Longitudinal study of sperm DNA fragmentation as measured by terminal uridine nick end-labelling assay

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BACKGROUND: One major limitation in the use of sperm DNA fragmentation as measured by the TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) assay is the paucity of solid data on the stability of this parameter. METHODS: The objective of our study was to evaluate variations in the degree of sperm DNA fragmentation, as measured by the TUNEL assay, over a 6 month period. Five donors provided semen samples (total 107) on the average three times per month, and 10 infertility patients provided semen samples every 4 weeks (total 58). RESULTS: The mean percentage of sperm DNA fragmentation for donors was 13.18%, the within-donor standard deviation (SDW = 3.79%) was small compared to between-donor (SDB = 17.56%). For the group of patients, the mean percentage of sperm DNA fragmentation was 22.44%, with SDW of 4.43% within patients and SDB of 29.48% between patients. No seasonal rhythm was observed during the study. The intra-class correlation coefficient for all subjects combined was 0.83. Compared to sperm concentration, individual coefficients of variation for sperm DNA fragmentation indicated less variability in four subjects, but were similar in the others. CONCLUSION: This longitudinal study shows that sperm DNA fragmentation is a parameter with good stability (repeatability) over time; it can be taken as a baseline both in healthy fertile men and in patients from infertility couples.

Key words: DNA fragmentation/longitudinal study/male infertility/sperm/TUNEL

Introduction

One in six couples of reproductive age presents with infertility (Hull et al., 1985). In addition, some 200 000 conceptions worldwide are lost yearly before the 20th week of pregnancy, and some 25 000 babies are born annually with defects; in most cases, the causes remain elusive (Gopalkrishnan et al., 2000). Concern is growing over the possible impact that environmental and industrial chemicals and lifestyle factors could have on reproductive outcomes. The contribution of the male partner is emerging as significant in many aspects. In infertile couples, a male infertility factor is found in close to 50% of cases; due to our lack of knowledge, the aetiology remains unknown in as many as 70% of affected men (Sokol, 1995).

It is virtually impossible to measure accurately the fertility potential of a man on a quantitative scale. In laboratory animals and livestock, mating or artificial insemination in a large number of females can serve to assess accurately male fertility. In men, conventional semen analysis (sperm count, motility and morphology) remains the most widely used method of evaluating male fertility in clinical andrology, toxicology, epidemiology and risk assessment. While some obvious defects such as azoospermia and necrozoospermia are strict causes of sterility, semen parameters are generally poor indicators of fertility. A rare instance where male fertility potential can be measured with relatively high precision and accuracy is in the setting of therapeutic donor insemination where a true fertility index can be calculated.

Several new approaches have been developed to measure sperm motility, capacitation and sperm–oocyte interaction as indicators of fertility potential (Irvine and Aitken, 1986). While some offer major advantages over conventional semen analysis, limitations have thus far precluded their use on a large scale.

The level of sperm DNA fragmentation reflects the integrity of genetic material of the gamete. This parameter is important since DNA lesions of many types induce mutations commonly observed in mutated oncogenes and tumour suppressor genes (Marnett, 2000). Transmission of oxidatively damaged DNA to the offspring, particularly at levels that exceed the DNA repair capacity of the oocyte, could have serious consequences (Ahmadi and Ng, 1999).

TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) is a useful and sensitive method for assessing DNA strand breaks in human sperm (for review, see Shen and Ong, 2000), and there is significant
correlation with other techniques that evaluate oxidative DNA damage (Donnelly et al., 2000). Several studies employing different assays, including the 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 (Hughes et al., 1996; Shen et al., 1997; Sun et al., 1997; Lopes et al., 1998; Gandini et al., 2000; Host et al., 2000; Irvine et al., 2000). Interestingly, sperm DNA fragmentation is inversely correlated with the percentage of oocytes fertilized after IVF, indicating that this parameter reflects sperm fertilizing ability (Sun et al., 1997; Lopes et al., 1998; Host et al., 2000).

