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Received: 9 July 2021 | Revised: 17 August 2021 | Accepted: 24 September 2021

DOI: 10.1111/and.14268

First International Journal of Andrology
aNDROLOGIa WILEY

SHORT COMMUNICATION

Association among sperm chromatin condensation, sperm DNA fragmentation and 8-OHdG in seminal plasma and semen parameters in infertile men with oligoasthenoteratozoospermia

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Funding information
Ondokuz Mayis University Research Foundation, Grant/Award Number: PYO.TIP.1904.17.012

Abstract

The present study aimed to investigate the clinical role of standard sperm diagnosis parameters (sperm concentration, motility, morphology) as well as aniline blue staining of histones, 8-OHdG, TUNEL assay were performed on semen samples in infertile men with oligoasthenoteratozoospermia (OAT). Thirty-two infertile and ten proven fertile men were included in the study. Chromatin condensation sperm in infertile men was significantly lower compared to the fertile men ($p < 0.0001$). Age, sperm concentration, morphology and motility were significantly negatively correlated with chromatin condensation ($p < 0.05$). However, no significant correlations among the chromatin condensation, SDF and sperm DNA damage were detected in terms of 8-OHdG concentration.

KEYWORDS

8-OHdG, chromatin condensation, OAT, semen parameters, sperm DNA fragmentation

1 | INTRODUCTION

Infertility is a heterogeneous and complex medical condition arising among others from genetic and epigenetic backgrounds. Globally, about 10%–15% of couples facing fertility problems at their reproductive age and the male factor is responsible for about half of these cases (Gunes & Esteves, 2020). Although the routine semen analysis is the most fundamental test for the initial evaluation of the fertility status of infertile men, it is insufficient to diagnose impaired sperm function and unreliable to predict male fertility (Esteves, 2016).

Despite all its criticism, standard semen analysis is still the 'gold standard' method used to diagnose male infertility. In semen analyses, sperm count, motility, normal sperm morphology and other essential parameters are assessed to evaluate the male fertility potential. However, semen analysis is not sufficient to explain the molecular basis of male infertility and is not only correctly reflecting fertility the male status but is also unable to predict sperm fertilising capacity. The subjectivity by which parameters such as sperm count,

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motility or normal sperm morphology are often determined (if not with computerised systems), the poor validation of these parameters as well as of the fact that fertilisation is essentially a multifactorial process (Amann, 1989; Henkel et al., 2005) and standard semen analysis only determines a few of the essential parameters.

Histone replacement by positively charged small proteins, protamines, in the sperm nucleus results in a denser and highly condensed sperm chromatin (Ward & Coffey, 1991). Normal condensation of sperm chromatin with histones and protamines is essential in proper sperm function, semen parameters, fertilisation rate and embryo quality after assisted reproduction techniques (ART) (Bichara et al., 2019; Gunes & Esteves, 2020). Sperm chromatin condensation defects make the sperm amenable to oxidative DNA damage (Vorilhon et al., 2018).

The present study show the relationship between sperm chromatin condensation, sperm DNA fragmentation and the level of 8-OH-deoxyguanosine (8-OHdG) residues in seminal plasma and their effects on sperm parameters of infertile men with oligoasthenoteratozoospermia (OAT).

2 | MATERIALS AND METHOD

2.1 | Study subjects and sample collection

This study has received ethical clearance from Medical Faculty Ethics Committee of Ondokuz Mayis University (OMU) with the reference number OMU KAEK: 2016/372. The present study consisted of 32 infertile men with OAT whose female partners had standard gynaecological evaluation and failure to achieve a clinical pregnancy after at least 1 year of unprotected intercourse. The group of study participants was selected among the infertile men attending the Urology Clinics of OMU. Ten healthy, age-matched fertile men who naturally fathered children within the last 2 years with normal semen parameters were included as a control group.

OAT diagnosis of patients was based on two or more semen analyses (sperm count $<15 \times 10^6$/ml, motility <32% and normal morphology <4%) according to World Health Organization (WHO, 2010) guidelines. All participants signed a written informed consent and provided medical history and lifestyle status data by a questionnaire. Exclusion criteria for the study were structural and numerical karyotype aberrations, AZF deletions, *CFTR* mutations, infections, testicular surgery, or trauma and other known causes of male infertility.

