BASIC RESEARCH

Pulmonary toxicity of chronic exposure to tobacco and biomass smoke in rats

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OBJECTIVE: The objective of this study was to examine the separate and combined effects of tobacco and biomass smoke exposure on pulmonary histopathology in rats.

INTRODUCTION: In addition to smoking, indoor pollution in developing countries contributes to the development of respiratory diseases.

METHODS: Twenty-eight adult rats were divided into four groups as follows: control group (Group I, no exposure to tobacco or biomass smoke), exposed to tobacco smoke (Group II), exposed to biomass smoke (Group III), and combined exposure to tobacco and biomass smoke (Group IV). After six months the rats in all four groups were sacrificed. Lung tissue samples were examined under light microscopy. The severity of pathological changes was scored.

RESULTS: Group II differed from Group I in all histopathological alterations except intraparenchymal vascular thrombosis. There was no statistically significant difference in histopathological changes between the subjects exposed exclusively to tobacco smoke (Group II) and those with combined exposure to tobacco and biomass smoke (Group IV). The histopathological changes observed in Group IV were found to be more severe than those in subjects exposed exclusively to biomass smoke (Group III).

DISCUSSION: Chronic exposure to tobacco and biomass smoke caused an increase in severity and types of lung injury.

CONCLUSION: Exposure to cigarette smoke caused serious damage to the respiratory system, particularly with concomitant exposure to biomass smoke.

KEYWORDS: Inhalation Toxicology; Lung Pathology; Pulmonary Disease; Rats; Smoke.

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INTRODUCTION

The harmful effects of tobacco smoke are well known and have been demonstrated by experimental trials.[1] Organic materials such as charcoal, dried dung, and firewood, which are derived from plant residues and used as fuel, are collectively called biomass. In developing countries, biomass fuel is burned to obtain energy for daily necessities, such as heating and cooking. The smoke from biomass fuel contains noxious materials, including carbon monoxide (CO), nitric oxide (NO), sulphur oxides (SO), formaldehyde, polycyclic organic matter (POM), and benzopyrene. More than 500 million people in the world are exposed to the smoke and noxious molecules emitted from burning these fuels.[2] Many studies reveal that exposure to biomass smoke can markedly increase the prevalence of respiratory disorders.[3-6]

In Turkey, there is a high frequency of chronic obstructive pulmonary disease (COPD) in women who have never smoked but were exposed to biomass smoke.[7] The potential mechanisms and radiological and functional effects of biomass smoke exposure are investigated at our clinic.[8-11] We have examined the relative importance of biomass and tobacco smoke exposures in the development of COPD.[12]

The impact of passive smoking along with biomass smoke exposure may have other implications in addition to COPD.[13,14] There is not sufficient data demonstrating the possible chronic effects of tobacco and biomass smoke exposure together on the respiratory apparatus. The objective of this study was to examine the separate and

combined effects of tobacco and biomass smoke exposure on pulmonary histopathology in rats.

MATERIALS AND METHODS

This study was approved by the Cumhuriyet University Faculty of Medicine Animal Research Ethics Committee and conducted in Cumhuriyet University laboratories between December 2008 and July 2009.

Experimental animals

Twenty-eight adult male Winstar rats, weighing about 300 g each, were obtained from the laboratories of Experimental Animals Research and Practice Centre, Cumhuriyet University, Sivas. The animals were housed in plastic cages with a floor area of 920 cm^2 (Type III, Allentown Inc., USA) under standard laboratory conditions (illuminated between 7:00 a.m. and 8:00 p.m., temperature of $21 \pm 2^{\circ}C$, and relative humidity of 55%) and maintained according to the recommendations in the 'Guide for the Care and Use of Laboratory Animals' of the National Institutes of Health.[15] When exposed to tobacco or biomass smoke, the subjects were placed in a different room with continuous ventilation. The study was initiated with four groups and each group consisted of nine rats. Group I was the control group, which was kept under standard laboratory conditions with ventilation and was not exposed to tobacco or biomass smoke, Group II was exposed to tobacco smoke, Group III was exposed to biomass smoke, and Group IV was exposed to tobacco and biomass smoke.

