Article

Cigarette smoke extract immobilizes human spermatozoa and induces sperm apoptosis

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Abstract

Cigarette smoking by the male partner adversely affects assisted reproductive techniques, suggesting that it may damage sperm chromatin/DNA and consequently embryo development. The effects of graded concentrations of research cigarettes smoke extract (CSE) on motility, mitochondrial membrane potential (MMP), chromatin integrity and apoptosis were evaluated in spermatozoa obtained from 13 healthy, non-smoking men with normal sperm parameters, by flow cytometry. CSE suppressed sperm motility in a concentration- and time-dependent manner and increased the number of spermatozoa with low MMP, the main source of energy for sperm motility. In addition, CSE had a detrimental effect on sperm chromatin condensation and apoptosis. Indeed, it increased the number of spermatozoa with phosphatidylserine externalization, an early apoptotic sign, and fragmented DNA, a late apoptotic sign, in a concentration- and time-dependent manner. These effects of CSE were of similar or even greater magnitude to those obtained following incubation with tumour necrosis factor-α, a cytokine known for its negative impact on sperm function, used as positive control. Since transmission of smoking-induced sperm DNA alterations has been found in pre-implantation embryos, and this may predispose offspring to a greater risk of malformations, cancer and genetic diseases, men seeking to father a child are recommended to give up smoking.

Keywords: *chromatin integrity, cigarette smoking extract, DNA fragmentation, mitochondrial membrane potential, phosphatidylserine externalization, sperm motility*

Introduction

Cigarette smoking is recognized worldwide as an important risk factor for many diseases. According to the World Health Organization (2002), approximately one-third of the world's male adult population (above 15 years of age) smokes. The combustion of tobacco yields about 4000 chemical compounds, some of which are deadly toxic. Given that cigarette smoke contains more than 30 compounds known to be mutagens, aneugens, or carcinogens, direct deleterious effects on human embryos and female and male germ cells are probable (reviewed in Zenzes, 2000).

Cigarette smoking has been reported to have a detrimental effect on male reproductive function. A meta-analysis including 27 studies showed that smoking is associated with the following sperm parameter modifications: −13% sperm density, −10% sperm motility and −13% of the normally shaped spermatozoa (Vine *et al.*, 1996). A significantly lower number of motile and viable spermatozoa have been found in smokers compared with non-smokers (Zavos

et al., 1998). Cigarette smoking has been reported to be associated with a significant reduction of sperm density (−15.3%), total number of spermatozoa (−17.5%) and total motility (−16.6%) (Kunzle *et al*., 2003), which are inversely related to the dose and length of cigarette smoking exposure (Zhang *et al*., 2002). Gaur and colleagues reported that 39% of non-smoking males are normozoospermics, whereas only 3% of smokers are normozoospermics. In addition, a significantly higher number of men with asthenozoospermia or astheno-teratozoospermia were found in smokers compared with non-smokers (Gaur *et al*., 2007). An inverse relationship has been reported between the number of cigarettes smoked and total spermatozoon number and motility and ejaculate volume (Ramlau-Hansen *et al*., 2007). Further evidence of the damage caused by cigarette smoking on spermatozoa can be inferred by the low success rate in assisted reproductive programmes when the male partner smokes (Zitzmann *et al*., 2003).

On the other hand, different authors failed to find any effect of smoking habit on conventional sperm parameters. A study, conducted in 517 non-smokers, 109 ex-smokers and 478 smokers did not show any effect of smoking on semen parameters, although smokers had a significantly higher number of round cells and leukocytes (Trummer *et al*., 2002). Accordingly, Belcheva *et al*. found no significant difference in standard sperm parameters between a group of healthy regularly smoking males and in healthy non-smoking donors (Belcheva *et al*., 2004). No differences were found in sperm motility grade a, c and d between 27 infertile men who smoked cigarettes, 79 infertile men who were non-smokers and 82 healthy non-smoking donors (Taszarek *et al*., 2005). In the same line, Pasqualotto *et al*. (2006) did not find any significant difference in sperm motility in 522 non-smokers, 143 mild smokers (<10 cigarettes/day), 154 moderate smokers (11–20/day) and 70 heavy smokers (>20/day).

