Cryodamage on Sperm Chromatin According to Different Freezing Methods, Assessed by AO Test

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Objective: *To evaluate cryodamage effects on human sperm chromatin, motility and cryosurvival rate after freeze-thawing, compared between liquid nitrogen vapour and computerized program freezer, assessed by acridine orange staining method (AO test).*
Material and Method: *Fifty semen samples were used. After semen analysis, each semen sample was mixed with cryoprotective media and divided into 2 straws. The first straw was frozen with liquid nitrogen vapour and the second with computerized program freezer. After 1 month of cryostorage, semen samples were thawed. Sperm chromatin integrity, motility, morphology, vitality and sperm cryosurvival rate were determined.*
Results: *DNA damage was significantly greater ($p < 0.001$) following freezing with liquid nitrogen vapour than with computerized program freezer. Furthermore, the computerized program freezing method significantly provided superior post-thaw sperm motility, vitality and cryosurvival rate, compared with the liquid nitrogen vapour freezing method.*
Conclusion: *Computerized program freezing is recommended as a first choice method for routine cryostorage.*

Keywords: *Liquid nitrogen vapour, Computerized program freezer, Cryopreservation, Sperm DNA integrity*

J Med Assoc Thai 2006; 89 (3): 306-13
Full text. e-Journal: *http://www.medassocthai.org/journal*

Since the establishment of technique for human sperm cryopreservation in 1960s, it has been refined and is currently performed in most assisted reproduction centers on a routine basis. However, during the cryopreserving process, osmotic effects of either freezing or thawing considerably lower the fertilizing capacity of the spermatozoa by damaging cell membranes[1] and by severely impairing sperm motility[2]. This creates morphology alterations such as coiled tails, and causing damage to acrosome as well as structural and functional integrity[3,4]. Spermatozoa further undergo tremendous chemical and physical abuse such as lethal intracellular ice crystal formation and dissolution, cellular dehydration, osmotic injury, and alteration of membrane permeability in the addition and removal of cryoprotectants during the freeze-thawing process[5-7]. Due to this problems, the technical aspects of freezing and thawing sperm, including the preparation of cryopreservation media have been refined over the years[8,9]. Nevertheless, the methods for freezing and thawing semen to optimize sperm chromatin recovery have not been established and, there is no standard method for cryopreservation of spermatozoa.

Interest in assessing the chromatin quality of human sperm has increased since DNA damage in sperm from infertile men was found to be associated with infertility[10,11]. No pregnancy was observed with ejaculation containing heavy loads of DNA damaged sperm[12]. The study by Virant-Klun et al (2002)[13] also reported that infertile patients with unsuccessful *in vitro* fertilization (IVF) had 65% of sperms with single-stranded DNA as detected by AO staining. The IVF results for those patients showed more embryo fragmentation, arrested embryonic development in spite of prolonged culture and increased spontaneous abortion rate.

Various methods of cryopreservation have been evaluated for their effects on sperm motility and

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sperm chromatin[14-16]. Staining of cells with acridine orange (AO) has been widely accepted as a predictor of DNA damage in many cell types including human sperm and fertilization rates[10,17]. AO fluorescence-staining appears when it intercalates as a monomer into native DNA (double stranded and normal) and appears red when it binds to denatured DNA (single stranded) as an aggregate. AO staining involves acid treatment (low pH) of sperm. This process dissociates thiols from DNA and increases DNA susceptibility to denaturation. Consequently AO competes for anionic binding sites by avoiding nonspecific aggregation[18,19].

Though sperm cryopreservation has been widely and routinely used, the consequences of cryo-preservation on the structure of sperm chromatin remain unclear. The objective of the present study, was to evaluate the cryodamage effects on human sperm chromatin, sperm motility and sperm cryosurvival rate after freeze-thawing, comparing between computerized program freezer and liquid nitrogen vapour, assessed by acridine orange (AO) staining method.

Material and Method

The present study was an experimental study performed at the infertility clinic, Siriraj Hospital, Bangkok, Thailand from January to September 2005. The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Siriraj hospital.

Patients

Semen samples were collected from 50 husbands of infertile couples who came to the infertility clinic, Siriraj Hospital for semen analysis by masturbation into sterile containers. Semen analysis had to have sperm concentrations exceeding 20 millions per milliliter, with greater than 50 percent of forward motility and than 15 percent of normal morphology according to strict Kruger criteria[20] 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 present study.