Two rats from Group II, one rat from Group III, and one rat from Group IV died for unknown reasons. One rat from Group III and one from Group IV were excluded from the study when it was noticed that they were sick. At the end of the study two rats from the control group were randomly excluded, equalizing the number of subjects in each group (n = 7).

Exposure to tobacco smoke

The rats in Group II were placed in another room every morning from 9:00 and 10:00 for 6 months. Smoke from 5 burning cigarettes was pumped into this ventilated room for the hour. Every cigarette was kept burning for about 15–20 minutes. Ambient CO concentration was measured as 116 ± 23 ppm and oxygen saturation was measured as 20% with the use of a GT series gas detector (Gas Measurement Instruments, UK).[16,17]

Exposure to biomass smoke

The rats in Group III were placed in another room every morning from 9:00 and 10:00 for 6 months. In this ventilated room, the smoke produced by burning approximately 500 grams of dried animal dung was dispersed into the environment with a manual bellow (Figure 1) modified from that used in previous studies.[18] The ambient CO concentration and oxygen saturation were measured as 228 ± 30 ppm and 20%, respectively.[16,17] Finally, the rats in Group IV were exposed to tobacco smoke between 11:00 a.m. and 12:00 a.m. and to biomass smoke between 14:00 p.m. and 15:00 p.m. every day for 6 months. Meanwhile, ambient CO concentration was detected at 116 ± 23 ppm and oxygen saturation was measured as 20%.[16,17] The United States Environmental Protection Agency states that the standard level for 8-hour average carbon monoxide concentrations is 9 ppm and it has been reported that this

Figure 1 - Biomass smoke exposure in rats.

level can reach 10-500 ppm during cooking.[18] At the end of six months, the rats in all 4 groups were sacrificed by intraperitoneal injection of 200 mg/kg sodium pentothal.

Pathological examination

Lung tissue samples were fixed in a 10% formaldehyde solution and embedded in paraffin blocks. From the paraffin block of each tissue sample, 4-μm slices were cut. After applying routine hematoxylin and eosin stain, prepared materials were examined by light microscopy (Nikon Eclipse 80i, Japan) by a pulmonary pathologist blinded to treatment groups. The severity of pathological changes was scored according to previously published methods that were modified for this study.[19-21]

The main parameters included perivascular and peri-bronchial inflammation, parenchymal infiltration and fibrosis, intraparenchymal vascular congestion and thrombosis, respiratory epithelial proliferation, nodular aggregation of inflammatory cells, alveolar destruction, emphysematous changes, alveolar macrophage count, interstitial deposit-containing macrophage count, vascular wall thickness, and tracheal alterations. These 13 pathological parameters were graded in parallel, where 0 indicated no injury, 1 indicated injury to 25% of the field, 2 indicated injury to 50% of the field, 3 indicated injury to 75% of the field, and 4 indicated diffuse injury, for a maximal total score of 52.

Lymphocytic infiltration was scored as 0 for no infiltration around small airways, 1 for minimal random infiltration, 2 (mild) for aggregate formation, 3 (moderate) for at least two aggregates per low power field, and 4 (severe) for more than two aggregates. Large lymphoid aggregates localized around bronchovascular ramification sites and large airways were not taken into consideration.

Macrophage count was scored as follows 1 for <5 cells in alveolar spaces, 2 for 5˚9 cells, 3 for ≥10 cells, and 4 for abundant cells. When needed Masson's trichrome stain was used to identify fibrosis.

Statistics

All data from control and experimental groups were transformed into numeric values and transferred to a statistical software package (Statistical Package for Social Sciences for Windows, version 14.0, SPSS Inc, USA). The Kruskal–Wallis test was used to the compare four groups and the Mann–Whitney U test was used to compare two groups. A p value <0.05 was considered statistically significant.