The reason for the discrepancy between studies showing a decrease in classical sperm parameters and those that have not replicated these findings may relate to several factors. The first factor to be taken into account is certainly the enrolment of smoking and non-smoking subjects without having excluded the presence of diseases that may alter sperm parameters. For example, the presence of asymptomatic male accessory gland infection may *per se* negatively affect sperm parameters through the release of cytokines (Perdichizzi *et al*., 2007) and/or radical oxygen species (Aitken and Krausz, 2003), independently of smoking. A second factor is represented by the lack, in many studies, of a quantification of the smoking habit by measuring specific markers. A third factor may be represented by the small number of subjects enrolled in some studies, and hence is not representative of the population findings.

Cigarette smoke has been associated with an increased number of seminal leukocytes (Trummer *et al*., 2002) and reactive oxygen species (Saleh *et al*., 2002), conditions able to cause oxidative stress-induced chromatin and/or DNA damage that can only be partially repaired because of the drastically reduced repair capacity of spermatozoa. Transmission of altered DNA by spermatozoa from smoking males has been demonstrated in pre-implantation embryos and in association with increased risk of childhood cancer (reviewed in Zenzes, 2000). Understanding the effects of cigarette smoking on sperm DNA/chromatin integrity is important not only for the success of assisted reproduction, but also for the possible health consequences in the offspring (reviewed in Aitken and Krausz, 2003).

This study investigates the effect of cigarette smoke extract (CSE) on motility, mitochondrial membrane potential (MMP), chromatin integrity and apoptosis in spermatozoa obtained from healthy non-smoking men with normal sperm parameters. So far as is known, this is the first report exploring the effect of cigarette smoking on sperm mitochondrial function, which supplies the energy necessary for sperm motility.

Materials and methods

Sperm preparation

Spermatozoa were obtained from 13 healthy, non-smoking, normozoospermic men. Semen was collected by masturbation after 3–5 days of abstinence from healthy subjects, and the sperm parameters were evaluated according to World Health Organization criteria (1999). After 30 min of liquefaction at 37°C, motile spermatozoa were isolated using a swim-up procedure. Briefly, the pellet was overlaid with 0.5 ml Biggers, Whitten and Whittingham (BWW) and left to incubate for 30 min, at the end of which the supernatant was collected. Sperm density was adjusted with BWW to a concentration ranging from 20 to 30 $\times$ 10^6 spermatozoa/ml.

An aliquot containing 5 $\times$ 10^6 spermatozoa was incubated with increasing concentrations of CSE (0%, 1%, 3% and 5%) in a water-jacketed incubator at 37°C under 5% CO_2 atmosphere for 3 and 24 h. Tumour necrosis factor-α (TNFα), 10 ng/ml, a concentration that can decrease total motility and induce sperm apoptosis (Perdichizzi *et al*., 2007), was used as a positive control for each experiment. At the end of the incubation, the following parameters were evaluated: total sperm motility and, by flow cytometry, the MMP was assessed by the cationic dye JC-1, sperm chromatin packaging quality following DNA staining with propidium iodide (PI), the presence of phosphatidylserine (PS) on the outer leaflet of the cell membrane using the annexin V/PI assay, and DNA fragmentation using the TUNEL assay, as previously reported (Perdichizzi *et al*., 2007).

