The in-vitro effects of nicotine and cotinine on sperm motility

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Swim-up spermatozoa from the seminal samples of non-smokers, usually not exposed to passive smoking, were treated in vitro with nicotine (NIC) and cotinine (COT) at the average levels found in smokers’ seminal plasma and at levels 500 times higher than this average. This was done to evaluate the action of these drugs on sperm motility. Each sample was allowed to swim up in Tyrode’s solution with or without the drug; the study was carried out at time 0 and +1, +2, +4, +8 and +24 h of incubation, using a light microscope and a CASA system (experiment 1). In addition, the direct action of smoke on spermatozoa was studied using aspirated cigarette smoke (experiment 2). Kinetic parameters were then measured at 30 min, 45 min and 60 min starting from the last smoke injection. The first experiment showed that NIC and COT at average levels did not produce statistically significant variations of the kinetic parameters studied up to 24 h. However, the much higher concentration significantly altered all the kinetic variables in relation to the time of incubation. The second experiment with smoke in toto demonstrated a sharp reduction in all the sperm kinetic parameters. This reduction was seen after 30 min exposure to smoke and increased progressively until almost complete immotility at 1 h of exposure. These results suggest that NIC and COT are not responsible for the harmful effects of cigarette smoke on sperm kinetic parameters reported in the literature.

Key words: CASA system/cotinine/human spermatozoa/kinetic parameters/nicotine

Introduction

The effects of smoking on male reproductive function have been widely studied in various mammalian species (Viczian, 1968; Stillman et al., 1986; Attia et al., 1989).

Smoke acts on sperm function, as demonstrated by several authors, even though the mechanisms by which tobacco smoke can affect spermatozoa have yet to be identified (Evans et al., 1981; Rodriguez-Rigau et al., 1982; Kulikauskas et al., 1985; Benowitz, 1988; El Mulla et al., 1995; Vine et al., 1996).

Tobacco combustion yields about 4000 compounds; the smoke can be divided into a gaseous phase and a phase made up of particles. The principal harmful components of the gaseous part are carbon monoxide, nitrogen oxide, ammonia, and volatile hydrocarbons. The main component of the particle phase is nicotine (NIC). This psychoactive substance is one of the few natural liquid alkaloids; it is a volatile base, colourless, water soluble and forms hydrosoluble salts. Its pharmacological action takes place at the level of the autonomic ganglia. The effects in the organism are due to the typically bi-phase action, both stimulating and depressive, that this alkaloid has on numerous neuro-effector chemical receptors (Benowitz, 1988).

NIC is quickly absorbed through the respiratory tract, mouth mucosa and skin. About 80–90% of the NIC is metabolized by the organism, mainly by the liver, but also by the kidney and lung (Armitage et al., 1975). Its major metabolite is cotinine (COT). NIC and its metabolites have been detected in serum, urine, saliva and milk (Weiss and Eckert, 1989; Pichini et al., 1992; Pacifici et al., 1993a), and more recently they have been found at significant levels in smokers’ seminal plasma and in subjects exposed to environmental tobacco smoke (Pacifici et al., 1993b, 1995; Vine et al., 1993).

The aim of this study was to evaluate the in-vitro effects of NIC and COT on sperm motility in normozoospermic semen samples of non-smokers.

Materials and methods

Subjects

Ten healthy non-smokers, aged 28–35 years, not normally exposed to passive smoking and not treated medically in the 6 months prior to the study, were selected from the men attending our Laboratory of Seminology and Immunology of Reproduction, Dept of Medical Pathophysiology, University of Rome ‘La Sapienza’.

The samples were collected by masturbation, after a 3-day period of sexual abstinence. Standard semen analyses (WHO standard procedure; WHO, 1992) and computer analysis of sperm motility (CASA System; Cell Soft, Cryo Resources, New York, NY, USA) (Ast and Rosemberg, 1986) were carried out. The variables taken into consideration were: volume of ejaculate (ml), sperm concentration (n×10⁶/ml), forward motility (%), morphology (% of atypical forms), curvilinear velocity (VCL) (µm/s), linearity (LIN) (index), amplitude of lateral head displacement (ALH) (µm) and beat cross-frequency (BCF) (Hz). The Automated Cell Soft System (Cryo Resources) was equipped with a heated stage (35°C). Parameter settings were as follows: number of frames to analyse: 25; number of frames/s: 25;
minimum track point for calculation of motility: 2; minimum track point for calculation of velocity: 8; maximum velocity: 150; threshold velocity: 10; pixel scale: 1.351; cell size range (low): 4; cell size range (high): 20; minimum track point for calculation of ALH: 8; minimum velocity for calculation of ALH: 20; minimum linearity for calculation of ALH: 3.5. At least 300 cells were examined in each sample.

