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Cryopreservation of human spermatozoa with minimal non-permeable cryoprotectant

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Abstract

Cryopreservation of human spermatozoa is a commonly used technique in assisted reproduction, however freezing low concentrations of sperm while maintaining adequate post-thaw motility remains a challenge. In an effort to optimize post-thaw motility yields, low volumes of human sperm were frozen in polyimide-coated fused silica micro-capillaries using 0.065 M, 0.125 M, 0.25 M, or 0.5 M trehalose as the only cryoprotectant. Micro-capillaries were either initially incubated in liquid nitrogen vapor before plunging into liquid nitrogen, or directly plunged into liquid nitrogen. Post thaw sperm counts and motility were estimated. Spermatozoa that were initially incubated in liquid nitrogen vapor had greater post thaw motility than those plunged immediately into liquid nitrogen independent of trehalose concentration. The protective effect of 0.125 M D-glucose, 3-O-methyl-D-glucopyranose, trehalose, sucrose, raffinose, or stachyose were evaluated individually. Trehalose and sucrose were the most effective cryoprotectants, recovering 69.0% and 68.9% of initial sperm motility, respectively.

1. Introduction

Male factor infertility is a salient issue with significant psychosocial impacts on the affected men. Recent advances in laboratory and surgical techniques have allowed the opportunity for men with severe sperm production issues to use their own gametes for reproduction. In addition, the growing success of oncologic therapies and resultant increased survival of

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Conflicts of interest

None.

cancer patients makes post-treatment preservation of reproductive potential a vital societal concern. In an effort to offer current and future fertility treatments in these groups of patients, sperm cryopreservation is often used. Current sperm cryopreservation techniques have been around for decades. However, they are not reliable for freezing very low numbers of sperm as is often the circumstance in patients with severe male factor infertility.

Cryoprotection covers a variety of techniques ranging from slow cooling to ultrafast freezing. There have been several studies seeking to determine the optimum technique for the cryopreservation of a very low number of human sperm; various protocols, freezing carriers, and cryoprotective agents have been described [1].

A review of the literature reveals that the common nonpermeable cryoprotective agent used to cryopreserve human sperm is sucrose (Table 1). Woelders et al. [26] reported a slight improvement in storage of bull sperm using sucrose rather than trehalose. A decade later, Hossain and Osuamkpe [15] supported the use of sucrose. We sought to compare the effect of various sugars on the recovered motility of thawed low-volumes of human spermatozoa after ultrafast freezing with a micro-capillary system.

2. Materials and methods

2.1. Study population

Men of subfertile couples referred for evaluation at a tertiary fertility center were requested permission to use discard semen samples for research purposes. This study was approved by the institutional review board of the Massachusetts General Hospital on the use of human subjects in research and written informed consent was obtained from all participants allowing research use of laboratory discard samples.

2.2. Semen samples

Fresh ejaculated semen samples were collected by masturbation from men who were seeking evaluation at a tertiary fertility center. A total of 34 randomly-selected samples with normal semen parameters were used. Motile sperm were isolated using a density gradient centrifugation method and sperm count and motility were quantified according to the 2010 guidelines of the World Health Organization (WHO) [28]. Sperm pellets were washed once and resuspended in test media as specified in the protocols below.

2.3. Reagents and media

Human tubal fluid (HTF) and human serum albumin (HSA) solutions were purchased from Irvine Scientific (Santa Ana, CA, USA). Trehalose was obtained from Ferro Pfanstiehl (Waukegan, IL, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Fused silica capillary tubes coated with a thin layer of polyimide were obtained from Postnova Analytics Inc. (UT, USA). Each micro-capillary was 6.5 cm in length, 200 μm in inner diameter, and 2 μl in capacity. Polyimide has a thermal conductivity about 6.5 times less than the fused silica (fused silica $\sim 1.35 \text{ W m}^{-1} \text{ K}^{-1}$ [6]; polyimide $\sim 0.2 \text{ W m}^{-1} \text{ K}^{-1}$ [20,23]). Heo et al. [13] have successfully used fused silica capillary tubes, after removing

the polyimide coat, in which to vitrify a variety of cell types. Part of their success is attributed to the high thermal conductivity of the wall of the fused silica tubes which allows very rapid cooling [22]. The polyimide was not removed in our work.

