



## Evaluation of Sperm DNA Fragmentation Using Halosperm Technique after the Freezing–Thawing Process in Men: A Study on the Validation of the SCD Protocol

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### Abstract

**Background:** DNA fragmentation index (DFI) enhances routine semen analysis by providing valuable insights into male reproductive potential. Utilizing Halosperm test, a sperm chromatin dispersion (SCD) assay based on induced condensation. The purpose of this study was to assess sperm DNA damage both before and after freezing. By following the specified kit instructions, an attempt was made to validate the SCD test protocol, with a particular emphasis on the implications of sperm freezing on its DNA integrity.

**Methods:** In total, 380 fresh human semen samples from normozoospermic patients were frozen at  $-20^{\circ}\text{C}$  for 10 days, using SCD cryopreservation reagent. Routine semen analysis and DNA fragmentation index (DFI) were determined for each sample before freezing and after thawing. Semen morphology and sperm DFI were compared before and after freezing/thawing process.

**Results:** There was a significant decrease in sperm normal morphology after thawing ( $9.31\pm 2.42\%$  vs.  $7.1\pm 1.53\%$ ,  $p<0.05$ , respectively). The sperm head, midpiece, and tail defect rate increased after freezing at  $-20^{\circ}\text{C}$ . Moreover, DFI was significantly higher after thawing compared to before freezing ( $20.71\pm 1.61\%$  before freezing vs.  $29.1\pm 0.21\%$  after thawing with  $p<0.001$ ).

**Conclusion:** Cryoconservation of semen samples at  $-20^{\circ}\text{C}$  for 10 days using SCD cryopreservation reagent seems to damage sperm morphology, resulting in a reduction in sperm DNA integrity. The measurement of DFI on a fresh sample remains the most reliable technique for obtaining accurate results.

**Keywords:** Cryopreservation, DNA fragmentation, Freezing, Halosperm test, Spermatozoa, Thawing.

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### Introduction

Male infertility, affecting 15% of reproductive-age couples globally, is predominantly caused by male factors, particularly sperm abnormalities (1). Factors influencing sperm quality in-

clude testicular failure, ejaculatory dysfunction, and a combination of genetic, environmental, and lifestyle influences (2). Sperm cryopreservation, a key component in assisted reproductive technolo-

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gy (ART), has a significant potential for treatment of infertility (3). It serves as a therapeutic option for cancer patients prior to undergoing chemotherapy or radiotherapy (4). Cryopreservation methods vary but typically involve cooling, freezing, and thawing. Despite their effectiveness, these methods often lead to reduced sperm motility, poor morphology, and decreased DNA integrity and viability post-thawing (5). Studies have shown that sperm DNA fragmentation is inversely correlated with ART success in infertile couples (6). Appropriate use of cryoprotectants and protocols for freezing-thawing procedures are useful for preventing DNA fragmentation in ART. However, many studies have shown that these processes may damage sperm function (7). Some research studies demonstrated that cryopreservation has been found to cause changes in membrane lipid composition and acrosome status, decreases in sperm motility and viability, and an increase in sperm DNA fragmentation (8). However, other studies have found that cryopreservation did not alter seminal fluid parameters or sperm DNA integrity (9). Thus, the issue of whether cryopreservation affects or causes fragmentation of sperm DNA remains an important research topic. Sperm DNA damage markers such as DNA fragmentation index, DNA denaturation, and chromatin disorders can serve as indicators of semen quality (10). There are several tests for assessing the integrity of sperm DNA, based on measurements of abnormalities in the sperm chromatin structure assay (SCSA) or through direct assessments of DNA strand breaks, including the sperm chromatin structure assay, acridine orange test, toluidine blue test, aniline blue test, terminal deoxynucleotidyl transferase dUTP (TUNEL), the Comet assay, sperm chromatin dispersion (SCD), or sperm DNA compaction (11). Halosperm test is an SCD test based on induced decompensation, which is directly related to sperm DNA fragmentation (12). This test is now performed using the sperm chromatin dispersion (SCD) assay and its application is easy and cost-effective in laboratories in developing countries such as Morocco. According to the instructions of this kit, the semen samples can be stored at  $-20^{\circ}\text{C}$  for a maximum period of 15 days, and the testing results will be the same with the fresh samples. This study was conducted to assess whether the storage conditions outlined in the kit have any detrimental effects on the quality of sperm, specifically in terms of sperm DNA integrity. Conventional freezing is the most popular

