

Comparison of the performance of targeted mitochondrial antioxidant mitoquinone and non-targeted antioxidant pentoxifylline in improving rooster sperm parameters during freezing and thawing

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ABSTRACT Oxidative stress is associated with impaired sperm quality after thawing. Since mitochondria are the main source of reactive oxygen species (ROS) in sperm, the aim of this study was to investigate the effects of targeted mitochondrial antioxidant mitoquinone (MitoQ) and non-targeted mitochondrial antioxidant pentoxifylline (PTX) during cooling and cryopreservation of rooster sperm. Sperm samples were collected from 15 roosters aged 28 wk and diluted with Beltsville extender. After dilution and addition of treatments (50, 100, and 200 pMol MitoQ and 0.5, 0.75, and 1 μ M PTX), samples were cooled for 2 h to 4°C and they were first analyzed at this stage and were frozen and re-evaluated after thawing. After the freezing and thawing, level of 100 pMol MitoQ significantly increased total

motility (TM), progressive motility (PGM), curvilinear velocity (VCL), membrane integrity, viability, total antioxidant capacity (TAC) and the glutathione peroxidase (GPx), as well as the level of 50 pMol significantly increased TM, PGM, average path velocity (VAP), straight-line velocity (VSL), membrane integrity, viability, and mitochondrial activity. Moreover, these 2 levels (50 and 100 pMol) decreased malondialdehyde and sperm with abnormal morphology. Addition of 0.75 μ M PTX also increased total motility compared to the control group and levels of 0.5 and 0.75 μ M decreased sperm with abnormal morphology. It could be concluded the addition of MitoQ and PTX can be useful for sperm cryopreservation industry and reduce the harmful effects of freeze-thawing.

Key words: sperm, mitoquinone, pentoxifylline, mitochondria activity

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INTRODUCTION

Artificial insemination in the poultry industry is an effective tool to speed up the reproduction process. To achieve the benefits of artificial insemination, long-term storage of sperm is essential. Frozen sperm storage is a remarkable development and a solution to the conservation of germ cell protoplasm that can be applied to biodiversity conservation (Mehdipour et al., 2021). One of the reasons for the reduced fertility of frozen sperm in addition to cold shock is the occurrence of oxidative stress (Mehdipour et al., 2016). The plasma membrane of sperm contains high unsaturated fatty acids that are prone to peroxidative damage and this can reduce membrane integrity, decrease kinetic and sperm fertility for artificial insemination (Mehdipour et al., 2020b; Zhandi

et al., 2020). One of the main factors in oxidative stress during the freezing and thawing process is the formation of free radicals. Enzymatic and non-enzymatic antioxidants can control or reduce the harmful effects of free radicals (Silva et al., 2010). Mitochondria play a key role in the apoptosis process. If the mitochondrial membrane is destroyed by ROS, cytochrome C is isolated from the mitochondria and induces caspase activation and induces apoptosis. Apoptosis can also exacerbate DNA damage caused by ROS (Reddy et al., 2011).

Recently, researchers have stated that mitochondria should be directly targeted by antioxidants (Jin et al., 2014). The targeted antioxidant mitoquinone (MitoQ) was designed to transfer the antioxidant portion of ubiquinol to the mitochondria (Smith and Murphy, 2010). In other term, MitoQ is an antioxidant that can be regenerated after some processes (Skulachev et al., 2009). MitoQ is an effective antioxidant against lipid peroxidation, whose rapid equilibrium across the plasma membrane is controlled by the plasma membrane potential. This antioxidant protects mitochondria from oxidative damage and destroys the mitochondrial membrane potential (Murphy and Smith, 2000; Smith and Murphy,

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2010). The targeted antioxidant MitoQ exerts its antioxidant defense by increasing ATP production and reducing ROS production. Experiments have clearly shown that MitoQ accumulated in mitochondria can be restored after oxidation by the electron chain and protect the cell against oxidative stress (Skulachev et al., 2009). Confirmatory evidence in bovine, buffalo, and goat spermatozoa showed that cold shock increases cAMP leakage and decreases cAMP synthesis capacity (Chaudhry and Anand, 1975).

Pentoxifylline (PTX) is a methylxanthine derived from phosphodiesterase inhibitors. Methylxanthine compounds have positive effects on sperm function by increasing the activity of the glycolysis pathway and producing large amounts of cAMP, which is inhibited by cAMP phosphodiesterase activity (Lardy et al., 1971). PTX has free radical scavenging properties. It can also increase sperm motility by inhibiting phosphodiesterase activity in human sperm (Schoenfeld et al., 1975). It was shown in a study that PTX reduces freezing damage in rat sperm (Esteves et al., 1998). It is proved that PTX and caffeine increase sperm motility and longevity in fresh and frozen sperm (Jenagrad et al., 2018; Gradil and Ball, 2000).

However, to our knowledge, there is no information on the use of MitoQ and PTX with rooster sperm during the pre-cryopreservation and post-cryopreservation processes. The present study aimed to determine the effects of MitoQ and PTX added to the diluent on increased motility, mitochondrial activity, and other enzymatic parameters (before freezing and after thawing).

MATERIALS AND METHODS

Principles of Animal Ethics and Chemicals

Experimental methods were approved by the Animal Ethics and Welfare Committee of Tabriz University. The chemicals used in this study were purchased from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany).

