

### IMPACT OF SINGLE AND MULTIPLE SPERM ABNORMALITIES AND LOW-LEVEL LEUKOCYTOSPERMIA ON SPERM DNA

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Submitted: 9 April 2019. Accepted: 27 May 2019. Published: 17 June 2019.

#### ABSTRACT

##### Background and objective

The aim of the present study was to identify the impact of defective standard sperm parameters individually and in combination on DNA damage in a large cohort of infertile men.

##### Material and methods

Retrospective analysis of semen characteristics was conducted on 436 patients. DNA fragmentation analysis was performed by using the terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-dUTP nick end labeling (TUNEL) assay. Sperm parameters were arranged into different categories such as normospermia asthenospermic, teratospermic, asthenoteratospermic, and oligoasthenoteratospermic. GraphPad Prism version 7 software was used for data analysis.

##### Results

Our results suggest that the mean percentage of DNA damage was proportionally higher than the semen abnormalities. Sperm with 3 abnormal parameters showed significantly higher DNA damage, suggesting that sperm having more than 2 abnormalities are more likely to have higher DNA damage.

##### Conclusion

Sperm motility had significant correlation and is supposed to be a predictor for these tests, while morphology was the second standard sperm parameter inversely correlated with sperm DNA damage.

J Mens Health Vol 15(2):e58-e65; 17 June 2019

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Patients demonstrating low levels of leukocytospermia should be advised sperm DNA testing before assisted reproductive technology treatment. However, there is a clear need for more research studies to further address these issues.

*Key Words: semen analysis, DNA fragmentation, male infertility, sperm abnormalities*

## BACKGROUND

Semen analysis is the first test prescribed in male infertility diagnosis and provides essential information about conventional sperm parameters, that is, sperm concentration, motility, morphology, and viability,<sup>1</sup> but may not give complete picture of male fertility potential.<sup>2</sup> Based on the cutoff values of conventional sperm parameters defined by World Health Organization,<sup>3</sup> semen analysis is reported as normal or abnormal. Oligospermia (concentration <15 million/mL), asthenospermia (total motility <40%), and teratospermia (morphology <4%) are the nomenclatures generally used to describe the individual sperm parameters abnormalities. Male factor infertility and defective sperm parameters contributes to 30%–50% infertile cases.<sup>4,5</sup> Various forms of assisted conception may be considered in the treatment of infertility due to abnormal semen parameter. However, the question whether sperm defect is limited to conventional sperm parameters or has compromised the sperm DNA is overlooked. Noteworthy, in mammals, fertilization events, subsequent early embryonic development, and genetic reprogramming in part are dependent on intact sperm DNA integrity.<sup>6</sup> High sperm DNA fragmentation can impact the fertilization process, embryonic development, implantation, and successful pregnancy outcome.<sup>7</sup>

High sperm DNA damage is reported in infertile men.<sup>8,9,10</sup> DNA fragmentation is negatively associated with fertility outcome as demonstrated both in vivo and ex vivo models.<sup>9,11,12</sup> To date, DNA fragmentation test is not included as a routine test in male infertility diagnosis particularly

when one or more quantitative sperm parameters are abnormal, the entire burden of infertility is shifted to the defective sperm parameters and sperm DNA investigations are mostly limited to idiopathic infertility cases. Male infertility cases with defective sperm parameters as well as unexplained or idiopathic are offered assisted reproductive technology (ART) treatment which bypasses several sperm function parameters (sperm capacitation, acrosome reaction, zona pellucida binding).<sup>13</sup> Sperm with defective parameters (motility, morphology and concentration) are more likely to carry defective sperm DNA.<sup>15</sup> Although ART overcomes the prerequisites of conventional sperm parameters, owing to the downstream role of sperm DNA in embryo development, significance of sperm DNA integrity cannot be ignored. Therefore, relation of defective sperm parameters to DNA quality must be ascertained before ART procedures. The aim of the present study was to identify the association of defective standard sperm parameters individually and in combination on DNA damage in a large cohort of infertile men.

## METHODS

After obtaining institutional ethical approval, retrospective analysis from 2012 to 2016 of semen characteristics was performed. Only samples from infertile men who were advised semen analysis after a minimum of 12 months of unprotected intercourse were included. The semen analyses were performed as per WHO 2010 guidelines.<sup>3</sup> Semen samples with less than 2 or more than 7 days of sexual abstinence were excluded.

Briefly, semen samples were collected by masturbation and liquified at 37°C for 30 minutes before analysis. Conventional semen analysis was performed manually; volume, concentration, motility, and morphology were recorded. Samples with more than 5 round cells per high magnification were further assessed for leukocytes count by peroxidase test as we have previously described.<sup>24</sup> Samples with leukocytes count  $>1 \times 10^6/\text{mL}$  were considered as high leukocytospermic, and samples with leukocytes count  $0.1\text{--}0.9 \times 10^6/\text{mL}$  were considered as low leukocytospermic. Sperm DNA fragmentation analysis was performed by using the terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-dUTP nick end labeling (TUNEL) assay.

