



## Impact of Temporary Energy Restriction on DNA Fragmentation in Cryopreserved Human Sperm

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**Received:** May 05, 2026

**Published:** May 19, 2026

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### Abstract

Cryopreservation is widely employed in assisted reproductive technology; however, it frequently leads to diminished sperm motility and impaired DNA integrity. The DNA Fragmentation Index (DFI) serves as an indicator of sperm DNA integrity, representing the proportion of sperm exhibiting fragmented genetic material. Elevated DFI values are closely associated with reduced fertilization rates, impaired embryo development, and lower chances of pregnancy, even in cases where sperm count and motility appear to be within normal ranges. In this study, cryopreservation significantly increased DNA fragmentation index (DFI) compared to baseline values. However, samples subjected to temporary energy restriction (TER) did not demonstrate a statistically significant reduction in DFI when compared to non-TER samples, indicating a negligible effect of TER on sperm DNA integrity under the conditions studied.

**Methods:** This study investigated semen samples obtained from 80 men. All samples were initially assessed for DNA fragmentation index (DFI) prior to cryopreservation. Following cryopreservation, the samples were randomly allocated into two groups (n = 40 each) using a computer-generated method: one group was evaluated without exposure to temporary energy restriction (TER), while the other was subjected to TER during the freeze-thaw process. In the TER group, cryopreserved sperm were exposed to a phase of nutrient deprivation followed by controlled nutrient introduction. DFI was subsequently assessed across all groups to evaluate sperm DNA integrity and quality. Comparative analysis enabled the determination of the effects of cryopreservation and TER on DNA fragmentation levels. Elevated DFI is strongly associated with reduced fertilization potential, even when conventional semen parameters such as sperm count and motility appear normal. All procedures were conducted in accordance with the guidelines established by the Indian Council of Medical Research (ICMR).

**Results:** TER-treated samples exhibited DFI values comparable to the non-TER group, indicating no measurable reduction in DNA fragmentation under the conditions studied.

**Conclusion:** Temporary energy restriction (TER) had a minimal effect on DNA fragmentation in cryopreserved sperm, with differences between groups being negligible. Under the conditions studied, TER does not appear to significantly influence sperm DNA integrity.

**Keywords:** DNA Fragmentation Index; Temporary Energy Restriction; Cryopreservation; Fertilization

## Introduction

Cryopreservation is routinely practiced in assisted reproductive technology (ART); however, the freeze-thaw process has been reported to impair sperm function and compromise DNA integrity [1]. Previous studies have demonstrated that cryopreservation can adversely affect sperm motility and induce structural and molecular alterations within the cell [3,7]. Cryopreservation followed by thawing has also been associated with irreversible damage to human spermatozoa [4].

Compared with many other cell types, spermatozoa display a degree of tolerance to cryopreservation-induced stress owing to their relatively high membrane fluidity and low intracellular water content (approximately 50%). To limit damage during freezing, cryoprotectants, also referred to as cryoprotective agents, are used to preserve cellular structure and function throughout the cryopreservation process [5].

During the freezing and thawing process, intracellular ice crystal formation, osmotic stress, and cold shock contribute to cellular injury [6]. In addition, generation of reactive oxygen species (ROS) during cryopreservation has been associated with membrane destabilization, mitochondrial dysfunction, and DNA damage [4]. These alterations collectively reduce sperm functional capacity and fertilization potential.

DNA integrity strongly influences reproductive outcome, and the DFI represents the proportion of spermatozoa containing fragmented genetic material. Elevated DNA fragmentation following cryopreservation has been reported in several studies [1,7,8], indicating susceptibility of sperm chromatin to freeze-thaw associated stress. Although cryopreservation supports long-term sperm storage, alterations in DNA structure may affect fertilization and subsequent embryo development [9]. Given these effects on sperm motility and genomic stability, investigation of post-thaw interventions remains relevant. The present study examines the effect of temporary energy restriction on cryopreserved human sperm, evaluating DFI following controlled nutrient introduction.

## Objective of the Study

This study aimed to assess the DNA fragmentation index (DFI) in cryopreserved semen samples subjected to temporary energy restriction (TER) post-thaw.

