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Cryopreservation of human spermatozoa by vitrification versus conventional rapid freezing: Effects on motility, viability, morphology and cellular defects☆

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ABSTRACT

Objectives: Sperm cryopreservation has great potential for male infertility treatment as used in assisted reproduction technology (ART). There are a variety of cryopreservation methods in order to preserve sperm in a long term. Although conventional freezing and vitrification now are used widely, they have damage on sperm parameters as well as sperm DNA integrity. It is necessary to answer which method is better and appropriate for sperm cryopreservation. The aim of this study was to compare the effect of conventional freezing and vitrification regarding to motility, vitality and morphology of sperm found in washed and unwashed samples.

Study design: One hundred and five human fresh semen samples were divided into washed and unwashed halves using density-gradient centrifugation. Each group then was split into two aliquots: one cryopreserved by conventional freezing and the other by vitrification, using SpermFreeze Solution™ (Vitrolife, Västra Frölunda, Sweden) containing glycerol as a cryoprotectant. The sperm parameters were analyzed and compared between six groups: washed fresh (FW), unwashed fresh (FU), washed conventional freezing (CfW), unwashed conventional freezing (CfU), washed vitrification (VitW) and unwashed vitrification (VitU) samples.

Results: Sperm progressive motility, vitality and normal morphology significantly decreased, together with an appreciable increase in sperm head, midpiece and tail defects when comparing to the fresh sperm parameters after thawing in all groups. In conventional freezing method groups, progressive motility and vitality were substantially higher than that in vitrification method groups. However, vitrification gave better results in normal morphology rates. Additionally, sperm head, midpiece and tail defects were significant lower in two vitrification groups in comparison with conventional freezing groups. Interestingly, washed groups had better sperm parameters than unwashed groups so that washing process before frozen seemed to improve sperm parameters.

Conclusion: Conventional freezing method resulted in better motility, viability in both washed/unwashed groups. On the contrary, spermatozoa undergoing vitrification were healthier regarding morphology with less defects than conventional freezing. Sperm washing before frozen was a beneficial preparation to sperm cryopreservation.

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Introduction

Sperm cryopreservation has great values for male infertility treatment as used in assisted reproduction technology (ART) [1]. The goal of sperm cryopreservation is to preserve sperm viability, motility, fertilizing ability and maintain original pre-freezing structural integrity.

Currently, there are several effective cryopreservation methods such as conventional freezing with or without programmable machine and vitrification that have been introduced for storage of male gametes. Slow freezing is the traditional technique, successfully employed for the cryopreservation of human sperm whereas vitrification, a fast alternative method of rapid freezing in nitrogen vapor, would provide significant benefits with regard to simple equipment and applicability to fertility centers [2]. However, sperm cryopreservation causes damage to plasma membrane, elevated production of ROS, loss of mitochondrial functionality, DNA fragmentation and chromatin decondensation [3]. Developed during the early 1970s, cryopreservation by slow freezing method has been found in recent research to induce the increase of apoptosis, sperm DNA damage and loss of the mitochondrial membrane potential [4,5].

Conventional freezing method does not require automatic equipment. The sample comes into direct contact with nitrogen vapors at −80 °C and then has been immersed in liquid nitrogen at −196 °C. This technique has such major drawbacks as being unable to control the temperature drop curve [6]. Tongdee and colleagues revealed that cryopreservation of human sperm by rapid freezing or slow programmable freezing resulted in a significant decrease in sperm motility, morphology and DNA integrity [7].

