Effects of long-term passive smoking on the mast cells in rat lungs

U. EREN*, S. KUM1, M. SANDIKCI1 and E. KARA2

SUMMARY
The effects of side stream smoking on histochemical properties and distributions of the mast cells in lungs of rats were investigated in this study. For these purposes lungs from 75 SpragueDawley rats with or without smoke exposure during 4 months were used. Lung samples were placed in 10% neutral buffered formalin (NBF) or in isotonic formaldehyde-acetic acid (IFAA) fixatives. To determine the mast cells, tissue sections were stained both with toluidine blue and alcian blue (AB) / safranin O (SO) methods. Mast cells were observed around vessels, in walls of bronchi and bronchioles, within the interseptal areas of the parenchyma and in the visceral pleurae of the peripheral lungs. Staining of the sections by the AB/SO method revealed that mast cells were AB+ and SO+, and most often AB/ SO+. Higher mast cell counts were found in the NBF fixed sections than those fixed with IFAA from both male and female rats (P<0.001), and the mean mast cell counts were higher in male rats than in females (P<0.001). Finally, a significant decline in mast cell numbers were observed in both female (P<0.001) and male (P<0.01) rats exposed to passive smoking, suggesting an intense degranulation of mast cells contributing to lung damage.

Keywords : Mast cell - passive smoking - lung - rat - female - male.

Introduction
Cigarette smoke consists of numerous vaporized chemicals (92 %) and particulates (8 %) suspended in a gaseous medium, i.e. an aerosol, formed by the combustion of tobacco. Most of the toxic organic components of smoke are contained in the particulate fraction. Mainstream smoke (45 % of the total) is that which is inhaled by puffing, whereas around 55% of cigarette smoke is side-stream smoke that plumes from the burning cigarette tip into the atmosphere. Nicotine is the component that causes clinical addiction; however, other irritants such as acrolein, formaldehyde, ammonia, oxides of nitrogen, toluene, phenol and pyridene are also present in microgram amounts per cigarette [14].

Epidemiologic studies have been established that environmental tobacco smoking has a negative health effect on those who are exposed. This was the reason why a wealth of investigations aimed to study the possible mechanisms of health impairment by passive smoking. These studies have given evidences that cigarette smoke can attract into and activate inflammatory cells within lung tissues, and cause inflammation and destruction of lung tissues [8, 18]. In this respect, many studies have focused on the possible roles of neutrophils [7, 18], macrophages [13], lymphocytes and their interactions [9]. Some studies also indicated that an increased number of mast cells are present in airways of smokers [1, 3, 12]. However, little information about changes in mast cell numbers in lung parenchyma of smoke-exposed individuals is available [10, 19, 20]. Thus, this study aimed to describe the changes of mast cells as well as their fixative and staining characteristics in lungs of female and male rats with or without side stream smoking early in their life.

Material and methods
The study has been approved by institutional Animal Ethics Committee. For this purpose 20 female and 10 male SpragueDawley rats from the Experimental Animal Unit of School of Veterinary Medicine were paired. Two female and one male rats were housed per cage in polycarbonate cages (28 x 28 x 16 cm in size) for one week. They were fed with rat chow from Best Yem (Gebze, Izmit, Türkiye) and water ad libitum. The baby rats, born 21 days later, were kept with their mothers for 3 weeks.

A total of 75 healthy newborn rats (38 males and 37 females) were included in the present study. Twenty male and twenty one female rats were exposed to passive smoking for 120 days, whereas 18 males and 16 females served as controls (they had no exposure to passive smoking). During smoke exposures the food was taken out of their cages so that the smoke did not contaminate it and the rats would not...
receive nicotine orally; their feed was taken out of the cages for 2 h every day to air. After the smoke exposure period was over, the rats were fed. At weekends, they were given unlimited feed.

