



## SPERMATOZOA DNA DAMAGE IN MEN WITH REPRODUCTIVE PROBLEMS

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### ABSTRACT:

Male infertility is a serious emerging problem of public health, with high social significance due to its direct connection to negative demographic trends in Bulgaria and in different regions of the world. The targeted and multifaceted studies in this field are contradictory and incomplete.

**Purpose:** The present study analyzes the degree of spermatozoa DNA damages in men with reproductive problems. The aim of the study was to investigate the spermatozoa DNA damage in men with reproductive problems by complex approach combining comet assay and conventional sperm analysis.

**Material and methods:** Totally 80 individuals have been studied by the usage of the conventional sperm analysis and the comet assay. The impact of environmental and lifestyle factors was analyzed based on completing the participants' voluntary questionnaire.

**Results:** About 21% of the participants among the studied group were undergoing occupational hazards, 22% were smokers, 42% – alcohol consumers, 13% – drug users, 7% taking anabolic steroids, 31% – taking medications and 5% work or live under stress. The results obtained show that about 55% of the subjects studied were with normozoospermia, 19% – with asthenozoospermia, 13% – with oligoasthenozoospermia, 10% – with oligoasthenoteratozoospermia and 3% – with asthenoteratozoospermia. Elevated levels of mean values of comet parameters were observed when the concentration, motility and percentage of cells with normal morphology were decreased ( $p < 0.05$ ).

**Conclusions:** The statistically significant relationships between the analyzed characteristics could be successfully used for prognostic purposes in characterizing male reproductive health. The results of our study also confirmed that some environmental factors affecting negatively sperm structure and function should continue to be a significant public health concern nowadays.

**Keywords:** male infertility, comet assay, semen quality.

### INTRODUCTION:

Male infertility is one of the most serious problems of public health, characterized by social significance with its direct connection with the negative demographic trends in Europe. It is known that various common biological, genetic and ecological factors are at its core [1, 2]. Different investigations indicate that environmental and lifestyle factors could significantly affect sperm quality. At the same time, conventional sperm analysis could not answer many of the issues related to male reproductive health and clarify the mechanisms of its deterioration [3, 4]. At present, the use of a variety of cytogenetic and molecular-genetic methods is a preferred way to study the genetic causes associated with male infertility. Many researches are focused on applying complex approaches providing greater accuracy and higher objectivity in assessing the male reproductive potential [5, 6]. In Bulgaria, the targeted and multifaceted studies in this field are incomplete. The analysis of the relations between sperm quality and male fertility shows that the causes leading to its deterioration are complex [7] and require a variety of detailed studies. In this aspect, we aimed to analyze the degree of spermatozoa DNA damage in men with reproductive problems and to examine the potential dependencies between the level of DNA damage and sperm quality.

### MATERIAL AND METHODS:

The study was done during the period from January 2018 to September 2019 accordingly to the ethical principles. It was approved by the Institutional Ethic Committee (Certificate N 2/16.01.2019). Informed consent to participate in the study was obtained from each patient. A comet assay was applied to the sperm of 79 individuals with pre-established conclusions by the conventional sperm analysis.

A physical examination was carried out by a medical doctor, and the patients with varicocele, cryptorchidism, parotiditis, azoospermia, genital trauma, infections and other genitourinary diseases were excluded from the study.

The impact of environmental and lifestyle factors was analyzed based on the completion of the participants' voluntarily questionnaire.

All participants provided a standardized semen sample. Spermatozoa concentration (in millions per mL) and motility (in percentage) were assessed in fresh native preparations by conventional methods in a Makler counting chamber. The obtained results were compared to the referent data for the Bulgarian population [8]. All morphological abnormalities were calculated as % of all spermatozoa cells analyzed per individual (morphological defects < 14% – norm) [8].

