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Human Sperm Cells After Purification Using SCLB Can Be Stored at 4°, -20°, or -80°C Before Small RNA Isolation

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ABSTRACT

There have been many studies about pre-analysis for sperm RNA examination which compared sperm purification methods, RNA isolation methods, sequencing methods, and semen storage before analysis. However, there is a lack of studies that determine the ideal storage temperature after sperm cell purification before RNA analysis, especially small RNA analysis. The aim of this study was to determine the preferred storage temperature for human sperm cells after sperm purification using Somatic Cell Lysis Buffer (SCLB) before sperm small ribonucleic acid (RNA) isolation and analysis. This study was a true laboratory experiment using the post-test only control group design. The samples were 13 fresh human semen that has been purified using SCLB. The sperm cells were then diluted and divided into four aliquots with different treatments. The first aliquot that served as a control group was immediately purified while the other three aliquots were stored for seven days at different temperatures as follows: 4°C, -20°, and -80°C. After the small RNA isolation, RNA level between each group was compared. Micro volume spectrophotometer measured RNA level. The median of small RNA yields of the control group was 49.8 (5.33-522.46) ng/10^6 sperm cells. There was no significant difference in median of small RNA yields of the control group and that of other groups. The median of the other groups with storage temperature of 4°C, -20°, and -80°C was 41.09 (7.03-1448.31), 65.95 (7.99-301.16), and 76.42 (10.45-434.25) ng/10^6 sperm cells, respectively (p-value= 0.314; α=5%). This condition suggested that after purification using SCLB, human sperm cells can be stored at temperatures of 4°C, -20°, or -80°C for seven days, depending on each laboratory facility.

Keywords: Sperm RNA analysis, purified sperm cells, storage temperature

INTRODUCTION

Infertility due to the male factors occurs in a range from 20 to 70% of the total infertility cases in the world.[1] The wide range of male infertility cases need to be supported by the best quality of management, including diagnosis and treatment. To diagnose male infertility, identifiable biomarkers in the male biospecimens, such as blood, urine, testes tissues, or semen. Semen contains sperm cells that are easier to collect than the other biospecimens. The biomarkers of male infertility that can be measured in semen or sperm cells are semen analysis, chromosomal aneuploidies, deoxyribonucleic acid (DNA) integrity, and ribonucleic acid (RNA) profile.[2] Chromosome analysis, DNA integrity, and RNA profile evaluate the genetic and epigenetic materials in sperm cells.[3-5]

The use of biomarkers, especially for genetic and epigenetic analysis, needs prior consideration about the laboratory methods, which consist of three aspects such as: pre-analysis, analysis, and post-analysis. The pre-analytical phase of the laboratory methods is one of the most important elements; however, it is still not carefully taken care of, despite its high contribution to biospecimen integrity and analysis results.[6] Thus, learning about the pre-analysis aspect of male infertility biomarkers in sperm cells is something uncommon yet very essential.

Facilities of fertility and andrology laboratory in Indonesia do not comprehensively support the genetic and epigenetic analysis of male infertility yet. Transport of the biospecimens from one laboratory to another is needed for advanced analysis. The sperm RNA profile, one of the male infertility biomarkers, will be more extensively explored from now on, especially in Indonesia. In this case, the biospecimen integrity, such as the sperm cells, must be carefully monitored in order to obtain the best analysis results.

There have been many studies about pre-analysis for sperm RNA examination which compared sperm purification methods, RNA isolation methods, sequencing methods, and semen storage before

analysis.[7-9] However, there is a lack of studies that determine the ideal storage temperature after sperm cell purification before RNA analysis, especially small RNA analysis. This study aimed to determine the preffered storage temperature of human sperm cells after sperm purification using Somatic Cell Lysis Buffer (SCLB) before sperm small RNA isolation and analysis. This study is important to begin other advance studies of human sperm RNA profile for male infertility biomarkers in Indonesia. In addition, it is also important as reference for fertility and andrology laboratory in Indonesia to improve their services.

METHODS

This study was a true laboratory experiment with a post-test only control group design performed in April - May 2019. 13 fresh human semen, which met the intrinsic criteria of concentration above 107 cells/mL, were used as a sample. Each sample was divided into four aliquots after the sperm cell purification. The first aliquot of each sample was isolated from their small RNA without storage, while the other 3 aliquots of each sample were stored at different temperatures for seven days and thawed for little RNA isolation.