The rats assigned to passive smoking were exposed to smoke in a unit for 120 min a day for 5 days a week for 4 months. The smoke entered from one side of the unit and the air in the unit was circulated by an aspirator (the power of which could be adjusted). The type of cigarette used was Birinci (85 mm; Tekel, Türkiye; Movas AS1, İzmir, Türkiye), which had a high rate of nicotine. The amount of smoke exposure was gradually increased: the animals were exposed to the smoke of six cigarettes for periods lasting 120 min, for two weeks, the smoke of nine cigarettes for the next 2 weeks, and the smoke of 13 cigarettes for the following 12 weeks. The percentages of CO and CO₂ were measured at certain intervals to evaluate the CO and CO₂ concentrations created by the smoke and to test the reliability of the test environment (Sun Modular Gas Analyser 1200; Amsterdam, Netherlands).

Four months later, the rats were weighed and anesthetized with ether and killed by cervical dislocation. Overall, right lungs were obtained from each rat. Tissue samples were placed in 10 % neutral buffered formalin (NBF) or isotonic formaldehyde acetic acid (IFAA) fixation [4] fluids and then embedded in paraffin. Five micrometer adjacent sections, were obtained from the paraffin-embedded tissues and stained with toluidine blue (TB, 0.5 %, pH 0.5) and alcian blue (AB, 0.5 %, pH 0.2)/saphranine O (SO 0.25 %, pH 1.0) techniques for histological evaluation [5].

Mast cell counts were determined on sections stained with TB. Images were transferred into a computer with the help of a camera. The number of cells observed in 10 image areas (0.13 mm²) from 10 sections obtained for each animal was counted. Data were analyzed by t-test for independent groups. The CO and CO₂ data were analyzed statistically using Kruskal Wallis one-way analysis of variance. If there were statistically significant differences, Mann-Whitney U test was used to determine from which group the difference originated. For this purpose SPSS 10.0 statistic package program for Windows® was used. Differences were considered as significant when P values were less than 0.05.

**Results**

The percentages of CO and CO₂ markedly increased in exposure room as the number of cigarettes used in each cycle increased (Table I). Thirteen cigarettes smoked in each cycle significantly produced more CO (P<0.001) and CO₂ (P<0.001) than 9 cigarettes smoked, and the combustion of 6 cigarettes gave intermediate values.

Examination of the sections using the TB and AB/SO staining method demonstrated that mast cells were present around vessels (Figure 1A, 1B), walls of bronchi and bronchioles (Figure 2A), in the interseptal areas of the parenchyma (Figure 2B) and in the visceral pleurae of the lungs (Figure 3A). In sections treated with the AB/SO stain some mast cells were found to be AB+ (Figure 1B) or SO+ while the majority was AB/SO+ (Figure 3B).

### Table I. — Percentages of carbon monoxide (CO %) and carbon dioxide (CO₂ %) in smoke exposure cycles. Results are expressed as means ± standard deviations.

<table>
<thead>
<tr>
<th>Number of cigarettes smoked in each cycle</th>
<th>CO (%)</th>
<th>CO₂ (%)</th>
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<tbody>
<tr>
<td>6 cigarettes</td>
<td>0.096 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.022 ± 0.002&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>9 cigarettes</td>
<td>0.011 ± 0.001&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.123 ± 0.004&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>13 cigarettes</td>
<td>0.092 ± 0.005&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.166 ± 0.006&lt;sup&gt;c&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>a, b, c</sup> means in a row with different superscript letters are statistically different.

### Table II. — Numbers of toluidine blue positive (TB+) mast cells in lungs of rats exposed (experimental groups) or not (control groups) to passive smoking during 4 months. Results are expressed as means ± Standards errors. Lungs were fixed in 10% neutral buffered formalin (NBF) or in isotonic formaldehyde acetic acid (IFAA).

**Asterisks**: **P<0.01; ***P<0.001** and different cd superscripts indicate significant differences between fixatives. Different ab superscripts indicate significant differences between males and females. Different A B C D superscripts indicate significant differences between control rats (♂♀) and passive smoking exposed rats (♂♀).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fixative</th>
<th>Numbers of TB+ cells</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Experimental group</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFAA</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>Male (n = 10)</td>
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<td>Total (n = 21)</td>
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<td></td>
<td>NBF</td>
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<td></td>
<td></td>
<td>Female (n = 8)</td>
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<td></td>
<td></td>
<td>Male (n = 9)</td>
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<tr>
<td></td>
<td></td>
<td>Total (n = 17)</td>
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<td></td>
<td></td>
<td>Female (n = 8)</td>
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<tr>
<td></td>
<td></td>
<td>Male (n = 9)</td>
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<tr>
<td></td>
<td></td>
<td>Total (n = 17)</td>
</tr>
</tbody>
</table>

