Vitrification of Neat Semen Alters Sperm Parameters and DNA Integrity

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Purpose: Our aim was to evaluate the effect of neat semen vitrification on human sperm vital parameters and DNA integrity in men with normal and abnormal sperm parameters.

Materials and Methods: Semen samples were 17 normozoospermic samples and 17 specimens with abnormal sperm parameters. Semen analysis was performed according to World Health Organization (WHO) criteria. Then, the smear was provided from each sample and fixed for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Vitrification of neat semen was done by plunging cryoloops directly into liquid nitrogen and preserved for 7 days. The samples were warmed and re-evaluated for sperm parameters as well as DNA integrity. Besides, the correlation between sperm parameters and DNA fragmentation was assessed pre- and post vitrification.

Results: Cryopreserved spermatozoa showed significant decrease in sperm motility, viability and normal morphology after thawing in both normal and abnormal semen. Also, the rate of sperm DNA fragmentation was significantly higher after vitrification compared to fresh samples in normal (24.76 ± 5.03 and 16.41 ± 4.53, $P = .002$) and abnormal (34.29 ± 10.02 and 23.5 ± 8.31, $P < .0001$), respectively. There was negative correlation between sperm motility and sperm DNA integrity in both groups after vitrification.

Conclusion: Vitrification of neat ejaculates has negative impact on sperm parameters as well as DNA integrity, particularly among abnormal semen subjects. It is, therefore, recommend to process semen samples and vitrify the sperm pellets.

Keywords: vitrification; humans; DNA damage; cryopreservation; methods; infertility; spermatozoa; semen preservation..

INTRODUCTION

Cryopreservation of human spermatozoa is performed routinely in assisted reproductive technology (ART) program. Sperm bank is mainly developed for men that are undergoing chemotherapy/radiotherapy, ART treatment cycles, or have ejaculation abnormalities and azoospermia. It has been reported that sperm cryopreservation might have several impacts on sperm cell, such as excessive dehydration, damage to plasma membrane and acrosome cap, mitochondria injury, apoptosis and sperm DNA fragmentation.[1-3] There are currently three methods of cryopreservation namely: slow freezing, rapid freezing and vitrification. The first two techniques have been in practice for decades. However, they have some drawbacks, such as requiring expensive equipment, are time and labor consuming and have limited efficacy.[4]

Vitrification is the freezing method based on ultra-rapid cooling of water to glassy state at the high viscosity with no intracellular ice formation.[5] Vitrification of sperm freezing was first introduced by the Isachenko's group, in which the samples were directly and quickly plunged into the liquid nitrogen (LN).[6,7] Sperm vitrification is fast, simple and more cost effective compared to slow freezing. Also, vitrification can prevent sperm cryo-injuries.[6-9] While, it is shown that slow freezing and thawing is associated with sperm DNA damage and apoptosis in human ejaculated spermatozoa, little is known about the effect of vitrification on induction of human sperm DNA fragmentation. Cryopreservation of raw or prepared semen has remained a matter of debate in the literature.[2] Nawroth and colleagues reported that recovery rate of motile spermatozoa as well as normal morphology after vitrification was higher in native spermatozoa in comparison to cryoprotectant used ones.[6] They also found that sperm recovery rate and normal morphology will be higher after vitrification in prepared spermatozoa compared to native group.[6] Recently, Satirapod and colleagues showed that the rate of DNA fragmentation will be reduced in cryopreserved raw semen with solid surface vitrification compared to standard freezing method.[10]

There are several techniques in order to determine sperm DNA fragmentation.[11-13] Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is a reliable technique to evaluate double strand DNA fragmentation.[14] The main goal of this study was to evaluate the effect of vitrification of neat semen samples in both normal and abnormal semen groups on the sperm parameters and DNA status using TUNEL assay.

