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Histological and biochemical effects of cigarette smoke on lungs

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In this study, rats were made to inhale cigarette smoke in a specifically prepared container for different periods. The lung tissue samples of the subjects were examined by light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). Malonaldehyde, one of the free oxygen radicals was determined in lungs and plasma. The catalase activity level of erythrocyte and arginase levels were determined. Three groups were formed. The rats in the Ist and IInd groups were made to inhale cigarette smoke for 30 and 60 minutes a day for a total period of 3 months. Control group, the rats in the IIIrd group (controls) were made to inhale clean air during the same periods. An increase in the number of macrophages was observed in the pulmonary tissue of the exposed groups. Especially in the group that inhaled the smoke for long periods, the number of macrophages and the inclusion bodies contained in them increased. These differences could easily be observed in TEM studies. In the light microscopy and SEM observations, it arouse attention that the alveolar macrophages occurred as sets and their activation increased. Depending on the length of the exposure to cigarette smoke, an increase in the number of macrophages was observed. Statistically significant increases were determined in the malonaldehyde levels of pulmonary tissue and plasma when compared to the control group. Besides significant increases were found in the catalase activity levels of erythrocytes in the experimental groups.

Keywords: lung, cigarette, TEM, SEM, malonaldehyde, catalase

It’s known that cigarette adversely influences the immune system negatively affects the cellular structure in respiratory system, increases the number of

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macrophages, PMN and lymphocytes in the parenchymal tissue of the lungs and causes pulmonary fibrosis, chronic bronchitis, emphysema and cancer in various organs, especially in the lungs (1–7). As the American Cigarette Association has stated, approximately 87% of the lung cancers in United States of America was caused by active cigarette smoking (8). Besides as a result of epidemiological studies it also has stated that passive cigarette smoking can be a risk factor for lung cancer in non-smokers (9).

Evidence has accumulated that cigarette smoke destroyed the endothelial structure of the blood vessels, increased the cholesterol, lipoprotein and endothelin-1 levels in serum and caused arteriosclerosis (10–12). It also has been observed that placental blood circulation in pregnant women was affected, the weight of the newborn babies was low (13) and these babies faced with learning difficulties later (14).

The accumulation of neutrophils has been observed in the pulmonary tissue of smokers. Nicotine has been described to induce apoptosis and to prolong the life period of neutrophils (15). Moreover the endothelium of pulmonary capillaries has been reported to be damaged, and PMN accumulation has been observed in alveoli and small blood vessels (16). It has been determined that number of the inclusion bodies in the cytoplasm of the increased macrophages also increased (17, 18). In biochemical studies, it has been observed that cigarette smoke contained many free radicals which can cause tissue damage (19).

The aim of this study was the light and electron microscopic (TEM and SEM) and the biochemical examination of changes occurring in the pulmonary tissues of rats exposed to cigarette smoke via inhalation.

Materials and Methods

Totally 15 Wistar type male rats weighing of 200–250 g were used. The subjects were divided into three groups. One group (n=5) was made to inhale cigarette smoke 30 minutes a day while the other group inhaled (n=5) 60 minutes a day in an inhalation cabin for a period of 3 months. The third group was evaluated as the control group (n=5), which inhaled clean air. A glass cabin was prepared and insulated with silicone (dimension: 50×35×36 cm, thickness 0.5 mm, inner volume 0.060 m^3). A short plastic pipe, with one end left outside, was inserted into the cabin.

The cigarette the was lighted during the experiment and placed to the end of this pipe and the entire cigarette was puffed. In the study, cigarettes without a filter tip (Bitlis-Tekel) were used. At the end of the study, blood and lung tissue samples were taken from the animals under general anesthesia (ketamin+rompun). The tissue were fixed with 2.5% Glutaraldehyde with 0.1 M phosphate buffer (pH 7.2); later they were

dehydrated by being passed through the graded ethanol series and at the last they were embedded in to Araldit Cy 212+DDSA+BDMA mixture. Semi-thin sections were prepared from a part of the tissues for light microscopic examinations. All the sections were examined with BH 2 Olympus photomicroscope and Zeiss 9S2 electron microscope.

