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Are Semen Quality Parameters Sufficient for Biomonitoring Spermatozoa DNA Integrity and Oxidatively Damaged DNA

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Are semen quality parameters sufficient for biomonitoring spermatozoa DNA integrity and oxidatively damaged DNA

Abstract: The present study aimed to investigate the relationship between semen quality parameters and DNA integrity, and determine whether semen quality parameters could serve as a reliable biomarker for monitoring sperm DNA damage. Conventional semen parameters from a total of 202 male human subjects were analyzed. DNA fragmentation and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxoGuo) were used to assess sperm DNA integrity. DNA fragmentation was analyzed by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and sperm chromatin structure assay (SCSA), while 8-oxodGuo was quantified by the liquid chromatography/tandem mass spectrometry (LC-MS/MS) coupled with an on-line solid phase system. The levels of 8-oxodGuo levels in sperm were related to the percentages of DNA fragmentation measured by both the TUNEL and SCSA ($r = 0.22$, $p = 0.048$; $r = 0.12$, $p = 0.039$). Sperm vitality, motility and morphology from all of the participants exhibited a weak correlation with the levels of 8-oxodGuo and the percentages of DNA fragmentation. Semen quality parameters may be independent of the formation of DNA fragmentation and oxidative adducts in sperm. Semen quality parameters may be insufficient to monitor sperm DNA fragmentation and oxidative damage. DNA damage in sperm is recommended to be included in routine measurements.

Keywords: Oxidative stress, semen quality, motility, 8-oxodGuo, DNA fragmentation

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1 Introduction

Semen quality parameters are typically the first diagnostic markers for male individuals or patients with fertility concerns. The percentages of abnormal sperm have served as a valuable predictor of sperm production, spontaneous pregnancies, and fertilization success in assisted reproductive technology [1-3]. Abnormal sperm morphology has been linked to a decrease in traditional parameters of semen quality [4-5], and to an increase in contemporary markers of sperm damage, such as DNA fragmentation [6], and overproduction [6]. Although semen quality parameters provide a general overview of the quality of sperm, that approach has been criticized for not consistently correlating with one of the most important components of the reproductive outcome, the male genome contained in the sperm head [7] Furthermore, studies have reported controversial results on the relationships between semen quality parameters and oxidative stress status of sperm. Sperm normal forms have negatively correlated with the level of oxidative stress in some studies [8-9] but not in others [10-11].

The chromatin of human spermatozoa has a highly condensed and organized structure, which protects sperm chromatin from oxidative damage [7]. However, in some cases where poor compaction and incomplete protamination of sperm chromatin exist, DNA is more vulnerable to oxidative damage and produces base-free sites, deletions, or frame-shift mutations [12]. Several studies have demonstrated that oxidative stress is a major etiology associated with sperm defective function and male infertility [13-15]. Emerging evidence has suggested that sperm DNA integrity may be a better predictor of male fertility potential than the semen quality parameters [16-17]. Sperm DNA fragmentation, measured by the terminal deoxynucleotidyl transferase dUTP nick end labeling

(TUNEL) assay, sperm chromatin structure assay (SCSA), and the Halosperm assay [18], has been used to examine sperm DNA integrity. However, DNA fragmentation may provide little specific information on the nature, mechanism, and severity of the DNA damage detected [13, 19].

The oxidized base adduct, 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-oxodGuo), has been recognized as a biomarker for oxidative DNA damage. 8-oxodGuo is significantly elevated in sperm of patients with infertility [20-21], and highly correlate with the DNA fragmentation of sperm [20, 22]. The quantified levels of sperm DNA extraction and 8-oxodGuo measurements have been doubted due to the possible spontaneous formation of 8-oxodGuo during the step of extraction/digestion of sperm DNA [23]. Recent DNA isolation procedures, developed and recommended by the European Standards Committee on Oxidative DNA Damage (ESCODD) [24], could significantly minimize and/or eliminate the artifactual formation of 8-oxodGuo. Also, chromatographic techniques for quantification have recently improved to address the weakness. For example, liquid chromatography/tandem mass spectrometry (LC-MS/MS) [25-27] has significantly increased selectivity and sensitivity more so than other chromatographic methods, such as gas chromatography and high performance liquid chromatography. The LC/MS system requires no derivatisation step [26,] to ensure accurate quantification of cellular 8-oxodGuo when low background levels of 8-oxodGuo in small quantities of DNA are measured [26-27].

