JRME

Journal of Reproductive Medicine and Embryology

# Cryopreservation of rare ejaculate and testicular spermatozoa, a novel simple technique; the culture dish slice freezing: A pilot study.

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## Abstract

**Objective:** The ability to cryopreserve small numbers of spermatozoa retrieved by testicular sperm extraction (TESE) in males having nonobstructive azoospermia avoids the requirement for repeated surgery and promotes the preservation of fertility. The same applies to men with cryptozoospermia who may show few sperms only after concentrating their semen samples. Many techniques were proposed for freezing rare motile spermatozoa. In this pilot study, we propose a new simple and safe technique that requires no specific commercial tools.

**Methodology:** A new method is suggested for cryopreserving rare human spermatozoa using the culture dish slice.

**Results:** Four cases were included in this pilot study. A total of 20 motile ejaculate sperms and 20 motile testicular sperms were frozen using the culture dish slice technique. Sperm retrieval, retrieval of motility and retrieval of usable sperms for ICSI were (100%, 25% and 70% respectively) for ejaculate sperms and (100%, 10% and 60% respectively) for testicular sperms.

**Conclusion:** The culture dish slice technique would represent an easy, non-expensive method for rare sperm cryopreservation that necessitates no special commercial devices.

**Abbreviations list:** ICSI: Intracytoplasmic sperm injection, DNA: Deoxyribonucleic acid, LN: Liquid Nitrogen, NOA: Non-obstructive azoospermia, OAT: Oligo-asthenoteratozoospermia, SV device: Sperm Vitrification device, TESE: Testicular sperm extraction, ZP: Zona Pellucida.

**Keywords:** Sperm, cryopreservation, functional azoospermia, testicular.

JRME® Volume. 1, Issue no. 2, July 2024


**Clinical trial**

## Introduction

Advancements in intracytoplasmic sperm injection (ICSI) technology provide the opportunity to overcome fertility issues in patients with azoospermia and cryptozoospermia. The ability to cryopreserve small numbers of spermatozoa is very crucial especially for those affected by non-obstructive azoospermia (NOA) to limit the need for repeated testicular sperm extraction (TESE) (1). In a recent meta-analysis, data were analyzed from 1261 cycles and proved that fertilization, good embryo, clinical pregnancy, implantation and live birth rates were similar when comparing fresh versus frozen testicular sperms (2). However, conventional sperm preservation techniques can result in sperm loss as a result of the centrifugation and washing procedures and sperm adherence to the carrier vessel used. Thus, the conventional techniques aren't practical in cases with few sperms (3).
In 1997, Cohen et al., described a new cryopreservation technique for limited sperm numbers using an empty zona pellucida (ZP) (4). They provided the technical and theoretical foundation for many subsequent techniques and introduced the concept of single sperm freezing. Since then, many techniques have been developed to improve sperm retrieval after thawing. In most methods, after thawing the sperms were not immediately available for micromanipulation and additional steps were required, which may cause post-thaw sperm loss (5).

## Aim of the study:

We intended to study a new technique that can safely cryopreserve rare spermatozoa without the need for any commercial devices and we compared our results with the previously reported methodology.

## Material and Methods:

The freezing dish slice was prepared by cutting a sector from the lid of a sterile dish (Falcon® 50 mm x 9 mm, Cat: 351006, Becton-Dickinson, USA) by a surgical scalpel blade (Size 10) warmed by Bunsen burner. The diameters of that sector and the detailed steps are shown in Figure 1.

The detailed steps were as follows: The freezing solution was prepared by using Glycerol based sperm freezing medium (SpermStore GM501: Gynemed-Germany) diluted vol. to vol. (20 µL each) with Sage sperm washing medium containing 5% HSA (Quinn's Advantage™ Sperm Washing Medium ART-1006, Cooper Surgical-USA). The freezing drop (1 µL drop) was pipetted into the center of the freezing dish slice while placed on a culture dish similar to the one used for the slice preparation.

Sperm collection was done using a Narishige micro-manipulator (Japan) mounted on an Olympus IX 71 inverted microscope (Japan) with the aid of a Micro-pipette (Origio, MIC 50-30, Cooper Surgical-USA) attached to a Cell Tram syringe (Eppendorf, Germany). Care during handling the sperms from the original sample dish (Falcon® 50 mm x 9 mm, Cat: 351006, Becton-Dickinson, USA) to the slice as it is located at a higher level than the dish (not to break the collecting needle). Oil (Lite Oil-LGOL-500, Cooper Surgical-USA) covering is mandatory all through the maneuver.

