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RESEARCH ARTICLE

Human sperm DNA Integrity: A Difference of Storage Temperature and Period of Freeze-Drying Process

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Abstract

The cryopreserve technique using liquid nitrogen is used to preserve human sperm. Nevertheless, this technique has several disadvantages, such as bacterial/viral contamination and its expensive cost. Freeze-drying process offers more simple and cheaper of sperm storage technique. This study aimed to investigate the effect of storage temperature and period of freeze-dried human sperm on DNA integrity. Human sperm samples of 15 donors were prepared with simple washing techniques. Post-washing samples (T0) were divided into 4 groups with the same volume. The four group of samples carried on the freeze-drying process and stored based on different temperature and period: 4°C for 1 week (T1), room temperature (24-25°C) for 1 week (T2), 4°C for 3 months (T3), and room temperature for 3 months (T4). Sperm DNA integrity was analyzed before and after the storage period of each group. There was a statistically significant difference of sperm DNA integrity of freezes - dried at 4°C in 1 week versus 3 months (T1 vs T3), at room temperature in 1 week versus 3 months (T2 vs T4) ($p < 0.05$)and between both temperatures in 3 months (T3 vs T4) ($p<0.05$).In conclusion, temperature at 4°C and period in 1 week is the best temperature and storage to store freeze-dried human sperm, based on sperm DNA integrity.

Keywords: *Freeze-drying process, Human sperm, Sperm DNA integrity.*

Introduction

Currently, the cryopreserve technique using liquid or vapor nitrogen is used to preserve human sperm. Unfortunately, this technique has several disadvantages. Cryopreserve techniques is capable in reducing human sperm DNA integrity [1-3].Bacterial, viral and fungal contamination of embryos and semen stored in liquid nitrogen have been reported by several researchers [4, 5].

In addition, expensive costs are also required for special containers, repetitive nitrogen filling and shipping. Some infertile patients who have financial problems such as suffering testicular cancer, undergoing routine hemodialysis, chemotherapy, and radiotherapy couldn't afford to fund this cryopreserve techniques [6, 7]. Therefore, simple and affordable cost methods are needed to develop for novel sperm preservation techniques. One of these techniques is freeze-drying (FD).

Freeze-drying can maintain chromosomal integrity of mammalian sperm in 4°C for several years and at room temperature for approximately 1 month [8].

However, the recent publications of freeze-drying on human sperm are still very limited. The freeze-drying technique allowed human sperm to be stored at 4°C for 1 week without a statistically significant change in DNA integrity (average: 81.06% ± 9.2 % and 81.3 % ± 3.5 % before and after FD) [9].

Another study showed no statistically significant change in freeze-dried human sperm DNA integrity (80.6 ± 11.3%), whereas DNA integrity significantly reduced after cryopreservation with liquid nitrogen (62.8 ± 10%) when compared with fresh samples (77.8 ± 12.2%) [10]. The different results performed in the study conducted by (Pramesti et al. 2010), in which the freeze-

dried human sperm storage for 7 days at 4ºC have more DNA damage (94.24 ± 5.58c) compared with fresh samples (70.62 ± 14.57%) [11]. In contrast, there has been no study of freeze-dried human sperm stored for more than 7 days. Therefore, in this study, we aimed to assess human sperm DNA integrity in freeze-dried which were stored at 4°C and room temperature (24-25ºC) for 1 week and 3 months.

Material and Methods

Human Sperm Collection

Human sperm samples were collected from 15 donors who signed informed consent forms to be used as research samples (mean age 33.6 ± 4.1 years). This study was approved by the Ethics Committee of Dr. Soetomo Hospital Surabaya.

Semen samples ejaculated by donors and collected in the non-toxic glass. Among the 15 samples, 14 teratozoospermia and 1 asthenoteratozoospermia based on World Health Organization (WHO) criteria [12]. Six donors were secondary infertility, 4 primary infertility, and the remaining still unmarried.

Human Sperm Preparation

Semen samples were placed in an incubator at 37°C until liquefaction. Sperm DNA integrity (T0) was counted from liquefied samples. Semen samples were washed using Sperm Rinse™ medium (Vitro life, Gothenburg, Sweden) by simple washing techniques according to WHO procedures [12]. Pellets were resuspended in 4 ml of Sperm Rinse™ medium and divided into four aliquots. Each aliquot was put into microtube with 1 ml volume.

Freeze-drying Process

All aliquots were freeze-dried using a freeze-drying machine (VirTis, SP Industries, USA) in Tissue and Cell Bank of Dr. Soetomo Hospital Surabaya. All aliquots were frozen at -80 ° C for at least 24 hours. Samples transferred into a freeze-drying machine after samples were frozen. Microtubes were opened, therefore frozen samples mayundergo sublimation and desorption process automatically for 48 hours.

