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Original article

Melatonin reduces oxidative damage and upregulates heat shock protein 90 expression in cryopreserved human semen

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ABSTRACT

Sperm cells can be damaged during the semen cryopreservation process, decreasing their fertilizing ability. Physical damage and oxidative stress may occur during the freeze–thawing process. Antioxidants such as the native antioxidant melatonin can potentially improve cryopreservation outcomes. In this study, we added melatonin to cryoprotectant to examine its effect on frozen–thawed human sperm. We found that adding 0.1 mM melatonin to cryoprotectant significantly increased sperm viability (24.80 ± 0.46% vs. 20.97 ± 1.27%, $P < 0.05$) and membrane integrity ($P < 0.05$), and decreased intracellular reactive oxygen species and lipid peroxidation damage. Furthermore, mRNA levels of the transcription factor NF-E2-related factor-2 and its downstream genes were significantly increased. Resistance to oxidative stress was enhanced and expression of the antiapoptotic gene *Bcl-2* was increased by inclusion of 0.1 mM melatonin in the cryoprotectant. Moreover, 0.1 mM melatonin upregulated the expression of heat shock protein 90 (HSP90), which confers resistance to stressors in frozen–thawed sperm. Results obtained upon addition of inhibitors of melatonin receptors (luzindole and 4-P-PDOT) and an HSP90 inhibitor (geldanamycin) in the cryoprotectant demonstrated that melatonin promoted HSP90 translation via the melatonin receptor MT1 and increased adenosine triphosphate levels, thus increasing the viability of thawed sperm.

1. Introduction

Semen cryopreservation is a procedure used to preserve sperm cells. However, the freeze–thawing process affects the physiological function and morphology of sperm. The cold shock and high concentrations of free radicals generated during the freeze–thawing process can damage sperm mitochondria, plasma, and acrosome membranes, and reduce DNA integrity. Thereby, sperm quality is reduced because of changes to the translation and function of sperm proteins [1]. Oxidative stress refers to an imbalance between the oxidant/antioxidant systems that is caused by the accumulation of radical molecules such as reactive oxygen species (ROS) and reactive nitrogen species [2]. At physiological levels, free radicals play critical roles in sperm hyperactivation, capacitation, and the acrosome reaction. However, during the sperm freezing process, excessive ROS production may result in high peroxide and free radical levels that cause lipid peroxidation and destroy the lipid bilayer structure of the spermatozoon membrane [3]. By influencing the relative activities of oxidative and anti-oxidative enzymes, ROS reduce sperm motility and oxidation resistance. Human sperm are very sensitive to free oxygen radical toxicity and reduced repair capacity [4]. The transcription factor NF-E2-related factor-2 (Nrf2) plays a critical role in the defense against oxidative stress by inducing the expression of antioxidant proteins and phase II detoxification enzymes as well as of genes encoding catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), and heme oxygenase-1 (HO-1). These genes are often used to monitor antioxidant effects because the proteins they encode are involved in the anti-oxidative responses that protect cells from oxidative stress [5].

Heat shock proteins (HSPs) are a group of molecular chaperones whose expression is related to sperm motility and fertility. HSP90 protects cells against oxidative stress by adjusting the folding of key proteins to maintain their three-dimensional structure. Furthermore, HSP90 has been shown to be involved in signal transduction related to sperm viability [6,7] and is associated with improved viability in

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frozen–thawed sperm [8]. In human sperm, HSP90 protein levels decrease after freezing, possibly as a result of protein degradation rather than loss into seminal plasma [9].

Adding antioxidants to mammalian sperm during cryopreservation has been shown to increase sperm motility [10,11]. Melatonin is a neuroendocrine hormone with a natural antioxidant activity. Melatonin is a potent free radical scavenger and interacts with antioxidant enzymes to promote their oxygen radical scavenging activities, thus protecting cells from oxidative stress [12]. Human seminal fluid contains melatonin and spermatozoa possess membrane melatonin receptors [13]. In humans, melatonin protects acrosomal integrity and promotes development and mitochondrial activity, and decreases capacitation and apoptosis, thus improving sperm quality [14,15]. A protective effect of melatonin during sperm cryopreservation has been reported for various species [16,17], and melatonin has been shown to upregulate the expression of HSP family genes [18]. In the present study, melatonin was added to sperm cryopreservation solution to investigate whether it can protect human sperm from the negative effects of cryopreservation. Furthermore, the pathways mediating the regulation of HSP90 by melatonin in thawed sperm were examined.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.2. Ethics statement

Human semen was collected at the experimental station of the Beijing Perfect Family Sterility Hospital, and all procedures were carried out in accordance with a protocol approved by the Ethics Committee of the Beijing Perfect Family Hospital.

2.3. Semen freeze–thawing

Semen was collected from 20 men (average age, 32 years). Semen samples were examined for volume and sperm concentration, morphology, and motility according to the 2010 World Health Organization (WHO) guidelines. Mean sperm density was 60×10^6 cells/mL, and mean sperm motility was > 30%. The semen samples were allowed to liquefy at room temperature. Samples from each donor were analyzed separately. Each sample was divided into 5 parts, to which cryoprotectant with different concentrations of melatonin (0, 0.001, 0.01, 0.1, and 1 mM) was added. Semen samples were mixed at a 1:1 ratio with TEST-yolk buffer (Irvine Scientific, Santa Ana, CA, USA) in 2-mL cryogenic vials and allowed to equilibrate to room temperature for 15 min. Then, the samples were suspended 10 cm above liquid nitrogen for 1 h and finally transferred to liquid nitrogen.

For the inhibitor experiment, melatonin (0.1 mM) with either 10^{-7} M luzindole (a nonselective melatonin receptor (MT1/MT2) inhibitor), 10^{-7} M 4-PPDOT (a specific MT2 inhibitor), or 10^{-7} M geldanamycin (GDA, an HSP90 inhibitor), was added to the semen extender. Semen extender without supplements was used as a control. Samples were cryopreserved as described above.

After 2 weeks, the samples were removed from the liquid nitrogen and thawed as follows: the cryotubes were maintained at room temperature for 2 min and were then transferred to a water bath at 37 °C and incubated for 5 min. The samples were centrifuged at 300 × *g*, and the supernatant was removed. Sperm-washing solution (HEPES-modified Biggers-Whitten-Whittingam with 5% albumin, Irvine Scientific) was added.

2.4. Sperm motility and viability analyses

A subjective method was employed for sperm motility analysis. When thawed semen reached room temperature, 10 μL of sperm suspension was dropped onto a slide and covered with a coverslip. The percentage of spermatozoa exhibiting forward progression was measured by optical microscopy at a magnification of 400×. At least 300 sperm were counted per sample and the counting was repeated thrice. Propidium iodide (PI) was used to assess sperm viability. Sperm samples (20 μL, 1×10^6 cells/mL) were mixed with 980 μL of PI at a final concentration of 10 μM and incubated at 37 °C for 10 min. Fluorescence at the single-cell level was quantified by flow cytometry (FACS Aria III; BD Biosciences, Franklin Lanes, NJ, USA). At least 10,000 spermatozoa were examined for each assay. PI stained the nuclei of dead spermatozoa red, whereas the nuclei of viable spermatozoa remained unstained.

