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LONG-TERM EFFECTS OF INHALED NICOTINE

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Summary

Tobacco smoking has been reported to be associated with increased risk of cardiovascular disease and cancer, particularly of the lungs. In spite of extensive research on the health effects of tobacco smoking, the substances in tobacco smoke exerting these negative health effects are not completely known. Nicotine is the substance giving the subjective pleasure of smoking as well as inducing addiction.

For the first time we report the effect on the rat of long-term (two years) inhalation of nicotine. The rats breathed in a chamber with nicotine at a concentration giving twice the plasma concentration found in heavy smokers. Nicotine was given for 20 h a day, five days a week during a two-year period. We could not find any increase in mortality, in atherosclerosis or frequency of tumors in these rats compared with controls. Particularly, there was no microscopic or macroscopic lung tumors nor any increase in pulmonary neuroendocrine cells. Throughout the study, however, the body weight of the nicotine exposed rats was reduced as compared with controls. In conclusion, our study does not indicate any harmful effect of nicotine when given in its pure form by inhalation.

Key Words: body weight, inhalation, nicotine, side effects, tumorigenesis

Smoking of tobacco increases the risk of lung cancer (1), bladder cancer (2) and cardiovascular diseases (3). Tobacco smoking, accordingly, results in major health problems. Whereas there is no doubt that nicotine is the substance in tobacco that elicits the stimulating effects, and also induces physical dependence (4), the role of nicotine in the negative health effects of tobacco is not known. The aim of the present study was to give nicotine as it is presented to smokers, that is by inhalation, to rats for a substantial proportion of their life time and assess its effect on the risk of developing neoplasia and atherosclerosis. We focused on the effect on the lungs and especially pulmonary neuroendocrine cells since oat cell carcinoma is a cancer type particularly increased in smokers (5). Oat cell carcinomas in the lungs are believed to be of neuroendocrine origin (6).

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Methods

The study was approved by the Animal Welfare Committee of the University Hospital of Trondheim.

Female Sprague-Dawley rats (approximately two months old) weighing 240 g at the start of the study (Møllegaard Breeding Centre, Denmark) were used. Female rats were chosen because females may be more disposed to tobacco induced lung carcinomas than males (7). The rats were housed in cages in three stainless steel chambers designed as a cube with a volume of 650 l with a conical top and bottom. In each chamber there were four cages with a maximum of eight rats in each. The main chamber air stream (hospital medical quality air) was drawn through the chamber from the top inlet and evacuated by suction at the bottom outlet. The laminar flow of air obtained created a slight negative pressure (1 - 2 mm H_2O) inside the chamber. The chamber air streams were 130 l/min, giving twelve changes of air per h. The temperature in the chambers was 20 ± 1°C and the humidity varied between 50% to 80% as influenced by the hospital medical quality air (8).

Nicotine exposure

Two of the chambers (A,B) were exposed to nicotine vapor by bubbling medical quality air (2.2 l/min) through a reservoir with approximately 60 ml of nicotine (> 99% pure) (Fluka AG, Buchs, Switzerland) at 25°C. The stream of air containing the nicotine vapor was passed through a drop catch bottle before being split into two and mixed with the main air streams of the chambers A and B, respectively. The nicotine concentration was determined in air and rat plasma extracts by gas chromatography/mass spectrometry (GC/MS) method using a Hewlett Packard HP 5890 Series II gas chromatograph equipped with HP MSD 5972, a mass selective detector and a HP 7673 autosampler (Hewlett-Packard, Palo Alto, CA., USA), using 2,6 butyl-4-methylphenol (BHT) (> 99% pure) (Fluka AG, Buchs, Switzerland) as standard (9).

Air was sampled from the exposure chambers by draining a small flow (1 l/min) of chamber air, and nicotine was extracted by passing the air through a gas washing bottle containing 40 ml of ethanol (96.9%, rectified) (Vinmonopolet AS, Oslo, Norway) (9).

