

Evaluation of Cryoinjury of Sperm Chromatin According to Liquid Nitrogen Vapour Method (I)

Thitikan Ngamwuttiwong MD*,
Somboon Kunathikom MD*

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

Objective: To evaluate cryodamage on human sperm motility, cryosurvival rate, and sperm chromatin assessed by acridine orange staining method (AO test) after a six-month freeze-thawing process using liquid nitrogen vapor.

Study design: Experimental study.

Material and Method: Twenty normal semen samples were obtained from the male partner of infertile couples attending the infertility unit, Siriraj Hospital. After semen analysis, each semen sample was frozen with liquid nitrogen vapor. The acridine orange test was used for assessment of chromatin structures. After 6 months of cryostorage, semen samples were thawed and the effects of cryopreservation on sperm chromatin integrity, motility, morphology, vitality, and cryosurvival rate were evaluated.

Results: The mean percentage of normally condensed sperm chromatin in the native semen sample decreased significantly (87.3 ± 9.1 vs 47.9 ± 26.2 ; $p < 0.001$) after the freeze-thawing process using liquid nitrogen vapor. Furthermore, the mean percentage of sperm motility and vitality also decreased significantly after the freeze-thawing process (52.6 ± 1.9 vs 23.2 ± 10.6 and 78.7 ± 5.6 vs 30.3 ± 8.8 respectively; $p < 0.001$). In contrast, the numbers of sperm with normal morphology after cryopreservation were not different from those before the procedure (21.4 ± 4.3 vs 24.2 ± 23.9 ; $p = 0.606$).

Conclusion: The freeze-thawing procedure using liquid nitrogen vapor had effects on chromatin, motility, and vitality of human spermatozoa. The six-month cryopreservation of semen is a good method for avoiding the window period of HIV; however, this can cause a lot of damage to spermatozoa, thus, limits their further use in the treatment of infertility.

Keywords: Liquid nitrogen vapour, Sperm DNA integrity, Cryopreservation, AO test

J Med Assoc Thai 2007; 90 (2): 224-8

Full text. e-Journal: <http://www.medassocthai.org/journal>

Human sperm cryopreservation has been performed in most of the assisted reproduction centers. It is a standard procedure used to avoid the window period of HIV infection in donor semen before being utilized⁽¹⁾. It is also a procedure that yields patient's convenience. Intrauterine insemination can be performed in the absence of a male partner with the presence of normal sperm parameter or help preserve reproductive capacity in men with various types of neoplasias before undergoing radical surgery and/or radiochemotherapy. It also aids in the management of infertile men undergoing vasectomy reversal or epididymo-vasostomy

when banking may provide a future sperm source for possible use in intrauterine insemination (IUI) or intracytoplasmic sperm injection (ICSI) treatment. Moreover, infertile men with a different degree of oligoastheno-teratozoospermia can now be offered the use of cryopreserved-thawed spermatozoa for assisted fertilization.

Success in cryopreservation of cells is always encountered with some adverse effects, especially osmotic effects of either freezing or thawing that considerably lower the fertilizing capacity of the spermatozoa by damaging the cell membrane and by impairing sperm motility⁽²⁾.

Acridine orange (AO) staining is one of the simple methods for assessing sperm nuclear nor-

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

mality⁽³⁾. This dye produces green fluorescence when bound to double-strand nucleic acids, whereas it produces red fluorescence when bound to single-strand nucleic acid. AO staining involves acid treatment (low pH) of sperm. This process dissociates thiol from DNA and increases DNA susceptibility to denaturation. Consequently, AO competes for anionic binding sites by avoiding nonspecific aggregation.

The objective of the present study was to evaluate the cryodamage effects on human sperm chromatin, motility and cryosurvival rate after freeze-thawing for 6 months. The authors performed a standard semen analysis, motility assessment, supravital staining for sperm viability, and AO test to define the relationship between pre-freeze semen parameters and post-thaw outcomes including sperm cryosurvival rate and post-thaw sperm motility.

Material and Method

The present study was an experimental study performed at the infertility clinic, Siriraj Hospital during the three months period starting from November 2005 to January 2006. The study protocol was approved by the Ethics Committee of the Faculty of Medicine Siriraj Hospital.

Semen samples were collected from 20 male partners, attending the infertility clinic, by masturbation into a sterile glass container. They were allowed to liquefy at room temperature for 30 minutes before being analyzed. Assessment of sperm motility was performed using WHO guideline⁽⁴⁾. Sperm morphology was assessed according to strict Kruger criteria⁽⁵⁾ and a part of the samples was stained with acridine orange for evaluation of sperm chromatin. After initial analysis, each sample was mixed with an equal volume of cryoprotective media (human sperm preservation medium that contains glycerol, glycine, and glucose). The media was added into the semen and swirled over 10-15 minutes. The mixture was drawn into a 0.25 ml straw which was later stored in the liquid nitrogen vapor freezer.

