Comparison of the Motility, Morphology, and DNA Integrity of Cryopreserved Human Spermatozoa from Processing Semen before and after Cryopreservation

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Background: *Cryopreservation of spermatozoa is an extremely important process in the field of male infertility. High quality preserved spermatozoa has been reported to retain better motility and DNA quality.*

Objective: *We showed the effect of semen preparation on the quality of cryopreservation of human spermatozoa.*

Material and Method: *We compared cryopreserved spermatozoa separated by Sil-Select density gradient centrifugation before or after cryopreservation in terms of spermatozoa motility, morphology, and DNA integrity. Spermatozoa's motility was determined by CASA; the morphology was determined by the eosin-methylene blue staining; and the DNA integrity was determined by TUNEL assay.*

Results: *We found that semen preparation before or after cryopreservation produced spermatozoa with comparable motility and morphology. However, semen preparation before cryopreservation showed a significantly higher total motile spermatozoa count (8.48 [2.47 to 37.87] vs. 5.35 [0.29 to 17.30], p-value <0.05) and significantly better DNA integrity as indicated by less DNA fragmentation (21.34±3.01 vs. 24.29±3.01, p<0.001).*

Conclusion: *We concluded that spermatozoa prepared by Sil-Select density gradient centrifugation before cryopreservation can improve quality of the preserved spermatozoa in terms of the total motile spermatozoa count and DNA integrity.*

Keywords: *Cryopreserved human spermatozoa, Sil-Select density gradient centrifugation, DNA integrity*

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Cryopreservation of human spermatozoa has been practiced for many years[1,2]. It is routinely used in assisted reproduction, e.g., preservation of male gametes for future fertility, storage of donated spermatozoa, and storage of spermatozoa retrieved from patients[1-3]. Presently, thawing semen is usually prepared before being used for infertility treatment such as intrauterine insemination (IUI), in vitro fertilization (IVF), or intracytoplasmic sperm injection (ICSI). The pregnancy rates after artificial insemination with cryopreserved semen often decrease due to poor spermatozoa quality after thawing[4]. The quality of frozen spermatozoa that has been reported to exhibit a mark reduction of motility and DNA integrity depends largely on the quality of spermatozoa before freezing[5-10]. The efforts to improve the fertilizing ability of cryopreserved spermatozoa will be useful for successful IUI[10-12].

One of the spermatozoa preparation techniques is to isolate the motile spermatozoa and remove their seminal plasma, which contains the factors that inhibit the acrosome reaction[13,14]. Preparation methods, e.g., Sil-Select density centrifugation, have been developed to improve the number of motile spermatozoa recovered, thus, increasing the assisted reproductive outcomes[15]. It has been shown that the pregnancy outcomes for IUI depended on the spermatozoa parameters such as the motility, the morphology, and the DNA damage[16-19].

We conducted the present study to compare the quality of post-thawing spermatozoa that were prepared by density gradient centrifugation with Sil-Select before or after cryopreservation in terms of their total motile spermatozoa count, morphology, and DNA fragmentation.

Material and Method

All procedures in the present study had been approved by the Ethical Committee of Faculty of

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Medicine, Ramathibodi Hospital, Mahidol University. All patients had been given a written informed consent to the use of their gametes for the research study.

Semen collection and assessment

We selected normal spermatozoas according to the World Health Organization Guidelines[20]. Routine semen analyses were performed using the computer assisted semen analysis (CASA) according to WHO 1999 to determine spermatozoa counts and motility.

Morphology assessment

Spermatozoa morphology was determined by using the strict criteria laid down by Kruger et al[21]. After preparation of slides, spermatozoas were stained using the eosin and methylene blue. Two hundred spermatozoas were counted at 400x magnification.

DNA integrity assessment

DNA integrity was determined by using an in-situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detection kit (In situ Cell Death Detection Kit, POD; Roche, Mannheim, Germany). All semen including the fresh semen and thawed semen were evaluated by using the same technique. Semen was centrifuged at room temperature for 10 minutes at 800x g. The supernatant was discarded, and the remaining pellet was washed twice in phosphate-buffered saline (PBS), pH 7.4, (Invitrogen Corporation, Scotland, United Kingdom), and resuspended in PBS. A droplet of the spermatozoa suspension was smeared onto a pretreated glass slides (Microscope slide, China), air dried, and fixed by immersion in freshly prepared 4% paraformaldehyde in PBS, pH 7.4 for one hour at room temperature. Next, the slides were rinsed in PBS for five minutes at room temperature, treated with pre-chilled 0.1% Triton X-100 in 0.1% sodium citrate for two minutes on ice, and rinsed twice with PBS for five minutes at room temperature. Excess liquid was removed by tapping the slides. Then, the staining was performed according to the manufacturer's instructions.

