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Aysel KÜKNER[1]
Hakan ÖNER[1]
Neriman ÇOLAKOĞLU[1]
Jale ÖNER[1]
Enver OZAN[1]
Sema OZAN[2]
Seval YILMAZ[2]

Observations of the Changes Occurring in the Rat Trachea Due to Inhalation of Cigarette Smoke

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[1]Department of Histology and Embryology Faculty of Medicine, [2]Department of Biochemistry Faculty of Veterinary, Firat University, Elazığ-TURKEY

Abstract: In this study, the tracheal tissues of rats inhaling cigarette smoke passively during different periods were examined by light and electron microscopy. Malonaldehyde, erythrocyte catalase and arginase levels were also measured. Three groups were formed and a total of 15 Wistar male rats weighing 200-250 g were used. Rats assigned to the first and second groups were made to inhale cigarette smoke for 30 and 60 minutes respectively for a total of 3 months. The subjects in the third group were made to inhale clean air as a control. Cigarettes without filter tips were used througout the study. Lit cigarettes were inserted into the chamber. At the end of the study period, blood and tissue samples were taken. The results revealed that the subjects which inhaled cigarette smoke for a longer period were affected more. Irregularities in epithelial cells, increase in secretion, and increases in mast cells in connective tissues, especially those which had migrated through the epithelial cells, were observed. The considerable increase in the separations between epithelial cells is of particular note. Malonaldehyde levels in tissues were lower than in the control group, but higher in plasma. The difference in plasma levels was found to be statistically significant. A significant increase was also observed in activity levels of erythrocyte catalase.

Key Words: cigarette, trachea, microscopy, malonaldehyde, catalase.

Introduction

It is known that cigarette smoke influences the occurrence of pulmonary cancer and diseases of the respiratory system, heart and vessels (1-3). Likewise, it is known that cigarettes contains more than 4000 chemical substances, most of which are mutagenic and carcinogenic (4,5). In epidemiological studies, it is stated that the passive inhalation of the cigarette smoke by non-smokers is a risk factor for pulmonary cancer (6). Many kinds of free radicals existing in cigarette smoke are proven to cause cellular damage in respiratory system diseases (7,8). The binding of asbestos fibers to the tracheal epithelium in groups with smoke exposure (9). In some studies, it has been found that preneoplastic changes occur in the tracheal epithelium (10,11). It is seen that the basal cells and mucous cells in the epithelial tissue increase in number, and expansions are also observed in the glands of submucosa (12). It was shown that no change occurred in the number of secretory cells that contained neutral glucoprotein, but that the cells containing acidic glucoprotein increased in number (12,13). Recent studies showed that the mucosal and connective tissue mast cells increased especially in allergic rhinitis due to external factors and that the mast cells migrated into the epithelium (14-16). The existence of intraepithelial mast cells related to smoking is not mentioned. In this work, light and electron microscopic and biochemical examination of the changes occurring in the tracheal tissue of rats inhaling cigarette smoke passively were carried out.

Materials and Methods

A total of 15 Wistar type male rats weighing 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 and the other group (n=5) 60 minutes a day in an inhalation chamber for a period of 3 months. The third group was the control group (n=5), which inhaled clean air. A glass chamber which had the dimensions 50x35x36 cm, glass thickness of 0.5 mm and interior volume of 0.060 m^3 was prepared and insulated with silicone. A short plastic pipe, one end of which was left outside, was inserted into the chamber.

During the experiment, a lit cigarette was placed in the end of this pipe and the entire cigarette was puffed. Cigarettes without filter tips (Bitlis-Tekel) were used. At the end of the study period, blood and tissue samples were taken from the subjects under general anesthesia (ketamine+Rompun). The tissue segments taken from the upper half of the trachea were determined through 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 embedded in araldit Cy 212+DDSA+BDMA mixture. Semi-thin sections were prepared from part of the tissues for light microscopic examinations. In order to determine the intracellular glucoprotein content, Alcian Blue (pH 2.5)/PAS staining was carried out on the paraffin sections. All the sections were examined by BH 2 Olympus photomicroscope and Zeiss 9S2 electron microscope.

