Chapter

A Method for Small Number of Human Sperm Cryopreservation

Xiangli Niu, Hua Huang, Yi Mo, Yan Sun and Wei-Hua Wang

Abstract

Recently, some sperm vitrification devices were developed to simplify the procedures to freeze small number of human sperm. In the present study, we used these devices to further examine some factors that affect sperm motility after fast freezing. Experiments were designed to examine the effects of 1) direct immersion of the devices to liquid nitrogen and indirect immersion of the devices to liquid nitrogen in which the devices were sealed in cryogenic storage vials; 2) different freezing volumes (1–5 μl); 3) different equilibration times (1–5 min); and 4) different ratio of freezing solution (0,1-5,1) on post thawing sperm motility. It was found that fast sperm freezing in the sealed vials had high post thawing sperm motility (91.3–93.7% of recovered sperm motility rates) while direct immersion of the devices to liquid nitrogen had 0% post thawing sperm motility. No differences in the recovered sperm motility rates were observed between different freezing solution volumes (87.4–90.5%), different equilibration times (89.5–94.0%), and different freezing solution ratios (90.8–94.6%). However, only 6.8% of recovered sperm motility rate was obtained if sperm were frozen in the medium without sperm freezing solution. These results indicate that human sperm can be rapidly frozen after the devices are sealed in the vials with different equilibration time in the medium containing sperm freezing solution. High post thawing sperm motility can be recovered with this method so that ~90% of sperm are usable after freezing.

Keywords: cryopreservation, fast freezing, human, motility, sperm

1. Introduction

Vitrification, as a fast cryopreservation method, has been used to cryopreserve human oocytes and embryos for many years and has become the routine and major method for cryopreservation of human oocytes and embryos as high survival rates can be obtained after warming [1, 2] However, for human sperm freezing, the current methods still mainly rely on slow freezing [3, 4]. Various methods for vitrification or fast freezing of human sperm have been tested, especially for cryopreservation of small number of sperm [5–12]. However, so far, no standard and practical vitrification/fast freezing method has been developed. This may be due to the facts that the number of sperm is quite large in most semen samples and current slow-programmed sperm freezing method can provide acceptable post recovery rate and survival rate. Furthermore, vitrification did not offer significantly superior results than slow freezing for sperm cryopreservation in the previous study [13].

Clinical uses of rare sperm or small number of sperm in human in vitro fertilization (IVF) are very common, especially when sperm are collected from testicle

biopsy. Due to very small number of sperm in these samples, many clinics chose to freeze the sperm during the initial semen analysis or testicle biopsy. However, sperm numbers and/or sperm motility may decrease after the traditional slow freezing. Occasionally, there are not enough sperm survival to inseminate all oocytes from a cycle. Therefore, methods for cryopreservation of small number of human sperm with high recovery and survival rates need to be investigated.

Currently, there are two methods for cryopreservation of small number of human sperm. One is to freeze all tissue or samples in cryogenic storage vials (1–2 ml) or straws (0.25–0.5 ml) by slow freezing [3]. Another is to find motile sperm and then the sperm were placed in some small devices to perform fast freezing or vitrification [5–8]. However, most protocols do not offer satisfactory recovery and motility rates after thawing. Because sperm are very small, it is difficult to process sperm for freezing. Devices and methods for oocyte and embryos vitrification are not suitable to freeze sperm. Therefore, it is necessary to develop devices and methods that are easy to use, and can also provide high recovery rate and good motility, to freeze small number of human sperm. Recently, a SpermVD, a sperm vitrification device, has been reported to freeze small number of human sperm from men with non-obstructive azoospermia [13]. Although it has been reported that high sperm recovery rates can be obtained with this device, sperm motility rates after thawing varied from 0 to 100% among samples from different patients [14]. It is still unknown whether the big differences between samples were resulted from freezing method, different samples, or technical difficulties. Therefore, in the present study, to avoid the differences among different samples, we used normal sperm samples to examine some factors that affect the post thawing sperm motility after fast freezing.