Only subjects with the following seminal characteristics were included in the study: volume ≥ 3.0 ml; sperm concentration/ml ≥ 50 × 10⁶; forward motility ≥ 60%; atypical forms ≤ 40%; VCL ≥ 55 μm/s; LIN ≥ 5; ALH ≥ 2.5; BCF ≥ 12.0.

Media
The basal solution was Tyrode’s (pH 7.5) (Sigma Chemical Co., T2397, St Louis, MO, USA) which was also used as a buffer in the preparation ofNIC and COT at various concentrations. The following solutions were prepared: A, NIC base 70 ng/ml (Sigma Chemical Co., St. Louis, MO, USA); B, COT base 300 ng/ml (Sigma Chemical Co.); C, NIC base 35 μg/ml and D, COT base 190 μg/ml.

The pH of solutions A, B, C and D was then confirmed as being the same as the basal solution.

The concentrations of NIC and COT in solutions A and B (low dosage) were chosen on the basis of results we obtained in a previous work, in which we measured these substances in the seminal plasma of a group of smokers by HPLC (Pacifici et al., 1993b). Solutions C and D (high dosage) were prepared at 500 times the average concentration found in our previous study, to evaluate sperm reaction to massive doses of these substances and to determine if the response differed and in what measure. Such high levels of NIC and COT have never been found in saliva, urine, milk of nursing smokers, cervical mucus and follicular fluid (Sasson et al., 1985; Weiss and Eckert, 1989).

Semen preparation
The samples, collected in sterile plastic jars, were allowed to liquefy for 30 min at room temperature (22°C). The kinetic parameters were then evaluated using both a light microscope and a CASA system. Each sample was then allowed to swim up in Tyrode’s solution with or without the drug as described in the following experimental protocols.

Experimental protocols

Experiment 1
Each seminal sample was divided into five aliquots of 0.5 ml and layering swim-up (Lopata et al., 1976) was carried out. Tyrode’s solution (0.5 ml; pH 7.5) was layered on the first aliquot and 0.5 ml after swim-up in Tyrode’s at time 0 were as follows; spermatozoa were allowed to migrate for 30 min at 37°C, in 5% CO₂. After migration, the five solutions were collected and incubated at 37°C for 24 h. Samples were examined with a light microscope and a CASA system at times 0 (the end of migration), +1, +2, +4, +8 and + 24 h of incubation, in order to determine the effect of NIC and COT on sperm motility, at low and high levels of concentration. As the control, we used spermatozoa migrated in Tyrode’s solution (T), Vital staining with eosin Y (Sigma Chemical Co.) (WHO, 1992) was carried out on the various solutions used. This test, which differentiates between living and dead cells, allows exclusion of a cytotoxic effect of the nicotine and cotinine on the spermatozoa.

Experiment 2
Five of the original ten donors provided a second semen sample. After liquefaction, these samples underwent swim-up in Tyrode’s solution according to the protocol previously set out (experiment 1). The suspensions obtained were divided into two aliquots, one of which was exposed to the effects of cigarette smoke in toto, the other was used as a control. The cigarette smoke was aspirated using a 10 ml syringe, inserting the needle directly into the cigarette filter, taking care not to go beyond the midpoint of the filter. At the end of each aspiration, the smoke collected in the syringe was allowed to bubble into the test tube containing the suspension of spermatozoa in Tyrode’s solution. This operation was repeated until the cigarette was completely finished. After the completion of this procedure, pH was evaluated. Kinetic parameters were then measured at 30 min, 45 min and 60 min starting from the last smoke injection. We used a cigarette brand with the following characteristics; NIC 1 mg and tar 11 mg. Yields of NIC and tar of each brand of cigarette were determined from lists made available by the Italian Ministry of Health.

Vital staining with eosin Y (WHO, 1992) was carried out on all the samples exposed to cigarette smoke 30, 45 and 60 min after exposure.

