Comparison of Cryopreserved Human Sperm between Ultra Rapid Freezing and Slow Programmable Freezing: Effect on Motility, Morphology and DNA Integrity

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Background: *Cryopreservation of sperm is common methods to preserve male fertility. Sperm freezing, suggest slow programmable freezing caused lower change of sperm morphology than sperm freezing in vapor of liquid nitrogen. Ultra rapid freezing is easy to be worked on, less time, low cost and does not need high experience.*
Objective: *To compare the effect on sperm motility, morphology and DNA integrity of post-thawed sperm after ultra rapid freezing and slow programmable freezing methods.*
Material and Method: *Experimental study at laboratory of infertility unit, Department of Obstetrics and Gynecology, Faculty of Medicine Ramathibodi Hospital. Thirty-seven semen samples with normal semen analysis according to World Health Organization (WHO) 1999 [normal sperm volume (≥2 ml) and normal sperm concentration (≥20x10^6/ml) and sperm motility (≥50%)]. Semen samples were washed. Then each semen sample was divided into six cryovials. Two cryovials, 0.5 ml each, were cryopreserved by slow programmable freezing. Four 0.25 ml containing cryovials, were cryopreserved by ultra rapid freezing method. After cryopreservation for 1 month, thawed process was carried out at room temperature. Main outcomes are sperm motility was determined by Computer-Assisted Semen Analysis (CASA), sperm morphology was determined by eosin-methylene blue staining and sperm DNA integrity was assessed by TUNEL assay.*
Results: *Sperm motility was reduced significantly by both methods, from 70.4 (9.0)% to 29.1 (12.3)% in slow programmable freezing and to 19.7 (9.8)% in ultra rapid freezing ($p<0.05$). Sperm motility decreased significantly more by ultra rapid freezing ($p<0.001$). The percentage of normal sperm morphology and DNA integrity were also reduced significantly by both methods. However, no significant difference between the two methods was found ($p>0.05$).*
Conclusion: *Cryopreservation of human sperm for 1 month significantly decreased sperm motility, morphology and DNA integrity in both methods. However, sperm motility was decreased more by ultra rapid freezing.*

Keywords: *Ultra rapid freezing, Slow programmable freezing, Sperm motility, Sperm morphology, Sperm DNA integrity*

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Infertility is an important problem that affects the marital life psychologically and causes familial and social stress. Treatment of infertility mainly depends on causes.

Cryopreservation of sperm, oocytes and embryos at different stages, are known as common methods in order to preserve fertility in the Assisted Reproductive Technology (ART). Cryopreservation of sperm is widely used for many reasons, for examples, in men who suffer from cancer before cytotoxic chemotherapy, radiotherapy, or certain surgical treatment that may lead to testicular failure or ejaculatory dysfunction such as testicular cancer[1]. The treatment of some non-malignant disease such as diabetes and an autoimmune disorder may also lead to testicular damage and cryopreservation of sperm should be advised[2]. Sperm cryopreservation allows enough time to screen donors for infectious agents such as human immunodeficiency virus and hepatitis B virus[3].

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Cryopreservation of sperm retrieved from testicular sperm extraction (TESE) or percutaneous epididymal sperm aspiration (PESA) could avoid the need for repeated biopsy or aspiration[4].

In 1954, Sherman JK reported the first baby that delivered from a mother who received intrauterine insemination (IUI) with cryopreserved sperm[5]. The technique of human sperm cryopreservation was established in 1960s. It was further developed to improve sperm quality in cryopreserved sperm. Because sperm is small with minimal cytoplasm, theoretically sperm cell damaging from freezing is lower than oocytes or embryos. However, during the cryopreserving process, an osmotic effect of either freezing or thawing considerably lower the fertilizing capacity of the spermatozoa by damaging cell membrane and by severely impairing sperm motility. This created morphologic alterations and caused damage to acrosome, functional and structural integrity[6]. Currently, several methods of sperm cryopreservation such as cryopreserving sperm in vapor of liquid nitrogen[7], in liquid phases of liquid nitrogen[7], slow programmable freezing or rapid freezing are available[8,9]. The recovery of motility is relatively low[10], with typically less than 60% of its fresh semen[3,13]. Nevertheless, the frozen-thawed method of semen to optimize sperm recovery has no standard method for cryopreservation of spermatozoa. The causes in loss of post-thawed survival sperm may be due to multiple factors such as intracellular ice crystal formation, high concentration of a cryoprotective agent that effect to cellular dehydration, osmotic injury and alteration of membrane permeability, chemical toxins from a cryoprotective agent during frozen-thaw process[12-14].

Embryo cryopreservation by slow programmable freezing is the standard method which is usually used in infertility and ART unit. Its advantages are giving high validity results and the temperature can be programmed and adjusted, but its disadvantage are spending a longer time freezing, much more embryological experience, complex equipment, which is expensive and needs to be well maintained. The same as sperm freezing, studies suggest slow programmable freezing caused lower change in sperm morphology than sperm freezing in vapor from liquid nitrogen[14,15].

