

## *Dependencies Between Some Sperm Quality Parameters and the DNA Integrity in the Spermatozoa Nuclei in Accordance with Environmental and Lifestyle Factors*

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**Abstract.** In order to establish DNA integrity in the sperm nuclei, acridine orange fluorescence test was performed in 87 males from Bulgaria in four age groups: up to 20 years; from 21 to 30 years; from 31 to 40 years and over 40 years. The results obtained were based on a four-step scale, depending on the percentage of damaged cells. At the same time, basic sperm counts, such as sperm concentration and motility, and sperm morphology, were analyzed by conventional sperm analysis. The individuals surveyed were also analyzed on the basis of reported harmfulness related to their lifestyle - environment and professional harms, use of tobacco, alcohol, drugs, anabolic steroids and medications. Statistically significant dependencies were found between the individual sperm counts and adverse environmental conditions, as well as between the presence of a high percentage of fragmented DNA in sperm nuclei and decreased sperm concentration and motility, and abnormal sperm morphology. Additional studies are needed to gain insight into the interdependencies between environmental and lifestyle factors and genetic factors that determine the reproductive health of men in Bulgaria.

**Key words:** male infertility, semen quality, sperm DNA integrity, environmental and lifestyle factors.

### **Introduction**

Human infertility affects about 10% - 15% of couples in reproductive age and is one of the main health problems of society, equally related to female and male. A clear tendency for deterioration in the quality of sperm indicators has been found worldwide. Different biological and environmental factors such as genetic reasons, problems in urogenital and reproductive system, abnormalities in fertilization and embryonic

development (MATZUK & LAMB, 2008; LI *et al.*, 2011) alcohol and cigarette consumption, use of androgenic anabolic steroids, various medications, occupational hazards and socio-psycho-behavioral aspects (RUBES *et al.*, 1998; TORRES-CALLEJA *et al.*, 2000; LI *et al.*, 2011; BREZINA *et al.*, 2012; YU *et al.*, 2014) are in association with male infertility.

It is known that conventional sperm quality analysis is based on microscopic assessments of sperm parameters, including

also subjective analyzes of sperm counts with normal morphology, motility and concentration. On the other hand, sperm samples that exhibit normal concentration, motility and morphology do not always achieve acceptable rates of fertilization because the sperm may have structural or functional abnormalities that may impair the sperm and egg interactions or the embryo development (CUNHA *et al.*, 2015). It is clear now that none of the methods for analysis applied alone is sufficiently sensitive to accurately determine the quality of sperm and the male reproductive potential. So, at present, the attention of many researchers is focused on the application of complex approaches, providing greater accuracy and higher objectivity to assess the male fertility (CELEGHINI *et al.*, 2007; CARVALHO *et al.*, 2010; HOSSAIN *et al.*, 2011; STANDERHOLEN *et al.*, 2014; SAPANIDOU *et al.*, 2015).

The integrity of sperm DNA is considered as a marker of the integrity of spermatogenesis and male fertility potential (BENCHAIIB *et al.*, 2003). Statistically, about 10% of fertile male spermatozoa and about 20-25% of infertile male spermatozoa have measurable levels of DNA damages (SMITH *et al.*, 2006). Associations between DNA damages in spermatozoa and some environmental and lifestyle factors have been researched by a number of authors (SAKKAS *et al.*, 1999; EVENSON & WIXON, 2005; YU *et al.*, 2014).

In Bulgaria, such complex studies have not been conducted. The influence of some environmental factors and some harmful habits on male fertility was partially analyzed by OUZOUNOVA-RAYKOVA *et al.* (2018) and a conclusion has been completed that the reasons for disruption of male fertility are multifaceted. Consequently, more complex researches are needed in this area in order to inform the population about the harmful environmental and lifestyle factors which impact the reproductive health as a whole, and the male fertility in particular.

This study aims to analyze the associations between DNA damages (fragmentations of DNA) in spermatozoa

nucleus, the deteriorated sperm quality parameters and the impact of some environmental and lifestyle factors on male reproductive health.