The dish with the slice was placed about 10 cm above the Liquid nitrogen (LN) level for 10 minutes. Then horizontal dipping of the dish slice after freezing into the LN bath was done to be inserted into a 1.8 ml sterile cryovial labeled by a crymarker pen (Cat: 43022, SPL Life Sciences Co. ®, Korea) under LN. The cryovials were kept in clips on metal canes into the storage tanks in the vapor phase.

At thawing (would be done on the day of ICSI), a new collection dish with 5 drops (1 µL each) of Sage sperm washing medium was prepared to keep the thawed sperms. Two hypo-osmotic drops (1 µL each) were added to the collection dish (prepared by diluting the sperm medium (1:2) with sterile water) to test the immotile sperms for viability (6). Then thawing of the dish slice was done.

After removing the cryovial from the LN tank, the slice was taken by forceps and put on a new dish (similar to the one used during freezing) on

a 37° heated surface to be covered immediately by pre-heated lite oil. After complete thawing was observed (mean time was 5 minutes), collection of motile sperms was done -with the help of the same equipment used in freezing- into the drop of sperm washing medium. Immotile sperms were tested for viability and viable sperms were collected in another drop.

Ten motile ejaculate sperms from two OAT semen samples (can't be counted by Makler Sperm Counting Chamber) were frozen by the above-described technique and were thawed on the same day to be assessed for motility and viability. The same was done for ten motile testicular sperms from two functional azoospermic cases (taken from the dishes that were prepared for sample assessment before usual traditional cryopreservation).

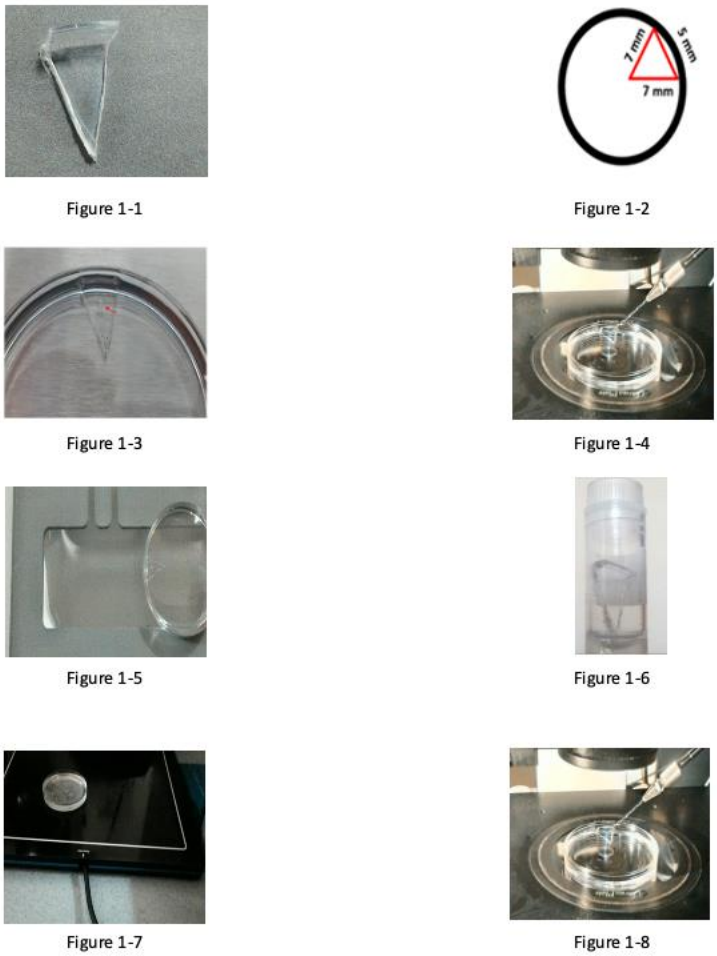


**Figure 1. Schematic illustration of spermatozoa freezing using the dish slice technique. 1,2- The culture dish slice 3- The slice with 1 ul freezing droplet while resting on the inner side of another culture dish covered with oil. 4- Sperm collection on the Slice while in a culture dish covered with oil. 5- Sperm Freezing in Nitrogen vapor. The slice is still on the culture dish and 10 cm above surface of LN for 10 minutes (to be dipped horizontally in the LN). 6- The slice in the cryovial to be stored in the LN tank. 7- Thawing of the Slice while in a new culture dish and covered with pre- heated oil on a 37°C surface. 8- Sperm retrieval from the Slice.**

## Results:

The results of sperm retrieval, retrieval of motility, and retrieval of viability were summarized in table 1.

