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Significant Effects of Smoking Habit on Male Fertility

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Background: Smoking is a common health problem worldwide. Many diseases and life threatening conditions have been linked to smoking habits, such as, lung cancer, oral cancer, bronchitis and several gastrointestinal disorders. Infertility link to smoking is still under intensive investigation.

Objective: To evaluate the effect of smoking on semen parameters of infertile Bahraini males.

Design: Open controlled trial.

Setting: Department of Molecular Medicine, CMMS, Princess Al-Jawhara Center for Molecular Medicine, Genetics and Inherited Diseases, Arabian Gulf University, Bahrain.

Method: Semen samples from 52 infertile patients were analyzed by conventional analysis methods, sperm chromatin dispersion test for sperm DNA integrity and colorimetric assay for total antioxidant capacity. Twenty-two (42.3%) were smokers of the study group.

Result: The data showed that smokers had more semen analysis abnormalities than the non-smokers. Smokers had more sperm with fragmented DNA than non-smokers (x^2=6.17; P<0.002).

Conclusion: Our study used conventional and molecular techniques to investigate male infertility in Bahraini patients and demonstrated that smoking is a contributing factor in the etiology of male infertility.

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The hope to have one's own offspring is an extremely strong human character. However, several couples fail to become parents because of fertility problems. Infertility is a growing medical and

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social problem in the world and approximately one of five couples has problems with conception[1]. Both male and female partners may be involved in the etiology of this problem. Many known diseases can disturb or even permanently impair male fertility; some are congenital, others are acquired. However, in several cases the exact cause is still unknown[2].

In the last few decades, there was growing concern about the possible threat of the environmental, chemical and physical agents to male fertility. Factors, such as, genital infection, smoking, and radiotherapy frequently disturb the biochemical events that occur during spermatogenesis[3]. In addition, many environmental agents can adversely affect the male sexual performance resulting in erectile dysfunction[4]. Therefore, social habits should be considered as a possible cause in any infertility case[1]. Cigarette smoking is an important environmental factor that adversely affects male fertility. It is one of the most extensively used, potentially hazardous social habits throughout the world. Cigarette smoke contains a number of mutagens and carcinogens, which could have adverse effects on male reproduction[5]. Several fertility workers have associated cigarette smoking with the alteration in semen analysis[6].

The aim of this study is to provide substantial scientific data on the possible relation of abnormal male fertility parameters with smoking habit.

METHOD

Semen samples were collected from infertile male patients attending Salmaniya Medical Complex (SMC). All samples were collected by masturbation into sterile containers after 3 to 4 days of sexual abstinence. After liquefaction, conventional analysis was performed according to World Health Organization guidelines[7]. The variables taken into account were volume of ejaculate (ml), round cells concentration ($\times 10^6$/ml), sperm concentration ($\times 10^6$/ml), forward motility (%) and morphology (% of normal forms). A leukocyte count ($\times 10^6$/ml) was carried out by using standard peroxidase test. The semen also assayed for sperm DNA condensation by sperm chromatin dispersion test (SCDT) and for seminal plasma total antioxidant capacity (TAC).

Sperm chromatin dispersion test (SCDT)

The method was performed according to Fernandez et al[8]. Low-melting point agarose in eppendorf tubes were placed in a water bath at 90°C then at 37°C, twenty-five µl sample was mixed with it. A slide with standard agarose was placed on a cold plate. Then 20 µl of the mixture was placed onto the cooled slide covered with a cover slip and left to solidify. Afterwards, the cover slip was removed, and the slide was immersed in an acid denaturizing solution and transferred to a neutralizing lysing solution. Finally, slides were washed and dehydrated in ethanol and air dried. For visualizing DNA, slides were stained with DAPI stain. By fluorescence microscopy, normal DNA showed bright bluish fluorescent halo, while abnormal DNA showed weak blue or even colorless.

Total antioxidant capacity assay of semen (TAC)

The samples were prepared according to Said et al (2003)[9]. Three hundred µl of the sample was centrifuged at 1337 rpm. The supernatant was recentrifuged at 1337 rpm, then frozen at -70°C. The colorimetric assay was performed according to Mahfous et al (2009)[10]. The frozen samples

were thawed at 37°C. TAC was measured using the Cayman's antioxidant assay kit. All samples were diluted 1:10 with the assay buffer. Ten µl of Trolox standard and samples were loaded onto the corresponding wells of 96-well plate. Then 10 µl of metmyoglobin and 150 µl of chromogen were added to the wells. The reaction was initiated by adding 40 µl of H_2O_2 as fast as possible. The plate was covered and incubated for 5 minutes on a shaker at room temperature. Absorbance was monitored at 750 nm using ELx800 Absorbance Microplate Reader.