Ultra rapid freezing is a method allowing for a rapid decrease in temperature for freezing sperm. It is easy to be worked on, less time consuming, of low cost and does not need highly experienced embryologist; however, higher concentrations of a cryoprotective agent can slow programmable freezing, but lower than vitrification needed.

Nowadays, sperm cryopreservation has been used widely and routinely in ART centers, but none is the best method for cryopreservation. The slow programmable freezing technique is the conventional method that was widely used in most ART centers. Previous studies showed that the results of sperm parameters from the frozen-thaw process by a slow programmable technique was better than the rapid freezing technique[16-18]; but a recent study showed that their in-house, rapid freezing method gave superior post-thawed motility and cryosurvival rates than a slow programmable freezing method[9]. Today, ART of Ramathibodi Hospital uses the slow, programmable freezing method and ultra rapid freezing but no previous study has been performed to compare these two methods for effective evaluation in prepared sperm before freezing.

The aim of this study was to compare post-thawed sperm motility, morphology and DNA integrity between sperm cryopreservation by ultra rapid freezing and the slow programmable freezing methods.

Material and Method

The study protocol was approved by the Ethical Clearance Committee on Human Rights Related to Researches Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. Informed consents from all participants were obtained.

Semen samples were obtained from the patients while attending the Infertility Clinic, Department of the Obstetrics and Gynecology, Faculty of Medicine Ramathibodi Hospital. Thirty-nine men partners consented to the use of their semen for research, started from June 2009 to September 2009. Thirty-seven specimens were completed the study.

Semen collection and assessment

The semen samples were collected by masturbation after 2-7 days abstinence period and ejaculated into a clean wide-mouth plastic container in a separate room close to the semen analysis laboratory, and allowed to liquefy and then immediately evaluated according to the WHO 1999 guideline. Semen volume $\geq$2 ml with sperm concentration of $\geq 20 \times 10^6$ spermatozoa/ml and motility >50% were included in this study.

Semen analysis

Semen parameters were evaluated after complete liquefaction at 37°C for 30-60 minutes. A routine semen analysis was performed using Computer-

Assisted Semen Analysis (CASA) according to the WHO 1999 guidelines to determine concentration and motility. Sperm count and motility were analyzed using the CASA (Hamilton Thorne Research version 12 IVOS, Bevery, MA, USA) by one observer. A 10-µl drop of sample was loaded onto Makler counting chamber (SefiMedical Instruments, Haifa, Israel) and placed on the pre-warmed stage (37°C) of the CASA. Analysis was performed using x10 objective lens on 5 random fields from each sample. The CASA settings were followed according to the manufacturer's standard setting: frames acquired 30; frame rate 60 Hz; minimum contrast 80; minimum cell size 3 pixels; static head size 1.00-2.90; static head intensity 0.60-1.40; and magnification 1.95. The motility defined as the sperm moving with rapid and medium velocity [rapid (grade a): average path velocity (VAP) >25 µm/s, medium (grade b): VAP 5-25 µm/s].

Semen preparation and cryopreservations

Semen preparation was performed with the washing technique at room temperature.

Two ml of FertiCult Flushing medium (FertiPro NV, Beernem, Belgium) was dispensed into a sterile conical-bottomed tube. One ml of semen was gently drop into the tube, then centrifuged at 1,500 rpm (400 g) for 7 minutes. The supernatant was removed and the remaining pellet was re-suspended with 1 ml of FertiCult Flushing medium.

The sperm suspension was mixed with equal volume (1:1) of sperm cryoprotective agent (Spermfreeze; FertiPro NV, Beernem, Belgium) in a cryovial. Frozen sperm was stored at 4°C but was allowed to equilibrate to room temperature before using. The mixture was kept at room temperature for 10 minutes and then was divided into six cryovials (Nunc, Roskilde, Denmark). The two cryovials, 0.5 ml each, were labeled as SL. The other four cryovials, 0.25 ml each, were labeled as UR. Then cryopreservation process was done by slow programmable freezing and ultra rapid freezing method, respectively.

Slow programmable freezing

The two cryovials were clipped to a cane. The cane was loaded into a controlled rate freezer (Planer Kryo 10 series III) and cooled from 25°C to 5°C at rate of -1°C/min. The cooled rate of -10°C/min to -85°C and then plunged into liquid nitrogen for storage.

Ultra rapid freezing

The four cryovials were clipped to a cane. The cane was quickly inserted into liquid nitrogen in the container for 10 minutes, then plunged into liquid nitrogen for storage. As Ramathibodi ultra rapid freezing method. All samples were stored 1 month before thawing and analysis.

Thawing of samples

After 1 month of storage, the samples were thawed. Samples were removed from liquid nitrogen, the caps of the each cryovials was loosened and left to thaw at room temperature for 15-20 minutes. When the samples were totally thawed; the thawed samples were assessed for sperm motility parameter using CASA, for morphology using eosin-methylene blue staining and DNA integrity using TUNEL assay.

Determination of sperm motility

Sample motility and kinetics of movement parameters before freezing and post-thawed samples were analyzed by CASA and Makler counting chamber.