2 Materials and methods

2.1 Human subjects

A total of 202 participants were recruited for this study as they met the eligibility criteria: no reproductive dysfunction that precluded a sperm sample, and no pre-existing urological and/or andrological disorders, including infertility, testicular injury, and surgery. Participants abstained from ejaculation for at least three days prior to the biological sampling. Urine and semen specimens were collected when they had their annual health examination at the Taiwan Kaohsiung Municipal Hsiao-Kang Hospital, Kaohsiung health clinic. It serves as the main municipal hospital system providing health care for general population and occupational workers in the southern region of Taiwan. The study was approved by the Institutional Research Board at the Kaohsiung Medical University. Each participant signed declaration of consent. A questionnaire was used to collect information pertaining to demographics and potential confounding factors. Demographic information included age, body mass index (BMI), education, marital status, smoking, alcohol consumption, and occupational history.

2.2 Semen quality analysis

After liquefaction of semen, the standard semen quality analysis was conducted according to World Health Organization recommendations [28]. Sperm concentration, motility, and vitality were assessed within one hour after the sampling of the ejaculates. A volume of 10 µl of semen was held in a Makler chamber (Irvine Scientific, Santa Ana, CA, USA) for sperm count and motility measurements. Manual evaluation of sperm concentration and motility were conducted using a Makler Counting Chamber. The eosin stained method was used to assess sperm vitality. At least 300 sperm per sample were assessed for vitality analysis. For the morphology assessment, two slide smears were prepared from each semen sample. Sperm morphology was evaluated on air-dried smears stained with air-dried Papanicolaou-stained preparations and scored at x1,000 magnification under a light microscope. At least 300 sperm per sample were categorized as normal or abnormal according to the presence or absence of head, midpiece, tail, as well as any defects in immaturity, according to the criteria recommended by the WHO [28].

2.3 Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) Assay

The TUNEL assay was used to detect sperm DNA fragmentation [29]. A sperm pellet was obtained after 200 µl of semen were centrifuged at 250 x g for 5 min. The pellet was re-suspended, washed with 1% human serum albumin in PBS, and spread onto slides. Then, cells were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate at 4 °C for 2 min. A nucleotide labeling mixture prepared according the Roche Diagnostic manufacturer's instruction was deployed onto sperm cells. After the cells were incubated for one hour at 37 °C, they were washed twice with 1% HAS in PBS. Each test included both positive and negative controls. Cells in the positive control were treated with 50 µl of DNase solution, while cells in the negative control were not treated with the nucleotide labeling mixture. Fluorescence in sperm cells recorded as a positive for the TUNEL assay was assessed using an Olympus BX61 fluorescence microscope (Center Valley, PA, USA). At least 300 sperm cells from each sample were

accounted for, and the percentage of TUNEL positive cells was calculated as the outcome of interest.

2.4 Sperm chromatin structure assay (SCSA)

The SCSA is a flow cytometric test to assess sperm DNA fragmentation [30-31]. The assay is based on the unique straining properties of acridine orange (AO) that emits green fluorescence (515-530 nm) when bound to normal dsDNA and red fluorescence (630 nm) when bound to damaged ssDNA. On the day of analysis, stored samples were thawed in a 37°C water bath for 30 seconds and used immediately. A total of 1 x 10^4 sperm were treated for 30 seconds with a detergent solution (pH 1.2) and then stained with 6 mg/L of purified AO in pH 6.0. Within 3 minutes after AO staining, the sperm were analyzed using a flow cytometer with a data handler (CellQuest software program, Becton Dickinson). The percentage of sperm with DNA fragmentation was calculated as the ratio of red to total fluorescence intensity [31].

2.5 Sperm DNA isolation

Sperm DNA were isolated according to the procedure recommended by the ESCODD [32] for DNA sperm extraction and hydrolysis, with modifications to minimize DNA oxidization during DNA isolation procedures. Briefly, sperm samples (15-30 × 10^6/ sperm) were washed with 1% HSA in PBS and centrifuged at 3000 x g for 5 min. The resulting pellet was added to 600 µl of ice-cold extraction buffer [10% (w/v) SDS and 1M DTT], 30 µl of proteinase K, 30 µl of RNase A (1 x 10^{-2} mg/l) and 8 µl of RNase T1 (1 U/µl). The mixture was incubated at 37 °C for 1 h and then cooled to 4 °C for 5 min. Subsequently, 1.2 ml of NaI solution and 2 ml of 2-propanol were added. After centrifugation at 5,000 g for 5 min, the DNA pellet was washed with 1 ml of ice-cold 40% (v/v) 2-propanol, collected by centrifugation and dissolved in 200 µl of 0.1 mM DFO overnight. DNA concentration was measured by absorbance at 260 nm. Protein contamination was checked using the absorbance ratio A260/A280. AN absorbance ration over 1.6 was acceptable.