Few investigations have been undertaken to verify the stability of parameters measuring sperm DNA status in a longitudinal protocol. Using the sperm chromatin structure assay (SCSA), Evenson et al. (1991) have shown that the sperm DNA fragmentation index is a highly stable parameter over time, more stable than the classical semen parameters. One major limitation in the field is the dearth of data on the stability of DNA fragmentation as measured by the TUNEL assay. The objective of this study was to evaluate variations in degree of sperm DNA fragmentation over a 6 month period. The results were compared with those obtained for sperm concentration as this semen parameter is the one with the greatest biological variation (Alvarez et al., 2003).

Materials and methods

Study participants

Five donors supplied 107 semen samples over a 6–8 month period during 2000–2001. The donors (median age 24 years; range 22–29) underwent a standardized screening protocol, including minimum semen criteria and a physical examination with review of medical, family and infectious disease histories. These are current procedures in our centre in accordance with Health Canada’s Therapeutic Products Program guidelines for the processing and distribution of semen for assisted reproduction. Before participating in this project, each donor signed an informed consent form approved by the Ethics Committee of the Centre hospitalier de l’Université de Montréal (CHUM)–Hôpital Saint-Luc. At each visit, the usual questionnaire was filled out concerning such items as time since last ejaculation. In addition, each donor was questioned about the occurrence of any unusual event 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.

Ten patients (median age: 33 years; range: 30–42) were recruited from infertile couples scheduled to undergo intrauterine insemination as part of a randomized double-blind placebo-controlled study (these men were found to belong to the placebo group; a detailed description of the protocol and results will be presented elsewhere). The patients supplied semen samples at regular 4 week intervals over a period of 6 months; at each visit a questionnaire was filled out to record any unusual event during the last month.

Sample collection and storage

Semen samples were obtained by masturbation into sterile polypropylene containers (Sarstedt, Canada) at the Andrology Laboratory after 2–4 days of sexual abstinence, the actual duration of abstinence was recorded. After semen liquefaction, sperm concentration was measured according to World Health Organization (1999) criteria; this evaluation was blinded vis-à-vis donor identity as well as patient status.

Sperm from fresh semen (5 × 10⁶ sperm) were separated from seminal plasma by centrifugation (600 g) at room temperature for 10 min. The pellets were then resuspended in 1 ml of Dulbecco’s phosphate-buffered saline (PBS; Gibco BRL, Life Technologies, USA) and fixed in 1% formaldehyde (BDH Inc., Canada) in PBS (pH 7.4) for 30 min at room temperature. The pellets were washed twice and then stored in PBS at 4°C for up to 6 months.

Assay for DNA fragmentation

A detailed protocol for the TUNEL assay of human sperm has been published previously (Sergerie et al., 2000). Briefly, after centrifugation of fixed sperm (5 min at 5000 g), the supernatant was removed and 100 µl of terminal deoxynucleotidyl transferase (TdT) buffer containing single-strength 1 mol/l Na-cacodylate, 150 mmol/l Tris (pH 7.4), 25 mmol/l CoCl₂, and 0.1% Triton X-100 (Sigma Chemical Co., USA) was added. The pellet was resuspended and incubated for 10 min at room temperature. After re-centrifugation (5 min at 5000 g), the TdT buffer was removed, and 50 µl of TdT buffer containing 3 µmol/l of biotin 16-deoxyuridine triphosphate (Boehringer Mannheim, Canada), 10 IU of TdT (Boehringer Mannheim) and 0.1% Triton X-100 were added. The sperm suspension was then incubated at 37°C for 60 min. After two washes with TN buffer (30 mmol/l Tris, pH 7.4, 300 mmol/l NaCl), the fixed, permeabilized sperm were treated with 100 µl of staining AP buffer (50 mmol/l Tris, pH 7.4, 150 mmol/l NaCl) containing 2% streptavidin–fluorescein, followed by incubation at room temperature in the dark for 45 min. The stained sperm were washed twice with AP buffer before analysis. The samples were stored at 4°C in the dark until analysis.