Table II. — Numbers of toluidine blue positive (TB+) mast cells in lungs of rats exposed (experimental groups) or not (control groups) to passive smoking during 4 months. Results are expressed as means ± Standards errors. Lungs were fixed in 10% neutral buffered formalin (NBF) or in isotonic formaldehyde acetic acid (IFAA).
FIGURE 1. — A. TB (toluidine blue) positive mast cells around the vessel. B. AB (alcian blue) positive mast cells around the vessel.

FIGURE 2. — A. TB (toluidine blue) positive mast cells around the bronchiole. B. TB (toluidine blue) positive mast cells in an interseptal area.

FIGURE 3. — A. TB (toluidine blue) positive mast cells in visceral pleura of the peripheral lung. B. AB/SO (alcian blue / safranin O) positive mast cells lung parenchyma.
The average mast cell counts in the sections treated with TB are shown in Table II. The numbers of TB+ mast cells were significantly higher in lungs fixed with 10% neutral buffered formalin (NBF) than in those fixed with isotonic formaldehyde acetic acid (IFAA) in the control group (no cigarette exposure) (P<0.001) as well as in the experimental group (passive smoking for 4 months) (P<0.001). Moreover, the determination of mast cell counts in lungs was significantly improved by NBF fixation exposed or not to cigarettes in both females and males (P<0.001).

On the other hand, whatever the fixative used, males significantly presented higher counts of mast cells than females in control (P<0.001) and experimental groups (P<0.001). In addition, the numbers of TB+ mast cells were significantly decreased in both females (P<0.001) and in males (P<0.01) exposed to passive smoking compared to the corresponding controls whatever the fixation methods chosen.

Discussion

Two distinct mast cell populations exist, with different phenotypes and functions, and likely different functional roles during inflammatory and immune responses [6]. While granules of mucosal mast cells contain chondroitin sulphate and RMCP-II (Rat Mast Cell Protease -II) which react positively with alcin blue (+) but negatively with formaldehyde fixation, the granules of mast cells found in connective tissues have been reported to contain heparin and RMCP-I that give positive reactions to safranin [4, 5, 11, 15]. In the same way, WILKES et al., [21] reported that AB+ cells contained RMCP-II, those with AB/SO+ staining granules contained RMCP-I in rats. However, there are some conflicted reports about this biochemical classification. In a study conducted on rats [17] mast cells of connective tissues were reported to contain chondroitin sulphate and KORETOU [11] mentioned the presence of a small number of AB+ granules in the peritoneal mast cells of rats. In the present study, the mean numbers of mast cells in both the experimental and control groups were higher in the 10% NBF solution than in the IFAA solution which has a lesser formaldehyde content. Also, with the AB/SO method, the mast cells were observed to be AB+ and SO+ with most of them being AB/SO+. These results agree with the findings of WILKES et al., [21] who reported connective tissue mast cell predominance in the parenchyma and visceral pleura.

The results of previous studies indicate that the distribution of mast cells in different organs of female and male rats show organ-dependent characteristics [2, 16]. However, no information could be found comparing the counts of mast cells in lungs of male and female rats. The mast cell counts from both experimental and control groups were lower in the females than in males in this study.

In addition, mast cell populations evidenced in lungs from male and female rats exposed to passive smoking for 4 months were markedly depressed compared to the respective control rats (not exposed to cigarettes) and the reduction of the mast cell counts would be resulted from intense cell degranulation. In a study carried out on monkeys, exposures to cigarette smoke led to mast cell degranulation [20]. In another study conducted on canine mast cells [19] cigarette smoke was suggested to lead to the release of mast cell mediators and to the inhibition of prostaglandin synthesis.

KALENDERIAN et al., [10] have supported that cigarette smoking may increase secretion of histamine by alveolar macrophages leading to subsequent degranulation of local mast cells. Mast cell discharge of inflammatory mediators (including neutrophil chemotactic factors and perhaps the elastolytic protease) could then participate in the destruction of alveolar walls [10].

References