Ten-year variation in semen parameters and sperm deoxyribonucleic acid integrity in a healthy fertile man

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Objective: To report parameters in semen samples and sperm DNA integrity in a healthy fertile volunteer over a 10-year period.
Design: Case report.
Setting: University-affiliated teaching hospital.
Intervention(s): None.
Patient(s): Semen samples from a nonsmoking healthy male volunteer of proven fertility aged from 40 to 50 years were collected and analyzed over a decade.
Main Outcome Measure(s): Semen parameters (sperm count, total sperm count, percentage of progressive motility grades a+b, morphology, and percentage of living spermatozoa) and sperm DNA integrity, measured by sperm chromatin structure assay (SCSA) and terminal uridine nick-end labeling (TUNEL) assay.
Result(s): Median (min–max) value of total sperm count was 330 (126–511) million. Motility and vitality presented a median of 50% (40%–75%) and 78% (53%–92%), respectively. Among semen parameters, morphology and vitality showed the lowest within-subject coefficient of variation (CV_w) and the total sperm count the highest (8.1% and 12.0% vs. 34.9%). Median values of DNA fragmentation index (DFI) and high DNA stainability (HDS) were 12.7% (7.9%–16.5%) and 6.5% (5.5%–8.2%), respectively. Sperm DNA fragmentation presented a median value of 8.9%, a minimum value of 1.4% and maximum value of 18.6%. Compared with TUNEL data, SCSA parameters (DFI and HDS) showed less variation over the data collection period (47.4% vs. 22.4% and 13.0%, respectively).
Conclusion(s): Our data show that in this healthy fertile volunteer, semen parameters and sperm DNA integrity remained normal, and no trend was observed over the study period. More interestingly, in this subject aged from 40 to 50 years old, sperm nucleus status presented less than 20% of sperm DNA fragmentation over a decade. (Fertil Steril® 2006;86:1513.e11–18. ©2006 by American Society for Reproductive Medicine.)

Key Words: Sperm DNA integrity, SCSA, TUNEL, semen variation, fertility

It has been reported that sperm DNA integrity is of paramount importance in the initiation and maintenance of a pregnancy in vivo and in vitro (1–4). Several studies using different assays, including measurement of DNA strand breaks, have shown that susceptibility to sperm DNA damage is higher in many cases of suspected male infertility than in fertile men(5, 6). The most commonly used techniques for assessing sperm DNA integrity are the terminal uridine nick-end labeling (TUNEL) assay, single-cell gel electrophoresis (also known as the Comet assay) and the sperm chromatin structure assay (SCSA). Recently, threshold values for DNA fragmentation have also been reported for SCSA and TUNEL in vivo (2, 4) and in vitro (1, 7). These threshold values are useful to set a level above which normal expression of the paternal genome, and thus pregnancy, could be compromised.

Although sperm DNA integrity promises to be a powerful biomarker of male fertility in vivo and in vitro, there have been few investigations of variation of sperm DNA status in a longitudinal protocol (less than 1 year). Using SCSA, Evenson et al. (8) measured individuality of DNA denaturation pattern in 45 unselected semen donors over an 8-month period. Interestingly, an intraclass correlation (ICC) was found for the mean and standard deviation of the sperm DNA fragmentation index (DFI) (previously named $COMP\alpha_t$) at 0.67. Compared with previous measures of semen parameters in a parallel study, ICC for sperm count was quite similar at 0.62 (9).

Recently, using TUNEL, we evaluated variations in the degree of sperm DNA fragmentation in 5 donors and 10 infertility patients over 6- and 8-month periods, respectively. We found that for infertile men and for men of proven fertility, sperm DNA fragmentation within-subject standard

Received December 1, 2005; revised and accepted March 7, 2006.
Supported by a grant from the Centre d'Etude et de Conservation des Oeufs et du Sperme Humain (CECOS) Toulouse Association, France. Dr. Sergerie is supported by a postdoctoral fellowship from Sidaction Organization (No. A015-2). His arrival in France was facilitated by the invaluable assistance of Kastler Foundation.
Presented in part at the 3rd International Congress on Male-Mediated Developmental Toxicity, Bradford, U.K., July 31–August 3, 2005.
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doi:10.1016/j.fertnstert.2006.03.067

deviation (SD_W) was small compared with between-subjects standard deviation (SD_B). In addition, the ICCs of sperm concentration and sperm DNA fragmentation were similar (0.86 and 0.83, respectively) for all subjects taken together. Our data also showed that sperm DNA fragmentation is a parameter with good stability over time and can be taken as a baseline in both healthy fertile men and patients from infertility couples (10). At the present time, there is a lack of information on variation of sperm DNA integrity, measured by SCSA and TUNEL, over a "very long" period of time.

