Effects of N-acetylcysteine on the lung histopathology and oxidant-antioxidant status in rabbits exposed to cigarette smoke

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ÖZET

N-asetilsisteinin sigara dumanına maruz bırakılan tavşanlarda akciğer histopatolojisi ve oksidan-antioksidan sistem üzerine etkileri

Bu çalışmamızda sigara dumanının akciğer histopatolojisi ve oksidan-antioksidan sistem üzerine etkilerini ve N-asetilsisteinin (NAC) oluşan bu değişiklikler üzerine etkisini değerlendirmeyi amaçladık. Bu amaçla bir grup tavşan cam bir kafes içinde bir ay boyunca günde bir saat sigara dumanına maruz bırakıldı. Bir grup tavşana sadece intraperitoneal NAC enjekte edildi. Bir grup tavşan hem sigara dumanına maruz bırakıldı hem de intraperitoneal NAC enjekte edildi. Kontrol grubu tavşanlara ise cam kafes içinde temiz hava verildi. Bir ayın sonunda hayvanlar sakrifiye edilerek akciğer dokuları histopatolojik olarak incelendi. Ayrıca, kan örneklerinde protein sülfhidrilleri, karboniller, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) ve malondialdehid (MDA) düzeyleri ölçüldü. Sigara grubunda intraparankimal vasküler konjesyon ve tromboz, intraparankimal hemoraji, respiratuar epitel proliferasyonu, alveoler ve bronşiyoler lümenlerdeki makrofajların sayısı, alveoler destrüksiyon, amfizematöz değişiklikler ve bronkoalveoler hemoraji skorları kontrol grubuna göre anlamlı olarak artmış olarak bulundu. Sigara grubu tavşanların kanında kontrol grubuna göre protein sülfhidrilleri anlamlı azalmış, karboniller, $PGF_{2\alpha}$ ve MDA düzeyleri anlamlı olarak artmış bulundu. Sigara dumanına maruz bırakılan tavşanlara NAC verilmesinin sadece bronkoalveoler hemoraji skorunda ve kan $PGF_{2\alpha}$ düzeylerinde anlamlı azalmaya neden olduğu görüldü. Diğer parametrelerde değişiklik saptanmadı. Sonuç olarak sigara dumanına maruziyet tavşan akciğerlerinde ciddi histopatolojik değişiklikler oluşturmakta ve oksidan-antioksidan sistemi negatif etkilemektedir. Günlük düşük doz NAC verilmesinin sigara dumanına bağlı gelişen histopatolojik değişiklikler ve oksidan-antioksidan sisteme sınırlı yararlı etkileri vardır.

Anahtar Kelimeler: *NAC, sigara dumanı, antioksidan, histopatoloji, akciğer, tavşan.*

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SUMMARY

Effects of N-acetylcysteine on the lung histopathology and oxidant-antioxidant status in rabbits exposed to cigarette smoke

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We aimed to evaluate the effects of smoking on the histopathology and the oxidant-antioxidant status of lungs and to test the effects of N-acetylcysteine (NAC) on the induced changes. Rabbits were exposed to cigarette smoke (CS) in a glass chamber for one hour daily for one month. An NAC control group was given intraperitoneal NAC only. CS + NAC rats were exposed to smoke and given intraperitoneal NAC. A control group was exposed to clean air only. At the end of one month, animals were sacrificed and lung tissues were examined histopathologically. Blood levels of protein sulfhydryls, carbonyls, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and malondialdehyde (MDA) were measured. Intraparenchymal vascular congestion and thrombosis, intraparenchymal hemorrhage, respiratory epithelial proliferation, number of macrophages in the alveolar and bronchial lumen, alveolar destruction, emphysematous changes and bronchoalveolar hemorrhage scores were significantly increased in rabbits exposed to CS compared with the control group. Protein sulfhydryls were significantly decreased; carbonyls, $PGF_{2\alpha}$ and MDA levels were significantly increased in the smoke exposed rabbits. Administration of NAC to rabbits exposed to CS caused a reduction in the bronchoalveolar hemorrhage score and blood $PGF_{2\alpha}$ levels. Other parameters were unaffected by NAC. Exposure to CS causes severe histopathological changes and negatively effects the oxidant-antioxidant status in the lungs of rabbits. A low daily dose of NAC has some ameliorative effects on histopathological changes and oxidant-antioxidant status of the lungs in smoke exposed rabbits.

