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ORIGINAL RESEARCH

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Effects of different storage conditions of semen samples on the detection results of sperm DNA damage

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Abstract

Standardizing the storage conditions of semen samples can improve the accuracy of detection results of sperm DNA fragmentation index (DFI) and reduce variability. This study aimed to investigate how different storage conditions affect the DFI results of sperm. To do this, thirty-five leftover semen samples were selected after routine testing. These samples had a sperm concentration of at least 10×10^6/mL, normal liquefaction, and no or few round cells. Each specimen was stored at room temperature (20 °C) for 2 and 4 hours, chilled (2–8 °C) for 1, 2 and 3 days, and frozen (−20 °C) for 3, 5 and 7 days, respectively. Each sample was frozen and thawed three times repeatedly. The sperm DFI at different time points was detected by sperm chromatin structure analysis (SCSA) based on flow cytometry. The results showed no significant differences in the sperm DFI of semen samples stored at room temperature for 0, 2 and 4 hours, chilled for 1, 2 and 3 days and frozen for 3, 5 and 7 days ($p > 0.05$). There were also no significant differences in the sperm DFI of semen samples frozen-thawed 1, 2 and 3 times repeatedly ($p > 0.05$). In conclusion, storage at room temperature for less than 4 hours, chilling for less than 3 days, freezing for less than 7 days and repeated freezing-thawing for 3 times have no significant impact on the sperm DNA damage of semen samples with sperm concentration $\geq 10 \times 10^6$/mL, normal liquefaction, and no or few round cells found in routine semen examination.

Keywords

Sperm DNA damage; Flow cytometry; Sample storage; Freeze-thaw times; Standardization

1. Introduction

The DNA fragmentation index (DFI) of sperm is a pivotal clinical index widely used to assess sperm DNA damage [1]. This index not only aids in evaluating the impact of male reproductive system disorders and their treatments on sperm DNA damage, but also serves as a predictive tool for the outcomes of natural pregnancy and *in vitro* fertilization. Furthermore, it plays a crucial role in monitoring the effects of various environmental pollutants, heavy metals, and carcinogens, as well as various intervention measures, such as semen refrigeration and treatment operations *in vitro*, on sperm DNA damage [2, 3]. Therefore, the accurate assessment of sperm DFI results is of utmost importance in our field.

The World Health Organization (WHO) recommends specific methods to detect sperm DFI. These methods include terminal deoxynucleotidyl transferase (dUTP) nick end labeling (TUNEL), single cell gel electrophoresis (Comet) assays, sperm chromatin structure analysis (SCSA), namely acridine orange flow cytometry (AO-FCM), and sperm chromatin dispersion test (SCD) [4]. Among these methods, AO-FCM has been widely used in clinical practice and accepted by most clinicians and technicians [5]. The detection principle of AO-FCM is as follows. The sperm with damaged DNA, such as single-strand DNA, will emit red fluorescence when combined with the fluorescent dye acridine orange (AO). In contrast, the sperm with intact DNA, such as double-strand DNA, will emit green fluorescence when combined with AO. The detection process of AO-FCM is derived from the literature published by Evanson and Jost in 2000 [6]. However, it has some unreasonable aspects, mainly manifested in the random setting of the gate, which lacks a theoretical basis, incorrect calculation of DFI, and a meaningless marker of high DNA stainability (HDS) [7, 8]. In order to correct the irrationality in the detection of AO-FCM, we have established a flow cytometry that can reflect the severity of human sperm DNA damage [9] and investigated preliminarily the standardization and quality control for the detection of sperm DNA damage [10]. In the detection of sperm DFI, sperm DFI exhibited an evident increase with the prolongation of the refrigeration of the semen samples at 2–8 °C, significantly at 2 days. Because the sample size of this study was small, only 10 cases, and possible confounding factors were not excluded [10], the impact of different storage conditions of semen samples on the results of sperm DFI still needs further exploration.