Nicotine in rat plasma was determined according to Feyerabend (10) using BHT as standard. Nicotine and the internal standard were detected by selected ion monitoring. For nicotine, a mass charge ratio (m/z) of 162 or 84 was used for quantitative calculations, while m/z 133 was used as qualifier ion. For the internal standard, m/z 220 was used quantitatively with m/z 205 as qualifier. Preliminary experiments were done to determine the nicotine vapor concentration in the chamber giving a plasma nicotine concentration exceeding that seen in heavy smokers (11). A chamber concentration of about 500 $\mu g/m^3$ was found to give a plasma nicotine concentration slightly above 100 ng/ml without affecting the rats' well-being, and was accordingly chosen for the exposure study.

Study design

Sixty-eight rats were exposed to nicotine (chamber A and B) with 34 rats serving as controls and placed in chamber C. The rats in chamber A and B were exposed to nicotine 20 h a day five days a week. On the nicotine exposure days the rats (nicotine exposed and controls) were fed only from eight o'clock to twelve noon each morning during which the cages were taken out of the chambers, and the rats were fed chow and tap water ad libitum. To prevent peroral ingestion of nicotine due to condensation on the chow, no food was given while the rats were in the exposure chambers.

During the weekends (Saturday and Sunday) nicotine vapor was turned off and the rats were fed freely. After five days of nicotine exposure, two controls and four nicotine exposed rats were anaesthetized with 0.2 ml/100 g body weight of a solution containing fluanisone 2.5 mg/ml, phentanyl 0.05 mg/ml and midazolam 1.25 mg/ml (Hypnorm/Dormicum), and blood was collected by heart puncture for determination of nicotine concentration in plasma. Similarly, nicotine

concentration was determined at the end of the study, that is after two years of nicotine exposure. Moreover, after six, twelve, and eighteen months control and nicotine exposed rats were similarly anesthetized, blood was drawn whereafter the rats were grossly examined for tumors in the brain, lungs, gastrointestinal tract, liver, kidney and ovaries, and tissues fixed in formalin for later histological examination after hematoxylin-eosin staining. At the end of the study (after 24 months) the remaining rats - seven controls and 22 nicotine exposed - were similarly sacrificed during anesthesia and examined for tumors as well as atherosclerosis (Table I). At that time the effect of nicotine on the pulmonary neuroendocrine cells was also examined. Thus, the right lung was immediately fixed by intratracheal installation with Bouin's fixative and subsequently fixed for approximately 18 h by immersion in the same fixative. Thereafter the right middle lobe was used for histological examination due to the convenient size of this lobe. It was rinsed in 50% ethanol and subsequently dehydrated through graded alcohol. The lobe was divided parallel to the bronchus and embedded in paraffin and sections were then cut parallel to this plane. Accordingly, the lobar bronchus and the largest branches of this bronchus were always present in at least one section. The neuroepithelial bodies (NEBs) (12) were identified by applying rabbit antiserum against calcitonin gene related peptide (CGRP) (Amersham, code RPN 1842) diluted 1:400 at room temperature for 1 h (13). After appropriate washing the sections were incubated with peroxidase conjugated porcine anti-rabbit immunoglobulins and subsequently visualized using 3,3'-diaminobenzidine. In each NEB identified on serial sections the number of cells with a visible nucleus was counted. This method was chosen to detect whether nicotine had stimulated the growth of the NEBs. At least 10 NEBs per lung were examined.

The rats from each cage were weighed in a lump once weekly. During the study the nicotine air concentration was determined once or twice a week throughout the two-year period of exposure with a fairly constant nicotine concentration in the air (501 ± 151 $\mu g/m^3$) (Fig. 1).

