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Evaluation of Chromosome Breakage and DNA Integrity in Sperm: An Investigation of Remote Semen Collection Conditions

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ABSTRACT: Collection of ejaculated semen at a remote site (outside of the laboratory) would facilitate participation rates and geographic diversity in reproductive epidemiology studies. Our study addressed concerns that remote collection and overnight mail return might induce chromosome/DNA damage. We collected semen from 10 healthy men. Part of each sample was snap frozen in liquid nitrogen and the rest held at 22 ± 1°C for 24 hours in a transport container (simulating ambient temperature during overnight return) then snap frozen. DNA breakage and fragmentation were measured using tandem-label sperm–fluorescence in situ hybridization (FISH), terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL), and neutral comet assay. Tandem-label sperm-FISH and TUNEL detected no statistically significant difference between sperm fresh frozen (FF) and those frozen after 24 hours (F24). The mean frequency of chromosome breakage per 10 000 cells scored in sperm-FISH for FF and F24 was 10.5 ± 1.3 breaks and 11.2 ± 1.1 breaks, respectively (P = .69, Student's t test). The mean frequency of TUNEL-positive cells per 2000 cells scored in FF and F24 was 136 ± 29 and 213 ± 28 cells, respectively, which approached but did not reach statistical significance (P = 0.07, Student's t test). The neutral comet assay detected a statistically significant difference in DNA strand breakage between the 2 groups (percentage of DNA in the tail P = 0.037; tail moment P = 0.006; and tail length P = 0.033, all Student's t test). The mean frequency of damage denoted by tail length in μm per 100 cells scored in FF and F24 was 175.0 ± 15.5 and 152.2 ± 17.6 μm, respectively. Tandem-label sperm-FISH, TUNEL, and neutral comet assay are useful analytical techniques for laboratory-based studies of human sperm genomic integrity; however, for field studies incorporating the nonrefrigerated return of semen after 24 hours, only chromosome breakage at a level that can be detected using tandem-label sperm-FISH was unaffected. TUNEL and neutral comet assay need further study before they are used in specimens collected at remote sites and transported to a central laboratory.

Key words: Snap-freezing, sperm-FISH, TUNEL, neutral comet assay.

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Occupational and epidemiological reproductive studies are hindered by low participation rates (30%–50%; Lahdetie, 1995) and the need to sample individuals from assorted locations (Royster et al, 2000). In addition to issues of privacy and embarrassment, low participation rates in semen collection studies may result from selection bias or loss to follow-up in longitudinal study designs. The ability to collect a sample in a private setting and ship it directly to the laboratory for analyses may improve participation rates (Royster et al, 2000). However, there are concerns regarding induced or endogenous chromosome breakage/DNA damage during semen collection and mail return. The aim of the present study was to evaluate the clinical usefulness of semen samples collected at remote sites and delivered to a central laboratory the next day after storage in a transport container. We measured genomic integrity using 1) tandem-label sperm-fluorescence in situ hybridization (sperm-FISH) to quantify chromosome breakage, 2) terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) to quantify endogenous DNA strand breakage, and 3) single-cell gel electrophoresis or ''comet'' assay (electrophoresis running solution, pH 9) to quantify double-stranded DNA breakage in male germ cells allowed to remain in seminal fluid at ambient temperature for 24 hours.

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Materials and Methods

Semen Collection

Semen samples were collected from 10 healthy men at Fertility Solutions Inc, Cleveland, Ohio, as a first step in evaluating re-

mote semen collection transport containers for a larger US Environmental Protection Agency study. Participants with known semen quality ranging from normal to oligozoospermic (World Health Organization, 1999) provided samples via masturbation into sterile 130-mL polypropylene jars after 2 to 5 days of abstinence from sexual activity. The median concentration was 55 million cells/mL (range, 12 to 203 million cells/mL). Three of 10 men were classified as oligozoospermic, with sperm concentrations less than 20×10^6 spermatozoa/mL (World Health Organization, 1999). All participants consented to the use of their semen for research purposes. After the semen liquefied, the specimen was mixed well and divided. One aliquot of each sample was immediately snap frozen (placed in a liquid nitrogen bath to induce rapid freezing), another aliquot was used for conventional semen analysis (Kinzer and Rothmann, 1998), and the remaining portion was placed in a TRANSEM semen transport container (Fertility Solutions Inc) and held at 22 ± 1°C for 24 hours to simulate overnight shipping and then snap frozen. Overnight shipping temperatures would be expected to vary in association with geographic location, season, or method of shipment. Royster et al (2000) determined that the TRANSEM semen transport container insulation protected specimens from the suboptimal temperatures that occur during air shipment. As a follow-up, our pilot study looked at the effects of ambient room temperature for 24 hours prior to snap freezing. Samples were stored in liquid nitrogen until being shipped overnight in a liquid nitrogen dry shipper (Taylor-Wharton) to the University of California, Los Angeles (UCLA), where they were coded and stored in −80°C without further treatment.