2. Materials and methods

2.1 Ethical statement

All patients undergoing sperm cryopreservation signed informed consents and the research was approved by ethics committee at the Reproductive Hospital of Guangxi Zhuang Autonomous Region.

2.2 Samples and devices

Semen samples were obtained from patients who were requested to freeze their semen for later IVF. A small portion of samples was used for fast freezing after swim-up for the study and the remaining was frozen by slow programmed freezing method. Samples were used after swim-up to obtain high percentage of motile sperm so that motility assessment is more accurate with swim-up samples than the original samples in which there were more immotile sperm and debris.

Two devices, as shown in **Figure 1**, SpermVD (MFC Global, Israel) and Microdevice (Xinchang Medical, China) were used in the experiments. For device holding, 2.0 ml externally threaded cryogenic storage vials (Fisher Scientific, USA) were used.

Sperm were washed and processed for swim-up with sperm washing medium (Fujifilm-Irvine Scientific, CA, USA) and then were frozen after being mixed with different ratios of Quinn's advantage sperm freezing medium (Origio, CT, USA). In the present study, to avoid the result variations between good samples and poor samples, we used normal sperm samples for all experiments. For loading the sperm to devices, we used 140 μl flexible pipette to add different volumes of samples to the devices and then processed the freezing.

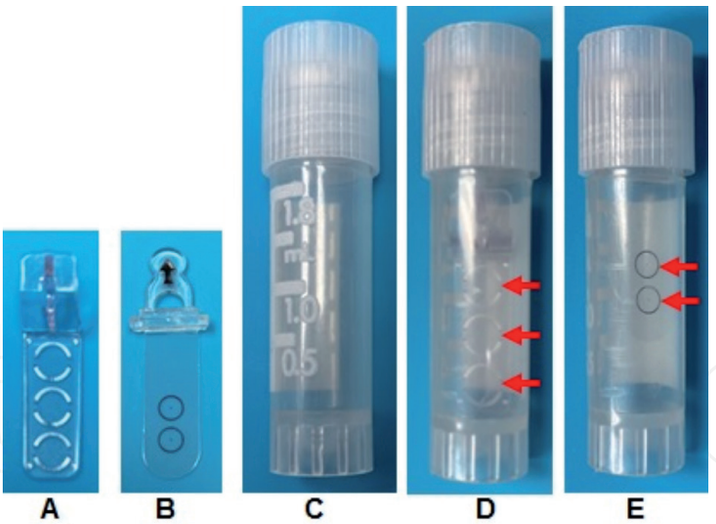

Figure 1.
Fast sperm freezing devices and set up for freezing. A: SpermVD, B: Microdevice, C: 2 ml externally threaded cryogenic storage vial. D: SpermVD in the vial, and E: Microdevice in the vial. Red arrows indicate the location of sperm drops in the devices.

2.3 Fast sperm freezing

Two methods were used for fast sperm freezing. Method I: after sperm were loaded to the devices and equilibrated in the sperm freezing solution for different times, the devices were directly immersed to liquid nitrogen and then each device was inserted to the cryogenic storage vial for storage. Method II: after sperm were loaded to the device, the device was inserted to the vial, the vial was tightly closed with the cap and then immersed to liquid nitrogen.

2.4 Sperm thawing and motility assessment

For sperm thawing, cryogenic vials were removed from liquid nitrogen, and the devices were taken out of the vials with forceps, placed on a 60 mm culture dish. After the frozen drop was thawed or melted within ~1 min at room temperature, the device was directly placed in a 60 mm culture dish with warm oil for sperm motility assessment. Because the volume is very small, it is not necessary to transfer sperm to other sperm count chamber to do motility assessment. Motility was assessed under a phase contrast microscope at ×200 magnifications.

2.5 Experiment design

In experiment 1, post thawing sperm motility was compared between Method I and Method II as well as between two devices, SpermVD and Microdevice. Each 1 μl of micro drop of equilibrated sperm for 5 min in 1:1 of sperm washing solution and sperm freezing medium was frozen in the SpermVD and Microdevice with either Method I or Method II. Three replications were done for this experiment. Temperature changes for two methods during sperm cooling were measured with a digital thermometer.