Vitrification is a simple and cost-effective method of freezing spermatozoa at an extremely high rate by directly plunging samples into liquid nitrogen. As a result, solidification of living cells has no intracellular ice crystallization [8,9]. Aizpurua et al. reported that the sperm vitrification method allows superior results on motility, vitality, DNA structure, acrosome, morphology and cytoskeleton compared with slow freezing method [10]. Slabbert et al. showed no statistically significant differences in the total motility or velocity parameters of post-thawing spermatozoa while significantly higher mitochondrial membrane potential and lower percentages of DNA fragmentation in spermatozoa cryopreserved by vitrification compared with vapor phases of liquid nitrogen cryopreservation method [9]

Conventional freezing in nitrogen vapor phase and vitrification in liquid nitrogen have been introduced to preserve human sperm. Darvishnia et al. compared the effects of two cryopreservation methods on semen samples from 31 men. The results showed that conventional freezing method was better than vitrification method in preserving sperm progressive motility [11]. However, other studies did not find significant differences between two cryopreservation methods in motility, viability, mitochondrial membrane potential, or percentages of normal sperm morphology [12–14]. Recently, some researchers demonstrated that the sperm vitrification protocol allowed superior results on sperm parameters such as motility, viability, DNA structure, acrosome, morphology compared with conventional freezing protocol [9,10,2]

Literaturelly, there are still controversies on which method is better and safer to choose in sperm cryopreservation. Therefore, it is really meaningful to make a comparison between the efficacies the two methods with respect to sperm motility, morphology and viability, especially in case of washed and unwashed human spermatozoa.

Material and methods

Population

In this study, a total of 105 semen samples were randomly collected from the male partners of couples seeking diagnosis of fertility at Hue Center for Reproductive Endocrinology and Infertility (HUECREI), Hue University of Medicine and Pharmacy, Vietnam, between October 2016 and April 2017.

Semen collection and assessment

Semen samples from patients were collected randomly by masturbation and ejaculation after abstinence for 2–7 days, exclusive of cryptozoospermia and azoospermia. A diagram of the experimental design is shown in Fig. 1. Samples were split into two groups of unwashed sperm and sperm washed by density-gradient centrifugation. Then each group was split into two aliquots: one cryopreserved by vitrification and the other by conventional freezing, using SpermFreeze Solution™ (Vitrolife, Västra Frölunda, Sweden). Semen analysis was done according to the 2010 WHO guidelines for examination and processing of human semen [15]. Comparisons of the sperm parameters were made between six study groups: fresh unwashed (FU), fresh washed (FW), conventional freezing unwashed (CfU), vitrification unwashed (VitU), conventional freezing washed (CfW), and vitrification washed (VitW).

Semen analysis

a. Sperm motility: The sperm motility parameter was analyzed by manual counting method under phase-contrast microscopy Primo Star (Zeiss, Jena, Germany) at 400 total magnification. The sperm motility is of two categories: progressive motility (PR) and nonprogressive motility (NPR). The PR sperm was moving actively in the way of linear or in a large circle. The NPR sperm performed moving in small circle, clear flagellar movement but without a change in position or immotile. In this study, the progressive motility of 200 sperms was assessed.

b. Sperm vitality: The vitality parameter was assessed by the eosin technique under light microscopy Primo Star (Zeiss, Jena, Germany) at 400 total magnification as recommended by the WHO. Death sperm was stained or partially stained red because of dye exclusion while alive sperm was white. Two hundred cells were counted immediately following liquefaction of the semen samples and the percentage of viable cells was calculated.

c. Sperm morphology: This parameter was estimated by Giemsa staining. Morphology of sperm head shape and size, acrosomal region, sperm neck, midpiece, tail and cytoplasmic droplets was determined under microscopy Primo Star (Zeiss, Jena, Germany) at 1000 total magnification, according to the 5th edition of the WHO guideline. At least 200 sperms were counted to calculate the percentage of both normal and abnormal morphology.