Key Words: *NAC, cigarette smoke, antioxidant, histopathology, lung, rabbit.*

Free radical species are known to cause oxidative damage to a number of different molecules in cells including membrane lipids, proteins, carbohydrates, and DNA. They are implicated in the pathogenesis of many diseases, including coronary heart disease and cancer (1). The tar and gas phase of cigarette smoke is a complex mixture of over 4,700 identified constituents (2). Enormous amounts of free radicals and reactive oxygen species, such as superoxide anion, peroxyl radical and nitrogen dioxide, are produced during cigarette smoking. Despite their short life time, the radical concentrations in cigarette smoke condensate are known to be maintained for a considerable time by redox reactions (3).

In humans, a major site of inflammatory action of cigarette smoke occurs in the small airways. Rubio et al. demonstrated in rats that the small bronchi wall thickness was significantly increased due to cigarette exposure (4). Emphysema is another free radical based debilitating condition associated with chronic cigarette smoking (5).

N-acetyl-L-cysteine (NAC), which was developed in the 1960s, is the N-acetyl derivative of the naturally occuring amino acid L-cysteine (6). Because of its ability to reduce disulphide bonds it is widely used to reduce viscosity and elasticity of mucus and is virtually non-toxic (7). NAC has the potential to interact directly with oxidants (7,8). Like many thiols, such as GSH, it is an excellent scavenger of hydroxyl radical (7). In addition to oxidant scavenger function, there is plenty of evidence showing that NAC promotes cellular glutathione production, and thus NAC reduced or even prevented oxidant mediated damage to cell culture or animals (8,9).

In the current study, we evaluated the effects of cigarette smoking on the histopathology and the oxidant-antioxidant status of the lungs and tes-

ted the effects of NAC in terms of its ability to prevent these changes.

MATERIALS and METHODS

All procedures were carried out with institutional review board approval. Adult male New Zealand White rabbits with weights ranging from 1.5 to 2.0 kg were used.

Four groups each with five rabbits were formed. Cigarette smoke rabbits were confined to a smoke filled chamber for one hour each day. NAC control rabbits were given the indole only. Cigarette smoke + NAC rabbits were both exposed to smoke and given NAC, finally, a control group of rabbits were exposed to clean air.

Cigarette Smoke Exposure

Rabbits were placed in a continuous air flow chamber (80 x 80 x 80 cm) in a seperate room for one hour daily, over a one month period with or without cigarette smoke exposure, as we described in a previous study (10). A lit cigarette was placed in the chamber, and fresh air was delivered constantly into the chamber with a flow rate of 78 mL/min. Each cigarette was allowed to burn out. The cigarette smoke and NAC + cigarette smoke groups were exposed to the smoke of four cigarettes per day. It took about 15-20 min for each cigarette to completely burn. The smoke exposure was well tolerated by the rabbits. Animals were fed rabbit chow and water. Carbon monoxide and oxygen levels in the chamber were measured. Carbon monoxide concentration in the chamber was 110 ± 41.8 ppm and the oxygen saturation was 19.4% ± 1.0. The temperature in the chamber was 27.0 ± 0.7°C. The control and NAC control groups were similarly placed in the chamber without cigarette smoke exposure.

Administration of NAC

During a one month period, 150 mg/kg per day of NAC, as used in some previous studies, was administered intraperitoneally to the rabbits in the NAC and NAC + cigarette smoke groups (11-13). The NAC + cigarette smoke group was exposed to the smoke immediately after the injection of NAC.

After the one month period, all rabbits were sacrificed by administration of 100 mg/kg sodium pentothal intraperitoneally.

Semiquantitative Evaluation of Lung Damage

A semiquantitative evaluation of the lung histological damage was accomplished by scoring its degree of severity according to previously published methods (14,15). The scorer was not aware of the slides identity. Lung tissue samples were fixed in 10% neutral buffered formaldehyde solution. After dehydration procedures, the samples were blocked in paraffin. 4 µm sections were cut and stained with hematoxylin-eosin. Mounted slides were examined under a light microscope (Nicon THP117).