A pooled standard reference for sperm DNA/chromatin quality evaluation was created by combining semen from 3 healthy men. Samples were delivered to the UCLA laboratory within 1 hour of ejaculation. Routine semen analysis was conducted according to World Health Organization guidelines (World Health Organization, 1999). The pooled specimen was aliquoted into 2 groups, 1 of which was immediately frozen at −80°C (negative control) and the other X-irradiated (ex vivo) at room temperature with a dose of 15 Gy then frozen (positive control). X-ray irradiation was done at a dose rate of 535 cGy/minute (Mark 1-30 x-ray irradiator; Shephard & Associates, San Fernando, Calif).

Sperm-FISH Analysis for Chromosome Breakage

We used tandem-label sperm-FISH for chromosome breakage, as described in Rupa and Eastmond (1997) and modified by Robbins et al (2001), for buccal cell analysis. The probe mixture was 26.4 μL Oncor hybrizol hybridization buffer (Oncor Inc, Gaithersburg, Md), 1.2 μL direct fluorescein isothiocyanate (FITC)–labeled alpha satellite probe (Vysis Inc, Dowers Grove, Ill) targeting a small centromeric region of chromosome I, and 2.4 μL direct labeled-Cy3 probe (provided by Dr. D. A. Eastmond, University of California, Riverside) targeting the adjacent classical satellite II region in the heterochromatin of chromosome I. Posthybridization washes were conducted according to the method of Robbins et al (2001). Slides were counterstained with 9 μL of 0.025 μg/mL DAPI (Sigma Chemical, St Louis, Mo), mounted in VectaShield mounting media (Vector Laboratories, Burlingame, Calif), covered by a 22 × 22 mm cover slip, and stored at 4°C until scoring.

Sperm nuclei were observed via fluorescence microscopy using a Zeiss Axiophot microscope with DAPI/FITC/Texas Red triple-bandpass filter setup (61002) for simultaneous visualization of DAPI plus fluorochromes FITC and Cy3 (Chroma Technology, Brattleboro, Vt). A single-bandpass emission filter for green (41001; exciter 480/40, and emitter 535/50) or for red (41004; exciter 560/55 and emitter 645/75) were used to confirm discrimination of only green or only red signals during scoring. A single scorer systematically analyzed 10 000 distinct sperm cells per sample for aberrations in the 1cen-1q12 region. Hybridized regions with juxtaposed alpha (green) and classical (red) satellite signals were scored as an intact 1cen-1q12 region of chromosome I. A clear separation of greater than or equal to the diameter of the probe region was scored as a break when separated signal intensities were equal. Sperm cells were scored for breaks within the classical satellite region or between the classical and alpha satellite region.

TUNEL Assay for DNA Fragmentation

We conducted the TUNEL assay using the Fluorescein FragEL DNA Fragmentation Detection Kit (Oncogene Research Products, Cambridge, Mass). In brief, sperm samples were centrifuged at 1000 × *g* for 5 minutes at 4°C, resuspended in 4% formaldehyde (1 × phosphate-buffered saline [PBS]) at a cell density of 1×10^6 cells/mL, and incubated at room temperature for 10 minutes. At the end of incubation, samples were centrifuged at 1000 × *g* for 5 minutes (at 4°C), resuspended in 80% ethanol, and stored at 4°C. On storage removal, 10 μL of well-mixed specimen was smeared onto a clean microscope slide and allowed to air dry at room temperature for 24 hours. Slides were immersed in 1× Tris-buffered saline (TBS; FisherBiotech) for 15 minutes at room temperature. Two micrograms of proteinase K (Amresco, Solon, Ohio; 2 mg/mL proteinase K diluted 1 : 100 in 10 mM Tris [pH 8]) was added to each sample prior to a 5-minute incubation at room temperature. Sperm was rinsed in 1× TBS and dried. Once dry, samples were incubated in 35 mL of 1× TdT equilibration buffer (Fluorescein FragEL) for 30 minutes at room temperature in a 75-mL coplin jar. Slides were removed, and 60 μL of TdT labeling reaction mix (57 μL Fluorescein FragEL TdT labeling reaction mix and 3 μl TdT enzyme) was applied to each slide; the slides were covered with parafilm and incubated at 37°C for 60 minutes in a humidified chamber. After incubation, the parafilm was removed, and slides were rinsed twice in fresh 1× TBS at room temperature for 1 minute. A 22 × 22 mm cover slip was mounted using Fluorescein FragEL Mounting Media, and slides were stored at 4°C. Each sample was treated in the absence of TdT to serve as its own control. A single scorer systematically analyzed 2000 cells per sample using a fluorescence microscope (Zeiss Inc, Thornwood, NY) equipped with a FITC filter (41001; exciter 480/40 and emitter 535/50) and categorized each cell as bright (a high concentration of TUNEL-positive signal within the sperm head), light (a low concentration of signal within the sperm head), or dark (no signal within the sperm head).

