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# Comparison of postthawing sperm motility recovery between cryopreserved with and without cryoprotective agent using 4 different cryopreservation methods

**Hilwah Nora[1*], Shofwal Widad[2], Irwan Taufiqur Rachman[2]**
[1]Department of Obstetrics and Gynecology, Faculty of Medicine, Syah Kuala University, Darussalam, Banda Aceh, [2]Department of Obstetrics and Gynecology, Dr. Sardjito General Hospital/Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

## ABSTRACT

Frozen-thawed human spermatozoa are routinely used for many assisted reproduction program. However, cryopreserved spermatozoa was reported to yield lower pregnancy rates compared to fresh semen in both intra uterine inseminations and *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) due to the reduction of sperm motility and viability induced by cryopreservation procedures. This study was aimed to evaluate the influence of cryoprotective agent (CPA) and cryopreservation methods on human sperm motility. This was a quasi experimental study. Thirty seven normozoospermic semen samples collected in Permata Hati Infertility Clinics of Dr. Sardjito General Hospital, Yogyakarta were recruited. Four different cryopreservation methods were applied using and without CPA (TEST-yolk buffer). In simple two steps freezing, cryostraw were gradually frozen from 8 to -4°C. In simple graduated freezing, cryostraus were directly frozen at -4°C. In vapor phase freezing method, the samples in cryostraw were placed 1 cm above liquid nitrogen. In the last method, the samples were directly submerged into liquid nitrogen. Thawing was conducted by incubation at 37°C for 5 minutes. The sperm motility recovery after cryopreservation in the 4 different cryopreservation methods was evaluated and analyzed by analysis of variance (ANOVA). The fresh sperm motility before cryopreservation was 52.9 ± 4.50%. The recovery of motile sperms was 17.00 ± 7.83%, 20.96 ± 5.81%, 15.06 ± 8.55% and 15.68 ± 8.3%, when using CPA and 5.63 ± 4.63%, 5.47 ± 3.95%, 4.45 ± 4.46% and 6.08 ± 5.06% when without CPA following direct plunge to liquid nitrogen freezing, vapor liquid nitrogen freezing, simple graduated freezing and simple 2-steps freezing, respectively. Among methods using CPA, the vapor phase method resulted in highest sperm motility recovery. In methods without CPA, no significant difference of sperm motility recovery was observed among the 4 different cryopreservation methods. In conclusion, the use of CPA for cryopreservation improves sperm motility recovery.

## ABSTRAK

Kriopreservasi spermatozoa manusia sudah rutin digunakan pada berbagai program reproduksi. Angka kehamilan lebih rendah telah dilaporkan, baik pada inseminasi intrauterine maupun *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) dengan menggunakan sperma beku dibandingkan sperma segar akibat adanya penurunan motilitas dan viabilitas akibat prosedur kriopreservasi. Penelitian ini bertujuan untuk mengkaji pengaruh pemakaian krioprotektan (*cryoprotective agent* = CPA) dengan berbagai metode kriopreservasi terhadap motilitas sperma. Penelitian ini merupakan penelitian kuasi eksperimental. Tiga puluh tujuh sampel semen *zoospermia* normal dari klinik infertilitas Permata Hati RSUP Dr. Sardjito Yogyakarta diikutkan penelitian. Motilitas sperma dievaluasi sebelum kriopreservasi. Selanjutnya sampel dibagi dua kelompok,