Bird Location, Sampling, and Adding Treatments

For this purpose, 15 adult Ross breeds aged 28 wk were used. The roosters were housed in solitary cages (85 × 70 × 85 cm) under 14 h light and 10 h dark and were given a diet for commercial poultry and free access to drinking water. The management of the roosters was by the main recommendations in the animal care guidelines. After a 2-wk acclimatization period, ejaculate samples were collected and tested in 5 replicates twice a week. Sperm collection was done by the dorso-abdominal massage method (Najafi et al., 2020). Sperm samples were transferred to the laboratory immediately after collection at 37°C, for initial evaluation. At first, the samples were examined for volume, concentration, and color

Table 1. Composition of the Beltsville extender.

Ingredient	Amount
Potassium citrate tribasic monohydrate (mM)	2.08
Sodium-L-glutamate (mM)	51.28
Magnesium chloride anhydrous (mM)	0.35
D(-)-Fructose (mM)	27.75
Potassium phosphate dibasic trihydrate (mM)	43.57
Potassium phosphate monobasic (mM)	5.14
N-[Tris (hydroxymethyl) methyl]-2 (mM)	13.95
Sodium acetate trihydrate (mM)	3.9
Purified water (mL)	100
PH	7.1
Osmolality (mOsm/kg)	310

and only samples with a volume of 0.2 to 0.7 mL and motility of more than 80%, concentrations greater than 3×10^9 sperm per mL, sperm content with healthy morphology more than 90% were used. Calibrated microtubes were used to measure the sample size. The color of the samples was measured visually and the mobility and concentration were evaluated using CASA. To eliminate the individual effects, the confirmed samples were mixed and combined into a single sample. Beltsville diluent (Najafi et al., 2019a; Table 1) was used to dilute the samples.

Cryopreservation Procedure

Semen was extended in 2 steps with equal volumes of Beltsville freezing extender. In the first step, semen was extended at 37°C in Beltsville extender (one part semen and 4 parts extender) containing MitoQ 50 pMol, MitoQ 100 pMol, MitoQ 200 pMol, PTX (Sigma, St. Louis, MO, P1784), 0.5 μM, PTX 0.75 μM, PTX 1 μM, and control group (the Beltsville extender with no antioxidant), and 2% (v/v) glycerol. then cooled for 2 h at 4°C. In the next step, the pre-diluted semen was further extended 1:1 with Beltsville extender, but containing 6% (v/v) of glycerol and no antioxidant, giving a final glycerol concentration of 3.8%.

Before drawing samples containing different levels of treatment into the straws, motility, viability, plasma membrane integrity, malondialdehyde content, and sperm with abnormal morphology were measured and then after equilibration time, immediately the samples were transferred to 0.25 mL straws (IVM, L'Aigle, France), and polyvinyl alcohol powder was used to seal the straws. After that, the straws were cryopreserved in liquid nitrogen vapors, located 7 min inside cryo-box (placed 4 cm above the surface of the liquid nitrogen), and then plunged into liquid nitrogen for storage. Following storing for 1 wk, the cryopreserved straws were thawed separately (37°C for 30 s) within a water bath and then examined separately (Mehdipour et al., 2020a).

Analyzing sperm motility was carried out using computer-assisted sperm analysis (CASA; 12.3 CEROS, Hamilton Thorne Biosciences, Beverly, MA). After

thawing, 3 μL of each sample was poured into the pre-warmed (37°C) chamber slide (Leja Products, Luzernesstraat, Holland). At least 5 fields were evaluated for each sample under a phase-contrast microscope (Labomed LX400; Labomed Inc., Culver City, CA), and the following parameters were analyzed: motility parameters including, total motility (TM, %) progressive motility (PM, %), average path velocity (VAP, $\mu\text{m/s}$), straight linear velocity (VSL, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, μm) (Abdalkarim Salih et al., 2021).

Sperm Membrane Functionality

Sperm membrane functionality was assessed by the hypo-osmotic swelling test (HOST). For this purpose, 9 g of fructose and 4.9 g of citrate were mixed with distilled water. Then, 10 μL of semen was mixed with 100 μL of the solution and placed in a warm water bath for half an hour. Then, 10 microliters of the sample were placed under a microscope at a magnification of $400\times$, and several fields were randomly photographed using the M-SHOT (Micro-shot Technology Co., Ltd, Guangzhou, China) system. Sperm ($n = 200/\text{slide}$) with swollen and non-swollen tails were subsequently recorded (Najafi et al., 2022).

Sperm Viability

Eosin nigrosine staining was used to evaluate the percentage of live and dead sperm. For this purpose, 10 μL of frozen sperm samples from each group was placed on a slide and mixed with 20 microliters of eosin nigrosine. Then, by another slide, the stained samples were spread on the slide and after drying, the percentages of live and dead spermatozoa were determined by counting 200 sperm under 400-magnification microscopy (Mehdipour et al., 2020a).

Sperm Morphology

To evaluate abnormal spermatozoa, 15 μL of each sample was added to microtubes containing 1 mL of Hancock solution (62.5 mL formalin (37%), 150 mL of sodium saline solution, 150 mL of buffer solution and 500 mL of double-distilled water), then a drop of this solution was placed on a slide and covered with a slide. Two hundred sperm were counted under a phase-contrast microscope and abnormal sperm percentage (head abnormalities, detached heads, abnormal mid-pieces, and tail defects) was calculated (Mehdipour et al., 2017).

Determination of Determination of Total Antioxidant Capacity, Superoxide Dismutase, and the Glutathione Peroxidase

To assess the activities of total antioxidant capacity (TAC), Superoxide Dismutase (SOD), and

Glutathione Peroxidase (GPx), semen specimens were centrifuged at $500 \times g$ for 5 min. After removing the supernatant, in order to separate seminal plasma, the samples were centrifuged at $10,000 \times g$ for 20 min at 4°C , and then stored at -20°C until the assessment of mentioned parameters (Najafi et al., 2020).

