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Full Length Research Paper

The effects of tobacco smoke generated from cigarettes exposed to pulsed electromagnetic field in the rat

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In this study, we have investigated the toxic effects of tobacco smoke obtained from cigarettes treated in pulsed electromagnetic field in the rat. The treatment of cigarettes was accomplished before the initiation of animal studies after exposure of standard cigarettes to pulsed electromagnetic irradiation of low power (>10^{-7}W) and wide frequency spectrum (30 Hz to 300 GHz) during 24 h according to the technology described in patents WO 01/26493, EP 1092354, US 2004/0206366A1 and AU2933700 entitled "Method for the Qualitative Improvement of the Products of Tobacco Plant". Rats of both sexes were exposed to tobacco smoke obtained after burning 16 or 32 cigarettes per day during 90 days. The toxicity was assessed on the basis of clinical appearance, changes in behavior, biomarkers of exposure to tobacco smoke (carboxyhemoglobin in blood and thiocyanates in serum) and histopathological and morphological analysis of the lungs of all animals included in the study. The results obtained have shown that, the smoke generated from treated cigarettes formed significantly lower amount of carboxyhemoglobin in rats of both sexes when compared to standard nontreated cigarettes and the effect was dose-dependent. Tobacco smoke obtained from standard nontreated cigarettes induced significant dose-dependent increase of thiocyanate concentration in serum. However, in rats exposed to the smoke of treated cigarettes, there were no differences in thiocynate concentration when compared to controls not exposed to tobacco smoke. The results of morphometrical analysis in the rats exposed to tobacco smoke generated from standard nontreated cigarettes have shown statistically significant and dose-dependent decrease in participation of the lung parenchima in total lung surface up to 34% in male rats and up to 18% in female rats exposed to the higher dose of nontreated cigarette smoke. In addition, in these rats the mean alveolar circumference was incresed for about 17%. In rats exposed to the smoke of treated cigarettes there were no histopathological changes and differences in morphometrical parameters when compared to control animals. In conclusion, the results of this study have shown that, after treatment of cigarettes in pulsed electromagnetic field, the cigarettes produced tobaco smoke that was much less toxic in rats of both sexes exposed by inhalation route during 90 days when compared to the same cigarettes that were not treated in electromagnetic field.

Key words: Tobacco smoke, carboxyhemoglobin, thiocyanates, pulsed electromagnetic field.

INTRODUCTION

The aim of the study was to investigate toxic effects of the tobacco smoke obtained from commercial cigarettes in rats exposed by inhalation route during 90 days. There were two types of tobacco smoke: the first was the smoke obtained from the cigarettes, and the second was the smoke generated from the same cigarettes that were, before the initiation of the study in animals, exposed to pulsed electromagnetic field. In this study, we have used two doses of the cigarettes 16 or 32 per day, in order to evaluate if there is dose dependence of the effects. The toxicity was assessed on the basis of clinical appearance, changes in behavior, biomarkers of exposure to tobacco

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smoke (carboxyhemoglobin and thiocyanates in blood) and histopathological and morphological analysis of organs and tissues of all animals included in the study.

MATERIALS AND METHODS

Cigarettes

The cigarettes had a filter and contained 8 mg of tar, 0.7 mg of nicotine and after burning they produced 10 mg of carbon monoxide. Cigarettes were kept at room temperature, protected from light and humidity according to manufacturer's instructions. The treatment of cigarettes was completed before the initiation of the study. The treated cigarettes were obtained after exposure of standard cigarettes to pulsed electromagnetic irradiation during 24 h according to the technology described in patents WO 01/26493, EP 1092354, US 2004/0206366A1 and AU2933700 entitled "Method for the qualitative Improvement of the Products of Tobacco Plant". The technology is patented in the USA, EU states and many other countries. The technology is based on the emission of pulsed electromagnetic irradiation of low power ($>10^{-7}$W) and wide frequency spectrum (30 Hz to 300 GHz).

Experimental animals

Experiments were done in Wistar rats of both sexes having body weight of 180 to 250 g. The rats were kept in ventilated rooms at the temperature of 22 to 26°C where the period day/night lasted 12 h each. The rats were kept in cages containing 5 animals of the same sex and had unrestricted access to water and food. The procedures with animals were in accordance with the Law on Animal Welfare of the Republic Serbia.