Laboratory technicians are often concerned with how long a laboratory should complete the detection of sperm DFI after semen collection and how to store the samples without affecting the detection results of sperm DFI. Unfortunately, there is very little research on this topic. To address this, we extensively investigated the impact of different storage conditions, such as room temperature, chilling, and freezing time, on the detection results of sperm DFI. We used a raw semen sample that covered almost all storage time points. Our research aims to clarify the impact of storage conditions on the AO-FCM detection results of sperm DFI in semen samples with sperm concentration $\geq 10 \times 10^6$/mL, normal liquefaction, and no or occasional round cells found in routine semen examination.

2. Materials and methods

2.1 Main reagents and instruments

Nanjing Xindi Biopharmaceutical Engineering Co., Ltd. (Nanjing, China) provided the sperm nuclear integrity staining kit (03122204), and Shenzhen Mindray Biomedical Electronics Co., Ltd. (Shenzhen, China) provided the BriCyte E6 flow cytometry.

2.2 Source of semen samples

Thirty-five patients visited our center for reproductive medicine during September 2022 and November 2022 were selected. These patients met the minimum sample size requirement of thirty, according to statistical guidelines [11]. After 2–7 days of abstinence, semen samples were collected in a sterile semen collection cup by masturbation. The remaining semen samples, after routine testing, were collected for use in this study. The patients were aged 25–50 years, with an average age of (32.17 $\pm$ 5.15) years. The semen samples with azoospermia, moderate and severe oligospermia (sperm concentration $<10 \times 10^6$/mL), poor liquefaction (liquefaction time exceeding 60 minutes), semen volume less than 0.5 mL, and the presence of obvious round cells (number of round cells $\geq$2/high power field) were excluded.

2.3 Routine analysis of semen samples

The semen samples were meticulously subjected to routine analysis using the standard methods outlined in the 6th edition of the WHO Laboratory Manual for the Examination and Processing of Human Semen [4]. After complete liquefaction for approximately 30 minutes, the routine semen analysis was performed immediately. The semen volume was measured using the weighing method, and the pH value of semen samples was measured using the precision pH test paper. Sperm concentration, motility, and the percentages of rapid and slow progressively motile sperm (PR) were detected by the CFT-9201 computer-assisted sperm analysis (CASA) system (Jiangsu Rich Life Science Instrument Co., Ltd., Xuzhou, Jiangsu, China). 5 μL of mixed semen sample was added into the Geoffrey sperm counting chamber with a depth of 10 μm, which was equipped in the CASA system directly. The chamber was then placed on the constant temperature stage and the CASA system automatically analyzed sperm concentration, motility and PR. The laboratory conducted intra-laboratory quality control of sperm concentration once a week, using quality control materials (latex beads solution, HARIOMED) provided by Hua Yue Medical Technology Co., Ltd. (Guangzhou, China). Our laboratory participated in the inter-laboratory quality assessment program twice a year organized by the Quality Control Center for Reproductive Testing of Jiangsu Provincial Maternal and Child Health Research Institute, and the results were qualified.

2.4 Grouping of semen samples

After conducting a routine semen analysis, each sample was thoroughly mixed and divided into 9 portions of 50 μL each. These portions were placed at room temperature (20 °C) for 0, 2 and 4 hours, refrigeration (2–8 °C) for 1, 2 and 3 days and freezing (−20 °C) for 3, 5 and 7 days, respectively. The semen sample placed at room temperature for 0 hours was equivalent to that detected promptly after routine semen examination. The semen samples frozen at −20 °C for 5 days were frozen-thawed 3 times repeatedly, and the sperm DFI was detected every other day to observe the effect of the number of freeze-thaw cycles on the results of sperm DFI. Once the frozen semen sample was taken out of the refrigerator, it was thawed at room temperature before immediately measuring the sperm DFI.

