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FULL PAPER

The effect of temperature and storage time off semen samples on the spermatozoa deoxyribonucleic acid (DNA) fragmentation

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DNA fragmentation analysis is essential for evaluating sperm DNA integrity, which affects fertilization, embryo development, and pregnancy outcomes. However, many Indonesian laboratories lack access to this testing due to limited availability of commercial kits. This study aimed to assess the effects of storage temperature and duration on sperm DNA fragmentation by comparing fresh semen samples with those stored under various conditions. This post-test-only experimental study was conducted at the Biomedical Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, from March to October 2023. Semen samples were collected from ten normozoospermic men aged 20-35 years. The samples were divided into four groups: fresh samples (P1), samples stored in a cool box (7-8 °C) for 3 hours (P2), and samples stored in a freezer at -16 °C for 3 days (P3) and 7 days (P4). DNA fragmentation was assessed using the Sperm Chromatin Dispersion (SCD) method. The Shapiro-Wilk test was used for normality. Depending on data distribution, paired t-tests or Wilcoxon signed-rank tests were applied. No significant differences were found in DNA fragmentation index between fresh and stored samples: P1 vs. P2 ($p = 0.257$), P1 vs. P3 ($p = 0.705$), and P1 vs. P4 ($p = 0.102$), indicating DNA stability. Sperm DNA fragmentation remains stable under the tested storage conditions. Semen samples can be stored at 7-8 °C for 3 hours or frozen at -16 °C for up to 7 days without compromising DNA integrity.

KEYWORDS

Sperm DNA fragmentation; temperature; semen storage; human and health; reproductive health.

Introduction

DNA fragmentation analysis is a crucial tool for assessing sperm DNA damage and serves as a predictor of fertilization success, implantation, pregnancy outcomes, and normal embryo development [1]. However, a survey of 37 respondents (73% andrologists and 27% clinical laboratory analysts) in Indonesia revealed that 54.1% were unable to perform DNA fragmentation testing due to the unavailability of testing kits (62.5%). Nevertheless, 87.5% of respondents expressed willingness to send semen samples to laboratories capable of performing the test, provided that adequate transportation facilities are available [2].

Fertility and andrology laboratories in Indonesia generally lack sufficient facilities for genetic and epigenetic analyses of spermatozoa, including DNA fragmentation analysis [2]. Spermatozoa are highly sensitive to temperature fluctuations, making optimal transportation and storage conditions crucial [3].

Studies indicate that air-drying methods and storing semen samples at -22 °C can maintain DNA stability for up to one month [4]. In contrast, the freeze-drying method followed by storage at 4 °C for seven days has been shown to increase DNA fragmentation [5].

Basic semen analysis often fails to distinguish between fertile and infertile men, with 15% of infertile men exhibiting normal semen parameters. This highlights the significane of nuclear and subcellular factors, including DNA integrity, in evaluating male infertility [1,4]. An increased DNA fragmentation index is associated with decreased pregnancy success rates, a decreased likelihood of live birth, and a higher risk of miscarriage [6]. Various methods, such as the Sperm Chromatin Structure Assay (SCSA), Comet assay, TUNEL assay, and SCD, have been developed for assessing DNA fragmentation. Among these, the SCD method offers simplicity, low cost, and high accuracy [7].

This study aims to analyze the impact of varying temperatures and storage durations on DNA fragmentation in semen samples by comparing fresh samples with those stored in cooled transport media and refrigerated for several days.

Experimental

Subjects and methods

This study employs an experimental post-test-only design with a single population. The objective is to compare the DNA fragmentation index of spermatozoa in fresh samples with those subjected to specific treatments.

The study population consists of semen samples from men of reproductive age (20-35 years) attending the Andrology Clinic at Dr. Soetomo General Academic Hospital, Surabaya, between August and October 2023. Samples were collected from men who met the inclusion criteria and provided informed consent for their participation.

The inclusion criteria required men aged 20-35 years with two to seven days of ejaculatory abstinence, semen analysis results indicating normozoospermia, and consent to participate. The exclusion criteria included semen volume <2 mL or incomplete collection of ejaculate samples. Sample collection was performed using a consecutive sampling method.

