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Effects of MTG and GSH on Human Sperm Motility and DNA Integrity during Vitrification in the Presence of Trehalose

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Abstract

Limited information exists about the effects of antioxidants on sperm motility and DNA integrity during vitrification in humans. This study compared the effects of reduced glutathione (GSH) and monothioglycerol (MTG) at different concentrations on post-thaw sperm motility and DNA integrity after vitrification in humans using 0.25 M trehalose as a cryoprotective agent, and found that supplementation of MTG or GSH at 0.5 mM resulted in significantly higher ($P < 0.05$) recovery rates of post-thaw total and progressive motilities. GSH was more powerful than MTG at the same concentration in cryoprotecting sperm motility (38.9% ± 3.6% vs 32.8% ± 2.4% compared to the control 26.8% ± 2.1% in recovery rate of progressive motility), but both had no significant influence on sperm DNA integrity during vitrification. It was concluded that sperm motility is more sensitive to oxidative stress during vitrification than sperm nuclear DNA, and supplementation of MTG or GSH in vitrification medium is beneficial in cryoprotecting sperm motility.

Keywords

Human, Sperm, Vitrification, Trehalose, Antioxidant

1. Introduction

Sperm cryopreservation has long been an important strategy in fertility preservation and assisted reproduction. Despite the extensive progress that has been made in the field, decreases in sperm motility and DNA integrity are commonly

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observed after cryopreservation [1] [2]. The mechanisms of cryodamage to human sperm have been linked to cold shock, osmotic stress, intracellular ice formation and oxidative stress [1] [3] [4] [5].

Antioxidant supplementation to cryopreservation media has been explored to minimize sperm cryodamage. Studies have shown that antioxidants quercetin, Vitamin E, melatonin, reduced glutathione (GSH) and MitoTEMPO are beneficial to improve post-thaw sperm motility after slow freezing [5] [6] [7] [8] and rapid freezing [9] [10] [11] [12]. Quercetin and GSH were also reported to be efficient in protecting the integrity of sperm nuclear DNA during slow freezing [6] [7] [8] [10] and rapid freezing [9] [11]. However, limited information exists about the use of antioxidants for human sperm cryopreservation by vitrification, although vitrification using sucrose or trehalose as non-permeating cryoprotective agents (CPA) has been demonstrated to be superior to slow freezing in cryopreserving sperm motility and DNA integrity [13] [14] [15]. So far, only butylhydroxytoluene (BHT, a synthetic analogue of vitamin E) and hypotaurine were reported to be beneficial in vitrifying human sperm motility and DNA integrity [16] [17]. In addition, antioxidant monothioglycerol (MTG) has been found to be extremely effective in cryoprotecting mouse sperm motility, DNA integrity and fertilization ability [18] [19] [20]; however, this antioxidant has not yet been tested for human sperm cryopreservation.

Thus, the objective of the present study was to examine and compare the efficacies of the antioxidants MTG and GSH in cryoprotecting sperm motility and nuclear DNA integrity during vitrification in humans using trehalose as a CPA.

2. Materials and Methods

2.1. Materials

All chemicals and reagents were purchased from Sigma-Aldrich Co. (Shanghai, China) unless otherwise stated. Human tubal fluid (HTF) medium containing 5 mg/ml human serum albumin (HSA) was prepared according to the method of Quinn *et al.* [21] GSH and MTG stock solutions were prepared in HTF medium.

2.2. Experimental Designs

Three experiments were performed, and each experiment was repeated at least 3 times using normozoospermic samples from different donors. Experiments 1 and 2 were to study the cryoprotective effects of MTG and GSH, respectively, during vitrification on recovery rates of sperm total motility and progressive sperm motility and sperm nuclear DNA integrity. Experiment 3 was to compare the cryoprotective effects of MTG and GSH on sperm motility and DNA integrity during vitrification.