Tebpajohan commercial kits (Tehran, Iran) were employed to assess SOD and TAC concentrations, according to the manufacturer's directions. Basis of SOD measurement by the kit was to measure SOD activity by production of formazan dye upon reducing the tetrazolium salt by superoxide anion. The formation rate of formazan is inhibited by the presence of SOD in environments and is measurable photometrically at 440 nm using a microplate reader. For measurement of TAC, the kit utilizes a peroxidase chromogenic substrate, producing a water-soluble chromogenic substrate upon oxidation by ferryl myoglobin radicals. The formation rate of the green chromogen is inhibited by presence of antioxidants in environments and is measurable photometrically at 412 nm using a microplate reader. In the assessment of mentioned parameters, all microplates were read by ELISA reader (TECAN, GmBH, Austria). GPx activity was assessed by Zellbio kit (Zellbio GmBH, Germany) and reading the absorbance with microplate reader at 412 nm according kit instruction

Malondialdehyde Concentration

Thiobarbituric acid reactive substances (TBARs) test was used to determine the level of sperm lipid peroxidation. In this test, malondialdehyde content was measured as an indicator of lipid peroxidation by reaction with thiobarbituric acid. First, to precipitate proteins, 1 mL of each treatment group after thawing at 37°C was mixed with 2 mL of chloroacetic acid in a sterile tube. Then to prevent lipid peroxidation during the test; 1 mL of butylated hydroxytoluene solution or 2% BHT in ethanol (plus 1 mL of EDTA) was added to the solution. Then, the samples were centrifuged for 15 min at $1,200 \times g$. After centrifugation, 1 mL of the supernatant was removed and mixed with 1 mL of 0.67% thiobarbituric acid solution in a tube and immersed in 95°C for 20 min. After cooling the samples, the absorbance of the samples was measured by spectrophotometer at 532 nm (Najafi et al., 2019b).

Mitochondrial Activity

Sperm mitochondrial activity was evaluated using Rhodamine-123 (RH-123, Sigma Aldrich). After thawing and diluting the samples, 250 μL of this sample was mixed with 5 μL of R123 solution and PI solution. Then prepared samples were incubated at 37°C for 15 min in a dark place. For determining the mitochondrial activity through flow cytometry, the percentage of sperm with R123 high fluorescence and no PI fluorescence was recorded (Mehdipour et al., 2021).

Table 2. Effect of MitoQ and PTX on sperm motility parameters during pre-freeze and thawing.

	Treatment	TM	PGM	VCL	VAP	VSL	ALH	BCF
Pre-freeze	Control	76.60	52.20 ^c	91.98	58.85	46.08	5.56	26.62
	MitoQ 50 pMol	76.60	52.20 ^c	94.46	61.51	50.48	5.70	26.76
	MitoQ 100 pMol	77.20	54.60 ^{abc}	93.76	63.40	48.24	6.33	27.10
	MitoQ 200 pMol	77.00	53.20 ^{bc}	94.02	62.88	47.65	6.22	25.61
	PTX 0.5 μ M	77.60	52.40 ^c	92.97	62.17	48.65	5.74	26.74
	PTX 0.75 μ M	80.00	56.20 ^a	95.10	63.57	49.97	6.22	27.38
	PTX 1 μ M	79.40	55.40 ^{ab}	97.69	65.33	50.81	6.12	27.04
	SEM	0.76	0.63	1.37	2.21	2.17	0.20	0.65
	<i>P</i> -value	0.0153	0.014	0.15	0.57	0.72	0.06	0.60
	Post-thaw	Control	55.00 ^b	32.00 ^b	58.14 ^{ab}	24.80 ^b	19.26 ^b	3.59
MitoQ 50 pMol		62.60 ^a	36.40 ^a	57.67 ^{ab}	30.16 ^a	23.95 ^a	4.66	13.11
MitoQ 100 pMol		62.60 ^a	36.80 ^a	65.92 ^a	26.59 ^{ab}	22.11 ^{ab}	4.17	14.34
MitoQ 200 pMol		59.80 ^{ab}	34.80 ^{ab}	60.11 ^{ab}	26.25 ^{ab}	20.47 ^{ab}	4.01	14.81
PTX 0.5 μ M		59.20 ^{ab}	33.20 ^{ab}	53.22 ^b	23.42 ^b	20.64 ^{ab}	4.33	12.88
PTX 0.75 μ M		60.60 ^a	34.60 ^{ab}	58.79 ^{ab}	28.36 ^{ab}	21.22 ^{ab}	3.58	14.58
PTX 1 μ M		58.20 ^{ab}	33.60 ^{ab}	53.07 ^b	24.81 ^{ab}	21.76 ^{ab}	3.89	14.94
SEM		1.23	0.978	2.130	1.305	1.033	0.32	0.71
<i>P</i> -value		0.002	0.018	0.003	0.018	0.092	0.215	0.072

Beltsville extender containing mitoquinone (MitoQ) 50 pMol, MitoQ 100 pMol, MitoQ 200 pMol, pentoxifylline (PTX) 0.5 μ M, PTX 0.75 μ M, PTX 1 μ M, and control group (the Beltsville extender with no antioxidant).

Abbreviations: ALH, lateral head displacement (μ m/s); BCF, beat cross frequency (Hz); PGM, progressive motility (%); TM, total motility (%); VAP, average path velocity (μ m/s); VSL, straight line velocity (μ m/s); VCL, curvilinear velocity (μ m/s).

^{abc}Different superscripts within the same column indicate significant differences among groups ($P < 0.05$).

