



Research Article

In-vitro alpha lipoic acid supplementation in freeze-dried human sperm: the impact on DNA fragmentation index

Rezia Octarina¹, M. P. B. Dyah Pramesti², Agustinus²

1) Andrology Specialist Program, Faculty of Medicine Universitas Airlangga/Dr. Soetomo General Hospital, Surabaya, Indonesia

2) Department of Medical Biology, Faculty of Medicine Universitas Airlangga, Surabaya, Indonesia

ARTICLE INFO

Submitted : February 2020

Accepted : August 2020

Published : January 2021

Keywords:

Freeze-drying, ALA, DNA Fragmentation Index, Human spermatozoa

*Correspondence:

rezia.octarina-2016@fk.unair.ac.id

ABSTRACT

The freeze-drying technique is an alternative method for sperm preservation. However, this process can result in DNA damage. Adding antioxidants before freezing can reduce the detrimental effects of ROS on spermatozoa. Alpha-lipoic acid (ALA) is one of the potent antioxidants that can be used to protect the sperm DNA. This study was aimed to determine the effect of ALA supplementation before freeze-drying on the DNA fragmentation index (DFI) of human spermatozoa. Nine semen samples were collected and evaluated for DFI (O1), then divided into 3 groups; freeze-drying without ALA (O2), freeze-drying with ALA 1,25 mg (O3), and freeze-drying with ALA 2,5 mg (O4). All three groups were stored at 4°C for 1 week. Sperm DFI was evaluated before and after freeze-drying. The result of this study showed a significant increase in sperm DFI in all three groups after freeze-drying ($p < 0,05$) when compared to DFI before freeze-drying ($14,67 \pm 3,295$). However, there was no significant difference between O2 ($26,90 \pm 9,180$) and O4 ($29,29 \pm 5,524$) group, where the supplementation of 2,5 mg ALA did not have a significant effect on protecting the DNA of freeze-dried human spermatozoa. The highest DFI was in the O3 group ($44,64 \pm 11,717$). Therefore, ALA supplementation before freeze-drying does not have a significant effect on protecting the DNA of freeze-dried human spermatozoa.



INTRODUCTION

Sperm preservation has been done to maintain male fertility and has facilitated assisted reproductive technology in humans. Men who will have chemotherapy or surgical therapy that can interfere with their fertility status can cryopreserve their sperm before the treatment (Zhu, Li, & Xiao, 2016).

The freeze-drying technique is an alternative method for sperm preservation. This technique is economically cheaper and allows sperm to be stored for a long period at room temperature or in the refrigerator at 4°C. In contrast with the currently used sperm preservation cryopreservation technique, this technique requires constant liquid nitrogen and special containers for its storage. Freeze-drying (FD) or lyophilization is a preservation method where a material undergoes freezing and sublimation process and results in a dry form end product (Olaciregui and Gil, 2017; Saili et al., 2006; Zhu, Li, & Xiao, 2016). The procedures during freeze-drying can damage sperm DNA. The process of freezing, drying, and exposure to the high pressure during the preparation of freeze-dried samples can endanger the sperm (Kusakabe, 2011). That damage occurs during the freeze-drying process through the activation of endonuclease and reactive oxygen species (ROS). In animal studies, one way to overcome this problem is to add antioxidants before the freezing process to reduce ROS's detrimental effects (Luño et al., 2014).

Alpha-lipoic acid (ALA) is a potent antioxidant that has four different roles: scavenging activity of free radicals, the capacity to regenerate endogenous antioxidants such as glutathione, vitamin C and vitamin E, chelating metal, and repair of oxidized protein (El Barky, Husein, & Mohamed, 2017; Haghghian et al., 2015). Exogenous ALA supplementation has been reported to increase unbound ALA levels,

which can act as a potent antioxidant and reduce oxidative stress both in vitro and in vivo (Canepa et al., 2018; Haghghian et al., 2015; Yeni et al., 2012). A previous study reported that the addition of ALA in sperm preparation could improve the sperm motility and vitality as well as the total motile sperm count (TMSC) (Buanayuda, Lunardhi, & Mansur, 2019). ALA supplementation in the swim up and density gradient centrifugation can select sperm with lower DNA fragmentation (Lestari, Lestari, & Pujiyanto, 2018). In the animal study, it was reported that supplementing ALA in *Boer bucks* semen before cryopreservation improves sperm motility and viability and minimizes DNA damage (Ibrahim et al., 2008).