Experimental design

The rats were exposed to tobacco smoke by inhalation route in a closed chamber containing four cages with five animals of each sex. For cigarette burning, we have used a specially designed device which "inhaled" the smoke every 15 s and transfered it via plastic tubes to the chamber. The cigarettes were burnt one after another. After the last cigarette was burnt, the animals were kept in the closed chamber for an additional 30 min. After treatment the animals were transferred to another room where they were breathing environmental air. The procedure was repeated for 90 days. The total number of cigarettes to which the animals were exposed daily was 16 or 32 depending on the dose group, which corresponded to the average number of cigarettes smoked by smokers.

The rats were divided into five dose groups each containing 10 animals of both sexes. Group I contained control animals that were not exposed to tobacco smoke. The group II included animals exposed to the smoke generated from 16 nontreated cigarettes. Group III included animals exposed to the smoke generated from 16 treated cigarettes. Group IV included animals exposed to the smoke generated from 32 nontreated cigarettes. Group V included animals exposed to the smoke generated from 32 treated cigarettes. The study protocol was approved by the Ethics Committee of the Faculty of Medicine at Nish, Serbia.

Treated animals were observed during exposure and two additional times during the day in order to reveal any symptoms of toxicity, clinical presentation, mortality, changes in skin color, spontaneous motoric activity, muscular tonus and other effects during the 90 days period.

Methods

At the end of the study, the rats were sacrificed by cervical dislocation 15 min after the treatment and blood samples from each animal was collected into test tubes. Concentration of carboxyhemoglobin was determined according to the method of Stankovic et al. (1970). Thiocyanate concentration in blood was determined according to the method of Elkins (1951), which was adopted for small sample volumes and reading was done on an Elisa Reader.

Morphological analysis

Morphological analysis of the rat lungs was performed using computer program Leica QWin.

Presentation of the results

The results obtained are presented as mean ± standard deviation (X±SD) for ten animals per dose group. The results were statistically evaluated according to Tukey HSD (Honestly Significant Difference) test using JMP® Pro Statistical discovery software. The level of statistical significance was $P<0.05$.

RESULTS

During the study there were no mortalities. The only sign of toxicity observed was hypersalivation during or just after exposure to tobacco smoke.

Biomarkers of exposure to tobacco smoke

In this study, in rats of both sexes exposed to tobacco smoke generated from nontreated cigarettes during 90 days, we have obtained dose-dependent increase in carboxyhemoglobin (COHb) concentration in the blood (Table 1). The differences in COHb concentration in the blood of the rats exposed to nontreated smoke and those from the control group were statistically significant. However, in rats exposed to the smoke of treated cigarettes the COHb concentration in the blood was significantly lower when compared to that of the rats exposed to the smoke of nontreated cigarettes. These results suggest the possibility that after burning treated cigarettes, a smaller amount of carbon monoxide (CO) is being formed when compared to nontreated cigarettes.

In this study in rats of both sexes exposed to tobacco smoke generated from nontreated cigarettes during 90 days, we have obtained statistically significant dose-dependent increase in thiocyanate (SCN) concentration in serum (Table 2). However, in rats exposed to the smoke of treated cigarettes there were no differences in SCN concentration when compared to controls. These results possibly suggest that after burning treated cigarettes, a smaller amount of hydrogen cyanide (HCN) is being formed compared to nontreated cigarettes or that HCN is not formed at all.

Table 1. The levels of COHb (in %) in rat blood at the end of the study (X±SD, n=10).

Group	Males		Females	
	COHb	%	COHb	%
I Control	2.1±0.2	100	2.0±0.2	100
II 16 cig. nontreated	12.3±1.1 [a]	597	14.2±1.6 [a]	715
III 16 cig. treated	10.4±0.6 [c]	495	11.1±0.7 [c]	555
IV 32 cig. nontreated	28.3±1.9 [a,b]	1373	31.3±3.1 [a,b]	1582
V 32 cig. treated	25.0±3.1 [c]	1190	24.3±2.3 [c]	1215

[a] P<0.05 compared to control. [b] P<0.05 between groups II and IV. [c] P<0.05 between groups II and III as well as between IV and V. cig. = cigarette.

Table 2. The concentration of thiocyanate (SCN) (µmol/L) in rat serum at the end of the study (X±SD, n=10).

Group	Males		Females	
	SCN	%	SCN	%
I Control	21.1±3.5	100	21.2±4.1	100
II 16 cig. nontreated	28.5±6.1 [a]	135	34.8±8.3 [a]	164
III 16 cig. treated	20.4±2.6 [c]	97	19.4±3.1 [c]	91
IV 32 cig. nontreated	33.8±6.2 [a,b]	160	34.5±7.5 [a,b]	163
V 32 cig. treated	22.6±3.1 [c]	107	21.3±4.3 [c]	100

[a] P<0.05 compared to control; [b] P<0.05 between groups II and IV; [c] P<0.05 between groups II and III and also between IV and V; cig. = cigarette.

