The effect of cryopreservation technique in a very low number sperm count at Soetomo Hospital, Surabaya, Indonesia

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ABSTRACT

Background: The massive development of Assisted Reproductive Technology (ART) offers new hope for the infertile couple. However, the advanced storage methods to preserve the quality of sperm has been raising by cryopreservation technique. This study aims to analyze the motility rate, recovery rate, and viability in subgroups of cryopreserved spermatozoa from the oligozoospermic patient as the quality parameter.

Method: A true-experimental study by pre-test and post-test group design was conducted among 13 oligozoospermic patients with a very low sperm counts at Medical Biology Laboratory Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. Parameters assessed in this study include motility, viability, and concentration of spermatozoa according to Laboratory Manual for the Examination and Processing of Human Sperm 5th edition. The samples were divided into 4 groups such as Group 1 (71-100 sperm), Group 2 (31-70 sperm), Group 3 (11-30 sperm), and Group 4 (1-10 sperm) based on sperm counts which underwent 30 repetitions of cryopreservation. Data were analyzed using SPSS version 17 for Windows.

Results: There was a significant difference in the recovery rate among Group 1 (34.88±1.87), Group 2 (44.77±1.89), Group 3 (61.83±2.09), and Group 4 (70.90±2.71) of cryopreservation technique (P<0.001). However, there was no significantly different in the motility rate of sperm-cryopreservation technique among Group 1 (65.60±14.63), Group 2 (52.50±26.28), Group 3 (39.90±9.95), and Group 4 (44.70±41.77) (p=0.070). Based on the sperm-viability parameter assessed in this study, there was no significant difference among Group 1 (56.40±3.89), Group 2 (58.60±5.99), Group 3 (59.60±9.96), and Group 4 (56.70±11.30) (p=0.504).

Conclusions: The sperm-cryopreservation technique significantly affected the recovery rate of very low sperm number count among groups. However, there was no significantly different in the motility rate and viability parameter in this study.

Keywords: Cryopreservation, Low Sperm Count, Motility, Recovery, Viability


INTRODUCTION

Infertility is a current matter of concern in the medical field. Couple with male infertility represents 30-40% of all cases of infertility.1 Azoospermia occupies 10% of all cases of male infertility.1 The massive development of Assisted Reproductive Technology (ART) offers new hope to have a biological child to the couple who has no hope before. Intracytoplasmic Sperm Injection (ICSI) makes possible to conceive by using only one living spermatozoa. Further, ICSI combined with another invasive spermatozoa recovery technique makes possible to use epididymal and testicular spermatozoa and it means even azoospermic patient has a possibility for his spermatozoa to be recovered with invasive or surgical or hormonal treatment.1,2 Cryopreservation of spermatozoa today is the widest procedure because by using cryopreservation it means that the necessary biological material can be available at the time it is needed and can be stored for a certain period of time so that spermatozoa collection and ovum pick up do not always have to be done on the same day. In men with severe oligoasthenoteratozoospermia occasionally can suddenly have transient Azoospermia on the day scheduled for ICSI so to overcome it is usually required cryopreservation prior to the ICSI day.3,4 The success rate of ICSI is 33.9%, so there is a possibility to do re-ICSI, and cryopreserved spermatozoa can be used for this purpose without doing a repetition of invasive surgery, treatment or high-cost hormonal treatment.5

Cryopreservation has its own limitations. Conventional cryopreservation is an obstacle to achieving sufficient harvest in cases of severe oligospermia.6 According to Koscinski et al., from 70 cryptozoospermia sufferers who came to the clinic for ART which was successfully carried out by cryopreservation were 44 people.4 In 26 patients, cryopreservation cannot be performed because a very low number of spermatozoa or the spermatozoa cannot pass the freeze-thaw procedure.4

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The principle of cryopreservation from physiological conditions to below-zero temperatures requires a biological understanding of this process that certainly varies between species and even between cells within a species.7 The formation of ice can occur at temperatures <0ºC to -130ºC. Usually at -10ºC – (-15ºC) crystallization occurs. The establishment of ice crystals can decrease the survival rate.7

Meanwhile, a systematic study by Abdelhafez et al. suggests that the output of the cryopreservation process is divided into primary output, the recovery rate and secondary output such as motility rate.4 Recovery rate is defined as the number of spermatozoa obtained post-thawing divided by the number of spermatozoa before cryopreservation multiplied by 100%.1 Motility rate is the percentage of motile spermatozoa divided by the percentage of motile spermatozoa before cryopreservation multiplied by 100%.3

Until now, there is no marker or cut-off value that can predict how much spermatozoa can be carried out by cryopreservation. In general, the survival rate of cryopreservation of spermatozoa is 50-60%. A previous study on predictors and cut-off values is necessary to avoid a useless cryopreservation procedure.4 Based on the mentioned above, this study aims to analyze the recovery rate, motility rate, and viability parameter in subgroups of cryopreserved spermatozoa from the oligozoospermic patient as a quality measurement.

