Effects of semen storage and separation techniques on sperm DNA fragmentation

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Objective: To determine the effect of semen storage and separation techniques on sperm DNA fragmentation.
Design: Controlled clinical study.
Setting: An assisted reproductive technology laboratory.
Patient(s): Thirty normoozospermic semen samples obtained from patients undergoing infertility evaluation.
Intervention(s): One aliquot from each sample was immediately prepared (control) for the sperm chromatin
dispersion assay (SCD). Aliquots used to assess storage techniques were treated in the following ways: snap frozen
by liquid nitrogen immersion, slow frozen with Tris-yolk buffer and glycerol, kept on ice for 24 hours or maintained
at room temperature for 4 and 24 hours. Aliquots used to assess separation techniques were processed by the
following methods: washed and centrifuged in media, swim-up from washed sperm pellet, density gradient
separation, density gradient followed by swim-up. DNA integrity was then measured by SCD.
Main Outcome Measure(s): DNA fragmentation as measured by SCD.
Result(s): There was no significant difference in fragmentation among the snap frozen, slow frozen, and wet-ice
groups. Compared to other storage methods short-term storage at room temperature did not impact DNA
fragmentation yet 24 hours storage significantly increased fragmentation. Swim-up, density gradient and density
gradient/swim-up had significantly reduced DNA fragmentation levels compared with washed semen.
Postincubation, density gradient/swim-up showed the lowest fragmentation levels.
Conclusion(s): The effect of sperm processing methods on DNA fragmentation should be considered when select-
ing storage or separation techniques for clinical use. (Fertil Steril® 2010;94:2626–30. ©2010 by American Society
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Key Words: Semen, storage, sperm, DNA fragmentation, sperm chromatin dispersion assay

Between 40% and 50% of all conception difficulties are associated with male-factor infertility (1–6). However, in many cases the cause of male infertility cannot be ascertained based on a conventional semen analysis (7, 8). Sperm DNA integrity is an important component of fertility not evaluated by a standard semen analysis. Levels of DNA fragmentation have been shown to correlate with success rates in natural reproduction, and intrauterine insemination (IUI) (9–13). The relationship to success of more advanced assisted reproductive technologies (ART) such as in

vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) is more controversial (14, 15). However, data do suggests that levels of DNA fragmentation can help guide whether IVF or ICSI is the more appropriate choice (12, 15).

Because DNA integrity plays an important role in both evaluation and treatment of male infertility, numerous tests have been developed for its assessment (9, 16–18). One of these is the sperm chromatin dispersion assay (SCD). This assay is based on induced DNA decondensation, which is directly related to levels of DNA integrity (19). Preparation of sperm for SCD results in nucleoids with a central core and a surrounding halo of dispersed DNA loops. Nonfragmented DNA produces large halos of dispersed DNA, whereas fragmented DNA produces little or no halo. After staining, halo presence, and therefore fragmentation, can be assessed with direct vision under bright-field or fluorescent microscopy. Sperm chromatin dispersion results correlate well with those obtained using other more complex and expensive fragmentation assays such as the sperm chromatin structure assay (SCSA) and terminal transferase-mediated DNA end-labeling (TUNEL) (20, 21). Because of its low cost, speed, and simplicity, SCD is an appealing option for assessment of DNA integrity.

Research into sperm DNA integrity has also focused on identifying the causes of fragmentation. Many etiologic factors have been