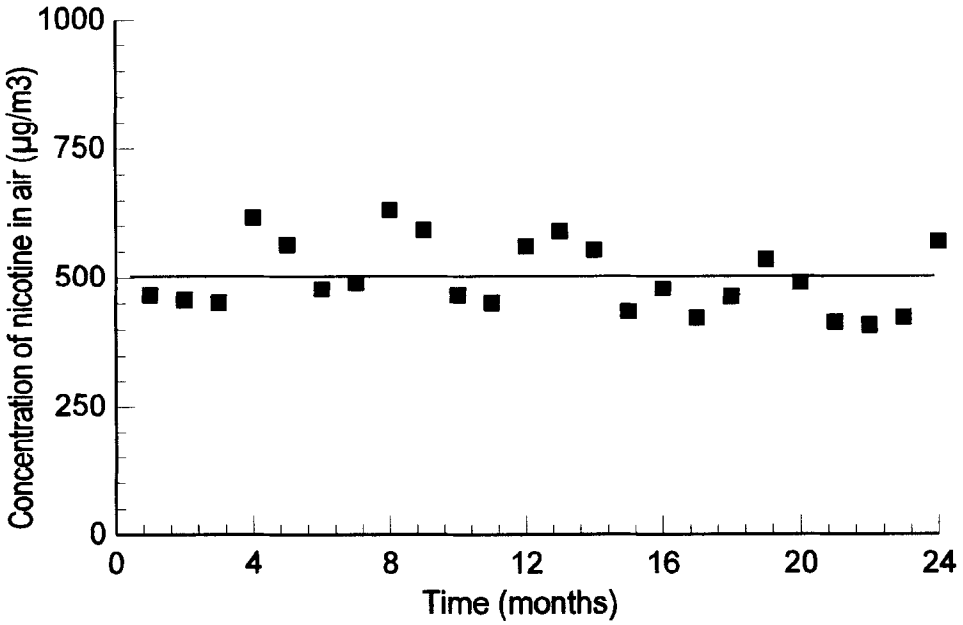

Fig.1
The concentration of nicotine in the exposure chamber during the study.

Rats showing general misthriving at observation (bristling fur, emaciation and shiny eyes) were withdrawn (seven controls (22%) and ten nicotine exposed (16%) rats). Whenever possible,

withdrawals were examined. Animals withdrawn out of schedule and not examined were classified as "Drop-outs" (Table I).

TABLE I

List of rats investigated and Drop-Outs

Week	Controls			Exposed to nicotine		
	Number of animals remaining	Number examined	Number of Drop-Outs***	Number of animals remaining	Number examined	Number of Drop-outs
0	34			68		
1	32	2**		64	4**	
12	31		1	64		
26	25	6		54	10	
39	24		1	54		
48	23		1	54		
53	17	6		44	10	
57	16		1	44		
58	16			43		1
64	16			42	1*	
65	16			41		1
67	16			40		1
71	15		1	40		
73	14		1	40		
74	14			39	1*	
76	14			38	1*	
77	9	5		30	8	
89	9			29	1*	
94	8		1	29		
98	8			24	5	
98	8			23		1
99	7	1*		22		1
103	0	7		0	22	

* Withdrawn out of schedule due to general misthriving and examined, **Preliminary test of animals for plasma nicotine concentration (not examined), ***Withdrawn out of schedule due to general misthriving, but not examined.

Examinations for atherosclerosis

The rat hearts were weighed, and inspected macroscopically and then fixed in a solution consisting of 5% acetic acid, 4% formaldehyde, and 85% ethanol. The specimens were kept in this solution for three days before changing to buffered formaldehyde (pH = 7). The hearts with the coronary arteries were cut in consecutive cross sections for microscopic examination.

The aorta was examined for fatty streaks and atherosclerotic plaques, then fixed as for the hearts, and cross sections from the ascending and abdominal regions were cut for microscopic analysis.

Statistics

Fischer's exact test and Mann-Whitney test were used to evaluate differences between the groups.

Results

The nicotine concentration in the inhalation chambers was kept constant during the whole study resulting in a nicotine concentration slightly above 100 ng/ml in the exposed rats, and not detectable in the controls (Table II).

TABLE II

Nicotine in rat plasma, ng/ml.

Week	Controls			Exposed		
	Mean	SD	n	Mean	SD	n
1	Not detectable*	-	2	108.4	55.1	4
103	Not detectable*	-	6	129.8	43.0	17

* Detection limit 2 ng/ml.

At the start of the study there was no difference between the weights of the controls and the rats to be exposed for nicotine (Fig. 2). Throughout the study the nicotine exposed rats weighed less than the controls (Fig. 2).