RESULTS

Data from all histopathological examinations are presented in Table 1. Group II (exposed to tobacco smoke) differed from Group I (control group) in all histopathological parameters except intraparenchymal vascular thrombosis. Vascular wall thickness, perivascular leukocyte-rich inflammation and interstitial inflammation were of moderate severity in subjects exposed to tobacco smoke. Peribronchial inflammation was prominent, especially the presence of aggregates (Fig. 2 & 3). Furthermore, the subjects in Group II exhibited alveolar destruction, emphysematous changes, epithelial proliferation, and increased alveolar macrophages, whereas control group did not.

As shown in Table 1, the subjects exposed to biomass smoke alone (Group III) manifested higher levels of perivascular inflammation, peribronchial inflammation, parenchymal infiltration and fibrosis, nodular aggregation, alveolar destruction, and emphysematous changes in comparison to the control group. However, there were no differences between the two groups in intraparenchymal vascular thrombosis and congestion, respiratory epithelial proliferation, alveolar and interstitial deposit-containing macrophage counts, or vascular wall thickness. Histopathological alterations were less severe for this group compared to those in the group exposed to tobacco (Fig. 4).

Histopathological changes in Group IV (exposed to both tobacco and biomass smoke) were more prominent than those in the control group. Significant differences were
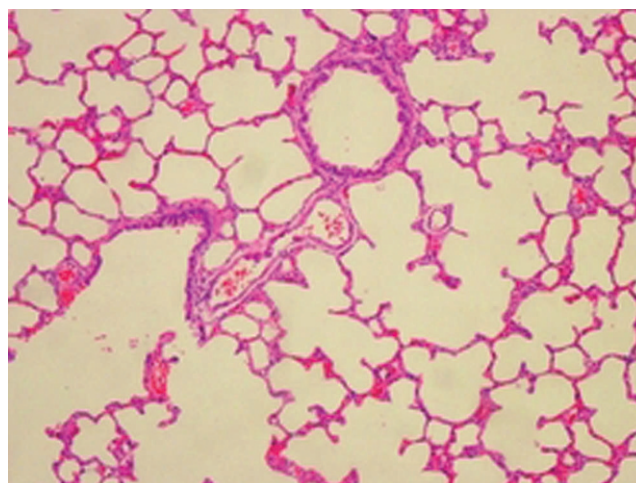

Figure 2 - Histopathological findings in the control group (Group I). No abnormalities were found (H&E, ×40).

observed in all the parameters except respiratory epithelial proliferation (Fig. 5).

With respect to histopathological changes, there was no significant difference between the subjects exposed exclusively to tobacco smoke (Group II) and those with combined exposure to tobacco and biomass smoke (Group IV). However, the histopathological changes observed in Group IV were more severe than in those exposed exclusively to

Table 1 - Histopathological examination data for all treatment groups.