Comet Assay for DNA Fragmentation

Modifications for the comet assay were based on existing methods described by McKelvey-Martin et al (1993), Hughes et al (1996), and Singh and Stephens (1998). All steps were conducted under reduced light, to prevent further DNA damage. Semen samples were thawed at room temperature, and approximately 1×10^5 cells were suspended in 500 μL 0.65% normal melting point (NMP) agarose (FisherBiotech) and Ca^{2+}- and Mg^{2+}-free PBS at 45°C. Partially frosted microscope slides were covered with 60 μL of 0.7% NMP agarose in 1× PBS, to permit the rigid attachment of subsequent agarose layers. A volume of 50 μL of the sperm cell suspension was layered on top and covered with a 24 × 50 mm cover slip. After solidification of the second layer, the cover slip was removed, and a third layer of 150 μL of 0.65% NMP agarose at 45°C was deposited and covered with a 24 × 50 mm cover slip. After solidification of the third layer and the removal of the cover slip, the slides were incubated in freshly prepared cold lysing solution (2.5 M sodium chloride, 10 mM Tris base, 100 mM EDTA disodium salt, 1% sodium lauryl sarcosine, and 1% Triton X-100, all Sigma Chemical [pH 10], with 3.5 mg/35 mL proteinase K, Amresco) for 4 hours at 4°C in a 75-mL coplin jar. Slides were drained and placed equidistant in a horizontal electrophoresis unit (Midi-Horizontal System; Fisher Scientific, Pittsburgh, Penn) filled with fresh, cold 1× neutral electrophoresis running buffer (pH 9; 10× stock solution consisted of 3 M sodium acetate from FisherBiotech and 1 M Tris base) to a level 0.25 cm above the slides. Slides remained immersed for 20 minutes, to allow DNA to unwind (McKelvey-Martin et al, 1993). Microgel electrophoresis was done using Electrophoresis Power Supply-EPS 601 (Amersham Pharmacia Biotech Inc, Piscataway, NJ) at 5°C for 20 minutes (final parameters, 20 V, 31 mA, and 1 W). Slides were transferred to the DNA fixing solution (5 ml 10× stock solution ammonium acetate; FisherBiotech) in 45 ml of reagent alcohol (90% anhydrous ethyl alcohol, 5% methyl alcohol, and 5% isopropyl alcohol [v/v]; FisherBiotech). After 15 minutes, slides were placed in 50 mL of reagent alcohol (for 30 minutes) then air-dried at room temperature. Samples were run in duplicate.

A fresh 50-μL application of 0.1% YOYO-1 iodide (Molecular Probes, Eugene, Ore) in 10% DMSO (FisherBiotech) and 2% sodium dihydrogen phosphate (Sigma) served as the intercalating DNA stain for comet analysis. Slides were viewed using a fluorescence microscope (Zeiss) equipped with a FITC filter combination (41001; exciter 480/40 and emitter 535/50) at ×250 magnification (×20 objective, 10 eyepiece, and 1.25 optovar). A single scorer randomly selected and captured 50 cells using the LAI Automated Comet Assay Analysis System (LACAAS) (Loats Associates Inc, Westmister, Md) from each replicate slide (i.e., 100 cells for a given sperm population). LACAAS calculated a corrected image by scanning the uninterrupted background signal above and below the comet being analyzed, to generate an approximation of the actual shape and magnitude of the background field and subtracted that value from the total image to define the comet. Quantitative measurements included comet tail length (the number of pixels from the back of the comet head to the end of the tail), tail moment (the product of migration distance and normalized intensity integrated over the tail length), and percentage of DNA in the tail (the integrated tail intensity × 100 divided by the total integrated cell intensity).

Table 1. *Selected semen parameters, by subject, in the pooled standard reference group for sperm quality evaluation*

Subject	Volume, mL	Concentration, Million Sperm Cells/mL	Total Count, Sperm Cells/ Ejaculate	Motility, %
1	2.1	135	283	75
2	5.3	75	395	15
3	3.2	230	734	90

Statistical Evaluation

The hypotheses that chromosome breakage and DNA fragmentation did not differ between fresh frozen (FF) semen and semen frozen after simulated shipping (F24) were tested using the 2-sided paired Student's *t* test. The 50 comet observations per subject, within each condition, were averaged prior to using the paired *t* test. Sperm-FISH data and comet data (tail moment and tail length) were transformed [reciprocal square root and $\log(x + 1)$, respectively]. TUNEL data were normally distributed. Within- and between-subject variability was determined using repeated-measures analysis of variance. Data are presented as group mean ± SE unless otherwise stated. Calculations were done using Microsoft Excel, Splus (Insightful, Seattle, Wash), and STATA7 (Stata Press, College Station, Tex) statistical software packages. $P \leq .05$ was considered to be statistically significant.

