Evaluation of Cryo-injury of Sperm Chromatin According to Computer Controlled Rate Freezing Method Part 2

Surachai Dejarkom MD*,
Somboon Kunathikom MD*

** Department of Obstetrics and Gynecology, Faculty of Medicine Siriraj Hospital, Mahidol, University*

Objective: *The present study was designed to determine the effect of the freeze-thawing procedure, computer controlled rate freezing and duration for six months, on human sperm chromatin (assessed by acridine orange test), vitality, motility, and morphology.*
Design: *Experimental study*
Material and Method: *Twenty semen samples were obtained from patients attending the infertility unit. The semen analysis was measured according to WHO criteria. Sperm morphology was evaluated by strict Kruger criteria and sperm chromatin were detected by acridine orange test. After semen analysis, each sample was mixed with cryoprotectant and divided into straw. The straw was frozen with computer controlled rate freezing method. After 6 months of cryostorage, semen samples were thawed and then the semen was analyed, and sperm chromatin and morphology were determined.*
Results: *After six months of cryostorage, the mean percentage of normal sperm chromatin decreased significantly (87.3 $\pm$ 9.0 vs. 51.9 $\pm$ 27.4, $p < 0.001$). Vitality, motility, and normal morphology of sperm decreased significantly (78.7 $\pm$ 1.9 vs. 32.8 $\pm$ 10.8, 52.6 $\pm$ 1.9 vs. 24.1 $\pm$ 10.9 and 21.4 $\pm$ 4.3 vs. 18.0 $\pm$ 4.4 respectively, $p < 0.001$).*
Conclusions: *The computer controlled rate freezing of sperm for six months and thawing process significantly decreased normal sperm chromatin, vitality, motility, and normal morphology.*

Keywords: *Cryopreservation, Sperm chromatin integrity, Computer program freezer*

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Donor sperm is one of many options for treatment in infertile couples who have azoospermia. Currently, AIDS era, donor sperm should be used very carefully due to possibility of HIV transmission. To decrease this problem from fresh donor sperm the authors use six-month cryopreserved semen. This is to avoid the window period in detection of HIV infection in donor semen.

In previous studies, Hammadeh et al[1,2] found that the freeze-thawing process had effects on chromatin, morphology, motility, and vitality of human spermatozoa. However, the duration of freezing time in those studies was less than six months. Thus, in the present study the authors used the freezing duration of six months to study effects on sperm chromatin, morphology, motility, and vitality.

Cryoinjury of spermatozoa can be evaluated from morphology, motility, and vitality. Chromatin staining of cell with acridine orange (AO test) has been widely accepted as a predictor of DNA damage in many cell types including human spermatozoa[1].

Hammadeh et al [1,2] found that computer controlled rate freezing method had less cryoinjury to spermatozoa than the conventional method. Thus, in the present study the authors decided to use the computer controlled rate freezing method to freeze the specimens.

The purpose of the present study was to evaluate the nuclear integrity, motility, vitality, and morphology of human spermatozoa after six months of freezing by using the computer controlled rate freezing method.

Correspondence to : Dejarkom S, Department of Obstetrics and Gynecology, Faculty of Medicine Siriraj Hospital, Bangkok 10700, Thailand. Phone: 0-2419-7000

Material and Method

The present study was an experimental study performed at the Infertility Unit, Siriraj Hospital, Bangkok. The study protocol was approved by the ethic committee of the Faculty of Medicine, Siriraj Hospital.

Semen samples, preparation and freeze-thawing

Semen samples were obtained, by masturbation into sterile container, from 20 patients attending Siriraj Infertility Unit for semen analysis. Each sample was allowed to liquefy at room temperature for 30 minutes before use.

Semen analysis was assessed according to WHO guideline[3]. Sample were not used in the present study unless there was sperm concentration exceeding 20 millions per milliliter, with greater than 50 percent of forward motility and more than 15 percent of normal morphology according to strict Kruger criteria[4].

Men who had an active sexual transmitted disease: HIV, syphilis, hepatitis B, hepatitis C and active prostatitis or urethritis were excluded from the present study.

After the examination of semen samples, some part of the samples was stained with acridine orange for sperm chromatin assessment.

After the initial analysis, each sample was mixed with an equal volume of sperm cryoprotectant (human sperm preserving medium that contains glycerol, glycine, and glucose). The media was added into the semen and swirled over 10-15 minutes. The mixture was divided into 0.25 milliliter straw and placed into the chamber of a computer program freezing machine (Sidney IVF program, freeze control model CL836, Cryologic PTY Ltd., Australia) and cooled in the following programs:

i) at cooling rate 6°C/minute from 24°C to 5°C, holding the temperature for 5 minutes

ii) 6°C/minute from 5°C to -8°C, holding the temperature 2 minutes

iii) 5°C /minute from -8°C to -30°C

iv) 4°C /minute from -30°C to -45°C

v) 25°C/minute from -45°C to -80°C, and then quickly plunged into liquid nitrogen for cryostorage.