Data analysis was performed using the SPSS 16. Data were expressed as percentage and mean ± SD. To find out the independent association between different seminal parameters and selected variables, logistic regression analysis was performed. Statistical significance was defined as $P<0.05$.

RESULT

The age range was 21-52 years; the mean age was 30.8±6.7 years. Forty-five (86.5%) patients presented with primary infertility while seven (13.5%) patients complained of secondary infertility. Twenty-two patients (42.3%) were smokers and 30 (57.7%) were non-smokers.

There was statistically significant alteration ($P<0.05$) in sperm count, vitality, motility, morphology and total antioxidant level, see table 1. Smokers' ejaculates had lower sperm concentration than those of non-smokers (16.09±1.86 versus 47.30±2.06). Likewise, the vitality of the spermatozoa was significantly lower in smokers compared to non-smokers (25.41±23.32 versus 67.90±22.16).

Table 1: Semen Characteristics and Quality of Smokers and Non-Smokers

Variable	Smoker	Non-smoker	X^2	df	P-value
Concentration (million/ml)	16.09 ± 1.86	47.30 ± 2.06	19.68	1	<0.05
Motility (% grade a)	19.86 ± 25.95	54.00 ± 20.99	18.07	1	<0.05
Vitality (% alive)	25.41 ± 23.32	67.90 ± 22.16	19.81	1	<0.05
Morphology (% normal form)	19.18 ± 7.53	57.50 ± 23.57	18.6	1	<0.05
Leukocyte (million/ml)	6.83 ± 4.70	1.2 ± 1.79	8.16	1	<0.05
TAC(mmol/l)	951.64 ± 21.91	1,362.53 ± 388.49	10.65	1	<0.05

In addition, the mean percentage of motile spermatozoa of smoker patients was much lower compared to non-smokers (19.86±25.95 versus 54.00±20.99; $P<0.05$). Moreover, the mean percentage of morphologically normal spermatozoa was significantly reduced in smokers than non-smokers (19.18±27.53 versus 57.50±23.57; $P<0.05$).

The level of TAC was dramatically lower in smokers than non-smokers (951.64±421.91 versus 1,362.53±388.49; $p<0.05$).

Seminal leukocytes number was found to be significantly higher ($P<0.005$) in smokers than non-smokers (3.41±3.09 versus 1.13±2.43).The study revealed a significant correlation between smoking and the DNA integrity; five dispersion patterns were clearly observed: (a) nuclei with

large DNA dispersion halos, (b) nuclei with medium-sized halos, (c) nuclei with very small-sized halos, (d) nuclei with no halo, (e) sperm cells without halos, degraded and weakly stained, see figure 1.

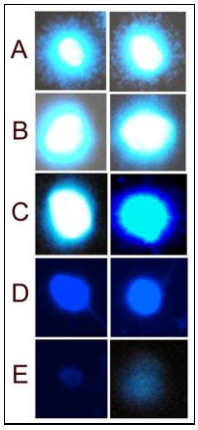

Figure 1: Nucleoids from Human Sperm Cells Obtained with SCD Test Stained with DAPI Staining for Fluorescence Microscopy. (A) Large Halo Nuclei, (B) Medium Sized Halo Nuclei, (C) Small Sized Halo Nuclei, (D) Sperm without Halo and (E) Weakly Stained Irregular Sperms

Any nuclei that do not morphologically correspond to sperms were separately scored. They could be big spermatids without tail or epithelial cells or leukocytes. According to the scoring system, the semen samples were either had normal or abnormal chromatin. Smokers had more sperm with fragmented DNA (abnormal chromatin condensation) than non-smoker, see table 2.

