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ORIGINAL ARTICLE

Sperm Biology

Improving native human sperm freezing protection by using a modified vitrification method

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Slow freezing is the most commonly used technique for the cryopreservation of spermatozoa in clinical practice. However, it has been shown to have a negative impact on sperm function and structure. Vitrification as a successful alternative method has been proved to have better protective effects on human embryos, but vitrification of spermatozoa is still subject to low recovery rates. In this study, a modified vitrification method for native spermatozoa was developed. A total of 28 semen samples were included; each sample was divided into three equal parts and assigned to fresh, slow freezing, and vitrification groups. Sperm vitality, motility, morphology, DNA integrity, and acrosome reaction were assessed for each of the groups. The results showed that vitrification achieves better results for several sperm protection parameters than slow freezing; vitrification achieves a higher recovery rate ($P < 0.05$), motility ($P < 0.05$), morphology ($P < 0.05$), and curve line velocity ($P < 0.05$) than slow freezing. Furthermore, DNA fragmentation was decreased ($P < 0.05$) and better acrosome protection ($P < 0.05$) was exhibited in the spermatozoa after vitrification. Principal component analysis of all sperm parameters revealed that the vitrification cluster was closer to the fresh cluster, indicating that spermatozoa are better preserved through vitrification. In conclusion, while both slow freezing and vitrification have negative effects on sperm function and structure, the vitrification protocol described here had a relatively better recovery rate (65.8%) and showed improved preservation of several sperm quality parameters compared with slow freezing.

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INTRODUCTION

Sperm freezing is the most efficient way to preserve male fertility and is an important procedure in assisted reproduction technologies (ART).¹ The first trial to cryopreserve spermatozoa dates back to 1938, when frog spermatozoa were successfully vitrified in liquid air.² However, subsequent attempts to vitrify mammalian spermatozoa failed. Eventually, the use of glycerol in slow freezing solved the problems associated with mammalian sperm cryopreservation.³

With continuous improvement, slow freezing became a conventional technique for sperm cryopreservation.⁴ The recovery rate of cryopreserved spermatozoa is usually 50% and shows a wide interindividual variability.^{5,6} Nevertheless, slow-freezing protocols have several problems because this methodology is based on programmed cooling, necessitating the use of an appropriate cryoprotectant. Several studies have demonstrated that slow freezing causes dramatic changes in sperm quality, both structurally and functionally.^{7–9} These impairments may come from the addition and removal of the cryoprotectant, which cause toxic and osmotic damage.^{10,11} In addition, the formation of ice crystals during sperm freezing can also cause damage to the sperm cytoskeleton, membrane, and DNA.^{8,10,11} Importantly, sperm DNA damage may play an important role in male infertility and recurrent miscarriage.^{12–15}

These inherent disadvantages of slow freezing necessitate the development of new alternative cryopreservation procedures.¹⁶ In recent years, vitrification has been shown to be a successful alternative to conventional freezing in the preservation of human oocytes and embryos. Thus, it represents a viable alternative method for improved sperm cryopreservation.^{17,18} During the vitrification procedure, water is cooled to a glassy state without intracellular ice crystallization, reducing cryopreservation damage. However, the classical vitrification technique cannot be used for spermatozoa cryopreservation, because the high concentrations of permeant cryoprotectants (30%–50%) can cause lethal osmotic shock to the spermatozoa.^{19,20} Thus, vitrification of spermatozoa remains a largely unexplored methodology. In fact, we think that those methods do not represent real vitrification because we have not observed the cryoprotectant being cooled to a glassy state.^{20,21} However, we habitually call it vitrification.

The first and most important improvement of sperm vitrification was developed in 2002. Nawroth *et al.*²⁰ reported a vitrification protocol and successfully applied it in sperm cryopreservation. Based on their research, many studies have described new methods of sperm vitrification using different combinations of cryoprotectants, devices, freezing rates, and volumes.^{8,16,22,23} However, most of these promising protocols have not been widely practiced in sperm banks or *in vitro*

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fertilization (IVF) labs. Thus, slow freezing remains the standard method of sperm cryopreservation.

Although several studies have demonstrated that vitrification is more effective than slow freezing for human sperm cryopreservation,^{8,24–26} its average recovery rate is not good compared with that of slow freezing.²⁷ Thus, improved vitrification methods that provide better protection of the quality and function of the spermatozoa than slow freezing are needed.

