiment. The owners of the animals provided an informed consent.

2.2. Semen collection and extension

In this study, three healthy Standardbred stallions with proven fertility, aged 10 to 13 years, were used. Stallions were kept in the same stud (Vigone, Turin, Italy) and were fed and exercised similarly. The stallions were fed with hay and concentrates. Water was provided *ad libitum*. The study was performed between March and April 2022. Semen samples were collected once weekly from each stallion for 6 consecutive weeks (a total of 18 ejaculates) using a pre-warmed (45–50°C) equine artificial vagina (INRA model; IMV-Technologies, L'Aigle, France). Semen samples were filtered immediately after collection to separate the gel fractions. Semen was evaluated for motility, viability, morphology, and concentration. Each stallion's sperm sample was diluted separately. Samples were diluted twice their volume with skimmed milk based extender and centrifuged at 600 g for 10 minutes at 24°c. After centrifugation and discarding the supernatant, the sperm pellets were re-suspended in a freezing medium (BotuCrio®, Botupharma, Botucatu, SP, Brazil) to a final concentration of 50×10^6 sperm/ml. The samples were divided into five groups and supplemented with Mitoquinone (MitoQ mesylate, MedChemExpress, Italy) at five different concentrations: 0 (control), 25, 50, 100, and 200 nM. Semen was packaged into 0.5 mL polyvinyl straws (IMV, France) and refrigerated at 5°C for 30 minutes to enable equilibration. For freezing, the straws were placed 4 cm above the level of the liquid nitrogen (LN2) in the vapor for 15 min before being directly dipped into the LN2 [36]. After 1 week, straws were thawed in a 37°C water bath for 40 seconds before being used in the various evaluations.

2.3. Semen evaluation

2.3.1. Assessment of sperm motility and velocity parameters

Using a Computer Assisted Sperm Analyzer (CASA; Hamilton Thorne, Inc., Beverly, MA, USA), the post-thawing sperm motility and velocity parameters were assessed after various periods of incubation (0, 30 minutes, 1, 1.5, and 2 hours (hrs) at 37°C. CASA was configured for stallion configuration (setup "equine species") following the manufacturer's instructions, as follows: 45 frames acquired; frame rate 60Hz; minimum contrast 80; minimum cell size 5 pixels.

The velocity variables including the curvilinear velocity (VCL, μm s^{-1}), straight linear velocity (VSL, μm s^{-1}), average path velocity (VAP, μm s^{-1}), beat cross frequency (BCF, Hz), amplitude of lateral head displacement, (ALH, μm), straightness (STR, [VSL/VAP] × 100) and linearity (LIN, [VSL/VCL] × 100) were measured. In each sample, eight randomly selected microscopic fields were examined.

If VAP > 90 μm s^{-1} and STR > 75 %, progressive cells were identified; the VAP cut-off was 20 μm /s, and the VSL cut-off was 0 μm m s^{-1}. A sperm specimen of 10 μl was loaded and evaluated in the pre-warmed Makler chamber. Motility parameters (Total, progressive, and rapid motility) were recorded and expressed as percentages.

2.3.2. Assessment of sperm viability

According to Łącka et al. [37], sperm viability was assessed using the eosin-nigrosine staining technique. Equal amounts of eosin-nigrosin and semen were mixed, smeared on a glass slide, and allowed to dry. The slides were examined under a microscope (Advanced Automated Research Microscope System, Nikon Eclipse E200, phase contrast at 40 and 100 magnifications). At least two hundred sperm were counted and the percentage of live (unstained white) and dead (eosin-stained red) spermatozoa was calculated.

2.3.3. Assessment of sperm acrosome intactness

According to Arruda de Oliveira et al. [2], sperm acrosome intactness was assessed using the Giemsa staining technique. Thawed semen (10 μl) was smeared and air-dried before being fixed in a neutral red solution for 4 minutes. The fixed smears were washed with distilled water, air dried, and then immersed in 7.5% Giemsa stain solution for 4 hours. At least two hundred spermatozoa were examined under a phase contrast microscope (400X) and analyzed for the percentage of cells with intact (pink or purple stained) and damaged (unstained) acrosome.

2.3.4. Assessment of sperm plasma membrane integrity

According to Nie and Wenzel [38], plasma membrane integrity was determined using a hypo-osmotic swelling assay (HOST ~100 mOsm/kg). In 50 mL of sterile deionized water, 1.712 g of sucrose was dissolved. The sperm sample was mixed 1:10 with pre-warmed (37°C) HOST and incubated for 60 minutes at 37°C. At the end of incubation, 10 μl semen was smeared on a glass slide and allowed to dry. At least two hundred spermatozoa were examined under a phase contrast microscope (400X) and analyzed for the percentage of cells with intact plasma membrane (curled tail; HOST positive).

2.3.5. Flow cytometric analysis

Using a FacsStar Plus flow cytometer (Becton Dickinson Immunochemistry, San Jose, CA, USA) equipped with standard optics and an air-cooled argon laser operating at 488 nm excitation and 15 mW, sperm samples were analyzed for DNA integrity (SCSA), mitochondrial membrane potential (JC-1), apoptosis (Annexin-V/PI binding assay) and ROS (H2DCFDA). CellQuest® software was used to collect the analyzed sperm cells at a flow rate of 200 events/s. A total number of 10.000 cells were analyzed per sample.