Fluorescence measurements

DNA fragmentation was measured with a FACScan flow cytometer (Becton Dickinson, USA) equipped with a 15 mW argon-ion laser for excitation at 488 nm. Flow during analysis was controlled at ~300 sperm/s, and 10 000 cells were analysed in each sample. Light-scattering and fluorescence data were obtained at a fixed gain setting in logarithmic mode. Debris were gated out by establishing a region around the population of interest, on the basis of light scatter characteristics of swim-up selected sperm. These characteristics were assessed in several swim-up selected sperm samples before starting the study (M.Sergerie et al., unpublished data). The same gates were applied to all samples. The percentage of labelled sperm was determined by setting a region that included >90% of events in the frequency histogram of the positive control (DNase I). Here, 10 000 events were collected and the coefficient of variation on this measure was 2.1% as evaluated from readings of the same sample within an assay occasion. The sperm gate was set to permit slight sample-to-sample variation, taking into account the light-scattering properties of the asymmetrical sperm head. The between-batch analytical coefficient of variation (CVₐ) was 3.7% as calculated from values obtained for six aliquots of a semen sample; these aliquots had been fixed, washed and stored before assay for DNA fragmentation as described above for the semen samples. For negative control, TdT was omitted from the reaction mixture. For positive control, the sperm were pretreated with 0.11U deoxyribonuclease (DNase I, RNase-free; Boehringer Mannheim) for 30 min at room temperature before labelling. Typical examples of such results are shown in Figure 1. Illustrations are presented of typical frequency histograms.
obtained by flow cytometry with markers (M1) for the detection of fluorescence; emission was monitored at 515–535 nm. Figure 1 illustrates a typical negative control (0% sperm labelling), and a positive control treated with DNase I (98% labelling).

Statistical analyses
SigmaStat 2.0 (Jandel Corp., USA) was used for data analysis. Sperm concentration for the donors did not show normal distribution, values were thus cube root-transformed. Reported means are back-transformations of mean cube roots; this value was then subtracted from the back-transformation of ‘mean cube root plus its SD’ to obtain the standard deviation above the mean. Sperm concentration for patients, and percentage DNA fragmentation for all subjects were normally distributed. Statistics (SPSS 9.0) for the latter parameters included between-subject standard deviation (SDb calculated by 2-way ANOVA without interaction); within-subject standard deviations (SDw also calculated by 2-way ANOVA without interaction); and within-subject coefficient of variation (CVw: SDw relative to mean, as percentage). The intra-class correlation coefficient for sperm DNA fragmentation (ICC: ratio of between-subject variance to the total variance; this is a measure of repeatability) was calculated after combining values for donors and patients. Since only one value per month was available for the patients, we chose the mean value of each month for the donors so as to avoid empty cells in the analysis; choosing the first value of each month had little effect on the ICC.

Results
The donors provided an average of three sperm samples per month; individually, their mean period of abstinence prior to sample collection ranged from 2.1 to 2.8 days. According to our Andrology Laboratory log, these donors had averaged 6.0 ± 4.5 fathered pregnancies. Each donor experienced at least one minor event (defined above) during the study period; however, no relationship was found between any of these events and semen parameters as well as sperm DNA fragmentation. For the patients, the mean duration of abstinence was 2.6 days (median 2.5; only one sample exceeded 4 days of abstinence), and only few minor events such as sore throat without bacterial infection and minor injuries were reported during the study period. For both groups, all semen samples had motile sperm and none showed significant leukocytospermia as per World Health Organization criteria (data not shown).