We reviewed numerous classic vitrification protocols.^{28–31} On the basis of those results, we developed a new sperm vitrification method that uses a nonpermeant cryoprotectant for native semen cryopreservation. After rigorous standardized optimization, the newly described method achieved better results in the protection of several sperm structure parameters than slow freezing, especially in terms of recovery rate.

MATERIALS AND METHODS

Sample collection

Semen samples were obtained from 28 healthy participants (age range 22–35 years) by masturbation after 2–5 days of sexual abstinence. Basic semen analysis, including sperm concentration, motility, and morphology assessment, was performed according to the 5th Edition of World Health Organization (WHO) Laboratory Manual for the Examination and Processing of Human Semen.³² The sperm concentration (mean \pm standard deviation [s.d.]) was $50.2 \times 10^6 \pm 38.7 \times 10^6$ cells per ml, and the semen volume (mean \pm s.d.) was 2.5 ± 1.7 ml.

Ethical approval

This study was carried out with approval from, and under the supervision of, the Ethics Committee of the Reproductive and Genetic Hospital of CITIC-Xiangya, Basic Medical Science School, Central South University, Changsha, China (Approval No. LL-SC-2017-015). All the samples used for the experiments were obtained from donors who provided informed consent.

Experimental design

Each semen sample was divided into three equal parts and assigned to fresh, slow freezing, and vitrification groups. Sperm structure and function analyses were performed on all three groups.

Slow freezing and thawing

According to the reported studies and our results, Sperm Freezing Medium™ (ORIGIO, Måløv, Denmark) was selected as the cryoprotectant for the slow freezing of spermatozoa.³³ The freezing medium was slowly added to the native semen sample to achieve 1:1 dilution, and the resultant mixture was packaged into 1.8 ml NuncCryotubes® (Thermo Scientific, Rockford, IL, USA). In order to reduce experimental error and guarantee quality control, a CryoMed™ Controlled-Rate Freezer (Thermo Scientific) was used for the programmed cryopreservation of spermatozoa. First, the mixture was incubated at 25°C for 5 min. Then, the temperature was decreased to –2°C at 1.2°C per min and thereafter to –45°C at 7.2°C per min. Finally, the temperature was reduced to –137°C at 25°C per min. The samples were transferred to liquid nitrogen for at least 48 h.

After storage, the samples were warmed in a 37°C water bath and shaken slightly. The postthaw sperm suspension was mixed with 5 ml G-IVF Plus medium (Vitrolife, Västra Frölunda, Sweden) and centrifuged (Centrifuge 5424 R, Eppendorf, Hamburg, Germany) at 300g for 5 min. Finally, the cell pellet was resuspended in 200 μ l G-IVF Plus medium.

Vitrification and thawing

For sperm vitrification, we tested different combinations of cryoprotectant, carrier (device for packing biological samples), and

freezing rate (**Supplementary Figure 1**). Cyoleaf (Medicult, Jyllinge, Denmark), Cryoloop (Hampton Research, Orange, CA, USA), and Straw (Cryo Bio System, Paris, France) were used as the carrier for the vitrification of spermatozoa. Finally, we chose trehalose (0.5 mol l^{-1}), glycine (100 mmol l^{-1}) and human serum albumin (1% [w/v]) as cryoprotective agents. In brief, the vitrification medium was slowly added to native semen sample to produce a 1:1 dilution, and the resultant suspension was incubated at 25°C for 5 min. Aliquots of the sperm suspension (approximately 25 μ l) were dropped directly into medical-grade liquid nitrogen free from contaminants. This process results in the immediate formation of a 25 μ l floating sphere that solidifies and sinks after about 25 s. This procedure was repeated to obtain a sufficient number of spheres (**Figure 1**). All the spheres were finally packed into a 1.8 ml cryovial and stored for at least 48 h in liquid nitrogen.

Sample warming was performed by quickly submerging the spheres in 5 ml G-IVF Plus medium prewarmed to 37°C accompanied by gentle agitation. After incubation at 37°C for 5 min, the postthaw sperm suspension was centrifuged at 300g for 5 min and resuspended in 100 μ l G-IVF Plus medium.