We determined the presence and the degree of peribronchial inflammation, perivascular inflammation, intraparenchimal infiltration and fibrosis, intraparenchimal vascular congestion, thrombosis and hemorrhage, respiratory epithelial proliferation, number of macrophages in alveolar and bronchiolar lumen, pneumocyte type 2, alveoli destruction, emphysematous changes and bronchoalveolar hemorrhage. The level of changes in each section is defined as fallows: no changes (0), minimal (1+), mild (2+), modarete (3+), severe (4+), very severe (5+).

Measurement of Oxidant/Antioxidant System Markers

Peripheral blood samples were drawn into tubes from each subject and analyzed same day. After centrifugating the samples at 1,000 rpm for 10 min at 4°C, the serum samples were removed and stored at -20°C for further analysis. To measure lipid peroxidation, malondialdehyde (MDA) levels were determined according to a previously described method (16). Oxidative damage to proteins were assessed by determination of protein carbonyls and total protein sulfhydryls (SH) levels (17,18). Plasma $PGF_{2\alpha}$ levels were measured using a Human ELISA kit (R&D Systems GmbH, Wiesbaden-Nordenstadt Germany).

Statistical Analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences SPSS 10.0. Appropriateness of data to normal ranges was controlled with Shapiro-Wilk test. Data of histopathological changes were found not to be appropriate to normal ranges. Comparison between groups was performed using Mann-Whitney U test. Data of biochemical changes were found to be appropriate to normal ranges. Comparison between groups was performed using independent sample t-test. Data were expressed as mean ± standard deviation. $p < 0.05$ was considered as statistically significant.

RESULTS

Histopathological changes were illustrated in Figure 1. Histopathological evaluation of rabbit lungs revealed that; intraparenchymal vascular congestion and thrombosis (p= 0.011), intraparenchymal hemorrhage (p= 0.008), respiratory epithelial proliferation (p= 0.015), number of macrophages in the alveolar and bronchial lumen (p= 0.008), alveolar destruction (p= 0.013), emphysematous changes (p= 0.013) and bronchoalveolar hemorrhage (p= 0.005) scores were significantly increased in rabbits exposed to cigarette smoke compared with the control group. Administration of NAC to rabbits exposed to cigarette smoke caused a reduction in bronchoalveolar hemorrhage only (p= 0.005). No significant changes were established in the other parameters (Table 1).

Protein sulphydryls (p= 0.020) levels activity was significantly decreased, carbonyl (p= 0.046), $PGF_{2\alpha}$ (p= 0.024) and MDA (p= 0.019) levels were significantly increased in rabbits exposed to cigarette smoke compared with the control group. Administration of NAC caused a significant reduction in $PGF_{2\alpha}$ (p= 0.029) levels only (Table 2).

DISCUSSION

Evidence suggests that the free radicals in cigarette smoke contribute to the adverse effects of smoking cigarettes (19-22). Cigarette smoke causes lipid peroxidation, oxidation of protein thiols, and alterations in protein carbonyls in plasma (23,24).

In the current study we investigated the histopathological changes in the lungs of rabbits exposed to cigarette smoke. We found that exposure to cigarette smoke in rabbits increased intraparenchymal vascular congestion and trombosis, intraparenchymal hemorrhage, respiratory epithelial proliferation, number of macrophages in alveolar and bronchiolar lumen, alveoli destruction, emphysematous changes and bronchoalveolar hemorrhage histopathologically compared to the control group. Our findings are compatible with the previous studies (4,25,26). Sekhon et al. demonstrated in rats that cigarette smoke exposure rapidly causes increased levels of cell proliferation in the epithelium and walls of bronchioles, and in the walls of the associated pulmonary arteries (25). In another study, Li et al. exposed rats to 20 cigarettes per day for five days per week in a period of six weeks time (26). They observed vasculitis and some hemorrhage in the lungs of the rats at 4^{th} week. At the end of six weeks they observed interstitial pneumonia and severe diffuse emphysema histopathologically in the lungs of the rats. The inflammatory process was characterized by alveolar septal thickening, septal infiltration by erythrocytes and chronic inflammatory cells (mostly macrophages), with a scattering presence of these cells within the alveolar space. A number of small caliber arteries also showed mild vasculitis, characterized by thickening of the artery wall (mostly the media), some periadventitial edema with few scattered macrophages, and a modest reduction of the arterial and arteriolar lumen. Rubio et al. demonstrated in rats that small bronchi wall thickness was significantly increased due to cigarette smoke (4).

