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ORIGINAL PAPER

Relationships between sperm DNA integrity and bulk semen parameters in Bulgarian patients with varicocele

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Summary ***Objective: This exploratory retrospective study aimed to compare the level of Sperm DNA Fragmentation (SDF) and investigate its association with bulk semen parameters, for the first time in Bulgarian patients with varicocele, using a distinct methodology.***
Material and methods: Standard semen analysis was performed according to the 2010 criteria of the European Society of Human Reproduction and Embryology - Nordic Association for Andrology (ESHRE-NAFA-2010) and DNA fragmentation was assessed using the Halosperm® kit. The total sample included 28 males: the control group consisted of men with normal genital examination and unknown fertility (n = 10), group one consisted of men with varicocele, normozoospermia and DNA fragmentation > 15% (n = 9) and group two consisted of men with varicocele, abnormal sperm parameters and DNA fragmentation > 15% (n = 9).
Results: DNA fragmentation was found to be higher in patients with abnormal sperm parameters (43.78 ± 30.78) compared to the normozoospermic group (21.22 ± 3.93) (p = 0.008). In normozoospermic patients, no statistically significant correlations were observed between SDF and bulk semen parameters. In patients with abnormal sperm parameters, DNA fragmentation exhibited significant very strong negative association with motility (a+b), vitality and typical morphology (p < 0.001).
Conclusions: DNA integrity assays could be used for a better evaluation and management of male infertility, particularly in normozoospermic varicocele patients.

KEY WORDS: Male Infertility; Sperm DNA fragmentation; Sperm DNA damage; Varicocele; Semen Analysis.

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INTRODUCTION

Varicocele, an abnormal dilation and tortuosity of the internal spermatic veins within the plexus pampiniformis, is the most frequently identified lesion in males undergoing infertility evaluation (1). It is a common condition in men with normal spermatogenesis but also in men with abnormal semen parameters. It is found in approximately 15% of the general population and is reported in 35% of men with primary infertility and 75 to 81% of men with secondary infertility (2).
Varicocele is a complex entity as its effects on sperm quality are both difficult to define and predict. Not all men with varicocele are infertile (3) and patients with clinically evident varicocele may present with normal values on primary semen assays. On the other hand, patients may present with altered spermatogenesis, poor bulk seminal parameters and *oligoasthenoteratozoospermia* (OAT syndrome) (4).
The mechanisms by which varicoceles lead to spermatogenic failure are not completely elucidated. However, most authors investigating how varicoceles impair the reproductive function consider the primary event to be an increase in intratesticular temperature secondary to interruption in the counter-current heat exchange provided in the plexus pampiniformis with opposing flow vectors in a central arterial system and surrounding veins. The proposed mechanisms by which male fertility is impaired by this effect mainly include DNA fragmentation, apoptosis and oxidative stress. Other associated factors include testicular hypoxia secondary to venous stasis, reflux of renal/adrenal toxic metabolites and hypertension in the internal spermatic veins (5).
Numerous researchers have recently examined the association between varicocele and *Sperm DNA Fragmentation* (SDF). Although a cause-and-effect relationship is not established, multiple reviews and meta-analyses conclude that there is indeed evident association between varicocele and increased DNA fragmentation (6).
Although sperm DNA damage represents a reproducible sperm function marker, the *American Society for Reproductive Medicine* and the *American Urological Association* (AUA) do not recommend routine clinical use of sperm DNA testing (7). The utility of SDF testing is currently appreciated by the AUA and the *European Association of Urology* (EAU) for the evaluation of *Assisted Reproductive Technology* (ART) success (8, 9).
Studies have shown that sperm DNA damage is associated with negative influence on embryo development and lower pregnancy rates (lower natural pregnancy rates and lower pregnancy rates after intrauterine insemination and in vitro fertilization) (10, 11).
A significant finding is the increased risk for spontaneous abortions with increasing sperm DNA fragmentation. Comprehensive meta-analyses have shown that higher levels of DNA fragmentation are linked with an approximately double *risk ratio* (RR) of miscarriages (12).
The current study included Bulgarian men clinically