2.5 Detection of sperm DFI

The detection of sperm DFI at different time points strictly followed the instructions of the sperm nuclear integrity staining kit. First, 10 μL of mixed semen samples were added into 100 μL of reagent A, which consisted of 0.01 mol/L Tris, 0.15 mol/L sodium chloride, and 1 mmol/L ethylenediaminetetraacetic acid disodium (Na_2EDTA). Then, 200 μL of reagent B containing 0.08 mol/L hydrochloric acid, 0.15 mol/L sodium chloride, and 1% Triton X-100 were added, and the suspension was immediately mixed on a mixer for 30 seconds. Next, 600 μL of reagent C, which consisted of 0.01 mg/mL OA, 37 mmol/L citric acid, 0.126 mol/L disodium hydrogen phosphate, 1 mmol/L Na_2EDTA, and 0.15 mol/L sodium chloride, were added into the above suspension. After staining in the dark for 5 minutes, the suspension was used to detect sperm DFI by the flow cytometer with 488 nm excitation light. At least 5000 sperm were captured for each sample. The subpackaged and frozen semen samples were used as intra-laboratory quality control samples to monitor the results of each batch of sperm DFI. Our laboratory participated in the inter-laboratory quality assessment program twice a year organized by the Quality Control Center for Reproductive Testing of Jiangsu Provincial Maternal and Child Health Research Institute, and the results were qualified.

2.6 Statistical analysis

The data were analyzed using the statistical software SPSS 25.0 (SPSS Inc., Chicago, IL, USA). The data were first performed using one-sample nonparametric tests (Kolmogorov-Smirnov test) to determine whether they were in normal distribution. The data conforming to normal distribution were expressed as mean $\pm$ SD (standard deviation), and those conforming to non-normal distribution were expressed as median

[P_{25}, P_{75}]. If the data conformed to a normal distribution, a paired *t*-test was used; if the data conformed to a non-normal distribution, the Wilcoson Signed Rank test was used. $p \leq 0.05$ was considered to be statistically significant.

3. Results

3.1 Results of routine semen examination of 35 patients

Table 1 shows the results of abstinence time, semen volume, sperm concentration, motility and PR in 35 patients.

3.2 Effects of semen samples placed at room temperature, chilling and freezing for different times on the results of sperm DFI

The results of sperm DFI of semen samples placed at room temperature for 0, 2 and 4 hours, chilled for 1, 2 and 3 days, and frozen for 3, 5 and 7 days are shown in Table 2. The results showed that there were no significant changes in sperm DFI of semen samples placed at room temperature for 2 and 4 hours, chilled for 1, 2 and 3 days, and frozen for 3, 5 and 7 days compared with those timely detected after routine semen examination ($p > 0.05$) and that there was no significant difference in sperm DFI between the semen samples placed at room temperature for 2 and 4 hours, chilled for 1, 2 and 3 days, and frozen for 3, 5 and 7 days ($p > 0.05$).

3.3 Effects of the number of freeze-thaw cycles of semen samples on the results of sperm DFI

The results of sperm DFI of semen samples frozen and thawed for 3 times repeatedly showed that there were no significant changes in sperm DFI of semen samples frozen and thawed for 1, 2 and 3 times compared with those timely detected after routine semen examination ($p > 0.05$, Table 3). There was no significant difference in sperm DFI among the semen samples frozen and thawed for 1, 2 and 3 times ($p > 0.05$).

In addition, all data from 11 different time points were compared and analyzed, and there were also no significant differences in the results of sperm DFI among all of the groups ($p = 0.997$).

4. Discussion

After comprehensively reviewing the literature related to sperm DNA damage, we found that there were many factors leading to sperm DNA damage, mainly including age, abstinence time, abnormal spermatogenesis and maturation, environmental pollutants, heavy metals, male reproductive system diseases or systemic diseases, obesity, season and temperature, lifestyle such as smoking and drinking, semen storage, *in vitro* procedures, administration of certain drugs, seminal plasma lipids, reproductive hormone levels, *etc.* [2, 12–14]. If the sperm DNA is damaged, this can lead to poor sperm maturation, increased apoptosis rates of germ cells or sperm, embryonic development arrest, or natural miscarriage [15, 16]. Therefore, detecting sperm DNA damage can help to evaluate a couple's fertility.

