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Comparison of Rapid Freezing and Vitrification for Human Sperm Cryopreservation Using Trehalose as a Cryoprotective Agent

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Abstract

Rapid freezing and vitrification are becoming popular for human sperm cryopreservation; however, it remains unclear which method is better. The aims of the present study were to determine the optimal trehalose concentration and to compare the cryoprotective effects of rapid freezing and vitrification. The results showed that: 1) The optimal trehalose concentration was 0.25 mol/L; 2) The post-thaw recovery rates of total and progressive sperm motilities after rapid freezing (38.6% ± 3.0% and 41.1% ± 5.0%) were significantly higher ($P < 0.05$) than that after vitrification (26.1% ± 3.1% and 27.2% ± 1.3%) when 0.5 mL straws were used; 3) However, the recovery rates of total and progressive motilities after rapid freezing in 0.5 mL straw (26.7% ± 9.6% and 26.8% ± 8.7%) were significantly lower ($P < 0.05$) than that after vitrification in a novel straw-in-straw system (43.1% ± 4.2% and 41.8% ± 15.5%); and 4) The post-thaw sperm nuclear DNA damage level after rapid freezing in 0.5 mL straw (8.7% ± 2.8%) was not significantly different from that of sperm after vitrification in the straw-in-straw system (9.2% ± 2.5%). It was concluded that rapid freezing is superior to vitrification when using 0.5 mL straws; however, vitrification is superior to rapid freezing when using the straw-in-straw systems.

Keywords

Human, Sperm, Rapid Freezing, Vitrification, Trehalose

1. Introduction

Sperm cryopreservation is essential to fertility preservation and assisted repro-

duction in humans. Currently, three cryopreservation methods are used including slow freezing, rapid freezing and vitrification. Since slow freezing is time consuming with the need for an expensive programmable freezer, research activities have increasingly been oriented towards the rapid freezing and vitrification methods in recent years [1]-[5]. Rapid freezing and vitrification are also superior to slow freezing in preserving sperm motility and DNA integrity [5]-[10]. In particular, rapid freezing and vitrification rely only on non-permeating cryoprotective agents (CPA), including natural sugars such as sucrose and trehalose, for sperm cryopreservation omitting the harmful effects of permeating CPA such as glycerol on human sperm [3] [4] [11] [12]. Rapid freezing is to freeze sperm in liquid nitrogen vapor followed by a rapid immersion in liquid nitrogen, and vitrification is to cryopreserve sperm by directly plunging/dropping sperm samples into liquid nitrogen [3] [6] [11] [12]. At the present, most reported applications of these two technologies describe cryopreservation of only very small volume of sperm suspension from 1 to 30 μL in closed or open systems, and standard protocols using sealed or closed systems (aseptic cryopreservation) have yet to be established [3]. In addition, few studies have been directed at comparing the safety and effectiveness of rapid freezing versus vitrification for human sperm cryopreservation, and it remains to be established which is better or whether they are equally effective. When cryoloops were used for comparison between rapid freezing (at −160˚C in liquid nitrogen vapor) and vitrification, it was found that both methods led to comparable results in terms of sperm motility, fertilization ability, and DNA integrity of the frozen-thawed sperm [13]. Studies of Agha-Rahimi *et al.* [14] also found that the post-thaw sperm motilities and DNA fragmentation levels were not significantly different between rapid freezing in cryovials and vitrification by direct dropping into liquid nitrogen. However, the study of Liu *et al.* [2] found that human sperm motility was cryopreserved significantly better by rapid freezing than vitrification using micro-capillaries as cryocontainers in the presence of different concentrations of sucrose or trehalose as CPA.

Sucrose at 0.25 mol/L concentration has been widely used for human sperm rapid freezing and vitrification [3] [7] [8] [15] [16], but the optimal concentration of trehalose for human sperm cryopreservation is still unclear. Recently, the study of Liu *et al.* [2] found that trehalose at 0.125 mol/L was better than 0.25 mol/L in cryoprotecting sperm motility by either rapid freezing or vitrification in micro-capillaries. However, the study of Schulz *et al.* [4] reported that 0.1 mol/L trehalose was better than 0.25 mol/L sucrose in cryopreserving human sperm motility in a closed straw system. Further research is needed to determine the optimal concentration of trehalose for the purpose to standardize the protocols of rapid freezing and vitrification.