CASE REPORT

This report describes, in a single volunteer, semen parameters recorded in native sperm (sperm count, total sperm count, progressive motility grades a+b, morphology, and vitality) and sperm DNA fragmentation, measured by SCSA and TUNEL, performed on frozen sperm over a 10-year follow-up.

MATERIALS AND METHODS

Semen samples from a nonsmoking healthy male volunteer of proven fertility were collected and analyzed from February 1995 to February 2005. At the end of the collection period, he was 50 years old. The healthy volunteer was enrolled to obtain a fertile control for our laboratory. He received minor compensation for the inconvenience (time, parking fees) of being involved as a semen volunteer. Medical examination was normal. The exclusion criteria for a healthy volunteer were occupational exposure to heavy metals, any previous treatment affecting spermatogenesis (such as chemotherapy, radiotherapy, and vasectomy), and the use of recreational drugs, including marijuana (THC), cocaine, or narcotics. Informed consent for voluntary participation was obtained.

At each visit, the usual questionnaire was completed concerning such items as time since last ejaculation. In addition, for each sample, the volunteer was questioned about any unusual events since his last visit to the laboratory, such as disease episodes, stressful condition(s), intake of medication(s), and/or dietary supplement(s) as well as any change in lifestyle habits. The time lapse between "event and effect" was taken into consideration with respect to the timing of spermatogenesis and epididymal sperm maturation. Factors known to contribute to variation in semen quality, such as abstinence, collection in a laboratory, medium, and laboratory technician, were minimized. An internal quality control has been carried out at the Centre d'Etude et de Conservation des Oeufs et du Sperme humain (CECOS) laboratory since 1992 to verify the intraobserver and interobserver variations for each technician. In the present study only one technician was involved in the semen analysis and presented acceptable agreement in intra- and interobserver variation (mean $<15\%$).

Sample Collection and Storage

Twenty semen samples with a mean ($\pm$SD) interval between each sample of 6.3 ($\pm$9.0) months were analyzed. Semen samples were collected by masturbation, after a recommended period of 3 to 6 days of sexual abstinence, into sterile polypropylene containers at the CECOS laboratory. Standard clinical semen analysis was performed according to World Health Organization (WHO) criteria (11) by a single technician who was blinded to the identity of the study subject. Semen samples were analyzed 30 minutes after the collection along with other routine specimens. Sperm count, total sperm count, motility, and sperm vitality were assessed according to previously published methods (12). Sperm morphology was analyzed according to the classification of David (13). This classification allows calculation of the Multiple Anomalies Index (MAI), which is the mean number of anomalies per abnormal sperm.

After sperm assessment, semen samples were cryopreserved within 1 hour of collection. For semen cryopreservation, a standard cryoprotectant (Freezing Medium; Irvine Scientific, Santa Ana, CA) was added in a single step at a ratio of 1:1 (vol/vol) of freezing medium to ejaculate. After mixing by repeated aspiration in and out of a 1-mL graduated pipette, the samples were transfered to 0.3-mL straws (Paillette CBS; CryoBio System, L'Aigle, France) and frozen in liquid nitrogen according to the standard procedures used for sperm banking in our laboratory until later "pooled" assessment of sperm DNA integrity.

One day before assessment of sperm DNA integrity, straws containing semen were removed from liquid nitrogen storage and thawed on ice. The stored sperm sample was suspended in 4.7 mL ice-cold TNE (0.01 mol/L Tris-HCl, 0.15 mol/L NaCl, and 1 mmol/L EDTA, pH 7.4) and centrifuged at 600*g* for 10 minutes. The pellets ($\sim 10 \times 10^6$ spermatozoa) were then resuspended in 1 mL ice-cold TNE and fixed with 4 mL 1% formaldehyde (Prolabo, Paris, France) in TNE (pH 7.4) for at least 30 minutes at 4°C. After centrifugation, the pellets were washed twice and each sample was then divided into 4 aliquots (2 for SCSA, 2 for TUNEL) of $\sim$2.5 million spermatozoa and then stored in ice-cold TNE with 0.1% sodium azide (Sigma Chemical Co., St. Louis, MO) at 4°C.

SCSA and Flow Cytometry

We used the SCSA procedure described by Evenson and Jost in 2000 (14), with minor modifications. Two hundred microliters of fixed sperm in TNE buffer ($\sim 2 \times 10^6$ cells/mL) were treated for 30 seconds with 400 μL of a solution containing 0.1% Triton X-100, 0.15 mol/L NaCl, and 0.08 N HCl, pH 1.2. After the 30-second acid treatment, 1200 μL of acridine orange (AO – chromatographically purified; Cat No. 04539, Polysciences, Warrington, PA) staining buffer (6 μg of AO/mL, 37 mmol/L citric acid, 126 mmol/L Na_2HPO_4, 1 mmol/L disodium EDTA, and 0.15 mol/L NaCl, pH 6.0) was admixed to the sperm cells before analysis by flow cytometry (14).