

## OXIDATIVE STRESS IS REDUCED IN WISTAR RATS EXPOSED TO SMOKE FROM TOBACCO AND TREATED WITH SPECIFIC BROAD-BAND PULSE ELECTROMAGNETIC FIELDS

V. BAJIĆ<sup>1</sup>, B. BAJIĆ<sup>2</sup>, ZORANA MILIĆEVIĆ<sup>3</sup>, SLAVICA RISTIĆ<sup>1</sup>, and A. NIKOLAU<sup>4</sup>

<sup>1</sup>*Institute for Biomedical Research, Galenika Pharmaceuticals, 11000 Belgrade, Serbia*

<sup>2</sup>*IBC Technologies, 11000 Belgrade, Serbia*

<sup>3</sup>*Vinča Institute for Nuclear Sciences, 11000 Belgrade, Serbia*

<sup>4</sup>*IBTec, 191 Megara, Athens, Greece*

**Abstract** — There have been a number of attempts to reduce the oxidative radical burden of tobacco. A recently patented technology, pulse electromagnetic technology, has been shown to induce differential action of treated tobacco products versus untreated products on the production of reactive oxygen species (ROS) in vivo. In a 90-day respiratory toxicity study, Wistar rats were exposed to cigarette smoke from processed and unprocessed tobacco and biomarkers of oxidative stress were compared with pathohistological analysis of rat lungs. Superoxide dismutase (SOD) activity was decreased in a dose-dependent manner to 81% in rats exposed to smoke from normal cigarettes compared to rats exposed to treated smoke or the control group. These results correspond to pathohistological analysis of rat lungs, in which those rats exposed to untreated smoke developed initial signs of emphysema, while rats exposed to treated smoke showed no pathology, as in the control group. The promise of inducing an improved health status in humans exposed to smoke from treated cigarettes merits further investigation.

**Key words:** Oxidative stress, tobacco smoke, broad-band electromagnetic waves, emphysema

UDC 599.323.4.591

### INTRODUCTION

More than 4000 constituents have been identified in tobacco smoke (Dube and Green, 1982). Also, the addition of ingredients, which are often complex chemical mixtures themselves, can either cause an increase or decrease in content of one or more of the smoke's constituents (Rustemeier et al., 2002) or else result in the formation of new constituents (Kapfe et al., 1989). This raises questions of toxicity control. The main toxicity of tobacco products and nicotine is through vast production of reactive oxygen species (ROS) in humans. Reactive oxygen species have been found to increase in mainstream smoke (Huang et al., 2005; Ou et al., 2006) and may produce inflammatory mediators (Facchinetti et al., 2007). Tobacco smoke contains free radicals and some published data indicate that their amount in one puff of smoke could be as high as  $10^{17}$ .

Among those in the gas phase, there are at least 10 low-molecular-weight carbon- and oxygen-centered radicals, plus nitric oxide (NO) and nitric dioxide ( $\text{NO}_2$ ). Tar contains a semiquinone radical that can reduce oxygen to superoxide and hence hydrogen peroxide and the hydroxyl radical (Pryor, 1997).

All aerobic biological systems produce free radicals, predominantly superoxide formed as a byproduct of electron transport in the mitochondria during the reduction of molecular oxygen. It has been estimated that 1% of respired oxygen will form  $\text{O}_2^-$ . Based on the average daily cell utilization of  $\text{O}_2^-$ , it has been estimated that approximately  $10^{11}$  superoxide radicals are produced each day in a cell. The production of radicals is heightened in the brain due to high oxygen metabolism in the neurons. While most of these radicals are sequestered in the mitochondria, oxidative insult can be exacerbated by age,

metabolic demand, and disease (Smith et al., 1992; Manton et al., 2004). The principal metalloenzymes responsible for cellular regulation of reactive oxygen are the Mn and CuZn superoxide dismutases (SOD2 and SOD1, respectively), which remove superoxide by converting it to hydrogen peroxide. Removal of ROS such as hydrogen peroxide in mitochondria requires removal of electrons by a functional Krebs tricarboxylic acid cycle. Superoxide dismutase (SOD) is therefore considered the primary defense against the buildup of reactive oxygen because it removes  $O_2^-$ , the initial form of metabolically produced reactive oxygen (Smith et al., 1992). Antioxidants and antioxidant enzymes present in the human body can be successful in preventing the toxic effects of free radicals, provided the number of cigarettes smoked per day is not too high. So how can we reduce the load of ROS in order to simulate lower consumption of cigarettes? We here present a patented, novel method of tobacco treatment by the use of specific pulse electromagnetic waves (PEW). The biomarkers of oxidative stress analyzed in this study are the activities of superoxide dismutase and glutathione peroxidase, which represent the first line of cellular defense against free radicals. Assessment of the concentration of malondialdehyde (as an index of lipid peroxidation) in rat plasma, which indicates the amount of tissue damage, is also included. In addition, a variety of biomarkers of tobacco smoke exposure were evaluated. Thiocyanate and carboxyhemoglobin (CHb) in blood, urine, and saliva have been used to distinguish smokers from nonsmokers (Jarvis et al., 1983, 1987; Benowitz, 1999). In this study, we assayed the concentration of thiocyanates and carboxyhemoglobin in blood as biomarkers of exposure to tobacco smoke. To establish possible correlation with SOD activity, CHb levels, and thiocyanate concentrations, samples of the heart, aorta, liver, lung and respiratory tissues, and peripheral nerves were evaluated for pathohistological changes caused by exposure to cigarette smoke.

Even after 90 days of being exposed to PEW-treated smoke, rats showed no differential expression of SOD, malondialdehyde, and glutathione peroxidase when compared to the control. These findings were also in concordance with the biomarkers

of exposure, i. e., there were no increased levels of thiocyanate and carboxyhemoglobin from treated smoke or any pathohistological findings. However, in the group of animals exposed to smoke from untreated cigarettes, we found a decrease to 81% of control levels of our main antioxidant (SOD), which shows that the animals are very vulnerable to the effects of smoke and suffer a decrease in their ability to cope with reactive oxygen species. This vulnerability was clearly emphasized by the presence of tissue changes in the lungs, showing initial phases of emphysema. We here demonstrate for the first time that a novel method using specific pulse electromagnetic radiation can induce an anti-oxidant effect in tobacco.

## MATERIAL AND METHODS

### *1. Test and control article and vehicle information*

Whole cigarette smoke was produced from treated versus untreated cigarettes. Storage: cigarettes were kept at room temperature, protected from direct light and humidity. Dosing was prepared by a standard protocol using an appropriate cigarette smoking machine. Cigarettes were a Macedonian brand of middle quality with 13 mg of tar and 2 mg of nicotine.

### *2. Brief description of the method*

Patent No. 1092354 (EU patent), USA patent No. 20040206366, Canada patent No. 2 388 627 (<http://patents.ic.gc.ca/cipo/cpd/welcome.html>).