MATERIALS AND METHODS

Sampling and Spermatozoa Evaluation

Ejaculates were obtained from men aged between 30-50 years old (17 normal and 17 abnormal semen samples) by masturbation after 48-hour of sexual abstinence. In normal semen group, the inclusion criteria was infertility due to female factor and in the infertile men the couples were infertile due to male factor. After liquefaction, semen analysis was performed according to World Health Organization (WHO) guidelines.[15] Sperm count and motility were assessed using Neubauer chamber under the light microscope ($\times$ 400). Motility types were categorized into: progressive, non-progressive, and immotile. The sperm viability was assessed using eosin-nigrosin staining protocol. The dead spermatozoa were stained red, while the live ones were unstained (Figure 1). Also, sperm morphology was evaluated by Papanicolaou staining procedure. At least, 200 spermatozoa were checked under light microscope for head, neck and tail abnormalities.

Vitrification and Warming

Vitrification method was according to previous reports with some modifications.[16] The semen was loaded on copper cryoloops of 2.5 mm diameter by dipping the loops in suspension to obtain a thin film of 8 $\pm$ 2 μL and the loaded loops were plunged in the LN. After storage for 7 days, the samples were warmed by plunging the loops into a tube containing 2.5 mL Ham's F10 at 37ºC. After warming of 10 loops in one tube, the tube was placed in a CO2 incubator for 5-10 min. Then, the spermatozoa were centrifuged at 300g for 10 min and the resultant pellet was resuspended in 100 μL of Ham's F10 and processed for further evaluation.

TUNEL Staining

In Situ Cell Death Detection Kit (Roche Diagnostics GmbH. Roche Applied Science. 68298 Mannheim, Germany) was applied for TUNEL assay. After providing the smear, the slides were fixed in 100% methanol solution for 4 min at room temperature. Blocking was performed by putting the slides in 3% H_2O_2 in methanol for 10 min in darkness. Before and after blocking, the slides were washed with phosphate buffered saline (PBS). For sperm permeabilization 0.1% Triton X-100 in 0.1% sodium citrate buffer was used (10 min

Table 1. Sperm parameters before and after vitrification in normozoospermic samples.*

Sperm Parameters	Before Vitrification	After Vitrification	P
Count (×10⁶/mL)	120.70 ± 68.14	89.00 ± 9.30	.134
Progressive motility (%)	56.11 ± 10.45	5.29 ± 5.05	.000
Total motility (%)	67.58 ± 10.01	8.64 ± 6.81	.000
Normal morphology (%)	46.05 ±10.46	37.00 ± 11.72	.024
Viability (%)	78.47 ± 9.38	11.05 ± 7.30	.000
TUNEL positive cells	16.41 ± 4.53	24.76 ± 5.03	.002

Key: TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
*Data are shown as mean ± SD.

on ice). The slides were incubated with TUNEL reaction mixture 1-hour with high humidity at 37°C. After washing with PBS, the slides were incubated with convertor-probe followed by incubation with 3,3'-Diaminobenzidine (DAB) (DAB, Roche, Mannheim, Germany) solution. Two hundred sperm cells were analyzed under the light microscope at × 1000. Abnormal spermatozoa had dark brown nuclear (Figure 2). For positive controls 0.1 IU DNase (Hoffmann-La Roche Diagnostics, Mannheim, Germany) was applied for 15 min at 37°C and the reaction mixture had no terminal deoxynucleotidyl transferase (TdT) for negative controls.

Statistical Analysis

The data are shown as mean ± SD. Sperm parameters before and after vitrification was analyzed using paired t test. Linear Pearson correlation test was applied to find out the correlation between the apoptosis and sperm parameters. The level of statistical significance was set at $P < .05$.

RESULTS

The sperm cell count were 120.70 ± 68.14 and 15.15 ± 2.58 (10⁶/mL) in normal and abnormal semen groups, respectively. Regarding sperm motility, 56.11 ± 10.45 and 28.11 ± 8.15 were progressive motility and 67.58 ± 10.01 and 35.58 ± 11.94 were total motility, respectively. Sperm viability were 78.47 ± 9.38 and 50.58 ± 15.15 and sperm morphology were 46.05 ± 10.46 and 12.52 ± 13.87, respectively.

