Original Article

Harmful Effects of Formaldehyde and Possible Protective Effect of *Nigella sativa* on the Trachea of Rats

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ABSTRACT

Objective: We aimed in this study to investigate the harmful effects of formaldehyde (FA) inhalation and possible protective effects of *Nigella sativa* (NS) on rats' trachea. **Materials and Methods:** In this study, 63 adult male rats were used. Animals were divided into nine groups. Group I was used as control group. All other groups were exposed to FA inhalation. Group III, V, VII, and IX were administered NS by gavage. Tissues were examined histologically, and immunohistochemical examination for Bax and caspase-3 immunoreactivity was carried out. **Results:** Our study demonstrated that FA caused apoptosis in the tracheal epithelial cells. The most apoptotic activity occurred at a 10 ppm dose in a 13-week exposure. Distortion of tracheal epithelium and cilia loss on epithelial surface was present in all groups. However, NS treated Groups VII and IX had decreased apoptotic activity and lymphoid infiltration and protected the epithelial structure, despite some shedded areas. Difference of tracheal epithelial thickness and histological score was statistically significant between Group VI–VII and VIII–IX. **Conclusion:** FA induces apoptosis and tracheal epithelial damage in rats, and chronic administration of NS can be used to prevent FA-induced apoptosis and epithelial damage.

Keywords: *Apoptosis, formaldehyde, Nigella sativa, rat, trachea*

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Introduction

Formaldehyde (FA) is the simplest member of aldehyde family; it is a chemical with high solubility and an irritant. FA that is found in the natural system of organism and it is used commonly in daily life for many purposes by reason of its chemical properties.[1-3] FA has detrimental effects on the human body, particularly on the ocular and respiratory system, but it also affects the nervous and genital system.[4-6] Previous studies claimed that FA demonstrates this effect causes the generation of reactive oxygen species (ROS) that cause apoptosis and necrosis, resulting in lipid peroxidation and metabolic alterations.[7,8] Further, the carcinogenic and immunosuppressive effects of FA are well known.[9-12]

Nigella sativa (NS) is a plant from *Ranunculaceae* family, it is used, especially in Middle Eastern, North Africa, and Asia.[13] NS has antimicrobial, anthelmintic antioxidant, and antiapoptotic specifications; it has also demonstrated antidiabetic, antitumoral, anti-inflammatory, bronchodilator, and neuroprotective specifications in animal experiments and various clinical studies.[14-19]

In this histopathological and immunohistochemical study, considering the antiapoptotic effects of NS, we aimed to determine the toxic effects of FA inhalation in the trachea of rats and NSs possible protective effects.

Materials and Methods

Animals and group design

Sixty-three Sprague-Dawley male rats weighing between 270 and 300 g were used in this experimental study. The Local Ethics Committee for Animal Research

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approved the experimental protocol for this study, and all animals received humane care in compliance with the European Convention on Animal Care. The rats were placed in glass cages in an air-conditioned room with automatically regulated temperature (22 + 1°C) and lighting (07.00–19.00 h). All the rats were acclimated for 1 week before the experimentation.

Type of exposure, dose level, and dose selection

FA was given by inhalation, and NS oil (Origo-Gaziantep, Turkey) was given (1 ml/kg/day) by gavage to the animals. FA concentrations and exposure periods were determined according to the previous studies.[20,21] FA concentration was determined with an FA meter device (Environmental Sensors Co., Boca Raton FL 33432 USA-Catalog No: ZDL-300). The rats were divided into nine groups, with a total of seven animals in each group. FA gas was obtained by heating paraformaldehyde at a temperature of 35–40°C in specifically prepared glass assembly. Each group (n = 7) were placed in glass cages (50 cm × 20 cm × 100 cm) for 8 h. There were two holes in the glass cages to allow the input and output of air. In inhalation chambers equipped with a trap and designed to sustain dynamic and adjustable airflow (11,250 mg/m^3) 8 h/day between 8:00 and 16:00. For the remaining 16 h of the day, the rats were placed back in their cages where they received laboratory animal feed and tap water.

