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Effect of the Heavy Cigarette Smoking on Sperm Chromatin, DNA Fragmentation and Methylation in Young Saudi Men

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ABSTRACT

Cigarette smoking is a lifestyle pattern. It is a major global health problem responsible for the decline in semen quality and an overall decline in male reproductive health. The main objective of the current study was therefore to evaluate the effect of cigarette smoking on sperm quality in young Saudi men living in the Western region of Saudi Arabia (Makkah). In this study, semen samples were collected from 100 men each (mild, moderate and heavy cigarette smokers) aged between 18 and 36 years with a mean age of 24.38 ± 9.58 years. All samples were collected by masturbation with the abstinence interval of sex or ejaculate of at least 72 hours, along with 100 aged matched healthy nonsmokers who served as controls. The percentage of abnormal sperm chromatin non-condensation, DNA fragmentation, and DNA methylation was found to be significantly higher in heavy smokers compared to mild / moderate / nonsmokers ($p < .0005$). These results indicate that heavy cigarette smoking negatively affects the conventional semen parameters including sperm chromatin condensation and sperm viability. We hypothesized that heavy cigarette smoking seems to be associated with sub-fertility in these males by reducing sperm quality in a dose-dependent manner.

Keywords: Cigarette smoking, Sperm chromatin, DNA fragmentation and methylation, young Saudi men.

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INTRODUCTION

Smoking is a major burden on the world's collective health system. Approximately 22.5 percent of adults in the world use tobacco products, of which, 32% are men and 7% are women[1]. Tobacco toxins expose the body to free radicals, leading to Deoxyribonucleic acid (DNA) damage and elevated oxidative stress.

Smoking indirectly decreased both *in vitro* and *in vivo,* neuronal nitrous oxides (NOS) activity through non-adrenergic non-cholinergic neurons due to enzymatic blockade. Interestingly, components of burned tobacco are responsible for this act but not the unburned part. It is thus well-established that cigarette smoke not only damages the endothelium but also impairs NOS mediated vasodilation. Intracellular nitrous oxide (NO) usually inhibits Rho-associated kinase (ROK), thus allowing for vasodilation. At the same time, decreased level of NO, which is secondary to smoking, disinhibit ROK and further worsen erectile dysfunctions[2].

In a 2016 meta-analysis composed of twenty studies with 5865 participants showed an association between cigarette smoking and reduced sperm count, motility, and abnormal morphology[3.] Another study showed an abnormal chromatin condensation and a decrease in viability among smoker men compared to non-smoker[4].

The underlying mechanisms of smoking's effect on semen parameters are not fully understood, but there are multiple known mechanisms which include sperm DNA fragmentation, caused by the increase in reactive oxygen species in the seminal fluid, and a decline in the sertoli and leydig cells secretions[5].

Although the reason for a decline in semen quality still remains unknown, a direct correlation between smoking and semen quality has been reported in many studies[6.] More recently, sperm nuclear DNA fragmentation assays have been developed. Results of these assays have shown a very strong and positive correlation with sperm fertility status, which is different from conventional semen morphological studies. These studies surround changes in sperm chromatin condensation or alteration which is necessary for chromatin packing[7.]

Many studies have shown a direct and positive correlation between DNA damage and chromatin integrity and abnormal sperm head morphology, but such a relationship in infertile patients due to an environmental factor such as heavy smoking has not been studied at a large population group[8.]

Cigarette smoke containing carbon monoxide, tar, formaldehyde, nicotine, lead, and cadmium, are reported to have a negative impact on spermatogenesis and spermatozoa function[9.] Moreover, studies have reported that cigarette smoke produces oxygen deficiency which, influences testicular functions, leading to impairment in spermatogenesis. As a result,

there is an ultimate impairment in sperm morphology, a sharp decline in the progressive sperm, and ultimate sperm death[10.] Several studies, in addition, have shown that tobacco cigarette smoking causes genetic alterations including chromosomal alterations, mutations, polymorphisms, DNA damage and epigenetic alteration, all influencing men's fertility[11.] Furthermore, tobacco smoking influences the sperm DNA methylation patterns which, may affect a male's fecundity and sperm phenotype[12.]