The data showed that sperm vitrification caused significant decrease in sperm motility, viability and morphology in normozoospermic samples (Table 1). Also, vitrification was involved with significant increase in sperm DNA fragmentation which was about 8% in abnormal semen group. In addition, there was significant reduction for all sperm parameters after
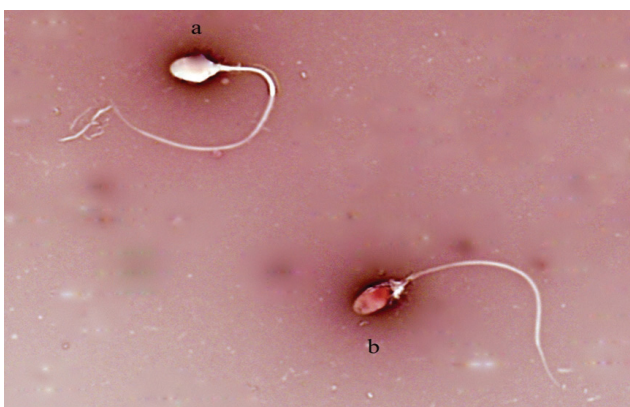

Figure 1. Evaluation of human sperm viability using eosin-nigrosin staining; (a) unstained (white) alive spermatozoa, (b) stained (red) dead spermatozoa.
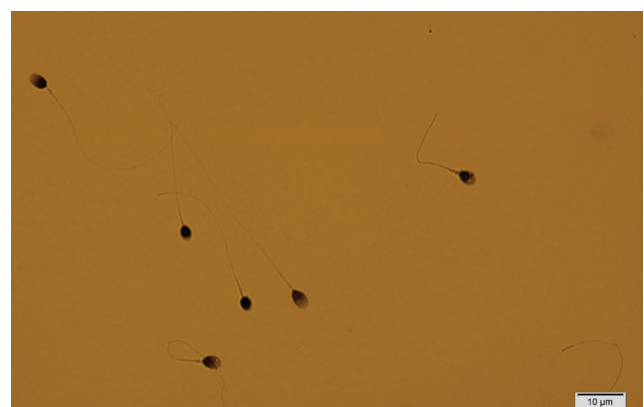

Figure 2. Evaluating the sperm DNA fragmentation using TUNEL test. Dark brown cells are abnormal spermatozoa.
Key: TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Table 2. Sperm parameters before and after vitrification in abnormal semen.*

Sperm Parameters	Before Vitrification	After Vitrification	P
Count (×10⁶/mL)	19.15 ± 2.58	17.82 ± 2.87	.806
Progressive motility (%)	42.11 ± 16.15	3.70 ± 2.35	.000
Total motility (%)	55.58 ± 18.94	7.88 ± 3.34	.000
Normal morphology (%)	18.52 ± 13.87	11.52 ± 9.57	.018
Viability (%)	60.58 ± 19.15	8.64 ± 3.66	.000
TUNEL positive cells	23.50 ± 8.31	34.29 ± 10.02	.000

Key: TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.
*Data are shown as mean ± SD.

vitrification in abnormal semen group (Table 2). DNA fragmentation was 11% higher in semen with abnormal sperm parameters compared to baseline. There was negative correlation between sperm DNA fragmentation and viability in normozoospermic samples after vitrification ($r = -0.6$, $P = .004$). The negative correlation was only observed between sperm DNA fragmentation and progressive motility after vitrification in normozoospermic men (Table 3). No significant correlation was found between abnormal sperm DNA, viability and morphology after vitrification in semen with abnormal sperm parameters (Table 4).

