INVESTIGATION OF SPERM DNA FRAGMENTATION BY SPERM DNA INTEGRITY ASSAY

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ABSTRACT

Male-factor infertility plays a role in approximately 50% of infertile couples. In at least 30% of cases, repeated standard semen analyses of the male partner of an infertile couple reveal normal results. When diagnostic work-up of the female partner is also normal, they are classified as unexplained or idiopathic. The objective of this study was to evaluate the levels of sperm DNA fragmentation, respectively a sperm nuclear DNA integrity, in normospermic infertile men, by a flow cytometric method. **Materials and methods**: Semen samples were obtained from 53 normospermic infertile men and 30 fertile donors. Flow cytometry was used to study sperm DNA fragmentation by Sperm DNA Integrity assay and the results were expressed as a percentage of sperm DNA fragmentation index (DFI). **Results**: Increase levels of DFI were observed in 21 of 53 (39,62%) normospermic infertile men. The %DFI in normospermic infertile men was significantly higher than fertile donors (P = 0.03). There were statistically significant negative correlations between DFI and sperm motility (r = –0.48, P = 0.0001) and normal morphology (r = –0.37, P = 0.022) in normospermic infertile men, but not in fertile donors. **Conclusion:** Our study indicates that the levels of sperm DNA fragmentation in normospermic infertile men were significantly higher compared with the levels in the fertile group. Sperm DNA Integrity assay is an independent test of sperm quality that may have better diagnostic capabilities than standard sperm parameters.

Key words: sperm DNA integrity, flow cytometry, male infertility, sperm

INTRODUCTION

Standard semen analysis consists of measuring a variety of semen parameters, including volume, pH, sperm concentration and motility, vitality and morphology, presence of leukocytes and immature germ cells [1]. Semen volume and pH level are an index of seminal vesicle and prostate function. Sperm concentration, motility and morphology are largely determined by testicular function and, to a lesser extent, by post-testicular (e.g., epididymal) genital tract function. However, in at least 30% of cases, repeated standard semen analyses of the male partner of an infertile couple reveal normal results. When diagnostic work-up of the female partner is also normal, they are classified as unexplained or idiopathic [2].

There is ongoing research in developing new methods to complement conventional semen analysis routinely used to assess infertility. One endpoint of interest is sperm DNA integrity, which can be assessed by a variety of assays, such as sperm chromatin structure assay (SCSA) [3,4], sperm DNA integrity assay (SDIa) [5], terminal deoxynucleotidyl-transferase-mediated dUDP nick-end labeling (TUNEL) assay [6], in situ nick translation (ISNT) [7], and single cell microgel electrophoresis (COMET) assay [8].

Fertilization involves the direct interaction of the sperm and oocyte, fusion of the cell membranes and union of male and female gamete genomes [9]. The completion of this process and subsequent embryo development depend in part on the inherent integrity of the sperm DNA [10]. Indeed, there appears to be a threshold of sperm DNA damage (i.e., DNA fragmentation, abnormal chromatin packaging, protamine deficiency) beyond which embryo development and pregnancy are impaired [10,11]. The study of sperm DNA integrity is particularly relevant in an era where advanced forms of assisted reproductive technologies (ART) are frequently used.

Methods focusing on the characterization of sperm chromatin condensation and stability are able to reveal hidden anomalies of the structural organization of sperm DNA and have been receiving growing attention. In particular, flow cytometric (FCM) approaches allow rapid measurements to be carried out with a high level of precision, objectivity, and consistency with reliable results [11,12]. SCSA and SDIa are FCM methods that may identify the

spermatozoa with abnormal chromatin packaging, the former being a result of DNA breaks and/or of derailments in protamine quantity and composition and/or of an insufficient level of disulfide groups [4,5,13,14]. Acridine orange staining is used, after a low pH challenge, to distinguish between native DNA and fragmented DNA. Furthermore, men with sperm DNA fragmentation (>27-30%) were placed into a statistical group taking a longer time to pregnancy, more in vitro fertilization (IVF) cycles, increased miscarriages or no pregnancy [3,4,5,11]. As a consequence, SCSA and SDIa have been recommended for routine use in ART programmes in order to predict a couple's probability of obtaining a pregnancy [3,4,5].

The objective of this study was to evaluate the levels of sperm DNA fragmentation, respectively a sperm nuclear DNA integrity, in normospermic infertile men and to compare the results with a control group of men with proven fertility.

MATERIALS AND METHODS

Study population

All infertile couples included in this study had at least one year of regular unprotected intercourse with no pregnancy achieved. A medical history was obtained from the infertile patients, and an urologist performed a genital examination. The participants in the study were infertile men with normal standard semen parameters on repeated analyses. The female partners of these men had no history of untreated female-factor infertility and had a normal reproductive and sexual history as well as normal diagnostic exams. Couples with untreated female-factor infertility and infertile men with an abnormality in one or more of the standard semen parameters were excluded from the study. A group of healthy donors (n = 30) with proven fertility (i.e., fathered a child within the last 12 months) and with normal genital examinations were included as controls.

