Original Article

Assessment of the Impact Induced by Different Incubation Time, Storage Time, Storage Medium and Thawing Methods on Sperm DNA Fragmentation Assay: A Before–After Study

Marjan Sabbaghian[1], Hani Hosseinifar[1], Alemeh Rafaee[1], Mohammad Ali Sadighi Gilani[1,2]

[1]Department of Andrology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran, [2]Department of Urology, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Background: The sperm DNA fragmentation has been considered an important index in the field of male infertility. **Aims:** Our study aims to evaluate the impact of different factors, including incubation time, storage time, storage medium and method of thawing, on DNA fragmentation of semen samples. **Settings and Design:** This study was designed as a before–after study in five experiments. **Materials and Methods:** Experiment 1 was conducted to assess the effect of storage time in liquid nitrogen on 15 semen samples. In experiment 2, DNA fragmentation was performed on 10 semen samples with different incubation times before freezing. In experiments 3, 4, two different storage media and thawing methods were applied respectively in two separate groups, each containing 30 samples and the DNA fragmentation index (DFI) was measured using the sperm chromatin structure assay method. **Statistical Analysis:** Data were analysed using Stata version 11. **Results:** There was a significant increase in sperm DNA fragmentation of samples stored in liquid nitrogen for 1 month. This increase occurred in the first 2 weeks. Furthermore, our results showed a significant increase in the DFI after 120 min of incubation at room temperature (RT) and also thawing in RT separately. **Conclusion:** It is better to use fresh samples to measure DNA fragmentation up to 2 h after ejaculation to achieve more accurate results. Furthermore, if sperm freezing is inevitable, the use of a water bath (37°C) to thaw will be the most appropriate option, as it can lead to less DNA damage.

Keywords: *DNA damage, environmental factors, sperm, sperm chromatin structure assay*

Introduction

In recent decades, the DNA fragmentation index (DFI) has been considered an appealing topic by researchers in the field of male infertility because conventional methods for assessing semen quality are variable and slightly unreliable. Although the traditional semen parameters such as concentration, motility and morphology are a golden standard in diagnosing male infertility, it has become shown that none of these parameters recommended by the World Health Organization[1] are sufficient for the prediction of male fertility capacity and their discriminative power regarding fertility is slightly low.[2,3] These methods usually involve a subjective assessment of a few hundred spermatozoa and quality assurance is rarely implemented in the laboratories performing such analysis.[4] As a result, there has for long been a search for better markers of male fertility. During the last decade, the search for

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Address for correspondence: Dr. Marjan Sabbaghian, Department of Andrology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, No. 2, Hafez Street, Banihashem Street, Resalat Ave., Tehran, Iran.
E-mail: marjan.sabbaghian@gmail.com

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better predictors of male fertility resulted in an increased focus on sperm DNA integrity.[5] The correlation between sperm DNA damage and male infertility has been widely studied. There are several methods to evaluate different aspects of sperm DNA integrity. These include terminal deoxynucleotidyltransferase-mediated nick end labelling,[6] *in situ* nick translation,[1,7] single-cell gel electrophoresis assay (Comet assay),[8] and the sperm chromatin structure assay (SCSA).[9] Among these methods, SCSA is an accurate and fast test that has been shown to be a good predictor of fertility.[10-14] Statistical thresholds have been established for fertility prognosis when using SCSA procedures in humans.[15] The different results obtained from SCSA procedures have also been shown to be independent of conventional semen quality measures, and the assay, therefore, makes a contribution to the semen analysis profile.[16]

The precision of the SCSA test may be influenced by several factors, including the storage medium, incubation time, thawing methods, the human error involved in running the test and the performance or variation of the instruments used. Therefore, high attention to the standardisation of methods and protocols could improve the accuracy of results and reduce variation.

There are many reports about the effects of cryopreservation and cryoprotectants on DNA integrity, but to our knowledge, the impacts of specific factors, including the storage medium, incubation and freezing time and thawing methods on DNA fragmentation of fresh and immediately frozen sperm without using cryoprotectant have not been reported so far. The present study was designed to assess the possible effects of these factors on DFI.