2.5. Plasma membrane, acrosomal, and mitochondrial membrane integrity analyses

After sperm were recovered, the group with the highest viability was evaluated for plasma membrane, and acrosomal, and mitochondrial membrane integrity. Acrosomal integrity was detected by the hypo-osmotic swelling method. Semen was diluted with a hypotonic solution (0.735 g sodium citrate and 1.35 g fructose dissolved in 100 mL water) in a 1:10 ratio. The mixture was incubated at 37 °C for 30 min, and then, 10 μL of mixture was observed by phase-contrast microscopy at a magnification of 200×. Sperm with a round tail loop were considered functional, and those without were considered to have a damaged plasma membrane. Mitochondrial function was examined by the succinate dehydrogenase (SDH) method. SDH activity was assessed according to the shade of bluish-violet granules that formed in the middle of the sperm tail. Acrosome integrity was assessed by fluorescein isothiocyanate-coupled peanut agglutinin (FITC–PNA; Sigma l7381) staining. A 10-μL drop of sperm suspension was placed on a slide, fixed with formalin, treated with 10 μL FITC–PNA, and incubated at 37 °C for 10 min. Sperm with intact acrosomes were counted under a fluorescence microscope at a magnification of 400×. A minimum of 100 spermatozoa were examined for each sample.

2.6. Assessment of sperm DNA fragmentation

Sperm DNA fragmentation was assessed using a human-sperm Halomax kit (Halotech DNA SL, Madrid, Spain) and the sperm chromatin dispersion test. Sperm DNA fragmentation was evaluated in frozen–thawed samples (5×10^6 sperm/mL) processed according to the manufacturer's instructions. A minimum of 100 spermatozoa were evaluated in each sample. Intact sperm exhibited a small, compact, intensely colored halo around the spermatozoon head. Spermatozoa with fragmented DNA exhibited a soft, widespread halo of dispersed chromatin. Spermatozoa exhibiting such a dispersed halo were considered to have a high DNA fragmentation index [19].

2.7. Quantitative real-time (q)PCR

Total RNA and protein were extracted from frozen–thawed sperm samples with an RNA/protein extraction kit (Tiangen, Beijing, China) according to the manufacturer's protocol. qPCR was used to analyze the mRNA expression of *HSP90AA1*, the apoptosis-related genes (*Bax* and *Bcl-2*), the oxidant-encoding gene *Nox5*, and the antioxidant-related genes (*HO-1*, *SOD2*, *GSTM1*, *CAT*, and *Nrf2* [20,21]). The primer sequences used for qPCR are listed in Table 1. cDNA was synthesized using 2 μL of total RNA using a commercial kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. qPCR reactions were conducted using a Real Master Mix SYBR Green kit (Tiangen) and a MX300 P quantitative PCR system (Stratagene; Agilent Technologies

Table 1
The primer sequences.

Gene (Accession no.)	Primer sequence	Product size (bp)
HSP90AA1 (KJ905786.1)	5′ AAGTCTGGGACCAAAGCGTTC 3′ 5′ GTTCCACGACCCATAGGTTCAC 3′	218
SOD2 (AY267901.1)	5′ CAAAGGGGAGTTGCTGGAAG 3′ 5′ AAACCAAGCCAACCCCAAC 3′	117
GSTM1 (CR541868.1)	5′ GGACTTTCCCAATCTGCCCT 3′ 5′ CTCCAAAATGTCCACACGAATCT 3′	139
CAT (NM_001752.3)	5′ ACCCCTCCTGGACTTTTTACATC 3′ 5′ GGGATGAGAGGGTAGTCCTTGTG 3′	115
HO-1 (NM_002133.2)	5′ ATCCCCTACACACCAGCCAT 3′ 5′ CAATGTTGGGGAAGGTGAAGA 3′	205
Nrf2 (S74017.1)	5′ ATTCCTTCAGCAGCATCCTCTC 3′ 5′ GCCCATTTAGAAGTTCAGAGAGTG 3′	192
Bax (KJ890756.1)	5′ ACGGCAACTTCAACTGGGG 3′ 5′ GCACTCCCGCCACAAAGAT 3′	237
Bcl-2 (NM_000633.2)	5′ CTTCTTTGAGTTCGGTGGGGT 3′ 5′ CCAGGAGAAATCAAACAGAGGC 3′	196
β-actin (HQ154074.1)	5′ TGCCCTGAGGCTCTTTTCC 3′ 5′ GGCATACAGGTCTTTGCGGAT 3′	117

Inc., Santa Clara, CA, USA). The fold-change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Expression levels in the treated samples were calculated relative to the expression level in the control sample.

2.8. Western blotting and immunofluorescence

Thawed semen were analyzed by western blotting with anti-MT2 (Abcam, Cambridge, UK; ab203346), anti-MT1 (Biorbyt, UK; orb11085), and anti-HSP90 (Abcam; ab13492) antibodies. β-Tubulin served as a control. The proteins were electrophoresed under reducing conditions on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. The blots were blocked in 5% bovine serum albumin and incubated overnight at 4 °C with primary antibody, then incubated with secondary antibody for 1 h at room temperature. The protein bands were visualized using enhanced chemiluminescence detection reagents (Applygen Technologies, Beijing, China) on X-OMAT BT film (Eastman Kodak, Rochester, NY, USA).

Additionally, thawed sperm were analyzed by immunofluorescence. Briefly, cells were fixed in 70% alcohol for 2 h, washed twice with phosphate-buffered saline (PBS), permeabilized with 0.5% Triton-X100 for 1 h and blocked with 1% bovine serum albumin for 1 h at room temperature. Anti-HSP90 antibody (final concentration 1:200) was added to the solution for 4 h, secondary antibody was added, and the cells were incubated for another 1 h. The cells were washed as described above, and the nuclei were stained with DAPI and visualized with a microscope.

2.9. ELISA and ATP and ROS assays

Thawed semen were prepared for HSP90AB1 (CSB-EL010808HU) and ATP2B1 (CSB-EL002335HU) detection using an ELISA kit (CUSABIO, Hubei, China), following the manufacturer's protocols. ATP content was determined according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute). Intracellular ROS were detected using PI and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) double fluorescence staining (Genmed Scientifics, USA) as follows: sperm were loaded with DCFH-DA at a final concentration of 40 μM and PI at a final concentration of 10 μM, incubated for 25 min at 37 °C, and centrifuged at 2000 rpm for 3 min. After two washes with PBS, at least 10,000 spermatozoa were examined for each assay by flow cytometry.

2.10. Measurement of malondialdehyde (MDA), lactate dehydrogenase (LDH), and oxidative stress-related enzymes

Thawed samples were subjected to biochemical assays. Semen samples were centrifuged at 800 × *g* for 10 min. The pelleted sperm cells were washed three times by resuspension in PBS and centrifugation. The supernatant was used for the assay. Total antioxidant capacity (T-AOC) and MDA, glutathione peroxidase (GSH-Px), GSH, total SOD (T-SOD), CAT, LDH, and NADPH oxidase concentrations were determined using commercial kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nangjing, China).

2.11. Statistical analyses

At least six samples of each group were used in all experiments. Experimental data were first analyzed by the homogeneity of variance test and normality test. Test data displayed normal distribution and homogeneity of variance. One-way ANOVA followed by Duncan's test for multiple comparisons was used to determine statistically significant differences between groups. Statistical analysis was conducted using Statistical Analysis System software (SAS Institute, Cary, NC, USA). All data are expressed as the mean ± standard error of the mean (SEM). Differences were considered significant when $P < 0.05$.