The set-up parameters of the system were as follows: acquisition rate (in hertz), 60; minimum contrast, 80; minimum size 3; low-size gate, 1; high-size gate, 2.9; low-intensity gate, 0.6; high-intensity gate, 1.4; and magnification factor, 1.95. The kinetics of sperm movement parameters measured were as follows: [1] progressive motility (those sperm which exhibit an actual space-gain motility), [2] average path velocity (VAP: the average velocity of sperm movement; cells were counted as exhibiting rapid progressive motility of VAP was >25 µm/s), [3] straight line velocity (VSL: the straight-line distance from the beginning to the end of a sperm track divided by the time taken; µm/s), [4] curvilinear velocity (VCL: a measure of the total distance traveled by a given sperm divided by the time elapsed; µm/s), [5] amplitude of lateral head movement (ALH: the mean width of sperm head oscillation; µm), [6] beat-cross frequency (BCF: the frequency of the sperm head crossing the sperm average path; Hertz), [7] linearity (LIN: the linearity of a curvilinear path; ratio of VSL/VCL), [8] straightness (STR: the linearity of a average path; ratio of VSL/VAP).

Determination of sperm concentration

Sperm concentration parameters of before freezing and post-thawed samples were analyzed by CASA and Makler counting chamber.

Determination of sperm morphology

Approximately 10 µl of fresh and thawed sperm samples were placed in the glass slide after had been

cleaned through with 70% ethanol before use and spread out. The slides were stained with eosin and methylene blue. Two hundred spermatozoa were evaluated morphologically by x100 oil-immersion brightened-field objective lens by two observers for every slide for morphology according to Kruger strict criteria.

Determination of sperm viability

Eosin-Y test: Sperm viability was assesses by staining with 0.5% eosin-Y test. After 1-2 minutes, 100 spermatozoa were counted as stained (dead) or unstained (viable).

Determination of sperm DNA integrity by TUNEL Assay

Determination of DNA integrity used In Situ Nick-end Labeling (TUNEL) assay by In Situ Cell Death Detection Kit, POD (Cat. No. 11 684 817 910, Roche, Thailand). Sperm samples were centrifuged at room temperature for 10 minutes at 1900 rpm. The supernatant was discarded, and the remaining pellet was washed twice in phosphate-buffered saline (PBS), pH 7.4 (Invitrogen corporation, Scotland, United Kingdom). All specimens were evaluated in the same technique. The process composed of:

Sperm head fixation and decondensation:

For sperm nuclei fixation, slides were incubated in Carnoy's solution [methanol: acetic acid (3:1)] for 20 minutes. The slides were washed twice for 5 minutes with PBS containing 1% Tween 20 [phosphate buffer detergent (PBD)] to permeate the cells. Fixed spermatozoa were incubated in 2xSSC, pH 7 at 37°C for 30 minutes and then washed twice in PBD for 5 minutes. For decondensation, slides were incubated in NaOH 1 N, at room temperature for 1 minute and 30 seconds. Slides were washed twice in 2xSSC, pH 7, for 5 minutes, to increase the stringency. Then, they were dehydrated through an ethanol series (70-90-100%) and air-dried.

Sperm nuclear DNA labeling:

The contents of the kit contains 5 blue vials of enzyme solution which was terminal deoxynucleotidyl transferase from calf thymus (EC 2.7.7.31), recombinant in *E. coli*, in storage buffer 50 µL/vial and 5 violet vials of label solution which was nucleotide mixture in reaction buffer 550 µL/vial. The first step was preparation TUNEL reaction mixture. One hundred µL of label solution was removed for two negative controls. And then add total volume (50 µL) of enzyme solution to the remaining 450 µL label solution to obtain 500 µL TUNEL reaction mixtures. Finally, mix well to equilibrate components. The TUNEL reaction mixture should be prepared immediately before use and should not be stored. TUNEL reaction mixture was kept on ice until use. Two negative controls and a positive control should be included in each experimental set up. For negative control, incubate fixed and permeabilized cells in 50 µL well label solution (without terminal transferase) instead of TUNEL reaction mixture. For positive control, incubate fixed and permeabilized cells with micrococcal nuclease or DNase I recombinant, grade I (3,000 U/ml-3 U/ml in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mg/ml BSA) for 10 min at 15-25°C to induce DNA strand breaks, prior to labeling procedures. After preparation of TUNEL reaction mixture, the step action of labeling was followed by: [1] Rinse slides twice with PBS, [2] Dry area around sample, [3] Add 50 µL TUNEL reaction mixture on sample. (For the negative control, add 50 µL label solution each). To ensure a homogeneous spread of TUNEL reaction mixture across cell monolayer and to avoid evaporative loss, samples should be covered with parafilm or coverslip during incubation, [4] Add lid and incubate for 60 minutes at 37°C in a humidified atmosphere in the dark area, [5] Rinse slide 3 times with PBS, [6] Add propidium iodide solution on sample for 1 minute for detected normal DNA, [7] Rinse slide 3 times with PBS, [8] Dehydrate smear slide through an ethanol series (70-90-100%) and air-dried (Caution: from step 4-8 must be made in the dark area for prevent colored label was fading), [9] Samples can be analyzed in a drop of PBS under a fluorescence microscope at this state. Use an excitation wave length in the range of 450-500 nm and detection in the range of 515-565 nm (green).

