

## IS CONCENTRATION AND MOTILITY OF MALE GAMETES RELATED TO DNA DAMAGE MEASURED BY COMET ASSAY?

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**Abstract:** Approximately 8% of couples in the World have problem with offspring production. Male infertility, which is the reason of the half of reproduction failures, is connected with diminished sperm production and deterioration of its quality. One important factor which affects fertility is the appearance of DNA damage in germ cells leading to enhanced frequency of mutations. This study aimed to compare the frequency of DNA damage in human spermatozoa in samples of different sperm concentration and motility. The anonymous sperm sample donors were men aged from 20–44 years, couples of pregnant females. Sperm concentration, motility and DNA damage measured by Comet assay were estimated. The following parameters were chosen for analysis of DNA damage: percent of DNA in comet head, comet tail length, tail moment. There were no differences in the mean DNA damage in male gametes between different age groups of donors, nor between samples of different sperm motility. The correlation between low sperm concentration in ejaculate and enhanced level of DNA damage was observed. The highest DNA damage was noted in samples with low sperm concentration. In gametes from this group, the lowest percent of DNA in comet head, the highest mean tail length, and the highest tail moment were observed.

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### INTRODUCTION

Infertility of a couple, i.e. lack of the offspring caused by impossibility to become pregnant, is recognized by the World Health Organization as a social disease. It is diagnosed when a couple have been unable to conceive after trying for at least 12-months [26]. Male infertility is most often connected with insufficient spermatozoa production or poor quality of sperm, e.g. weak motility and abnormal morphology of gametes. Permanent infertility is usually the result of spermatogonia stem cells death; however, temporary infertility is caused by damage to male germ cells in post-spermatogonial stages of development or spermatogenesis disorders.

At the end of last century, many studies regarding the problem of fertility deterioration in contemporary men compared to the generation of their grandfathers were published. On the basis of 61 papers published between 1938–1990, examining data of 14,947 men, Carlsen *et al.* [6] showed that the average sperm concentration was diminished from 113 mln/ml in 1940 to 66 mln/ml in 1990. In the same period, male sperm sample volume was reduced from 3.40 ml–2.75 ml. These data were confirmed by later study covered analysis of 101 papers describing research results for 1931–1994, published between 1934–1996 [33]. Simultaneously, deterioration of sperm quality, e.g. increase in frequency of abnormal spermatozoa and decrease in sperm motility were observed. At the same



time, an increase in male sex-related typical cancers, such as testis and prostate cancers, was noted. A two-fold increase in the incidence of congenital malformations of the genital tract, such as cryptorchidism and hypospadias in newborn boys, were observed [5, 6, 14].

Decrease in spermatozoa concentration in the sperm of contemporary males, accompanied by its poorer quality, leads to reduction in the chance of reproduction which may be considered as probably one of the reason negative birth-rate in numerous countries of Europe. According to a report of the World Health Organization published in 1993, it has been estimated that approximately 7%, e.g. 50–80 mln people on the World, have problem with procreation [26]. The problem of infertility concerned 1 in 20 males and was the reason for 50% of the failures in reproduction. For example, in the area of Upper Silesia in Poland, the fertilization potential of donors' semen is low, and seems to be diminished due to high degree of industrial pollution [16].

The following risk factors are regarded responsible for reduced male fertility: toxins, cytokinins, free oxygen radicals, deficiency of vitamins and mineral salts, inadequate nutrition, nicotine, alcohol, drugs, medications (anabolites), high temperatures, vibrations, ionising radiation and chemical agents, predominately endocrine disruptors. It has been observed recently that a decrease in sperm viability and progressive motility is correlated with the frequency of usage of mobile phones, whereas an increase in the level of abnormal spermatozoa is associated with the duration of exposure to waves emitted by GSM equipment [37].

Except for reduced sperm concentration and deterioration of sperm quality, one of the most important agents which affects fertility is the occurrence of DNA damage in germ cells, leading to enhanced level of mutations. Spermatozoa contribute a half of the genetic information to the genome of a developing foetus; therefore, DNA damage in germ cells may be reflected in the health of the offspring. Fertilization of the egg by damaged spermatozoa carries the risk of spontaneous abortion, congenital malformations observed at birth, and genetic diseases, as well as increased frequency of childhood cancers.

This study aimed at comparison of DNA strand break frequency in human spermatozoa in sperm samples of different concentration and motility of the gametes.

