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ORIGINAL ARTICLE

Assessing Sperm DNA Fragmentation Post-Cryopreservation in Infertility Cases

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Abstract

Background/Aim: Sperm cryopreservation is an important practice utilised in many assisted reproductive techniques. The potential biological effects of cryopreservation on sperm DNA damage in infertile males need to be better understood for therapeutic optimisation. This study aimed to explore the relationship between different types of infertility, sperm DNA damage and the post-thaw survival of cryopreserved sperm.

Methods: Present study initially included a total of 31 patients, wherein only 21 patients with either primary or secondary infertility and have consented for sperm cryopreservation were included, whereas patients with conditions like oligozoospermia, azoospermia and necrozoospermia were not included in the study. After a 7-days abstinence period, semen samples (from 21 patients) were collected and routine semen parameters (eg, semen volume, pH and motility) were evaluated, along with sperm DNA fragmentation analysis before cryopreservation. A few weeks later, post-thaw sperm motility was assessed for all samples.

Results: The semen characteristics, including volume, pH, leukocyte count, the concentration levels of sperm and their motility were found to be similar in patients with primary and secondary infertility. However, post-thaw sperm motility was significantly lower in secondary infertility patients compared to those with primary infertility. Specifically, post-thaw motility in secondary infertility patients decreased to 43 %, a substantial reduction from 66 % in their pre-freeze state. In contrast, semen cryopreservation led to a 28 % reduction in sperm motility overall, regardless of infertility type. The freshly ejaculated semen samples showed higher sperm DNA fragmentation in case of secondary infertility patients as compared to those with primary infertility.

Conclusion: The reduced post-thaw sperm motility observed in secondary infertility patients may be attributed to increased sperm DNA fragmentation in these individuals. Therapeutic interventions, such as the administration of synthetic additives to fortify semen samples, could help reduce DNA damage and improve outcomes in assisted reproductive techniques for secondary infertility patients.

Key words: Cryopreservation; Infertility; DNA fragmentation; Sperm motility; Reproductive techniques, assisted.

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Introduction

Infertility is a public health concern affecting nearly 10-15 % of couples falling in the reproductive age group worldwide. Nearly 50 % of them are reported to be caused due to the male factor infertility (MFI).[1, 2] The term infertility, refers to the inability of a couple to accomplish a natural

pregnancy following 12 or more months of regular unprotected sexual intercourse.[3] Based on the World Health Organization's (WHO) guidelines, it has been categorised in two divisions, terms as primary infertility (PI) and secondary infertility (SI). PI is referred to as the infertility conditions when the woman among the couple has not been able to conceive even once, as compare to SI where the couple is not able to achieve a second conception, after having achieved at least one successful conception.[4] As per studies and the current practices, male infertility has been primarily related to hormonal imbalances and sperm abnormalities.[5, 6] Hence, the evaluation of male infertility is evaluated based on routine semen analysis. However, routine semen analysis-based information is found to be insufficient in terms of certain functional aspects of the sperm and is still considered to be unsatisfactory in predicting the success of the assisted reproductive technology (ART) based procedures, hence the male fertility potential.[7, 8] Contrary to this, reports also suggest nearly 15 % of patients with male infertility are shown to have normal results of the routine semen analysis.[9]

On the other hand, sperm DNA fragmentation is another process which is known to have detrimental effects on the normal fertilisation, embryo development and the success of ART procedures.[10] Hence, the sperm DNA fragmentation (SDF) test, is a recent addition to the battery of already existing tests, which evaluates sperm quality as a measure of sperm's ability to act as the DNA carrier is considered as a substantial parameter in routine semen analyses.[11] The utilisation of damaged sperm DNA in ART is a potential risk factor which could account for undesirable results leading to low success rate in terms of pregnancy, miscarriages, chromosomal abnormalities, as well as other genetic and birth defects in the offsprings.[12]