This study was aimed to determine the effect of ALA supplementation before freeze-drying on the DNA fragmentation index (DFI) of human spermatozoa.

METHODS

Semen Sample Collection

Semen samples were obtained from 9 patients who came to Andrology Clinic Dr. Soetomo General Hospital and signed the informed consent protocol approved by the ethical committee of Dr. Soetomo General Hospital Surabaya (1324/KEPK/VII/2019). Patients who met inclusion criteria which are men age 25-40 years, abstinence for 48 hours – 7 days, semen volume ≥ 2 ml, sperm concentration > 10 million/ml, progressive motility $> 40\%$, did not take any antioxidants before (more than 7 days) and exclusion criteria (leukospermia and haemospermia) were included to this research. According to the World Health Organization (WHO) laboratory manual (World Health Organization, 2010), the samples were analyzed for routine examination and examined for DNA fragmentation index.



Sperm Preparation

Simple Washing

Semen was mixed with a 1:1 ratio of *SpermRinse*TM, then centrifuged at 500 g for 10 minutes. Supernatant was removed and the pellets were mixed with 1 ml of *SpermRinse*TM and re-centrifuged at 500 g for 5 minutes. Supernatant was removed and the pellets were suspended with 3 ml of *SpermRinse*TM and divided into 3 aliquots of 1 ml to be put into microtubes.

ALA Supplementation

Treatment group was supplemented with ALA 1,25 mg (O3) and 2,5 mg (O4) and incubated for 1 hour at 37°C.

Freeze-drying, rehydration and storage

All sample units were frozen in -80°C for 24 hours. After samples have been frozen, the microtubes were transferred to the freeze-drying machine (VirTis, SP Industries, USA) at the Tissue and Cell Bank Dr. Soetomo General Hospital. Microtubes were opened and the frozen samples undergo an automated sublimation and desorption process for 48 hours. The microtubes then vacuumed and stored in the refrigerator at 4°C for 1 week. After one week, samples were rehydrated by adding 1 ml of distilled water into the microtubes and DNA fragmentation index were re-evaluated.

DNA Fragmentation Index Evaluation

DNA fragmentation were evaluated using Sperm Chromatin Dispersion (SCD) technique according to *SpermFunc*[®] DNA fragmentation

kit (BRED Life Science Technology Inc.). At least 500 spermatozoa in each sample were evaluated using microscope with 400x magnification. The normal reference value is if the percentage of spermatozoa with fragmented DNA is less than 25%.

Statistical Analysis

Comparative test between before and after freeze-drying groups was done by using paired t test. The comparison between three treatment groups was measured by same subject Anova test. Data analysis was performed using SPSS 16th.

RESULTS

In the sperm DNA fragmentation test, an image of the halo will appear as shown in Figure 1, between before freeze-drying (O1) group, after freeze-drying without ALA (O2) group, after freeze-drying with ALA 1,25 mg (O3) group and after freeze-drying with ALA 2,5 mg (O4) group. These figures showed various halo sizes (large, medium, small, no halo, and degraded spermatozoa), nonetheless, O1 group has more extensive and medium halos compared to O2, O3 and O4 group.

Same subject Anova test results showed a significant difference between 4 groups ($p < 0,05$). The results of further tests using LSD showed that before freeze-drying group (O1) has significant difference with all three treatment groups; however there was no significant difference between after freeze-drying without ALA (O2) group with after freeze-drying with ALA 2,5 mg (O4) group.

