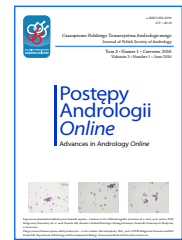




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# RELATIONSHIP BETWEEN ALCOHOL CONSUMPTION AND SPERM NUCLEAR DNA FRAGMENTATION AND PREGNANCY

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## Abstract

**Background and objectives.** In adults, the possible effects of alcohol consumption on semen parameters during spermatozoa production have been described by a limited number of studies with conflicting results. According to some reports alcohol consumption unfavorably affects sperm chromatin integrity. Research was to designed evaluate the influence of risky alcohol consumption on the sperm nuclear DNA fragmentation and the effectiveness of infertility treatment.

**Material and methods.** The study enrolled 499 couples being treated with intrauterine insemination. The Alcohol Use Disorders Identification Test was applied to select a group of men who consumed alcohol in a risky manner ( $n = 154$ ) and a group of subjects who consumed alcohol without risk ( $n = 345$ ). The sperm nuclear DNA fragmentation was identified by chromatin dispersion test (Halo sperm test). The sperm DNA fragmentation index (DFI) was expressed as the percentage of cells that had detectable sperm fragmentation (little or no halo) and calculated from the total number of sperm observed.

**Results.** The compared groups differed in DFI. The percentage of sperm cells with nuclear DNA strand breaks was significantly higher ( $p < 0.001$ ) in men with risky alcohol consumption (median 42.50% vs 21.00%) (Mann–Whitney U-test). The completion of successful infertility treatment was most often ( $p = 0.004$ ) observed in the group of men who did not consume alcohol in a risky way (achieved pregnancy 29.77% vs. 17.65%) (Chi-square test). The DFI was significantly higher ( $p = 0.001$ ) in the group of men with risky alcohol consumption from couples who did not achieve pregnancy compared to men from couples who achieved pregnancy (median 45.00% vs. 38.00%). Furthermore, the proportion of sperm cells with nuclear DNA strand breaks slightly increased with men age independently of alcohol consumption (Spearman rank correlation test).

**Interpretation and conclusions.** Obtained findings indicate that the burden of risky alcohol consumption result in an intensification of sperm DNA fragmentation and a decrease in the effectiveness of infertility treatment. Moreover, it seems that sperm nuclear DNA integrity may decrease with age of men.

**Key words:** alcohol consumption, sperm DNA fragmentation, infertility, age

## Abbreviations

AMH – anti-Müllerian hormone, AUDIT – Alcohol Use Disorders Identification Test, BMI – body mass index, BRFSS – Behavioral Risk Factor Surveillance System, DFI – DNA fragmentation index, FSH – follicle stimulating hormone, GSH – glutathione, ICSI – intracytoplasmic sperm injection, IVF – *in vitro* fertilization, ROS – reactive oxygen species, SCD – sperm chromatin dispersion test, WHO – World Health Organization

## Introduction

Epidemiological data show that human reproductive disorders are a common problem worldwide, affecting one-sixth of all couples. Infertility is thus considered a social disease by the World Health Organization (WHO). Recently, a number of reports have been published concerning the considerable decrease observed in male fertility (*Andersson et al., 2008; Carlsen et al., 1992; Cissen et al., 2016; Esteves and Chan, 2015; Feki et al., 2009; Rolland et al., 2013; Sripada et al., 2007; Swan et al., 2000*). The low sperm count of contemporary males, apart from the deterioration of the quality of the semen, reduces the chance of reproduction, constitutes a major problem for public health, and may be among the causes of the negative birth rates in many European countries (*Jørgensen et al., 2012; Macaluso et al., 2010*).