The current clinical management and treatment strategies for the MFI, involve extensive utilisation of ART procedures. Wherein, *in-vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) have led to significant advancements in the treatment of MFI. The freezing of spermatozoa is a key procedure in several ART techniques, wherein, freezing of the human semen sample, acts as a backup in patients undergoing infertility treatment and as a fertility preservation method in patients receiving anti-cancer therapy.[13, 14] In addition, sperm cryopreservation is also useful in people with diabetes and autoimmune conditions, wherein their therapeutic treatments are anticipated to affect their reproductive functions with the advancing therapy.[15] The primary goal of cryopreserving semen samples aims at maintaining optimal activity of the functional parameters including good motility and fertility of spermatozoa for a long period of time.[16] Moreover, the decline in the male reproductive health over time, accounts to be an essential indication aiming to enhance the focus on sperm banking requirements.[17, 18] Each step of cryopreservation has been shown to damage different components of the sperms like the cell membrane, as well as other cellular structural components. Hence affecting the normal sperm functions, resulting into reduced sperm motility and induction of capacitation like changes.

The thawing procedures have shown to result in significant decrease in sperm viability, motility, plasma membrane functionality and acrosome integrity in the semen samples.[19] It was reported earlier that, the sperm motility is significantly declined from 50.6 % to 30.3 % in cryopreserved samples.[20] Therefore, the present study aimed to determine the association of sperm DNA damage and post-thaw sperm cryo-survival and their relationship with the primary and secondary infertility condition.

Methods

Study design

The present study initially included a total of 31 infertile couples, visiting the Institute of Reproduction and Child Cares (IRCC) and IRCC IVF Centre, at Panchkula (Haryana, India). The study commenced after obtaining a letter of approval (024/IRCCPKL/2020, dated 01/06/2020) from the Institutional Ethical Committee of IRCC. The male partners with less than 7 days (mean: 3.19 ± 3.8 days) of sexual abstinence, appropriate sperm count and sperm motility participated in the study. Those with conditions like oligozoospermia, azoospermia and necrozoospermia were not included in the study. Ejaculates of 21 out of 31 patients (~ 67.74 %), evaluated for semen analysis were considered optimal for semen freezing. Based on the type of infertility, male patients were categorised into primary (n = 14) and secondary infertility (n = 7).

Semen evaluation

The selected ejaculates were subjected to routine semen analysis procedure as described in the manual of semen examination published by WHO in 2010.[21] All the samples were analysed using the single semen freezing medium (*FertiPro*, Belgium). The post-thaw sperm motility was analysis after two weeks of semen freezing. The necessary consent of all the patients for this study was taken.

Sperm DNA fragmentation test

The sperm DNA fragmentation test was performed by using commercially available QwickCheck DFI kit (*Medical Electronics Systems* India Pvt. Ltd.) as per the manufacturer's instructions. Briefly, the swim-up sperms were mixed in melted agarose gel by maintaining 20 million sperm count at 37 °C. The DFI stained slide were observed under the 40x objective lens and 500 sperms per slide were counted and percentage of sperms as degraded (type a), without halo (type b), medium halo (type c) and big halo (type d) were calculated. The non-fragmented and fragmented DNA were differentiated based on the visual presence and absence of a core/peripheral halo of DNA dispersion loop and small or no halo of dispersion respectively.

Semen cryopreservation

The semen samples were collected in a sterile container and kept at 37 °C for liquefaction. Following liquefaction, the routine examination consisting of sperm concentration, motility and other parameters were performed. One mL of each semen sample was cryopreserved by dropwise adding the prewarmed (37 °C) and equilibrated freezing medium at 37 °C with continuous gentle shaking for 10 minutes. Following this the suspensions were loaded into the cryovials and subjected to vapour freezing in LN_2 for 10 minutes. Thereafter, the vials were stored into canisters in LN_2 (-196 °C) until thawing.

Semen thawing

The cryopreserved vial was placed for 5 min in water at room temperature, followed by thawing in the incubator at 37 °C and then evaluated for sperm motility of the semen samples.

Statistical analysis

The SPSS statistical software (version 20) was utilised for data analysis. Two groups were analysed for comparison using the Mann Whitney test, wherein a p value < 0.05 was considered to be statistically significant.

Results

The mean age of patients, female partner's age and active marriage life was found to be 32.00 ± 1.14 and 34.14 ± 1.67, 29.57 ± 1.20 and 31.43 ± 1.13 and 2.86 ± 0.70 and 8.14 ± 1.53 years in case of both the primary and secondary infertility cases. No significant difference ($p > 0.05$) was observed in the semen parameters such as volume, pH, leukocytes percentage, sperm concentration before freezing and the motility of the sperms the patients with primary and secondary infertility (Table 1). Sperm motility evaluations irrespective of groups (pooled data), showed significant ($p < 0.05$) drop (28.8 %) in sperm motility in the cryopreserved samples as compared to the fresh ejaculates. Interestingly, sperm motility was found to be significantly lowered after the thawing process ($p < 0.05$) in patients with SI as compared to PI.