Evaluation of sperm vitality, motility, morphology, and recovery rate

Eosin-nigrosin staining was used to assess sperm vitality (WHO 2010),³² and at least 200 spermatozoa per sample were assessed.³¹ Sperm motility and morphology were assessed immediately after liquefaction for fresh samples and after warming for the slow-freezing and vitrification samples. In order to reduce experimental error, computer-assisted sperm analysis (CASA) system (SAS medical, Beijing, China) and SAS-II® version 2.3 software (SAS medical) were applied to evaluate sperm motion parameters, including concentration, progressive motility (PR), nonprogressive motility (NP), immotility (IM), straight line velocity (VSL), and curve line velocity (VCL). Our CASA system is calibrated for concentration each month by using standard concentration

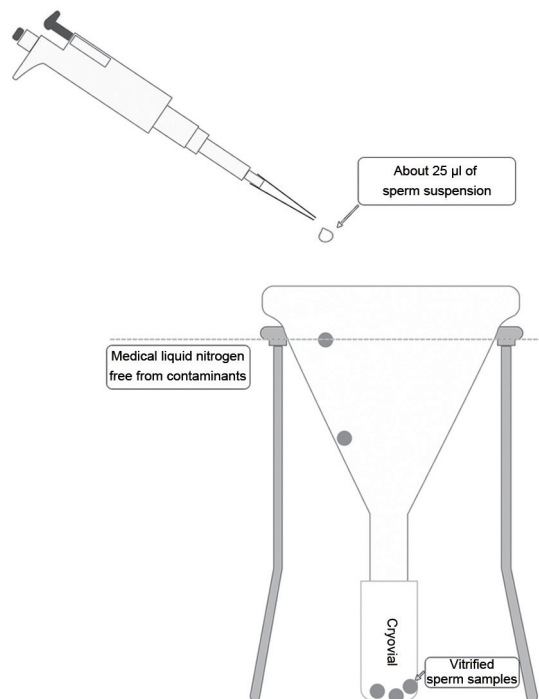


Figure 1: Illustration of the vitrification process and apparatus.

beads, a video-recording was used for quality control of analysis of sperm motility, and error analysis was made by comparing results of CASA system and manual methods. Sperm morphology was assessed according to the WHO (2010).³² At least 200 spermatozoa for each sample were analyzed for head damage, mid-piece damage, tail damage, and excess residual cytoplasm (ERC). The recovery rate of motile sperm was calculated according to the WHO (2010);³² it is the value of the treated group as a percentage of that of the control.

Recovery rate = total sperm motility after freezing/total sperm motility before freezing × 100%.

Evaluation of sperm DNA fragmentation

Sperm DNA fragmentation was analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; *In Situ* Cell Death Detection Kit, Roche, Mannheim, Germany) according to the manufacturer's protocol. At least 200 spermatozoa were evaluated per sample in a Nikon fluorescence microscope (Nikon 80i, Nikon, Tokyo, Japan) at 100 × magnification.

Evaluation of spontaneous acrosome reactions

Fluorescein isothiocyanate-*Pisum sativum* agglutinin (FITC-PSA, Sigma-Aldrich, Steinheim, Germany) was used following the protocol described by Lybaert *et al.*³⁴ In brief, 1 ml of phosphate buffer saline (PBS) was added to 100 µl native semen sample and centrifuged at 300g for 5 min. After discarding the supernatant, the pellet was gently resuspended in 100 µl PBS, and 5 µl sperm suspension was placed in the center of a slide. After being dried at room temperature, the spermatozoa were fixed with 4% (*w/v*) paraformaldehyde (PFA) for 15 min, washed in PBS for 5 min three times, and incubated with FITC-PSA (15 µg ml⁻¹) at 4°C for at least 1 h. Spermatozoa were incubated for 15 min at room temperature in 100 nmol l⁻¹ 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Finally, the slides were rinsed in ddH₂O and mounted with fluorescence mounting medium (DAKO, Glostrup, Denmark). At least 200 spermatozoa were evaluated in each sample in a Nikon fluorescence microscope at 100 × magnification.

Statistical analyses

Descriptive and statistical analyses were performed using SAS University Edition (SAS, Wake County, NC, USA) and the GraphPad Prism 7.0 (GraphPad software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant. Data distribution was tested

by D'Agostino and Pearson and Shapiro–Wilk normality test to establish the type of statistical tests (parametric or non-parametric). The data which compared PR, NP, IM, VSL, VCL, motility, head damage, mid-piece damage, and recovery rate were subjected to the paired *t*-test. Wilcoxon matched-pairs signed rank test was used to calculate the statistical difference of vitality, normal morphology, ERC, tail damage, auto-acrosomal reaction, and DNA fragmentation index (DFI). The statistical difference among three groups was compared by using one-way repeated measures ANOVA and Friedman's test for normal or nonnormal data, respectively. Principal component analysis (PCA) and Spearman correlation analysis were performed by using R version 3.5 (R Foundation, Vienna, Austria). Correlation significance was assessed at the 5% level.