Semen samples

Semen samples were obtained from normospermic infertile men (n = 53) and from fertile donors (n = 30) during March 2007 through April 2008. All samples were collected by masturbation, after a period of 3 to 5 days of sexual abstinence. After 30 min from the time of ejaculation (for semen liquefaction at room temperature), 500 µl of the raw semen sample was frozen into a LN_2 tank at –196°C for later SDIa.

Standard semen analysis

Following liquefaction, semen specimens were evaluated for semen volume, appearance, pH, and viscosity. Manual semen analysis was performed according to World Health Organization (WHO) guidelines [1] to determine sperm concentration and motility. Five microliter aliquot of liquefied semen was loaded on a Cell-VU counting chamber (Millennum Sciences, Inc., NY, USA) and examined under x 200 magnification. Sperm concentration was expressed as $1x10^6$/mL semen, while motility was expressed as a percentage. Smears of the raw semen were stained using the Spermac stain kit (FertiPro N.V., Belgium) for assessment of sperm morphology using the WHO classification [1]. Immediately after staining, the smears were rinsed in distilled water, air-dried, and scored. In this study, normal values were: sperm concentration $\geq 20x10^6$/mL semen, motility ≥50% and normal sperm forms ≥14% [1].

Sperm DNA Integrity assay

Sperm DNA damage was determined by acridine orange staining method on flow cytometry [5]. On the day of analysis, the samples were thawed and the test was performed as soon as possible. All buffers and samples need to be kept at 4 to 6ºC and the test to be run in box with ice. After wash with TNE (0.15M NaCL, 0.01M TrisHCL, 1mM EDTA, pH 7.4) buffer, the sperm concentration was adjusted to approximately $1\text{-}2x10^6$ cells/ml and 200ìl was placed in 12x75 flow cytometer tubes. 400 µl of low-pH (pH 1.2) detergent solution containing 0.1% Triton X-100, 0.15M NaCl and 0.08M HCl was added for 30 sec. immediately followed by 1.2 ml staining buffer (phosphate-citrate buffer, pH 6.0) with 6 mg/l acredine orange (AO) (chromatographically purified; cat. No. 318337-1G, Sigma-Aldrich, USA). Cells were acquired on FACScan flow cytometer (Becton Dickinson). A total of 5000 events were accumulated for each measurement at a low flow rate. Under these experimental conditions, when excited with a 488 nm light source, AO intercalated with double-stranded DNA emits green fluorescence and AO associated with single-stranded DNA emits red fluorescence. Thus, sperm chromatin damage can be quantified by the flow cytometric measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red (fragmented DNA) versus green (DNA stainability) fluorescence intensity cytogram patterns. Data for each acquired specimen was saved and analyzed by FlowJo software (TreeStar, Inc.,Ashland,OR,). Computer gates are used to determine the proportion of spermatozoa with increased levels of red fluorescence (fragmented single-stranded DNA) and green fluorescence (native double-stranded DNA). The results were presented as a percentage of sperm DNA fragmentation index (DFI).

Statistical analysis

Comparisons of donors and normospermic infertile men were performed using Mann-Whitney test for continuous variables. Spearman correlation coefficients were used to evaluate relationships between continuous variables. Summary statistics were presented as median and inter-quartile values (25th and 75th percentiles). All hypothesis testing was two-sided with a probability value of $P < 0.05$ deemed as significant. Calculations were performed with SPSS version 13.0 software for Windows.

RESULTS

All fertile donors and infertile men had normal

standard semen parameters according to the WHO standards [1].

From extensive animal and human studies, Evenson and co-authors, who first described the SCSA, suggested that thresholds of 0-15%, 16-29% and >30% for the DNA fragmentation index (DFI) relate to high, moderate and low fertility potential, respectively [4]. These thresholds also were applied to the SDIa [5].

Among the fertile donor group, the highest observed %DFI was 29.97%. Levels of DFI higher than 30% were observed in 21 of 53 (39,62%) normospermic infertile men. A comparison of standard semen parameters and DFI in fertile donors and in normospermic infertile men is shown in Table 1.

The only significant difference between fertile donors and normospermic infertile men was observed in the levels of DFI ($P = 0.03$).

We identified statistically significant negative correlations between the percentage of DFI and sperm motility ($r = -0.48$, $P = 0.0001$) and normal morphology ($r = -0.37$, $P = 0.022$) in normospermic infertile men, but not in fertile donors.

The median age as well as the 25th and 75th percentile values of normospermic infertile men were not significantly different from fertile men ($P = 0.85$) (Table 1).

DISCUSSION

The results from this study indicate that the levels of SDIa-defined DFI in sperm from the infertile men with normal standard sperm parameters (i.e., concentration, motility, and normal forms) were significantly higher than the levels of the fertile donors. Saleh et al. [12] also demonstrated a significant increase in SCSA-defined DFI in sperm from infertile men with normal standard sperm parameters.