Group design

Group 1 was selected as control group. The rats in the other eight groups were given FA for 8 h a day in the glass cages. The rats in Group 2 (5 ppm FA 4 week) were exposed to FA for 4 weeks at a dose of 5 ppm. The rats in Group 3 (5 ppm FA 4 week + NS) were given NS at a dose of 1 ml/kg/day with exposure to 5 ppm FA. The dose of NS was based on a previous study.[22] Group 4 (10 ppm FA 4 week) was exposed to 10 ppm FA for 4 weeks. Group 5 (10 ppm FA 4 week + NS) was given 1 ml/kg/day NS with exposure to 10 ppm FA 8 h a day. Group 6 (5 ppm FA 13 week) was exposed to 5 ppm FA for 13 weeks. Group 7 (5 ppm FA 13 week + NS) was given 1 ml/kg/day NS with exposure to 5 ppm FA for 13 weeks. Group 8 (10 ppm FA 13 week) was exposed to 10 ppm FA for 13 weeks. Group 9 (10 ppm FA 13 week + NS) was given 1 ml/kg/day NS with exposure to 10 ppm FA for 13 weeks. At the end of the experiment, the rats were decapitated. The trachea tissues were rapidly removed for histopathological and immunohistochemical evaluation.

Histological analysis

The trachea tissues were placed in a 10% formalin solution. After dehydration and clearing, tissues were embedded in paraffin. Sections 5–6 μm thick were obtained using a microtome and were stained with periodic acid-Schiff + hematoxylin (PAS-H). Tracheal sections for histopathological evaluation were examined for shedding of epithelial cells, loss of cilia, metaplasia, lymphoid cell infiltration, and PAS(+) staining degree. Microscopic injury was evaluated as none (0), mild (1), moderate (2), and severe (3), the maximum score was 15.[23] Each rat was scored, and values were determined for each group. In addition, tracheal epithelial thickness (TET) was measured with a ×40 objective lens with a Leica Q-Win Image Analysis System. TET was measured as the distance between the basal membrane and the ciliary surface in the anterior part of the trachea. Sections were examined using a Leica DFC-280 research microscope.

Immunohistochemical analysis

Immunohistochemical (IHC) staining was performed to determine the expression of Bax and caspase-3 protein according to the instructions of the manufacturer. First, paraffin-embedded trachea sections (4–5 μm thick) were placed on poly-L-lysine-coated coverslips, then deparaffinized in xylene and rehydrated in a series of graded concentrations of ethanol. Protein antigenicity was enhanced by boiling the sections in a citrate buffer in a microwave oven at 95°C for 15 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phosphate-buffered saline (PBS) at 37°C for 20 min. Background staining was prevented using Ultra V Block solution (Ultra V Block, TA-125-UB, Thermo Fisher Scientific Inc., USA). Afterward, tissue sections were covered with biotinylated primary antibody (ab77566, Anti-Bax antibody, Abcam, Germany; ab2302, antiactive Caspase-3 antibody, Abcam, UK) and incubated for 60 min in a humidity chamber. Subsequently, the tissue sections were covered with streptavidin peroxidase (biotinylated anti-mouse IgG, Diagnostic BioSystems, KP 50A, Pleasanton, USA), and incubated for 30 min. Then, the slides were covered with 3-amino-9-ethylcarbazole chromogen solution and counterstained with hematoxylin. For negative controls, an equivalent volume of PBS was used in place of the specific primary antibodies.

Evaluation of immunohistochemistry

For the evaluation of the immunoreactivity of Bax and caspase-3, H-score analysis was used.[24] Cytoplasmic staining intensities of Bax and caspase-3 were evaluated in four categories during the H-score analysis. According to the evaluation, (0) was considered as no stain, (1+) as poor but detectable staining, (2+)

moderate staining, and (3+) as intense staining. Cells were detected according to each staining intensity category and percentage values were obtained by rating the number of cells in the category to the total number of cells under the ×40 objective. The total score was obtained by multiplying these percentage values with their own staining grade. When we formulize this as H-SCORE = ∑Pi (i + l), 'i' is staining grade and "Pi" is the percentage of cells in this staining intensity category. Evaluation of score was repeated at five different areas for each tissue slice, and a mean score value was calculated.

