Original Article

Comparison of Conventional Slow Freeze versus Permeable Cryoprotectant-Free Vitrification of Abnormal Semen Sample: A Randomized Controlled Trial

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Abstract

Background: The cryopreservation of semen samples by slow freezing remains as standard protocol. Recently, vitrification of spermatozoa was successfully reported with superior outcome. Till date, there is no randomized trial comparing the two different protocols. **Aim:** The aim of the present study is to evaluate the slow freezing with vitrification of the subfertile men spermatozoa to evaluate the progressive motility, vitality, and chromatin integrity. **Setting:** The study was conducted at University teaching hospital. **Design:** Study design involves randomized control trial. **Materials and Methods:** Twenty subfertile men with semen characteristics of severe oligoasthenozoospermia (SOA) and very SOA (VSOA) randomized to undergo slow freezing and vitrification protocol and cryopreserved at 1-month and 6-month storage interval, postthawed or warmed, samples were assessed for progressive motility, vitality, and hyaluronan binding. SPSS version 14 software was used for statistical analysis. **Results:** The SOA samples at 1 month revealed significantly higher motility (42% [22%–74%] vs. 7% [1%–13%]; $P = 0.015$) and vitality (57% [45%–78%] vs. 34.5% [27–42]; $P < 0.001$) following vitrification compared to slow-freeze method. For Very severe oligoasthenozoospermia (VSOA), the motility was significantly higher following vitrification (14.5% [2%–32%] vs. 2.5% [0%–4%]; $P = 0.007$). At 6 months, no statistically significant difference in motility was found between the two groups for Severe Oligoasthenozoospermia (SOA) samples (27% [13%–62%] vs. 8% [0%–11%]; $P = 0.066$), but motility was significantly higher following vitrification for VSOA samples (12.5% [3%–32%] vs. 2% [1%–5%]; $P = 0.019$). The hyaluronan-binding assay was comparable in both the groups at 6 months. **Conclusions:** The current study found the vitrification method involving the use of only nonpermeable cryoprotectants for cryopreservation of abnormal semen sample to be an effective alternative to the conventional slow-freeze technique.

Keywords: *Cryopreservation of spermatozoa, slow freezing, vitrification*

Introduction

Assisted reproductive technology (ART) treatment often involves cryopreservation of spermatozoa for various reasons such as fertility preservation, semen collection issues due to anxiety, the presence of very few sperms in the ejaculate or poor seminal parameters, and surgically retrieved sperm in men with abnormally high-follicle-stimulating hormone levels.[1-3] The use of donor sperm in ART also involves cryopreservation during the quarantine period.[4] Since semen cryopreservation entails the handling of human gamete which is likely to be used therapeutically for

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Quick Response Code:

Website: www.jhrsonline.org

DOI: 10.4103/jhrs.JHRS_154_18

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How to cite this article: Karthikeyan M, Arakkal D, Mangalaraj AM, Kamath MS. Comparison of conventional slow freeze versus permeable cryoprotectant-free vitrification of abnormal semen sample: A randomized controlled trial. J Hum Reprod Sci 2019;12:150-5.

developing embryos, the methods involved should be safe, efficient, and reproducible. While oocyte and embryo cryopreservation methods have been refined with good ART outcomes, sperm cryopreservation techniques have largely been neglected and still need considerable improvement.[5]

The main method of semen cryopreservation in contemporary practice is conventional slow freezing;[6,7] however, more recently, vitrification has also been used.[8,9] The conventional slow freeze can be done using programmable and nonprogrammable methods, and the slow-freeze method has been associated with a decline in sperm quality and is considered time-consuming and labor-intensive.[7,8,10,11] The deleterious effect of the slow-freeze method varies according to semen parameters. The damaging effect is more pronounced in the semen sample with severe oligoasthenoteratozoospermia than normozoospermia samples.[12,13] Efforts have been made to refine the semen cryopreservation techniques, and the rapid freezing protocols were introduced. However, the results following rapid freezing have been inconsistent.[14]

Over the past few years, the vitrification technique for sperm cryopreservation has been explored. Few studies using different carriers and open system of vitrification for sperm cryopreservation have been published.[13-15] A recent study compared a novel cryoprotectant-free sperm vitrification protocol versus conventional slow freeze and reported good sperm recovery with the former method.[16] Another alternative semi-closed system of vitrification of spermatozoa was reported using a solid surface vitrification (SSV) method.[15]

In view of the paucity of data, we decided to perform a randomized trial on the effectiveness of semen cryopreservation using the vitrification method compared to conventional slow freeze. Further, we decided to explore a novel method of vitrification and planned to evaluate its effectiveness in the semen sample with severe abnormal parameters.

