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Original Article

Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa

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Abstract

Appropriate semen processing and assessment are critical for successful infertility treatment. We investigated whether laboratory procedures including semen preparation and incubation affect sperm DNA integrity. A total of 153 infertile men were involved. Conventional semen parameters and sperm chromatin structure assay (SCSA) parameters, that is, DNA fragmentation index (%DFI) and high DNA stainability (%HDS), were assessed on the fresh ejaculated semen samples, which were treated and incubated under different conditions. Negative correlations were identified between the %DFI and sperm concentration, motility, progressive motility and morphology. A lower percentage of DFI was detected in spermatozoa when density gradient centrifugation (DGC) was followed by swim-up treatment in comparison with DGC alone ($P < 0.01$). Although the %DFI increased in a time-dependent manner with incubation both at room temperature (RT) and at 37ºC in air, the %DFI after 24 h at RT was significantly lower than that at 37ºC ($P < 0.05$). Incubation with 5% CO_2 was effective in maintaining sperm motility ($P < 0.01$); however, it induced further elevation of %DFI ($P < 0.001$). Thus, sperm DNA damage was associated with longer incubation periods. Interestingly, common culture conditions, such as maintaining pH and temperature, compromised the sperm DNA integrity.

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1 Introduction

Appropriate semen analysis is a mandatory practice in infertility clinics. Spermatozoa used for assisted reproductive technique (ART) are mostly prepared by either density gradient centrifugation (DGC) or a swim-up method, both aiming to enrich mature, motile and morphologically normal spermatozoa. It was previously demonstrated that long-term *in vitro* incubation at 37ºC reduced the motility and viability of spermatozoa [1, 2]. Nevertheless, incubation of ejaculated spermatozoa at 37ºC has been recommended and widely practiced in ART laboratories [3]. Several studies have shown that both DGC and swim-up methods are effective in separating spermatozoa with poorly condensed chromatin and with fragmented nuclear DNA as detected by the terminal transferase-mediated DNA end-labeling (TUNEL) method [2] or the sperm chromatin structure assay (SCSA) [4–6].

Sperm nuclear condensation is a complex process, which involves a series of critical events, such as rearrangements of chromosomes, transition of nuclear binding proteins and alteration of gene transcription [7].

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Any failure(s) in this process during spermatogenesis may have a negative effect on male fertility as the highly organized and condensed sperm chromatin is known to have a crucial role in the normal fertilization process and embryo development [8–10].

The SCSA, introduced by Evenson *et al.* [11], utilizes the metachromatic properties of the fluorescent stain, acridine orange (AO), and evaluates the extent of DNA denaturation at sites of acid-induced DNA strand breaks, which is determined by measuring the shift from green (double-stranded, native DNA) to red (single-stranded, denatured DNA) fluorescence by flow cytometry [11, 12]. The SCSA parameters, that is, DNA fragmentation index (%DFI) and high DNA stainability (%HDS), indicate the fraction of defective spermatozoa showing either the presence of DNA breaks or poorly condensed chromatin [11]. It has been shown that the chance of spontaneous pregnancy decreases when the DFI exceeds 20%–30% [12, 13]. Recent reports have confirmed the utility of SCSA and concluded that the SCSA is the only method that can provide clear clinical threshold levels and can be recommended to provide robust evaluation of sperm DNA integrity [13, 14]. Furthermore, it has been demonstrated that the SCSA can predict ART outcomes [4, 12]. The etiology of sperm DNA damage appears to be multifactorial, including intrinsic factors such as DNA condensation failures and extrinsic factors such as air pollution, urogenital infections, fever, varicocele, certain prescription medications and chemotherapy, the exact mechanisms of which have not been elucidated [15].

As the main objective of this study, we tested whether *in vitro* incubation, which is routinely practiced in clinical andrology and embryology laboratories, affects sperm DNA integrity. Using fresh semen, we compared the SCSA parameters between samples prepared by different methods and exposed to different incubation conditions.