Samples preparation and sperm cell purification were carried out in Medical Biology Laboratory, Faculty of Medicine, Airlangga University. Sperm cells storage and small RNA isolation were carried out in Rumah Sakit Khusus Infeksi (RSKI) Airlangga University. This study protocol was approved by the Local Ethics Committee or Komisi Etik Penelitian Kesehatan (KEPK) Faculty of Medicine, Airlangga University, Surabaya (Ethical Clearance Reference No. 114/EC/KEPK/FKUA/2019).

The concentration of thirteen fresh semen samples was firstly determined using the WHO protocol for semen analysis.[10] The semen samples were then purified from somatic cells using Phosphate Buffer Saline (PBS) and SCLB (0.1% SDS and 0.5% Triton X in DEPC H_2O). The concentration of the purified sperm cells was determined after purification and diluted to 2 mL of volume. The dilution of each purified sperm cells was divided into four 0.5 mL aliquots. The first isolated aliquot was as a control group. The other aliquots were stored at 4^0C, -20^0C, and -80^0C for seven days and thawed for small RNA isolation. Small RNA was isolated using the Hybrid-R™ miRNA kit (Gene All®, Korea) and resulted in 60 uL of small RNA. Low RNA yields were measured using a micro volume spectrophotometer (Nano Drop® ND-1000). Low RNA yields (ng/uL) were then converted to ng/10^6 sperm cells divided by purified sperm concentration.

The quantitative data were analyzed using Shapiro-Wilk for normality test. Normally distributed data were shown as mean ± SD, and the others were shown as median (minimum–maximum). To determinethe differences between the four groups, repeated ANOVA was used for the normally distributed data, and Friedman test was used for the abnormally distributed data. The statistic analysis was performed using SPSS software version 23, with a significance level of 0.05.

RESULTS AND DISCUSSION

The results of this study revealed that 13 samples had a concentration above 10^7 sperm cells/mL as the criteria of this study. Samples were purified using SCLB, and the level after purification was determined. Purified sperm cells were diluted to 2 mL and were divided into four aliquots. The total sperm number was obtained by multiplication of concentration and volume. The characteristic of the samples before and after sperm cell purification can be seen in Table 1.

Somatic cell lysis buffer was the buffer with the ability to lysis the somatic cells in the semen to purify sperm cells. The recovery rate after the sperm cell purification using SCLB was 35.15 $\pm$ 29.631 %. It was different from the previous study using the same purification method, which found the recovery rate of 74.5 to 100%.[7] This difference might be due to the number of samples. The previous study used 4 samples, each of them was stored in two different forms of pellet and liquid,whilethis study used 13 samples stored in the same way.[7] Likewise, this difference was possibly related to sperm cell counting methods.

Table 1. The characteristics of samples before and after sperm cells purification

Variables	Before Purification (n=13)	After Purification (n=13)
Volume	2.6 $\pm$ 0.9309* mL	diluted to 2 mL
Concentration	29 (14 – 82)** x 10^6 cells/mL	20 (1.3 – 65)** x 10^6 cells/mL
Total sperm count	78.4 (12.5 – 246)** x 10^6cells	40 (2.6 – 130)** x 10^6 cells

Table 2. Small RNA yields from each group

Group	Small RNA Yields (ng/10^6 cells)	P-value
Control (without storage)	49.8 (5.33 – 522.46)	0.314
Storage at 4°C	41.09 (7.03 – 1448.31)	
Storage at -20°C	65.95 (7.99 – 301.16)	
Storage at -80°C	76.42 (10.45 – 434.25)	

The purified sperm cells were divided into four aliquots and were allocated in four different groups. One group was as a control group without storage, and the other groups were with storage for seven days at different storage temperatures. Small RNA was isolated in each aliquot and was measured using a micro volume spectrophotometer. Low RNA yields were converted from a measurement unit of ng/uL to ng/10^6 cells, as seen in Table 2.