Materials and Methods

Ethics approval

The study was conducted in an infertility unit of a tertiary level hospital between November 2013 and June 2014. Approval was obtained from the Institutional Review Board and trial was registered under the Clinical Trial Registry (CTRI/2015/01/005391).

The eligible men who were undergoing diagnostic semen analysis were enrolled in the study after obtaining written informed consent. The inclusion criteria were semen sample with oligoasthenozoospermia. We excluded normozoospermia, cryptozoospermia, and surgically retrieved sperms.

Randomization

Randomization was done using computer-generated sequence. Block randomization was done with half getting randomized into a block of four and the other half, into a block of two. Allocation concealment was done using sequentially numbered, opaque envelopes.

Two protocols for semen cryostorage were evaluated. One was conventional slow freeze (Group 1) and another one was vitrification (Group 2). The effectiveness of both these methods was tested using abnormal semen samples which were divided into two subgroups depending on the level of abnormality are as follows: severe oligoasthenozoospermia (SOA) (concentration 1–10 million, progressive motility 10%–30%, and vitality <78%) and VSOA (concentration <1 million, progressive motility <10%,). Each group (n = 20) had half of SOA (n = 10) samples and half were VSOA (VSOA, n = 10) [Figure 1].

Semen wash and assessment

The fresh semen sample was scored for progressive motility and vitality by phase-contrast microscopy. The vitality assessment was made using eosin-nigrosin staining. The semen sample was washed by the density gradient (40 and 80%; Sydney IVF, Cook, Australia) method and a soft pellet was obtained. The pellet was resuspended in 1 mL of buffer hydroxyethylpiperazine ethane sulfonic acid (HEPES) and scored again for progressive motility and vitality before cryopreservation. The progressive motility of spermatozoa was scored by preparing a wet preparation using a manual method.[17] A 10 μL drop of washed sperm preparation was covered

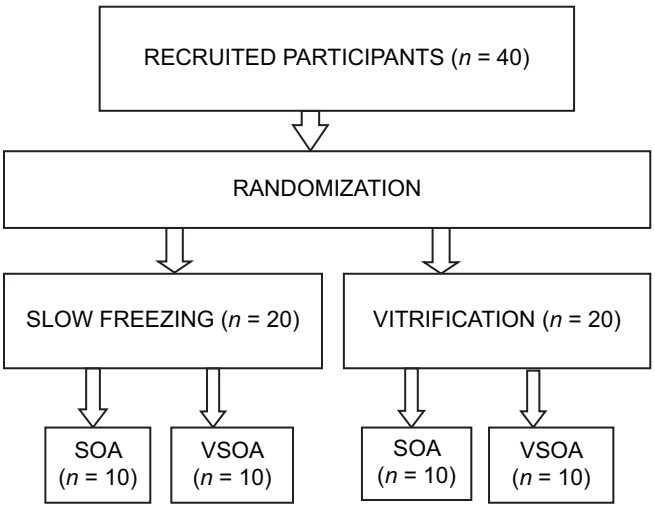

Figure 1: Flow chart. SOA = Severe oligoasthenozoospermia, VSOA = Very severe oligoasthenozoospermia

by a coverslip (22 mm × 22 mm) on a clean glass slide and kept in a humid chamber for 10 min. The slide was focused under phase-contrast microscope (Nikon Corporation, Tokyo, Japan) and only the forward progressive motile sperms were scored using blood cell counter (Fisher Scientific, USA) manually. The counting was done at room temperature by two experienced persons in an independent manner and about 100 spermatozoa were counted by each one. Average motility was calculated and noted for each sample. The inter-observer variation limit was set for <10% for each sample.[17] The maturity of spermatozoa was assessed using hyaluronan-binding assay (HBA) (Origio, Malov, Demark) for SOA samples the following wash in each group. The viability and HBA could not be carried out for VSOA sample since the wash recovery was poor and numbers of spermatozoa were insufficient to perform the test. After the wash process, the samples were cryopreserved randomly either by conventional slow freeze or vitrification methods. The samples were thawed/warmed at 1 and 6 months interval and assessed for motility and vitality. The HBA was done only for five samples in each group (SOA sample) instead of ten due to restricted fund availability.