2.3. Semen Preparation

Semen samples were obtained by masturbation in a private room near the laboratory from 15 healthy volunteer donors from the ages of 21 to 35 years old after

3 - 7 days of sexual abstinence. Written informed consent was obtained from all semen donors before the procedure. The study was approved by the Ethics Committee of Xinxiang Medical University. The semen collection, liquefaction and analysis for volume, sperm concentration, motility and morphology were carried out according to the guidelines and protocols recommended by the World Health Organization (WHO, 2010) [22]. Ejaculates with volume < 2 ml, concentration < 5×10^7/ml, progressive motility < 50% and normal sperm morphology < 30% were excluded from the study. Upon liquification at 37˚C, semen was diluted with 5% CO_2 pre-equilibrated warm HTF medium at 1:2 ratio and then the sperm were washed twice by centrifugations (400 × g for 10 min each) and resuspensions. Washed sperm were incubated at 37˚C in 5% CO_2 prior to cryopreservation.

2.4. Sperm Cryopreservation

Washed sperm in HTF medium from each ejaculate were assessed immediately for sperm quality, and then mixed thoroughly and divided into equal aliquots using a large orifice pipet tip. Each aliquot was diluted 1:1 with aqueous solution of trehalose at 0.5 mol/L, so that the final concentration of sperm was 15 - 20 × 10^6/ml and the final concentration of trehalose was 0.25 mol/L.

Immediately, each of the sperm aliquots was supplemented with different concentrations of an antioxidant (MTG or GSH) by adding 2.5 µL of 200 × stock solution of each concentration to be tested to each sperm aliquot and mixing thoroughly. The concentrations tested for each of the two antioxidants were selected on the basis of previous studies [6] [8] [19] [20]. Then, the sperm samples were loaded into straw-in-straws systems (**Figure 1**). The straw-in-straw system was made by loading a shortened (2 cm cut off from the open end) standard 0.25 mL straw (IMV Technologies, USA, catalog # 005565) with 50 µl sperm sample by aspiration to the middle part of the straw. Then, the loaded straw was placed inside a standard 0.5mL straw (IMV Technologies, USA, catalog # 005569) with their plug ends on the same side. After the plug end of the 0.5 mL straw was heat-sealed, the open ends of both the 0.25 and 0.5 mL straws were heat-sealed together using an impulse sealer. Finally, the sperm samples were equilibrated in vitrification media for 10 min at room temperature before vitrification.

Vitrification was performed by submerging sperm samples directly and horizontally into liquid nitrogen (LN2, −196˚C). Sperm samples were stored in LN2 for 1 week before analysis. All cryopreserved sperm samples were thawed by submerging a straw-in-straw system in a 37˚C water bath for 2 min before the heat seals at both ends were cut off and the sperm suspension was expelled into 1 mL pre-warmed HEPES-buffered HTF medium for dilution.

Figure 1. The straw-in-straw system loaded with 50 µl sperm and heat-sealed at both ends. The straw-in-straw system was made by inserting a 0.25 mL straw loaded with sperm into a 0.5 mL straw with their plug ends placed on the same side.

2.5. Sperm Concentration and Motility Assessments

Sperm concentration, total motility (% of motile sperm) and progressive motility (% of sperm with curvilinear velocity > 25 μm/s and straightness ≥ 0.8) at 37˚C were measured immediately before vitrification and post-thaw using counting chambers with 20-μm depth and a WLJY-9000 computer-assisted sperm analyzer (Weili New Century Science & Tech, Beijing, China). At least 2000 sperm per sample from randomly selected fields were examined. Sperm motility recovery rates including total motility recovery rate and progressive motility recovery rate were calculated and used to evaluate the cryoprotective effects of different treatments during vitrification. Motility recovery rate = (post-thaw motility ÷ pre-freeze motility) × 100%.

2.6. Sperm DNA Damage Assessments

The nuclear DNA integrity of post-thawed sperm samples was assessed by the sperm chromatin dispersion (SCD) test as described by Fernández *et al.* [23] with some modifications. Briefly, 30 μL of sperm suspension from a sample was mixed with 70 μL of 1% low melting agarose in Ca/Mg-free PBS at 37˚C, and then 30 μl of mixture was added onto a slide and spread with a 22 × 22 mm cover glass. After solidification of the agarose at 4˚C for 5 min, the cover glass was removed, and the slide was immersed in 0.08 mol/L HCl for 7 min in the dark at room temperature. Then, the slide was treated in neutralizing and lysing solution containing 0.4 mol/L Tris-HCl, pH 7.5, 0.1 mol/L dithiothreitol (DTT), 0.5% sodium dodecyl sulfate (SDS) and 0.005 mol/L ethylenediaminetetraacetic acid disodium salt solution (EDTA) for 20 min at room temperature. Further, the slide was dehydrated in 70%, 90% and 100% ethanol and air-dried. After being mounted with VECTASHIELD® containing DAPI (Vector Laboratories, Inc., USA), the slide was scored under an epifluorescence microscope (Nikon Instruments, Japan) at 1000× magnification. At least 200 sperm were examined per sample, and sperm DNA fragmentation index (DFI), *i.e.* the percentage of sperm with non-dispersed chromatin (with fragmented DNA), was calculated (**Figure 2**).

