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Cotinine Characterization and Quality Effect of Sperm for Smoking and Nonsmoking Students

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In recent years, there has been increasing concern about the possible deleterious effects of environmental factors on sperm quality (Zenses et al. 1999; Oldereid et al. 1989). Cigarette smoking in women has been associated with decreased fertility (Jensen et al. 1998; Howe et al. 1985). Some evidence shows that cigarette smoke could affect male fertility and in particular influence sperm quality (Vine 1996). Cigarette smoking has been associated with adverse effects on sperm quality, including reduced sperm density and motility and abnormal morphology (Vine 1996; Zavos et al. 1998). The chemicals in cigarette smoke can harm spermatozoa by damaging DNA (Bos and Henderson 1984; Aitken 1999). The result of oxidative DNA damage could be sperm dysfunction, infertility, birth defects, genetic disease, and cancer in offspring by increasing the rate of heritable mutations and chromosome abnormalities (Field et al. 1994; Cummins et al. 1994).

Cotinine is the major metabolite of nicotine, which is the major psychoactive substance found in cigarette smoke (Hulka 1991; Zenzes et al. 1996). Because it is easily detectable in human body fluids, such as urine, saliva (Zenzes et al. 1996; Wall et al. 1988), and seminal plasma (Pacifici et al. 1993), cotinine has been used as a specific biomarker of cigarette smoking (Hulka 1991). Cotinine with a half-life in sperm of 5–7 days is a better indicator of long-term exposure to cigarette smoke than urinary metabolites index (Jarvis et al. 1988).

A 2005 survey in Taiwan on the "knowledge, attitude, and behavior about health promotion" found that 50.0% of men and 5.8% of women smoked, which is higher than the corresponding rates in Korea, Japan, and other countries, and 32.37% of the interviewees had environmental tobacco smoke (ETS) exposure (HPB Taiwan 2006). College students, in particular, have a higher smoking rate than any other group. Among college students, significantly higher mean concentration of urinary cotinine has been found in smokers than non-smokers (3,055 vs. 15 ng/mL) (Yang 2000). This is a critical public health problem in Taiwan. Assay of seminal plasma cotinine has not been used in the study of cigarette smoking and male fertility. The purpose of this study is (1) to document the presence of cotinine in the semen of smoking and non-smoking students, (2) to compare the sperm quality of smoking and non-smoking students, (3) to analyze the relationship between semen cotinine levels and sperm quality.

Materials and Methods

A cross-sectional epidemiological study was conducted of 35 smoking students (mean ± SD age, 22.0 ± 2.0 years; the exposed group) and 30 non-smoking students (mean ± SD age, 22.2 ± 1.8 years; the control group) from Yuanpei University, Hsinchu, Taiwan. Standard measures of sperm quality were evaluated (volume, sperm density, total sperm count, percentage of motile sperm, viability, and total percentage of abnormal sperm). A questionnaire was administered when the students arrived to donate their samples. The questionnaire was self-administered and

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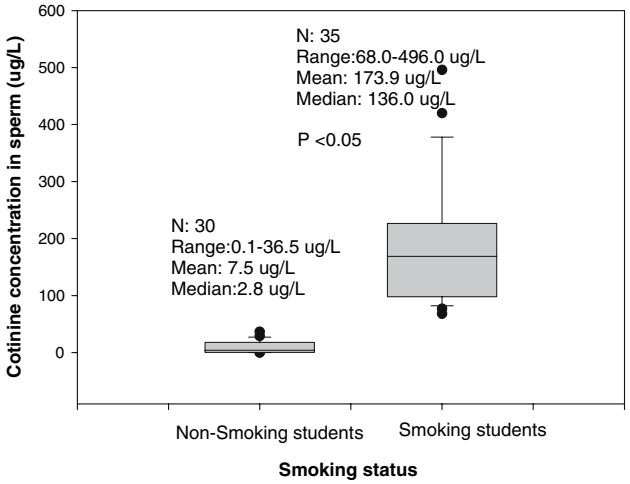

Fig. 1 Box-plots showing the mean, median, and range of semen cotinine concentration of smoking and non-smoking students

designed to determine current smoking behavior. The participants were divided on the basis of average number of cigarettes smoked per day into three groups: 1–10, 11–20 and 21–40.