DISCUSSION

The data showed a significant decrease in sperm parameters as well as significant increase in sperm DNA fragmentation after vitrification in both groups of normal and abnormal semen samples. Commonly, cryopreservation has negative impact on sperm motility and viability. Our data were similar to others that cryopreservation caused decrease in sperm motility and viability.[8,10] Satirapod and colleagues investigated the efficacy of new vitrification method on normozoospermic samples. They evaluated the role of raw semen solid surface vitrification in comparison to rapid freezing method on sperm parameters. Their data showed that sperm motility, viability, morphology and DNA integrity were noticeably reduced after

vitrification.[10] In comparison to our study, their sperm recovery rate as well as DNA damage was higher. One probable cause would be the method of sperm vitrification. Also they used commercial cryoprotectant, while we used cryoprotectant free method. Nawroth and colleagues also reported reduced sperm parameters after neat semen vitrification of normal donors. The data showed that sperm recovery would be much higher after swim up compared to native spermatozoa.[6] Formation of lethal intracellular ice crystal sand osmotic stress may be the main cause for reduction in sperm cell motility and viability during cryopreservation.[17] Isachenko and colleagues compared sperm motility after four different cryoprotectant-free vitrification techniques and showed that cryoloop method resulted in a lower sperm motility compared to droplets, open pool straws and open straws.[18] Our data also showed that vitrification impairs sperm normal morphology in normal and abnormal semen groups. The findings were similar to other reports in terms of negative effects of cryopreservation on normal sperm morphology.[1,10] It appears that the most probable reason for the effect of freezing on sperm morphology is the formation of ice crystals outside the sperm cell which can alter sperm architecture.[1]

Generally, it is believed that normozoospermic semen samples may be more resistant to cryo-injury compared to abnormal oligozoospermic or asthenozoospermic specimens.

Table 3. Correlation between sperm DNA integrity and sperm parameters in normal semen before and after vitrification.

Variables		Progressive Motility	Morphology	Viability	Count
TUNEL positive spermatozoa (%)	Before vitrification	$r = -0.23$ $P = .35$	$r = -0.85$ $P = .000$	$r = -0.03$ $P = .89$	$r = 0.15$ $P = .54$
	After vitrification	$r = -0.49$ $P = .04$	$r = 0.45$ $P = .06$	$r = -0.6$ $P = .004$	$r = -0.53$ $P = .02$

Key: TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Table 4. Correlation between apoptosis and sperm parameters in abnormal semen.

Variables		Progressive Motility	Morphology	Viability	Count
TUNEL positive spermatozoa (%)	Before vitrification	$r = -0.73$	$r = 0.15$	$r = -0.67$	$r = 0.18$
		$P = .001$	$P = .54$	$P = .003$	$P = .48$
	After vitrification	$r = -0.6$	$r = -0.37$	$r = -0.32$	$r = -0.12$
		$P = .01$	$P = .13$	$P = .2$	$P = .62$

Key: TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Our results showed that sperm parameters and DNA fragmentation decreased at the same manner in both groups of normal and abnormal semen groups, which is in conflict with some reports. Donnelly and colleagues found that spermatozoa from infertile men would be less resistant to cryo-injuries compared with spermatozoa of fertile men.[19] One probable causes of this discrepancy may be method of cryopreservation. They used rapid freezing method (freezing in LN vapor), while our cryopreservation method was typical vitrification. Also, we used no cryoprotectant in the freezing method. Permeable cryoprotectant is used in slow freezing in order to reduce cell shrinkage during cryopreservation. Using permeable and non-permeable cryoprotectants in sperm cryopreservation not only may have no beneficial effects, but also can induce damage even at room temperature.[16] It has been reported that cryoprotectants have some cell toxicity such as osmotic damage and chemical toxicity.[20] Regarding probable effect of cryoprotectants on sperm DNA, it is shown that presence of cryoprotectants has no negative impact on sperm DNA integrity.[16] Our data showed that vitrification can significantly increase sperm DNA fragmentation in both normal and abnormal semen groups. The results were similar to the Brazilian group. They found cryopreservation induced DNA fragmentation in both oligozoospermic and normozoospermic samples.[21] and colleagues also showed that 84.66% of spermatozoa show undamaged DNA following vitrification/warming in swim-up prepared normal specimens.[22] The effect of cryopreservation on sperm DNA status has remained controversial. Some investigators believe that cryopreservation has no negative effect on sperm DNA status.[16,23] While, others have shown that the cryopreservation is associated with negative effect on sperm DNA integrity and chromatin stability.[2,24] Oxidative stress may be one of the important causes of increasing sperm DNA fragmentation.[25] Cryopreservation may change the fluidity of sperm mitochondrial membrane and consequently increase the potential of mitochondrial membrane, finally reactive oxygen species (ROS)