Results

Pooled Standard Reference

All subjects in the pooled standard reference group exceeded the minimum World Health Organization reference values (World Health Organization, 1999) for volume, sperm concentration, morphology, and total count. One man fell below the more than or equal to 50% motility reference (Table 1).

Pooled standard reference specimens, exposed to x-irradiation (1, 5, 10, and 15 Gy) and measured by neutral comet assay, exhibited dose-dependent DNA damage (Table 2). Interslide variation between the means of control slides showed coefficients of variation ranging from 0.10% to 0.86% in irradiated cells and 1.6% to 3.5% in nonirradiated cells. (Table 3).

The reproducibility of the comet software was studied using the irradiated and nonirradiated semen references. Twenty cells were chosen at random from positive (Figure 1) and negative (Figure 2) control slides. Each cell was analyzed 10 times (Hughes et al, 1997). Coefficients of variation less than 2.4% for repeated comet tail-length analysis of individual irradiated cells demonstrated reproducibility of the image analysis software for tail length. Coefficients of variation ranged 2.9% to 13.0% for per-

Table 2. *DNA damage detected by tandem-label sperm–FISH and neutral comet assay in pooled reference sperm cells exposed ex vivo to X-irradiation*

Dose, Gy	Total Number of Chromosome Breaks*	DNA in Tail, %	Tail Moment†	Tail Length, mm
0	6	3	1	22
1	6	23	21	121
5	11	35	36	165
10	10	44	52	217
15	16	51	67	250

* Sum of the breaks within and between classical and alpha satellite DNA on chromosome I.

† Product of the distance of migration and normalized intensity, integrated over the tail length.

Tandem-label FISH breakage is reported as events per 3000 cells scored. The test for linear trend was significant at $P = .02$. Neutral comet parameters are reported per 200 cells scored. The test for linear trend was significant at $P = .03$, $P < .01$, and $P = .03$ for percentage DNA, tail moment, and tail length, respectively.

centage of DNA in the tail and 11.0% to 55.0% for tail moment, which suggests these measures may be less reproducible than comet tail length. Coefficients of variation for tail length in nonirradiated cells averaged 10.2% (data not shown).

The tandem-label sperm-FISH assay also detected dose-dependent DNA damage in the pooled standard reference exposed ex vivo to x-irradiation. (Table 2). Induced breakage was detected at a lower dose by comet than by sperm-FISH assay. However, both assays demonstrated statistically significant increases in DNA damage with increasing doses of x-irradiation.

Immediate Versus Delayed Freezing

Tandem-label sperm-FISH detected no significant difference in chromosome breakage between FF and F24 cells; neither breaks within the classical satellite region ($P = .22$, Student's t test), nor breaks between classical and alpha satellite region ($P = .71$, Student's t test). Mean frequency of total chromosome breakage in FF sperm was 10.5 ± 1.3 breaks per 10 000 cells scored versus 11.2 ± 1.1 breaks per 10 000 cells in F24 sperm ($P = .69$, Student's t test). (Table 4). Sperm-FISH hybridization efficiency was 99.5%.

DNA fragmentation detected by TUNEL increased for F24 compared with FF, but this did not reach statistical significance. (Table 5). The mean frequency of DNA strand breakage in FF sperm was 136 ± 29 TUNEL-positive cells per 2000 cells scored versus 213 ± 28 TUNEL-positive cells per 2000 cells in F24 sperm ($P = .07$, Student's t test). Differences between cells exhibiting a slight signal (light; $P = .15$, Student's t test) and no signal (dark; $P = .07$, Student's t test) also did not reach statistical significance as based on sample age.

One semen sample in the study population had a low sperm concentration (11.6×10^6 cells/mL), which was problematic for the assessment of multiple DNA assays; thus, although all 10 samples were analyzed by sperm-FISH and TUNEL, only 9 samples had adequate cells remaining for the comet assay. Selected comet parameters of the study population are shown in Figure 3. Seven semen samples exhibited less DNA damage (depicted as a decrease in the percentage of DNA in the tail and tail moment) and shorter comet tail lengths after 24 hours of exposure to ambient temperature.