The levels of malonaldehyde (17,18), a free oxygen radical, arginase levels (19) and the catalase activity in erythrocytes (20) were biochemically examined in the tissue. The malonaldehyde level was determined as nmol/ml in the blood and as nmol/g in the tissue. Arginase activity was measured spectrophotometrically by the thiosemicarbazide diacetylmonoxime urea (TDMV) method. The unit for erythrocyte arginase is the g-Hb value of the enzyme activity that produces mikromol 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 and 0.400 absorbencies was measured and evaluated. The results were evaluated by the Mann - Whitney U test.

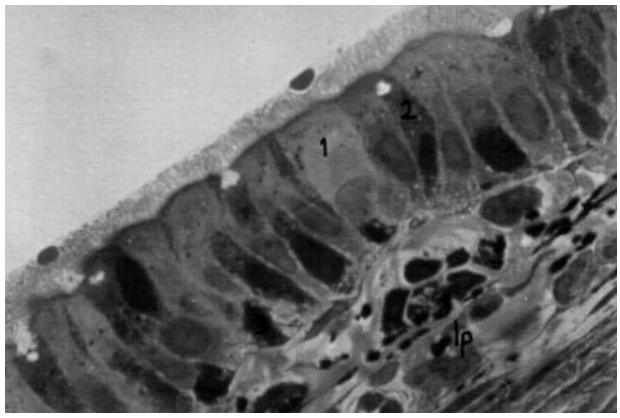


Figure 1. Control Trachea. The cells with cilia, the nuclei and cytoplasm of which are dyed a light colour (1); mucus secretory cells, the nuclei of which are situated near basal and dyed a dark colour (2) and the epithelial cell layer in which basal cells (3) occur, and underneath, lamina propria (lp) are observed. Toluidine Blue X100.

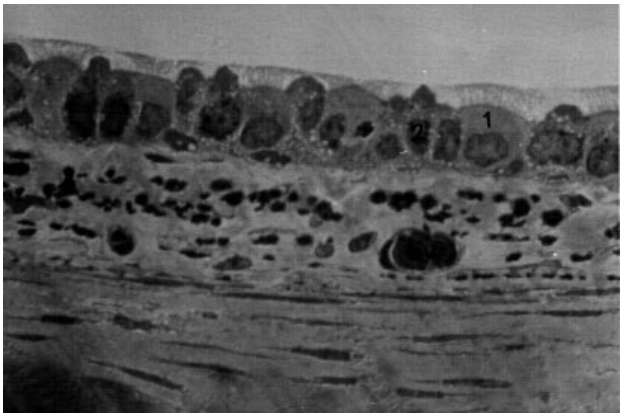


Figure 2. The trachea of the first experiment group. Among the cells with cilium (1), the secretory cells that have vacuolization projecting towards the lumen (2) are observed. In the connective tissue under the epithelial layer blood vessels (bv) are seen. Toluidine Blue X100.

Results

In the light microscopic examination, more cells with cilia, mucus secretory cells and basal cells in the epithelium of the two experiment groups were seen than in the control group (Figure 1), and that their cells formed a bud-shaped projection towards the lumen. The vacuolated appearance was determined in the cells. The fullness of the blood vessels and the cellular condensation were obvious in the connective tissue under the epithelium (Figures 2,3). In the second group the increase in the number of cells and the cellular irregularity in the epithelial layer were of particular note. Another finding of interest in the same group of subjects was that the mast cells were observed to be intense among epithelial cells (Figure 3). In addition, mast cells had increased in number in the lamina propria and submucosa layers (Figures 4 a, b).

At the transmission electron microscope levels when compared to the control groups, the secretory granules had increased in the region above the nucleus in the experiment groups (Figures 5,6). In the first experiment group, secretory granules were observed to be of different densities and smaller amounts (Figure 5). Electron-dense stained secretory granules was observed, particularly in the second group. The apical parts of these cells contained short microvilli and were recognized as being arched towards the lumen. Moreover, the

