



RESEARCH PAPER

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Chromatin integrity in human ejaculate sperm of smokers and non-smokers patients and its relationship to seminal oxidative stress parameters

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Key words: Human sperm, chromatin condensation, DNA fragmentation, oxidative/smoking biomarkers.

<http://dx.doi.org/10.12692/ijb/4.9.126-141>

Article published on May 07, 2014

Abstract

Accumulative indications propose that sperm DNA damage has a negative impact on male fertility. Therefore Cigarette smoking may be a possible source of chemicals capable of causing sperm DNA damage. The purpose of the present study was to evaluate the consequences of cigarette smoking on the sperm chromatin integrity of infertile patients undergoing IVF/ICSI therapy. Ejaculates from 795 patients of couples consulting for infertility were divided into smokers (n=340) and non-smokers (n=455). Sperm chromatin integrity was evaluated using Chromomycin A₃ (CMA₃) and Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) Assay methods. The smoking metabolite (Cotinine) was evaluated in seminal plasma by Enzyme Linked Immunosorbant Assay (ELISA) technique and Malondialdehyde (MDA) levels determined by chemical reactions. Results showed that, both parameters CMA₃ positive and TUNEL were significantly higher ($p < 0.001$) in smokers ($33.47 \pm 11.37\%$, $20.06 \pm 7.78\%$) than non-smokers ($26.12 \pm 9.36\%$, and 12.68 ± 5.43 respectively). Besides, both parameters were negatively associated with the sperm parameters. Moreover, the MDA (μM), and cotinine (ng/ml) levels were significantly higher ($p < 0.001$) in smokers (7.97 ± 1.56 , 65.56 ± 35.72) than that of non-smokers (5.45 ± 1.50 , 2.24 ± 2.14 , respectively). Likewise, these oxidative stress biomarkers were inversely ($p < 0.001$) correlated with the sperm parameters. In conclusion, these outcomes propose that smoking may a risk factor of oxidative stress elevation and therefore deteriorate semen quality especially sperm nuclear integrity that disrupting sperm functions.

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Introduction

During spermatogenesis process protamines replaces histones in a process involving rearrangement and remodeling of DNA, ending with highly organized chromatin structure of human sperm (Ward and Coffey, 1989). This highly compact chromatin structure protects the integrity of the paternal genome during transportation of sperm in the male and female reproductive tracts (Brewer *et al.*, 2002). In addition, protamines may have a role in silencing of the paternal genome and the imprinting pattern of the gamete (Aoki and Carrell, 2003). Abnormalities in sperm chromatin packaging (less compact chromatin) due to protamine deficiency are more vulnerable to DNA damage that may lead to poor fertility of human sperm (Carrell and Liu, 2001, Aoki, *et al.*, 2005, Aoki, *et al.*, 2005). However, fertile men ejaculates may contain some sperms with detectable levels of DNA damage (Zini, *et al.*, 2001).

Furthermore, significant correlations were found between protamine deficiency, poor fertilizing ability of sperm and poor quality of embryos (Carrell and Liu, 2001; Aoki, *et al.*, 2005). Also, infertile men with protamine deficiency display lower sperm counts, motility, and penetration ability and sperm with higher abnormal morphology (Aoki, *et al.*, 2006). Chromomycin A₃ (CMA₃) staining method was used to evaluate sperm protamines. Many studies reported significant association between CMA₃ staining and the protamine1/protamine2 ratio assessed by electrophoresis (Zubkova, *et al.*, 2005, Hammadeh, *et al.*, 2010).

Cigarette contains many hazardous elements that have been founding in seminal plasma of smokers, including nicotine and some of its' metabolites; cotinine, trans-3'hydroxycotinine (Pichini, *et al.*, 1994), mutagenic nitrosamines compounds (Hoffmann, *et al.*, 1994), and (Notarianni, *et al.*, 1996). Studies revealed the presence of about 4000 chemicals in cigarettes (Kumosani, *et al.*, 2008).

It is therefore possible that seminal plasma of smokers contains chemicals capable of causing sperm

DNA damage *in situ*. In addition, polycyclic aromatic hydrocarbons, one of the compounds of cigarette smoke, has been found to reduce fertility in both male and female mice (MacKenzie and Angevine, 1981), have a negative impact on Sertoli cell function (Raychoudhury and Kubinski, 2003), and impair the production of testosterone from Leydig cell (Inyang, *et al.*, 2003). It was shown that late stages of spermatogenesis are testosterone-dependent (De Gendt, *et al.*, 2004, Said, *et al.*, 2004), and sperm production might, therefore, be impaired due to lower intra testicular testosterone levels. Cigarette smoking also was found to have an anti-androgenic effect (Kupeli, *et al.*, 1997).