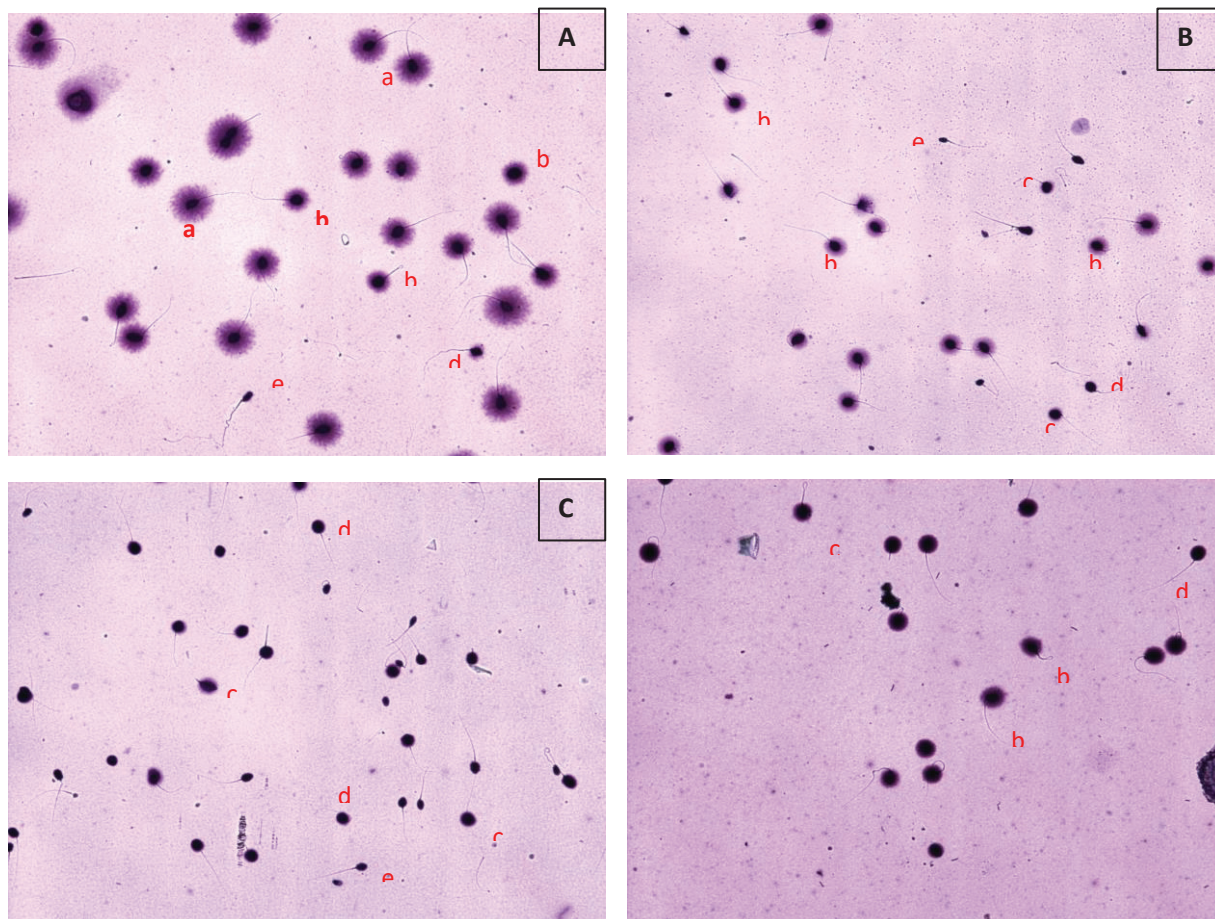


Figure 1. Halo image from DNA fragmentation evaluation in group A. Before freeze-drying (O1), B. After freeze-drying without ALA (O2), C. After freeze-drying with ALA 1,25 mg (O3) and D. After freeze-drying with ALA 2,5 mg (O4). Note: a. Large halo, b. Medium halo, c. Small halo, d. No halo, e. Degraded spermatozoa. Observation using a 400x magnification light microscope.

The paired t-test result showed an increase in DFI in all three treatment groups ($p < 0,05$).

Table 1. Paired t-test result between before and after freeze-drying groups

Treatment Group	n	Mean ± Standard Deviation with pre-freeze-drying	p value
After freeze-drying without ALA	9	12,23 ± 9,128	0,004
After freeze-drying with ALA 1,25 mg	9	29,98 ± 13,463	< 0,001
After freeze-drying with ALA 2,5 mg	9	14,62 ± 5,989	< 0,001