Samples for scanning electron microscopy were fixed in 2.5% Glutaraldehyde with 0.1 M phosphate buffer (pH 7.2); later they were post-fixed in a phosphate-buffered solution of 1% Osmium tetroxide at 37 °C for 2 hours. The specimens were then dehydrated in a graded ethanol series. After serial dehydration and immersion into isoamyl acetate they were dried using a CO_2-critical point drying method and coated with gold. Finally, the specimens were examined under a scanning electron microscope (Jeol JSM-35 SEM at 15 kV).

The levels of malonaldehyde (19, 20), one of the free oxygen radicals, arginase levels (21), and the catalase activity in erythrocytes (22) were examined in the tissue biochemically. The malonaldehyde level was determined as nmol/ml in the blood and as nmol/gr in the tissue. Arginase activity was measured spectrophotometrically with thiosemicarbaside diacetylmonoxime urea (TDMV) method. The unit for erythrocyte arginase is the g-Hb value of the enzyme activity that produces l μmol urea from L-arginin substrate at 37 °C in 1 minute. For the catalase activity, tissue homogenate was centrifuged at +4 °C, 3000 rpm for 15 minutes, and the time between 0.450–0.400 absorbancies was measured and evaluated. The results were evaluated with Mann-Whitney *U* test.

Results

Light microscopic examination revealed that macrophages in the inter-alveolar parenchyma tissue increased in number when compared to the control group and that the cytoplasmic granules in the macrophages especially in experimental Group II. were stained more obviously. The appearance of type I and type II pneumotocytes resembled to those of the control group. The macrophages that had thinner cytoplasmic granules could also be seen (Figs 1, 2a and 3a). In both of the experimental groups, free macrophages were observed in the alveoli (Figs 2b and 3b). The number of free alveolar macrophages had increased very much experimental group II and their coming together and forming sets aroused attention (Fig. 3b). The activation of these macrophages that increased in number and their moving through the spaces among alveoli were clearly seen (Fig. 3c).

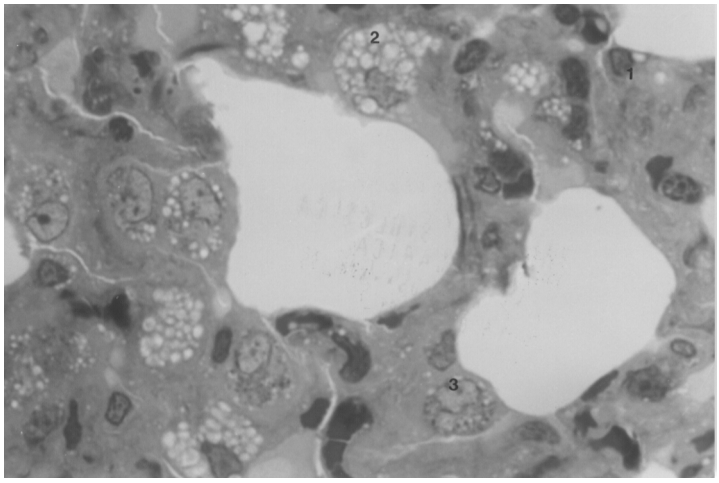

Fig. 1. Control lung. Type I pneumocytes (1), type II pneumocytes (2) and macrophages (3) has been observed. Toluidine Blue ×100