method for cryopreservation in Morocco due to its convenience, ease of implementation, and cost-effectiveness. In general, the freezing procedure for the SCD test begins by placing the sample in a freezer at a temperature of  $-20^{\circ}\text{C}$ , in accordance with the recommended storage conditions specified by the kit. The existing literature indicates that there are still disagreements about the negative effects of freezing on DNA integrity (13). Therefore, the objective of the current study was to evaluate the impact of freezing on sperm DNA fragmentation using the SCD test. This was accomplished by examining 380 fresh human semen samples obtained from normozoospermic patients to validate the SCD test protocol.

### Methods

**Study population:** A total of 380 normozoospermic patients (ages 28 to 60 years, mean age of  $42.25 \pm 1.66$  years), who visited Reproductive Center at Mohammed VI University Hospital Center of Oujda, Morocco, between November 2022 to May 2023 were included in the present study. Individuals with familial diseases, genital trauma, organic lesions, urological and reproductive disorders (such as varicocele, cryptorchidism, and azoospermia) as well as those with occupational exposure to zinc, high-density radiation, chemicals, and high temperature were excluded from the study.

The information on sociodemographic characteristics collected consisted of patient's age, type of infertility (primary or secondary), duration of infertility, body mass index, occupations, history of smoking, and alcohol consumption.

**Semen collection and assessment:** Samples were analyzed and classified according to the 2021 World Health Organization (WHO) guidelines for examination and processing of human semen (1). Sperm volume, concentration, motility, and morphology in the semen were examined. A clean sterile plastic container confirmed to be non-toxic for spermatozoa was given to each participant to produce semen samples by masturbation (after 2-3 days of sexual abstinence). Each semen sample was directed into a sterile plastic cup and liquefied in an incubator at  $37^{\circ}\text{C}$ . To minimize temperature fluctuations and ensure timely analysis, all samples were collected in our unit. Macroscopic analysis of the sperm included the assessment of liquefaction time, viscosity, semen volume, color, and pH.

First, a Pasteur pipette was used to homogenize the semen samples. Subsequently, the evaluation of spermatozoa parameters, including concentration and motility, was conducted using a Computer Assisted Sperm Analyzer (CASA; MICROTIC, Spain). For each measurement, a 2.5  $\mu$ l aliquot of sperm was loaded into a standard four-chamber slide (Leja Products B.V., the Netherlands). The spermatozoa exhibiting fast and slow progressive motility were counted as category A+B, followed by the non-progressive motile spermatozoa as category C, and finally the non-motile spermatozoa as category D. Sperm concentration count and sperm motility were determined using  $\times 10$  magnifications.

The evaluation of the spermatozoa morphology was made based on David's modified classification using Diff-Quik kit (Dade Behring Diagnostics AG, Switzerland) which includes one stain fixative and two types of stain (A and B) (14). Morphological assessment was performed using Nikon microscope with oil immersion (Nikon Corporation, Japan). A minimum of 100 sperm was counted. Men were classified as having normal or abnormal morphology based on David's modified classification, utilizing strict scoring criteria (1).

**Normal parameters:** According to 2021 World Health Organization (WHO) guidelines for examination and processing of human semen, the following parameters were considered: semen volume  $\geq 1.4$  ml; pH  $\geq 7.2$ ; liquefaction time  $< 60$  min; sperm count  $\geq 16 \times 10^6$ /ml; progressive motility  $\geq 30\%$ ; total motility  $\geq 40\%$ ; vitality  $\geq 56\%$ ; proportion of sperm with normal morphology  $\geq 15\%$ , based on David's modified classification; and DNA fragmentation index  $\geq 25\%$ .

