Original Articles/นิพนธ์ต้นฉบับ

A Comparision of the Cryodamage on Human Sperm in Vapor and Slow Freezing.

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Abstract

Objective: To evaluate the percentage of motile, vitality, morphology, and DNA integrity change on human sperm after freeze-thawing compared between vapor and slow freezing method.

Setting: The Infertility and ART Unit, Department of Obstetrics and Gynaecology, Faculty of Medicine Ramathibodi Hospital.

Materials and Methods: In this experimental study, forty-five normozoospermic volunteer donors were used in the study. After swim up of the sperm, the sperm suspensions were divided into two equal parts, one part was kept as a control (frozen with vapor phase liquid nitrogen) and the other was used for the frozen with slow freezing. After one month of cryostorage, the sperm samples were thawed. The percentage of motile, vitality, morphology, and DNA integrity change were determined. The data was analyzed by a paired t-test.

Results: The percentage of the sperm motility with slow freezing was observed to decrease from 85.9 to 45.4 while that of the control declined to 40.7. The percentage of motile change of human sperm was significantly better following freezing with slow freezing than the control. The percentage of vitality change on human sperm assessed with slow freezing was significantly higher than in the vapor phase liquid nitrogen method. No significant differences in post thaw normal morphologically sperm and DNA integrity were compared between the vapor and slow freezing method.

Conclusion: The percentage of motile and vitality change of human sperm with slow freezing is superior to that of the vapor phase liquid nitrogen method.

Keywords: sperm cryopreservation, vapor phase liquid nitrogen, slow freezing

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Introduction

The cryopreservation of human sperm is commonly used to preserve male fertility in assisted reproduction technology. Since the establishment of this technique for human sperm cryopreservation in 1960s, it has been an important tool to preserve male fertility using assisted reproduction technology on a routine basis. However it may cause damage to the spermatozoa.

The donor sperm is one of many options for treatment in infertile couples who have azoospermia. Cryodamage of sperm can be evaluated from motility, vitality, morphology, and DNA integrity. Chromatin staining of sperm cells with acridine orange has been widely accepted as a predictor of DNA integrity in many cell types including human sperm[1]. Current methods of human sperm cryopreservation are crude compared with those for human embryos. Various methods of cryopreservation have been evaluated for their effects on sperm motility and sperm chromatin[2-5]. Though cryopreservation has been widely and routinely used, the effect of cryopreservation on the structure of sperm chromatin remain unclear.

The aim of this experimental study was to compare the cryodamage of human sperm in the vapor and slow freezing method on the sperm motile, vitality, morphology, and DNA integrity.

Materials and Methods

This prospective experimental study was performed at the Infertility and ART Unit, Department of Obstetrics and Gynaecology, Faculty of Medicine Ramathibodi Hospital. The protocol was approved by the Ethics Committee of the Faculty of Medicine Ramathibodi Hospital, Mahidol University.

Semen samples

Human semen samples were collected from 45 volunteer donors by masturbation in a sterile container after 2 to 7 days of sexual abstinence. The characteristics of semen parameters were evaluated according to the World Health Organization (WHO) guideline[6]. Sperm morphology was assessed by the Kruger's strict criteria. The sperm vitality was determined using eosin-Y test. The semen samples were stained with acridine orange for evaluation of DNA integrity[1,7].

Selection criteria

Semen inclusion criteria were required to contain 20 million or more spermatozoa per milliliter, and to show at least 50 percent progressive motility to be included in the study.

Sperm preparation

After semen analysis, each semen sample was prepared by swim up and divided into two equal parts, one part was kept as a control (frozen with vapor phase liquid nitrogen) and the other part was frozen with slow freezing.

Sperm freeze-thawing

Vapor freezing

The aliquot was a mixed homogenous drop with an equal volume of sperm cryopreservation medium (SpermFreeze FertiPro N.V. Beemem Belgium). The mixed sperm vial was left at room temperature for 10 minutes. The vial was placed at 15 centimeters above the level of the liquid nitrogen for 15 minutes, and then was quickly plunged into liquid nitrogen for storage.

Slow freezing

The aliquot was a mixed homogenous drop with an equal volume of sperm cryopreservation medium (SpermFreeze FertiPro N.V. Beemem Belgium). The mixed sperm vial was left in room temperature for 10 minutes. The vial was loaded into a controlled rate freezer (Planer Kryo 10 series III) and cooled from 20°-5°C at a rate of -1°C/min. The vial was then cooled at a rate of -10°C/min to -80°C/min, and then was

quickly plunged into liquid nitrogen for storage.