2.2 | Semen analysis

Semen samples were obtained from each individual by masturbation in a sterile container after 2–5 days of sexual abstinence and kept at 37°C for 20–30 min to liquefy. Following liquefaction, standard semen analysis was performed to evaluate the semen parameters (total sperm count and concentration, ejaculate volume, sperm motility and morphology) according to WHO guidelines (2010). Motility and sperm concentration were evaluated with a MicroCell counting chamber (Vitrolife, San Diego, CA) using a phase-contrast microscope.

After semen analyses, the semen samples were divided into the three aliquots to determine the chromatin condensation levels of sperm using the aniline blue staining, sperm DNA fragmentation using the TUNEL assay and 8-OHdG levels in seminal plasma by an ELISA method. Samples with a sperm concentration less than 1.4×10^6/ml were excluded from the study due to insufficient sperm count. The TUNEL assay was performed for the evaluation of sperm DNA fragmentation (SDF) in 25 specimens (16 patients and nine fertile donors) and the determination of 8-OHdG was used to identify sperm DNA damage in 36 samples (26 patients and ten fertile donors).

2.3 | Aniline blue staining

Sperm chromatin condensation was evaluated with the aniline blue staining (Terquem & Dadoune, 1983), staining the histone-rich spermatozoa. Fresh semen samples were washed twice with 0.2 M phosphate buffered saline (PBS) at 1,200 *g* for 7 min at room temperature. Ten microliters of the sperm pellet were spread on a slide and allowed to dry in the air. Then, the smear was fixed for 30 min at room temperature with 3% glutaraldehyde (Sigma-Aldrich, Darmstadt, Germany) in 0.2 M PBS. Finally, slides were stained for 10 min with 5% aqueous aniline blue solution in 4% acetic acid. About 200 spermatozoa were counted for each sample under the light microscope (CX31, Olympus Life and Material Sciences, Hamburg, Germany) at 1,000× magnification and the percentage of aniline blue-positive spermatozoa was calculated. Dark stained spermatozoa or partially stained spermatozoa were assessed as aniline blue-positive (immature, rich in histone), while unstained or only slightly stained spermatozoa were evaluated as aniline blue-negative spermatozoa (mature).

2.4 | TUNEL assay

SDF was evaluated using an in-situ cell death detection kit TUNEL assay (Roche Diagnostics GMbH, Mannheim, Germany). The seminal plasma was removed from neat semen samples by centrifugation of the samples at 250 *g*. The pellets containing spermatozoa were washed twice with PBS, fixed with 3.6% paraformaldehyde for 1 min at room temperature and stored at 4°C (up to 1 week) until analysis. The spermatozoa have been isolated from paraformaldehyde by centrifugation at 250 *g* for 7 min. Cells were spread on poly-L-lysine-coated slides and incubated at 4°C overnight. Then, cells were permeabilised with 0.1% triton X-100 in 0.1% sodium citrate solution and then incubated with the TUNEL reaction mixture in a humidified chamber at 37°C for 1 hr in the dark. Finally, spermatozoa were counterstained with an anti-fade, including DAPI. Each slide was immediately visualised under a fluorescent microscope at

400 X magnifications (Nikon Eclipse E600, Tokyo, Japan) and 200 spermatozoa were evaluated. Spermatozoa showing green FITC-fluorescence were assessed as TUNEL positive, while those sperm showing blue DAPI-fluorescence were evaluated as TUNEL negative. A positive control pre-treated with 200 mM H_2O_2 for an hour and a negative control without TdT enzyme was included in each run (Kabartan et al., 2019).