Statistical Analysis

All data were analyzed for normal distribution by PROC UNIVARIATE and the Shapiro–Wilk test using SAS software (version 9.1). The data were analyzed using the GLM procedure. Differences among groups were evaluated by Tukey’s test. Results are shown as a mean \pm SEM. $P < 0.05$ was considered to be significant.

RESULTS

According to the results of Table 2, PTX at 0.75 level improved total motility. The results highlighted that addition of 0.75 and 1 μ M PTX were able to significantly increase the progressive motility compared to the

control group during the cooling phase. Moreover, 50 and 100 pMol MitoQ levels increased total and progressive motility compared to the control group. Moreover, 100 pMol MitoQ level increased curvilinear velocity, Average path velocity and straight-line velocity parameters compared to the control group ($P < 0.05$). No significant difference was observed for the rest of the motility parameters.

The results of data analysis of the survival and integrity of plasma membranes and malondialdehyde levels of sperm are reported in Table 3. Based on the data, none of the treatment levels could have a significant effect on these parameters during the cooling process, compared to the data obtained after freezing. The highest viability percentage among the treatment groups was related to 50 pMol treatment (63.85 ± 1.0) and 100 pMol level

Table 3. Effect of MitoQ and PTX on viability, plasma membrane functionality, abnormality, and malondialdehyde during pre-freeze and thawing.

	Treatment	Viability (%)	Plasma membrane integrity (%)	Abnormal forms (%)	Malondialdehyde (nmol/mL)
Pre-freeze	Control	82.90	73.33	5.72	0.90
	MitoQ 50 pMol	84.85	75.87	5.08	0.88
	MitoQ 100 pMol	85.57	76.17	5.15	0.75
	MitoQ 200 pMol	85.03	74.29	4.84	0.72
	PTX 0.5 μ M	84.99	74.46	4.76	0.83
	PTX 0.75 μ M	85.22	74.88	4.93	0.75
	PTX 1 μ M	84.98	76.86	4.89	0.75
	SEM	0.63	0.88	0.51	0.04
	<i>P</i> -value	0.12	0.10	0.86	0.03
	Post-thaw	Control	57.75 ^c	50.46 ^b	23.07 ^a
MitoQ 50 pMol		63.85 ^a	56.04 ^a	18.73 ^b	1.63 ^b
MitoQ 100 pMol		63.45 ^{ab}	57.22 ^a	18.63 ^b	1.60 ^b
MitoQ 200 pMol		60.95 ^{abc}	55.27 ^{ab}	19.48 ^{ab}	1.90 ^{ab}
PTX 0.5 μ M		60.27 ^{abc}	53.08 ^{ab}	18.75 ^b	1.69 ^{ab}
PTX 0.75 μ M		62.30 ^{abc}	53.91 ^{ab}	18.87 ^b	2.09 ^{ab}
PTX 1 μ M		59.06 ^{bc}	52.91 ^{ab}	20.10 ^{ab}	2.14 ^{ab}
SEM		1.02	1.09	0.86	0.15
<i>P</i> -value		0.001	0.003	0.01	0.012

Beltsville extender containing mitoquinone (MitoQ) 50 pMol, MitoQ 100 pMol, MitoQ 200 pMol, pentoxifylline (PTX) 0.5 μ M, PTX 0.75 μ M, PTX 1 μ M, and control group (the Beltsville extender with no antioxidant).

^{abc}Different superscripts within the same column indicate significant differences among groups ($P < 0.05$).

Table 4. Effect of MitoQ and PTX on GPX, TAC, SOD and mitochondria activity after freeze-thawing.

Treatment	GPX (IU/g protein)	TAC (mmol/l)	SOD (U/mg)	Mitochondria activity (%)
Control	56.34 ^c	1.173 ^{ab}	109.75	45.45 ^b
MitoQ 50 pMol	60.09 ^{ab}	1.31 ^{ab}	112.31	52.01 ^a
MitoQ 100 pMol	61.36 ^a	1.41 ^a	113.74	48.45 ^{ab}
MitoQ 100 pMol	58.74 ^{abc}	1.18 ^{ab}	111.82	7.71 ^{ab}
PTX 0.5 μ M	58.96 ^{abc}	1.19 ^{ab}	110.55	47.85 ^{ab}
PTX 0.75 μ M	58.88 ^{abc}	1.30 ^{ab}	111.80	46.80 ^b
PTX 1 μ M	56.85 ^{bc}	1.14 ^b	110.31	46.50 ^b
SEM	0.73	0.05	1.12	1.13
P-value	0.0006	0.023	0.214	0.011

Abbreviations: GPx, glutathione peroxidase; SOD, superoxide dismutase; TAC, total antioxidant capacity.

Beltsville extender containing mitoquinone (MitoQ) 50 pMol, MitoQ 100 pMol, MitoQ 200 pMol, pentoxifylline (PTX) 0.5 μ M, PTX 0.75 μ M, PTX 1 μ M, and control group (the Beltsville extender with no antioxidant).

^{abc}Different superscripts within the same column indicate significant differences among groups ($P < 0.05$).

(63.45 \pm 1.0) which had a significant difference with the control group.

As illustrated in Table 3, adding different concentrations of PTX could not significantly affect viability compared to the control group. Regarding the sperm plasma membrane integrity after freezing and thawing, levels of 200 (57.22 \pm 1.0) and 100 (56.04 \pm 1.0) had significant increase compared to the control group. The results related to the sperm with unhealthy morphology are shown in Table 3. Based on the results, adding 50 (18.73 \pm 0.86) and 100 (18.63 \pm 0.86) pMol MitoQ and 0.75 (18.75 \pm 0.86) and 1 (18.87 \pm 0.86) μ M PTX reduced the amount of sperm with unhealthy morphology compared to the control group.