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diagnosed with varicocele and SDF level higher than 15%, which is defined as the normal threshold for in vivo fertilization using the *Halosperm® kit* (13).
The objective was to compare the levels of DNA fragmentation between varicocele patients with normozoospermia and abnormal sperm parameters and to investigate the correlation of SDF and bulk semen parameters as well as describe the relationships between them, for the first time in the Bulgarian population.
At the same time a secondary aim was to collect data using a distinct methodology which is not utilized as frequently in literature. Namely, the normozoospermic patients in the current study are defined according to the ESHRE-NAFA-2010 criteria which are stricter in defining normal parameters and make the additional distinction of borderline parameters in comparison to the WHO manual.
Finally, there is great variability in literature in the methods used to determine SDF, which renders making correlations and drawing conclusions difficult (14). SDF in the present study was analyzed using the *Halsoperm® kit*, aiming to add to the pool of data acquired via *Sperm Chromatin Dispersion*.

Materials and methods

Study group

The study included 28 men assessed by the andrology laboratory department at *Genika, Genetic and Medico-Diagnostic Laboratory in Sofia, Bulgaria*. A retrospective study was designed involving one control group of men with unknown fertility, normal genital examination and normal bulk semen parameters (n = 10) and two groups of patients with clinically diagnosed varicocele and infertility. The varicocele was diagnosed by palpation and Doppler ultrasound examination. Among the patients with varicocele, the first group (n = 9), presented with normozoospermia and levels of DNA fragmentation higher than 15%. The second group (n = 9) presented with abnormal sperm parameters and levels of DNA fragmentation higher than 15%. In all men, medical history was obtained, including occupation, smoking habits, alcohol intake and the use of prescription medication. Exclusion criteria for all groups were: current or previous systemic diseases that would lead to testicular alterations such as cancer and endocrinopathies (and their treatments) and a history of excessive alcohol and drug consumption.
According to the laboratory's protocol, informed consent is acquired from all patients at the time of registration for the anonymous inclusion of their sperm analysis results in potential medical studies and research.

Sperm collection and semen analysis

Semen samples were collected by masturbation after three to seven days of ejaculatory abstinence and were analyzed within one hour of collection. Additionally, patients were instructed to abstain from alcohol consumption for three to seven days and maintain a good general status for three months before the examination as well as present afebrile on the day of the analysis, without having consumed any antibiotics (they were instructed to report any received medications). In all patients, the semen sample was (up to 25 minutes post ejaculation) retained in a 37 °C thermostat for circa 15 minutes. After complete semen liquefaction, pH and volume were measured and a standard seminal analysis was performed according to the criteria of the *European Society of Human Reproduction and Embryology - Nordic Association for Andrology 2010* (ESHRE-NAFA-2010). Analysis was performed on the computer microscopic fluorescent platform *Sperm Class Analyzer* (SCA - v.5.0) and the parameters measured included total sperm count, motility, vitality and morphology. Sperm morphology was assessed using Kruger's strict criteria. Sperm vitality was assessed using Eosin Y to stain spermatozoa and Negrosin to stain the background. In comparison to WHO-5, 2010, the criteria of ESHRE-NAFA-2010 interpret results in three categories: normal, borderline and pathologic. The borderline results represent a buffer zone of clinical importance. Patients in this zone are regarded as having the potential to shift to either normal or pathological spectra depending on the persistence of offending factors.