Semen collection and processing

The semen samples were liquefied at room temperature. Semen analysis was performed within 20-30 minutes after delivery to the laboratory. Semen volume, sperm concentration, percentage of sperm motility and viability were measured according to the World Health Organization (WHO) guideline[21]. Sperm morphology was assessed according to strict criteria[20,22], and a part of the samples was stained with acridine orange for sperm chromatin assessment. After initial analysis, the sample was mixed with equal volume of cryoprotective media. (Human sperm preservation medium (HSPM) which contains glycerol, glycin and glucose)[23] The media was added into the semen and swirled over 10-15 minutes. The mixture was divided into 2 parts equally and each was drawn into a 0.25 ml straw and subjected to one of the two techniques of cryopreservation; liquid nitrogen vapour or computerized program freezer.

The first straw was frozen with liquid nitrogen vapour according to Mahadevan (1983)[23,24]. In brief, the straw was placed in horizontal position at 15 centimeters above, and parallel to the surface of liquid nitrogen for 30 minutes to cool down to -80 C, and thereafter was quickly plunged into liquid nitrogen for cryostorage. The second straw was frozen using computer-program controlled freezing (Sidney IVF programme, Freeze control Model CL 836 , Cryologic PTY Ltd., Australia). Briefly, the straw was placed into the chamber of a computerized program freezing machine. Temperature was reduced stepwise according to the preset program: (i) at cooling rate 6 C/min from 24 C to 5 C, holding the temperature for 5 minutes; (ii) 6 C/min from 5 C to -8 C, holding the temperature for 2 minutes; (iii) 5 C/min from -8 C to -30 C; (iv) 4 C/min from -30 C to -45 C; (v) 25 C/min from -45 C to -80 C, and then, quickly plunged into liquid nitrogen for cryostorage.

Following 1 month of cryostorage, both straws were taken out of liquid nitrogen and thawed in room temperature for 10 minutes and in a warming chamber (37 C) for another 10 minutes. Post-thaw sperm concentration, motility, morphology, vitality, and sperm chromatin were assessed the same way as before freezing. The comparison was made between pre-freezing and post-thaw with liquid nitrogen vapour and post-thaw with the computerized program method.

Initial semen analysis

Sperm concentration, motility, vitality were assessed according to the WHO laboratory manual[21]. Sperm morphology was assessed according to Kruger strict criteria[20-22]. 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[21]. The cryosurvival rate was accordingly calculated as follows[25,26]:

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

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 1:3 ratios, and then was taken out to be air-dried and stained in acridine orange at pH 2.5 for 5 minutes at room temperature, in a dark atmosphere. The stained slide was kept until evaluated on the same day using the fluorescence microscope, equipped with 490 nm excitation filter and 530 nm barrier. Totally, 300 spermatozoas were monitored per smear. Normal DNA content exhibited green fluorescence over the head region while any abnormality of DNA content was possibly specified by a spectrum of fluorescence, varying from yellow to red.

$$\text{Sperm cryodamage (\%)} = \text{Post-thaw DNA integrity (\%)} - \text{Pre-freeze DNA integrity (\%)}$$

Data analysis

The data was analyzed by paired 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 $\pm$ SD).

Results

Age of the donor volunteers was 30.02 $\pm$ 4.16 years old in average. The semen characteristics before freezing presented normal as shown in Table 1.

The data in Table 2 shows significant decrease of post-thaw sperm DNA integrity, sperm motility, vitality, and normal morphology, compared with pre-freeze, in both cryopreservation methods used. In contrast, the number of normal morphological spermatozoa after freeze-thawing was not different from before freezing. Furthermore, no significant difference was observed in the percentage of normal sperm morphological when comparing the two freezing methods (-2.34 $\pm$ 5.04% vs -1.73 $\pm$ 4.39% in vapour freezing and computerized freezing, respectively). Table 3 shows that the cryo-damage of DNA after vapour freezing was significantly greater ($p < 0.001$) than after computerized freezing (24.79 $\pm$ 14.23% vs 8.92 $\pm$ 8.81%, respectively). Post-thaw sperm motility was significantly lower ($p < 0.001$) in vapour freezing than in computerized freezing (-22.23 $\pm$ 10.08% vs -17.93 $\pm$ 11.13%, respectively), either in grade A ($p < 0.05$) or in grade B ($p < 0.05$) (Table 4). Correspondingly, cryosurvival rate after vapour freezing was significantly lesser ($p < 0.001$) than after computerized freezing (60.75 $\pm$ 18.11% vs 68.31 $\pm$ 19.90%, respectively) as well as post-thaw vitality ($p < 0.01$) (-25.21 $\pm$ 10.71% vs -21.16 $\pm$ 11.81%, respectively).