Visualization and evaluation of spermatozoa DNA fragmentation was performed by using a Nikon (Tokyo, Japan) photomicroscope equipped with epifluorescent light under appropriate filters. Spermatozoa heads were examined at 1,000x magnification, and each was recorded as either green staining on the spermatozoa head (positive, spermatozoa with fragmented DNA), or red staining (negative, spermatozoa without fragmented DNA). Two hundred spermatozoas were assessed from each subject, and the percentages of spermatozoa cells with positive staining were calculated.

Sil-Select density gradient centrifugation

Semen preparation was performed with the density gradient centrifugation technique at room temperature. A 1.5 ml of 40% Sil-Select Stock solution (FertiPro NV, Belgium) was dispensed into a sterile conical-bottomed tube and underlay 1.5 ml of 90% Sil-Select Stock solution. The semen was gently overlaid on a two-layer discontinuous gradient. The tube was centrifuged at 350x g for 10 minutes. The supernatant was removed, and the remaining pellet was transferred to a fresh tube containing 1.5 ml of FertiCult Flushing medium (FertiPro NV, Belgium) and then centrifuged at 300x g for five minutes twice. The last pellet was resuspended by transferring to a fresh tube containing 1 ml of FertiCult Flushing medium.

Cryopreservation of samples

Both processed and unprocessed semen was frozen by the same standard method. The semen volume was mixed with equal volume (1:1) of Spermfreeze® cryoprotectant (FertiPro NV, Beernem, Belgium). The mixture was swirled gently and kept at room temperature for 10 minutes and then the total volume 0.5 ml of mixture was measured into standard 0.5 ml insemination straws (Cryo Bio System, Paris, France). The straws were exposed in the liquid nitrogen vapor (10 cm above the level of liquid nitrogen; -80°C) for 10 minutes and then placed in cassettes (MTG Medical Technology, Altdort, Germany) before transferring into liquid nitrogen (-196°C) (Fig. 1).

Thawing of samples

After four to five weeks of storage, all samples were removed from liquid nitrogen and left to thaw at room temperature for 15 to 20 minutes. The thawed samples were then assessed for spermatozoa's motility, morphology, and DNA integrity as prior described. The processed samples were assessed first while the unprocessed samples were prepared with Sil-Select before being assessed for all parameters (Fig. 1).

Statistical analysis

Data were analyzed by using the program STATA® version 9 (StataCorp, TX, USA). The data were presented as mean ± SD. Data with normal distribution were analyzed by the paired Student's

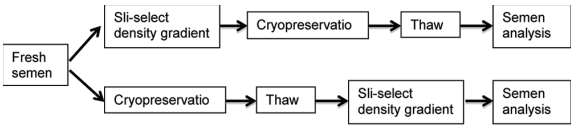

Fig. 1 Show both method of experiments.

t-test. Data without normal distribution (motility) were analyzed by using the Wilcoxon signed-ranks test. A *p*-value below 0.05 was considered as statistically significant.

Results

Thirty-eight semen samples were collected and analyzed prior to any cryopreservation procedures to give a baseline data. The mean of total spermatozoa count, total motile spermatozoa count, motility, progressive motility, normal morphology, and DNA fragmentation for these fresh samples were 106.49±85.43(x10^6), 76.46±69.64(x10^6), 67.45±9.97%, 41.45±11.41%, 10.31±4.15%, and 30.24±2.58%, respectively (Table 1). After Sil-Select density gradient centrifugation, we found that spermatozoa showed 22.82% higher motility (82.84±9.10% vs. 67.45±9.97%, p<0.0001), 23.93% more spermatozoa with better morphology (12.79±5.90% vs. 10.32±4.15%, p<0.005), and 38.46% less spermatozoa with DNA damage (18.61±2.86% vs. 30.24±2.58%, p<0.0001) when compared with spermatozoa from fresh semen (Fig. 2).

Table 1. Spermatozoa parameters of fresh semen

Spermatozoa parameters	Fresh semen
Total sperm count (x10^6)	106.49±85.43
Total motile sperm count (x10^6)	76.46±69.64
Motility (%)	67.45±9.97
Progressive motility (%)	41.45±11.41
Normal morphology (%)	10.31±4.15
DNA fragmentation (%)	30.24±2.58

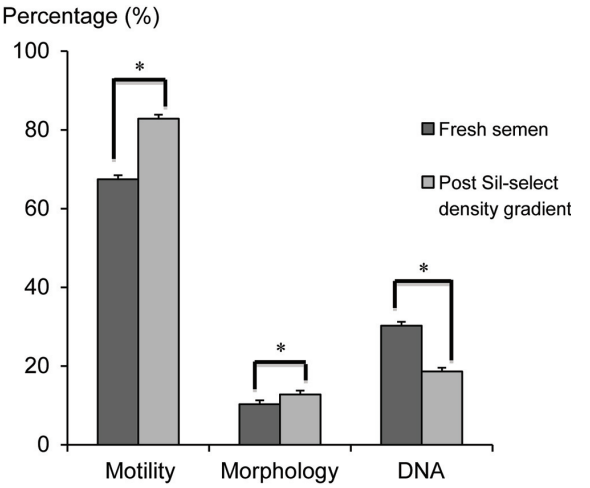

Fig. 2 Percentage of spermatozoa parameter compared the baseline with prepared spermatozoa, * p<0.05.