With the advent of IVF and intracytoplasmic sperm injection (ICSI), the concern over using damaged DNA has become apparent. In natural conception, a DNA-damaged sperm would likely be unsuccessful in fertilizing an egg. Human spermatozoa that bind to oviduct (fallopian tube) cells have better DNA integrity than spermatozoa that do not bind to these cells, which suggests that nature can select spermatozoa with enhanced DNA integrity during natural fertilization. However, ART bypass this selection process. It is probable that spermatozoa selected for ARTs may originate from samples with high percentages of sperm with damaged DNA [11]. ICSI is the technique used primarily for the treatment of infertile men with poor sperm quality, a major concern would be the use of DNA-damaged spermatozoa to fertilize the oocyte, which may have adverse consequences such as fertilization failure, early embryo death, spontaneous abortion, childhood cancer, and infertility in the offspring [4,5,6,11].

Our data on the associations between the sperm quality (sperm motility and morphology) and DFI are consistent with those of Zini et al. [13] and Giwercman et al. [14], who reported significant negative correlations between DFI (assessed by SCSA) and sperm quality. Irvine et al. [7] also demonstrated a negative correlation between sperm DNA integrity (assessed by COMET and ISNT assays) and sperm quality. The association between DFI and motility indicates that normally motile sperm may or may not have an intact DNA molecule. On the other hand, it is possible to has an association between a sperm nuclear DNA damage and a sperm mitochondrial DNA damage. Although the bulk of the sperm DNA is in the nucleus, a small fraction is of mitochondrial origin (within the sperm midpiece). The sperm mitochondrial DNA is a small, circular DNA that is not bound to proteins. Mitochondrial DNA exhibits a high rate of mutation. Sperm motility is related to the mitochondrial volume within the sperm midpiece, and mutations or deletions in the mitochondrial DNA have been associated with reduced sperm motility. Although inheritance of mitochondrial DNA is primarily maternal, paternal transmission of mitochondrial DNA mutations have been reported (but no more than 1% of inheritance). The examination of mitochondrial DNA may gain some importance in the evaluation of male infertility, particularly in relation to ART.

Potential mechanisms of sperm DNA damage include (1) defective chromatin condensation during spermatogenesis, (2) initiation of apoptosis during spermatogenesis or during transport of sperm through male or female genital tracts, and (3) oxidative stress by reactive oxygen species produced internally or externally [11]. These three mechanisms might independently or codependently be responsible for sperm DNA damage. The first mechanism postulates that sperm does not mature completely during spermiogenesis and that DNA breaks are therefore unable to physiologically re-ligate when chromatin rearrangement occurs during the replacement of histones by protamines [11]. Furthermore, many investigators have correlated the presence of DNA damage with poor chromatin packaging [3,5,11,13]. The second hypothesized mechanism relates to apoptotic DNA cleavage in germ cells [11]. Sperm DNA damage might result from an “abortive apoptosis”, which occurs in the later stages of germ cell development, resulting in dying sperm cells not being properly eliminated by the testis. Finally, a third hypothesized mechanism attributes sperm DNA damage to excessive reactive oxygen species production in testicular and posttesticular sites [11].

Based on the results of this and other recent independent studies, the integrity of the sperm DNA may be tested to predict pregnancy outcomes as follows:

- in couples who do not know their fertility potential (i.e., first pregnancy) [5,13]. Couples in whom the man has a high percentage of spermatozoa with DNA damage have very low potential for natural fertility and will have to wait a long time before conceiving. For those normospermic men

who are suspected to have high levels of sperm DNA fragmentation, antioxidant supplements can be considered;

- counseling couples planning to undergo intrauterine insemination. SDIa and SCSA are good predictors of negative pregnancy outcome [11]. If the male partner has high levels of sperm DNA fragmentation, the couple should consider IVF or ICSI instead;

- in couples who are planning to undergo IVF or ICSI. SDIa and SCSA are only fair predictors of negative or positive pregnancy outcomes [5,11].

In conclusion, our study indicate that the levels of sperm DNA fragmentation in normospermic infertile men were significantly higher compared with to the levels in the fertile group. SDIa may reveal a hidden abnormality of sperm nuclear DNA in infertile men classified as idiopathic, based on apparently normal standard sperm parameters. This test has an important diagnostic and prognostic value in the evaluation of male infertility, particularly in relation to assisted reproductive technologies.

Table 1. Age, standard sperm parameters and the percentage of sperm DNA fragmentation index (DFI) in fertile donors and in normospermic infertile men. Values are median (25^{th} and 75^{th} percentiles). Mann-Whitney test was used for comparison and statistical significance was assessed at $P < 0.05$.

Variable	Fertile donors (n = 30)	Normospermic infertile men (n = 53)	*P* value
Age	34 (31, 37)	35 (31, 37)	0.85
Concentration ($x10^6$/mL)	63 (51, 84)	74 (46, 94)	0.89
Motility (%)	60 (50, 68)	55 (51, 61)	0.34
Morphology (%)	30 (20, 33)	24 (16, 32)	0.07
DFI (%)	7 (4, 11)	13 (5, 43)	0.03

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