2.4. Trehalose freezing protocols

Sperm suspension was diluted 1:1 with freezing medium consisting of HTF, 5% HSA, and varying concentrations of trehalose to obtain a final trehalose concentration of 0.0 M, 0.065 M, 0.125 M, 0.25 M, or 0.5 M. After 1.5 min of incubation, each sperm-containing solution was loaded by capillary action into a silica micro-capillary. Both capillary ends were then sealed with Tygon tubing. At the time of cooling, sperm had been mixed and incubated with freezing medium for 4–5 min. Two different cooling methods were used in this study. First, micro-capillaries were held in liquid nitrogen (LN₂) vapor for 3–5 s before placing on a metal rack in the vapor phase for 5 min; then the capillaries were plunged into LN₂. Placing the sample directly on a rack in the vapor is likely to yield a slightly faster cooling rate compared to holding in the vapor. However, this decision was merely for practical purposes to always place the sample in the same location in the vapor phase. Second, each capillary was plunged directly into LN₂ without prior incubation.

To thaw, each capillary was quickly immersed into a room temperature (~22 °C) water bath. Capillary content was expelled into a 12 µl drop of HTF with 5% HSA on a glass slide. The glass slide was then covered with a coverslip, and motility of the sperm was examined. Sham controls consisted of loading the mixture of sperm and freezing medium suspension into capillaries then immediately expelling the sample for assessment without interval freezing.

2.5. Different saccharides as cryoprotectant

Sperm suspension was mixed 1:1 with media containing different saccharides. Final concentrations of these media were HTF supplemented with 5% HSA, and 0.125 M of one of the following sugars: D-glucose, 3-O-methyl-D-glucopyranose (3-OMG), trehalose, sucrose, raffinose, or stachyose. Cooling method one stated above was used to freeze the capillary content. To thaw, the same thawing procedure described above was applied.

2.6. Sperm motility

Pre-freeze and post-thaw sperm motility was counted according to the method described in WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th edition [28]. A minimum of 200 sperm per replicate was counted and the numbers of motile and immotile sperm were recorded. Recovered motility was calculated as a percentage of post thaw motility divided by pre-freeze motility [10,21]. Pre-freeze motility is the motility obtained after Percoll separation. Our average pre-freeze motility for the 34 samples used in this study was 88.0%.

2.7. Statistical analysis

In all experiments the sperm specimen obtained from each patient formed a replicate. To avoid the use of P-values as the only test of significance to summarize results and to provide

additional information of the variation between the original observations, results were summarized by including “effect sizes and uncertainty metrics” as confidence intervals [4].

The population mean of each group is estimated by the arithmetic mean. The variability of each group is given by the 95% confidence limits which indicate that there is a 95% chance of the population mean lying within this interval. The following inferences are then made: (1) if a pair of confidence limits do not overlap the difference between the groups is clinically significant, (2) if a pair of confidence limits slightly overlap the difference may be worth further study. All computations were done using Stata 12 (StataCorp, College Station, TX).

3. Results

Preliminary experiments examining the effect of HSA concentration on post thaw sperm motility showed no difference between 5% and 10% HSA in the freezing medium, so all the media used in this study contained only 5% HSA.

Two cooling methods were used to freeze sperm in media containing 0.0 M, 0.065 M, 0.125 M, 0.25 M, or 0.5 M trehalose: direct plunging into LN₂ and precooling in LN₂ vapor prior to direct plunging into LN₂. The results demonstrate a much larger recovery of motility when the LN₂ vapor method is used for all concentrations of trehalose (Table 2, Fig. 1). The percent motility of spermatozoa that recovered after thawing is dependent on the concentration of trehalose using both methods. When the spermatozoa were frozen with the LN₂ vapor method the maximum motility was obtained using 0.125 M trehalose (67.8%) while much lower motility was observed after thawing when 0.0 M and 0.5 M trehalose was used (41.0% and 29.1% respectively). A similarly shaped concentration-response relation was also observed when spermatozoa were frozen using direct plunging into LN₂. Maximum motility was obtained using 0.125 M trehalose (10.9%) while lower motility rates were observed when 0.0 M and 0.5 M trehalose was used (2.5% and 3.3%, respectively). Incubation in LN₂ vapor at temperatures ranging from -90°C to -130°C and durations from 3 to 20 min had no effect on post thaw sperm motility (Data not shown). Loading then immediately unloading of sperm in media containing 0.25 M or 0.5 M trehalose without freezing (sham control) negatively affected sperm motility.

The effect of six different saccharides as cryoprotectants (D-glucose, 3-OMG, trehalose, sucrose, raffinose, stachyose) on the motility of human spermatozoa thawed after ultrafast freezing with the LN₂ vapor method are compared in Table 3 (also see Fig. 2).