The objectives of the present study were to determine the optimal concentration of trehalose for sperm cryopreservation and compare the cryoprotective effects of rapid freezing versus vitrification using trehalose in a closed standard

straw and a novel straw-in-straw system on sperm motility and nuclear DNA integrity in humans.

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) media containing 5 mg/ml human serum albumin (HSA) were prepared according to the method of Quinn *et al.* [17].

2.2. Experimental Designs

Four experiments were performed, and each experiment was repeated at least 3 times using normozoospermic samples from different donors.

Exp. 1 was to compare the cryoprotective effects of vitrification with different concentrations of trehalose on post-thaw recovery rates of total and progressive sperm motilities using 0.5 mL standard straws to determine the optimal concentration of trehalose for sperm cryopreservation.

Exp. 2 was to compare rapid freezing versus vitrification using the optimal concentration of trehalose determined by Exp. 1 and 0.5 mL standard straws in terms of the post-thaw recovery rates of total and progressive sperm motilities.

Exp. 3 was to compare rapid freezing in a 0.5 mL straw versus vitrification in the straw-in-straw system (**Figure 1**) using the optimal concentration of trehalose in terms of the post-thaw recovery rates of total and progressive motilities.

Exp. 4 was to compare rapid freezing in a 0.5 mL straw versus vitrification in the straw-in-straw system using the optimal concentration of trehalose in terms of post-thaw sperm DNA damage level.

2.3. Semen Preparation

Semen samples were obtained by masturbation in a private room near the laboratory from 18 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 [18]. Ejaculates with volume < 2 ml, concentration < 3.5×10^7/ml, progressive motility < 50% and normal sperm morphology < 30% were excluded from the study, and 13 normal ejaculates were used. 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.

Figure 1. The straw-in-straw system loaded with 100 μl sperm and heat-sealed at both ends.

2.4. Sperm Cryopreservation

Washed sperm in HTF medium from each ejaculate were assessed immediately for sperm quality, and then divided into aliquots according to the design of each experiment. Each aliquot was diluted 1:1 with aqueous solution of trehalose at 0.5 mol/L or other concentrations, so that the final concentration of sperm was 15×10^6/ml - 20×10^6/ml and the final concentration of trehalose was 0.25 mol/L or other tested concentrations. Then, the sperm samples were loaded into straws by aspiration to the middle part of the straw (100 μl each). Standard 0.5 mL straws heat-sealed at both ends and straw-in-straw systems were used as cryocontainers. 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 100 μl sperm sample by aspiration to the middle part of the straw, then the loaded straw was placed inside a standard 0.5 mL 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 (**Figure 1**). Sperm samples were equilibrated in freezing media for 10 min at room temperature before rapid freezing or vitrification.

Rapid freezing was performed by placing straws horizontally in the liquid nitrogen (LN2) vapor at 5 cm above the LN2 surface (−130˚C) for 10 min before being plunged into liquid nitrogen. Vitrification was performed by submerging straws or the straw-in-straw systems directly and horizontally into LN2 (−196˚C). Sperm samples were stored in LN2 for 3 - 5 days before being analyzed. All cryopreserved sperm samples were thawed by submerging a straw or a straw-in-straw in 37˚C water bath for 2 min before the heat seals at both ends were cut off and the sperm suspension was expelled into 2 mL pre-warmed HEPES buffered HTF medium for dilution.

2.5. Sperm Concentration and Motility Assessments

Sperm concentration, total motility and progressive motility at 37˚C were measured immediately before freezing/vitrification and after thawing/warming using a WLJY-9000 computer-assisted sperm analyzer (CASA, Weili New Century Science & Tech, Beijing, China). The counting chambers with 20-μm depth were from the same CASA manufacturer. At least 2000 sperm per sample from randomly selected fields were examined. Motility recovery rates (= post-thaw motility ÷ pre-freeze motility × 100%) including total sperm motility recovery rate and progressive sperm motility recovery rate were calculated to compare the cryoprotetcive effects of different treatments.

