EVALUATION OF THE OUTCOMES OF THREE DIFFERENT COOLING METHODS FOR HUMAN SPERM CRYOPRESERVATION

Pham Hong Minh[1,✉], Dao Thi Thuy Phuong[2]

[1]Haiphong University of Medicine and Pharmacy
[2]Hanoi Medical University

A laboratory experiment was conducted on 30 normal semen samples according to the WHO 2010 criteria to evaluate the ability to preserve sperm quality of three cryopreservation methods using freezers or liquid nitrogen vapor in the cooling process. Each sample was mixed well with cryoprotectant and divided into three equal aliquots in order to be cryopreserved with three different cooling methods. These methods are: placing in ECM2900WA-XE - 20ºC freezer (Electrolux) for 30 minutes and subsequently MDF-U74V-PE - 80ºC freezer for 30 minutes (group 1); MDF-U74V-PE - 80ºC freezer (PHCbi) for 15 minutes (group 2), and liquid nitrogen vapor for 15 minutes (group 3). Afterwards, all samples were stored in liquid nitrogen. After 30 days of cryopreservation, the quality of three groups was analysed after thawing by the same method. There was a statistically significant decrease in sperm viability, motility and progressive motility after cryopreservation. Group 2 had the best post-thaw sperm quality: total motility and progressive motility rates were 41.17 ± 7.81% and 30.60 ± 7.14%, respectively. However, no statistically significant difference in sperm quality was noted between group 2 and group 3, assessed by normal morphology rate and the cryo-survival factor (CSF). Group 1 showed the lowest sperm quality amongst the three groups, this result was statistically significant.

Keywords: Sperm, cryopreservation, ultralow freezer, liquid nitrogen vapor

I. INTRODUCTION

Infertility is a growing concern because of its profound impact on the quality of life of many couples around the world. Infertility rate ranges from 8 to 10% of reproductive-aged couples globally and stands at 7.7% in Vietnam.[1,2] Sperm cryopreservation is routinely performed in assisted reproductive technology (ART) centers and andrology laboratories to assist in the treatment of infertility. The principle of this technique is to transform the intracellular and extracellular fluid into a solid-state, thus stopping the molecular movement and biological processes in the cell. Cryopreservation should assure that the cells are unaffected structurally and functionally after thawing and returning to 37ºC.[3,4] There are currently two main sperm freezing techniques: slow freezing and vitrification; in particular, the former is still more popular in ART centers. The slow freezing process can be achieved through an automatic, semi-automatic or manually controlled machine in liquid nitrogen vapor. The use of freezers was introduced by the WHO in the "Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction" in 2010.[5,6] Available cooling devices have been implemented in a number of studies.[7-9] However, there is no standard freezing method, and the method for freezing semen that optimizes motility recovery has not been firmly established. Our study is to discover how to utilize the available devices, simplify the freezing procedure and save costs. At the Center of IVF and Tissue Engineering – Hanoi Medical University Hospital, we conducted this

Corresponding author: Pham Hong Minh
Haiphong University of Medicine and Pharmacy
E-mail: phminh@hpmu.edu.vn
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study to determine the cryopreservation method wih the best post-thaw sperm quality after using either freezers or liquid nitrogen vapor in the cooling process.

II. SUBJECTS AND METHODS

1. Subjects

30 normal semen samples were selected based on 2010 WHO criteria at the Center of IVF and Tissue Engineering – Hanoi Medical University Hospital from 8/2019 to 4/2020.

The samples should meet all of the following criteria: Semen samples with volume > 3 ml, sperm concentration within 50 - 200 million/ml, taken from of 20 - 50 year-old males who had semen analysis at the Center.

2. Methods

The study was designed as a laboratory experiment. The used equipment include: sterile sperm containers, incubator, glass slides and coverslips, eosin, Giemsa stain, optical microscope, Makler® counting chamber, Pasteur pipettes, white blood cell counter, cryotubes, cryocanes, ruler, foam boxes, MDF-U74V-PE - 80ºC freezer, ECM2900WA-XE - 20ºC freezer, liquid nitrogen, liquid nitrogen tanks, SpermFreeze™ (FertiPro, Belgium), Ferticult™ Flushing (FertiPro, Belgium). The data were collected based on the research form. Research design: Laboratory experiment research

Data collection: based on the research form

2.1. Semen collection

The samples were collected in a private room of the Center by masturbation after 2 - 7 days of sexual abstinence.