Freeze-dried Sperm Storage

After the freeze-drying process, microtubes were closed and stored into different storage temperature and periods 4°C for 1 week (T1), room temperature (24-25ºC) for 1 week (T2), 4°C for 3 months (T3), room temperature for 3 months (T4). T1 and T3 were put in the refrigerator and kept stable at 4°C. T3 and T4 were put in a room with the air conditioner (AC) which was set at 24-25ºC.

Rehydration

After 1 week and 3 months later, microtubes containing freeze-dried sperm were resuspended 1 ml of distilled water and analyzed for DNA integrity.

Sperm DNA Integrity Test

Sperm DNA integrity was analyzed using SpermFunc® kit (BRED Life Science Technology Inc., China) based on Sperm Chromatin Dispersion (SCD) method.Sperm DNA was considered normal if the halo size was at least more than one third the shortest diameter of the sperm head. In contrast, sperm DNA was considered abnormal or fragmented if the halo size was smaller or have no halo at all. It was observed under light microscope in 500 sperm cells, by two observers. Sperm DNA integrity was defined as the percentage of normal sperm DNA.

Result

Human sperm DNA integrity in each groups was shown in Figure 1 and Figure 2. Sperm DNA integrity in all groups (T1, T2, T3, T4) was statistically significantly reduced compared with the fresh sample before freeze-drying process (T0) ($p < 0.05$). Furthermore, the DNA integrity of freeze-dried human sperm showed a statistically significant change at 4°C in 1 week versus 3 months (T1 vs T3) and at room temperature in 1 week versus 3 months (T2 vs T4) ($p < 0.05$).

In addition, there was also statistical significant different between both temperatures in 3 months (T3 vs T4) ($p<0.05$).Meanwhile, there was no statistical significant difference in sperm DNA integrity between 4°C and room temperature in 1 week (T1 vs T2) ($p>0.05$). Sperm DNA at room temperature in 3 months (T4) was totally fragmented in all samples.

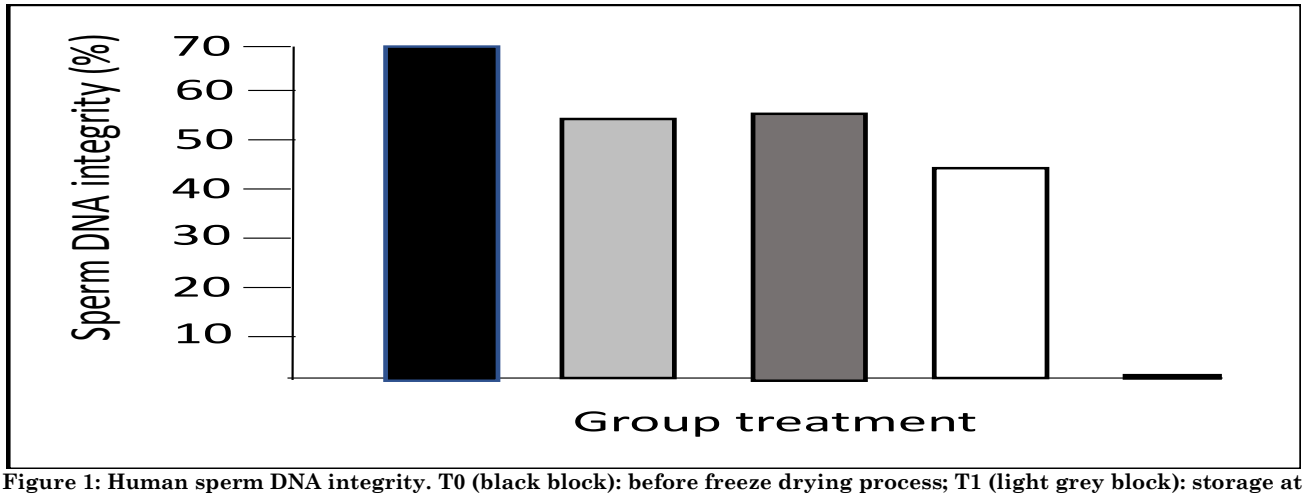

Figure 1: Human sperm DNA integrity. T0 (black block): before freeze drying process; T1 (light grey block): storage at 24-25°C in 1 week; T2 (dark grey block): storage at 4°Cin 1 week; T3 (white block): storage at 4°Cin 3 months and T4 (black line): storage at 24-25°C in 3 months

