

SHORT COMMUNICATION

RELATIONSHIP BETWEEN THE LEVEL AND TIME OF EXPOSURE TO TOBACCO SMOKE AND URINE NICOTINE AND COTININE CONCENTRATION

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The study attempts to evaluate whether it is possible to determine time and level of exposure of rats to tobacco smoke based on nicotine and cotinine content in urine.

The animals were exposed to tobacco smoke by inhalation in a specially designed experimental chambers. The exposure to three different tobacco smoke levels (500, 1000 and 1500 mg CO/m³ of air) lasted 6 h per day, for one, three and five days. Nicotine and cotinine concentrations were measured in daily urine using high performance liquid chromatography procedure developed by the authors.

It has been shown that cotinine but not nicotine can be used as a biomarker of time and extent of exposure to tobacco smoke.

Key words: *cotinine, nicotine, tobacco smoke, biomarkers*

INTRODUCTION

Tobacco smoking is one of few well-known hazardous factors people are voluntarily exposed to. Biological co-influence of many carcinogenic and toxic substances combined with addictive character of nicotine causes the tobacco to be considered dangerous poison of the twentieth century. Moreover, it is a factor that might be avoided. Epidemiological data obtained from many countries clearly indicate close correlation between tobacco smoking and occurrence of various dangerous diseases [18, 24]. Harmful influence of tobacco smoking on human body affects practically all its organs and its effects depend on intensity and on the method of smoking [3, 5, 8]. Today tobacco smoking is not only the highest preventable cause of premature death in adult population and other demographic problems, but also one of the most important elements of attitudes toward health and lifestyle of people in Poland [25]. As tobacco smoking, due to its effects, became not only a health problem, but also a social and economic one, an emphasis should be placed on scientific research which results might aid anti-tobacco prophylaxis, which is much more effective than fighting smoking [6].

To date, over 4000 components of tobacco smoke have been identified: about 400–500 appear in the gaseous phase and about 3500 in particulate phase of tobacco smoke. Components of tobacco smoke are present in inhaled smoke and may be detected in smokers and in passive smokers [1, 10, 21]. Indicators frequently used as markers of tobacco smoke exposure include carbon oxide (CO) in exhaled air, cotinine in plasma, urine, saliva and milk, carboxyhemoglobin (HbCO) in blood, nicotine in plasma and urine and thiocyanate in plasma, urine and saliva [2, 12, 15, 19].

Among the abovementioned biochemical markers of tobacco smoke exposure, nicotine and cotinine fulfil the condition of specificity. Nicotine use for that purpose may be limited by its short biological half-life. Cotinine seems to be more suitable, as its $t_{0.5}$ values in humans and rats are several times higher than those of nicotine [2, 7, 8, 13, 15, 20, 22]. The purpose of the study was establishing the correlation between level and duration of exposure to tobacco smoke and concentration of nicotine and cotinine in urine.

MATERIAL and METHODS

Animals

We used male white Wistar rats with body weight 250 ± 10 g bred at Department of Toxicology of the University of Medical Sciences (Poznań, Poland). To adapt the animals to the new environment, the rats were kept at our facilities for 3–5 days in groups of 2–3 animals. The 12/12 hours light/dark cycle was maintained throughout the study. The animals were housed in Tecniplast (1291H001) stainless-steel cages, which were maintained at $20 \pm 1^\circ\text{C}$ and $60 \pm 10\%$ relative humidity. The animals were fed standardized normal Labofeed (Feeds and Concentrates Production Plant, Certificate of Quality System No 181/1/98, Kcynia, Poland). The animals were fed every day at the same time in the morning with 10% surplus to the amount consumed on the previous day. Water was available *ad libitum*. The total number of animals was 54.

Experimental procedure

The rats' whole body was exposed in toxicological dynamic chamber [4] to the tobacco smoke generated from Polish brand of cigarettes without a filter tip. One cigarette contained 0.93 mg of nicotine, 11.6 mg of tar, and tobacco smoke generated from it contained 10.71 mg of CO. To mimic the exposure to environmental tobacco smoke (ETS), the tobacco smoke generated by a smoking machine at the rate corresponding to smoking 15, 25 or 35 cigarettes per hour was mixed with the atmospheric air. The concentration of tobacco smoke within the chamber was continuously controlled by the concentration of CO, and was maintained at the level of 500, 1000 and 1500 mg CO/m³ of air, respectively, for three different doses of generated tobacco smoke. The oxygen concentration was kept at the level of $20 \pm 0.5\%$ of the volume. There was continuous airflow within the chamber with the tenfold air exchange per hour. The temperature and humidity were maintained at the standard level. During exposure in one toxicological chamber there were no more than eighteen animals in cages (no more than three animals in one cage). Animals were exposed 6 h a day.