2.3.5.1. Assessment of DNA integrity using SCSA. The sperm chromatin structure assay (SCSA), which distinguishes between denatured and native DNA depending on the acridine orange (AO, Sigma-Aldrich, St. Louis, MO, USA) metachromatic properties, was used to determine the integrity of sperm DNA, according to Evenson and Jost [39]. Thawed semen was washed in 1 ml phosphate-buffered saline (PBS) solution by centrifugation at 500 *g* for 10 minutes. The sperm pellets were diluted to a final concentration of 2×10^6 sperm with TNE buffer (0.01 M Tris-Cl, 0.15 M NaCl, 1 mM EDTA, and disodium pH 7.4) and mixed with 400 µL acid detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% [w/v] Triton X-100, and pH 1.2). Thirty seconds later, the sperm cells were stained with 1200 µL of Acridine Orange (AO, Sigma-Aldrich, USA) staining solution (600 µL AO, 6 mg/mL) in 100 ml staining buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0). The flow cytometric assessment of red and green fluorescence was performed three minutes after the AO staining. FL1 (530/15 nm filter) and FL3 (650 nm long pass filter) fluorescence measurements were performed.

2.3.5.2. Assessment of sperm mitochondrial membrane potential (MMP). JC-1, a lipophilic cation, was used to measure the mitochondrial membrane potential of sperm. According to the manufacturer (MitoProbe™ JC-1 Assay Kit for Flow Cytometry [M34152], Fisher Scientific - Scheepsbouwersweg 1b - Postbus 4 - 1120 AA Landsmeer), increasing the mitochondrial membrane potential changes the JC-1 fluorescence reversibly from green (monomeric status) to orange (multimeric status). Thawed sperm with a final concentration of 1×10^6 was washed by centrifugation for 10 min at 500 g in 1 mL PBS. The sperm pellets were resuspended in 1ml PBS and mixed with 10 µl JC-1 (JC-1 200 µM in DMSO), and incubated for 30 minutes at 37°C, 5% CO2. After incubation, the sperm was washed by centrifugation in 2 ml PBS followed by resuspension in 500 µl PBS and assessment. JC-1 was excited by A 488 nm laser, and cells with green and orange fluorescence were identified using the 535 nm and 595 nm emission filters. The frequency plots of FL1 (green) and FL2 (orange) were used to assess the percentage of green and orange-stained spermatozoa. The percentage of orange-stained sperm was recorded as sperm cells with high mitochondrial membrane potential (HMMP).

2.3.5.3. Assessment of sperm apoptosis. The Alexa Fluor 488 Annexin-V Apoptosis Kit (INVITROGEN - V13245) and propidium iodide were used to evaluate the sperm plasma membrane integrity and apoptosis, according to Anzar et al. [40]. Thawed sperm with a final concentration of 1×10^6 was washed in 1 ml PBS by centrifugation at 500*g* for 10 minutes. The sperm pellets were resuspended in 100 µL of annexin-V binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES, and pH 7.4), mixed gently with 5 µL of annexin-V and 1 µL of PI (100 µg/mL), and incubated for 15 minutes at room temperature in the dark. After incubation, 400 µL of annexin-V binding buffer was added and the samples were examined flow cytometrically. The forward light scatter (FSC), orthogonal light scatter (SSC), FITC fluorescence (FL1), and PI fluorescence (FL3) were assessed. The analysis was restricted to spermatozoa by using an acquisition gate in the FSC/SSC two-dimensional histogram. Four different kinds of gated sperm were evaluated: the viable (annexin V^- and PI^-), necrotic (annexin V^- and PI+), apoptotic (annexin V+ and PI+), and early apoptotic (annexin V+ and PI^-) spermatozoa percentage.

2.3.5.4. Assessment of sperm intracellular ROS concentrations. The sperm intracellular ROS level was measured using the 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Abcam Cellular ROS Assay Kit, ab113851) method according to Romero et al. [41]. After thawing, the sperm sample with a final concentration of 1×10^6 was washed in 1 ml PBS by centrifugation at 500 *g* for 10 minutes. The sperm pellets were resuspended in 500 µl of freshly diluted stain (10 µM H2DCFDA) and incubated in the dark at 37°C for 30 minutes. After incubation, the samples were analyzed. The H2DCFDA was excited by the 488 nm laser and the fluorescence intensity was measured from 500 to 560 nm (typically FL1).

2.4. Statistical analysis

Using SPSS (Ver. 25), normality of the data was ascertained. Semen data were analyzed and presented as mean ± SEM. Using the general linear model (GLM) multivariate, the multiple means comparisons were carried out, followed by the Dunnett post-hoc test. The statistical significance was set at $P<0.05$.

3. Results

3.1. Effect of MitoQ on stallion sperm motility parameters

The effect of MitoQ on sperm motility parameters assessed by CASA

Table 1
Effect of MitoQ on motility parameters of frozen-thawed stallion semen after different incubation periods.