Materials and instruments

The study utilized the following materials: a DNA fragmentation kit (SpermFunc®, BRED Life Science Technology Inc.), phosphate buffer solution (pH 6.8; Merck), ethanol (70%, 90%, and 100%; Merck), safranin solution (Merck), aquabides (WIDA WITM UNICAP), diluent solution (NaCl + Ortho-toluidine + H_2O_2), and crystal violet solution (Merck).

The instruments used included semen collection containers, glass slides, cover slips, micropipettes, disposable tips, microtubes, a microscope (Olympus CX 31), a cell counter, an Improved Neubauer hemocytometer, a 37 °C incubator, a thermometer, test tubes, a water bath, a refrigerator (7-8 °C), a freezer (-16 °C), a cool box, ice packs, a slide barrel, a timer, and trays.

Location and ethical approval

The present study was conducted at the Biomedical Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, from March to October 2023. Ethical approval was obtained from the Health Research Ethics

Committee of the Faculty of Medicine, Universitas Airlangga (No. 252/EC/KPEKFKUA/2023).

Data collection procedure

Prospective participants were provided with detailed information about the study's objectives. Participants who consented to the study provided their signatures on an informed consent form. Sample collection was conducted via masturbation into sterile, labeled containers, and the time of ejaculation was recorded. Semen volume was determined by measuring the weight difference of the container before and after sample collection. Samples were allowed to liquefy completely at room temperature before undergoing basic semen analysis in accordance with the 2010 WHO guidelines.

The semen samples were assigned into four groups:

- *P1*: Samples analyzed immediately after complete liquefaction.
- *P2*: Samples stored in a cool box (7-8 °C) for three hours.
- *P3*: Samples mixed with a preservative solution and preserved in a freezer (-16 °C) for three days.
- *P4*: Samples mixed with a preservative solution and preserved in a freezer (-16 °C) for a week.

DNA Fragmentation Measurement

DNA fragmentation was measured using the Sperm Chromatin Dispersion (SCD) method by analyzing 500 spermatozoa. The DNA fragmentation percentage was calculated using Equation (1):

$$DFI = \frac{Fragmented\ Spermatozoa + Degraded\ Spermatozoa}{Total\ Observed\ Spermatozoa} \times 100\% \qquad (1)$$

Assessments were conducted by three independent observers in a random and non-sequential manner. The average DNA fragmentation results from the three observers were statistically analyzed.

Data analysis

Data analysis was performed using SPSS version 25.0 in two stages. Univariate analysis was conducted to evaluate sample characteristics, while bivariate analysis was used to compare DNA fragmentation indices between groups. Data normality was assessed using Shapiro-Wilk test. For data that follow a normal distribution ($p > 0.05$), a paired t-test was applied, whereas for data that do not follow a normal distribution ($p < 0.05$), Wilcoxon signed-rank test was applied.

Results

The study involved ten semen samples from men aged 20-35 years attending the Andrology Clinic at Dr. Soetomo General Academic Hospital, Surabaya, from August to September 2023. Samples were collected through masturbation in a designated collection room and left at ambient temperature to fully liquefy before semen analysis. Normozoospermic samples were divided into four groups: P1 (DNA fragmentation analysis immediately after liquefaction), P2 (analysis after preserving the samples in a cool box with ice packs at 7-8 °C for three hours), P3 (analysis after preserving the samples in a freezer at -16 °C for three days), and P4 (analysis after preserving the samples in a freezer at -16 °C for a week). Table 1 summarizes the characteristics of the study samples.

TABLE 1 Characteristics of the study samples

Characteristics	Mean ± SD (n = 10)	Median (Q1–Q3) (n = 10)
Age (years)	29.40 ± 3.06	
Ejaculatory Abstinence (days)	3.2 ± 1.14	
Liquefaction Time (minutes)	36.00 ± 9.07	
pH		7.35 (7.20-7.55)
Volume (mL)		2.60 (2.30-4.43)
Concentration (10^{-1}/mL)	98.01 ± 32.90	
Total Sperm Count (10^{-1}/ejaculate)		330.50 (165.75-397.00)
Progressive Motility (%)	52.80 ± 9.10	
Non-progressive Motility (%)	20.00 ± 7.07	
Immotility (%)	27.20 ± 11.60	
Total Motility (%)	72.80 ± 11.60	
Normal Morphology (%)		4.00 (4.00-5.00)

The mean spermatozoa DNA fragmentation indices across the groups were similar, as presented in Table 2.