**STATISTICAL ANALYSIS**

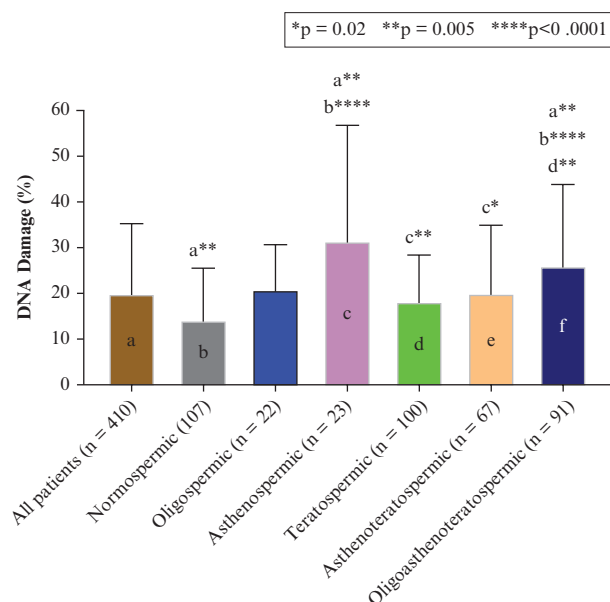
The data were analyzed by GraphPad Prism version 7 software (GraphPad Software Inc., USA). Means of quantitative semen parameters and DNA fragmentation among groups were observed. The data was analyzed using one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test to compare the differences between groups. A p value of  $\leq 0.05$  was considered significant with 95% confidence interval. Individual participant consent and local institutional ethical review board approval were sought and granted for this project.

**RESULTS**

Out of the studied 436 men with complete semen profile, 410 had been investigated for DNA damage. Data were grouped as *all patients* that include the means of all 436 patients for age (37 years), BMI (29.7 kg/m<sup>2</sup>), sperm concentration (42 million/mL), motility (44.678%), morphology (3.171%), volume (3.09 mL), and leukocytes count (0.444 million/mL) (Table 1). Next, the data were segregated into *normospermia* that represents a group with all normal semen parameters. Further segregation of data was done based

**TABLE 1** Distribution of Patients into Different Groups Based on Abnormal Sperm Parameters as per WHO 2010 Guidelines

	All patients (n=436)	Normospermic (n=108)	Oligospermic (n=22)	Asthenospermic (n=36)	Teratospermic (n=100)	Asthenoteratospermic (n=67)	Oligoasthenoteratospermic (n=93)
Age (years)	37	37.47	37	39.91	36.83	37.82	37.14
BMI (kg/m <sup>2</sup> )	29.7	29.21	29.36	29.81	29.02	29.59	30.22
Volume (mL)	3.09	2.91	3.12	2.92	3.14	3.24	3.08
Concentration (million/mL)	42	80.08	7.32	47.33	58.07	42.33	5.96
Total motility (%)	44.678	60.56	51.68	29.43	56.47	28.70	24.56
Normal morphology (%)	3.171215	6.84	5.90	5.82	1.53	1.79	1.10
Abstinence time (days)	3.838675	3.85	3.90	4	3.66	4.13	3.84
Leukocytes (million/mL)	0.444	0.62	0.3	0.32	0.30	0.47	0.26



**FIG. 1** Impact of individual and combined abnormal sperm parameters on sperm DNA damage.

Key: \*Normospermic=All semen parameters are normal as per WHO 2010 guidelines; Oligospermic=Abnormal sperm concentration (<15 million/mL); Asthenospermic=Abnormal motility (<40%); Teratospermic=Abnormal morphology (normal forms <4%); Asthenoteratospermic=Abnormal motility and morphology; Oligoaasthenoteratospermic=Abnormal concentration, motility, and morphology.