Semen preparation and cryopreservations

Semen was divided into washed and unwashed halves, each then taken to be frozen by means of vitrification or conventional freezing. The washing procedure was as follows: semen was layered on top of 45%/90% discontinous gradients Sil-Select Plus™ (REF: SIP050LO/SIP050UP, Fertipro, Beernem, Belgium)and then centrifuged at 1500

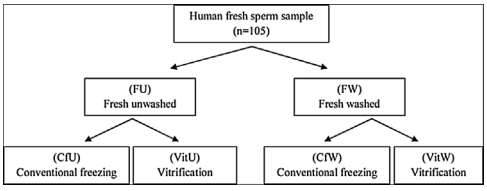

Fig. 1. Diagrammatic representation of the experimental design. Comprehensive sperm analysis was performed in all six study groups: Fresh unwashed (FU), Fresh washed (FW), Conventional freezing unwashed (CfU), Vitrification unwashed (VitU), Conventional freezing washed (CfW), Vitrification washed (VitW).

rounds per minute (RPM) for 10 min. The pellet was resuspended in Ferticult™ Flushing (REF: FLUSH050, Fertipro, Beernem, Belgium) and centrifuged at 1500RPM for 10 min. After washing, the samples were suspended in 0.5 ml Ferticult™ Flushing.

Both two cryopreservation methods performed in this study used SpermFreeze Solution™ (REF: 10137, Vitrolife, Västra Frölunda, Sweden). This commercial medium was bicarbonate and MOPS buffered medium containing glycerol which played a permeating cryoprotectant role. It was used widely for human sperm cryopreservation to protect against thermal shock. In addition, SpermFreeze Solution™ was egg york free in order to avoid potential risks of viral contamination. To supply cholesterol which improve sperm survival rate due to membrane stabilizing function, the product contained free fatty acids.

a. Vitrification: The semen samples were diluted at a ratio of 1:1 with the SpermFreeze Solution™ (Vitrolife, Västra Frölunda, Sweden) at room temperature in 10 min for equilibration and then sperm diluted was dropped directly into the liquid nitrogen by a micropipette (30 μl/drop). The solid mixture was transferred into Nunc cryotube vial 1.8 ml (Catalog number: 375418, Thermo Fisher Scientific, Jiangsu, China) using forcep embedded into liquid nitrogen and stored in tank for at least 24 h [13].

b. Conventional freezing: The freezing procedure was done according to SpermFreeze Solution™ guideline. SpermFreeze Solution™ was added slowly and dropwise to the sample in Nunc cryotube vial 1,8 ml at a ratio of 1:1 and carefully and gently mixed after each drop added. The process was 10 min at room temperature. Then the cryotube was placed horizontally at 3 cm above the liquid nitrogen surface. During cooling in vapors status, the cryotube was prefer to place in horizontal position to minimize the heat difference between the two ends and increase contact surface with nitrogen vapors. Inside nitrogen vapor, there is a thermal gradient, approximately 20 °C/min (Paoli et al., 2014). The freezing temperatures was estimated approximately from -70, -80 and −90 °C [6]. After 15 min, it was submerged into liquid nitrogen and stored at −196 °C in tank for at least 24 h.

Thawing of samples

Thawing was performed in a modified way as described in SpermFreeze Solution™ guideline (Vitrolife, Västra Frölunda, Sweden). It is an equally step at 37 °C to avoid abrupt thermal changes [6] and the thawing procedure must be fast to avoid the formation of larger ice crystals (Paoli et al., 2014). Therefore, the cryotube was taken out of liquid nitrogen and submerged in warm water (37 °C) for 5 min. After using clean paper towel to wipe cryotubes dry, post-thawed sperm parameters (motility, concentration, vitality and abnormal morphology) were analyzed.

Statistical analysis

All analyses were performed using the Statistical Product and Service Solutions (SPSS) version 2.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as means and standard deviations (SD) or absolute numbers and percentages. The *t*-test was used to compare the means of normally distributed continuous variables. Wilcoxon non-parametric test was used to compare the means of non-normally distributed variables. A statistical value of $p < 0.05$ was considered significant.

Results

Characteristics of study population

One hundred and five specimens included in this study were from patients attending HUECREI. The characteristics of study population were shown in Table 1. The mean age was 33.28 ± 6.15 years, with a range from 23 to 54 years old. Most of them had primary infertility (65.7%). Their mean duration of infertility was 2.58 ± 2.11 years, with a range from 1 to 11 years. 34.3% of the subjects had over 3 years of infertility duration, and men with under 3 years of infertility accounted for 65.70%. Most of the patients had no history of mumps virus infections (89.5%) and normal ejaculation (91.40%), normal erectile function (90.50%). It is found that there were no significant differences on sperm progressive motility, viability and normal morphology rates between groups regarding to basic characteristics.