Concentrations of 0.125 M trehalose and 0.125 M sucrose were most efficient among all sugars, with 69.0% and 68.9% recovered sperm motility. D-glucose and 3-OMG may be almost efficient and raffinose and stachyose had lower motility rates than trehalose and sucrose (58.8% and 55.4% respectively).

4. Discussion

We achieved 67.8%–69.0% recovered sperm motility after ultrafast freezing using 0.125 M trehalose or 0.125 M sucrose as the only cryoprotectants. This is among the highest post thaw motility rates reported for human sperm (Table 1).

The maximum motility of human spermatozoa was observed when a medium containing 0.125 M trehalose was used. Concentrations of trehalose above and below 0.125 M were detrimental particularly at the higher concentrations. Others have studied different concentrations of sucrose as the only cryoprotectant for human sperm and the optimal concentrations were reported to be 0.1 M using petri dishes and micro centrifuge tubes [15], or 0.2 M with cryogenic vials as the carrier [29]. The monosaccharides (D-glucose and 3-OMG), disaccharides (trehalose and sucrose), trisaccharide (raffinose), and tetrasaccharide (stachyose) were compared for their ability to protect human sperm during freezing and thawing using concentrations of 0.125 M. The disaccharides were more effective than monosaccharides, trisaccharides, or tetrasaccharides.

The results shown in Table 2 also demonstrate that there is a large population of spermatozoa that can survive freezing when a permeating and a non-permeating protective agent are excluded from the medium. This phenomenon is particularly evident when the sperm samples are exposed to LN₂ vapor before plunging into LN₂ (41.0%), but it is also observed at a much lower level when the sperm samples are plunged directly into LN₂ (2.5%). The capillary tube used to hold the sperm is coated by a sleeve of a polymer, polyimide, which has a thermal conductivity about 6.5 times less than the fused silica. Thus the rate of heat loss through the capillary wall will be limited by the thermal conductivity of the outer coat as well as the temperature gradient determined by the position of the capillary in the LN₂ vapor. By chance the protocol we used may have created conditions that facilitate the extrusion of intracellular water thereby preventing the formation of intracellular ice during the initial cooling period. The results also demonstrate that the addition of 0.125 M trehalose in the medium greatly enhances the survival rate of the spermatozoa (an increase of 26.8% when exposed to LN₂ vapor and 7.3% when directly plunged into LN₂).

When a dry cell is warmed its cell membrane undergoes a thermotropic phase transition in which the lipid bilayer transitions from an ordered “gel” structure to a disordered liquid structure, during which the membrane becomes temporarily leaky [7]. The temperature at which the transition occurs is called the melt point (T_m). Beattie et al. [3] made use of the leakiness associated with T_m to introduce trehalose into the cells of pancreatic islets. It has also been shown that trehalose can enter red blood cells [14], and blood platelets [11,27] in significant concentrations when the temperature is lowered through T_m . The cell membrane of mammalian spermatozoa also undergoes a phase transition, associated with leakiness, as the temperature is lowered through T_m [9,24]. Although we have no direct evidence, trehalose might have entered our spermatozoa at a critical T_m when they were cooling in the LN₂ vapor where it would protect the cell membranes and later participate in the formation of a favorable glass in the specimen [12,19], possibly protecting cells from subsequent chilling and warming injuries.

Without the need of expensive equipment, our protocol using trehalose is potentially helpful for preserving small numbers of sperm, for example, from men with severe male factor infertility. An advantage of our procedure is that it eliminates washing after thawing, avoiding unintended sperm loss, an important consideration when processing small samples of spermatozoa. These results do not demonstrate any practical difference in motility of spermatozoa after thawing using either trehalose or sucrose as a cryoprotectant. The

glycosidic bond linking the two glucose monomers in trehalose is very stable, far more stable than the bond in sucrose [9]. Trehalose also has a higher glass transition temperature than sucrose which results in increased stability of biomaterials stored with trehalose in less desirable conditions [8]. Crowe [9] concluded that if the glycosidic bond is protected and the temperature is low enough to maintain the glassy state, other sugars can be as effective as trehalose. Nevertheless, trehalose could prove to be a better choice upon further investigation due to its special properties. In summary, the use of minimal amounts of non-permeating cryoprotectants (disaccharides) in a micro-capillary system could provide a reliable technique to store low concentrations of human spermatazoa as part of treatment options for severe male factor infertility.

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Fig. 1. Recovered motility (%) of human sperm frozen in the vapor phase or by direct plunging into LN₂. Upper and lower lines of the bars are maximum and minimum observations. Diamonds inside of the bars indicate mean values.