Slow-freeze protocol

The sperm suspension of 0.5 mL volume was slowly mixed with equal volume of sperm freezing medium (Spermfreeze, Ferti Pro, Beernem, Belgium) at room temperature and loaded into 0.5 ml French straws (IMV Technologies, Cryo biosystem, Rambouillet, France). The straws were sealed with sealant powder and straws were kept at −85°C in a deep freezer (Nuaire, Inc., Plymouth, USA) for 1 h and then shifted to liquid nitrogen banks. The thawing process involved keeping the straws at room temperature for 10 min after removing it from the liquid nitrogen tank. The wick end of the straw was cut to release the stored sample into a sterile tube (6 ml) and warm buffer solution of HEPES (SAGE, Origio, Malov, Denmark) was added. A wet preparation was made to assess progressive motility and viability as done for the fresh sample. The HBA was performed only for SOAS group using Cell-VU slide (Origio, Malov, Denmark).

Vitrification protocol

Aseptic vitrification was done using a solid surface methodology (Cryologic PTY Ltd., Victoria, Australia) and a stripper was used as a carrier to load the sperm suspension. The sperm suspension (4 μl) was mixed with equal volume of 0.5 M sucrose (in house preparation) at 1:1 ratio in a sterile Petri dish (Falcon, BD Biosciences, New Jersey, USA) for a maximum of 2 min and then loaded onto a 275 μL stripper or denuding pipette (Denupet, Vitromed GmbH, Jene, Germany) using a stripper handle (Origio, Midatlantic, Malov, Denmark) [Figure 2]. A droplet was formed by bringing the loaded suspension in contact with a precooled metal block bathed in liquid nitrogen to form a glassy bead. The stripper with the bead was inserted into a french straw (0.25 ml) (IMV Technologies, Cryo biosystem, Rambouillet, France) and stripper handle is removed to facilitate loading of the stripper into the sleeve. A silicone seal (Narishige Co. Ltd., Tokyo, Japan) helped to secure the stripper onto the straw. The process of warming was done by keeping the stripper with the droplet in 40 μL of warm HEPES buffer solution (SAGE, Origio, Malov, Denmark) at 37°C. The sample was then assessed for progressive motility, viability, and hyaluronan-binding assay for SOA group and for progressive motility for VSOA group.

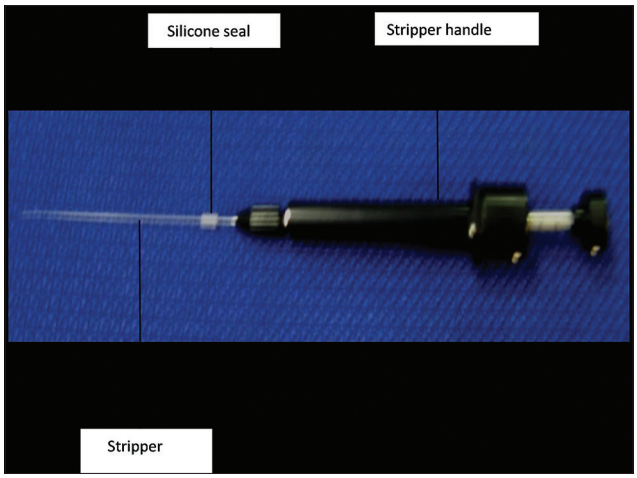

Figure 2: Denuding stripper handle with stripper

Statistical analysis

Sample size calculation was done using two means hypothesis based on the difference in progressive motility of semen sample following cryopreservation using the two-freezing protocols reported in a previous study.[18] With an alpha error of 5% and beta error at 80%, a total sample size of 40 participants (20 in each arm) was needed. Descriptive statistics were done using median along with range and the nonparametric test of the Mann–Whitney U test was used to compare the difference between the outcomes of the two interventions. The value of $P < 0.05$ was considered to be statistically significant. Statistical analysis was performed using SPSS version 20 software (IBM SPSS, IBM Corporation, Armonk, New York, USA).