Production of cigarette smoke extract

CSE was generated from two standard research cigarettes (Kentucky 2R4 F; Tobacco Research Institute, University of Kentucky, Lexington, KY, USA). This item is sold for research purposes only and it is characterized by very strict criteria for the cigarette content (tar 9 mg; nicotine 0.8 mg; particulate 11.7 mg) with an overall variability of < 10%. Cigarettes, without filters, were attached to tube connected to a Buchner flask containing 50 ml of PBS 1× buffer. The smoke derived from cigarettes was drawn into the flask under a vacuum generated by a nickel-plated water aspira-

tor pump (maximum vacuum 100 mmHg). The solution of CSE was filtered with Millex-GV (0.22 µm, PVDF 4 mm) aqueous solution filters (Millipore S.p.A., Vimodrone, MI, Italy) and diluted as necessary. This extract contains the major components in the smoke of burning cigarettes (Chow *et al.*, 1997). The nicotine concentrations in this extract, ranging from 10 to 100 µg/ml, were similar to the blood concentrations measured in light (1% CSE) and heavy smokers (3% CSE), respectively (Lawson *et al.*, 1998).

Flow cytometry

The EPICS XL flow cytometer (Coulter Electronics, IL, Italy), equipped with a 488 nm argon laser as light source, was used for flow cytometry analysis. Three fluorescent detectors were used according to the assay utilized to measure the fluorescence corresponding to the green colour (FL-1 detector 525 nm wavelength band), orange colour (FL-2 detector 620 nm wavelength band) and red colour (FL-3 detector 620 nm wavelength band). In all, 20,000 events were measured for each sample at a flow rate of 50–100 events/s and analysed using the SYSTEM II™ Software, 3.0 Version (Coulter Electronics). The debris was gated out, by drawing a region on forward versus side scatter dot plot enclosing the population of cells of interest, as previously reported (Perdichizzi *et al.*, 2007). The compensations and the settings were adapted according to the assay utilized.

JC-1 staining

To evaluate a decrease in MMP, a marker of early apoptosis, the cell suspension was adjusted to a density of 1×10^6 cells/ml and incubated for 10 min at 37°C in the dark with the JC-1 probe (Molecular Probes, Milan, Italy). This molecule, which is able selectively to enter the mitochondria, exists in a monomeric form emitting at 527 nm after excitation at 490 nm. However, depending on the membrane potential, JC-1 is able to form aggregates that are associated with a large shift in emission (590 nm). Thus, the colour of the dye changes reversibly from green to greenish orange as the mitochondrial membranes become more polarized. At the end of the incubation period, cells were washed in PBS and analysed. For the analysis of the cells stained with JC-1, the photomultiplier (PMT) value of the detector in FL-1 was set at 529 V, FL-2 PMT at 543 V; FL1-FL2 compensation was 12%, FL2-FL1 compensation was 28%, as previously reported (Perdichizzi *et al.*, 2007).

Annexin V/PI assay

Staining with annexin V/PI was performed using a commercially available kit (Annexin V-FITC Apoptosis detection kit; Sigma Chemical, Italy) as previously reported (Perdichizzi *et al.*, 2007). Briefly, an aliquot containing 0.5×10^6 spermatozoa/ml was re-suspended 0.5 ml of binding buffer, labelled with 10 µl of annexin V-FITC plus 20 µl of PI, incubated for 10 min in the dark, and immediately analysed. Signals were detected through FL-1 (FITC) and FL-3 (PI) detectors. The cell population of interest was gated on the basis of the forward (FSC) and side-scatter (SSC) properties. The different labelling patterns in the bivariate PI/annexin V analysis identify the different cell populations where FITC negative and PI negative were designated as viable cells; FITC positive and PI negative as apoptotic cells, and FITC positive and PI positive as late apoptotic or necrotic cells.

PI staining

PI staining was performed following cell membrane permeabilization to allow fluorochrome nuclear penetration, as previously reported (Perdichizzi *et al.*, 2007). Briefly, 1×10^6 spermatozoa were incubated in 1 ml PBS containing 50 µg/ml of PI (Sigma Chemical), 0.1% sodium citrate, and 0.1% Nonidet P40 (Sigma Chemical), 100 Kunitz units/ml of RNAse type A (Sigma Chemical) in the dark, at room temperature. After 30 min, flow cytometry analysis was performed. In this assay, only the FL3 detector was used. To gate out and thus exclude from the analysis doublets and cell aggregates, a 'doublet discrimination module' was used. The peak width was estimated using the coefficient of variation (CV) of the signals within each peak.