DNA methylation occurs at the 5th carbon atom of a cytosine as epigenetic mechanism that is followed by guanine residues (CpG) to form 5-methylcytosine (5mC). This whole process occurs through DNA methyltransferases (DNMTs) enzyme. CpG dinucleotides in humans are present in clusters known as CpG islands, which are found mostly in the promoter region and remain unmethylated, while the majority of all other CpG islands are methylated. As a result of CpG island methylation in promoter regions, the transcription activity of the corresponding gene is suppressed, thus playing a significant role in gene repression and its activation[13.] DNA methylation is an important step which, facilitates the chromatin in the sperm head, thus permanently silencing the promoters of genes involved in genetic imprinting[14.] It is thus suggested that in humans, correct sperm DNA methylation is extremely essential for fertilization as well as early fetus viability[15.]

A strong correlation between cigarette smoking and an altered pattern in the spermatozoa DNA methylation has been reported in literature[16, 17.] However, the influence of smoking on sperm DNA methylation and gene transcription level in sperm remains debatable.

In Saudi Arabia, the prevalence of smoking is reported to be 2.4-52.3% (median: 17.5%). Of this, it ranges from 12-29.8% (median: 16.5%) among school students and 2.4-37% (median: 13.5%) among university students[18.] The studies regarding the effect of smoking on semen parameters on the Saudi population are limited in number. Data collected by a fertility clinic in Riyadh, Saudi Arabia, AlEnezi et al, have reported that smokers have a higher chance of having abnormalities in semen fluid and sperm function compared to non-smokers[19]. Another study had reached the same conclusion in a sample of infertile men[20.]

Previous studies that targeted the Saudi population were limited in number and, more importantly, only focused on infertile people and not the general population, especially those who are younger in age. Moreover, only a few studies have reported regarding correlation of sperm head morphology parameters with DNA fragmentation analysis and chromatin integrity in the Saudi younger aged population[21]. The objective of the current study is therefore to evaluate the smoking effect on sperm DNA fragmentation / chromatin integrity/ methylation, patterns in the younger aged Saudi population.

MATERIALS AND METHOD

Study Design

For this cross-sectional study, samples were collected from the Makkah Region of Saudi Arabia. The ethical and Protocol review committee of Umm Al Qura University, Faculty of Medicine, reviewed and approved the study (Approval No. HAPO-02-K-012-2022-05-1075). Prior to the study, all the subjects were provided with written informed consent.

In total of, 510 men in communities within the Makkah region were spoken. Any subject visiting a fertility clinic for the treatment of sexual dysfunctions or occupational exposure or with a history of chronic urinary tract infection or testicular injury or with disorders such as diabetes mellitus, hypertension and coronary heart disease were excluded from the study. After completing screening, finally, 100 men each (mild, moderate and heavy smokers) ages of 18 and 36 years, with a mean age of 24.38 ± 9.58 years along with 100 ages matched healthy nonsmokers (control group) agreed to participate in the study.

Smokers are defined as subjects who have smoked cigarettes (and are still smoking) continuously for at least 5 years. Smokers who smoked less than five (<5) sticks of cigarette per day were classified as mild smokers, between 5 and 10 sticks of cigarette per day as moderate smokers and more than ten (>10) sticks per day as heavy smokers.

Sample Collection

Subjects were requested to collect the ejaculates by masturbation with the abstinence interval of sex or ejaculate of at least 72 hours. Seminal fluid was collected in a pre-warmed clean sterile plastic container (Olympic Plastics, Los Angeles, CA, Lot # 10050, and USA). From the time of collection to the time of examination, all samples were protected from extreme temperatures, not less than 22 °C and not more than 45 °C. All samples were allowed to liquefy in an incubator at 37°C prior to examination and the analysis was done according to the method described previously[22].