Briefly, the straw was placed in the horizontal position at 15 cm. above and parallel to the surface of liquid nitrogen for 30 minutes to cool down to -80°C. Then it was quickly plunged into liquid nitrogen for cryostorage. After 6 months of cryostorage, the straws were taken out and thawed in room temperature for 10 minutes and in a warming chamber for another 10 minutes. Post-thaw sperm concentration, motility, morphology, vitality, and sperm chromatin were assessed in the same way as before freezing. After that, the com-

parison was made between the pre-freezing and the post-thaw sperm.

Selection criteria

After obtaining the semen samples, semen analysis was performed. Each sample must have sperm concentrations exceeding 20 million per milliliter, with greater than 50 percent forward motility and greater than 15 percent normal morphology, in order to meet the criteria of normal semen. Men who had active sexually transmitted diseases (STD) such as HIV, syphilis, hepatitis B, hepatitis C, and active prostatitis or urethritis, were excluded from the present study.

Sperm motility assessment

Sperm motility was assessed according to the WHO laboratory manual, with the aid of the grid on an eyepiece graticule using phase contrast optics by counting the number of motile spermatozoa in several randomly selected fields until at least 100 spermatozoa were visualized. Sperm motility was graded into four categories including category A: rapid forward progression spermatozoa, category B: movement with forward motion spermatozoa, category C: motion with no forward progression, and category D: no motion. Counting of 100 spermatozoa was performed twice, then the average number for each category was calculated. The value was expressed as percentage

Sperm morphology assessment

Sperm morphology, including head, midpiece, and tail, was assessed according to strict Kruger criteria

Chromatin condensation assessment

Each semen sample was smeared onto a pre-cleaned glass slide and allowed to air dry for 20 minutes. The smear was then fixed overnight in Carnoy's solution (methanol:acetic acid = 3:1), which was daily prepared. After fixation, the slide was allowed to dry for a few minutes before staining. The acridine orange staining solution was prepared as follows: 10 ml of 1% AO in distilled water was added to a mixture composed of 40 ml of 0.1M citric acid and 2.5 ml of 0.3M Na₂HPO₄·7H₂O, pH 2.5.

The smear was stained for 5 minutes, and gently rinsed and mounted with distilled water. Each slide was interpreted on the same day by a fluorescence microscope using a 490-nm excitation filter and a 530 nm barrier filter. Two hundred cells were counted on each slide with the duration no longer than 40

seconds by the same investigator. Spermatozoa with normal DNA content exhibited green fluorescence of the heads, while those with abnormal DNA content expressed a spectrum varying from yellow green to red.

Data analysis

Sperm characteristics were analyzed and expressed by means, standard deviation, and percentage. Paired t-test was used for the comparison of sperm characteristics before and after the thawing process. P value of less than 0.05 was considered statistically significant.

Results

Semen characteristic of 20 samples before the freeze-thawing process are shown in Table 1. All of them met the criteria for normal semen analysis. However, most of these parameters had undergone significant changes after being frozen and thawed (Table 1). Sperm concentration had a moderate decrease from 35.5 to 26.1 million per milliliter, ($p = 0.04$) and percentage of sperm vitality, motility and DNA integrity had decreased significantly when compared to fresh semen ($p < 0.001$). In contrast, the percentage of normal morphology of spermatozoa had no obvious change after the freeze-thawing process (21.4 ± 4.3 vs 24.2 ± 23.9 ; $p = 0.606$).

For details of sperm morphology, there was some alteration in various parts of the sperm, including head, midpiece, tail, and cytoplasmic droplet. However, only head defect had a significant change after the freezing procedure ($p < 0.05$), as shown in Table 2.

Discussion

From previous studies, cryopreservation of human semen resulted in a significant loss of sperm motility and viability^(2,6), with considerable variation between ejaculates of different individuals. Only semen from a highly selected population of men is suitable for treatment purposes after cryopreservation^(6,7).

In the present study, semen concentration as well as most of the sperm qualities, such as DNA integrity, motility, and vitality in the native semen, decreased significantly ($p < 0.001$) after the six-month freeze-thawing procedure with liquid nitrogen vapor. The effect on sperm DNA, which corresponded to a previous study, might delay paternal nuclear de-condensation during fertilization⁽⁸⁾.