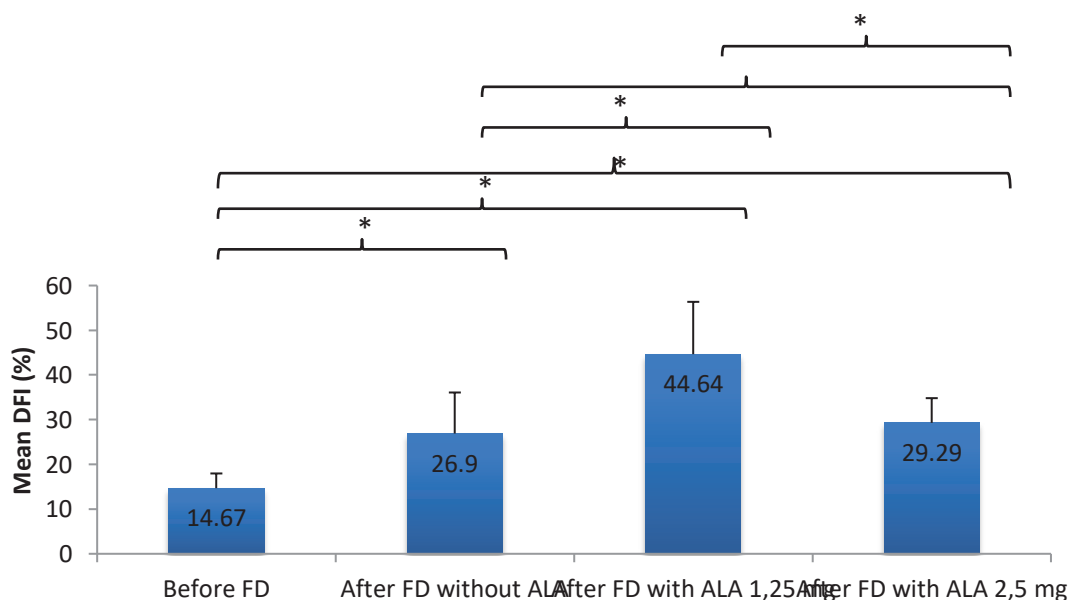


Figure 2. Comparative test between all groups. Note: * = sign of significance ($p < 0,001$)

DISCUSSION

Sperm DNA fragmentation after freeze-drying

The study result showed an increase in sperm DNA fragmentation in all three treatment groups. This result was in line with a previous study by Pramesti et al. (2010), which stated that freeze-dried human sperm stored at 4°C for 1 week has an increased DNA fragmentation ($38,43 \pm 9,46$) compared to fresh semen samples ($18,83 \pm 2,71$) and freeze-dried-only human sperm ($20,64 \pm 3,69$). Peitao et al. (2008) reported that freeze-dried sperm without a protective agent has more DNA damage ($78,29 \pm 3,24$) compared with a protective agent ($5,28 \pm 1,42$). Our study's most probable explanation is the lack of protective agents used in the freeze-drying process.

In other human studies, DNA integrity of freeze-dried human sperm stored at 4°C for 1 week ($81,3\% \pm 3,5\%$) has no significant difference compared to DNA integrity before freeze-drying ($81,06\% \pm 9,2\%$) (Patrizio et al., 2016). Gianaroli et al. (2012) also reported that the proportion of sperm with intact DNA

was $77,8\% \pm 12,2\%$ in fresh semen samples and $80,6\% \pm 11,3\%$ in freeze-dried sperm, indicating that the proportion of freeze-dried sperm with fragmented DNA did not have any significant changes after freeze-drying. Both studies used LyoS; alpha-MEM Eagle-0.25M sucrose, 0.25M trehalose and 0.6% (w/v) HSA and 10 mM Tris-HCl and 1 mM of ethylenediaminetetraacetic acid [EDTA]) consecutively as a freeze-drying solution. Freeze-drying solution act as a protective medium with an important role in maintaining sample integrity. Therefore, this solution must be able to support the integrity of the sperm nucleus as in normal conditions (Gil et al., 2014). The addition of chelating agents such as EDTA or EGTA to the freeze-drying solution can inactivate DNase and protect sperm from DNA damage during the freeze-drying process and subsequent storage (Olaciregui & Gil, 2017).