Table 1. Descriptive statistics of sperm DNA fragmentation for donors and patients

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Mean (SD) (%)</th>
<th>Median (%)</th>
<th>Interquartile range (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donors</td>
<td></td>
<td>(SD) (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>23</td>
<td>17.0 (3.53)</td>
<td>16.4</td>
<td>14.0–18.8</td>
<td>20.7</td>
</tr>
<tr>
<td>B</td>
<td>16</td>
<td>12.5 (4.52)</td>
<td>14.5</td>
<td>9.3–15.8</td>
<td>36.1</td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>15.1 (4.58)</td>
<td>13.9</td>
<td>12.3–17.4</td>
<td>30.3</td>
</tr>
<tr>
<td>D</td>
<td>25</td>
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<td>6.4</td>
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</tr>
<tr>
<td>E</td>
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<td>13.7 (2.77)</td>
<td>13.5</td>
<td>11.1–16.1</td>
<td>20.3</td>
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<tr>
<td>Patients</td>
<td></td>
<td>(SD) (%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>6</td>
<td>17.5 (7.15)</td>
<td>17.3</td>
<td>12.2–19.4</td>
<td>40.9</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>12.3 (1.77)</td>
<td>12.6</td>
<td>11.0–13.5</td>
<td>14.4</td>
</tr>
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</tr>
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<td>25.4–49.9</td>
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</tr>
<tr>
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<td>6</td>
<td>13.7 (1.76)</td>
<td>14.4</td>
<td>11.8–14.8</td>
<td>12.9</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>21.8 (7.09)</td>
<td>20.6</td>
<td>16.5–29.2</td>
<td>32.6</td>
</tr>
<tr>
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<td>6</td>
<td>40.6 (5.85)</td>
<td>40.4</td>
<td>37.3–44.4</td>
<td>14.4</td>
</tr>
</tbody>
</table>

aPercentage of sperm DNA fragmentation.

n = number of semen samples over study period; CV = coefficient of variation.
Basic descriptive statistics for percentage of sperm DNA fragmentation are presented in Table I. The overall mean for all semen samples from donors was 13.18%; fragmentation SDW (3.79%) was small compared to SD B (17.56%). The evolution of sperm DNA fragmentation for each donor during the study period is shown in Figure 2; individual values ranged from 3.07 to 26.74%. It can be noted that the values for within individuals are more tightly clustered than those between individuals. No seasonal rhythm was observed over this period: the median percentages of sperm DNA fragmentation ranged from 11.69% in March to 15.90% in September.

Corresponding values for patients are presented in Table I; the overall mean percentage of fragmentation was 22.44%. Individual means varied over a wider range than in donors, with three patients showing relatively high values. However, as in the case of the semen donors, SDW (4.43%) was small compared to SD B (29.48%). Here also, no seasonal rhythm was found.

An ICC of 0.83 was calculated for combined data from donors and patients (total n = 15) over a 6 month period.

**DNA fragmentation**

![Figure 2. Evolution of sperm DNA fragmentation (%) for each donor during the study period.](image)

**Sperm concentration**

The evolution of sperm concentration for each donor is presented in Figure 3. All five donors maintained high sperm counts throughout the study period, with mean values >100 × 10^6/ml; interquartile values ranged from 88 to 313 × 10^6/ml (Table II). Individual coefficients of variation varied between 25.6 and 41.3%. No component rhythm was observed: median sperm concentrations ranged from 130 × 10^6/ml in March to 103 × 10^6/ml in September, and values tended to be lower during the fall (September to November) compared to the other seasons, but the differences were not statistically significant.

As expected, the means of sperm concentration for patients were much lower than for donors (Table II). However, the intra-class correlation coefficient (ICC = 0.86) and interquartile range indicate that the patients maintained their original sperm count value at fairly repeatable levels over time. Assessing for possible seasonal changes in patients, we found that mean monthly values tended to be higher in April, but no statistically significant difference was found. Here also, individual coefficients of variation were quite large: form 16.7 to 63.2%.

