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Mitochondria-targeted antioxidant MitoTEMPO improves the post-thaw sperm quality

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ABSTRACT

Human spermatozoa cryopreservation is an important means of assisted reproductive technology and male fertility preservation. Although this technique is particularly useful, sperm cryopreservation significantly reduces the quality of spermatozoa after freezing and thawing. The objective of the study is to examine the efficacy of mitochondria-targeted antioxidant MitoTEMPO in improving sperm quality during semen cryopreservation processes. Semen samples were collected and cryopreserved in extenders containing different concentrations (0.0, 0.5, 5, 50, and 500 μM) of MitoTEMPO. Sperm motility, viability, membrane integrity, mitochondrial membrane potential and antioxidant activities were measured and analyzed. The results showed that the addition of MitoTEMPO (5–50 μM) significantly improved post-thaw sperm motility, viability, membrane integrity and mitochondrial membrane potential ($P < .05$). Meanwhile, antioxidant enzymes activities were enhanced and MDA content were decreased in the group supplemented with MitoTEMPO. In conclusion, mitochondria-targeted antioxidant MitoTEMPO improves the post-thaw sperm quality and antioxidant enzymes profile.

1. Introduction

Cryopreservation is a key technique for stable long-term storage of human sperm, which is widely used in conservation of male reproductive capacity. It is a good choice for some patients with fertility requirements to preserve fertility prior to chemotherapy or radiotherapy treatments and surgical infertility interventions. Moreover, donor semen was also stored in sperm banks by cryopreservation, and the post-thaw donor sperm is now clinically widely used in oligospermia and azoospermia patients with fertility requirements [5,19,20]. However, during semen cryopreservation processes, damage usually occurs due to ice crystal formation, osmotic shock, and oxidative stress, showing diminished sperm motility, deteriorated sperm viability and poor membrane stability [10,22].

Oxidative stress is a recognized major factor that affects sperm quality due to reactive species (ROS) generation and lipid peroxidation during semen cryopreservation. Many studies have reported that supplementation of antioxidants during semen freezing process can improve frozen-thawed sperm quality in varying degrees [2,8,9,15,16], but there is still a lack of a recognized effective antioxidant.

MitoTEMPO, a novel cell permeable ROS scavenger, is comprised of the piperidine nitroxide TEMPOL unit and the lipophilic triphenylphosphonium (TPP) moiety which endows MitoTEMPOL with the ability to rapidly pass through Lipid bilayer membranes and massively accumulate in energized mitochondria, the major site of ROS generation [14]. This makes it a useful molecular sensor for detection of intracellular superoxide production, attenuation stress-induced apoptosis and necrosis [12,27]. Until now, there are few studies about the effects of supplemental mitochondria-targeted antioxidant on semen cryopreservation, and no reports have evaluated the protective effects of MitoTEMPO on sperm quality parameters during cryopreservation. Therefore, the aim of the present study was to examine the role of various concentrations of MitoTEMPO in the cryopreservation of human sperm using a commercially available cryopreservation solution.

2. Materials and methods

2.1. Semen samples

This study was approved and monitored by the Ethics Committees of Jinan Central Hospital. Volunteers were the men with normal sperm parameters who came to our laboratory for pre-pregnancy examination, and were given informed consent before recruitment into the study. Samples were collected into sterile disposable containers with lid by masturbation after 2–7 d of sexual abstinence. All sperm parameters

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were evaluated using a computer-assisted system based on World Health Organization (WHO) guidelines [30].

2.2. Sperm cryopreservation

Sperm freezing was performed using a commercialized cryoprotectant medium, SpermFreeze SolutionTM (Vitrolife, Goteborg, Sweden). After semen liquefaction, samples were diluted 1:1 with SpermFreeze Solution supplemented with 0.0, 0.5, 5, 50, and 500 μM of MitoTEMPO (Enzo Life Sciences, Farmingdale, NY). The equilibrated samples were transferred to cryovials (Corning, Corning, NY) and placed horizontally for 30 min at 1–5 cm above the liquid nitrogen surface, then plunged into liquid nitrogen and stored for a week. In the thawing procedures, Cryovials were taken out of liquid nitrogen and thawed in a 35 ± 2 °C water bath for at least 30 s. After thawing, semen samples were immediately evaluated for motility, vitality, mitochondrial membrane potential and ROS status.

2.3. Measurement of sperm motility and vitality

Sperm motility was analyzed using the computer-assisted sperm motility analysis program (CASA, Tsinghua Tongfang, Shanghai, China) for all samples. Sperm viability was assessed using eosin-nigrosin stain. Briefly, 10 μL of each sample was mixed with the staining solution, and then drop the mixture onto a glass slide. Thin smears were made and allowed to air-dry, and 200 sperms were counted and the percent of stained and unstained sperms was calculated on each slide under a phase-contrast microscope. The unstained sperms were considered as live [29].