The neutral comet assay detected a statistically significant difference in DNA damage between FF and F24 sperm (Table 5). FF cells revealed more strand breaks. The mean frequency of double-stranded DNA damage (denoted by tail length in μm) for FF sperm was 175.0 ± 15.5 μm per 100 cells scored versus 152.2 ± 17.6 μm per 100 cells in F24 sperm ($P = .033$, Student's t test). Differences between FF and F24 sperm were also significant as based on the frequency of double-strand breaks expressed as percentage of DNA in the tail ($P = .037$, Student's t test) and tail moment ($P = .006$, Student's t test). Within-subject cell-to-cell variability for moment, percentage of DNA in the tail, and length was approximately 4 times as large as the between-subject variability for these same comet parameters.

Discussion

The TRANSEM100 semen collection and transportation kit (Fertility Solutions) was designed to improve geographic diversity and participation rates in population-based environmental studies evaluating reproductive effects (Royster et al, 2000). A previous study revealed moderate compliance with instructions resulting in more than 90% of the kits being correctly assembled and 65%

Table 3. *Selected DNA integrity measurements by neutral comet assay (pH 9) in the positive and negative pooled reference groups for sperm quality evaluation (100 sperm cells scored per sample) (n = 4)*

Pooled Reference	DNA in Tail, % Mean ± SD	CV, %	Tail Moment,* Mean ± SD	CV, %	Tail Length, μm Mean ± SD	CV, %
Positive	30.81 ± 0.27	0.86	32.91 ± 0.04	0.10	202.19 ± 0.70	0.34
Negative	8.61 ± 0.30	3.5	13.84 ± 0.26	1.9	128.36 ± 2.00	1.6

* Product of the distance of migration and normalized intensity, integrated over the tail length.

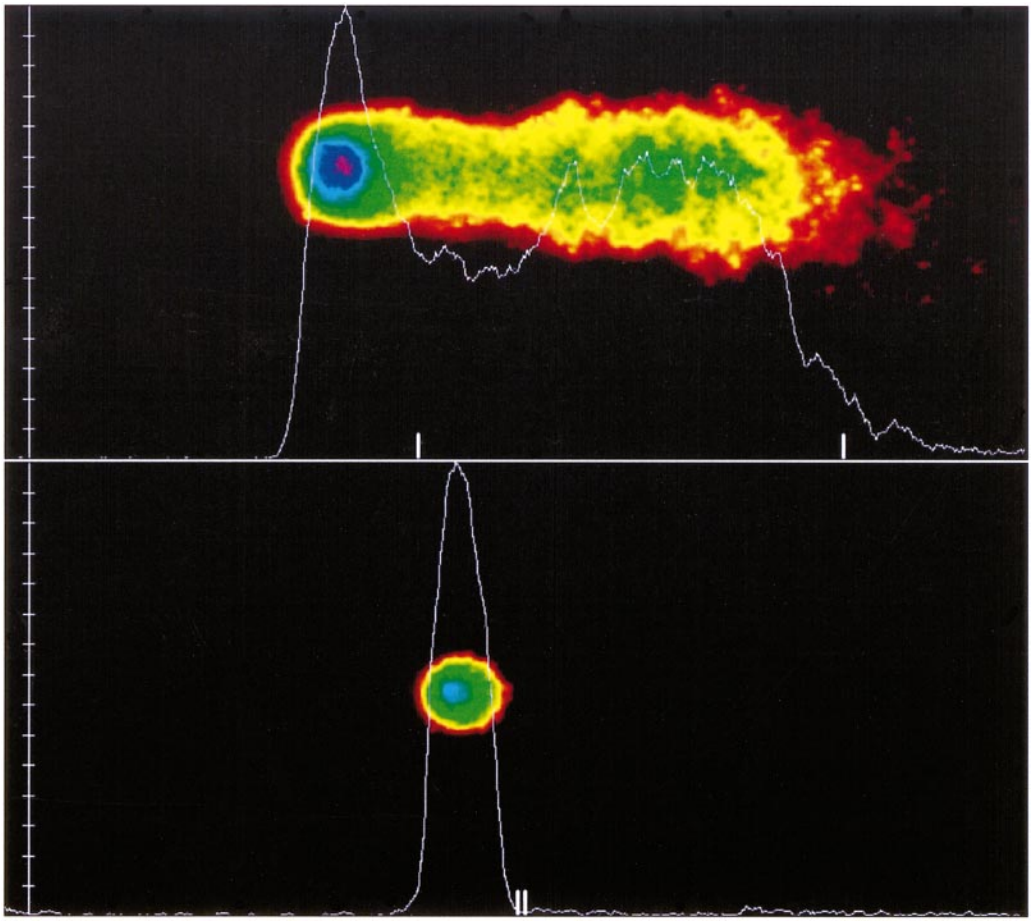

Figure 1. Fluorescence photomicrograph of human sperm (×250) after exposure to 15 Gy x-irradiation, single-cell microgel neutral electrophoresis, and YOYO-1 iodide staining. The electrical field drew negatively charged DNA strand breaks away from the nucleus toward the anode. The tail represents the extent of migration from the sperm nucleus in micrometers. Hatch marks on the scale bar are equivalent to approximately 8 μm. Selected LACAAS measurements were 70.9% DNA in the tail, 73.2 (tail moment), and 213.0 μm (tail length).