3. Results

3.1. Melatonin increases motility and membrane integrity of frozen–thawed sperm

Western blot analysis indicated that the melatonin receptors MT1 and MT2 are expressed in human sperm (Fig. 1A). Semen extender supplemented with 0.1 mM melatonin significantly increased motility as compared with the melatonin-free control (38.03 ± 7.06% vs. 19.49 ± 4.24%, $P < 0.05$) (Fig. 1B). Supplementation with 0.1 mM melatonin significantly increased frozen–thawed sperm viability (24.80 ± 0.46% vs. 20.97 ± 1.27%, $P < 0.05$; Fig. 1C and D) and sperm membrane integrity ($P < 0.05$; Fig. 1E, F and G) after cryopreservation, as compared with the control.

3.2. Melatonin decreases oxidative lipid damage in frozen–thawed sperm

Lipid peroxides are derived from the peroxidation of polyunsaturated fatty acids. The most abundant lipid peroxide is MDA [22]. Levels of MDA after thawing were significantly lower in the 0.01 mM and 0.1 mM melatonin-treated groups than in the melatonin-free group ($P < 0.05$) (Fig. 2A), while 0.001 and 1 mM had no significant effect. There was no obvious difference in DNA fragmentation between the 0.1 mM melatonin-treated group and the melatonin-free group ($P > 0.05$; Fig. 2B).

3.3. Melatonin promotes Bcl-2 expression in frozen–thawed human sperm

Relative transcript levels of the pro-apoptotic gene *Bax* and the anti-apoptotic gene *Bcl-2* were measured by qPCR. Supplementation of the cryopreservation medium with 0.1 mM melatonin significantly increased *Bcl-2* expression and decreased *Bax* expression when compared with the melatonin-free control ($P < 0.05$; Fig. 3).

3.4. Melatonin decreases the ROS content of frozen–thawed human sperm

Expression of NADPH oxidase, which promotes ROS generation, is regulated by Nox5. ROS levels were significantly lower in all melatonin-treated groups than in the melatonin-free group, with the largest effect being observed for 0.01 mM melatonin ($P < 0.05$; Fig. 4A). Additionally, NADPH oxidase activity was suppressed by 0.1 mM

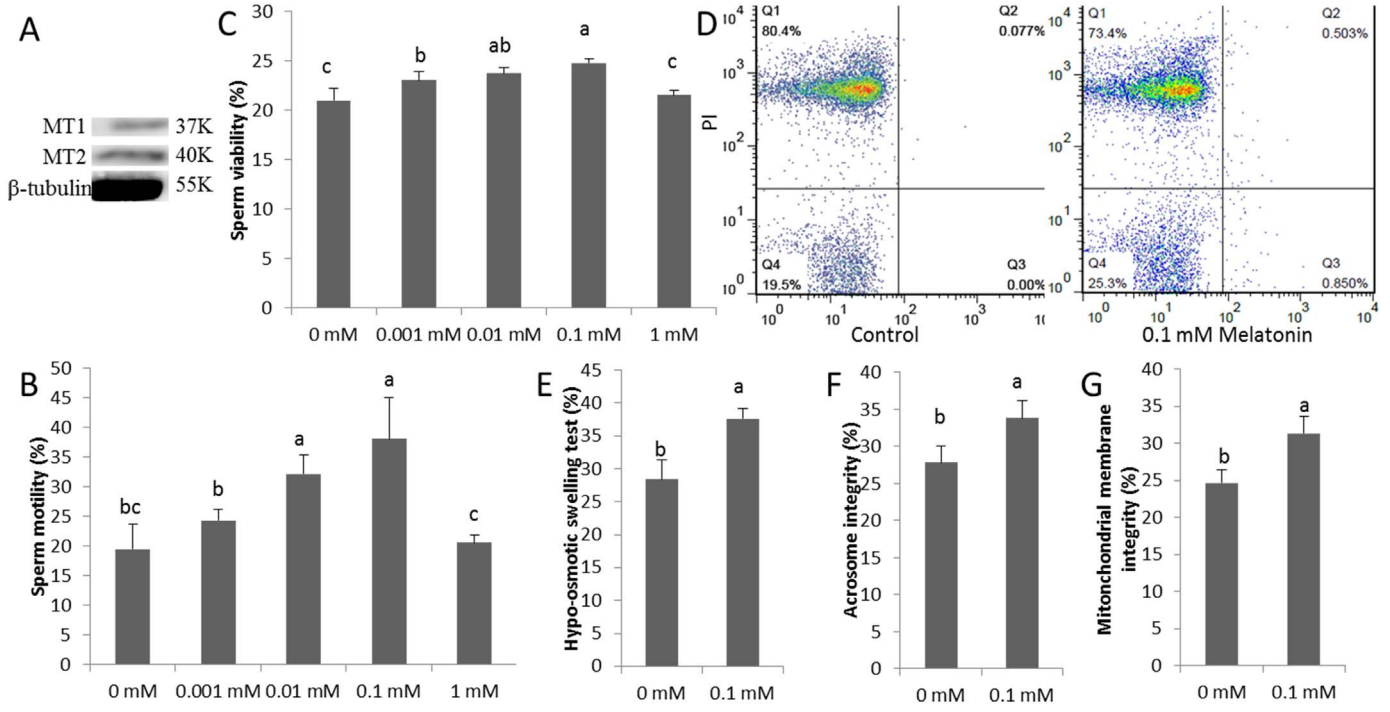

Fig. 1. Sperm motility, viability, mitochondrial integrity, membrane integrity, and acrosome integrity in frozen–thawed human semen. A) Protein expression of the melatonin receptors MT1 and MT2 in human sperm, detected by western blotting. B) Evaluation of the effects of the indicated concentrations of melatonin on sperm motility after cryopreservation. C) Evaluation of the effects of the indicated concentrations of melatonin on sperm viability after cryopreservation using a fluorescence-activated cell sorting (FACS) system. D) Viability of frozen–thawed sperm was detected by flow cytometry in the 0.1 mM melatonin-treated group; cryopreservation medium without melatonin was used as a control. Dead spermatozoa were labeled with PI (Q1), viable spermatozoa were non-fluorescent (Q4). E) Plasma-membrane integrity assessed by the hyperosmotic swelling test. F) FITC-PNA staining to assess sperm acrosomal integrity. G) Succinate dehydrogenase assay to detect mitochondrial membrane integrity. Control, 0 mM melatonin; melatonin-treated group, 0.1 mM melatonin. n = 15 in each experiment. Data are shown as the mean ± SEM. Different superscript letters (a–c) in each column represent statistically significant differences ($P < 0.05$).

melatonin (Fig. 4B). Addition of 0.1 mM melatonin to the cryopreservation medium significantly downregulated Nox5 transcription ($P < 0.05$; Fig. 4C). We concluded that 0.1 mM melatonin effectively decreased the free radical content of semen, and thus potentially reduced ROS damage to sperm.