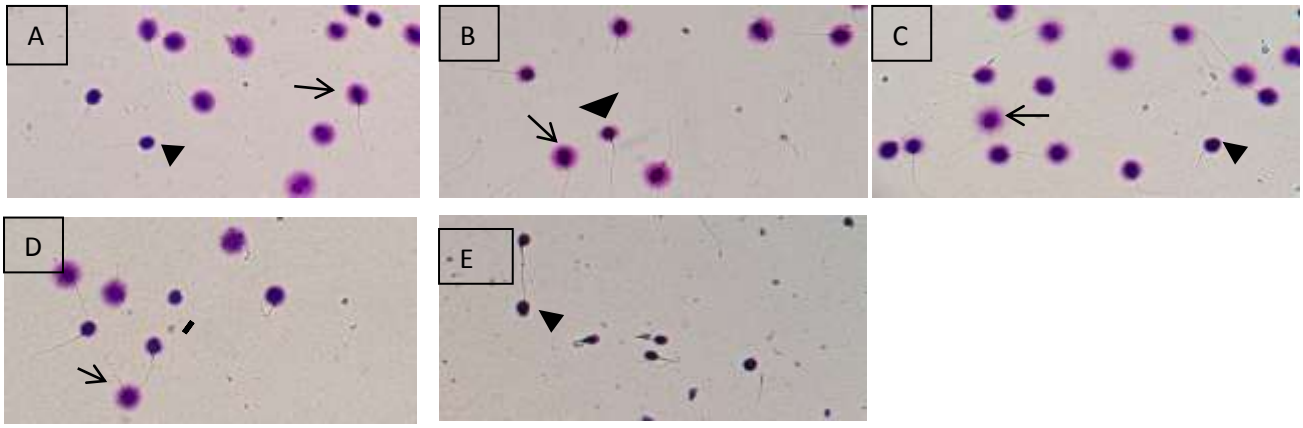

Figure 2: The images of sperm DNA integrity. [A] Before freeze-drying (T0), [B] After freeze-drying and storage at 4°C in 1 week (T1), [C] After freeze-drying and storage at room temperature (24-25°C) in 1 week (T2), [D] After freeze-drying and storage at 4°C in 3 months (T3), [E] After freeze-drying and storage at room temperature (24-25°C) in 3 months (T4). Caption: → arrow showedsperm with normal DNA integrity, ► arrow showed sperm with fragmented DNA

Discussion

This study showed that sperm from fresh samples showed better DNA integrity compared to sperm which had undergone the freeze-drying process. Our study is consistent with (Pramesti, et al, 2010) who showed DNA integrity freeze-dried sperm stored at 4°C for 1 week had more DNA damage compared to fresh samples [11].In addition, this study also showed that the storage process at different temperatures (4C and room temperature) and at the same period (1 week), did not significantly affect the integrity of sperm DNA.

In contrast, storage processes at the same temperature and at different periods (1 week and 3 months) showed significant differences in sperm DNA integrity. In other words, this study proves that the freeze-drying process does not affect the integrity of sperm DNA, but the storage process of sperm after freeze drying which may affect the integrity of sperm DNA.

This can be due to the storage process which does not ensure that it is free from oxygen, so it may cause extracellular oxidative stress (11). Furthermore, the most likely mechanism for the reduction of freeze-dried sperm DNA integrity in all sample groups (T1, T2, T3, T4) is the lack of protective agents such as ethylenglycolbis (b-aminoethyl ether) -N, N, N0, N0-tetraacetic acid (EGTA), ethylene diamine tetra-acetic acid(EDTA) and EGTA Tris-HCl buffered solution (ETBS) pH 8.2 to 8.4, antioxidants, and trehalose [13-15].

Previous studies using protective agents before freeze-drying have shown better results compared to those without protective agents. One study showed that the use of ETBS, fucose and egg yolk (5.20 ± 1.38%) as protective agents showed better results than without the protective agent (83.70 ± 2.89%) [15].

However, there are other studies showing that the DNA integrity of human sperm after (80.6 ± 11.3%) and before freeze drying (77.8 ± 12.2%) did not significantly differ using EDTA as a protective agent [10]. EGTA can bind to Ca^{2+} ions, thereby inhibiting endogenous Ca^{2+}-dependent nuclease activity in sperm.

Meanwhile, EDTA can also bind various divalent cations, such as Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+}. Zn^{2+} ion has an important role to stabilize the chromatin structure of human sperm [13]. Moreover, previous studies did not show statistically significant changes in DNA integrity of human sperm dried after storage at 4 ° C for 7 days (81.3 ± 3.5%) compared with fresh samples (81.06 ± 9.2%) [9].