Sperm thawing

After at least one month of cryostorage in liquid nitrogen, the sperm samples were thawed in room temperature (25°-28°C) for 5 minutes. Each thawed sample was washed with 1.5 ml of Flushing medium and centrifuged at 250 x g for 7 minutes to remove the cryoprotectant. The sperm pellet was mixed with 0.5 ml of Flushing medium. The post thaw sperm concentration, motility, vitality, morphology, and DNA integrity were assessed in the same method as after preparation (Prefreeze). The comparison was made between the post thaw sperm with vapor freezing and slow freezing.

Data and statistical analysis

The data was analyzed by a paired t-test. Statistical calculations were performed using the computer program SPSS Version 11.5 (SPSS Inc., Chicago. IL, USA). The level of significance was set at $p<0.05$. The results were presented as mean and standard deviation (mean ± SD).

Results

The characteristics of semen parameters in before and after preparation (Prefreeze) presented as normal as shown in Table 1.

For post thaw sperm, the mean percentage of the sperm motility with slow freezing was observed to decrease from 85.9 to 45.4 while that of the vapor freezing declined to 40.7. The data in Table 2 shows significant decrease of post thaw sperm motility, and vitality. The percentage of post thaw sperm motile and vitality were higher in the slow freezing method compared with the vapor freezing method (45.4 ± 7.7 versus 40.7 ± 9.7 and 47.8 ± 6.6 versus 43.5 ± 8.5). No significant differences in post thaw normal morphologically sperm and DNA integrity were compared between vapor and slow freezing method.

Table 1. Characteristics of semen parameters in before and after preparation

Parameters	Before preparation	After preparation (Prefreeze)
Volume (ml)	2.7	1.0
Concentration (mil/ml)	79.4	47.0
Motility (%)	59.9	85.9
Vitality (%)	65.3	87.8
DNA integrity (%)	84.5	90.7
Normal Morphology (%)	12.8	15.9

Table 2. The post thaw sperm parameters with vapor and slow freezing (mean ± SD)

Parameters	Vapor freezing	Slow freezing	p value
Motility (%)	40.7 ± 9.7	45.4 ± 7.7	$p < 0.05$
Vitality (%)	43.5 ± 8.5	47.8 ± 6.6	$p < 0.05$
DNA integrity (%)	87.4 ± 4.0	88.5 ± 3.7	NS
Normal Morphology (%)	15.2 ± 3.7	15.3 ± 3.8	NS

NS = Not significance

The slide was viewed under the fluorescence microscope (Nikon E80i, Tokyo, Japan). At least 200 spermatozoa were counted to determine the percentage. As shown in figure 1, The sperm samples were stained with acridine orange (AO) to stain all sperm chromatin, prior to evaluation. The percentage of spermatozoa with DNA damage was determined by red fluorescence (positive sperm) and AO for negative sperm was determined by green fluorescence in figure 2. The DNA integrity was 87.4 ± 4.0 % versus 88.5 ± 3.7 %, so the DNA integrity in the vapor freezing was lower than the slow freezing method as shown in figure 3 although, there was no significant difference in the DNA integrity between the two groups.

This study suggests that even the percentage of DNA fragmentation (DNA damage) showed statistical difference between before and after preparation (15.5 % and 9.3 % respectively) as shown in figure 4. The DNA fragmentation (DNA damage) in the vapor freezing was higher than the slow freezing method (12.6 % and 11.5 % respectively) as shown in figure 5 although, there was no significant difference in the DNA damage between the two groups.

Discussion

Since the establishment of a technique for human sperm cryopreservation, this has been refined and is routine by used in of assisted reproduction