Statistical analysis

For statistical evaluation, a Windows compatible IBM-SPSS 20 package (SPSS Inc., Chicago, Illinois, USA) program was used. The results were given as mean ± standard deviation differences among two groups in terms of H-score values, histological scores, and TET were determined using the independent samples *t*-test. In addition, the one-way analysis of variance was used in comparisons between three or more independent groups and Tukey's test was used as *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Histopathologic results

In the control group, normal histological structure of the trachea was observed. It was covered with pseudostratified columnar epithelium cells. Purple-magenta color stained secretions and distinguished goblet cells were located among epithelial cells individually or in groups [Figure 1].

Table 1: Tracheal epithelium thickness of the groups (micrometer) and histological evaluation results

Groups	TET	HE
Group 1 (control)	137.6±3.2	0.1±0.1
Group 2	94.9±2.9[a]	5.0±0.2[f]
Group 3	121.9±12.3[b]	4.30±0.2[f,g]
Group 4	119.5±3.2	8.0±0.4[f]
Group 5	125.3±6.3[c]	8.2±0.3[f,c]
Group 6	72.6±6.7[a,c]	8.1±0.3[f]
Group 7	110.5±2.0[a,d]	5.1±0.4[f,j]
Group 8	50.8±4.8[a]	9.8±0.3[f,i]
Group 9	88.6±6.7[a,e]	6.0±0.3[f,h]

[a]Significant decreased to control group, $P<0.05$; [b]No statistical significance to Group 2, $P>0.05$; [c]No statistical significance to Group 3, $P>0.05$; [d]Significant increase to Group 6, $P<0.05$; [e]Significant increase to Group 8, $P<0.05$; [f]Significant increase to control group, $P<0.05$; [g]No significant decrease to Group 2, $P>0.05$; [h]Significant decrease to Group 8, $P<0.05$; [i]No statistical significance to Group 4, $P>0.05$; [j]Significant decrease to Group 6, $P<0.05$. TET=Tracheal epithelium thickness; HE=Histological evaluation

While a clear increase in goblet cell secretion was observed in Group 2 [Figure 1c], In Group 3, the histological appearance of the epithelium was similar to control group, except partly epithelial splits, [Figure 1d]. The most conspicuous changes in Group 4 were noticeable leukocyte infiltration in some parts of lamina propria and cytoplasmic residues in the lumen; due to superficial loss of the