The comet assay was performed on sperm cells according to a neutral version of Haines et al. [9], with slight modifications. A mixture (100 µL) of sperm sample and 0.7% low melting point agarose, diluted in PBS, was added to the microscope slide, pre-coated with 1% normal melting point agarose. Slides were submersed in lysis buffer (2.5M NaCl, 100mM Na<sub>2</sub>-EDTA, 10mM Tris, 0.25M NaOH, pH 10.0) containing 1% Triton X-100 and 40mM dithiothreitol for 1 h at room temperature. Proteinase K (100 µg/mL) was added to the lysis solution, following the initial period of lysis. Then additional lysis was performed at 37°C for 3 h. The unwinding of DNA was done by 20 min incubation in TBE buffer (pH 8.5), and electrophoresis was conducted (20 min, 25 V, 300 mA). The steps above were carried out at 4°C provided by a cooling apparatus and under the yellow light to avoid additional temperature- and UV-induced DNA damage. After electrophoresis, slides were neutralized (0.4M Tris-HCl, pH 7.4), and DNA was then stained with SYBR Green I fluorescent dye (1:10 000 dilution). Individual cells or “comets” were viewed using a Leica DM 1000 microscope with a filter and camera at 400× magnification. 100 cells were analyzed per slide, and comet analysis was performed with Comet Assay Score Program (CASPlab, Poland). Three comet parameters were used to assess DNA damage in sperm cells: % tail DNA – %TDNA

(the amount of DNA in the tail, in %), tail length – TL (distance of DNA migration in the comet tail, in µm), and tail moment – TM (this parameter takes into account both the distance of DNA migration and the amount of DNA in the tail of the comet).

Descriptive statistics were used to describe the characteristics of studied parameters, such as a variable's mean, standard deviation, standard error or frequency. Differences between groups were analyzed using a t-test. Statistical significance was defined as P<0.05 and P<0.001. The one-way analysis of variance (ANOVA) was used to determine whether there are any statistically significant differences between the means of the investigated parameters in the groups studied.

## RESULTS:

The data indicated that among the studied group, about 21% (N=16) of the participants were undergoing occupational hazards, 22% (N=17) were smokers, 42% (N=33) – alcohol consumers, 13% (N=9) – drug users, 7% (N=5) taking anabolic steroids, 31% (N=24) – taking medications and 5% (4) work or live under stress. The total percentage exceeds 100% because some of the men surveyed are influenced by multiple of the mentioned factors.

The results obtained show that about 55% (N=44) of the subjects studied are with normozoospermia, 19% (N=15) - with asthenozoospermia, 13% (N=10) - with oligoasthenozoospermia, 10% (N=8) - with oligoasthenoteratozoospermia and 3% (N=2) - with asthenoteratozoospermia.

The established relationships between the mean values of the studied comet assay parameters (%TDNA, TL and TM) and sperm quality parameters (sperm concentration, motility and morphology) are presented in Table 1.

Dependencies established between the comet assay parameters and the conclusions done by conventional sperm analysis are presented in Table 2.

**Table 1.** Dependencies between the mean values of the comet assay parameters (%TDNA, TL and TM) and the sperm quality parameters (sperm concentration, motility and morphology).

Comet assay parameter	Sperm quality parameter	N	Mean	SD	SE
<b>Sperm concentration</b>					
<b>%TDNA</b>	Under the norm	18	15.632*	7.211	1.700
	In the norm	61	8.725	4.823	0.618
<b>TL</b>	Under the norm	18	15.550*	8.809	2.076
	In the norm	61	6.982	4.810	0.616
<b>TM</b>	Under the norm	18	3.997*	3.618	0.853
	In the norm	61	1.284	1.432	0.183
<b>Sperm motility</b>					
<b>%TDNA</b>	Under the norm	35	12.920*	6.723	1.136
	In the norm	44	8.213	4.760	0.718
<b>TL</b>	Under the norm	35	12.375*	7.904	1.336
	In the norm	44	6.198	4.472	0.674
<b>TM</b>	Under the norm	35	2.910*	3.080	0.521
	In the norm	44	1.100	1.194	0.180

Sperm morphology					
%TDNA	Under the norm	10	16.998*	9.153	2.894
	In the norm	69	9.328	4.963	0.597
TL	Under the norm	10	16.818*	10.988	3.475
	In the norm	69	7.792	5.327	0.641
TM	Under the norm	10	4.774*	4.599	1.454
	In the norm	69	1.486	1.5487	0.186

N - Number of individuals; SD - standard deviation; SE - standard error; \* - $P \leq 0.05$ ; %TDNA - % tail DNA; TL - tail length ( $\mu\text{m}$ ); TM – tail moment ( $\mu\text{m}$ )