Some studies had concluded that sperm membrane liquid peroxidation was correlated with abnormal sperm morphology. Furthermore, many reports had described an association between disturbances in morphology and male fertility^(9,10) However, the authors found no significant change in overall morphology of spermatozoa in the present study. Verheyen et al

Table 1. Semen parameters before and after freeze-thawing (mean \pm SD)

Parameter	Before freezing n = 20	After freezing n = 20	p-value
Concentration ($\times 10^6$ /ml)	35.5 \pm 18.1	26.1 \pm 12.4	0.04
Vitality (%)	78.7 \pm 5.6	30.3 \pm 8.8	<0.001
Motility (%)	52.6 \pm 1.9	23.2 \pm 10.6	<0.001
Morphology (%)	21.4 \pm 4.3	24.2 \pm 23.9	0.606
DNA integrity (%)	87.3 \pm 9.1	47.9 \pm 26.2	<0.001

Table 2. Alteration of sperm morphology after freeze-thawing process

Sperm morphology (%)	Mean change n = 20	Standard deviation	p-value
Normal	-2.8	23.4	0.606
Head defect	-6.3	12.7	0.038
Midpiece defect	0.2	4.8	0.891
Tail defect	3.4	8.1	0.075
Cytoplasmic droplet	-0.03	2.1	0.536

demonstrated that motility and morphology of normal ejaculated semen was reduced by 50% after cryopreservation in comparison to native semen⁽¹¹⁾. This decrease of sperm motility was reflected in the present study.

The percentages of post-thaw sperm vitality also decreased significantly. This was similar to a study by Check et al, who reported that sperm vitality decreased from 70.0% to 33.7% post thaw possibly due to membrane damage⁽¹²⁾. This might be related to the composition of sperm membrane where a reduction in the fluidity of the membrane and the association between membrane disruption and lower sperm survival during cryopreservation was noted.

For details about morphology defects, Stanic et al⁽¹³⁾ and Hammadeh et al^(14,15) had found midpiece morphological defects during cryopreservation, however, head defects were the only abnormality that showed significant change in the present study.

From a previous study, it seemed that a severe alteration of sperm vitality, DNA integrity, morphology, and motility, after the freeze-thawing procedure might influence their capability to fertilize. Perderson and Lebech⁽¹⁰⁾, as well as Keel et al⁽²⁾, suggested that the decreased fecundity of cryopreserved sperm was due to structural damage. A small number of studies suggested that although cryopreserved semen was equally as fertile as fresh semen when used for in vitro fertilization (IVF)⁽¹⁶⁾, there was still a significant decrease in conception rates observed *in vivo*⁽¹⁷⁾. In the present study, the authors found a significant decrease in the mean percentage of sperm DNA integrity, motility, and vitality after cryopreservation of semen samples. This probably caused some problem to sperm fecundity and capability to fertilize.

Conclusion

The present study demonstrated a significant deterioration in chromatin integrity, vitality, and motility of post thaw spermatozoa after 6 months of cryopreservation with liquid nitrogen vapor. This effect may reduce future pregnancy rate after insemination of cryopreserved sperm.

Finally, the authors can conclude that although the six-month cryopreservation of semen is a good method for avoiding the window period of HIV, it can cause a lot of damage to spermatozoa. Therefore, this limits their further use in the treatment of infertility.

Acknowledgements

The authors wish to thank Professor Somboon

Kunathikom, head of the infertility unit and Clinical Professor Manee Piya-anan. The authors also wish to thank all the staff of the infertility clinic for their support with this work.

References

1. The American Fertility Society. Guidelines for therapeutic donor insemination sperm. Guidelines for gamete donation: 1993. *Fertil Steril* 1993; 59: 1S-4S.
2. Keel BA, Webster BW, Roberts DK. Effects of cryopreservation on the motility characteristics of human spermatozoa. *J Reprod Fertil* 1987; 81: 213-20.
3. Hoshi K, Katayose H, Yanagida K, Kimura Y, Sato A. The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. *Fertil Steril* 1996; 66: 634-9.
4. World Health Organization. Collection and examination of human semen. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge: Cambridge University Press; 1999: 4-33.
5. Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S. Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertil Steril* 1988; 49: 112-7.
6. McLaughlin EA, Ford WC, Hull MG. Motility characteristics and membrane integrity of cryopreserved human spermatozoa. *J Reprod Fertil* 1992; 95: 527-34.
7. Heuchel V, Schwartz D, Czyglik F. Between and within subject correlations and variances for certain semen characteristics in fertile men. *Andrologia* 1983; 15: 171-6.
8. 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.
9. Foresta C, Zorzi M, Rossato M, Varotto A. Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. *Int J Androl* 1992; 15: 330-7.
10. Pedersen H, Lebech PE. Ultrastructural changes in the human spermatozoon after freezing for artificial insemination. *Fertil Steril* 1971; 22: 125-33.
11. Verheyen G, Pletinckx I, Van Steirteghem A. Effect of freezing method, thawing temperature and post-thaw dilution/washing on motility (CASA)