Determination of DNA integrity

Sperm nuclear DNA integrity was evaluated by use of the *Halosperm® kit* (*Halotech® DNA SL, Madrid, Spain*).
This test is a modified and improved version of the *sperm chromatin dispersion* (SCD) test (15). The clinical threshold for percentage of spermatozoa with DNA fragmentation has been established as (1) < 15% for in vivo fertilization, (2) < 30% for in vitro fertilization. The kit included: 10 Super Coated Slides, 10 Eppendorf agarose tubes, one tube with denaturizing solution (1 mL), and two bottles of Lysis solution (60 mL ×2). The first step included inserting the semen sample in agarose microgel. The lysis solution was first left in room temperature. The semen sample was diluted with an extender or PBS until a concentration of 5-10 $\times 10^6$/ml. The Eppendorf tube with the agarose was placed in a float for five minutes at 90 to 100 °C until the agarose melted. The tube was then moved along with the float into a water bath with a temperature of 37 °C and was left there for five minutes. 60 mL of the semen sample were transferred to the Eppendorf tube and were gently mixed. The Super-Coated Slide to be used was placed on a cold surface area (glass plate) at 4 °C. Once the slide had been cooled, cell suspension was transferred from the Eppendorf tube to the slide, towards the side that was treated and marked by a dark point. A coverslip was placed on top very carefully, to avoid creating bubbles.
A drop of 14, 20 or 50 µL for the slides is used for the respective magnifications of 18×18mm, 22×22 mm or 24×60 mm. The slides were maintained in horizontal orientation throughout the whole procedure. The cooled glass plate with the slide was then placed in the fridge and the sample was left to jellify for five minutes. Processing the sample followed. The denaturizing agent was prepared by adding 80 µL from the acid denaturizing solution to 10 µL distilled water and was placed in an incubator tray. The cover slip was slid off the slide and the slide was immediately immersed in the denaturizing solution in horizontal orientation, leaving it to incubate for seven minutes at room temperature (22°C). After putting on gloves, the slide was picked up with the help of pincers. While remaining in horizontal position it was placed

in another incubation tray containing 10 µL of lysis solution. It was left to incubate for 25 minutes. The slide was picked up and placed in a Petri dish filled with distilled water in order to wash off the lysis solution. It was left to incubate for five minutes. The slide was picked up and was placed horizontally in a Petri dish with 70% ethanol for two minutes, 90% ethanol for two minutes and 100% ethanol for two minutes. The slide was left to dry and once completely dried the prepared slide was stained for observation under light microscopy. Wright's staining solution was mixed with *Phosphate-buffered saline* (PBS) in 1:1 ratio and one layer of the staining solution was placed horizontally in order to cover the wet slide. The slide was left to stain for five to 10 minutes. The slide was carefully rinsed with running water and was left to dry. Subsequently direct microscopic visual analysis was performed. The nucleoid that corresponds to deproteinized nuclei of spermatozoa is made up of two parts: a core located centrally and a peripheral halo of chromatin/DNA dispersion. The spermatozoa tails are also visible. A minimum of 500 spermatozoa are studied per sample and scored according to the following criteria.

Sperm classification

The categorization of the different halo sizes is performed using the minor diameter of the core from the own nucleoid as a reference to which the halo width is compared. The SCD patterns established are the following (15):

Sperm cells without DNA fragmentation

a) Sperm cells with large halo: those whose halo width is similar or higher than the minor diameter of the core.
b) Sperm cells with medium-size halo: the halo size is between: maximum one third of the minor diameter of the core and minimum the length of the minor diameter of the core.

Sperm cells with DNA fragmentation

c) Sperm cells with small halo: the halo width is similar or smaller than one third of the minor diameter of the core. The core may have irregular form or barely distinguishable (< 150 µm^2).
d) Sperm cells without halo
e) Sperm cells without halo-degraded: there is no halo and the core presents granule-like fragmentation and is weakly stained.

"Others"

Nucleoids that do not correspond to spermatozoa. One of the morphological characteristics that distinguish them is the absence of tail. These cells are not included in the estimation of frequency of sperm with fragmented DNA.

Statistical analysis

Data analysis was performed using the R software environment for statistical computing and graphics. For each variable the mean, the standard deviation and *interquartile range* (IR) are presented to indicate the relevant distribution. The Shapiro-Wilk test was applied to test whether variables are normally distributed. For normally distributed data a Student's test was conducted to test whether means differ across groups.