Improper storage of samples may have an impact on the detection results of certain items. There was limited and controversial research on whether different storage conditions of semen samples had impacts on the detection results of sperm DFI. Sabbaghian *et al.* [5] detected sperm DFI in 10 human semen samples placed at room temperature for 0, 30, 60, 90, 120, 180, 210, 240, 270 and 330 minutes, respectively, and found that sperm DFI had a significant increase in semen samples placed at room temperature for more than 120 minutes. However, Sadeghi *et al.* [17] reported no significant changes in the results of sperm DFI when goat semen samples were stored at 17 °C and 5 °C for 48 hours. Therefore, the impacts of different storage conditions of semen samples on the detection results of sperm DFI deserve to be further investigated.

Different clinical laboratories varied in the detection time of sperm DFI, which generally depended on the sample size of a laboratory. Some laboratories conducted the centralized testing of sperm DFI on the same day, and semen samples needed to be stored at room temperature for a period of time. Some laboratories conducted the test twice or once a week, and semen samples needed to be refrigerated or frozen for storage. Standing at room temperature, refrigeration, and freezing were the most common methods of sample storage, and the impacts of correct storage of samples on test results could not be ignored. The periods of clinical semen sample storage were almost covered in our study. Semen samples required to be stored for more than 4 hours generally needed to be stored in refrigeration. Those required at refrigeration for more than

TABLE 1. Results of routine semen examination of 35 patients.

Variable	Value	Range
Abstinence time (d)	4.11 ± 1.55	2–7
Semen volume (mL)	3.51 ± 1.47	0.80–6.30
Sperm concentration ($\times 10^6$/mL)	52.6 [40.8, 77.4]	14.40–122.00
Sperm motility (%)	44.1 ± 19.7	1.0–74.2
Percentage of progressively motile sperm (%)	35.0 ± 16.5	0.5–67.5

The semen volume was measured using the weighing method, while the sperm concentration, motility, and PR were detected using a computer-assisted sperm analysis (CASA) system. The results of abstinence time, semen volume, sperm motility and PR conformed to a normal distribution, and were expressed as mean ± standard deviation (SD). However, the results of sperm concentration did not conform to a normal distribution and were expressed as median [P_{25}, P_{75}].

TABLE 2. Comparisons of sperm DFI of semen samples placed at room temperature, chilled and frozen for different times.

Storage conditions	N	DFI (%)	p^a
Room temperature			
0 h	35	17.5 [10.5, 28.0]	
2 h	35	18.5 [11.1, 29.0]	0.660
4 h	35	17.5 [10.7, 28.4]	0.801
Chilled			
0 d	35	17.5 [10.5, 28.0]	
1 d	35	17.8 [12.1, 33.5]	0.601
2 d	35	17.9 [11.5, 31.6]	0.549
3 d	35	17.9 [11.9, 34.9]	0.327
Frozen			
0 d	35	17.5 [10.5, 28.0]	
3 d	35	19.0 [12.7, 32.1]	0.350
5 d	35	19.7 [10.7, 30.0]	0.609
7 d	35	18.1 [11.3, 30.1]	0.573

DFI: DNA fragmentation index; [a]: Compared with that placed at room temperature for 0 hours, chilled for 0 days, and frozen for 0 days. The sperm DFI was detected by acridine orange flow cytometry (AO-FCM). All the results of sperm DFI conformed to non-normal distribution and were expressed as median [P_{25}, P_{75}]. The Wilcoxon Signed Rank test showed that there were no significant differences in the results of sperm DFI among different groups ($p > 0.05$).

TABLE 3. Comparisons of sperm DFI results of semen samples frozen and thawed for different times.

Number of freeze-thaw cycles	N	DFI (%)	p^a
0	35	17.5 [10.5, 28.0]	
1	35	19.7 [10.7, 30.0]	0.609
2	35	18.9 [12.4, 30.0]	0.333
3	35	18.7 [12.6, 31.0]	0.388

DFI: DNA fragmentation index; [a]: Compared with that frozen and thawed for 0 time. The sperm DFI of semen samples frozen and thawed 1, 2 and 3 times was detected using acridine orange flow cytometry (AO-FCM). All the results of sperm DFI conformed to non-normal distribution and were expressed as median [P_{25}, P_{75}]. The Wilcoxon Signed Rank test showed that there were no significant differences in the results of sperm DFI among different groups ($p > 0.05$).