Results

The baseline characteristics which included clinical variables and semen parameters were similar in both the groups [Table 1]. No significant difference

was noted between semen parameters such as fresh ejaculate volume, concentration, progressive motility, and morphology between the two groups. However, the postwash progressive motility was significantly higher in Group 2 for both subgroups.

The progressive motility of the cryopreserved semen sample following warming/thawing after 1 and 6 months duration, differed significantly between the two groups. In the SOA subgroup, postthawing/warming assessment revealed significantly higher progressive motility in the vitrification group at 1-month assessment (42% [22%–74%] vs. 7% [1%–13%]; $P = 0.015$). However, the difference in progressive motility was not statistically significant between the two groups at 6 months interval (27% [13%–62%] vs. 8% [0%–11%]; $P = 0.066$) as shown in Table 2.

In VSOA group, vitrification yielded significantly higher progressive motility at 1 month (14.5% [2%–32%] vs. 2.5% [0%–4%]; $P = 0.007$) and 6 months (12.5% [3%–32%] vs. 2% [1%–5%]; $P = 0.019$) compared to slow freeze [Table 2].

Postthawing/warming vitality testing was done only for SOA subgroup and significantly higher vitality scoring was observed in vitrification compared to slow freeze [Table 3]. In addition, the functional integrity as assessed by HBA was not significantly different in both the groups [Table 4].

Discussion

The current study found that the vitrification method involving the use of only nonpermeable cryoprotectants for cryopreservation of abnormal semen sample to be an effective alternative to the conventional slow-freeze technique. The postwarming/thawing assessment of the samples showed significantly better results in SOA and VSOA subgroups the following vitrification compared to slow-freeze method.

A previous study compared vitrification versus slow freeze for normozoospermia samples.[16] The investigators used permeable cryoprotectant-free vitrification technique and assessed samples for concentration, motility, and DNA fragmentation index. They found significantly higher motile sperms (18.17 ± 2.70% vs. 11.33 ± 2.70%; $P < 0.05$) following vitrification compared to conventional slow freeze. Sperm motility, which is partially dependent on mitochondrial function, is particularly affected by the slow-freeze method.[19] Earlier studies have demonstrated deleterious effects of slow freeze and thaw on postfreeze sperm motility and viability.[6,20] The damaging effect of slow freeze is mainly attributed to ice-crystal formation, osmotic shock, and toxicity of cryoprotectants.[20] The addition of antioxidants and cryopreserving the spermatozoa along with seminal plasma also has not been able to reduce

Table 1: Baseline patient characteristics and semen analysis parameters

Parameters	Median (minimum-maximum)		*P*
	Slow freezing	Vitrification	
Age	35 (25-44)	36 (25-49)	0.21
Abstinence (days)	4 (2-8)	4 (2-8)	0.76
Volume (ml)	2.5 (0.5-5)	2 (0.5-6)	0.45
Concentration (million/ml)	5.6 (1.1-9.8)	4.2 (1.9-8)	0.93
Progressive motility (%)	26.5 (10-29)	28 (25-29)	0.23
Morphology (%)	2 (1-4)	2 (1-4)	0.99

Table 2: Pre- and post-cryopreservation motility assessment (*n*=10)

Parameter	Median (minimum-maximum)		*P*
	Slow freezing	Vitrification	
SOA subgroup			
Prefreeze motility	43.5 (14-58)	55.55 (40-80)	0.0031
Postthaw/warming motility (1 month)	7 (1-13)	42 (22-74)	0.0151
Postthaw/warming motility (6 months)	8 (0-11)	27 (13-62)	0.0669
VSOA subgroup			
Prefreeze motility	15 (9-28)	21 (4-46)	0.003
Postthaw/warming motility (1 month)	2.5 (0-4)	14.5 (2-32)	0.007
Postthaw/warming motility (6 months)	2 (1-5)	12.5 (3-32)	0.019