The causes of the decrease in male infertility have not been fully recognized. This is due to the fact that it is very difficult to select individual hazardous factors. The main factors that have been considered are exposure to plant protection products, exposure to heavy metals, the effect of the nearby sources of electromagnetic waves, the increasingly frequent occurrence of overweight and obesity among males, sedentary lifestyles causing overheating of the testicles, tobacco smoking, narcotics, and alcohol consumption (*Carpenter et al., 2013; Condorelli et al., 2014; Den Hond et al., 2015; Fronczak et al., 2012; Kanter et al., 2013; Kumar et al., 2014; Leisegang et al., 2014; Mehrpour et al., 2014; Safarinejad et al., 2013; Taha et al., 2013; Yao and Milis, 2016; Zhang et al., 2013*).

At present, Poland is among the countries with the highest consumption of alcoholic beverages worldwide. The mean consumption of spirit drinks in Poland is among the highest, approximately 0.9 million people are addicted to alcohol, and the number of those who undertake risky alcohol consumption is estimated to be on the level of 2–3 million. As many as 10–15% of the adolescent population (14–18 years) is threatened with the negative effects of drinking alcohol. These data show how important the problem of alcohol consumption is for the health and reproductive capabilities of males (*Kłós, 1997; [www.who.int/substance\\_abuse/publications/global\\_alcohol\\_report/msb\\_gsr\\_2014\\_3.pdf](http://www.who.int/substance_abuse/publications/global_alcohol_report/msb_gsr_2014_3.pdf)*).

In adults, the possible effects of alcohol consumption on semen parameters during spermatozoa production have been described by a limited number of studies, with conflicting results: some indicate a detrimental effect, but others indicate no effect, or even a protective effect from moderate amounts of alcohol (*Marinelli et al., 2004; Yao*

*and Milis, 2016*). Excessive alcohol consumption causes an increase in systemic oxidative stress as ethanol stimulates the body's production of reactive oxygen species (ROS), and many alcohol abusers also have diets deficient in protective antioxidants (*Koch et al., 2004; Kumar et al., 2014; Nordmann, 1994; Nordmann et al., 1990; Rosenblum et al., 1989; Pasqualotto and Pasqualotto, 2012; Yao and Milis, 2016*). Finally imbalance between generation of ROS and the protective effects of antioxidant system can result in damage of sperm DNA. Therefore, alcohol consumption also unfavorably affects sperm chromatin integrity (*Frączek and Kurpisz, 2015; Gavrieliouk and Aitken, 2015; Komiya et al., 2014; Kumar et al., 2014; Pourentezari et al., 2016*). Therefore, our research was designed to evaluate the influence of risky alcohol consumption on the sperm nuclear DNA fragmentation and the effectiveness of infertility treatment.

## Material and methods

### Subjects

The study<sup>1</sup> involved 499 couples being treated by intrauterine insemination and who had been diagnosed with infertility, according to the WHO definition. Patients were treated over the 2011–2014 period in the Ovum Centre for Infertility Treatment, Lublin.

Men aged 23–57 were enrolled into the study group, but those with BMI (body mass index) <17 or >30, as well as those with metabolic diseases, severe asthenoteratozoospermia symptoms of systemic diseases, clinical confirmation of an inflammatory state of the reproductive organ, or a smoking habit were excluded from the study. Also not qualified to the study group were patients with the presence of clinically confirmed varices of the spermatic cord (varicocele), and those who, within the three months prior to the study, had taken medicines that might affect the quality and density of sperm.

The AUDIT (Alcohol Use Disorders Identification Test) was administered to the male participants, and on the basis of its results, a group of patients who consumed alcohol in a risky manner was selected. The test consisted of two parts: an interview about alcohol and a clinical examination. All participants were asked about the number of alcoholic drinks (12 oz. beer, 8 oz. wine cooler, 4 oz. wine, or 1 oz. liquor) consumed each week. The total number of alcoholic drinks was divided by 7 to obtain an estimated daily number of alcoholic drinks consumed. In the BRFSS (Behavioral Risk Factor Surveillance System),