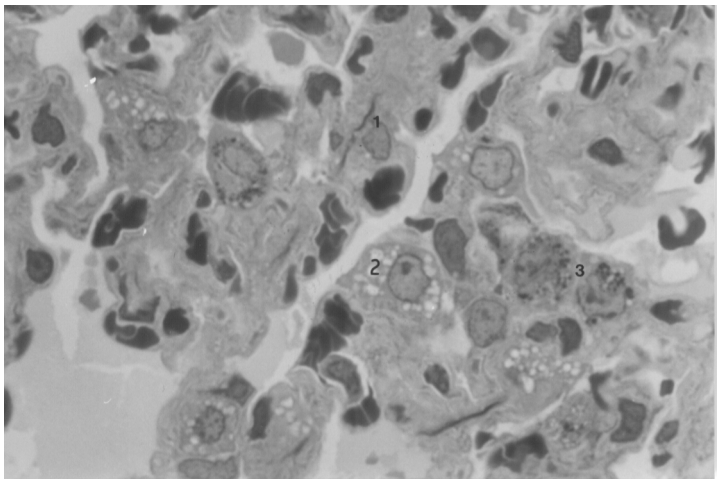

Fig. 2a. Appearance of the lung of the first experimental group resembling the control group. Type I pneumocytes (1), type II pneumocytes (2), macrophages (3). Toluidine Blue ×100

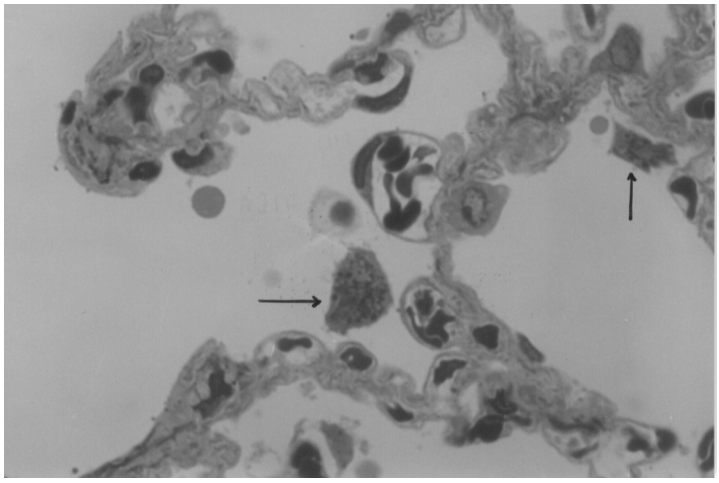

Fig. 2b. At the same group another aspect. A few alveolar macrophages (arrows). Toluidine Blue ×100

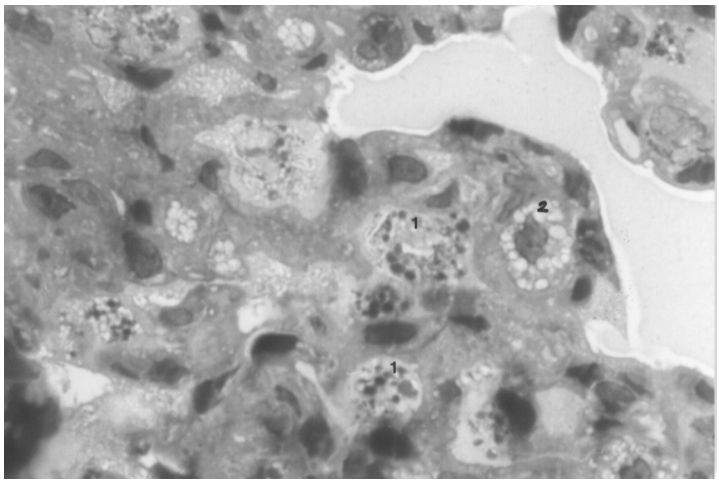

Fig. 3a. The lung tissue of the second experimental group. It can be seen that the increased macrophages with intracytoplasmic granules in the lung parencyma (1), type II pneumocytes (2). Toluidine Blue ×100

