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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.

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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 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 non-permeable 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

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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 ml 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].

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 re-suspended 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 ~1.35 $Wm^{-1}K^{-1}$ [6]; polyimide ~0.2 $Wm^{-1}K^{-1}$ [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 (LN_2) vapor for 3–5 s before placing on a metal rack in the vapor phase for 5 min; then the capillaries were plunged into LN_2. 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 LN_2 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

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