2 Materials and methods

2.1 Patients

The study involved a total of 153 men who visited our clinic for infertility screening between May 2008 and December 2008. Following the initial basic semen analysis, aliquots of each of the 153 semen samples were assigned to assess the correlations between basic semen parameters and SCSA values (n = 153), DGC/swim-up (n = 31), temperature (n = 24) and CO_2 (n = 14) experiment, as described later. The study design was approved by the internal review board. Informed consent was obtained from all participants.

2.2 Semen collection and analysis

Semen samples were collected by masturbation, and processed for semen analysis according to the World Health Organization guidelines [16]. The interval of ejaculatory abstinence of each patient was between 3 and 7 days. Only samples with a sperm concentration of at least 2×10^6 mL^{-1} and leukocytes below 1×10^6 mL^{-1} were used for further analysis because leukocytes may affect the SCSA results [17]. After the conventional semen analysis, the remaining raw semen sample was incubated at room temperature (RT; 25 ± 1ºC) or at 37ºC without CO_2 for up to 24 h. We did not add HEPES to the culture medium even though samples were incubated without CO_2 because the pH-buffering ability of the HEPES is compromised after long-time incubation in HEPES buffer [18]. Before and after incubation in each condition, an aliquot was examined for sperm motility, and another was used for the SCSA. The pH of each aliquot was measured using a Twin pH B-211 pH meter (Horiba Inc., Kyoto, Japan).

2.3 Density gradient centrifugation (DGC)

A three-layer gradient was prepared by using SpermGrad™ (Vitrolife Inc., Englewood, CO, USA) diluted to 50%, 70% and 90% fractions. Using a sterile pipette, a 0.5-mL sample of liquefied semen sample was placed on top of the upper layer into a 15-mL conical Falcon tube [19]. The tube was centrifuged at 500 × g for 20 min. The supernatant was then discarded and the pellet was washed twice with 5 mL G-IVF™ (Vitrolife Inc.) followed by centrifugation at 500 × g for 10 min. The pellet was resuspended in a volume of 0.5 mL G-IVF™. This medium is designed and recommended for use at 37ºC and in a 5% (v/v) CO_2 atmosphere. An aliquot was incubated at RT for 60 min in a 2-mL microcentrifuge tube, and another aliquot was further processed for swim-up as described below.

Some aliquots of samples were divided into several 2-mL culture tubes and incubated either in a thermal box in which the temperature was maintained at 37ºC without CO_2, or in a 5% (v/v) CO_2 incubator at 37ºC.

2.4 Swim-up protocol

In our clinic, semen is processed using DGC

followed by swim-up (DGC + swim-up) for conventional *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) in order to enrich motile spermatozoa. After DGC, the sample pellet was gently immersed in 0.5 mL G-IVF^{TM} in a conical tube. The tube was inclined at an angle of 45º. Following the incubation at RT for 60 min, the tube was set upright and the 50 μL of the upper interface was then gently aspirated with a Pasteur pipette [20] and examined for sperm concentration, motility and the SCSA.

2.5 SCSA procedure

Sperm DNA integrity was evaluated by SCSA as described elsewhere [12, 21]. Briefly, fresh semen samples were incubated for 5 min in an ice-bath and an aliquot diluted to a sperm concentration of 1×10^6–2×10^6 sperm per mL in TNE buffer (0.01 mol L^{-1} Tris-HCl, 0.15 mol L^{-1} NaCl, 1 mmol L^{-1} EDTA disodium, pH 7.4) in a total volume of 100 μL. This cell suspension was admixed with 200 μL of acid detergent solution (0.08 mol L^{-1} HCl, 0.15 mol L^{-1} NaCl, 0.1% [v/v] Triton X-100, pH 1.2) for exactly 30 s at RT, and then 600 μL of 6 μg mL^{-1} AO-staining solution (0.037 mol L^{-1} citric acid, 0.126 mol L^{-1} Na_2HPO_4, 1.1 mmol L^{-1} EDTA disodium, 0.15 mol L^{-1} NaCl, pH 6.0) was added. When frozen samples were subjected to the SCSA, they were rapidly thawed in a 37ºC water bath and used immediately.