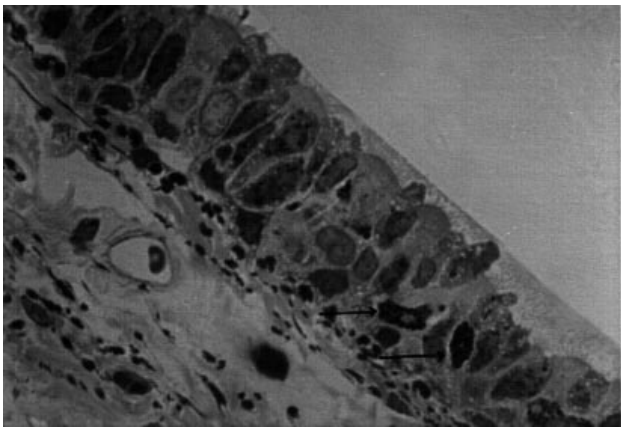
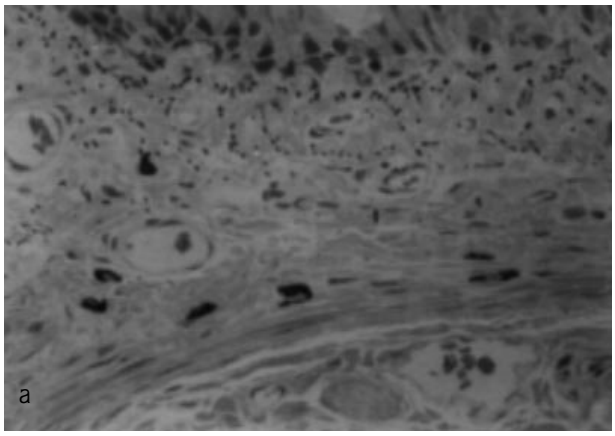


Figure 3. The trachea of the second experiment group. In the epithelial layer, cellular condensation and irregularity are observed. The increase in secretory cells and contents and the existence of mast cells (arrows) with their obvious granule structures among the other cells are noteworthy. Toluidine Blue X100.



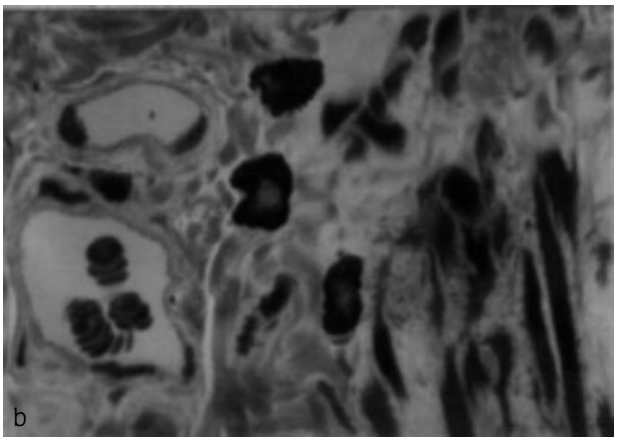


Figure 4. The mast cells in the connective tissue of Group II subjects, which inhaled cigarette smoke for a longer period, increased in number. a.Lamina propria, Toluidine Blue X40 b. Submucosa, Toluidine Blue X100.

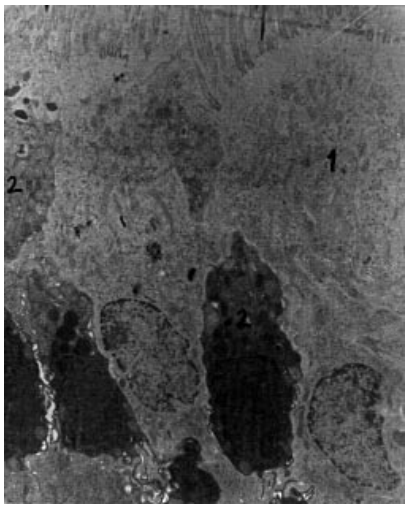


Figure 5. The electron microscopic appearance of Group I. Cells with cilium (1), secretory cells (2) are observed. The separations between the cells are distinguished. Lead citrate-uranyl acetate, original magnification 3000.

separations and the space between the cells were observed to be greater in this group (Figure 6). The mast cells, the cytoplasm of which was filled with secretory granules of different densities and sizes, were also of note among the epithelial cells (Figure 7).