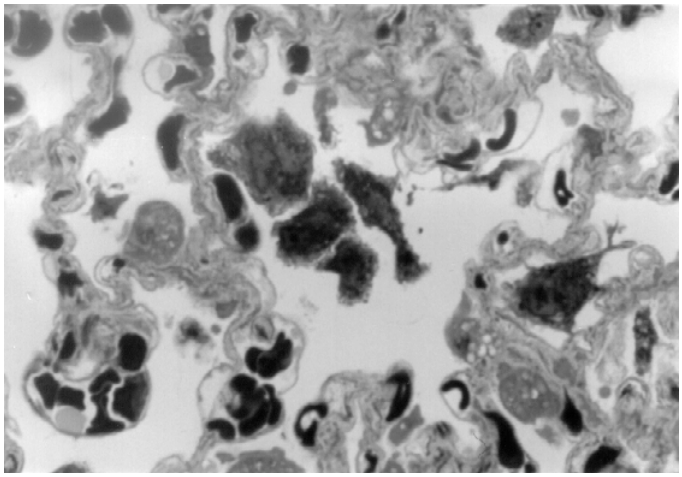

Fig. 3b. It is observed that the alveolar macrophages (arrows) come together and form sets in the experimental group II. Toluidine Blue ×100

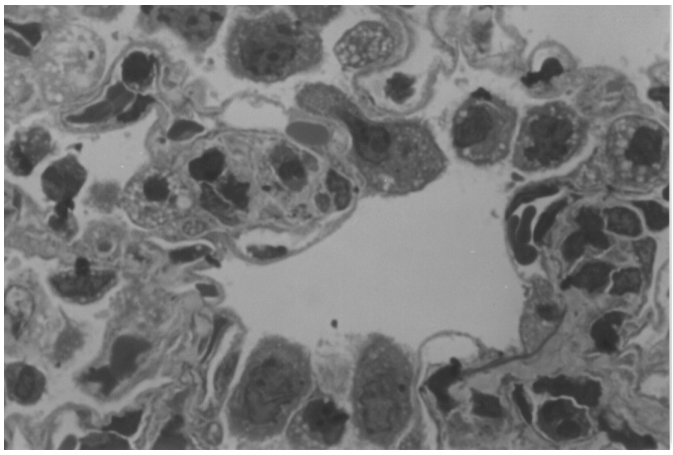

Fig. 3c. The activated alveolar macrophages at the same group move through interalveolar spaces. Toluidine Blue ×100

When examined at electron microscopic (TEM) level inclusion bodies in the cytoplasm of macrophages were seen to have had different shapes and sizes (Figs 4, 5 and 6). The diameters and numbers of the inclusion bodies in the macrophages of the smoking group had increased and it was observed that they were stained dark.

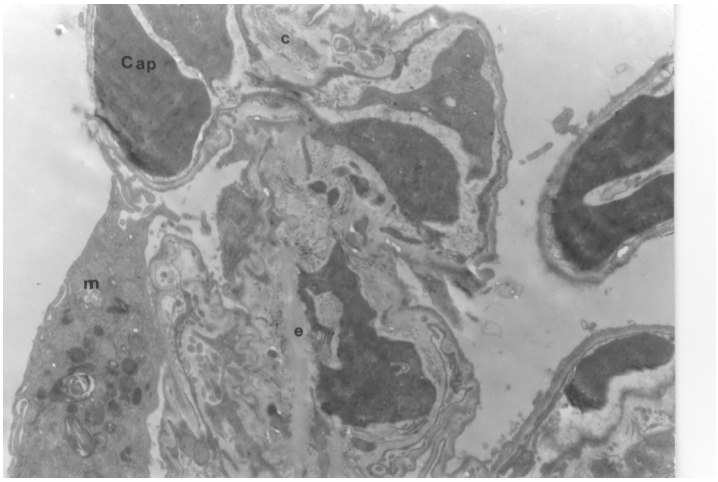

Fig. 4. The TEM appearance of the control lung tissue. Capillaries (Cap), Elastic fibers (e), Collagen fibers (c), Alveolar macrophages (m). Lead citrate-uranyl acetate. Original magnification ×4400