After AO staining, the fluorescence intensity of individual sperm cells was analyzed using an EPICS ALTRA flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA) with a solid-state laser (Coherent Inc., Santa Clara, CA, USA) operated at 488 nm and a power of 15 mW. Under these experimental conditions, when excited with a 488 nm light source, AO intercalated with double-stranded DNA emits green fluorescence (525 ± 30 nm), while that with single-stranded DNA generates red fluorescence (> 640 nm). A total of 20 000 events were accumulated for each sample at a flow rate of about 200 cells per second, because it is suggested that at least 5 000 events are required for consistent, reliable flow cytometric assays [22]. To minimize the inter-assay co-efficients of variation, AO equilibration buffer (400 μL of acid detergent solution and 1.2 mL of AO-staining solution) was run between every new assay as described previously [23].

Scattergram data, with each point representing the coordinate of red and green fluorescence intensity values for individual spermatozoa, were analyzed by using Expo32 software (Beckman Coulter Inc.) (Figure 1). Events corresponding to sample debris were excluded from the analysis. Spermatozoa with increased levels of red fluorescence corresponding to increased levels of sperm DNA fragmentation were distributed outside the main population (green fluorescence; double-stranded DNA) and quantified on the basis of the percentage of spermatozoa with increased red fluorescence (%DFI). %HDS represents spermatozoa with incomplete chromatin condensation [9] and is expressed as the percentage of spermatozoa with high levels of green fluorescence (> 600 channels of fluorescence in this study, Figure 1).

2.6 Statistical analysis

Statistical analysis was performed using the SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Correlations between SCSA parameter values and semen characteristics were analyzed by Pearson's coefficient test. Paired and unpaired corresponding differences in the sperm motility and SCSA parameter values after different preparation methods and incubation conditions were examined using one-way

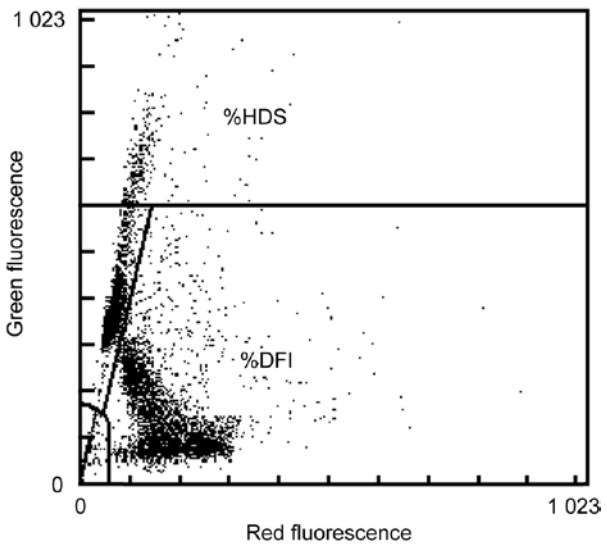

Figure 1. Representative cytogram of sperm chromatin structure assay (SCSA). Green (double-stranded DNA: ordinate) versus red fluorescence (single-stranded DNA: abscissa) showing DNA fragmentation index (%DFI); the percentage of spermatozoa with high levels of DNA fragmentation, and high DNA stainability (%HDS); the percentage of spermatozoa with high DNA stainability. Each cytogram dot represents a single spermatozoon with dual-parameter green and red fluorescence values. The debris (bottom-left corner) was excluded from the analysis.

repeated-measures analysis of variance (ANOVA) or two-way repeated-measures ANOVA as appropriate as stated in the text. Tukey–Kramer test was performed as a *post-hoc* test. The term 'statistically significant' was used to denote a two-sided *P*-value being less than 0.05.