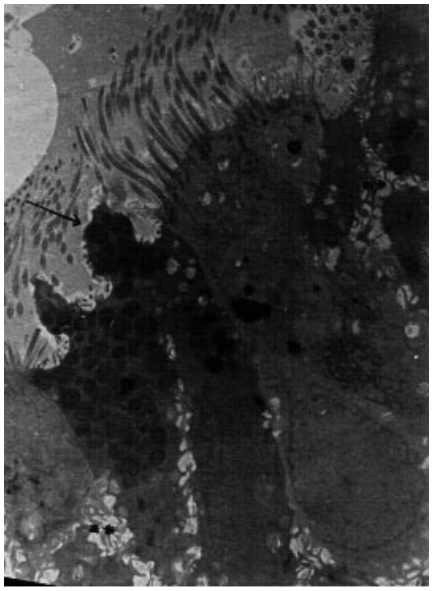


Figure 6. In Group II, the serous secretory granules are observed to form buds (arrow) quite intensively towards the lumen in the cellular apex. In this group, the separations between the cells are noteworthy (**). Lead citrate-uranyl acetate, original magnification 3000.

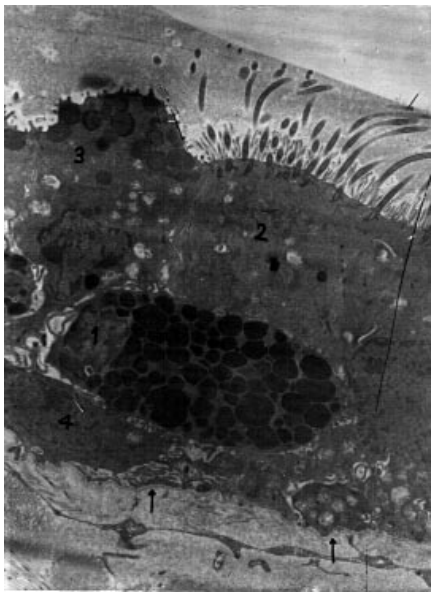


Figure 7. Another aspect of the same experiment group. Mast cells, the cytoplasm of which is filled with secretory granules of different diameters, are observed among epithelial cells (1). Cell with cilium (2), secretory cell that has short microvillus (3), basal cell (4), basal lamina (arrows). Lead citrate-uranyl acetate, original magnification 3000.

The data obtained biochemically are given in Tables 1-3. The data obtained as a result of all these procedures were evaluated statistically. The increases in plasma malonaldehyde levels and erythrocyte catalase activity in both groups inhaling cigarette smoke were statistically significant when compared to the control group (Tables 1, 2). The malonalaldehyde levels in the tracheal tissue were insignificantly lower than in the control (Table 1). Similarly, there was no significant difference in arginase levels between the groups inhaling cigarette smoke and the control group (Table 3).

Table 1. Malonaldehyde level in trachea (nmol/g protein; Mean±SD) and plasma (nmol/ml; Mean±SD). Group I and Group II were composed of rats exposed to cigarette smoke inhalation for 30 min/day and 60 min/day for 3 months respectively. *p<0.05. n: number of observations in each group.

	n	Trachea	Plasma
Control	5	1798+44	1.63+0.05
Group I	5	1178+53.3	4.13+0.1*
Group II	5	895.6+22	4.10+0.65*

Table 2. 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 for 3 months respectively. *p<0.05. n: number of observations in each group.

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

Table 3. Activity of arginase in trachea (U: µmol/urea/gprotein/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 for 30 min/day and 60 min/day for 3 months respectively. p>0.05. n: number of observations in each group.

	n	Trachea	Erythrocytes
Control	5	0.31+0.18	18.75+8.45
Group I	5	1.74+0.25	23.45+3.95
Group II	5	3.12+0.01	26.23+7.87

Discussion

In this study, the structural effects that occur in the tracheas of rats which inhale cigarette smoke for 30 and 60 minutes a day were examined during a 3 month period by light and electron microscopy. Inhalation of smoke for a 90-day period results in hyperplastic and metaplastic epithelial changes in the larynx (21). In another study on the effects of cigarette smoking on tracheal epithelial cells, some changes were observed in cytokeratin expression (22). Smoking increases the production of eicosanoids (PGE2, 6-keto PGF-alfa and TxB2) (23). Hyperplasia, which is observed in mucus secretory cells in diseases such as asthma and chronic bronchitis, can also occur due to smoking. Smoking stimulates the division of basal and mucus secretory cells and thus causes the formation of new cells (13). It has been demonstrated by histochemical methods that the cellular damage in smokers is related to the dose, that there is a budding outside of the apical membrane in the cell, loss of cilia occurred and cell death, and that the cigarette tar sticks to the cells' surface (8). Different studies demonstrate that hyperplasia and mitosis increase in secretory cells (13). The hypertrophy of the