1 The study was approved by the local Ethics Committee

**Table 1.** Descriptive statistics of standard seminological parameters (n = 499)

Parameter	median (range)	$\bar{X}$ (SD)
Age (years)	35.00 (24.00–58.00)	35.07 (4.82)
Sperm concentration ( $\times 10^6$ /mL)	37.00 (9.80–85.70)	37.67 (16.61)
Normal sperm morphology (%)	8.00 (0.00–17.00)	8.35 (4.45)
Progressive motility (%)	45.00 (5.00–65.00)	44.23 (14.80)
Sperm vitality (% eosine-negative sperm cells)	80.00 (7.00–98.00)	73.67 (19.81)
Sperm vitality (% Host-test-positive sperm cells)	71 (3.00–96.00)	62.21 (24.01)

$\bar{X}$  (SD) – arithmetic mean (standard deviation)

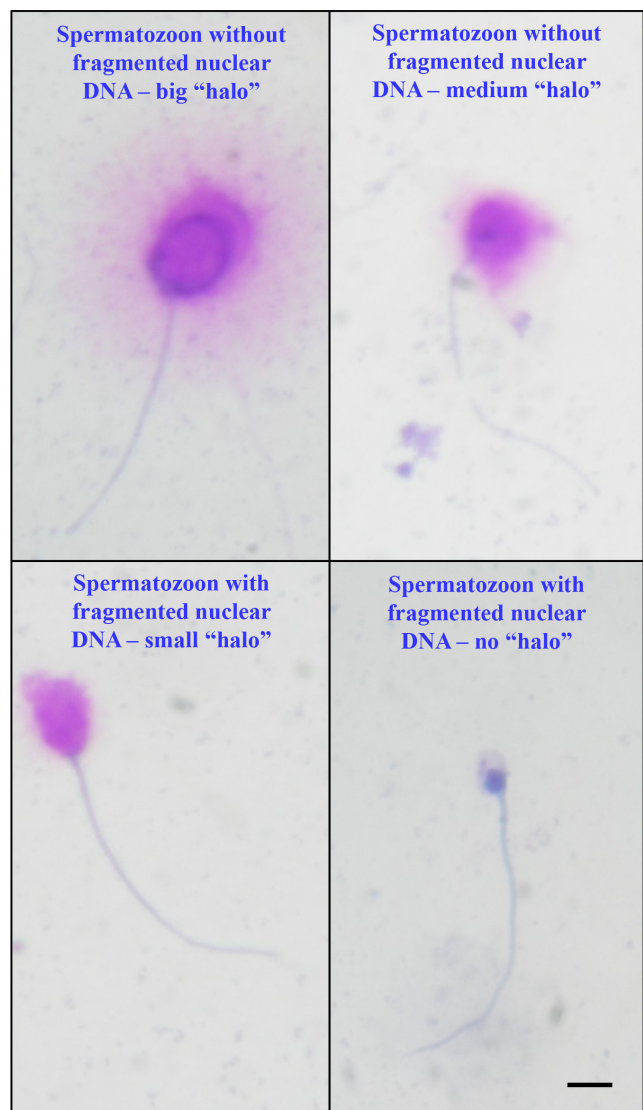
one alcoholic drink was measured as a 12 oz. beer, a 5 oz. glass of wine, or one shot of liquor. For both samples, heavy alcohol use for males was identified as having more than two drinks per day and for females having more than one drink per day (CDC, 2009).

**Standard semen analysis and sperm chromatin dispersion test (Halo sperm test)**

Sperm was obtained by means of masturbation and was examined directly after liquidation, according to the WHO criteria (WHO, 2010) (table 1). Prior to the examination, the men had been abstinent from sex for 4 days. In order to determine the percentage of fragmented nuclear DNA in sperm, the sperm chromatin dispersion test (SCD) was used, according to the instructions provided by the producers (Dyn-Halosperm Kit, Halotech DNA SL, Madrid, Spain; Fernández et al., 2005). Sperm cells suspended in agarose gel were treated with an acidic solution and then a lysing solution, and finally staining solution. The sperm cells with small ( $\leq 1/2$  of the width sperm head) or no “halo” of decompressed DNA were considered as cells with fragmented DNA. Whereas those with no DNA fragmentation had long loops forming a rich nucleic acid “halo” (medium and big “halo”) (figure 1). From each sample 300 sperm cells were scored under the 100 $\times$  objective of the microscope (CX31, Olympus Optical Co., Japan). The sperm DNA fragmentation index (DFI) was expressed as the percentage of cells that had detectable sperm fragmentation (small or no “halo”) and calculated from the total number of sperm observed. The DFI was determined immediately after semen liquefaction.