METHOD

A true-experimental study by pre-test and post-test group design was conducted among 13 oligozoospermic patients with a very low sperm count at Medical Biology Laboratory Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. Sample of the study was semen obtained from patients attending Ferina Center for Reproductive Medicine, Surabaya, Indonesia. The inclusion criteria used in this study were men with sperm oligoastenoteratozoospermia sperm analysis with concentrations range 7–9 million/ml and age 21–40 years. The exclusion criteria were hematospermia and leukospermia condition.

All samples were examined for motility and concentration of spermatozoa with 5th edition WHO’s method. First, the semen preparation by using simple washing technique was conducted. Spermatozoa need to be separated from the plasma seminal to avoid negative consequences and ensure good ICSI results. Spermatozoa move to the lowest part. Although this technique can not be used if the goal is to concentrate spermatozoa, this technique is best to obtain spermatozoa with high motility.8 The simple washing procedure is as follows: 1) Mix the semen by stirring; 2) Insert into the tube; 3) Add the medium equivalent as the semen; 4) Stir by closing and tilting the tube; 5) Centrifuge 300-500 g for 2-10 minutes; 6) Gradually take the supernatant, and 7) Take the pellet and put in the middle of drop-side migration technique.9

Human Tubal Fluid (HTF) with 5% albumin is used as a medium for washing and also cryopreservation. There were 4 subgroups in this study, such as Group 1 (71–100 spermatozoa), Group 2 (31–70 spermatozoa), Group 3 (11–30 spermatozoa), and Group 4 (1–10 spermatozoa). For each group, there were 30 drops. It means there was 30 repetition for each group. Thirty drops contained 71–100 spermatozoa for Group 1, thirty drops contained 10 spermatozoa for Group 2 and so on. Moving spermatozoa were aspirated into pipette in a certain amount and put into 2 µl micro drops of freezing medium (HTF: Sucrose 0,1M=1:1). Sucrose 0,1 M was used as Cryoprotective Agent (CPA). The drops were put in the tip of Cryologic® and left in liquid nitrogen vapour for 1 min prior to being plunged in it.

For thawing, Cryologic® was warmed in room temperature and the recovered sperm were being observed in an inverted microscope to assess recovery and motility. All this procedure did not require micromanipulator due to a pulled glass Pasteur pipette was used over a flame to take and put spermatozoa into drops.

Statistical analysis was performed using Statistical Package for the Social Sciences version 17.0 (SPSS) for Windows. The Kolmogorov Smirnov and Levene’s test was performed to test the parameter distribution. Recovery test was analyzed using Kruskal Wallis because it has normal and non-homogenous distribution. For motility test, the non-parametric Kruskal Wallis test was used due to its abnormal and nonhomogeneous distribution. A P-value of less than 0,05 was considered statistically significant.

RESULTS

Several parameters have been assessed in this study, such as recovery rate, motility rate, and viability of sperm. Based on the recovery rate, there was a statistically significant average score among Group 1 (134.88±1.87), followed by 44.77±1.89 in Group 2, 61.83±2.09 in Group 3, and 70.90±2.71 in Group 4 (p<0.001). However, in the motility rate evaluation, there was no significant difference between Group 1 (65.60±14.63), Group 2 (52.50±26.28), Group 3 (39.90±9.95), and Group 4 (44.70±41.77) (p=0.070) (Table 1).

Table 1
According to the viability test, some variables have been assessed in this study. Those variables include living motile, living immotile, non-living, progressive motility (PR), non-progressive motility (NP), immotility (IM), and viability evaluation (Table 2). According to the viability assessment, there was no statistically different between Group 1 (56.40±3.89%), Group 2 (58.60±5.99%), Group 3 (59.60±9.96%), and Group 4 (56.70±11.30) (p=0.504) (Table 2). Most of the viability indicators were tended to be higher in Group 1 for living motile (6.69±8.84), non-living (7.54±4.59), NP (9.90±2.33), and IM (9.70±8.00) but not statistically significant (Table 2).