## MATERIALS AND METHODS

**Sample collection.** Anonymous sperm donors were men aged between 20–44 years, the spouses of pregnant women. Sexual abstinence time before collecting sample was not less than 2 days. Protocols of sperm sampling (sperm sample volume, motility and sperm concentration) were performed according to the World Health Organization recommendations [38].

Each donor filled in the questionnaire, describing diseases suffered from and medicines given during the last 6 months. For Comet assay, samples were selected from

healthy donors who had not received medicines within the 6 months before sampling. Donors agreed to donate a part of their samples for scientific research. The permission of the Bioethical Commission was also granted for this study.

Samples were taken in previously described and pre-weighed containers. The weight of each sample was calculated on the basis of the difference between the weight of the container before and after addition of the sample. Sample attributes (colour, stickness, consistency, aggregation, agglutination, and presence bands of mucus) were recorded. Immediately after sampling, the specimens were placed on a mixer and incubated at 37°C to mix and liquidate of samples. Then, parts of sperm samples were taken to estimate motility; the remaining sperm samples were placed in test tubes in a water bath. Sixty two sperm samples were collected.

**Sperm motility.** Sperm motility was determined by counting all motile and immotile spermatozoa in randomly selected fields of view under a contrast phase microscope, using a 40× magnification, only free spermatozoa were scored.

Motility analysis was started immediately after the incubation. Six µl of undiluted semen was placed on a microscope slide and covered with an 18 × 18 coverslip. The wet slide was placed on a heated (37°C) microscope table. A hundred spermatozoa on each of 2 independent slides were scored. Spermatozoa were scored on each microscope field according to the classes described by the World Health Organization. The first, spermatozoa of class a – rapidly progressive, and class b – slowly progressive, were scored. Class c is characterized by non-progressive movements (spermatozoa remaining in place, only circular and/or oscillatory movements observed), class d – immobile sperm. To calculate the percentage of motile spermatozoa, sperm of groups a+b were taken into consideration.

**Sperm concentration.** Samples were mixed accurately before analysis. High density samples were diluted (dilution was taken into consideration during calculation). Suspension was again accurately mixed before pouring into a Neubauer chamber. Scoring of spermatozoa in the squares of the chamber was carried according to procedures recommended by the World Health Organization [38].

**Comet assay.** Parts of individual samples assigned to DNA damage analysis were frozen at -70°C and kept in this conditions for several months. They were defrosted immediately before analysis of DNA damage frequency. For this analysis, suspension of single cells on the agarose gel is required, according to the modified comet assay [7]. Samples with a very high concentration (over  $700 \times 10^6$ ) were diluted 10-fold. From the remaining samples, depending on the concentration, 1 µl–30 µl of semen to Eppendorff test tubes were taken. 80 µl of low melting



**Table 1.** Correlation between the level of DNA damage in male gametes, age of donors, and concentration and motility of spermatozoa.

	Number of samples	Percentage of DNA in comet head	Comet tail length ( $\mu\text{m}$ )	Tail moment
Mean values	62	70.53 $\pm$ 10.32	28.24 $\pm$ 6.20	10.49 $\pm$ 4.94
Age of donors (years):				
20–29	31	69.79 $\pm$ 10.18	28.52 $\pm$ 6.28	10.79 $\pm$ 5.17
30–35	26	71.07 $\pm$ 10.68	28.19 $\pm$ 6.56	10.53 $\pm$ 5.06
>35	5	72.26 $\pm$ 11.07	26.92 $\pm$ 4.57	8.65 $\pm$ 2.71
Sperm concentration				
< 20 $\times$ 10 <sup>6</sup> /ml	10	66.19 $\pm$ 12.33	32.24 $\pm$ 7.78*	13.30 $\pm$ 6.77
20–100 $\times$ 10 <sup>6</sup> /ml	30	70.67 $\pm$ 10.34	27.44 $\pm$ 5.00	10.06 $\pm$ 4.32
>100 $\times$ 10 <sup>6</sup> /ml	22	72.54 $\pm$ 9.52	27.46 $\pm$ 6.47	9.98 $\pm$ 4.40
Sperm motility				
<50%	14	73.35 $\pm$ 7.97	28.29 $\pm$ 4.67	9.82 $\pm$ 3.86
>50%	48	69.91 $\pm$ 10.88	28.58 $\pm$ 6.77	10.79 $\pm$ 5.33

\*Statistical significance compared to other groups by Student t-test,  $p < 0.05$ .