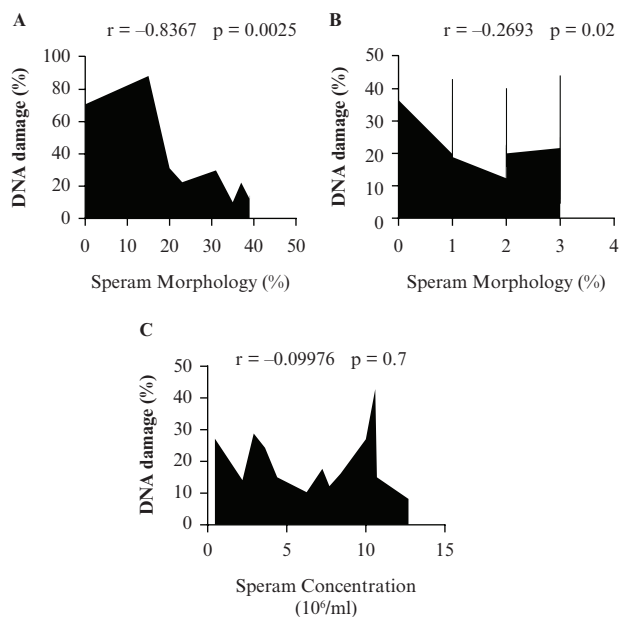
on individual and combined abnormal sperm parameters, that is, *oligospermic* (abnormal sperm concentration <15 million/mL), *asthenospermic* (abnormal total motility <40%), *teratospermic* (abnormal morphology <4%), *asthenoteratospermic* (abnormal motility and morphology), and *oligoasthenoteratospermic* (abnormal concentration, motility, and morphology) (Table 1).

Sperm DNA damage was among all groups. The mean percentage of DNA damage (TUNEL positive cells) was significantly higher in all patients group than normospermic group ( $p=0.005$ ; Figure 1). Further analysis was carried out based on the DNA damage values of normospermic group that were considered as the base values to compare groups with individual and

combined abnormal sperm parameters. Oligospermic group presented discernibly higher, but nonsignificant increase in the sperm DNA damage when compared with normospermic group (Figure 1). Asthenospermic group showed higher significance in sperm DNA damage ( $p<0.0001$ ) when compared with normospermic group. Although teratospermic and asthenoteratospermic groups showed higher DNA damage, yet did not achieve significance when compared with normospermic group. *Oligoaasthenoteratospermic* group which represents the combined 3 abnormalities (concentration, motility, and morphology) showed significant increase ( $p<0.0001$ ) in sperm DNA damage when compared with normospermic group, suggesting that sperm with multiple anomalies carry more DNA damage.

To assess whether the studied variables (concentration, motility, morphology) are linearly related to DNA damage, Pearson correlation was calculated which revealed significant inverse correlation with motility ( $p=0.0025$ ;  $r=-0.83$ ) and morphology ( $p=0.02$ ;  $r=-0.26$ ), but not with sperm concentrate (Figure 2).

As per WHO 2010 guidelines, semen samples with more than 1 million leukocytes/mL of samples are considered leukocytes positive, indicating infection that may require further treatment. To investigate if low concentrations of leukocytes (<1 million/mL) do impact the sperm DNA quality, we further categorized the analysis based on the leukocyte numbers, that is, low-level leukocytes (0.1–0.9 million/mL) and high-level leukocytes ( $\geq 1$  million/mL). We also compared the sperm DNA damage with patients without leukocytes (Figure 3). Interestingly, low-level leukocytospermic samples showed significantly higher DNA damage when compared with samples without leukocytes (Figure 2). DNA damage in high-level leukocytes was markedly higher than no leukocytes, but was not statistically significant. There was no statistical difference between

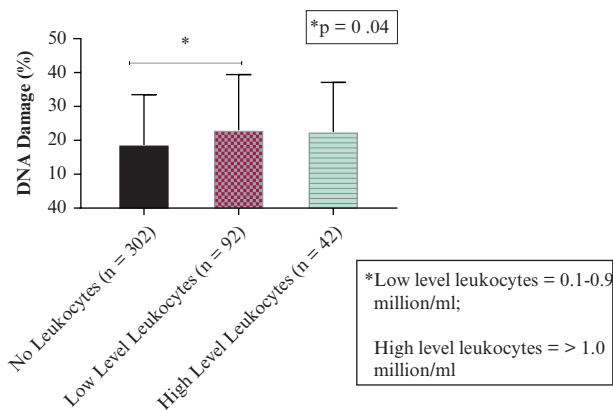


**FIG. 2** Pearson correlation of sperm DNA damage with standard sperm parameters.  
Key: \*a (motility); b (morphology) and c (concentration).

low-level leukocytes and high-level leukocytes DNA damage.

### DISCUSSION

Sperm DNA integrity is crucial to successful fertilization, embryo development, and well-being of the offspring. Although sperm have highly compacted chromatin, yet it has to travel a long way to reach oocytes which expose the sperm to internal and external insults leading to sperm DNA damage.<sup>14</sup> Internally, sperm DNA can be compromised during spermatozoa developmental and differentiation stages, epididymal transit, and male reproductive tract infection. Externally, environmental insults such as testicular hyperthermia, radiations, pollutants, toxins, smoking, alcohol, and endocrine disrupting chemicals can cause DNA damage.<sup>14-17</sup> In addition, these factors equally impact the conventional sperm parameters. However, in general clinical practice the decision to test sperm DNA damage is not made based on defective sperm



**FIG. 3** Impact of low-level leukocytes on sperm DNA damage.

parameters and most commonly it is advised in cases of unexplained/idiopathic infertility. In case of defective sperm parameters, the major focus of infertility is attributed to the abnormal sperm parameters and DNA investigations may be overlooked and the couple can go through ART treatment. This treatment strategy can benefit patients by increasing the chances of conception, but still carries the risk of retarded embryo development, recurrent pregnancy losses, and developmental abnormalities resulting from damaged sperm DNA.<sup>17,18</sup> Therefore, assessment of sperm DNA quality with relation to abnormalities in standard sperm parameters is important in cases of male infertility.