2.6. Sperm DNA Damage Assessments

The nuclear DNA damage of post-thawed sperm was assessed by the sperm chromatin dispersion test (SCDT) as described by Fernández *et al.* [19] with some modifications. Briefly, 30 μl of sperm suspension-low melting agarose mixture at 37˚C 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 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

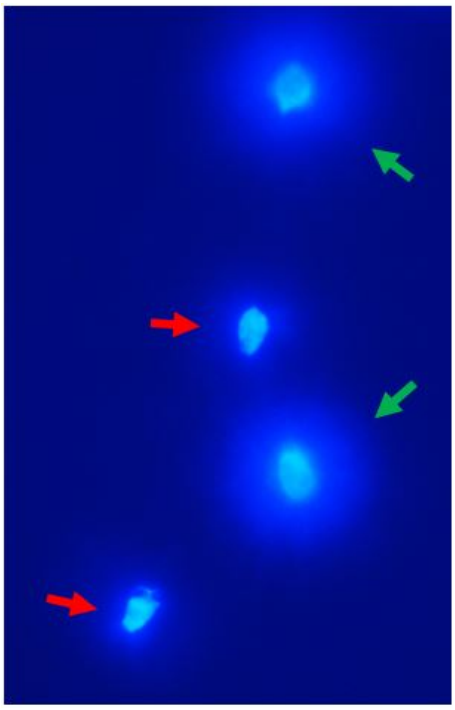

Figure 2. Human sperm SCDT. Sperm pointed by red arrows had small haloes indicating DNA fragmentation and the sperm pointed by green arrows had large haloes indicating intact DNA. Original magnification 1000×.

DNA fragmentation were arcsine transformed, and then group differences were detected by both one-way ANOVA followed by Tukey HSD tests and t-tests, where $P < 0.05$ was considered significant. Data are expressed as mean (M) ± standard deviation (SD).

3. Results

3.1. Comparison of Different Trehalose Concentrations for Sperm Vitrification

To determine the optimal concentration of trehalose for sperm vitrification, concentrations of trehalose at 0, 0.125, 0.25 and 0.5 mol/L were tested, and the post-thaw sperm motility recovery rates are summarized in **Figure 3**. The post-thaw total motility recovery rates were 3.8% ± 1.5% at 0 mol/L, 13.9% ± 2.7% at 0.125 mol/L, 22.7% ± 3.1% at 0.25 mol/L and 16.1% ± 3.9% at 0.5 mol/L, and the post-thaw progressive motility recover rates were 3.3% ± 1.5% at 0 mol/L, 15.4% ± 6.6% at 0.125 mol/L, 24.5% ± 5.7% at 0.25 mol/L and 17.5% ± 6.5% at 0.5 mol/L. The data indicate that trehalose significantly cryoprotected both sperm total motility and progressive motility compared with the control (without trehalose), and the optimal concentration of trehalose among the concentrations tested was 0.25 mol/L.

3.2. Comparison of Sperm Rapid Freezing Versus Vitrification in 0.5 mL Straws

To compare sperm rapid freezing versus vitrification using standard 0.5 mL straws and 0.25 mol/L trehalose as CPA, sperm were loaded into 0.5 mL straws with 100 µl sperm each followed by heat-sealing at both ends, and then the straws were placed horizontally in LN2 vapor at 5 cm above LN2 surface for 10 min before being plunged into LN2 (rapid freezing) or plunged directly into LN2 for vitrification. The results are summarized in **Figure 4**. The recovery rates of post-thaw total motility and progressive motility of rapid freezing were 38.6% ± 3.0% and 41.1% ± 5.0%, respectively, which were significantly higher ($P < 0.05$) than that of vitrification (26.1% ± 3.1% and 27.2% ± 1.3%, respectively).

3.3. Comparison of Rapid Freezing in 0.5 mL Straw and Vitrification in the Straw-in-Straw

To compare sperm rapid freezing in standard 0.5 mL straw and vitrification in the straw-in-straw system using 0.25 mol/L trehalose as CPA, sperm of 100 µl were loaded into each container and sealed, and then the sperm samples were placed at 5 cm location in LN2 vapor for 10 min before being plunged into LN2 (rapid freezing) or plunged directly into LN2 for vitrification. As shown in **Figure 5**, the post-thaw recovery rates of total motility and progressive motility of the sperm vitrified in the straw-in-straw systems were 43.1% ± 4.2% and 41.8% ± 15.5%, respectively, which were significantly higher ($P < 0.05\%$) than that of sperm cryopreserved by rapid freezing in the 0.5 mL straws (26.7% ± 9.6% and 26.8% ± 8.7%, respectively (**Figure 5**). However, the post-thaw sperm nuclear