DNA fragmentation and Sperm Chromatin analysis

Semen samples were diluted with phosphate buffer saline (PBS) to get an sperm concentration ranging between 5 and 10 million and the suspensions were mixed with 1 % agarose (final agarose concentration of 0.7 %) at 37 °C. 25 µl aliquots of the above mixture were placed onto a pre-coated agarose glass slide (0.65 %), covered with coverslip and slides were dried and solidified for 5 min at 4 °C. Slides were immersed in freshly prepared denaturation solution (0.08 N Hydrochloric acid [HCl]) for 7 min at room temperature. Slides were transferred to a freshly prepared 10 ml lysis buffer [0.4 m Tris, 0.4 m DTT (Dithiothreitol), 50 mm EDTA (Ethylenediaminetetraacetic acid), 0.3 % SDS (Sodium dodecyl sulfate) and 1 % Triton X-100] and incubated for 25 min at room temperature to stop

denaturation. After through wash and dehydration for 2 min in each of 70 %, 90 % and 100 % ethanol slides were air dried and stained with Giemsa and about 500 spermatozoa per sample were examined to determine the degree of DNA dispersion by observing the relative halo size under bright field microscopy (Zeiss Axioskope 2 plus; Carl Zeiss, Gottingen, Germany). Sperm having large and medium sized halos were considered non-fragmented, while sperm with a small sized halo or no halo were considered to have significant DNA fragmentation.

Sperm Chromatin non-condensation Analysis

Using chromomycin A3 (CMA3), stain four semen smears were prepared and fixed, by using a fixative solution (methanol-glacial acetic acid, 3:1 respectively) at 4°C for 20 min. After air dries at room temperature, smears were incubated in a dark place at room temperature for 20 minutes immersed in 50 µl of staining solution (Sigma-Aldrich, USA). After thorough washing with phosphate-buffered saline (PBS) the slides, were mounted with 1:1 (v/v) glycerol/PBS and incubated overnight at 4°C. Using a fluorescence microscope (Zeiss Photomicroscope III, Germany), 300 spermatozoa on each smear were evaluated by differentiating the stained spermatozoa: bright yellow (positive, bad spermatozoa), dull yellow (negative, good spermatozoa).

Sperm DNA Methylation Analysis

Sperm DNA methylation (5-methylcytosine) was evaluated according to the guidelines using MethylFlash™ Methylated DNA Quantification ELISA (Epigentek Group Inc, USA). Extracted DNA (100 ng) was incubated with the DNA binding buffer solution at 37°C for 90 minutes (blank, a positive, and negative control have been used in triplicate during this assay). After 3 times washing of micro-well, the methylated DNA capture solution was added to each well and incubated for 60 min at room temperature, detection antibodies were added to each well and further incubated at room temperature for 30 minutes. Another three-time washing was done, a developing solution was added to each well and again incubated at room temperature in the dark place for 10 minutes. A Stop solution was then added and microplate ELISA reader was used to assess the absorbance at 450 nm. The DNA methylation status (ng) was calculated using the equation: 5-mC (ng) = [(sample OD − blank OD)/100].

Statistical analysis

Comparisons between mild, moderate and heavy smokers were performed by Student *t* tests using SPSS program 17.0 (SPSS Institute, Inc.; Chicago, IL, USA) software. All results were tabulated as mean ± standard deviation. A p value < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

The estimated values of sperm chromatin non-condensation of the smoker groups and their age matched non-smoker controls are presented in figure-1.