Parameter	Group	Incubation (37°c) durations				
		Post-thawing	30 mints	1h	1.5h	2h
Total motility (%)	Control	79.49 ±0.98	70.74 ±1.16^{b}	68.28 ±1.14^{a}	64.06 ±1.16^{a}	53.00 ±1.46
	MitoQ 25 nM	78.51 ±0.88	75.22 ±1.06^{a}	68.56 ±1.31^{ab}	64.74 ±1.32^{ab}	54.69 ±1.66
	MitoQ 50 nM	80.41 ±0.97	74.24 ±1.08^{a}	67.82 ±1.26^{ab}	65.12 ±1.32^{ab}	54.33 ±1.36
	MitoQ 100 nM	80.18 ±0.89	74.82 ±1.05^{a}	66.81 ±1.29^{ab}	65.89 ±1.31^{ab}	55.00 ±1.44
	MitoQ 200 nM	77.08 ±1.04	70.22 ±1.36^{b}	64.36 ±1.49^{b}	59.93 ±1.39^{b}	53.60 ±1.43
	P-value	NS	0.01	0.05	0.01	NS
Progressive motility (%)	Control	24.85 ±0.50^{a}	19.55 ±0.53	14.11 ±0.54^{a}	10.29 ±0.47^{a}	5.61 ±0.35
	MitoQ 25 nM	24.81 ±0.52^{ab}	18.74 ±0.54	13.94 ±0.58^{ab}	10.28 ±0.50^{ab}	6.22 ±0.35
	MitoQ 50 nM	24.52 ±0.61^{ab}	19.81 ±0.64	13.40 ±0.55^{ab}	10.04 ±0.43^{ab}	5.67 ±0.31
	MitoQ 100 nM	24.85 ±0.53^{ab}	19.17 ±0.51	13.00 ±0.48^{ab}	9.45 ±0.42^{ab}	5.45 ±0.34
	MitoQ 200 nM	22.44 ±0.64^{b}	18.07 ±0.64	12.26 ±0.52^{b}	7.99 ±0.45^{b}	5.63 ±0.31
	P-value	0.01	NS	0.01	0.01	NS
Rapid motility (%)	Control	34.63 ±0.81^{a}	26.76 ±0.79	20.01 ±0.77^{a}	14.51 ±0.66^{a}	8.00 ±0.51
	MitoQ 25 nM	35.33 ±0.92^{ab}	26.56 ±0.88	19.38 ±0.83^{ab}	14.42 ±0.69^{ab}	8.86 ±0.50
	MitoQ 50 nM	35.03 ±1.03^{ab}	27.73 ±1.02	19.13 ±0.89^{ab}	14.49 ±0.69^{ab}	8.23 ±0.49
	MitoQ 100 nM	34.40 ±0.83^{ab}	26.31 ±0.78	18.98 ±0.76^{ab}	13.63 ±0.65^{ab}	8.08 ±0.49
	MitoQ 200 nM	30.77 ±0.96^{b}	25.13 ±0.96	17.74 ±0.81^{b}	11.88 ±0.68^{b}	7.94 ±0.48
	P-value	0.01	NS	0.05	0.01	NS

Values are presented as mean ± SEM; NS: Not Significant. The superscripts within the same column indicate statistically significant differences versus controls.

is shown in Table 1. Treatments with MitoQ at different concentrations (25, 50, and 100 nM) in the extender medium during cryopreservation significantly increased the total sperm motility evaluated 30 minutes after thawing, compared with controls (P<0.01). On the other hand, the highest tested concentration of MitoQ (200 nM) significantly decreased total motility after 1 (P <

0.05) and 1.5 hours (P < 0.01) of incubation. In addition, it impaired progressive (P < 0.01, 0.01, 0.01) and rapid (P < 0.01, 0.05, 0.01) motility after thawing, and after 1 and 1.5 hours of incubation, respectively, compared to the control group (Table 1).

3.2. Effect of MitoQ on stallion sperm velocity parameters

As shown in Table 2, increased values of ALH were obtained in semen treated with 25 nM or 200 nM MitoQ and assessed after 30 minutes of incubation, compared to the control group (P < 0.01). Whereas, VAP, VSL, and VCL were negatively affected by 200 nM MitoQ immediately after thawing (P<0.01) or at different incubation durations (1 or 1.5 hours), compared with controls (P<0.05). The STR and LIN values increased with a concentration of 200 nM after thawing (P < 0.01; Table 2).

3.3. Effect of MitoQ on sperm viability, acrosomal intactness, and plasma membrane integrity

As presented in Table 3, the highest concentration of MitoQ (200nM) decreased (P< 0.01) the percentage of sperm viability compared with the values for the control group. Whereas, no difference was found regarding the plasma membrane and acrosomal integrity between the different experimental groups.

3.4. Effect of MitoQ on sperm mitochondrial membrane potential (MMP), intracellular ROS concentrations, DNA integrity, and sperm apoptosis

No differences were found between the different experimental groups regarding the MMP, ROS concentrations, DNA integrity (Table 4), and sperm apoptosis (Table 5).

4. Discussion

Stallion semen cryopreservation process leads to an increased level of ROS due to the reduced sperm antioxidant capacity because of the elimination of seminal plasma [16,42]. The increased level of ROS during the sperm freeze-thaw process enhances lipid peroxidation which negatively affects the sperm fertilizing potentials and increases the damage of DNA [43]. Stallion sperm depends on the oxidative phosphorylation process for ATP production [22]. Therefore, after cryopreservation, sperm require a highly active mitochondrial activity to have the ATP concentrations required for fertilizing activities such as capacitation, hyperactivation, and acrosome reaction [44]. In the present study, we tried to improve the frozen-thawed sperm mitochondrial activity by incorporating different concentrations of mitochondria-targeted antioxidants MitoQ in the semen extender of frozen-thawed stallion sperm.

Sperm motility and kinematics are crucial for fertilization [45]. A positive correlation has been reported between sperm motility/velocity characteristics and fertility in stallions [46]. In the present study, MitoQ, mainly at the concentration of 25, 50, and 100 nM slightly improves the total sperm motility and ALH parameter, particularly after 30 min post-thawing, while, progressive motility and other sperm velocity parameters were not affected. On the contrary, the addition of 200 nM MitoQ negatively affected the stallion sperm quality. Our data could be discussed on the context of the results obtained in different species in which, semen cryopreservation with nanomolar concentrations of MitoQ have been tested. In detail, some studies have demonstrated a positive effect of nM concentrations of MitoQ added during

Table 2
Effect of MitoQ on velocity parameters of frozen-thawed stallion semen after different incubation periods.