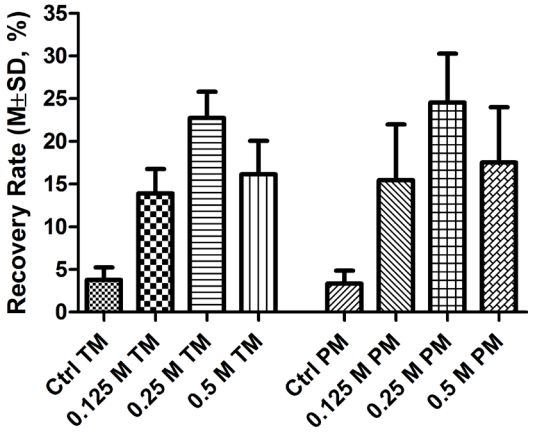

Figure 3. Cryoprotective effects of different concentrations of trehalose by vitrification in 0.5 mL straws on the recovery rates of total sperm motility (TM) and progressive motility (PM). The optimal trehalose concentration was 0.25 mol/L at which both TM and PM were significantly higher than that of all other concentrations tested ($P < 0.05$). Ctrl = 0 mol/L.

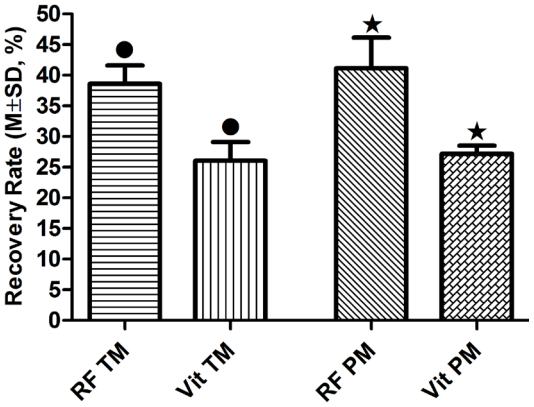

Figure 4. Comparison of rapid freezing (RF) and vitrification (Vit) in standard 0.5 mL straws. Rapid freezing provided significantly better cryoprotection on sperm total motility (TM) and progressive motility (PM) than vitrification ($P < 0.05$). Bars with the same symbols represent significant difference.

DNA damage levels after rapid freezing in 0.5 mL straws (8.7% ± 2.8%) and that of sperm after vitrfication in the straw-in-straw systems (9.2% ± 2.5%) were not significantly different ($P > 0.05$, **Figure 6**).

4. Discussion

Trehalose has the highest glass transition temperature in disaccharides [20] [21] and has been used for sperm cryopreservation in humans [2] [4] [5]. Sucrose at 0.25 mol/L concentration has been widely used for human sperm cryopreservation by rapid freezing and vitrification [3] [7] [8] [15] [16], but the optimal concentration in trehalose is still unclear. Study of Liu *et al.* [2] reported that trehalose at 0.125 mol/L was better than 0.25 mol/L in cryoproserving motility of

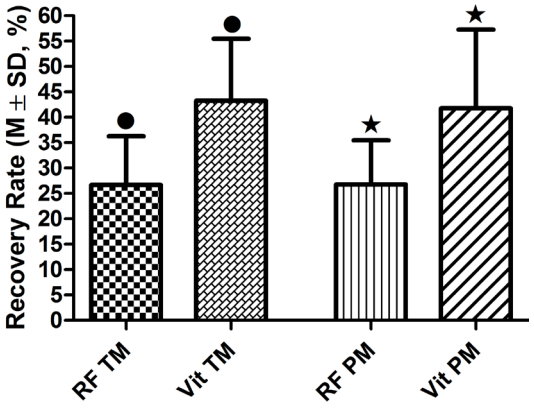

Figure 5. Comparison of rapid freezing (RF) in standard 0.5 mL straw and vitrification (Vit) in the straw-in-straw system. Vitrification provided significantly better cryoprotection on sperm total motility (TM) and progressive motility (PM) than rapid freezing ($P < 0.05$). Bars with the same symbols represent significant difference.

human sperm by either rapid freezing or vitrification in sealed silica micro-capillary tubes. Study of Schulz *et al.* [4] reported that 0.1 mol/L trehalose was better than 0.05 mol/L trehalose and 0.25 mol/L sucrose in cryopreserving human sperm motility by vitrification in an open straw system, but concentrations of trehalose higher than 0.1 mol/L were not tested in the study. In the present study, we found that 0.25 mol/L was the optimal concentration of trehalose protecting both the total motility and progressive motility during cryopreservation among the tested concentrations of 0, 0.125, 0.25 and 0.5 mol/L using closed 0.5 mL standard straws containing 100 µl sperm each.