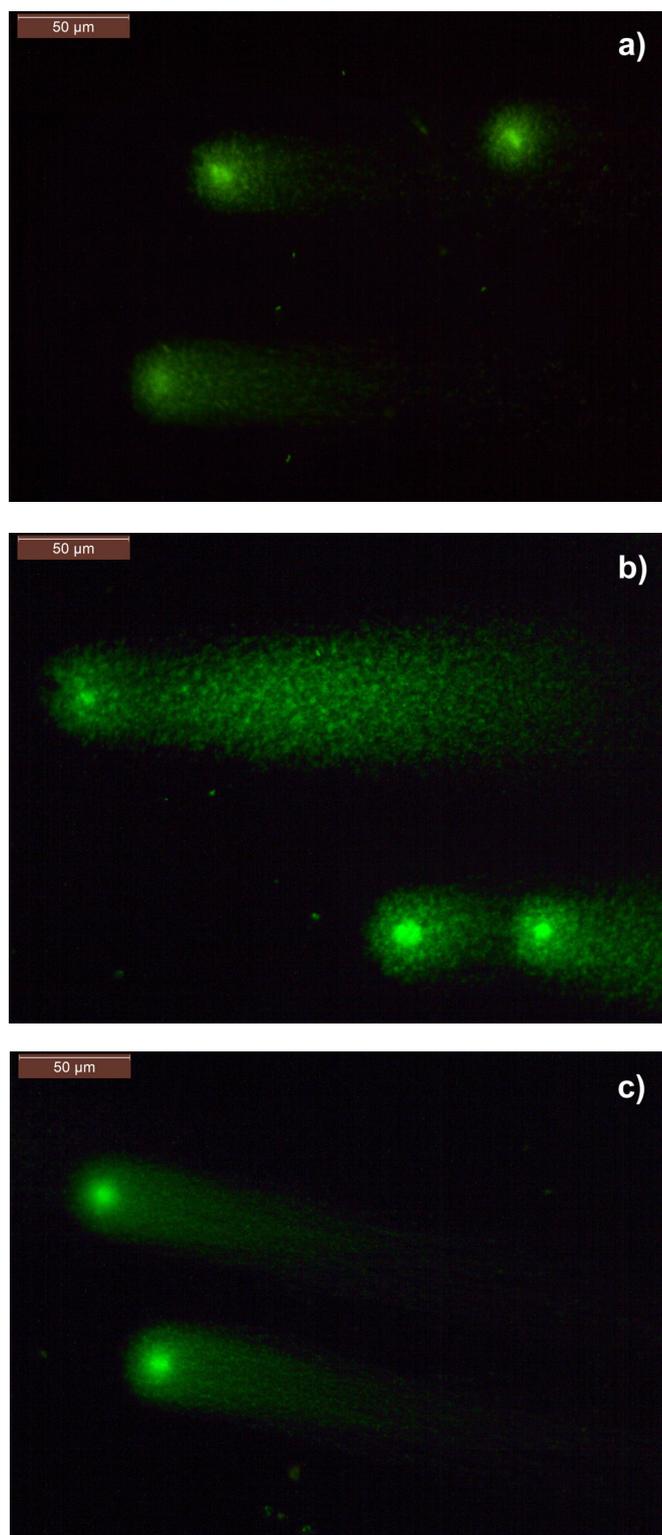
**Table 2.** Established dependencies between the mean values of the comet assay parameters and the conclusions based on the conventional sperm analysis.

Conclusions	N	Mean	SD	SE	95% confidence interval for mean value		Min. value	Max. value
					Lower boundary	Upper boundary		
<b>%TDNA</b>								
Normozoospermia	44	8.213	4.760	0.718	6.766	9.660	1.752	21.148
Asthenozoospermia	15	9.944	4.619	1.193	7.387	12.503	4.606	19.782
Oligoasthenozoospermia	10	13.304	4.519	1.429	10.072	16.537	9.418	22.194
Oligoasthenoteratozoospermia	8	18.542	9.0889	3.213	10.943	26.140	5.413	31.418
Asthenoteratozoospermia	2	10.822	8.966	6.340	-69.734	91.377	4.482	17.161
<b>Total</b>	79	10.298	6.142	0.691	8.923	11.674	1.753	31.418
<b>TL</b>								
Normozoospermia	44	6.198	4.472	0.674	4.839	7.558	0.012	17.429
Asthenozoospermia	15	8.489	4.437	1.146	6.032	10.946	2.162	18.654
Oligoasthenozoospermia	10	13.758	5.954	1.883	9.499	18.017	6.518	27.754
Oligoasthenoteratozoospermia	8	17.791	11.512	4.070	8.166	27.415	4.594	39.985
Asthenoteratozoospermia	2	12.928	11.000	7.778	-85.906	111.762	5.149	20.706
<b>Total</b>	79	8.934	6.913	0.778	7.386	10.483	0.012	39.985
<b>TM</b>								
Normozoospermia	44	1.100	1.194	0.180	0.737	1.463	0.000	4.373
Asthenozoospermia	15	1.669	1.821	0.470	0.661	2.678	0.387	7.711
Oligoasthenozoospermia	10	2.909	1.764	0.558	1.647	4.171	1.228	6.789
Oligoasthenoteratozoospermia	8	5.358	4.897	1.732	1.264	9.451	0.609	13.419
Asthenoteratozoospermia	2	2.438	2.981	2.108	-24.343	29.218	0.330	4.545
<b>Total</b>	79	1.902	2.396	0.270	1.365	2.439	0.000	13.419