will be produced and released.[2] We verified that semen samples and non-sperm cells in seminal fluid are potential sources of ROS production. Also, it is shown that presence of seminal leukocytes is associated with more ROS production during cooling to 4°C.[26] The thawing seems to have more important role in induction of DNA damage in sperm cells. It was reported that the highest degree of sperm DNA fragmentation will be occurred during the first 4-hour of incubation after thawing in fertile donors.[27]

Another finding was the negative correlation between sperm progressive motility and DNA fragmentation after vitrification in normozoospermic men. It was shown that there is a negative relationship between sperm motility, vitality or concentration and sperm DNA damage.[28,29] But, there was no significant correlation between sperm morphology and DNA integrity, which was similar to other findings.[19] It seems that the sperm morphological feature is not representative of sperm DNA quality. Cryopreservation of raw or prepared semen has remained matter of debate in the literature.[2] It is believed that seminal plasma contains natural antioxidants which can protect spermatozoa from cyro-injuries during cryopreservation and these seminal plasma antioxidants will be eliminated with sperm preparation methods. Neat semen cryopreservation would be rapid and cost-effective as well. In this study we cryopreserved normal and abnormal raw semen. Maybe, ROS production by non-sperm cells in seminal plasma is higher than seminal plasma antioxidants capacity, especially in sub normal specimens.

CONCLUSION

Vitrification of human neat semen can impair vital sperm parameters of motility, viability, morphology as well as DNA integrity. It might be better to vitrify the processed semen, especially for cases with male factor infertility.

CONFLICT OF INTEREST

None declared.

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Editorial comment on: Vitrification of Neat Semen Alters Sperm Parameters and DNA Integrity

Vitrification has brought about important changes in cryopreservation and human fertility preservation. Easiness and speed and no need for costly freezing technologies are reasons for its rapid development. Vitrification is the solidification of a liquid without crystallization. As cooling continues, however, the molecular waves in the liquid permeating the tissue decline. Finally, an "arrested liquid" state known as a glass is attained. Vitrification has been demonstrated to afford higher preservation for a number of cells, including monocytes, ova and early embryos and pancreatic islets.[1]

There are a number of major contests for performing of vitrification for tissue engineered medical products. Without adhering to these standards, certainly the process of vitrification will fail. The first one is vitreous state. There is no explanation about vitreous state in this study. Stability of the vitreous state is critical for the maintenance of vitrified tissue integrity and viability. In present study the method of vitrification has not been explained in details and it seems most of standards for vitrification have not been considered. Vitrification methods to preservation have some of the limitations associated with conventional freezing methods.[2] First, both methods entail low temperature storage and transportation conditions. Neither can be stored above their glass transition temperature for long without significant risk of product damage due to inherent instabilities resulting to ice formation and growth. Both methods use cryoprotectants with their associated problems and necessitate experienced technical support during rewarming and cryoprotectant elution phases. The very high concentrations of cryoprotectants needed to facilitate vitrification are potentially toxic since the cells may be exposed to these high concentrations at higher temperatures than in freezing methods of cryopreservation. Cryoprotectants can kill cells by direct chemical toxicity, or indirectly by osmotically-induced stresses during suboptimal addition or removal.[3] Upon complete achievement of warming, the cells should not be exposed to temperatures above 0ºC for more than a few minutes before the glass-forming cryoprotectants are removed. It is possible to employ vitrified products in highly controlled environments, such as a commercial manufacturing facility or an operating theater, but not in an outpatient office. There isn't any data about above mentioned points in this study.[4] Another issue is heat transfer. Heat transfer issues are the primary problem for scaling up the successes in somewhat small tissue specimens to larger tissues and organs. The limits of heat and mass transfer in bulky systems result in non-uniform cooling and leads to stresses that might begin cracking. In fact, the higher cooling rates that facilitate vitrification will typically lead to higher mechanical stresses.[5] In present study there is no information on the used material properties of vitreous aqueous solutions. Material properties such as thermal conductivity and fracture strength of vitreous aqueous solutions have many connections with their inorganic analogues that happen at normal temperatures. Any material that is unrestricted will undergo a change in size (thermal strain) when subjected to a change in temperature. Additional important issue that has not been addressed, is the stresses that arise to billet the differential shrinkage. Thermal stress can definitely reach the produced strength of the frozen tissue resulting in plastic deformations or fractures.[6] One more major obstacle for performing