Fig.1 *Acridine Orange for positive sperm (red fluorescence:DNA damage)*



Fig.2 *Acridine Orange for negative sperm (green fluorescence:DNA normal)*

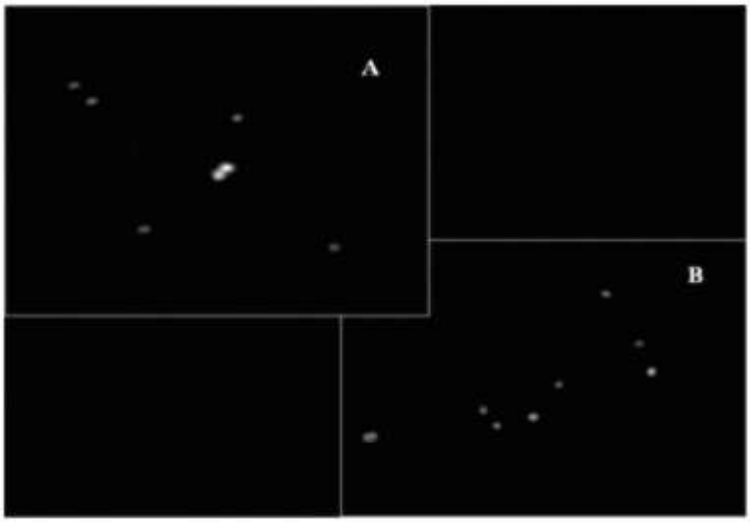


Fig.3 *Acridine Orange for evaluation of DNA integrity in the vapor freezing (A) and the slow freezing method (B)*

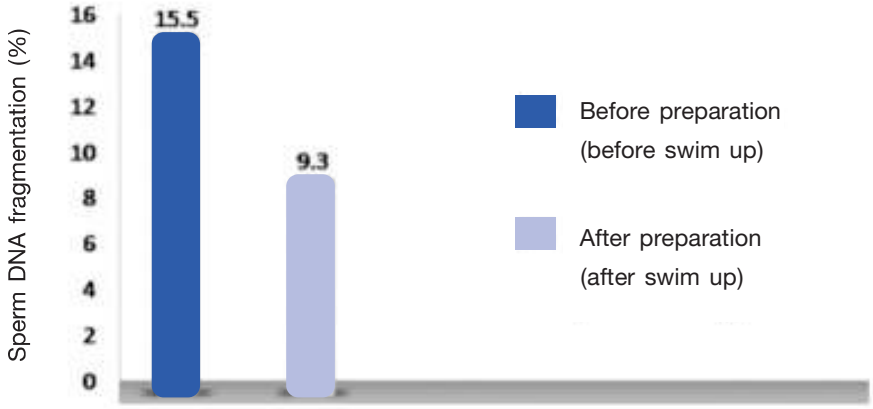


Fig.4 *The percentage of DNA fragmentation (DNA damage) showed no statistical difference between before preparation (before swim up) and after preparation (after swim up).*

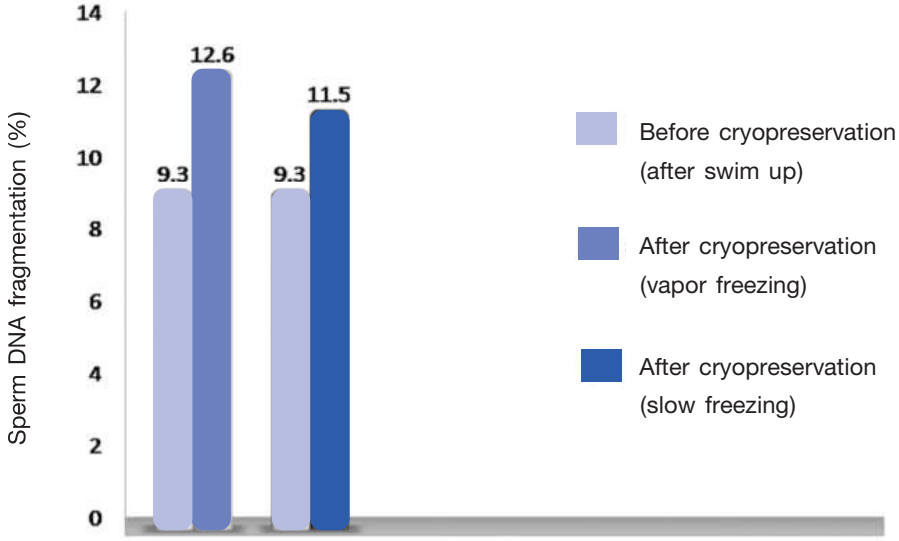


Fig.5 *The percentage of DNA fragmentation (DNA damage) in the vapor freezing and the slow freezing method*

centers. Cryodamage of sperm can be evaluated from motility, vitality, morphology, and DNA integrity. Though cryopreservation has been widely and routinely used, the effect of cryopreservation on the structure of sperm chromatin remain unclear. Sperm cryopreservation has long been an important tool to preserve male fertility using assisted reproduction technology. Also sperm DNA fragmentation impact on reproductive outcome, and decreases the pregnancy rate outcome which depends on oocyte quality.[12-17] The chromatin staining of cells with acridine orange (AO) has been widely accepted as a predictor of DNA damage in many cell types including human sperm.[1,7] The most important factor in the reduction of sperm viability was osmotic shock, due to changes in the physical properties of the extracellular environment during warming.