* corresponding author: nora.hilwah@yahoo.com

yakni sampel dengan krioprotektan dan tanpa krioprotektan (TEST-yolk buffer) dan diawetkan dengan 4 metode kriopreservasi yang berbeda. Pada metode s*imple two steps*, sampel dimasukkan *cryostraw* lalu dibekukan bertahap pada suhu 8°C hingga -4°C, sedangkan pada metode *simple graduated* sampel dibekukan pada suhu -4°C saja. Pada metode *vapour phase freezing*, sampel diletakkan 1 cm diatas permukaan nitrogen cair, sementara pada metode *direct plunge*, *cryostraw* langsung dicelupkan ke dalam nitrogen cair. Proses *thawing* dilakukan dengan pemaparan sampel suhu 37°C selama 5 menit. Selanjutnya motilitas sperma *post-thawing* dievaluasi dan dianalisis dengan ANOVA. Motilitas sperma sebelum kriopreservasi adalah 52,9 ± 4,50%. Rerata motilitas sperma *post-thawing* untuk metode d*irect plunge to liquid nitrogen, vapor liquid nitrogen freezing, simple graduated freezing,* dan *simple 2- steps freezing* dengan krioprotektan masing-masing adalah 17,00 ± 7,83%, 20,96 ± 5,81%, 15,06 ± 8,55% dan 15,68 ± 8,3%. Pada kelompok tanpa krioprotektan masing-masing 5,63 ± 4,63%, 5,47 ± 3,95%, 4,45% ± 4,46 dan 6,08 ± 5,06%. Dari analisis statistik dengan ANOVA didapatkan bahwa metode *vapor phase freezing* menghasilkan perolehan kembali motilitas sperma tertinggi dibandingkan metode lainnya, pada kelompok krioprotektan. Pada kelompok tanpa krioprotektan, tidak ada perbedaan bermakna pada perolehan kembali motilitas sperma. Dapat disimpulkan, kriopreservasi dengan penambahan krioprotektan menghasilkan perolehan kembali motilitas sperma lebih baik dibandingkan dengan tanpa krioprotektan.

***Keywords*** : cryopreservation - fertility - sperm mortality - cryoprotective agent - semen

## INTRODUCTION

Semen cryopreservation offers possibility to maintain fertility over a long time period. Frozen-thawed human spermatozoa are routinely used for many assisted insemination and fertilization programs, either by donor or husband. It is also used for self-preservation program for men undergoing vasectomy or therapeutic procedures leading to infertility as a result of surgery or chemo-radio-therapy.[1] The use of frozen-thawed spermatozoa in human reproduction has increased since the introduction of intracytoplasmic sperm injection (ICSI) that allows the use of few spermatozoa obtained by testicular biopsy or needle aspiration for *in vitro* fertilization (IVF).[2,3] However, using cryopreserved spermatozoa has been reported to yield lower pregnancy rates in both intra uterine inseminations (IUI) and IVF/ICSI program, compared to those using fresh semen. These lower pregnancy rates are generally attributed to a reduction of sperm motility and viability induced by cryopreservation procedures. Damage to plasma membrane structures may explain the lower functionality of frozen-thawed spermatozoa.[4,5] Many different techniques have been designed to decrease the cryopreservation stress and different types of cryoprotective agents were developed to minimize the effects of cryopreservation.

Semen freezing is currently used worldwide. There is still no established method that can be referred to as an entranced standard for routine laboratory use. The success of cryopreservation is affected by cryoprotective agent as well as technique of freezing. The damage is due to freezing result in significant motility reduction of cryopreserved spermatozoa after thawing compared to those of prefreezing motility. However, it shows wide inter-individual variability. Therefore, many studies are constantly being done to come up with the best technique to cryo-preserve semen with zero alteration in its original quality. It is essential to look for the best method of preservation which has minimum effects on the sperm function. This study is important to find out the most beneficial method used in preserving the sperm, in term of post-thawing motility recovery rate. The information derived

from this research may also be used as a basis for further study in cryopreservation technique in local clinical setting (climate, humidity, temperature and the facilities availability).