In experiment 2, post thawing sperm motility was compared among different volumes of micro drops. Each 1 μl, 2 μl and 5 μl of micro drops of equilibrated sperm (5 min in 1:1 of sperm washing solution and sperm freezing medium) was frozen in the SpermVD with the Method II. Five replications were done for this experiment.

In experiment 3, post thawing sperm motility was compared after different equilibration times. Each 1 μl of micro drop of sperm equilibrated for 1, 2 and 5 min

in 1:1 of sperm washing solution and sperm freezing medium was frozen in the SpermVD with the Method II. Three replications were done for this experiment.

In experiment 4, post thawing sperm motility was compared among different ratios of sperm freezing solutions. Each 1 μl of micro drop of equilibrated sperm for 5 min with different proportions of sperm freezing solution (0:1, 1:1, 2:1 and 5:1 of sperm freezing medium and sperm washing solution) was frozen in SpermVD with the Method II. Five replications were done for this experiment.

2.6 Statistical analysis

Each experiment was repeated three to five times, and each time with a different semen sample being used. For motility assessment, 100 sperm in each sample were counted. Mean ± SD of percentages of motile sperm before freezing and after thawing were obtained with replications. The recovered rates of sperm motility were calculated after dividing the post thawing sperm motility by the original sperm motility. All data were analyzed by ANOVA. If there were differences among groups, the differences between groups were further compared with chi-square test. If the P value was less 0.05, the difference was considered statistically significant.

3. Results

3.1 Effects of direct and indirect exposure of sperm to liquid nitrogen on the post thawing sperm motility

In the first study, we compared sperm motility after freezing with the Method I and Method II, i.e. direct and indirect immersion of sperm freezing devices (**Figure 1**) to liquid nitrogen and found that no sperm were motile if the devices were directly immersed to liquid nitrogen, while indirect immersion had high post thawing sperm motility. As shown in **Table 1**, the original motility was 85.0 ± 1.0%, and post thawing motilities were 79.7 ± 1.5% and 77.7 ± 2.1% with SpermVD and Microdevice, respectively. The recovery rates (post thawing motility/original motility) of motility were 93.7 ± 0.7% and 91.3 ±1.4% with SpermVD and Microdevice, respectively. No statistical differences were found between the devices (**Table 1**). Based on these findings, all following experiments were done in the SpermVD with the Method II.

After temperature changes were examined during direct and indirect immersion of devices to liquid nitrogen, as shown in **Figure 2**, we found that direct immersion has a 107°C/second cooling rate, or 2 seconds from 25°C to −190°C, while indirect

Devices	Methods	Original motility	Post thawing motility	Recovered motility***
SpermVD	I*	85.0 ± 1.0	0[a]	0[a]
	II**	85.0 ± 1.0	79.7 ± 1.5[b]	93.7 ± 0.7[b]
Microdevice	I*	85.0 ± 1.0	0[a]	0[a]
	II**	85.0 ± 1.0	77.7 ± 2.1[b]	91.3 ± 1.4[b]

Method I: Direct immersion of sperm freezing devices to liquid nitrogen.
**Method II: Sperm freezing devices were placed cryogenic storage vial, vials were sealed and then immersed to liquid nitrogen.*
****Percentage of post thawing sperm motility/original motility.*
[ab]*Values are significantly different in the same column, P < 0.00001.*

Table 1.
Effects of direct and indirect exposure of sperm to liquid nitrogen on the post thawing sperm motility.