**DNA fragmentation assessment:** DNA fragmentation was assessed by sperm chromatin dispersion (SCD) assay. After denaturation and the removal of nuclear proteins, dispersed DNA loops exhibited a distinctive halo, provided there was no significant sperm DNA breakage. Sperm containing fragmented DNA either does not develop such a halo, or the halo it produces is small in size. DNA fragmentation test was performed using the sperm chromatin dispersion assay by SpermFunc® (BRED Life Science Technology Inc., China). The agarose was placed at a temperature of 90-100°C for 20 min and heated at 37°C for 5 min. Then, the semen was added to the agarose and mixed well. The suspension was poured onto aga-

rose-coated slides and subsequently covered with a 20x20 mm cover glass. The slides were cooled at 4°C for 5 min, then were slowly opened. They were then incubated in a denaturation solution for 7 min, followed by treatment with a lysis solution at room temperature for 25 min. Following a 5-min wash with H<sub>2</sub>O, the slides were subjected to dehydration by sequential immersion in graded ethanol solutions (70%, 90%, and 100%) for 2 min each. Subsequently, the slides were allowed to air-dry and were then stored at room temperature within opaque closed boxes. The slides were dried and stained with Wright's solution for 25 min. This was followed by the examination of the slides under a light microscope, where various halo images were observed, including those classified as large, medium, small, no halo, and degraded sperm. The large and medium halos were classified as unfragmented DNA, while others as fragmented DNA. Two observers independently made observations by randomly determining the presence of fragmented and unfragmented DNA in 500 sperm. These observations were used to calculate DNA fragmentation index (DFI), where a DFI of less than 25% was considered within the normal range. Normal sperm DNA presented radiate halos, whereas damaged sperm DNA displayed either no halos or small halos. Fragmented sperm were defined as those having a small or no halo. The thickness of the halo on one side was observed to be less than one-third of the diameter of the thinnest part of the sperm head. The DNA fragmentation index (DFI) was calculated using the following formula:

DFI (%) =  $100 \times (\text{Number of spermatozoa with fragmented DNA} / \text{Total number of spermatozoa})$ .

**Freezing of samples:** The freezing procedure was followed according to kit instructions (SpermFunc®; BRED Life Science Technology Inc., China). SCD preservative reagent was used as a cryoprotectant agent of sperm. SCD preservative reagent was added slowly by drops to the sample at a ratio of 3:1 and gently mixed after each drop was added. The process took 10 min at room temperature. Next, Eppendorf tubes were stored at -20°C for testing later; according to the kit instructions, they can be stored for at least 15 days.

**Thawing of samples:** After 10 days of freezing at 20°C, thawing was performed following the same procedure outlined in the kit guidelines. Eppendorf tubes were removed from the freezer and allowed to equilibrate at room temperature (37°C)

for 5 min. The post-thaw sperm parameters, including morphology and DFI were analyzed. Before testing, the stored samples should be equilibrated at 37°C and the sperm concentration should be calculated. The semen sample should be diluted or concentrated using a washing solution to achieve a final sperm concentration of 5-10 million sperm per milliliter.

The study was approved by the Research Ethics Review Committee of the Faculty of Medicine and Pharmacy of Mohammed VI University Hospital Center of Oujda. All subjects enrolled into the study provided written formal consent to participate. The subjects were informed about the scientific nature of the study.

**Statistical analysis:** Statistical analyses were conducted using SPSS version 20.0 (IBM Corp., USA). The data are presented as means±standard deviations or as absolute numbers and percentages, as appropriate. A paired-samples t-test was utilized to compare pre-freezing and post-thawing results. Differences between groups were assessed using independent-samples t-tests and analysis of variance (ANOVA), with post-hoc comparisons performed via the Tukey test. A p-value of less than 0.05 was initially set as the threshold for statistical significance. Univariate and multivariate logistic regressions were used to examine the percentage of DFI in patients, both before freezing and after thawing, within each group.

