Freezing Effect on Post-Thawed Sperm Characteristics Especially Sperm DNA Integrity Comparing between Liquid Nitrogen Vapour and Computerized Program Freezer

Somsin Petyim, M.D., Roungsin Choavaratana, M.D., Somboon Kunathikom, M.D., Pitak Laokirkkiat, M.D., Japarath Prechapanich, M.D.

Department of Obstetrics & Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

ABSTRACT

The aim of the study was to evaluate the cryodamage effects on human sperm characteristics, especially on sperm DNA integrity, after 6 months of freezing comparing between using liquid nitrogen vapour (LNV) and using computerized program freezer (CPF). Forty normal semen samples were collected for semen analysis. Each sample was mixed with cryoprotective media and divided into 2 straws. The first straw was frozen with LNV and the second one with CPF. After 6 months of cryostorage, semen samples were thawed, and sperm chromatin integrity as well as sperm motility, morphology, vitality and cryosurvival rate were determined. Percentages of DNA damage were higher ($p<0.01$) following freezing with LNV than with CPF. Sperm vitality was greater ($p<0.05$) after CPF than after LNV, as well as cryosurvival rate ($p<0.001$). Post-thawed sperm motility was greater after CPF than after LNV, either in grade A ($p<0.001$) or in grade B ($p<0.05$). No significant difference was observed in the percentage of normal sperm morphology comparing the two freezing methods. The current study demonstrated a post-thawed decrease in sperm DNA integrity as well as other sperm characteristics after freezing in both methods. The CPF significantly provided superior results in post-thawed sperm DNA integrity, sperm motility and vitality than LNV did. In case of 6 months of cryostorage, therefore, we recommend the computerized program freezer as a preference for sperm cryopreservation.

Keywords: Cryopreservation, computerized program freezer, liquid nitrogen vapour, sperm characteristics, sperm DNA integrity

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The cryopreservation of human sperm is now taking an important part to increase the successfulness in the treatment of infertility. The goals of cryopreservation is to meet patient's convenience in providing assisted fertilization such as, intrauterine insemination, *in vitro* fertilization, intracytoplasmic sperm injection with a different degree of oligoasthenoteratozoospermia, and in preserving reproductive capacity in men with various types of neoplasias before undergoing radical surgery or chemotherapy. It also supplies banking for donor sperm. Currently, sexually transmitted disease such as HIV, hepatitis B, or syphilis has been widely spread, so the donor semen must be managed to avoid HIV transmission. In this case, the cryopreservation for at least 6 months is a standard procedure used to overcome the window period of HIV infection in the donor semen before utilization.

Until now, many technical aspects of freezing and thawing process, including the preparation of cryopreservation media have been refined over the years[1]. However, during the cryopreserving process which includes the addition and the removal of cryoprotectants, spermatozoa will undergo tremendous chemical and physical insults such as lethal intracellular ice crystal formation and dissolution, cellular dehydration, osmotic injury, and alteration of membrane permeability[2,3]. The adverse effects of freezing and thawing can lower the fertilizing capacity of the spermatozoa by damaging cell membranes[4] and by severely impairing sperm motility[5], creating morphology alterations such as coiled tails, and by causing the damages to

Correspondence to: Somsin Petyim
E-mail: sispyi@mahidol.ac.th

acrosomes as well as to structural and functional integrity[6]. As described elsewhere, DNA damaged sperm from infertile men was claimed to be associated with infertility and poor outcome in vitro fertilization (IVF) such as embryo fragmentation, arrested embryo and increased spontaneous abortion rate[7]. There are several methods for detecting the quality of sperm chromatin. Acridine orange (AO) staining has been widely accepted as a method for detection of DNA damage in many cell types including human sperm and also for predicting fertilization rates[8]. AO-fluorescence staining appears green when it intercalates as a monomer into native (double stranded and normal) DNA and appears red when it binds to denatured (single stranded) DNA as an aggregate. The staining involves acid treatment of sperm, which dissociates thiols from DNA in order to increase DNA susceptibility to denaturation and as a consequence, AO competes for anionic binding sites by avoiding nonspecific aggregation[9].