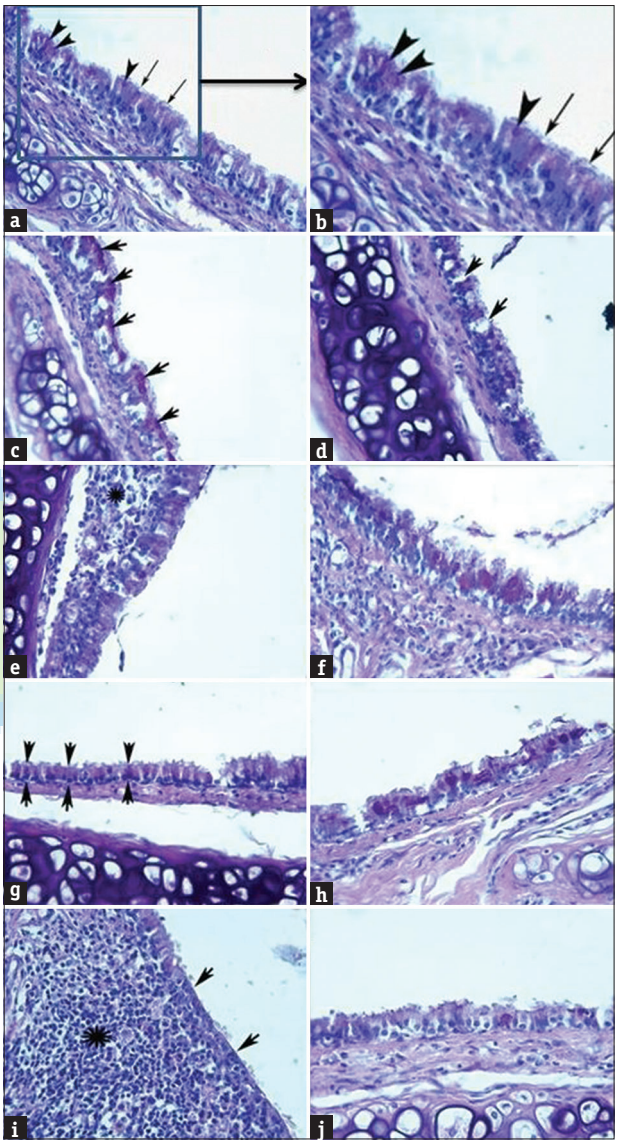

Figure 1: Histopathological examination of all groups. (a and b) control group; sills observed on the surface of epithelial cells (arrows) and goblet cells distinguished with purple excretes (arrowhead). (c) Goblet cells secretion is increased in Group 2. (d) Group 3, loss of tracheal epithelium in places. (e) Group 4, diffuse lymphoid infiltration on lamina propria (star). (f) Group 5, loss of cilia and irregularities in epithelium due to the loss of the apical epithelial surface. (g) Group 6, single-layered appearance of the tracheal epithelial (arrows). (h) Group 7, increase in goblet secretion and minimal disruption of the epithelial layout. (i), Group 8, single-layered appearance of epithelial, clear loss of cilia (arrows), and lymphoid infiltration followed on lamina propria (star). (j) In Group 9, it was observed that tracheal epithelium is preserved except the epithelial sheddings in the apical surface of the epithelial cells and loss of cilia in some areas PAS-H, ×40

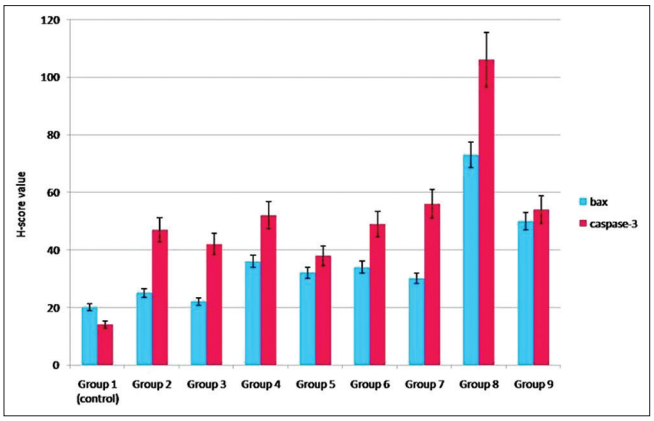

Figure 2: H-score values of Bax and caspase-3 immunoreactivity

epithelium [Figure 1e]. Administration of NS did not reveal any microscopic improvement. As a matter of fact, in terms of the histological scoring difference between Group 4 and Group 5, there was no statistical significance ($P > 0.05$).

It was observed that tracheal epithelial cells of the rats in Group 6 turned into partly single epithelial and cilias were sparse and irregular [Figure 1g]. These changes were not observed in Group 7. In terms of the histological score, there was statistically significant difference in between Group 6 and 7 ($P < 0.05$).

Among the groups given FA, the highest histological damage (9.8 ± 0.3) was detected in Group 8. With the loss of the pseudostratified columnar features, the tracheal epithelium turned into a single-layer cubic epithelium. In this group, the both the loss of cilia on epithelial surfaces and a diffuse lymphoid infiltration in the lamina propria were observed [Figure 1i]. Furthermore, in Group 9, it was observed that NS prevents lymphoid infiltration and protects epithelial structure except for epithelial shedding in some areas. The difference between Group 8 and Group 9 in terms of TET and histological scores was significant ($P < 0.05$).

Tracheal epithelium was found to be the thinnest in Group 8 (50.8 ± 4.8). The thickest epithelium (125.3 ± 6.3), not including the control group, was identified in Group 5. Tracheal epithelium thickness and histological evaluation results of all groups are given in Table 1.