3 days generally needed to be stored in freezing. Almost all the test items of semen samples could be completed within one week, so the storage time of semen samples in our study was observed till one week of freezing. Moreover, since some semen samples may require re-examination or re-checking, which involves repeated freeze-thaw cycles, we observed the effect of the number of freeze-thaw cycles on the results of sperm DFI.

Clinical practice and relevant literature showed that poor liquefaction and low sperm concentration in semen samples could affect the accurate detection of sperm DFI [18]. Moreover, semen samples with low sperm concentration were often required to centrifuge to concentrate sperm to ensure a sufficient number of sperm for the detection of sperm DFI based on flow cytometry. However, the mechanical effects of centrifugation and bottom compaction of sperm, as well as severe resuspension, might reduce sperm quality, increasing in sperm DFI [19]. The higher the centrifugal force and the longer the centrifugal time, the greater the damage to sperm DNA [20]. The increase of reactive oxygen species (ROS) in semen could cause an increase in sperm DFI [21]. The excessive production of ROS in semen mainly came from white blood cells and abnormal sperm [5]. Research [22] showed that UU (Ureaplasma urealyticum) infection [23], HPV (human papillomavirus) infection [24], bacteriospermia [25], and bacterial contamination during semen collection [19] could affect sperm DNA integrity by increasing ROS levels. Therefore, in order to avoid the influence of the above factors on our study's results, semen samples with poor liquefaction, low sperm concentration, and white blood cells were excluded. At the same time, we investigated the impacts of different storage conditions on the detection results of sperm DFI based on quality control, aiming to obtain relatively reliable results.

Reports indicated that the sperm DFI of semen samples placed at 37 °C was significantly higher than those kept at room temperature, and that the DNA stability of boar sperm at 16 °C was significantly lower than at 5 °C [19]. In stallions, the temperature of about 5 °C had been determined as the best

storage temperature to maintain sperm vitality and fertility, which might be related to the fact that low temperature can reduce the metabolic activity of sperm by reducing enzyme reactions [26] and lower microbial growth and activity [19]. Lowering the storage temperature below body temperature was a common strategy to reduce cell metabolism and increase storage time [17]. Therefore, we first observed the impacts of room temperature and refrigeration on sperm DFI. The results showed that sperm DFI of semen samples stored at room temperature for 4 hours and 2–8 °C for 3 days were not increased. These were similar to the research results of Jackson *et al*. [27] and Sadeghi *et al*. [17]. The former stored human semen at room temperature for 4 hours without affecting DNA breakage, while the latter stored goat semen at 17 °C and 5 °C for 48 hours, respectively, without significant changes in sperm DFI. It was reported that horse semen stored at 5 °C could maintain sperm motility, membrane integrity, and DNA integrity for up to 40 hours [19]. Although it was shown that the removal of seminal plasma was beneficial for maintaining sperm quality, there were also inconsistent conclusions [19, 28]. The semen samples used in this study were all original, and storage at room temperature for 4 hours and 2–8 °C for 3 days did not affect the integrity of sperm DNA. These might be related to the protective effects of antioxidant substances and buffer systems in seminal plasma on sperm, but further research was needed to confirm. In addition, the storage time observed in this study was limited. If the storage time at room temperature or refrigeration had to be extended, antibiotics might need to be added. It was shown that storing semen under aerobic conditions at 15 °C did not significantly reduce bacterial growth, while under anaerobic conditions at 5 °C, bacterial growth was minimal [19].