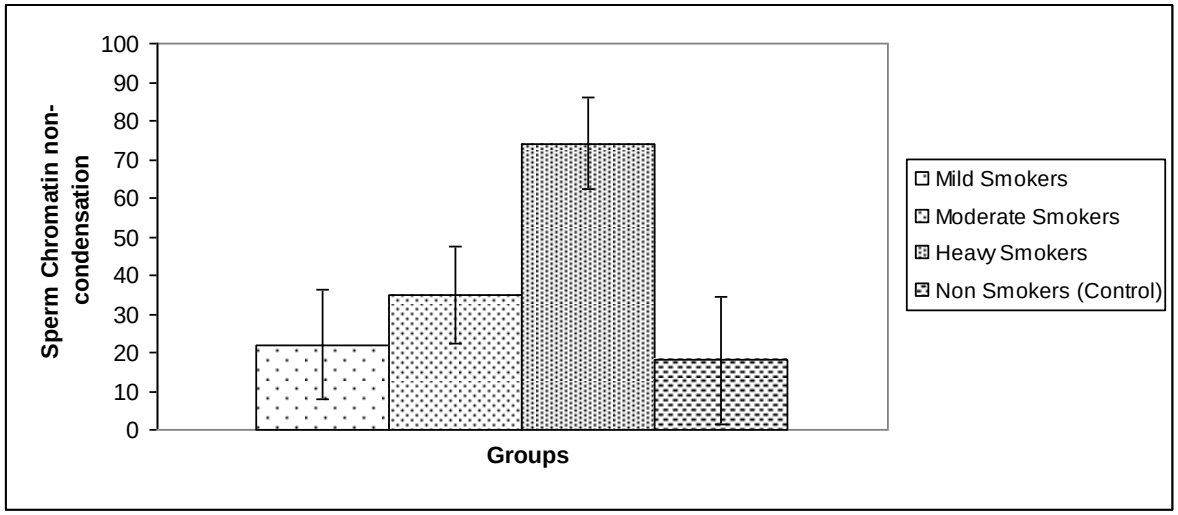

Figure 1: Measurement of sperm chromatin non-condensation in mild, moderate, heavy cigarette smokers compared with the age matched nonsmokers control group in Saudi young men. Values are Mean ± SD, (n = 100). *Note:* n = Total number of subjects examined.

Almost all the specimens of the smoker groups showed a consistently increased level of sperm chromatin non-condensation. In moderate smokers, this level was found to be significant ($p<0.005$), whereas in heavy smokers sperm chromatin non-condensation was found to be highly significant ($p<0.0005$) respectively than their respective controls.

However, this difference was found to be non-significant in the mild smoker group when compared with nonsmoker controls.

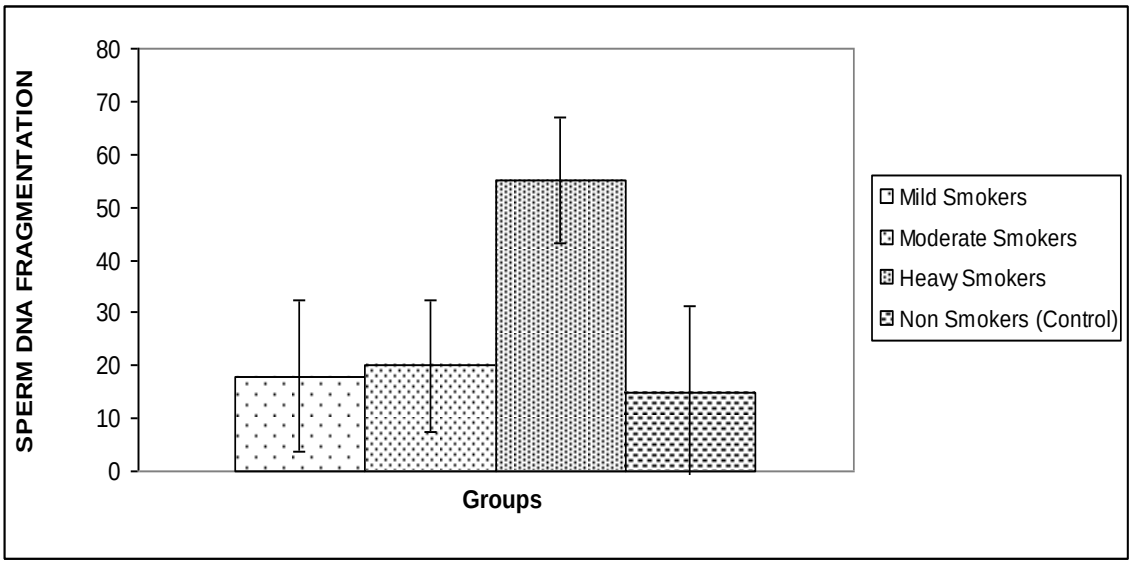

Figure 2: Measurement of sperm DNA fragmentation in mild, moderate, heavy cigarette smokers compared with the age matched nonsmokers control group in Saudi

young men. Values are Mean ± SD, (n = 100). *Note:* n = Total number of subjects examined.