3.5. Melatonin promotes antioxidant enzyme expression in frozen–thawed human sperm

Melatonin increased the T-AOC of frozen–thawed semen only when used at a concentration of 0.1 mM (Fig. 5A). GSH and GSH-Px activities were significantly higher in the 0.01 and 0.1 mM melatonin-treated groups than in the melatonin-free group, with the strongest effect noted for 0.1 mM ($P < 0.05$; Fig. 5B and C). CAT and T-SOD activities were also higher in the melatonin-treated group than in the melatonin-free group ($P < 0.05$; Fig. 5D and E). Furthermore, expression levels of the antioxidant-related gene *Nrf2* and its downstream genes *SOD2*, *CAT*,

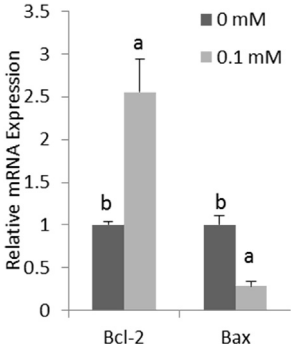

Fig. 3. Melatonin decreases *Bax* expression and increases *Bcl-2* expression in frozen–thawed human semen. n = 6 in each experiment. Relative expression data obtained by qPCR are shown as the mean ± SEM. Different superscript letters (a, b) in the 0.1 mM and 0 mM melatonin-treated groups represent statistically significant differences ($P < 0.05$).

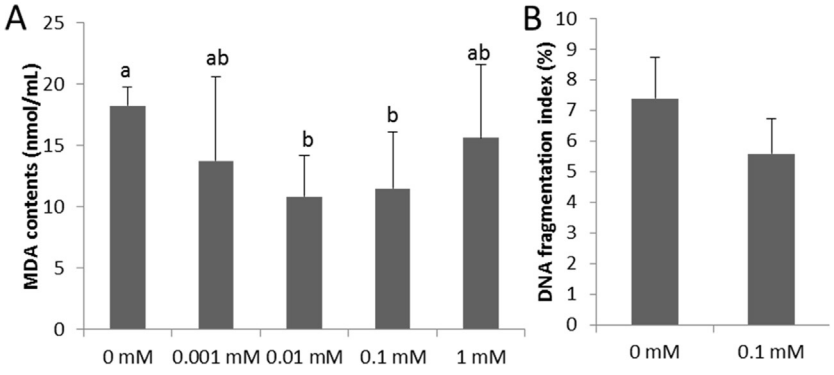

Fig. 2. Melatonin decreases lipid oxidative damage in frozen–thawed human semen. A) MDA levels in sperm after cryopreservation with the indicated concentrations of melatonin. B) Evaluation of DNA fragmentation. n = 6 in each experiment. Data are shown as the mean ± SEM. Different superscript letters (a, b) in each column represent statistically significant differences ($P < 0.05$).

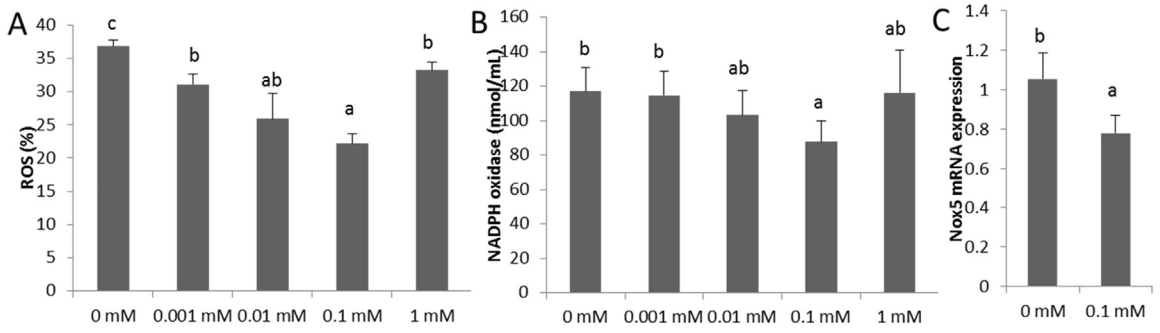

Fig. 4. Melatonin decreases ROS generation in frozen–thawed human semen. A) Evaluation of the effects of the indicated concentrations of melatonin on sperm ROS levels after cryopreservation using a FACS system. B) NADPH oxidase activity in frozen–thawed human sperm. C) qPCR detection of Nox5 expression. n = 6 in each experiment. Data are shown as the mean ± SEM. Different superscript letters (a, b) in each column represent statistically significant differences ($P < 0.05$).

HO-1, and *GSTM1* were significantly upregulated in the melatonin-treated group ($P < 0.05$) (Fig. 5F). This suggests that melatonin promoted antioxidant enzyme expression and increased Nrf2 expression, and consequently enhanced antioxidant capacity in the frozen–thawed sperm.

3.6. Melatonin promotes HSP90 expression in frozen–thawed human sperm via MT1

Before the acrosome reaction, HSP90 was detected in the sperm neck and tail, whereas after acrosome reaction, HSP90 was observed in the sperm equatorial region. Immunofluorescence analysis revealed HSP90 in the neck region of the frozen–thawed sperm (Fig. 6A). HSP90 expression was higher in the 0.1 mM melatonin-treated group than in the melatonin-free group (Fig. 6B). Moreover, *HSP90AA1* transcript levels were higher in the 0.1 mM melatonin-treated group than in the melatonin-free group ($P < 0.05$; Fig. 6C). This suggests that melatonin promoted *HSP90* expression in the frozen–thawed sperm.

Addition of the MT1/MT2 inhibitor luzindole to the semen extender decreased sperm viability as compared with treatment with melatonin alone ($P < 0.05$). GDA, an inhibitor of HSP90, also decreased sperm viability ($P < 0.05$; Fig. 7A) and resulted in higher MDA levels than those observed in sperm treated with melatonin alone (Fig. 7B). HSP90AA1 and HSP90AB1 transcript levels were similar in the two groups (Fig. 7C and D). GDA suppressed HSP90AA1 transcription and HSP90AB1 translation, and HSP90AB1 expression was downregulated in the melatonin- and luzindole-treated groups ($P < 0.05$). There was no significant difference in HSP90AB1 between the group treated with melatonin plus the specific MT2 inhibitor 4-PPDOT and the group treated with melatonin alone ($P > 0.05$). ATP2B1 expression and ATP content were significantly higher in the melatonin-alone group and the GDA-treated group compared with the control group ($P < 0.05$) (Fig. 7E and F). These results revealed that melatonin promoted HSP90 expression and increased ATP expression via MT1.