FIGURE 1

A schematic showing the study design for evaluation of sperm storage and separation techniques. The schematic for the evaluation of storage techniques is on the left in green, and the schematic for evaluation of semen separation techniques is on the right in blue. Aliquots from each sample were divided into treatment groups. After processing, DNA integrity in each group was assessed by SCD. For semen separation technique evaluation, sperm from each treatment group were then cultured for 24 hours. DNA integrity was then reassessed and motility was measured. PC = permeating cryoprotectants.
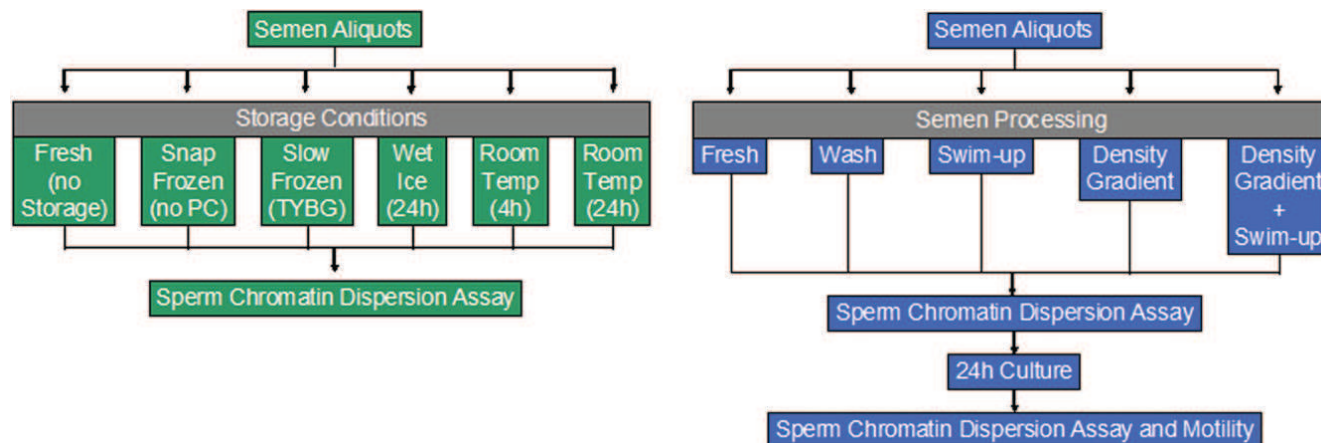
Jackson. Storage, processing, and sperm DNA integrity. Fertil Steril 2010.

identified including health conditions such as cancer, infection, and varicoceles (10, 22, 23), and environmental exposures such as smoking or radiation (24, 25). Recent studies have indicated that some changes in DNA integrity may be iatrogenic. Sperm storage techniques such as cryopreservation have been shown to increase DNA damage (26–28). Sperm separation techniques have also been shown to have an impact of on DNA integrity, although the results are less clear cut, with some studies showing decreased levels of fragmentation after processing (29, 30), and other studies showing varying levels of fragmentation depending on the separation technique in question (28, 31–33). To our knowledge, none of these studies have looked comprehensively at multiple processing techniques spanning the entire range from cryopreservation through semen separation and preparation for ART. Additionally, none of these other studies have used the SCD assay to evaluate a range of different sperm processing methods. The aim of this study was to evaluate the impact of various short-term storage and separation methods on sperm DNA integrity using the SCD test.

MATERIALS AND METHODS
Semen Samples

All samples were obtained from patients who presented for a semen analysis at the assisted reproduction technology laboratories at Huntington Center for Reproductive Medicine in Sao Paulo, Brazil. Semen analysis was performed to assess pH, semen volume, sperm concentration, percentage sperm motility, percentage forward progression, and percentage normal morphology. Samples found to be normozoospermic by World Health Organization standards (34) were subsequently used in this study. Institutional review board exemption was obtained to record deidentified results using samples that would otherwise be discarded after semen analysis. Ten samples were analyzed for evaluation of different storage techniques, and 20 samples were analyzed for evaluation of separation techniques.

Processing for Evaluation of Storage Techniques

Each sample was divided into six equivalent size aliquots (n = 60 aliquots). A control aliquot was immediately prepared for SCD. The other aliquots were handled in one of five ways before preparation for SCD analysis: [1] snap frozen by immersion in liquid nitrogen, [2] cryopreserved with TEST-yolk buffer with glycerol (TYBG), [3] kept on ice for 24 hours, [4] maintained at room temperature for 4 hours, or [5] maintained at room temperature for 24 hours (Fig. 1).

Semen aliquots cryopreserved with TYBG (Irvine Scientific, Santa Ana, CA) were mixed in a dropwise fashion to reach a 1:1 volume:volume (v:v) solution over 10 minutes in a cryovial. Cryovials with semen/TYBG were cooled to 4°C in ice water for 10 minutes, incubated in vapor nitrogen at a level between 38 and 29 cm above liquid nitrogen providing between −88°C and −93°C for 20 minutes, then plunged into liquid nitrogen where they remained immersed until thawed. After 24 hours of cryostorage, samples were removed from the liquid nitrogen, allowed to thaw at room temperature for 30 minutes, and then assessed for DNA fragmentation.