Immunohistochemical results

H-score results of Bax and caspase-3 immunoreactivity are presented in Figure 2. H-score analysis revealed that Bax and caspase-3 immunoreactivity significantly increased in the Group 8 compared to all other groups ($P < 0.001$ for all comparisons) [Figures 3 and 4]. When compared the control group, all groups had higher

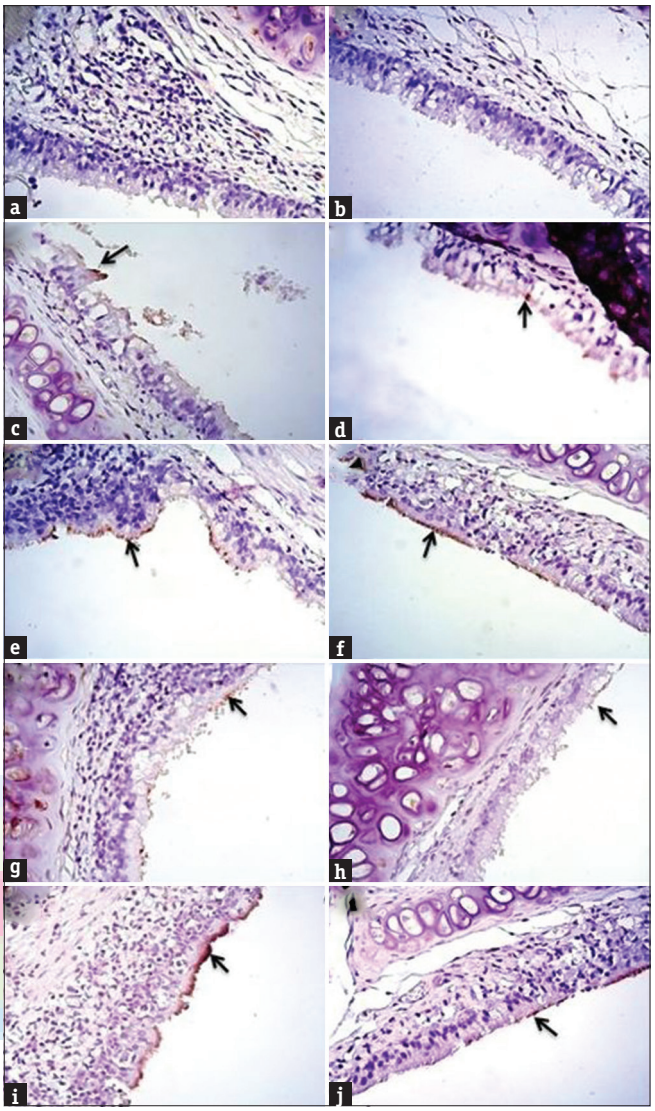

Figure 3: Bax immunohistochemical staining intensity of all groups. (a) Group 1 (control), weak immunohistochemical staining (±); (b) negative control group, no immunohistochemical staining (-); (c) Group 2 (5 ppm FA, 4 weeks), mild immunohistochemical staining (+); (d) Group 3 (5 ppm FA + NS, 4 weeks), mild immunohistochemical staining (+); (e) Group 4 (10 ppm FA, 4 weeks), moderate immunohistochemical staining (++); (f) Group 5 (10 ppm FA + NS, 4 weeks), moderate immunohistochemical staining (++); (g) Group 6 (5 ppm FA, 13 weeks), moderate immunohistochemical staining (++); (h) Group 7 (5 ppm FA + NS, 13 weeks), moderate immunohistochemical staining (++); (i) Group 8 (10 ppm FA, 13 weeks), strong immunohistochemical staining (+++); (j) Group 9 (10 ppm FA + NS, 13 weeks), moderate immunohistochemical staining (++). FA = Formaldehyde; NS = *Nigella sativa*

expression levels of caspase-3 protein ($P < 0.01$ for comparison between Group 5 and control group; $P < 0.001$ for other comparisons). When compared the FA and treatment groups for Bax immunoreactivity, statistically significant difference was determined only between Group 8-9 ($P < 0.01$) but did not in Group 2–3, Group 4–5, and Group 6–7.