Assessment of DNA damage under fluorescence microscopy:

Evaluation of sperm DNA damage was performed by one investigator using an Axioplan Ziess (Gen, Germany) photomicroscope equipped with epifluorescent light and appropriate filters. Single sperm head were examined at x1,000 magnification and each was recorded either for green staining on the sperm head (positive), or red staining (negative). Two hundred spermatozoa were assessed for each subject, and the proportion of sperm cells with positive staining was calculated. For positive control, sperm cells were pretreated with 0.1 IU DNase 1 (Pharmacia LKB Biotech) per slide for 1 hour at 37°C in reaction buffer containing

500 µL 1 M Tris-HCl pH 7.5, 100 µL 1 M $MgCl_2$, 10 µL 5% BSA, 9,390 µL of DD H_2O. For negative control, the enzyme terminal transferase was omitted from reaction solution.

Statistical analysis

Statistical analysis was performed using the computer program STATA version 11 (State corp, TX, USA). The mean and standard deviation (SD) or median and range were used to describe continuous data. Data with normal distribution were analyzed by paired Student's t-test. Data without normal distribution were analyzed by sign test. The *p*-value <0.05 was considered as statistical significance.

Results

Thirty-nine specimens were included in this study from patients attending infertility clinic, one was excluded due to loss of a cryovial into slow programmable freezer and other was loss when storage. Before freezing, the mean (SD) sperm count was 124.6 (66.1) x10^6/ml. The mean (SD) of progressive motility was 70.4 (9.0)% and of normal morphology according to Kruger strict criteria was 14.6 (5.5)% (Table 1).

Effect of cryopreservation by ultra rapid freezing and slow programmable freezing on sperm motility

After the frozen-thawed process of human sperm by slow programmable freezing, mean (SD) sperm progressive motility decreased to 29.1 (12.3)%, which represents a decline of 57.9 (19.6)% from baseline. While cryopreservation by ultra rapid freezing, mean (SD) sperm progressive motility decreased to 19.7 (9.8)% which was 71.9 (14.3)% of reduction. Ultra rapid freezing method had significant greater reduction in sperm motility than slow programmable freezing method ($p<0.001$). Both cryopreservation methods showed significant decreased in VAP, VSL, VCL, BCF and LIN from before freezing method ($p<0.05$) (Table 2).

Table 1. Baseline sperm parameters

Sperm parameters	Mean ± SD
Volume (ml)	3.2±1.3
Concentration (10^6/ml)	124.6±66.1
Motility (%)	70.4±9.0
VAP	67.2±8.7
VSL	57.0±8.4
VCL	105.6±13.1
ALH	4.5±0.7
BCF	30.5±2.4
STR	82.2±3.7
LIN	53.9±5.8
Morphology (% of normal morphology)	14.6±5.5
Viability (%)	74.2±9.9
DNA integrity (%)	94.6±3.5

VAP = average path velocity; VSL = straight line velocity; VCL = curvilinear velocity; ALH = amplitude of lateral head movement; BCF = beat-cross frequency; LIN = linearity; STR = straightness

Effect of cryopreservation by ultra rapid freezing and slow programmable freezing on sperm morphology

The mean (SD) percentage of normal sperm morphology before freezing was 14.6 (5.5)%. After the frozen-thaw process by slow programmable freezing, mean (SD) of normal sperm morphology decreased to 8.4 (6.5)%. In addition, the mean (SD) percentage of normal sperm morphology by ultra rapid freezing was reduced in the same direction to 8.9 (5.8)%. There were no statistically significant difference between the two methods ($p = 0.265$) (Table 3).

Effect of cryopreservation by ultra rapid freezing and slow programmable freezing on sperm DNA integrity

The mean (SD) of sperm DNA integrity in both cryopreservation method groups were decreased significantly from before freezing by 94.6 (3.5)% to 90.9 (8.1)% in slow programmable freezing and 87.6 (13.4)% in ultra rapid freezing, respectively. There was no significant difference between slow programmable and ultra rapid freezing (p = 0.205) (Table 3).

Discussion

The present study is an experimental one, to determine the effects of frozen-thaw processes on sperm motility, morphology and DNA integrity in normozoospermic men, compared between slow programmable freezing and ultra rapid freezing methods of human sperm. Thirty-nine specimens were included in this study from patients attending infertility clinic, one was excluded due to loss of a cryovial into slow programmable freezer and the other one during storage. Before freezing, the mean (SD) sperm concentration was 124.6 (66.1) x10^6/ml. The mean (SD) progressive motility was 70.4 (9.0)% and the mean (SD) of normal morphology according to Kruger strict criteria was 14.6 (5.5)%.

