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Melatonin and selenium supplementation in extenders improves the post-thaw quality parameters of rat sperm

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Objective: The aim of this study was to determine the effects of melatonin and selenium in freezing extenders on frozen-thawed rat sperm.
Methods: Semen samples were collected from 20 adult male Wistar albino rats. Following dilution, the samples were divided into six groups: four cryopreserved groups with 1 mM and 0.5 mM melatonin and selenium supplements, and two fresh and cryopreserved control groups. The rapid freezing technique was used to freeze the samples. Flow cytometry was used to assess plasma membrane integrity, mitochondrial membrane potential, and DNA damage, while computer-assisted sperm analysis was used to assess motility.
Results: Total motility was higher in the 1 mM melatonin supplementation group than in the cryopreserved control group (mean±standard error of the mean, 69.89±3.05 vs. 59.21±1.31; $p \leq 0.05$). The group with 1 mM selenium had the highest plasma membrane integrity (42.35%±1.01%). The cryopreserved group with 0.5 mM selenium had the highest mitochondrial membrane potential, whereas the cryopreserved control group had the lowest (45.92%±4.53% and 39.45%±3.52%, respectively).
Conclusion: Cryopreservation of rat semen supplemented with 1 mM melatonin increased sperm motility after freeze-thawing, while supplementation with 0.5 mM selenium increased mitochondrial activity.

Keywords: Computer-assisted sperm analysis; Flow cytometry; Melatonin; Selenium

Introduction

Changes in sperm occur during the cryopreservation cycle, including membrane lipid peroxidation, membrane integrity changes, mitochondrial damage, acrosome damage, DNA denaturation, and chromatin damage [1]. The harmful changes in the semen samples of different species are caused by reactive oxygen species (ROS) and free radicals during the freezing process [2,3]. ROS, along with low antioxidant levels in seminal plasma, causes oxidative stress, which leads to a reduction in semen quality [4]. Enzymatic and antioxidant systems in sperm and seminal plasma are responsible for reducing or neutralizing free radicals, but these systems are not always sufficient for protection [5]. To reduce oxidative damage during the freezing process, an extender supplement with an appropriate antioxidant system is required [4]. Melatonin (N-acetyl-5-methoxytryptamine; MT) is an indoleamine hormone that is synthesized from tryptophan in the pineal gland [6-8]. MT neutralizes the toxic effects of ROS by isolating reactive free radicals. This compound helps maintain cellular function by stimulating antioxidant enzymes and neutralizing toxic species such as nitric oxide, peroxynitrite anion, and hydrogen peroxide [9,10].

Evidence has shown that MT has the ability to improve sperm parameters, including total motility, plasma membrane integrity (PMI), survival rate, and DNA integrity, and decrease lipid peroxidation [4,11]. Selenium (Se) is a rare biological mineral that regulates mito-

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chondrial function, thyroid hormone synthesis, and phagocytic function, among other metabolic functions [10]. Se acts as part of antioxidant enzymes such as glutathione peroxidase (GPx) [12]. By detoxifying hydrogen peroxide, this enzyme protects against peroxidative damage [13]. Several endogenous antioxidants protect epididymal sperm from oxidative stress [14]. Studies have shown that Se can improve the quality of sperm in impotent camels [15]. As a result of its antioxidant properties, this compound has been studied extensively in animal research [16]. The aim of the present study was to evaluate the antioxidant effect of MT and Se on the functional parameters of epididymal rat sperm, including motility, cell membrane and mitochondrial integrity, and DNA damage during freezing.

Methods

1. Animals

A total of 20 adult male Wistar albino rats weighing 150–200 g (10–12 weeks old) were used as sperm donors. The animals were obtained from the Animal Center at Yasuj University of Medical Sciences. All animal housing and surgical procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Yasuj University of Medical Sciences, Yasuj, Iran. The rats were housed in a pathogen-free environment in animal cages with standard food and water at a temperature of 23°C ± 1°C with a relative humidity of 55% ± 10% under a 14-hour light/10-hour dark cycle.

2. Semen collection

The rats were killed using cervical dislocation. During surgery and under sterile conditions, the tails of the epididymis that contained sperm were collected and placed in a 35 mm culture dish containing 5 mL of HEPES buffered Tyrode's lactate (TL-HEPES) solution with 3 mg/mL bovine serum albumin supplementation. Incisions were made at the tail of the epididymis using insulin injection needles to extract the sperm. The samples were then incubated at 37°C with 5% CO_2 for 5 minutes to remove the sperm from the epididymis. The sperm suspension was poured into a 5 mL conical tube and stored at 22°C for further experiments. The TL-HEPES extender solution was prepared as follows: 2.3 mM KCl, 114 mM NaCl, 0.4 mM $NaH_2PO_4 \cdot H_2O$, 0.2 mM $CaCl \cdot H_2O$, 0.5 mM $MgCl \cdot 6H_2O$, 2 mM $NaHCO_3$, 10 mM lactic acid, 10 mM HEPES, and 10 mL/L penicillin-streptomycin [17].