TUNEL assay

TUNEL assay was carried out using the commercially available Apoptosis Mebstain kit (Beckman Coulter, IL, Milan, Italy), as previously reported (Perdichizzi *et al.*, 2007). To obtain a negative control, TdT was omitted from the reaction mixture. The positive control was obtained by pre-treating spermatozoa with 1 µg/ml deoxyribonuclease I, RNAse-free (Sigma Chemical) at 37°C for 60 min before labelling. The debris was eliminated following the same procedure described above. Light-scattering and fluorescence data were obtained at a fixed gain setting in logarithmic mode. The percentage of FITC-labelled spermatozoa was determined in the FL-1 channel of the flow cytometer.

Statistical analysis

Results are reported as means ± SEM throughout the study. The data were analysed by one-way analysis of variance (ANOVA) followed by the Duncan's Multiple Range test. The software Statistical Package for Social Sciences (SPSS) 9.0 for Windows was used for statistical evaluation (SPSS Inc., Chicago, IL, USA). A statistically significant difference was accepted when the *P*-value was lower than 0.05.

Results

CSE had a dose- and time-dependent inhibitory effect on total sperm motility (**Figure 1**). After 3 h of incubation, the effect became significant from the lowest concentration tested (1%) ($P < 0.05$ versus CSE 0, one-way ANOVA). The inhibitory effect became significantly stronger at the concentrations of 3% and 5% ($P < 0.05$, versus CSE 1%, one-way ANOVA). TNF-α, at the concentration of 10 ng/ml, was as effective as 1% CSE by decreasing sperm motility on average by 13.3 ± 4.1% (**Table 1**). After 24 h of incubation, CSE had a profound negative impact on total sperm

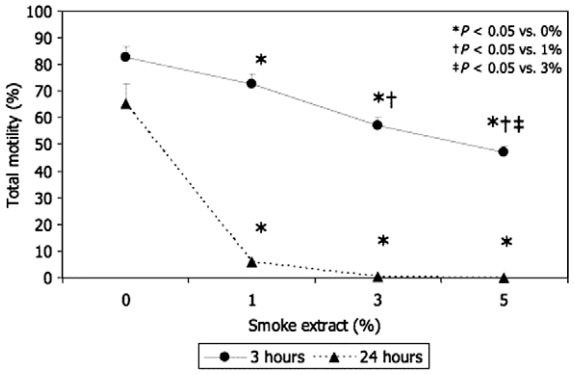

Figure 1. Time-course and dose–response of the effects of cigarette smoke extract on total motility of spermatozoa separated by swim-up from 13 healthy, non-smoking, normozoospermic men.

Table 1. Effects of tumour necrosis factor (TNF)-α, used as the positive control for each experiment, after 3 and 24 h of incubation, on total motility, mitochondrial membrane potential (MMP), chromatin integrity, phosphatidylserine (PS) externalization, and DNA fragmentation of spermatozoa separated by swim-up from 13 healthy non-smoking normozoospermic men.