#### *2.1. Brief description*

The method belongs to the field of electronic science and is applied to the field of the manufacture of products deriving from the tobacco plant, such as cigarettes, cigars, pipe tobacco, and tobacco in general and achieves their qualitative improvement. The qualitative improvement is realized through the pulsatory emission of electromagnetic waves towards the tobacco products. The waves are produced by electromechanical or electronic devices (3), are pre-programmed, and cover wide ranges of wavelengths from 1 mm to 11,000 Km, together with their harmonic frequencies, which are generated by the de-

vice and emitted either at all wavelengths from 1 mm to 11,000 Km or at one or more parts of particular areas with controlled potency, controlled application time, and controlled application result. The method is applied in industries and commercial enterprises manufacturing final or intermediate tobacco products, either at the processing stage, after the completion of their manufacture, or during their storage, regardless of the way the materials are packaged.

### 2.2. Description of the device

Emission of electromagnetic waves is programmed not as continual, but in an impulse mode. Programmed impulse emission of electromagnetic waves towards the treated matter has a constant intermittent mode.

### 2.3. Description of the emitter and its characteristics

Voltage for the emitter passes through a grid of 110/240 V.

Composition:

- Stable source of voltage
- Main oscillator
- Impulse-broad range modulator
- Frequency generator
- Broad-band amplifier
- Cable
- Output antenna

A stable source of 30V/10A voltage on its output supplies a constant output of voltage in conditions of changes in the voltage grid.

The main oscillator determines a working regime with very strict and defined conditions and accuracy of the frequency signal. The modulator makes possible suitable coordination of the working regime with needs of the output signal.

A well defined and accurate signal provides the basis of the working regime of the frequency generator and is introduced to the broad-band amplifier.

The broad band is realized in a way to ensure unaltered frequency and amplitude of the output signal.

The antenna of the emitter is connected with a high-quality coaxial cable. The emitter works in a regime of from - 40 to 60°C.

This emitter is adapted for treating tobacco, which means it has a frequency range of from 100 Hz to 2.5 GHz. The output power of the emitted signal is 5 W.

## 3. Experimental animals

The rat is used as a generally accepted rodent species for toxicity studies.

We used 100 adult Wistar rats (obtained from the animal facility of Galenika Pharmaceuticals, Belgrade) of both sexes, aged 10 to 12 weeks and with body weights ranging between 180 and 250 g at the beginning of the study. All animals were kept in a ventilated (artificial ventilation with 16 air changes per hour) room specially designed for work with small rodents. The room was not air-conditioned and had temperatures of from 20 to 26°C and relative humidity of 55-75%. Animals were kept in macrolone cages, five animals per cage, in compliance with accepted standards for animal caging. Sterile woodchips were used for bedding and changed twice a week. All animals were fed a standard pelleted diet for rats, supplied by Vet. Zavod Subotica, Serbia. This food was available *ad libitum* throughout the study. Animals were supplied with tap water in standard bottles, also available *ad libitum* throughout the study. Precautions were taken to eliminate any possible sources of contamination of the food, water, and housing by physico-chemical or biological factors that might interfere with the conduct of the experiment and its results. All animals were observed during a routine veterinary check-up prior to their selection and allocation to groups.

### 3.1. Animal welfare provisions

This study was conducted in accordance with current guidelines for animal welfare. The protocol was reviewed by the Institutional Animal Care and Use

Committee and complies with accepted standards of animal welfare and humane care.

### 3.2. Experiment design

Allocation to treatment groups:

Animals were randomly allocated to test and control groups. The animals were exposed to smoke generated from one cigarette at a time. The cigarettes were burned one after another, the total number of cigarettes per day being 16 or 32, depending on the dose group. The exposure period was 90 days. This dosing regimen was chosen in order to imitate the real situation of human smokers, who are repeatedly exposed to the smoke produced from one cigarette at a time for several hours a day.

Group	Treatment Number of cigarettes	No. of animals per dose group
I Control	0	10 ♂ + 10 ♀
II Normal cigarettes	16	10 ♂ + 10 ♀
III Normal cigarettes	32	10 ♂ + 10 ♀
IV Treated cigarettes	16	10 ♂ + 10 ♀
V Treated cigarettes	32	10 ♂ + 10 ♀

During exposure, two groups of cages (four for males and four for females) were placed in a gas-tight glass exposure chamber with dimensions of 60 x 80 x 50 cm. A special smoking device was then used to introduce the smoke from one cigarette at the time (16 or 32 cigarettes per day, one after another) into the chamber. After the last cigarette from the test groups was burned, the rats were kept in a closed inhalation chamber for an additional 15 minutes.

### 3.3. Blood collection

Blood samples for analysis of biochemical parameters were collected on day 90 of the experiment approximately 15 minutes after termination of exposure.

#### 3.3.1. Hematology

Values of the following indexes were determined:

Hemoglobin (HGB)

Mean corpuscular hemoglobin (MCH)

Mean corpuscular hemoglobin concentration (MCHC)

Hematocrit (HCT)

Red blood cell count and morphology (RBC)

Mean corpuscular volume (MCV)

White blood cell count (WBC)

Platelet count (PLT)

Hematology analysis was performed at the Center for Biomedical Research in a GLP hematological laboratory using an ACTDIFF automatic analyzer from Beckmann Coulter.

### 3.4. Biomarkers of exposure to tobacco smoke

Thiocyanate and carboxyhemoglobin in blood, urine, and saliva have been used to distinguish smokers from nonsmokers (Jarvis et al., 1987; Benowitz, 1999). Thiocyanate concentration in blood was determined according to the spectrophotometric method of Elkins (1951).

The concentration of carboxyhemoglobin in blood was determined according to the method of Stankovic and Milic (1970).

### 3.5. Status of biomarkers of oxidative stress

Blood samples were centrifuged at 3000 rpm for 15 min to remove the plasma and buffy coat (consisting of leukocytes and platelets). Erythrocytes were washed three times in buffered saline (0.9% saline in 0.01 M phosphate buffer, pH 7.4), and the packed cells were suspended in an equal volume of buffered saline. The methods used for antioxidant status determination were described elsewhere, i. e., malondialdehyde (MDA) (Ohkawa et al., 1979) and glutathione (GSH) (Beutler et al., 1963) levels, along with glutathione peroxidase (GSH-Px) (Paglia and Valentine, 1967) and superoxide dismutase (SOD) (Beauchamp and Fridovich, 1971) activities. The antioxidant activities of the mentioned enzymes were estimated in red cell lysates. These parameters were assayed in blood of control rats and animals exposed to cigarette smoke. Blood samples were taken at the end of the study on day 90.