## Results

**Characteristics of the study population:** The characteristics of the study population are shown in table 1. The mean age was 42.25±1.66 years, with a range from 28 to 60 years. Primary infertility was present in 57.89% of the patients. The mean duration of their infertility was 7.9±1.3 years, with a range from 3 to 14 years. Among the men included in the study, 28.42% were cigarette smokers, and 23.59% reported alcohol consumption. The mean BMI of these men was 25.3±1.2 kg/m<sup>2</sup>.

**The effect of SCD kit freezing on sperm parameters:** The baseline semen analysis results are shown in table 2. Our study population (n=380) exhibited a wide range of concentrations, progressive motility, viability, normal morphology, and DNA fragmentation index, which were compared based on the guidelines of World Health Organization (WHO), 6th edition (2021).

**Comparing DNA fragmentation index in fresh semen and after thawing:** Pre-freezing and post-

**Table 1.** Basic characteristics of the study population (n=380)

| Characteristics                      | No (%)      |
|--------------------------------------|-------------|
| Age (yr)                             |             |
| ≥35                                  | 174 (45.79) |
| <35                                  | 206 (54.21) |
| Infertility type                     |             |
| Primary                              | 220 (57.89) |
| Secondary                            | 160 (42.10) |
| Infertility duration (yr)            |             |
| >5                                   | 300 (78.95) |
| ≤5                                   | 80 (21.05)  |
| Body mass index (kg/m <sup>2</sup> ) |             |
| <18.5                                | 15 (3.95)   |
| 18.5–22.9                            | 100 (26.31) |
| 23–24.9                              | 220 (57.89) |
| ≥25                                  | 45 (11.84)  |
| Occupation                           |             |
| Office                               | 98 (25.8)   |
| Manual                               | 210 (55.26) |
| Others                               | 72 (18.95)  |
| Smoking                              |             |
| Yes                                  | 108 (28.42) |
| No                                   | 272 (71.58) |
| Alcohol consumption                  |             |
| Yes                                  | 91 (23.95)  |
| No                                   | 289 (76.05) |

**Table 2.** Fresh semen analysis (n=380)

| Sperm parameters           | Mean±SD    | WHO criteria (2021) |
|----------------------------|------------|---------------------|
| pH                         | 7.12±0.61  | ≥7.2                |
| Volume (ml)                | 2.62±1.61  | ≥1.4                |
| Concentration (million/ml) | 22±2.5     | ≥16                 |
| Progressive motility (%)   | 36.1±3.8   | ≥30                 |
| Total motility (%)         | 48.02±0.71 | ≥42                 |
| Viability (%)              | 60.01±1.81 | ≥54                 |
| Normal morphology (%)      | 8.51±2.8   | ≥15                 |
| DFI (%)                    | 31±2.31    | ≥25                 |

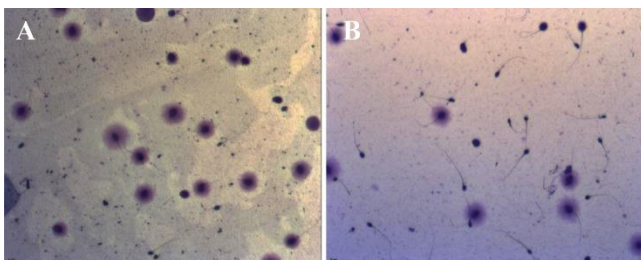
SD: Standard Deviation, WHO: World Health Organization, DFI: DNA fragmentation index

thawing sperm parameters, including sperm morphology and DNA fragmentation index values are displayed in table 3. As demonstrated, there was a significant decrease in the percentage of sperm showing normal morphology and an increase in spermatozoa defects after thawing. In fact, DNA fragmentation index after thawing showed a statistically significant increase compared to DNA fragmentation index before freezing.