RESULTS

Sperm vitality, motility, and morphology

To assess the toxicity of cryoprotectants used for vitrification, ten semen samples were incubated in vitrification solution or Biggers-Whitten-Whittingham (BWW) medium for 30 min at 37°C under 5% (*v/v*) CO₂, after which sperm motility, acrosome reaction, and vitality were analyzed (**Supplementary Figure 2**). There were no significant differences between the vitrification solution and BWW group in the parameter values ($P > 0.05$).

The basic sperm parameters, including vitality, motility, and morphology, were assessed immediately after the sperm samples were liquefied or thawed. The vitality of the spermatozoa was decreased upon cryopreservation ($P < 0.05$) compared with that of fresh samples, but there was no significant difference between the slow-freezing and vitrification groups ($P > 0.05$). CASA was used to evaluate sperm motion parameters. All the sperm parameter values are shown in **Table 1**. Most of the sperm parameters were worse in the vitrification and slow-freezing groups than those of the fresh sample. However, most of the parameters of the vitrification group were better than those of the slow-freezing group.

The recovery rate of motile sperm (the value of the treated group as a percentage of that of the control) of vitrification was better than that of slow freezing (mean ± s.d.: 65.8% ± 12.6% vs 59.3% ± 16.9%, $P < 0.05$, paired *t*-test). The total motility of vitrified spermatozoa was also higher than that of slow-frozen spermatozoa (mean ± s.d.: 49.6%

Table 1: Sperm quality parameters for fresh, slow freezing and vitrification groups (n=28)

Parameters	Fresh	Slow freezing	Vitrification
Progressive motility (%), mean±s.d.	50.1±12.0	23.7±13.4 [#]	23.1±10.0 [#]
Nonprogressive motility (%), mean±s.d.	25.1±9.3	21.4±7.2 [*]	26.5±9.8 [*]
Immotility (%), mean±s.d.	24.3±12.0	54.4±15.3 ^{*,#}	49.6±12.3 ^{*,#}
Curve line velocity (µm s ⁻¹), mean±s.d.	65.5±10.0	60.0±9.2 ^{*,#}	69.9±11.1 [*]
Straight line velocity (µm s ⁻¹), mean±s.d.	51.4±9.2	39.6±9.2 [#]	40.8±9.0 [#]
Motility (%), mean±s.d.	75.2±12.1	45.1±15.5 ^{*,#}	49.6±13.1 ^{*,#}
Vitality (%), median (IQR)	90.4 (88.4–92.0)	79.1 (75.1–80.5) [#]	78.3 (74.7–81.2) [#]
Nomal morphology (%), median (IQR)	11.5 (4.7–15.8)	7.6 (3.4–11.4) ^{*,#}	9.1 (4.0–12.6) ^{*,#}
Head damage (%), mean±s.d.	48.3±5.7	54.9±5.9 ^{*,#}	52.4±6.2 ^{*,#}
Mid-piece damage (%), mean±s.d.	28.7±5.0	34.4±5.6 ^{*,#}	32.6±5.0 ^{*,#}
ERC (%), median (IQR)	2.4 (1.6–3.2)	1.4 (1.0–1.5) ^{*,#}	1.5 (1.4–1.9) ^{*,#}
Tail damage (%), median (IQR)	28.8 (26.8–29.7)	39.5 (36.2–42.8) ^{*,#}	35.5 (32.3–37.6) ^{*,#}
Auto-acrosomal reaction (%), median (IQR)	19.4 (14.7–27.4)	68.8 (64.2–72.6) ^{*,#}	57.6 (44.8–63.8) ^{*,#}
DFI (%), median (IQR)	7.1 (6.0–9.7)	16.5 (12.9–18.2) ^{*,#}	13.1 (10.9–16.1) ^{*,#}

^{*}Experimental groups (slow freezing and vitrification) are significantly different between them ($P < 0.05$). [#]Groups are significantly different to control (fresh; $P < 0.05$). Data that follow a normal distribution are expressed as mean±s.d., and tested by paired *t*-test. Data that follow a nonnormal distribution are expressed as median (IQR), and tested by Wilcoxon matched-pairs signed rank test. ERC: excess residual cytoplasm; DFI: DNA fragmentation index; s.d.: standard deviation; IQR: 1st–3rd quartile range



$\pm 13.1\%$ vs $45.1\% \pm 15.5\%$, $P < 0.05$, paired *t*-test). In addition, VCL ($P < 0.05$) was also better in the vitrification group (Table 1).