The rats were divided into 9 experimental groups of 6 animal each and exposed in dynamic toxicological chamber [4] to tobacco smoke in the condition described below.

- A. Animals exposed to tobacco smoke containing 500 mg CO/m³ of air: Group I – one-day exposure, Group II – three-day exposure, Group III – five-day exposure.
- B. Animals exposed to tobacco smoke containing 1000 mg CO/m³ of air: Group I – one-day exposure, Group II – three-day exposure, Group III – five-days exposure.
- C. Animals exposed to tobacco smoke containing 1500 mg CO/m³ of air: Group I – one-day exposure, Group II – three-day exposure, Group III – five-days exposure.

During exposure, the appearance and the behavior of the animals have been observed. After the last day of exposure, the animals were separately placed in metabolic cages where the urine samples were collected over a period of 24 h. Biomarkers of exposure to tobacco smoke nicotine and its major metabolite cotinine were measured in urine of rats.

Analytical method

Nicotine and cotinine analysis was carried out using a high performance liquid chromatography (Beckman System Gold) method developed by us and described below. Two milliliters of urine sample were mixed with an internal standard (anti-pyrene) and 1 ml of saturated borate buffer pH 9. The prepared sample was transferred into a tandem of OCTYL C-8 and SILICA GEL columns (J.T. Baker). Cotinine was eluted from the columns with 1ml aliquots of 70% methylene chloride and 30% methanol containing 1% ammonium hydroxide [11]. The extract was dried under nitrogen stream and then reconstituted in 0.2 ml of mobile phase. Nicotine and cotinine levels were determined by HPLC with Bakerbond Silica Gel column (4.6 × 250 mm), maintained at a temperature 25°C, with spectrophotometric detection ($\lambda = 259$ nm). The mobile phase contained 95% of dichloromethane, 5% of methanol containing 1% of ammonium hydroxide. The range of determination was 0.1–50 µg/ml. The retention times were 2.79 for nicotine and 4.02 for cotinine. The detection limit for both compounds was 20 ng/ml, and the coefficient of variation was below 10%.

Statistical analysis

For comparisons of the concentrations of nicotine and cotinine in different experimental groups, the analysis of variance (ANOVA) and Students' *t*-test were used [16].

RESULTS and DISCUSSION

Tobacco smoke is one of the major civilizational hazards of our times [25]. Still large part of population are smokers and almost everyone is exposed to ETS, so-called passive smoking, at home, work and entertainment places [14, 23]. Tobacco smoke is a well-documented factor of many illnesses (e.g. heart, lung diseases).

For monitoring the exposure to tobacco smoke, many biomarkers are used [2, 15, 17] including carbon monoxide, thiocyanates, protein and DNA adducts, nicotine and the most popular cotinine.

The latter two indicators were chosen as biomarkers of tobacco smoke exposure in current studies. Nicotine and cotinine were measured in urine by high performance liquid chromatography (HPLC) method developed and validated in our laboratory. The animals were exposed to tobacco smoke by inhalation in specially designed experimental chamber. The exposure has been performed at three different levels of tobacco smoke based on concentration of carbon monoxide in air: 500, 1000 and 1500 mg/m³. The exposure lasted 6 h per day during 1, 3 and 5 days. The concentration of nicotine and cotinine were measured in daily urine.

In the course of the experiment, appearance and behavior of the animals were observed. All rats behaved similarly: after the inhalation they showed anxiety, which subsided after more than 10 min. Then the animals fell asleep. After the exposure ended, they demonstrated abnormal mobility.