### 3.6. Sacrifice and post-mortem sampling

All animals were sacrificed by cervical dislocation and subjected to standard necropsy procedures. The appearance and macroscopic changes of *in situ* organs, mucosae, and serose membranes were observed.

#### 3.6.1. Histopathological evaluation

Samples of the heart, aorta, liver, lung, and respiratory tissues and peripheral nerves were taken for pathohistological evaluation of possible changes caused by exposure to cigarette smoke. All samples were preserved in 4% aqueous paraformaldehyde solution.

Elastine breakdown/emphysema in smoke-exposed rats was quantitatively measured by morphometric analysis of alveolar mean intercepts and alveolar tissue density in fixed lungs.

Briefly, 5- $\mu$ m sections were cut from midsagittal blocks of paraffin-embedded fixed left and right lungs and stained with hematoxylin and eosin, after which 10 randomly selected fields were sampled by projecting a microscope image of the lung section onto a screen. The images were analyzed using the Image-Pro Plus for Windows program. Pictures were taken with an Olympus dp 12 microscope digital camera system. The mean linear intercept, as a measure of interalveolar wall distance, and the alveolar tissue density were measured as previously described (Thurlbeck, 1967a) with slight modifications as described by Kawakami et al. (1984) and Niewoehner (1988). The number of intersections of alveolar walls with test lines were counted and used to quantitate the mean linear intercept (the average distance between alveolar walls). Increased average distance was taken as evidence of air space enlargement (Thurlbeck, 1967b; Wright and Churg, 1990; Escolar et al., 1995). The number of test points falling on alveolar wall tissue in relation to the reference lattice was also counted and used to determine alveolar wall tissue volume density (% P). Decreasing % P was taken as evidence of lung parenchyma destruction (Escolar, 1995). All quantitations were

corrected for tissue shrinkage during processing.

### 3.7. Statistics

Data from concurrent controls were used for evaluation of the effects of cigarette smoke exposure. Arithmetic means and standard deviations of biometric parameters were calculated for each group where appropriate. On the basis of the type of distribution for each biometric parameter, appropriate statistical tests for significance of differences between means such as the Tukey HSD test were applied.

## RESULTS

There were no mortalities or signs of morbidity, even though the animals had been under constant stress for 90 days. Animals experienced signs of hyper-salivation during and/or immediately after exposure. This effect has been described in the literature and is thought to be a reflex produced by irritation of the upper respiratory tract by smoke, rather than a systemic effect of nicotine (Taylor, 1996). There were no differences between the effects of normal and treated cigarettes in relation to salivation. The data presented in Fig. 1 show the weights of male animals included in the study. In the group of animals treated with smoke generated from normal cigarettes, the rats gradually lost weight and this effect apparently was not dose-related. In the group of rats exposed to smoke from 16 treated cigarettes, the animals did not lose any weight, which was similar to that of control animals. This difference in weight between groups of rats treated with 16 normal and 16 PEW-treated cigarettes was statistically significant ( $p < 0.05$ ), indicating only that the rats exposed to smoke generated from treated cigarettes resembled control animals, while those exposed to 16 and 32 normal cigarettes displayed some unspecific signs of toxicity. This effect was observed in both male and female rats (data not shown for female rats). Although the data were collected individually, for print purposes we used average body weight to show changes in treated versus untreated groups (Fig. 1).

The results presented in Table 1 show the hematology data for male and female rat blood samples