Processing for Evaluation of Separation Techniques

Each sample was equally divided into six aliquots (n = 120 aliquots). These were processed using the following treatments: [1] semen mixed v:v with 2% H_2O_2 (positive control for high levels of DNA fragmentation), [2] fresh sample, [3] sperm washed and centrifuged in human tubal fluid medium with HEPES (wash), [4] swim-up from washed pellet of sperm, [5] 45/90% ISolate density gradient centrifugation, and [6] 45/90% ISolate density gradient centrifugation followed by swim-up from density gradient pellet (DG/SU). Immediately following sperm separation, DNA integrity was then assessed using the SCD assay (Fig. 1).

Following separation, 0.1×10^6 sperm/mL were then taken from each sample in treatment groups 2–6 and were cultured in human tubal fluid + 10% (v:v) serum substitute supplement at 37°C in 5% CO_2 for 24 hours. Deoxy-ribonucleic Acid fragmentation levels were then reevaluated, again using SCD, and motility for each group was recorded.

Sperm Chromatin Dispersion Assay

The SCD assay was performed as described by Fernadez and colleauges (17): fresh semen samples were diluted in PM to a concentration of 5–10 million

Levels of DNA fragmentation following treatment of sperm by different storage methods represented as mean ± standard error. There were no significant differences between freezing methods. The mean for all freezing methods was significantly less than for the 24-hour room temperature group.
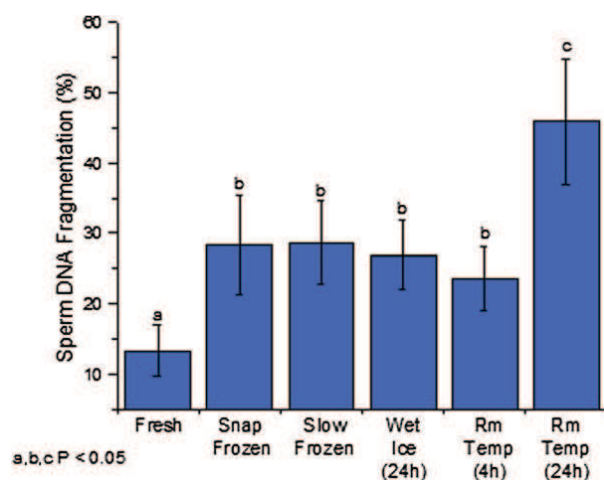
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Levels of DNA fragmentation following treatment of sperm by different separation methods represented as mean ± standard error. The mean for swim-up, density gradient centrifugation, and density gradient centrifugation + swim-up groups was significantly less than for fresh and wash groups.
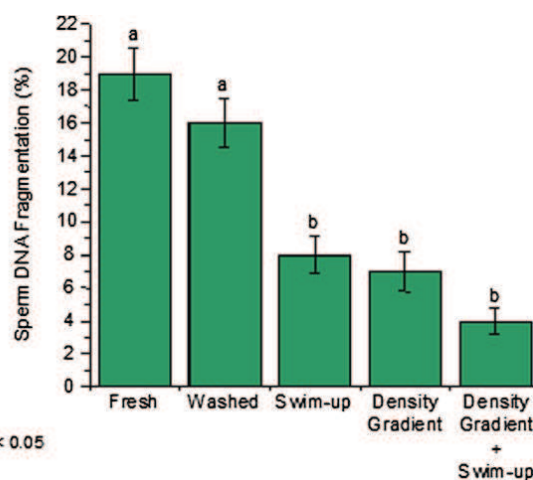
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sperm/mL. At 37°C, 60 μL of the diluted sample was added to 140 μL of 1% low melting point agarose to obtain a 0.7% final agarose concentration. Fifty microliters of the semen agarose solution was pipetted onto slides precoated with 0.65% agarose and covered with a 24 × 60 mm coverslip. Slides were then placed on a cold plate at 4°C for 5 minutes to allow the samples to gel. Slide covers were then removed and slides were immediately immersed horizontally in 0.08 N HCl denaturation solution for 7 minutes at room temperature 22°C. Slides were then horizontally immersed in a lysis solution of 0.4 M Tris, 0.4 M Dithiothreitol, 1% sodium dodecyl sulfate, 50 mM ethylenediaminetetracetic acid, pH 7.5 for 25 minutes. Slides were then washed with distilled water for 5 minutes before sequential dehydration with 70%, 90%, and 100% ethanol for 2 minutes each. Slides were allowed to air dry before flourescent staining with Hoechst 33258. A minimum of 500 sperm per sample were then scored under the 100× objective lens and results were expressed as the percentage of sperm with fragmented DNA. Halos were scored as large, medium-size, small, or absent as previously defined (17). Sperm with small or absent halos were considered to have DNA damage.