Figure 2. Fluorescence photomicrograph of healthy human sperm (×250) after single-cell microgel neutral electrophoresis and YOYO-1 iodide staining. The sperm nucleus is seen as a bright fluorescent nuclear core. Selected LACAAS measurements were 0.2% DNA in the tail, 0.0 (tail moment), and 4.0 μm (tail length).

being returned within the overnight timeframe (Royster et al, 2000). Postshipping semen analysis confirmed that count and morphology remained within World Health Organization reference guidelines (1992), which suggested that the TRANSEM100 kit could provide a reliable method for semen transport from field studies (Royster et al, 2000).

Table 4. *Chromosome breakage measured by tandem-label sperm–FISH (10,000 sperm cells scored per sample)*

	Fresh Frozen, Mean ± SE	Frozen After 24 h, Mean ± SE	*P*
Within*	5.6 ± 1.5	6.0 ± 1.6	.22
Between†	4.9 ± 0.6	5.2 ± 0.8	.71
Sum‡	10.5 ± 1.3	11.2 ± 1.1	.69

* Number of breaks within the classical satellite DNA on chromosome I.
† Number of breaks between the classical and alpha satellite DNA on chromosome I.
‡ Sum of breaks within and between the classical and alpha satellite DNA on chromosome I.

No previous studies have evaluated the clinical or research usefulness of DNA damage parameters in semen samples collected at remote sites and delivered to a central laboratory via overnight shipper. In the current study, our hypothesis was that chromosome/DNA damage in ejaculated spermatozoa would not increase significantly if cells remained in seminal fluid at ambient temperature for 24 hours, a first step in assessing the potential use of remote semen collection for DNA biomarker studies.

Tandem-label sperm-FISH is an efficient and reliable method to detect breakage in sperm chromosomes (Robbins et al, 1997, 2001; Sloter et al, 2000; Ong et al, 2002). The method reduces analysis time while enhancing sensitivity to particular regions that are prone to breakage (Rupa et al, 1995). However, hybridization is limited to

Table 5. *DNA strand breakage, measured by TUNEL assay (2000 sperm cells scored per sample) and neutral comet assay (100 sperm cells scored per subject)*

	Fresh Frozen, Mean ± SE	Frozen After 24 h, Mean ± SE	*P*
TUNEL			
Bright*	136 ± 29	213 ± 28	.07
Light†	292 ± 48	370 ± 27	.15
Dark‡	1572 ± 75	1417 ± 48	.07
Comet			
DNA in tail, %	38.1 ± 5.1	29.3 ± 4.3	.037
Tail moment§	36.0 ± 5.4	27.8 ± 6.8	.006
Tail length, μm	175.0 ± 15.5	152.2 ± 17.6	.033

* Number of cells with high concentration of TUNEL-positive signal within the sperm head.

† Number of cells with low concentration of TUNEL-positive signal within the sperm head.

‡ Number of cells with no TUNEL-positive signal within the sperm head.

§ Product of the migration distance and normalized intensity, integrated over the tail length.

only one region, and breakage induced at other sites on chromosome I or throughout the genome will go undetected (Rupa et al, 1995), which is a limitation of this assay if other targeted breakage sites are expected. Exposures that induce breakage randomly across the genome would not be affected by this limitation. Estimations are that 85% of chromosome aberrations found in newborns occur de novo in parental germ cells (Mohrenweiser 1991), with the majority of genetic mutations in sperm due to breakage rather than rearrangement (Donnelly et al, 2001). The ability to detect modifications at breakage-prone areas suggests that tandem-label sperm-FISH is a useful method to quantify early chromosomal alterations in human sperm.

No previous investigations comparing chromosome breakage in fresh frozen semen versus aged specimens were identified. In the current study, our focus was on breakage-prone areas in the pericentric and centromeric regions of chromosome I, in an effort to assess whether conditions during transport would induce damage. The number of chromosome breaks (within the classical satellite region and between the classical and alpha satellite regions) were not statistically different between FF and F24 sperm, which suggests that breakage in this region did not increase under these conditions. The mean frequency of total chromosome breakage in our study was consistent with the results of a recent report of one breakage event in chromosome I per 1000 sperm cells scored (Robbins et al, 2001). Finding no difference in chromosome breakage between FF and F24 sperm suggests that remote semen collection and transport to a central laboratory (using a container such as TRANSEM100) would be possible for field studies looking at chromosome breakage at a level detectable by the tandem-label sperm-FISH assay.