Table 1: Semen characteristics in patients with primary and secondary infertility

Category	Semen volume (mL)	Leukocytes (%)	pH of semen	Sperm concentration (million/mL)	Sperm motility Type-a + type-b (%)	
					Fresh ejaculate	Post thawing
Primary infertility	2.98 ± 0.36	1.13 ± 0.29	7.32 ± 0.04	110.07 ± 13.98	60.31 ± 5.35	51.29 ± 4.43^{B}
Secondary infertility	3.04 ± 0.56	0.67 ± 0.36	7.33 ± 0.04	105.86 ± 15.31	58.00 ± 6.85^{b}	32.96 ± 3.63^{aA}

a:b ($p < 0.05$) within row indicates significant difference; A:B ($p < 0.05$) within column indicates significant difference;

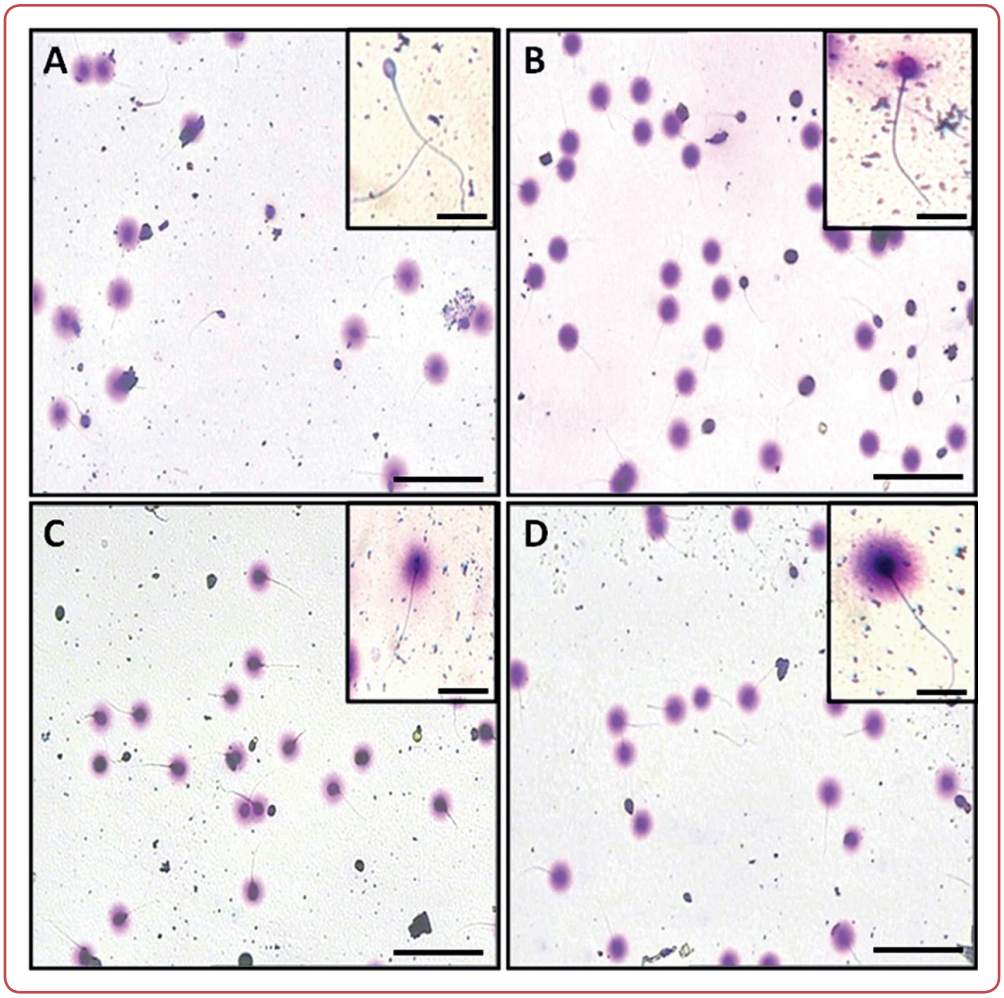

Figure 1: Sperm DNA fragmentation test: The Sperm DNA fragmentation index was observed for degraded (A), without halo (B), medium halo (C) and big halo (D) in the infertility patients at 10 x and 40 x (as shown in inset).