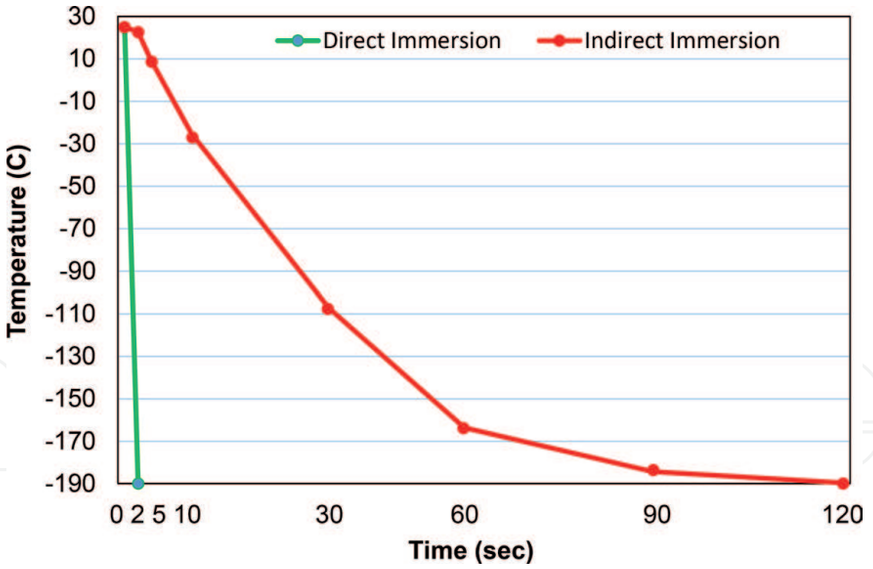

Figure 2.
Temperature changes during direct and indirect immersion of sperm freezing devices to liquid nitrogen. Times were recorded at 0, 2, 5, 10, 30, 60, 90 and 120 seconds during cooling from room temperature (25°C) to −190°C.

immersion has slow cooling rates with a 2 min of total cooling time from 25°C to −190°C. However, the cooling rates were further examined during this period and we found that it was 1.2°C/second during the first 2 seconds (25°C to 22.6°C), 4.6°C/second from 22.6°C to 8.7°C, 7.1°C/second from 8.7°C to −27°C, 4.03°C/second from −27°C to −107.6°C, 1.87°C/second from −107.6°C to −163.8°C, 0.66°C/second from −163.8°C to −183.6°C and 0.22°C/second from −183.6°C to −190°C. The temperature and time were not examined after the temperature reached to −190°C as it showed very slow changes after −190°C.

3.2 Effects of freezing volumes on the post warming sperm motility

As shown in **Table 2**, we found that there were no significant differences in the post thawing sperm motility between 1, 2 and 5 μl of freezing volumes. The original motility was 95.6 ± 1.7%, and post thawing motilities were 83.5 ± 5.5%, 88.9 ± 1.3%, and 86.4 ± 5.0%, respectively. The recovery rates of motility were 87.4 ± 6.2, 93.0 ± 1.9 and 90.5 ± 5.2%, respectively.

Volume (μl)	Original motility	Post thawing motility	Recovered motility**
1	95.6 ± 1.7	83.5 ± 5.5	87.4 ± 6.2
2	95.6 ± 1.7	88.9 ± 1.3	93.0 ± 1.9
5	95.6 ± 1.7	86.4 ± 5.0	90.5 ± 5.2

**Method II and SpermVD were used to freeze sperm.*
***Percentage of post thawing sperm motility/original motility.*

Table 2.
Effects of freezing volumes on the post warming sperm motility.*

3.3 Effects of equilibration times on post thawing sperm motility

As shown in **Table 3**, we found that equilibration of 1, 2 and 5 minutes of sperm in the freezing solution had similar post thawing sperm motility. Original motility was 94 ± 2%, and post thawing motilities were 88.3 ± 2.1, 85.4 ± 1.6 and 84.1 ± 0.9%, respectively. The recovery rates of motility were 94.0 ± 1.6, 90.9 ± 3.0 and 89.5 ± 2.4%, respectively.