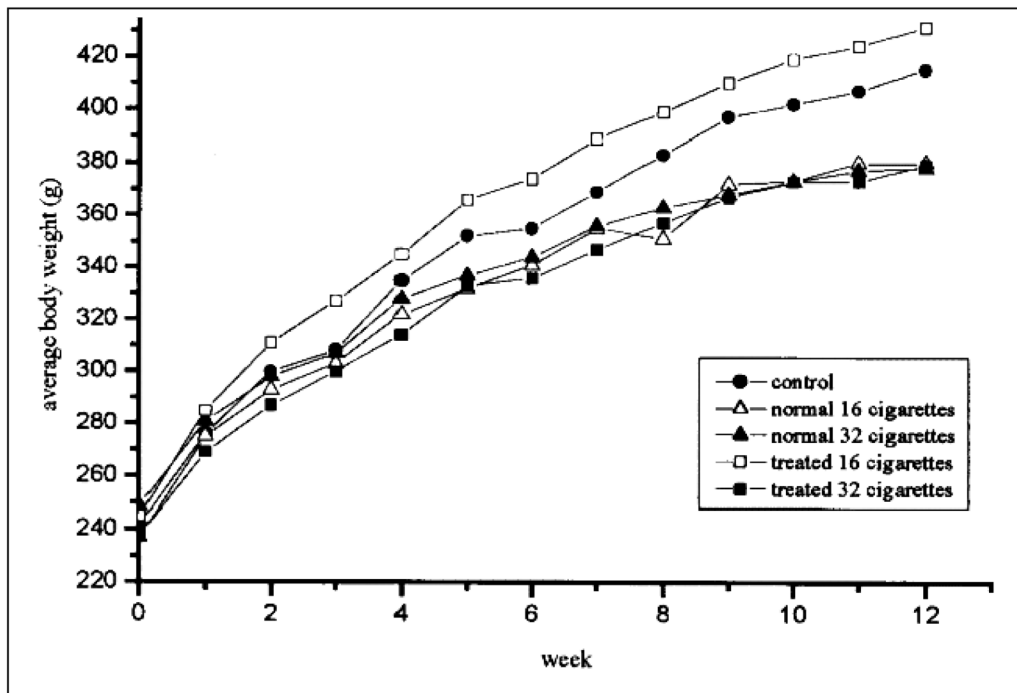


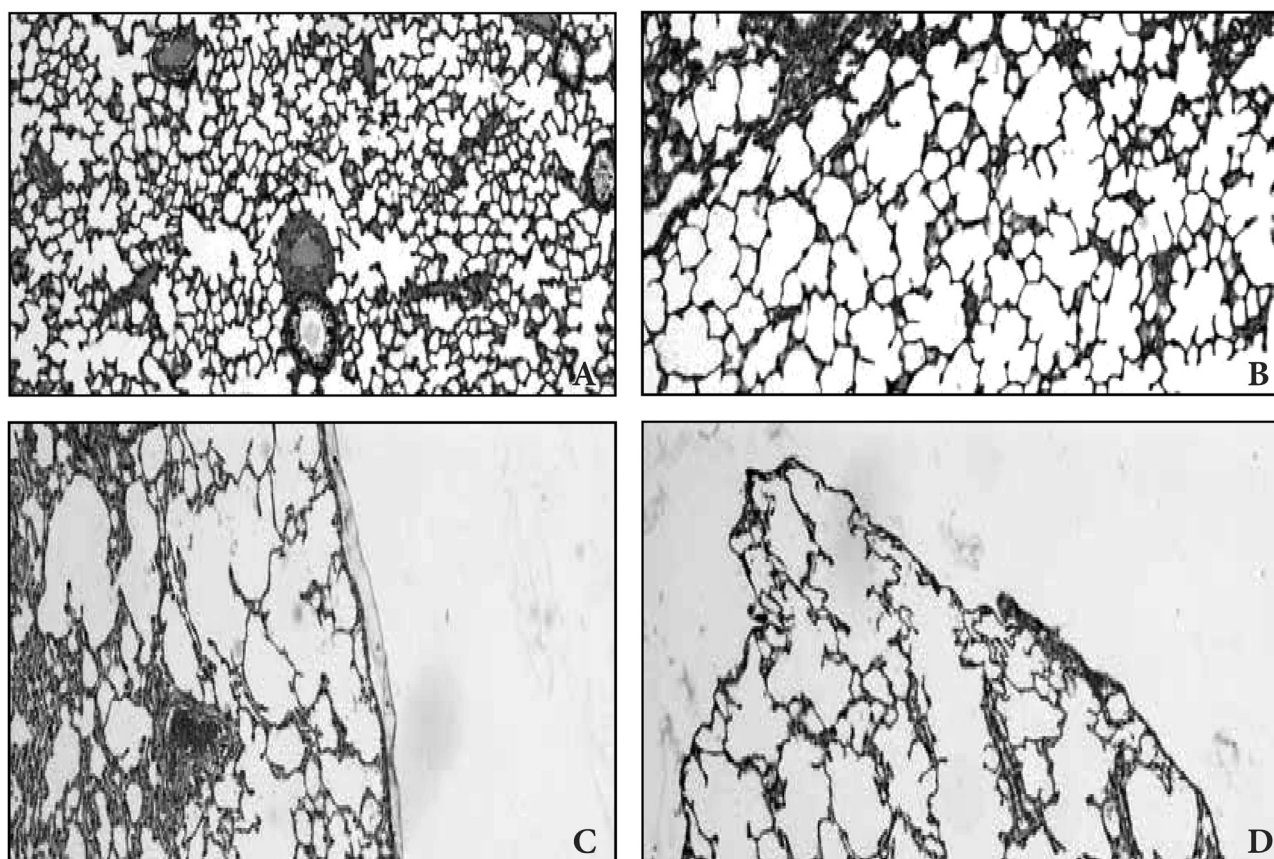
Fig. 1. Average body weights of male rats during the study. Comparison between experimental groups (16 cigarettes) shows a statistical difference ( $p < 0.05$ ) according to the Tukey HSD test.

collected after termination of the experiment on day 90. In male rats, exposure to smoke generated from normal cigarettes reduced the number of white blood cells to about 43% of the control (Table 1). In male rats exposed to smoke from treated cigarettes,

this decrease was to about 60% of the control. These results indicate that, owing to their reduced white blood cell count, male rats exposed to smoke from normal cigarettes could be much more susceptible to the influence of environmental factors such as infec-

**Table 1.** Summary of mean values of hematology data in male rat blood samples collected after completion of the experiment (on day 90). The numbers represent the mean value derived from 10 animals from the same experimental group. (WBC - white blood cells, RBC - red blood cells, HGB - hemoglobin, HCT - hematocrit, MCV - mean corpuscle volume, MCH - mean corpuscular hemoglobin, MCHC - mean corpuscular hemoglobin concentration, PLT - platelets). Units: WBC (109/L), RBC (1012/L), HGB (g/dL), MCV (fl), MCH (pg), MCHC (g/dL), PLT (109/L). ap < 0.05 compared to control according to Tukey HSD test, bp < 0.05 compared to 16 normal cigarettes according to Tukey HSD test.

Group/Males	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	PLT
Control	9.2	7.67	140	0.410	53.4	18.2	341	640
16 normal cigarettes	4.0 <sup>a</sup>	7.00 <sup>a</sup>	137	0.392	56.0	19.6	350	668
16 treated cigarettes	5.2 <sup>a</sup>	6.11 <sup>b</sup>	129	0.362	59.3	21.1	357	678
32 normal cigarettes	4.2 <sup>a</sup>	7.58	146	0.418	55.2	19.3	350	671
32 treated cigarettes	5.4 <sup>a</sup>	6.5	132	0.371	57.1	20.3	355	683
Group/Females	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	PLT
Control	8.0	7.05	131	0.381	54.1	18.6	343	718
16 normal cigarettes	6.0	7.48	139	0.393	54.5	18.7	346	656
16 treated cigarettes	5.2	6.39	135	0.376	58.8	21.1	359	696
32 normal cigarettes	6.2	6.50	133	0.374	57.6	20.5	356	719
32 treated cigarettes	6.2	6.56	137	0.379	57.7	20.8	361	675



**Fig. 2.** Histological analysis of the lungs from the rats included in the study. A - control animals, B - slight emphysema, C - moderate emphysema, D - severe emphysema.

**Table 2.** Summary of thiocyanates and carboxyhemoglobin in rat blood samples collected after completion of the experiment (on day 90). The numbers represent the mean value derived from 10 animals from the same experimental group. SCN# (thiocyanates), COHb\* (carboxyhemoglobin), ap < 0.05 compared to control according to Tukey HSD test, bp < 0.05 between 32 normal and 16 normal or 32 treated and 16 treated cigarettes according to Tukey HSD test, cp < 0.05 between 32 treated and 32 normal or 16 treated and 16 normal cigarettes according to Tukey HSD test.

Group	Male rats		Female rats	
	SCN# ( $\mu\text{M}$ )	% of control	SCN ( $\mu\text{M}$ )	% of control
Control	21.15	100.0	21.22	100.0
16 normal cigarettes	28.54 <sup>a</sup>	134.9	34.78 <sup>a</sup>	163.9
16 treated cigarettes	20.38 <sup>c</sup>	96.4	19.43 <sup>c</sup>	91.6
32 normal cigarettes	33.85 <sup>a</sup>	160.0	34.46 <sup>a</sup>	162.4
32 treated cigarettes	22.59 <sup>c</sup>	106.8	21.28 <sup>c</sup>	100.0
Group	Male rats		Female rats	
	COHb*	%	COHb*	%
Control	2.06	100	1.98	100
16 normal cigarettes	12.30 <sup>a</sup>	597	14.16 <sup>a</sup>	715
16 treated cigarettes	10.40 <sup>a</sup>	504	11.10 <sup>a, b, c</sup>	560
32 normal cigarettes	28.29 <sup>a, b, c</sup>	1373	31.32 <sup>a</sup>	1582
32 treated cigarettes	24.96 <sup>a, b, c</sup>	1211	24.36 <sup>a, c</sup>	1230

tions, toxic agents, microorganisms, etc. They also indicate that the rats exposed to smoke from treated cigarettes could be more resistant to environmental factors and in better health than those exposed to smoke from normal cigarettes. In female rats, the reduction of white blood cell count was about 25% in all experimental groups, and there were no differences among them that could be attributed to dosing regimen (Table 1). There were no differences of other hematological parameters (red blood cells, hemoglobin, hematocrit, mean corpuscle volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelets) in experimental groups compared to untreated control groups of male or female rats.