## MATERIALS AND METHODS

### Samples

Human semen samples were obtained, with informed consent, from healthy and fertile donors by masturbation into sterile containers after 2 to 7 days of sexual abstinence. The men were confirmed to be fertile by selecting only men who had previously produced 2 or more children from his marriage. The semen samples were allowed to liquefy for 30 min at room temperature prior to analysis. Standard semen analyses were performed manually by a single investigator and consisted of assessment of semen volume, pH, viscosity, liquefaction, sperm count, sperm motility, sperm morphology and white blood cell count. Only normal quality of sperm according to the WHO guidelines (volume 2-6 mL, pH 7.2-8.0, sperm count >20 x 106/mL, sperm motility >50% type a+b or >25% type a, sperm morphology >30%, sperm vitality >75%, white blood cell counts <1 x 106/mL) were selected for further process of cryopreservation. Approval for utilizing semen samples for all experiments was obtained prior to the study from the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

### Treatment

A quasi experimental design was used in this study. After assessing sperm motility in a fresh semen sample, each semen sample was divided into 2 parts. The first part was mixed with cryoprotective agent (using CPA) and the other part without any cryoprotectant (wihtout CPA). Each part was then divided into 4 equal fractions which cryopreserved with 4 different preservation methods and placed in sterile cryotubes at a volume of 0.25 mL semen sample per tube.

Cryoprotective agent TEST-yolk buffer {zwitterion–citrate–egg yolk extender containing TES [N-tris (hydroxymethyl) methylaminoethane sulfonic acid] and tris [(hydroxymethyl) aminomethane]} was added to group using CPA at a semen-to-medium ratio of 1:1. The ends of all the straws were plugged with polyvinyl chloride to prevent leakage and were then subjected to freezing procedures. Fraction 1 was rapidly frozen in liquid nitrogen vapor. Tubes were horizontally placed 1cm above the liquid nitrogen surface (-180°C) for 45 min and then plunged into liquid nitrogen (-196°C). Fraction 2 and 3 were placed into the chamber of freezer and cooled in the following programmes. Fraction 2 (simple two steps freezing): (1) cooling at 8°C for 30 min; (2) freezing at -4°C for 30 min; (3) placed into liquid nitrogen (-196 °C). Fraction 3 (simple graduated freezing): (1) freezing at -4°C for 45 min; (2) placed into liquid nitrogen (-196°C). Fraction 4 directly submerged into liquid nitrogen. After at least 24 h in liquid nitrogen, samples were thawed by plunging the straws into a water bath at 37°C for 5 min after exposed the cryo-straws to room temperature for about 1 min. Once totally thawed, CPA was removed by adding a sperm wash medium and centrifuging under intense agitation for 5-10 min.

Straws from each experimental treatment were pooled for estimation of sperm motility. Sperm motility was assessed immediately after liquefaction by a single individual. The Makler chamber was used for motility scoring. Motility was estimated under the light microscope using the x400 magnification by counting 100 sperm. Only spermatozoa with progressive motility (WHO categories 'a' and 'b') were assessed. Recovery of motile spermatozoa was defined as the percentage of post-thaw motility x 100% divided by the percentage of pre-freezing motility.

### Statistical analysis

SPSS 15.0 software was used for data entry and analysis. The outcome measured was the mean sperm motility. Analysis of variance (ANOVA) was applied to compare: (1) the post-thaw motilities recovery of sperm frozen using CPA following four different freezing techniques (2) the post-thaw motilities recovery of sperm frozen without CPA after four different freezing techniques. To compare the post-thaw motility recovery of the sperm frozen in TEST-yolk and frozen without CPA for each freezing technique, t-test was used. A p value of less than 0.05 was accepted as statistical significant.

## RESULTS

The sperm motility in 37 fresh semen samples before cryopreservation was 44–64% (52.9 ± 4.5%). The motility of semen after cryopreservation was significantly reduced ($p < 0.01$) when compared with the fresh semen values. FIGURE 1 and 2 show sperm motility determined before and after cryopreservation using and without CPA in 4 different cryopreservation methods.