	Control	Cigarette	Dried dung	Cigarette + Dried dung	P[a]	P[b]	P[c]	p[d]	P[e]	P[f]
perivascular inflammation	0.00±0.00	2.43±0.79	1.57±0.54	2.86±0.38	**<0.001**	**0.001**	**0.001**	**0.001**	0.383	**0.002**
peribronchial inflammation	0.86±0.38	2.71±0.49	2.29±0.49	2.71±0.49	**<0.001**	**0.001**	**0.001**	**0.001**	1.00	0.209
parenchymal infiltration and fibrosis	0.00±0.00	2.43±0.79	1.71±0.76	3.00±0.58	**<0.001**	**0.001**	**0.001**	**0.001**	0.259	**0.011**
intraparenchymal vascular congestion	1.14±0.38	2.43±0.54	1.57±0.54	2.57±0.54	**0.001**	**0.002**	0.209	**0.001**	0.710	**0.017**
intraparenchymal vascular thrombosis	0.00±0.00	0.71±0.76	0.29±0.49	1.14±0.90	**0.025**	0.073	0.383	**0.026**	0.383	0.097
respiratory epithelial proliferation	0.00±0.00	1.71±1.11	0.71±1.11	0.43±1.13	**0.009**	**0.004**	0.209	0.710	0.053	0.456
nodular aggregation	0.00±0.00	1.43±1.13	1.14±0.90	1.71±0.76	**0.004**	**0.026**	**0.004**	**0.001**	0.710	0.209
alveolar destruction	0.00±0.00	1.43±0.54	0.71±0.49	1.57±1.13	**0.001**	**0.001**	**0.026**	**0.004**	0.902	0.165
emphysematous changes	0.00±0.00	1.57±0.54	0.71±0.49	1.71±0.95	**<0.001**	**0.001**	**0.026**	**0.001**	1.00	0.073
alveolar macrophages (n)	0.00±0.00	1.29±0.76	0.57±0.79	1.57±0.54	**0.002**	**0.004**	0.209	**0.001**	0.535	**0.038**
interstitial deposit-containing macrophages	0.00±0.00	0.86±0.90	0.29±0.49	1.14±0.90	**0.027**	0.073	0.383	**0.026**	0.620	0.097
vascular wall thickness	0.00±0.00	2.43±0.54	0.14±0.38	2.43±0.54	**<0.001**	**0.001**	0.710	**0.001**	1.00	**0.001**
trachea	1.86±1.46	0.71±0.49	0.71±0.95	0.86±1.07	0.310					
Mean score[g]	4.43±0.61	24.29±2.19	13.14±1.24	25.71±2.31	**<0.001**	**0.001**	**<0.001**	**0.001**	0.710	**0.001**