Parameter	*TNFα concentration (ng/ml)*			
	0	*10*	*0*	*10*
	3 h Incubation		*24 h Incubation*	
Total motility (%)	83.8 ± 3.0	69.6 ± 1.4[a]	65.3 ± 2.6	59.1 ± 2.3[a]
Spermatozoa with low MMP (%)	14.1 ± 1.1	24.4 ± 1.9[a]	18.1 ± 0.9	45.8 ± 2.0[a]
Chromatin integrity (%)	7.5 ± 1.4	11.6 ± 1.0[a]	8.8 ± 1.2	18.7 ± 1.6[a]
PS externalization (%)	11.4 ± 1.9	19.2 ± 3.5[a]	21.4 ± 4.3	45.6 ± 7.9[a]
DNA fragmentation (%)	7.1 ± 1.5	17.6 ± 2.9[a]	12.2 ± 2.2	27.8 ± 5.7[a]

Values are means ± SEM.
[a] $P < 0.05$ versus TNFα 0 ng/ml.

motility starting from the concentration of 1% ($P < 0.05$ versus CSE 0, one-way ANOVA). At higher concentrations, no motile spermatozoa were found. TNF-α, at the concentration of 10 ng/ml, decreased total sperm motility by 11.2 ± 3.4% (**Table 1**).

CSE increased the percentage of spermatozoa with low MMP in a dose- and time-dependent manner (**Figure 2**). After 3 h of incubation, the effect became significant from the concentration of 1% ($P < 0.05$ versus CSE 0, one-way ANOVA). The inhibitory effect became significantly higher at the concentration of 5% ($P < 0.05$, versus CSE 1% and 3%, one-way ANOVA). TNF-α, at the concentration of 10 ng/ml, was as effective as 1% CSE and increased the percentage of spermatozoa with low MMP by 53.5 ± 12.5% (**Table 1**). After 24 h of incubation, CSE increased significantly the percentage of spermatozoa with low MMP starting from a concentration of 1% ($P < 0.05$ versus CSE 0, one-way ANOVA). At concentrations of 3% and 5%, the effect was significantly higher ($P < 0.05$ versus CSE 1% and 3%, respectively, one-way ANOVA). TNF-α, at the concentration of 10 ng/ml, increased significantly the percentage of spermatozoa with low MMP by 144.5 ± 13.5% (**Table 1**).

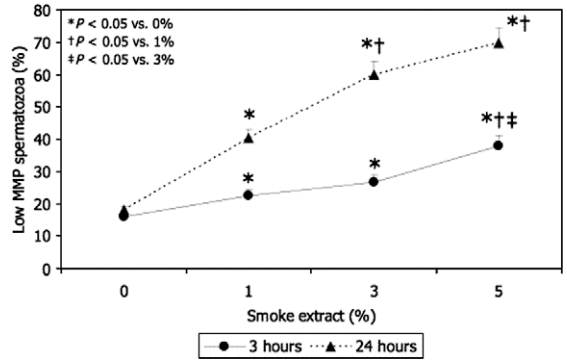

Figure 2. Time-course and dose–response of the effects of cigarette smoke extract on spermatozoa with low mitochondrial membrane potential (MMP) separated by swim-up from 13 healthy, non-smoking, normozoospermic men.

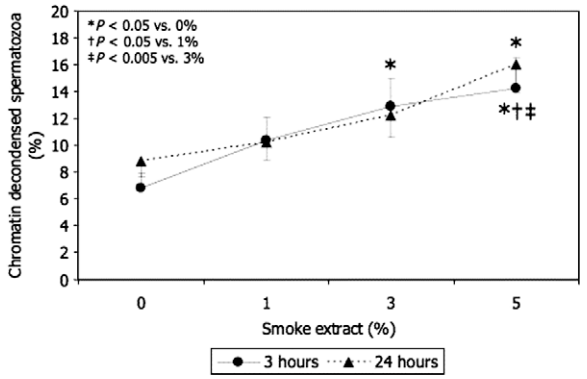

Figure 3. Time-course and dose–response of the effects of cigarette smoke extract on the chromatin integrity of spermatozoa separated by swim-up from 13 healthy, non-smoking, normozoospermic men.