The normal morphology of the spermatozoa was changed after cryopreservation ($P < 0.05$), especially in terms of damage to the head and tail (Table 1). Vitrification achieved better preservation of normal sperm morphology than slow freezing (median [1st–3rd quartile range (IQR)]: 9.1% [4.0%–12.6%] vs 7.6% [3.4%–11.4%], $P < 0.05$, Wilcoxon matched-pairs signed rank test). Damage to the head, mid-piece, and tail were significantly increased in the slow-frozen samples compared with the vitrified spermatozoa (Table 1). From the modified Papanicolaou staining of the spermatozoa, the number of head vacuoles and curled tails were higher for the slow-frozen spermatozoa (Supplementary Figure 3).

Sperm DNA fragmentation

The results of TUNEL to assess sperm DNA fragmentation are shown in Figure 2. The sperm DFI was increased after freezing compared with that of the fresh semen ($P < 0.05$). Furthermore, the vitrification group exhibited a lower DFI than the slow-freezing group (median [IQR]: 13.1% [10.9%–16.1%] vs 16.5% [12.9%–18.2%], $P < 0.05$).

Acrosomal integrity

FITC-PSA was used to detect the acrosomal integrity of sperm (Figure 3). The percentage of acrosome-reacted spermatozoa was higher in both the vitrification and slow-freezing groups than that in the fresh samples ($P < 0.05$). However, the vitrification method provided stronger protection against cryodamage, and the number of acrosome-reacted spermatozoa was lower in the vitrified sample than that in the slow-frozen sample (median [IQR]: 57.6% [44.8%–63.8%] vs 68.8% [64.2%–72.6%], $P < 0.05$).

Synthetic analysis of sperm quality

To evaluate the sperm quality after freezing more holistically, we performed PCA based on the sperm motility, morphology,

and structure parameters for the fresh and cryopreserved groups (Table 2 and Figure 4). We chose two components with eigenvalues > 1 that account for 59.0% of the variance. Significant differences among the three groups ($P < 0.01$) were observed for Principal component 1 (PC1). The PC1 (accounting for 45.3% of variance) distinguished the fresh, slow-frozen, and vitrified samples mainly with negative values for several biomarkers, including PR, vitality, VSL, VCL, and normal morphology ($r < -0.5$, $P < 0.01$). Other biomarkers such as head damage, tail damage, mid-piece damage, IM, DFI, and auto-acrosomal reaction show significant positive correlations ($r > 0.5$, $P < 0.01$). PC2 (13.8% of the variance) discriminated semen samples mainly by NP ($r = 0.861$, $P < 0.01$), normal morphology ($r = -0.521$, $P < 0.01$), VCL ($r = 0.422$, $P < 0.01$), and head damage ($r = 0.463$, $P < 0.01$).

The PCA scatter showed that the frozen group's parameters were partially separated from the fresh groups, but the separation between the two cryopreservation groups was not complete. In addition, values in the vitrification group exhibited considerable overlap with those of the fresh group (Figure 4). Thus, the cryopreserved samples were distinguishable from the fresh sample, with vitrification performing better in the preservation of sperm function and structure.

DISCUSSION

Numerous cryobiological investigations of spermatozoa have focused on vitrification, revealing that it is less effective than slow freezing owing to low recovery rates of motile sperm after thawing.³⁵ Vitrification of spermatozoa is different from conventional cell vitrification because the high permeant of the cryoprotectant and the high freezing rate needed for conventional vitrification are lethal for human spermatozoa.^{21,36} Accordingly, we tested different combinations of cryoprotectant, carrier, and freezing rate, on the basis of previously reported results. Nonpermeant cryoprotectants have been reported to be suitable,^{16,20} but trehalose and glycine have been reported to provide better protection of cell membrane structures during the procedure of freezing than sucrose.^{37–39} Unlike conventional vitrification, a high