### **Materials and Methods**

This investigation was done during the period from 2014 to 2018 accordingly to ethical principles and norms. Each participant has given informed consent to participate in the study. Based on a completed voluntarily questionnaire of 1540 individuals from Bulgaria (mean age  $33 \pm 7.4$  years), who visited a reproductive health office in Plovdiv for prophylaxis or for a reproductive problem, the impact of environmental and lifestyles factors was analyzed. The information was collected concerning occupational hazards, working and living under stress, cigarette smoking, alcohol and drugs consumption, anabolic steroids and medications taken. 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 comparisons in the research.

Apart from this, in order to establish DNA fragmentations in the sperm nuclei, acridine orange fluorescence test was performed in 87 males (the smaller group), randomly separated from the total studied group of 1540 individuals (the whole group). These patients were clustered in four age groups: up to 20 years; from 21 to 30 years; from 31 to 40 years and over 40 years. Among the population surveyed, the percentage of males aged between 31 and 40 years was 52.9% and the percentage of men aged between 21 and 30 years was 34.5%. The surveyed individuals aged up to 20 years were 3.3% and those aged over 40 years - 12.8%.

All participants in the study provided a standardized semen sample by masturbation in sterile containers after sexual abstinence of 3 to 5 days. Semen samples were supplied for laboratory activities within 30 minutes of their collection. Spermatozoa concentration and motility were assessed by conventional

methods in a Makler counting chamber by preparing fresh, native preparations. Spermatozoa concentration was calculated in millions per ml and spermatozoa motility was considered in percentage (%). The spermatozoa concentration and motility results obtained in the study were compared to the referent data for Bulgarian population, presented in [STANISLAVOV & NIKOLOVA \(2013\)](#) and in the Fourth edition of the [WHO \(1999\)](#). Microcephalic, macrocephalic, bicephalous and bicaudal spermatozoa, or cells possessing a coiled tail or a deformed or abnormally small acrosome were all classified as abnormal. All morphological abnormalities were calculated as % of all spermatozoa cells analyzed per an individual (morphological defects < 14% - norm) according to [STANISLAVOV & NIKOLOVA \(2013\)](#) and [WHO \(1999\)](#).

Acridine orange fluorescence test was performed for establishing DNA integrity in the sperm nuclei. Two smears from each sample were prepared on glass slides and air-dried. Each smear was fixed overnight in Carnoy's solution, freshly prepared with methanol and glacial acetic acid in a 3:1 proportion. The slides were stained with acridine orange at the time of analysis according to [VIRANT-KLUN et al. \(2002\)](#) and evaluated with an epifluorescence microscope (Leica DM 1000) equipped with an appropriate filter and photo camera. One hundred cells were analyzed in each treatment slide. As a result of acridine orange metachromasia sperm with normal (double stranded) DNA content presents a green fluorescence, whereas sperm with abnormal (single stranded) DNA content emits fluorescence in a spectrum varying from yellow-orange to red - Fig. 1. The obtained results were based on a four-step scale, depending on the percentage of damaged cells ([EVENSON et al., 2002](#)). Fertility potential in this four-step scale was determined as follows: excellent - when damaged spermatozoa cells are less than 15%; good - when damaged cells are between 15% and 20%; fair - when damaged cells are between 25% and 30% and poor - when damaged spermatozoa cells are

more than 30%. The percentage of damaged DNA cells was determined according to [EVENSON et al. \(1999\)](#) as the ratio between the number of cells with damages (which fluoresce in red) and the total number of cells (which fluoresce in red and green). Descriptive statistics was used to characterize the groups compared. Data were presented as the mean  $\pm$  standard deviation (SD), number of participants and a percentage of the entire cohort. For non-parametric analysis, the Chi-square test for orderly ranging data was used, and Gamma coefficient was applied to determine the direction and the strength of the relationship between the parameters studied. Statistical significance was defined as  $P < 0.05$  (for the whole group) and  $P < 0.001$  (for the smaller group).