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of vitrification is the technique used for warming. This issue also has been ignored in present study. The warming technique should be highly effective to prevent devitrification and ice growth by recrystallization.

The rational for vitrification of neat semen has not been mentioned. What are the advantages of vitrification of semen instead of sperm? Is there any scientific background for this procedure? For vitrification, it is recommended that, even the plasma of sperm should be removed. For vitrification the sperm plasma is removed, it means that by using this technique many infecting agents such as HIV, hepatitis and other viruses will be removed from the sperm, and therefore these infectious microorganism cannot be transmitted via sperm. Hence HIV+ men will have the chance to father children without the risk of passing infectious organisms to baby and mother. After separation of plasma from the sperm, the vitrified sperm should be stored in an ultra-cold deep freeze at -86ºC environment. This method has several advantages compared to other methods, first the motility of rethawed sperm increases significantly (75% using this method vs. 31% using conventional methods) second a higher number of viable sperm can be achieved and this can result in higher chance of fertilization in ARTs, such as IVF and ICSI.[7] However, two decades past the first live-birth from vitrified embryos, there are still some uncertainties on the safety of these techniques and its possible toxic effects on the health of children born from vitrified embryos or oocytes. There is fear that use of high concentrations of cryoprotectants may result in genetic or epigenetic abnormalities with ensuing inborn malformations. Therefore, there is no agreement or scientific recommendations for the replacement of slow freezing method with vitrification universally.

The techniques for performing vitrification are evolving. Recently vitrification of metaphase II oocytes has been described to hold ability for oocyte preservation, which can be vital in countries where a limited number of oocytes can be inseminated and embryo cryopreservation is illegal, as well as in oocyte donation and fertility preservation prior to cancer treatment.[8]

The two most commonly used tests to determine sperm DNA damage are the TUNEL assay and the sperm chromatin structure assay (SCSA).[9] the TUNEL assay has never been adjusted for use with human spermatozoa and lower normal threshold values have not been obviously recognized. DNA testing by SCSA has been widely standardized. TUNEL test has not been standardized to the same level as SCSA. TUNEL assay cannot selectively differentiate clinically significant DNA fragmentation from clinically insignificant fragmentation. The assay also cannot differentiate normal DNA grooves from pathologic grooves. Moreover, the TUNEL test does not give any information concerning the particular genes that may be affected by DNA fragmentation. This assay can only determine the amount of DNA fragmentation that ensues, with the hypothesis that higher levels of DNA fragmentation are pathologic. [10] Nowadays, the only reliable test to determine sperm DNA fragmentation is SCSA. This test has validated clinical reference range and criteria to interpret the yielded results precisely. Using the SCSA test one can test 5,000 individual sperm with a high-precision flow cytometer. To interpret the results of SCSA test DNA fragmentation index (DFI) is used, which represents the population of cells with DNA damage.[11,12]

Finally a major limitation of present study is absence of pictures both from TUNEL results and vitrified sperms.

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