Effects of cryopreservation by vitrification and conventional freezing on sperm motility

The value of sperm motility parameters before and after cryopreservation was shown in Table 2 and Fig. 2. The washing process increased sperm motility parameter before cryopreservation. Sperm PR motility in the fresh washed group (FW) was significantly higher than that in the fresh unwashed group (FU) (29.49 ± 9.68 and 25.75 ± 9.01, respectively). After the frozen-thawed process, sperm PR motility significantly decreased compared with fresh groups. Cryopreservation by conventional freezing showed sperm motility parameters (14.76 ± 6.16 in case of unwashed and 14.91 ± 6.25 in case of washed), which were significantly higher than those of vitrification groups (8.67 ± 5.31 in case of unwashed and 9.78 ± 5.33 in case of washed). In vitrification groups, sperm motility parameters in washing sample were significantly higher than those of unwashed samples. However, there is no significant difference between washed and unwashed in conventional freezing groups ($P = 0.57$).

Effects of cryopreservation by vitrification and conventional freezing on sperm viability

The process of conventional freezing and vitrification had marked effects on the sperm viability as shown in Table 2 and Fig. 3. In fresh groups, sperm viability in the washed group (FW) was significantly higher than that of the unwashed group (FU) (86.24 ± 5.88 and 81.90 ± 5.90, respectively). After cryopreservation by conventional freezing and vitrification, sperm viability significantly decreased compared with fresh groups. Sperm viability in vitrification groups (38.99 ± 8.62 in case of unwashed and 41.35 ± 9.10 in case of washed) were significantly lower than conventional freezing groups (53.10 ± 9.65 in case of unwashed and 55.46 ± 8.00 in case of washed). Sperm washing process significantly increased viability after cryopreservation by either conventional freezing or vitrification.

Effects of cryopreservation by vitrification and conventional freezing on sperm normal morphology and sperm defects

Effects of cryopreservation on normal morphology of washed and unwashed sperm were shown in Table 2 and Fig. 4. The washing process significantly increased the average percentage of normal morphology spermatozoa in fresh samples (3.13 ± 1.61 in case of unwashed and 3.66 ± 1.51 in case of washed). Percentage of sperm having normal morphology in all cryopreservation groups was significantly reduced in comparison with fresh groups. Unlike motility and viability parameters, the average percentage of normal morphology in vitrification groups (1.95 ± 1.08 in case of unwashed and 1.87 ± 1.09 in case of washed) was significantly higher than conventional freezing groups (1.36 ± 1.02 in case of unwashed and 1.43 ± 0.94 in case of washed). There was no significant difference between washed and unwashed in

Table 1
Basic characteristics of study population (n = 105).