3 Results

3.1 Semen parameters and the SCSA

A total of 153 fresh semen samples were assessed for conventional semen parameters and the SCSA (Table 1). The mean (range) age of men was 38.5 ± 5.3 (29–62) years. The mean ± SD (range) %DFI was 28.7 ± 15.3 (4.3–87.2), and their %HDS was 4.5 ± 4.3 (0.1–24.0). A negative correlation was observed between the %DFI and all the basic semen parameter values except for semen volume (Table 1). %HDS was negatively correlated with sperm concentration, motility and progressive motility, but not with semen volume or the percentage of normal morphology (Table 1).

3.2 Effect of the semen preparation methods

Compared with the raw semen incubated at RT for 60 min, spermatozoa prepared by both simple DGC and DGC + swim-up treatment exhibited high percentages of motile spermatozoa (one-way repeated-measures ANOVA, Table 2). DGC + swim-up improved the motility more than DGC alone. As expected, the percentages of spermatozoa with HDS were significantly lower after the DGC as well as the DGC + swim-up treatment, although there was no difference in the %HDS between the DGC and the DGC + swim-up groups (one-way repeated-measures ANOVA, Table 2). The %DFI was significantly increased only after DGC, and was remarkably reduced by the subsequent swim-up treatment (one-way repeated-measures ANOVA, Table 2).

3.3 Effect of temperature and incubation time

To investigate the effect of culture conditions on the %DFI and sperm motility, raw semen samples were examined after 0, 3, 5 and 24 h at RT or 37ºC in 2-mL cylindrical tubes. The mean pH values of semen samples following a 5-h incubation at RT and 37ºC without CO_2 were identical (pH 8.3 ± 0.4 each). Both at RT and 37ºC, a significant decrease in the motility of spermatozoa was observed in a time-dependent manner (Figure 2A, Tukey–Kramer test, $P < 0.001$, 3 and 5 h; $P < 0.01$, 24 h). The 37ºC treatment reduced sperm motility to a greater extent and more rapidly

Table 1. Correlations between the SCSA and semen parameters ($n = 153$).

Semen parameters	Values (mean ± SD)	Pearson's correlation coefficients	
		With %DFI	With %HDS
Volume (mL)	3.9 ± 1.7	−0.130[a]	−0.156[a]
Concentration (10^6 mL^{-1})	69.0 ± 51.7	−0.207[b]	−0.250[c]
Motility (%)	44.2 ± 16.8	−0.624[d]	−0.206[b]
Progressive motility (%)	5.7 ± 5.1	−0.407[d]	−0.216[c]
Normal morphology (%)	15.4 ± 6.9	−0.386[d]	−0.086[a]

Abbreviations: %DFI, DNA fragmentation index; %HDS, high DNA stainability; SCSA, sperm chromatin structure assay.
[a]not significant, [b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$.

Table 2. Sperm motility and SCSA parameters in different semen preparation methods ($n = 31$).

Parameters (mean ± SD)	Raw semen	Semen preparation methods	
		DGC	DGC + swim-up
Sperm motility (%)	47.2 ± 10.8[a]	60.0 ± 11.5[b]	90.8 ± 10.9[c]
%DFI	15.8 ± 9.6[d]	29.7 ± 20.4[e]	8.9 ± 6.7[f]
%HDS	2.2 ± 1.0[g]	0.8 ± 0.5[h]	0.6 ± 0.6[h]

Abbreviations: DGC, density-gradient centrifugation; %DFI, DNA fragmentation index; %HDS, high DNA stainability; SCSA, sperm chromatin structure assay.
In the same rows, different superscripts indicate significant differences. [a] *vs.* [b], $P < 0.05$; [b] *vs.* [c], $P < 0.001$; [a] *vs.* [c], $P < 0.001$; [d] *vs.* [e], $P < 0.05$; [e] *vs.* [f], $P < 0.01$; [d] *vs.* [f], $P < 0.05$; [g] *vs.* [h], $P < 0.001$.

than that at RT (two-way repeated-measures ANOVA, $P < 0.05$, 24 h; $P < 0.01$, 3 and 5 h). A time-dependent increase in the %DFI was observed at both RT and 37ºC (Figure 2B, Tukey–Kramer test, $P < 0.05$, 3 h; $P < 0.01$, 5 and 24 h). The %DFI at RT was significantly lower than that at 37ºC after 24 h (two-way repeated-measures ANOVA, $P < 0.001$). %HDS values were not influenced by these conditions (data not shown).