Statistical analysis
Descriptive statistical analysis was performed on each seminal variable; mean and standard deviation were computed. Then an analysis of variance for repeated measures was carried out separately on the following variables: forward motility, VCL, LIN, ALH and BCF, in order to evaluate the significance of the differences between the mean values obtained in the five groups (T, A, B, C, D). Finally, the same differences were tested by multiple comparison methods to check type I errors. The Bonferroni test was computed on the pairwise comparisons for each variable and Dunnnett procedure was carried out to compare the four treatments (A, B, C, D) to the single control (T). The Bonferroni test was also performed to compare the mean values of each variable observed in spermatozoa exposed to smoke in toto after 30 min, 45 min and in T used as the control (Winer et al., 1991).

Results
The 10 seminal samples from selected subjects showed the following mean values by light microscopy: sperm concentration 85.0 ± 12.2 × 10⁶/ml (range 70–105 × 10⁶/ml), forward motility 64.5 ± 0.04% (range 60–70) and atypical forms 37.4 ± 0.02% (range 34–40). The kinetic parameters evaluated by the CASA system were as follows; VCL 62.3 ± 2.6 μm/s (range 66.2–58.9), LIN 7.6 ± 0.2 (range 6.0–6.6), ALH 4.2 ± 0.2 μm (range 3.8–4.5), BCF 14.1 ± 0.3 Hz (range 13.7–14.5).

The mean ± SD of the sperm parameters of the 10 samples after swim-up in Tyrode’s at time 0 were as follows; sperm concentration 23.6 ± 4.17 × 10⁶/ml (range 18–30 × 10⁶/ml), forward motility 85.5 ± 0.05% (range 80–90), atypical forms 17.0 ± 0.02% (range 15–20), VCL 79.8 ± 1.68 μm/s (range 72.1–84.2), LIN 7.6 ± 0.16 (range 7.3–8.0), ALH 4.9 ± 0.25 μm (range 4.5–5.3) and BCF 15.0 ± 0.35 Hz (range 14.5–15.5). The results at +1, +2, +4, +8 and +24 h were used as the control for the subsequent experiments. The analysis of the results demonstrated that kinetic parameters at +1 and +2 h were similar, but that there was a statistically significant decrease starting at +4 h. The significance varied according to the variables considered from P < 0.05 to P < 0.001.

Experiment 1
Incubation at low and high dosage of NIC and COT
The analysis of the results for A and B demonstrated that kinetic parameters at +1 and +2 h did not differ greatly, but
that a statistically significant decline started at +4 h. The significance varied according to the variables considered; for treatment A, LIN, ALH and BCF $P < 0.001$, VCL $P < 0.01$, forward motility $P < 0.05$; for treatment B, LIN, VCL, ALH and BCF $P < 0.001$, forward motility $P < 0.05$.

The analysis of the results for C and D demonstrated that the percentage of forward motility, VCL, and LIN started declining significantly from +1 h ($P < 0.001$), whereas ALH and BCF showed significant change only after +4 h ($P < 0.001$).

The analysis of variance for repeated measures performed on the five groups (T, A, B, C, D) showed a highly significant statistical difference between the mean values of each considered variable caused by the influence of treatment and time. Multiple comparison tests showed that this significance was due only to the data for treatments C and D.

The results of A and B versus T, referring to the various times of incubation are reported in Figure 1. It can be seen that at time 0 the data were very similar, and at +1, +2, +4, +8 and +24 h they did not show a statistically significant modification for the variables considered as demonstrated by both Bonferroni and Dunnett procedures.

Figure 2 shows the comparison between C and D versus T, over the various times of incubation. At time 0 the data are similar. Multiple pairwise comparisons by the Dunnett method showed a significant statistical difference ($P < 0.01$) between mean values of the variables forward motility, VCL and LIN seen in the three groups, after 1 h or more of incubation. These variables decreased with the time of incubation. The eosin sperm vitality test, performed at all the various times of incubation, showed values similar to the control.

For the variable ALH, a significant increase between C, D and T was found after 4 h of incubation ($P < 0.01$). For the variable BCF, a significant decrease between C, D and T was reached only after 8 h of incubation ($P < 0.01$).