Characteristic	Total		Progressive motility (%)	Viability (%)	Normal morphology (%)
	n	%			
Total	105	100	25.07 ± 9.01	81.90 ± 5.90	3.13 ± 1.61
Age					
≥ 35 years	37	35.2	24.45 ± 7.76	81.59 ± 4.96	3.27 ± 1.45
< 35 years	68	64.8	25.40 ± 9.66	82.06 ± 6.39	3.06 ± 1.70
Mean ± SD	33.28 ± 6.15				
P value			0.61	0.70	0.52
Infertility type					
Primary	69	65.7	25.45 ± 8.98	82.25 ± 6.14	3.20 ± 1.75
Secondary	36	34.3	24.33 ± 9.16	81.22 ± 5.44	3.00 ± 1.31
P value			0.55	0.40	0.54
Infertility duration (years)					
≥ 3 years	36	34.3	27.33 ± 9.58	81.33 ± 5.65	3.33 ± 1.62
< 3 years	69	65.7	23.88 ± 8.53	82.19 ± 6.05	3.03 ± 1.61
Mean ± SD	2.58 ± 2.11				
P value			0.06	0.48	0.36
History of mumps					
Yes	11	10.5	28.00 ± 12.95	81.18 ± 7.35	2.45 ± 0.93
No	94	89.5	24.72 ± 8.46	81.98 ± 5.75	3.21 ± 1.66
P value			0.26	0.67	0.14
Erectile dysfunction					
Yes	10	9.5	26.90 ± 7.17	83.50 ± 3.34	3.20 ± 0.79
No	95	90.5	24.87 ± 9.19	81.73 ± 6.10	3.13 ± 1.68
P value			0.50	0.37	0.89
Ejaculation dysfunction					
Yes	9	8.6	27.22 ± 9.55	84.56 ± 4.07	3.67 ± 2.50
No	96	91.4	24.86 ± 8.98	81.65 ± 6.00	3.08 ± 1.51
P value			0.46	0.16	0.30

cryopreservation groups (P = 0.324 in case of conventional freezing groups and P = 0.124 in case of vitrification groups).

The process of cryopreservation caused an increase in the average percentage of sperm head defects and midpiece and tail defects as shown in Table 2, Figs. 5 and 6 . Sperm washing significantly reduced the average percentage of sperm defects in fresh samples (from 92.50 ± 4.44 to 91.47 ± 3.76 in case of head defect and from 64.50 ± 4.30 to 64.10 ± 4.38 in case of midpiece and tail defect). The average percentage of sperm defects in almost all cryopreservation groups significantly increased compared with fresh groups. Only the average percentage of head defect in vitrification unwashed group was not significantly different compared with that of fresh unwashed (P = 0.03). In contrast to normal morphology parameter, the percentage of sperm head defects and midpiece and tail defects in vitrification groups were significantly lower than conventional freezing groups. Sperm washing process significantly decreased head defects in conventional freezing washed group compared with unwashed group (94.67 ± 3.41 in case of unwashed and 94.24 ± 3.54 in case of washed). Similarly, vitrification washed group had sperm midpiece

Table 2
The analyses of the human sperm parameters before and after freezing by conventional rapid freezing and vitrification of washed or unwashed human spermatozoa (n = 105).

Group n = 105	Sperm parameters				
	Motility (%)	Viability (%)	Normal morphology (%)	Head defect (%)	Midpiece and tail defect (%)
Fresh					
Unwashed (FU)	25.75 ± 9.01	81.90 ± 5.90	3.13 ± 1.61	92.50 ± 4.44	64.50 ± 4.30
Washed (FW)	29.49 ± 9.68[a]	86.24 ± 5.88[a]	3.66 ± 1.51[a]	91.47 ± 3.76[a]	64.10 ± 4.38[a]
Conventional freezing					
Unwashed (CfU)	14.76 ± 6.16[a]	53.10 ± 9.65[a]	1.36 ± 1.02[a]	94.67 ± 3.41[a]	68.48 ± 4.31[a]
Washed (CfW)	14.91 ± 6.25[b]	55.46 ± 8.00[b,c]	1.43 ± 0.94[b]	94.24 ± 3.54[b,c]	68.40 ± 4.41[b]
Vitrification					
Unwashed (VitU)	8.67 ± 5.31[a,c]	38.99 ± 8.62[a,c]	1.95 ± 1.08[a,c]	93.15 ± 3.65[c]	67.92 ± 4.41[a,c]
Washed (VitW)	9.78 ± 5.33[b,d,e]	41.35 ± 9.10[b,d,e]	1.87 ± 1.09[b,d]	92.95 ± 3.50[b,d]	67.27 ± 4.29[b,d,e]

Values are mean ± SD; n, number of semen samples.
[a] p value < 0.001 versus fresh unwashed (FU).
[b] p value < 0.001 versus fresh washed (FW).
[c] p value < 0.001 versus conventional freezing unwashed (CfU).
[d] p value < 0.001 versus conventional freezing washed (CfW).
[e] p value < 0.001 versus vitrification unwashed (VitU.