According to Table 4, biochemical traits and mitochondrial activity of rooster sperm were measured after freezing and thawing. It was conclusively shown that 100 pMol MitoQ enhanced TAC and GPX, but none of the levels could have a significant effect on increasing SOD. Regarding PTX antioxidant, although the addition of this antioxidant had beneficial effects on the increase of antioxidant parameters, but this increase was not significant.

For mitochondrial activity, only increasing the level of 50 pMol was able to improve mitochondrial activity after freezing and thawing.

DISCUSSION

The results of the present study showed that the addition of targeted antioxidant MitoQ at levels of 50 and 100 pMol had significant effects on improving sperm motility parameters, increasing survival, plasma membrane integrity and reducing sperm with unhealthy morphology. Addition of targeted antioxidant, MitoQ significantly reduced lipid peroxidation and increased viability in frozen sperm of roosters, indicating lipid peroxidation and consequently ROS production significantly induce cryopreservation damage. Methods that can effectively prevent ROS production or reduce lipid peroxidation can reduce cryopreservation damage and subsequently improve sperm viability. It is widely acknowledged that MitoQ indirectly reduces ROS production by reducing lipid peroxidation of frozen semen

samples. Moreover, the present study shows that reducing lipid peroxidation increases the viability of frozen sperm. The favorable effects of targeted antioxidants are due to the protection of mitochondrial function (Tyurin et al., 2007; Skulachev et al., 2009; Galley, 2010; Fang et al., 2014).

Sperm contain large amounts of mitochondria, which are the main source of active oxygen species. It is demonstrated in different studies that sperm are sensitive to lipid peroxidation due to the high levels of unsaturated fatty acids in the plasma membrane (Abdalkarim Salih et al., 2021). High levels of ROS can damage sperm protein, fat and DNA, thereby reducing the viability of frozen sperm. This fact is the basis of the use of antioxidants to improve cryopreservation protocols (Oehninger et al., 2000). Mitochondria play an essential role in maintaining normal sperm function and energy homeostasis by oxidative phosphorylation and ATP synthase (Ruiz-Pesini et al., 2007). Studies have shown that sperm mitochondria are severely damaged after freezing. Therefore, ATP transport processes are disrupted and as a result, sperm motility is reduced (Fang et al., 2014). In this study, the addition of MitoQ to the extender improved plasma membrane integrity, and sperm viability compared to the control group. The mechanism of targeted antioxidant activity of MitoQ and all other targeted antioxidants is through increased ATP production, decreased ROS production, and increased antioxidant defense (Fang et al., 2014). Levels of 50 and 100 pMol MitoQ improved total motility compared to the control group, also these levels increased the percentage of progressively motile sperm compared to the control group. It was shown in a study that adding four levels (200, 20, 2, 0.2 nM) of MitoQ to yellow catfish *Pelteobagrus fulvidraco* sperm led to an increase in post-thaw ATP production and kinematics and viability at the level of 20 nM. They observed MitoQ inhibited lipid peroxidation in a dose-dependent manner while simultaneously increasing sperm viability (Fang et al., 2014) which is in line with the present study. In the present study, sperm mitochondrial activity increased after cryopreservation and the addition of MitoQ (50 pMol) significantly reduced the dead sperm.

In sperm, cAMP has been shown to activate protein kinase (PKA), which is responsible for regulating

tyrosine phosphorylation, which is an important regulatory pathway in events related to sperm capacity (Yovich, 1993). The mechanisms of action of PTX on sperm status can be summarized in several categories: inhibition of phosphodiesterase, increase in ATP to cAMP conversion, effect on intracellular calcium transport and elimination of free radicals due to degeneration and necrosis of sperm (Yovich, 1993). PTX increases cAMP levels in the cell by inhibiting the phosphodiesterase enzyme. This increase leads to ATP enhancement, which in turn results in cellular glycolysis, energy production, increased sperm motility, and increased ATP production (Park et al., 2000), which can be satisfactory explanation for the results in the present study. The present results affirm that adding PTX to the extender could increase progressive motility before cryopreservation. In the present study, the level of 0.75 μM PTX in rooster sperm extender during freezing and thawing, increased total motility, which is consistent with previous studies which have reported addition of 3.5 to 7 mM of this antioxidant increased the progressive motility of horse sperm (Stephens et al., 2013). It is shown in another study that PTX and caffeine increased sperm motility and longevity in fresh and frozen sperm in different mammalian species (Jenagrad et al., 2018). By adding PTX to stallion sperm before cooling, a significant reduction in the percentage of sperm with normal morphology was observed. Sperm tail abnormalities can lead to deficiencies in energy sources and impaired motility (Guasti et al., 2017). PTX can be effective in maintaining plasma membrane integrity because it has no effect on tyrosine phosphorylation status (Jenagrad et al., 2018). In a study, PTX increased sperm strength in human sperm, after 5 min of incubation, which peaked in 30 min and lasted up to 3 h (PATRIZIO et al., 2000). In another study, an increase in total and progressive motility has been observed in bovine sperm by adding PTX to the cryopreservation environment (Barakat et al., 2015), which is in line with the present study. In thawed frozen semen, the need for motile and live sperm for fertilization is much higher than fresh semen, because freezing significantly reduces the population of motile and live sperm. MitoQ supplementation had a superior effect than PTX on maintaining progressive motility and viability in semen after cryopreservation and thawing, but at pre-cryopreservation level of 0.75 μM PTX had a significant effect on progressive motility. PTX is reported to have an immediate effect on the surface of cAMP that justifies changes in the movement patterns at different times (Guasti et al., 2013) that may justify this result. A study showed that PTX is a potent stimulant for sperm motility, which is consistent with our study (Banihani and Abu-Alhayjaa, 2016). In humans, PTX is used as a sperm motility stimulator (for reproductive technology) that has positive effects on fertility rates (Herbement and Sifer, 2015).