An inverse impact of smoking on human sperm parameters, associated with cigarettes smoking duration. Many studies verified lower semen volume, sperm count, motility and viability in smokers compared to non-smokers (Hammadeh, *et al.*, 2010). Adding to that, smokers ejaculates contains high percentage of abnormal morphologically sperm, and sperm with cytoplasmic droplets and high levels of seminal leukocytes (Colagar, *et al.*, 2007, Ramlau-Hansen, *et al.*, 2007, Hassan, *et al.*, 2009, Mostafa, 2010).

Recently,(Joo, *et al.*, 2012) reported that heavy smoking was linked with decreased sperm counts and alcohol drinking was linked with high rates of morphologically abnormal sperm. Another study conducted by (Nadeem, *et al.*, 2012) concluded that cigarette smoking negatively affects male fertility by decreasing the sperm motility and levels of normal sperm shape. These abnormalities are also correlated to the number of cigarettes smoked per day. (Zhang, *et al.*, 2013) findings suggested that smoking cause a significant decline in semen quality and higher levels of leukocytes, thus smoking may affects the fertilization efficiency. In another study it was concluded that smoking caused a negative impact on sperm progressive motility, HOS test, seminal Zn and positive impact on sperm DNA fragmentation, semen ROS level (Taha, *et al.*, 2013).

The imbalance between ROS production and antioxidant capacity place the sperm of smokers under additional risk of oxidative stress. (Pasqualotto, *et al.*, 2008) observed that cigarette smoking may impair sperm motility and decrease the antioxidant activity (negative correlation with superoxide dismutase) in the seminal plasma.

Besides, accumulating evidence now suggesting that reactive oxygen species (ROS) mediated damage to sperm is significantly contributing pathology in 30-80% of cases (Iwasaki and Gagnon, 1992, Shekarriz, *et al.*, 1995, Agarwal, *et al.*, 2006). Also, high levels of ROS are toxic to sperm quality and function (Saleh, *et al.*, 2002, Hammadeh, *et al.*, 2006).

Many studies showed that oxidative stress appears to be the major cause of DNA damage in the male germ cells (Aitken, *et al.*, 2003, Aitken, *et al.*, 2003, Aitken and Sawyer, 2003, Saleh, *et al.*, 2003).

Similarly, (Sepaniak, *et al.*, 2006) showed that smoker's sperm have a significantly higher DNA fragmentation than non-smokers. (Elshal, *et al.*, 2009) also found that cigarette smoking of idiopathic infertile men was significantly associated with DNA fragmentation index (DFI %), high DNA stain ability (HDS %) as indicator of immature sperm and lipid peroxidation (Malondialdehyde, MDA) of sperm plasma membrane and decreased superoxide dismutase (SOD) levels. These findings suggested that cigarette smoking may have deleterious effects on sperm nuclear DNA.

The particular pathophysiology through which cigarette smoking deteriorates sperm is unclear. The suggested mechanisms proposed a negative effect of cigarette smoke on sertoli and Leydig cell and testicular microcirculation functions (Collin, *et al.*, 1995). Also, congenital abnormalities, childhood cancer, and sperm mutagenicity of smoking is proposed (Marinelli, *et al.*, 2004). As many reports suggested that DNA damage may be one of the factors that might affect male fertility and smoking is widely spread and may be a risk factor of elevation oxidative

stress which may inversely affect the integrity of sperm DNA. Therefore, the purposes of the present study were done to determine the relationship between smoking and the levels of oxidative stress biomarkers in seminal plasma and their effect on chromatin integrity, DNA strand breaks, and other sperm parameters.

Materials and methods

In order to study the chromatin status of human sperm, ejaculates from male partners (n=795 patients) of couples consulting for infertility at department of obstetrics and gynecology, university of Saarland/Homburg/Saar were collected. Those patients were divided into two groups: (i) smokers (n=455) and (ii) non-smokers (n=340). All chemicals used in this study were bought from Sigma, Germany unless other sources mentioned.

Sperm collection and preparation

Semen samples were collected by masturbation after 3-5 days of sexual abstinence in a sterile container, then allowed to liquefy for 30 minute (min) at 37 °C and examined within 2 hours after collection. A standard semen analysis was performed according to WHO criteria (WHO, 2010). Chromatin condensation was evaluated by Chromomycine test (CMA₃-test) and DNA fragmentation was evaluated by (Tunnel) assay. Liquefied semen samples were prepared by discontinuous PureSperm gradient (Nidacon International AB, Sweden). The supernatant (seminal plasma) was immediately separated and examined under microscope in order to rule out the presence of sperm. Thereafter, seminal plasma was aliquot into storage ampoules and stored at -80°C until used.

Sperm Vitality Assay (Eosin Test)

Test was carried out taken 5 µl of liquefied seminal fluid was mixed with 5 µl of 0.5% aqueous yellowish eosin Y solution and mixed on a glass slide. The mixture covered with a cover slide, then evaluated after 1-2 min by distinguishing between the dead sperm (Red stained) and the live sperm (white). 100 sperm from each slide were evaluated and percentage of vital calculated.