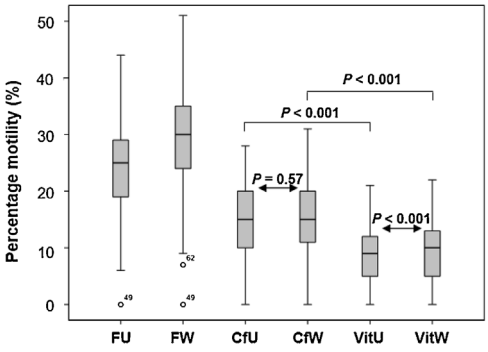

Fig. 2. Effects of cryopreservation by conventional freezing and vitrification on motility parameters of washed and unwashed sperm.

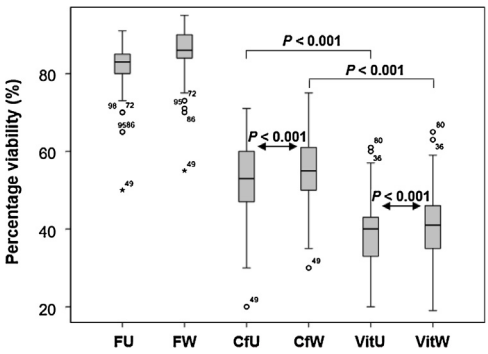

Fig. 3. Effects of cryopreservation by conventional freezing and vitrification on viability parameters of washed and unwashed sperm.

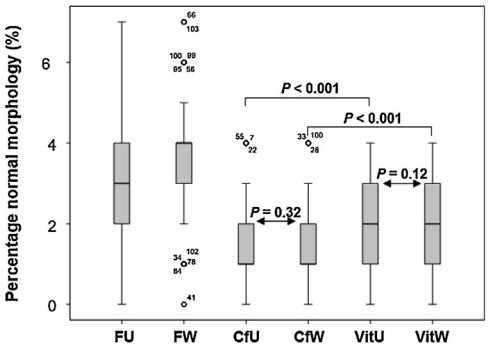

Fig. 4. Effects of cryopreservation by conventional freezing and vitrification on normal morphology of washed and unwashed sperm.

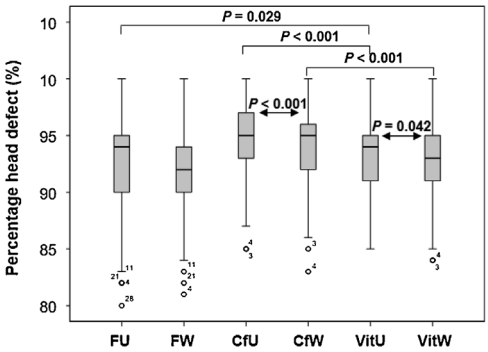

Fig. 5. Effects of cryopreservation by conventional freezing and vitrification on head defect of washed and unwashed sperm.

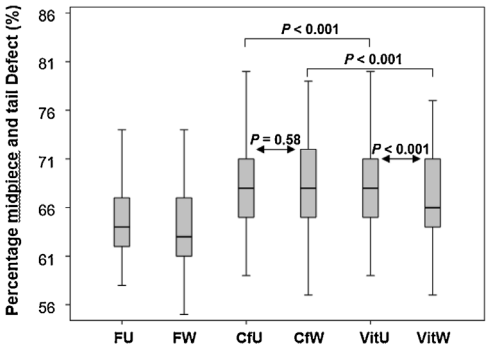

Fig. 6. Effect of cryopreservation by conventional freezing and vitrification on midpiece and tail defect of washed and unwashed sperm.

and tail defects significantly lower than unwashed group (67.92 ± 4.41 in case of unwashed and 67.27 ± 4.29 in case of washed).

