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Protective effect of aerobic exercise on the vocal folds against cigarette smoke exposure

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Abstract

Purpose Laryngeal pathologies due to cigarette smoking vary among individuals, whereas some smokers remain disease free. These differences can be explained by multiple factors among individuals. In this context, an animal study was designed to determine if there is any protective effect of aerobic exercise against the detrimental effects of cigarette smoke on laryngeal tissues.

Methods A total of 24 male Wistar albino rats were divided into three groups of eight animals each: control (no smoke exposure), smoking (smoke exposure), and exercise (smoke exposure and exercise) groups. Histopathological (light and electron microscopy) and immunohistochemical (GSTA1, CYP1A1, CYP2E1) evaluations of the vocal folds were performed at the end of experimental period.

Results Exercise group revealed statistically significant decrease in edema ($p=0.03$) and inflammatory cell infiltration ($p=0.02$) compared to smoking group. In electron microscopic evaluation; cytoplasmic vacuoles were also present in exercise group, but were smaller than smoking group. Edema and swollen mitochondria were also less prominent in exercise group. Condensed chromatin material in the periphery of nucleus was observed only in few cells in exercise group, and observed in more cells in smoking group. GSTA1 expression was higher ($p=0.047$) and CYP1A1 expression was lower ($p=0.01$) in exercise group than smoking group.

Conclusions Our results indicate that aerobic exercise has a protective role on the larynx against the damaging effect of cigarette smoke. Smokers who exercise regularly may be at a lower risk of cigarette smoke-related laryngeal diseases, as compared with those who do not exercise.

Keywords Aerobic exercise · Rat · Smoke · Vocal fold

Introduction

Cigarette smoke contains numerous noxious chemicals, or xenobiotics, such as nicotine, carbon monoxide, polycyclic hydrocarbons, tar, and ammonia, which cause tissue damage via increased production of reactive oxygen species and degeneration of the inflammatory response, thereby acting as a major cause of morbidity and mortality worldwide [1].

In the absence of xenobiotic metabolism, the abundance of xenobiotics, such as cigarette smoke, in the body accumulate and cause cellular damage. Xenobiotics are generally metabolized through phase I reactions via the activities of cytochrome P450 enzymes found in the membrane of the smooth endoplasmic reticulum, which oxidize lipophilic substances to electrophiles. Electrophiles are the more polar forms of lipophilic xenobiotics, but are still toxic, and are eliminated by phase II reactions via the activities of

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glutathione S-transferases (GSTs), which facilitate the excretion of these electrophiles by catalyzing the conjugation of the reduced form of glutathione and forming less toxic and more hydrophilic substrates. The most studied classes of the GST superfamily are alpha, mu, theta, and pi [2].

Cigarette smoking has been correlated with chronic obstructive pulmonary disease, cardiovascular disease, cancer, autoimmune disorders, and infertility, and has an especially important role in the etiology of head and neck cancers [3]. Smoking-related lesions of the larynx are characterized by dysplasia, leukoplakia, Reinke's edema (RE), and the presence of abnormal squamous cells. The majority of patients with laryngeal cancer and almost all with RE have a history of smoking. In most RE cases, there are no or minimal alterations to the epithelium and primary epithelial lesions generally do not appear edematous [4]. Although epithelial lesions have the potential for malignant transformation, RE is not considered to be a premalignant lesion. The mRNA expression level of the heme oxygenase-1 gene was found to be four times higher in the laryngeal tissues of RE patients and may be associated with a chemoprotective effect against malignant transformation [5].

Laryngeal pathologies due to cigarette smoking vary among individuals, whereas some smokers remain disease free. These differences can be explained by genetic, behavioral, and environmental factors among individuals. Therefore, the aim of this study was to determine whether regular aerobic exercise protects the larynx from the damage of cigarette smoke. In this context, an animal study was designed to determine if there is any protective effect of aerobic exercise against the detrimental effects of cigarette smoke on laryngeal tissues by histopathological evaluation and immunohistochemical analysis of the expression profiles of cytochrome P450 family 1 subfamily A polypeptide 1 (CYP1A1), cytochrome P450 family 2 subfamily E polypeptide 1 (CYP2E1), and glutathione S-transferase alpha 1 (GSTA1).

Materials and methods

Approval

The study protocol was approved by the Experimental Animal Research Ethics Committee (Ankara, Turkey). All applicable international guidelines for the care and use of animals were followed.

Animals

A total of 24 male Wistar albino rats were obtained and maintained at a constant temperature of 22 °C under a 12 h light/dark cycle with ad libitum access to food and water. The rats were divided into three groups of eight animals each: those in the control group were left untreated, whereas those in the smoking group were exposed to the equivalent of smoke from 15 cigarettes in an isolated cigarette smoke cabin for 30 min twice per day, 5 days per week, for 3 months, and those in the exercise group were also exposed to cigarette smoke as in the smoking group and were forced to run at a medium speed of 18 m/min on a treadmill (four-path treadmill for rats) for approximately 40 min per day, 5 days per week, for 3 months. At the end of the 3-month experimental period, the rats were sacrificed, and the larynxes were removed for assessment of the vocal folds. Histopathological and immunohistochemical evaluations were conducted by specialists who were blinded to the experimental groups.

Light microscopy

Laryngeal specimens were fixed in 10% buffered formalin, dehydrated with a graded series of alcohol, and embedded in paraffin blocks. Then, 5-µm-thick sections were cut and stained with hematoxylin and eosin, Alcian blue-periodic acid Schiff, and Masson trichrome according to standard protocols.