Sperm Membrane Integrity Assay (Hypo-Osmotic Test; HOS)

HOS test performed by taken 0.1 ml of fresh ejaculate and mixed with 1.0 ml of the hypo-osmotic solution (equal parts of 150 mOsmol fructose and 150 mOsmol sodium citrate solution) then mixture incubated for 30–60 min at 37°C. 10 µl of the mixture was taken and spread on a slide and tested under phase contrast microscope. A total of 200 sperms were examined per slide. Then calculate the percentage of sperm that showed typical tail abnormalities (good sperms).

Sperm Morphology Assay

Sperm morphology was evaluated according to WHO guideline (WHO, 2010). Smears were prepared by spreading 10 µl of semen on a glass slide and stained using Papnucleou method. A total of 100 sperm from each slide were evaluated under oil immersion at a magnification of 1000 X using bright field illumination. At least 10 high power fields from various areas of the slide were evaluated.

Chromatin Condensation Assay: Chromomycin A₃, CMA₃-test

Chromomycin A₃ (CMA₃) staining performed as following: semen samples were washed twice in phosphate-buffered saline (PBS, Ca²⁺ and Mg²⁺-free), (pH = 7.4). Thin smears of washed sperms were spread and fixed with ethanol:acetone (1:1) solution, then dried for 30 minutes at room temperature, then each slide was treated with 100 µL of CMA₃ solution for 20 minutes [0.25 mg/mL in 0.15 M PBS, pH = 7.4, containing 10 mM MgCl₂] at 25°C in the dark. Slides then washed in PBS and air dried. Microscopic analysis of the slides was performed using a fluorescent microscope (Zeiss Photomicroscope III) with suitable filters (excitation:460–495 nm, emission: 510 nm). For each slide 200 sperm were assessed to calculate the percentage of sperms with bad chromatin condensation (CMA₃ positive). CMA₃ staining was evaluated by differentiating between bright yellow-green staining sperms (CMA₃-positive, bad sperm) and dull yellow-green staining sperms (CMA₃-negative, good sperms).

DNA Fragmentation Assay (Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling, TUNEL Test)

DNA damage was assessed using the Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. The TUNEL assay was performed using the In-Situ Cell Death Detection Kit: Fluorescein following the manufacturer's guidelines (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, smears from semen samples were spread on microscopic slides, air dried then fixed in 4% paraformaldehyde in PBS, pH 7.4 and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, pH 6.0. Fragmented DNA was detected by TUNEL kit following the manufacturer's guidelines. For evaluation, a total of 200 sperm were analyzed on each slides, by distinguishing sperm stained bright green (TUNEL positive, Fragmented DNA) from those stained dull green (TUNEL negative, with good DNA). A Zeiss Photomicroscope III used for the Fluorochrome evaluation via a combination of exciter dichromic barrier filter of BP 436/10: FT 580: LP 470. A negative control was performed for each sample by using fluorescein-isothiocyanate-labelled dUTP without enzyme.

Measurement of Malondialdehyde (MDA)

Lipid peroxidation level of semen samples (seminal plasma,) was assessed by measuring the malondialdehyde (MDA) level using thiobarbituric acid (TBA) method: 250 µl of seminal plasma was added to 2.0 ml of the Thiobarbituric acid - Trichloroacetic acid (TBA–TCA) Extraction reagent (15% (w/v) TCA (Trichloroacetic acid), 0.375% (w/v) TBA (Thiobarbituric acid) in 0.25 N HCl (Hydrochloric acid)). The mixture then boiled in water bath at 95 °C for 30 min. After cooling samples were centrifuged at 3000 rpm for 10 min at RT and absorbance was measured at 535 nm against blank. Concentration of MDA (µM) then calculated from the equation of the plotted standard curve.

Cotinine measurement

The levels of Cotinine in seminal plasma were measured using the Calbiotec Cotinine Direct ELISA

Kit (Calbiotech, CA, USA) which is design to detect the presence of Cotinine in serum and urine and other fluids, as previously described by Hammadeh *et al.*, (2008). Assay procedures were applied according to the manufacturer's guidelines. Briefly: standards, controls and seminal plasma samples were pipette into selected well in duplicate, then Enzyme Conjugate was added to each well and plates were incubated for 60 min in dark at room temperature. After washing the wells with distilled water the substrate reagent was added to each well then after 30 min incubation at room temperature in the dark, stop solution was added and absorbance was read on ELISA reader at 450nm. The concentration of cotinine (ng/ml) was calculated against the standard curve generated from the standards applied in the kit in the same plate. Samples with concentrations higher than 100 ng/ml were diluted and measured once more.