The values of sperm DNA fragmentation were estimated in all types of smoker groups and their age matched nonsmoker control subjects and the results are enlisted in figure-2.

In all the experiments on smoker groups, the value of sperm DNA fragmentation was found to be significantly higher ($p<0.0005$) in heavy smokers from the specimens of their respective age matched control subjects. However a non-significant difference was observed between the values of mild smokers / moderate smokers and their respective control subjects.

The estimated DNA methylation level (ng / ul) of the smoker groups and their age matched non-smoker controls are presented in figure-3

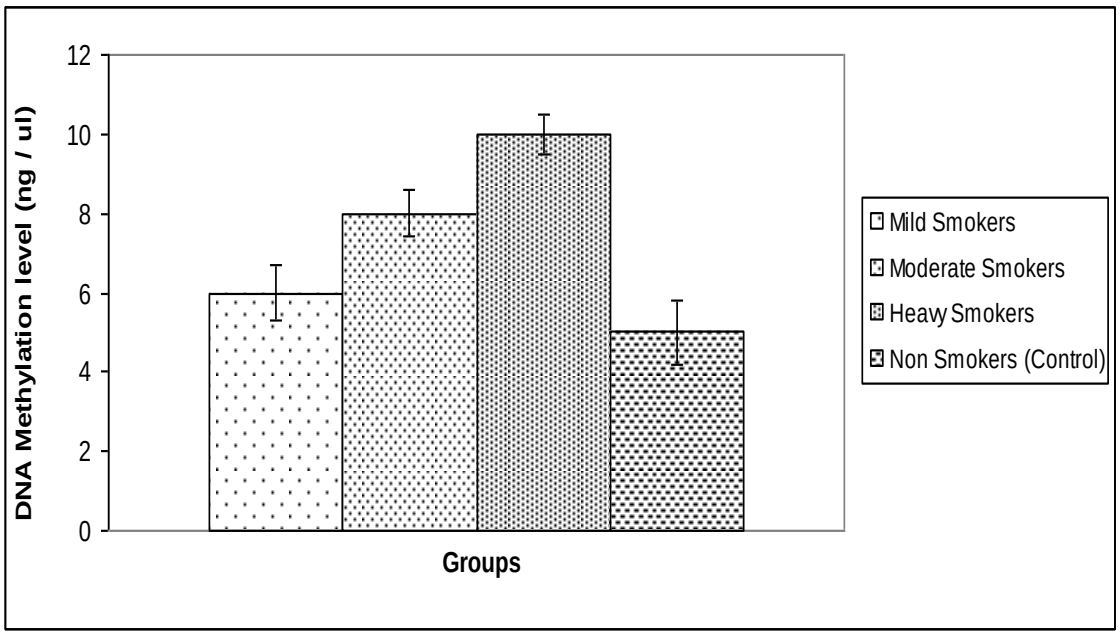

Figure 3: Measurement of DNA methylation level (ng / ul) in mild, moderate, heavy cigarette smokers compared with the age matched nonsmokers control group in Saudi young men. Values are Mean ± SD, (n = 100). *Note:* n = Total number of subjects examined.

Almost all the specimens of the smoker groups showed a consistently increased level of DNA methylation. In moderate smokers the level of DNA methylation was found to be significant ($p<0.005$), whereas in heavy smokers levels of DNA methylation were found to be highly significant ($p<0.0005$) respectively than their respective controls.

However, this difference was found to be non-significant in the mild smoker group when compared with nonsmoker controls.