Statistical Analysis

Differences between treatments were analyzed using analysis of variance statistics and Turkey's test for means. Differences were considered significant at $P<.05$.

RESULTS
Storage Treatments

The fresh (control) samples had an average DNA fragmentation level of 13 ± 3.6%. For all five storage methods evaluated, the level of sperm DNA fragmentation increased after storage. Levels of fragmentation were as follows: snap frozen (28.3 ± 7.1%; mean ± SE), cryopreserved with TYBG (28.7 ± 5.9%) placed on ice for 24 hours (26.9 ± 4.8%), maintained at room temperature for 4 hours (23.5 ± 4.6%), and maintained at room temperature for 24 hours (45.9 ± 8.9%). The percentage of induced DNA damage was not significantly different among the snap frozen, cryopreserved, wet ice, and 4-hour room temperature groups. However, samples stored at room temperature for 24 hours had a significantly higher percentage of DNA fragmentation compared with all other storage methods (Fig. 2).

Separation Treatments

For separation techniques, levels of DNA fragmentation immediately following processing were significantly lower for swim-up (8.3 ± 1.5%), density gradient centrifugation (7.1 ± 2.2%), and DG/SU (4.0 ± 1.0) than for fresh (17.8 ± 2.2%) and washed samples (15.9 ± 2.0%) (Fig. 3). After 24-hour culture, there was no significant difference in DNA fragmentation rates between the wash and swim-up groups. Compared with results from the wash and swim-up treatments, fragmentation following culture was significantly lower in the density gradient centrifugation group. In the postculture DG/SU treatment, fragmentation was significantly lower than all other treatments analyzed. Additionally, motility 24 hours after semen processing was not significantly different between washed and swim-up treatments. Motility for sperm separated by density gradient centrifugation was not significantly higher than for washed semen. Motility following DG/SU treatment was significantly higher than for all other treatments analyzed (Table 1).

DISCUSSION

Many centers are not currently equipped to offer DNA fragmentation analysis. Therefore, short-term storage and shipping remain most clinicians' primary means of performing these tests, and acquiring the potential benefits they afford to patients. Any DNA damage caused by storage could skew results and should therefore be minimized. Our data indicate that any sample that will not be analyzed within 4 hours of collection should be frozen to prevent increasing DNA damage. Among the different methods of freezing, there was no statistically significant difference in the resultant amount of DNA fragmentation. Both wet ice and snap freezing

TABLE 1

Percent sperm DNA fragmentation and motility 24 hours after semen processing by either washing, swim-up, density gradient separation, or density gradient separation followed by swim-up.

24-h postprocessing evaluation	Wash (n = 20)	Swim-up (n = 20)	Density gradient (n = 20)	Density gradient + swim-up (n = 20)
Sperm DNA fragmentation (%)	26 ± 1.8[a]	20 ± 3.0[a]	12 ± 1.7[b]	6 ± 1.1[c]
Sperm motility (%)	38 ± 4.2[a]	53 ± 6.5[a,b]	67 ± 2.7[b]	84 ± 1.7[c]

Note: Values are mean ± SE.

[a,b,c] Different letters within an assessment significantly different at $P < .05$.

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were therefore found to be equivalent to the more expensive option of cryopreservation with TYBG.

Results from our samples maintained at room temperature were consistent with the recent findings by Gosalvez and coworkers (35), who found a progressive decrease in DNA quality over time, when analyzing samples incubated at 37°C over a 24-hour period. In their study the largest increase in DNA damage occurred within the first 4 hours, and the rate decreased over time to around 1% per hour at 24 hours. We observed similar trends with the rate of fragmentation greatest in the first 4 hours and then slowing over the remainder of the 24-hour period. This underscores that samples intended for diagnostic assessment should be used, or cryopreserved, as quickly as possible to minimize levels of DNA degradation. Additionally, these finding may also translate to timing of sample preparation and use for IUI.