2.7. Statistical Analysis

GraphPad Prism 5 (GraphPad Software, Inc., San Diego, USA) was used for statistical analysis. Sperm motility recovery rates and percentages of sperm with DNA fragmentation were arcsine transformed, and then group differences were detected by t tests and one-way ANOVA followed by Tukey HSD tests, where $P < 0.05$ was considered significant. Data are expressed as mean (M) ± standard deviation (SD).

3. Results

3.1. Cryoprotective Effect of MTG on Sperm Motility and DNA Integrity

In this experiment, sperm were exposed to 0 (Ctrl), 0.25, 0.5, 1.0 and 2.0 mM

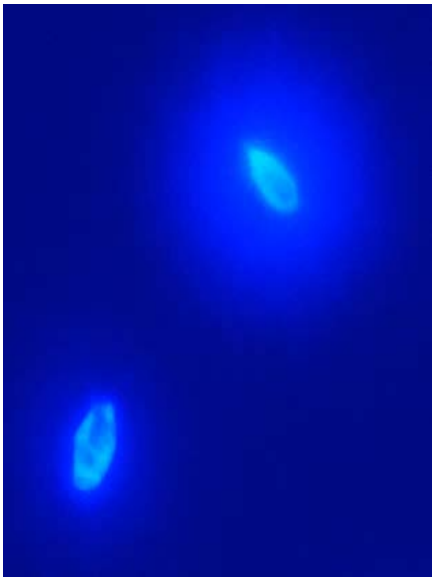

Figure 2. Human sperm SCD test. The bottom sperm with small halo indicating DNA fragmentation and the upper sperm with large halo indicating intact DNA. Original magnification 1000×.

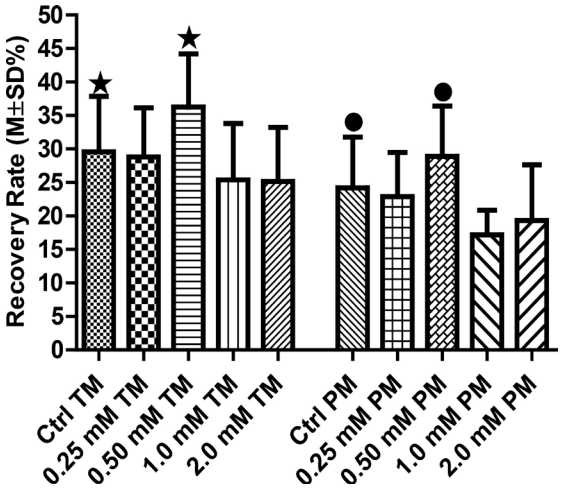

Figure 3. Effect of different concentrations of MTG on the recovery rate of sperm total motility (TM) and progressive motility (PM). $P < 0.05$ between bars with the same symbols.

MTG during vitrification. The data shown in **Figure 3** indicate that MTG at 0.5 mM significantly ($P < 0.05$) protected sperm total motility (36.3% ± 7.9% vs 29.6% ± 8.3% in recovery rate) and progressive motility (28.9% ± 7.5% vs 24.2% ± 7.6% in recovery rate) compared to the controls, but lower and higher concentrations of MTG tested had no significant effect on both total and progressive motilities ($P > 0.05$).

We also measured DFI of post-thawed sperm and the data summarized in **Figure 4** indicates that MTG had no significant effect ($P > 0.05$) on sperm DNA integrity during vitrification at the concentrations tested compared to the control DFI (13.5% ± 1.8%).