In the case of not normally distributed variables a Mann-Whitney-Wilcoxon test was applied to test whether the distribution across two groups differs. For both tests, the Null-Hypothesis (difference in means/populations) was rejected if the p-value was smaller than 0.05. Correlation coefficients were calculated according to the Pearson method. Statistically significant correlation coefficients were interpreted as: weak from 0.20 to 0.39 (or -0.20 to -0.39); moderate from 0.40 to 0.59 (or -0.40 to -0.59); strong from 0.60 to 0.79 (or -0.60 to -0.79); very strong from 0.80 to 1.0 (or -0.80 to -1.0). If the p-value was smaller than 0.05 then the correlation coefficient was interpreted as being statistically significant.

Results

All 18 patients entering the study presented varicocele in the left testis, which was detected by physical examination and confirmed by Doppler ultrasound. The median age (years) was 31 ± 6.182 (26.25-34.5) for controls, 32 ± 6.648 (30-37) for patients with normal semen parameters (G1) and 36 ± 6.464 (35-42) for patients with abnormal semen parameters (G2). No statistically significant difference was found between the groups with respect to age apart from between the controls age and the G2 age, p = 0.01905.

Standard semen analysis parameters

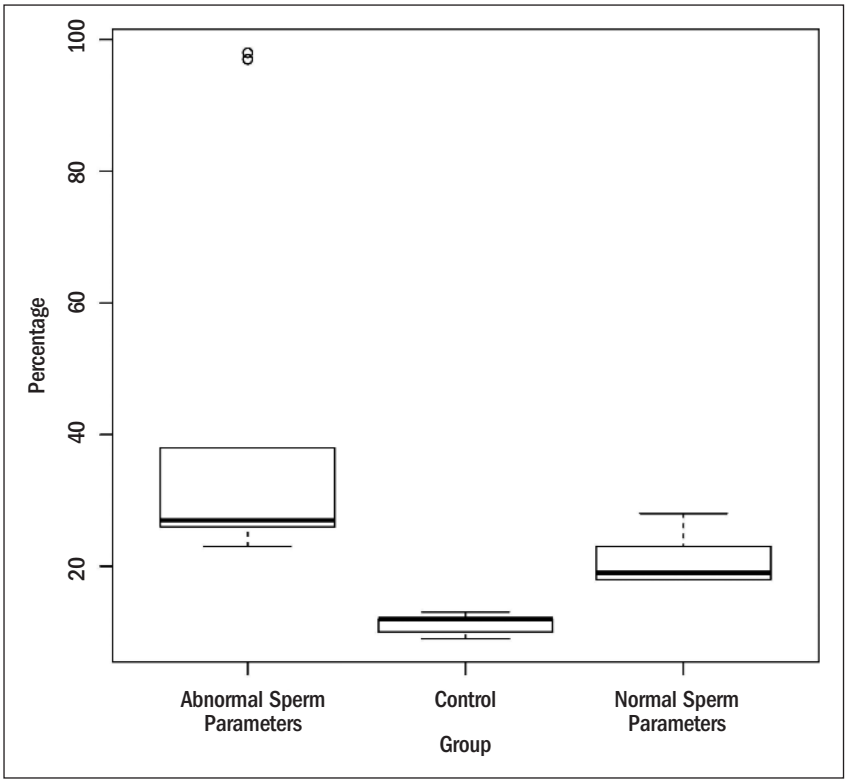
The main sperm parameters of controls and varicocele patients are presented in Table 1. G1 (n = 9) had normal

Table 1.

Comparison of standard sperm parameters between control subjects, varicocele patients with normal sperm parameters (G1) and varicocele patients with abnormal sperm parameters (G2). Numbers represent mean ± standard deviation and range is shown in parentheses.