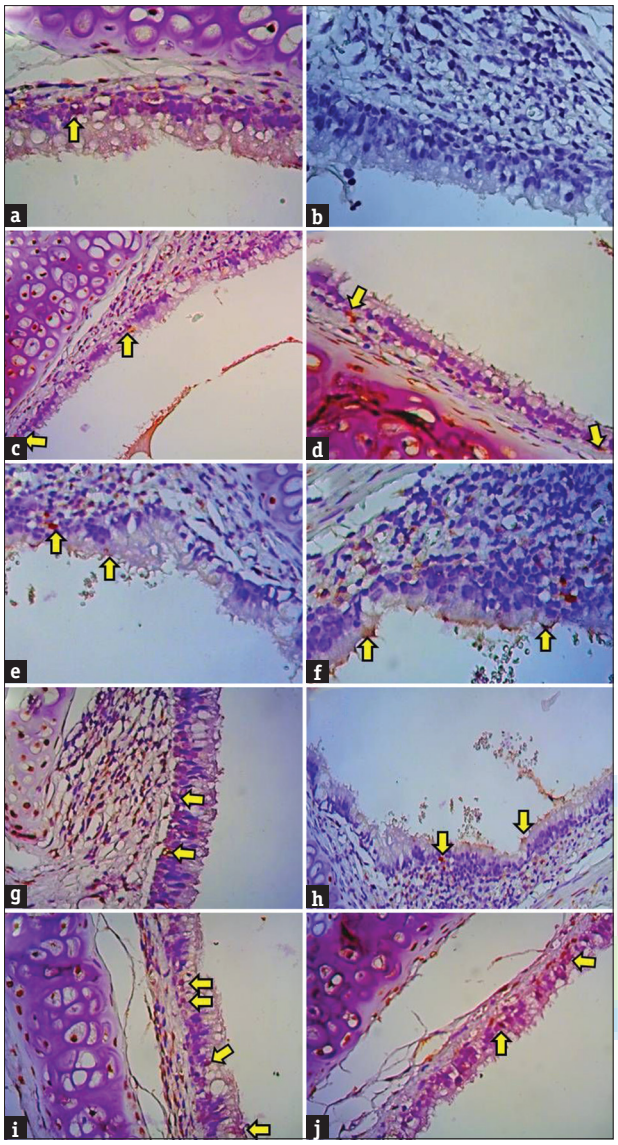

Figure 4: Representative photomicrographs of cytoplasmic immunoreactivity for activated caspase-3 of all groups. (a) Group 1 (control); (b) negative control group, no immunohistochemical staining is seen in tracheal tissue; (c) Group 2 (5 ppm FA, 4 weeks), mild immunohistochemical staining (+); (d) Group 3 (5 ppm FA + NS, 4 weeks), mild immunohistochemical staining (+); (e) Group 4 (10 ppm FA, 4 weeks), mild immunohistochemical staining (+); (f) Group 5 (10 ppm FA + NS, 4 weeks), mild immunohistochemical staining (+); (g) Group 6 (5 ppm FA, 13 weeks), mild immunohistochemical staining (+); (h) Group 7 (5 ppm FA + NS, 13 weeks), mild immunohistochemical staining (+); (i) Group 8 (10 ppm FA, 13 weeks), ciliated cells and basal cells of the respiratory epithelium is strongly immunohistochemical staining for active caspase 3; (j) Group 9 (10 ppm FA + NS, 13 weeks), after the NS treatment it is seen that the reduction in the number of immunopositive cells. FA = Formaldehyde; NS = *Nigella sativ*

Discussion

Experimental and clinical studies have revealed that FA can cause metaplastic changes in the epithelial layer in the nasal mucosa.[25,26] When the trachea is exposed to glutaraldehyde, a member of the same aldehyde family, minimal changes are observed only in the larynx histopathology, whereas no changes are observed in the main bronchus or even in the lung.[27] The cause of metaplasia of goblet cells is linked to the inhibition of mucociliary activity. The changes were more pronounced in the alveolar epithelium as marked emphysema, increased cellularity and thickness of alveolar wall, accumulation of inflammatory cells, mild edema, congestion, and hemorrhages.[28] It has been reported that FA causes the loss of the pseudostratified columnar characteristic in tracheal epithelium, change to a single-layered cubic epithelium, loss of cilia on the surface of epithelial cells, and diffuse lymphoid infiltration in the lamina propria.[29] Furthermore, we observed similar findings in the FA groups.