3.7. Melatonin has no obvious cytotoxic effect in frozen–thawed human sperm

LDH is released from damaged sperm plasma membranes into the extracellular fluid. The cytotoxic effect of melatonin was therefore evaluated by assessing LDH leakage (Fig. 8). The mean level of LDH leakage slightly but insignificantly ($P > 0.05$) increased in the

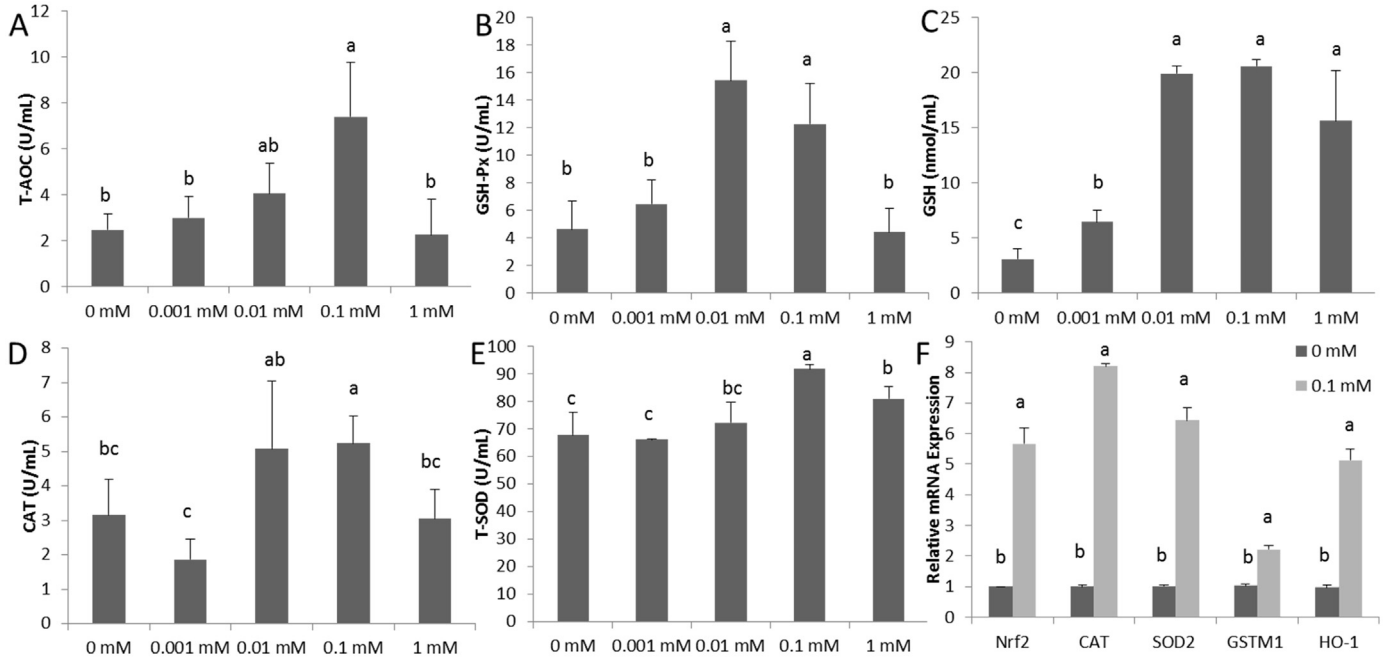

Fig. 5. Melatonin promotes antioxidant enzyme expression in frozen–thawed human semen. A) T-AOC, B) GSH-Px, C) GSH, D) CAT, and E) T-SOD concentrations in sperm after cryopreservation with melatonin at the indicated concentrations. n = 6 in each experiment. Data are shown as the mean ± SEM. Different superscript letters (a–c) in each column represent statistically significant differences ($P < 0.05$). F) qPCR detection of *Nrf2*, *CAT*, *SOD2*, *GSTM1*, and *HO-1* mRNA expression in sperm cryopreserved in the presence of 0.1 mM melatonin. Different superscript letters (a, b) in the 0.1 mM and 0 mM melatonin-treated groups represent statistically significant differences ($P < 0.05$).

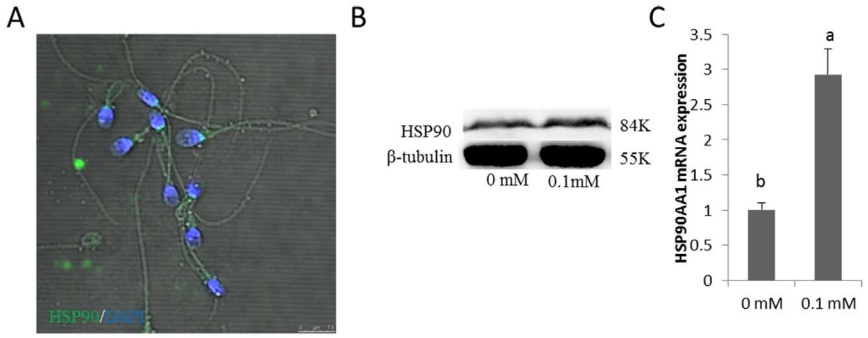

Fig. 6. Melatonin promotes HSP90 expression in frozen–thawed human semen. A) Immunofluorescence analysis of HSP90 in frozen–thawed sperm. Green fluorescence represents HSP90; blue fluorescence represents nuclei. B) HSP90 expression as evaluated by western blotting. C) qPCR detection of *HSP90AA1* mRNA expression. n = 6 in each experiment. Data are shown as the mean ± SEM. Different superscript letters (a, b) in each column represent statistically significant differences ($P < 0.05$).