Materials and Methods

Setting and study design

This research was designed as a before–after study in 2013. This study was conducted in accordance with principles of Helsinki Declaration and approved by the Ethics Committee of the Royan Institute (EC/93/1103). All participants were informed about the programme and informed consent were obtained from all individuals.

The study randomly included a single ejaculation from men after 3 days of abstinence without any knowledge about the fertility situation. Semen samples with sperm concentration <1 million per ml were excluded from the study. A sample size calculation was not performed.

Experimental design

Experiment 1

To compare the effects of freezing time on the sperm DFI, 15 semen samples were categorised into eight subgroups based on storage time (fresh, 1 min, 1, 2, 3 weeks and 1, 2, 3 months after storing in liquid nitrogen). All samples stored in liquid nitrogen were thawed in a water bath at 37°C for 30 s. It should be noted that except for the variable evaluated during each experiment, all other factors were constant for all the specimens. Besides, all experiments were performed twice.

Experiment 2

DFI levels were measured in 10 semen samples after 0, 30, 60, 90, 120, 180, 210, 240, 270 and 330 min of ejaculation and also an overnight incubation at room temperature (RT).

Experiment 3

To determine the possible effects of the storage medium on the sperm DFI, 30 semen samples were collected. 15 of 30 were stored in semen plasma, and the remaining 15 were stored in human tubal fluid (HTF). DFI was evaluated at two different times (fresh and 1 week after freezing).

Experiment 4

To compare the effects of thawing methods on SCSA results, 30 semen samples were categorised into two following groups: 15 semen samples were thawed in a water bath at 37°C for 30 s and 15 semen samples were thawed at RT.

Sperm chromatin structure assay

The procedure to measure sperm DNA damage by flow cytometry (FCM) SCSA was performed as described by Evenson *et al*.[10] with few modifications. Briefly, on the day of analysis, fresh and frozen samples were analysed immediately. An aliquot of unprocessed semen (~2–8 ml) was diluted to a concentration of 1–2 × 10^6 sperm/ml with phosphate buffer saline. This cell suspension was treated with an acid detergent solution (pH, 1.2) containing 0.1% Triton X-100, 0.15 M NaCl and 0.08 N HCl for the 40s, and then stained with 6 mg/l purified Acridine Orange (AO) in a phosphate-citrate buffer (pH, 6.0).

Cells were analysed using a FACSCalibur flow cytometer, equipped with an air-cooled argon ion (488 nm) laser (BD, San Jose). A total of 10000 events were accumulated for each measurement at a flow rate of 200–300 cells/s. AO, intercalated in double-stranded DNA, emits green fluorescence, and emission was detected using 530/30 nm bandpass filter (Fl-1), whereas AO associated with single-stranded DNA emits red fluorescence and emission was detected with 670 bandpass filter (Fl-3). Data were collected and analysed using the CellQuest programme. The percentage of abnormal sperm with detectable DFI (DFI%) was

calculated from the DFI frequency Dot plot obtained from the ratio between the red and total (red plus green) fluorescence intensity.[17] For the flow cytometer set-up and calibration, a reference sample was used from a normal donor ejaculate sample retrieved from the laboratory repository. It should be noted that SCSA was performed by one technician.

Statistical analysis

Data were analysed using Stata version 11 (Stata Corp., College Station, TX, USA). All statistics are presented as mean ± standard error. Randomisation was done by the Balance Block Randomisation method. Results were analysed by variance analysis and paired *t*-test. $P < 0.05$ were considered statistically significant.

Results

There was a significant increase in the mean of DFI between fresh samples and those being stored for 1 min in liquid nitrogen (22.93 ± 1.01 vs. 25.67 ± 1.08, $P < 0.05$). The mean of DFI continued to increase until 2 weeks after storage (31.24 ± 3.74) but did not show any significant increase after the 3rd week.