One semen specimen was provided by each participant. Semen specimens were collected via masturbation after an abstinence period of 3–5 days. The samples were collected in separate polypropylene containers and assayed within 2 h after collection. The cotinine in 1 ml samples from each participant was detected using a column-switching high performance liquid chromatography system with a programmable UV detector module (254 nm) (Kuo and Teng 2001). Cotinine and other chemicals were purchased from Sigma (Munich, Germany). Semen specimens were centrifuged (2,000×*g*). The seminal plasma was extracted with 6 mL of methanol: sodium hydroxide: chloroform = 1:1:4, v/v and recentrifuged (10 min). Two milliliters of the organic phase was transferred to a vial and dried under nitrogen. The residue was dissolved in 0.5 mL of methanol and the solution was injected into our HPLC-UV system for analysis. The HPLC eluent consisted of 85% buffer solution (20 mM potassium dihydrogen phosphate +20 mM sodium-1-decanesulfonate, pH 3.0) and 15% acetonitrile. The range of calibration of working standards was 0.07–450 ng/mL and the detection limit of cotinine was ≤ 6 ng/L. The recovery yield was 92%.

Semen analysis was performed according to World Health Organization criteria (WHO 1992). The volume of semen specimens was measured in a graduated collection vessel. The pH value was measured with special indicator paper manufactured by E. Merck (Darmstadt, FRG). Sperm motility in diluted ejaculate was determined under negative phase-contrast microscopy and recorded onto videotape. The videotapes were replayed later for analysis of sperm motion, sperm density, and sperm mobility using an HTM-IVOS semen analyzer (Hamilton-Thorn Research, Beverly, MA, USA). The percentage of motile sperm and the quality of motility were recorded within detection limits. The percentage of progressively motile sperm was determined from the videotapes after scoring at least 100 sperm per sample. If <50% of the sperm were motile, a vitality test was carried out. If no spermatozoa were found, the sperm was centrifuged and the sediment again subjected to microscopic examination. The proportion of misshapen spermatozoa was expressed as a percentage.

Mann–Whitney *U* test was used for analysis of continuous variables (i.e., cotinine distribution and semen parameters). Differences in cotinine distribution and characteristics of sperm quality were evaluated between smokers and nonsmokers. Statistical significance was defined as $p < 0.05$.

Results and Discussion

Box-plots in Fig. 1 show the difference in semen cotinine levels between smoking and non-smoking students. The average levels in smoking and non-smoking groups was 173.9 and 7.5 μg/L, respectively, representing a 23-fold difference, and their ranges were 68.0–496.0 and 0.1–36.5 μg/L, respectively. The difference was significant ($p < 0.05$). These results demonstrate cigarette smoking is the main contributor to semen cotinine. Moreover, semen cotinine levels in non-smokers appear to be skewed toward the upper end of the range, with 23% showing extremely high mean values (>7.5 μg/L) attributable to inhaled environmental tobacco smoke (ETS). Semen cotinine was higher in non-smoking students who lived with smokers than non-smoking students who lived with nonsmokers. Seven students lived in households where both parents smoked or with smoking classmates. The semen cotinine positively correlated with the degree of ETS exposure. These results agree with Yang's results (Yang 2000) showing that urinary cotinine levels of non-smokers are related to second-hand smoke exposure ($r_s = 0.58$). According to a 2005 survey in Taiwan, 32.37% of interviewees had ETS exposure. Thus, second-hand smoke is an important potential health risk in Taiwan and its impact on health needs further investigation.

Figure 2 summarizes the smoking status of smoking students. In our study, only 14.3% of smoking students smoked 21–40 cigarettes per day, while the majority (54.3%) smoked 1–10 cigarettes per day. Self-reported number of cigarettes smoked per day (by category) was highly correlated with semen cotinine ($r_s = 0.70$). The mean semen cotinine level of smokers of 1–10, 11–20, 21–40 cigarettes per day was, respectively, 100.5, 198.7, and

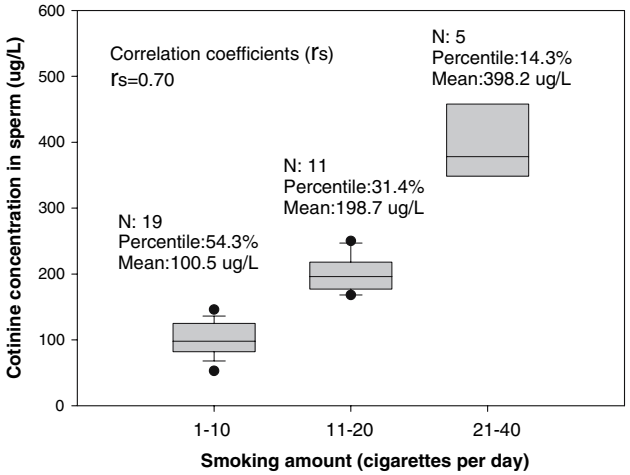

Fig. 2 Percentage of smoking students in each group and correlation between smoking amount and semen cotinine concentration of smoking students