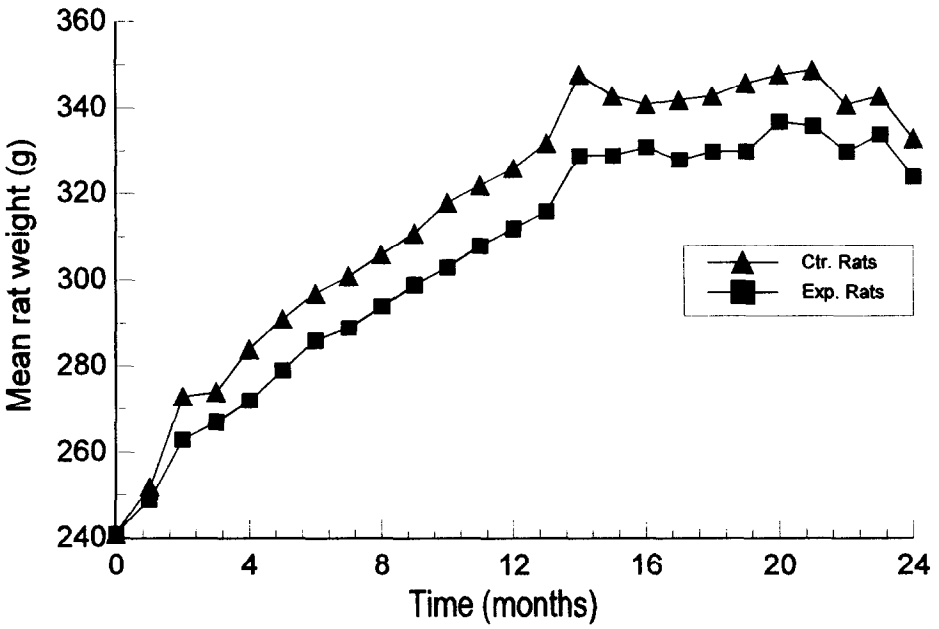

Fig. 2
The mean weight of nicotine exposed and control rats during the study.

The number of withdrawals was similar in the nicotine exposed (22%) and control (25%) group (Table I).

Tumors

Fibroadenomas of the mammary gland were the most frequent tumor, occurring with a similar frequency among controls and exposed rats (Table III).

TABLE III

Occurrence of tumors in female Sprague-Dawley rats exposed to nicotine for up to 24 months and controls.

Tumors site and type	Controls (n = 25)	Nicotine exposed (n=59)
Mammary gland	6	9
Fibroadenoma	0	1
Adenocarcinoma		
Pituitary gland		
Adenoma	0	4
Atypical adenoma	0	1
Ovary		
Granulosa-theca cell tumor	0	1
Adenocarcinoma	0	2
Skin		
Histiocytoma	0	1
Metastasis (origin unknown)		
Liver	0	1
Abdominal cavity	0	1
Total number of rats with tumors	6	21 (36 %)

Tumors of the anterior pituitary gland were encountered among the nicotine exposed rats only. The tumors ranged in size from 3 - 7 mm causing compression of the normal glandular elements. Microscopically, most tumors were composed of well differentiated cells forming nodules and occasional tubular structures, often with extensive signs of recent or old hemorrhages. In one of the tumors there was a marked cellular atypia with frequent mitosis. Tumors of the ovary were seen in three instances among the exposed rats, two tumors representing moderately differentiated adenocarcinomas and one showing the features of a granulosa-thecal cell tumor.

Furthermore among the exposed rats there were two cases in which metastatic tumors from adenocarcinomas were found in the liver and in the abdominal cavity, respectively, and where the origin of the primary tumor could not be identified. However, there was no significant difference in the occurrence of tumors between the two groups.

Lungs

No tumor was detected either on macroscopic or on microscopic examination of the lungs. Furthermore, in a total of 119 sections from 28 rats (six controls and 22 nicotine exposed), 84 and 295 NEBs were examined in the control and nicotine exposed group, respectively. The mean number ± SEM. of nuclei per NEB was 7.9 ± 0.8 in the controls and 6.8 ± 0.3 in the nicotine exposed rats (N.S.).