After six months of cryostorage, each sample was thawed in room temperature for 10 minutes and in a warming chamber (37°C) for another 10 minutes. Then sperm concentration, motility, vitality, morphology, and sperm chromatin was re-assessed by the same method as before the freezing process.

The semen analysis, before and after freeze, in this study was assessed by two well-trained technicians in andrology laboratory. The acridine orange test was performed by the author.

Semen analysis

Sperm concentration, motility, vitality were assessed according to the WHO laboratory manual[3]. Sperm morphology was assessed according to strict Kruger criteria[4].

Assessment of DNA normality (Acridine orange staining)

Nuclear integrity of the spermatozoa was assessed by acridine orange fluorescence method[5]. The smears were fixed overnight in freshly prepared Carnoy's solution (3 parts of methanol and 1 part glacial acetic acid) and let to air-dry for a few minutes before being stained with acridine orange. The smears were stained for 5 minutes, gently rinsed under tap water, and mounted. The slides were evaluated immediately using fluorescence microscope equipped with a 490-nm excitation filter and 530-nm barrier. A normal DNA content exhibited green fluorescence over the head region, while abnormalities of DNA content were indicated by spectrum of fluorescence varying from yellow to red. Two hundred spermatozoa were evaluated per smear.

Statistical analysis

The data was statistically analyzed by paired t-test which was performed by computer program SPSS for Microsoft Window version10.0 (Chicago, IL). A p-value of less than 0.05 was considered statistically significant.

Results

The mean percentage of vitality of spermatozoa in native semen samples decreased significantly after freeze-thawing process (78.7 ± 5.5 vs. 32.8 ± 10.8, $p < 0.001$). Furthermore, the mean percentage of forward motility of spermatozoa was also decreased significantly (52.6 ± 1.9 vs. 24.1 ± 10.9, $p < 0.001$). Additionally, significant decreased of normal sperm chromatin, as assessed by acridine orange test, was observed in semen samples after freeze-thawing process (87.3 ± 9.0 vs. 51.9 ± 27.4, $p < 0.001$) (Table 1).

In Table 2, the authors also found a significant decrease of normal morphology after the freeze-thawing process when compared with the value observed in the native semen samples (21.4 ± 4.3 vs. 18.0 ± 4.4, $p = 0.007$). Obvious changes were observed in

Table 1. Alteration of semen parameters after the freeze-thawing process (mean ± SD)

Parameter	Before freezing	After freezing	p-value
Vitality (%)	78.7 ± 5.5	32.8 ± 10.8	<0.001
Motility (%)	52.6 ± 1.9	24.1 ± 10.9	<0.001
Morphology (%)	21.4 ± 4.3	18.0 ± 4.4	0.007
Normal chromatin (%)	87.3 ± 9.0	51.9 ± 27.4	<0.001

Table 2. Effect of the freeze-thawing process on morphology of human spermatozoa (mean ± SD)

Morphology (%)	Before freezing	After freezing	p-value
Normal	21.4 ± 4.3	18.0 ± 4.4	0.007
Head defect	57.0 ± 11.8	64.9 ± 4.7	0.013
Midpiece defect	4.5 ± 4.1	4.9 ± 2.9	0.665
Tail defect	15.6 ± 7.7	9.9 ± 3.3	0.008
Cytoplasmic droplet	1.6 ± 1.7	1.7 ± 2.7	0.899

head (57.0 ± 11.8 vs. 64.9 ± 4.7, p = 0.013) and tail (15.6 ± 7.7 vs. 9.9 ± 3.3, p = 0.008); however, no significant defect was observed in the midpiece (4.5 ± 4.1 vs. 4.9 ± 2.9, p = 0.665) and cytoplasmic droplet (1.6 ± 1.7 vs. 1.7 ± 2.7, p = 0.899).

Discussion

Several reports had shown that the freeze-thawing process reduced the metabolic state of spermatozoa and damaged their plasma membranes[6,7]. Other investigators had placed more emphasis on morphology alteration of spermatozoa such as coiled tails, damaged membranes, and acrosomes[8,9].

In the present study, the mean percentage of spermatozoa that were alive, normal chromatin and normal morphology in the native semen decreased significantly after the freeze-thawing process (p < 0.001). The acridine orange test revealed that the freeze-thawing process could cause deterioration of the chromatin structure of spermatozoa. The authors found that the mean percentage of normal sperm chromatin had decreased significantly after this procedure (p < 0.001). Previous studies demonstrated that the effect of freezing also led to chromatin damage and to decrease in chromatin instability including DNA denaturation[10,11]. However, further studies are required to confirm the effect of cryostorage for 6 months on sperm chromatin assessed by other advance methods such as flow cytometry.

The mean percentage of vital spermatozoa in the native semen samples had decreased significantly after freeze-thawing process (p < 0.001). This finding was consistent with the results of a previous study by Check et al[9] who reported that vitality of spermatozoa decreased from 70.0% to 33.7% post thaw, which was possibly due to membrane damage.

Cryopreserved spermatozoa often had lower progressive motility[12]. In the present study, a significant decrease in the percentage of sperm motility was also observed after the freeze-thawing process (p < 0.001). The explanation of this effect was that cryopreservation induced mitochondrial damage[13] and alteration of sperm morphology such as coil tails defect[14].