Table 3. Morphometrical parameters in the lungs of male rats at the end of the study (X±SD, n=10).

Group	%P	%	MCA	%
I Control	0.47±0.05	100	303±40	100
II 16 cig. nontreated	0.36±0.06 [a]	77	336±47 [a]	111
III 16 cig. treated	0.42±0.02	90	314±36	103
IV 32 cig. nontreated	0.31±0.02 [a]	66	354±52 [a]	116
V 32 cig. treated	0.46±0.04	98	310±17	102

%P – The percent of lung parenchima compared to the total lung surface; MCA – The mean alveolar circumference (in µm); [a] P<0.05 when compared to controls according to Tukey HSD test. cig. = cigarette.

Histopathology

Histopathological analysis of the lung tissue of the rats exposed to tobacco smoke during 90 days have shown the effects that varied from mild emphysema (group II) to serious emphysema (group III) including the collapse of the lung tissue in some animals. In the rats from group V there were no histopathological differences in lung tissue when compared to that of nonexposed animals (group I). Histopathological data on rat lung were analyzed using morphometrical methods. Two parameters were followed: 1) the percent of lung parenchima compared to the total surface of the lungs (%P) and 2) the mean alveolar circumference (MCA). The results are presented in Tables 3 and 4.

The results of morphometrical analysis in the rats exposed to tobacco smoke generated from nontreated cigarettes during 90 days have shown statistically significant and dose-dependent decrease in participation of the lung parenchima in total lung surface (%P) up to 34% in male rats and up to 18% in female rats exposed to the higher dose of nontreated cigarette smoke (Tables 3 and 4). In addition, in these rats the mean alveolar circumference (MCA) was incresed for about 17%.

However, in rats exposed to the smoke of treated cigarettes there were no histopathological changes and differences in morphometrical parameters when compared to control animals (Tables 3 and 4).

DISCUSSION

Biomarkers of exposure to tobacco smoke

CO is a product of incomplete burning of organic material. After burning a single cigarette between 0.5 and

Table 4. Morphometrical parameters in the lungs of female rats at the end of the study (X±SD, n=10).

Group	%P	%	MCA	%
I Control	0.39±0.03	100	312±23	100
II 16 cig. nontreated	0.36±0.04	92	344±62 [a]	110
III 16 cig. treated	0.39±0.04	100	320±40	102
IV 32 cig. nontreated	0.32±0.03 [a]	82	365±38 [a]	117
V 32 cig. treated	0.39±0.02	100	306±50	98

%P – The percent of lung parenchima compared to the total lung surface; MCA – The mean alveolar circumference (in µm); [a] P<0.05 when compared to controls according to Tukey HSD test. cig. = cigarette.

13 mg CO is being formed which corresponds to 0.2 to 4.5% of CO in the primary cigarette smoke. According to EU regulations, the primary cigarette smoke, in cigarettes produced after 2004, is allowed to contain up to 10 mg CO per cigarette (Scherer, 2006). Inhaled CO is absorbed in lung alveoli and bound to hemoglobin forming COHb. The affinity of CO for hemoglobin is 200 to 250 higher than for oxygen and the ability of COHb to bind and transport oxygen from the lungs to the rest of the body is limited. CO is also bound to other proteins cointaining heme such as myoglobin, but to a much lesser extent (10 to 15%). CO is eliminated mainly via exhaled air. COHb has a half-life in the blood of 4 to 6 h. In the blood of smokers, the levels of COHb are higher (4 to 7%, >12% in heavy smokers) when compared to the levels in nonsmoker's blood (1 to 2% COHb).

In this study, rats of both sexes exposed to tobacco smoke generated from nontreated cigarettes during 90 days, we have obtained dose-dependent increase in COHb concentration in blood (Table 1). The differences in COHb concentration in the blood of the rats exposed to nontreated smoke and those from the control group were statistically significant. However, in rats exposed to the smoke of treated cigarettes the COHb concentration in the blood was significantly lower when compared to that of the rats exposed to the smoke of nontreated cigarettes. These results suggest the possibility that after burning treated cigarettes, a smaller amount of CO is being formed when compared to nontreated cigarettes. The results obtained in this study (Table 1) are in agreement with those published by Theophilus et al. (2007) who found increased concentration of COHb in the blood of Sprague-Dawley rats exposed to tobacco smoke during 13 weeks. SCN in blood are present as a metabolite of HCN which is formed from proteins and nitrates after cigarette burning at temperatures higher than 700°C in the absence of oxygen. The primary smoke of a single cigarette contains 150 to 300 mg of cyanides. HCN is absorbed in the lungs, oral mucosa, stomach and in the skin. Absorbed HCN binds to Fe(III)-hemoglobin. HCN is metabolized in mitochondria in the presence of thiocyanate sulfurtransferase (rhodanese) which transfers sulfur from thiosulfate to cyanide ion forming a far less toxic metabolite thiocyanate. The half life of thiocyanates in blood is 10 to 14 days (Scherer, 2006).