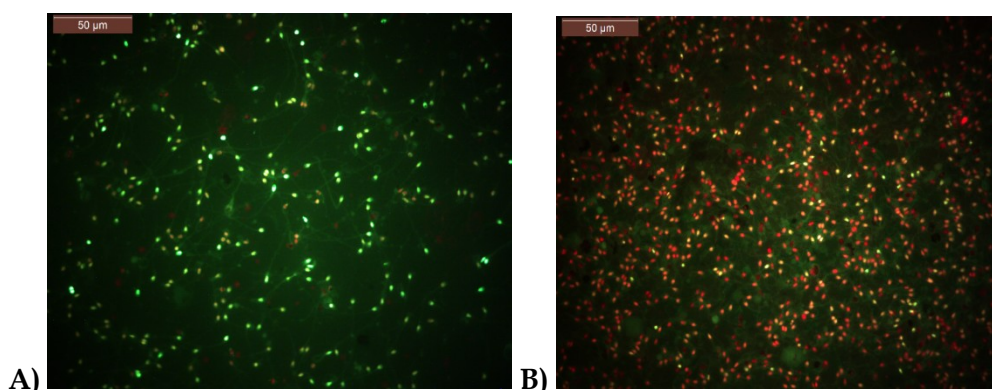
### **Results**

Results of this study for the whole group (1540) indicated that 13% of individuals have been working in an environment with occupational hazards, 5.2% of them have been working or living under stress, 21% have been smokers, 21% - have been consuming alcohol, 5.9% - have used drugs, 3.9% of them have been taking anabolic steroids and 16% - medications. Data from the conventional sperm analysis for the whole group revealed that 23% of the male individuals tested have a decreased spermatozoa concentration, 54% of them have spermatozoa motility lower than normal. In 16.6% of the person's studied morphological defects in spermatozoa with frequency more than 14% among the analyzing cells were detected. Among the smaller group which was studied by means of the acridine orange fluorescence test (87), these values were lower (Table 1).

The results received in the present study for the whole group showed that factors as stress, medications, cigarette smoking and alcohol consumption have a statistically significant impact on the semen quality. Statistically significant relationship was found also between the professional hazards and the spermatozoa motility ( $P < 0.05$ ). Data concerning frequency and valid percent of

participants in the study with DNA denaturation in the sperm nuclei are presented in Table 2. Statistical data regarding the

relations between the semen parameters and the levels of DNA denaturation in the sperm nuclei are included in Table 3 ( $P < 0.001$ ).



**Fig. 1.** Acridine orange fluorescence test: sperm with normal (double stranded) DNA – a fluorescence in green; sperm with abnormal (single stranded) DNA – a fluorescence in yellow-orange to red – A) - damaged spermatozoa cells less than 15% B) damaged spermatozoa cells more than 30% (400×).

**Table 1.** Number of individuals (N) and valid percent (%) of participants studied by means of the acridine orange fluorescence test with information concerning semen quality parameters (spermatozoa concentration, motility and morphology) in or out of the norm \*according to the reference values (STANISLAVOV & NIKOLOVA, 2013; WHO, 1999).

N / %	Semen quality	Spermatozoa concentration (10 <sup>6</sup> /ml)	Spermatozoa motility (% progressive)	Spermatozoa morphology (% abnormal cells)
Referent data*	In the norm	>20	>50	< 14
	Under of the norm	< 20	< 50	> 14
Number/Valid Percent	In the norm	69/79.3	51/58.6	77/88.5
	Under of the norm	18/20.7	36/41.4	10/11.5
<b>Total</b>		<b>87/100</b>	<b>87/100</b>	<b>87/100</b>

### Discussion

The results of our study show the presence of statistically significant dependencies between some of the studied seminal parameters (spermatozoa concentration and motility) and environment and lifestyle factors like stress, medications, cigarette smoking, alcohol consumption and professional hazards. These results are in agreement with data reported by some authors (IAZZO *et al.*, 2010; YU *et al.*, 2014) and are in contrast to the

results discussed by others (WONG *et al.*, 2000; MARTINI *et al.*, 2004).