point agarose (LMPA) was mixed with the semen and then pipetted onto each microscope slide covered previously with agarose of normal melting point (NMPA). Slides were covered with cover slips and kept at 4°C for 5 min to solidify the agarose. After removing the cover slips, the next layer of LMPA agarose were added and allowed to solidify again. Then the cells on slides were immersed in lysing solution containing proteinase K in the presence of detergent. Lysis was conducted at 35°C for 20 h. On the next day, the cells were incubated in electrophoresis solution for 20 min. Low voltage electrophoresis was conducted in alkaline condition for 10 min using 24 V and 300 mA. After finishing the electrophoresis, the slides were neutralised and stained with ethydyne bromide (EtBr). Slides were examined using a fluorescence microscope. Images were registered and then examined using computer software CASP [20] enabling various DNA damages analysis. A hundred cells from each donor were scored. Parameters chosen for DNA damage estimation were “percentage of head DNA”, “comet tail length” and “tail moment”. Statistical analysis was performed using Student t-test.

## RESULTS

The mean values of DNA damage in genetic material are shown in Table 1. DNA damages were estimated taking into account the age of donors (20–29, 30–35, over 35 years); depending on the sperm concentration (below 20  $\times$  10<sup>6</sup>, from 20  $\times$  10<sup>6</sup> to 100  $\times$  10<sup>6</sup>, over 100  $\times$  10<sup>6</sup>; and depending on motility (under 50% and over 50%).

Percentages of head DNA in 62 semen samples differed, and varied from 46.17% in a 27-year-old donor (concentration 12.8  $\times$  10<sup>6</sup>, motility 74%) to 94.20% in a 31-year-old donor (concentration 319  $\times$  10<sup>6</sup>, motility 72%). Mean percentage of head DNA was 70.53%. There were no significant differences between mean DNA damage in samples of donors from different age groups.

Mean percentage of DNA in comet heads from samples of concentrations below 20  $\times$  10<sup>6</sup>/ml was 66.19%, in samples of concentrations from 20  $\times$  10<sup>6</sup>/ml to 100  $\times$  10<sup>6</sup>/ml – 70.67%, whereas in samples of concentration over 100  $\times$  10<sup>6</sup>/ml – 72.54%. There was a slightly higher percentage of DNA in comet heads in samples with less motile spermatozoa, but the results were not statistically significant.

The mean comet tail length was 28.24  $\pm$  6.20  $\mu\text{m}$  and varied between 9.91  $\mu\text{m}$  in a 31-year-old donor (sperm concentration 319  $\times$  10<sup>6</sup>/ml, motility 72%) to 44.5  $\mu\text{m}$  in a donor 27-year-old (sperm concentration 12.8  $\times$  10<sup>6</sup>/ml, motility 74%). Results for donors aged 20–29 years and aged 30–35 years were similar. In the group of the oldest donors (over 35 years old) the average comet tail length was the lowest, 6.92  $\pm$  4.57  $\mu\text{m}$ . There were no statistical differences between tail lengths in different age groups. The mean comet tail length was the highest in donors having a sperm concentration below 20  $\times$  10<sup>6</sup>. The results observed in this group were statistically significant compared to the results from other groups (20–100  $\times$  10<sup>6</sup>/ml and over 100  $\times$  10<sup>6</sup>/ml). There were no statistically differences in comet tail lengths between samples of different mobility of gametes.

The values of the mean tail moment value were calculated as 10.49  $\pm$  4.94, and varied from 0.94 in a donor aged 31 years (sperm concentration: 319  $\times$  10<sup>6</sup>/ml, motility 72%) to 2.53 in a 27-year-old donor (sperm concentration: 12.8  $\times$  10<sup>6</sup>/ml, motility 74%). The lowest values were observed in donors aged over 35 years, but the results were not statistically significant compared to other experimental groups. Some, however not statistically significant differences in the comet tail moment values in samples of different sperm concentration, were noted. The highest values were observed in samples of the lowest sperm concentrations, whereas the lowest values were in samples of the highest sperm concentrations. Nevertheless, no statistically significant differences in comet tail moment values between samples of different motility were noted.

## DISCUSSION

It is estimated that 25–50% of couples' infertility is attributed to the male factor [34, 38].