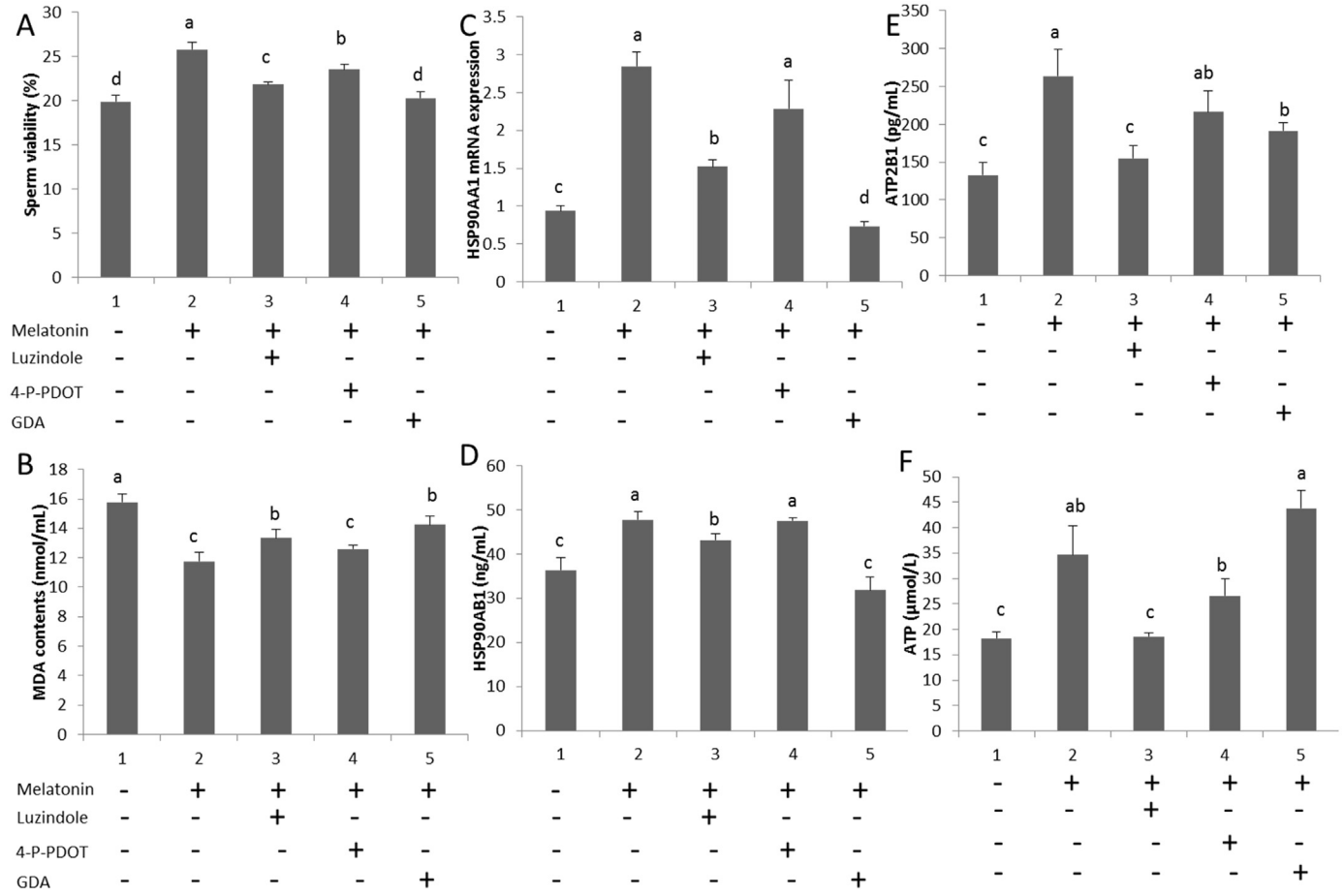

Fig. 7. Melatonin promotes HSP90 expression in frozen–thawed human semen via MT1. A) Sperm viability, B) MDA concentration, C) HSP90AA1 mRNA transcription, D) HSP90AB1 protein content, E) ATP2B1 protein content, and F) ATP content in frozen–thawed sperm treated with the indicated combinations of melatonin, luzindole, 4-P-PDOT and GDA. n = 6 in each experiment. Data are shown as the mean ± SEM. Different superscript letters (a–d) in each column represent statistically significant differences ($P < 0.05$).

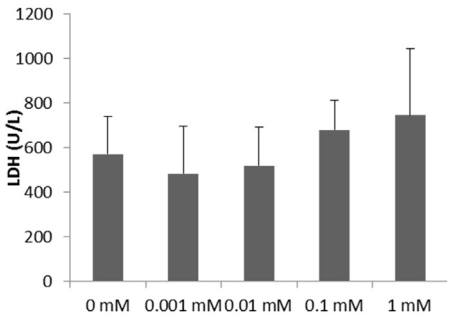

Fig. 8. Toxicity assay. Melatonin in cryopreservation medium at the indicated concentrations is not cytotoxic. n = 6 in each experiment. Data are shown as the mean ± SEM.

melatonin-supplemented media in a concentration-dependent manner as compared with the melatonin-free media. This result indicated that cryopreservation medium supplemented with 0.1 mM melatonin is not cytotoxic to frozen–thawed human sperm.

4. Discussion

Under normal physiological conditions, low levels of ROS are generated by oxidation–reduction reactions in the mitochondrial respiratory chain and play important roles in sperm motility, capacitation, and the acrosome reaction. However, high ROS levels may impair sperm motility, damage acrosomal membranes, and oxidize DNA, rendering sperm cells unable to fertilize oocytes [23]. During the freeze–thawing process, excess ROS are produced [24]. The plasma

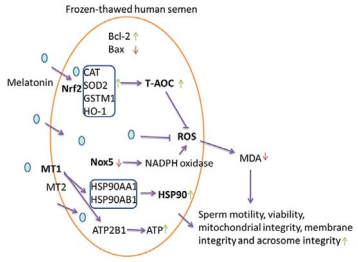

Fig. 9. Schematic illustrating the proposed mechanism by which melatonin protects against oxidative damage and upregulates HSP90 expression in cryopreserved human semen.

membrane and acrosomal and mitochondrial membranes can therefore be damaged by freeze–thawing. ATP-dependent translocase functions to maintain the integrity of sperm membranes [25]. It has been reported that ROS induce mitochondrial membrane lipid peroxidation, thus altering ATPase activity, impairing mitochondrial respiratory function, reducing the energy produced by the tricarboxylic acid cycle, and ultimately impairing sperm motility [26]. It has been shown that melatonin increased the activity of mitochondrial respiratory complexes I and IV in a time- and dosage-dependent manner [27] and enhanced the efficiency of the membrane electron transport chain, resulting in reduced ROS generation [28]. Furthermore, Succu et al. demonstrated that supplementation of cryopreservation medium with 1 mM melatonin significantly enhanced ram sperm motility and survival rate, decreased oxidative damage to the sperm plasma membrane, and increased intracellular ATP concentration [29]. Because melatonin receptors may be desensitized by exposure to melatonin at low and high concentrations, it is possible that the reduction of the effects of exogenous melatonin in the antioxidant defense system occurs via melatonin receptor desensitization [30]. In the current study, ROS levels were lower in sperm cryopreserved in semen extender containing 0.1 mM melatonin after freeze–thawing than in sperm cryopreserved in the absence of melatonin. Melatonin treatment significantly increased sperm motility, sperm viability, and membrane integrity, and promoted ATPase expression, which could increase ATP levels in sperm.

SOD and GSH-Px are two important antioxidant enzymes, which may help sperm to resist oxidative damage and maintain the dynamic ROS equilibrium in semen under normal physiological conditions [31,32]. Relatively high levels of SOD and GSH-Px activities are found in highly motile sperm [33]. MDA levels are closely related to SOD activity in sperm and thus, indirectly reflect the degree of ROS damage [34]. Melatonin promotes GSH-Px activation and enhances GSH cycle efficiency [35]. In the present study, melatonin induced SOD, GSH-Px, and CAT activation, increased levels of antioxidant substances, and decreased ROS concentration and MDA levels in frozen–thawed sperm. Consequently, melatonin effectively inhibited sperm plasma membrane lipid peroxidation. It has been suggested that melatonin treatment might upregulate Nrf2-mediated HO-1 activity and increase the Bcl-2/Bax ratio [36]. Here, we found that melatonin promoted expression of *Bcl-2* and of *Nrf2* and its downstream genes, and thus enhanced resistance to damage caused by freezing. Apoptosis destroys the integrity of DNA [37]. Sperm DNA fragmentation is associated with an increase in oxidative stress during cryopreservation [38]; melatonin may be useful in reducing this effect [39]. However, in this study, 0.1 mM melatonin had no significant effect on DNA fragmentation in frozen–thawed sperm.

HSPs protect cell function, and HSP90 content is correlated with sperm vitality [40,41]. HSP90 levels in bull spermatozoa gradually decline following freeze–thawing, which may affect sperm plasma membrane integrity and acrosome integrity [42]. HSP90 has been shown to repair chromosome damage caused by freeze–thawing and to maintain DNA integrity [43]. Low concentrations of melatonin induce HSP90 production [44,45], and HSP90 recruits ATPase, thus promoting ATP degradation [46]. In this study, we found that melatonin promoted HSP90 expression via MT1, and that higher HSP90 levels were present in sperm with higher mobility. This may be a result of rapid HSP90-induced ATP hydrolysis providing energy for motility.