Cardiovascular system

Macroscopic examination of the hearts did not reveal any abnormalities, neither in the controls nor in the nicotine exposed rats. The median weight of the hearts from the control group was 1.24 g (range 1.20 - 1.49 g), and for the nicotine group 1.25 g (range 0.90 - 1.58 g) ($p>0.1$). The thickness of the ventricular walls did not differ between the two groups. Microscopically no difference between the hearts from the two groups was observed. The myocardium appeared normal with no inflammation, atrophy, hypertrophy, or infarction. Furthermore, the coronary arteries were macroscopically and microscopically normal in both groups.

No atherosclerotic lesions were found in cross sections from the aorta. Microscopic examination did not reveal any differences between the control or the test group. The endothelium appeared normal. Foam macrophages, proliferation of myointimal cells, fibrosis, inflammation, or destruction of the lamina elastica interna were not observed.

Discussion

In the present study we did not find any tumorigenic effect of nicotine on the lungs. This is in accordance with previous studies in man where only smoking of tobacco (1) and not oral tobacco intake has been associated with lung tumors. On the other hand, using nicotine inhalation the nicotine concentration in the lungs would be expected to be higher locally in the lungs than what is found after oral tobacco use. Therefore, our study shows for the first time that nicotine itself shows no tumorigenic effect on the rat lung. It may be argued that there may be species differences which may reduce the importance of the present findings. It should, however, be recalled that species differences most often are of quantitative and not qualitative nature. Moreover, the smoking induced lung tumors are not seldomly of the small cell type (5), probably derived from the Kultschitzky neuroendocrine cell (6). Taking into consideration the similarities between the neuroendocrine cells of the foregut derived organs, the lungs and the stomach (14) and the similar regulation of the gastric neuroendocrine cells in the rat and man (15), it may be assumed that the findings from our rat study have relevance also for man. The lack of any tumorigenic effect on the lungs of inhaled pure nicotine supports the theory that tar and other hydrocarbons, and perhaps particularly nitrosamines, in the tobacco smoke are responsible for the tumorigenic effect (16). Since tobacco smoke inhalation may induce pulmonary neuroendocrine hyperplasia (17) and neuroendocrine cells are important in the tumorigenesis in smokers (5,6), the lack of neuroendocrine hyperplasia recorded in the present study also supports the view that nicotine itself does not have any tumorigenic effect on the lungs.

We did not find any tumorigenic effect of nicotine on any organ in the body. The frequencies of tumors in the female nicotine exposed rats were in the same range as found spontaneously in female Wistard rats (18) and also not significantly different from our control group. Although there was no significant difference in the frequency of pituitary tumors between nicotine exposed and control rats, such tumors were only found in the nicotine exposed animals (Table III). The neuroendocrine actions of nicotine have previously been focused on (19), and it is possible that the recorded tendency to an increase in pituitary tumor frequency in nicotine exposed rats may indeed reflect such an action. A slight tumorigenic effect of nicotine on the pituitary may have been missed in this rather small series. The effect of nicotine on the process of atherosclerosis has hitherto been unclear (20). Smoking has been reported to induce coronary vasospasm (21). The mechanism behind this effect has not been revealed (21). We did not find any atherogenic effect of nicotine. Neither did we see any increase in mortality in the nicotine exposed rats.

Throughout the study, nicotine exposed rats weighed less than the controls. An increase in body weight after cessation of smoking is well known. Moreover, in a more short-term study where nicotine was administered via osmotic minipumps placed subcutaneously, a similar effect on the body weight as found in the present study, was recorded (22). The effect of nicotine on the body weight has been suggested to be caused by a stimulation of the metabolic rate (23).

Recently it has been reported that smoking may reduce the tendency to develop Alzheimer's disease (24), Parkinson's disease (25) and ulcerative colitis (26). In ulcerative colitis the beneficial component of smoking seems to be nicotine, since transdermal nicotine treatment actually has been shown to be beneficial to patients with ulcerative colitis (27). It is also reason to believe that the possible positive effect of tobacco smoking on Alzheimer's disease (24) and Parkinson's disease (25) is mediated by nicotine since nicotine has a well known effect on cerebral activity (28). The results of the present study showing no negative effect of nicotine on healthy rats during a nearly lifelong exposure may indicate that nicotine, if used, should be inhaled as a pure substance (29).

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