Time (min)**	Original motility	Post thawing motility	Recovered motility***
1	94.0 ± 2.0	88.3 ± 2.1	94.0 ± 1.6
2	94.0 ± 2.0	85.4 ± 1.6	90.9 ± 3.0
5	94.0 ± 2.0	84.1 ± 0.9	89.5 ± 2.4

*Method II and SpermVD were used to freeze sperm.
**Sperm equilibration time in the freezing solution before freezing.
***Percentage of post thawing sperm motility/original motility.

Table 3.
Effects of equilibration times on post thawing sperm motility.*

3.4 Effects of freezing solution ratios on the post warming sperm motility

In this experiment, no differences were observed in the post thawing sperm motility between different ratios of sperm freezing medium (1:1, 2:1 and 5:1). As shown in **Table 4**, the original sperm motility was 91.4 ± 2.4%, and post thawing motility was 83.0 ± 3.7, 86.8 ± 2.8 and 85.2 ± 1.8%, respectively. The recovery rates of sperm motility were 90.9 ± 5.4, 94.6 ± 3.7 and 93.1 ± 4.2%, respectively. However, if sperm were frozen in the solution without sperm freezing medium (0:1), only 6.2 ± 1.1% motility, or 6.8 ± 1.2% of recovery rate of motility was obtained (**Table 4**).

Ratio**	Original motility	Post thawing motility	Recovered motility***
0:1	91.4 ± 2.4	6.2 ± 1.1[a]	6.8 ± 1.2[a]
1:1	91.4 ± 2.4	83.0 ± 3.7[b]	90.9 ± 5.4[b]
2:1	91.4 ± 2.4	86.8 ± 2.8[b]	94.6 ± 3.7[b]
5:1	91.4 ± 2.4	85.2 ± 1.8[b]	93.1 ± 4.2[b]

*Method II and SpermVD were used to freeze sperm.
**Ratio of sperm freezing solution to sperm washing medium.
***Percentage of post thawing sperm motility/original motility.
[ab]Values are significantly different in the same column, $P < 0.00001$.

Table 4.
Effects of freezing solution ratios on the post warming sperm motility.*

4. Discussion

Vitrification has been used to cryopreserve human oocytes and embryos in human infertility clinics for many years. For oocyte and embryo cryopreservation, vitrification requires high concentration (30%) of cryoprotectants in the vitrification solutions [1, 2]. Cryoprotectants are macromolecules added to the freezing medium to protect cells from the detrimental effects of intracellular ice crystal formation during the process of freezing and thawing. When embryo vitrification solution is cooled to −196°C in liquid nitrogen, substances in the solution is transformed into a glass, not ice crystal. However, in the present study, we used slow sperm freezing solution with low concentration of cryoprotectant (7.5% glycerol) and sperm still survived after freezing. It is still unknown whether ice crystal is formed inside sperm or not.

Although the procedure used in the present study is similar to vitrification, cooling time from room temperature to −196°C is longer (~2 minutes) than vitrification, thus the cooling rate is lower than vitrification. In fact, direct immersion of sperm to liquid nitrogen (that is similar as vitrification) did not support sperm

survival, which is completely different from vitrification of oocytes and embryos. Our data indicate that this fast sperm freezing method with regular concentration of cryoprotectant in the freezing solution can bring about ~90% recovered rate of sperm motility after thawing. This rate is higher than those reported in the previous studies with other methods [4–12]. The results in our study were very stable after freezing of a total of 16 semen samples, further indicating that this method is practicable in human IVF clinics.

A few previous publications indicated that vitrification or fast sperm freezing with different concentrations of sucrose as cryoprotectant also supported sperm motility after thawing, but the overall sperm motility rates were less than 40% [4–12]. Reduced sperm motility after freezing/thawing is mainly caused by the injury of sperm membrane [12–15]. Sperm DNA can also be further damaged by different cryopreservation methods or cryopreservation medium [12, 16, 17]. We did not examine sperm DNA fragmentation before vitrification and after warming in the present study, it remains necessary to further examine whether high survival rate is correlated with low DNA fragmentation.