**Statistical analysis**

The measurable parameters included in the analysis were expressed as mean(standard deviation) values, median (range) values, while non-measurable parameters were given as a percentage. The conformity of the variables with the normal distribution was examined using Shapiro–Wilk test. Mann–Whitney U-test was employed to evaluate statistical differences between compared groups. Spearman rank correlation coefficients ( $r_s$ )



**Fig. 1.** Results of sperm chromatin dispersion test. Micrographs by PhD, prof. of PUM Małgorzata Piasecka and PhD Kamil Gill, Department of Histology and Developmental Biology, Pomeranian Medical University in Szczecin

test was used to determine the relationships between obtained variables. The prevalence of categorical variables were compared using Chi-square test. Statistical significance was considered at  $p < 0.05$ . The database and

**Table 2.** Descriptive statistic and comparison of sperm DNA fragmentation index (DFI) between men with risky alcohol consumption and without risky alcohol consumption

Subjects	Parameter	DFI (%)
	median (range)	28.00 (6.00–91.00)
Total (n = 499)	$\bar{X}$ (SD)	29.91 (15.11)
Subjects with risky alcohol consumption (n = 154)	median (range)	42.50 <sup>a</sup> (14.00–84.00)
	$\bar{X}$ (SD)	44.56 (12.26)
Subjects without risky alcohol consumption (n = 345)	median (range)	21.00 <sup>a</sup> (6.00–91.00)
	$\bar{X}$ (SD)	23.37 (11.17)

$\bar{X}$  (SD) – arithmetic mean (standard deviation); common superscripts denote significant difference between compared groups: a –  $p < 0.001$ ; Mann–Whitney U-test

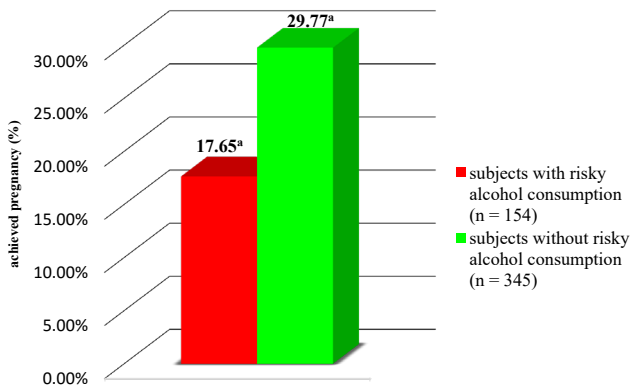


Fig. 2. Comparison of the percentage of achieved pregnancy between study groups. Common superscripts denote significant difference between compared groups: a – p = 0.004, Chi-square test

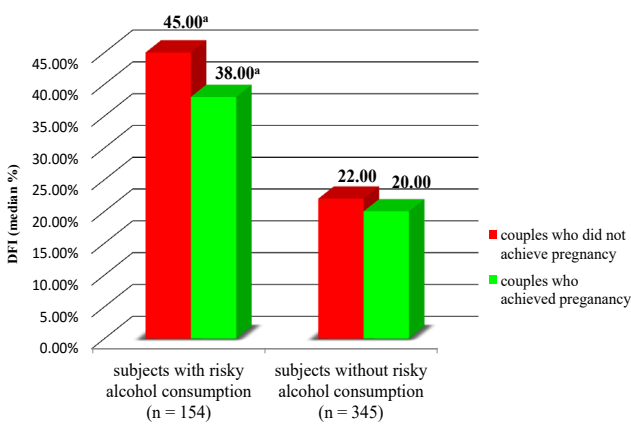


Fig. 3. Comparison of the percentage of sperm DNA fragmentation index (DFI) between study groups. Common superscripts denote significant difference between compared groups: a – p < 0.001, Mann-Whitney U-test

statistical analysis were performed using Statistica 9.1 (StatSoft, Poland).