No significant difference in post-thaw normal morphologically sperm was observed compared between vapour freezing and computerized freezing methods (Table 5). There was, however, a significantly higher ($p < 0.05$) number of post-thaw sperms with tail defect following freezing with vapour freezing, compared with computerized freezing. (4.79 $\pm$ 6.51% vs 3.43 $\pm$ 6.59%, respectively)

Table 1. Semen characteristics before freezing (mean $\pm$ SD) (N = 50)

Parameter	Value
Volume (ml)	2.12 $\pm$ 4.5
Count (x10^6)	37.13 $\pm$ 16.62
Vitality (%)	75.18 $\pm$ 8.05
Normal morphology (%)	23.70 $\pm$ 3.63
Motility (%)	57.00 $\pm$ 4.04
DNA integrity (%)	88.02 $\pm$ 8.89

Table 2. Semen parameters before and after freeze-thawing (mean $\pm$ SD)

Parameter	Before freezing	Vapour freezing	Computerized freezing
Count (x10^6)	37.13 $\pm$ 16.62	23.13 $\pm$ 7.34	23.5 $\pm$ 7.45
Vitality (%)	75.18 $\pm$ 8.05	49.96 $\pm$ 7.50	54.02 $\pm$ 8.44
Normal morphology (%)	23.70 $\pm$ 3.63	21.36 $\pm$ 4.97	21.96 $\pm$ 4.16
Motility (%)	57.00 $\pm$ 4.04	34.77 $\pm$ 11.09	39.07 $\pm$ 12.09
DNA integrity (%)	88.02 $\pm$ 8.89	63.94 $\pm$ 15.15	79.09 $\pm$ 10.34

Table 3. Semen parameter alteration after freeze-thawing (mean ± SD)

Parameter	Vapour freezing	Computerized freezing	Significance (p value)
Cryodamage (%)	24.79 ± 14.23	8.92 ± 8.81	p<0.001
Morphology (%)	-2.34 ± 5.04	-1.73 ± 4.39	NS
Motility (%)	-22.23 ± 10.08	-17.93 ± 11.13	p<0.001
Vitality (%)	-25.21 ± 10.71	-21.16 ± 11.81	p<0.01
Cryosurvival rate (%) (A + B)	60.75 ± 18.11	68.31 ± 19.90	p<0.001
Cryosurvival rate (%) (A + B + C)	63.28 ± 16.76	66.81 ± 16.08	NS

NS = Not significance

Table 4. Effects of freezing method on human sperm motility alteration (mean ± SD)

Motility (%)	Vapour freezing	Computerized freezing	Significance (p value)
category A	-16.69 ± 6.35	-15.29 ± 7.14	p<0.05
category B	-5.54 ± 10.11	-2.64 ± 10.62	p<0.05
category C	-5.77 ± 7.60	-7.43 ± 6.79	NS
category D	28.00 ± 12.95	25.36 ± 12.58	NS

Table 5. Effects of freezing method on human sperm morphology alteration (mean ± SD)

Morphology (%)	Vapour freezing	Computerized freezing	Significance (p value)
Normal	-2.34 ± 5.04	-1.73 ± 4.39	NS
Head defect	1.50 ± 7.59	2.48 ± 7.01	NS
Mid piece defect	-2.71 ± 5.39	-2.93 ± 5.16	NS
Tail defect	4.79 ± 6.51	3.43 ± 6.59	p<0.05
Cytoplasmic droplet	-1.23 ± 1.36	-1.25 ± 1.21	NS

Discussion

The goal of cryopreservation is to obtain a high number of post-thawing survival normal sperms which maintain structural integrity, viability and fertilization potential as before freezing[16]. Currently, the standard methods for freezing-thawing semen which well optimize sperm recovery have not been firmly established yet. There are many factors during cryopreservation process can affect the post-thawing outcome including freezing method, cooling rate, thawing method and type of cryopreservative media. Various methods of cryopreservation including technical aspects of freezing and thawing sperm and of preparing cryopreservation media have been evaluated for their effects on post thawing sperm quality[15,26-29].

In the present study, the authors determined the cryodamage effects on human sperm chromatin integrity, as well as on sperm motility, vitality, survival rate, and morphology in a population of men with normal semen quality after freeze-thawing. Those effects were compared between computerized program and ordinary liquid nitrogen vapour freezing method.