2.5 | ELISA for 8-OHdG

8-OHdG which is a major biomarker of oxidative DNA damage, was measured in seminal plasma using the colorimetric DNA/RNA oxidative DNA damage ELISA kit according to the manufacturer's instruction in a 96-well microplate (Cayman, Ann Arbor, USA) (Hosen et al., 2015). Briefly, the semen samples were centrifuged for 10 min at 300 *g* to separate seminal plasma from spermatozoa and somatic cells. The seminal plasma was stored at −80°C until used. Fifty microliters of diluted seminal plasma, positive and negative controls and blank were added to each well of a microtiter plate. Then, the wells were washed five times with 200 µl Ellman's solution provided in the kit, 5 µl of tracer added and incubated for 90 min on an orbital shaker. After adding of the stop solution, the absorbance in each well was measured at a wavelength of 420 nm by using a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific, Vantaa, Finland). A standard curve was generated by serial dilutions of the standards and based on the standard curve, a linear regression equation ($r^2 = 0.9945$) was received. The 8-OHdG concentrations were calculated using the equation obtained from standard curve plot and the OD values of the samples.

2.6 | Statistical analysis

The statistical analyses were performed with MedCalc Statistical Software version 19.2 (MedCalc Software Ltd, Ostend, Belgium). The normal distribution of the data was determined using the Shapiro–Wilk test. Independent *t*-test and Pearson correlation coefficients were calculated. A p-value of less than 0.05 was considered statistically significant.

3 | RESULTS

Chromatin condensation, SDF and 8-OHdG level in seminal plasma were evaluated in infertile men with OAT and normozoospermic fertile controls. Table 1 shows sperm concentration, motility and morphology and ejaculate volume of infertile men and fertile controls. However, sperm count and motility were significantly higher in fertile than infertile men with OAT ($p < 0.0001$), no significant difference was observed between the groups concerning patients age ($p = 0.2285$) and semen volume ($p = 0.6728$) (Table 1). Among the participants, 12 reported being smokers while 19 indicated that they do not smoke. There was no significant correlation between smoking status and aniline blue staining ($p = 0.670$), asthenoteratozoospermia ($p = 0.472$), oligozoospermia ($p = 1.000$), teratozoospermia ($p = 1.000$), TUNEL positive ($p = 0.733$).

Sperm chromatin condensation in terms of aniline blue-positivity, sperm DNA fragmentation and 8-OHdG level in seminal plasma in the patient and control groups are provided in Table 2. The percentage of aniline blue-positive spermatozoa in the fertile men was significantly lower compared to the infertile men with OAT

TABLE 1 Results of basic semen analysis of fertile and infertile men

Parameters	Infertile men with OAT ($n = 32$)	Fertile men ($n = 10$)	*p*-value
Age			
Mean ± *SD*	32.4 ± 5.7	34.8 ± 4.5	0.2285
Median (min–max)	31 (20–48)	35.5 (26–40)	
Volume (ml)			
Mean ± *SD*	2.6 ± 0.8	2.6 ± 1.1	0.6728
Median (min–max)	2.0 (2.0–5.0)	2.5 (1.6–5.0)	
Normal morphology (%)			
Mean ± *SD*	2.7 ± 1.7	6.9 ± 1.9	<0.0001
Median (min–max)	3.0 (0.0–6.0)	6.0 (5.0–12.0)	
Sperm Concentration (10^6/ml)			
Mean ± *SD*	5.1 ± 2.8	51.6 ± 16.2	**<0.0001**
Median (min–max)	5.5 (0.5–10.0)	56.0 (21.0–70.0)	
Motility			
Mean ± *SD*	26.2 ± 15.8	54.8 ± 5.9	**0.0001**
Median (min–max)	25.5 (3.0–59.0)	56.0 (45.0–63.0)	

Note: OAT, oligoasthenoteratozoospermia; *SD*, standard deviation.
$p < 0.05$ was considered statistically significant by independent samples *t* test.
Statistical significance is indicated in bold font.

TABLE 2 Comparison of different parameters of semen sample in fertile and infertile men

Parameters	Infertile men with OAT	Proven fertile men	(95% CI) for difference	*p*-value
Aniline blue-positive sperm Mean ± *SD*	57.50 ± 18.57	13.20 ± 5.20	(−56.41 to −32.19) −44.30	**<0.0001**
TUNEL Mean ± *SD*	24.8 ± 20.2	26.7 ± 21.3	(−16.33 to −20.18 1.92	0.839
8-OHdG Mean ± *SD*	0.19 ± 0.008	0.25 ± 0.20	(−0. 017 to 0.143) 0.063	0.120

Note: CI, confidence interval; 8-OHdG, 8-OH-deoxyguanosine; OAT, oligoasthenoteratozoospermia; *SD*, standard deviation.