## Materials and Methods

Semen samples were obtained from 80 men as part of routine diagnostic procedures. Written informed consent was obtained from all participants prior to sample collection, and the study was conducted with institutional authorization in accordance with established clinical and ethical protocols. Key aspects of cryopreservation and temporary energy restriction (TER) were adapted in brief from our previous study [2].

Following collection, samples were analyzed according to World Health Organization (WHO) guidelines for standard semen parameters. Baseline DNA fragmentation index (DFI) was assessed prior to cryopreservation in all samples.

For cryopreservation, semen samples were mixed with cryoprotectant (1:1 ratio) and stored in cryovials at  $-196^{\circ}\text{C}$  in liquid nitrogen. Post-storage, samples were thawed by bringing cryovials to room temperature to allow liquefaction, followed by removal of cryoprotectants through centrifugation using a 1ml buffered medium.

The samples were randomly divided into two equal groups ( $n = 40$  each) using a computer-generated method. The first group underwent direct DFI assessment after thawing (non-TER group). The second group was subjected to temporary energy restriction (TER), involving a defined 30-minute period of nutrient deprivation, followed by controlled nutrient introduction prior to DFI assessment.

Thus, three groups were analyzed: pre-cryopreservation ( $n = 80$ ), post-thaw without TER ( $n = 40$ ), and post-thaw with TER ( $n = 40$ ).

DFI was evaluated using the Sperm Chromatin Dispersion (SCD). The kit used in this study, obtained from Canfrag (Shivani Scientific), contained agarose gel tubes, agarose-coated slides, diluent tubes, staining solution, buffer solution, dehydrating agents (70%, 90%, and 100%), lysis solution, and denaturation solution.

The DFI assessment procedure began with the evaluation of sperm concentration and motility. Semen samples with sperm concentrations exceeding 100 million/mL were diluted prior to analysis, and dilution was also performed for samples with concentrations around 50 million/mL to ensure optimal cell

distribution. In contrast, samples with sperm concentrations below 20–25 million/mL were analyzed without dilution.

A total of 40 µL of processed semen sample was added to the agarose gel tube, mixed thoroughly with the melted agarose gel, and then transferred onto an agarose-coated slide. The slide was then placed in a refrigerator for 5 minutes to allow solidification. After solidification, the coverslip was carefully removed without disturbing the agarose gel layer. Denaturation solution (1 mL) was applied for 7 minutes, followed by lysis solution (1 mL) for 20 minutes. The excess solution was then drained. Subsequently, approximately 20 mL of distilled water was added. Dehydrating agents (70%, 90%, and 100%) were then applied sequentially, approximately 1 mL each for 2 minutes. For staining, 0.3 mL of stain solution was mixed with 0.1 mL of buffer in the diluent tubes. Approximately 0.25 mL of this mixture was applied to the agarose slide for staining for about 5 minutes, after which the slide was rinsed with water and examined under a microscope at 40× magnification to evaluate DFI.

The principle of the SCD test is that the denaturation solution induces separation of DNA strands in sperm cells, while the lysis solution removes nuclear proteins. This process allows visualization of sperm with intact DNA. The integrity of sperm DNA is determined by evaluating the halo patterns around sperm nuclei, which are categorized as large halo, medium halo, small halo, no halo, fragmented, or degraded sperm.

DFI was calculated using the following formula:

$$DFI (\%) = \left( \frac{\text{Number of fragmented sperm}}{\text{Total sperm counted}} \right) \times 100$$

This calculation provides the percentage of DNA fragmentation in the semen sample, allowing assessment of sperm DNA quality. A DFI value above 25% is generally considered indicative of high DNA fragmentation and may be associated with reduced fertility potential.

Data were expressed as mean ± standard deviation (SD). Statistical comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test for multiple pairwise comparisons. A p-value < 0.05 was considered statistically significant. All statistical analyses were conducted using Python (version 3.13).

The study aimed to assess the DFI in cryopreserved semen samples subjected to TER followed by nutrient introduction. DFI values were also measured prior to cryopreservation to establish baseline DNA integrity and to evaluate the effect of the freeze-thaw process on sperm DNA quality.

Group	Sample Size	Mean DFI (%)	SD
Before Cryopreservation	n=80	24.83	5.36
After Cryopreservation (Without TER)	n=40	32.88	6.13
After Cryopreservation (With TER)	n=40	33.53	6.86

Table 1: DNA Fragmentation Index Across Study Groups.