2.6 8-oxodGuo in sperm

The LC-MS/MS was used with an online solid-phase extraction for direct determination of 8-oxodGuo and dG in DNA hydrolysates [26]. The LC/MS system consists of a switching valve (two-position microelectric actuator; Valco) and a SPE cartridge (Inertsil, ODS-3 column). The switching valve function was automatically controlled using PE-SCIEX control software (Analyst; Applied Biosystems). The online SPE can divert away dG to avoid artifacts during the ionization process. Meanwhile the rest of the dG can be accurately quantified because the loss of dG during online SPE is compensated by the use of an [$^{15}N_5$]-dG internal standard. Also, the optimization of online-SPE conditions was accomplished by modifying the washing time of the trap column to eliminate the artifactual response in mass spectrometry. Briefly, sperm DNA (20 **µg**) was spiked with isotopic internal standards ($^{15}N_5$-8-oxodG and $^{15}N_5$-dG, Cambridge Isotope Laboratories, Inc.) and enzymatically digested to nucleosides with nuclease P1 and alkaline phosphatase. The resulting DNA hydrolysates were quantified using LC-MS/MS with an on-line SPE system. After automatic sample cleanup, DNA samples were injected into an Agilent 1100 series HPLC system interfaced with a PE-SCIEX API 3000 triple quadrupole mass spectrometer with an electrospray ion source. Detection was performed in the positive ion multiple reaction monitoring mode for simultaneous quantification of 8-oxodGuo and dG. Transitions of the precursors to the product ions were as follows: 8-oxo-dGuo (m/z 284→168), [$^{15}N_5$]- 8-oxo-dGuo (m/z 289→173), dG (m/z 268→152), and [$^{15}N_5$]-dG (m/z 273→157). With the use of isotopic internal standards and on-line SPE, this method exhibited a low detection limit of 1.8 fmol for 8-oxodGuo, which corresponds to 0.13 adducts 10^6 /dG when using 20 µg of DNA per analysis. A DNA calibration curve was established by addition of a fixed amount of [$^{15}N_5$]-8-oxodG or [$^{15}N_5$]-dG internal standard with various amounts of 8-oxodG (0.02–10.6 pmol) or dG standard solutions (0.7–22.4 nmol). The recovery and imprecision of this method have been estimated to be 99.7% and < 10% (CV), respectively. The limit of detection was 1.8 fmol for 8-oxodG.

2.7 Statistical analysis

Data analysis was performed using a SAS version 9.1 (SAS Institute Inc., Cary, NC) with the results expressed as the mean ± standard deviation (SE). Frequency distribution analyses were also conducted, including calculation of a normal distribution based on each variable's mean and standard deviation. Log transformation was used to normalize the distribution of the readings from the TUNEL and SCSA. Paired comparisons of means of semen quality and sperm DNA integrity were conducted using a paired *t*-test at the 0.05 level of significance. Non-paired comparisons were conducted with a non-paired *t*-test and confirmed with a non-parametric test (Mann–Whitney *U*). Multivariate regression analysis was conducted to

determine the correlation between sperm DNA integrity parameters and sperm quality, while controlling for confounding factors. All hypothesis testing was two-sided with a probability value of 0.05 or less being considered significant.

3 Results

Mean age of the subjects was 42 years old. 29% of the subject smoked, while 15% of them drank regularly. 64% of sperm were motile. Among them, 45% of sperm moved progressively, while 10% and 8% of sperm moved non-progressively and non-linear. 79% of sperm were vital (Table 1).

Table 2 summarizes the readings of the two groups of DNA fragmentation and 8-oxodGuo grouped based on the cut-off values of sperm concentration (≥15 10^6/ml), motility (≥40%), vitality (≥58%), and normal forms (≥4%) based on the WHO criteria. Means of the outcomes of DNA fragmentation measured by the TUNEL assay and SCSA were 35% and 12%, respectively, while mean level of 8-oxodGuo in sperm was 18/10^6 dG. There was no significant difference of DNA fragmentation and 8-oxodGuo between sperm concentrations ≥15 10^6/ml and <15 10^6/ml, motility ≥40% and <40%, vitality ≥58% and <58%, and normal forms ≥4% and <4%.