Post-thaw sperm motility was affected by frozen-thaw processes in both cryopreservation

Table 2. Comparison of sperm motility parameters between ultrarapid freezing and slow programmable freezing

Sperm parameters	Before freezing	Ultrarapid freezing	Slow programmable freezing
Motility (%)	70.4 (9.0)	19.7 (9.8)[a]	29.1 (12.3)[b,c]
VAP	67.2 (8.7)	48.5 (8.7)[a]	55.7 (9.1)[b,c]
VSL	57.0 (8.4)	40.2 (8.1)[a]	46.5 (8.3)[b,c]
VCL	105.6 (13.1)	84.7 (13.6)[a]	96.2 (16.6)[b,c]
ALH	4.5 (0.7)	4.6 (0.7)	4.9 (0.8)[b]
BCF	30.5 (2.4)	26.0 (3.3)[a]	26.0 (3.0)[b]
STR	82.2 (3.7)	80.2 (4.4)[a]	81.1 (3.7)
LIN	53.9 (5.8)	47.9 (5.3)[a]	49.1 (5.2)[b]

[a] $p<0.05$ ultrarapid freezing compared with before freezing, [b] $p<0.05$ slow programmable freezing compared with before freezing, [c] $p<0.05$ ultrarapid freezing compared with slow programmable freezing

Table 3. Comparison of sperm parameters between ultrarapid freezing and slow programmable freezing

Sperm parameters	Before freezing	Ultrarapid freezing	Slow programmable freezing
Morphology (% of normal morphology)	14.6 (5.5)	8.9 (5.8)[a]	8.4 (6.5)[b]
DNA integrity (%)	94.6 (3.5)	87.6 (13.4)[a]	90.9 (8.1)[b]

[a] $p<0.05$ ultrarapid freezing compared with before freezing, [b] $p<0.05$ slow programmable freezing compared with before freezing, [c] $p<0.05$ ultrarapid freezing compared with slow programmable freezing

methods. An average reduction in sperm motility was 57.9% after slow programmable freezing [from 70.4 (9.0)% to 29.1 (12.3)%] which was comparable to the results from other studies[7,9,14,19-21]. However, ultra rapid freezing had obvious reduction of sperm motility of 71.9% [from 70.4 (9.0)% to 19.7 (9.8)%], this reduction was statistically significant more than slow programmable freezing method. These results were in the same direction as previous study[8,16].

The reduction of sperm motility after cryopreservation is an important topic of current research. These mechanisms, which decrease sperm motility, are still unclear. This mechanisms may be mechanical, physical or chemical factors. The mitochondrial activity is directly related to the sperm motility. Energy, which is necessary for sperm motility and fertilization, is supplied in the form of ATP partly synthesized through oxidative phosphorylation in the mitochondria[22]. Conventional freezing causes extensive chemical and physical damage to the extracellular and intracellular membranes of sperm that attribute to change in lipid phase transition and/or increased lipid peroxidation during frozen-thawed process. The consequence is the decrease in both sperm velocity and sperm motility[10]. Other possible mechanism for reduction in sperm motility after freezing is an irreversible looping of the sperm flagellum, corresponding to the studies by Hammadeh ME et al[17] and O'Connell M et al[22] or adding of cryoprotective agent to semen samples.

Previous study showed that in sperm preparation for intrauterine insemination (IUI), the success rate or pregnancy rate depends on total motile sperm count (TMSC). TMSC greater than 10 millions sperm had higher pregnancy rate and live birth rate after preparation than TMSC less than 10 millions sperm[23]. According to this, sperm preparation by slow programmable freezing method should be better for IUI than our ultra rapid freezing method. However, in ART, which required only 50,000-100,000 sperm per one oocyte, this could have a less effect on outcome.

Vutyavanich T et al reported the cryopreservation techniques caused change in sperm kinematics or severe loss of sperm motility. The most sensitive parameters were the percentage of progressive motile sperm, VAP, VSL and VCL, that were significantly reduced by their in-house rapid freezing technique less than by slow programmable freezing

technique, whereas ALH or LIN were unchanged[9]. In our study, using the same CASA system and analysis, the results were in similar effect of cryoinjury on sperm motility, VAP, VSL and VCL. The percentage of progressive motile sperm, VAP, VSL and VCL that were reduced by ultra rapid freezing technique were greater than by slow programmable freezing technique. It was concluded that the percentage of progressive motile sperm and motility parameters (VAP, VSL and VCL) was useful indicators to detect effects on sperm motility. The difference in the results may be due to longer duration of storage in our study and difference rapid freezing technique and media between the two studies. Previous study that evaluated the effect of long-term storage on cryopreserved human sperm and found that sperm motility was most sensitive parameter with markedly decreased gradually within the first 3 years of storage and sperm density decreased hardly at all[24].

Sperm morphology is frequently used to assess men fertility. Some studies concluded that sperm membrane lipid peroxidation was correlated with abnormal morphology[25] and showed that cryopreservation had some effects[7,14,17] or no effect on normal morphologically sperm[9,15]. In our study, which correlated with previous study, the percentage of morphologically normal sperm according to Kruger strict criteria in post frozen-thaw process samples was not significant difference between the two methods, but significantly decreased after frozen-thaw process. These results may be the effect of cryopreservation and the frozen-thaw process that disrupts the outer and inner membranes, resulting in alteration of sperm morphology such as coiled tails[26-28]. Our study had longer duration of storage than other that could have more alteration of post thaw sperm morphology[7].