Discussion

Sperm cryopreservation is a technique of great potential in management of male infertility in ART, but freezing and thawing processes may result in cellular damage compromising sperm function. The human sperm cryopreservation method normally involves the freezing and storage of human semen in liquid nitrogen ($-196\,^{\circ}C$). At this temperature, water molecules are aligned in a glassy and crystalline structure, and there is not sufficient energy available for most physiological reactions. The potential damage to sperm stored at this temperature is DNA breakdown due to cryodamage [16].

The decline in sperm parameters after freezing and thawing is currently a study topic of interest. Therefore, it is necessary to conduct further studies on sperm cryopreservation so as to identify the best method. Conventional freezing technique is a routinely applied method of sperm cryopreservation whereas the ultra-fast freezing method through vitrification has recently been introduced. Vitrification is based on the ultra-rapid freezing of the cell by direct immersion in liquid nitrogen (LN2), the viscosity of cell increase fast and become to glass-like state, thereby avoiding the formation of ice crystals [13]. This study has made initial comparisons between the two methods of conventional rapid freezing and vitrification in terms of motility, viability and morphology of post-thawing sperm (after one-month freezing) in unwashed and washed samples.

Cryopreservation of semen samples has potential effects upon the sperm parameters such as motility, viability and morphology [6,17]. This study showed that sperm cryopreservation by conventional freezing and vitrification consistently caused a reduction in post-thawing motility, viability, normal morphology and an increase in sperm defects. Compared with fresh groups, all cryopreservation groups suffered a significant reduction in motility, viability and normal morphology plus an increase in sperm head defects, midpiece and tail defects. The findings suggest that freezing and thawing processes may have exposed sperm to osmotic stress with CPA high concentration and ice crystal formation in cooling and warming may have resulted in cellular damage compromising sperm function. Isachenko and colleague optimized vitrification process with small volume of semen suspension (10 μl) and only using sucrose 0.25 M (nonpermeable cryoprotectant) in oder to having improved high integrity sperms recover rate. High rate recover sperm with high integrity. Motility, the percentage of spermatozoa with plasma membrane integrity and acrosome intact after vitrification were significantly higher when compared to slow-conventional freezing [18]. Vitrification without permeable

cryoprotectants could perform on spermatozoa because spermatozoa wassmallest cell in body, had high level of protein, sugar and other component. The large amount of component in spermatozoa are natural cryoprotectant in cell. Therefore, using proteins and sugar as nonpermeable cryoprotectants prevents ice crystal formation in extracellular [19]. Vitrification sperm with highly concentrated cryoprotectants especially the permeable cryoprotectants arenot suitable for mammalian spermatozoa because of effect of osmotic shock. The small droplet containing low level nonpermeable cryoprotectants achieves by vitrification with sperm mixed solution. This drop-wise technique is the key technical advance, sperm mixed drop achieved highly cooling rate 2000–10000 °C/min by direct plunging into liquid nitrogen. Successful vitrification of spermatozoa without the use of permeable cryoprotectants is important to maintain physiological parameters such as capacitation, acrosome reaction, and the integrity of cytoplasmic and mitochondrial membrane, fertilization of spermatozoa like fresh sperm [20]. Another study of Agha-Rahimi used SpermFreeze Solution™ as a permeable cryoprotectant and protocol to cryopreservation was similar to our study. They found that the sperm parameters, such as motility, viability and normal morphology were similar in cryopreservation groups by freezing or vitrification with/without permeable cryoprotectant. Freezing made more stress for sperm after cryopreservation than vitrification so that DNA fragmentation rate increased but hyaluronan-binding assay (HBA) showed the similar results in the percent sperm bound rate. In addition, the presence or absent of permeable cryoprotectants were also no significant differences in motility, viability normal morphology, DNA fragmentation index and HBA between the two vitrification subgroups. However the parameters associated with sperm quality after cryopreservation significantly reduced when compared with fresh samples [13]. The temperature decrease results in an increase of lipid peroxidation, reduction of antioxidant defenses and formation of reactive oxygen species (ROS) in human spermatozoa [21]. Human sperm ROS formation and the resulting oxidative stress during cryopreservation is associated with increasing apoptosis leading to spermatozoa DNA fragmentation [22]. Osmotic stress leads to ice crystal formation, cellular dehydration and cell shrinkage. The consequences could be sperm lethality [23].

