INITIAL EVALUATION OF THE EFFICIENCY OF CRYOPROTECTANT-FREE VITRIFICATION IN PCR TUBES ON A LOW NUMBER OF HUMAN SPERMATOZOA

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Abstract

Objectives: To inititally evaluate the efficiency of cryoprotectant-free vitrification in PCR tubes on a low number of human spermatozoa. ***Methods:*** A laboratory experimental study was conducted on 30 severe oligospermia treated at the Military Institute of Clinical Embryology and Histology, Vietnam Military Medical University. The semen samples were vitrified and thawed at the following times, then sperm concentration, morphology, viability, and motility were evaluated after thawing. ***Results:*** The viable CSF, motile CSF when vitrifing without using CPAs were 50.45 ± 4.63; 43.35 ± 4.81 (1-week storage), 45.24 ± 4.33; 37.24 ± 4.39 (4-week storage) and 40.69 ± 3.96; 33.96 ± 4.14 (8-week storage), respectively. ***Conclusion:*** Vitrification of human sperm can be achieved without cryoprotectants and could be recommended for routine assisted reproductive technology.

Keywords: Sperm vitrification; Oligospermia; Free-cryoprotectants.

INTRODUCTION

Sperm cryopreservation was first introduced by Lazaro Spallanzani in 1976 by cryopreserving semen in snow. After that, the first baby born from cryopreserved sperm was reported in 1953 by Bunge and Sherman [1]. The principle of this kind of technique is to transform both the extracellular and intracellular fluid into a solid state; therefore, pausing the molecular movement and biological processes in the cell. An ideal cryopreservation technique should ensure that the cells

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are structurally unaffected and functionally post-thawing. Recently, fertility centers often use two main sperm cryopreservation techniques: Vitrification and slow freezing. The slow freezing technique is more popular and has been used for a long time in laboratories, despite the fact that the rate of sperm recovery after thawing with this technique is usually 50%, varying greatly depending on the sample. In addition, numerous studies around the world have proven that this technique causes major changes in both sperm structure and function [2, 3]. Applying slow cryopreservation techniques for semen samples with a low number of spermatozoa is still a big challenge. Sperm vitrification can overcome the disadvantages of the slow sperm freezing technique for the above sample types because it is quick and simple to perform. Moreover, sperm quantity and quality recover well after thawing, but the temperature reduction rate of up to several tens of thousands of degrees per second can also create ice crystals in sperm cells. Besides, the use of cryoprotective agents (CPAs) during sperm cryopreservation is still controversial among studies. According to Mochida (2014), the use of high-concentration cryopreservation CPA with a small quantity of sperm samples is necessary for sperm cryopreservation [4]. However, human sperm is very sensitive to CPA, so some authors have proposed a sperm cryopreservation method that does not use CPA [5, 8]. Therefore, we conducted this study: *To evaluate the efficiency of cryoprotectant-free vitrification in PCR tubes on a low number of human spermatozoa*.

MATERIALS AND METHODS

1. Materials

* *Research subjects*: 30 oligospermia semen samples at the Military Institute of Clinical Histology and Embryology, Vietnam Military Medical University

* *Inclusion criteria*: Oligospermia semen samples (< 1 million sperm/mL); patients agreed to participate in the study.

* *Exclusion criteria*: Patients tested positive with HIV, HBsAg, and HCV.

* *Location and time*: Conducted at the Military Institute of Clinical Histology and Embryology, Vietnam Military Medical University from September 2022 to September 2023.

2. Methods

* *Research design*: A laboratory experimental study.

* *Data collection*:

- Sample size: 30 severe oligospermia semen samples after analysing according to the WHO-Laboratory manual for the examination and processing of human semen 6th 2021.

- Sampling: Convenience sampling from September 2022 to September 2023.

* *Research process:*

After collecting, the samples were vitrified and thawed at the following times, and sperm concentration, morphology, viability, and motility were evaluated after thawing:

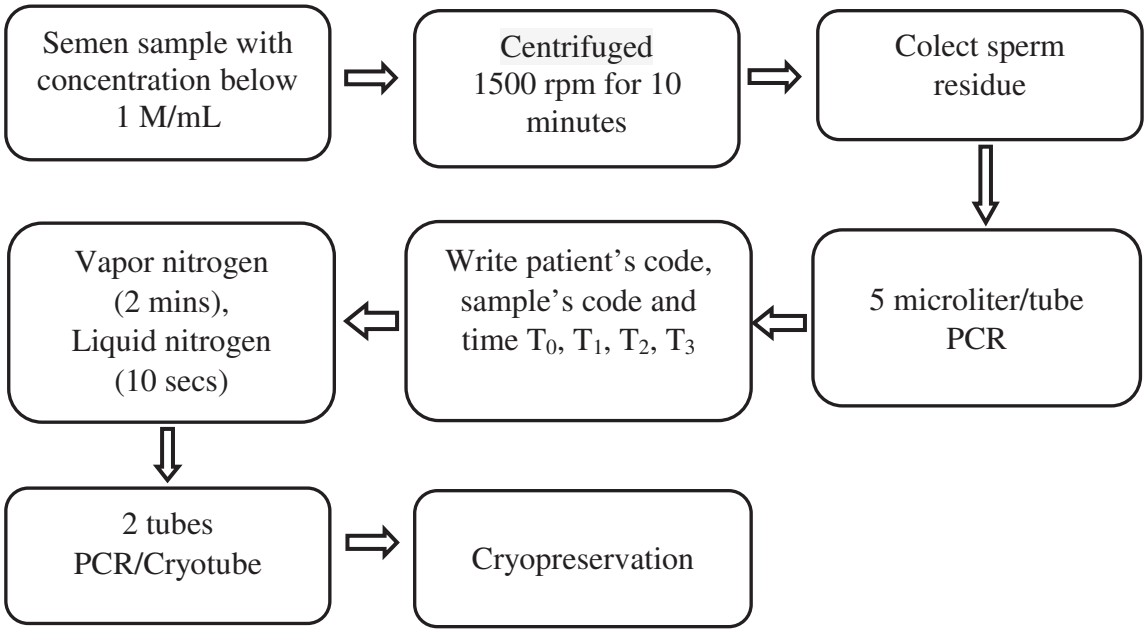
- T_0: After centrifuging the sample, cryopreserved the sperm sample by vitrifying a low number of spermatozoa without using cryoprotective agents (CPAs) in a PCR tube.

- T_1: After 1 week, the stored samples were thawed and compared with T_0.

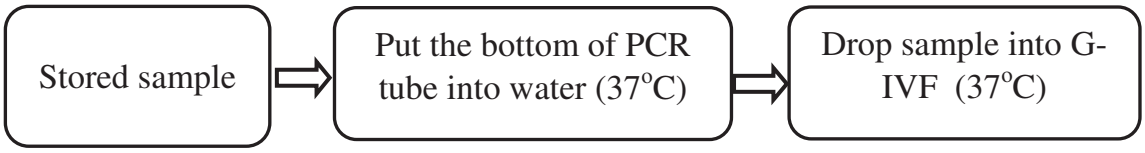
- T_2: After 4 weeks, the stored samples were thawed and compared with T_0 and T_1.

- T_3: After 8 weeks, the stored samples were thawed and compared with T_0, T_1, and T_2.

Sperm sample vitrification process:



Thawing stored sample process:



** Evaluating parameters:*

- Sperm concentration (million/mL).
- Sperm viability (percentage).
- Total motility (PR + NP) and progressive motility (PR) (percentage).
- Sperm morphology (percentage of normal sperm).
- CSF (Cryo-survival Fator):

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

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

** Data processing methods:* Research data were processed with the Stata 14.0 using a paired t-student test. The difference is statistically significant when the value is < 0.05.

3. Ethics

The study was approved by the Ethics Committee for Biomedical Research of the Military Institute of Clinical Histology and Embryology, Vietnam Military Medical University. Information relating to the research is kept strictly confidential and used only for scientific purposes. Semen samples were disposed of immediately after the study.

RESULTS

Compare sperm quality before and after thawing.

Table 1. Comparison of sperm quality without using CPAs before and after thawing.

	T_0 (0)	T_1 (1)	T_2 (2)	T_3 (3)	p
Concentratrion (milion/mL)	2.78 ± 1.68	2.78 ± 1.66	2.78 ± 1.67	2.79 ± 1.67	$p_{0-1} = 0.491$ $p_{1-2} = 0.587$ $p_{2-3} = 0.424$ $p_{1-3} = 0.217$
Sperm viability (%)	40.54 ± 20.90	20.33 ± 10.31	18.28 ± 9.26	16.44 ± 8.42	$p_{0-1} = 0.000$ $p_{1-2} = 0.000$ $p_{2-3} = 0.000$ $p_{1-3} = 0.000$
PR(%)	4.19 ± 4.97	1.38 ± 1.63	1.18 ± 1.39	1.06 ± 1.26	$p_{0-1} = 0.0001$ $p_{1-2} = 0.0002$ $p_{2-3} = 0.0002$ $p_{1-3} = 0.0001$
NP(%)	12.75 ± 11.27	8.96 ± 12.99	7.69 ± 11.18	7.02 ± 10.09	$p_{0-1} = 0.0153$ $p_{1-2} = 0.0007$ $p_{2-3} = 0.0027$ $p_{1-3} = 0.0011$
PR + NP (%)	16.95 ± 13.35	10.33 ± 12.89	8.87 ± 11.09	8.09 ± 10.01	$p_{0-1} = 0.0026$ $p_{1-2} = 0.0001$ $p_{2-3} = 0.0006$ $p_{1-3} = 0.0002$
Sperm morphology (%)	2.70 ± 0.92	2.67 ± 0.96	2.60 ± 0.81	2.53 ± 0.82	$p_{0-1} = 0.573$ $p_{1-2} = 0.326$ $p_{2-3} = 0.326$ $p_{1-3} = 0.103$