2.2. Semen analysis

After liquefaction in an incubator (37ºC), macroscopic and microscopic assessments according to the 2010 WHO manual wasere carried out to select the appropriate samples for the study. The percentage of viable sperm was assessed with 0.5% eosin-alone staining technique. 10 µl of semen was placed onto the lame, then 20 µl of 1% eosin was added to the mix. This was mixed for 30 seconds and covered with a lamelle. Under the x40 objective lens, 200 spermatozoa were counted and calculated for the percentage of alive (unstained) / death (stained). Assessment of sperm motility and concentration was performed with Makler® counting chamber. 10 µl of sperm was transferred with a pipette into the center of the counting chamber and assessed under the 20x objective lens. The white blood cell counter was used to classify sperm. 200 spermatozoa were counted and calculated for the percentage of progressive motile, non-progressive motile and immotile sperm. Sperm concentration was examined by counting the number of spermatozoa on multiple fields. Examination of sperm morphology was done with Giemsa staining technique. A drop of 10 µl semen was placed onto a glass slide to make a smear. The smear was fixed with absolute ethanol and stained with 10µl Giemsa then washed under running tap water and dried at room temperature. Sperm morphology was observed at 400x magnification.

2.3. Semen cryopreservation: Semen cryopreservation

Immediately after semen analysis, each sample was equilibrated with Sperm Freeze Solution™ to reach a final ratio of 1:0.7 (vol/vol) of freezing medium to semen. The medium was added by dropping into the semen sample and haking for 7 seconds before another drop. After adding cryoprotective agents, the specimen was allowed to settle for 10 minutes. and the The specimen was divided into three cryotubes with 0.85 ml in volume. The cryotubes were then adopted divided into three groups: 1, 2 or 3. After keeping at room temperature for 10 minutes, three groups were cooled by three different methods. as following:

• Group 1 was placed in ECM2900WA-XE freezer (- 20ºC) for 30 minutes and

subsequently in MDF-U74V-PE freezer (- 80°C) for 30 minutes.

• Group 2 was placed in MDF-U74V-PE freezer (- 80°C) for 15 minutes.

• Group 3 was attached onto a cryocane and placed in liquid nitrogen vapor within a foam box at 13 cm above the surface of the liquid nitrogen infor 15 minutes. The box is 38 cm in length, 28.5 cm in width and 27 cm in height.

Afterwards, the all groups 1, 2 and 3 were immersed into liquid nitrogen at - 196°C for storage until analysis.

Greisinger GMH 3200 Thermometer was used to ensure a correct temperature of the devices where the samples would be placed. In regard to Group 3, we determined a temperature of - 80°C was determined at 16 cm from above the bottom of the box.

Thawing of the samples was carried out after 30 days of cryopreservation: the samples were kept at room temperature for 5 minutes and then in a 37°C water bath for 20 minutes. The thawed samples after thawing were mixed with Ferticult™ Flushing medium in a 1:1 ratio, then centrifuged at 1500 rpm for 10 minutes. After centrifugation, the medium was aspirated to leave exactly 0.5 ml to and performed semen analysis.

2.4. Data collection and analysis

The following parameters were evaluated: sperm concentration (million/ml), sperm viability (percentage), total motility and progressive motility (percentage), sperm morphology (percentage of normal sperm) and CSF (cryosurvival fator).

$$\text{Motile CSF} = \frac{\%\ \text{motile sperm after cryoprervation}}{\%\ \text{motile sperm before cryoprervation}} \times 100\%$$

$$\text{Progressive motile CSF} = \frac{\%\ \text{progressive motile sperm after cryoprervation}}{\%\ \text{progressive motile sperm before cryoprervation}} \times 100\%$$

$$\text{Viable CSF} = \frac{\%\ \text{viable sperm after cryoprervation}}{\%\ \text{viable sperm before cryoprervation}} \times 100\%$$

Data analysis with SPSS 20.0, using paired t-test and Wilcoxon test to compare the results. The difference is statistically significant when $p < 0.05$.