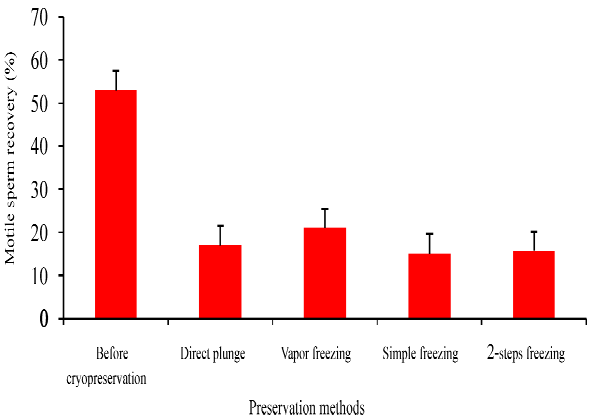


FIGURE 1. Comparison of thawed sperm motility recovery before and after four different freezing protocols using CPA

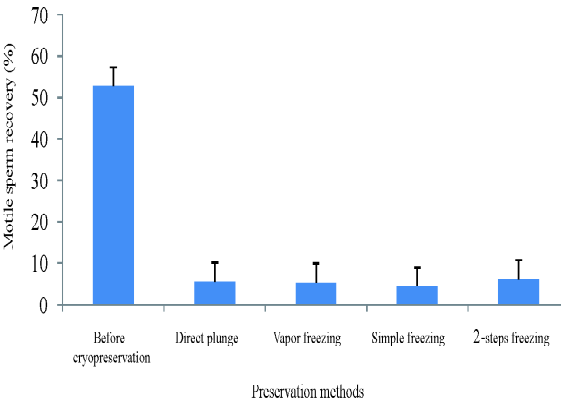


FIGURE 2. Comparison of thawed sperm motility recovery before and after four different freezing protocols without CPA

These data showed a reduction in the motility of spermatozoa after cryopreservation in both groups, indicating a detrimental effect of the freezing. However, the reduction was more prominent in without CPA group (FIGURE 3).

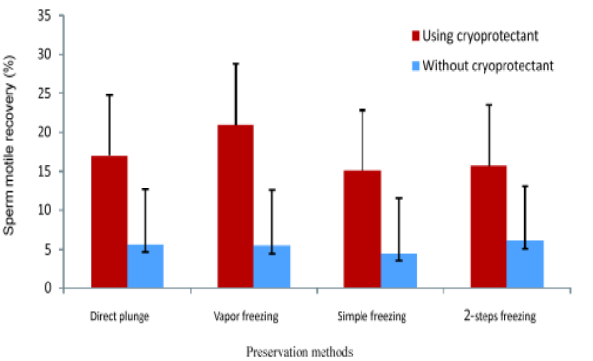


FIGURE 3. Comparison between mean sperm motility recovery of semen with CPA versus without CPA

The mortility of the sperm after cryopreservation using CPA in all of the 4 different cryopreservation methods was significantly hinger than cryopreservation wihtout CPA ($p<0.01$) as shown in TABLE 1-4. The mortality of the sperm after cryopreservation using CPA compared to without CPA after direct plunge to liquid nitrogen freezing was 17.00 ± 7.83%

versus 5.63 ± 4.63%, after liquid nitrogen vapor freezing was 20.96 ± 5.81%, versus 5.47 ± 3.95%, after simple graduated freezing was 15.06 ± 8.55% versus 4.45 ± 4.46% and after simple two steps freezing was 15.68±8.3% versus 6.08±5.06%.

Sperm motility before cryopreservation was significantly lower than after cryopreservation both using CPA and without CPA ($p < 0.05$). Morover, sperm motility after cryopreservation using CPA was significantly better than without CPA ($p < 0.05$). Among the 4 different cryopreservation methods using CPA, the vapor phase freezing method yielded the highest sperm motility recovery ($p<0.01$). However, no significant difference in sperm motility recovery was observed in all of the 4 different cryopreservation methods without CPA.