Table 2: Sperm DNA fragmentation index in patients with primary and secondary infertility

Category	Type of DNA fragmentation (%)			
	Degraded (type a, %)	Without Halo (type b, %)	Medium Halo (type c, %)	Big Halo (type d, %)
Primary infertility	3.76 ± 1.45[A]	8.24 ± 3.25	60.43 ± 4.03	27.57 ± 3.28
Secondary infertility	10.11 ± 5.00[B]	7.57 ± 4.60	50.63 ± 6.05	31.69 ± 3.76
Mann Whitney U-test	$p < 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$

A:B ($p < 0.05$) within column indicates significant difference;

Out of 21 patients, 14 had history of primary infertility and 7 had secondary infertility. In couples experiencing primary infertility, the mean sperm DNA fragmentation indices of male partners (Figure 1) were found to be 3.76 % (type-a), 8.24 % (type-b), 60.43 % (type-c) and 27.57 % (type-d) respectively (Table 2).

Similarly, in couples with secondary infertility, the sperm DNA fragmentation indices of male partner were found to be: 10.11 % (type-a), 7.57 % (type-b), 50.63 % (type-c) and 31.68 % (type-d). The sperm DNA fragmentation was found to be significantly ($p < 0.05$) higher in semen sample of patients with secondary infertility as compared to those with primary infertility.

Discussion

Cryopreservation is a well-known technique that is utilised for preserving male gametes in various fertility treatment procedures. This technique involves considerable changes in spermatozoa which may impact their fertilising capability. Such changes are principally attributed to the formation of ice crystals and the use of cryoprotective agents.[22] Therefore, in order to maintain the essential characteristics of sperm, required for successful fertilisation process, it is fundamental to meticulously understand the sperm physiology during such cryopreservation process and to consider the environmental condition/s to which sperm are stored. The key attributes commonly considered includes: motility, viability, acrosomal integrity and DNA integrity. Particularly, spermatozoa are known to have significant flexibility compared to other cell types, chiefly due to their structural features, such as their small size and high surface area, aiding to minimise cryopreservation-induced damages. The scope and importance of sperm cryopreservation is expanding with the advancement in assisted reproductive technologies. Sperm cryopreservation is the only method that has epitomised as the primary preventive measure utilised in fertility conservation methods helping many of the infertile men to be able to father children.[19, 23] The present study observed a 28.8 % decline in sperm motility after cryopreservation/freezing irrespective of the type of infertility. These results are in accordance with an earlier report, wherein, a significant decrease was observed in parameters such as sperm concentration, progressive motility, along with a normal morphology post cryopreservation.[22] On the other hand, in another study the freezing-thawing process was reported to cause a 37 % reduction in the typical morphological forms of sperm.[24]

DNA fragmentation acts as one of the primary causes of male infertility, leading to poor fertility oucomes. Present study reports a significant relationship between sperm DNA fragmentation index and different parameters of sperm quality, thereby indicating a possible involvement of sperm DFI as an important factor leading to the decline of the sperm parameters. During cryopreservation, the use of a suitable cryo-protectant and an efficient sperm selection technology seems to a have notable influence on the prevention of DNA fragmentation, hence improvement in the cryo-survival rates of the sperm.[25] Interestingly, in the present study, the DNA fragmentation index (type-a) of fresh ejaculate was higher in secondary infertility patients, a drastic drop in post cryopreservation sperm motility in such patients was also observed. This has been attributed to increased oxidative stress induced following cryopreservation in earlier reports and has also been shown to cause loss of sperm motility/ vitality, enhanced membrane damage, induced acrosome reaction and increase apoptosis. Similarly, exposure to different freeze-thawing methods have also been reported to cause alterations in the sperm DNA integrity.[26] Hence, increased sperm DNA fragmentation post cryopreservation still stands as a matter of concern in ART as it is found to be related to reduced full term pregnancy rates and increased risk of miscarriages, while a healthy offspring remains the primary goal of various ART services.[27-29] Thus, the extent of DNA fragmentation in male patients with secondary infertile in present study may get increased by the process of cryopreservation. Therefore, reduction of oxidative burden in patients with secondary infertility prior should be undertaken to lower the subsequent sperm DNA damage caused by semen cryopreservation.