Data of the present study demonstrate lower values for the analyzed parameters of the conventional sperm analysis among the group which was studied by means of the acridine orange fluorescence test (87) – Table 1, in comparison with the whole group (1540). These differences we associate with the random nature of the construction of the smaller sample group, analyzed by the acridine orange test.

As could be seen from our data, most of the analyzed individuals have indicated the influence of some environment or lifestyle factor in their life. At the same time between 11.5% and 41.5% of the studied male individuals in the smaller acridine orange tested group have non-normative sperm counts (Table 1) and 41.4% of them have poor and fair DNA fragmentation level based on a four-step scale, depending on the percentage of damaged cells (EVENSON *et al.*, 2002) – Table 2.

Analyzing the relationship between the studied seminal parameters and the percentage of DNA damaged cells indicative statistically significant dependencies have been found in our investigation. Clear relation between the sperm motility, concentration and morphology, from one hand, and the acridine orange DNA fragmentation level, from the other hand, has been demonstrated by the results of the Gamma test: -0.929; -0.947 and -1.000, respectively (Table 3). As could be realized from the Table 3, 69.4% of the individuals with low spermatozoa motility, 94.4% of the men with sperm concentration under the

norm and 100% of these with abnormal spermatozoa morphology have been with poor DNA levels. At the same time, the male individuals with normal sperm parameters – motility, concentration and morphology of spermatozoa have had 5.9%, 15.9% and 23.4% poor DNA levels, respectively (Table 3).

**Table 2.** Frequency and Valid % for individuals with DNA fragmentations in the sperm nuclei based on a four-step scale, depending on the percentage of damaged cells (Evenson *et al.*, 2002): Excellent – damaged spermatozoa cells less than 15%; Good – damaged cells between 15% and 20%; Fair – damaged cells between 25% and 30%; Poor – damaged spermatozoa cells more than 30%.

DNA fragmentation level	Frequency	Valid %
Excellent	43	49.4
Good	8	9.2
Fair	8	9.2
Poor	28	32.2
<b>Total</b>	<b>87</b>	<b>100.0</b>

**Table 3.** Differences between the groups analyzed and statistically significant relations between the semen parameters and the levels of DNA fragmentation in the sperm nuclei studied by usage the Chi-Square and Gamma\* exact tests. \*Gamma test is used to indicate the direction of the influence if the factor is significant (P<0.001).

DNA fragmentation level > Semen quality V	Excellent DNA	Good DNA	Fair DNA	Poor DNA	$\chi^2$	Gamma
	<b>Spermatozoa motility</b>					
Under the norm	8.4%	2.8%	19.4%	69.4%	<b>0.000</b>	<b>-0.929</b>
In the norm	78.4%	13.7%	2.0%	5.9%		
	<b>Spermatozoa concentration</b>					
Under the norm	5.6%	0.0%	0.0%	94.4%	<b>0.000</b>	<b>-0.947</b>
In the norm	60.9%	11.6%	11.6%	15.9%		
	<b>Spermatozoa morphology</b>					
Under the norm	0.0%	0.0%	0.0%	100.0%	<b>0.000</b>	<b>-1.000</b>
In the norm	55.8%	10.4%	10.4%	23.4%		

Our results demonstrate that the measured by the acridine orange test sperm chromatin integrity is much indicative for the quality of sperm parameters. Differently, than results of some other authors who reported only slightly relation between acridine orange DNA status and sperm parameters (TEJADA *et al.*, 1984) or relation only with sperm morphology (CLAASSENS *et al.*, 1992), the relationships identified in our study are enough clear and distinct.

### Conclusions

The results received in the present study for the whole group show that factors as stress, medications, cigarette smoking, alcohol consumption and professional hazards have a statistically significant impact on the semen quality. Based on the established clear dependencies between the analyzed environmental and lifestyle factors and the quality of male sperm, from one hand, and between the semen quality parameters tested and DNA fragmentations in spermatozoa found, from the other hand, the conclusion could be done that these factors significantly affect the spermatozoa chromatin integrity and are associated with the fragmentation of DNA in the sperm nuclei. More complex researches are needed in the future to characterize in details the parameters of the male reproductive health and to inform the population for the potential risks of deterioration.

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