Sperm DNA fragmentation with ALA supplementation

In an animal study, Ibrahim et al. in 2008 reported that ALA administration in *Boer*



bucks semen at a certain dose could increase the sperm DNA fragmentation index. Their review stated that the administration of ALA in small dosage (0,00625 mmol/ml) increased sperm DNA fragmentation up to 6 times (4,59) when compared to the control group (0,69) (Ibrahim et al., 2008). This result was in accordance with our research in which an increase of sperm DNA fragmentation was found in low dose ALA supplementation (1,25 mg).

In other animal studies, Shen et al. reported that sperm motility of boar spermatozoa after cryopreservation is decreased in groups with high concentrations of ALA, possibly because exorbitant ALA concentrations. Antioxidant activity of ALA decreases at higher concentrations, and this may occur because the addition of antioxidants in greater amounts can damage the mitochondria of spermatozoa (Hu et al., 2013; Shen et al., 2016). Because ALA stabilizes most intracellular antioxidant systems, its administration has a beneficial effect on mitochondrial function and oxidative stress associated with several diseases and aging processes. However, proper plasma levels must be achieved to ensure maximum therapeutic effects (Tibullo et al., 2017). It must be realized that antioxidant therapy is like a double-edged knife with undesirable effects if the safe dose threshold is passed (Donnelly, McClure, & Lewis, 1999).

The limitation of this study was the small number of samples. However, this is the first study conducted to determine the effect of ALA supplementation on DNA fragmentation of freeze-dried human sperm. In previous studies, both in animal and human studies, it was found that ALA can improve sperm parameters in motility, viability, and DNA integrity, but ALA may not be able to provide sufficient protection to human sperm that undergo freeze-drying.

CONCLUSION

There is an increase in the DNA fragmentation index of freeze-dried human sperm stored for 1 week at 4°C. Alpha-lipoic acid (ALA) supplementation before freeze-drying does not provide a significant protective effect on human sperm DNA.

REFERENCES

- Buanayuda, G. W., Lunardhi, H., & Mansur, I. G. (2019). Effect of in-vitro alpha lipoic acid addition on spermatozoa motility in sperm preparation process. *Fol Med Indones*, 55(4), 246-50. <http://dx.doi.org/10.20473/fmi.v55i4.17309>
- Canepa, P., Dal Lago, A., De Leo, C., Gallo, M., Rizzo, C., Licata, E., Anserini, P., Rago, R., & Scaruffi, P. (2018). Combined treatment with myo-inositol, alpha-lipoic acid, folic acid and vitamins significantly improves sperm parameters of sub-fertile men: A multi-centric study. *Eur Rev Med Pharmacol Sci*, 22, 7078-85.
- Donnelly, E. T., McClure, N., & Lewis, S. E. M. (1999). The effect of ascorbate and α -tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis*, 14(5), 505-11.
- El Barky, A. R., Hussein, S. A., & Mohamed, T. M. (2017). The potent antioxidant alpha lipoic acid. *J Plant Chem and Ecophysiol*, 2(1), 1-5.
- Gianaroli, L., Magli, M. C., Stanghellini, I., Crippa, A., Crivello, A. M., Pescatori, E. S., & Ferraretti, A. P. (2012). DNA integrity is maintained after freeze-drying of human spermatozoa. *Fertility and Sterility*, 97(5), 1067-1073. <https://doi.org/10.1016/j.fertnstert.2012.02.014>