Fig. 2. Recovered motility (%) of human sperm frozen in media containing different saccharides. Upper and lower lines of the bars are maximum and minimum observations. Diamonds inside of the bars indicate mean values.

Table 1
Comparison of pre-freeze and post thaw motility of human sperm cryopreserved solely with sucrose.

Reference	Sucrose concentration	Freezing device	Sample volume	Freezing method	Pre-freeze motility	Post thaw motility of frozen sample
[18]	0.25 M	Cut standard straw inside a 0.5 ml straw	10 µl	Directly plunged into LN ₂	Progressive motility: 90% ^b	Progressive motility: 60%
[29]	0.20 M	2.0 ml cryogenic vial	0.5 ml	Directly plunged into LN ₂	Motility: 95.7± 2.0% ^b Progressive motility: 92.2± 3.8% ^b	Motility: 58.5± 6.3% Progressive motility: 47.5± 6.8%
[16]	0.25 M	droplet	30 µl	Directly plunged into LN ₂	Not shown ^b	Motility: 57.1± 3.2%
[2]	0.25 M	droplet	30 µl	Directly plunged into LN ₂	Total motility: 96.2± 2.5% ^b Progressive motility: 86.6± 5.9% ^b	Total motility: 53.9± 9.5% Progressive motility: 41.9± 10.3%
[15]	0.1 M	Petri dish or microcentrifuge tube	10 µl	Directly plunged into LN ₂	Motility: 90% ^a	Motility: 30± 3%
[5]	0.25 M	Cryotop	0.5 µl	Placed at 4 cm above LN ₂ for 2 min before plunging into LN ₂	Motility: > 99% ^{ab}	Motility: ~30%
[17]	0.25 M	50 µl plastic capillary inside of a 0.25 ml straw	10 µl	Directly plunged into LN ₂	Motility: 35.0± 9.5% ^b	Motility: 28.0± 6.0%
[25]	0.25 M	0.5 µl straw	300 µl	Directly plunged into LN ₂	Total motility: 74.47% ^a	Total Motility: 20.48% Rapid progressive Motility: 11.52%

Note:^a, ^b and ^{ab} indicate that motile sperm were prepared by density gradients^a, the swim up procedure^b, or density gradients followed by a swim up procedure^{ab}.

Table 2

Recovered motility (%) of human sperm after freezing and thawing in media containing 5% HSA and different concentrations of trehalose (n = number of patients).

Summary statistics						
Treatment		Sham controls		Vapor phase		Direct plunging
HSA (%)	Trehalose (M)	n	Mean (confidence limits, P = 0.95)	n	Mean (confidence limits, P = 0.95)	n Mean (confidence limits, P = 0.95)
5	0.0	16	98.0 (96.5–99.5)	24	41.0 (35.2–46.0)	24 2.54 (1.82–3.27)
5	0.065	16	98.9 (97.3–100)	24	62.0 (58.6–65.5)	24 8.08 (6.27–9.89)
5	0.125	16	98.9 (97.3–100)	24	67.8 (65.3–70.3)	24 10.88 (8.85–12.91)
5	0.25	16	93.8 (92.0–95.7)	24	52.3 (49.3–55.3)	24 7.08 (5.27–8.89)
5	0.5	16	84.2 (82.2–86.2)	24	29.1 (25.5–32.7)	24 3.30 (2.09–4.52)
Analysis of paired differences						
Comparison				Mean difference		
0.065 v. 0.0				21.0		
0.125 v. 0.065				5.8		
0.25 v. 0.125				–15.5		
0.5 v. 0.25				–23.2		
				Confidence limits, P = 0.95		
				19.7 to 22.3		
				4.28 to 7.17		
				–0.059 to –3		
				–21.6 to –24.85		

Table 3

Recovered motility (%) of human sperm frozen and thawed in media containing different saccharides.

Summary statistics		
Saccharide	No. patients	Mean (confidence limits, P = 0.95)
D-glucose	30	63.3 (60.2–66.5)
3-OMG	30	64.2 (60.8–67.5)
Trehalose	30	69.0 (66.0–72.1)
Sucrose	30	68.9 (65.9–71.9)
Raffinose	30	58.8 (54.4–63.2)
Stachyose	30	55.4 (51.2–59.7)
Analysis of paired differences		
Comparison	Mean difference	Confidence limits, P = 0.95
Trehalose v. glucose	5.69	2.48 to 8.90
Trehalose v. 3-OMG	4.84	1.53 to 8.18
Trehalose v. sucrose	0.14	–3.19 to 3.48
Trehalose v. raffinose	10.21	6.26 to 14.15
Trehalose v. stachyose	13.58	9.71 to 17.46