In experiment 2, the mean of DFI in fresh samples was measured at 19.65 ± 2.95; and after 2 h of incubation in RT, there was a significant increase in DFI (22.73 ± 2.73). Moreover, it increased sharply when samples were incubated overnight at RT (34.32 ± 3.19) [Figure 1].

As shown in Figure 2, no significant increase was observed between the mean of DFI in samples stored with semen plasma in comparison with those stored with HTF.

In experiment 4, the values of DFI of frozen samples thawed by water bath at 37°C were significantly lower than those which were melted in RT (22.83 ± 3.83 and 30.92 ± 4.93, respectively [Figure 3].

Discussion

Today, the SCSA is the only sperm DNA integrity assessment method that has demonstrated clear and clinically useful cut-off levels for calculating male fertility potential.[12,17-19] The SCSA is a standardized test that is performed according to a strict protocol[15] and apart from being subject to a very limited intralaboratory variation,[20] it has shown to be very robust to variation between laboratories. Moreover, the clear cut-off levels concerning fertility are maybe the most obvious benefit compared to other sperm DNA integrity tests.[19] The disadvantage of this method includes the need for expensive tools, particularly a flow cytometer, to run the analysis. Furthermore, this method irreversibly damages

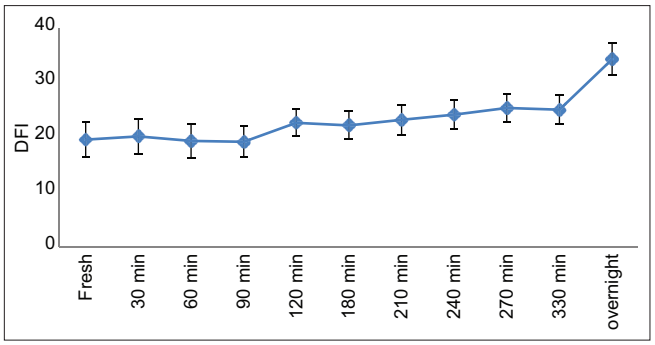

Figure 1: Comparison of mean of DFI in different incubation time in RT. RT: Room temperature, DFI: DNA fragmentation index

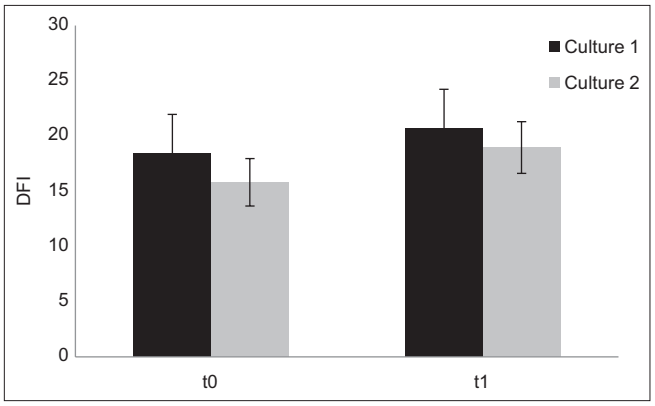

Figure 2: Mean of DFI in two cultures (culture 1, semen plasma and culture 2, HTF); t0, fresh samples; t1, 1 week after freezing samples ($P > 0.05$). DFI: DNA fragmentation index, HTF: Human tubal fluid

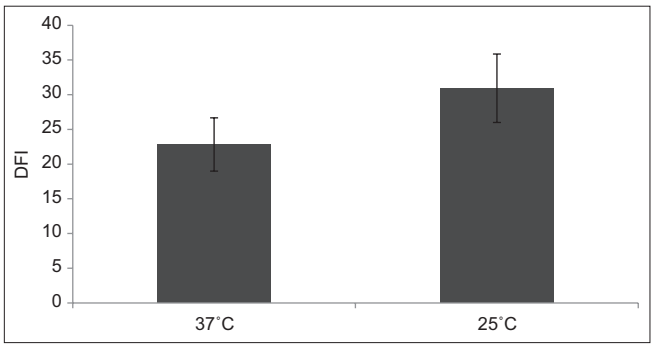

Figure 3: Comparison of DFI levels in two different thawing methods (water bath 37°C and room temperature 25°C) ($P < 0.05$). DFI: DNA fragmentation index

the spermatozoa and they cannot be used for fertilisation purposes anymore.