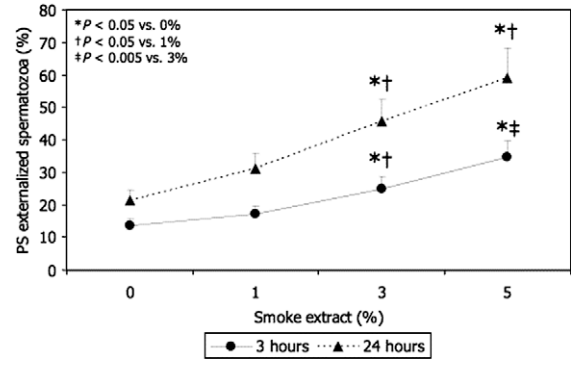

Figure 4. Time-course and dose–response of the effects of cigarette smoke extract on phosphatidylserine (PS) externalization of spermatozoa separated by swim-up from 13 healthy, non-smoking, normozoospermic men.

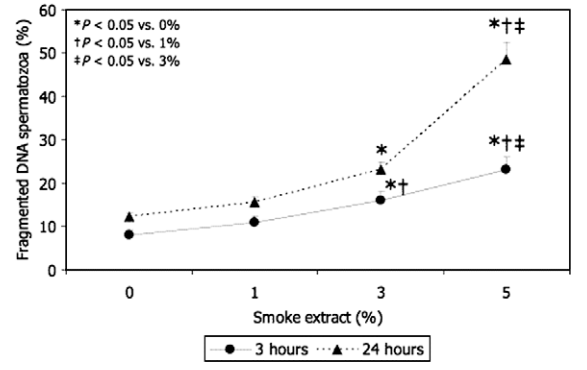

Figure 5. Time-course and dose–response of the effects of cigarette smoke extract on DNA fragmentation of spermatozoa separated by swim-up from 13 healthy, non-smoking, normozoospermic men.

A tiny, but significant, effect of CSE was observed on sperm chromatin condensation both after 3 and 24 h of incubation (**Figure 3**). The effect became significant from the concentration of 3% after 3 h of incubation ($P < 0.05$ versus CSE 1%, one-way ANOVA) and at the concentration of 5% after 24 h ($P < 0.05$ versus CSE 1%, one-way ANOVA). TNF-α, at the concentration of 10 ng/ml, increased the percentage of spermatozoa with decondensed chromatin by 129.1 ± 33.9% after 3 h (**Table 1**) and by 165.4 ± 20.2% after 24 h (**Table 1**).

The effect of CSE on PS externalization was more pronounced. It was dose-dependent and became statistically significant from the concentration of 3% both after 3 and 24 h of incubation ($P < 0.05$ versus CSE 0% and 1%, one-way ANOVA) (**Figure 4**). TNF-α, at the concentration of 10 ng/ml, increased the percentage of spermatozoa with PS externalization by 66.2 ± 9.5% after 3 h (**Table 1**) and by 126.6 ± 30.9% after 24 h (**Table 1**).

CSE increased the percentage of spermatozoa with DNA fragmentation in a dose-dependent manner (**Figure 5**). After 3 and 24 h of incubation, the effect became significant from the concentration of 3% ($P < 0.05$ versus CSE 0% and 1%, one-way ANOVA). The effect became significantly stronger at the concentrations of 5% ($P < 0.05$, versus CSE 1% and 3%, one-way ANOVA). TNF-α, at the concentration of 10 ng/ml, increased the percentage of spermatozoa with fragmented DNA by 140.2 ± 47.1% after 3 h (**Table 1**) and by 131.8 ± 17.3% after 24 h (**Table 1**).

Discussion

For the present study, a defined aqueous CSE obtained from standard research cigarettes (Kentucky 2R4F) was

used. Although compound concentrations in the 1% CSE solution approximately relates those in the blood of a mild smoker (Lawson *et al*., 1998), it is clear that CSE does not constitute the identical smoke compound distribution as in smoker's blood, where cigarette smoke is pre-filtered by the extremely complex lung/blood barrier. Moreover, numerous factors affect the variety of compound distributions and compound concentrations among smokers (e.g. puffing habits, puffing intensity, lung surface), making smokers' blood a highly variable source for cigarette smoke constituents (Jarvis *et al*., 2001). However, aqueous tobacco smoke extract showing comparatively constant chemical compositions offers a useful, widely employed alternative system (Yang and Liu, 2003, 2004; Van der Deen *et al*., 2007).