This difference occurs because this study uses a lyophilized solution (LyoS: a-MEM Eagle-sucrose 0.25M, trehalose 0.25M and 0.6% (w / v) HSA) which functions as a protective agent. In addition, antioxidants are also needed to ward off and prevent oxidative stress in the freeze drying process.

Rosmarinic acid is an antioxidant that has been shown to be a protective agent in the freeze drying process (14,16). Trehalose has also been reported to protect sperm DNA in animals and humans in dehydrated conditions [14, 17]. This study also showed that although the freeze-dried sperm storage process was assisted by the addition of protective agents to help maintain the integrity of sperm DNA, the storage process at non-optimal temperatures (room temperature) and long periods(3 months) could not afford to avoid sperm DNA fragmentation.

Conclusion

Temperature at 4°C and periode in 1 weeknis the best temperatureand storage to store freeze-dried human sperm, based on sperm DNA integrity.

References

1. Raja DR, Natrajamani S (2013) Sperm DNA Integrity before and After Cryopreservation, 2(4):15.
2. Le MT, Nguyen TTT, Nguyen TT, Nguyen TV, Nguyen TAT, Nguyen QHV, et al (2019) Does conventional freezing affect sperm DNA fragmentation? Clin Exp. Reprod Med., 1:46(2):67-75.
3. Cankut S, Dinc T, Cincik M, Ozturk G, Selam B (2019) Evaluation of Sperm DNA Fragmentation via Halosperm Technique and TUNEL Assay Before and After Cryopreservation. Reprod Sci., 1: 26(12):1575-81.
4. Joaquim DC, Borges ED, Viana IGR, Navarro PA, Vireque AA (2017) Risk of Contamination of Gametes and Embryos during Cryopreservation and Measures to Prevent Cross-Contamination [Internet]. BioMed Research International. [cited 2019 Mar 20]. Available from: https://www.hindawi.com/journals/bmri/2017/1840417/
5. Molina I, Mari M, Martínez JV, Novella-Maestre E, Pellicer N, Pemán J (2016) Bacterial and fungal contamination risks in human oocyte and embryo cryopreservation: open versus closed vitrification systems. Fertility and Sterility, 106(1):127-32.
6. Nangia AK, Krieg SA, Kim SS (2013) Clinical guidelines for sperm cryopreservation in cancer patients. Fertility and Sterility, 1: 100(5):1203-9.
7. Rozati H, Handley T, Jayasena C (2017) Process and Pitfalls of Sperm Cryopreservation. J Clin Med [Internet]. Sep 19 [cited 2019 Mar 25];6(9). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5615282/
8. Keskintepe L, Eroglu A (2015) Freeze-drying of mammalian sperm. Methods in molecular biology (Clifton, NJ). 1 (1257): 489-97.
9. Patrizio P, Natan Y, Barak Y, Levi Setti P, Arav A (2016) A simple new method for the freeze-drying and storage of human sperm. Fertility and Sterility, 106(3): e307.
10. Gianaroli L, Magli MC, Stanghellini I, Crippa A, Crivello AM, Pescatori ES, et al (2012) DNA integrity is maintained after freeze-drying of human spermatozoa. Fertility and Sterility, 97(5): 1067-1073.e1.
11. Pramesti MB, Lunardhi H, I'tishom R (2010) Human Sperm Preservation Using Freeze-Drying an Experimental Laboratory Study, 9.

12. World Health Organization, editor (2010) WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization, 271.

13. Kusakabe H (2011) Chromosomal integrity and DNA damage in freeze-dried spermatozoa. Reprod Med. Biol., 10(4): 199-210.

14. Olaciregui M, Gil L (2017) Freeze-dried spermatozoa: A future tool? Reproduction in Domestic Animals, 52: 248-54.

15. Peitao W, Cuihua S, Daqing S, Li Q, Liu Z, Xiuyan F (2008) The Effect of Freeze Drying on the Deoxyribonucleic Acid Chain of Human Sperm. In: 2008 2nd International Conference on Bioinformatics and Biomedical Engineering [Internet]. Shanghai, China: IEEE; 2008 [cited 2019 Oct 14]. 919-23. Available from: http://ieeexplore.ieee.org/document/4535106/

16. Domingo P, Olaciregui M, González N, De Blas I, Gil L (2018) Long-term preservation of freeze-dried rabbit sperm by adding rosmarinic acid and different chelating agents. Cryobiology, 81: 174-7.

17. Zhang M, Oldenhof H, Sydykov B, Bigalk J, Sieme H, Wolkers WF (2017) Freeze-drying of mammalian cells using trehalose: preservation of DNA integrity. Scientific Reports, 24: 7(1):6198.