![Figure 3. Evolution of sperm counts (10^6/ml) among donors during the study.](image)
Patients

Discussion

In light of the increasing interest in evaluating the integrity of the genetic material of sperm, the most interesting observation in this study was the within-subject stability of sperm DNA fragmentation. This indicates that the TUNEL assay can provide a baseline measurement of semen quality for an individual. The results proved repeatable both in semen donors with high-quality semen as well as in men from infertile couples presenting with low sperm count and/or motility. This allows baselines to be established against which deviations can be appreciated as indicative or not of a physiological or pathological condition affecting the sperm.

We calculated the number of tests needed to estimate the homeostatic set point for sperm DNA fragmentation. We used the formula derived by Fraser and Harris (1989), with an analytical coefficient of variation (CVₐ) of 3.7% and a biological within-subject CV of 27.9%. At 80% confidence, one would require three semen samples to obtain an estimate within ±20% of the true set point (e.g. if true set point is 50%, the mean value of triplicates should statistically be in the range of 40–60% sperm DNA fragmentation). Using this number of tests is feasible in many research projects; however, if small deviations from set point are expected, it can be limiting, mostly in studies on large cohorts. In a clinical context, with such variability and assuming a normal value of <25% DNA fragmentation, our data suggest that a result <20% would not require a retest. On the other hand, an abnormal high value needs confirmation.

It is difficult to explain the variability observed in this study. One main reason is that the causes of sperm DNA fragmentation are still unclear. Three main hypotheses have been proposed: oxidative damage to sperm DNA, incomplete maturation during spermiogenesis, and possibly apoptotic degeneration. It is well established that reactive oxygen species, produced by the sperm themselves or originating from other sources such as leukocytes, can damage sperm and induce oxidative damage to DNA. A number of review articles have comprehensively discussed these issues (Griveau and Le Lannou, 1997; Storey, 1997; Aitken, 1999; Sakkas et al., 1999). Evidence from the literature indicates that DNA in the sperm of infertile men is less well-protected and could suffer more damage in vitro than the sperm of fertile men (Shen and Ong, 2000). A high degree of sperm DNA fragmentation could also result from imperfect spermiogenesis: nicks within DNA of elongating spermatids, elicited by an endogenous nuclease, are seen during the replacement of histones by transition proteins; these could persist if not repaired prior to the completion of spermiogenesis (Manicardi et al., 1998). Therefore, the presence of DNA fragmentation in ejaculated sperm might correlate with defects in spermatogenesis, as suggested by Gandini et al. (2000). DNA fragmentation is more evident in atypical forms (Lopes et al., 1998), confirming that sperm count and morphology correlate with testicular function. It has also been reported that poor sperm DNA integrity, as assessed by the TUNEL assay, can negatively influence sperm-fertilizing capacity in vitro (Sun et al., 1997; Lopes et al., 1998), although sperm DNA damage does not preclude IVF (Twigg et al., 1998).

Conditions that induce apoptosis (Gandini et al., 2000) or a defect in the normal apoptotic process during spermatogenesis (Sakkas et al., 1999) were thought to account for part of the DNA fragmentation observed in ejaculated sperm. However, the contribution of this mechanism would be minimal (Henkel et al., 2004; McVicar et al., 2004; Moustafa et al., 2004).

In the framework of our study, several factors could contribute to the within-subject variation of sperm DNA fragmentation. One of them is a strong fever that can induce changes in sperm chromatin composition and structure (Evenson et al., 2000). Despite testing over a 6 month period, and even though all donors reported at least one unusual ‘event’ during the study, none of the subjects suffered from very high fever. After its acute effects on spermatogenesis (over a few months), a high fever is unlikely to cause irreversible change in the baseline level of sperm DNA fragmentation. Measuring this parameter at defined periods after the fever episode should readily evaluate its impact.