Parameter	Group	Incubation (37°c) durations				
		Post-thawing	30 mints	1h	1.5h	2h
VAP (μm s^{-1})	Control	84.09 ±0.50^{a}	79.70 ±0.51	75.78 ±0.66^{a}	70.93 ±0.83^{a}	64.95 ±0.94
	MitoQ 25 nM	84.01 ±0.53^{ab}	78.67 ±0.58	75.06 ±0.69^{ab}	70.49 ±0.77^{ab}	65.51 ±0.86
	MitoQ 50 nM	83.46 ±0.57 ^{ab}	79.46 ±0.63	74.70 ±0.74^{ab}	70.13 ±0.76^{ab}	65.01 ±0.84
	MitoQ 100 nM	83.03 ±0.47 ^{ab}	78.77 ±0.57	75.13 ±0.62^{ab}	69.03 ±0.83^{ab}	64.08 ±0.88
	MitoQ 200 nM	80.51 ±0.62^{b}	78.31 ±0.63	73.47 ±0.78^{b}	68.74 ±0.83^{b}	63.53 ±0.86
	P-value	0.01	NS	0.05	0.05	NS
VSL (μm s^{-1})	Control	66.42 ±0.40^{a}	64.23 ±0.42^{a}	61.19 ±0.48	58.26 ±0.60^{a}	54.22 ±0.71
	MitoQ 25 nM	66.85 ±0.35 ^{ab}	62.92 ±0.39^{b}	61.06 ±0.49	57.92 ±0.56^{ab}	55.12 ±0.64
	MitoQ 50 nM	65.93 ±0.42 ^{ab}	64.13 ±0.45^{ab}	60.67 ±0.48	57.44 ±0.55^{ab}	54.12 ±0.61
	MitoQ 100 nM	65.82 ±0.38 ^{ab}	63.53 ±0.44^{ab}	60.66 ±0.46	56.74 ±0.61^{ab}	53.13 ±0.67
	MitoQ 200 nM	64.92 ±0.43^{b}	62.87 ±0.51^{b}	60.02 ±0.57	56.51 ±0.57^{b}	52.99 ±0.68
	P-value	0.01	0.05	NS	0.05	NS
VCL (μm s^{-1})	Control	140.30 ±0.90^{a}	133.00 ±0.87	127.99 ±1.14^{a}	119.81 ±1.31	110.82 ±1.51
	MitoQ 25 nM	139.31 ±0.95 ^{ab}	131.48 ±0.90	126.51 ±1.18^{ab}	119.09 ±1.23	110.88 ±1.33
	MitoQ 50 nM	138.46 ±0.87 ^{ab}	132.48 ±1.00	125.57 ±1.20^{ab}	119.23 ±1.26	111.41 ±1.34
	MitoQ 100 nM	137.97 ±0.80 ^{ab}	131.00 ±0.89	126.79 ±1.09^{ab}	117.13 ±1.37	110.24 ±1.36
	MitoQ 200 nM	133.47 ±1.15^{b}	131.97 ±1.00	124.20 ±1.28^{b}	116.96 ±1.34	109.08 ±1.33
	P-value	0.01	NS	0.05	NS	NS
ALH (μm)	Control	6.35 ±0.06	6.15 ±0.07^{a}	6.26 ±0.09	6.20 ±0.07	5.78 ±0.20
	MitoQ 25 nM	6.29 ±0.07	6.42 ±0.08^{b}	6.34 ±0.08	6.08 ±0.08	5.42 ±0.17
	MitoQ 50 nM	6.26 ±0.06	6.22 ±0.09^{ab}	6.20 ±0.08	6.25 ±0.09	5.69 ±0.14
	MitoQ 100 nM	6.43 ±0.07	6.26 ±0.08^{ab}	6.23 ±0.08	6.15 ±0.08	5.69 ±0.16
	MitoQ 200 nM	6.28 ±0.07	6.38 ±0.08^{b}	6.14 ±0.09	6.30 ±0.10	5.96 ±0.11
	P-value	NS	0.01	NS	NS	NS
BCF (Hz)	Control	35.10 ±0.30	35.51 ±0.33	35.98 ±0.36	36.58 ±0.37	37.51 ±0.38
	MitoQ 25 nM	35.29 ±0.34	35.21 ±0.32	35.80 ±0.39	36.35 ±0.36	37.48 ±0.40
	MitoQ 50 nM	35.33 ±0.30	35.57 ±0.33	36.07 ±0.36	36.40 ±0.37	37.34 ±0.37
	MitoQ 100 nM	35.33 ±0.30	35.52 ±0.35	35.93 ±0.39	36.60 ±0.37	37.79 ±0.39
	MitoQ 200 nM	35.51 ±0.30	35.54 ±0.32	35.72 ±0.37	36.44 ±0.37	37.39 ±0.36
	P-value	NS	NS	NS	NS	NS
STR (%)	Control	78.01 ±0.44^{a}	79.99 ±0.49	80.63 ±0.56	82.29 ±0.52	83.88 ±0.60
	MitoQ 25 nM	78.72 ±0.49 ^{ab}	79.23 ±0.44	81.17 ±0.56	82.28 ±0.55	84.67 ±0.57

(continued on next page)

Table 2 (*continued*)