3. Freezing and thawing procedure

Semen samples with a concentration of 20×10^6 sperm/mL to 30×10^6 sperm/mL were diluted in a TL-HEPES extender solution, and the semen of each rat was divided into six groups, with four groups containing MT and Se at concentrations of 0.5 mM and 1 mM and two groups containing no added material, which were used as fresh and cryopreservation control groups, respectively [18,19]. The samples were cooled to 4°C and equilibrated at this temperature for 2 hours. Next, 0.5 mL of each sample was placed on French straws and sealed using a sealing device. They were then cooled for 10 minutes in nitrogen vapor (suspended 3 cm above liquid nitrogen) before being poured into liquid nitrogen at −196°C. The frozen straws were thawed for 30 seconds in a 37°C water bath before evaluation [20].

4. Semen evaluation

1) *Computer-Assisted Sperm Analysis*

Sperm motility analysis was performed using computer-assisted sperm analysis (CASA; SCA, Microptic Co., Barcelona, Spain). In this study, a 10 µL thawed semen sample was placed on a Makler chamber slide that had already been preheated to 37°C. The evaluated parameters included motility (%), progressive motility (%), average path velocity (µm/sec), curvilinear velocity (µm/sec), linearity (%), straight-line velocity (µm/sec), straightness (%), wobble (%), and beat cross-frequency (Hz), as shown in Table 1.

2) *Integrity of the DNA*

The sperm DNA integrity was assessed using acridine orange (AO) staining. The heated samples were first put in a centrifuge for 5 minutes at 500 ×*g*. The pellets were then mixed with a null ethylenediaminetetraacetic acid (EDTA) buffer solution containing 10 mmol Tris, 0.15 mol NaCl, and 1 mmol EDTA and were dissolved at a concentration of 5×10^6 sperm/mL. A total of 400 µL of acid detergent solution and 1,200 µL of AO staining solution were added to the mixture. After 15 seconds, the sperm were evaluated using flow cytometry [21].

3) *Plasma membrane integrity*

The hypo-osmotic test was used to evaluate the sperm PMI. First, 50 µL of thawed semen was mixed with 50 µL of HOS solution (1.35 g fructose, 0.735 g sodium citrate with 100 mL distilled water at an osmotic pressure of 190 mOsmol). The resulting solution was then incubated in an incubator (37°C, 45 minutes). Finally, 10 µL of the sample was placed on a dry and preheated slide and covered with a slip cover. A contrast phase microscope (Olympus BX20) with ×400 magnification was used for microscopic evaluation. In each slide, 200 spermatozoa were studied, and sperm with swollen and twisted tails were considered to have integrated membranes [22].

5. Mitochondrial membrane integrity

Molecular probes of 5,5′, 6,6′-tetrachloro1,1′3,3′-tetramethyl benzimidazolyl-carbocyanine iodide (JC-1)-PI were used to evaluate the mitochondrial membranes of thawed sperms. For this purpose, thawed semen samples were put in a centrifuge for 5 minutes at 500 ×*g*. The spermatozoa were then dissolved in 487 µL of phosphate-buffered

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Table 1. Effects of a complemented semen extender with nothing added (control) or two concentrations of melatonin or selenium on various CASA kinetic parameters in frozen/thawed rat sperm

Group	Fresh control	Freezing control	Melatonin		Selenium	
			0.5 mM	1 mM	0.5 mM	1 mM
Total motility (%)	87.32±4.21	59.21±1.31[a]	67.00±2.63	69.89±3.05[b]	65.01±4.14	61.09±4.21
Progressive motility (%)	59.44±2.81	39.30±2.05[a]	45.21±2.00	46.83±3.14	39.11±4.11	43.62±2.12
VCL (μm/sec)	95.15±3.03	83.30±3.31[a]	89.21±1.76	93.33±1.69	81.36±5.72	84.70±3.09
VSL (μm/sec)	70.00±4.22	45.01±4.42[a]	48.52±2.27	56.81±3.45	39.03±6.30	51.73±1.25
VAP (μm/sec)	62.15±3.15	52.66±4.33[a]	57.02±2.69	64.07±3.72	48.68±6.00	57.33±2.70
LIN (%)	73.60±5.01	50.42±5.17[a]	51.05±2.55	57.42±4.09	43.66±5.25	58.03±1.56
STR (%)	80.40±3.20	71.09±3.67[a]	71.21±1.52	75.43±1.76	66.92±4.47	76.71±1.04
WOB (%)	72.32±3.77	62.81±3.11[a]	63.47±2.17	67.51±4.07	59.22±3.59	67.03±2.12
BCF (Hz)	14.54±1.01	10.97±0.35[a]	9.52±0.63	9.31±1.07	10.85±0.61	10.79±0.40