In summary, our study showed that melatonin had multiple effects on frozen–thawed sperm, including enhancing sperm motility and vitality, protecting sperm membrane integrity, enhancing antioxidative enzyme activation, effectively suppressing ROS generation, and decreasing oxidative damage in frozen–thawed sperm (Fig. 9). Furthermore, 0.1 mM melatonin had no cytotoxic effect on frozen–thawed sperm. Therefore, adding melatonin to cryoprotectant may help to protect human sperm during cryopreservation.

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Competing financial interests

The authors declare that they have no competing interests.

Author contributions

Conceived and designed the experiments: Shou-Long Deng and Yi-Xun Liu. Performed the experiments: Shou-Long Deng, Zhi-Peng Wang, Kun Yu and Tie-Cheng Sun. Analyzed the data: Shou-Long Deng, Bao-Lu Zhang and Zhengxing Lian. Contributed reagents/materials/analysis tools: Xiu-Xia Wang and Tie-Cheng Sun. Wrote the paper: Shou-Long Deng and Yi-Xun Liu.

References

[1] D.G. Valcarce, F. Cartón-García, M.P. Herráez, V. Robles, Effect of cryopreservation on human sperm messenger RNAs crucial for fertilization and early embryo development, Cryobiology 67 (2013) 84–90.
[2] S. Deng, K. Yu, Q. Wu, Y. Li, X. Zhang, B. Zhang, G. Liu, Y. Liu, Z. Lian, Toll-like receptor 4 reduces oxidative injury via glutathione activity in sheep, Oxid. Med. Cell. Longev. 2016 (2016) 9151290.
[3] W. Zhang, F. Li, H. Cao, C. Li, C. Du, L. Yao, H. Mao, W. Lin, Protective effects of l-carnitine on astheno- and normozoospermic human semen samples during cryopreservation, Zygote 24 (2016) 293–300.
[4] S. Wang, W. Wang, Y. Xu, M. Tang, J. Fang, H. Sun, Y. Sun, M. Gu, Z. Liu, Z. Zhang, F. Lin, T. Wu, N. Song, Z. Wang, W. Zhang, C. Yin, Proteomic characteristics of human sperm cryopreservation, Proteomics 14 (2014) 298–310.
[5] S. Deng, K. Yu, W. Jiang, Y. Li, S. Wang, Z. Deng, Y. Yao, B. Zhang, G. Liu, Y. Liu, Z. Lian, Over-expression of Toll-like receptor 2 up-regulates heme oxygenase-1 expression and decreases oxidative injury in dairy goats, J. Anim. Sci. Biotechnol. 8 (2017) 3.
[6] S. Park, J.A. Park, H. Yoo, H.B. Park, Y. Lee, Proteasome inhibitor-induced cleavage of HSP90 is mediated by ROS generation and caspase 10-activation in human leukemic cells, Redox Biol. 13 (2017) 470–476.
[7] S. Volpe, G. Galeati, C. Bernardini, C. Tamanini, G. Mari, D. Zambelli, E. Seren, M. Spinaci, Comparative immunolocalization of heat shock proteins (Hsp) − 60, − 70, − 90 in boar, stallion, dog and cat spermatozoa, Reprod. Domest. Anim. 43 (2008) 385–392.
[8] S.Y. Huang, Y.H. Kuo, H.L. Tsou, Y.P. Lee, Y.T. King, H.C. Huang, P.C. Yang, W.C. Lee, The decline of porcine sperm motility by geldanamycin, a specific inhibitor of heat-shock protein 90 (HSP90), Theriogenology 53 (2000) 1177–1184.
[9] W.L. Cao, Y.X. Wang, Z.Q. Xiang, Z. Li, Cryopreservation-induced decrease in heat-shock protein 90 in human spermatozoa and its mechanism, Asian J. Androl. 5 (2003) 43–46.
[10] J.C. Martinez-Soto, J. de DiosHourcade, A. Gutiérrez-Adán, J.L. Landeras, J. Gadea, Effect of genistein supplementation of thawing medium on characteristics of frozen human spermatozoa, Asian J. Androl. 12 (2010) 431–441.
[11] N. Zribi, N.F. Chakroun, F. Ben Abdallah, H. Elleuch, A. Sellami, J. Gargouri,