The same significant differences were also found, by the Bonferroni test, between treatments A, B and treatments C and D. No difference was found between A versus B and C versus D respectively.

**Experiment 2**

**Incubation with cigarette smoke in toto**

The pH value varied over time as follows: 0 min: 7.5; 30 min: 7.3; 45 min: 8.0; 60 min: 8.5.

To check whether pH can affect sperm kinetics, we used an additional control with spermatozoa in Tyrode’s solution at pH 7.5 and 8.5. This control showed that motility is unaffected within this range of values.

The eosin sperm vitality tests, performed at the various times of incubation, all showed similar mean values: 85% at +30 min, 84% at +45 min and 86% at +60 min.

The mean ± SD of the five samples after swim-up in Tyrode’s solution were as follows; sperm concentration 23.6 ± 4.17 × 10^9/ml (range 18–30 × 10^9/ml), forward motility 86.0 ± 0.05% (range 80–90%), atypical forms 17.0 ± 0.02% (range 15–20), VCL 78.6 ± 2.23 µm/s (range 75.7–81.3), LIN 7.7 ± 0.21 (range 7.5–8.0), ALH 4.7 ± 0.16 (range 4.5–4.9) and BCF 14.7 ± 0.30 (range 14.3–15.1).

Table I shows data on the kinetic parameters of the spermatozoa 30 min and 45 min after treatment with smoke. The data referring to spermatozoa migrated in Tyrode’s after 30 min and 45 min are not different from baseline.

We carried out all the possible pairwise comparisons (Bonferroni test) on all the parameters taken into consideration and found a highly significant difference ($P < 0.001$) in the mean values detected in the three groups (Tyrode’s, smoke +30 min, smoke +45 min). In particular, at +30 min there was a reduction in the percentage of forward motility, VCL, LIN and BCF, and an increase of ALH. At +45 min there was a further significant worsening of the kinetic parameters.

The evaluation carried out by light microscopy after 60 min of incubation with cigarette smoke showed the complete lack of forward motility. The mean values seen were 5% non-forward progression and 10% non-progressive motility, i.e. only intensive flagellar beating. Because of the kind of movement and the low percentage of motility, the evaluation of the kinetic parameters gave unreliable readings; in fact, with the CASA system a spermatozoon must achieve a minimum VCL to be motile (e.g. 10 µm/s).

**Discussion**

Cigarette smoke is a complex mixture of gas and organic compounds. NIC is one of the most abundant organic substances found both in tobacco and cigarette smoke. It is this substance that is responsible for the inhibiting and stimulating effects on the various organs. Each cigarette contains about 5–15 mg of NIC and for each cigarette smoked about 1 mg of NIC can be absorbed. Inhaled NIC is swiftly oxidated to its main metabolite, cotinine. COT is eliminated more slowly, having a half-life of 19 h versus 2 h for NIC; for this reason COT is a better marker of the smoke absorbed. Cigarette smoke contains a mixture of harmful components such as carbon monoxide (CO), hydrogen cyanide (HCN), ammonia, volatile hydrocarbons, alcohol, aldehydes and ketones (acetalddehyde, formaldehyde, acrolin); some of these substances act as powerful ciliary inhibitors.

There is much debate regarding the toxic effect of cigarette smoke on human reproduction (Fredricsson and Gilljam, 1992). In the literature there are several studies that demonstrate that cigarette smoking is not associated with a reduction of fertilization rates in couples undergoing IVF (Hughes et al., 1994) or with modifications of seminal parameters (Dunphy et al., 1991). However, other authors have reported a reduction in fertilization rates (Rosevear et al., 1992) and in semen volume (Holzki et al., 1991), concentration and motility (Shaarawy and Mahmoud, 1982; Vine et al., 1996). A worsening of sperm morphology has been shown as well as a reduced capacity of spermatozoa to undergo the acrosome reaction (Evans et al., 1991; El Mulla et al., 1995). In addition, mutagenic effects on germ cells (Schmidt, 1986) have been observed, and mutations in spermatozoa have been noted that could lead to cancer and genetic diseases (Fraga et al., 1996). A recent study evaluating testicular function and semen quality demonstrated a decrease in sperm fertilizing potential in smokers. The authors stated that this effect could be ascribed to...
to the detrimental effects of smoking on the spermatozoal cytoskeleton, deriving from disturbances during spermatogenesis, and the epididymal sperm maturation process (Sofikitis et al., 1995).