Table 3 shows DNA fragmentation and 8-oxodGuo negatively correlated with semen quality parameters except total count and concentration. However, all of the correlations were not of statistical significance, when adjusted for potential confounders, including smoking status, age, and alcohol consumption. 8-oxodGuo was significantly correlated with the outcomes of DNA fragmentation measured both by TUNEL and SCSA (Ps = 0.048 and 0.039, respectively).

4 Discussion

This study is one of the few examining DNA fragmentation and 8-oxodGuo formation in concert with the simultaneous assessment of semen quality parameters in a general population. The study has employed new, reliable, and sensitive methods for quantification of DNA extraction and DNA oxidative adduct quantification. A combination of these assays offered a comprehensive way for assessment of relationships between semen parameters and sperm DNA integrity, with an understanding of their biological significance in sperm function and male reproductive health. Such outcomes could be useful in determining a clinical diagnosis for male reproductive function and fertility.

Readings of semen quality parameters from the sample population were comparable with the general

Table 1. Characteristics of study subjects and semen quality*

	Human subjects (n=202)	General population[a] Mean range
Age (yr)	42	
BMI (kg/m^2)	36.6	
Smoking (%)	23	
Alcohol consumption (%)	19	
Semen quality**		
Total sperm count (10^6/ejaculated)	289 ± 123	
Concentration (10^6/ml)	105.2 ± 94.6	78.5 – 103.6
Motility (%)	63.4 ± 20.3	55.0 – 66.3
Progressive	46.4 ± 10.3	
Non-linear	10.2 ± 8.3	
Non-progressive	6.8 ± 3.3	
Vitality (%)	79.1 ± 14.5	78.8 – 93.3
Morphology (%)		
Normal form	27.2 ± 8.2	7.9 – 70
Head defects	63.1 ± 12/3	
Coiled tail	1.4 ± 0.9	

[a]General population: Rubes et al.2005; Zribi et al., 2011; Singh et al., 2011

*Sampling size was 200

**presented in Mean ± SD

Table 2. Levels of DNA fragmentation and 8-oxodGuo as presented by WHO reference values*

	TUNEL Assay (%)	SCSA Assay (%)	8-oxodGuo (/10^6 dG)
Sperm concentration (10^6/ml)	33.2%	10.5%	18
≥ 15	32.3	10.4	17.8
< 15	34.9	9.6	18.5
Motility (%)			
≥ 40	30.5	8	17
< 40	36.9	12	19
Vitality (%)			
≥ 58	28.6	7	17
<58	37.8	14	19
Normal forms (%)			
≥ 4	31.5	8	17
< 4	35.9	12	19

*Cutoff reference values for semen characteristics as recommended by the WHO (WHO 2010)
Concentration: all of the subjects met the WHO reference value.
Mortality: 10% of the subjects did not meet the WHO reference value.
Vitality: 13% of the subjects did not meet the WHO reference value.
Normal forms: 18% of the subjects did not meet the WHO reference value.

Table 3. Correlations between DNA integrity and semen quality

	TUNEL Assay	SCAC Assay	8-oxodGuo
TUNEL assay	--	0.15 (0.031)	0.22 (0.049)
SCSA assay	0.15 (0.031)	--	0.12 (0.039)
8-oxodGuo	0.22 (0.048)	0.12 (0.039)	--
Concentration	0.028 (0.79)	0.028 (0.79)	0.038 (0.83)
Motility	-0.188 (0.11)	-0.058 (0.34)	-0.058 (0.34)
Progressive	-0.188 (0.11)	-0.058 (0.34)	-0.058 (0.34)
Non-linear	-0.188 (0.11)	-0.058 (0.34)	-0.058 (0.34)
Non-progressive	-0.188 (0.11)	-0.058 (0.34)	-0.058 (0.34)
Vitality	-0.184 (0.09)	-0.044 (0.45)	-0.044 (0.45)
Morphology			
Normal forms	-0.069 (0.53)	-0.079 (0.38)	-0.079 (0.38)
Head defects	-0.069 (0.53)	-0.079 (0.38)	-0.079 (0.38)
Coiled tail	-0.069 (0.53)	-0.079 (0.38)	-0.079 (0.38)

population cited in other studies (Table 1). Although DNA fragmentation and 8-oxodGuo were detected in all of the subjects, the DNA fragmentation readings measured by the TUNEL and SCSA suggested that a majority of the studied subjects had good to fair fertility potential [28].