#### *Biomarkers of exposure to tobacco smoke*

##### *Thiocyanates*

The results shown in this section represent the most important findings in this study. It is well known from the literature that thiocyanates and carboxyhemoglobin can be regarded as biomarkers of exposure to tobacco smoke (Jarvis et al., 1987; Benowitz, 1999). In human studies, thiocyanates are one of the most important biomarkers for inhalation of tobacco smoke (Table 2).

Rats exposed to smoke generated from normal cigarettes showed a dose-dependent increase of thiocyanate concentration in blood, and these differences were statistically significant compared to the control values (i. e., thiocyanate concentration

in blood of rats not exposed to tobacco smoke). However, in rats exposed to smoke generated from either 16 or 32 PEW-treated cigarettes, no differences of thiocyanate concentration in blood were found compared to the control animals. These results indicate, at least in relation to this specific biomarker, that the rats exposed to smoke from treated cigarettes for 90 days resembled the control animals and showed no signs of toxicity and exposure to tobacco smoke. This effect was observed in both male and female rats to a similar extent.

##### *Carboxyhemoglobin (COHb)*

Exposure of rats to smoke generated from both normal and treated cigarettes resulted in a dose-dependent increase of carboxyhemoglobin content in blood, and the differences were statistically significant compared to the control values (Table 2). However, in rats exposed to smoke generated from 16 or 32 treated cigarettes, the carboxyhemoglobin content in blood was significantly lower than in the group of animals exposed to smoke from normal cigarettes. These results possibly indicate that treated cigarettes produce less carbon monoxide than normal cigarettes. It should be noted that, according to published data, after cessation of exposure to carbon monoxide, carboxyhemoglobin is rapidly converted to normal hemoglobin (oxyhemoglobin) within several hours (Klaassen, 1996).

##### *Status of biomarkers of oxidative stress*

The results presented in Table 3 show the activities of superoxide dismutase (SOD) and glutath-

**Table 3.** Summary of superoxide dismutase (SOD) (U/gHb), glutathione peroxydase (GPx) (U/gHb), and malondialdehyde (MDA,  $\mu$ moles/mg of protein) in rat blood samples collected after completion of the experiment (on day 90). The numbers represent the mean value derived from 10 animals from the same experimental group. ap < 0.05 compared to control according to Tukey HSD test, bp < 0.05 between 32 treated and 32 normal or 16 treated and 16 normal cigarettes according to Tukey HSD test.

Group	Male rats						Female rats					
	SOD	%	GPx	%	MDA	%	SOD	%	GPx	%	MDA	%
Control	1678	100	604	100	115	100	2040	100	602	100	181	100
16 normal cigarettes	1526	90.9	511 <sup>a</sup>	84.6	158	137.4	1563 <sup>a</sup>	76.6	548	91.0	143	79.0
16 treated cigarettes	1363 <sup>b</sup>	81.1	539	89.3	159	138.3	1334 <sup>a, b</sup>	65.4	516 <sup>a</sup>	85.7	167	92.3
32 normal cigarettes	1362 <sup>b</sup>	81.1	512 <sup>a</sup>	84.8	130	113.0	1556 <sup>a</sup>	76.3	548	91.0	162	89.5
32 treated cigarettes	1854	110	540	89.4	147	127.8	2015	98.8	495 <sup>a</sup>	82.2	151	83.4



ione peroxidase (GPx) and the concentration of malondialdehyde (MDA) in blood of rats included in the study. Activity of SOD in male rats exposed to smoke from normal cigarettes was decreased in a dose-dependent manner to 81.1% of control levels. However, in animals exposed to smoke from PEW-treated cigarettes, there were no differences of SOD activity in rats exposed to even 32 cigarettes/day compared to the controls, and concentrations of the superoxide radical in the experimental group exposed to 32 treated cigarettes were similar to those recorded in the controls. This pattern was also seen in female rats.

The slightly decreased activity of glutathione peroxidase found in male and female rats in all experimental groups, but without any statistical significance compared to the controls, indicated that there were still some reactive oxygen species (such as hydrogen peroxide) that react with GPx, while probably not inducing any damage in the tissues. A slight and statistically insignificant change of the MDA level in animals exposed to cigarette smoke also indicated that there was no lipid peroxidation in the tissues.

#### *Pathohistological results*

There were no differences in the heart, aorta, liver, and peripheral nerves between experimental groups exposed to tobacco smoke and controls that could be attributed to treatment, indicating that exposure to tobacco smoke did not induce any change in these tissues.

#### *Morphometric analysis of lung tissue*

Repeated exposure to cigarette smoke can induce lung elastine breakdown and thus emphysema. Elastine breakdown/emphysema in smoke exposed rats was quantitatively measured by morphometric analysis of alveolar mean intercepts and alveolar tissue density in fixed lungs.

The data presented in Table 4 show the results of morphometric analysis of the lungs from rats included in the study in either control or smoke-exposed experimental groups. Two typical parameters were studied: the percentage of lung parenchyma in total surface of the lungs (% P) and mean circumference of alveoles (MCA).

In male rats exposed to smoke generated from normal cigarettes, we observed a 20-23% decrease in involvement of lung parenchyma in total surface of the lungs compared to the controls. This effect could be related to emphysema induced by tobacco smoke from normal cigarettes. In male rats exposed to smoke generated from treated cigarettes, there were no such changes and this parameter was the same as in the control animals. In female rats, however, there were no changes induced by exposure to smoke from normal cigarettes that would have any biological significance. We also observed an up to 20% increase in the mean circumference of alveoli in male rats exposed to smoke generated from normal cigarettes. As in the case of % P, this effect could be related to emphysema induced by tobacco smoke from normal cigarettes. In male rats exposed to

**Table 4.** Summary of mean values of morphometric parameters in rat lungs at the end of the experiment (on day 90). The numbers represent the mean value derived from 10 animals from the same experimental group. % P = percentage of lung tissue in total surface of the lungs, MCA = mean circumference of alveoles (in  $\mu\text{m}$ ).