We also investigated the effects of cigarette smoke on oxidant/antioxidant status. We found that cigarette smoke causes a significant reduction in SH levels and a significant increase in carbonyl, $PGF_{2\alpha}$ and MDA levels. Protein-SH levels in smokers were found to be decreased in several studies (27,28). Eiserich et al. reported that protein carbonyl formation, a measure of

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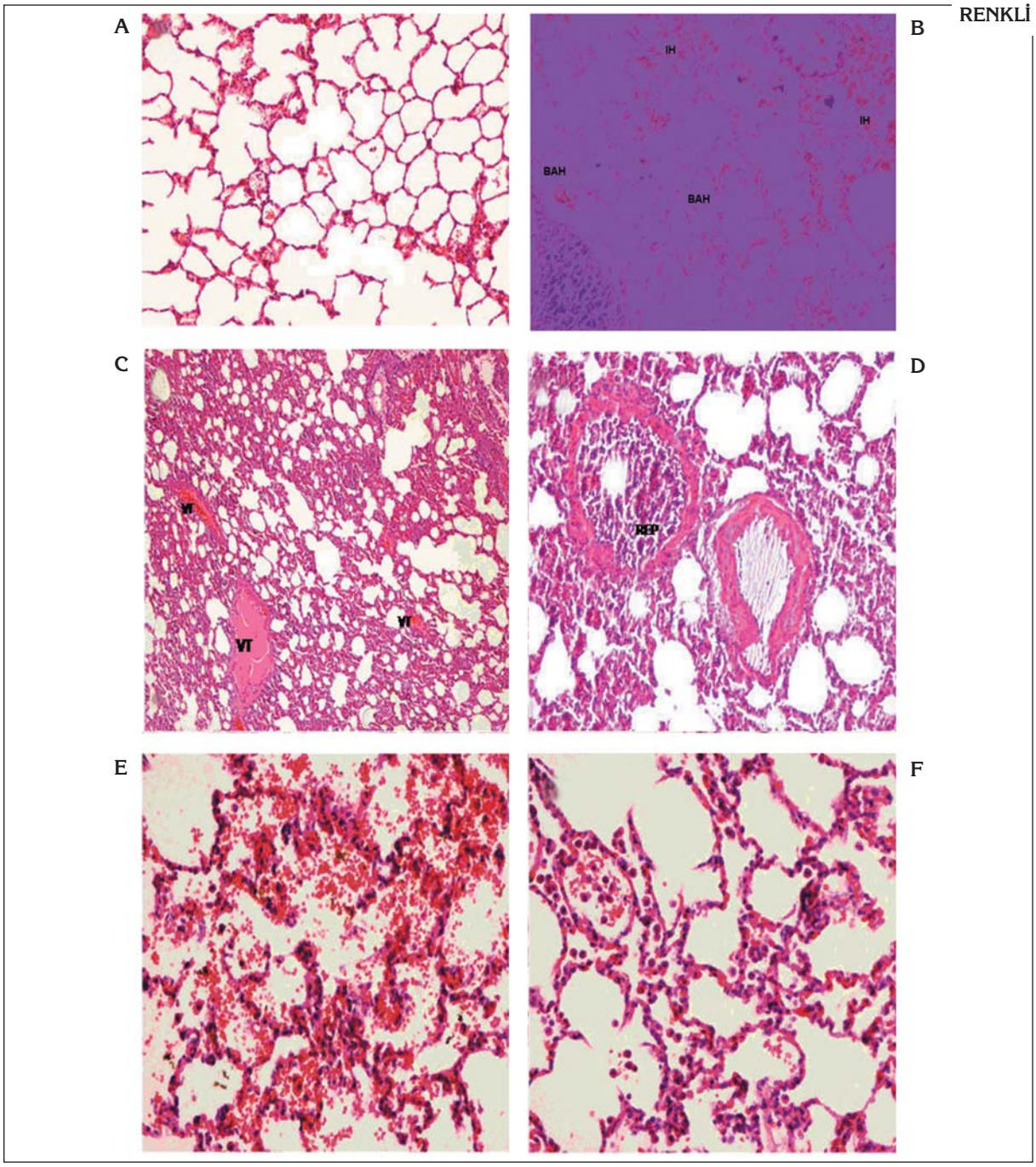