Conclusion

In summary, the study observed a considerable decline in sperm motility after sperm cryopreservation in patients with secondary infertility and this is attributed due to the presence of already existing higher sperm DNA fragmentation in their fresh ejaculates. Future studies are required to develop therapeutic/ intervention-based modules that could target improvement or preservation of the semen quality parameters. These might include therapeutic interventions involving supplements and/or fortification of semen samples with appropriate additives to prevent sperm DNA damage for better post-thaw survival, hence improved ART outcomes.

Ethics

The ethical approval (decision No: 024/IRCCP-KL/2020, dated 01 Jun 2020) for the study was obtained from the Institutional Ethics Commit-

tee, from the Institute of Reproduction and Child Cares (IRCC) and IRCC IVF Centre, at Panchkula (Haryana, India).

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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References

1. Agarwal A, Baskaran S, Parekh N, Cho CL, Henkel R, Vij S, et al. Male infertility. Lancet. 2021 Jan 23;397(10271):319-33. doi: 10.1016/S0140-6736(20)32667-2.
2. Khalafalla K, Majzoub A, Elbardisi H, Bhathella A, Chaudhari A, Agarwal A, et al. The effect of sperm DNA fragmentation on intracytoplasmic sperm injection outcome. Andrologia. 2021 Nov;53(10):e14180. doi: 10.1111/and.14180.
3. Brannigan RE, Hermanson L, Kaczmarek J, Kim SK, Kirkby E, Tanrikut C. Updates to male infertility: AUA/ASRM guideline (2024). J Urol. 2024 Dec;212(6):789-99. doi: 10.1097/JU.0000000000004180.
4. Vander Borght M, Wyns C. Fertility and infertility: Definition and epidemiology. Clin Biochem. 2018;62:2-10. doi: 10.1016/j.clinbiochem.2018.03.012.
5. Sanderman EA, Willis SK, Wise LA. Female dietary patterns and outcomes of in vitro fertilization (IVF): a systematic literature review. Nutrit J. 2022;21(1):5. doi: 10.1186/s12937-021-00757-7.
6. Chorosho SH, Malik N, Panesar G, Kumari P, Jangra S, Kaur R, et al. Phytochemicals: alternative for infertility treatment and associated conditions. Oxid Med Cell Longev. 2023 May 11;2023:1327562. doi: 10.1155/2023/1327562.
7. Jeyendran RS, Caroppo E, Rouen A, Anderson A, Puscheck E. Selecting the most competent sperm for assisted reproductive technologies. Fertil Steril. 2019 May;111(5):851-63. doi: 10.1016/j.fertnstert.2019.03.024.
8. Upreti K, Singh D, Singh A, Vats P, Bhardwaj R, Kapoor S. Discovering patterns of live birth occurrence before in vitro fertilisation treatment using association rule mining. Int J Electr Healthcare. 2023;13(4):275-94. doi: 10.1504/IJEH.2023.138250.
9. Kadioglu A, Ortac M. The role of sperm DNA testing on male infertility. Transl Androl Urol. 2017 Sep;6(Suppl 4):S600-S603. doi: 10.21037/tau.2017.03.82.
10. Marinaro JA. Sperm DNA fragmentation and its interaction with female factors. Fertility and Sterility. 2023;120(4):715-719. doi: 10.1016/j.fertnstert.2023.06.001.
11. Conti D, Calamai C, Muratori M. Sperm DNA fragmentation in male infertility: tests, mechanisms, meaning and sperm population to be tested. J Clin Med. 2024;13(17):5309. doi: 10.3390/jcm13175309.
12. Kim GY. What should be done for men with sperm DNA fragmentation? Clin Experim Reprod Med. 2018;45(3):101. doi: 10.5653/cerm.2018.45.3.101.
13. Huang C, Tang YL, Hu JL, Zhou WJ, Huang ZH, Luo XF, et al. Update on techniques for cryopreservation of human spermatozoa. Asian J Androl. 2022 Nov-Dec;24(6):563-9. doi: 10.4103/aja20229.