Group	Male rats			Female rats				
	% P	%	MCA	%	% P	%	MCA	%
Control	0.470	100	303.24	100	0.388	100	312.84	100
16 normal cigarettes	0.366	77.6	363.86	120	0.376	96.9	344.87	110.2
16 treated cigarettes	0.418	88.9	331.93	109.4	0.392	101.3	320.84	102.5
32 normal cigarettes	0.376	80	354.00	116.7	0.369	95.1	335.53	107.3
32 treated cigarettes	0.465	98.9	310.23	102.3	0.391	100.8	306.44	98.0

smoke generated from treated cigarettes, there were no such changes and this parameter was similar to that in the control animals. In female rats, there was a slight increase of MCA in animals exposed to smoke from normal cigarettes. However, there were no differences of MCA in rats exposed to smoke from treated cigarettes compared to the control animals. Taken together, the results of morphometric analysis of lung tissue indicate that exposure of rats to smoke generated from normal cigarettes for 90 days induced changes characteristic of emphysema that were mainly at the initial stage. But in rats exposed to smoke generated from treated cigarettes, such changes were not observed.

#### DISCUSSION

A novel method of utilizing PEW has shown that it can induce changes in the response of animals to exposure to cigarette smoke from treated tobacco. Generally, the changes involved different levels of antioxidant enzymes due to ROS from cigarette smoke and pathological changes in the lungs or the occurrence of emphysema in animals exposed to smoke from untreated cigarettes.

During the 90-day exposure to cigarette smoke, there were neither mortalities nor significant signs of acute toxic exposure to cigarette smoke. In the group of animals treated with smoke generated from normal cigarettes, rats gradually lost weight and this effect apparently was not dose-related. In the group of rats exposed to smoke from 16 treated cigarettes, the animals did not lose any weight, which was similar to that of the control animals.

Results of our investigation of hematological parameters showed a significantly decreased white blood cell count (by 57%) in male rats exposed to normal smoke and a less pronounced decrease (up to 40%) in animals exposed to smoke from PEW-treated cigarettes. This indicates better health of male animals exposed to smoke from treated cigarettes compared to normal cigarettes and possibly a lower susceptibility to the influence of environmental factors. In female rats, the differences were within 25% of the control. There were no significant differences of clinical chemistry data within the

studied groups (data not shown). Specific biomarkers of exposure to tobacco smoke (thiocyanates and carboxyhemoglobin) were highly positively correlated with antioxidant biomarkers in the group of animals exposed to smoke from cigarettes treated by specific pulse electromagnetic radiation.

Exposure of rats to smoke generated from normal cigarettes resulted in a dose-dependent increase of thiocyanate concentration in blood, and these differences were statistically significant compared to the control values (i. e., thiocyanate concentration in blood of rats not exposed to tobacco smoke). However, in rats exposed to smoke generated from either 16 or 32 treated cigarettes per day, there were no differences in the concentration of thiocyanates in blood compared to the control animals. These results indicate, at least in relation to this specific biomarker, that the rats exposed to smoke from treated cigarettes for 90 days resembled the control animals and showed no signs of toxicity and exposure to tobacco smoke. This effect was observed in both male and female rats to a similar extent. Exposure of rats to smoke generated from both normal and treated cigarettes resulted in a dose-dependent increase of carboxyhemoglobin content in blood, and the differences were statistically significant compared to the control values. However, in rats exposed to smoke generated from 16 or 32 PEW-treated cigarettes per day, the carboxyhemoglobin content in blood was significantly lower than in the group of animals exposed to smoke from normal cigarettes. It has been questioned if food can enhance the levels of thiocyanate. In human studies, caution is taken in interpretation of data on thiocyanate, since its levels can be influenced by variations in food consumption, especially if onions or cyanogenic glycosides (such as those in fruit seeds) are frequently eaten, it being known that glycosides can be metabolized in the body to thiocyanates. However, the rats in our study were fed the same food and such variations of this parameter were thereby avoided. Interestingly, the results on the thiocyanate biomarker correspond to normal SOD activity in the control and the group exposed to smoke from treated cigarettes, but not in the experimental group exposed to smoke from normal cigarettes. Superoxide dismutase is the main

scavenger for ROS. It is very important that we have no increase or decrease in levels of SOD. Decreased SOD levels in red blood cells indicate decreased ability of the tissues to handle  $O_2^-$  radicals (Patel et al., 1998). Similar findings on SOD have been reported in the tissues of mice exposed to high fluoride intake (Shivarajashankara et al., 2001, 2002). Increased levels of oxidative radicals have been found in diseases such as Alzheimer's (Smith, 1998; Ouri et al., 2002; Roizen, 2005), COPD, and emphysema (Kasahara et al., 2000; Yoshida et al., 2007). Our results clearly show that PEW-treated tobacco does not impair the SOD activity in rats exposed to smoke from it. The status of biomarkers of oxidative stress in male rats, specifically SOD activity, in rats exposed to smoke from normal cigarettes was decreased in a dose-dependent manner to 81% compared to the controls. However, in animals exposed to smoke from treated cigarettes, there were no differences of SOD activity even in rats exposed to smoke from 32 cigarettes/day compared to the controls, indicating similar concentrations of superoxide radical activation. This pattern was also discernible in female rats. Slightly decreased activity of glutathione peroxidase (GPx) in male and female rats was found in all experimental groups, but without any statistical significance compared to the controls, indicating that there were still some reactive oxygen species that react with GPx, while probably not inducing any damage in the tissues. A slight, but statistically insignificant change of the malondialdehyde level in animals exposed to cigarette smoke also indicated that there was no lipid peroxidation in the tissues.

Previous results and research show that homeostasis of SOD activity must be maintained, and that only normal levels of SOD indicate good health. Increased levels like those recorded in AD and Down's syndrome point to increased oxidation on the levels of protein, RNA, and DNA (Roizen et al., 2005) and enhanced expression of heme oxygenization (Müller et al., 1994, 1998; Müller and Gebel, 1997).

This 90-day study corroborates some previous work in our lab indicating that exposure to smoke can induce emphysema. Emphysema induced by tobacco smoke is a subject that has been thoroughly

explored in the tobacco research community, and tobacco smoke is known to be an agent of toxicity in humans (Niewoehner, 1988; Ofulue and Ko, 1999; Kasahara et al., 2000; Yoshida and Tuder, 2007). Some latest research (Banerjee et al., 2007) has shown that by using high levels of antioxidants from black tea, apoptosis and lung damage can be prevented in an animal model. Many strategies over the years have been employed to lower the oxidative load from cigarettes, from using pycnogenol in filters (Zhang et al., 2002) to using antioxidants as supplements (De Waart et al., 2000). The possibility of an economical and effective way to directly reduce toxicity of tobacco products by using PEW is here explored. Morphological analysis of the lungs revealed that in male rats exposed to smoke generated from normal cigarettes, there was about a 20-23% decrease in involvement of lung parenchyma in total surface of the lungs compared to the controls. We also observed an up to 20% increase in the mean circumference of alveoli in male rats exposed to smoke generated from normal cigarettes. These effects could be related to emphysema induced by tobacco smoke from normal cigarettes. In male rats exposed to smoke generated from PEW-treated cigarettes, there were no such changes and this parameter was similar to that in the control animals. In female rats, there was a slight increase of MCA in animals exposed to smoke from normal cigarettes. However, there were no differences of MCA in rats exposed to smoke from treated cigarettes compared to the control animals.