Statistical Analysis

Data were expressed as mean \pm SD and range. The relationships between conventional sperm parameters, DNA fragmentation, chromatin condensation and oxidative stress markers MDA and smoking marker Cotinine were analyzed using nonparametric methods. Statistical analysis was done using Sperman's correlation test and Mann-Whitney *U* test for examining differences between samples from smoking and non-smoking patients, where a probability value of $p < 0.050$ was considered significant and $p < 0.010$ was considered highly significant. The statistical analyses were carried out using the SPSS 19 for Windows Software Package (SPSS Inc., Chicago, IL, USA).

Results

The semen sample included in the present study (n=795) were divided into two groups: 355 non-smokers (mean age 36.52 ± 7.38) and 440 smokers (mean age 36.69 ± 7.48). The age of patients was not significantly ($p > 0.05$) different in both groups.

Table 1. Sperm and seminal plasma parameters of all volunteers (non-smokers and smokers).

Parameters	All (N=795)	Non-Smokers (N=355)	Smokers (N=440)	p-value
Age (year)	36.61 \pm 7.42	36.52 \pm 7.38	36.69 \pm 7.48	0.850
Volume (ml)	2.92 \pm 1.58	3.04 \pm 1.67	2.82 \pm 1.49	0.131
Count (mill/ml)	60.67 \pm 35.85	66.57 \pm 35.48	55.77 \pm 35.51	0.0001
Motility (% motile)	34.33 \pm 20.16	38.55 \pm 20.06	31.06 \pm 19.62	0.0001
Morphology ((%)Normal)	27.03 \pm 18.11	29.65 \pm 18.57	24.92 \pm 17.53	0.0001
Sperm vitality (Eosin) (%)	37.89 \pm 18.48	39.67 \pm 19.52	36.57 \pm 17.43	0.0170
Membrane integrity (HOS) (%)	57.44 \pm 20.40	59.38 \pm 20.57	55.72 \pm 20.09	0.0040
Non Condensed Chromatin (positive CM A3) (%)	30.22 \pm 11.19	26.12 \pm 9.36	33.47 \pm 11.37	0.0001
DNA Fragmentation (TUNEL) (%)	16.57 \pm 7.74	12.68 \pm 5.43	20.06 \pm 7.78	0.0001
Malondialdehyde (MDA) (μ M)	6.85 \pm 1.99	5.45 \pm 1.50	7.97 \pm 1.56	0.0001
Cotinine (ng/ml)	37.38 \pm 41.44	2.24 \pm 2.14	65.56 \pm 35.72	0.0001

Sperm parameters

Standard semen analysis was carried for all samples following the guidelines of WHO. The levels of morphologically normal sperm, motility, and count were significantly lower ($p < 0.001$) in smokers (24.92 \pm 17.53%, 31.06 \pm 19.62%, 55.77 \pm 35.51%) in comparison to that of non-smokers (29.65 \pm 18.57%,

38.55 \pm 20.06%, 66.57 \pm 35.48% respectively). In addition, A significant ($p < 0.050$) differences were established between the smokers versus non-smokers groups for the sperm vitality (36.57 \pm 17.43% vs. 39.67 \pm 19.52%) and membrane integrity (55.72 \pm 20.09 vs. 59.38 \pm 20.57). the volume (ml) of the semen fluid

was non-significant ($p > 0.050$) lower in smokers in comparison to non-smokers (Table 1).

In addition, significant ($p < 0.001$) associations were found between the number of sperm ($10^6/\text{ml}$) with sperm motility (%) ($r = 0.340$), morphology (%) ($r = 0.303$), vitality (%) ($r = 0.230$), and ($r = 0.126$). Also, motility of the sperms was correlated positively high with normal morphology ($r = 0.241$, $p < 0.001$), with vitality (%) ($r = 0.397$, $p < 0.001$) and membrane

integrity ($r = 0.273$, $p < 0.001$). Similarly, morphologically normal sperm was highly and positively ($p < 0.001$) correlated with sperm vitality ($r = 0.184$) and membrane integrity ($r = 0.2167$). Same result was found between sperm vitality and membrane integrity ($r = 0.441$, $p < 0.001$). In addition, semen volume (ml) was significantly ($p < 0.050$) associated with sperm motility, morphology, vitality and membrane integrity (Table 2).

Table 2. Correlations of sperm and seminal plasma parameters of patient samples (n =795).