Some devices for embryo vitrification have been used for vitrification/fast freezing of sperm but they are not the ideal devices for sperm freezing [5, 6]. Cryogenic storage vials may be suitable for normal sperm freezing because large volume is required for these kinds of freezing, but not suitable for small number of sperm freezing [10, 12–15]. Recently, a report indicated that direct pellet vitrification of human sperm with 0.25 M sucrose and thawing at 42°C increased sperm motility to ~70% and the authors considered that their high post thawing survival rate was resulting from a higher thawing temperature [10]. However, this method may not be suitable for freezing of small number of sperm.

Based on our study, we found that the following technical aspects are important to obtain high rates of sperm motility after fast freezing:

First, the traditional slow sperm freezing solution containing glycerol and sucrose works well for fast sperm freezing. It is not necessary to use higher concentrations of cryoprotectants, like those used for oocyte and embryo vitrification. However, cryoprotectants are still necessary for fast sperm freezing because very low motile sperm was obtained in the solution without cryoprotectants. This result indicates that the current commercial sperm freezing solution is appropriate for fast sperm freezing.

Second, time for equilibration of sperm in the freezing solution and volume of solution did not affect the post thawing sperm motility. These advantages allow this fast sperm freezing procedure to be easily used in human infertility clinics. For example, if there are very few sperm, freezing solution drop can be as small as 1 μl, and if there are more sperm, the volume of micro drop can be increased to 5 μl. However, it may not be necessary to freeze more than 20 sperm (if there is small number of sperm in a sample) in one device as high recovery and survival rates can be obtained with this method. Also, the time range (1–5 min) for equilibration of sperm in the sperm freezing solution allows laboratory technicians to process the freezing without rushing. We did not examine whether longer equilibration time has similar post thawing sperm motility or not, but it appears that up to 5 minutes is sufficient for technicians to load and freeze sperm.

Third, direct immersion of sperm to liquid nitrogen does not support post thawing sperm motility. This result indicates that fast cooling rate, like that for embryo vitrification, does not work for sperm freezing. By contrast, slow cooling rate is better than rapid cooling rate. When the devices are sealed in the cryogenic storage vial and then immersed to liquid nitrogen, cooling rate is reduced 60 times (120 sec vs. 2 sec) as compared with direct immersion of device to liquid nitrogen. These results indicate that vitrification does not work for human sperm freezing with the

current sperm freezing solutions. When the device was placed in the vial and then immersed to liquid nitrogen, the cooling rate was much slower than vitrification.

High survival rates (>90%) can be obtained when human oocytes and embryos were vitrified [1, 2]. Here we provide evidence that ~90% of recovered sperm motility can also be obtained with this fast sperm freezing method. In the present study, we examined sperm motility, but did not examine sperm vitality because ~90% of sperm were motile after thawing. Usually, sperm survival rates after freezing and thawing are higher than motility, indicating that sperm membrane has been injured in some sperm and these sperm cannot move but they are still live [4–12]. However, in the present study, high post thawing sperm motility indicates that sperm membrane was not injured during freezing and thawing, thus the current method may have less injury to sperm and/or sperm membrane than the previous methods [4–12].

The advantage of the devices used in the present study is that these devices can be directly placed in a culture dish, so sperm loading before freezing and sperm picking up after thawing can be done directly under a phase contrast microscope. Therefore, it is not necessary to transfer sperm between devices or wash sperm by centrifugation.

Furthermore, this method is a closed system. The vials must be closed tightly before immersion to liquid nitrogen to avoid liquid nitrogen entering the vials. Therefore, this method not only guarantees high rate of sperm motility after freezing, but also ensures samples not to have cross contamination during freezing and storage.

5. Conclusion

In conclusion, our data indicate that high post thawing sperm motility can be obtained after fast sperm freezing with the current commercial sperm freezing solution and commercially available devices, such as SpermVD and Microdevice. Our results also indicate that this method is safe as the devices are sealed in the storage vials during freezing and storage, which can avoid cross contamination. Furthermore, this method is simple and easy to learn.

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Conflict of interest

The authors declare that they have no conflict of interests.

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