**Table 1:** Summary of the results

| Case | Sperm Source | Original frozen motile sperms | Time needed to relocate the sperms | Post -Thawing data | | | |
|---|---|---|---|---|---|---|---|
| | | | | Retrieved sperms | Motile sperms | HOS positive sperms | Usable sperms |
| 1 | Ejaculate | 10 | 4 minutes | 10 | 3 | 4 | 7 |
| 2 | Ejaculate | 10 | 3 minutes | 10 | 2 | 5 | 7 |
| 3 | Testicular | 10 | 2 minutes | 10 | 1 | 6 | 7 |
| 4 | Testicular | 10 | 2 minutes | 10 | 1 | 4 | 5 |

Using this technique for ejaculate sperms (severe OAT samples), we retrieved all the cryopreserved sperms 100% (20 out of 20). The retrieval of motility was 25% (5 out of 20), while the retrieval of HOS positive sperms was (9 out of 20) with retrieval of usable sperms for ICSI of 70% (14 out of 20). Regarding testicular sperms (from functional azoospermic cases), we retrieved all the cryopreserved sperms 100% (20 out of 20). The retrieval of motility was 10% (2 out of 20), while the retrieval of HOS positive sperms was (10 out of 20) with retrieval of usable sperms for ICSI of 60% (12 out of 20).

## Discussion:

Cryopreservation of testicular sperms is generally recommended for fear of future failure to obtain suitable spermatozoa for ICSI specially in NOA patients (1). While for a cryptozoospermic patient, he would have few sperms in the ejaculte in rare occasions in the laboratory and in that event sperm cryopreservation is mandatory (7).

An advantage of sperm cryopreservation for those cases is that the couple will know in advance that sperms are available and not to worry about the possibility of useless ovarian stimulation for ICSI and financial loss. Cryopreserved sperms also allow the embryologist to know whether sperms are available for ICSI before egg retrieval (1).

However conventional cryopreservation techniques are inadequate for preserving limited number of sperms, but single-sperm freezing technology gives hope to these patients.

From the use of the empty ZP biocarriers, studies on carriers and freezing methods have improved the recovery of sperms after cryopreservation. The use of non-human or human zonae with possible remnant host DNA had discouraged the continued use of such techniques to avoid the potential risk of contamination and disease transmission.

Later, the non-biological carriers, were developed from polystyrene and polypropylene materials. Single-sperm microdroplet freezing methods are the core of all these methods, the micromanipulation process is convenient and sperm recovery, motility rates and fertilization rates are high (3).

An ideal container that can be universally used is yet to be developed. The sperm retrieval and motility after thawing are the most important factors that would determine the efficacy of any cryopreservation procedure. Also the fast

**Table 2:** Comparison between the currently available, safe cryostorage sperm vehicles

| | Consumables | Freezing droplet preparation | Freezing method | Search time after thawing | Techniqual / Special Storage demands / Risk of cross - contamination | Clinical outcome when used in ICSI procedures |
|---|---|---|---|---|---|---|
| Cell Sleeper (Ejaculate Sperm) (Endo et al., 2012) | Commercial device | A 3.5 µl droplet of Sperm Freeze-based cryopreservation medium. | The device while secured in cryopreservation cane was placed about 0.5 cm above the LN level for 2.5 minutes. | approximately 30 minutes | No | 10 infertile men and did not evaluate fertilization and delivery rates. |
| Cell Sleeper (Testicular Sperm) (Coetzee et al., 2016) | | A 2 µl droplet of a cryopreservation medium . | The device while secured in cryopreservation cane was placed about 4-5 cm above the LN level for 2 minutes then in LN for 1 minute before storage. | Not exceeding 40 minutes | | 12 ICSI cycles, fertilisation rate 65.9% and pregnancy rate 58.3%. |
| Cryopiece (Ejaculate and testicular sperms) (Sun et al., 2017) | Commercial device | A freezing medium was used | Holding the Cryopiece upon liquid nitrogen vapor for 15 min, followed by putting it into a cell freezing tube and storing it in the liquid nitrogen tank. | Minutes | No | 4 ICSI cycles, Fertilization Rate 73%, 2 of those cases had a singlton delivery and a twin live birth inanother one. |
| Sperm Vitrification device (Ejaculate and testicular sperms) (Berkovitz et al., 2018) | Commercial device | A 0.8–1 µl droplet of a 50/50 v/v mixture of Quinn's Advantage Sperm Freezing Medium and Quinn's Advantage Sperm Washing Medium. | The vial containing the SpermVD was immediately loaded onto a labeled aluminum holder and submerged into liquid nitrogen. | Minutes | No | 44 ICSI cycles, Fertilization Rate 59%, Pregnancy Rate 55% , Delivery Rate with ongoing pregnancies 39%, Miscarriage Rate 29% |
| Culture Dish Slice (Ejaculate and testicular sperms) (Current study) | Usual labware | A 1 µl droplet of a 50/50 v/v mixture of Sperm Store, Gynemed and Quinn's Advantage Sperm Washing Medium. | The dish with the slice in it were placed about 10 cm above the LN level for 10 minutes. | Less than 5 minutes | No | Not tried |

recovery of the preserved sperms is highly crucial in a busy IVF laboratory. An easy manueavre that requires minimal skills is also an advantage. It would be advatageous if no specific storage facility is needed other than the ordinary cryotank accessories. Storing samples