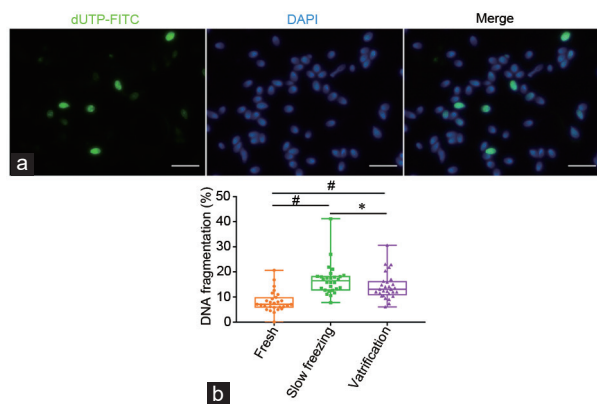


Figure 2: Impact on sperm DNA of slow freezing and vitrification. (a) Sperm DNA fragmentation assessment was performed by using TUNEL. Sperm with DNA fragments are labeled by dUTP-FITC (green). Nuclei counterstained with DAPI (blue). (b) Frequencies of DNA-damaged sperm in the slow freezing, vitrification and fresh groups are shown in the box plot (min to max, show all points), the upper and lower error bars represent 95th and 5th centiles respectively, the upper, middle and lower lines of each box represent the 75th centile, median and 25th centiles, respectively. Wilcoxon matched-pairs signed rank test was used. *Significant differences between slow freezing and vitrification groups ($P < 0.05$). #Groups are significantly different from control (fresh; $P < 0.05$). Scale bars = 10 μm . TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP end labelling; dUTP: 2'-deoxyuridine 5'-triphosphate; FITC: fluorescein isothiocyanate; DAPI: 4',6-diamidino-2-phenylindole.

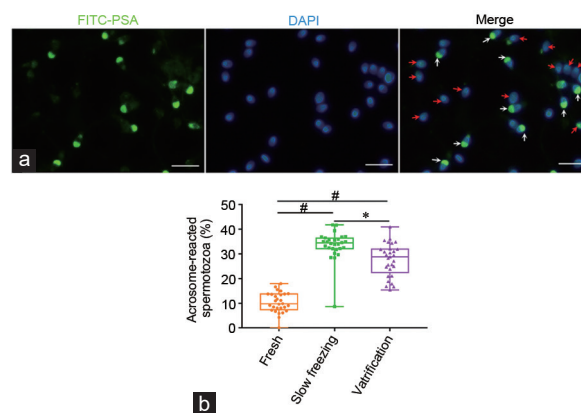


Figure 3: Impact on sperm acrosome of slow freezing and vitrification. (a) Representative images of acrosome detection by FITC-PSA (green). Nuclei counterstained with DAPI (blue). Different staining of the acrosome is visible, *i.e.*, acrosome-reacted (red arrow) and acrosome-intact (white arrow). (b) Frequencies of acrosome reacted spermatozoa in the fresh, slow-freezing, and vitrification groups are shown in the box plot (min to max, show all points), the upper and lower error bars represent 95th and 5th centiles respectively, the upper, middle and lower lines of each box represent the 75th centile, median and 25th centiles, respectively. Wilcoxon matched-pairs signed rank test was used. *Significant differences between slow freezing and vitrification groups ($P < 0.05$). #Groups are significantly different from control (fresh; $P < 0.05$). Scale bars = 10 μm . FITC: fluorescein isothiocyanate; PSA: *Pisum sativum* agglutinin; DAPI: 4',6-diamidino-2-phenylindole.

Table 2: Factor loadings for the first two principal components

Parameters	Principal component 1	Principal component 2
Progressive motility	-0.862*	-0.027
Nonprogressive motility	-0.022	0.861*
Immotility	0.811*	-0.365*
Curve line velocity	-0.299	0.422*
Straight line velocity	-0.772*	-0.299
Vitality	-0.818*	0.243
Morphology	-0.597*	-0.521*
Head damage	0.725*	0.463*
Mid-piece damage	0.583*	-0.086
ERC	-0.422*	0.081
Tail damage	0.828*	0.026
Acrosome reaction	0.742*	-0.308*
DFI	0.709*	0.042

*Significant correlation (Pearson correlation, $P < 0.01$). ERC: excess residual cytoplasm; DFI: DNA fragmentation index

cooling rate is not necessary and may even be harmful for human spermatozoa.⁴⁰ The optimal cooling rate for spermatozoa is about 500°C per min, which is achieved by using microdroplets. The highest freeze rate (about 10 000°C per min) is achieved by the Cryoleaf and Cryoloop system,^{20,22} but they also exhibit the lowest freezing efficiencies (Supplementary Figure 1).