Figure 1. Intraparenchymal vascular congestion and thrombosis, intraparenchymal hemorrhage, respiratory epithelial proliferation, number of macrophages in the alveolar and bronchial lumen, alveolar destruction, emphysematous changes and bronchoalveolar hemorrhage scores were significantly increased in rabbits exposed to CS compared with the control group.
A: (control); H/E x40.
B: (cigarette smoke); BA (bronchoalveolar hemorrhage), IH (intraparenchymal hemorrhage); H/E x100.
C: (cigarette smoke); VK (vascular congestion), VT (vascular thrombosis); H/E x40.
D: (cigarette smoke); REP (respiratory epithelial proliferation); H/E x100.
E: (cigarette smoke); alveolar hemorrhage, H/E x200. Administration of NAC to rabbits exposed to CS caused a reduction in the bronchoalveolar hemorrhage score, other parameters were unaffected.
F: (cigarette smoke + NAC); decrease in alveolar hemorrhage, H/E x200.

Table 1. Mean histopathological scores of the groups.

	Control	Cigarette	NAC	Cigarette + NAC	p*	p**
Peribronchial inflammation	1.4 ± 1.3	2.2 ± 0.5	1.2 ± 0.8	1.4 ± 0.9	0.343	0.095
Intraparenchimal infiltration	1.8 ± 1.1	1.6 ± 0.9	1.4 ± 0.6	1.0 ± 1.0	0.606	0.282
Intraparenchimal fibrosis	1.6 ± 0.9	1.2 ± 1.1	1.2 ± 0.8	0.8 ± 0.8	0.513	0.502
Intraparenchimal vascular congestion and trombosis	1.4 ± 1.3	4.2 ± 0.8	1.2 ± 1.1	3.4 ± 0.6	0.011	0.118
Intraparenchimal hemorrhage	1.2 ± 1.1	4.2 ± 0.8	1.0 ± 1.0	3.4 ± 0.6	0.008	0.118
Respiratory epithelial proliferation	0.4 ± 0.9	2.2 ± 0.5	0.4 ± 0.8	1.6 ± 0.9	0.015	0.180
Macrophages in alveolar and bronchiolar lumen	1.6 ± 0.9	4.2 ± 1.1	0.4 ± 0.9	4.0 ± 0.7	0.008	0.658
Alveoli destruction	0.4 ± 0.6	2.2 ± 0.8	0.8 ± 0.5	1.2 ± 08	0.013	0.100
Emphysematous changes	0.4 ± 0.6	2.0 ± 0.7	0.4 ± 0.5	1.2 ± 0.8	0.013	0.142
Bronchoalveolar hemorrhage	0.0 ± 0.0	4.2 ± 1.3	0.0 ± 0.0	2.0 ± 0.0	0.005	0.005

NAC: N-acetylcysteine.

* Comparison between control and cigarette groups.

** Comparison between cigarette and cigarette + NAC groups.

Table 2. Mean oxidant/antioxidant marker levels of the groups (mean ± SD).