TABLE 2 Spermatozoa DNA fragmentation results

Group	Mean ± SD (%)
P1	12.30 ± 7.53
P2	12.00 ± 6.91
P3	12.50 ± 7.53
P4	12.70 ± 7.00

To ensure data normality, the Shapiro-Wilk test was applied before analysis. For groups P2 and P4, where the data distribution was not normal, the Wilcoxon signed-rank test was employed. Meanwhile, the data for groups P1 and P3 followed a normal distribution. Therefore, a paired t-test was used for statistical analysis.

TABLE 3 Comparison of DNA fragmentation indices between groups

Group Comparison	N	Median (Q1–Q3)	Mean ± SD	Mean ± SD *Difference*	*P*-value
P1	10	9.00 (5.75–20.25)			0.257
P2	10	9.50 (5.75–19.25)			
P1	10		12.30 ± 7.53	-0.20 ± 1.62	0.705
P3	10		12.50 ± 7.53		
P1	10	9.00 (5.75–20.25)			0.102
P4	10	9.50 (6.75–19.5)			

The statistical analysis revealed no significant differences in spermatozoa DNA fragmentation between fresh samples (P1) and the other treatment groups. The Wilcoxon signed-rank test revealed no substantial difference between P1 and samples stored in a cool box with ice packs at 7-8 °C for three hours (P2), with a p-value of 0.257 (Table 3). Similarly, the paired t-test indicated no significant difference between P1 and samples preserved in a freezer at -16 °C for three days (P3), with a p-value of 0.705 (Table 3). Furthermore, Wilcoxon signed-rank test also revealed no substantial difference between P1 and samples preserved in a freezer at -16 °C for a week (P4) shown in Figure 1, with a p-value of 0.102 (Table 3).

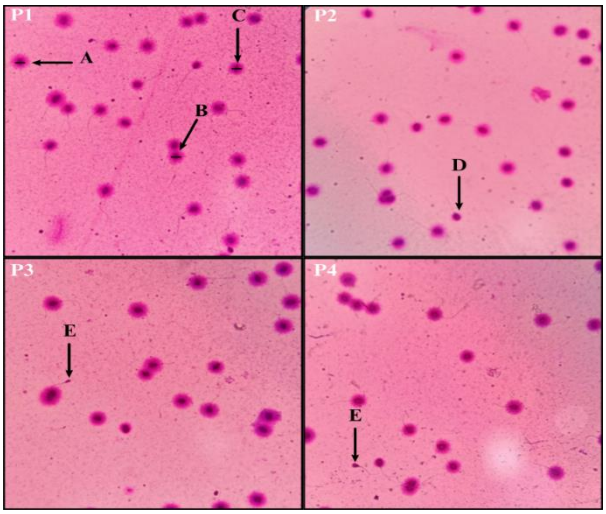

FIGURE 1 Visualization of spermatozoa DNA fragmentation results for fresh samples (P1), samples preserved in a cool box with ice packs for three hours (P2), samples preserved in a freezer at -16 °C for three days (P3), and samples preserved in a freezer at -16 °C for a week (P4). *(A), halo (B), large halo (C), small halo (D), and without halo (E)

Discussion

This study analyzed the impact of temperature and storage duration on spermatozoa DNA fragmentation. Samples were divided into four groups: the baseline group (fresh samples analyzed immediately after complete liquefaction), samples preserved in a cool box at 7-8 °C for three hours, samples preserved in a freezer at -16 °C for three days, and samples preserved in a freezer at -16 °C for a week. The methodology was selected based on previous studies demonstrating that temperature changes affect spermatozoa DNA degradation [4-5,8]. Given its superior sensitivity and suitability as a standard procedure, the SCD method was employed for detecting DNA fragmentation [9].

Participants in this study had a mean age of 29.4 years, consistent with findings indicating that semen quality begins to decline after the age of 35, with increased DNA fragmentation reported in men aged 26–30 years [10,11]. Semen characteristics, including total motility (72.8%), total sperm count (330.5 million/ejaculate), and sperm concentration (98.01 million/mL), were within the normal range according to the 2010 WHO guidelines. Studies show that higher levels of sperm DNA damage are correlated with both asthenozoospermia and morphological abnormalities in sperm, which can affect DNA methylation in specific genes [12].