N - Number of individuals; SD - standard deviation; SE - standard error;  $P < 0.001$ ; %TDNA - % tail DNA; TL - tail length ( $\mu\text{m}$ ); TM - tail moment ( $\mu\text{m}$ )

Under the fluorescent analysis, an increased expression of comets was observed in cases of decreased sperm concentration, motility and percentage of cells with normal morphology (fig.1).

**Fig. 1.** Expression of comets at cases of: a) decreased sperm concentration; b) decreased sperm motility; c) decreased percentage of sperm cells with normal morphology (magnification 400×).



The comparative analysis of the obtained data affirmed high statistically significant differences between the values of the three comet parameters for the groups compared (ANOVA:  $P < 0.001$ ). At the same time, the t-test indicated statistically significant relationships between the comet char-

acteristics of %TDNA, TL and TM, on the one hand, and sperm quality parameters – spermatozoa concentration, motility and morphology, on the other hand ( $P \leq 0.05$ ).

#### DISCUSSION:

As could be seen from the obtained results, a high percentage of the examined individuals have poor sperm quality, as well as clear indications for DNA fragmentations in the spermatozoa nuclei. This result could be related to the fact that the men included in the study are influenced by various adverse effects, the result of work or living in adverse environmental conditions, or their harmful individual lifestyle-related habits, which may have a direct or indirect genotoxic effect [1, 2].

DNA migration resulting from the genotoxic effect of different factors is reported based on the %TDNA and the TL. It is believed that the %TDNA has an advantage because it could be standardized in the course of the study and compared with values obtained in other studies [10]. This parameter is considered to be the most appropriate in evaluating the genotoxic effect since it increases in parallel with an increase in the intensity of the effect of the toxic agents and is independent of the visual analysis conditions. It is not recommended to use the TL as the only criteria for assessing the genotoxic effect, as such may increase at low levels of damage under different electrophoretic conditions [10]. On the other hand, the TL, because of its sensitivity, allows creation of a quick visual representation of a particular situation, and its reading is important for calculating the tail moment.

The TM indicates both the migration of damaged DNA in the tail and the relative amount of DNA in the tail. At low levels of genotoxicity, its value deviates from the established linear dose dependence and lacks strictly defined standard units. This parameter reveals the dependence between a particular class of comets observed and the direct damage to the cells. The use of TM is advisable for conducting qualitative genotoxic studies [11].

In the course of our study, the values of all three comet parameters were analyzed. As can be seen in Table 1, the %TDNA observed in men with a sperm concentration under the norm was almost twice as high (%TDNA=15.632) compared to those with normal sperm concentration (%TDNA=8.725). The differences between the two compared groups – “under the norm” and “in the norm” were also clear for the other two comet parameters – TL (“under the norm”=15.550 and “in the norm”=6.982) and TM (3.997 and 1.284), respectively. Statistically significant dependencies between the comet parameters studied, and the other two sperm parameters – spermatozoa motility and morphology have also been established. The data presented in Table 1 shows that in the case of reduced spermatozoa motility, the mean values of the three comet parameters were higher (%TDNA=12.920, TL=12.375, and TM=2.910) than for men with normal spermatozoa motility (%TDNA=8.213, TL=6.198 and TM=1.100), respectively. The relationship between them and the sperm morphology was statistically significant ( $P < 0.001$ ). The %TDNA, TL and TM were significantly higher in men with high levels of morphological abnormalities (normal morphology below normal) – %TDNA=16.998, TL=16.818 and TM=4.774, respectively,

compared to those with normal morphology cells (%TDNA=9.328, TL=7.792 and TM=1.486, respectively).