The results from our study show that the new vitrification protocol provides a significantly better preservation of sperm of structure and function than slow freezing.^{7,8} Several studies have demonstrated that cryodamage to membranes, the cytoskeleton, and DNA, upon slow freezing may result in poor IVF fertilization rates.⁴¹ Improved preservation of sperm quality and function can be achieved through vitrification.

Ice crystal formation during the thawing-freezing process is thought to be the most important factor physically injuring cell membrane and cytoskeleton structure.^{10,42} This is in agreement with the higher rate of tail and head damage for sperm in the slow-freezing group than that in the vitrification group, especially for the head vacuoles and tail coiling. We also observed a decrease in acrosomal reaction in the vitrification group compared with the slow-frozen spermatozoa, which may result from a decrease in ice crystal formation, which can cause cytoskeleton impairment and membrane alteration. The temperature decrease may stimulate cells to increase the levels of cytoplasmic calcium, which then induces acrosomal content loss and acrosomal exocytosis.⁴³ In the vitrification group, the temperature drop is faster and hence there may not be enough time for acrosome content release. This may also explain the higher percentage of spontaneous acrosome reaction detected in the slow-frozen spermatozoa than that in the vitrified spermatozoa.

Motility is a key indicator for assessing the fertilization ability of spermatozoa. We observed a decrease in motility for both cryopreservation protocols, but the reduction was significantly higher for frozen than for vitrified spermatozoa.

A number of researchers have reported that the rapid osmolality changes caused by the addition and removal of cryoprotectants may also be responsible for sperm damage,^{10,11} together with the dehydration and rehydration resulting from water-cryoprotective agent (CPA) exchange and intracellular crystal formation or ice recrystallization.⁴² These factors may help explain why the damage was reduced in the vitrified group. Because only nonpermeant cryoprotectant was used in the vitrification protocol, there could be no water-CPA exchange or rehydration.

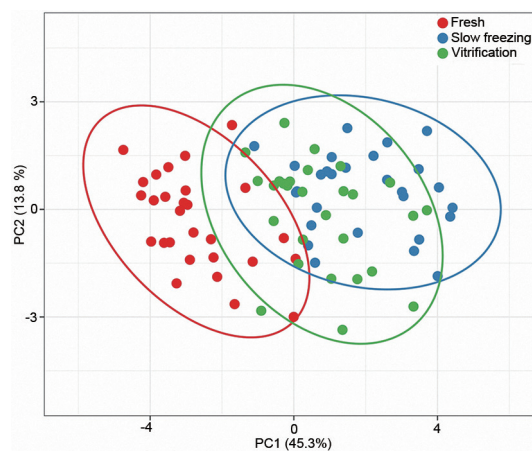


Figure 4: Scatter plot of the first two principal components. X and Y axes show PC1 and PC2, which account for 45.3% and 13.8% of the total variance, respectively. The vitrification group exhibits significant overlapping with the fresh group. PC1: principal component 1; PC2: principal component 2.

Recent studies have revealed that DNA integrity is crucial for safe transmission of paternal genetic information to the embryo.¹³ The presence of DNA fractures in spermatozoa can have negative effects if the damage is severe enough, arresting embryo development and even causing birth defects.¹⁵ Thus, it is crucial to maintain DNA integrity during the freezing and thawing processes. A significant increase in DFI was observed after cryopreservation compared with that of the fresh samples, but there was no significant difference between the vitrification and slow-freezing groups.

Several studies have demonstrated that oxidative stress is the main factor causing sperm DNA breakage during cryopreservation. Furthermore, elevated reactive oxygen species (ROS) contribute to the activation of caspases, which can ultimately lead to DNA fragmentation. Thus, the addition of antioxidant to the CPA may improve the protection of DNA integrity.⁴⁴ The synthetic analysis of sperm quality parameters performed in this study through PCA indicated that the new vitrification protocol could provide better structural and functional protection of spermatozoa during the freezing-thawing process. From the PCA scatter plot, the cryopreservation groups presented parameters that allowed their separation from the fresh group, and the vitrification cluster was only partially, instead of entirely, separated from the fresh cluster (as the slow freezing group was). This means the parameter values for the spermatozoa in the vitrification group were closer to those of the fresh group.

CONCLUSION

Our study presents a new vitrification protocol that provides improved preservation in terms of many sperm function and structure parameters. We employed a more standardized method involving CASA, a controlled-rate freezer, and sperm freezing medium to reduce random errors and ensure an efficient and reliable vitrification method. However, we need to perform more research to determine whether this method can be applied to the freezing of spermatozoa from the epididymis or testicular tissue and whether the new vitrification method can improve clinical ART outcomes.