TUNEL can be used to quantitate endogenous DNA strand breaks in sperm nuclear chromatin (Sailer et al, 1995; Smith and Haaf, 1998). No previous investigations comparing endogenous DNA breakage in sperm FF versus F24 specimens were identified. Our focus was to assess whether endogenous DNA damage would increase significantly because of conditions during remote collection and transport. TUNEL approached but did not reach a statistically significant difference in endogenous DNA fragmentation between FF and F24 sperm. The percentage of positive cells detected by TUNEL that have been reported in other laboratories ranged 1% to 40% (Aravindan et al, 1997; Sun et al, 1997). The percentage of TUNEL-positive cells in the current study ranged 2.8% to 18.2% across donors and freezing conditions. TUNEL is specific for endogenous breakage, given that undamaged cells and cells in necrosis or that have been exposed to irradiation are only minimally labeled (Gorczyca et al, 1993). The ability to detect endogenous DNA damage suggests that TUNEL is a useful method to quantify DNA breakage in sperm. Because the power to detect a statistically significant difference between groups was only 75%, further investigation using larger sample sizes is necessary to determine the utility of remote collection kits with overnight return to a central laboratory for TUNEL analysis.

Numerous reports have been published that have used the comet assay to quantitate double-stranded DNA breaks in somatic cells (Östling and Johanson, 1984; Olive et al, 1991, 1995; McKelvey-Martin et al, 1993; Fairbairn et al, 1995; Collins et al, 1997a,b; Marples et al, 1998; Olive, 1999; Rojas et al, 1999; Tice et al, 2000), but it has only recently been adapted for use in human sperm (Singh et al, 1989; Hughes et al, 1996, 1997, 1999; Duty et al, 2002; Morris et al, 2002). A comparison of DNA damage between somatic and germ cells have shown elongated spermatids, and spermatozoa exhibit more damage (Hughes et al, 1996, 1999; Anderson et al, 1997) because the damage is not repaired (Van Loon et al, 1993) because of the loss of cytoplasm and repair enzymes during spermiogenesis (Sega 1974, 1976; Singh et al, 1989; Sega and Generoso 1990; Genesca et al, 1992). Another consideration when using comet analysis for sperm cells is the lack of stoichiometric staining between DNA complexed in the head, with strands of DNA more accessible to stain because they are pulled out in the comet tail. The comet assay continues to undergo modification for studies of human sperm (Perreault et al, 2003).

The ability to detect sperm DNA integrity without introducing further damage through analytical preparation (as might occur during remote collection and overnight shipper to a central laboratory) suggests that the neutral comet assay (pH 9) may be a robust method for assessing