Another predictor that crucial to ability of sperm to fertilize with oocyte is sperm DNA integrity[29-31]. Sperm nucleus integrity is very important causes. Recently described, chromatin abnormalities affect sperm quality and men fertility status[32]. The previous study correlated damaged sperm DNA with mutagenic effects[33]. Frozen-thaw process had significant effects on sperm morphology and membrane integrity, and leads to significant chromatin damage. Unfortunately, sperm with DNA damage are still capable of fertilization and mutation defects, which may not become evidence until the embryo has divided to blastocyst stage or the fetus[34-36].

In this study, the effect of cryopreservation on sperm DNA was determined by using In Situ Nick-end Labeling (TUNEL) assay by In Situ Cell Death Detection Kit, POD in array to monitor qualification of sperm chromatin DNA. After sperm nuclear DNA labeling by TUNEL assay, evaluation of sperm DNA damage or sperm chromatin integrity was performed by one investigator using fluorescence microscopy that is limitation of this study. The visual counting method using fluorescence microscope was performed for assessing DNA integrity of 200 spermatozoa. Both cryopreservation techniques, slow programmable freezing and ultra rapid freezing obviously affected on sperm nuclear DNA integrity irrespective of the methods of freezing and may caused by adding of cryoprotective agent to semen samples. This suggests that cryopreserved sperm by ultra rapid freezing technique affecting sperm DNA from cryodamage similar to slow programmable freezing technique.

The results from previous studies of cryopreservation of human sperm by ultra rapid freezing had conflict data. Some of these studies cryopreserved storage of semen samples for a short duration, but in our study, sperm were stored for longer duration approximately 1 month. In 2003, the study on comparison of sperm freezing in oligospermic men between rapid freezing and slow programmable freezing showed no difference result in post-thawed sperm motility[8]. The present study was done in normal semen, the result could not be extrapolated to abnormal semen and these are the intermediate outcomes for infertility treatment that may not reflect pregnancy outcomes.

In conclusion, cryopreservation of human sperm for 1 month significantly decreased sperm motility, morphology and DNA integrity in both methods. However, sperm motility decreased more by ultra rapid freezing. Further study should evaluate these effects clinically in IUI or IVF outcomes and in abnormal sperm parameters specimens such as oligozoospermia.

Conclusion

Cryopreservation of human sperm for 1 month significantly decreased sperm motility, morphology and DNA integrity in both methods. However, sperm motility decreased more by ultra rapid freezing.

What is already known on this topic?

Past studies obtained data from other methods than sperm cryopreservation such as vapor nitrogen sperm freezing or vitrification sperm freezing. The different time for sperm freezing before the thawing process from previous studies. Some study did not report on sperm DNA integrity.

What this study adds?

This study had longer time to sperm freezing that before the thawing process, that nearly true using in infertility couple treatment in infertility unit. The techniques of ultra rapid sperm freezing in this study difference from other studies.

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Potential conflicts of interest

None.