AUTHOR CONTRIBUTIONS

DZ and LQF participated in the design of the study and the preparation of manuscript. XMW participated in data analysis and figures preparation. ZZL, YCC, RXL, and YZW collected and analyzed the data.

ZHH and HCN supervised sample collection. WBZ and YQT helped perform the analysis with constructive discussions and contributed analysis tools. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

All authors declared no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

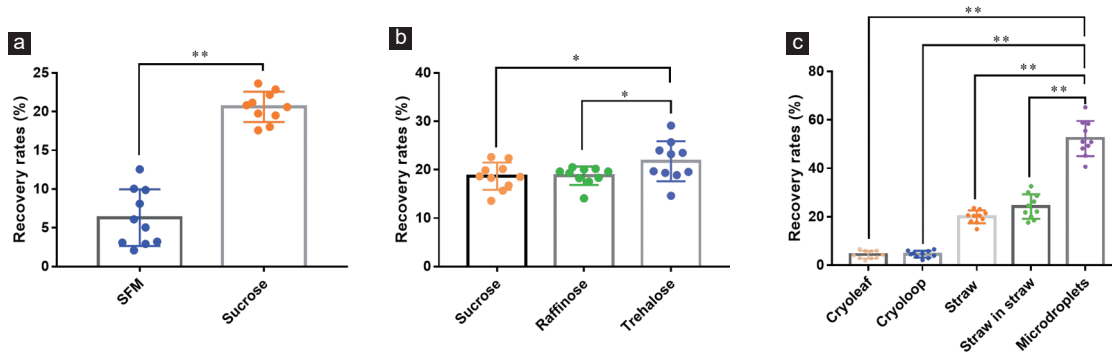
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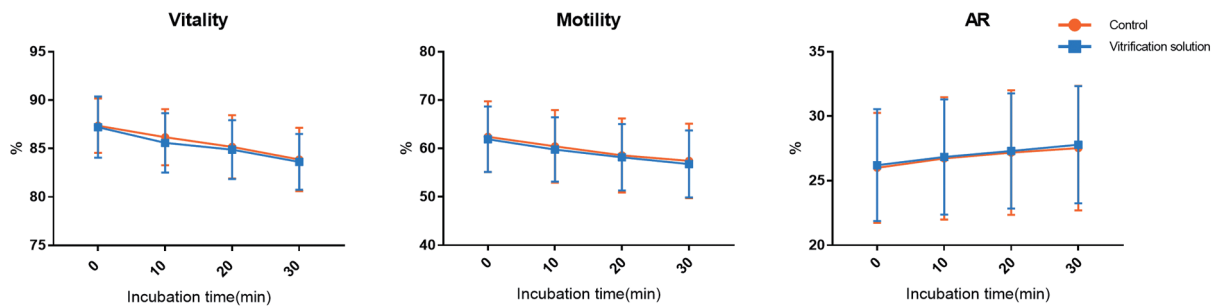
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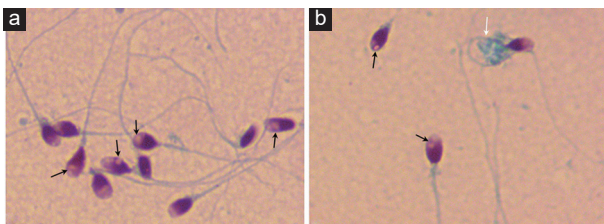




Supplementary Figure 1: The selection of different combinations for sperm vitrification. (a) The recovery rate of motile spermatozoa with permeable cryoprotectant or non-permeable cryoprotectant following vitrification using straw as a standard carrier. (b) The recovery rate of different nonpermeable cryoprotectants using straw as a standard carrier. (c) The recovery rate of different carriers using trehalose as a cryoprotectant. SFM: sperm freezing medium.



Supplementary Figure 2: Vitrification solution toxicity test. The effect of cryoprotectants on sperm vitality, motility, and acrosome reaction. Ten semen samples were incubated for 30 min at 37°C under 5% CO₂.



Supplementary Figure 3: Modified Papanicolaou staining of spermatozoa after slow freezing. (a) Representative images of head vacuoles (black arrow). (b) Representative images of tail curl (white arrow). The frequency of those damages increased after slow freezing compared to vitrification.