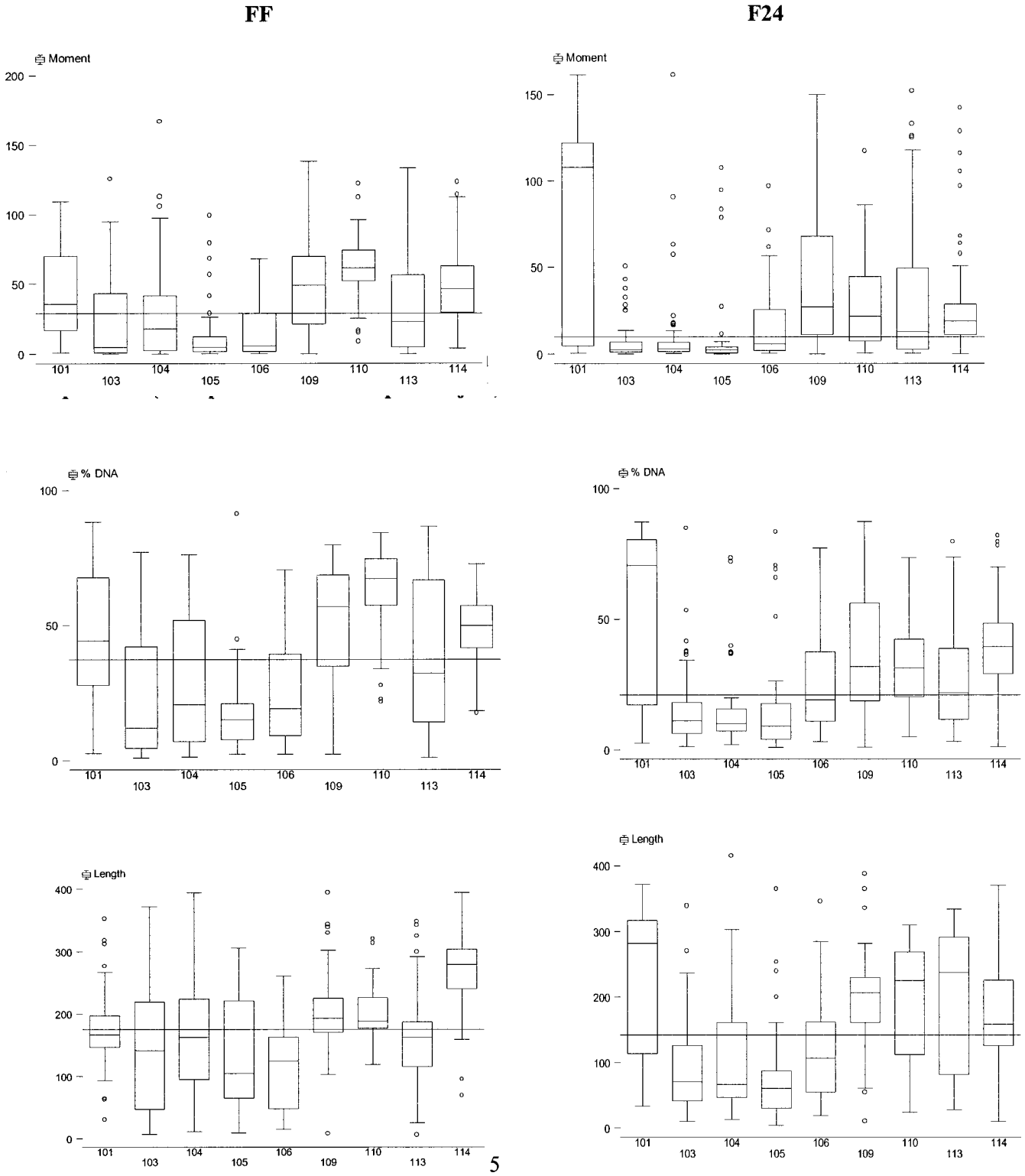

Figure 3. Selected DNA Integrity measurements by neutral comet assay of the study population (100 sperm cells scored per subject).

double-strand breaks (Singh and Stephens, 1998; Olive, 1999) and thus led us to use it for this first test of remote collection for DNA outcomes. The current study assessed whether DNA damage significantly increased because of conditions during transport compared with immediate freezing. The neutral comet assay detected a significant difference in DNA fragmentation between FF and F24 sperm, measured as tail moment, tail length, and percentage of DNA in the tail. Tail moment, a function of the percentage of DNA in the tail and tail length, was the most sensitive indicator in this data set. The mean frequency of double-stranded DNA damage was lower in aged specimens than in FF sperm.

The discrepancy between statistically significant findings in the comet versus TUNEL data may be due to the small sample size or may stem from a confounding variable such as an alteration in chromatin stability. Exposure to ambient temperature (for 24 hours) may increase the opportunity for sulfhydryl bond formation, yielding a hyperstabilized nucleus that may resist strand separation during electrophoresis. If hyperstabilization occurs, then the addition of dithiothreitol prior to electrophoresis should reduce the S-S bonds and allow proper migration. An increase in sample stabilization may also be achieved by keeping the samples on ice (4°C; Duty et al, 2002) instead of at ambient temperature.

In the current study, standard reference aliquots were developed from pooled semen samples, and a portion were treated with x-ray (15 Gy) to create a positive external standard for comet analysis. The results of numerous studies have confirmed dose-dependent DNA damage using x-rays (Olive et al, 1990, 1995; Hughes et al, 1996, 1999; Singh, 1996; Duty et al, 2002). In general, when running the sperm comet assay, neighboring comets differ markedly in tail moment, tail length, and percentage of DNA in the tail, which suggests that the majority of variation in parameters is due to inherent cell-to-cell differences within specimens (Singh et al, 1989; Duty et al, 2002; Morris et al, 2002). However, as is seen in Table 3, cell-to-cell variability was lower in the irradiated cells. Interslide variation between the means of control slides suggested that variation in nonirradiated cells is the result of biological differences between cells (ie, live vs dead or mature vs immature cells), which is overcome by the damage induced after exposure to x-irradiation. Measurements between slides were highly reproducible, as has been seen in other studies (Hughes et al, 1997; Morris et al, 2002). We found comet tail length to be the most reproducible measurement, which agrees with the results of a recent study by Duty et al (2002).

The tandem-label sperm-FISH, TUNEL, and neutral comet assays are useful analytical techniques for laboratory-based studies of genomic integrity. DNA damage at a level detectable by TUNEL and neutral comet assay, but not tandem label sperm-FISH, may be affected by the remote collection and overnight return of semen specimens to a central laboratory and will require further study using larger sample sizes before their incorporation into reproductive epidemiology studies of ejaculated semen collected at sites remote from the laboratory.

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