- and morphology characteristics of high-quality human sperm. Hum Reprod 1993; 8: 1678-84.
12. 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.
 13. Stanic P, Tandara M, Sonicki Z, Simunic V, Radakovic B, Suchanek E. Comparison of protective media and freezing techniques for cryopreservation of human semen. Eur J Obstet Gynecol Reprod Biol 2000; 91: 65-70.
 14. Hamamah S, Royere D, Nicolle JC, Paquignon M, Lansac J. Effects of freezing-thawing on the spermatozoon nucleus: a comparative chromatin cytophotometric study in the porcine and human species. Reprod Nutr Dev 1990; 30: 59-64.
 15. Barthelemy C, Royere D, Hammah S, Lebos C, Tharanne MJ, Lansac J. Ultrastructural changes in membranes and acrosome of human sperm during cryopreservation. Arch Androl 1990; 25: 29-40.
 16. Mahadevan MM, Trounson AO, Leeton JF. Successful use of human semen cryobanking for in vitro fertilization. Fertil Steril 1983; 40: 340-3.
 17. Richter MA, Haning RV Jr, Shapiro SS. Artificial donor insemination: fresh versus frozen semen; the patient as her own control. Fertil Steril 1984; 41: 277-80.

ประเมินความเย็นที่มีต่อโครมาตินของตัวอสุจิในการแช่แข็งน้ำเชื้ออสุจิโดยการใช้อะไรของไนโตรเจนเหลว (I)

จิตติภัฏ งามวุฒิวงศ์, สมบูรณ์ คุณาธิคม

วัตถุประสงค์: เพื่อศึกษาผลกระทบจากความเย็นต่อโครมาตินของตัวอสุจิในการแช่แข็งน้ำเชื้ออสุจิโดยวิธีแช่แข็งโดยใช้อะไรของไนโตรเจนเหลวเป็นระยะเวลา 6 เดือน ประเมินโดยย้อมสี acridine orange รวมทั้งศึกษาถึงผลของความเย็นต่อการเคลื่อนไหวและอัตราการรอดของตัวอสุจิ หลังจากแช่แข็งน้ำเชื้ออสุจิโดยวิธีดังกล่าว

ชนิดของการวิจัย: การวิจัยชนิดการทดลอง

วัสดุและวิธีการ: กลุ่มศึกษาคือสามีของคู่สมรสที่มารับการรักษาเรื่องมีบุตรยากจำนวน 20 รายที่มาตรวจน้ำเชื้ออสุจิที่หน่วยผู้มีบุตรยาก โรงพยาบาลศิริราช และมีผลการตรวจน้ำเชื้ออสุจิอยู่ในเกณฑ์ปกติ โดยตรวจความเข้มข้นของน้ำเชื้อ ตรวจดูการเคลื่อนไหว ตรวจหาความผิดปกติของตัวอสุจิ และดูความผิดปกติของโครมาตินที่ตัวอสุจิ โดยย้อมสี acridine orange ก่อนและหลังการแช่แข็ง 6 เดือน

ผลการศึกษา: ผลการศึกษาพบว่าน้ำเชื้ออสุจิที่ผ่านการแช่แข็งด้วยวิธีใช้อะไรของไนโตรเจนเหลวเป็นระยะเวลา 6 เดือน มีความผิดปกติของโครมาตินอย่างมีนัยสำคัญ ($p < 0.001$) นอกจากนี้พบว่า การเคลื่อนไหวและอัตราการรอดของตัวอสุจิก็น่าจะลดลงอย่างมีนัยสำคัญเช่นกัน ($p < 0.001$) แต่รูปร่างของตัวอสุจิหลังการแช่แข็งลดลงอย่างไม่มีนัยสำคัญทางสถิติ ($p = 0.606$)

สรุป: น้ำอสุจิที่ผ่านการแช่แข็งโดยวิธีควบคุมอัตราการลดอุณหภูมิด้วยไนโตรเจนเหลวเป็นระยะเวลา 6 เดือน จะได้น้ำเชื้อที่คุณภาพลดลงอย่างชัดเจน ดังนั้นในการให้บริการอสุจิบริจาคโดยการแช่แข็งอสุจิไว้ 6 เดือนเพื่อตรวจการติดเชื้อ HIV ของผู้บริจาคจึงอาจมีผลทำให้อัตราการตั้งครรภ์ลดลง