Chapter 5

Slow Freezing of Human Sperm

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Abstract

Cryopreservation of human spermatozoa is a highly efficient procedure for managing male fertility, and much of its successful application seems to have a crucial impact on the reproductive outcome of assisted reproduction technologies. Here, we present, explain, and describe the slow freezing method for preserving human spermatozoa, which is currently the most commonly used freezing technique in most clinical andrology laboratories.

Key words Human spermatozoa, Slow freezing, Cryodamage, TEST yolk buffer, Cryopreservation

1 Introduction to Sperm Cryopreservation: Why and Who?

Cryopreservation is the collection, freezing, and long-term storage of structurally intact living cells and tissues [1]. Historically, spermatozoa were the first mammalian cells to be successfully cryopreserved [2], due to the discovery of the cryoprotectant glycerol. Since then, many advances in cryopreservation technology have led to the evolution of different methods that allow low-temperature maintenance of a variety of cells, tissues, and organs [3]. Much progress in this field has originated from empirical work and fundamental cryobiology. Furthermore, increased awareness of the causes and consequences of cryo-injury has continuously helped to improve distinct cryopreservation methods [4].

Human sperm cryopreservation has become a widely accepted procedure in the contemporary world [5]. The importance of sperm banking following cryopreservation lies in its potential to preserve the future fertility of men who temporarily or permanently lose their fertility due to a variety of reasons, including cancer [6]. Malignant diseases are associated with fever, malnutrition, altered immune response and hormone imbalance, stress, and inflammation leading to destructive consequences on testicular and sperm function [7, 8]. In addition, cancer treatments are highly toxic to the male reproductive system. Chemotherapy is particularly
harmful during the early stages of spermatogenesis, especially to the proliferating and differentiating spermatogonia, and could lead to temporary or permanent azoospermia or severe oligozoospermia [9, 10]. Radiotherapy is similarly deleterious to spermatogenesis, generally causing sperm DNA fragmentation, damage to Sertoli and Leydig cells, and obstruction of the ejaculatory ducts, leading to reduced semen volume [11, 12]. Patients with other diseases, such as lupus, multiple sclerosis, or ulcerative colitis, can also benefit from sperm banking before treatment [13, 14].

Sperm cryopreservation is often recommended to patients with urological diseases and pathologies, including varicocele and testicular torsion [15]. Men awaiting urogenital surgical procedures should consider sperm banking as a precaution as well as those men opting for surgical contraception (bilateral vasectomy) [14].

Healthy men may choose sperm cryopreservation to prevent health problems from occupational exposure to toxic chemicals, ionizing radiation, or biological contaminants [15, 16]. Males undergoing gender reassignment through hormonal or surgical therapy may also be a potential target population [17].

Cryopreservation is also widely used to store spermatozoa retrieved from oligozoospermic and azoospermic patients as well as from men with ejaculatory dysfunction or spinal cord injury. In the latter cases, testicular sperm extraction or percutaneous epididymal sperm aspiration is used, both of which avoid the need for repeated biopsies and aspiration [5].

Finally, cryopreservation of spermatozoa is mandatory in donor insemination programs. All frozen samples are screened for infectious diseases including HIV, herpes, and hepatitis before being released [5, 14, 16].

Whatever the reason for sperm cryopreservation, when pregnancy is desired, the semen sample is thawed and used in a number of assisted reproductive techniques including intracytoplasmic sperm injection, intrauterine insemination, and in vitro fertilization [6].

This chapter will discuss slow freezing of human spermatozoa, which is currently regarded as a safe, cost-effective, and useful strategy to preserve male fertility.

2 Slow Freezing: Principles and Overview

Slow freezing is a cryopreservation technique based on dehydration. A small amount of cryoprotectant is added to a sperm sample, and the mixture is slowly cooled to −196 °C. Thus, the actual dehydration occurs during cooling [6]. At a certain point, ice masses containing pure crystalline water are created. An unfrozen fraction remains between the growing ice masses, where all cells and solutes are retained. The concentrations of sugars, salts, and
cryoprotectant increase while the volume of the unfrozen fraction declines. Increasing osmotic pressure causes an efflux of water from the cells, which minimizes intracellular ice formation. As cooling continues, the viscosity of the unfrozen fraction becomes too high for further crystallization. The remaining unfrozen fraction ultimately becomes an amorphous solid with no ice crystals [4].

Slow freezing is the preferred method for cryopreservation of human spermatozoa. Raw or washed fresh semen samples are generally used. At the same time, different techniques are available to preserve surgically retrieved spermatozoa [6]. For example, isolated sperm can be injected into an empty zona pellucida of a hamster oocyte and placed between two air bubbles inside a straw [19, 20]. Other studies have proposed freezing under a layer of paraffin oil with glycerol [21]. A frozen “testicular pill” combining a mixture of sperm and testicular tissue may be possible as well [22].