DNA integrity & Cryopreservation

In the present study, the effect of cryopreservation on sperm DNA was determined by using metachromatic properties of acridine orange (AO) in order to monitor the susceptibility of sperm chromatin DNA to in situ acid denaturation. Additionally, Carnoy's solution, which provides a better predictive value for DNA damage in sperm than the other solution[7], was used for sperm fixation. After AO staining, sperm chromatin integrity can be analyzed under numerous methods including microscopic acridine orange test either by visual counting method or computer-assisted counting method, and flow cytometry. In the present study, the visual counting using fluorescence microscope was performed for assessing chromatin integrity on 300 sperms by a single person[30,31].

The results indicated that freezing procedure obviously affected on DNA integrity irrespective of the cryopreservation method used. Chromatin condensation is vital for sperm because spermiogenesis results in discarding of cytoplasm, causing cessation of transcription and leaving the sperm incapable of undertaking DNA repair[16]. Previous studies demonstrated that the effect of freezing also led to chromatin damage and to decrease in chromatin instability including DNA denaturation[32-34]. Sperm DNA denaturation was previously shown to correlate with male fertility potential by impairing the fertilizing ability of human sperm[10-12], and further with mutagenic events[16]. Unfortunately, sperms with damaged genetic material are still capable of fertilization and mutations and defects may not become evident until the embryo has divided or the fetus develops[13,35].

Corresponding to previous studies[15,32], the present study clearly illustrated that to cryopreserve semen by computerized program freezing could protect sperm DNA from cryodamage better than by liquid nitrogen vapour freezing method. Nevertheless, further large studies are required to prove conclusively the effects of cryopreservation on sperm DNA assessed by other advanced methods such as flow cytometry, and to clarify the cryo-effects on sperm mitochondria including DNA comparing between different freezing methods.

Motility & Cryopreservation

In the present study, post-thaw sperm motility and cryosurvival rate were clearly affected by freezing-thawing process during both cryopreservation methods. Such effect was explained in many ways including cryopreservation-induced mitochondrial damage[36] and alteration of sperm morphology such as coiled tails[3,37,38].

It is well accepted that mitochondrial activity is directly related to the sperm motility. Sperm are made up for several compartments enclosed within plasma membrane which must remain intact and permit cell competence. Energy which is necessary for sperm motility and fertilization is supplied in the form of ATP partly synthesized through oxidative phosphorylation [OXPHOS) in the mitochondria[39]. To be able to drive motility, such ATP must be transferred from the inner mitochondria to the microtubules[40]. Hence, reduced sperm motility may be associated with mitochondria damage. Other possible mechanism for reduction in sperm motility after freezing is an irreversible looping of the sperm flagellum[38], corresponding to the studies by O'Connell et al[36] and Hammadeh et al[15,28] on the increase in tail abnormalities in human sperm after freezing. The present result also demonstrated that freezing with computerized freezer provided less cryodamage effects on sperm motility and cryosurvival rate based on the number of forward progression spermatozoa, compared to freezing with vapour freezing method, as well as on sperm tail defects. The latter may explain the different sperm motility alterations after freezing of 2 methods.

Owing to bad motility of thawed sperm, an intracytoplasmic sperm injection (ICSI) technique has widely been considered as a gold standard for treatment of male factor pathologies which make a post thawing non-progressive motile spermatozoa extremely valuable. If putting non-progress motile together with forward progression sperms in the calculation of cryosurvival rate, such rate seemed to be similar in the 2 freezing methods. This partly indicates that the effects of cryopreservation have no impact on sperm cryosurvival rate.

Vitality & Cryopreservation

In the present study, certain percentages of post-thaw sperm vitality decreased after freezing-thawing in both cryopreservation methods especially in vapour freezing. An explanation may be related to the composition of sperm membranes. Sperm cells present highly specific lipidic composition[41], which has very high levels of phospholipids, sterols, saturated and polyunsaturated fatty acids. Sperm plasma membrane lipids exist in two phases, fluid and gel. At physiological temperatures, the two forms coexist but in case of the lower temperature, a phase transition occurs in favour of the gel form[42]. This may lead to a reduction in the fluidity of the membrane associated with sperm membrane disruption and lower sperm survival during cryopreservation[30,43]. In addition, human sperm membranes have unusually high cholesterol contents which act as membranes stabilizer during cooling [44]. Correspondingly, some studies reported that the susceptibility of the sperm to cryodamage during the freezing process appeared to be related to a high ratio of saturated VS unsaturated fatty acids, together with low cholesterol content[44,45].