SOA=Severe oligoasthenozoospermia, VSOA=Very severe oligoasthenozoospermia

Table 3: Postthawing/warming vitality testing severe oligoasthenozoospermia subgroup (*n*=10)

Semen parameter (SOA)	Median (minimum-maximum)		*P*
	Slow freezing	Vitrification	
Prefreeze vitality (%)	71.5 (64-78)	73 (60-81)	0.76
Postthaw/warming vitality (%) (1 month)	34.5 (27-42)	57 (45-78)	<0.001
Postthaw/warming vitality (%) (6 months)	20.5 (2-28)	42 (28-68)	<0.001

SOA=Severe oligoasthenozoospermia

Table 4: Hyaluronan-binding assay outcome for severe oligoasthenozoospermia subgroup (*n*=5)

Semen parameter (SOA)	Median (minimum-maximum)		*P*
	Slow freezing	Vitrification	
Prefreeze HBA (%)	44 (33-49)	46.79 (30-52)	0.17
Postthaw/warming HBA (%) (6 months)	27.5 (20-32)	33 (28-38)	0.91

SOA=Severe oligoasthenozoospermia, HBA=Hyaluronan-binding assay

the cryodamage.[21] It is suggested that vitrification without permeable cryoprotectants is associated with less cryodamage due to the lack of ice-crystal formation and osmotic shock.[22,23] The current study finding of comparable results the following vitrification compared to the slow-freeze technique are in broad agreement with the findings of the previous studies.[15,24]

In the study, only nonpermeable cryoprotectant was used in the vitrification protocol instead of traditional permeable cryoprotectants such as glycerol, ethylene glycol, or dimethylsulphoxide, which are considered toxic to highly sensitive spermatozoa. In contrast to earlier studies which employed cryotop and cryo-loop as carriers in open system for vitrification, the current study employed a novel stripper as a carrier.[13,14] In addition, a SSV method which is a semi-closed system was employed. An earlier study which also employed SSV for cryopreserving spermatozoa found it a quicker and more practical way compared to the conventional slow-freeze method.[15] The use of denuding pipette or stripper for sperm vitrification has been sparsely reported in the literature.[25] During the course of the current study, cryotolerance of the stripper was tested under extreme temperature in liquid nitrogen and it withstood the temperature stress, thereby validating its use as a carrier.

An indirect assessment of DNA integrity using HBA was employed in the study for SOA samples and comparable results were obtained in both the groups. This is an important finding since the cryopreserved sperms recovered following warming are likely to be used for intracytoplasmic sperm injection, and chromosomal integrity has prognostic implications.[26] These findings help in the process of validating the use of this novel method of vitrification for cryopreserving spermatozoa.

The current study remains the first-randomized controlled trial comparing SSV and conventional slow freeze for cryopreserving abnormal semen sample. It also used nonpermeable cryoprotectant and semi-closed system along with a stripper as a carrier. The small sample size of the study is an important limitation of the study. The postwash motility of the sample in vitrification group was significantly higher, thereby introducing a selection bias and influencing the final results. Further, the differences in motility outcome after vitrification at 1 and 6 months interval in cryopreservation duration could be due to the smaller number of included cases or samples which could lead to a possible Type II error. Since the study was not designed to test therapeutic samples, further validation is needed with clinical outcomes as end points before it can be employed routinely for cryopreservation. Future studies should employ larger sample size and should involve different centers to further validate the current study findings.

Conclusions

Cryopreservation of abnormal semen sample using permeable cryoprotectant-free SSV when compared to the conventional slow- freeze method gives comparable results. The toxic effect of permeable cryoprotectant can be avoided with this SSV protocol and does not require expensive programming equipments. While this method of SSV seems promising with advantages over conventional slow freeze, it still needs further validation before it can be used routinely for cryopreserving semen sample.

Financial support and sponsorship

This study is supported by Fluid Research Grant, Christian Medical College, Vellore, Tamil Nadu, India.

Conflicts of interest

There are no conflicts of interest.

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