Values are presented as the mean±standard error of the mean.
CASA, computer-assisted sperm analysis; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; BCF, beat cross frequency.
[a]$p<0.05$, significant differences vs. fresh control group; [b]$p<0.05$, significant differences vs. frozen control group.

Table 2. Effects of complemented semen extender with nothing added (control) or two concentrations of either melatonin or selenium on plasma membrane integrity, MMP, and DNA damage in frozen/thawed rat sperm

Group	Fresh control	Freezing control	Melatonin		Selenium	
			0.5 mM	1 mM	0.5 mM	1 mM
PMI (%)	51.70±2.26	40.51±1.43[a]	36.15±1.49[b]	35.20±0.27[b]	39.93±1.30	42.35±1.01
High MMP (%)	60.31±3.54	39.45±3.52[a]	43.51±1.09	44.00±1.15	45.92±4.53[b]	44.16±1.03
DNA damage (%)	5.03±3.12	9.61±3.25[a]	4.22±1.50[b]	3.66±1.50[b]	3.15±1.01[b]	3.42±0.91[b]

Values are presented as the mean±standard error of the mean.
MMP, mitochondrial membrane potential; PMI, plasma membrane integrity.
[a]$p<0.05$, significant differences vs. fresh control group; [b]$p<0.05$, significant differences vs. frozen control group.

saline solution. Then, 10 μL of a JC-1 solution and 3 μL of a PI solution were added. Finally, they were incubated in a warm water bath (37°C for 30 minutes) in a dark room. Mitochondrial membrane potential (MMP) analysis was performed using CytExpert 2.2 software [23].

6. Flow cytometry

Flow cytometry was performed using a Cytoflex flow cytometer (Beckman Coulter). This system contains a 50 MW laser output (488 nm laser beam) with 610±20 nm, 585±42 nm, and 525±40 nm emission filters. 10×10^3 spermatozoa were collected for each analysis.

7. Statistical analysis

The Shapiro-Wilk test was used to analyze the normality of data distribution. One-way analysis of variance and the Tukey test were used to detect differences between the groups and to determine the locations of differences. Statistical analysis was carried out using IBM SPSS Statistics for Windows ver. 20 (IBM Corp., Armonk, NY, USA). The results are presented as the mean percentage and the standard error of the mean, and $p<0.05$ was considered to indicate statistical significance.

Results

The addition of 1 mM and 0.5 mM MT and Se to the extender led to improved total motility compared to the frozen control group. In addition, 1 mM MT was associated with more total motility than the other groups. However, no statistically significant differences between the groups were observed for the other dynamic parameters (Table 1). A higher PMI was observed in the 1 mM Se group than in either MT group. In addition, the highest percentage of sperm with high MMP was observed in the 0.5 mM Se group, and the lowest was observed in the control group (45.92%±4.53% and 39.45%±3.52%, respectively). MT and Se reduced DNA damage at both concentrations (Table 2).

Discussion

Sperm cytoplasm is prone to the overproduction of ROS and exposure to severe oxidative stress during the freezing process due to an insufficient immune response and concentration of antioxidants [21,22]. Balanced and optimal amounts of ROS are necessary for im-