		Age (year)	Volume (ml)	Count (mill/ml)	Motility (% motile)	Morphology (%)	Sperm vitality (Eosin) (%)	Membrane integrity (HOS,%)	Non Condensed Chromatin (positive CM A ₃) (%)	DNA Fragmentation (TUNEL) (%)	Malondialdehyde (MDA) (µM)	Cotinine (ng/ml)
Age (year)	R	1.000	-.163**	-.047	-.145**	-.155**	-.124**	-.043	.067	.057	.001	.000
	P	.	.000	.182	.000	.000	.000	.230	.058	.111	.985	.993
Volume (ml)	R	-.163**	1.000	.051	.095**	.092**	.078*	.076*	-.038	-.045	-.029	-.036
	P	.000	.	.147	.007	.010	.027	.033	.289	.201	.408	.304
Count (mill/ml)	R	-.047	.051	1.000	.340**	.303**	.230**	.126**	-.202**	-.169**	-.16**	-.171**
	P	.182	.147	.	.000	.000	.000	.000	.000	.000	.000	.000
Motility (% motile)	R	-.145**	.095**	.340**	1.000	.241**	.397**	.273**	-.226**	-.116**	-.141**	-.214**
	P	.000	.007	.000	.	.000	.000	.000	.000	.001	.000	.000
Morphology (%)	R	-.155**	.092**	.303**	.241**	1.000	.184**	.167**	-.155**	-.187**	-.191**	-.171**
	P	.000	.010	.000	.000	.	.000	.000	.000	.000	.000	.000
Sperm vitality (Eosin) (%)	R	-.124**	.078*	.230**	.397**	.184**	1.000	.411**	-.277**	-.074*	-.173**	-.145**
	P	.000	.027	.000	.000	.000	.	.000	.000	.038	.000	.000
Membrane integrity (HOS, %)	R	-.043	.076*	.126**	.273**	.167**	.411**	1.000	-.231**	-.037	-.199**	-.172**
	P	.230	.033	.000	.000	.000	.000	.	.000	.299	.000	.000
Non Condensed Chromatin (positive CM A ₃) (%)	R	.067	-.038	-.202**	-.226**	-.155**	-.277**	-.231**	1.000	.298**	.323**	.397**
	P	.058	.289	.000	.000	.000	.000	.	.000	.000	.000	.000
DNA Fragmentation (TUNEL) (%)	R	.057	-.045	-.169**	-.116**	-.187**	-.074*	-.037	.298**	1.000	.321**	.442**
	P	.111	.201	.000	.001	.000	.038	.299	.000	.	.000	.000
MDA (µM)	R	.001	-.029	-.165**	-.141**	-.191**	-.173**	-.199**	.323**	.321**	1.000	.648**
	P	.985	.408	.000	.000	.000	.000	.000	.000	.000	.	.000
Cotinine (ng/ml)	R	.000	-.036	-.171**	-.214**	-.171**	-.145**	-.172**	.397**	.442**	.648**	1.000
	P	.993	.304	.000	.000	.000	.000	.000	.000	.000	.000	.

Chromatin condensation

Sperm samples were assessed after staining with CMA₃. The mean number of non-condensed chromatin (CMA₃ % positive) was significantly higher in smokers than that of non-smokers ($33.47 \pm 11.37\%$ vs. $26.12 \pm 9.36\%$, $p < 0.001$) (Table 1). Moreover, in table 2; the mean value of chromatin condensation

(CMA₃ positive %) showed significantly high ($p < 0.001$) negative correlations with sperm's count (mill/ml) ($r = -0.202$), motility (%) ($r = -0.226$), normal morphology (%) ($r = -0.155$, $p < 0.010$), vitality (%) ($r = -0.277$), and membrane integrity (%) ($r = -0.231$). In contrast, chromatin condensation (CMA₃ %) highly and positively ($p < 0.001$) associated with DNA

integrity (TUNEL %) ($r=0.298$, Figure 1).

Non-smokers sperms showed higher chromatin condensation than smokers that reflects an adverse effect of smoking and oxidative stress on sperm chromatin condensation.

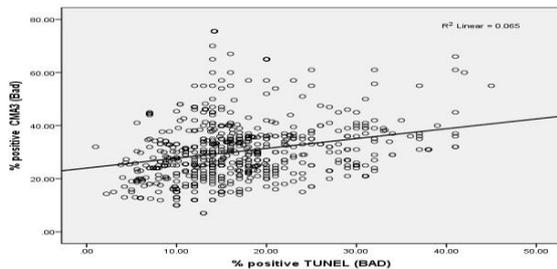
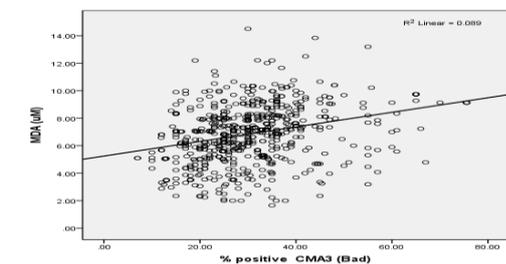
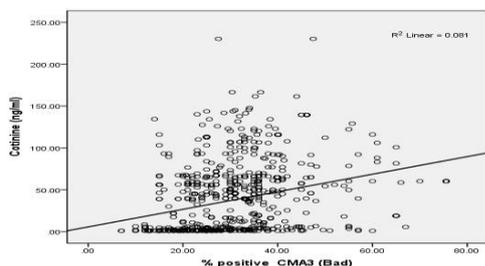


Fig. 1. Scatter plot of correlation between sperm chromatin condensation (CMA₃, %) and sperm DNA fragmentation (TUNEL, %) of patients. A significant positive correlation was found ($r=0.298$, $p<0.001$).

