Intra-individual variation of the sperm chromatin structure assay DNA fragmentation index in men from infertile couples

K. Oleszczuk*, A. Giwercman, and M. Bungum

Reproductive Medicine Centre, Skåne University Hospital, Lund University, SE-205 02 Malmö, Sweden

*Correspondence address. Tel: +46-40-338282; Fax: +46-40-338286; E-mail: krzysztof.oleszczuk@skane.se

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BACKGROUND: The sperm chromatin structure assay (SCSA) is a valuable tool for prediction of fertility in vivo, with DNA fragmentation index (DFI) of 30% as a clinically useful cut-off level. Previous studies on fertile men have shown a high level of repeatability, with an intra-individual variability in DFI of ≈10%. However, conflicting data on how much the DFI fluctuates within individuals exist. The aim of the present study was to investigate the intra-individual variation of DFI in order to further evaluate the clinical use of SCSA.

METHODS: Among 2409 consecutive men under infertility investigation, repeated SCSA analyses were performed on 616 samples from men between 18 and 66 years of age. The coefficient of variation (CV) for DFI was calculated. For each patient, we also analyzed whether the DFI value in tests I and II switched the category from <30 to ≥30, or vice versa.

RESULTS: Mean CV for DFI for men with at least two SCSA analyses within a 30-month period was 30.1% (SD 21.5). Compared with the first test, 85% (95% confidence interval: 82–87%) of the men remained on the same side of the cut-off point of 30%.

CONCLUSIONS: Despite showing a high intra-individual CV for DFI, 85% of the men from infertile couples did not change category between tests, with respect to the cut-off level of 30%. Thus, using the previously established DFI cut-off value of 30%, a single SCSA analysis has a high predictive value for assessing fertility in vivo.

Key words: sperm DNA / DNA fragmentation index / infertility / intra-individual variation

Introduction

Conventional semen analysis, including assessment of sperm counts, morphology and motility, is a standard laboratory test of male fertility, according to the World Health Organization (2010). However, these parameters are not sufficient to interpret the fertility status or chance of pregnancy in a couple (Bonde et al., 1998; Auger et al., 2000; Guzick et al., 2001; Jequier, 2004), regarding neither natural nor assisted conception. A search for better predictors of fertility has brought the genomic integrity of the male gametes in focus (Reviewed in Agarwal and Said, 2003; Erenpreiss et al., 2006b) and during the last decades several methods to assess sperm DNA damage have been developed. The sperm chromatin structure assay (SCSA), a flow cytometric technique first described by Evenson et al. (1980), is one such test that provides additional information about the fertility capacity of the sperm. With SCSA the proportion of spermatozoa with impaired DNA integrity, expressed numerically as the DNA fragmentation index (DFI), is measured. SCSA was shown to be an independent marker of fertility in vivo, defined as the capability to get pregnant by either intercourse (in unstimulated cycle or after ovulation stimulation) or by intrauterine insemination (Evenson et al., 1999; Spanò et al., 2000; Bungum et al., 2004; Evenson and Wixon, 2006b; Giwercman et al., 2010). The SCSA has also a potential to contribute to more efficient use of in vitro assisted reproduction techniques (ARTs) in the future (Evenson and Wixon, 2006a; Bungum et al., 2007).

A well-known problem with using conventional semen analysis as a diagnostic tool is the high intra-individual variation reported for sperm concentration, motility and morphology (Mallidis et al., 1991; Amann and Hammerstedt, 1993; Alvarez et al., 2003; Keel, 2006). In contrast, previous studies on men who had a DFI of ≈10% have shown a high level of repeatability (Evenson et al., 1991, 2002). A comprehensive study on the variation of multiple SCSA measures for non-infertility patients showed SCSA measures which were significantly lower than those derived using common semen measures. In a study by Evenson et al. (1991), semen samples collected once per month for...
were included in the study. Clinically applicable fertility marker (Evenson et al., 2000; Spano et al., 1998; Bungum et al., 2010). In addition, more recently a single study has reported a significant intra-individual variation in infertile men with a CV of ~30% (Erenpreiss et al., 2006a), corresponding to the magnitude of intra-individual variation reported for other standard sperm parameters (Erenpreiss et al., 2008; Castilla et al., 2010).

However, unlike other sperm parameters, DFI has a distinct cut-off value for infertility in vivo (when exceeding 30%) and is, therefore, a clinically applicable fertility marker (Evenson et al., 1991, 1999; Spanò et al., 2000; Bungum et al., 2007; Gwercman et al., 2010). Thus, from a clinical point of view, the proportion of subjects who are switching between levels above and below 30% is more important than the magnitude of the intra-individual CV. In order to further elucidate this issue, we aimed to investigate the variation of DFI in repeated tests from the same patient, both in fertility work-up and during ART treatment. In particular, the study was aimed at assessing the feasibility of using SCSA in a clinical environment where the control of patient behavior, access to patient information and opportunity to maintain the highest levels of assay control and standardization may not be possible.