QANUN MEDIKA

JURNAL KEDOKTERAN FKUM SURABAYA

<http://journal.um-surabaya.ac.id/index.php/qanunmedika>



- Gil, L., Olaciregui, M., Luño, V., Malo, C., González, N., & Martínez, F. (2014). Current Status of Freeze-Drying Technology to Preserve Domestic Animals Sperm. *Reproduction in Domestic Animals*, 49, 72–81. <https://doi.org/10.1111/rda.12396>
- Haghighian, H. K., Haidari, F., Mohammadiasl, J., & Dadfar, M. (2015). Randomized, triple-blind, placebo-controlled clinical trial examining the effects of alpha-lipoic acid supplement on the spermatogram and seminal oxidative stress in infertile men. *Fertility and Sterility*, 104(2), 318–324. <https://doi.org/10.1016/j.fertnstert.2015.05.014>
- Hu, J.-H., Sun, X.-Z., Li, Q.-W., Zhang, T., Hu, X.-C., Hu, J.-H., & Wang, L.-Q. (2013). The effect of Laminaria japonica polysaccharide on sperm characteristics and biochemical parameters in cryopreserved boar sperm. *Animal Reproduction Science*, 139(1–4), 95–100. <https://doi.org/10.1016/j.anireprosci.2013.03.015>
- Ibrahim, S. F., Osman, K., Das, S., Othman, A. M., Majid, N. A., & Rahman, M. P. A. (2008). A study of the antioxidant effect of alpha lipoic acids on sperm quality. *Clinics*, 63(4), 545–550. <https://doi.org/10.1590/S1807-59322008000400022>
- Kusakabe, H. (2011). Chromosomal integrity and DNA damage in freeze-dried spermatozoa. *Reproductive Medicine and Biology*, 10(4), 199–210. <https://doi.org/10.1007/s12522-011-0092-7>
- Lestari, S. W., Lestari, S. H., & Pujianto, D. A. (2018). Sperm quality after swim up and density gradient centrifugation sperm preparation with supplementation of alpha lipoic acid (ALA): A preliminary study. *AIP Conference Proceedings 1933*, 030015-1-6. <https://doi.org/10.1063/1.5023962>
- Luño, V., Gil, L., Olaciregui, M., González, N., Jerez, R. A., & de Blas, I. (2014). Rosmarinic acid improves function and in vitro fertilising ability of boar sperm after cryopreservation. *Cryobiology*, 69(1), 157–162. <https://doi.org/10.1016/j.cryobiol.2014.07.002>
- Olaciregui, M., & Gil, L. (2017). Freeze-dried spermatozoa: A future tool? *Reproduction in Domestic Animals*, 52, 248–254. <https://doi.org/10.1111/rda.12838>
- 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. <https://doi.org/10.1016/j.fertnstert.2016.07.871>
- 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. *2008 2nd International Conference on Bioinformatics and Biomedical Engineering*, 919–923. <https://doi.org/10.1109/ICBBE.2008.226>
- Pramesti, M. P. B. D., Lunardhi, H., & I'tishom, R. (2010). Human sperm preservation using freeze-drying an experimental laboratory study. *Jurnal Kesehatan Reproduksi*, 1(1), 14–22.
- Saili, T., Prasetyaningtyas, W. E., Setiadi, M. A., Agungpriyono, S., Agungpriyono, S., & Boediono, A. (2006). Status DNA spermatozoa domba setelah proses pengeringbekuan. *JITV*, 11(3), 215–21.
- Shen, T., Jiang, Z.-L., Li, C.-J., Hu, X.-C., & Li, Q.-W. (2016). Effect of alpha-lipoic acid on boar spermatozoa quality during freezing–thawing. *Zygote*, 24(2), 259–265. <https://doi.org/10.1017/S0967199415000155>



QANUN MEDIKA
JURNAL KEDOKTERAN FKUM SURABAYA
<http://journal.um-surabaya.ac.id/index.php/qanunmedika>



- Tibullo, D., Li Volti, G., Giallongo, C., Grasso, S., Tomassoni, D., Anfuso, C. D., Lupo, G., Amenta, F., Avola, R., & Bramanti, V. (2017). Biochemical and clinical relevance of alpha lipoic acid: Antioxidant and anti-inflammatory activity, molecular pathways and therapeutic potential. *Inflammation Research*, 66(11), 947–959. <https://doi.org/10.1007/s00011-017-1079-6>
- World Health Organization (Ed.). (2010). *WHO laboratory manual for the examination and processing of human semen* (5th ed). World Health Organization.
- Yeni, D., Fidan, A. F., Ciğerci, I. H., Konuk, M., Avdatek, F., & Gündoğan, M. (2012). Effect of α -lipoic acid on sperm quality, reproductive tract measures in thinner exposed rats: α -lipoic acid sperm quality thinner induced rats. *Andrologia*, 44, 74–80. <https://doi.org/10.1111/j.1439-0272.2010.01140.x>
- Zhu, W., Li, J., & Xiao, L. (2016). Changes on membrane integrity and ultrastructure of human sperm after freeze-drying. *Journal of Reproduction & Contraception*, 27(2), 76-81. <https://doi.org/10.7669/j.issn.1001-7844.2016.02.0076>