**Table 3.** Comparison of sperm parameters prior to freezing and following thawing in the same patient (n=380)

| Sperm parameters            | Before freezing | After thawing | p-value |
|-----------------------------|-----------------|---------------|---------|
| DFI (%)                     | 20.71±1.61      | 29.1±0.21     | <0.001  |
| Normal morphology (%)       | 9.31±2.42       | 7.1±1.53      | <0.001  |
| Head defect (%)             | 81.41±0.81      | 85.63±1.67    | <0.001  |
| Midpiece or tail defect (%) | 63.01±4.9       | 66.61±3.43    | <0.001  |

Values are presented as mean±standard deviation. DFI: DNA fragmentation index. The paired-samples t-test was used to compare the results before freezing and after thawing. Comparisons between groups were evaluated by the independent-samples t-test and analysis of variance, followed by the Tukey test for post-hoc analysis



**Figure 1.** The effects of freezing at 20°C, as assessed by Halosperm technique, on DFI before cryopreservation (A) and after thawing (B)

Similarly, the normal morphology rate alters after thawing. In addition, the head defect rate and the midpiece or tail defect rate increased after freezing.

Spermatozoa without DNA fragmentation can be identified by the presence of either large halos or medium-sized halos. However, spermatozoa with DNA fragmentation are sperm cells with a very small-sized halo or without halos. Figure 1 shows the effects of freezing at 20°C, as assessed by Halosperm technique, on DFI before cryopreservation (Figure 1A) and after thawing (Figure 1B).

In the multivariate logistic regression model, sperm progressive motility and morphology were identified as independent factors that significantly influenced the DNA fragmentation index for the

validation of the SCD protocol in patients prior to freezing (Table 4). After thawing, the independent factors were spermatozoa morphology (head defect, midpiece, or tail defect).

### Discussion

Sperm DNA fragmentation index (DFI) is proven to be an important adjunct to routine semen analysis. DFI provides additional insights into men's reproductive potential compared to other parameters (15). Furthermore, sperm DNA integrity is essential for precise genetic transmission to a developing embryo and has been proposed as a biomarker for fertilization (16). This prospective study provides the first preliminary evidence on the comparative measurement reliability of the DNA fragmentation index before cryopreservation and after thawing at -20°C for 10 days using the SCD cryopreservation reagent. Therefore, the objective of this study was to investigate whether the conditions of sperm freezing, as specified in the package of the SCD kit (-20°C for a maximum period of 15 days), have any detrimental effect on the genetic material of sperm. This was accomplished by comparing DNA fragmentation rate of fresh sperm before cryopreservation with that after thawing, in order to determine if these storage conditions have any negative impact on sperm quality. A statistically significant variation in

**Table 4.** Multivariate logistic regression analysis for evaluation of sperm DNA fragmentation via Halosperm technique after the freezing– thawing process in men

| Sperm parameters (before freezing) | OR       | Sperm parameters (after thawing) | OR       |
|------------------------------------|----------|----------------------------------|----------|
| Male age                           | 7.1 *    | Normal morphology                | 1.64 *** |
| Total motility                     | 0.32 **  | Head defect                      | 2.43 **  |
| Viability                          | 0.84 *** | Midpiece or tail defect          | 3.73 *** |
|                                    |          | Smoking                          | 2.93 **  |

OR: Odds Ratio, DFI: DNA fragmentation index  
\* p<0.05; \*\* p<0.01; \*\*\* p<0.001

DNA fragmentation index (DFI) was observed between fresh samples and those subjected to freezing/thawing. The impact of cryopreservation on critical sperm characteristics, such as DNA integrity, acrosomal integrity, and morphology is significant, given their essential role in oocyte fertilization. Freezing and thawing processes are known to adversely affect seminal fluid parameters, particularly leading to a reduction in progressive motility, viability, and normal morphology (7).