References

1. Sanger WG, Olson JH, Sherman JK. Semen cryobanking for men with cancer—criteria change. Fertil Steril 1992; 58: 1024-7.
2. Anger JT, Gilbert BR, Goldstein M. Cryopreservation of sperm: indications, methods and results. J Urol 2003; 170: 1079-84.
3. Morris GJ, Acton E, Avery S. A novel approach to sperm cryopreservation. Hum Reprod 1999; 14: 1013-21.
4. Tournaye H. Surgical sperm recovery for intracytoplasmic sperm injection: which method is to be preferred? Hum Reprod 1999; 14 (Suppl 1): 71-81.
5. Sherman JK. Freezing and freeze-drying of human spermatozoa. Fertil Steril 1954; 5: 357-71.
6. Donnelly ET, Steele EK, McClure N, Lewis SE. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod 2001; 16: 1191-9.
7. Punyatanasakchai P, Sophonsritsuk A, Weerakiet S, Wansumrit S, Chompurat D. Comparison of cryopreserved human sperm in vapor and liquid phases of liquid nitrogen: effect on motility parameters, morphology, and sperm function. Fertil Steril 2008; 90: 1978-82.
8. Schuster TG, Keller LM, Dunn RL, Ohl DA, Smith GD. Ultra-rapid freezing of very low numbers of sperm using cryoloops. Hum Reprod 2003; 18: 788-95.
9. Vutyavanich T, Piromlertamorn W, Nunta S. Rapid freezing versus slow programmable freezing of human spermatozoa. Fertil Steril 2010; 93: 1921-8.
10. Critser JK, Huse-Benda AR, Aaker DV, Arneson BW, Ball GD. Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility. Fertil Steril 1988; 50: 314-20.
11. Isachenko E, Isachenko V, Katkov II, Rahimi G, Schondorf T, Mallmann P, et al. DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. Hum Reprod 2004; 19: 932-9.
12. Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995; 7: 871-91.
13. Thurston LM, Siggins K, Mileham AJ, Watson PF, Holt WV. Identification of amplified restriction fragment length polymorphism markers linked to genes controlling boar sperm viability following cryopreservation. Biol Reprod 2002; 66: 545-54.
14. Hammadeh ME, Dehn C, Hippach M, Zeginiadou T, Stieber M, Georg T, et al. Comparison between computerized slow-stage and static liquid nitrogen vapour freezing methods with respect to the deleterious effect on chromatin and morphology of spermatozoa from fertile and subfertile men. Int J Androl 2001; 24: 66-72.
15. Petyim S, Choavaratana R. Cryodamage on sperm chromatin according to different freezing methods, assessed by AO test. J Med Assoc Thai 2006; 89: 306-13.
16. Rota A, Rota A, Martini M, Milani C, Romagnoli S. Evaluation of dog semen quality after slow (biological freezer) or rapid (nitrogen vapours) freezing. Reprod Nutr Dev 2005; 45: 29-37.
17. Hammadeh ME, Szarvasy D, Zeginiadou T, Rosenbaum P, Georg T, Schmidt W. Evaluation of cryoinjury of spermatozoa after slow (programmed biological freezer) or rapid (liquid nitrogen vapour) freeze-thawing techniques. J Assist Reprod Genet 2001; 18: 364-70.
18. Di Matteo O, Langellotti AL, Masullo P, Sansone G. Cryopreservation of the Mediterranean mussel (Mytilus galloprovincialis) spermatozoa. Cryobiology 2009; 58: 145-50.
19. World Health Organiztion. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge: Cambridge University Press; 1999.
20. Kruger TF, Menkveld R, Stander FS, Lombard CJ, Van der Merwe JP, van Zyl JA, et al. Sperm morphologic features as a prognostic factor in in vitro fertilization. Fertil Steril 1986; 46: 1118-23.
21. Donnelly ET, McClure N, Lewis SE. Cryopreservation of human semen and prepared

sperm: effects on motility parameters and DNA integrity. Fertil Steril 2001; 76: 892-900.
22. O'Connell M, McClure N, Lewis SE. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod 2002; 17: 704-9.
23. Van Voorhis BJ, Barnett M, Sparks AE, Syrop CH, Rosenthal G, Dawson J. Effect of the total motile sperm count on the efficacy and cost-effectiveness of intrauterine insemination and in vitro fertilization. Fertil Steril 2001; 75: 661-8.
24. Bolten M, Weissbach L, Kaden R. Cryopreserved human sperm deposits: usability after decades of storage. Urologe A 2005; 44: 904-8.
25. Hammadeh ME, Askari AS, Georg T, Rosenbaum P, Schmidt W. Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men. Int J Androl 1999; 22: 155-62.
26. Check ML, Check JH, Long R. Detrimental effects of cryopreservation on the structural and functional integrity of the sperm membrane. Arch Androl 1991; 27: 155-60.
27. Woolley DM, Richardson DW. Ultrastructural injury to human spermatozoa after freezing and thawing. J Reprod Fertil 1978; 53: 389-94.
28. Holt WV. Alternative strategies for the long-term preservation of spermatozoa. Reprod Fertil Dev 1997; 9: 309-19.
29. Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil Steril 1998; 69: 528-32.
30. Gandini L, Lombardo F, Paoli D, Caruso F, Eleuteri P, Leter G, et al. Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. Hum Reprod 2004; 19: 1409-17.
31. Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, et al. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biol Reprod 1998; 59: 1037-46.
32. Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. Fertil Steril 2010; 93: 1027-36.
33. Jacob RA, Kelley DS, Pianalto FS, Swendseid ME, Henning SM, Zhang JZ, et al. Immunocompetence and oxidant defense during ascorbate depletion of healthy men. Am J Clin Nutr 1991; 54 (6 Suppl): 1302S-9S.
34. Dumoulin JC, Coonen E, Bras M, van Wissen LC, Ignoul-Vanvuchelen R, Bergers-Jansen JM, et al. Comparison of in-vitro development of embryos originating from either conventional in-vitro fertilization or intracytoplasmic sperm injection. Hum Reprod 2000; 15: 402-9.
35. Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. Fertil Steril 2004; 82: 378-83.
36. Shoukir Y, Chardonnens D, Campana A, Sakkas D. Blastocyst development from supernumerary embryos after intracytoplasmic sperm injection: a paternal influence? Hum Reprod 1998; 13: 1632-7.