Materials and Methods

Patients

The study is based on a database of 2409 men aged between 18 and 66 years (mean 34.3 ± SD 6.3) who underwent infertility investigation and/or ART treatment at the Reproductive Medicine Centre, Skåne University Hospital, Malmö, Sweden, during the period May 2007 to November 2009. Six hundred and sixteen men with at least two SCSA (2–7) analyses were included in this retrospective observational descriptive study. In order to obtain sufficient numbers of sperm for SCSA analysis, only men having a sperm concentration of at least 1 × 10⁹/ml in neat semen were included in the study.

Semen collection and standard sperm analysis

Semen samples were collected by masturbation after the recommended abstinence period of 2–7 days. Standard semen analysis was performed according to the WHO guidelines (WHO, 1999).

Sperm chromatin structure assay

The principles and procedure to measure sperm DNA damage by flow cytometry SCSA are described in detail elsewhere (Evenson and Jost, 2000; Spanò et al., 2000; Bungum et al., 2004). In brief, the SCSA is based on the phenomenon that a 30 s treatment with a pH 1.2 buffer denatures the DNA at the sites of single- or double-strand breaks, whereas normal double-stranded DNA remains intact. Thereafter, the sperm cells are stained with the fluorescent DNA dye Acridine orange, which differentially stains double- and single-stranded DNA. After blue light excitation in a flow cytometer, the intact (double-stranded) DNA emits green fluorescence, whereas denaturated (single-stranded) DNA emits red fluorescence. Sperm chromatin damage is quantified by the flow cytometry measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red versus green fluorescence intensity cytogram patterns. The extent of DNA denaturation is expressed as the DFI, which is the ratio of red to total fluorescence intensity i.e. the level of denatured DNA over the total DNA. The frequency histogram of DFI provides a more precise calculation of percentage DFI than the use of computer gating on the green versus red cytogram.

Five thousand cells were analyzed by FACSort (Becton Dickinson, San Jose, CA, USA). Analysis of the flow cytometric data was carried out using dedicated software (SCSASoft; SCSA Diagnostics, Brookings, SD, USA) which implies that the DFI histogram is used to precisely determine the percentage DFI. All SCSA measurements were performed on raw semen, which on the day of analysis was quickly thawed and analyzed immediately. For the flow cytometer setup and calibration, a reference sample was used from a normal donor ejaculate retrieved from the laboratory repository (Evenson and Jost, 2000). The same reference sample was used for the whole study period. A reference was run for every fifth sample. The intra-laboratory CV for DFI analysis was found to be 4.5%. A single SCSA measurement was made for each reference sample.

Statistical analysis

Results were expressed as mean (± SD). The CV for DFI in each man was calculated using the formula (SD/mean) × 100.

According to previous reports suggesting 30% DFI as a cut-off value for achieving a pregnancy in IVF, the patients were dichotomized according to whether the DFI in raw semen was < 30% (Category I) or > 30% (Category II). Subsequently, the proportion of men switching from one category in the first test to the other category at the second examination was calculated, with 95% confidence interval (CI), based on the assumption of binomial distribution. Subsequently, the same calculation was performed using an interval of 29–31% instead of the 30% cut-off value (switch from < 29 to > 31% or vice versa). For the subjects for whom the date of the delivery of the first and the second ejaculate were computed in the database, the correlation between the length of the interval between sampling and CV of DFI was calculated using Spearman’s ρ-test.

Statistical analysis was performed using the Statistical Package for the Social Sciences 14.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance was regarded as a two-sided P < 0.05.

Results

Mean CV for DFI of all repeated SCSA measurements in the study group was 30.1% (SD: 21.5%); median 26.9% (range: 0–130%). Of the 616 patients included, 521 (85%; 95% CI: 82–87%) did not change DFI category from first to second sample (Category I: DFI ≤ 30% versus Category II: DFI > 30%). Ninety percents (95% CI: 87–93%) of these men had DFI ≤ 30% and the remaining 10% of the men had a DFI > 30% (95% CI: 7.4–13%). Sixty-eight patients (11%; 95% CI: 8.6–14%) had belonged to Category I in test 1 and to Category II in test 2. The DFI in those subjects was 31–71% (mean 40%, SD 10%). Twenty-seven patients
(4.4%; 95% CI: 2.8–6.0%) switched from Category II in test 1 to Category I in test 2. Of these, 19 had a DFI between 20 and 30% and 8 had a DFI between 15 and 20% (mean for DFI in test 2 was 22%, SD 4.4%). These results are summarized in Fig. 1.

When the DFI interval 29–31% was used instead of the 30% cut-off level, 12% of the subjects (95% CI: 9.2–14.2) switched from a value <29 to >31, or vice versa.

For 141 of the 616 men (23%), the date of both measurements was registered in the database. The mean for CV for the two DFI assessments in this group was 29.5%, whereas the mean CV for the remaining 475 subjects was 25.7%. The mean time interval between the two samples for this subgroup of men was 134 days. There was no significant correlation between the intra-individual CV and time interval between samples (Spearman’s ρ-test; ρ = 0.19; P = 0.82).