Spermatozoa are considered as motile and viable when they have an integrity of sperm membranes [24]. When semen is cryopreserved, sperms are exposed to a cold shock, formation of ice crystals and cellular dehydration, which result in irreversible damage [14]. The disadvantages of vitrification are adverse changes in the composition of membrane lipid which caused increasing membrane damage, inducing acrosome reaction and apoptosis [2]. The results of this study showed that the ultra-fast freezing method through vitrification significantly reduced sperm motility and viability parameters compared with conventional rapid freezing method and that vitrification method may have induced more cell membrane damage compared with rapid freezing method, which caused a decrease in sperm parameters of motility and viability.

DNA integrity is crucial for the maintenance of sperm reproductive potential. However, sperms displaying altered genome and DNA damage are able to achieve fertilization, thereby posing concerns about the transmission of abnormal genetic material to the offspring [25]. It is widely reported that sperm cryopreservation causes an increase in sperm DNA damage [26]. Highly significant correlations were observed between sperm DNA damage and sperm morphology [27,28]. In our results, the average percentage of normal morphology in vitrification groups had significantly higher than that of rapid freezing groups while sperm head defects and midpiece and tail defects in vitrification groups were significantly lower than those of conventional rapid freezing groups. The findings suggest that vitrification may be less DNA damaging than conventional rapid freezing and may also have caused less changes to sperm morphology and less sperm defects. Satirapod and colleagues also reported similar results that vitrification caused less DNA damage in spermatozoa than rapid freezing [29]. According to these authors, there are two main mechanisms for damaged DNA in cryopreservation: mechanical injuries due to ice crystal formation and oxidative stress. Recently, Rahiminia and colleagues reported that there was a negative correlation between sperm motility, vitality and sperm DNA damage [2].

In general, sperm washing before cryopreservation improves the percentage of normal morphology, sperm motility, sperm viability, and non-apoptotic spermatozoa in post-thawing sperm [30]. It is known that washing process eliminates a variety of dead sperms, non-motile sperms and abnormal sperms so that after washing, sample has better motility, viability and sperm defects decrease [30]. Hence, the results of this study demonstrated these parameters in washed groups were higher than that in unwashed group after thawing no matter what cryopreservation method we used. This investigation indicated that sperm washing before cryopreservation should be done because of its improvement of post-thawing sperm parameters, which may lead to an increasingly successful rate of assisted reproductive treatment.

In conclusion, the present study revealed significant effects on the progressive motility, viability, normal morphology and cellular defects of human spermatozoa undergoing cryopreservation. The conventional freezing of human sperm is indeed feasible and better at saving sperm motility and viability, whereas the sperm vitrification protocol, as described in this study, allows superior results in terms of normal morphology and sperm defects, thereby, constituting an efficient and reliable alternative to conventional freezing method. However, the implication of cryo-injury mechanism in cryopreservation of human sperm by vitrification and conventional rapid freezing method remains a critical unanswered question that requires further investigation. Furthermore, washed groups had better sperm parameters than unwashed groups so that washing process before frozen seemed to improve sperm parameters.

Ethics approval and consent to participate

This study was approved by the Hue University of Medicine and Pharmacy Ethics Committee.

Consent for publication

Not applicable

Availability of data and material

The dataset used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LMT/NTTT/NVT/NTTA developed the study concept and designed the study; LMT/NTTT/NVT/NTT acquired the data for

analysis; NTTT/NTT/NVT performed the statistical analysis; NTTT/NTT/LMT drafted the first manuscript; LMT/NTTT/NTT/NVT contributed to the interpretation of the data and provided critical revision for important intellectual content. All authors reviewed and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ejogrb.2019.01.001.

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