3 Materials and Equipment, Samples, and Reagents

3.1 Equipment

1. Phase contrast microscope.
3. Automatic pipettor.
4. Vortex.
5. 
-20 °C freezer.
6. LN2 dewar with 11" canisters.
7. Sterile specimen container.
8. Sterile 15 mL centrifuge tubes with screw caps.
9. Sterile serological pipettes (2 mL capacity).
10. Sterile cryovials (1.8 mL capacity).
11. Colored cryomarkers.
12. Test tube racks (for 15 mL test tubes).
13. Cryovial racks.
15. Plastic cryosleeves.
17. Protective goggles.
18. Vinyl/nitrile gloves.
19. 37 °C incubator.
20. LN2 from supplier.
3.2 Reagents
Freezing medium (TEST yolk buffer; TYB). Each bottle is sterile and for onetime use. Stored frozen at \(-20^\circ\text{C}\) until time of use.

3.3 Specimen
A fresh semen sample is collected in a sterile plastic container by masturbation in the collection room. The client should abstain from ejaculation between 48 and 72 h before collection (see Note 1).

Even semen samples with poor sperm quality can be cryopreserved. Specimens with poor semen quality can be successfully used by in vitro fertilization or intracytoplasmic sperm injection technique.

4 Methods and Procedures

1. The collected sample is labeled and placed in a 37 \(^\circ\text{C}\) incubator for at least 20 min (see Note 2). Simultaneously, a bottle of TEST yolk buffer is thawed in the 37 \(^\circ\text{C}\) incubator for at least 20 min (see Fig. 1).

2. After liquefaction, the sample is drawn into a 2 mL sterile pipette, leaving at least 50 \(\mu\text{L}\) in the specimen container. The volume is measured and recorded, and any unusual consistency is noted (see Note 3).

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Fig. 1 37 \(^\circ\text{C}\) incubator used for sample incubation and thawing of TEST yolk buffer. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2010–2013. All Rights Reserved
3. The specimen is placed into a labeled, sterile 15 mL conical tube previously checked for cracks or defects.

4. The remaining aliquot in the specimen container is analyzed to assess sperm motility (see Note 4).

5. Within 1 h of collection, an aliquot of freezing medium equal to 25% of the original specimen volume is added to the centrifuge tube with a sterile pipette.

6. The specimen is gently rocked with the freezing media for 5 min on an aliquot mixer (see Fig. 2).

7. Steps 5 and 6 are repeated three times or until the volume of freezing medium added is equal to the original specimen volume (see Fig. 3).

8. During the mixing steps above, cryomarkers are used to label the cryocanes. An additional 1.8 mL cryovial is labeled—this will contain a leftover aliquot of the cryodiluted specimen to be assessed for cryosurvival 24 h after freezing.

9. The cryodiluted specimen is visually inspected for motility. Percent motility is assessed using a MicroCell chamber and phase microscope. The percent motility is documented as “pre-cryo motility %.”

10. The well-mixed, cryodiluted sample is evenly distributed into the pre-labeled vials using a 2 mL sterile serological pipette. At least 0.2 mL is added to the post-thaw test cryovial (see Fig. 4).
11. Labeled vials are placed into the labeled cryocane(s) (see Fig. 5). A maximum of two cryovials are placed into bottom slots of canes right-side up, while the canes are held upside down (i.e., labeled portion of cryocanes will be facing the ground while placing the cryovials upright).

12. The cryocane(s) is covered with a cryosleeve(s) and placed in the \(-20^\circ\text{C}\) freezer for 8 min (see Note 5).

13. The cryocane(s) is then removed from the \(-20^\circ\text{C}\) freezer and placed into a LN\(_2\) vapor tank for at least 2 h (see Note 6). Thereafter, the vials are frozen by the LN\(_2\) vapors (\(-80^\circ\text{C}\)).
14. After at least 2 h of incubation in the LN2 vapors (−80 °C), the canes are turned upside down and immersed into LN2 (−196 °C) (see Fig. 6).

15. At least 24 h in LN2 thereafter, the aliquot is thawed in the 1.8 mL post-thaw cryovial. The cane containing the cryovial is removed and snapped out. The cap is loosened and placed in the 37 °C incubator for 20 min.

16. The vial is mixed well and analyzed using a computer-assisted semen analyzer for spermatozoa motility parameters.

17. The results are recorded and the cryosurvival is evaluated using the following formula (see Note 7):  

\[
\frac{\% \text{ motility of post-thaw specimen}}{\% \text{ motility of pre-freeze specimen}}
\]

18. For IUI, the thawed specimen is pipetted into a sterile non-spermatotoxic centrifuge tube and rinsed with an equal volume of sperm washing media containing human albumin.