In addition, chromatin condensation (CMA₃ positive %) was positively and significantly correlated ($p<0.001$) with the levels of oxidative stress biomarkers in seminal plasma Malondialdehyde (MDA, μM) ($r=0.323$), and smoking biomarker cotinine (ng/ml) ($r=0.397$) (Table 2, Figure 2).



2A

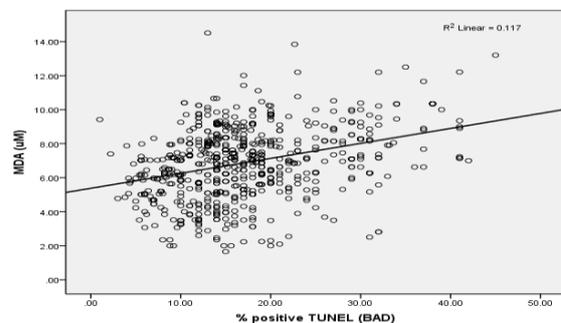


2B

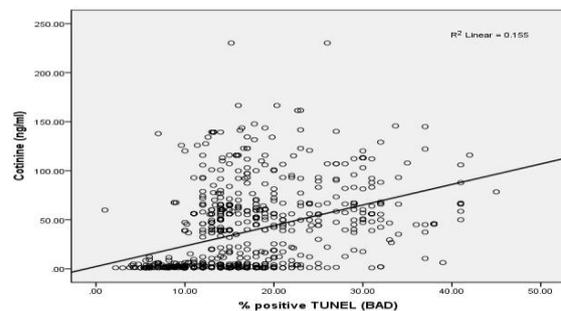
Fig. 2 (A-B). Scatter plot of correlation between sperm chromatin condensation (CMA₃, %) with concentrations of (2A) MDA (μM) and (2-B) Cotinine (ng/ml) in samples of both smokers and non-smokers. Significant positive correlation were found ($r=0.323$, $r=0.397$, $p<0.001$, respectively).

DNA fragmentation

Sperm DNA fragmentation was evaluated using TUNEL assay. The average number of DNA fragmentation showed significant ($p<0.001$) difference between smokers ($20.06\pm 7.78\%$) and non-smokers ($12.68\pm 5.43\%$) (Table1). Besides, significant negative correlations were detected between DNA fragmentation (%) and sperm's concentration (%) ($r=-0.169$, $p<0.0010$), motility (%) ($r=-0.116$, $p=0.001$), morphologically normal sperm (%) ($r=-0.187$, $p<0.001$, and vitality (%) ($r=-0.074$, $p=0.038$), (table 2). The above results reveal that DNA integrity and sperm parameters were higher in samples of non-smokers compared to smokers, which clarifies inverse effect of smoking on sperms.



3A



3B

Fig. 3 (A-B). Scatter plot of correlation between sperm DNA fragmentation (TUNEL, %) with concentrations of (2A) MDA (μM) and (2-B) Cotinine (ng/ml) in samples of both smokers and non-smokers. Significant positive correlation were found ($r=0.321$, $r=0.442$, $p<0.001$, respectively).

Sperm DNA fragmentation (%) also showed significant positive correlations ($p<0.001$) with the rates of oxidative stress biomarkers MDA and smoking biomarker cotinine (ng/ml) (μM), ($r=0.321$,

$r=442$, respectively) (Table 2, Figure 3). These findings showed the negative effect of oxidative stress and smoking on DNA integrity.

Oxidative stress biomarkers

The concentrations of oxidative stress biomarkers MDA and cotinine were measured in seminal plasma of smokers; MDA ($7.97 \pm 1.56 \mu\text{M}$), and cotinine ($65.56 \pm 35.72 \text{ ng/ml}$) and found to be significantly higher ($p < 0.001$) than that detected in non-smokers group (5.45 ± 1.50 and 2.24 ± 2.14 , respectively) (Table 1). Furthermore, rates of oxidative stress biomarkers (MDA, Cotinine) revealed high ($p < 0.001$) inverse correlations with sperm parameters (count, motility, morphology, vitality, and membrane integrity) (Table 2). Where, significant positive correlations ($p < 0.0010$) were found between levels of cotinine and MDA ($r=0.648$) (Figure 4). These findings designate higher oxidative stress in semen samples of smokers compared to non-smokers.

Discussion

The findings of the current study illustrated a significant decline in sperm's count, motility, vitality, membrane integrity, and normal morphology in smokers when likened with non-smokers, while non-significant decline found in semen volume. In addition, significant higher proportions of DNA fragmentation and poor chromatin condensation (protamine deficiency) and concentrations of oxidative stress biomarkers (MDA and Cotinine) were seen. The current results revealed that sperm DNA from smokers is more sensitive to damage and contains more DNA strand breaks than that from non-smokers (Table 1).