การศึกษาผลการแช่แข็งอสุจิมนุษย์โดยวิธีการแช่แข็งแบบเร็วมากเปรียบเทียบกับวิธีการลดอุณหภูมิอย่างช้า ๆ โดยโปรแกรมคอมพิวเตอร์ต่อการเคลื่อนที่ รูปร่าง และความสมบูรณ์ของสารพันธุกรรมของอสุจิ

ปัทมา ทองดี, มัชชุพร สุขประเสริฐ, ชลธิชา สถิรพจน์, แอนนา วงศ์กุหลาบ, วิชาญ โชคธนะศิริ

ภูมิหลัง: การแช่แข็งอสุจิเป็นวิธีการเก็บอสุจิในผู้ชายที่มีภาวะมีบุตรยาก ซึ่งการแช่แข็งโดยวิธีการลดอุณหภูมิอย่างช้า ๆ โดยโปรแกรมคอมพิวเตอร์มีผลต่อรูปร่างของอสุจิน้อยกว่าการแช่แข็งโดยวิธีใช้ไอไนโตรเจน วิธีการแช่แข็งแบบเร็วมากเป็นวิธีที่ทำง่าย ใช้เวลาน้อย ค่าใช้จ่ายน้อย และอาศัยประสบการณ์การทำน้อย

วัตถุประสงค์: เพื่อเปรียบเทียบผลต่อการเคลื่อนที่ ลักษณะรูปร่าง และความสมบูรณ์ของสารพันธุกรรมของอสุจิที่ละลายหลังจากการแช่แข็งอสุจิมนุษย์ระหว่างวิธีการแช่แข็งแบบเร็วมากเปรียบเทียบกับวิธีการแช่แข็งโดยลดอุณหภูมิลงอย่างช้า ๆ ด้วยโปรแกรมคอมพิวเตอร์

วัสดุและวิธีการ: การวิจัยเชิงทดลอง สถานที่ทำวิจัย ห้องปฏิบัติการของหน่วยรักษาผู้มีบุตรยากและเทคโนโลยีช่วยการเจริญพันธุ์ ภาควิชาสูติศาสตร-นรีเวชวิทยา คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี กลุ่มตัวอย่าง น้ำอสุจิ 37 ตัวอย่าง ที่มีปริมาตร จำนวนอสุจิ และการเคลื่อนที่ของอสุจิปกติตามเกณฑ์ WHO 1999 (ปริมาตร ≥2 มิลลิลิตร ความเข้มข้น ≥20 ล้านตัว/มิลลิลิตร และการเคลื่อนที่ของอสุจิ ≥50 เปอร์เซ็นต์) โดยนำน้ำอสุจิมาล้างแล้วแบ่งน้ำอสุจิแต่ละตัวอย่างเป็น 6 หลอด 2 หลอดบรรจุน้ำอสุจิหลอดละ 0.5 มิลลิลิตร ถูกแช่แข็งโดยวิธีลดอุณหภูมิลงอย่างช้า ๆ ด้วยโปรแกรมคอมพิวเตอร์ หลอดที่เหลือบรรจุน้ำอสุจิ 0.25 มิลลิลิตร 4 หลอด ถูกแช่แข็งด้วยวิธีการแช่แข็งแบบเร็วมาก หลังจากแช่แข็งเป็นระยะเวลา 1 เดือน นำมาละลายโดยการวางไว้ที่อุณหภูมิห้อง ตัววัดที่สำคัญ คือ จำนวนอสุจิที่มีการเคลื่อนที่ ตรวจด้วย Computer-Assisted Semen Analysis (CASA) ลักษณะรูปร่างของอสุจิย้อมด้วยสี eosin และ methylene blue และความสมบูรณ์ของสารพันธุกรรมทำการตรวจด้วย TUNEL assay

ผลการศึกษา: การเคลื่อนที่ของอสุจิมีการลดลงอย่างมีนัยสำคัญทางสถิติโดยทั้ง 2 วิธี จาก 70.4 (9.0) เปอร์เซ็นต์ เป็น 29.1 (12.3) เปอร์เซ็นต์ ในวิธีการแช่แข็งโดยลดอุณหภูมิลงอย่างช้า ๆ ด้วยโปรแกรมคอมพิวเตอร์และเป็น 19.7 (9.8) เปอร์เซ็นต์ ในวิธีการแช่แข็งแบบเร็วมาก ($p<0.05$) การเคลื่อนที่ของอสุจิมีการลดลงมากกว่าอย่างมีนัยสำคัญโดยวิธีการแช่แข็งแบบเร็วมาก ($p<0.001$) เปอร์เซ็นต์ของลักษณะรูปร่างของอสุจิปกติ และความสมบูรณ์ของสารพันธุกรรมลดลงอย่างมีนัยสำคัญทางสถิติโดยทั้ง 2 วิธี แต่อย่างไรก็ตามไม่พบความแตกต่างอย่างมีนัยสำคัญจากทั้ง 2 วิธี ($p>0.05$)

สรุป: การแช่แข็งอสุจิมนุษย์เป็นระยะเวลา 1 เดือน ทั้ง 2 วิธี ทำให้มีการลดลงของการเคลื่อนที่ของอสุจิ ลักษณะรูปร่างและความสมบูรณ์ของสารพันธุกรรมของอสุจิอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับก่อนการแช่แข็ง แต่การแช่แข็งแบบเร็วมากมีการลดลงของการเคลื่อนที่ของอสุจิมากกว่าอย่างมีนัยสำคัญ