## Results

The men were divided into two groups: subjects with risky (n = 154) and without risky alcohol consumption (n = 345). The compared groups differed in DFI. The percentage of sperm cells with nuclear DNA strand breaks was significantly higher (p < 0.001) in men with risky alcohol consumption (median 42.50% vs 21.00%) (table 2). In the group of men with risky alcohol consumption, 27 pregnancies were achieved (17.65%) as a result of the infertility treatment, whereas in the group of men without risky alcohol consumption 103 pregnancies were achieved (29.77%). The completion of successful infertility treatment was most often (p = 0.004) observed in the group of men who did not consume alcohol in a risky way (figure 2). The percentage of sperm cells with fragmented DNA was significantly higher (p < 0.001) in the group of men with risky alcohol consumption from couples

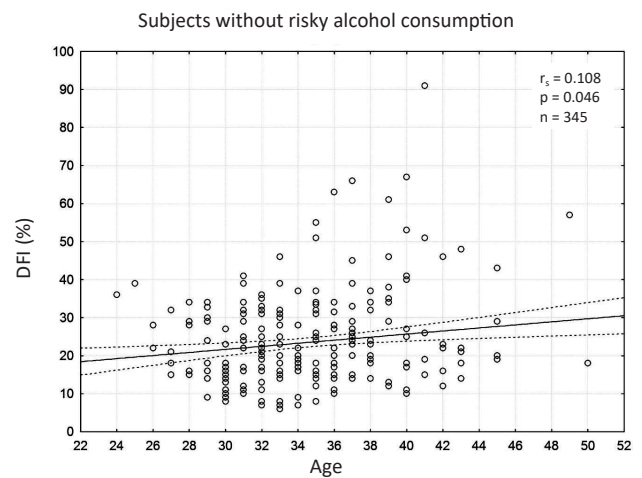
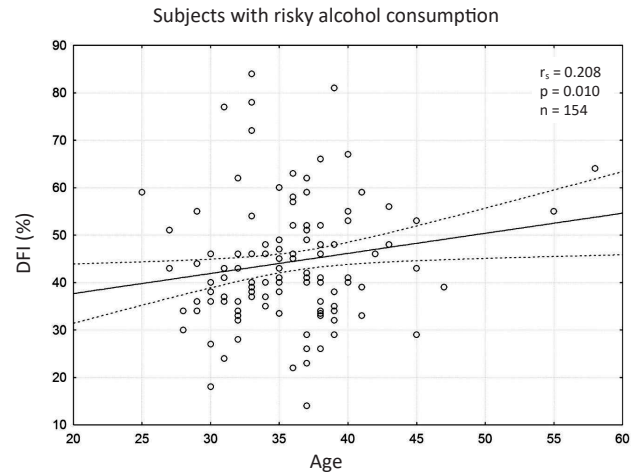


Fig. 4. Correlations between DNA fragmentation index (DFI) and age of subjects

who did not achieve pregnancy compared to men from couples who achieved pregnancy (median 45.00% vs. 38.00%). There was no statistical difference (p = 0.349) in DFI between men without risky alcohol consumption from couples who did not achieve pregnancy and men from couples who achieved pregnancy (median 22.00% vs. 20.00%) (figure 3). The percentage of sperm cells with fragmented DNA increased with men age independently from alcohol consumption. However, a slight intensification in DNA fragmentation was found. The values of Spearman rank correlation coefficients were low (group with risky alcohol consumption:  $r_s = 0.208$ , p = 0.010; group without risky alcohol consumption:  $r_s = 0.108$ , p = 0.046) (figure 4).