3. Research ethics

The study was approved by the leaders of Center of IVF and Tissue Engineering – Hanoi Medical University Hospital. The purpose of the research was fully informed and the study was carried out with the approval of the participants. Information relating to the research is kept strictly confidential and used only for scientific purposes. Semen samples were disposed immediately after the study.

III. RESULTS

1. Sperm concentration and viability after cryopreservation

Table 1. Sperm concentration and viability after cryopreservation using 3 methods of cooling (n = 30)

Parameter	Before cryopreservation (1)	After cryopreservation		
		Group 1 (2)	Group 2 (3)	Group 3 (4)
Sperm concentration (million/ml)	84.20 ± 14.62	80.90 ± 17.28	81.47 ± 13.71	83.27 ± 15.54
Sperm viability (%)	84.77 ± 8.85	36.53 ± 7.88	53.13 ± 8.51	53.90 ± 9.47

Sperm concentration: $p > 0.05$.

Sperm viability: $\mathbf{p_{1/2}, p_{1/3}, p_{1/4}, p_{2/3}, p_{2/4} < 0.001.}$

$P_{3/4} > 0.05$.

There was no significant difference in sperm concentration before and after cryopreservation in all three groups; however, group 3 (cooled with liquid nitrogen vapor) had the highest concentration of 83.27 million/ml. Sperm viability decreased significantly in all three groups compared to before cryopreservation with $p < 0.001$; group 1 (cooled with two freezers via two steps) had the lowest percentage of viable sperm (Table 1).

2. Sperm motility after cryopreservation

Table 2. Sperm motility after cryopreservation using 3 methods of cooling (n = 30)

Parameter	Before cryopreservation (1)	After cryopreservation		
		Group 1 (2)	Group 2 (3)	Group 3 (4)
PR (%) ($\overline{X}$ ± SD)	57.20 ± 10.29	20.37 ± 5.30	30.60 ± 7.14	29.67 ± 7.99
PR + NP (%) ($\overline{X}$ ± SD)	72.60 ± 13.41	29.37 ± 4.85	41.17 ± 7.81	40.87 ± 8.98

$p_{1/2}$, $p_{1/3}$, $p_{1/4}$, $p_{2/3}$, $p_{2/4}$ < 0.001.

$P_{3/4} > 0.05$.

The results illustrated in table 2 clearly showed that after cryopreservation, group 1 had the highest percentages of sperm motility and progressive motility: 41.17 ± 7.81% and 30.60 ± 7.14%, respectively, which were closely followed by those of liquid nitrogen vapor cooling method. Group 1 was seen with the lowest rates of 29.37 ± 4.85 in motility and 20.37 ± 5.30% in progressive motility. However, after cryopreservation, the results of all three groups decreased significantly compared to before cryopreservation with $p < 0.001$.

3. The percentage of sperm with normal morphology after cryopreservation

Table 3. The percentage of sperm with normal morphology after cryopreservation using 3 methods of cooling (n = 30)

Parameter	Before cryopreservation (1)	After cryopreservation		
		Group 1 (2)	Group 2 (3)	Group 3 (4)
% sperm with normal morphology	5.07 ± 1.02	4.43 ± 0.68	4.80 ± 0.96	4.83 ± 1.02

$p_{1/2}$ < 0.05.

$p_{1/3}$, $p_{1/4}$, $p_{2/3}$, $p_{2/4}$, $p_{3/4} > 0.05$.

There was no significant change in the rates of normal morphological sperm compared to before cryopreservation in group 2 and 3. Group 1 was noted with a lower percentage of normal morphological sperm (4.43 ± 0.68%) than before cryopreservation (5.07 ± 1.02%), this difference was statistically significant ($p < 0.05$) (table 3).