Ribonucleic acidis a nucleic acid degraded by ribonuclease A (RNase A) enzyme. This enzyme is activated at 215K or -58°C.[11] Pre-analysis factors in the laboratory must be carefully taken care of, due to their potential intervention to the quantity and quality of the results. These factors include tube type (must be RNase free), sterility, biospecimens type, volume, and short-term storage temperature. RNA was obtained in small yields, so it must be handled carefully to avoid contamination and degradation.[6] Storing and processing under the activated temperature of RNase can minimalize RNA degradation. Based on this study, there was no different small RNA yields among all group (p-value = 0.314, α = 0.05). The highest yields were found in the -80°C storage group [76.42 (10.45 – 434,25) ng/10^6 cells] and the second was found in the -20°C storage group [65.95 (7.99 – 301,16) ng/10^6 cells], suggesting a possible role of RNase activation or the freezing stage that can influence the biospecimens concentration, including nucleic acid.[11]

Aprevious study about sperm RNA with a different forms of sample for storage and isolation methods reported that there was no difference in the total RNA yields between the study groups, but there was a difference in RNA expression in different sperm RNA isolation methods.[7] No variation of small RNA yields in this study probably led to the finding of different RNA expressions in advance little RNA analysis, suggesting a need for further studies.

The previous studies of the pre-analysis aspect in Japan had been performed to determine the ideal temperature and length of storage for whole blood, urine, and serum. Each of specimens was stored at room temperature (23°C), 4°C, -20°C, and -80°C for 1, 3, and 7 days. The result showed that serum was stable after storage in -80°C for seven days, while the other temperatures led to increased or decreased result.[12]

Temperature and length of storage of biospecimens must be entirely concerned for RNA analysis. Suboptimal temperature and period of storage might influence RNA integrity. Low concentration of specimen, long-term storage, and suboptimal temperature would disrupt the RNA integrity.[13]

CONCLUSION AND SUGGESTION

Human sperm cells after sperm purification using SCLB can be stored at 4°, -20°, or -80°C for seven days before small RNA analysis, depending on each laboratory facilities. This conclusion was merely based on the low RNA yields. Further studies were needed to determine the length of storage influencing the small RNA expression.

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REFERENCES.

1. Agarwal A, Mulgund A, Hamada A, Chyatte MR. A unique view on male infertility around the globe. Reprod Biol Endocrinol, 2015; 13(37): 1-9.
2. Anton E, Krawetz SA. Spermatozoa as biomarkers for the assessment of human male infertility and genotoxicity. Syst Biol Reprod Med, 2012; 58: 41-50.
3. Ramasamy R, Scovell JM, Kovac JR, Cook PJ, Lamb DJ, Lipshultz LI. Fluorescence in situ hybridization detects increased sperm aneuploidy in men with recurrent pregnancy loss. Fertil Steril, 2015; 103: 906-10.
4. Agarwal A, Majzoub A, Esteves SC, Ko E, Ramasamy R, Zini A. Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios. Transl Androl Urol, 2016; 5(6): 935-50.
5. Jodar M, Sendler E, Moskovtsev SI, Librach CL, Goodrich R, Swanson S, *et al*. Absence of sperm RNA

elements correlates with idiopathic male infertility. Sci Transl Med, 2015; 7(295): 1-8.
6. Ellervik C, Vaught J. Preanalytical Variables Affecting the Integrity of Human Biospecimens in Biobanking. Clin Chem, 2015; 61(7): 914-34.
7. Mao S, Goodrich RJ, Hauser R, Schrader SM, Chen Z, Krawetz SA. Evaluation of the effectiveness of semen storage and sperm purification methods for spermatozoa transcript profiling. Syst Biol Reprod Med, 2013; 59: 287-95.
8. Barragan M, Martinez A, Llonch S, Pujol A, Vernaeve V, Vassena R. Effect of ribonucleic acid (RNA) isolation methods on putative reference genes messenger RNA abundance in human spermatozoa. Andrology, 2015; 3: 797-804.
9. Mao S, Sendler E, Goodrich RJ, Hauser R, Krawetz SA. A comparison of sperm RNA-seq methods. Syst Biol Reprod Med. 2014; 60(5): 308-15.
10. WHO. WHO laboratory manual for the Examination and processing of human semen. 5th., Geneva, WHO, 2010; 32-44.
11. Hubel A, Spindler R, Skubitz APN. Storage of human biospecimens: Selection of the optimal storage temperature. Biopreserv Biobank. 2014; 12(3): 165-75.
12. Ikeda K, Ichihara K, Hashiguchi T, Hidaka Y, Kang D, Maekawa M, *et al*. Evaluation of the short-term stability of specimens for clinical laboratory testing. Biopreserv Biobank, 2015; 13(2): 135-43.
13. Olivieri EHR, Franco L de A, Pereira RG, Mota LDC, Campos A nio HJFM, Carraro DM. Biobanking practice: RNA storage at low concentration affects integrity. Biopreserv Biobank, 2014; 12(1): 46-51.