It was found that CSE is a potent inhibitor of sperm motility. This was associated with an increased percentage of spermatozoa with reduced MMP, which plays a relevant role in sperm fertilizing capacity. Indeed, it has been shown that spermatozoa with high MMP are a subpopulation of spermatozoa with better fertility performance because they have normal morphology, higher motility values, and calcium ionophore-induced acrosome reaction (Gallon *et al*., 2006). So far as is known, this is the first study showing a detrimental effect of CSE on sperm mitochondrial activity. Damaging effects of CSE have, on the other hand, already been reported in mitochondria of mouse brain and human umbilical vein endothelial cells (HUVEC). CSE inhibits mitochondrial ATPase and cytochrome C oxidase activities in mouse brain mitochondria in a dose-dependent manner, whereas nicotine, the major component of cigarette smoke, did not have any significant effect (Yang and Liu, 2003). A decreased MMP has been reported in HUVEC exposed to CSE (5-20%) *in vitro*. The study suggested also that mitochondria seem to play a key role in the apoptosis of HUVEC induced by exposure to CSE (Yang and Liu, 2004).

A reduced sperm motility following CSE exposure *in vitro* finds its counterpart in *in vivo* studies. Vine *et al*. reported a negative correlation between sperm motility and the number of cigarettes smoked per day, years smoked, and cotinine concentrations. Potential confounders included in regression models did not diminish the association (Vine *et al*., 1996). A significant decrease (about −17%) in the total number of motile spermatozoa was reported in a large number of smokers ($n = 655$) compared with non-smokers ($n = 1131$) (Kunzle *et al*., 2003). Cigarette smoking correlated negatively with progressive motile sperm count in a total of 223 sperm samples (126 smokers and 97 nonsmokers) obtained from infertile men during routine diagnostic work-up (Hassa *et al*., 2006). Gaur and colleagues reported that light smokers predominantly have asthenozoospermia, whereas heavy smokers have asthenozoospermia, teratozoospermia and oligozoospermia. Statistical analysis showed that the incidence of both isolated asthenozoospermia and asthenozoospermia plus teratozoospermia among smokers was significantly higher in comparison with non-smokers. Asthenozoospermia, the most common semen variable observed in this study, can be therefore regarded as an early indicator of smoke-induced reduction of semen quality (Gaur *et al*., 2007). Likewise, Ramlau-Hansen *et al*. observed an inverse dose–response relationship between smoking and percentage of motile spermatozoa after adjusting for age and other covariates (Ramlau-Hansen *et al*., 2007). A recent prospective study confirmed that cigarette smoking decreases sperm motility and the antioxidant activity (negative correlation with superoxide dismutase) in the seminal plasma (Pasqualotto *et al*., 2008).

Cigarette smoking by one or both partners can adversely affect ART outcome, including ICSI which bypasses the initial steps of fertilization and does not relate to sperm motility. Zitzmann and colleagues reported a clinical pregnancy rate of 22% by ICSI in women with smoking male partners compared with the 38% achieved with non-smoking partners. Similar results were seen for IVF, with 18% versus 32%, respectively. Multinominal logistic regression analysis showed smoking in men to be a significant predictor of ICSI and IVF outcome. The odds ratio for failure for male smokers to impregnate their partners was 2.95 for ICSI and 2.65 for IVF compared with non-smoking males. On the basis of these results, the authors postulated that sperm DNA alterations might hamper embryo development (Zitzmann *et al*., 2003). Accordingly, it was found that the smoke extract had a deleterious effect on sperm DNA/chromatin integrity. Exposure of spermatozoa obtained from healthy men with normal sperm parameters to CSE resulted in sperm apoptosis, suggested by the increased number of spermatozoa with PS externalization and with fragmented DNA (sign of late apoptosis) after both short- and long-term incubation. The externalization of PS identifies cells with deteriorated membrane, one of the earliest features of cells undergoing apoptosis (Vermes *et al*., 1995), which marks them for phagocytosis by macrophages. A number of cigarette combustion products may explain such an effect, which was of similar magnitude to that observed with TNF-α, a cytokine known for its pro-apoptotic effects in several cell types, including spermatozoa (Perdichizzi *et al*., 2007).