Parameter	Group	Incubation (37°c) durations				
		Post-thawing	30 mints	1h	1.5h	2h
	MitoQ 50 nM	78.08 ±0.42 [ab]	80.42 ±0.46	81.42 ±0.60	81.99 ±0.60	83.77 ±0.52
	MitoQ 100 nM	78.04 ±0.44 [ab]	79.76 ±0.43	80.71 ±0.57	82.25 ±0.56	83.08 ±0.53
	MitoQ 200 nM	79.76 ±0.45[b]	79.63 ±0.51	81.69 ±0.57	82.55 ±0.54	83.44 ±0.53
	P-value	0.01	NS	NS	NS	NS
LIN (%)	Control	48.23 ±0.39[a]	49.61 ±0.47	49.67 ±0.52	50.80 ±0.53	51.53 ±0.65
	MitoQ 25 nM	48.98 ±0.40 [ab]	48.97 ±0.41	50.16 ±0.55	50.78 ±0.52	52.17 ±0.61
	MitoQ 50 nM	48.55 ±0.39 [ab]	49.92 ±0.45	50.34 ±0.54	50.26 ±0.60	51.06 ±0.54
	MitoQ 100 nM	48.48 ±0.40 [ab]	49.69 ±0.47	49.82 ±0.59	50.69 ±0.57	50.43 ±0.59
	MitoQ 200 nM	50.01 ±0.47[b]	48.96 ±0.49	50.51 ±0.56	50.83 ±0.56	50.95 ±0.60
	P-value	0.01	NS	NS	NS	NS

Values are presented as mean ± SEM; NS: Non-Significant. VAP: Average path velocity (μm s^{-1}). VSL: Straight linear velocity (μm s^{-1}). VCL: Curvilinear velocity (μm s^{-1}). ALH: Amplitude of lateral head displacement (μm). BCF: Beat cross frequency (Hz). STR: straightness ([VSL/VAP] × 100). LIN: Linearity ([VSL/VCL] × 100). The superscripts within the same column indicates statistically significant differences versus controls.

Table 3
Effect of MitoQ on frozen-thawed stallion semen viability, plasma membrane, and acrosomal integrity.

Group	Sperm Viability (%)	Plasma membrane Integrity (%)	Acrosomal intactness (%)
Control	70.17±0.70[a]	58.03±0.82	71.37±0.81
MitoQ 25 nM	69.03±0.69 [ab]	59.43±0.90	71.43±0.74
MitoQ 50 nM	69.23±0.73 [ab]	58.40±0.83	71.73±0.89
MitoQ 100 nM	68.93±0.63 [ab]	58.67±0.90	71.30±0.72
MitoQ 200 nM	67.83±0.72[b]	57.87±0.98	71.27±0.64

Values are presented as mean ± SEM Values with different superscripts within the same column differed significantly at P< 0.01.

Table 4
Effect of MitoQ on frozen-thawed stallion semen mitochondrial membrane potential, DNA integrity, and intracellular reactive oxygen species (ROS) concentrations.

Group	HMMP (%)	DNA Integrity (%)	ROS (%)
Control	84.54±2.64	88.78±2.04	28.34±1.34
MQ25 nM	86.41±2.11	88.68±2.10	28.43±1.55
MQ50 nM	86.51±1.99	89.57±2.16	27.90±1.34
MQ100 nM	86.84±1.62	91.26±1.01	27.67±1.38
MQ 200 nM	85.50±2.67	91.19±1.17	28.73±1.46

Values are presented as mean ± SEM. HMMP: High Mitochondrial membrane Potential. ROS: Reactive Oxygen Species.

cryopreservation of catfish [29], humans [30], buffalo [32], rooster [33], and goat [34] spermatozoa. However, other studies have demonstrated no influences of MitoQ on humans [47] and bull semen [48]. Furthermore, some studies have shown negative effects such as decreased sperm motility and disruption of membrane integrity with the use of 200 nM MitoQ [33], which is consistent with the results of our study.

MitoQ has been stated to support ATP synthesis and ROS neutralization continuously, therefore preserving sperm motility for longer

Table 5
Effect of MitoQ on frozen-thawed stallion semen apoptosis.

Group	Normal viable Sperm (%)	Necrotic Sperm (%)	Total apoptotic Sperm (%)
Control	51.79±1.50	23.10±2.73	25.11±3.54
MQ25 nM	53.90±2.39	16.81±3.00	29.54±2.61
MQ50 nM	51.95±2.54	18.70±3.28	29.36±3.61
MQ100 nM	51.25±1.50	17.20±2.90	31.56±3.01
MQ 200 nM	50.52±4.04	17.32±4.99	32.16±2.54

Values are presented as mean± SEM.

periods [47]. The MitoQ ubiquinone (coenzyme Q) penetrates the mitochondrial membranes and accumulates inside the mitochondria [26,27] and improves the bioenergetics function through protons and electrons transfer in the electron transport chain of mitochondria, leading to the synthesis of ATP required for sperm motility [47]. The effects of MitoQ on the rate of oxygen consumption of different cell types range from no influence [49] to oxidative phosphorylation impairment [50,51].

Stallion semen cryopreservation has been reported to adversely affect sperm viability and plasma membrane integrity [2]. In this study, we observed a negative impact of the highest concentration of MitoQ (200nM) on sperm viability which is consistent with that reported by Sun et al. [33]. Moreover, in ram semen, Lu et al. [31] stated that the highest concentration (400nM) of MitoQ decreased sperm vitality and plasma membrane integrity. However, Câmara et al. [48] did not record any effect of MitoQ addition in bull semen extender on post-thawing sperm viability and membrane intactness. On the other hand, in buffalo, Tiwari et al. [32] reported a positive effect of MitoQ on sperm vitality and membrane intactness in a dose-dependent manner. In humans, it has been described that MitoQ at a concentration of 20 nM improved sperm vitality and membrane integrities without any effect at the concentration of 200 nM [47]. The adverse effect of the highest MitoQ concentration (200 nM) on sperm motility and viability may be attributed to the weak antioxidant enzyme system of post-thawed spermatozoa under the effect of excessive MitoQ [52]. The excess MitoQ has been stated to induce the release of one-electron oxidizing species such as $O_2^{\bullet-}$ [50], which is a crucial factor in determining the total ROS effects [53]. Moreover, excess MitoQ (200nM) adversely affects the sperm plasma membrane integrity leading to mitochondrial dysfunctions [32].