Since Sil-Select density gradient centrifugation can enrich healthy spermatozoa, we tested whether using Sil-Select density gradient centrifugation prior to cryopreservation (Method 1, Fig. 1) can improve overall spermatozoa quality. In addition, we used Sil-Select density gradient centrifugation after thawing the cryopreserved semen (Method 2, Fig. 1).

Importantly, we found that spermatozoa from Method 1 and Method 2 showed comparable morphology and motility. All motility parameters were not changed (data not shown). Interestingly, the total number of spermatozoa count and the total number of motile spermatozoa count were significantly higher in spermatozoa recovered from Method 1 when compared with Method 2 (16.09 [4.50 to 74.25] vs. 9.56 [1.25 to 27.90], p-value <0.05 and 8.48 [2.47 to 37.87] vs. 5.35 [0.29 to 17.30], p-value <0.05, respectively). Moreover, DNA fragmentation in spermatozoa recovered from Method 1 appeared to be slightly but significantly lower than that from Method 2 (21.34±3.01 vs. 24.29±3.01, p<0.001, Table 2).

Table 2. Post-thawed parameters of spermatozoa in preparation before cryopreservation group (Method 1) and preparation after cryopreservation group (Method 2)

Sperm parameters	Semen sample		*p*-value
	Method 1	Method 2	
Total sperm count (x10^6)*	16.09 (4.50 to 74.25)	9.56 (1.25 to 27.90)	0.02
Total motile sperm count (x10^6)*	8.48 (2.47 to 37.87)	5.35 (0.29 to 17.30)	0.03
Motility (%)**	54.50±9.89	52.82±19.99	0.64
Normal morphology (%)**	10.39±3.85	9.68±3.60	0.41
DNA Fragmentation (%)**	21.34±3.01	24.29±3.01	<0.001

* Median (range): comparison by Wilcoxon sign-rank test
** Mean ± SD: comparison by Paired t-test

Discussion

We have shown that the preparation of spermatozoa by Sil-Select density gradient technique before freezing give a better quality of post-thawed spermatozoa than direct cryopreservation of fresh semen for total spermatozoa count, total motile spermatozoa count and DNA integrity. The quality of the preserved spermatozoa from our study is clinically acceptable. The optimal concentration of total motile spermatozoa for successful fertilization and pregnancy in IUI varies. Previous studies found that spermatozoa motility[12,22-24], degree of motility[11,12] and total motile spermatozoa[16,18,19] with varying cut-off threshold can be predictors for IUI success. In our result, Sil-Select density centrifugation of semen before cryopreservation can improve the total motile spermatozoa count from $5.35x10^6$ to $8.48x10^6$. This difference might be more meaningful in infertile men who have mild oligozoospermia. However, further studies need to confirm such effect.

The etiology of DNA damage in spermatozoa appears to be multifactorial and may be due to intrinsic or external factors. Our study demonstrated that semen specimens processed by Sil-Select density gradient centrifugation before cryopreservation resulted in a significantly lower DNA fragmentation (21.34%) compared to conventional method (24.29%), because intact ejaculated spermatozoa may be damaged by endogenous ROS from seminal leukocytes and other damaged spermatozoa. The stress caused by cryopreservation procedure itself may further damage spermatozoa. Therefore, enriching a highly motile spermatozoa population before freezing enhances the overall spermatozoa quality recovered after cryopreservation. It has been suggested that abnormal DNA integrity may adversely affect fecundity in couples having natural intercourse and in those treated by IUI, IVF, and ICSI[19,25-27].

From our study, the motility and the morphology of the cryopreserved spermatozoas in both groups appeared to be comparable. Many studies have observed strong correlation between strict spermatozoa morphology and IUI success[28-31], although others have not[11,32]. Meta-analysis of predictive values of normal spermatozoa morphology using the strict criteria in IUI showed a significant improvement in pregnancy rate above 4% threshold.

Since the present study used normal semen, the results cannot be extrapolated to abnormal semen or male infertility. Further studies are needed to these directions. The present study was designed to examine major parameters of interest (spermatozoa motility and DNA integrity), but these were the intermediate outcome for infertility treatment that might not reflect ultimate outcome, the pregnancy rate. However, the benefits of this method are less storage volume and improve spermatozoa quality from cryopreservation. Since Sil-Select density centrifugation is conducted prior to cryopreservation, thawed spermatozoa can be used promptly and directly for IUI or IVF and ICSI.