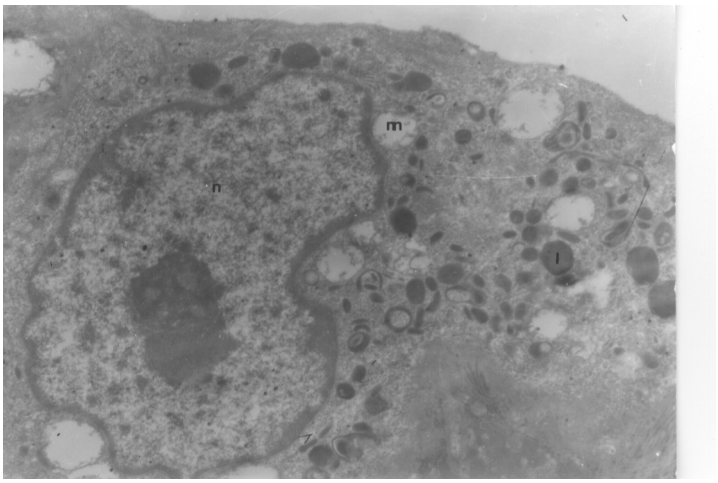

Fig. 5. Macrophages structure in the lung of the experimental groups I. Nucleus (n), mitochondria (m), lysosomes (l). Lead citrate-uranyl acetate. Original magnification ×7000

Especially the middle parts of the inclusion bodies, there were regions in the shape of straight lines, which were not stained. The macrophage nucleus had a deep notch and there were quite widened structures in the regions near the nucleus (Fig. 6).

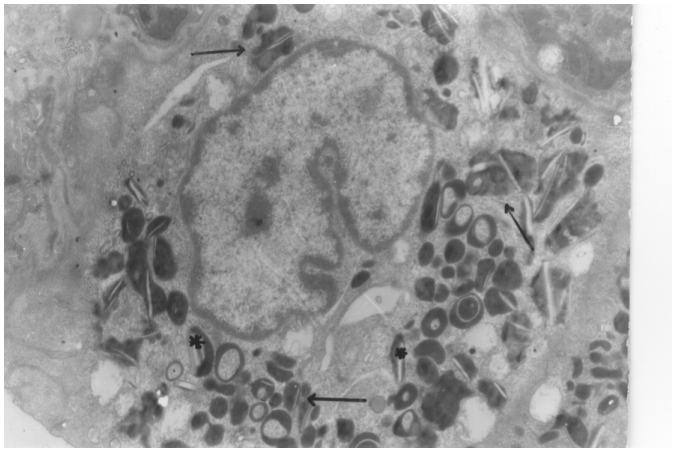

Fig. 6. The diameters and numbers of the inclusion bodies in the macrophages of the experimental group II had increased more than experimental group I and the control group (arrows). In the middle of the inclusion bodies there were regions in the shape of straight lines, which were not stained (*).
Lead citrate-uranyl acetate. Original magnification ×7000

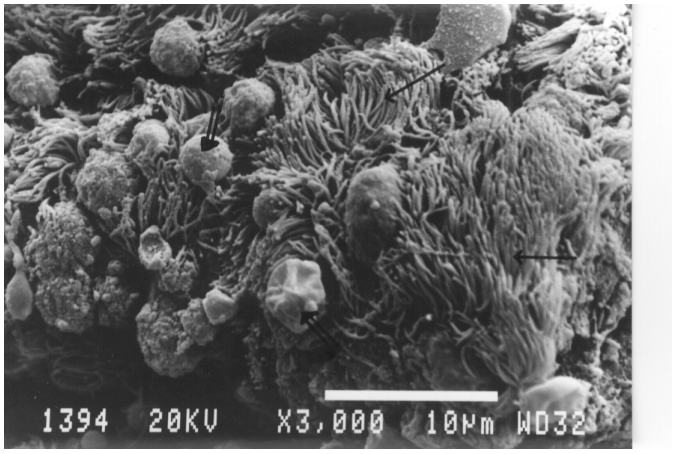

Fig. 7. The SEM appearence of control group of the rat bronchiole. The regular cilium structure is seen (arrows) and among it the various stage secretion of Clara cells (double arrows).
Original magnification ×3500

In the SEM studies, it was seen that the cilium structures in the bronchioles of the experimental groups had been destroyed when compared to the control group and the group which inhaled the smoke for longer periods were more obviously affected.