	Control	Cigarette	Cigarette + NAC	p*	p**
SH (µmol/L)	469.2 ± 31.7	325.4 ± 90.4	390.6 ± 54.8	0.020	0.205
Carbonyl (µmol/L)	76.4 ± 11.2	90.5 ± 3.0	77.9 ± 21.5	0.046	0.233
MDA (µmol/L)	1.8 ± 0.3	2.7 ± 0.6	2.4 ± 0.3	0.019	0.299
$PGF_{2\alpha}$	15377.2 ± 2795.0	33633 ± 11823.2	16698.0 ± 4501.4	0.024	0.029

NAC: N-acetylcysteine, SH: Sulphydryls, MDA: Malondialdehyde, $PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$.

* Comparison between control and cigarette groups.

** Comparison between cigarette and cigarette + NAC groups.

protein modification, was increased by approximately 400 mmol/L after nine puffs of cigarette smoke (29). Hong et al. exposed guinea pigs to low-nicotine cigarette smoke and found that $PGF_{2\alpha}$, prostaglandin D_2 and thromboxane A_2 levels were increased (30). The most widely used index of lipid peroxidation is MDA formation, often assayed with the thiobarbituric acid (TBA) assay. Bingol et al. reported that chronic smoking causes peroxidation reactions in both plasma and erythrocytes which leads to increased MDA levels (31). Unlu et al. exposed 15 rabbits to cigarette smoke two hours per day for six weeks (32). They found significantly increased plasma and lung homogenate MDA levels in rabbits exposed to cigarette smoke compared to the control group. Our findings are compatible with these results.

NAC, which is a scavenger of hydrogen peroxide, hypochloric acid and hydroxyl radical in vitro has been used in various disease settings. Rubio et al. showed in rats that the cigarette smoke induced alterations of the small bronchi can be avoided by concomittant oral NAC administration (4). Neal et al. irradiated mice using a 9 MeV beam and administered 500 mg/kg/day NAC intraperitoneally (33). They showed that NAC has some radioprotective effect on lung, spleen, liver and red blood cells. In an animal model Fan et al. demonstrated that liposome encapsulated NAC is able to provide sustained protection against acute respiratory distress

syndrome (34). It has been demonstrated that NAC is able to attenuate cellular infiltration and collagen deposition in a model of bleomycin-induced lung fibrosis (35-37). When administered together with steroids, it improves the lung function index in patients with idiopathic pulmonary fibrosis (38).

In the current study we administered 150 mg/kg/day NAC intraperitoneally to cigarette smoke exposed rabbits. We observed some amelioration in peribronchial inflammation, intraparenchimal vascular congestion and thrombosis, intraparenchimal hemorrhage, respiratory epithelial proliferation, macrophages in alveolar and bronchiolar lumen, alveoli destruction, emphysematous changes and bronchoalveolar hemorrhage but the change was statistically significant in only bronchoalveolar hemorrhage. Rubio et al. administered 200 mg/day of NAC for each rat that was exposed to two cigarettes, three times daily for a total period of 10 weeks and found that the small airway wall thickening, caused by cigarette smoke exposure, was disappeared with administration of NAC (4). Rogers et al. exposed rats to 25 cigarettes daily for 14 days and concurrently gave NAC with an average daily dose of 973 mg/kg (39). They found that NAC inhibited cigarette smoke induced epithelial thickening. Balansky et al. exposed rats to cigarette smoke once daily for 40 consecutive days (40). They observed a severe inflammation of bronchial and bronchiolar mucosae, with multiple hyperplastic and metaplastic lesions and foci of micropapillomatous growth as well as emphysema, with extensive disruption of alveolar walls by cigarette smoke exposure. Then they administered daily NAC by gavage and observed that all histopathological changes were efficiently prevented. The discordance between these studies and the current study was considered to be due to the animal model used and to the relatively low dose of NAC used in this current study.

We also investigated the effects of NAC on oxidant/antioxidant status. Administration of NAC significantly reduced $PGF_{2\alpha}$ levels and did not cause any significant alterations in the other markers. To our knowledge, there are no studies studying the effects of NAC on these oxidants and antioxidants.

As a conclusion, cigarette smoke exposure was found to cause severe histopathological injury and negatively effects the oxidant/antioxidant status in rabbits. Low dose NAC has limited ameliorating effect on the histopathological changes and oxidant/antioxidant status. Studies evaluating the effects of higher doses of NAC on cigarette smoke exposure will provide useful information.

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