[a]Comparison among four groups (Kruskal-Wallis test).
[b]Comparison between control and cigarette groups (Mann-Whitney U test).
[c]Comparison between control and dried dung groups (Mann-Whitney U test).
[d]Comparison between control and cigarette + dried dung groups (Mann-Whitney U test).
[e]Comparison between cigarette and cigarette + dried dung groups (Mann-Whitney U test).
[f]Comparison between dried dung and cigarette +dried dung (Mann-Whitney U test).
[g]Changes of each histopathological parameters were scored from 0 to 4, then summed for a maximum total score of 42. Data are shown as mean ± SEM.
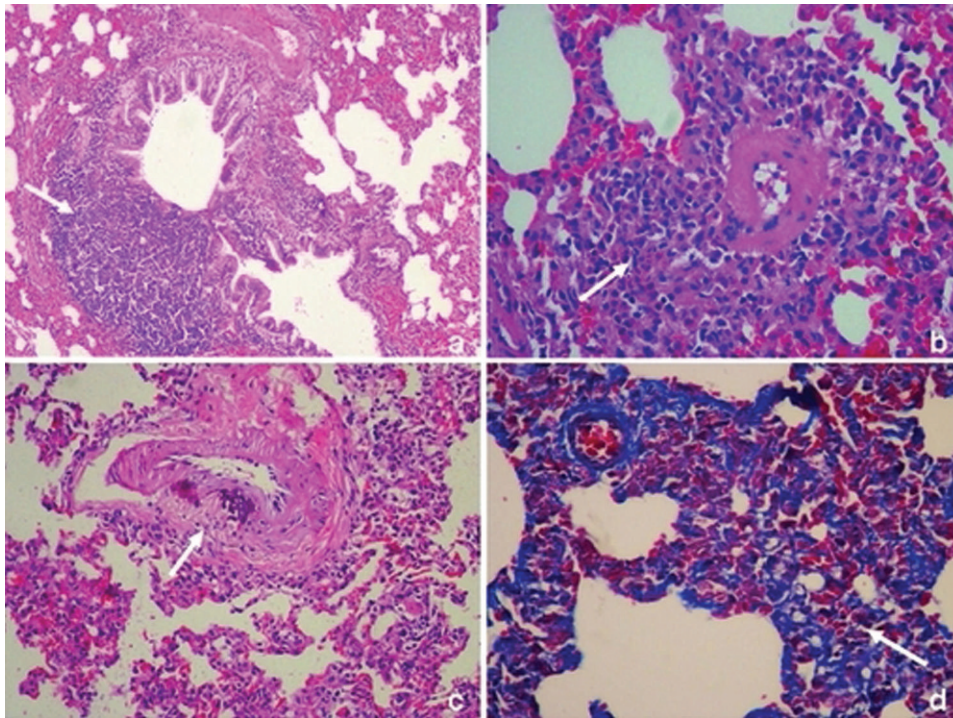
Figure 3 - Histopathological findings in the cigarette smoke-exposed group (Group II). (a) peribronchial inflammation (arrow) (H&E, ×20). (b) perivascular inflammation and vascular wall thickness (arrow) (H&E, ×20). (c) vascular wall thickness and calcification (arrow) (H&E, ×20). (d) parenchymal infiltration and fibrosis (arrow) (Masson's trichrome, ×20).
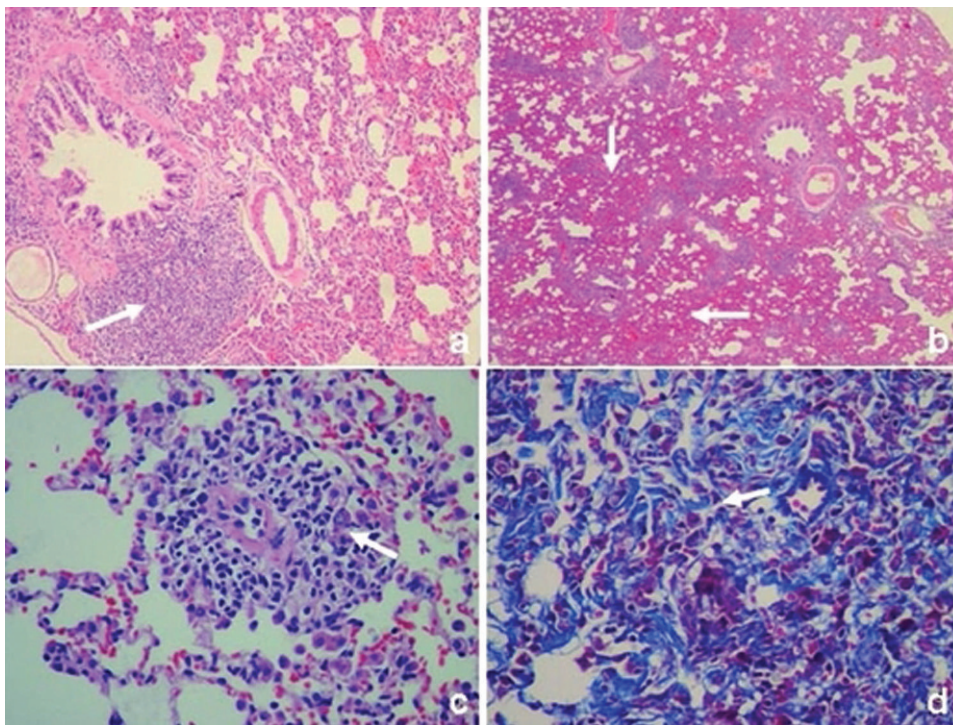

Figure 4 - Histopathological findings in the biomass smoke-exposed group (Group III). (a) peribronchial inflammation (arrow) (H&E, ×10). (b) peribronchial, perivascular, and interstitial inflammation (arrows) (H&E, ×40). (c) perivascular inflammation (arrow) (H&E, ×40). (d) parenchymal infiltration and fibrosis (arrow). (Masson's trichrome, ×40).
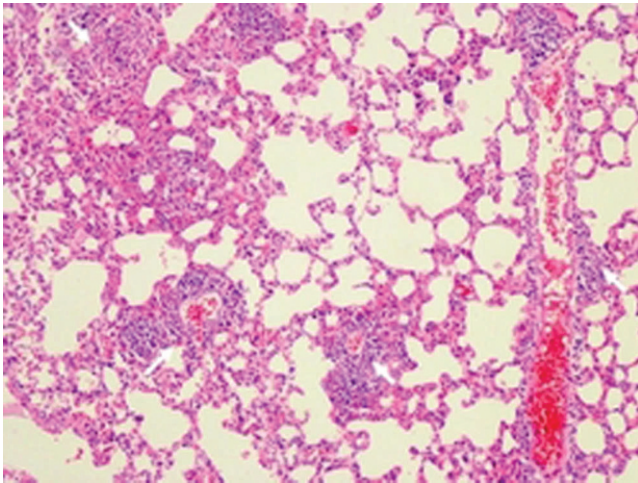
Figure 5 - Histopathological findings in the cigarette and biomass smoke-exposed group (Group IV). Parenchymal, peribronchial, and perivascular inflammation (arrow). (H&E, ×10).