	Control (n = 10)	Normal Sperm Parameters (G1) n = 9	Abnormal Sperm Parameters (G2) n = 9	A: p value between controls and G1	B: p value between controls and G2
pH	7.54 ± 0.07 (7.5-7.575)	7.611 ± 0.078 (7.6-7.7)	7.733 ± 0.132 (7.6-7.8)	0.052	0.002
Volume (mL)	3.87 ± 2.465 (2.525-4.4)	3.944 ± 1.474 (2.7-5.1)	3.889 ± 2.070 (2.6-5.5)	0.595	0.744
Number (x 10^6/mL)	448.4 ± 317.518 (251.2-651.7)	293.9 ± 109.582 (214.6-362.3)	117.3 ± 115.782 (41.1-169.3)	0.488	0.008
Motility (% a+b)	57.8 ± 3.225 (56-60.5)	57 ± 3.640 (55-58)	24.89 ± 13.897 (26-32)	0.594	<0.001
Vitality (%)	78.2 ± 3.676 (78-81)	76.67 ± 3.464 (73-79)	48.89 ± 19.127 (46-62)	0.337	< 0.001
Morphology: Typical (%)	17.7 ± 1.947 (16-18)	16.56 ± 1.667 (16-17)	4.111 ± 2.028 (4-5)	0.256	< 0.001

Figure 1.
DNA fragmentation by group.



sperm parameters. G2 (n = 9) showed an abnormality in one or more of the bulk semen parameters. Particularly, five patients had oligoasthenoteratozoospermia and four had asthenoteratospermia. Furthermore, six patients presented with more than 20% decapitated forms.

Sperm DNA fragmentation measured by the Halosperm® kit

In the control group the mean SDF percentage was calculated as 11.4 ± 1.35 (10.25-12). This was lower in comparison to G1 ($p < 0.001$) and G2 ($p < 0.001$). SDF was found to be higher in patients with abnormal sperm parameters 43.78 ± 30.78 (26-38) compared to the normozoospermic group 21.22 ± 3.93 (18-23) ($p = 0.008$). SDF by group is presented in Figure 1.

Relationships between DNA fragmentation and bulk semen parameters

In patients with varicocele and normal sperm parameters, DNA fragmentation was negatively correlated with sperm pH ($r = -0.54$, $p = 0.135$) and motility (a+b) ($r = -0.31$, $p = 0.410$), and positively correlated with sperm volume ($r = 0.10$, $p = 0.799$), number ($r = 0.14$, $p = 0.713$), vitality ($r = 0.05$, $p = 0.894$) and typical morphology ($r = 0.38$, $p = 0.314$). In all cases the strength of association ranges from very weak to moderate only, of which none is significant (i.e., $p > 0.05$).

On the other hand, in the group of patients with abnormal sperm parameters, DNA fragmentation was positively correlated with pH ($r = 0.08$, $p = 0.840$) and volume ($r = 0.15$, $p = 0.706$); and negatively correlated with number ($r = -0.47$, $p = 0.204$), motility (a+b) ($r = -0.94$; $p < 0.001$), vitality ($r = -0.93$; $p < 0.001$) and typical morphology ($r = -0.89$; $p < 0.001$). The latter three indicate a very strong association.

Discussion

This exploratory study aimed to answer the following question: do varicocele patients with abnormal sperm parameters have higher levels of SDF than varicocele patients with normozoospermia?

According to the literature research performed by the authors, it was impossible to identify other studies using the same design: directly comparing the DNA fragmentation levels, by use of the *Halosperm® kit*, between varicocele patients with normozoospermia and varicocele patients with abnormal sperm parameters, while performing semen analysis according to the ESHRE-NAFA-2010 criteria.

The results of our study indicate that there is clearly a higher degree of SDF in patients with varicocele. Furthermore, in our study varicocele patients with abnormal sperm parameters were found to have higher levels of SDF than varicocele patients with normozoospermia.

This finding is consistent with what other authors have also demonstrated regarding infertile men (16). The fact that the levels of fragmentation in the group of patients with abnormal sperm parameters are higher, could reflect the more advanced effects of varicocele on spermatogenesis.

Moreover, in men with abnormal sperm parameters, we established statistically significant, very strong correlations between DNA fragmentation and three semen parameters: progressive motility, vitality and typical morphology. These results are comparable to what other authors have observed (17).

Weaknesses of this study are the limited number of participating patients as well as the absence of data regarding the grade of varicocele which do not allow the drawing of any major conclusions. This being an exploratory study, the authors urge other researchers both in Bulgaria and abroad to collect more data using the methodology described, to obtain a more thorough understanding of the correlations between SDF and bulk semen parameters in patients with varicocele.