14. Ledesma B, Campbell K, Muthigi A, Ila V, Nassau DE, Ibrahim E, Ramasamy R. Semen cryopreservation in men with cancer: identifying patterns and challenges. J Fertil Preserv. 2023;3. doi: 10.32371/jfp/246156.
15. Huang R, Chen J, Guo B, Jiang C, Sun W. Diabetes-induced male infertility: potential mechanisms and treatment options. Mol Med. 2024;30(1):11. doi: 10.1186/s10020-023-00771-x.
16. Gupta S, Sharma R, Agarwal A. The process of sperm cryopreservation, thawing and washing techniques. The Complete Guide to Male Fertility Preservation. 2018:183-204. doi. 10.1007/978-3-319-42396-8_14.
17. De Jonge CJ, Barratt CLR, Aitken RJ, Anderson RA, Baker P, Chan DYL, et al. Current global status of male reproductive health. Hum Reprod Open. 2024 Apr 12;2024(2):hoae017. doi: 10.1093/hropen/hoae017.
18. Huang B, Wang Z, Kong Y, Jin M, Ma L. Global, regional and national burden of male infertility in 204 countries and territories between 1990 and 2019: an analysis of global burden of disease study. BMC Public Health. 2023 Nov 8;23(1):2195. doi: 10.1186/s12889-023-16793-3.
19. Tamburrino L, Traini G, Marcellini A, Vignozzi L, Baldi E, Marchiani S. Cryopreservation of human spermatozoa: functional, molecular and clinical aspects. Int J Mol Sci. 2023 Feb 28;24(5):4656. doi: 10.3390/ijms24054656.
20. Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaeili V, et al. Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. Reprod Biomed Online. 2018;37(3):327-39. doi: 10.1016/j.rbmo.2018.05.012.
21. Björndahl L, Brown JK. The sixth edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen: ensuring quality and standardization in basic examination of human ejaculates. Fert Seril. 2022;117(2):246-51. doi: 10.1016/j.fertnstert.2021.12.012.
22. Karabulut S, Demiroğlu-Zergeroğlu A, Yılmaz E, Kutlu P, Keskin İ. Effects of human sperm cryopreservation on apoptotic markers in normozoospermic and non-normozoospermic patients. Zygote. 2018;26(4):308-13. doi: 10.1017/S0967199418000254.
23. Wang S, Wang W, Xu Y, Tang M, Fang J, Sun H, et al. Proteomic characteristics of human sperm cryopreservation. Proteomics. 2014;14(2-3):298-310. doi: 10.1002/pmic.201300225.
24. Tamburrino L, Traini G, Ragosta ME, Dabizzi S, Vezzani S, Scarpa F, et al. Semen cryopreservation and storage in liquid nitrogen: Impact on chromatin compaction. Andrology. 2024. doi: 10.1111/andr.13806.
25. Ozimic S, Ban-Frangez H, Stimpfel M. Sperm cryopreservation today: Approaches, efficiency, and pitfalls. Current Issues Mol Biol. 2023;45(6):4716-34. doi: 10.3390/cimb45060300.
26. Hai E, Li B, Zhang J, Zhang J. Sperm freezing damage: the role of regulated cell death. Cell Death Discovery. 2024;10(1):239. doi: 10.1038/s41420-024-02013-3.
27. Sugihara A, Punjabi U, Chimienti T, Goovaerts I, Peeters K, Bouziotis J, et al. Sperm DNA fragmentation after cryopreservation and sperm selection has no implications for clinical pregnancies and live births after intrauterine insemination with donor sperm. J Pers Med. 2023 Nov 28;13(12):1668. doi: 10.3390/jpm13121668.
28. Esteves SC, Zini A, Coward RM, Evenson DP, Gosálvez J, Lewis SEM, et al. Sperm DNA fragmentation testing: Summary evidence and clinical practice recommendations. Andrologia. 2021;53(2):e13874. doi: 10.1111/and.13874.
29. Evans EPP, Scholten JTM, Mzyk A, Reyes-San-Martin C, Llumbet AE, Hamoh T, et al. Male subfertility and oxidative stress. Redox biology. 2021;46:102071. doi: 10.1016/j.redox.2021.102071.