proved performance and enhance motility with increased cyclic adenosine monophosphate and protein phosphorylation; however, high amounts of ROS resulting from a lack of sufficient antioxidant capacity can have harmful and toxic effects [24]. Oxidative stress caused by free radicals produced in the sperm cytoplasm has a significant role in reducing sperm fertility [25]. During freezing, the antioxidant capacity of frozen sperm decreases. However, semen dilution processes can reduce or eliminate antioxidant compositions in semen and lead to increased oxidative stress [24]. Several main endogenous antioxidant systems such as GPx, glutathione reductase (GSH), and superoxide dismutase (SOD) have been introduced for sperm [26]. Studies have shown that MT has a positive effect on the activity of antioxidant enzymes and increased proteins of SOD, GPx, GSH and catalase by increasing the synthesis of intracellular antioxidants [27,28]. Se can also improve the antioxidant activity of GPx enzymes [10]. Sperm mitochondria have the ability to produce some amount of ROS products in response to oxidative phosphorylation functions [29]. Freezing increases the release of ROS, especially superoxide anion and hydrogen peroxide, by altering the structure of the mitochondrial membrane [30]. According to the results, MT and Se improved sperm MMP in the frozen groups by increasing the activity of antioxidant enzymes and decreasing intracellular ROS. The presence of MT in freezing solutions was associated with a higher frequency of samples with a high MMP and a lower rate of PMI (as an indicator of a healthy plasma membrane). This study observed a correlation between sperm MMP and PMI. These results are comparable to a study by Gungor et al. [23] that found that gallic acid gave sperm more energy, thereby improving MMP in comparison to the control group, but resulted in a significant reduction in PMI compared to the control group. The highest percentage of high MMP was observed in sperm from the 0.5 mM Se group. Mitochondrial activity was higher in the 0.5 mM Se group than in the frozen control group, but there were no significant differences between the MT and Se groups. It is notable that MT can protect the mitochondrial structure by reducing oxygen consumption and O_2 production and, as a result, reduce lipid peroxidation and play an important role in mitochondrial activation [31]. In addition, supplementation with Se has the ability to improve the mitochondrial activity of sperm [32].

Proper motility is one of the essential characteristics of sperm related to fertility, and any disorder related to motility can prevent sperm from reaching the fertilization site [33]. Our study showed that the presence of 1 mM MT in the extender caused a significant increase in total motility. In addition, the highest degree of progressive motility corresponded to this same concentration of MT; however, no significant difference was observed in the frozen control group. The results of this part of the study are consistent with those of a study by Fadl et al. [18] that found that concentrations of 1 mM MT corresponded to the highest degree of progressive motility compared to other doses as well as the control group. In addition, the present study showed that adding Se to the extender did not significantly improve rat sperm motility after thawing. These results contradict those of a study by Khalil et al. [19] that found that Se at concentrations of 1 and 1.5 mM significantly increased sperm motility. Dorostkar et al. also evaluated different doses of Se (1 mg/mL, 2 mg/mL, 4 mg/mL, and 8 mg/mL) in buffalo and found that doses of 1 mg/mL and 2 mg/mL significantly improved sperm motility compared to the control group, while doses of 4 mg/mL and 8 mg/mL showed a decrease in sperm motility compared to the control group [34]. These contradictions might be explained by differences in sperm type, supplement concentrations, and sperm preparation and freezing processes.

The plasma membrane is involved in protecting the physiology of sperm cytosol. In freezing, osmotic changes influence membrane integrity and sperm homeostasis by changing salt concentrations [35]. In the current study, although the PMI in the group containing 1 mM Se did not differ from that of the frozen control group, it had the highest amount compared to the other groups. The motility scores for the Se groups were not higher than those of the MT group, but better and more acceptable results were observed for PMI parameters than in the MT group. In addition, PMI in the group with 0.5 mM Se concentration was not statistically significant compared to the frozen control group, while the concentration of 1 mM Se had the highest PMI. This finding can probably be attributed to the ability of Se (at a concentration of 0.5 mM) to reduce lipid peroxidation and regulate osmotic balance and pH.

The last part of the results of this study showed that adding MT and Se to the freezing medium significantly reduced DNA damage after thawing, which is consistent with the results of a study by Rezaeian et al. [32] and Breininger et al. [36] that found that Se concentrations (5 μL and 1 mg and 2 mg, respectively) reduced DNA damage after freezing. Pool et al. also observed that all doses of supplementary MT (0.1 μM, 1 μM, 10 μM, and 100 μM) helped reduce DNA damage compared to the control group [31]. Adding these supplements to increase the antioxidant capacity of freezing solutions may increase the density of chromatin structure and protect sperm from freezing damage.

In this study, the presence of 1 mM MT in the extender corresponded to the highest degree of motility, and the addition of 0.5 mM Se was associated with the highest degree of mitochondrial function in post-thaw rat sperm. However, more extensive studies that examine a wider range of concentrations are required to further understand the impact of these factors on fertility.

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Conflict of interest

No potential conflict of interest relevant to this article was reported.

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Conceptualization: ES, FB. Data curation: TM, MG. Formal analysis: TM, MG. Methodology: MG. Project administration: ES. Visualization: TM. Writing–original draft: FB. Writing–review & editing: ES.

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