TABLE 1. Comparison of motile sperm recovery post thawing between CPA-added and non CPA in direct plunge to liquid nitrogen freezing

| Cryopreservation method | n | Mean (SD) | Δ mean | 95%CI | p |
|---|---|---|---|---|---|
| CPA | 37 | 17.01 (7.83) | 11.34 | 8.34-14.35 | < 0.01 |
| Non CPA | 37 | 5.65 (4.63) | | | |

TABLE 2. Comparison of motile sperm recovery post thawing between CPA-added and non CPA in vapor phase liquid nitrogen freezing

| Cryopreservation method | n | Mean (SD) | Δ mean | 95%CI | p |
|---|---|---|---|---|---|
| CPA | 37 | 20.96 (5.81) | 15.50 | 13.19-17.80 | < 0.01 |
| Non CPA | 37 | 5.47 (3.95) | | | |

TABLE 3. Comparison of motile sperm recovery post thawing between CPA-added and non CPA in simple graduated freezing

| Cryopreservation method | n | Mean (SD) | Δ mean | 95%CI | p |
|---|---|---|---|---|---|
| CPA | 37 | 15.06 (8.55) | 10.60 | 7.43-13.78 | < 0.01 |
| Non CPA | 37 | 4.45 (4.46) | | | |

TABLE 4. Comparison of motile sperm recovery post thawing between CPA-added and non CPA in simple 2-steps freezing

| Cryopreservation method | n | Mean (SD) | Δ mean | 95%CI | p |
|---|---|---|---|---|---|
| CPA | 37 | 15.68 (8.31) | 9.60 | 6.40-12.80 | < 0.01 |
| Non CPA | 37 | 6.08 (5.05) | | | |

## DISCUSSION

This study showed that vapor liquid nitrogen freezing was superior to direct plunge freezing, simple graduated freezing and simple two steps freezing as a method of cryo-preservation of high-quality semen. Some authors reported that the computer-controlled freezing methods preserve sperm quality better than vapor freezing[6,7] but others have found no beneficial effects, at least for human spermatozoa.[8] Suspending samples at a fixed height above liquid nitrogen surface in vapor phase can provide controlled-rate freezing conditions. However, it does not produce a linear cooling rate and there is evidence that this nonlinear cooling actually increases the functional survival of spermatozoa.[9] Whatever freezing procedure is used, there is damage to the cells producing either structural or functional changes.[5] The most commonly described adverse effect of cryopreservation is severe impairment of sperm motility.[10] Other investigators have emphasized sperm morphology alterations, coiled tails, or damage to membranes and acrosomes.[5,11,12] Because of the membrane damage and diminished motility, the efficacy of cryopreserved–thawed spermatozoa in achieving pregnancy has been thought to be lower compared to that of fresh semen.[13,14]

Rapid freezing will lead to intracellular ice formation because higher cooling rates lead to supercooling of cells below their nucleation temperature. In this case water will be kept in the cell, whence eventually either large lethal or small less damaging ice crystals will be formed within. Rapid freezing may also lead to large gradients in osmotic pressure across the plasma membrane, which will become damaged at a critical pressure gradient, and intracellular freezing will also occur as a result of this damage.[15,16] The building of large intracellular ice crystals is associated with cell death. Although more moderate freezing rates are associated with exposure to smaller ice crystals, these later can re-crystallize upon rewarming and become lethal. However, if freezing progresses at very slow rates, dehydration will take place over a longer period of time. During this slow freezing, the osmolality of the external medium increases as water-ice is precipitated. The cells will be exposed to high salt concentrations in the external medium and dehydrate to a degree of shrinking associated with serious cellular disruption. Cryoinjury due to the cell's sensitivity to high solute concentrations is referred as to a "solution effect". Thus, freezing rates should be slow enough to allow the cells to minimize chemical potential and osmolality gradients across the plasma membrane and to dehydrate without being exposed to lethal salt concentrations. This "equilibrium" freezing leads to tolerable cell shrinkage, to an increase in the intracellular chemical potential, and to depression of the freezing temperature. Due to the decreased amount of intracellular water and the freezing point depression, the potential risk of intracellular ice formation is reduced, so that only extracellular ice will be formed.