The general assumption was that the fertility of semen stored at room temperature or refrigerated at 5 °C was higher than that of frozen-thawed semen because frozen-thawed semen samples could cause DNA damage more quickly and strongly than refrigerated samples [28]. However, the primary defect of refrigerated semen was its short shelf life, which was unsuitable for long-term storage or long-distance transportation. Therefore, cryopreservation of semen was widely used in clinical practice. Although it was reported that drastic changes in temperature during the freezing-thawing process might cause sperm DNA damage [28], our results showed that cryopreservation of semen samples for one week did not significantly increase sperm DFI and that three cycles of freeze-thaw had no significant effect on the results of sperm DFI. These might be related to the fact that the semen samples we selected avoided the production of ROS by white blood cells, had not undergone any treatment, and had a relatively short storage time. Sperm DNA damage after cryopreservation was generally the indirect result of stressors related to temperature fluctuations, changes in osmotic pressure, and changes in plasma membrane instability [28]. The increase in sperm DFI during storage was mainly caused by oxidation factors [21]. Avoiding the generation of ROS might be the critical factor for long-term storage of semen samples. In our study, the key reasons why the frozen storage of semen samples did not change sperm DFI might be attributed to: First, the semen samples containing white blood cells were not selected; Second, repeated blowing or adding enzymes to treat semen samples with poor liquefaction were avoided; Third, operations of concentrating sperm such as centrifugation were avoided; Fourth, the change of temperature of freezing storage at −20 °C was relatively gentle compared with that of liquid nitrogen cryopreservation; Last, the retention of seminal plasma maintained the original osmotic pressure and redox equilibrium. All these measures have avoided the generation of ROS as much as possible. However, it should be noted that although the increase of sperm DFI was not significant in semen samples with shorter storage times, subsequent treatment of sperm at 37 °C may lead to a rapid increase in sperm DFI [19], indicating that such sperm may be unsuitable for further fertilization with eggs, as the relatively short refrigeration and freezing processes may have already induced apoptosis through oxidative stress.

It is worth noting that different species of sperm may have different responses to storage conditions [17, 28], so the storage conditions and duration of semen samples for detecting sperm DFI should be investigated separately for different species. The results of sperm DFI vary significantly among different semen samples, and the sensitivity of different semen samples to different storage temperatures also varies significantly [17, 28]. There are few reports on whether these are related to some particular samples. It has been found in clinical practice and relevant literature that some particular semen samples, such as those with leukocytospermia, severe oligospermia, and poor liquefaction, could potentially increase sperm DFI. Therefore, for these particular semen samples, it is necessary to investigate the potential impact of various treatment measures on the detection results of sperm DFI and develop the corresponding detection scheme of sperm DFI, which is a limitation of this study and also one of the future research topics. After packaging and freezing or refrigeration, we do not conduct further testing of the main seminal parameters in all storage steps, which is also one of the limitations of our study. In addition, the storage duration we observed was limited. For human semen samples, how long does it take for sperm DFI to significantly increase at room temperature, refrigeration, or freezing? What is the mechanism of the increase? These are also some of the future research topics. In addition, this is a single-center study with a limited sample size, and further confirmation from more sample sizes and centers is needed.

It is important to note that this study only tested the storage conditions for the AO-FCM method. Further confirmation is required to determine whether these conditions suit the TUNEL, Comet and SCD methods. The SCSA and SCD methods detect sperm DNA damage indirectly through acid denaturation [4], and the SCD method may apply to the storage conditions used in this study. However, the TUNEL and Comet methods detect sperm DNA damage directly [4]. Therefore, verifying whether the storage conditions used in this study are also appropriate for these methods is necessary.

5. Conclusions

Our study shows that storage at room temperature for less than 4 hours, chilling for less than 3 days, and freezing for

less than 7 days, and repeated freezing-thawing for 3 times have no significant impact on the AO-FCM detection results of sperm DFI in semen samples with sperm concentration $\geq 10 \times 10^6$/mL, normal liquefaction, and no or occasional round cells found in routine semen examination. However, for semen samples with low sperm concentration, increased white blood cells, and poor liquefaction, further clarification is needed on the potential impact of various treatment measures on the detection results of sperm DFI to develop the corresponding detection protocol of sperm DFI. The correct storage of semen samples not only ensures the accuracy of detection results of sperm DFI but also provides an essential basis for the development of standardization and quality control measures of sperm DFI testing.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

JCL—conceived the idea and directed the writing of our manuscript. YHX, SST and YMG—collected and analyzed the data and wrote the manuscript. YJL—directed the analysis of the data. All authors had made a substantial, direct, and intellectual contribution to the work and approved it for publication.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Independent Ethics Committee for Clinical Research of Zhongda Hospital, Affiliated to Southeast University (No. 2022ZDSYLL133-P01), and all patients signed the informed consent form.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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