$p < 0.05$ was considered statistically significant by independent samples *t* test.

Statistical significance is indicated in bold font.

($p < 0.0001$) (Table 2). No significant difference was observed for the seminal levels of 8-OHdG between infertile patients and controls ($p = 0.135$) (Table 2). There were also no significant differences for SDF between the patient and fertile donor groups ($p = 0.839$). The percentages of TUNEL-positive sperm in the infertile and fertile men were 24.8% and 26.7% respectively (Table 2).

The age of patients and controls was negatively correlated with chromatin condensation ($r = -0.3932$, $p = 0.011$). Sperm concentration, normal morphology and motility were found to be significantly negatively correlated with chromatin condensation ($p < 0.0001$, $p < 0.0001$ and $p = 0.0011$, respectively) (Table 3, Figure 1). However, there were no significant correlation between the chromatin condensation, SDF ($p = 0.657$) and 8-OHdG levels in seminal plasma ($p = 0.137$) (Table 3).

No significant difference was observed for the seminal plasma levels of 8-OHdG between infertile patients with OAT and controls ($p = 0.135$) and also no association with 8-OHdG and semen parameters (Table 3).

No significant differences were observed for SDF between the patient and fertile donor groups ($p = 0.839$). The percentage of TUNEL-positive sperm in the infertile and fertile men was 24.8% and 26.7% respectively (Table 2). SDF was not correlated with chromatin condensation ($\rho = -0.0543$; $p = 0.7967$). However, a correlation was close to the significance level between SDF and 8-OHdG level in seminal plasma ($\rho = 0.401$; $p = 0.052$). However, with a reasonably larger sample size of a total of 46 subjects, the correlation between SDF and 8-OHdG level would have reached significance. There was no significant association between SDF and semen parameters (Table 3).

4 | DISCUSSION

The present study confirms that chromatin condensation is strongly associated with OAT (Hammadeh et al., 1996; Irez et al., 2018; Nasr-Esfahani et al., 2006; Ni et al., 2016). Patients with OAT showed to have significantly more aniline blue-positive sperm than the fertile controls. The association between chromatin condensation and male infertility or assisted reproduction outcomes has been demonstrated in other studies using various assays including aniline blue staining, chromomycin-A3 (CMA3) or toluidine blue (Pourmasumi et al., 2019; Rogenhofer et al., 2013; Simon et al., 2011). Additionally, the present study's findings confirm a link between decreased chromatin condensation and semen parameters including sperm concentration, morphology, motility and men's age. Although Hammadeh and colleagues reported a significant association between patients and controls concerning the percentage chromatin condensation by aniline blue staining, they did not show correlations between chromatin integrity with morphology, motility and count (Hammadeh et al., 2001).

Infertile patients with abnormal protamine content are known to have chromatin disturbances and are more vulnerable to DNA damage (Agarwal & Said, 2003). Approximately 8% of infertile patients with normal semen parameters show an abundance of abnormal chromatin/DNA (Schulte et al., 2010). In this study, SDF and DNA damage in terms of 8-OHdG in seminal plasma as a byproduct of oxidative DNA damage did not show any difference between infertile and fertile men. There was also no significant difference among chromatin condensation, SDF and 8-OHdG levels in seminal plasma between the two study groups.

Athsan and colleagues demonstrated a positive correlation between DNA fragmentation index (DFI) and the level of chromatin packing in teratozoospermic and normozoospermic men using CMA3. A significantly higher percentage of positive CMA3 spermatozoa was similar to our results (Atshan et al., 2020). Similarly, De Iulliis and colleagues showed a direct relationship between 8-OHdG deposition and chromatin re-modelling in 130 infertile and 95 students with unknown fertility status (De Iuliis et al., 2009). A meta-analysis analysing the sperm protamine deficiency in seven studies demonstrated a higher protamine deficiency rate in subfertile men then the fertile controls and a significant correlation between protamine deficiency measured by CMA3 staining and general sperm DNA damage (Ni et al., 2016). Meanwhile, a recent study demonstrated a correlation between chromatin condensation and sperm DNA fragmentation in patients with OAT (Metin Mahmutoglu et al., 2021).