We found that emphysema in animals exposed to smoke from untreated cigarettes increased from day 70 and corresponds to the oxidative status in animals of that group. The antioxidant properties of treated tobacco versus untreated tobacco were correlated with the onset of emphysema. The results of morphometric analysis of lung tissue indicate that exposure of rats to the smoke generated from normal cigarettes for 90 days induced changes characteristic of emphysema that are mainly at the initial stage. But in rats exposed to the smoke generated from treated cigarettes, such changes were not observed. Even though these results of using PEW in treatment of tobacco products are promising, we need to further

expand these findings by using more species (such as hamsters) (Banerjee, 2007) and eventually test the oxidative status in humans smoking PEW-treated cigarettes. In mice (data not published), the content of antioxidant enzymes showed a lower level of oxidative stress when exposed to smoke from treated cigarettes, but the difference was not statistically significant. Still, mice exhibited significant changes of behavior, i. e., a significantly lower stress response was observed in mice that were exposed to smoke from treated cigarettes. In the present study, no difference of behavior was seen. In toxicology, a difference of responses in different species is not uncommon, so more research needs to be done before our data can be extrapolated to humans, especially since these are the first data showing that PEW can induce lower toxicity in such a complex compound as tobacco.

### CONCLUSION

On the basis of the results obtained in this study, it can be concluded that smoke generated from cigarettes exposed to a specific pulsed electromagnetic field is significantly less toxic than smoke generated from normal cigarettes, which caused significant toxic effects such as emphysema in the lungs and decreased levels of SOD.

*Acknowledgments* — We thank IBC Technologies for providing a grant for this research and the inventor Athanasios Nikolaou for supplying the PEW machine.

### REFERENCES

- Banerjee, S., Maity, P., Mukherjee, S., Sil, A. K., Panda, K., Chattopadhyay, D., and I. B. Chatterjee (2007). Black tea prevents cigarette smoke-induced apoptosis and lung damage. *J. Inflamm. (Lond.)* **4**, 1-12.
- Beauchamp, C., and I. Fridovich (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gel. *Anal. Biochem.* **44**, 276-287.
- Benowitz, N. L. (1999). Biomarkers of environmental tobacco smoke exposure. *Environ. Health Perspect.* **107**, 349-355.
- Beutler, E., Duron, O., and B. M. Kelly (1963). Improved method for the determination of blood glutathione. *J. Lab. Clin. Med.* **61**, 882-888.
- De Waart, F. G., Smilde, T. J., Wollersheim, H., Stalenhoef, A. F., and F. J. Kok (2000). Smoking characteristics, antioxidant vitamins, and carotid artery wall thickness among life-long smoking. *J. Clin. Epidemiol.* **53**, 707-714.
- Dube, M. F., and C. R. Green (1982). Methods of collection of smoke for analytical purposes. *Rec. Adv. Tobacco Sci.* **8**, 42-102.
- Elkins, H. (1951). Determination of thiocyanates in biological materials, 360-364. In: *The Chemistry of Industrial Toxicology*. New York.
- Escolar, J. D., Martinez, M. N., Rodriguez, F. J., Gonzalo, C., Escobar, M. A., and P. A. Roche (1995). Emphysema as a result of involuntary exposure to tobacco smoke: morphometrical study of the rat. *Exp. Lung Res.* **21**, 255-273.
- Facchinetti, F., Amadei, F., and P. Geppetti (2007). Alpha, beta-unsaturated aldehydes in cigarette smoke release inflammatory mediators from human macrophages. *Am. J. Respir. Cell Mol. Biol.* **5**, 617-623.
- Huang, M. F., Lin, W. L., and Y. C. Ma (2005). A study of reactive oxygen species in the mainstream in ovaries of mice and its reversal. *Indoor Air.* **15**, 135-140.
- Jarvis, M. J., Russell, M. A., and C. Feyerabend (1983). Absorption of nicotine and carbon monoxide from passive smoking under natural conditions of exposure. *Thorax* **38**, 829-833.
- Jarvis, M. J., Tunstall-Pedoe, H., Feyerabend, C., Vesey, C., and Y. Sallojee (1987). Comparison of tests used to distinguish smokers from nonsmokers. *Am. J. Public Health* **77**, 1435-1438.
- Kapfer, G. F., Nitz, S., and F. Drawert (1989). Bound aroma compounds in tobacco smoke condensates. *Z. Lebensmitt. Forsch.* **188**, 512-516.
- Kasahara, Y., Tuder, R. M., and L. Taraseviciene-Stewart (2000). Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J. Clin. Invest.* **106**, 1311-1319.
- Kawakami, M., Paul, J. L., and W. M. Thurlbeck (1984). The effect of age on lung structure in male BALB/cNnIa inbred mice. *Am. J. Anat.* **170**, 1-21.
- Klaassen, C. D. (1996). Nonmetallic environmental toxicants. Air pollutants, solvents, and vapors, and pesticides. In: *The Pharmacological Basis of Therapeutics* (Eds. J. G. Hardman, L. E. Limbird, P. B. Molinoff, R. W. Ruddon, Goodman, and A. Gilman), Ninth Edition, Chapter 67, p. 1678. McGraw-Hill, New York.
- Manton, K. G., Volovik, S., and A. Kulminski (2004). ROS effects on neurodegeneration in Alzheimer's disease and related disorders: on environmental stresses of ionizing radiation. *Curr. Alzheimer Res.* **4**, 277-293.
- Müller, T., and S. Gebel (1994). Heme oxygenase expression in Swiss 3T3 cells following exposure to aqueous cigarette smoke fractions. *Carcinogenesis* **15**, 67-72.
- Müller, T., and S. Gebel (1998). The cellular stress response induced by aqueous extracts of cigarette smoke is criti-