This study showed that the sperm motility recovery after cryopreservation using CPA was significantly higher compared to wihout CPA. It indicated that the addition of CPA increased the probability of survival of sperm compared to without CPA added, for all cryopreservation methods. Lucena and Obando[17] showed that cell survival is less than 15% when human spermatozoa are subjected to the freeze thaw process without the addition of a cryoprotectant. CPA protects cells from cryodamage by reducing intracellular water through diffusion, which helps to minimize intracellular ice formation during freezing. Exposure of spermatozoa to cryoprotectant solution initially causes them to

shrink because of losing intracellular water due to increased extracellular osmolarity. The sperm cells return to its original volume by allowing the penetration of cryoprotectant into their intracellular space. In actual freezing, the addition of a cryoprotectant is usually accompanied by the reduction of temperature. In the cooling process, when the temperature reaches -5 to -15 ºC, extracellular ice formation occurs and it induces the development of an extracellular solid phase.[18] The sperm cell remains unfrozen but supercooled. The supercooled intracellular water diffuses out of the cell osmotically, and freezing continues extracellularly, resulting in hypertonicity and a further reduction of water from sperm cell, leading to dehydration in fulfilling the goal of freezing. At the time of thawing, the sperm cells undergo the same hydration/dehydration process but in reverse. Spermatozoa that have undergone a slow freezing rate should also be warmed slowly during thawing, while sperms that have been frozen rapidly should be thawed rapidly.[18,19]

Moreover egg yolk, a compound of lipoproteins, phospholipids, cholesterol, and various other less-abundant components, is also used in combination with Tris buffers to constitute TEST-yolk buffer for sperm cryopreservation with a good postthaw recovery rate.[20-22] Egg yolk may play a role in reducing the deleterious effects on membrane structures of hyperosmotic salt solutions that occur during rapid cooling.[23,24] It is the phospholipid portion of the low-density protein of yolk that affords the protection to sperm during the exposure to low temperature.[23] This is possibly due to an exchange of lipids between spermatozoa and egg yolk or to the influence of saturation of fatty acid. Many investigators[22,23,25] have pointed out that certain lipid elements of egg yolk may combine with cell membranes, altering their molecular composition and maintaining their fluidity. According to Paulson *et al.*[26] TYB pretreatment of sperm for 24 hours results in higher fertilization rates during IVF among suspected patients with male factor infertility. Therefore, use of a cryoprotective agent is indispensable in preventing injury to human spermatozoa during the cryopreservation process.

This study showed that the motile sperm recovery may not be sufficient for certain assisted reproductive technology procedures that require or depend on total viable sperm count, such as artificial intrauterine insemination (IUI) and invitro fertilization (IVF). However procedures such as IVF with intracytoplasmic sperm injection (ICSI) only requires a minimum of 1 surviving spermatozoa to proceed. Therefore freezing procedures can be followed by IVF-ICSI. The motility of spermatozoa in this study could probably be improved with strict protocols followed. Processing time for the samples, from sample collection to freezing, may have varying duration. Increased time decreases motility. However, a study showed that only a proportion (16%) of samples demonstrated a decreased in 1 to 2 h motility and in that proportion, samples with a higher total motile sperm count have a significantly lower reduction in motility.[27] Considering samples of this study were normozoospermic, overall motility deterioration may only be trivial. Variability in the incubation environment may also contribute to the loss of motility because of the difference in temperature and climate. These results can serve as a basis of further studies aiming to compare other probable methods in hoping to optimize a single processing protocol with the highest success rate.

## CONCLUSION

In conclusion, cryopreservation of semen using CPA results has higher motility recovery compared to that without CPA. In addition, vapor liquid nitrogen freezing is more superior than other freezing method for sperm cryopreservation and offers better recovery of human motile spermatozoa.

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