The relatively low proportion of survival and plasma membrane integrity in the groups treated with PTX levels in the control ratio indicates that PTX supplementation has no effect on membrane integrity. The

results obtained by Mirshokraei et al. (2011), showed that PTX had a beneficial effect on sperm motility but the harmful effect of PTX on sperm capacity was also proven.

Esteves et al. (2007), stated that sperm pretreated with PTX before cryopreservation showed no improvement in motility and viability. They also reported loss of acrosomal reaction during the freezing process. Our results also showed that PT improved sperm motility but had no significant effect on sperm capacity and acrosomal reaction after thawing. As reported by Aliabadi et al. (2018), PTX increased sperm motility but failed to protect the integrity of sperm plasma membranes during the freeze-thaw process, which is consistent with the results of the present study. A healthy sperm plasma membrane is essential for the sperm to attach to the egg and initiate the acrosome reaction. Physical and osmotic stress during freezing separates the oxidation and phosphorylation processes in the mitochondria, stimulates electron leakage and the formation of superoxide radicals, and thus deteriorates sperm quality.

MitoQ supplementation significantly improved membrane integrity after freezing and thawing compared to control, which is in line with previous findings. Fu et al. (2019), stated that MitoQ supplementation in ram semen improves sperm membrane integrity. Moreover, Tiwari et al. (2021), reported that MitoQ had beneficial effect on buffalo sperm membrane integrity.

Mitochondria are the source of ATP production, which is necessary for metabolic processes and sperm motility (Mehdipour et al., 2018). The process of freezing and thawing of sperm leads to the loss of a dense sheath around the mitochondria and the formation of bumps or thickening of the plasma membrane. There is a positive relationship between sperm motility and mitochondrial activity (Barbagallo et al., 2020). In this study, both mitochondrial activity and motility parameters were improved at the levels of MitoQ used. In our study, the improvement in sperm ratio with active mitochondria was significantly greater for PTX than MitoQ supplementation.

ROS production during freezing is a major cause of abnormalities in plasma membrane structure (Mehdipour et al., 2017) that can reduce the strength of antioxidant defense system (Najafi et al., 2020) such as intracellular glutathione activity (Gadea et al., 2004). It is shown in a study that PTX improved the physiological state of sperm as well as increase in IVF success (Rizk et al., 1995). Progressive sperm motility is dependent on cell ATP level. Sperm motility is one of the main parameters of the quality of frozen semen samples for artificial insemination (Najafi et al., 2021a,b; Van den Berghe et al., 2018). Mitochondrial dysfunction causes the production of more reactive oxygen species, resulting in damage to the mitochondrial membrane. However, the inner membrane of the mitochondria is impermeable to most molecules, and as a result many antioxidants cannot enter the inner membrane of the mitochondria (Mehdipour et al., 2018). Therefore, based on our result, the use of antioxidants that have the

ability to penetrate the mitochondria and protect it against ROS damage, can improve sperm quality parameters. In a study, adding 20 nM MitoQ in cryopreservation of yellow catfish sperm (Fang et al., 2014) reduced ROS production, lipid peroxidation and increased sperm viability after thawing which is consistent with the current study.

CONCLUSIONS

In this study, levels of 50 and 100 pMol MitoQ improved the parameters of motility and viability and plasma membrane integrity, mitochondrial activity, GPX, and TAC of sperm, reduced malondialdehyde levels and sperm with abnormal morphology. PTX reduced the mass of sperm with abnormal morphology pre-cryopreserved and also post-cryopreservation and thawing. Therefore, these 2 antioxidants can be useful for sperm cryopreservation industry and reduce the harmful effects of freeze-thawing.

DISCLOSURES

None of the authors have any conflict of interest to declare.