It was found that when the concentration, motility, and percentage of cells with normal morphology were decreased, the expressed comets had a significantly longer tail (fig. 1) –  $P \leq 0.05$ .

Data from other authors indicate a 4-fold higher rate of DNA damage in infertile males and a negative correlation with sperm motility and morphology without establishing similar dependence on the sperm count [12], as well as a negative correlation between sperm counts and the level of DNA damage, which decreases when spermatozoa motility and normal morphology increase [13]. The inverse correlations between DNA damage and motility were also observed with alkaline comet assay in the research of Simon et al. [14], indicating that sperm DNA damage is important to determine male factor infertility.

In contrast to these cited studies, where no correlation with sperm concentration was found, our results indicate such a statistically significant dependence.

The use of the One-way test in the course of this study showed statistically significant dependencies between the three comet parameters and the conclusions found by the usage of the conventional sperm analysis (Table 2) ( $P < 0.001$ ). The data showed that the %TDNA in the group with normozoospermia (%TDNA=8.213) was the lowest. At the same time, this parameter was the highest in the individuals with oligoasthenoteratozoospermia (%TDNA=18.542) and oligoasthenozoospermia (%TDNA=13.304). The data are similar with respect to the other two comet parameters – TL and TM – with lowest values in men with normozoospermia and highest – in those with oligoasthenoteratozoospermia. The findings of Ribas-Maynou et al. [15] also demonstrated also a high percentage of single and double strand DNA breaks both at neutral and alkaline comet assay in patients with oligoasthenoteratozoospermia and asthenoteratozoospermia. The statistically significant dependencies between the conclusions done and the comet parameters quality ( $P < 0.001$ ) were logically based on the correlations between %TDNA, TL and TM, on the one hand, and spermatozoa concentration, motility and morphology, on the other. In this aspect, the results obtained in our study are similar to those of another author [16], who found a negative correlation between the level of DNA damage and sperm concentration but also a high level of DNA abnormalities (more than 32%) in cases of oligoasthenoteratozoospermia.

Previous studies based on comet assay [17] have found that all comet parameters were predictive of male infertility and clinical pregnancies following assisted reproductive technology (ART) procedures. The data from the re-

gression analysis in our study concerning the three comet parameters are categorical that by knowing the value of one of the parameters, it is possible to predict the values of the other two with high statistical reliability ( $P = 0.00$ ).

Our study revealed sperm DNA damage through comet assay in men with reproductive problems, and with worse sperm quality parameters, for the first time in Bulgaria. These data are important given the fact that the impact of sperm DNA damage among infertile men remains the main factor that influences natural and assisted reproduction. Furthermore, an overall detrimental effect of sperm DNA damage on clinical pregnancy rate after *in vitro* fertilization (IVF) and/or Intracytoplasmic Sperm Injection (ICSI) was identified by Simon et al. [18] after a systematic review and meta-analysis of studies on sperm DNA damage and reproductive outcome after IVF and/or ICSI. According to PannerSelvam et al. [19]: “it is important to understand the etiologies associated with sperm DNA damage and correlate it with various male infertility scenarios.” It is clear that the mechanisms involved in sperm DNA damage can be activated by exogenous factors like radiation, environmental toxins (including alcohol, tobacco, drugs, etc.) accompanying harmful habits and may induce poor lifestyle sperm DNA fragmentation by different pathways [20]. Exposure to chemical toxins may affect semen quality, measured by conventional sperm analysis, as well as DNA integrity in spermatozoa nuclei, studied by various molecular genetic analyses [21]. In addition, there is evidence that smoking and alcohol intake leads to the worse quality of the spermatozoa DNA [22, 23]. The results of our study also confirmed that some environmental factors have a negative impact on sperm structure and function and continue to be a significant public health concern nowadays.

## CONCLUSION:

The clear statistically significant relationships between the studied comet assay parameters and between them and the studied sperm quality indicators could be used for prognostic purposes.

Different environmental and lifestyle factors may have potential risks for male reproductive health because of their possible negative effect on the sperm quality indicators and increase the degree of genetic instability in sperm nuclei.

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