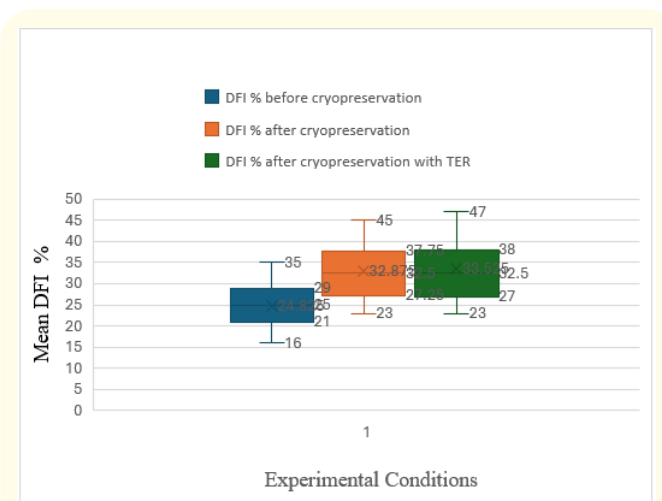


Figure 1: Comparison of DNA fragmentation index (DFI) across study groups. Cryopreservation significantly increased DFI compared to baseline (p < 0.001). No significant difference was observed between samples with and without temporary energy restriction (TER) (p = 0.656). Data are presented as mean ± SD.

### Results

A total of 80 semen samples were analyzed to evaluate DNA fragmentation index (DFI) across three experimental conditions: prior to cryopreservation (n = 80), post-cryopreservation without temporary energy restriction (TER) (n = 40), and post-cryopreservation with TER (n = 40). One-way ANOVA demonstrated a statistically significant difference in DFI among the study groups.

Baseline assessment demonstrated relatively low DNA fragmentation, with a mean DFI of  $24.83 \pm 5.36\%$ . Following cryopreservation, a marked increase in DNA fragmentation was observed in samples that did not undergo TER, with mean DFI rising to  $32.88 \pm 6.13\%$ . This increase was statistically significant when compared to pre-cryopreservation values ( $p < 0.001$ ), confirming the detrimental impact of the freeze-thaw process on sperm DNA integrity.

Samples subjected to TER after thawing exhibited a mean DFI of  $33.53 \pm 6.86\%$ , which was comparable to that observed in the non-TER group. Statistical analysis revealed no significant difference between the TER-treated and non-TER samples ( $p = 0.656$ ), indicating that the application of temporary energy restriction did not result in a measurable reduction in DNA fragmentation under the conditions studied. The comparative DFI values across the study groups are summarized in Table 1, while the graphical representation of DFI distribution is presented in Figure 1. Overall, while cryopreservation significantly increased DNA fragmentation relative to baseline, the implementation of TER post-thaw did not demonstrate a significant protective or restorative effect on sperm DNA integrity.

## Discussion

This study evaluated the effect of temporary energy restriction (TER) on DNA integrity in cryopreserved semen samples from individuals diagnosed with asthenozoospermia. The findings confirmed that cryopreservation significantly increases DNA fragmentation index (DFI), consistent with previous reports demonstrating the detrimental effects of freeze-thaw processes on sperm chromatin stability and integrity [1,3,7]. However, the application of TER following thawing did not result in a statistically significant reduction in DFI when compared to samples processed without TER.

The lack of a measurable difference between TER-treated and non-TER groups suggests that, under the present experimental conditions, metabolic modulation through transient energy restriction does not substantially influence DNA fragmentation outcomes. While prior studies have reported that temporary starvation or controlled nutrient exposure can improve certain sperm functional parameters, particularly motility and capacitation-related responses [10,11], these effects may not directly translate

to improvements in DNA integrity. This distinction highlights the possibility that TER primarily affects functional metabolic activity rather than structural DNA stability.

Cryopreservation-induced DNA damage is widely attributed to oxidative stress, membrane disruption, and intracellular ice formation, all of which can compromise chromatin organization [4,6,12]. Although TER has been proposed as a strategy to reduce metabolic stress and limit reactive oxygen species (ROS) generation [10,11], the current findings suggest that such modulation may be insufficient to counteract the extent of DNA damage induced during the freeze-thaw process. It is therefore likely that DNA fragmentation is influenced more strongly by physical and biochemical cryoinjury mechanisms than by post-thaw metabolic conditions.