Many studies reported detrimental effects of cryopreservation on human sperm[10,11], but the duration of freezing under liquid nitrogen in those studies was less than 6 months. Various methods of cryopreservation including technical aspects of freezing and thawing sperm and of preparing cryopreservation media have also been evaluated for their effects on post thawing sperm quality.[3,12,13] However, the preferred methods for freezing semen for at least 6-month long which suitably optimize sperm chromatin recovery have not been firmly established. The objective of our study, therefore, was to evaluate the cryoinjury on human sperm characteristics especially on chromatin integrity, and sperm motility and cryosurvival rate, after six months of freezing comparing between liquid nitrogen vapour and computerized program freezer.

MATERIALS AND METHODS

Patients

Semen samples were collected from 40 husbands of infertile couples who came to the infertility clinic at Siriraj Hospital for semen analysis by masturbation into sterile containers. Semen analysis had to have sperm concentration exceeding 20 x106 sperms/ml, with greater than 50% of forward motility and more than 15% of normal morphology according to strict Kruger criteria[14] and leucocytes not greater than a million per milliliter. Men who showed active sexual transmitted diseases; HIV, Syphilis, Hepatitis B, Hepatitis C, and active prosatitis or urethritis were excluded from the study.

Semen collection and processing

The semen samples were liquefied at room temperature. Semen analysis was performed within 20-30 minutes after delivery to laboratory. Semen volume, sperm concentration, percentage of sperm motility and viability were measured according to World Health Organization (WHO) guidelines[15]. Sperm morphology was assessed according to strict criteria, and a part of each sample was stained with acridine orange for sperm chromatin assessment. After initial analysis, the sample was mixed with an equal volume of the cryoprotective media (Human sperm preservation medium (HSPM) which contains glycerol, glycin and glucose). The media was added dropwisely into the semen and swirled over 10-15 minutes. Then the mixture was divided into 2 equal parts and each was drawn into a 0.25-ml straw and subjected to 2 different techniques of cryopreservation; liquid nitrogen vapour and computerized program freezer.

The first straw was frozen with liquid nitrogen vapour according to Mahadevan[16]. In brief, the straw was placed in a horizontal position at 15 centimeters above, and parallel to the surface of liquid nitrogen for 30 minutes to cool down to -80 ^{o}C, and thereafter was quickly plunged into liquid nitrogen for cryostorage. The second straw was frozen with computer-program controlled rate freezing (Sidney IVF programme, Freeze control Model CL 836®, Cryologic PTY Ltd., Austraria). Briefly, the straw was placed into the chamber of a computerized program freezing machine. The temperature was reduced stepwise according to the preset program: (i) at cooling rate 6 ^{o}C/min from 24 ^{o}C to 5 ^{o}C, holding the temperature for 5 minutes; (ii) 6 ^{o}C/min from 5 ^{o}C to -8 ^{o}C, holding the temperature for 2 minutes; (iii) 5 ^{o}C/min from -8 ^{o}C to -30 ^{o}C; (iv) 4 ^{o}C/min from -30 ^{o}C to -45 ^{o}C; (v) 25 ^{o}C/min from -45 ^{o}C to -80 ^{o}C, and after all quickly plunged into liquid nitrogen for cryostorage.

After 6 month of cryostorage, both straws were taken out of liquid nitrogen and thawed in room temperature for 10 minutes and in warming chamber (37 ^{o}C) for a continuing 10 minutes. Post-thaw sperm concentration, motility, morphology, vitality, and sperm chromatin were assessed by the same manner as before freezing. The comparison was made between pre-freezing, after freezing with liquid nitrogen vapour and after freezing with computerized program method.