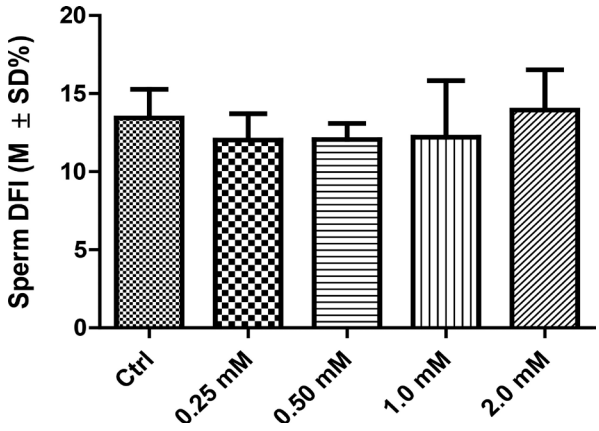

Figure 4. Effect of different concentrations of MTG on post-thaw sperm DFI. No significant difference was found among the tested concentrations.

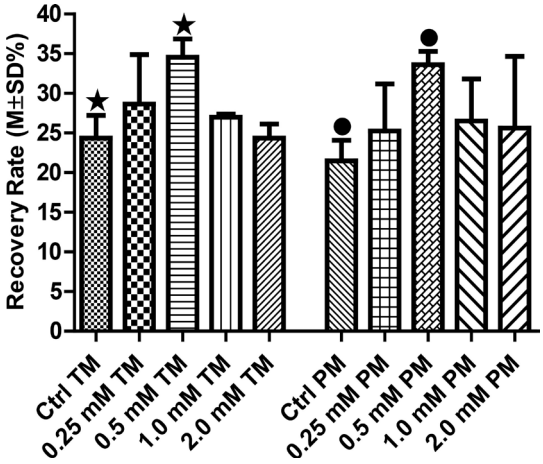

Figure 5. Effect of different concentrations of GSH on the recovery rate of sperm total motility (TM) and progressive motility (PM). $P < 0.05$ between the bars with the same symbols.

3.2. Cryoprotective Effect of GSH on Sperm Motility and DNA Integrity

In this experiment, sperm were exposed to 0 (Ctrl), 0.25, 0.5, 1.0 and 2.0 mM of GSH during vitrification. The data summarized in **Figure 5** show that 0.5 mM GSH was significantly efficient ($P < 0.05$) in cryoprotecting both total motility (34.5% ± 2.3% vs 24.4% ± 2.9% in recovery rate) and progressive motility (recovery rate 33.6% ± 1.7% vs 21.5% ± 2.6%) compared to the controls, but GSH had no significant effect on cryoprotecting sperm nuclear DNA integrity ($P > 0.05$, **Figure 6**) at the concentrations tested compared to the control DFI (8.4% ± 2.8%).

3.3. Comparison of MTG and GSH in Cryoprotecting Sperm Motility

To compare the cryoprotecting effects of MTG and GSH on sperm motility, sperm samples were exposed to MTG or GSH at the same concentration (0.5 mM) for vitrification. The post-thaw recovery rates of sperm total motility and progressive motility results are summarized in **Figure 7**. Both MTG and GSH

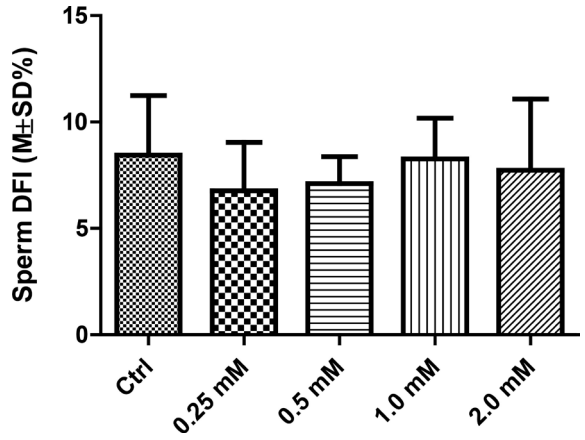

Figure 6. Effect of different concentrations of GSH on post-thaw sperm DFI. No significant difference was found among the tested concentrations.