REFERENCES

- Abdalkarim Salih, S., H. Daghigh-Kia, M. Mehdipour, and A. Najafi. 2021. Does ergothioneine and thawing temperatures improve rooster semen post-thawed quality? *Poult. Sci.* 100:101405.
- Aliabadi, E., S. Jahanshahi, T. Talaei-Khozani, and M. Banaei. 2018. Comparison and evaluation of capacitation and acrosomal reaction in freeze-thawed human ejaculated spermatozoa treated with L-carnitine and pentoxifylline. *Andrologia* 50:e12845.
- Banihani, S., and R. Abu-Alhayjaa. 2016. The activity of seminal creatine kinase is increased in the presence of pentoxifylline. *Andrologia* 48:603–604.
- Barakat, I. A., M. A. Danfour, F. A. Galewan, and M. A. Dkhil. 2015. Effect of various concentrations of caffeine, pentoxifylline, and kallikrein on hyperactivation of frozen bovine semen. *BioMed Res. Int.* 2015.
- Barbagallo, F., S. La Vignera, R. Cannarella, A. Aversa, A. E. Calogero, and R. A. Condorelli. 2020. Evaluation of sperm mitochondrial function: a key organelle for sperm motility. *J. Clin. Med.* 9:363.
- Chaudhry, P., and S. Anand. 1975. Enzyme activities regulating adenosine 3', 5'-cyclic monophosphate of buffalo, bull and goat spermatozoa. *Indian J. Biochem. Biophys* 56:133–139.
- Esteves, S. C., R. K. Sharma, A. Thomas Jr, and A. Agarwal. 1998. Cryopreservation of human spermatozoa with pentoxifylline improves the post-thaw agonist-induced acrosome reaction rate. *Hum. Reprod. (Oxford, England)* 13:3384–3389.
- Esteves, S. C., D. M. Spaine, and A. P. Cedenho. 2007. Effects of pentoxifylline treatment before freezing on motility, viability and acrosome status of poor quality human spermatozoa cryopreserved by the liquid nitrogen vapor method. *Braz. J. Med. Biol. Res.* 40:985–992.
- Fang, L., C. Bai, Y. Chen, J. Dai, Y. Xiang, X. Ji, C. Huang, and Q. Dong. 2014. Inhibition of ROS production through mitochondria-targeted antioxidant and mitochondrial uncoupling increases post-thaw sperm viability in yellow catfish. *Cryobiology* 69:386–393.
- Fu, L., W. Fan, S. Zhang, C. Wu, J. Xu, and D. Zhang. 2019. Protective effect of mitochondria-targeted antioxidant Mitoquinone on the damage of Hu sheep frozen sperm. *Acta Vet. Zootech. Sin* 50:2554–2559.
- Gadea, J., E. Sellés, M. A. Marco, P. Coy, C. Matás, R. Romar, and S. Ruiz. 2004. Decrease in glutathione content in boar sperm after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders. *Theriogenology* 62:690–701.
- Galley, H. F. 2010. Bench-to bedside review: targeting antioxidants to mitochondria in sepsis. *Crit. Care* 14:1–9.
- Gradil, C., and B. Ball. 2000. The use of pentoxifylline to improve motility of cryopreserved equine spermatozoa. *Theriogenology* 54:1041–1047.
- Guasti, P., G. Monteiro, R. Maziero, M. Carmo, J. Dell'Aqua Jr, A. Crespilho, E. Rifai, and F. Papa. 2017. Pentoxifylline effects on capacitation and fertility of stallion epididymal sperm. *Anim. Reprod. Sci.* 179:27–34.
- Guasti, P. N., G. A. Monteiro, R. R. Maziero, I. Martin, B. R. Avanzi, J. A. Dellaqua Jr, and F. O. Papa. 2013. Effects of pentoxifylline on equine epididymal sperm. *J. Equine Vet. Sci.* 33:1153–1156.
- Herbement, C., and C. Sifer. 2015. How to select the spermatozoon for intracytoplasmic sperm injection in 2015? *Minerva Ginecol* 67:185–193.
- Jenagrad, P. A., H. D. Kia, G. Moghaddam, and M. Ebrahimi. 2018. Evaluating caffeine antioxidant properties on Ghezel ram sperm quality after freeze-thawing. *Rev. Med. Vet.* 169:233–240.
- Jin, H., A. Kanthasamy, A. Ghosh, V. Anantharam, B. Kalyanaraman, and A. G. Kanthasamy. 2014. Mitochondria-targeted antioxidants for treatment of Parkinson's disease: preclinical and clinical outcomes. *Biochim. Biophys. Acta (BBA)-Mol. Basis Dis.* 1842:1282–1294.
- Lardy, H. A., D. L. Garbers, W. Lust, and N. L. First. 1971. Effects of phosphodiesterase inhibitors and cyclic nucleotides on sperm respiration and motility. *Biochemistry* 10:1825–1831.
- Mehdipour, M., H. Daghigh Kia, G. Moghaddam, and H. Hamishehkar. 2018. Effect of egg yolk plasma and soybean lecithin on rooster frozen-thawed sperm quality and fertility. *Theriogenology* 116:89–94.
- Mehdipour, M., H. Daghigh-Kia, A. Najafi, and F. Martinez-Pastor. 2021. Type III antifreeze protein (AFP) improves the post-thaw quality and in vivo fertility of rooster spermatozoa. *Poult. Sci.* 100:101291.
- Mehdipour, M., H. Daghigh Kia, A. Najafi, H. Mohammadi, and M. Alvarez-Rodriguez. 2020b. Effect of crocin and naringenin supplementation in cryopreservation medium on post-thaw rooster sperm quality and expression of apoptosis associated genes. *PLoS One* 15:e0241105.
- Mehdipour, M., H. Daghigh Kia, M. Nazari, and A. Najafi. 2017. Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic and oxidative parameters in ram semen. *Cryobiology* 78:34–40.
- Mehdipour, M., H. D. Kia, A. Najafi, H. V. Dodaran, and O. García-Álvarez. 2016. Effect of green tea (*Camellia sinensis*) extract and pre-freezing equilibration time on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology* 73:297–303.
- Mehdipour, M., H. Daghigh Kia, and F. Martinez-Pastor. 2020a. Poloxamer 188 exerts a cryoprotective effect on rooster sperm and allows decreasing glycerol concentration in the freezing extender. *Poult. Sci.* 99:6212–6220.
- Mirshokraei, P., H. Hassanpour, A. Mehdizadeh, and M. A. Taheri. 2011. Pentoxifylline induces capacitation and acrosome reaction and improves quality of motility in canine ejaculated spermatozoa. *Res. Vet. Sci.* 91:281–284.
- Murphy, M. P., and R. A. Smith. 2000. Drug delivery to mitochondria: the key to mitochondrial medicine. *Adv. Drug Del. Rev.* 41:235–250.
- Najafi, A., H. Daghigh Kia, H. Hamishehkar, G. Moghaddam, and S. Alijani. 2019a. Effect of resveratrol-loaded nanostructured lipid carriers supplementation in cryopreservation medium on post-thawed sperm quality and fertility of roosters. *Anim. Reprod. Sci.* 201:32–40.
- Najafi, A., H. Daghigh-Kia, M. Mehdipour, H. Mohammadi, and H. Hamishehkar. 2022. Comparing the effect of rooster semen extender supplemented with gamma-oryzanol and its nano form on post-thaw sperm quality and fertility. *Poult. Sci.* 101:101637.