Morphology & Cryopreservation

Some studies concluded that sperm membrane lipid peroxidation was correlated with abnormal morphology[33,46], as well as midpiece morphological defects during cryopreservation[47]. Similar to Stanic et al[27]

and Hammadeh et al[6], the present study however showed that cryopreservation had no effect on normal morphologically sperm. On the other hand, population of post-thaw sperms with tail defects was higher after freezing with liquid nitrogen vapour than with computerized freezing. As mentioned elsewhere, cryopreservation and the freeze-thaw process disrupts the outer and inner membranes, resulting in alteration of sperm morphology such as coiled tails[3,37,38].

Conclusion

The present study demonstrated a significant post-thaw decrease in sperm chromatin integrity, sperm vitality, sperm motility, sperm cryosurvival rate and percentages of normal morphological sperms following both cryopreservation methods. Despite this, freezing with a computerized program seemed to optimize post-thaw sperm DNA integrity, motility, vitality better than freezing with liquid nitrogen vapour. Therefore, the authors recommend the computerized program freezer as a first choice for routine cryostorage.

Acknowledgement

The authors wish to thank Professor Somboon Kunathikom, head of infertility unit and assistant Professor Manee Piya-anan, head of Department of Obstetrics and Gynecology for permission to do this research. The authors also wish to thank all officers of infertility clinic for their support with this work. This work was supported by a research grant from Faculty of Medicine, Siriraj Hospital.

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ผลกระทบจากความเย็นต่อโครมาตินของตัวอสุจิในการแช่แข็งน้ำเชื้ออสุจิเปรียบเทียบระหว่างเทคนิคการแช่แข็ง 2 วิธี

สมสิญจน์ เพ็ชรยิ้ม, เรืองศิลป์ เชาวรัตน์

วัตถุประสงค์*: เพื่อศึกษาผลกระทบจากความเย็นต่อโครมาติน การเคลื่อนไหวและอัตราการรอดของตัวอสุจิ ในการแช่แข็งน้ำเชื้ออสุจิโดยเปรียบเทียบระหว่างเทคนิคการแช่แข็ง 2 วิธี คือ ใช้เครื่องแช่แข็งซึ่งควบคุมอัตราการลดอุณหภูมิด้วยเครื่องคอมพิวเตอร์ และ วิธีแช่แข็งโดยใช้ไอระเหยของไนโตรเจนเหลว ประเมินโดยย้อมสี acridine orange*

วัสดุ*: ตัวอย่างน้ำเชื้ออสุจิ 50 ราย*

วิธีการ*: แบ่งตัวอย่างน้ำเชื้อเป็น 2 ส่วน เพื่อทำการแช่แข็งทั้ง 2 วิธี เก็บนาน 1 เดือน หลังจากละลายน้ำเชื้อแล้วทำการตรวจความผิดปกติของโครมาติน ดูการเคลื่อนไหว และอัตราการรอดของตัวอสุจิ ทำการเปรียบเทียบกับผลก่อนการแช่แข็ง*

ผลการวิจัย*: ผลการวิจัยพบว่าน้ำเชื้ออสุจิที่ผ่านการแช่แข็งโดยวิธีไอระเหยของไนโตรเจนเหลว จะพบความผิดปกติของโครมาตินที่ตัวอสุจิมากกว่าวิธีแช่แข็งด้วยเครื่องคอมพิวเตอร์ อย่างมีนัยสำคัญทางสถิติ ($p < 0.001$) และในทำนองเดียวกัน น้ำเชื้ออสุจิที่ผ่านการแช่แข็งโดยใช้เครื่องคอมพิวเตอร์ จะมีการเคลื่อนไหวและอัตราการรอดของตัวอสุจิมากกว่า วิธีไอระเหยของไนโตรเจนเหลว*

สรุป*: น้ำเชื้ออสุจิที่ผ่านการแช่แข็งโดยวิธีควบคุมอัตราการลดอุณหภูมิด้วยเครื่องคอมพิวเตอร์ จะได้น้ำเชื้อที่มีคุณภาพดีกว่าวิธีแช่แข็งโดยใช้ไอระเหยของไนโตรเจนเหลว ดังนั้นจึงเป็นวิธีที่เหมาะสำหรับการเก็บแช่แข็งน้ำเชื้ออสุจิ*