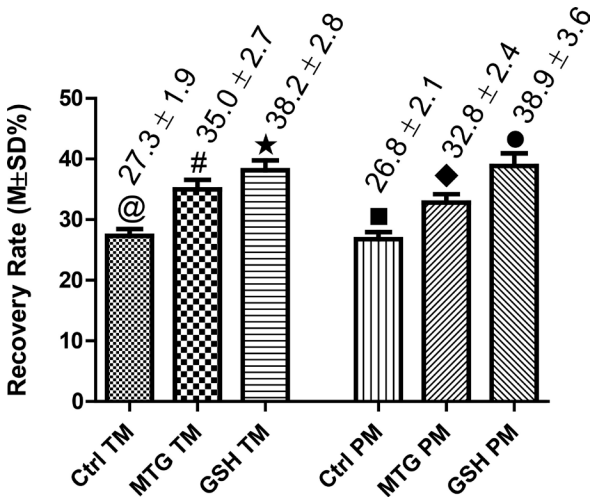

Figure 7. Comparison of MTG (0.5 mM) and GSH (0.5 mM) in cryoprotecting sperm total motility (TM) and progressive motility (PM). $P < 0.05$ between bars with different symbols in the same group (TM or PM).

had significant cryoprotective effects ($P < 0.05$) on total motility and progressive motility compared to the controls, and GSH was more efficient ($P < 0.05$) than MTG.

4. Discussion

Sperm cryopreservation significantly reduces motility, DNA integrity and fertility in humans [1] [2]. The mechanisms of sperm cryodamage are most likely multifactorial, but oxidative stress has been recognized as a significant cause [1]. Excess reactive oxygen species (ROS) produced during cryopreservation impair sperm motility and fertilization ability through membrane lipid peroxidation, protein thiol group oxidation and other mechanisms [24]. Oxidative stress can also induce DNA fragmentation in sperm nuclear genome [4] [16] [17].

Oxidative stress is caused by any imbalance between prooxidants and antioxidants in which the former prevail. The presence of an appropriate concentra-

tion of antioxidants in sperm freezing medium can neutralize the oxidative stress; however, excessive antioxidants can stop the normal sperm functions, including sperm motility and fertility, associated with ROS [25] [26] [27], and this is most likely why we found in the present study that MTG and GSH at concentrations higher than 0.5 mM had no protective effects on sperm motility during vitrification.

GSH at 1 mM and 5 mM was reported to cryoprotect human sperm motility during slow freezing [6] [7] [28], but in the present study we found 0.5 mM was the optimal concentration for human sperm vitrification. It is possible that a larger amount of ROS is produced during slow freezing than vitrification (ultra-rapid freezing) and a higher concentration of antioxidant is thus needed to protect sperm from ROS attacks.

Antioxidant MTG at 0.477 mM or 0.5 mM has been demonstrated to be effective in protecting mouse sperm motility during cryopreservation by rapid freezing [18] [19] [20]. In the present report, we found for the first time that MTG at 0.5 mM is also effective in protecting human sperm motility during vitrification. We also found MTG at 0.5 mM was less efficient in cryoprotecting both total motility and progressive motility than GSH at the same concentration.

Interestingly, we did not find significant effects of MTG and GSH on cryoprotecting human sperm DNA integrity during vitrification in the present study, and this observation is consistent with the results of Taylor *et al.* (2009) [29] and Banihani *et al.* (2014) [30] that vitamin E and L-carnitine did not affect human sperm DNA during slow freezing. Recently, antioxidants GSH, vitamin E and BHT were also found to be inefficient in cryoprotecting sperm DNA integrity during rapid freezing in boars [31]. However, studies of Merino *et al.* (2015) [16] and Seify *et al.* (2019) [17] reported that antioxidants BHT and hypotaurine were beneficial in cryoprotecting human sperm DNA during vitrification of human sperm. GSH at high concentrations (5 mM) were also reported to be efficient in cryoprotecting human sperm DNA integrity during slow freezing [6] [7] [8].

5. Conclusion

In conclusion, this study compared the effects of GSH and MTG on post-thaw sperm motility and DNA integrity after vitrification in humans and found that supplementation of MTG or GSH at 0.5 mM resulted in significantly higher post thaw total and progressive motilities. GSH is more powerful than MTG at the same concentration in protecting sperm motility, but neither had any significant influence on sperm DNA integrity during vitrification.

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Authors' Contributions

All authors have accepted responsibility for the entire content of this submitted manuscript and approved its submission.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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