biomass smoke (Group III). These changes included perivascular inflammation, parenchymal infiltration and fibrosis, intraparenchymal vascular congestion, increased alveolar macrophages, and vascular wall thickness.

DISCUSSION

Cigarette smoking, especially in industrialized countries, is an important risk factor for COPD, coronary artery disease, lung cancer, and several other diseases. Outdoor pollution in developed countries and indoor pollution in developing countries also contribute to the development of respiratory diseases. The ongoing use of biomass as solid fuel for heating and cooking in developing countries has been associated with an increased risk for chronic respiratory diseases by numerous clinical and epidemiological studies conducted in Turkey and other countries.[22-27] Cigarette and biomass smoke exert their harmful effects via oxidative stress.[9,28,29] Acute massive exposure and chronic exposure to inhalational irritants may have diverse pathological outcomes. Significantly increased lung damage due to chronic exposure has been demonstrated. Many experimental animal studies reveal that the chronic effects of smoke, particularly from cigarettes, cause very severe pathological changes.[30-34] In our study, exposure to tobacco smoke (Group II) caused differences in all histopathological parameters except intraparenchymal vascular thrombosis and bronchoalveolar hemorrhage. Vascular wall thickness, perivascular leukocyte-rich inflammation, and interstitial inflammation were of moderate severity in this group. Peribronchial inflammation was prominent, in particular with the formation of aggregates (Fig. 3). Alveolar destruction, emphysematous changes, epithelial proliferation, and alveolar macrophages were increased in this group, consistent with the literature. When guinea pigs were exposed to the smoke of 5 cigarettes a day for 3 months lesions similar to human emphysema were found.[31] In addition, when guinea pigs were exposed to cigarette smoke for 12 months, functional losses and structural emphysematous changes were found when lung tissue was viewed ultra-microscopically.[33] Our study reveals the development of alveolar destruction and emphysema, which is in agreement with previous studies.

Exposure of rats to cigarette smoke results in small airway wall muscular hyperplasia and fibrosis and vascular changes associated with pulmonary hypertension.[32] Vascular changes due to exposure of rats to cigarette smoke are associated with pulmonary hypertension,[34,35] and these changes likely result from hypertrophy of the vascular wall due to inflammation. We found similar alterations, including perivascular inflammation, vascular congestion, and vascular wall thickness, in the group exposed to cigarette smoke. In the biomass smoke-exposed group, only perivascular inflammation was present. It was interesting to observe that intravascular thrombosis occurred solely in the group with combined exposure but not in the groups with exclusive exposures, suggesting that multiple exposures may have more damaging effects and have a synergistic effect on the development of pulmonary hypertension.

Rats heavily exposed to cigarette smoke (20 cigarettes a day) for 6 weeks develop diffuse emphysema and interstitial pneumonia characterized by macrophage-rich inflammation.[36] We also found parenchymal infiltration and increased macrophage count in alveolar spaces in the group exposed to tobacco smoke.