In general, the longer the storage period, the lower the sperm quality. Specifically, the average sperm viability, progressive motility rate, non-progressive motility rate, and total motile sperm rate gradually decreased over

time of thawing; the difference was statistically significant, with $p < 0.05$. The average sperm concentration and the percentage of sperm with normal morphology did not change significantly over the stages, the difference was not statistically significant, with $p > 0.05$.

Table 2. Viable CSF after cryopreservation.

Time	Viable CSF (%)
T_1	50.45 ± 4.63
T_2	45.24 ± 4.33
T_3	40.69 ± 3.96

When evaluating post-thaw the stored samples, the viable CSF index decreased overtime after 1 week, 4 weeks, and 8 weeks; the difference was statistically significant with $p < 0.05$.

Table 3. Motile CSF after cryopreservation.

Time	Motile CSF(%)
T_1	43.35 ± 4.81
T_2	37.24 ± 4.39
T_3	33.96 ± 4.14

When evaluating post-thaw the stored samples, the motile CSF index decreased overtime after 1 week, 4 weeks, and 8 weeks; the difference was statistically significant with $p < 0.05$.

DISCUSSION

Dramatic changes during cryopreservation have detrimental effects on the sperm membrane, resulting in a large increase in the percentage of poorly motile sperm or sperm with abnormal morphology. The negative effects related to high-speed temperature decrease, such as intracellular ice crystal formation, osmotic injury, cellular dehydration, and oxidative stress, can also injure the sperm in ways that affect reproductive outcomes. Sperm cells are rich in mitochondria because a constant supply of energy is required for their motility, and mitochondrial damage during cryopreservation processes is

linked with a loss of membrane permeability. This confirms the fact that cryopreservation affects motility because of mitochondrial damage and also because of physical changes to the tail. Also, human spermatozoa contain large quantities of proteins, sugars, and other components that may act as natural cryoprotectants. It is known that the loss of sperm quality is more significant in patients whose sperm parameters are poor. Therefore, there is a significantly lower post-thaw percent motility, motile sperm concentration, and cryo-survival rate, especially in a group of OAT patients, as in our study (concentration < 1 million/mL). Our group's results on motile CSF and viable CSF are similar to the studies of Widyastuti R (2017) [10] and Nguyen Thi Hang (2018) with the crystal freezing method [7]. In Widyastuti R's study (2017), samples were divided into three groups: The control group, samples mixed with a basic solution, and samples mixed with a vitrification solution. The straws (carriers) were vaporized in liquid nitrogen for 5 seconds, then plunged into liquid nitrogen directly and stored for 24 hours. Sperm motility and viability were observed to evaluate sperm quality before and after vitrification. Overall, vitrified products without preservative agents had a proportion of sperm motility and viability rates of 35% and 48%, respectively [10]. However, in that study, the authors evaluated motile CSF and viable CSF right after 24 hours of storage, earlier than the time T_1, T_2, and T_3 in our study. In this research, we have not explored the stability of epigenetics information in human spermatozoa. However, Wang M and et al. (2022) analyzed epigenetic differences between fresh and cryopreserved spermatozoa using high-throughput RNA sequencing in three groups: Fresh spermatozoa (control group), frozen spermatozoa, vitrified spermatozoa, and concluded that cryopreservation of human spermatozoa is an epigenetically safe method for male fertility preservation; cryoprotectant-free vitrification can induce minor biological changes in human spermatozoa, in comparison with conventional freezing. [9].

CONCLUSION

Vitrification of human sperm can be achieved without intracellular cryoprotectants and could be recommended for routine assisted reproductive technology. Carriers are PCR tubes in a cryotube, which will refrigerate samples in very small volumes, safely in laboratories, and limit infection.

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