Both rapid freezing and vitrification methods are fast, simple and cost-effective sperm cryopreservation technologies, but it is necessary to know which one is better or if they are equally efficient in cryopreserving human sperm using aseptic technology. Few studies have been directed at comparing the safety and effectiveness of rapid freezing versus vitrification for human sperm cryopreservation. Study of Isachenko V. *et al.* [13] using cryoloops and a commercial preservation medium (SPM, Scandinavian IVF Science, Gothenburg, Sweden) found that rapid freezing (1 cm above liquid nitrogen surface) and vitrification methods led to comparable results in terms of sperm motility, fertilization ability, and DNA integrity frozen-thawed sperm. Agha-Rahimi *et al.* [14] also reported that the post-thaw sperm motilities and DNA fragmentation levels were not significantly different between the sperm samples cryopreserved by rapid freezing at 3 cm above liquid nitrogen surface in cryovials using a sample size of 1 mL sperm per cryovial and by vitrification by directly dropping 30 µL of sperm into liquid nitrogen. Unfortunately, the study used Vitrolife Sperm Freeze Solution for the rapid freezing and 0.25 M sucrose as CPA for the vitrification, and therefore the results are difficult to compare.

Study of Liu *et al.* [2] found that human sperm motility was cryopreserved

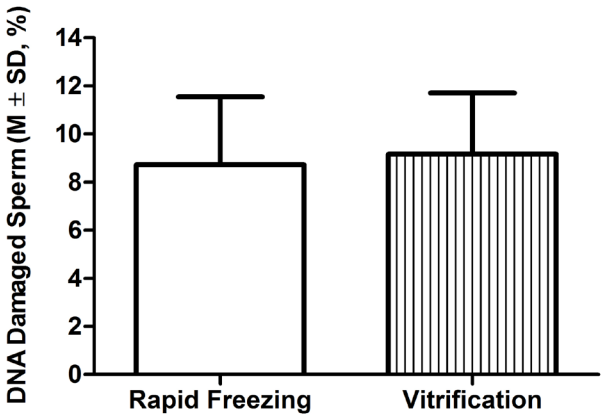

Figure 6. Comparisons of DNA damage levels after rapid freezing in 0.5 mL straw or vitrfication in the straw-in-straw system ($P > 0.05$).

Table 1. Differences between the straw-in-straw system and the open straw system.

	Straw-in-Straw	Open Straw
Inner 0.25 mL straw	Straw end heat-sealed together with the outside 0.5 mL straw end	Straw end open and separated from the outside 0.5 mL straw
Thawing method	Thaw the straw-in-straw in 37˚C water bath, then cut off heat seals and expel thawed sperm into culture medium	Remove the inner 0.25 mL open straw from the 0.5 mL straw and plunge into culture medium to thaw sperm

significantly better by rapid freezing than vitrification using sealed micro-capillaries as cryocontainers in the presence of different concentrations of sucrose or trehalose as CPA, and the results are consistent with our findings that both total motility and progressive motility were cryopreserved significantly better by rapid freezing than vitrification when using standard 0.5 mL straws and 0.25 M trehalose as CPA (data in Figure 4). However, when the vitrification was performed in the straw-in-straw packing system and the rapid freezing was still performed in the 0.5 mL standard straw using the same cryopreservation medium, it was found that both total motility and progressive motility were cryopreserved significantly better by vitrification than rapid freezing (data in Figure 5), although no difference was found in sperm DNA fragmentation level between the two cryopreservation methods (data in Figure 6). These data indicate that sperm cryopreservation by direct plunging into liquid nitrogen or vitrification using 0.25 M trehalose and a 100 μL sample size needs an appropriate combination of cooling rate and thawing rate, and the cooling rate and/or thawing rate obtained in the straw-in-straw system were significantly better than that of the standard 0.5 mL straw.

Both the straw-in-straw system used in the present study and the open straw system described by Isachenko V. *et al.* are different, although both systems were made by inserting a 0.25 mL standard straw into a 0.50 mL standard straw. The differences between the systems are summarized in Table 1. Since sperm sam-

ples in the straw-in-straw systems are thawed directly in a water bath and there is no need to remove the inner straw from the outside straw before thawing, it is easier to thaw sperm compared with the open straw system.

In conclusion, the present study demonstrated that rapid freezing is superior to vitrification for human sperm cryopreservation when using 0.5 mL standard straws, 0.25 M trehalsoe as CPA and 100 μL sample size; however, vitrification is superior to rapid freezing when using the novel straw-in-straw system.

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Authors' Contributions

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

Authors' Disclosure Statement

All authors declare no competing interests.

Conflicts of Interest

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

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