The levels of DNA fragmentation and non-condensed chromatin showed a statistically significant ($p < 0.001$) elevation in smoker's sperms compared to that in non-smokers and associated positively in both groups (Figure 1). Studies conducted by (Fraga, *et al.*, 1996) and (Shen, *et al.*, 1999) reported a high oxidative damage to sperm DNA and reduction in the seminal plasma antioxidant levels in cigarette smokers.

In addition, many studies had indicated a significant correlation between DNA damage and high levels of oxidative stress in infertile patients (Sun, *et al.*, 1997, Twigg, *et al.*, 1998, Twigg, *et al.*, 1998, Barroso, *et al.*, 2000, Aitken and Baker, 2004). Furthermore, (Potts, *et al.*, 1999) recorded a negative effect smoking on sperm DNA, as evidenced from the increased level of 8-hydroxydeoxyguanosine (a marker of DNA damage). Moreover, the significance effect of oxidative stress on sperm DNA integrity was supported by (Loft, *et al.*, 2003) who reported that pregnancy occurring in a single menstrual cycle was inversely associated with the level of 8-hydroxy-2'-deoxyguanosine. It has, also, been demonstrated that the levels of sperm DNA fragmentation were significantly higher among infertile men when compared to fertile sperm donors (Irvine, *et al.*, 2000). Besides, Smokers' sperm have a significantly higher DNA fragmentation than those of non-smokers (Sepaniak, *et al.*, 2006).

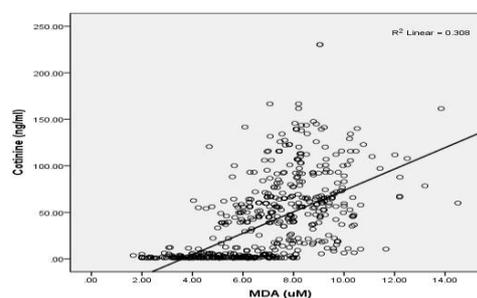


Fig. 4. Scatter plot of correlation between Cotinine (ng/ml) and Malondialdehyde (MDA) (μM) concentrations in seminal plasma of smokers and non-smokers. A significant positive correlation was illustrated ($r=0.648$, $p < 0.001$).

Nevertheless, DNA strand breaks between the smokers and non-smokers groups have many reasons, including incomplete replacement of histones by protamines, abnormal ratios of protamine 1 to protamine 2, high levels of non-oxidized SH groups in protamine molecules, or the occurrence of DNA breaks (de Yebra, *et al.*, 1993, Bench, *et al.*, 1996). A study by (Erenpreiss, *et al.*, 2006) illustrated a defect in spermatid protamination and disulphide bridge formation due to inadequate oxidation of thiol-groups resulted in sperm chromatin packaging; making

sperm cells more susceptible to ROS induced DNA fragmentation. Also, it should be emphasized that cigarette smoke was associated with high frequency of aneuploidy in sperm (Twigg, *et al.*, 1998, Twigg, *et al.*, 1998).

In addition, in this study, statistically significant negative correlations between semen parameters and chromatin condensation as well as DNA fragmentation that analyzed in smokers and nonsmokers group we reported (Table 2). Other study by (Tarozzi, *et al.*, 2009) showed a significant negative correlations ($p < 0.050$) between CMA₃ positivity and sperm concentration, motility, and morphology. (Lopes, *et al.*, 1998) showed that increase in DNA fragmentation detected by using TUNEL-assay was negatively correlated with sperm motility and morphology. Previous study by (Zini, *et al.*, 2001) confirmed the negative relation between motility, morphology and DNA fragmentation with the same technique as well as with the SCSA. Recent study conducted by (Taha, *et al.*, 2013) demonstrated a correlation between high levels of ROS and DNA fragmentation and sperm parameters in smokers. Whereas, some studies did not find correlations between sperm DNA damage and conventional sperm parameters (Saleh, *et al.*, 2002, Henkel, *et al.*, 2004, Sepaniak, *et al.*, 2006). Furthermore, the present data are in agreement with results of other previous studies analysing the relationship between abnormal protamination and conventional sperm parameters and between under-protamination and sperm DNA fragmentation (Carrell and Liu, 2001, Aoki, *et al.*, 2005, Aoki, *et al.*, 2005, Borini, *et al.*, 2006, Torregrosa, *et al.*, 2006, Tarozzi, *et al.*, 2009).

Normal sperm function requires a balance between the ROS produced during sperm maturation process in the epididymis and the antioxidants levels found in the secretions of the genital male tract, but several forms of sperm DNA damage are caused by high ROS levels, for example, chromatin cross-linking, chromosome deletion, DNA strand breaks, and base oxidation (Agarwal, *et al.*, 2003). Moreover, induction of apoptosis through cytochrome c and

caspases 3 and 9, which resulted in high frequency of single- and double-stranded DNA breaks (Agarwal and Allamaneni, 2004, Said, *et al.*, 2004) may be mediated by ROS. Therefore, in the circumstance of male infertility, seminal oxidative stress, sperm DNA damage, and apoptosis are interlinked and establish a unified pathogenic molecular mechanism (Agarwal, *et al.*, 2003, Agarwal, *et al.*, 2005).