- cally dependent on the intracellular glutathione concentration. *Carcinogenesis* **19**, 797-801.
- Müller, T., Haussmann, H. J., and G. Schepers (1997). Evidence for peroxynitrite as an oxidative stress-inducing compound of aqueous cigarette smoke fractions. *Carcinogenesis* **18**, 295-301.
- Niewoehner, D. E. (1988). Cigarette smoking, lung inflammation, and the development of emphysema. *J. Lab. Clin. Med.* **111**, 15-27.
- Ofulue, F., and M. Ko (1999). Effects of depletion of neutrophils or macrophages on development of cigarette smoke-induced emphysema. *Am. J. Physiol. Lung Cell Mol. Physiol.* **277**, 97-105.
- Ohkawa, H., Ohishi, N., and K. Yagi (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95**, 351-358.
- Ou, B., and D. Huang (2006). Fluorescent approach to quantitation of reactive oxygen species in mainstream cigarette smoke. *Anal. Chem.* **78**, 3097-3103.
- Oury, T. D., Schaefer, L. M., and C. L. Fattman (2002). Depletion of pulmonary EC-SOD after exposure to hyperoxia. *Am. J. Physiol. Lung Cell Mol. Physiol.* **283**, 777-784.
- Pagilia, D. E., and W. N. Valentine (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* **70**, 158-169.
- Patel, P. D., and N. J. Chinoy (1998). Influence of fluoride on biological free radical reactions in ovary of mice and its reversal. *Fluoride* **31**, S27.
- Pryor, W. A. (1997). Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. *Environ. Health Persp. Suppl.* **105**, 875-883.
- Roemer, E., Tewesa, F. J., Meisgena, T. J., Veltela, D. J., and E. L. Carmines (2002). Evaluation of the potential effects of ingredients added to cigarettes. Part 3: *In vitro* genotoxicity and cytotoxicity. *Food Chem. Toxicol.* **40**, 105-111.
- Roizen, N. J. (2005). Complementary and alternative therapies for Down's syndrome. *MRDD Res. Rev.* **11**, 149-155.
- Rustemeier, K., Stabbert, R., Haussmann, H. J., Roemer, E., and E. L. Carmines (2002). Evaluation of the potential effects of ingredients added to cigarettes. Part 2: Chemical composition of mainstream smoke. *Food Chem. Toxicol.* **40**, 93-104.
- Shivarajashankara, Y. M., Shivashankara, A. R., and P. Gopalakrishna (2001). Effect of fluoride intoxication on lipid peroxidation and antioxidant systems in rats. *Fluoride* **34**, 108-113.
- Shivarajashankara, Y. M., Shivashankara, A. R., Gopalakrishna, B. P., and R. S. Hanumanth (2002). Brain lipid peroxidation and antioxidant systems of young rats in chronic fluoride intoxication. *Fluoride* **3**, 197-203.
- Smith, C. D., Carney, J. M., Tatsumo, T., Stadtman, E. R., Floyd, R. A., and W. Markesbury (1992). Protein oxidation in the aging brain. *Ann. N. Y. Acad. Sci.* **663**, 110-119.
- Smith, M. A. (1998). Alzheimer's disease. *Int. Rev. Neurobiol.* **42**, 1-54.
- Stankovic, M., and M. Milic (1970). *Analysis of Biological Material in Industrial Toxicology* (in Serbian), pp. 123. Serbian Institute of Health and Work Management, Belgrade.
- Taylor, P. (1996). Agents acting on the neuromuscular junction and autonomic ganglia, In: *The Pharmacological Basis of Therapeutics* (Eds. J. G. Hardman, L. E. Limbird, P. B. Molinoff, R. W. Ruddon, Goodman, and A. Gilman), Ninth Edition, Chapter 9, p. 192. McGraw-Hill, New York.
- Thurlbeck, W. M. (1967a). Measurement of pulmonary emphysema. *Am. Rev. Respir. Dis.* **95**, 752-764.
- Thurlbeck, W. M. (1967b). Internal surface area and other measurements in emphysema. *Thorax* **22**, 483-496.
- Toshinori, Y., and R. M. Tuder (2007). Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. *Physiol. Rev.* **87**, 1047-1082.
- Tschumperlin, D. J., and S. S. Margulies (1999). Alveolar epithelial surface area-volume relationship in isolated rat lungs. *J. Appl. Physiol.* **86**, 2026-2033.
- Wright, J. L., and A. Churg (1990). Cigarette smoke causes physiologic and morphologic changes of emphysema in the guinea pig. *Am. Rev. Respir. Dis.* **142**, 1422-1428.
- Zhang, D. L., Tao, Y., Duan, S. J., Rohwald, P., and B. L. Zhao (2002). Pycnogenol in cigarette filters scavenges free radicals and reduces mutagenicity and toxicity of tobacco smoke *in vivo*. *Toxicol. Indus. Health* **18**, 215-224.

## ОКСИДАТИВНИ СТРЕС ЈЕ РЕДУКОВАН У W1STAR ПАЦОВА КОЈИ СУ ЕКСПОНИРАНИ ДИМУ ИЗ ДУВАНА ПРЕТХОДНО ТРЕТИРАНОГ СПЕЦИФИЧНИМ ШИРОКО ПОЈАСНИМ ЕЛЕКТРОМАГНЕТНИМ ПОЉЕМ

В. БАЈИЋ<sup>1</sup>, Б. БАЈИЋ<sup>2</sup>, ЗОРАНА МИЛИЋЕВИЋ<sup>3</sup>, СЛАВИЦА РИСТИЋ<sup>1</sup> и А. НОКОЛАУ<sup>4</sup>

<sup>1</sup>Институт за биомедицинска испитивања, Галеника а. д., 11000 Београд, Србија

<sup>2</sup>ИБЦ Технологије, 11000 Београд, Србија

<sup>3</sup>Институт за нуклеарна истраживања »Винча«, 11000 Београд, Србија

<sup>4</sup>IVSTec, 191 Мегара, Атина, Грчка

Током деведесетодневног експеримента респираторне токсичности, пацови Wistar соја су били изложени дуванском диму који је настао од сагоревања цигарета претходно процесираних специфичним ултра-широкопојасним пулсним електромагнетним пољем *vs.* нетретираним цигаретама. У оквиру експеримента пратила се активност антиоксидативних ензима супероксид дисмутазе (SOD); глутатион пероксидазе (GPx) и малондиалдехида (MDA), маркера изложености диму (тиоцијанате и карбоксихемоглобин), хематолошких параметара и на крају патоморфолошка анализа плућа. Резултати су показали да постоје статистички значајне разлике у вредности SOD, тиоцијаната и карбоксихемоглобина између групе пацова која је била изложена дуванском диму који је настао од третираних (процесираних) цигарета од групе где цигарете нису биле процесиране. Резултати третиране групе (процесиране) су пока-

зале да се вредности приказаних параметара могу поредити са контролном групом. Такође, добијени резултати су били у кореспонденцији са патоморфолошким анализом плућа која је указала на појаву почетка емфизема код пацова изложених диму нетретираних цигарета. Емфизем је изостао у експерименталној групи пацова који су били изложени диму третираних (процесираних) и код пацова који уопште нису били изложени диму (контролна група). Укупни резултати указују на појаву смањења оксидативног стреса код пацова који су изложени диму третираних цигарета специфичним електромагнетним пољем. Могућност да се направе промене у метаболичком одговору биолошких система у смислу смањења оксидативног стреса приликом изложености токсичних материја из дима добијеног од цигарета процесираних ЕМ пољем јесте важан правац за будућа истраживања.