To our knowledge, there are only two previous animal studies on exposure to biomass smoke, one of which examined the acute effects of cigarette and biomass smoke[13] and the other focused on the chronic effects of only biomass smoke.[14] Fidan et al. exposed rabbits to cigarette and biomass smoke for one month.[13] Intraparenchymal vascular congestion and thrombosis, intraparenchymal hemorrhage, respiratory epithelial proliferation, macrophages within alveolar and bronchial lumina, and bronchoalveolar hemorrhage were higher in the cigarette-exposed group than in the control group.[13] Apart from intraparenchymal vascular thrombosis and bronchoalveolar hemorrhage, we had similar pathologic findings. However, we found that perivascular inflammation, peribronchial inflammation, parenchymal infiltration, and fibrosis were also significantly increased. This difference can be attributed to the longer exposure time to cigarette smoke in our study, and our results reveal that chronic exposure causes an increase in severity and types of lung injury. In the study mentioned above, the biomass smoke-exposed group showed respiratory epithelial proliferation, alveolar destruction, and emphysematous changes exclusively.[13] In our study, the similarly treated group showed no respiratory epithelial proliferation, but differed from the control group with respect to perivascular inflammation, peribronchial inflammation, parenchymal infiltration and fibrosis, and nodular aggregation. This difference is again attributable to chronic exposure. Both studies indicate that acute or chronic exposure to cigarette smoke causes more pathological alterations than biomass smoke. Pathological changes in the group with combined exposure to cigarette and biomass smoke were more prominent than those in the group with exclusive exposure to biomass smoke, but were similar to those in the group with exclusive exposure to cigarette smoke. There are no other studies on concurrent and chronic exposure to tobacco and biomass with which to compare our findings.

Ozbay et al. exposed rat groups to biomass smoke for 3, 6, or 9 months.[14] They demonstrated that exposure to the smoke of dried dung caused chronic inflammatory and premalignant changes in various parts of the respiratory system, including trachea, bronchioles, pulmonary

interstitium, and lung vessels. Perivascular inflammation, peribronchial inflammation, and parenchymal fibrosis were of moderate severity by the end of three and six months.[14] Our biomass smoke-exposed group presented similar pathological findings. However, we found severe perivascular inflammation, peribronchial inflammation, and parenchymal fibrosis at the end of the sixth month but no changes in the trachea. Ozbay et al. identified hypertrophic changes in vessel walls accompanied by perivascular infiltration of lymphocytes, macrophages, and eosinophils and they suggested that this could result in pulmonary hypertension.[14] While we found intense perivascular inflammation in the biomass smoke-exposed group, there were no increases in alveolar or interstitial macrophage counts or in vascular wall thickness. There were increases in alveolar and interstitial deposit-containing macrophage counts, vascular wall thickness, and intraparenchymal vascular thrombosis in the group with combined exposure to biomass and cigarette smoke. We believe that pulmonary hypertension may result from this process.

Several studies on experimental animals have demonstrated that exposure to cigarette smoke can cause histopathological alterations in lungs, such as emphysema, small airway remodeling, vascular remodeling (pulmonary hypertension), and mucus hypersecretion in large airways (chronic bronchitis). Exposure to cigarette smoke must last for at least 6 months for these changes to occur.[37] In their study on induced myocardial infarction in rats, Duarte et al. also exposed rats to tobacco smoke for 6 months.[38] The fact that long term biomass smoke exposure can lead to these changes has only been shown by one previous study[14] and there is no existing data about the damage to respiratory system in the setting of coexisting, long-term exposure to cigarette and biomass smoke. Therefore, our study is the first to investigate this issue and has significant impact for women and children in developing countries that are subjected to long-term passive exposure to tobacco and biomass smoke.

The major limitation of this study involves the interpretation of emphysematous changes. In order to perform a precise assessment of emphysema, lung tissues should be inflated under a constant pressure.[39] This methodology was not followed in our study since the primary objective was not to obtain an emphysema model. Our aim was to examine, without additional interference, the possible impact of chronic passive exposure to indoor fumes by creating a simulation setting.

CONCLUSION

This study demonstrates that exposure to biomass smoke causes serious damage to the respiratory system, especially with concomitant cigarette smoke exposure. Therefore, it is recommended that in developing countries the use of biomass for heating and cooking purposes should be limited. When it is absolutely necessary, it should be used in ventilated areas. Furthermore, smoking should be avoided to reduce combined long-term effects.

ACKNOWLEDGEMENTS

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