Initial semen analysis

Sperm concentration, motility, and vitality were assessed according to WHO laboratory manual[15]. Sperm morphology was assessed according to Kruger strict criterias. Sperm motility was graded into 4 categories; category A -rapid forward progression spermatozoa, category B -movement with forward motion spermatozoa, category C -motion with no forward progression, category D -no motion[15]. The cryosurvival rate was accordingly calculated as follow 1:

$$\text{Percentage of cryosurvival} = \frac{\text{Post-thaw sperm motility} \times 100}{\text{Pre-freeze sperm motility}}$$

Assessment of chromatin condensation

A smeared slide of semen sample was fixed overnight with freshly prepared Carnoy's solution, comprising glacial acetic acid and absolute methanol as a 1:3 ratio, and was taken out for air-drying and stained in acridine orange at pH 2.5 for 5 minutes at room temperature, in the dark atmosphere. The stained slide was kept dark until evaluated on the same day using the fluorescense microscope, equipped with a 490-nm excitation filter and 530-nm barrier. Totally, 300 spermatozoas were monitored per smear. Normal DNA content exhibited green fluorescense over the head region while any abnormality of DNA content was possibly specified by a spectrum of fluorescence, varying from yellow to red.

Data analysis

The Data was analyzed by student's t-test. Statistical analysis was performed by Computer program SPSS for Microsoft Windows version 10.0 (Chicago, IL). The level of significance was set at $p<0.05$. The results were presented as mean and standard deviation (mean ± SD).

RESULTS

The mean ages of the donor volunteers were 29.5 ±

TABLE 1. Semen characteristics before freezing (mean ± SD).

Parameter	Value
Volume (ml)	1.6 ± 0.6
Count (x10^6)	34.6 ± 13.8
Vitality (%)	77.9 ± 6.1
Normal morphology (%)	20.0 ± 4.8
Motility (%)	51.7 ± 1.6
DNA integrity (%)	90.8 ± 7.5

7.6 years old. The semen characteristics before freezing were summarized in Table 1. All parameters: sperm vitality, normal morphology, motility, vitality, and sperm DNA integrity of the post-thawed sperm significantly decreased when compared with pre-freezing in both freezing methods (Fig 1). Comparing the two freezing methods, the cryo-damage of sperm DNA after computerized freezing was significantly lesser ($p<0.01$) than after vapour freezing (34.8 ± 25.1% vs 44.1 ± 26.6%, respectively). Sperm vitality was significantly greater ($p<0.05$) after computerized freezing than after vapour freezing (-47.3 ± 8.8% vs -44.5 ± 7.6%, respectively) as well as cryosurvival rate ($p<0.001$), (61.7 ± 20.6% vs 44.6 ± 19.4%, respectively) (Table 2). The post-thawed sperm motility was significantly greater ($p<0.001$) after computerized freezing than after vapour freezing (-19.9 ± 11.0 % vs -28.7 ± 10.3%, respectively), either in grade A ($p<0.001$) or in grade B ($p<0.05$) (Table 3).

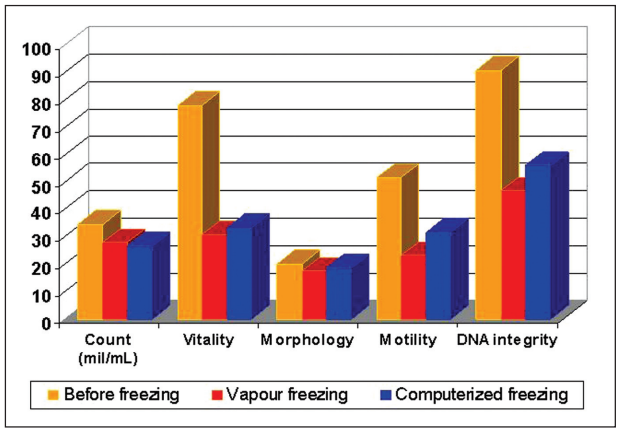

Fig 1. Semen parameters before and after freeze-thawing.