4. CSF indexes after cryopreservation

Table 4. CSF indexes after cryopreservation using 3 methods of cooling (n = 30)

Parameter	After cryopreservation			p
	Group 1 (1)	Group 2 (2)	Group 3 (3)	
Progressive motile CSF (%)	35.99 ± 13.01	57.59 ± 17.67	52.28 ± 13.55	**$p_{1/2}$ < 0.05** **$p_{1/3}$ < 0.05** $p_{2/3} > 0.05$

Motile CSF (%)	41.43 ± 8.66	57.09 ± 13.17	56.94 ± 11.39	**$p_{1/2} < 0.05$** **$p_{1/3} < 0.05$** $p_{2/3} > 0.05$
Viable CSF (%)	46.94 ± 9.26	63.10 ± 10.49	63.73 ± 9.87	**$p_{1/2} < 0.05$** **$p_{1/3} < 0.05$** $p_{2/3} > 0.05$

After 30 days of cryopreservation, both motile CSF and progressive motile CSF were the lowest in group 1, while these indexes were the highest in group 2. The differences in all three indexes between group 2 and 3 were not statistically significant ($p > 0.05$). However, these indexes in group 1 were significantly lower than the other two groups ($p < 0.05$).

IV. DISCUSSION

After experimental cryopreservation of 30 normal semen samples using three different cooling methods, we noted that there was a statistically significant decline in sperm viability, motility and progressive motility compared to before cryopreservation ($p < 0.001$). When using freezers for cooling, group 2 which was cooled via one step (- 80°C freezer) showed the best results of all three methods while the cooled method via two steps (- 20°C and - 80°C freezers) showed the worst results. Vaz[10] (2018) experimented with the temperature of - 80ºC to preserve human sperm for 24-96 hours, and concluded that it was not possible to preserve sperm at this temperature long term due to the significant decline in sperm viability and mobility.[10] Regarding the decreased viability of spermatozoa, Karow[11] (1974) explained that the recrystallization of intracellular ice occurring at - 87ºC led to irreversible cell damage, which was very close to - 80ºC used in Vaz's and this studies. However, since the time of exposure to - 80°C in this research was only 15 minutes (in group 2 and 3), the post-thaw samples had higher rates of sperm viability and motility than Vaz's results ($p < 0.001$).

The principle of sperm cooling and cryopreservation is to transform the intracellular and extracellular fluid into a solid state, causing the biochemical reactions inside the cell to stop. However, the fluctuation of temperature can lead to stress as well as the release the water-soluble molecules which changes the osmotic pressure, resulting in cell damage. Thermal fluctuations may occur when handling samples, changing the storage location, or transportation.[12] The two-step cooling method (group 1) involved greater temperature fluctuation in the samples than the one-step cooling with a freezer (group 2) or liquid nitrogen vapor (group 3). Therefore, we did not obtain good results in sperm cryopreservation when using the two-step cooling with two freezers.

According to the American Association of Tissue Banks (AATB), the sperm quality to be achieved after cryopreservation includes the percentage of motile sperm ≥ 50% compared to before cryopreservation.[13] Our results revealed that the samples cooled via one step with MDF-U74V-PE freezer and liquid nitrogen vapor showed motile CSF and progressive motile CSF were reduced but still met the requirement of the AATB. Thus, the deep freezers at - 80ºC can be used to cryopreserve normal semen sample. Moreover, the cooling procedure is very simple and less time-consuming with no special tool needed. However, this method is only suitable for centers with freezers that can cool down to - 80ºC such as tissue banks, stem cell banks, etc. Meanwhile, assisted reproductive technology centers now rarely utilize this type of freezers. The center of IVF and Tissue

Engineering of Hanoi Medical University is a center where the combination of assisted reproductive techniques and tissue engineering takes place. It is very convenient for the center to apply the freezer method.

As for the group 1, these indexes in comparison with before cryopreservation did not reach the goal issued by the AATB. The decrease in sperm motility is believed to result from mitochondrial membrane damage, which impaired the oxidative phosphorylation pathway that produced adenosine triphosphate (ATP) – energy for microtubule-mediated motility of the sperm. Moreover, intracellular ice recrystallization caused by thermal fluctuation also contributed to the reduced sperm motility. Although intracytoplasmic sperm injection (ICSI) can solve the problem of poor or even absent sperm motility, motility is still an important factor in sperm quality assessment.

V. CONCLUSION

Our study results have demonstrated that:

- Sperm cryopreservation with one-step one-freezer cooling gave the best results of post-thaw sperm quality, but there was no statistically significant difference compared to liquid nitrogen vapor cooling.

- Freezers can be a simple and effective option to freeze normal semen samples. The two-step two-freezer cooling method should not be used in sperm cryopreservation in this situation.

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