## Discussion

The present study showed that, sperm nuclear DNA fragmentation is considerably higher among males suffer from alcohol abuse compared to males without risky alcohol consumption (median 42.50% vs 21.00%). Our data



were agreement with studies by *Komiya et al.* (2014) conducted among 54 males being treated for infertility. The researchers revealed that chronic alcohol use gave a DFI of  $49.6 \pm 23.3\%$ , as compared with  $33.9 \pm 18.0\%$  in non-drinkers. Similar results were obtained by *Anifandis et al.* (2014) who found that the percentage of spermatozoa with small “halo” significantly associated with drinking behavior. Likewise, *Vellani et al.* (2013) observed the effect of ethanol consumption on the intensification of sperm DNA fragmentation. Similar relationships between the consumption of alcohol and chromatin integrity have been also confirmed in experimental studies conducted on rats (*Talebi et al.*, 2011) and mice (*Pourentezari et al.*, 2016). The researchers administered alcohol to these animals and found that ethanol abuse results in the production of spermatozoa with less chromatin quality.

The effect of alcohol consumption on sperm chromatin integrity most probably results from oxidative stress generated by the ethanol. Experimental studies carried out on rats subjected to acute and chronic intoxication with ethanol showed that alcohol-induced oxidative stress might affect not only the liver, but also various extrahepatic tissues in which the active oxidation of ethanol took place. The oxidation mostly concerned the heart, kidneys, central nervous system, and testes (*Galicia-Moreno and Gutiérrez-Reyes*, 2014; *Ozbek*, 2012; *Rosenblum et al.*, 1989; *Zakhari*, 2006). The ethanol is known to be a toxin which influences on the testes, and its chronic use leads to both endocrine and reproductive failure. Described reports suggest that ethanol abuse may lead to testicular lipid peroxidation. Testicular membranes are rich in polyenoic fatty acids susceptible to peroxidative decomposition, and it is likely therefore that the abuse and chronic use of alcohol is related to lipid peroxidation which contributes to membrane injury and dysfunction of the gonads. The observed reductions in the content of polyenoic fatty acids and glutathione (GSH) of the testes in animals administered alcohol, compared to the isocalorically fed control group, are in accordance with the mechanism of alcohol-related testicular toxicity (*Dosumu et al.*, 2014; *Himabindu et al.*, 2015; *La Vignera et al.*, 2013; *Nordman et al.*, 1990). The increased conversion of xanthine dehydrogenase into xanthine oxidase, and the activation of peroxisomal acyl CoA-oxidase linked to the consumption of ethanol, may be a contributing factor to oxidative stress. Chronic administration of alcohol results in an increase in mitochondrial lipid peroxidation and a decrease in the GSH level in the testes. It seems to be related to the observed considerable testicular atrophy (*Nordmann et al.*, 1994; *Pasqualotto and Pasqualotto*, 2012).

Our study confirmed an unfavorable effect of risky alcohol use on the effectiveness of infertility treatment with intrauterine inseminations. Literature reports concerning the effect of alcohol consumption on the outcomes of infertility treatment are equivocal. *Dunphy et al.* (1991) evaluated the relationship between male ethanol