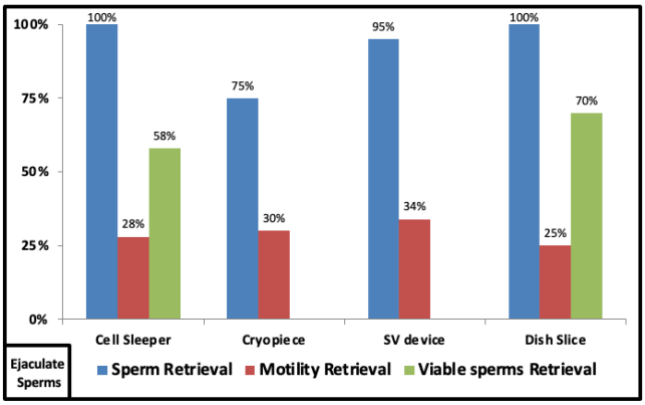


**Figure 2. Comparison between the currently available, safe cryostorage sperm vehicles regarding ejaculate sperm retrieval after thawing**

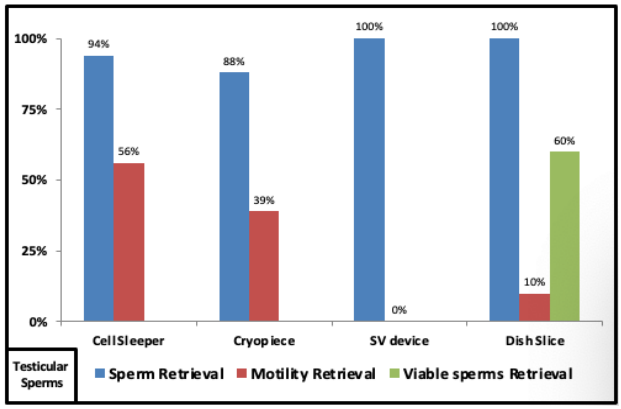


**Figure 3. Comparison between the currently available, safe cryostorage sperm vehicles regarding testicular sperm retrieval after thawing**

in the cryobank by any method of those should be ideally aseptic. Current evidence fails to completely support the benefit of one technology over the other.

Whole culture dish freezing was first described in 2000 by Quintans et al. (8). and was later tried by two other groups (9,10). This method is simple and easy, but the culture dishes are problematic due to their size and difficult storage in the liquid nitrogen and they cannot be sealed with the risk of potential cross-contamination. However, by introducing just a slice of the culture dish into the usual cryovial, we managed to solve those problems.

The addition of a glycerol-based cryoprotectant Vol/Vol to a sperm washing medium which creates the same cryopreservation environment as in bulk freezing followed by freezing in liquid nitrogen vapor at different distances from the surface of liquid nitrogen were used by similar researches. The small size of the drop used to store the sperms minimized the time needed to relocate the sperms after thawing from hours to minutes and allows a nearly 100% recovery rate.

The use of immotile sperms for ICSI is generally not recommended, however if only immotile spermatozoa are available then they can be used after adding motility stimulants, such as Pentoxifylline (11) or else checking their viability. Different methods can be used to detect live but immotile spermatozoa as the hypo-osmotic swelling test, the sperm tail flexibility test or the use of Laser. So we included the presence of immotile but viable sperms in our study as usable sperms (12).

We compared our results of sperm retrieval with the previously described methods (Figures 3-4) putting in mind the requirements of each technique (Table 2). We only included current methodologies that used properly sealed containers (no human or animal sources) to avoid any cross-contamination hazards.Those studies also share the step that the microdroplet in which the sperms were placed during cryopreservation is the same one from which the spermatozoa will be recovered after thawing without the need for a new recovery microdroplet, medium containing well or intermediary carrier. It can be assumed that the culture dish slice technique is a promising and safe method that can be easily integrated into the routine ICSI programme without the need of any commercial devices other than the usual labware and without the need to modify the internal storage systems.

The major drawbacks to our study are the small number of cases included and the lack of an appropriate control group. Further studies are needed to validate the use of the culture dish slice technique in larger number of cases. Also clinical trials are mandatory by using the recovered sperms in injecting mature oocytes that were retrieved for ICSI in such difficult cases to validate that new technique.

**Declarations of Interest:** none.

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