- Najafi, A., H. Daghigh Kia, M. Mehdipour, M. Shamsollahi, and D. J. Miller. 2019b. Does fennel extract ameliorate oxidative stress frozen-thawed ram sperm? *Cryobiology* 87:47–51.
- Najafi, A., H. D. Kia, M. Mehdipour, H. Hamishehkar, and M. Alvarez-Rodriguez. 2020. Effect of quercetin loaded liposomes or nanostructured lipid carrier (NLC) on post-thawed sperm quality and fertility of rooster sperm. *Theriogenology* 152:122–128.
- Najafi, A., M. Mehdipour, and H. D. Kia. 2021a. Effects of melatonin on the rooster sperm quality during cryopreservation. *Cryobiology* 103:208.
- Najafi, A., M. Mehdipour, and H. D. Kia. 2021b. Effects of rutin on the quality of rooster sperm during cryopreservation. *Cryobiology* 103:202.
- Oehninger, S., N. K. Duru, C. Srisombut, and M. Morshedi. 2000. Assessment of sperm cryodamage and strategies to improve outcome. *Mol. Cell. Endocrinol.* 169:3–10.
- Park, W. S., Y. S. Chang, and M. Lee. 2000. The efficacy of pentoxifylline as an anti-inflammatory agent in experimental *Escherichia coli* meningitis in the newborn piglet. *Neonatology* 77:236–242.
- Patrizio, P., Y. Liu, G. J. Sonek, M. W. Berns, and Y. Tadir. 2000. Effect of pentoxifylline on the intrinsic swimming forces of human sperm assessed by optical tweezers. *J. Androl.* 21:753–756.
- Reddy, A. R. N., M. V. Rao, D. R. Krishna, V. Himabindu, and Y. N. Reddy. 2011. Evaluation of oxidative stress and anti-oxidant status in rat serum following exposure of carbon nanotubes. *Regul. Toxicol. Pharmacol.* 59:251–257.
- Rizk, B., S. Fountain, S. Avery, C. Palmer, M. Blayney, M. Macnamee, C. Mills, and P. Brinsden. 1995. Successful use of pentoxifylline in male-factor infertility and previous failure of in vitro fertilization: a prospective randomized study. *J. Assist. Reprod. Genet.* 12:710–714.
- Ruiz-Pesini, E., C. Díez-Sánchez, M. J. López-Pérez, and J. A. Enríquez. 2007. The role of the mitochondrion in sperm function: is there a place for oxidative phosphorylation or is this a purely glycolytic process? *Curr. Topics Dev. Biol.* 77:3–19.
- Schoenfeld, C., R. D. Amelar, and L. Dubin. 1975. Stimulation of ejaculated human spermatozoa by caffeine. *Fertil. Steril.* 26:158–161.
- Silva, F., A. Marques, and A. Chaveiro. 2010. Reactive oxygen species: a double-edged sword in reproduction. *Open Vet. Sci. J.* 4:127–133.
- Skulachev, V. P., V. N. Anisimov, Y. N. Antonenko, L. E. Bakeeva, B. V. Chernyak, V. P. Elichev, O. F. Filenko, N. I. Kalinina, V. I. Kapelko, and N. G. Kolosova. 2009. An attempt to prevent senescence: a mitochondrial approach. *Biochim. Biophys. Acta (BBA)-Bioenerg.* 1787:437–461.
- Smith, R. A., and M. P. Murphy. 2010. Animal and human studies with the mitochondria-targeted antioxidant MitoQ. *Ann. NY Acad. Sci.* 1201:96–103.
- Stephens, T. D., R. M. Brooks, J. L. Carrington, L. Cheng, A. C. Carrington, C. A. Porr, and R. K. Splan. 2013. Effects of pentoxifylline, caffeine, and taurine on post-thaw motility and longevity of equine frozen semen. *J. Equine Vet. Sci.* 33:615–621.
- Tiwari, S., T. Mohanty, M. Bhakat, N. Kumar, R. Baithalu, S. Nath, H. Yadav, and R. Dewry. 2021. 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*.
- Tyurin, V., Y. Tyurina, A. Osipov, N. Belikova, L. Basova, A. Kapralov, H. Bayir, and V. Kagan. 2007. Interactions of cardiolipin and lyso-cardiolipins with cytochrome c and tBid: conflict or assistance in apoptosis. *Cell Death Differ* 14:872–875.
- Van den Berghe, F., M. C. J. Paris, M. B. Briggs, W. K. Farstad, and D. B. B. P. Paris. 2018. A two-step dilution tris-egg yolk extender containing Equex STM significantly improves sperm cryopreservation in the African wild dog (*Lycan pictus*). *Cryobiology* 80:18–25.
- Yovich, J. L. 1993. Pentoxifylline: actions and applications in assisted reproduction. *Hum. Reprod.* 8:1786–1791.
- Zhandi, M., A. Talebnia-Chalanbar, A. Towhidi, M. Sharafi, A. R. Yousefi, and S. M. H. Hussaini. 2020. The effect of zinc oxide on rooster semen cryopreservation. *Br. Poult. Sci.* 61:188–194.