Taking into considering our results and the cost and complexity of the different storage methods, wet-ice storage is recommended as the simplest and most cost-effective option for short-term storage. Samples that are unable to be shipped and/or analyzed within 24 hours can be snap frozen and stored in liquid nitrogen without higher incidence of sperm DNA fragmentation compared with cryopreservation with TYBG.

The effects of sperm separation techniques on DNA integrity have been the subject of a number of studies over the past decade. Our preincubation swim-up data were consistent with observations from a large number of studies using SCSA and TUNEL, which showed decreases in sperm DNA fragmentation after swim-up (28, 29, 33, 36–38). In contrast to these, a 2006 study by Muriel and colleagues (19) using SCD, found no significant improvement in DNA integrity after swim-up in samples of males from couples undergoing IUI.

For density gradient centrifugation, most studies showed results similar to ours, with DNA integrity improving following processing (29, 37, 39, 40). However, other studies demonstrated postcentrifugation fragmentation levels to be unchanged or increased compared with those from raw semen (31–33). Possible explanations for contradictions among these studies include differences in technique (i.e., speed and duration of centrifugation, type of media, number of gradient layers), and small sample sizes of the studies in question, ranging from 7 to 44 patients, which leave room for possible type II error.

Two studies, both using SCSA, have compared the predictive value of DNA fragmentation analysis before processing to that following density gradient centrifugation (41, 42). Both studies showed a significant negative correlation between successful ART and the fragmentation level of neat semen. Fragmentation levels postcentrifugation were not predictive of ART outcome, despite significant decreases in fragmentation level. One of the possible explanations offered for this lack of correlation is that postcentrifugation cohorts are uniformly characterized by very low levels of fragmentation that are not contrasted enough to allow the detection of a difference between samples and a resultant correlation to ART outcomes (41, 42). The significant reduction in fragmentation noted in our density gradient centrifugation and DG/SU groups lends credence to this possibility.

A large 2007 study of almost 1,000 ART cycles, including almost 400 IUI cycles found a significant correlation between high DNA fragmentation index, as measured by SCSA, and IUI outcomes including biochemical pregnancy, clinical pregnancy, and delivery. Numerous other studies have reported similar correlations (9–13, 41). Given the evidence that levels of fragmentation influence IUI outcomes, the use of swim-up and density gradient centrifugation techniques alone, or in sequence, is recommended for those samples with sufficient total/motile sperm, over sperm wash for separation before IUI, as these techniques do a better job selecting a sperm cohort with minimal chromatin damage, thereby increasing the chance of reproductive success.

For IVF, as noted above, the importance of DNA fragmentation remains controversial. However, the possibility that poor DNA integrity adversely affects IVF outcomes has not been ruled out. Density gradient centrifugation followed by swim-up can be used to select a postincubation cohort of sperm with both high DNA integrity and high motility, minimizing any potentially negative fragmentation effects, and optimizing potential for fertilization.

Some studies indicate that patients with poor DNA integrity have a higher likelihood of reproductive success with ICSI compared with conventional IVF. Intracytoplasmic sperm injection has therefore been suggested as the treatment of choice for those with high sperm DNA fragmentation levels (12, 13). However, there is some concern that ICSI bypasses the genetic safeguards provided by natural selection. A number of studies have suggested a link between sperm DNA damage and morbidities including cancer and infertility, and an increase in genetic imprinting disorders such as Angelman's syndrome and Beckwith-Wiedemann syndrome (43–45). Our data indicate that density gradient centrifugation and swim-up techniques can reduce DNA fragmentation rates, significantly reducing the chance that sperm with low DNA integrity will be selected for ICSI. However, it is recognized that this combination approach may not be practical when processing severely oligoasthenozoospermic samples.

Our data illustrate the impact of sperm storage and separation techniques on DNA integrity, and highlight that different treatments result in differing levels of fragmentation. For storage techniques,

levels of fragmentation after wet-ice freezing and snap freezing are equivalent to those found after cryopreservation with TYBG, indicating the utility of these short-term storage techniques. For separation techniques, density gradient centrifugation, swim-up, and DG/SU yielded significant reductions in fragmentation levels. After 24-hour culture DG/SU was superior to other treatments evaluated in terms of both fragmentation and motility. The ability of these processing methods to isolate sperm cohorts with reduced levels of DNA damage should be taken into consideration when selecting processing modalities.

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