Our study did not find a significant association between SDF and semen parameters (sperm concentration, motility and normal morphology), chromatin condensation and seminal 8-OHdG level.

TABLE 3 Correlations among age, semen parameters, SDF, 8-OHdG level in semen, and chromatin condensation

Parameters	Age	Aniline blue-positive spermatozoa	TUNEL	8-OHdG	Motility	Concentration
Age	1.000					
Aniline blue-positive spermatozoa (%)	**ρ = −0.3932** **p = 0.011***	1.000				
TUNEL	ρ = 0.022 p = 0.920	ρ = −0.0543 p = 0.7967	1.000			
8-OHdG	ρ = −0.156 p = 0.369	ρ = −0.252 p = 0.138	ρ = 0.401 p = 0.052	1.000		
Motility	ρ = 0.187 p = 0.2425	**ρ = −0.485** **p = 0.0011**	ρ = −0.321 p = 0.117	ρ = 0.112 p = 0.514	1.000	
Concentration	ρ = 0.241 p = 0.129	**ρ = −0.643** **p < 0.0001**	ρ = 0.034 p = 0.870	ρ = −0.003 p = 0.986	**ρ = 0.661** **p < 0.0001**	1.000
Morphology	ρ = 0.251 p = 0.114	**ρ = −0.611** **p < 0.0001**	ρ = −0.077 p = 0.7156	ρ = −0.123 p = 0.475	**ρ = 0.763** **p < 0.0001**	**ρ = 0.742** **p < 0.0001**

Note: SDF, sperm DNA fragmentation.
Pearson correlation, statistical significance is indicated in bold font.
*Statistically significant.

FIGURE 1 (a) Correlation between sperm concentration and aniline positive staining ($\rho = -0.643$, $p < 0.0001$); (b) Correlation between sperm motility and aniline positive staining ($\rho = -0.485$, $p = 0.0011$) (c) Correlation between normal morphology and aniline positive staining ($\rho = 0.611$, $p < 0.0001$)

However, a linkage that is close significance between SDF and 8-OHdG level in seminal was found. If a slightly larger sample size had been used, this association would be significant. Sharma et al. (2016) conducted a study using a flow cytometric TUNEL assay analyzing

infertile men and controls both unproven and proven fertile men showed that SDF levels in infertile men are higher than in the controls (Sharma et al., 2016).

This study shows the relationship between age, normal morphology, sperm concentration and motility, sperm chromatin condensation, sperm DNA fragmentation and the level of 8-OHdG residues in seminal plasma from the same semen sample in infertile men with OAT. As a result, we could analyse the correlations between these parameters in detail. The major limitation of the present study is its small size, which we tried to compensate for by doing a sample size calculation, which was successful in the correlation between 8-OHdG and TUNEL. In addition, the heterogeneous nature of male infertility and other factors such as unknown fertility causes, heterogeneity in patients and control group, age, and lifestyle factors are limiting factors.

In conclusion, our study showed that chromatin condensation has a detrimental effect on male fertility potential as it is negatively associated with semen parameters, especially sperm morphology. Moreover, it appears that chromatin condensation in terms of chromatin compaction is a parameter that needs to be taken into consideration apparently, more in patients undergoing ICSI as in this patient group, a significantly higher frequency of chromatin condensation defects was detected.

ACKNOWLEDGEMENTS

The present was supported by Ondokuz Mayis University Research Foundation (Project no PYO.TIP.1904.17.012).

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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How to cite this article: Hologlu, D., Gunes, S., Asci, R., Henkel, R., & Guvenc, T. (2022). Association among sperm chromatin condensation, sperm DNA fragmentation and 8-OHdG in seminal plasma and semen parameters in infertile men with oligoasthenoteratozoospermia. *Andrologia*, 54, e14268. https://doi.org/10.1111/and.14268