In this study, in rats of both sexes exposed to tobacco smoke generated from nontreated cigarettes during 90 days, we have obtained statistically significant dose-dependent increase in SCN concentration in serum (Table 2). However, in rats exposed to the smoke of treated cigarettes there were no differences in SCN concentration when compared to controls. These results possibly suggest that after burning treated cigarettes a smaller amount of HCN is being formed compared to nontreated cigarettes or that HCN is not formed at all. The results obtained in this study related to increased SCN concentrations in rat serum were in concordance with those published by other authors. Increased SCN concentrations in plasma were found in male Wistar rats exposed to tobacco smoke for 2 h per day during 60 days (Isik et al., 2004).

In the study conducted in 25300 volunteers, Foss and et al. (1986) found significantly higher SCN levels (up to 300%) in serum of smokers of both sexes when compared to nonsmokers. Similar increase of the SCN concentration in serum of normotensive and hypertensive pregnant women compared to nonsmokers was reported by Kosanović et al. (2007). The results of this study related to higher levels of COHb and SCN in rats exposed to nontreated tobacco smoke are also in agreement with the study of Cohen et al. (1980) who reported such results in 191 non-smokers and 426 cigarette smokers.

Histopathological analysis

The results of morphometrical analysis in the rats exposed to tobacco smoke generated from nontreated cigarettes during 90 days have shown statistically significant and dose-dependent decrease in participation of the lung parenchima in total lung surface up to 34% in male rats and up to 18% in female rats exposed to the higher dose of nontreated cigarette smoke (Tables 3 and 4). In addition, in these rats the mean alveolar circumference was incresed for about 17%. However, in rats exposed to the smoke of treated cigarettes there were no histopathological changes and differences in

morphometric parameters when compared to control animals (Tables 3 and 4).

The results of morphometric analysis (Tables 3 and 4) were in agreement with those of Zheng et al. (2009) who reported the increase in mean alveolar circumference for 25 and 45%, after exposure of rats to tobacco smoke during 24 and 36 weeks, respectively. The results obtained in this study have shown an increase of mean alveolar circumference of 17% after 90 days exposure to the smoke generated from 32 nontreated cigarettes. In addition, Zheng et al. (2009) reported that in rats exposed to tobacco smoke during 36 weeks, air spaces were markedly enlarged, the thickness of alveolar walls was greatly increased, some alveoli were merged and large number of accumulated cells was observed.

The mechanisms involved in pathological changes in the respiratory system of smokers were discussed by Bohadana et al. (2004) and Molfino et al. (2007). The initial phase involves airway inflammation and the reactions of neutrophils and alveolar macrophages that cause damage to the lung epithelium and its protein structure. Peptides are formed that cause activation and proliferation of T cells which can further attract neutrophils, macrophages and eosinophiles to the site of inflammation. After some time, structural changes in the airways appear as thickening of the walls and narrowing of the lumen of the airways. As a consequence, the forced expiratory volume of the lungs is decreased proportionally to the number of cigarettes smoked.

Gaworski et al. (1997) and Coggins (2007) have studied histopathological changes in rats exposed to high doses of tobacco smoke. The rats had a significant increase of squamous metaplasia, basal hyperplasia and hyperkeratosis of the nasal, laryngeal and tracheal epithelium. They have also described degeneration of olfactory epithelium with the loss of sensory cells and the disappearance of the thin eosinophilic film lining the olfactory epithelium. Finally, they have found hyperplasia of the lung epithelium with macrophage influx. These changes were observed mostly in rats exposed to the highest doses of tobacco smoke.

Conclusion

The results of this study have shown that, after treatment of cigarettes in pulsed electromagnetic field, the cigarettes produced tobaco smoke that was much less toxic in rats of both sexes exposed by inhalation route during 90 days when compared to the same cigarettes that were not treated in electromagnetic field.

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