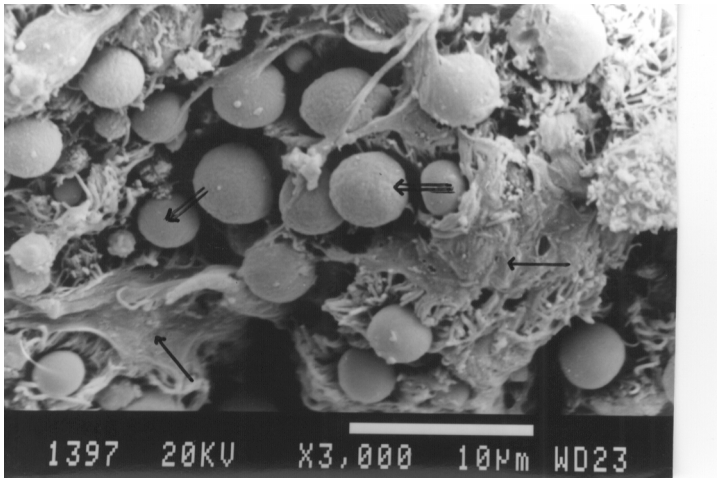

Fig. 8. In the experimental group I, the cilium structure is irregulate (arrows) and activated Clara cells (double arrows) are seen. Original magnification ×3000

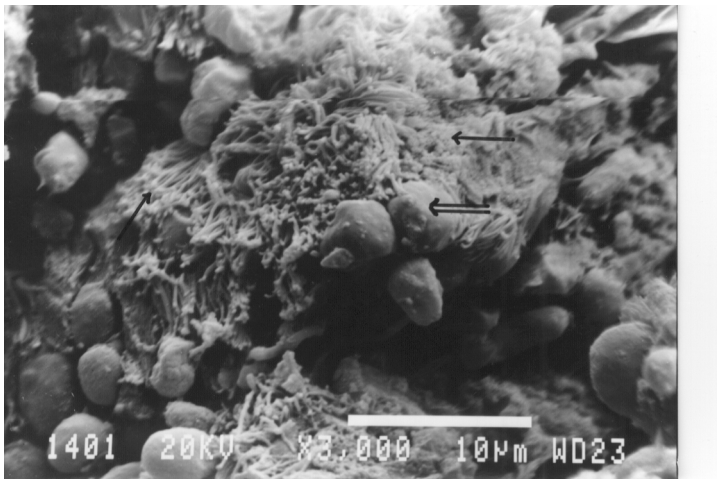

Fig. 9. The bronchiole of the experimental group II. It is seen that cilium degeneration degree (arrows) increases. Clara cells (double arrows). Original magnification ×3000

It was observed that the abundant Clara cells became activated especially in experimental group I (Figs 7, 8 and 9). In the experimental group II there were free macrophages which had quite evident prolongations and which were gathered together, while free alveolar macrophages were not observed in the control and experimental group I (Fig. 10).