Conclusion

Sil-Select density gradient centrifugation processing before cryopreservation (Method 1) improves total spermatozoa count, total motile spermatozoa count, and gives less DNA fragmentation than conventional practice (Method 2).

What is already known on this topic?

The sperm cryopreservation method is routinely used in various circumstances such as in an assisted reproduction and to preserve male gametes for future fertility. Nowadays, the thawing semen is usually prepared before being used for infertility treatment such as IUI and IVF. Qualities of the preserved spermatozoa are a mark reduction of motility and DNA integrity after thawing.

What this study adds?

The findings support the spermatozoa prepared by Sil-Select density gradient centrifugation before cryopreservation can improve the total motile spermatozoa count and DNA integrity after thawing.

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Potential conflicts of interest

None.

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การศึกษาอสุจิมนุษย์หลังแช่แข็งเปรียบเทียบอสุจิที่ผ่านขบวนการการเตรียมอสุจิก่อนกับหลังการแช่แข็ง

รวิวรรณ เจียมอนุกูลกิจ, ฉัตรชัย ตรีธรรมพินิจ, มัธชุพร สุขประเสริฐ,ศศิวิมล รัตนสิริ, วิชาญ โชคธนะศิริ, ชลธิชา สถิระพจน์

ภูมิหลัง: *ขบวนการการแช่แข็งอสุจิถือเป็นขั้นตอนที่มีความสำคัญในการรักษาภาวะมีบุตรยากที่เกิดจากฝ่ายชาย การแช่แข็งอสุจิที่มีคุณภาพดีมีรายงานว่าหลังการละลายตัวแล้ว ควรตรวจพบว่าอสุจิยังคงมีการเคลื่อนที่ดี ร่วมกับมีการรักษาคุณภาพของ DNA ไว้ได้*

วัตถุประสงค์: *เพื่อเปรียบเทียบผลการเตรียมอสุจิ ด้วยวิธี Sil-Select density gradient centrifugation ก่อนและหลังการแช่แข็งของอสุจิมนุษย์*

วัสดุและวิธีการ: *การศึกษานี้ได้ทำการศึกษาผลการเตรียมอสุจิ ด้วยวิธี Sil-Select density gradient centrifugation ก่อนและหลังการแช่แข็งของอสุจิมนุษย์ ต่ออัตราการเคลื่อนที่ ความผิดปกติของรูปร่าง รวมถึงปริมาณความสมบูรณ์ของ DNA ของตัวอสุจิหลังผ่านการแช่แข็งด้วยวิธีมาตรฐาน ซึ่งการตรวจวัดคุณภาพของอสุจิมีวิธีการดังนี้ การตรวจวัดการเคลื่อนที่ของอสุจิด้วยคอมพิวเตอร์ (CASA) การตรวจนับร้อยละของอสุจิที่มีรูปร่างที่ผิดปกติ ด้วยการนับตัวอสุจิที่ติดสีด้วยการย้อม eosin-methylene blue และการตรวจความสมบูรณ์ของ DNA ของอสุจิด้วยวิธี TUNEL assay*

ผลการศึกษา: *พบว่าการเตรียมอสุจิทั้งก่อนและหลังการแช่แข็งด้วยวิธีมาตรฐานนั้นเมื่อละลายอสุจิมาตรวจพบว่าร้อยละของอสุจิที่มีการเคลื่อนที่และร้อยละของอสุจิที่มีรูปร่างผิดปกติมีค่าใกล้เคียงกันมาก แต่พบว่าในกลุ่มที่มีการเตรียมอสุจิก่อนการแช่แข็งหลังจากการละลายพบว่าจำนวนตัวอสุจิเคลื่อนที่ทั้งหมดมากกว่ากลุ่มที่มีการเตรียมอสุจิหลังผ่านการแช่แข็งอย่างมีนัยสำคัญทางสถิติ (8.48 [2.47-37.87] vs. 5.35 [0.29-17.30], p-value <0.05) และพบว่าปริมาณการทำลาย DNA อสุจิลดลงอย่างมีนัยสำคัญทางสถิติ (21.34±3.01 vs. 24.29±3.01, p<0.001)*

สรุป: *การเตรียมอสุจิด้วยวิธี Sil-Select density gradient centrifugation ก่อนการแช่แข็งอสุจิสามารถเพิ่มคุณภาพของอสุจิหลังการละลายในด้านจำนวนอสุจิโดยรวมและความสมบูรณ์ DNA ของอสุจิ หลังผ่านขบวนการแช่แข็งด้วยวิธีมาตรฐาน*