Rapid freezing versus slow programmable freezing of human spermatozoa

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Objective: To compare the efficacy of rapid freezing (RF) and slow programmable freezing (SPF) of human spermatozoa.

Design: Experimental study.

Setting: University-based assisted conception laboratory.

Patient(s): Semen from 30 normospermic men.

Intervention(s): Semen was processed through density gradients and divided into three groups: nonfrozen control, RF, and SPF.

Main Outcome Measure(s): Sperm motility, kinematics, and morphology were assessed by a computer-assisted semen analyzer; viability by eosin-Y staining and the hypo-osmotic swelling test; DNA integrity by comet assay; and sperm binding by hemi-zona assay.

Result(s): Post-thaw sperm motility (53.9%, 37.0%, and 75.5% for RF, SPF, and controls, respectively) and sperm vitality (hypo-osmotic swelling test 60.1%, 44.1%, and 77.9%; eosin-Y staining 64.8%, 50.4%, and 81.8% for RF, SPF, and controls, respectively) were higher in the RF than in the SPF group, but lower than in the nonfrozen control group. There was no significant difference in post-thawed normal sperm morphology (14.9%, 14.4%, and 16%) and sperm DNA integrity by comet assay (93.6%, 94.5%, and 94.2%) in the RF, SPF, and controls, respectively. The hemi-zona index was no different between the two cryopreserved groups.

Conclusion(s): The RF gave superior post-thaw motility and cryosurvival than SPF. (Fertil Steril® 2010;93:1921–8. ©2010 by American Society for Reproductive Medicine.)

Key Words: Comet assay, hemi-zona assay, rapid freezing, semen preservation, slow programmable freezing

Sperm freezing is commonly used to preserve fertility in men with cancer, before they receive cytotoxic chemotherapy, radiotherapy, or surgical treatment that may induce testicular failure (1). The treatment of some nonmalignant diseases, such as diabetes and autoimmune disorders, may also lead to testicular damage, and cryopreservation should be advised as well (2). In donor insemination programs, cryopreservation is necessary to allow time to screen donors for infectious agents, such as human immunodeficiency virus (HIV) and hepatitis B virus, before the cryopreserved semen is released for clinical use (3). Cryopreservation is also indicated in cases of surgical sperm retrieval, to avoid the need for a repeat biopsy or aspiration (1).

Current methods of human sperm cryopreservation are crude compared with those for human embryos. The recovery of motility is relatively low, with typically less than 60% of them regaining motility upon thawing (3). Improvements in sperm cryopreservation have been attempted by various means, such as the use of different cryoprotective agents (CPAs) and change in cooling rate. However, human spermatozoa have an unusual cryobiological behavior and improvements in their survival have not been achieved by conventional approaches (3). Computer models of sperm osmotic behavior during freezing have suggested that human spermatozoa should survive cooling rates of up to 10,000°C/min. In actual experiments, there is little difference in their observed survival when cooling at 1°C/min up to 100°C/min (4), but the survival rate begins to decline beyond 100°C/min (5).

Vitrification is a process that produces a glasslike solidification of living cells, without ice crystal formation during cooling (6). The conventional method of vitrification requires a relatively high cooling rate and high CPA concentration (30%–50% compared with 5%–7% for slow freezing). Such a high concentration of CPA is damaging to cells, causing both biochemical changes and lethal osmotic injury (7). As a result it has not been possible to effectively cryopreserve the sensitive mammalian spermatozoa by conventional vitrification. This prompts the idea of exploring vitrification methods that do not require a high concentration of potentially toxic CPA. One approach is to use very rapid cooling and warming rates and a very small drop size. A new method of sperm vitrification, without the use of conventional

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TABLE 1

Composition of rapid freezing medium (10 mL).

Component	Amount
Trehalose (100 mM)	0.38 g
D-glucose (5.5 mM)	0.01 g
Sodium pyruvate (12.2 mM)	0.014 g
Glycine (133 mM)	0.10 g
HEPES (20 mM)	0.05 g
Glycerol 10% (vol/vol)	1.0 mL
Human serum albumin 10% (wt/vol)	1.0 g
Phosphate-buffered saline	9.0 mL

Vutyavanich. Rapid freezing of human spermatozoa. Fertil Steril 2010.

permeable CPA, has been described by Nawroth et al. (8) and Isachenko et al. (9, 10). The method involves the use of a thin film of sperm on a cryoloop and an extremely rapid freezing rate ($\geq$50,000°K/min) (7). The drawback is that only a very small volume of sperm ($\leq$20 μL) can be vitrified at one time. The method is, therefore, not suitable for routine cryopreservation of human semen for artificial insemination (AID) or IUI.