DNA fragmentation measured by the TUNEL and SCAS correlated with 8-oxodGuo formation ($r = 0.22$, $p = 0.048$; $r = 0.12$, $p = 0.039$). Similar results were observed

in other studies [Kothari et al., 2010; Montjean et al, 2010]. 8-oxodGuo formation more highly correlated with DNA fragmentation measured by using both the TUNEL and SCSA. A significant proportion of the DNA damage seen in the studied cells was oxidatively induced, and occurred in early spermatogenesis. DNA fragmentation measured by the TUNEL assay included both single- and double-strand breakages, while the SCAS assay mainly detected single-breakage, which likely occur upstream of DNA fragmentation and could be repaired by the enzyme pathway. The generation of TUNEL-reactive 3'-OH termini in sperm DNA has been suggested to be a perimortem change that takes place sometime after the initial oxidative insult, while the DNA denaturation, a single-strand break, is detected in the early stage of spermiogenesis [32]. Our study suggested that the cascade of causes leading to 8-oxodGuo could occur in early spermatogenesis.

The readings from the TUNEL and SCSA exhibited an inverse correlation with all semen quality, except sperm concentration and total count. Also, the 8-oxodGuo levels had an inverse correlation with sperm motility, vitality, and morphology. However, all of these correlations did not reach statistical significance. Sperm vitality correlated with the outcome of the TUNEL assay, but not with the results of the SCSA and the 8-oxodGuo. In the individuals with sperm vitality less than 50%, the correlation between the outcome of the TUNEL assay and sperm viability increased. The correlation may explain that both measurements detected the cell deaths. Thus, vitality may not be sufficient to detect oxidative damage in the relatively early upstream of DNA fragmentation [35]. Also, the study demonstrated that dysfunction of membrane permeability associated with sperm viability doesn't result in DNA fragmentation and thus must be considered as an independent parameter of semen quality. That could explain the lack of correlation with vitality in the outcome of SCSA and 8-oxodGuo levels [33].

Sperm motility from the entire study subjects weakly correlated with the outcome of the TUNEL assay [$r = -0.188$, $p = 0.11$]. Also, sperm motility did not correlate with the 8-oxodGuo levels. As the study subjects were divided into two groups based on the cut-off point of motility > 40%, we did not observe a significant correlation between motility and the TUNEL assay, and 8-oxodGuo level. Similar observations were also reported in other studies [34]. However the study's results contrasted with [10], which used infertility patients with a semen quality profile that significantly differed from the general population. Among the semen parameters, sperm motility has been repeatedly observed in correlating with lipid peroxidation in a variety different species. ROS-generating systems have also clearly demonstrated the susceptibility of sperm motility to oxidative attack and identified hydrogen peroxide as the most cytotoxic oxygen metabolite in this context [36]. However, it has been suggested that there are two types of origins of sperm oxidative damage, which are two independent steps in generating sperm DNA damage and lipid peroxidation [34], and that nuclear alterations and lipid peroxidation don't seem to be synchronous. Reactive oxygen species, e.g. hydrogen peroxides, could induce oxidative attack on the sperm membrane lipids and polyunsaturated fatty acid leading to lipid peroxidation cascade [37-38]. This results in a subsequent loss in membrane integrity and impaired sperm motility [39]. Also, other mechanisms, including oxidative damage to the axoneme and depletion of intracellular adenosine triphosphate could reduce sperm motility [40].

Recent interest in sperm DNA integrity has raised concerns that, while sperm may appear motile, those with fragmented or decondensed DNA may still be selected for intracytoplasmic sperm injection [13]. Such sperm have been linked to poor embryonic development and an increased risk of miscarriage [41-42]. Our study showed that DNA fragmentation assessed by both TUNEL and SCSA did not significantly correlate with normal forms and head defects. The findings suggested that DNA fragmentation may be independent from sperm morphology. Vernocchi et al. also reported the TUNEL readings did not correlate with sperm head morphology and recommended that the evaluation of the DNA status of spermatozoa is included in the standard analysis of fresh semen used in assisted reproductive technologies. Also, Casssuto et al. reported that DNA fragmentation measured by TUNEL did not correlate with sperm head morphology [43]. In contrast, some studies reported a significant correlation between DNA fragmentation and several forms of morphologically abnormal profiles, such as the combined presence of tapered heads [44] and multiple tails with disomy [45].

In conclusion, the study confirms that oxidative stress was associated with DNA fragmentation. Semen parameters did not correlate with sperm DNA fragmentation and 8-oxodGuo as a semen parameter could be independent from sperm DNA integrity. Thus, semen parameters may not be sufficient to assess sperm DNA integrity.

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