**Discussion**

The present study demonstrated that in men from infertile couples the variation of DFI in repeated samples is approximately of the same magnitude as for standard sperm parameters, previously being estimated as ~30% for concentration, motility and morphology (Leushuis et al., 2010).

However, using the DFI of 30% as a clinical cut-off level, the result of the SCSA analysis is relatively robust, since 85% of the men, when repeating the analysis, were still in the same DFI category. This figure is similar to the previously reported 82% in another cohort of men under infertility assessment (Erenpress et al., 2006a). Furthermore, there was no correlation between the length of the time period between the delivery of the two semen samples and the intra-individual CV, indicating that a single SCSA analysis is equally predictive for the DFI level some days, as well as several months, after the first sampling.

A strength of this study is the high number of subjects included. Furthermore, it is based on men coming for investigation owing to infertility problems, thereby representing the target group for which the prediction of chances of fertility in vivo is of the greatest interest. Previous studies have shown that variation might be lower for non-infertile men in contrast to the men from infertile couples studied here (Evenson et al., 1991).

Although data from the present study demonstrated a high intra-individual DFI variation, this does not invalidate the use of the test in clinical practice. The reason is robustness of the estimation based on one analysis in relation to whether the patient belongs to the DFI category below or above 30%, the clinically significant cut-off for predicting in vivo infertility (Evenson et al., 1999; Spanò et al., 2000; Bungum et al., 2007; Evenson and Wixon, 2008).

A major weakness of the study is lack of information about changes in life style and health during the follow-up of the men included in the study. Factors such as smoking, medication and fever were previously suggested to have a possible influence on sperm DNA integrity (Evenson et al., 1991, 2000; Niu et al., 2010; Eshal et al., 2009; Rubes et al., 2010). However, use of medication is not that common in men belonging to the age group seeking help for infertility. Change of smoking habits during infertility investigation, if occurring, most often implies that the patient stops smoking, which might explain the observed lowering of DFI between tests I and II. However, Spano et al. (1998) found no significant impact of smoking, alcohol consumption, fever or genital viral infection on DFI, which recently was confirmed by Smit et al. (2007), who demonstrated that neither life style nor occupation had any influence on the intra-individual variation of chromatin fragmentation.

All the patients were asked to keep an abstinence period of 2–7 days. In principle, the DFI outcome can be compromised by the presence of older spermatozoa that still remain after previous

**Figure 1** Variation in DFI between tests 1 and 2, in relation to the two categories of DFI (Category I: DFI ≤ 30% and Category II: DFI > 30%), which are used in the assessment of male fertility. Based on 616 patients. CI, confidence interval.
ejaculations. Although we did not correct for the actual length of the abstinence period, the DFI was found to increase by 0.45% per day of increase of the abstinence period (Richthoff et al., 2002). Furthermore, our set up reflects the daily situation where one analysis of semen quality is supposed to predict the chance of the couple to achieve pregnancy during the following months, and for each subject a day-to-day variation in abstinence period can be expected.

Apart from the clinical implications of our finding, the high intra-individual variation in the DFI raises some questions related to biological aspects of regulation of semen quality. As the intra-laboratory CV for determination of DFI was as low as 4.5%, the variation in the results of the SCSA analysis can hardly be explained by technical aspects of the analysis, although we only measured each reference sample once with no repeat measurement to verify that sample debris caused no artifact in the measurement. As for other sperm parameters, our knowledge of biological factors which may have a major impact on the intra-individual variation in DFI is limited. Sperm, during its development, transport and storage, can be negatively affected by different mechanisms (Sakkas et al., 2010); abortive apoptosis during spermatogenesis, DNA strand breaks during the remodeling of sperm chromatin under the spermiogenesis process, and oxidative stress caused by reactive oxygen species, which may lead to post-testicular DNA fragmentation (Aitken et al., 1998). Moreover, DNA fragmentation can be induced by endogenous caspases and endonucleases, or external factors, such as radiotherapy, chemotherapy and environmental toxicants. Although the highly organized, compact and insoluble nature of the sperm chromatin with its protective system of histones and protamines (Erenpreis et al., 2006b; Shamsi et al., 2008), make the spermatozoa exposed for conspicuous disintegration. It appears plausible that the factors which cause an increase in DFI are more pronounced in subfertile men, thereby also leading to higher intra-individual variation in infertile subjects as compared with men without fertility problems.

In conclusion, this study describes a considerable intra-individual variability in sperm DNA damage within a large group of infertile men. However, in the vast majority of the subjects, repeated SCSA testing does not result in a switch in DFI category, in relation to the clinical cut-off level of 30%. This finding adds to the utility of SCSA DFI as a valuable tool in the investigation of men from infertile couples.

Authors’ roles
K.O., A.G. and M.B. have all given substantial contributions to conception and design of the present study. All authors have contributed to acquisition of data, analysis as well as interpretation of data. K.O. has drafted the manuscript and A.G. and M.B. have revised the content critically. All three authors have made final approval of the version to be published.

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