In the geographical region of Montreal, seasonal extremes in environmental temperature cover the range of −30°C to +30°C, and differences in length of the daylight period are considerable. In addition, the composition of the diet and the origin of food (e.g. fresh vegetables) vary with seasons. This raised concern about possible seasonal variations in sperm DNA fragmentation. The stability of this parameter over time, as observed in this longitudinal study, indicates no significant effect of season. These results are well in line with those of Evenson et al. (1991) who reported no significant seasonal effect on the parameters of the SCSA. The situation is less clear in the case of sperm concentration. Our results tend to indicate lower values in the fall, but the differences did not reach statistical significance. Most other longitudinal

### Table II. Descriptive statistics of sperm count for donors and patients

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Mean (SD)a (10⁶/ml)</th>
<th>Median (10⁶/ml)</th>
<th>Interquartile range (10⁶/ml)</th>
<th>CV (%)</th>
</tr>
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<tbody>
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<td>Donors</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>23</td>
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<td>122.0</td>
<td>97.5–144.5</td>
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<tr>
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<td>116.1 (40.5)</td>
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<td>91.0–130.0</td>
<td>34.9</td>
</tr>
<tr>
<td>C</td>
<td>25</td>
<td>127.2 (40.1)</td>
<td>122.0</td>
<td>95.5–160.0</td>
<td>31.5</td>
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<tr>
<td>D</td>
<td>25</td>
<td>249.9 (102.1)</td>
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<td>199.5–313.0</td>
<td>40.9</td>
</tr>
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<td>88.0–122.0</td>
<td>41.3</td>
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<td></td>
</tr>
<tr>
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<td>37.0</td>
<td>30.0–41.0</td>
<td>27.6</td>
</tr>
</tbody>
</table>

*aMean for each donor is back-transformation of mean cube root; SD above mean was calculated after back-transformation of cube root values (see text). n = number of semen samples over study period.*
studies also failed to find statistically significant seasonal changes in sperm concentration (Malladis et al., 1991; Carlsen et al., 2004), even at 69° N latitude (Malm et al., 2004). On the other hand, significant seasonal variations were reported in sperm donors but not in andrology patients (Centola and Eberly, 1999).

Small differences in numbers of days of sexual abstinence can have significant effect on sperm concentration. Hence, confirming previous reports, Carlsen et al. (2004) found increments in sperm concentration of 25% per day during the first 4 days of abstinence. In contrast, Evenson et al. (1991) and De Jonge et al. (2004) found no influence of length of abstinence period on the DNA fragmentation index (using SCSA), while Richthoff et al. (2002) reported a relatively weak positive correlation between these two parameters. In our study, 90% of the semen samples were produced after either 2 or 3 days of abstinence; a comparison on paired data of these two intervals did not reveal any significant difference in percentage DNA fragmentation as measured by the TUNEL assay. Values at other intervals were generally within the interquartile range. We may conclude that length of abstinence contributes very little, if any, to the biological variation of sperm DNA fragmentation. This represents one of the advantages of this method over the measurement of sperm concentration. In clinical field studies, sperm count remains the most widely used parameter for the assessment of male reproductive potential (Zinaman et al., 2000). However, variability is a problem due to large inter-sample and inter-observer differences for a given subject at different time-points.

In our study, the within-subject variation was quite small and tended to be lower in donors compared to infertility patients. In this regard, it was reported that the repeatability of values for sperm DNA stability, as measured by the SCSA, is higher in men with high compared to low quality semen (Evenson et al., 1999).

The variation of sperm DNA fragmentation between subjects was much larger than within subjects. As expected, this is more evident in the group of patients: elevated mean values in some of the patients, combined with small within-subject variations, indicate a high set point, possibly associated with a cause of male infertility.

In conclusion, the results of this study may be relevant to the investigation of donors for optimized quality control. The degree of DNA fragmentation in sperm from semen donors is highly stable and our accumulating statistics indicate a correlation between the fertility index (pregnancy per cycle of therapeutic donor insemination) and the degree of sperm DNA fragmentation for these semen donors (M.Sergerie et al., unpublished data). The data also have clinical importance for the assessment of patients on an individual basis, as well as in the design of protocols using DNA fragmentation as a variable.

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