### DISCUSSION

Vitrification or rapid freezing is considered superior to slow freezing. Vitrification eliminates the formation of intracellular ice crystals, although in practice it will be necessary to adjust the technique and the amount of CPA. When it was compared between vitrification with rapid freezing, there were no differences in motility rate means that vitrification is not better than rapid freezing. For the fewer amount of spermatozoa, vitrification is very advantageous. This research uses the ultra-rapid freezing method. The frozen material is placed in liquid nitrogen vapour for 1 minute. This method was chosen because, in the preliminary study, ultra-rapid freezing yielded better post thawing result compared to vitrification and slow freezing. This preliminary study provides similar results with another study where cryopreservation of 100 spermatozoa in Cryoloop with ultra-rapid freezing obtain a good post thawing result. We used sucrose as a CPA in this study. The advantage of using sucrose is that post-thawing washing is not required. From the preliminary study, it was concluded that too high concentration of sucrose would result in poorly marked post thawing results. The concentration of 0.1 M sucrose made the best post thawing result. The supplementation of albumin to HTF resulted in a better post thawing result than without albumin. Therefore, this study used HTF with 5% Human Serum Albumin (HSA) and sucrose with a ratio of 1:1.

This research is highly dependent on operator skills. Comparison of sperm-CPA-HTF should be precise because if there is too much CPA, motility will be reduced and many spermatozoa will lead to immotile prior to the beginning of...
cryopreservation. It will undoubtedly affect the calculation of results and data analysis. The size of the drop should also be precisely 2 μl or a maximum of 2.5 μl because if it is too small, it will affect sperm parameters, especially the number 71-100 which will be crammed inside a slight drop. In addition, it also will affect the post-thawing parameters, and if the drop is too significant, then it will not be able to fit in Cryologic®’s straw.

The technique for placing cryopreservation drops in Cryologic® should be accurate and correct, no spermatozoa should be left on the plate, and the plate itself should not be scratched in order not to affect spermatozoa’s recovery and motility rate. To put a drop at the end of Cryologic® use Pasteur pipettes or micropipettes needs extra attention when we have to suck the drop carefully, so there is no residual drop left and affect the recovery rate. We also need to empty the pipette shortly before sucking up to another drop. The motility of spermatozoa will be reduced by 50% after thawing. Damage to spermatozoa after passing through cryopreservation process occurs because of ice crystals in intra-cell or extracellular, cell dehydration, osmotic shock and recrystallization after thawing. This whole process can damage the integrity of the cell membrane, and if it destroys the mitochondria, the motility rate will be impaired.10 In this study, the motility rate after thawing showed no significant difference between the groups because of the large standard deviation.

The semen parameters prior to cryopreservation will affect post-thawing motility and viability.17 Motility will be high if the number of frozen spermatozoa is also high. When the initial semen parameters before cryopreservation are not proper, the recovery, motility, and viability of sperm will not be functional either.

The limitation of this study is the intra-individual bias. This research is highly dependent on the skill of the researcher. Early semen parameters are also very influential. This study used samples from 13 different subjects with no controls. Parameters in this study were restricted to recovery rate, motility rate and viability rate but not fertilization rate, cleavage rate, clinical pregnancy and live birth. Technically this study used only one nitrogen storage. Ideally, there should be two nitrogen storage, one for storing the sample and one for the storage of liquid nitrogen that will be required during the cryopreservation process. The constant temperature must be maintained. The specimen inside the storage must not be disturbed but frequent opening or closing the seal.18

CONCLUSION

The recent findings suggest the effect of cryopreservation technique in a very low number of sperm count in different results. There was a statistically significant difference in the recovery rate among groups, but not statistically significant was found in the motility rate and viability test evaluation.

ETHICAL CLEARANCE

This study has been reviewed by the Ethics Committee of Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia and approved in December 22th 2017 with number 309/EC/KEPK/FKUA/2017.

CONFLICT OF INTEREST

The author declares there is no conflict of interest regarding all aspects of the study.

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AUTHOR CONTRIBUTION

RSM has performed all freezing procedures in the frame of her brevet. Besides, RSM also wrote this manuscript under the supervision of A and AH. All authors read and approved the final manuscript.

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