TABLE 2. Semen parameter alteration after freeze-thawing (mean ± SD).

Parameter	Vapour freezing	Computerized freezing	Significance (p value)
Cryodamage (%)	44.1 ± 26.6	34.8 ± 25.1	0.002
Morphology (%)	-2.5 ± 5.1	-1.4 ± 4.9	0.226
Motility (%)	-28.7 ± 10.3	-19.9 ± 11.0	p<0.001
Vitality (%)	-47.3 ± 8.8	-44.5 ± 7.6	0.021
Cryosurvival rate (%) (A+B)	44.6 ± 19.4	61.7 ± 20.6	p<0.001

No significant difference between the two freezing methods was observed in the percentages of normal morphological sperm (Table 4). There was however a significantly higher ($p<0.05$) number of post-thawed sperms with tail defect after freezing with vapour freezing, comparing with after computerized freezing. (0.1 ± 8.5% vs -3.8 ± 7.9%, respectively).

TABLE 3. Effects of freezing method on human sperm motility alteration (mean ± SD).

Motility (%)	Vapour freezing	Computerized freezing	Significance (p value)
Category A	-15.4 ± 5.9	-9.2 ± 7.3	p<0.001
Category B	-13.3 ± 6.3	-10.7 ± 6.6	0.023
Category C	-2.6 ± 4.4	-3.8 ± 5.5	0.201
Category D	32.0 ± 12.8	29.9 ± 15.5	0.28

TABLE 4. Effects of freezing method on human sperm morphology alteration (mean ± SD).

Morphology (%)	Vapour freezing	Computerized freezing	Significance (p value)
Normal	-2.5 ± 5.1	-1.4 ± 4.9	0.226
Head defect	4.7 ± 11.5	5.2 ± 12.1	0.611
Mid piece defect	-1.1 ± 5.8	0.2 ± 5.0	0.064
Tail defect	0.1 ± 8.5	-3.8 ± 7.9	p<0.001
Cytoplasmic droplet	-0.9 ± 3.4	-0.8 ± 3.9	0.948

DISCUSSION

Cryopreservation has been widely used in infertility treatment program as a routine procedure in most infertile clinics. The objective of cryopreservation is to preserve male gametes especially their reproductive potential for future conception. It is also beneficial for azoospermic patients in order to allow donor sperm screening of infection such as HIV, Hepatitis B prior to collection of semen for insemination. The cryopreservation of donor sperms for at least 6 months is routinely performed as a standard method to avoid the window period of infection before insemination. Nowadays, there are many freezing-thawing techniques, but the standard methods for freezing-thawing semen which well optimize sperm recovery have not been firmly established yet. This study was designed to evaluate the cryoinjury on post-thawing human sperm chromatin integrity, as well as on sperm characteristics including sperm motility, vitality, morphology, and survival rate in a population of men with normal semen quality after six months of freezing compared between two different freezing methods; computerized program and ordinary liquid nitrogen vapour freezing method.

Sperm DNA is organized in a specific manner to keep the chromatin in the nucleus compact and stable. During spermiogenesis, the replacement of somatic type histones by protamines leading to highly packaged chromatin[17], and discarding of cytoplasm, causes cessation of transcription and leaves the sperm incapable of undertaking DNA repair[18]. While sperm transit through the epididymis, sperm epididymal maturation implies a final stage of chromatin organization involving protamine cross-linking by disulfide bond formation. Spermiogenesis especially epididymal maturation makes the most condensed eukaryotic sperm DNA and makes high sperm chromatin stability[17]. Several studies showed that the effect of freezing caused chromatin damage, decrease in chromatin instability, and DNA denaturation[10,11,19], which was previously showed to correlate with impairing the fertilizing ability of human sperm[20], and further with mutagenic events[18].