This study revealed no substantial differences in the mean DNA fragmentation of spermatozoa across all groups, with minimal variation. This is likely due to the study participants being men aged 20-35 years with normozoospermia. Men over the age of 35 tend to experience a decline in semen quality, including reductions in ejaculate volume, sperm count, morphology, motility, vitality, and chromatin stability. This decline is attributed to sperm damage from oxidative stress, limited antioxidant defense mechanisms in spermatozoa, and increased reactive oxygen species (ROS) production. DNA fragmentation in older men is also associated with disruptions in DNA remodeling mechanisms, leading to reduced chromatin packaging and impaired DNA repair capabilities [13].

This study revealed no substantial difference in spermatozoa DNA fragmentation among samples stored in a cool box at 7-8 °C for three hours and fresh samples (p = 0.257). This aligns with findings indicating that semen storage at room temperature for four hours or on wet ice for 24 hours does not significantly affect DNA fragmentation results [14]. Wet ice is recommended as a simple and economic short-term storage method. Similarly, studies have reported that storing ejaculates at 4 °C for 48 hours does not result in significant differences compared to fresh samples [15]. Conversely, reports indicate higher DNA fragmentation in semen analyzed after one and two hours at 37 °C compared to the prompt analysis [3,16]. Other research has revealed that temperature reduction can damage spermatozoa membranes, while high temperatures (37 °C) can cause membrane protein denaturation, impair membrane integrity, and alter spermatozoa morphology [3].

This study revealed no substantial difference in spermatozoa DNA fragmentation in samples preserved in a freezer at -16 °C for three days (p = 0.705). This supports findings that semen storage using methods such as snap freezing, cryopreservation, or wet ice is more effective than ambient temperature storage for 24 hours, which results in higher DNA fragmentation [14]. Additionally, other recommendations suggest storing and transporting ejaculates at temperatures less than 4 °C or freezing them at -20 °C to -70 °C for one to two days as an adequate method for evaluating spermatozoa DNA fragmentation [15].

This study revealed no substantial difference in spermatozoa DNA fragmentation in samples preserved in a freezer at -16 °C for a week (p = 0.102). However, other studies have shown that storage at higher temperatures, such as 24-25 °C or 4 °C, can increase DNA fragmentation due to oxidation during storage [5,17]. Another study reported that spermatozoa viability and motility remained more stable at 4-6 °C compared to 25 °C during five to seven days of storage, with the optimal temperature for maintaining motility being 20 °C [18]. Research also indicated that storing mouse spermatozoa at 4-6 °C for up to 20 days did not increase DNA damage [19]. Similarly, dog semen stored at 5 °C showed no DNA damage for up to 14 days [20]. In forensic DNA analysis, studies have concluded that -20 °C is the optimal temperature for preserving DNA integrity, while storage at ambient temperature or 4 °C leads to significant degradation due to microbial activity. These findings highlight the significance of low temperatures in maintaining DNA integrity for both clinical and forensic applications [21].

Conclusion

This study concludes that the DNA fragmentation index of spermatozoa in samples stored in a cool box at 7-8 °C for three hours, a freezer at -16 °C for three days, and a freezer at -16 °C for seven days showed no significant differences compared to fresh samples. Based on these findings, andrology practitioners without in-clinic DNA fragmentation testing facilities are advised to transport semen samples using a cool box with ice packs if the laboratory can be reached within three hours. Additionally, clinical laboratories without access to -22 °C freezers can use -16 °C freezers to store semen samples for up to seven days prior to DNA fragmentation analysis. Further research is necessary to determine the optimal storage duration for maintaining spermatozoa DNA integrity at temperatures above -22 °C.

Ethical Issues

Ethical approval was obtained from the Health Research Ethics Committee of the Faculty of Medicine, Universitas Airlangga (No. 252/EC/KPEKFKUA/2023).

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Authors' Contributions

WSP and JN; researched, designed, and conducted the study, and also analysed and interpreted data; JN prepared and revised the manuscript; ND, PN, IGNP, and MI MT reviewed the manuscript and provided critical input.

Concflict of Interest

The authors declared that they do not have any conflict of interest regarding this study.

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