T. Rebai, F. Fakhfakh, L.A. Keskes, Effect of freezing-thawing process and quercetin on human sperm survival and DNA integrity, Cryobiology 65 (2012) 326–331.
[12] U. Kilic, A.B. Caglayan, M.C. Beker, M.Y. Gunal, B. Caglayan, E. Yalcin, T. Kelestemur, R.Z. Gundogdu, B. Yulug, B. Yılmaz, B.E. Kerman, E. Kilic, Particular phosphorylation of PI3K/Akt on Thr308 via PDK-1 and PTEN mediates melatonin's neuroprotective activity after focal cerebral ischemia in mice, Redox Biol. 12 (2017) 657–665.
[13] R. Luboshitzky, Z. Shen-Orr, P. Herer, Seminal plasma melatonin and gonadal steroids concentrations in normal men, Arch. Androl. 48 (2002) 225–232.
[14] A. Ortiz, J. Espino, I. Bejarano, G.M. Lozano, F. Monllor, J.F. García, J.A. Pariente, A.B. Rodríguez, High endogenous melatonin concentrations enhance sperm quality and short-term *in vitro* exposure to melatonin improves aspects of sperm motility, J. Pineal Res. 50 (2011) 132–139.
[15] J. Espino, I. Bejarano, A. Ortiz, G.M. Lozano, J.F. García, J.A. Pariente, A.B. Rodríguez, Melatonin as a potential tool against oxidative damage and apoptosis in ejaculated human spermatozoa, Fertil. Steril. 94 (2010) 1915–1917.
[16] I. Ashrafi, H. Kohram, F.F. Ardabili, Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa, Anim. Reprod. Sci. 139 (2013) 25–30.
[17] Y. Zhao, L.W. Qi, W.M. Wang, P.K. Saxena, C.Z. Liu, Melatonin improves the survival of cryopreserved callus of *Rhodiola crenulata*, J. Pineal Res. 50 (2011) 83–88.
[18] H. Shi, D.X. Tan, R.J. Reiter, T. Ye, F. Yang, Z. Chan, Melatonin induces class A1 heat-shock factors (HSFA1s) and their possible involvement of thermotolerance in Arabidopsis, J. Pineal Res. 58 (2015) 335–342.
[19] S. Varesi, V. Vernocchi, M.G. Morselli, G.C. Luvoni, DNA integrity of fresh and frozen canine epididymal spermatozoa, Reprod. Biol. 14 (2014) 257–261.
[20] M.T. Abdel Aziz, T. Mostafa, N. Roshdy, H. Hosni, L. Rashed, D. Sabry, T. Abdel Nasser, O. Abdel Azim, O. Abdel Gawad, Heme oxygenase enzyme activity in human seminal plasma of fertile and infertile males, Andrologia 40 (2008) 292–297.
[21] K. Chen, Z. Mai, Y. Zhou, X. Gao, B. Yu, Low NRF2 mRNA expression in spermatozoa from men with low sperm motility, Tohoku J. Exp. Med. 228 (2012) 259–266.
[22] J. Cadet, K.J.A. Davies, M.H. Medeiros, P. Di Mascio, J.R. Wagner, Formation and repair of oxidatively generated damage in cellular DNA, Free. Radic. Biol. Med. 107 (2017) 13–34.
[23] C. Zerbinati, L. Caponecchia, R. Puca, M. Ciacciarelli, P. Salacone, A. Sebastianelli, A. Pastore, G. Palleschi, V. Petrozza, N. Porta, R. Rago, A. Carbone, L. Iuliano, Mass spectrometry profiling of oxysterols in human sperm identifies 25-hydroxycholesterol as a marker of sperm function, Redox Biol. 11 (2017) 111–117.
[24] M. Gavella, V. Lipovac, Antioxidative effect of melatonin on human spermatozoa, Arch. Androl. 44 (2000) 23–27.
[25] N.K. Duru, M. Morshedi, A. Schuffner, S. Oehninger, Cryopreservation-thawing of fractionated human spermatozoa and plasma membrane translocation of phosphatidylserine, Fertil. Steril. 75 (2001) 263–268.
[26] S. Kim, S. Hooper, C. Agca, Y. Agca, Post-thaw ATP supplementation enhances cryoprotective effect of iodixanol in ratspermatozoa, Reprod. Biol. Endocrinol. 14 (2016) 5.
[27] M. Martín, M. Macías, G. Escames, R.J. Reiter, M.T. Agapito, G.G. Ortiz, D. Acuña-Castroviejo, Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red *in vivo*, J. Pineal Res. 28 (2000) 242–248.
[28] M.H. Karimfar, F. Niazvand, K. Haghani, S. Ghafourian, R. Shirazi, S. Bakhtiyari, The protective effects of melatonin against cryopreservation-induced oxidative stress in human sperm, Int. J. Immunopathol. Pharmacol. 28 (2015) 69–76.
[29] S. Succu, F. Berlinguer, V. Pasciu, V. Satta, G.G. Leoni, S. Naitana, Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner, J. Pineal Res. 50 (2011) 310–318.
[30] M.A. Geihs, M.A. Vargas, F.E. Maciel, S.S. Caldas, B.P. Cruz, E.G. Primel, J.M. Monserrat, L.E. Nery, Effect of melatonin in the antioxidant defense system in the locomotor muscles of the estuarine crab *Neohelice granulata* (Decapoda, Brachyura), Gen. Comp. Endocrinol. 166 (2010) 72–82.
[31] M.B. Shamsi, S. Venkatesh, M. Tanwar, P. Talwar, R.K. Sharma, A. Dhawan, R. Kumar, N.P. Gupta, N. Malhotra, N. Singh, S. Mittal, R. Dada, DNA integrity and semen quality in men with low seminal antioxidant levels, Mutat. Res. 665 (2009) 29–36.
[32] D.S. Salmanoglu, T. Gurpinar, K. Vural, N. Ekerbicer, E. Darıverenli, A. Var, Melatonin and L-carnitin improves endothelial disfunction and oxidative stress in Type 2 diabetic rats, Redox Biol. 8 (2016) 199–204.
[33] J. Gadea, M. Molla, E. Selles, M.A. Marco, F.A. Garcia-Vazquez, J.C. Gardon, Reduced glutathione content in human sperm is decreased after cryopreservation: effect of the addition of reduced glutathione to the freezing and thawing extenders, Cryobiology 62 (2011) 40–46.
[34] M.G. Buffone, J.C. Calamera, S. Brugo-Olmedo, S. De Vincentiis, M.M. Calamera, B.T. Storey, G.F. Doncel, J.G. Alvarez, Superoxide dismutase content in sperm correlates with motility recovery after thawing of cryopreserved human spermatozoa, Fertil. Steril. 97 (2012) 293–298.
[35] C.M. da Silva, B. Macías-García, A. Miró-Morán, L. González-Fernández, A. Morillo-Rodriguez, C. Ortega-Ferrusola, J.M. Gallardo-Bolaños, G. Stilwell, J.A. Tapia, F.J. Peña, Melatonin reduces lipid peroxidation and apoptotic-like changes in stallion spermatozoa, J. Pineal Res. 51 (2011) 172–179.
[36] A.K. Singh, C. Haldar, Melatonin modulates glucocorticoid receptor mediated inhibition of antioxidantresponse and apoptosis in peripheral blood mononuclear cells, Mol. Cell. Endocrinol. 436 (2016) 59–67.
[37] U. Paasch, R.K. Sharma, A.K. Gupta, S. Grunewald, E.J. Mascha, A.J. Thomas Jr., H.J. Glander, A. Agarwal, Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa, Biol. Reprod. 71 (2004) 1828–1837.
[38] R.J. Aitken, G.N. De Iuliis, J.M. Finnie, A. Hedges, R.I. McLachlan, Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria, Hum. Reprod. 25 (2010) 2415–2426.
[39] I. Bejarano, F. Monllor, A.M. Marchena, A. Ortiz, G. Lozano, M.I. Jiménez, P. Gaspar, J.F. García, J.A. Pariente, A.B. Rodríguez, J. Espino, Exogenous melatonin supplementation prevents oxidative stress-evoked DNA damage in human spermatozoa, J. Pineal Res. 57 (2014) 333–339.
[40] P. Wang, Y.F. Wang, H. Wang, C.W. Wang, L.S. Zan, J.H. Hu, Q.W. Li, Y.H. Jia, G.J. Ma, HSP90 expression correlation with the freezing resistance of bull sperm, Zygote 22 (2014) 239–245.
[41] I. Casas, S. Sancho, J. Ballester, M. Briz, E. Pinart, E. Bussalleu, M. Yeste, A. Fàbrega, J.E. Rodríguez-Gil, S. Bonet, The HSP90AA1 sperm content and the prediction of the boar ejaculate freezability, Theriogenology 74 (2010) 940–950.
[42] X.G. Zhang, S. Hu, C. Han, Q.C. Zhu, G.J. Yan, J.H. Hu, Association of heat shock protein 90 with motility of post-thawed sperm in bulls, Cryobiology 70 (2015) 164–169.
[43] E. Flores, D. Cifuentes, J.M. Fernández-Novell, A. Medrano, S. Bonet, M.D. Briz, E. Pinart, A. Peña, T. Rigau, J.E. Rodríguez-Gil, Freeze-thawing induces alterations in the protamine-1/DNA overall structure in boar sperm, Theriogenology 69 (2008) 1083–1094.
[44] Y. Wei, W. Hu, Q. Wang, H. Zeng, X. Li, Y. Yan, R.J. Reiter, C. He, H. Shi, Identification, transcriptional and functional analysis of heat-shock protein 90s in banana (*Musa acuminata* L.) highlight their novel role in melatonin-mediated plant response to Fusarium wilt, J. Pineal Res. 62 (2017).
[45] A. Leja-Szpak, P. Pierzchalski, M. Goralska, K. Nawrot-Porabka, J. Bonior, P. Link-Lenczowski, M. Jastrzebska, J. Jaworek, Kynuramines induce overexpression of heat shock proteins in pancreatic cancer cells via 5-hydroxytryptamine and MT1/MT2 receptors, J. Physiol. Pharmacol. 66 (2015) 711–718.
[46] S. Polier, R.S. Samant, P.A. Clarke, P. Workman, C. Prodromou, L.H. Pearl, ATP-competitive inhibitors block protein kinase recruitment to the Hsp90-Cdc37 system, Nat. Chem. Biol. 9 (2013) 307–312.