3.4 Effect of 5% CO_2

In order to investigate the effect of pH stabilization on SCSA values, two aliquots of unprocessed semen were incubated for up to 24 h at 37ºC with or without 5% CO_2. The mean pH of semen samples was 8.1 ± 0.1 (0 h), 8.3 ± 0.1 (without CO_2, 3 h), 7.3 ± 0.2 (with CO_2, 3 h), 8.5 ± 0.1 (without CO_2, 24 h) and 7.2 ± 0.1 (with CO_2, 24 h), respectively. Although at 3 h there was no difference in sperm motility, sperm quality in the presence of CO_2 incubation was significantly better than that without CO_2 after 24 h (Tukey–Kramer test, $P < 0.001$; two-way repeated-measures ANOVA, $P < 0.001$, Figure 3A). However, the %DFI both at 3 and 24 h was significantly higher in the CO_2 incubation than in the counterparts (Tukey–Kramer test, $P < 0.01$, 3 h; $P < 0.001$, 24 h; two-way repeated-measures ANOVA, $P < 0.01$, 3 h; $P < 0.001$, 24 h, Figure 3B). %HDS values were comparable between the two groups (data not shown).

4 Discussion

Although several studies have compared the effects of DGC and swim-up or both on sperm DNA integrity, the findings have been discordant [20, 24]. Chiamchanya *et al.* [25] compared the sperm quality

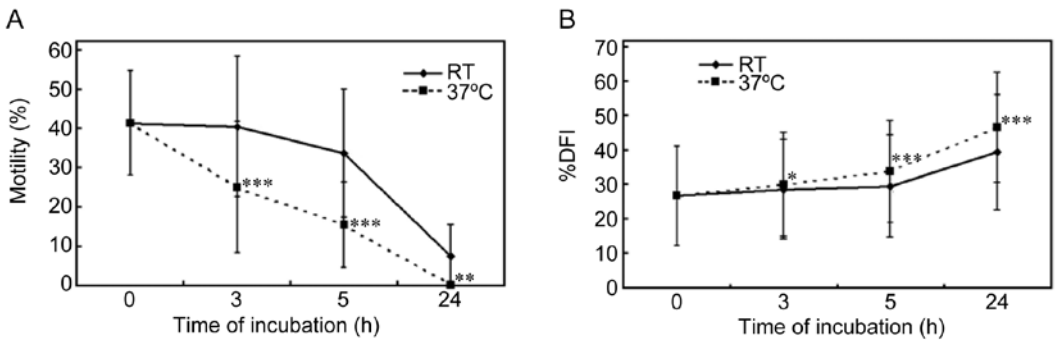

Figure 2. Effect of incubation temperatures and time on the motility and DNA fragmentation index (%DFI) of spermatozoa in raw semen (not frozen and thawed). Sperm motility (A) and DFI (B) as a function of incubation time (abscissa) at room temperature (RT; diamonds) and 37ºC (squares) ($n = 24$) are given on the ordinate. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the values at RT.