In this study, we analyzed the relation of single and multiple abnormal sperm parameters with DNA quality. In addition, impact of low-level leukocytes on sperm DNA quality was also assessed. Percentage values of sperm DNA damage were higher in all individual abnormal sperm parameters than normospermic group, but significance was limited to motility only. Nonetheless, in addition to motility a significant negative correlation was noted with morphology. Sperm motility seemed to be strongly related and predictive of sperm DNA damage followed by morphology, but concentration reflected poor

relation. DNA damage in sperm with 2 abnormalities (motility and morphology) was markedly higher than normospermic group ( $19.96 \pm 14.71$  vs.  $14.13 \pm 11.35$ , respectively) but was not statistically different. Notably, sperm with 3 abnormal parameters showed significantly higher DNA damage when compared with normospermic group as well as single/double abnormal sperm parameters, suggesting that sperm having more than 2 abnormalities are more likely to have higher DNA damage.

Inverse correlation of total sperm motility and DNA fragmentation has been reported previously.<sup>19</sup> Similarly, low progressive sperm motility (<32%), but not concentration and morphology, has been reported to be inversely correlated with high sperm DNA fragmentation in relatively older men ( $\geq 40$  years).<sup>20</sup> Erenpreiss et al.<sup>21</sup> reported significantly higher odds ratios for having higher DNA fragmentation in men with lower sperm motility and morphology. Others have reported strong inverse correlation of all 3 standard sperm parameters (concentration, motility, and morphology) with sperm DNA fragmentation and showed significantly higher association of DNA fragmentation with multiple (more than 2) sperm parameters abnormalities compared with single or double defective parameters.<sup>22</sup> The results of the current study are in accordance with the above reports, more closely to the work of Moskovtsev et al.,<sup>22</sup> except that in our results total motility was the single most parameter significantly inversely associated with sperm DNA damage. One of the possible reasons of strong association of sperm motility with sperm DNA fragmentation is the common origin of both processes during spermiogenesis when sperm nucleus is being matured (histones are replaced with protamines) and flagellum is formed.<sup>20</sup>

With the notion that samples with leukocytes  $\geq 1 \times 10^6/\text{mL}$  are considered infectious and men are recommended antibiotic treatment, we compared the DNA damage in low-level leukocytes and

high-level leukocytes with samples without leukocytes that revealed significantly higher DNA damage in low-level leukocytes. This is interesting to note that even mild leukocytospermia taken as single casual factor showed increased DNA damage. Leukocytes are a major source of reactive oxygen species (ROS) production in semen, which leads to DNA damage and impacts the male fertility potential. Low-level leukocytes can also produce detectable levels of ROS, which can impair sperm function by affecting DNA quality.<sup>23</sup>

## CONCLUSION

Taken together, our data suggest that sperm motility is the most notable, while morphology is the second standard sperm parameter inversely correlated with sperm DNA damage. Low-level leukocytospermia does produce DNA damage and such patients may be advised sperm DNA testing before ART treatment. Although sperm DNA is not a part of conventional semen analysis, based on our and previous evidence, it may be advisable to suggest DNA testing in couples planned for ART cycles particularly with low or poor motility. If high DNA fragmentation is identified, the cause of damage may be explored to improve the fertilization success, embryo development, and to minimize the developmental complications.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Individual participant consent and local Institutional Review Board approval were sought for this project.

## CONSENT FOR PUBLICATION

Not applicable. The manuscript does not contain patient identifiable data.

## AVAILABILITY OF DATA AND MATERIAL

The datasets analyzed/generated during the current study are not publicly available due to patient confidentiality.

### COMPETING INTERESTS

The authors declare that they have no competing interests.

### FUNDING

We are thankful for the support of this project (No. 2017/03/7524) by the deanship of scientific research in Prince Sattam bin Abdulaziz University, Al-Kharj, SA.

### AUTHORS' CONTRIBUTIONS

All authors have made equal and important contributions to the manuscript.

### ACKNOWLEDGEMENT

This project no. 2017/03/7524 was funded by the deanship of scientific research at Prince Sattam Bin Abdulaziz University, Alkharj, KSA. We are thankful to the deanship for this support.

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