Therefore, the slow freezing causes extensive chemical and physical damage to sperm cell membranes[8]. In our study, the results demonstrate that the motile change of human sperm was significantly better following freezing with slow freezing than the vapor freezing. The percentage of vitality change on human sperm assessed with slow freezing was significantly higher than the vapor freezing.[7-11] We found no significant changes in sperm morphology and DNA integrity after cryopreservation. Although sperm motility and morphologically are frequently used to assess male fertility, these parameters are not accurate predictors[8]. As well as sperm motility after cryo-preservation being markedly improved.

Conclusion

The percentage of post thaw sperm motility was significantly higher in the slow freezing method than that in the vapor freezing method. Sperm viability, significantly decreased after thawing in both groups. The decreased in viability was significantly higher in the slow freezing method than that in the vapor freezing method. No significant differences in post thaw DNA integrity was compared between vapor and slow freezing method. The mean percentage of morphologically normal sperm did not change significantly after thawing in both groups. The mean percentage of the sperm motile and vitality with slow freezing is superior to that of the vapor phase liquid nitrogen method. Therefore, the slow freezing method resulted in significantly better sperm motile and vitality. Our results suggest that swim up separation of motile sperm from normal semen, does not increase the level of DNA fragmentation (DNA damage).

Acknowledgement

The authors would like to thank Associate Professor Sawaek Weerakiet, head of Department of Obstetrics and Gynaecology for permission to do this research. The authors also wish to thank all officers of infertility and ART unit for their support with this work.

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Original Articles/นิพนธ์ต้นฉบับ

ความเสียหายของอสุจิมนุษย์หลังการแช่แข็งโดยวิธีอังไอไนโตรเจนเหลวเปรียบเทียบกับวิธีการลดอุณหภูมิด้วยเครื่องแช่แข็ง

แอนนา วงษ์กุหลาบ, วท.ด., วิชาญ โชคธนะศิริ, พบ., อร่าม โรจนสกุล, พบ.

หน่วยเอนโดครีนการเจริญพันธุ์และภาวะมีบุตรยาก ภาควิชาสูติศาสตร์-นรีเวชวิทยา
คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล กรุงเทพฯ 10400

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์ เพื่อประเมินความเสียหาย (ร้อยละของการเคลื่อนไหว ร้อยละของการมีชีวิต ร้อยละของรูปร่างที่ปกติและร้อยละของดีเอ็นเอที่ปกติ) ของอสุจิมนุษย์หลังจากทำการแช่แข็งวิธีอังไอไนโตรเจนเหลวเปรียบเทียบกับวิธีการลดอุณหภูมิด้วยเครื่องแช่แข็ง จากการหลั่งน้ำอสุจิของผู้ชาย 45 คน ที่มีความเข้มข้นมากกว่าหรือเท่ากับ 20 ล้านตัวต่อมิลลิลิตรและตัวเคลื่อนที่มากกว่าหรือเท่ากับร้อยละ 50 โดยทำการเตรียมอสุจิแบบว่ายขึ้นก่อนทำการแบ่งน้ำอสุจิออกเป็นสองส่วน ส่วนที่ 1 ทำการแช่แข็งโดยวิธีอังไอไนโตรเจนเหลว (กลุ่มควบคุม) ส่วนที่สองทำการแช่แข็งวิธีลดอุณหภูมิด้วยเครื่อง (กลุ่มศึกษา) ผลการศึกษา พบว่า จากการละลายอสุจิหลังการแช่แข็ง 1 เดือน ค่าร้อยละของการเคลื่อนไหวจาก 85.9 ลดลงเหลือ 45.4 ในกลุ่มศึกษา ส่วนกลุ่มควบคุมเหลือ 40.7 ซึ่งมีความแตกต่างอย่างมีนัยสำคัญทางสถิติ และค่าร้อยละของการมีชีวิตที่เปลี่ยนแปลงกลุ่มศึกษาให้ผลดีกว่ากลุ่มควบคุมเช่นกัน ส่วนร้อยละของรูปร่างที่ปกติและดีเอ็นเอที่ปกติพบว่าไม่มีความแตกต่างทางสถิติ จากการศึกษากล่าวได้ว่าการแช่แข็งวิธีลดอุณหภูมิด้วยเครื่องแช่แข็ง (กลุ่มศึกษา) เป็นทางเลือกหนึ่งของการแช่แข็งอสุจิมนุษย์