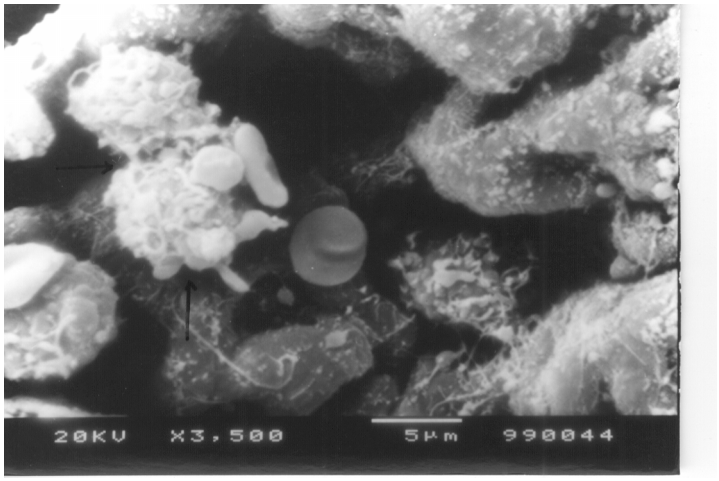

Fig. 10. The SEM appearance of the experimental group II. Alveolar macrophages (arrows) increased. Original magnification ×3500

The biochemically obtained data are shown in Tables I, II and III. The malonaldehyde levels in the pulmonary tissue and in the plasma of the experimental groups increased. These increases were significant when compared to the control group. The difference between the experimental groups were statistically non-significant (Table I). The catalase activity of erythrocyte of the experimental groups increased when compared to the control group, and these results had statistical significance (Table II). While the arginase activity levels in the pulmonary tissue of the experimental groups decreased when compared to the control, these levels increased in erythrocyte and such changes were not significant (Table III).

Table I

Malonaldehyde level in lung (nmol/g protein; Mean ± SD) and erythrocytes (nmol/ml; mean ± SD). Group I and group II were composed of rats exposed to cigarete smoke inhalation for 30 min/day in 3 month, respectively

	n	Lung	Plasma
Control	5	513.73 ± 99	1.63 ± 0.05
Group I	5	790.8 ± 181*	4.13 ± 0.1*
Group II	5	641.20 ± 59.39*	4.10 ± 0.65*

*$p<0.05$, n: number of observations in each group

Table II

Activity of catalase in erythrocytes (U/g Hb; mean ± SD). Group I and group II were composed of rats exposed to cigarette smoke inhalation for 30 min/day and 60 min/day in 3 months, respectively

	n	Erythrocytes
Control	5	0.03 ± 00
Group I	5	0.06 ± 0.02*
Group II	5	0.05 ± 00*

*p<0.05, n: number of observations in each group

Table III

Activity of arginase in lung (U: μmol/urea/g protein/h; mean ± SD) and erythrocytes (U: μmol/urea/g Hb/h; mean ± SD). Group I and group II were composed of rats exposed to cigarette smoke inhalation for 30 min/day and 60 min/day in 3 months, respectively

	n	Lung	Erythrocytes
Control	5	0.61 ± 0.29	18.75 ± 8.45
Group I	5	0.13 ± 0.04	23.45 ± 3.95
Group II	5	0.18 ± 0.03	26.23 ± 7.87

p>0.05, n: number of observations in each group

Discussion

In this study, the changes that occur in pulmonary tissues of the rats which inhale cigarette smoke for 30 and 60 minutes a day for a period of 3 months were examined.

In chronic smokers who start smoking from early youngages, the risks of lung cancer chronic obstructive pulmonary disease and emphysema is higher. The N-Methyl carbazole that has a strong mutagen effect. It is produced by the lung and liver microsomes, depending on the period and the amount of the inhaled nicotine (24). Neutrophil infiltration in the bronchial epithelium and an increase in the number of eosinophils in the sub-epithelial connective tissue were observed (25). Cigarette shortens the period of PMN transition from the bone marrow to the periphery. It causes damage in the pulmonary capillary endothelium and thus neutrophil accumulation occurs in the tissue, around the vessels (16, 26). In pulmonary tissues of smokers, macrophages have been reported to be increased in number (27) and to be activated (28). It was also stated that the number of neutrophils and lymphocytes increased in addition to the increase of the alveolar macrophages, fibrosis occurred and emphysema