We had developed a method to cryopreserve human sperm in conventional straws by rapid freezing. In this study, we compared the efficacy of our in-house method of rapid freezing with that of the conventional slow programmable freezing. A comparison of costs and time involved was also included.

MATERIALS AND METHODS

Samples

The Ethics Committee of the Faculty of Medicine, Chiang Mai University, approved this study. Semen samples were obtained from 30 male partners of infertile couples who consented to the use of their spermatozoa for research. These couples were undergoing infertility evaluation at Maharaj Nakorn Chiang Mai University Hospital. The man produced semen by masturbation after an abstinence of 2–7 days. A routine semen analysis was performed within 1 hour of collection, according to the methods described by the World Health Organization (WHO) (11). Semen with a concentration of 20 million spermatozoa/mL or more, and at least 50% progressive motility was included in the study.

Sperm Preparation Before Cryopreservation

Semen samples were layered on top of 80%/40% discontinuous Percoll gradients (Pharmacia Fine Chemical AB, Uppsala, Sweden) and centrifuged at 350 × *g* for 10 minutes. The pellet was resuspended in Earle's balanced salts solution (EBSS; Biological Industries, Kibbutz, Israel), supplemented with 0.3% human serum albumin (HSA; LifeGlobal, Guilford, CT), 0.03 M sodium pyruvate (Sigma P5280, St. Louis, MO) and 1 M HEPES (Sigma H0887), and centrifuged at 200 × *g* for 5 minutes. After two washes, the final pellet was resuspended in 600 μL of the same medium, and divided into three aliquots. The first aliquot served as a nonfrozen control and was immediately assessed for motility, kinematics of movement, and morphology, using a computer-aided semen analyzer (CASA; HTM-IVOS, Hamilton Thorne Biosciences, Beverly, MA), equipped with a clinical human motility program version 12 and dimension morphology software. Sperm viability was assessed by the hypo-osmotic swelling test (HOST) and eosin-Y staining. DNA integrity was checked by comet assay, and sperm zona interaction was evaluated by hemi-zona-binding assay. The second and third aliquots were cryopreserved by standard slow programmable freezing (SPF) and our in-house method of rapid freezing (RF), respectively.

Sperm Cryopreservation

Slow programmable freezing An equal volume of sperm cryopreservation medium (SpermFreeze; FertiPro NV, Beernem, Belgium) was added drop-wise to an aliquot of processed semen sample. The mixture was drawn into a 0.25-mL straw and left at room temperature for 10 minutes. The straw was loaded into a controlled rate freezer (Planer Kryo 10 series III) and cooled from 20°–5°C at a rate of -1°C/min. The straw was then cooled at a rate of -10°C/min to -80°C/min and then plunged into liquid nitrogen for storage.

Rapid freezing The aliquot was mixed drop-wise with an equal volume of cold cryopreservation medium (4°C). The mixture was loaded into a precooled 0.25-mL straw and left to incubate at 4°C for 10 minutes. The cryopreservation medium for rapid freezing was a modified human sperm preservation medium (12), in which 50 mM of sucrose was replaced by 100 mM of trehalose. The concentration of glycerol was decreased to 10%, whereas that of HSA was increased 20-fold (Table 1). After 10 minutes, the straw was inserted into a hole in a precooled homemade aluminum block, previously immersed in liquid nitrogen (Fig. 1).

After at least 1 week of storage in liquid nitrogen, the sample was thawed in running tap water (temperature 25°–28°C). The thawed sample was washed with 4 mL of EBSS and centrifuged at 250 × *g* for 5 minutes to remove the cryoprotectant.

Determination of Sperm Motility Parameters

Control and post-thaw samples were assessed for sperm motility and kinematics of movement using a disposable counting chamber (20-μm depth; Leja, Niew-Vennep, The Netherlands) and a CASA, equipped with a clinical human motility program version 12. The following parameter settings were used: frame rate, 60 Hz; 30 acquisition frames; straightness threshold, 80%; minimum contrast, 80; minimum cell size, 3 pixels; nonmotile head size, 6 pixels; nonmotile head intensity, 160; illumination intensity, 2,128; magnification, 1.9; temperature, 37°C; and chamber depth, 20 μm. At least 400 sperm trajectories were analyzed.

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