Table 2: **Chromatin Condensation in Smokers and Non-Smokers**

Chromatin Condensation	**Smokers**	**Non-smokers**
Abnormal	15	10
Normal	7	20

X^2=6.17 df =1 P-value<0.02

DISCUSSION

This study showed that smokers had low sperm density, low motility, low percentage of morphologically normal sperm and poor DNA integrity. The results suggest that smoking is a possible contributing factor in male infertility. Studies about smoking and semen quality reveal conflicting results[11]. Some studies had shown that the production and function of healthy normal spermatozoa can be affected by the number of cigarettes smoked per day and the level of nicotine present in the body fluids[1,5]. Other publications described the damaging effect of smoking on the function of accessory glands (prostate and seminal vesicles) and subsequent infertility[12]. In addition, smoking increases the production of free radicals (ROS), which have damaging effect on sperm[11-13].

Other studies have not found any significant association between smoking and male infertility[14,15]. In the present study, sperm concentration in the ejaculate of smokers was significantly lower than non-smokers; it is similar to the findings of Chen et al and Mehrannia[5,16]. The vitality of the spermatozoa was significantly low in smokers compared to non-smokers. Several publications have suggested a harmful effect of Cotinine on sperm membrane permeability and sperm membrane function[19]. Therefore, the vitality of spermatozoa may be reduced and may not have optimal ability to undergo capacitation and hyperactivation within the female reproductive tract[17,18]. Likewise, the mean percentage of motile spermatozoa of smokers was significantly lower compared to non-smokers; it is similar to the findings of Wang et al and Collodel et al[6,19]. Moreover, the mean percentage of morphologically spermatozoa was significantly reduced. Ozgur et al found a significant alteration in the morphology of sperm (head and tail abnormalities) with subsequent reduction in sperm function[20].

Seminal leukocytes number was found to be significantly higher in smokers' semen than non-smokers. Saleh et al found that semen samples of smoker had higher leukocyte content than non-smokers[21].

Although the etiology of sperm DNA fragmentation is still poorly understood, but the relation between smoking and sperm DNA damage in infertile smokers had been illustrated in several studies[22,23]. Cigarette smoking has mutagenic properties; other possible mechanism is by increasing leukocyte-induced oxidative stress on developing or mature sperms, which in turn increase DNA breaking strands[24].

The present study, applied the SCDT sperm DNA evaluation. The basis of this technique lies in the different response of sperm nuclei with fragmented DNA compared to those with intact DNA. The controlled denaturation of the DNA followed by the extraction of the nuclear proteins, gives rise to partially deproteinized nucleoids in which the DNA loops expand, forming halos of chromatin dispersion. Large and medium size halos represent normal chromatin, while small no halo or weakly stained sperm DNA are considered abnormal, see figure 1. This method is simple, reliable, reproducible, and without the need for complex instrumentation[25,26]. Yet, the heterogeneous staining of slides and the rapid fading were encountered in the present study. These technical problems were also reported in other studies[27]. According to this method, DNA integrity had shown significant difference between smokers and non-smokers. Smokers had more sperm DNA fragmentation than non-smokers. Evenson et al and Sepaniak et al found that smoker spermatozoa have significantly higher DNA fragmentation than non-smokers[28,29].

Low seminal TAC level is frequently linked to male infertility[12]. The relation between smoking and increase seminal ROS level was of considerable importance during the last years. This can be attributed in part to the smoking associated increase in seminal WBCs concentration in smokers' semen. In addition, semen of cigarette smokers contains a variety of ROS that have harmful effect on sperm[11]. The main ROS which create this problem are superoxide anion (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH-). Moreover, cigarette smoking decrease the TAC in semen making the developing or mature sperm more vulnerable to the toxic effect of ROS[30].

The present study measured the seminal TAC level by using colorimetric assay. Said et al had recommended this technique for seminal TAC measurements and concluded that this method is as accurate as enhanced chemiluminescent assay; it is simple, rapid, cheap and can be applied in epidemiological study[9]. The result of the present study demonstrated a significant correlation between smoking habit and seminal TAC. The semen of infertile smokers had lower amount of TAC than non-smokers. This result is similar to previous studies where smokers demonstrated lower seminal TAC level than non-smokers[11,30].

CONCLUSION

This study used conventional and molecular techniques to investigate male infertility in Bahraini patients and demonstrated that smoking is a contributing factor in the etiology of male infertility because smokers had low sperm density, low motility, low percentage of morphologically normal spermatozoa and poor DNA integrity.

Author contribution: All authors share equal effort contribution towards (1) substantial contributions to conception and design, acquisition, analysis and interpretation of data; (2) drafting the article and revising it critically for important intellectual content; and (3) final approval of the manuscript version to be published. Yes

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