submucosal glands is sometimes seen together with the hyperplasia of secretory cells. As a result of this, stimulation as well as cellular division may occur. The epithelial thickening becomes more than 71% and the number of secretory cells increases to 400% that of the control values (12). In this study there were generally 3 types of cells in the tracheal epithelial cell layer. In the experimental groups, it was seen that the secretory cells became arched towards the lumen due to the increase in their secretion. Secretion in the tracheal epithelium of the subjects that inhaled more cigarette smoke was more active. In the same group, irregularity in the epithelial cell layer and increase in the cells were observed. No change was recorded in the submucosal glands. While there was no change due to cigarettes in the number of neutral glucoprotein secretory cells, the increase in the number of acidic glucoprotein secretory cells was determined through PAS/Alcian Blue. Neutral glucoprotein secretory cells were stained with PAS whereas acidic glucoprotein secretory cells were stained with Alcian Blue (13). In another study, a different way of staining in the secretory cells of the epithelial tissue was not identified (10). And in our study, PAS (+) was clearly determined in the epithelial secretory cells of the experiment groups as a result of PAS/Alcian Blue staining, which was used to identify the content of the intracellular glucoproteins. No increase in cells containing acidic glucoprotein was observed.

When the tracheal tissue samples of the experiment groups were examined by electron microscopy, it was observed that the electron dense secretory granules had increased considerably in the secretory cells. The dense secretory granules were observed to be more intense in the second group, which inhaled cigarette smoke for a longer period. There exists information that supports this observation. It was stated that the electron intense serious secretory granules were densely observed in mucous cells due to smoking. This event was interpreted as the beginning of the mucous cellular transformation and metaplasia (12).

With the H-3 thymidine marking, it was observed that the cellular division increased within 1-3 days after smoking and that it drew near to the control group within 7 days (24). Although we found irregularity and cellular condensation in the epithelial cells of the subjects inhaling cigarette smoke when compared to the control group, we did not encounter any mitotic figures in the cells.

Among the junction units between the tracheal epithelial cells, gap junctions were affected most by smoking and deteriorations occurred as well (25). We observed these effects of cigarettes on epithelial cells and the separations between the cells, especially in the second group subjects.

The mast cells in the tissues of respiration channels that are continuously in contact with the external environment have been identified by various researchers (26,27,16). In the larynx epithelial tissue of rats, intraepithelial granulated cells (mast cells) are distinguished by 5 HT (28). Especially in allergic rhinitis, the migration of mast cells to the epithelium has been observed (14-16). In addition, there was an increase in eosinophils (15,29). In the semi-thin sections stained with Toluidine Blue, we observed the existence of intraepithelial mast cells, particularly in the group which inhaled cigarette smoke for the longest. In both of the experiment groups, the increase in the condensation of mast cells in the lamina propria and submucosa was noteworthy. This increase in mast cells and their existence in the epithelium indicated that allergic sensitivity occurred against the cigarette smoke.

In biochemical studies, it has been observed that cigarette smoke contains many types of free radicals, and these were significant in the occurrence of pulmonary diseases due to cigarettes (8,30). While some researchers stated that malonaldehyde, one of the lipid peroxidation products, increases (30,31), Chow et al. reported that the malonaldehyde level decreases together with smoking (32). Antioxidants have been used against these destructive effects of free radicals, leading to a decrease in damage (33). It has also been shown that the catalase enzyme is protector against cellular damage (8). According to the biochemical results that we obtained, a decrease was seen in the malonaldehyde levels in the tracheal tissue of the two experiment groups. There was also an obvious increase in the malonaldehyde levels of plasma. An increase was recorded in the catalase activity levels in erythrocytes. The differences between the levels of plasma malonaldehyde and erythrocyte catalase were found to be statistically significant when compared to the control group.

The effects of cigarette smoke on tracheal epithelium change according to the period of cigarette smoke inhalation. As the inhalation period is prolonged, cells are afffected more. The epithelium becomes irregular. The

secretory cells in which the serous secretory granules are intense, especially the intraepithelial mast cells, increase. The biochemical data which were obtained also show that the groups were affected differently.

Correspondence author:
Aysel Kükner
Department of Histology and Embryology,
Faculty of Medicine, Fırat University,
23100 Elazığ-TURKEY

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