Triphenylphosphonium (TPP+) is a molecular vector for selective mitochondria delivery [54]. Therefore, MitoQ accumulates inside the inner mitochondrial membrane through its conjugation with the TPP cation and preserves the mitochondrial function by the continuous ubiquinol supply and free radicals scavenging [55]. In this study, MitoQ did not affect the MMP, DNA integrity, or ROS level, which is in agreement with that reported by Câmara et al. [48] in the bull sperm. On the contrary, a positive effect of MitoQ on frozen-thawed sperm MMP and ROS concentrations has been reported in frozen-thawed ram [31], rooster [33], and buffalo [32] sperm. The minimal positive influence of MitoQ on sperm quality parameters in this study could be explained by the fact that we used a BOTU freeze extender that has been shown to preserve mitochondrial health and maintain maximal Mitochondrial oxygen consumption [8].

5. Conclusion

In conclusion, supplementation of Mitoquinone in stallion semen extender before cryopreservation has a mild positive effect at concentrations of 25, 50, and 100 nM, and the most significant effect was found at 25 nM. The highest tested concentration (200nM) had a detrimental effect on different motility and velocity parameters and sperm viability percentages of frozen-thawed stallion sperm. Further studies using lower concentrations than 25 nM are still needed.

Institutional review board statement

Not applicable.

Informed consent statement

Not applicable.

Data availability statement

Data sharing is not applicable.

Ethics in publishing statement

I testify on behalf of all co-authors that our article submitted followed ethical principles in publishing.

Funding

This research received no external funding.

CRediT authorship contribution statement

Ahmed R. Elkhawagah: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Gian Guido Donato:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Mariagrazia Poletto:** Writing – review & editing, Investigation, Formal analysis. **Nicola A. Martino:** Writing – review & editing, Investigation, Formal analysis. **Leila Vincenti:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Laura Conti:** Writing – review & editing, Investigation. **Denis Necchi:** Writing – review & editing, Investigation. **Tiziana Nervo:** Writing – review & editing, Investigation, Conceptualization.

Declaration of competing interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgments

The authors are especially grateful to all staff at FONTANETTE farm, Vigone, Turin, Italy, for their assistance in collecting semen samples.

The authors would like to thank Prof. Laura Conti, Department of Molecular Biotechnology and Health Sciences, Torino University, for her assistance in the flow cytometric evaluation of semen parameters.

References

[1] Freitas ML, Bouéres CS, Pignataro TA, Gonçalves de Oliveira FJ, de Oliveira Viu MA, Oliveira RA de. Quality of fresh, cooled, and frozen semen from stallions supplemented with antioxidants and fatty acids. J Equine Vet Sci 2016;46:1–6. https://doi.org/10.1016/j.jevs.2016.07.003.

[2] De Oliveira RA, Wolf CA, De Oliveira Viu MAÔ, Gambarini ML. Addition of glutathione to an extender for frozen equine semen. J Equine Vet Sci 2013;33:1148–52. https://doi.org/10.1016/j.jevs.2013.05.001.

[3] Aurich JE. Artificial Insemination in Horses-More than a Century of Practice and Research. J Equine Vet Sci 2012;32:458–63. https://doi.org/10.1016/j.jevs.2012.06.011.

[4] Miller CD. Optimizing the use of frozen-thawed equine semen. Theriogenology 2008;70:463–8. https://doi.org/10.1016/j.theriogenology.2008.04.037.

[5] Elkhawagah AR, Nervo T, Poletto M, Martino NA, Gallo D, Bertero A, et al. Effect of relaxin on semen quality variables of cryopreserved stallion semen. Anim Reprod Sci 2020;216. https://doi.org/10.1016/j.anireprosci.2020.106351.

[6] Blottner S, Warnke C, Tuchscherer A, Heinen V, Torner H. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. Anim Reprod Sci 2001;75:75–88.

[7] Al-Kass Z, Morrell JM. Freezing stallion semen—what do we need to focus on for the future? Vet Sci 2024;11. https://doi.org/10.3390/vetsci11020065.

[8] Darr CR, Cortopassi GA, Datta S, Varner DD, Meyers SA. Mitochondrial oxygen consumption is a unique indicator of stallion spermatozoal health and varies with cryopreservation media. Theriogenology 2016;86:1382–92. https://doi.org/10.1016/j.theriogenology.2016.04.082.

[9] Ugur MR, Saber Abdelrahman A, Evans HC, Gilmore AA, Hitit M, Arifiantini RI, et al. Advances in Cryopreservation of Bull Sperm. Front Vet Sci 2019;6. https://doi.org/10.3389/fvets.2019.00268.

[10] Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;60–61:481–92.

[11] Yeste M, Estrada E, Rocha LG, Marín H, Rodríguez-Gil JE, Miró J. Cryotolerance of stallion spermatozoa is related to ROS production and mitochondrial membrane potential rather than to the integrity of sperm nucleus. androl 2015;3:395–407. https://doi.org/10.1111/andr.291.

[12] Macías García B, González Fernández L, Ortega Ferrusola C, Morillo Rodríguez A, Gallardo Bolaños JM, Rodríguez Martinez H, et al. Fatty acids and plasmalogens of the phospholipids of the sperm membranes and their relation with the post-thaw quality of stallion spermatozoa. Theriogenology 2011;75:811–8. https://doi.org/10.1016/j.theriogenology.2010.10.021.