19. After centrifugation at 300 × g for 7 min, the supernatant is discarded and the pellet is resuspended to a volume of 0.5 mL using sperm wash medium. Insemination should be performed as soon as possible.
5 Notes

1. Sterile technique should be used throughout the sample processing. Gloves are mandatory. At the same time, latex can be toxic to sperm. Therefore, it is important to prevent contamination of the specimen with latex or talc or, preferably, not use them at all. Vinyl or nitrile gloves are recommended.

2. Only one sample is processed at a time to avoid mix-up or possible contamination. If more than one patient is scheduled for a specific time slot, then a second technologist should process the other sample.

3. Specimens that have a moderate or high viscosity that do not liquefy after incubation can be manipulated by pipetting them up and down with a sterile pipette or adding viscosity treatment system (VTS) whenever necessary.

4. The presence of round cells in the semen sample is counted, and a peroxidase staining test for white blood cells is done if ≥0.20 M/mL round cells are seen under wet preparation. At this point, any bacteria or foreign cells should be noted [23].

5. Exposure to freezing conditions should occur within 1.5 h following specimen collection [24].

6. Wear cryogloves and protective goggles when exposed to LN$_2$. 

Fig. 6 Short-term storage of semen samples in liquid nitrogen tanks. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2010–2013. All Rights Reserved
7. Quality control of the protocol consists of a pre-freeze and post-thaw evaluation of a new lot number of TEST yolk buffer using a donor sample that meets the following criteria:

50% survival, calculated by the following formula:

\[
\frac{\text{% post-thaw motility}}{\text{% pre-freeze motility}}
\]

6 Slow Freezing Versus Alternative Techniques: Pros and Cons

Slow, controlled sperm freezing is the cryopreservation method of choice for human spermatozoa (see Figs. 7, 8). The protocol [25, 26] results in thawed spermatozoa with excellent motion parameters including curvilinear velocity, straight-line velocity, and average path velocity [25].

However, in spite of reports showing successful sperm freezing with manual techniques, the reproducibility of this procedure could pose a variety of problems, especially to unexperienced technologists. For this reason, automated, computerized methods are...

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**Fig. 7** Long-term storage of semen sample in liquid nitrogen tank. The figure shows a metal canister containing boxes filled with cryovials. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2010–2013. All Rights Reserved
thought to be more exact and have been reported to limit cryodamage of low-quality spermatozoa [27]. Furthermore, the programs are usually simple to use and do not require continuous operator intervention [28]. On the other hand, automated freezers are often time-consuming and expensive to purchase and also use up to five times more liquid nitrogen [29].

Some authors argue that slow freezing, either manual or automated, may cause extensive chemico-physical damage to the sperm because of extensive ice crystallization [30].

Rapid freezing may be used as an alternative option [3]. This technique requires direct contact between the cryotubes and the nitrogen vapors for 8–10 min only, followed by a rapid immersion in liquid nitrogen. Rapid sperm freezing has several advantages over conventional slow freezing: no programmable freezer is required and the entire freezing/thawing procedure takes less than 15 min [31]. On the other hand, the technique has low reproducibility, and it is difficult to control the drop in the freezing temperatures [3].

A new and promising freezing technique is vitrification, based on cell preservation at extremely low temperatures without freezing. Vitrification involves the formation of an amorphous solid state which, unlike freezing, does not involve the formation of ice crystals, which can damage delicate cellular structures. Modern vitrification techniques are ice- and cryoprotectant-free, in which a sperm suspension is plunged directly into liquid nitrogen [32, 33]. It is a relatively simple and straightforward approach to preserve sperm.

Fig. 8 Freeze racks containing boxes filled with cryovials. Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2010–2013. All Rights Reserved
motility and fertilizing ability, as it omits the need for permeable cryoprotectants, preventing the lethal effects of osmotic shock [34]. Sperm vitrification is, however, a relatively new technique and has not yet been standardized for use in clinical andrology laboratory.

7 Concluding Remarks

The impact of disease, environment, and medical treatment may impair sperm quality and lead to male infertility. Therefore, effective promotion of sperm banking is crucial, based on adequate counseling focused on the severity and estimated risk for infertility, assessment of the patient’s plans to have children in the future, and emphasis on the benefits of banking as well as on possible difficulties such as cost or cultural factors.

Continuing research on developing fast, simple, and cost-effective protocols for semen cryopreservation is needed. The potential of cryopreservation continues to be explored currently, focusing on cryoprotectant-free sperm vitrification and refining the freezing and thawing protocols to obtain optimal outcomes.

References