It should be noted that in the present study, all steps of the FCM procedure were performed by one technician to minimise human performance errors.

Although there are several studies about the usage of SCSA in immediately frozen semen,[12,21,22] the effect of sperm freezing without cryoprotectants on DFI results has not been evaluated so far. Our results showed

that sperm storage in liquid nitrogen during 1 min to 2 weeks causes an increasing DFI in comparison to fresh samples. To our knowledge, no report exists about the role of immediate freezing on sperm DNA damage and most studies have been focused on the effect of cryopreservation on DFI.

In experiment 2, our results showed that SCSA values increase significantly after 2 h of ejaculation and also an overnight incubation at RT as a result of the presence of oxidants in the seminal plasma, so based on our obtained data, it is better to perform DFI analysis until 2 h of sampling. Previous studies confirmed that reactive oxygen species play a role in sperm DNA damage.[14,23-26] In 2010, Zribi *et al*. evaluated the effects of cryopreservation on human sperm DNA integrity and demonstrated that oxidation could cause DNA damage.[27] Peris *et al*. showed that ROS promotes DNA instability in ram sperm.[23] Moreover, Smith *et al*. indicated that oxidative damage is associated with sperm DNA damage in patients.[28,29] In fact, ROS have an adequate time to damage sperm chromatin with poor compaction and incomplete protamination. The main sources of excess ROS generation in semen are spermatozoa with abnormal morphology and leucocytes.[29] Well-known variations of semen variables overtime can theoretically lead to variable levels of ROS in semen, and, subsequently, to the variation of sperm DNA damage levels overtime in some men.[5]

In the other part of our research, semen plasma and HTF were considered two medium for sperm storage in liquid nitrogen. In the initial hypothesis, we assumed that seminal plasma oxidants might affect DNA integrity. Although our results represented an increase in the mean of DFI in sperm stored in semen plasma compared with those in HTF, the difference was not statistically significant. We propose that urgent freezing may prevent the damaging effects of oxidants on sperm DNA integrity.

In experiment 4, two methods were applied for thawing frozen samples. As we expected, the levels of DFI in samples thawed at 37°C were significantly less than those thawed at RT. This finding is consistent with earlier reports by Boe-Hansen *et al*., showing that slow incubation of samples for thawing has a significant adverse effect on the DFI.[30] Before we assumed that the increase of DFI levels in samples thawed at RT would result from a longer time of thawing in RT condition.

Since, in recent years, DFI has been considered an effective parameter as well as sperm parameters in spontaneous abortion and ART outcomes and was prescribed by clinicians, reproducibility and accuracy of the test are extremely important. In the present study, it was demonstrated that incubation and freezing time and thawing methods could affect sperm DNA integrity, and thus, optimisation of the whole conditions is required.

Conclusion

According to our results, it can be concluded that sperm storage without cryoprotectants in liquid nitrogen can lead to an increase in DFI levels; therefore, it is better to use fresh samples for sperm DNA fragmentation measurement. Moreover, SCSA should be performed until 2 h after ejaculation to achieve more accurate results. Furthermore, if sperm freezing is inevitable, the use of a water bath (37°C) to thaw will be the most appropriate option, as it can lead to less DNA damage. Finally, to our knowledge, it is the first study about optimisation of SCSA and this approach will help the experts and improve quality control and validity of the test.

Authors' contributions

M. S and M. S. G developed the concept. H. H performed the experiments and participated in drafting the manuscript. A. R analysed the data.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Data availability statement

All data generated or analysed during this study are included in this published article.

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