Criticisms regarding the utility of SDF tests to evaluate male infertility include: that the minimum number of spermatozoa without fragmentation required for in vivo conception is unknown, that a simple sperm vitality assessment could suffice given its correlation to SDF (18), and that more studies investigating the cost effectiveness of SDF testing are required to determine its role alongside the standard sperm analysis. While the *Halosperm® kit* is only available in a few laboratories in

our country, it can be argued that it is cost-and-time effective, since the results are produced on the same day and the average price of 100 EUR for the combined standard semen and SDF analysis is affordable.

In conclusion, it has already been proven by numerous studies that sperm DNA damage is associated with decreased pregnancy rates in both *in vivo* and *in vitro* fertilization. There is an association of SDF with miscarriage and some studies have demonstrated the potential of reversing it by varicocelectomy (19). In patients with abnormal sperm parameters, SDF levels could be expected to be higher. In normozoospermic patients, SDF testing could potentially alter management as increased levels of SDF could present a therapeutic target. DNA fragmentation testing can be another arrow in the quiver of the clinician for the better understanding of male infertility and the management of selected patients with varicocele.

References

1. Jarow J. Effects of varicocele on male fertility. Hum Reprod Update. 2001; 7:59.

2. Gorelick J, Goldstein M. Loss of fertility in men with varicocele. Fertil Steril. 1993; 59:613.

3. Kursh E. What is the Incidence of Varicocele in a Fertile Population?. J Urol. 1988; 139:665.

4. World Health Organization. The influence of varicocele on parameters of fertility in a large group of men presenting to infertility clinics. Fertil Steril. 1992; 57:1289.

5. Masson P, Brannigan R. The Varicocele. Urol Clin North Am. 2014; 41:129.

6. Zini A, Dohle G. Are varicoceles associated with increased deoxuribonucleic acid fragmentation?. Fertil Steril. 2011; 96:1283

7. Practice Committee of American Society for Reproductive Medicine. The clinical utility of sperm DNA integrity testing. Fertil Steril. 2008; 90:178.

8. Jarrow J, Sigman M, Kolettis P, et al. Optimal Evaluation of the Infertile Male. AUA Best Practice Statement reviewed and validity confirmed. 2011.

9. Jungwirth A, Giwercman A, Tournaye H, et al. European Association of Urology guidelines on Male Infertility: the 2012 update. Eur Urol. 2012; 62:324.

10. Giwercman A, Lindstedt L, Larsson M, et al. Sperm chromatin structure assay as an independent predictor of fertility in vivo: a case-control study. Int J Androl. 2010; 33:221.

11. Collins J, Barnhart K, Schlegel P. Do sperm DNA integrity tests predict pregnancy with in vitro fertilization?. Fertil Steril. 2008; 89:823.

12. Robinson L, Gallos I, Conner S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod, 2012; 27:2908.

13. Evenson D, Wixon R. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. Reprod Biomed Online. 2006; 12:466.

14. Evgeni E, Lymberopoulos G, Touloupidis S, et al. Sperm nuclear DNA fragmentation and its association with semen quality in Greek men. Andrologia. 2015; 47:1166.

15. Fernandez J, Muriel L, Goyanes V, et al. Halosperm® is an easy, available, and cost-effective alternative for determining sperm DNA fragmentation. Fertil Steril. 2005; 84:860.

16. Moskovtsev S, Willis J, White J, et al. Sperm DNA Damage: Correlation to Severity of Semen Abnormalities. Urology. 2009; 74:789.

17. Omran H, Bakhiet M, Dashti M. DNA integrity is a critical molecular indicator for the assessment of male infertility. Mol Med Rep. 2013; 7:1631.

18. Mitchell LA, De Iuliis GN, Aitken RJ. The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. Int J Androl. 2011; 34:2.

19. Roque M, Esteves S. Effect of varicocele repair on sperm DNA fragmentation: a review. Int Urol Nephrol. 2018; 50:583.

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