In a previous study, we tested the seminal plasma of smokers and non-smokers for NIC and COT, and showed that there are high levels of these substances in smokers’ seminal plasma (at the same level as that present in blood). The comparison of the seminal parameters of smokers and non-smokers clearly indicates a statistically significant reduction of the percentage of motile spermatozoa in smokers (Pacifici et al., 1993b).

In this study, we used an in-vitro model to establish whether...
the reduction of the motility previously demonstrated was due to the direct effect of NIC and COT on the sperm kinetic parameters or to other substances present in the cigarette smoke. Considering the results of our previous paper (Pacifici et al., 1993b), we utilized NIC and COT at the same levels found in the seminal plasma of smokers, and also at a dose 500 times more concentrated. Since the results obtained with the higher dose showed continued motility even after the final incubation, we decided not to seek further intermediate dose–response data.
In addition, we evaluated the effect of the cigarette smoke in toto, directly at the level of the motile spermatozoa.

The most interesting finding of this study is the demonstration that, in vitro, NIC and COT, at the levels found in the seminal plasma of smokers, do not affect sperm motility. In fact, the values of VEL, LIN, ALH and BCF vary during the experiment in the same way as the control. When we used a dose 500 times more concentrated than the previous level, we saw a sharp reduction of all sperm parameters except ALH, which increased. Despite the impact seen on these parameters, there was still 30% forward motility after 24 h of incubation. On the basis of these data, we can postulate that this is a consequence of a toxic aspecific effect of the high concentration of this substance, rather than a specific mechanism acting on the structure responsible for the flagellar motion.

With regard to the action of the smoke in toto (experiment 2), our data, in agreement with the literature (Makler et al., 1993), showed a sharp reduction in all the sperm kinetic parameters. This was not due to pH variation, as shown by the control carried out in Tyrode’s solution. This great reduction, after 30 min of exposure to smoke, progressively increased until absolute immotility after 1 h. Using the eosin vitality test (WHO, 1992), we demonstrated that the effect of these substances is on the moving apparatus, excluding a cytotoxic action of the smoke on the spermatozoa.

In conclusion, we have shown in vitro that the harmful effect of cigarette smoke on sperm kinetic parameters is not a consequence of NIC and COT. In-vivo conditions are obviously somewhat different and the chronic exposure of spermatozoa to smoke could produce different results. However, this is a common limitation in all toxicological in-vitro studies. Our results on NIC and COT would suggest that other compounds (i.e. hydrocarbons, aldehydes, ketones, etc.) found in the gaseous phase are responsible for smoke-induced sperm motility damage. In fact, these substances have been shown to be responsible for the total inhibition of ciliary movement at the bronchial level (Marchese-Ragona and Johnson, 1990). These inhaled compounds are absorbed at the level of the blood circulation and, after passage through the blood–testis barrier, can act on mature spermatozoa and on their precursors, provoking the alterations of the seminal fluid found in heavy smokers (Rantal a and Koskimies, 1987; Makler et al., 1993). This research indicates the need for further studies on the other compounds found in the gaseous phase in order to understand better the harmful effects of smoke on spermatozoa.

**Table I.** Means of kinetic parameters of spermatozoa migrated in Tyrode’s solution 30 min and 45 min after treatment with smoke

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>Tyrode</th>
<th>Smoke 30 min</th>
<th>Smoke 45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward motility (%)</td>
<td>86.0 ± 0.05</td>
<td>40.0 ± 0.04</td>
<td>23.0 ± 0.04</td>
</tr>
<tr>
<td>Velocity (µm/s)</td>
<td>78.6 ± 2.2</td>
<td>56.5 ± 2.5</td>
<td>34.0 ± 3.0</td>
</tr>
<tr>
<td>Linearity (index)</td>
<td>7.8 ± 0.2</td>
<td>5.0 ± 0.1</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td>ALH (µm)</td>
<td>4.7 ± 0.3</td>
<td>5.5 ± 0.3</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>14.7 ± 0.3</td>
<td>13.5 ± 0.3</td>
<td>11.4 ± 0.5</td>
</tr>
</tbody>
</table>

ALH = amplitude of lateral head displacement; BCF = beat cross-frequency.

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