Furthermore, many reports had described an association between disturbances in morphology of spermatozoa after freeze-thawing process[15,16]. The present study demonstrated a significant decreased of the mean percentage of morphologically normal spermatozoa and tail defect (p = 0.007 and 0.008). Head defect was increased significantly (p = 0.013). However, midpiece defect and cytoplasmic droplet defect had not changed significantly (p = 0.665 and 0.899). The previous study investigated this effect by using light and electron microscopy, they concluded that freeze-thaw effect damaged to membranes and acrosome of spermatozoa[16].

In view of these findings, it seems that normal chromatin, vitality, forward motility, and normal morphology of spermatozoa underwent a severe alteration under the freeze-thawing technique, which might be limit the usage of frozen semen in treatment of infertile couples such as intrauterine insemination.

Conclusion

The present study demonstrated that the mean percentage of normal chromatin, vitality, motility, and morphology of spermatozoa had decreased significantly after being frozen for six months and then thawed. The data from the present study revealed the deterioration of many sperm characteristics after the process of six-month frozen sperm, to avoid the possibility of HIV transmission. These findings showed the limitation of frozen semen in the treatment of infertile couples.

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ผลกระทบจากความเย็นต่อโครมาตินของตัวอสุจิในการแช่แข็งน้ำเชื้ออสุจิโดยการใช้คอมพิวเตอร์ควบคุมการลดอุณหภูมิ (II)

สุรชัย เดชอาคม, สมบูรณ์ คุณาธิคม

วัตถุประสงค์: *เพื่อศึกษาผลกระทบจากความเย็นต่อโครมาตินของตัวอสุจิในการแช่แข็งน้ำเชื้ออสุจิเป็นระยะเวลา 6 เดือน โดยการใช้คอมพิวเตอร์ควบคุมการลดอุณหภูมิ ประเมินผลด้วยการย้อมสี acridine orange และศึกษาผลกระทบจากความเย็นต่อการอยู่รอด การเคลื่อนไหวและลักษณะทางกายภาพของตัวอสุจิ*

ชนิดของการวิจัย: *การวิจัยแบบทดลอง*

วัสดุและวิธีการ: *เก็บตัวอย่างน้ำเชื้ออสุจิจำนวน 20 ตัวอย่างจากผู้ป่วยที่มาตรวจน้ำเชื้ออสุจิที่คลินิกผู้มีบุตรยาก โรงพยาบาลศิริราชที่มีผลการตรวจอยู่ในเกณฑ์ปกติ ตรวจวิเคราะห์น้ำเชื้ออสุจิโดยวิธีมาตรฐาน และตรวจหาความผิดปกติของโครมาตินที่ตัวอสุจิโดยการย้อมสี acridine orange หลังจากนั้นนำตัวอย่างน้ำเชื้ออสุจิที่ผสมน้ำยาแช่แข็งมาทำการแช่แข็งโดยวิธีการใช้คอมพิวเตอร์ควบคุมการลดอุณหภูมิ เก็บน้ำเชื้ออสุจิที่ทำการแช่แข็งไว้เป็นเวลา 6 เดือน แล้วจึงนำตัวอย่างน้ำเชื้ออสุจิดังกล่าวมาละลาย และตรวจน้ำเชื้ออสุจิตามวิธีมาตรฐานและ ตรวจหาความผิดปกติของโครมาตินที่ตัวอสุจิโดยการย้อมสี acridine orange ทำการแปลผลเปรียบเทียบกันระหว่างก่อนและหลังการแช่แข็ง*

ผลการศึกษา: *หลังแช่แข็งน้ำเชื้ออสุจิเป็นระยะเวลา 6 เดือน โครมาตินปกติของตัวอสุจิลดลงอย่างมีนัยสำคัญทางสถิติ (87.3 $\pm$ 9.0 และ 51.9 $\pm$ 27.4, $p < 0.001$) การรอดชีวิตของตัวอสุจิลดลงอย่างมีนัยสำคัญทางสถิติ (78.7 $\pm$ 1.9 และ 32.8 $\pm$ 10.8, $p < 0.001$) การเคลื่อนไหวไปข้างหน้าของตัวอสุจิลดลงอย่างมีนัยสำคัญทางสถิติ (52.6 $\pm$ 1.9 และ 24.1 $\pm$ 10.9, $p < 0.001$) และลักษณะปกติของตัวอสุจิลดลงอย่างมีนัยสำคัญทางสถิติ (21.4 $\pm$ 4.3 และ 18.0 $\pm$ 4.4, $p < 0.001$)*

สรุป: *การแช่แข็งน้ำเชื้ออสุจิโดยวิธีใช้คอมพิวเตอร์ควบคุมการลดอุณหภูมิเป็นระยะเวลา 6 เดือนมีผลลดจำนวนโครมาตินปกติ การรอดชีวิต การเคลื่อนไหวไปข้างหน้า และลักษณะปกติของตัวอสุจิอย่างมีนัยสำคัญทางสถิติ*