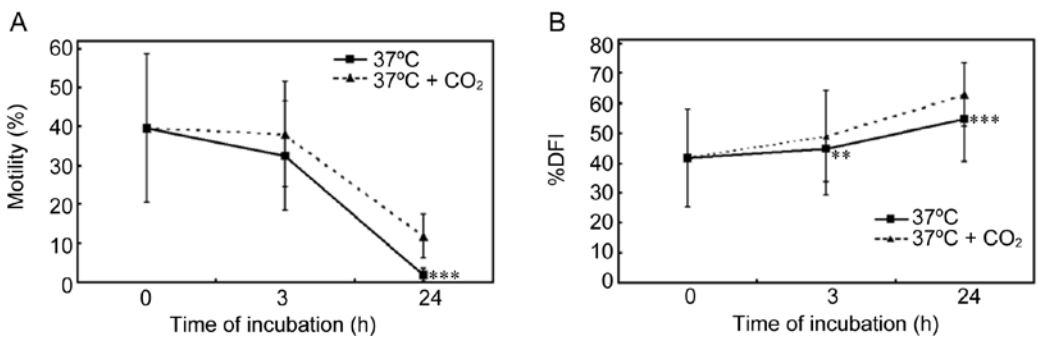

Figure 3. Effect of CO_2 incubation and time on the motility and DNA fragmentation index (%DFI) of spermatozoa obtained after density-gradient centrifugation treatment (not frozen and thawed). Sperm motility (A) and DFI (B) as a function of time (abscissa) at 37ºC without CO_2 (squares) and at 37ºC with CO_2 (triangles) ($n = 14$) are shown on the ordinate. $^{**}P < 0.01$, $^{***}P < 0.001$. compared with the values at 37ºC with CO_2.

and extent of DNA damage after incubation in different media, and found that the SpermGrad™ preparation selected a population of spermatozoa that had a high percentage of normal morphology and also a high percentage of DNA damage, as assessed by TUNEL. Similarly, in this study with SCSA, spermatozoa with high %DFI were observed after the SpermGrad™-based DGC treatment, compared with the raw semen sample. Subsequently, the percentage of the spermatozoa with high %DFI was reduced when further selected by swim-up. Thus, the swim-up treatment is effective in isolating spermatozoa with low %DFIs.

The study by Dalzell *et al.* [26] showed that DNA fragmentation in sperm cells increases after 4 h of incubation at 37ºC. In addition, Hammadeh *et al.* [27] reported a significant increase in spermatozoa with decondensed chromatin upon *in vitro* incubation at 37ºC for 24 h. Furthermore, it has been suggested that prolonged sperm cell manipulations should be performed at RT (21ºC) rather than at 37ºC, because extended handling of spermatozoa at 37ºC is detrimental to fine sperm nuclear morphology [28]. In the present study, sperm motility decreased and %DFI increased in a time-dependent manner at both RT (25ºC) and 37ºC. However, we found that the incubation at RT was better than that at 37ºC in terms of sperm motility and DNA integrity. This observation might be associated with the fact that lowering the temperature reduces the metabolic activity of spermatozoa and sustains their viability *in vitro* [29].

In general, spermatozoa are usually incubated at 37ºC in an atmosphere of 5% CO_2 for at least 1–2 h before intracytoplasmic sperm injection or conventional insemination. In our study, although incubation under 5% CO_2 maintained pH and sperm motility, the %DFI was also concomitantly increased. It is known that there is a relationship between the pH of semen and sperm motility and metabolic activity [30]. Thus, together with our finding regarding incubation under the conventionally considered 'appropriate' conditions, maintaining pH as well as the temperature may also induce sperm DNA fragmentation. Although we did not examine the %DFI at RT in an atmosphere of 5% CO_2, it remains possible that these incubation conditions might be applicable to ART. In the present study we have identified a significant association between compromised semen parameters, especially sperm motility, and %DFI. However, we found the opposite result in the case of DGC treatment and incubation at 37ºC with 5% CO_2. These results suggest that it is important to evaluate both two parameters, sperm motility and DFI, as reported previously [31].

In conclusion, our data demonstrated that sperm DNA damage is enhanced by prolonged *in vitro* incubation. The commonly used preparation and culture conditions impair sperm DNA integrity. Any improvements in semen handling before fertilization are expected to reduce the negative effects of DNA damage in the fertilizing paternal genome.

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