2.4. Measurement of sperm membrane integrity

Sperm membrane integrity (PMI) was evaluated using the hypo-osmotic swelling test (HOST). Briefly, 20 μL semen sample was added to 200 μL hypo-osmotic solution containing fructose (9.0 g/L) and sodium citrate (4.9 g/L) and incubated at 37 °C for 30 min. After that, a minimum of 200 sperms were counted under a phase-contrast microscope, and the swollen sperm with coiled or looped tail was considered to have an intact plasma membrane [4].

2.5. Measurement of sperm mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨm) was evaluated by dual fluorescent staining, JC-1 and PI (Beyotime, China), following a previously described method [6]. JC-1 selectively enters mitochondria as monomers and emits green fluorescence when ΔΨm is relatively low. Alternatively, JC-1 forms multimers of J-aggregates and fluoresce red in cells with high ΔΨm. Approximately 2×10^6 sperms from each sample were mixed with 50 μL JC-1 staining working solution (10 μg/mL) and incubated at 37 °C for 30 min, and then counterstained with PI (0.5 mg/ml). After staining, the samples were examined by fluorescence microscope. Sperm cells exhibiting only orange/red fluorescence were considered to have complete mitochondrial function (higher ΔΨm) and the proportion was used to represent the level of ΔΨm in each sample. For each group, three slides were assessed per sample, and about 200 sperms were evaluated per slide.

2.6. Western blotting

Immunoblotting was performed as previously described [28]. Briefly, protein extracts of human sperm were prepared and the protein concentrations were determined by bicinchoninic acid assays (Thermo Scientific, USA). Equal amounts of protein were electrophoresed on 10% SDS-PAGE and then transferred onto PVDF membranes. Membranes were blocked with 5% nonfat dried milk in Tris-buffered saline for 1 h at room temperature and then incubated overnight with antibodies to GPI (sc-3650; Santa Cruz,USA), and Beta-actin (CoWin, China). Subsequently, the membranes were incubated with HRP-conjugated secondary antibody and finally visualized using an enhanced chemiluminescence method.

2.7. Biochemical assays

Semen samples were centrifuged at 1000 g for 10 min to remove the supernatants, and the pellets were then washed three times with phosphate-buffered saline buffer (PBS). After that, the pellets were re-suspended in lysis buffer containing 0.2% Triton X-100 and incubated on ice for 20 min. The obtained supernatant was then collected for the biochemical analyses.

Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GSh-Px) activities were determined with spectrophotometric method using commercially available kits (Jiancheng Bioengineering Institute, Jiangsu, China) according to the manufacturer's protocol which were described in detail by Hu et al. [13]. Malon-dialdehyde (MDA) content was analyzed by the thiobarbituric acid (TBA) method using the test kits (Jiancheng Bioengineering Institute, Jiangsu, China). Briefly, MDA reacted with TAB to form a stable chromophoric production with a maximum absorption at 532 nm. The spermatozoa samples MDA levels were measured at the wavelength and expressed as nmol/mL.

2.8. Statistical analyses

The experiment was repeated five times, results were expressed as mean ± SEM (standard error of the mean) and analyzed using one-way analysis of variance (ANOVA) to assess differences. The value of $P < .05$ was regarded as statistically significant.

3. Result

3.1. Effects of MitoTEMPO on sperm characteristics

The effects of MitoTEMPO supplementation on the parameters (vitality, motility and membrane integrity) of frozen–thawed sperms were analyzed and the results were listed in Table 1. The parameters of sperm were significantly reduced after cryopreservation compared to the fresh state ($P < .05$). The addition of different concentrations of MitoTEMPO (0.5–500 μM) improved sperm parameters in varying degrees when compared to the group without MitoTEMPO addition (0.0 μM), and the difference of each parameters in the 5 and 50 μM groups was significant ($P < .05$).

Table 2 shows the effects of MitoTEMPO supplementation on post-thawed sperm mitochondrial membrane potential (ΔΨm). ΔΨm was greater in the extenders supplemented with 5, 50 and 500 μM MitoTEMPO, compared to the group without addition ($P < .05$). Glucose-6-phosphate isomerase, a proven new molecular marker for cryogenic damage in sperm, was significantly reduced after freezing ($P < .01$). For frozen sperm, the GPI protein was significantly increased in the group with MitoTEMPO (50 μM) addition compared with the non-added group (Fig. 1).

3.2. Effects of MitoTEMPO on antioxidant activities

The effects of MitoTEMPO supplementation on SOD, CAT and GSH-Px activities and the MDA content of frozen–thawed semen were presented in Table 3. After cryopreservation a significant decrease in antioxidant enzymes activities and a significant increase in MDA concentration were observed ($P < .05$), adding MitoTEMPO partially reversed this trend. The CAT activity increased and MDA concentration decreased with the increase of concentration (0.5–50 μM) and the difference was significant (5 and 50 μM), however, the improvement showed a downward trend when MitoTEMPO concentration was higher