[13] Gibb Z, Butler TJ, Morris LHA, Maxwell WMC, Grupen CG. Quercetin improves the postthaw characteristics of cryopreserved sex-sorted and nonsorted stallion sperm. Theriogenology 2013;79:1001–9. https://doi.org/10.1016/j.theriogenology.2012.06.032.

[14] Lone SA, Mohanty TK, Baithalu RK, Yadav HP. Sperm protein carbonylation. Andrologia 2019;51. https://doi.org/10.1111/and.13233.

[15] Neild DM, Gadellab BM, Graciela Chaves M, Miragayaa MH, Colenbranderb B. Agtiero Abstract A. Membrane changes during different stages of a freeze-thaw protocol for equine semen cryopreservation 2003;59.

[16] Ball BA. Oxidative stress, osmotic stress and apoptosis: Impacts on sperm function and preservation in the horse. Anim Reprod Sci 2008;107:257–67. https://doi.org/10.1016/j.anireprosci.2008.04.014.

[17] Marzano G, Moscatelli N, Di Giacomo M, Martino NA, Lacalandra GM, Dell'aquila ME, et al. Centrifugation force and time alter CASA parameters and oxidative status of cryopreserved stallion sperm. Biology (Basel) 2020;9. https://doi.org/10.3390/biology9020022.

[18] Liu T, Han Y, Zhou T, Zhang R, Chen H, Chen S, et al. Mechanisms of ROS-induced mitochondria-dependent apoptosis underlying liquid storage of goat spermatozoa. Aging 2019;11:7880–98.

[19] Moraes CR, Meyers S. The sperm mitochondrion: Organelle of many functions. Anim Reprod Sci 2018;194:71–80. https://doi.org/10.1016/j.anireprosci.2018.03.024.

[20] Saraste M. Oxidative Phosphorylation at the fin de siècle. Science (1979) 1999;283:1488–93 (1979).

[21] Peña FJ, O'Flaherty C, Ortiz Rodríguez JM, Martín Cano FE, Gaitskell-Phillips GL, Gil MC, et al. Redox regulation and oxidative stress: The particular case of the stallion spermatozoa. Antioxidants 2019;8. https://doi.org/10.3390/antiox8110567.

[22] Gibb Z, Lambourne SR, Aitken RJ. The paradoxical relationship between stallion fertility and oxidative stress. Biol Reprod 2014;91. https://doi.org/10.1095/biolreprod.114.118539.

[23] Barbagallo F, Vignera S La, Cannarella R, Aversa A, Calogero AE, Condorelli RA. Evaluation of sperm mitochondrial function: A key organelle for sperm motility. J Clin Med 2020;9. https://doi.org/10.3390/jcm9020363.

[24] Kasai T, Ogawa K, Mizuno S, Nagai S, Uchida S, Ohta M, et al. Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. Asian J Androl 2002;4:97–103.

[25] Smith RAJ, Porteous CM, Coulter CV, Murphy MP. Selective targeting of an antioxidant to mitochondria. Eur J Biochem 1999;263:709–16. https://doi.org/10.1046/j.1432-1327.1999.00543.x.

[26] Severin FF, Severina II, Antonenko YN, Rokitskaya TI, Cherepanov DA, Mokhova EN, et al. Penetrating cation/fatty acid anion pair as a mitochondria-targeted protonophore. Proc Natl Acad Sci USA 2010;107:663–8. https://doi.org/10.1073/pnas.0910216107.

[27] Asin-Cayuela J, Manas ARB, James AM, Smith RAJ, Murphy MP. Fine-tuning the hydrophobicity of a mitochondria-targeted antioxidant. FEBS Lett 2004;571:9–16. https://doi.org/10.1016/j.febslet.2004.06.045.

[28] Alleva R, Scararmuccib A, Manterob F, Bompadrec S, Leonic L, Littarrua GP. The Protective Role of Ubiquinol-10 against Formation of Lipid Hydroperoxides in Human Seminal Fluid. vol. 18. 1997.

[29] Fang L, Bai C, Chen Y, Dai J, Xiang Y, Ji X, et al. Inhibition of ROS production through mitochondria-targeted antioxidant and mitochondrial uncoupling increases post-thaw sperm viability in yellow catfish. Cryobiology 2014;69:386–93. https://doi.org/10.1016/j.cryobiol.2014.09.005.

[30] Liu L, Wang MJ, Yu TH, Cheng Z, Li M, Guo QW. Mitochondria-targeted antioxidant Mitoquinone protects post-thaw human sperm against oxidative stress injury. National Journal of Andrology 2016;22:205–11.

[31] Lu S, Fu L, Fan W, Zhang S, Wu C, Xu J, et al. Protective effect of mitochondria-targeted antioxidant Mitoquinone on the damage of Hu sheep frozen sperm. Acta Veterinaria et Zootecnica Sinica 2019;50:2554–9.

[32] Tiwari S, Mohanty TK, Bhakat M, Kumar N, Baithalu RK, Nath S, et al. Comparative evidence support better antioxidant efficacy of mitochondrial-targeted (Mitoquinone) than cytosolic (Resveratrol) antioxidant in improving in-vitro sperm functions of cryopreserved buffalo (Bubalus bubalis) semen. Cryobiology 2021;101:125–34. https://doi.org/10.1016/j.cryobiol.2021.04.007.

[33] Sun L, He M, Xu J, Wu C, Zhang S, Zhang D, et al. Does Antioxidant Mitoquinone (MitoQ) Ameliorate Oxidative Stress in Frozen–Thawed Rooster Sperm? Animals 2022;12. https://doi.org/10.3390/ani12223181.