Table 1 Comparison of semen characteristics of smokers and nonsmokers

Semen characteristics	Smokers	Nonsmokers	*p* value
No. of subjects	35	30	
pH	7.4 ± 0.3	7.2 ± 0.9	NS
Volume (mL)	4.4 ± 0.6	4.6 ± 0.8	NS
Density ($\times 10^6$/mL)	69.2 ± 18.5	105.0 ± 10.2	<0.05
Total sperm count ($\times 10^6$/mL)	157.4 ± 32.5	268.9 ± 28.7	<0.05
Progressive motility (%)	37.2 ± 5.8	38.9 ± 4.4	NS
Total motile sperm count ($\times 10^6$/mL)	96.8 ± 14.7	155.2 ± 10.8	<0.05
Viability (%)	31.2 ± 2.4	31.4 ± 1.6	NS
Abnormal morphology (%)	29.0 ± 1.7	28.2 ± 0.6	NS
Citrate concentration (μmol/L)	80.0 ± 13.7	113.0 ± 12.6	<0.05
Fructose concentration (μmol/L)	46.9 ± 7.7	48.0 ± 8.1	NS

Values are mean ± SD

NS is not statistically significant ($p > 0.05$)

398.2 μg/L. This finding suggests that nicotine from cigarettes is actively transferred from the arteriae testicularis via the Sertoli cell into the seminiferous tubules and seminal plasma. Similarly, Vine et al. (1993) found nicotine and cotinine in the seminal plasma of smokers and suggested that other harmful components of tobacco smoke would pass through the blood–testis barrier.

Table 1 compares semen characteristics of smokers and nonsmokers. Conventional semen quality analyses use World Health Organization (WHO) criteria. Statistical analysis demonstrated significantly lower semen quality, significantly lower sperm density, no effect on ejaculate volume, nonsignificantly lower percentage of sperm with progressive motility, and significantly lower total number of motile sperm in the ejaculates of smokers. Smoking had clearly deleterious effects on sperm morphology. The ejaculate pH was slightly higher for smokers than nonsmokers, which may be due to reduced citrate concentration. Smoking habits did not directly affect the fructose concentration. Moreover, the smoker–nonsmoker differences in sperm density, total sperm count, and total motile sperm became more significant after logarithmic transformation of the raw data. Semen cotinine concentration was clearly correlated with sperm quality (Table 2). Semen cotinine concentration was highly negatively correlated with total number of motile sperm ($r_s = -0.69$; $p < 0.05$), significantly correlated with percentage of sperm with abnormal morphology ($r_s = 0.45$; $p < 0.05$), but not correlated with volume, density, progressive motility and viability. The relation of semen cotinine characteristics to sperm quality in our study is consistent with past reports. Pacifici et al. (1993) found that total motility of spermatozoa was significantly negatively correlated with concentrations of cotinine and hydroxycotinine. Zavos et al. (1998) found smoking affected sperm viability and had a strong detrimental effect on motility of spermatozoa. Moreover, alcohol and caffeine consumption were important confounders when we evaluated the relation of semen cotinine level to sperm quality. Alcohol consumption could not be separated from smoking because all smokers consumed moderate to high amounts of alcohol, whereas most nonsmokers were also nondrinkers. Smoking remained strongly associated with increase in the frequency of disomy Y sperm. In this study, we minimized the effects of the two confounders by forbidding alcohol and caffeine consumption for 3–5 days before the semen samples were collected.

Cigarette smoking exposure appears to be an important risk factor for male infertility. Significant smoker-nonsmoker differences were found in semen cotinine concentration and sperm quality. Cotinine may decrease male

Table 2 Correlation coefficient of cotinine concentration and sperm quality

Variables	Volume	Density	Progressive motility	Total motile sperm	Viability	Abnormal morphology
Cotinine in semen (μg/L)	–0.09	0.32	–0.19	–0.69	0.03	0.45
p value	NS	NS	NS	<0.05	NS	<0.05

fertility by inhibiting density, reducing total progressively motile sperm count, and increasing the percentage of sperm with abnormal morphology. These results can be used as a reference for revision of governmental regulation and to develop a tobacco control strategy for protecting public health. To minimize health risks, long-term monitoring of cotinine and sperm quality should be carried out and health education of students should be continued.

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