Generally, the concentration of nicotine did not increase in urine of animals, which were repeatedly exposed to tobacco smoke at the studied concentrations. Only after exposure to the smoke level corresponding to 1000 CO/m³ of air, the concentrations of nicotine were higher after 3 and 5 days of exposure compared to one-day exposure (Tab. 1, Fig. 1). In case of one-day experiment, only after exposure to the highest smoke concentration, i.e. 1500 mg CO/mg³, significant increase in nicotine content in urine was detected in comparison with the lower smoke concentrations (500 and 1000 mg CO/m³). On the average, the concentrations were three times higher (Tab. 1). Repeated exposure to tobacco smoke (3- and 5-day exposure) caused two- and three-fold increase in nicotine concentration in animals inhaling smoke at a concentration corresponding to 1000 or 1500 mg CO/m³ of air in comparison to one-day exposure. The values were comparable re-

Table 1. Concentration of nicotine and cotinine in the urine of rats depending on the tobacco smoke concentration and time of exposure

	CO concentration in air [mg/m ³]	Urine concentration \pm SD [μ g/ml]					
		Nicotine			Cotinine		
		Time of exposure [day]					
		1	3	5	1	3	5
	500	8.09 \pm 2.14	9.77 \pm 2.87	9.16 \pm 2.34	3.36 \pm 0.17	4.35 \pm 1.42 ^c	10.72 \pm 1.39 ^{c,d}
	1000	7.57 \pm 1.63	19.51 \pm 4.23 ^{a,c}	16.92 \pm 2.23 ^{a,c}	4.32 \pm 1.18	7.33 \pm 1.81 ^{a,c}	11.79 \pm 4.33 ^{c,d}
	1500	23.38 \pm 6.18 ^a	28.05 \pm 7.08 ^{a,b}	26.91 \pm 6.88 ^{a,b}	6.07 \pm 1.91 ^a	10.65 \pm 1.02 ^{a,b,c}	15.08 \pm 3.99 ^{a,c,d}

a – significant vs 500 mg CO/m³, $p < 0.001$; b – significant vs 1000 mg CO/m³, $p < 0.001$; c – significant vs one-day exposure, $p < 0.001$; d – significant vs three-day exposure, $p < 0.001$