features were observed in some regions (29). The cigarette smoke effects the neuroepithelial cells in bronchi and increases the secretion of peptides such as Bombesin. And this influences the production of the granulocyte/macrophage colony stimulating factor by monocytic cells and causes the macrophage, neutrophil, eosinophil cells to increase in number (30). Heckman and Dalbly (31) observed an increase of lymphoreticular cells around the vessels and bronchi in pulmonary tissues of the smokers that were made to smoke 7 cigarettes a day for 1–2 years, fibrosis, and an increase of the number of type II pneumotocytes. In emphysema, the activated neutrophils or macrophages produce protease in large amounts resulting in damage to the pulmonary tissue. While a positive correlation is found between the number of T lymphocytes and that of the alveolar macrophages, there existed a negative correlation between T lymphocytes and neutrophils. Therefore, it can be concluded that it is the macrophage that can be made mainly responsible for pulmonary damage (32).

In our observations it has been found that macrophages in the pulmonary tissues of smoking groups increased rather than neutrophils, and especially alveolar macrophages formed sets in the group that inhaled the smoke for a long time. The alveolar macrophages that were in the activated groups were also observed through SEM. The migration of macrophages through the septal spaces among alveoli could easily be seen. There was no change such as fibrosis or emphysema in any of our experimental groups.

The inclusion bodies that were included by macrophages the number of which increased due to smoking and the structure of lysosomes were examined. These structures, which are surrounded by a single membrane, are also named as “smokers” inclusions (17, 18). It was observed that the number and the diameters of secondary lysosomes increased (33). Structurally 15 different inclusion bodies were found in macrophages. Lipid inclusions, myelin structures, erythrocytes were defined as kaolin crystals (34).

At the end of this study, the findings about macrophages were consistent with the observations stated. Especially in the experimental group II the inclusion bodies in the macrophages were in various shapes and intensely dyed. This was observed both in light and in TEM. Although type I pneumatocytes and damages in endothelial cells were seen, depending on the amount of the inhaled nicotine (35), we didn’t observe any type I, type II diseases or any structural destruction in endothelial cells. In the distal air track, respiratory and terminal bronchiole epithelia of the smokers, many intra-epithelial mast cells were found (36). We didn’t observe intraepithelial mast cells in bronchi and pulmonary air track. However, we found intraepithelial mast cells in the tracheas of the rats that inhaled cigarette smoke (37).

In SEM studies, the diameters of interalveolar pores in the areas that were near to respiratory bronchioles and alveolar ducts were found to be greater than the diameters of the pores in distal regions (38). It was also observed that the pores increased in number and most of them existed in the bottom parts of alveolar walls (39). The expansion of the alveolar pores after a short-term (2–4 months) smoking was states to be dependent on the destruction in alveolar walls (40). The increase in the number and diameter of the pores was evaluated as a step of emphysema development (38). The number and the diameter of the interalveolar pores of the experimental groups that inhaled the smoke for a short and long period were like those of control group. We found that the cilium structure was more and more affected and alveolar macrophage sets occurred in experimental groups. We considered the increase of secretion that protects the surface epithelial cells as the reason of the cell activity.

In biochemical studies it is stated that the free radicals in cigarette smoke caused tissue damage (19, 41). Researchers have shown that the malonaldehyde level of smokers increased (19). In our biochemical data, a statistically significant increase was found in the malonaldehyde and erythrocyte catalase levels of their pulmonary tissues and plasms when compared to the control group. Some researchers have observed an inverse proportion between the malonaldehyde level and the erythrocyte catalase level. The arginase activity levels were not statistically significant.

As a result, it is considered that the lack of an increase in bronchus and bronchiole epithelia, the lack of fibrosis and emphysema in experimental groups is related with the period of smoking, that the macrophage increase and activation are changes at the beginning, and that the defense system becomes active. The macrophage increase which is observed in subjects that in hale the cigarette smoke for longer periods proves the direct relation between the pulmonary tissue damage and the smoking period once again.

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