intake and fertility in 258 couples attending an infertility clinic, but found no significant association between the amount of alcohol consumed per week and the fertility outcome. A multicenter research project was carried out in Europe covering a population of women aged 25–44, randomly selected based on census records and electoral registers in various European countries, as well as pregnant women who reported for prenatal care visits (*Olsen et al.*, 1997). More than 4,000 couples were included in study, and 10 different regions in Europe took part in the data collection. The results of the survey did not confirm any significant relationship between alcohol consumption and subfertility. Similar results were obtained by *Curtis et al.* (1997). The authors analyzed the effect of alcohol on the fertility of males and females using data from the Ontario Farm Family Health Study (*Curtis et al.*, 1997). This retrospective cohort study was conducted during the period 1991–1992 among rural men and women and provided information concerning 2,607 planned pregnancies achieved within 30 years. Fertility rates were calculated by means of the Cox Proportional Hazards model. The results of the investigation indicate that alcohol consumption among males and females was not related to fertility. The relationship between the amount and timing of alcohol consumption by men and women undergoing IVF therapy and the reproductive outcome was also analyzed. It was discovered that, in males who consumed one additional drink daily, the risk of not achieving live birth increased by 2.28 (1.08–4.80) to 8.32 (1.82–37.97) times, and drinking beer also exerted an effect on live births (odds ratio 55.49–45.64) (*Klonoff-Cohen et al.*, 2003). *Huang et al.* (2012) identified risk factors for pregnancy outcomes in couples treated with intracervical or intrauterine insemination, with or without superovulation for unexplained or male-factor infertility. Out of 932 couples randomized to four treatment groups, 664 couples who had completed the lifestyle questionnaires were assessed for occurrence of pregnancy and live birth. Couples in which the female partners drank coffee, tea, or alcoholic beverages in the past had higher pregnancy and live birth rates compared with those who had never used these or currently used them. In discontinuing these habits, they might have made other lifestyle changes to improve pregnancy outcome. The considerable discrepancies concerning the effects of alcohol consumption on infertility therapy may be due to the difficulties resulting from the actual assessment of alcohol consumption. Despite using standardized instruments, it may be expected that some respondents will hide an alcohol problem, and the effect of ethanol on the body may be conditioned by individual predispositions and diseases which have not always been diagnosed.

The findings presented in this paper showed a slight effect of increasing age on the intensification of DNA fragmentation. Similar results were obtained by *Schmid et al.* (2007), who examined non-clinical sperm samples from non-smoking, healthy, and active employees and

retirees. The authors found a relationship between the age of subjects and the damage to the sperm DNA strand. Significantly higher frequencies of sperm DNA fragmentation measured under alkaline conditions (Comet assay) were found in the sperm of older man. The higher frequencies might indicate alkali-labile DNA sites and single-strand DNA breaks. However, under neutral conditions, age was not related to sperm DNA damage, which is thought to represent double-strand DNA breaks. The effects of age on genomic damage has been confirmed by another authors ([Morris et al., 2002](#); [Sharma et al., 2015](#); [Singh et al., 2003](#); [Spano et al., 1998](#); [Wyrobek et al., 2006](#); [Zitzmann, 2013](#)) who discovered a strong relationship between DFI and age in men aged 18–55 ([Spano et al., 1998](#)) and positive correlation between sperm DNA damage and age of man who were enrolled in an *in vitro* fertilization program as well as impairment of post-fertilization embryo cleavage following intracytoplasmic sperm injection (ICSI) ([Morris et al., 2002](#)).

Regarding age-related oxidative stress in the reproductive tract, older males may produce more sperm with DNA damage ([Barnes et al., 1998](#); [Barroso et al., 2000](#); [Sharma et al., 2015](#)). Oxidative stress may cause damage to sperm DNA and mitochondrial and nuclear membranes ([Agarwal et al., 2014](#); [Aitken et al., 2003](#); [Frączek and Kurpisz, 2015](#); [Gavriliouk and Aitken, 2015](#); [Kodama et al., 1997](#); [Lobascio et al., 2015](#); [Walczak-Jędrzejowska, 2015a, b](#)). A relationship between oxidative sperm DNA damage and male fertility was noted by [Kodama et al. \(1997\)](#). However, in older males, the apoptotic functions of spermatogenesis may be less effective and may result in the release of more DNA damaged sperm ([Brinkworth et al., 1997](#); [Print and Loveland, 2000](#)).

## Concluding remarks

Obtained findings indicate that the burden of risky alcohol consumption result in an intensification of sperm DNA fragmentation and a decrease in the effectiveness of infertility treatment. Moreover, it seems that sperm nuclear DNA strand breaks may increase with age of men. Reports in the literature suggest many factors that might be responsible for sperm DNA fragmentation, and also emphasize the importance of this phenomenon in human reproduction ([Anifandis et al., 2014](#); [Frączek and Kurpisz, 2015](#); [Gavriliouk and Aitken, 2015](#); [Morris et al., 2002](#); [Singh et al., 2003](#)). There is thus a need for further studies into the conditioning of this phenomenon.

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