Moreover, the extent of damage incurred during these processes may be influenced by the cryopreservation methodology, including the specific techniques used, the volume of semen samples, and the application of cryoprotective agents (17). One of the factors contributing to the degradation of sperm quality, particularly the integrity of sperm DNA, is thermal shock. Thermal shock induces the formation of intracellular and extracellular ice crystals, cell dehydration, and osmotic shock (18). Furthermore, the movement of water from inside to outside the cell increases the solute concentration and osmotic pressure, resulting in toxic damage, dehydration, and cell volume changes (19). Our study findings were similar to those of other reports, showing a significant decrease in human sperm DNA fragmentation following repeated cycles of freezing and thawing (20). In many previous studies, motility decreased after thawing based on different cryopreservation methods (7). In our investigation, alterations in normal sperm morphology were observed post-thawing, with significant defects in the head and tail regions. Previous literature suggests that these tail defects may stem from plasma membrane destruction in the tail area, a consequence of cryopreservation (21). Our study employed a cryoprotective agent containing glycerol from the provided kit. Cryoprotectants play a crucial role in maintaining membrane integrity by lowering the freezing point and minimizing ice formation. Nevertheless, despite the presence of cryoprotectants, freezing can still partially damage cells, potentially impairing sperm parameters and DNA integrity (22). Significantly, a notable increase in the DNA fragmentation index (DFI) was detected after freezing at  $-20^{\circ}\text{C}$  and subsequent thawing, indicating compromised DNA integrity. This aligns with the findings of Spano et al. (23), who also reported worsened sperm quality, including DNA integrity, after thawing. Conversely, other studies, like those conducted by Lusignan et al. (17) and

Isachenko et al. (24), found no significant impact of cryopreservation on sperm chromatin or DNA fragmentation. The presence of partially decondensed chromatin in poor-quality sperm is a result of reduced protamination. Thus, chromatin condensation may result in functional cell maturity. Duru et al. (25) found that cryopreservation was associated with factors other than DNA fragmentation, namely disruption of mitochondrial membrane and activation of caspase. Several studies have also described the relationships between phosphatidylserine externalization, caspase activation, and DNA fragmentation (26). Halosperm test has recently become popular due to its simplicity, affordability, and cost-effectiveness, making it a suitable choice to assess DNA fragmentation, especially in developing countries like Morocco. Moreover, it eliminates the need for expensive and complex equipment, as it can be performed using equipment readily available in andrology laboratories. Halosperm test is an indirect technique that quantifies the amount and magnitude of halos formed around the head of sperm during the removal of nuclear proteins. This measurement is directly correlated with sperm DNA damage (27). The application of Halosperm test can be challenging when measuring the rate of DNA fragmentation in patients with severe oligozoospermia or cryptozoospermia. Furthermore, among the various methods available for measuring the rate of DNA fragmentation, Halosperm test was selected for our study. This choice was made based on the validated nature of the SCD test and its consistent validation by the technician, thus confirming the reliability of the results obtained in our fertility center. Numerous studies demonstrated significant correlations between various techniques for measuring DFI, such as TUNEL, SCD, Comet assay, acridine orange, and SCSA tests (28).

### Conclusion

In conclusion, the present study revealed statistically significant deleterious effects of freezing/thawing on sperm morphology and DNA fragmentation when adhering to storage conditions and cryopreservation protocol described in the instructions of the SCD kit. The objective of this study was to underscore the significance of assessing the DNA fragmentation index (DFI) in fresh semen samples, rather than frozen samples, to ensure accurate and reliable results for patients undergoing assisted reproductive technology pro-

cedures. This investigation highlights the need for rigorous validation of new protocols for measuring DNA fragmentation in frozen specimens. Further comprehensive studies are recommended to validate these methodologies, thereby contributing to the improvement of reproductive outcomes.

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### Conflict of Interest

Authors declare no conflict of interest.

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