Our study suggested that that cryopreservation of semen caused DNA integrity impairment, irrespective of the cryopreservation method used, corresponding to the previous studies[10,11,18,19]. Moreover, our results clearly indicated that freezing by computerized program could prevent post thawing sperm DNA from cryoinjury better than freezing by liquid nitrogen vapour, regardless of the length of freezing.

Cryopreservion of semen is generally claimed to have impaired sperm motility, normal morphology, vitality and cryosurvival rate, irrespective of the cryopreservation method used[10,11,18]. The results obtained in the present study confirm those findings. On the other hand, the value for post-thawed sperm motility, normal morphology, vitality and cryosurvival rate were significantly higher following the freezing-thawing process with a computerized freezer, compared to freezing with vapour freezing method. The detrimental effect of cryopreservation may be explained in many ways i.e., cryopreservation-induced mitochondrial damage[21], outer and inner sperm membranes disruption and alteration of sperm morphology such as coiled tails[6, 22]. Our study confirmed that cryopreservation had a slightly detrimental effect on normal morphological sperm of which the population of post-thawed sperms with tail defects was higher after freezing with liquid nitrogen vapour than with computerized freezing. Post-thawed sperm motility and cryosurvival rate after computerized freezing, therefore, were significantly higher than in the liquid nitrogen vapour method. Our result also demonstrated that the percentages of post-thawed sperm vitality dramatically decreased after freezing-thawing with both cryopreservation methods. However, freezing with computerized freezer provided less cryodamage effects on sperm vitality when compared to freezing with vapour freezing. The decreased sperm vitality during cryopreservation might be related to sperm membrane disruption. The explanation might be related to the alteration of the sperm membrane's composition such as the reduction in the fluidity of the membrane[6,23].

Although various methods of cryopreservation have been evaluated on post thawing sperm quality, no method has been firmly established as a standard method. Details of the freezing-thawing procedure such as the optimum falling rate of temperature during freezing remains controversial. Several studies reported the results in favor of the fast freezing method[24], or in favor of slow staged cooling method[10-12,25]. On the contrary, some studies showed no difference between two freezing methods[13]. Further, in most of the previous studies, sperms were kept freezing under liquid nitrogen less than 6 months, and also there was no comparative study between various freezing techniques of human sperm cryopreservation especially on DNA integrity. Our study has conclusively confirmed that computerized slow rate freezing provided a good quality of post thawing sperm: chromatin integrity, vitality, motility, cryosurvival rate and normal morphology after 6 months of freezing, compared with vapor freezing. It could also be assumed that the long duration of freezing did not increase the detrimental effect to the sperms. In addition, various types of cryoprotectant media are available in able to protect sperm from cryodamage. Several studies tried to compare the cryoprotective effect of other cryoprotectants, but conflict in the results were reported, irrespective of the freezing-thawing method used[3,12]. Therefore, further studies should be conducted to conclusively prove the effect of cryopreservation on post-thawed sperm such as potential for fertilization and sperm mitochondria include mitochondrial DNA changes compared between various types of freezing methods and cryoprotectants.

Further, to routinely apply any freezing methods in an infertility clinic, the economic consideration of each method such as time-consumed and the cost of instruments should be made. The duration as well as the cost for freezing with computerized freezer was higher than with nitrogen vapour. However, from our study, the computerized freezer offered the better quantitative and qualitative outcomes of post-thawing sperm, including a high number of survival sperms, normal structural integrity and possibly improved fertilization potential especially for donor semen. Hence, the computerized program freezer should not be discarded but instead may be considered as a procedure for keeping such a valuable sperm.

CONCLUSION

The current study demonstrated a post-thawed decrease in sperm chromatin integrity as well as sperm motility, cryosurvival rate, vitality and a number of normal morphological sperms after freezing in both cryopreservation methods. However, the computerized freezing method significantly provided superior results in post-thawed sperm DNA integrity, sperm motility and vitality than liquid nitrogen vapour did, in the case of 6 months of cryostorage, especially in donor semen. Therefore, we recommend the computerized program freezer as a preference for sperm cryopreservation.

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