The deciliation or clumping of cilia of tracheal epithelium may impair the functional activity of cilia required for the continuous movement of glandular secretions toward pharynx.[30] FA maximizes its own toxic effect by inhibiting mucociliary function. To minimize the carcinogenic effect, mucociliary function must be intact. Vitamin A deficiency in the tracheal epithelium of rats has been reported to cause squamous metaplasia.[31] It is suggested that vitamin A controls gene expression, which is related to squamous differentiation via the retinoic acid receptor.[32] A reduction of damage was observed in rats that had been exposed to benzopyrene when given vitamin A.[33] In another study, FA exposure caused bronchoconstriction and hyperreactivity at lower concentrations when exposure duration was extended from 2 to 8 h. Exposure to ≥ 0.3 ppm FA for 8 h was sufficient to produce a significant increase in airway reactivity, whereas similar effects only occurred after > 9 ppm FA for 2 h.[34] In our study, the most damage occurred at a 10 ppm dose in a 13-week exposure. We observed that subepithelial lymphocytic infiltration and superficial loss of the epithelium similar to the results of the study carried out by Davarian *et al*.[35]

FA causes the generation of ROS that cause apoptosis and necrosis, resulting in lipid peroxidation and metabolic alterations.[7,8] Apoptosis is a form of programmed cell death characterized by DNA fragmentation, cytoplasmic shrinkage, membrane changes, and cell death without damage to neighboring cells.[36] Proapoptotic members of the Bcl-2 family (Bax, Bak, and Bad) are localized in the cytosol and transmigrate from the cytosol to the mitochondria during apoptosis and increase cytochrome c release.[37] Increased cytochrome c triggers, the apoptosome complex and executioner caspases are then activated, especially caspase-3, which are responsible for morphological changes such as chromatin condensation and DNA breakdown.[38] To sum up, increased Bax and caspase-3 immunoreactivity in the cytoplasm of

the cells indicates increased apoptotic activity.[8] In the present study, we showed that FA caused apoptosis in the trachea epithelial cells. Especially, the most apoptotic activity occurred at a 10 ppm dose in a 13-week exposure. Likewise, in previous studies, FA has been reported to cause DNA damage, thereby leading to apoptosis.[8,39] Some studies have shown that FA caused more severe damages with a shorter exposure time at high concentrations in comparison to long-term exposure in low concentrations.[26] The most apoptotic activity and tissue damage were observed in the group that was exposure to the longest and highest concentration.

A study reported that FA given to rats caused a tracheal hyperactivity, and it was found that estradiol prevents this but does not affect progesterone.[40] In addition, the previous study demonstrated that some antioxidant has protective effects against FA toxicity.[7,41] NS has been used as antioxidant in many studies, its protective effect in the liver, stomach, and brain tissue have previously been shown.[19,42,43] Furthermore, it was reported that it prevents allergic inflammation in trachea.[43,44]

In our study, we showed that FA caused apoptosis in the tracheal epithelial cells. Especially, the most apoptotic activity occurred at long period exposure. Cellular damage of tracheal epithelium was present in all groups. However, NS treatment decreased apoptotic activity and lymphoid infiltration and protected the epithelial structure.

Conclusion

These findings suggest that the duration of exposure is important for the induction of airway hyperactivity. Furthermore, prolonged, high-level exposures generate abnormal histological, and IHC responses in the airway and NS serve to help prevent some of this damage, especially long period.

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Nil.

Conflicts of interest

A part of manuscript was presented at International Symposium of Clinical and Applied Anatomy in 2012, Ankara, Turkey.

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