Importantly, the absence of a significant difference between TER and non-TER groups does not negate the potential utility of TER in assisted reproductive technologies (ART). Previous work, including our earlier study on motility enhancement [2], has demonstrated that TER can improve post-thaw sperm motility. This suggests that TER may still hold value as a functional optimization strategy, even if its impact on DNA integrity is limited. Given that sperm function and DNA integrity represent distinct but complementary aspects of fertility, these findings emphasize the need to evaluate multiple parameters when assessing post-thaw sperm quality.

The present study is limited to the assessment of DFI and does not include evaluation of downstream reproductive outcomes. Future studies should investigate whether TER influences fertilization rates, embryo development, or clinical pregnancy outcomes. Additionally, mechanistic investigations focusing on oxidative stress markers, mitochondrial function, and apoptotic pathways may provide further insight into why TER does not significantly alter DNA fragmentation despite its effects on sperm physiology.

## Conclusion

The findings of this study demonstrate that cryopreservation significantly increases DNA fragmentation index (DFI) in semen samples, indicating a detrimental effect of the freeze-thaw process on sperm DNA integrity. However, the application of temporary energy restriction (TER) following thawing did not result in a

statistically significant difference in DFI when compared to samples processed without TER. Although minor variations in DFI were observed between the groups, these differences were negligible and did not indicate a measurable improvement in DNA integrity.

Overall, the results suggest that while cryopreservation adversely affects sperm DNA quality, the implementation of TER under the present experimental conditions does not provide a significant protective or restorative effect. Further studies with optimized protocols may be required to better understand the potential role of metabolic interventions in preserving sperm DNA integrity.

### Bibliography

1. De Paula TS., *et al.* "Effect of cryopreservation on sperm apoptotic deoxyribonucleic acid fragmentation in patients with oligozoospermia". *Fertility and Sterility* 86 (2006): 597-600.
2. Krithika ST and Karigar CS. "Cryopreserved human sperm motility enhancement via temporary energy restriction". *Acta Scientific Womens Health* 8.5 (2026): 1-6.
3. Hammadeh ME., *et al.* "Effect of freeze-thawing procedure on chromatin stability, morphological alteration and membrane integrity of human spermatozoa in fertile and subfertile men". *International Journal of Andrology* 22 (1999): 155-162.
4. Said TM., *et al.* "Implication of apoptosis in sperm cryoinjury". *Reproductive BioMedicine Online* 21 (2010): 456-462.
5. Abou-El-Naga AM., *et al.* "Impact of cryopreservation agents on sperm quality, DNA fragmentation, and apoptotic markers in fertile and infertile males". *Scientific Report* (2025).
6. Muldrew K and McGann LE. "Mechanisms of intracellular ice formation". *Biophysics Journal* 57 (1990): 525-532.
7. Zribi N., *et al.* "Effects of cryopreservation on human sperm deoxyribonucleic acid integrity". *Fertility and Sterility* 93 (2010): 159-166.
8. Peris SI., *et al.* "Cryopreservation of Ram Semen Facilitates Sperm DNA Damage: Relationship between Sperm Andrological Parameters and the Sperm Chromatin Structure Assay". *Journal of Andrology* 25 (2025): 224-233.
9. Pravardhan V., *et al.* "Innovative strategies in sperm cryopreservation: Overcoming challenges and enhancing fertility preservation-A narrative review". *Asian Pacific Journal of Reproduction* 14 (2025): 146-152.
10. Marín-Briggiler CI., *et al.* "Human Sperm Remain Motile After a Temporary Energy Restriction but do Not Undergo Capacitation-Related Events". *Frontiers in Cell and Developmental Biology* 9 (2021).
11. Navarrete FA., *et al.* "Transient Sperm Starvation Improves the Outcome of Assisted Reproductive Technologies". *Frontiers in Cell and Developmental Biology* 7 (2019).
12. Wang Y., *et al.* "Mechanisms of oxidative stress-induced sperm dysfunction". *Frontiers in Endocrinology* 16 (2025).