In vivo studies have also examined whether smokers have an increased number of spermatozoa with DNA abnormalities and/or apoptosis signs. It has been shown that benzo(a)pyrene, a carcinogenic polycyclic aromatic hydrocarbon resulting from cigarette combustion, is able to bind covalently to DNA forming adducts. These adducts have been reported to be increased in spermatozoa of smoking men compared with non-smoking and show a direct correlation with seminal plasma concentration of cotinine, a stable metabolite of nicotine. The presence of adducts in spermatozoa has been postulated to be a potential source of transmissible prezygotic DNA damage, since the DNA repair capacity is low in spermatozoa (Zenzes *et al*., 1999). Sun and colleagues reported that 35 smoking males undergoing IVF had a significantly increased percentage of spermatozoa with fragmented DNA ($4.7 \pm 1.2\%$) as compared with 78 non-smokers ($1.1 \pm 0.2\%$) (Sun *et al.,* 1997). Accordingly, Potts *et al*. (1999) reported higher levels of DNA strand breaks in spermatozoa of non-smokers (Potts *et al*., 1999). Sepaniak *et al*. reported that smokers' spermatozoa had a significantly higher DNA fragmentation than non-smokers. The degree of sperm DNA fragmentation did not correlate with

any of the conventional parameters, suggesting that cigarette smoking may have deleterious effects on sperm nuclear quality, which may be regarded as an independent parameter with diagnostic, prognostic, and strategic value in the treatment of male infertility (Sepaniak *et al*., 2006). Belcheva and collaborators reported an increased number of spermatozoa with PS externalization, but not with DNA fragmentation, in a group of smokers (>20 cigarette/day for >1 year) (Belcheva *et al*., 2004). An elevated number of spermatozoa with fragmented DNA has also been found in swim-up separated samples of smokers compared with non-smokers. This suggests that smoking habit alters the sperm swim-up selection process with the consequent implications for ICSI or IVF procedures (Viloria *et al*., 2007). On the other hand, Sergerie and colleagues did not find any increased percentage of DNA-fragmented spermatozoa in the heavy or light smokers compared with non-smokers (Sergerie *et al*., 2000). Since smoking-related DNA adducts have been detected in pre-implantation embryos and given that transmission of altered DNA from smoking by spermatozoa has been demonstrated in pre-implantation embryos (reviewed in Zenzes, 2000), it can be postulated that these changes may result in sperm DNA mutations that predispose offspring to greater risk of malformations, cancer and genetic diseases (Sorahan *et al*., 1997; Potts *et al*., 1999; Zenzes, 2000).

It was found that smoke extract also increases the number of spermatozoa with damaged chromatin, a parameter that plays an important role in sperm fertilizing ability (reviewed in Erenpreiss *et al*., 2006). Accordingly, Potts and colleagues reported that spermatozoa from subjects who smoked were significantly more sensitive to acid-induced denaturation than non-smokers evaluated by the sperm chromatin structure assay, which measures the sensitivity of sperm DNA to acid induced denaturation (Potts *et al*., 1999).

In conclusion, this study showed that CSE has a negative impact on sperm motility and DNA/chromatin integrity. Given the relevant role played by these parameters on male reproductive ability, men seeking to father a child are recommended to give up smoking.

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