Furthermore, in the present study, the levels of oxidative stress and smoking biomarkers in seminal plasma showed higher levels of MDA, and cotinine in samples of smokers compared to that of non-smokers (Table 1, Figure 4) and showed an inverse correlations with conventional sperm parameters (Table 2). Whereas, significant positive correlations ($p < 0.010$) were found within the levels of these oxidative stress biomarkers (Table 2). The results of this study suggest the consequence of cigarette smoking in the stimulation of sperm chromatin abnormalities and DNA fragmentation in infertile patients (Figures 2 and 3). Accordingly (Armstrong, *et al.*, 1998) demonstrated that high levels of seminal oxidative stress correlated with sperm impairment of sperm metabolism, motility, and fertilizing capacity. Oxidative stress may increase in smokers seminal plasma due to the presence of high levels of ROS like hydrogen radicals (OH[•]), hydrogen peroxide (H₂O₂), and superoxide anion (O₂⁻) (Saleh, *et al.*, 2002, Kunzle, *et al.*, 2003) and from the inflammatory reaction initiated in male reproductive tract from smoking metabolites causing production of high levels of leukocytes that can produce high levels of ROS (Saleh, *et al.*, 2002, Saleh, *et al.*, 2002). Smoking metabolites also, may deteriorate spermatogenesis as an excess of free radical synthesis often involves in an error in spermiogenesis which results in release of sperm with excess of cytoplasmic retention (Aitken and Sawyer, 2003, Said, *et al.*, 2004).

Many studies had proposed that effect of smoking on sperm parameters may be a dose-dependent effect, and high levels of cigarette smoking positively associated with low sperm parameter quality (Martini, *et al.*, 2004, Pasqualotto, *et al.*, 2004,

Colagar, *et al.*, 2007, Hammadeh, *et al.*, 2010). Furthermore, seminal plasma cotinine levels were found to be significantly higher ($p < 0.001$) in smokers than non-smokers and correlated inversely with sperm count, motility, vitality, membrane integrity, morphology, chromatin condensation and DNA integrity (Tables 1 and 2). These findings are in agreement with previous reports by (Zavos, *et al.*, 1998, Wong, *et al.*, 2000) who found that cotinine concentration impaired sperm motility, vitality membrane function, and their ability to undergo capacitation. The current results are also in accordance with the finding of (Chen and Kuo, 2007), who established that cotinine may decrease male fertility by declining sperm density, reducing motile sperm count, and elevating the number of sperms with abnormal morphology and confirm our previous studies (Hammadeh, *et al.*, 2008, Hammadeh, *et al.*, 2010).

MDA levels were significantly lower in non-smokers compared to that of smokers and has a negatively affect all evaluated sperm parameters (Table 1). The significant positive correlation ($p < 0.010$) between MDA and cotinine pointed out the negative effect of smoking on lipid peroxidation of sperm. These results (Figure 4, Table 2) approve earlier studies conducted by Elsaied *et al.*, (2006) who showed a significant negative correlation between MDA and sperm concentration, motility, and percentage of normal forms. Likewise, (Saraniya, *et al.*, 2008) found a significant correlation between MDA concentrations and sperm motility and concentration in sub-fertile patients compared to normal. Moreover (Nabil, *et al.*, 2008), reported that lipid peroxidation plays a significant role in disrupting sperm functions and semen quality especially sperm motility and morphology and may account for some cases of male infertility. Furthermore, (Tavilani, *et al.*, 2008), observed a higher content of lipid peroxidation product malondialdehyde (MDA) in sperm of asthenozoospermic compared with normozoospermic samples ($p < 0.05$).

In conclusion, these findings suggest that prompted oxidative stress by smoking may have a significant

role in disturbing sperm functions and semen quality especially sperm nuclear integrity and stability, same results were revealed by many studies (Joo, *et al.*, 2012, Nadeem, *et al.*, 2012, Taha, *et al.*, 2013, Zhang, *et al.*, 2013). Cigarette smoke contains many toxins that may have harmful effects on male fertility. Therefore, it could be suggested that infertile patients should stop smoking before undergoing ART therapy.

Acknowledgement

The authors would like to thank **Professor Dr. Dr. ME Hammadeh** from Department of 1Department of Obstetrics and Gynaecology, IVF & Andrology Laboratory, Faculty of Medicine, of Saarland, Germany, for a place and supplying chemical material and constructive discussion concerning this work. Also, the author would like to thank Dr. Mutasim Albady, Department of Basic Sciences, Collage of Science and Health Professions, King Saud Bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia for performing statistical analysis of the data.

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