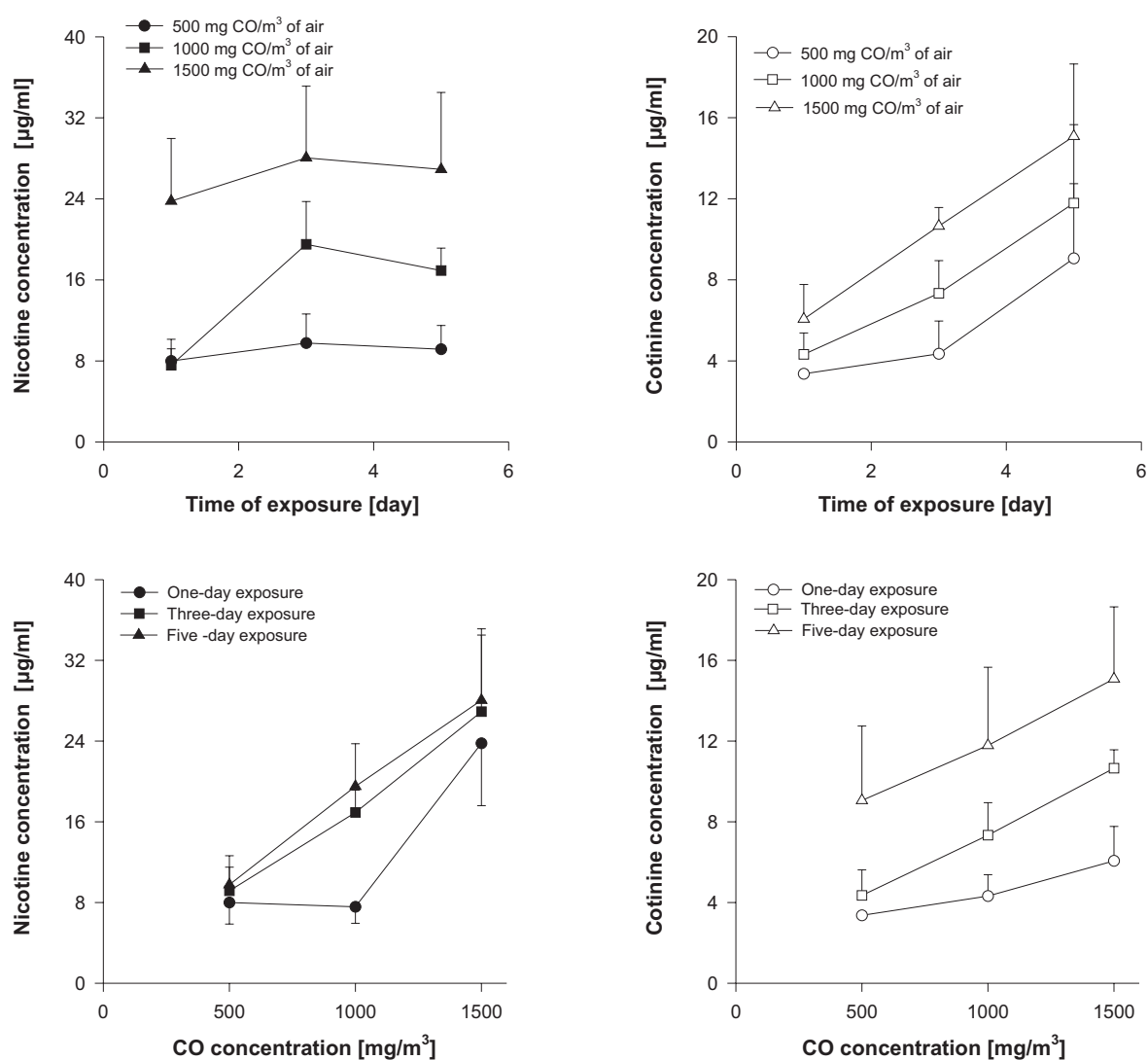


Fig. 1. The effect of the level (upper panels) and duration (lower panels) of exposure to tobacco smoke on nicotine and cotinine level in urine of rates

ardless of the exposure time and averaged: 16.92 $\mu\text{g}/\text{cm}^3$ (for 1000 mg CO/m^3 of air) and 26.91 $\mu\text{g}/\text{cm}^3$ (for 1500 mg CO/m^3 of air) (Tab. 1).

No correlation between the exposure time to tobacco smoke and amount of excreted unchanged nicotine in urine was observed. The exposure time did not influence the nicotine level in urine because of short biological half-life of nicotine, which for rats is equal approximately to 0.9 h, for humans 1–2 h. It does not accumulate and is quickly eliminated from an organism [13, 22].

Research done by other authors determined that after a person smokes one cigarette, nicotine in his blood reaches 20–30 ng/cm^3 and then drops rapidly, which is caused by distribution to body tissues and elimination (metabolism and excretion). In research conducted on people smoking over 16 cigarettes per day, nicotine concentrations ranged 0.2–20 $\mu\text{g}/\text{ml}$. [9]. The results obtained in this experiment are very similar.

Results concerning concentration of cotinine, the main metabolite of nicotine, in urine of rats exposed to tobacco smoke are presented in Table 1 and Figure 1. Research results have shown that both the quantity of tobacco smoke and time of exposure influence cotinine excreted in urine. An increase in metabolite concentration paralleled an increase in smoke concentrations, although statistically significant differences might be seen only in several cases. The level of cotinine ranged between 3.36 $\mu\text{g}/\text{ml}$ (for 500 mg CO/m^3 of air) to 6.07 $\mu\text{g}/\text{ml}$ (for 1500 CO/m^3 of air) in case of one-day exposure (Tab. 1, Fig. 1). Three- and five-day exposure caused an increase in metabolite quantity to 11.79 $\mu\text{g}/\text{ml}$ and 15.08 $\mu\text{g}/\text{ml}$, respectively.

The experiment was design also to evaluate the influence of time of exposure on quantity of excreted cotinine. When smoke concentrations were constant, prolongation of exposure caused statistically significant increase in the metabolite level in urine (Fig. 1). This relationship was observed at all levels of exposure. For dose of smoke corresponding to 500 mg CO/m^3 , the increase was from 3.36 $\mu\text{g}/\text{ml}$ (one-day) exposure, to 10.72 $\mu\text{g}/\text{ml}$ (5-day exposure), and for a dose of 1500 mg CO/m^3 , the level dropped from 6.07 $\mu\text{g}/\text{ml}$ to 15.05 $\mu\text{g}/\text{ml}$, respectively. The concentration of cotinine in rat urine was well correlated not only with the time of exposure (as nicotine) but also with the number of smoked cigarettes (concentration of carbon monoxide) (Fig. 1).

Comparing the possibility to apply nicotine and cotinine as biomarkers of tobacco exposure (active and passive smoking), it can be concluded that nicotine reflects only level of exposure to tobacco smoke within short time after inhaling, while cotinine can be used as a marker of time and intensity of smoking or exposure to tobacco smoke.

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