[34] Rezaei A, Bahmani HR, Mafakheri S, Farshad A, Nazari P, Masoudi R. Protective effects of different doses of MitoQ separately and combined with trehalose on

oxidative stress and sperm function of cryopreserved Markhoz goat semen. Cryobiology 2023;110:36–43. https://doi.org/10.1016/j.cryobiol.2022.12.019.
[35] Masoudi R, Dadashpour-Davachi N, Asadzadeh N, Hatefi A. Alipour-Jenaghard P. MitoQ preserves the quality and fertility of liquid-preserved ram sperm. Theriogenology 2024;216:8–11. https://doi.org/10.1016/j.theriogenology.2023.12.023.
[36] Cristanelli MJA, Amann RP, Squires EL, Pickett BW. Effects of egg yolk and glycerol level in lactose-EDTA-egg yolk extender and of freezing rate on the motility of frozen-thawed stallion spermatozoa. Theriogenology 1985;23:25–38.
[37] Łacka K, Kondracki S, Iwanina M, Wysokińska A. Assessment of stallion semen morphology using two different staining methods, microscopic techniques, and sample sizes. J Veterinary Res (Poland) 2016;60:99–104. https://doi.org/10.1515/jvetres-2016-0014.
[38] Nie GJ, Wenzel JGW. Adaptation of the hypoosmotic swelling test to assess functional integrity of stallion spermatozoal plasma membranes. Theriogenology 2001;55:1005–18.
[39] Evenson D, Jost L. Sperm chromatin structure assay is useful for fertility assessment. Methods Cell Sci 2000;22:169–89.
[40] Anzar M, He L, Buhr MM, Kroetsch TG, Pauls KP. Sperm Apoptosis in Fresh and Cryopreserved Bull Semen Detected by Flow Cytometry and Its Relationship with Fertility 1. vol. 66. 2002.
[41] Romero Tisot C, Zanella R, Souza AP, Mallmann Lof L, Mendes Soares JC, Marques MG, et al. Melatonin effect on cryopreserved sperm cells of crioulo stallions. Open Access J Veterinary Sci Res 2020;5:1–8. https://doi.org/10.23880/oajvsr-16000202.
[42] Schober D, Aurich C, Nohl H, Gille L. Influence of cryopreservation on mitochondrial functions in equine spermatozoa. Theriogenology 2007;68:745–54. https://doi.org/10.1016/j.theriogenology.2007.06.004.
[43] Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod 2009;24:2061–70. https://doi.org/10.1093/humrep/dep214.
[44] Gibb Z, Aitken RJ. The Impact of Sperm Metabolism during in Vitro Storage: The Stallion as a Model. Biomed Res Int 2016:2016. https://doi.org/10.1155/2016/9380609.
[45] Li Y, Lu T, Wu Z, Wang Z, Yu T, Wang H, et al. Trends in sperm quality by computer-assisted sperm analysis of 49,189 men during 2015–2021 in a fertility center from China. Front Endocrinol (Lausanne) 2023;14. https://doi.org/10.3389/fendo.2023.1194455.
[46] Love CC. Relationship between sperm motility, morphology and the fertility of stallions. Theriogenology 2011;76:547–57. https://doi.org/10.1016/j.theriogenology.2011.03.007.
[47] Kumar P, Wang M, Isachenko E, Rahimi G, Mallmann P, Wang W, et al. Unraveling subcellular and ultrastructural changes during vitrification of human spermatozoa: effect of a mitochondria-targeted antioxidant and a permeable cryoprotectant. Front Cell Dev Biol 2021;9. https://doi.org/10.3389/fcell.2021.672862.
[48] Câmara DR, Ibanescu I, Siuda M, Bollwein H. Mitoquinone does not improve sperm cryo-resistance in bulls. Reproduction in Domestic Animals 2022;57:10–8. https://doi.org/10.1111/rda.14024.
[49] Gottwald EM, Duss M, Bugarski M, Haenni D, Schuh CD, Landau EM, et al. The targeted anti-oxidant MitoQ causes mitochondrial swelling and depolarization in kidney tissue. Physiol Rep 2018;6. https://doi.org/10.14814/phy2.13667.
[50] Pokrzywinski KL, Biel TG, Kryndushkin D, Rao VA. Therapeutic targeting of the mitochondria initiates excessive superoxide production and mitochondrial depolarization causing decreased mtDNA integrity. PLoS One 2016;11. https://doi.org/10.1371/journal.pone.0168283.
[51] Sun C, Liu X, Di C, Wang Z, Mi X, Liu Y, et al. MitoQ regulates autophagy by inducing a pseudo-mitochondrial membrane potential. Autophagy 2017;13:730–8. https://doi.org/10.1080/15548627.2017.1280219.
[52] Shi L, Zhang Y, Huang X, Shi M, Sun D, Zhang Y, et al. Effects of mitoquinone (MitoQ) supplementation during boar semen cryopreservation on sperm quality, antioxidant status and mitochondrial proteomics. Anim Reprod Sci 2022;247. https://doi.org/10.1016/j.anireprosci.2022.107099.
[53] Li X, Chen B, Xie H, He Y, Zhong D, Chen D. Antioxidant structure–activity relationship analysis of five dihydrochalcones. Molecules 2018;23. https://doi.org/10.3390/molecules23051162.
[54] Tsepaeva OV, Nemtarev AV, Pashirova TN, Khokhlachev MV, Lyubina AP, Amerkhanova SK, et al. Novel triphenylphosphonium amphiphilic conjugates of glycerolipid type: synthesis, cytotoxic and antibacterial activity, and targeted cancer cell delivery. RSC Med Chem 2023;14:454–69. https://doi.org/10.1039/d2md00363e.
[55] Oyewole AO, Birch-Machin MA. Mitochondria-targeted antioxidants. FASEB J 2015;29:4766–71. https://doi.org/10.1096/fj.15-275404.