Findings from this study underscore the fact that cigarette smoking has adverse reproductive outcomes for semen quality. Nicotine present in cigarettes could be responsible for this reduction, which affects the functioning of accessory sex glands (seminal vesicle, prostate and urethral glands), that control semen volume through their secretions[23].

Several studies have reported that the mutagenic components of cigarette smoke adversely and rapidly affect dividing cells, including germ cells in testis[24]. Polycyclic aromatic hydrocarbons and nicotine are present in cigarette smoke which can cause atrophy of seminiferous tubules, testis and reduce or block spermatogenesis (MacKenzie and Angeline)[25].

Semen abnormalities (oligospermia, asthenozoospermia and teratozoospermia) were present in this study (data not shown). More over semen abnormalities were more prevalent among heavy smokers compared to moderate and mild smokers. These abnormalities were dose-dependent and in agreement with prior studies[26].

In our studies, the percentage of abnormal sperm chromatin condensation, DNA fragmentation, and DNA methylation was found to be significantly higher in heavy smokers compared to moderate smokers ($p < .005$). These results indicate that heavy cigarette smoking negatively affects all conventional semen parameters in addition to sperm chromatin condensation and sperm viability.

Our results are thus supported by Abdelrazik et al.[27] , who found that abnormal forms of sperm especially amorphous types as well as micro head sperms, have higher DNA fragmentation rates. Similar results have been confirmed by Sheikh et al.[28], who confirmed higher DNA fragmentation rate in sperm samples having abnormal morphology. Recent studies further confirmed elevated degree of DNA fragmentation and immature chromatin especially in amorphous head sperms[29, 30].

It is now established that heavy smoking causes sperm DNA damage by apoptosis, which leads to DNA strand breakage, and a defective sperm maturation process due to excessive reactive oxygen species (ROS) production.

An increase in ROS as well as nitrogen production alters the entire process of spermatogenesis by producing various forms of modified DNA bases, thus causing extreme mutagenicity as well as carcinogenicity.

It is well established that smoking caused about 48% increase in leukocyte levels in sperm as compared to non-smokers[31]. As a result, the DNA fragmentation index significantly rises in smokers from 14.51% in non-smokers to 37.66% in smokers as observed in our studies too.

In addition, an increased ROS production causes oxidative stress, which on one hand affects the sperm nuclear DNA, and on the other hand alters the entire endocrine function and sperm mitochondrial respiratory activity, thus, leading to male infertility by changing the sperm quality and integrity. Nitrous oxide and decreased level of glutathione also result in the loss of sperm cell integrity[32]. Taken together, the results of the present study suggest a negative biological effect of cigarette smoking on sperm parameters and DNA fragmentation.

Our findings showed that heavy tobacco smoking causes severe deleterious effects on the sperm parameters, especially in the sperm head. These results are in conformity with previous finding[33], thus concluding that moderate to heavy smoking is proportional to deterioration in sperm quality.

Many studies have reported that quitting the smoking and a change in lifestyle like losing weight due to exercise and less eating, reducing caffeine intake, and minimum exposure to harmful toxins like phthalate improve fertility outcomes[34]. Since our study has some limitations, like we studied a relatively smaller number of cigarette smokers, the limitation of clinical trials and obtaining the cigarette smoking subjects from a questionnaire, further large scale investigations are recommended.

FOOT NOTE

This study is a part of on-going M.Sc. Research project, Department of Physiology, College of Medicine, Umm-Al- Qura University, Makkah, Saudi Arabia.

CONCLUSION

This study showed that moderate to heavy smoking significantly increased the sperm chromatin non-condensation and fragmentation as well as DNA methylation level. Higher incidence of chromatin packing abnormalities and DNA fragmentation in heavy smokers might have an adverse impact on the process of fertilization and embryo development, leading to an increase in abortion rates. In our results, an altered DNA methylation level in the sperm of the heavy smoker group also indicates a negative effect on semen quality which, may significantly affect men's fertility. This reduction in semen quality was always found to be associated in a dose-dependent manner. We conclude that cigarette smoking has a significant adverse reproductive outcome in younger aged Saudi men.

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