Damage to sperm nuclear chromatin, such as DNA fragmentation may influence male fertility, successful fertilization and sustained pregnancy [1, 11, 21, 22, 36]. DNA integrity in the male gamete is of great importance for proper transmission of genetic information to the offspring genome, and in the proper development of the embryo [9]. It is generally recognized that damage to 30% or more sperm DNA causes impossibility to become pregnant in a natural way [11, 12].

The enhanced frequency of DNA damages measured, among others, by comet assay in infertile patients has been observed by numerous authors [18, 21, 28]. Such an increase in DNA damage may be also connected with exposure to genotoxic agents [15] or with consumption of 3 or more cups of coffee per day [27].

Studies on DNA damage in samples from fertile normospermic, infertile normospermic and infertile asthenospermic men showed no differences in spontaneously occurred DNA damages. However, spermatozoa of asthenospermic men were more susceptible to induction of DNA damage by X-rays and hydroxyl peroxide [17, 24]. Induction of DNA damage in male gametes may be connected with enhanced risk of congenital malformations in the offspring, and with mutations leading to childhood cancers and infertility in the offspring [2].

In this study, for the estimation of DNA damages, 3 parameters were used: percentage of head DNA, comet tail length and tail moment. A high value of the first parameter provides information about minimal damage DNA of genetic material because undamaged DNA remains bonded to the nuclear matrix forming the head DNA, whereas broken DNA strands are taken from the nucleus towards the anode, forming characteristic DNA tail during electrophoresis. A higher level of DNA damage means the longer the DNA tail, and the higher the value of tail moment, which combines with distribution DNA in the tail and tail length. All parameters testified for minimal DNA damage in the same donor, a man aged 31 years (sperm concentration  $319 \times 10^6$  and sperm motility 72%). In another man aged 27 years all parameters testified for maximal DNA damage (sperm concentration  $12.8 \times 10^6$ , sperm motility 74%) were noted. Each of the tested parameters may therefore be used to estimate the DNA damage in male gametes.

Analysis of over 60 papers regarding the parameters of male semen showed that there were big differences between the quality of semen in men below 30 years and over 50, whereas there were no differences in the sperm count. Kidd *et al.* [19] found that the semen volume, percentage of morphologically normal and mobile spermatozoa decreases together with male age. On the other hand, Schmidt

*et al.* [27] found that in a population of men aged 22–80 years, the frequency of DNA damage measured by alkaline comet assay increased with age. They were not able, however, to show such a relationship when measurements were performed under neutral conditions. An increase in DNA damage in semen of men over 35-year-old was also observed by Trisini *et al.* [35]. Morris *et al.* [25] observed that in patients aged 29–44 the frequency of DNA damage increased with age. Other authors also stated that in the semen of older men, highly damaged gametes were more frequent [32]. This may be caused by oxidative stress in the male reproductive system [3, 4]. Significantly higher DNA damage in semen of men aged 36–57 years, compared to those aged 20–35 years, stated Singh *et al.* [31]. The results presented here did not show correlation between the frequency of DNA damage measured by alkaline comet assay and the age of men. This could be due to the fact that the group of the oldest men (35–44 years old) enrolled to the study was relatively small, men were relatively young compared to previously cited papers, and because they were spouses of pregnant females.

Numerous authors confirmed a correlation between the frequency of DNA damage measured by alkaline comet assay and sperm count [8, 10, 17, 21, 27, 30]. They observed an enhanced level of gametes with damaged DNA in men with a sperm concentration below  $20 \times 10^6$ /ml [23]. In our studies, a statistically significant correlation between the frequency of DNA damage and sperm concentration was noted. The frequency of DNA damage in samples with a sperm concentration below  $20 \times 10^6$  was significantly higher compared to samples with higher concentrations. Other author however, did not observe a correlation between sperm concentration and the incidence of DNA damage measured by comet assay [18, 25, 35].

Reference evidences indicate that the frequency of DNA damage increased in less motile gametes [23]. The increase in DNA damage dependent on motility and frequency of abnormal gametes was observed by Morris *et al.* [25]. The correlation between the spermatozoa motility and DNA damages frequency was also noted by other authors [13, 17, 30, 39]. Results presented above did not confirmed such correlation. Similarly, such dependence did not observe Schmidt *et al.* [27].

## CONCLUSIONS

Spermatozoa of oligozoospermic (low sperm concentration) donors are characterized by a significantly higher level of DNA damage.

In the examined population (20–44 years old), there was no correlation between the frequency of damaged DNA and donors' age.

The Comet assay may be used in biomonitoring of quality of human male gametes.

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