The specimens were examined under a light microscope (Leica DM6000B, Wetzlar, Germany) fitted with a digital camera (DC490, Leica, Wetzlar, Germany). Images were used to identify morphological changes to the epithelium and the presence of edema, congestion, and inflammation. Each specimen was scored on a scale of 0–3 as follows: absent = 0, mild = 1, moderate = 2, and severe = 3. The mean values of the scores were used for statistical analysis.

Electron microscopy

For transmission electron microscopic examination, the vocal folds of the rats were dissected and sectioned. The sections were fixed in the 2.5% glutaraldehyde for 48–72 h, post-fixed with 1% osmium tetroxide in phosphate buffer (pH 7.4) for 2 h, dehydrated in increasing concentrations of alcohol, washed with propylene oxide, and embedded in epoxy resin-embedding media. Semi-thin sections of about 2 mm in thickness and ultra-thin sections of about 60 nm in thickness were cut with a glass knife and an ultramicrotome (LKB-Produkter AB, Bromma, Sweden). The semi-thin sections were stained with methylene blue and examined under a light microscope (Nikon Optiphot; Nikon Corporation, Tokyo, Japan). Following this examination, the tissue blocks were trimmed and ultra-thin sections were obtained using the same ultramicrotome and then stained with uranyl acetate and lead citrate. Following staining, the ultra-thin sections were examined using a transmission electron microscope (JEM-1200EX; Jeol Ltd., Tokyo, Japan).

Immunohistochemical staining

Specimens were fixed in 10% buffered formalin and embedded in paraffin blocks. Sections were cut at a thickness of 4 μm, and one section was stained with hematoxylin and eosin to observe tissue morphology. For immunohistochemical analysis, endogenous peroxidase activity was blocked by incubating the sections in 1% hydrogen peroxide (v/v) in methanol for 10 min at room temperature (RT). The sections were subsequently washed in distilled water for 5 min, and antigen retrieval was performed for 3 min using 0.01 M citrate buffer (pH 6.0) in a domestic pressure cooker. After washing with distilled water, the sections were transferred to 0.05 M Tris–HCl (pH 7.6) containing 0.15 M sodium chloride (TBS) and incubated at RT for 10 min with super block reagent [SHP125; (ScyTek Laboratories, Inc., Logan, UT, USA)] to block nonspecific background staining. The sections were then incubated with primary antibodies against GSTA1 (dilution, 1:500), CYP1A1 (1:200), and CYP2E1 (1:500) in TBS at 4 °C overnight. After washing with TBS for 15 min, the sections were incubated with biotinylated secondary antibodies at RT (ScyTek Laboratories, Inc.), followed by streptavidin covalently conjugated to horseradish peroxidase (ScyTek Laboratories, Inc.). Diaminobenzidine was used to visualize peroxidase activity in the tissues, and the nuclei were lightly counterstained with hematoxylin. Then, the sections were dehydrated and mounted. Both positive and negative controls were included in each run.

Light microscopy of immunohistochemically stained sections was performed by two pathologists who were blinded to all clinical data. The distribution, localization, and characteristics of immunostaining were recorded. Brown staining of the cytoplasm and/or nuclei of the epithelial cells was considered positive. Scoring was also performed by observers unaware of the patient data. For each antibody, the intensity of the reaction (negative = 0, weak = 1, moderate = 2, and strong = 3) was determined to describe the immunoreactions.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corporation, Armonk, NY, USA). Normality of distribution was determined using the Kolmogorov–Smirnov test. Quantitative data were evaluated using one-way analysis of variance and subjected to post hoc analysis with Tukey's test. A probability (p) value of < 0.05 was considered statistically significant.

Results

Light microscopy

The resected larynxes were sectioned for examination of the vocal folds. Histological examination of the vocal folds revealed the presence of stratified squamous epithelium in the vocal folds and pseudostratified columnar epithelium in the false vocal folds of the control group. The lamina propria under the stratified squamous epithelium was composed of collagen fibers, elastic fibers, fibroblasts, and blood vessels. Seromucous glands were prominent in the lamina propria of the false vocal folds.

In the smoking group, the epithelium was notably thinned and detected as a single epithelial cell layer in the wall of the ventricle and vocal folds with moderate edema, severe congestion, and infiltration of inflammatory cells in the lamina propria, whereas the epithelium of the false vocal folds was composed of stratified squamous cells.

In the exercise group, the vocal folds were composed of stratified squamous epithelium with mild edema, moderate congestion, and less infiltration of inflammatory cells in the lamina propria (Fig. 1). As compared with the smoking group, there was less edema and inflammatory cell infiltration in the exercise group ($p = 0.03$ and 0.02, respectively), but no difference in vascular congestion ($p = 0.476$). The scores for histopathological changes are shown in Table 1.

Electron microscopy

Ultrastructural examination of the vocal folds of the control group revealed that the epithelial cells, connective tissue components, microvascular structures, intercellular distances, and connections and continuity of the basement membrane were normal.

In the smoking group, the cytoplasm of the squamous cells contained very large vacuoles with swollen mitochondria and edematous areas. In some of these cells, the chromatin material was condensed in the periphery of the nucleus.

In the exercise group, the vacuoles in the cytoplasm of the squamous cells were comparatively small. However, swelling of the mitochondria and nuclear irregularities were observed in this group. In very few cells, the chromatin material was condensed in the periphery of the nuclei (Fig. 2).

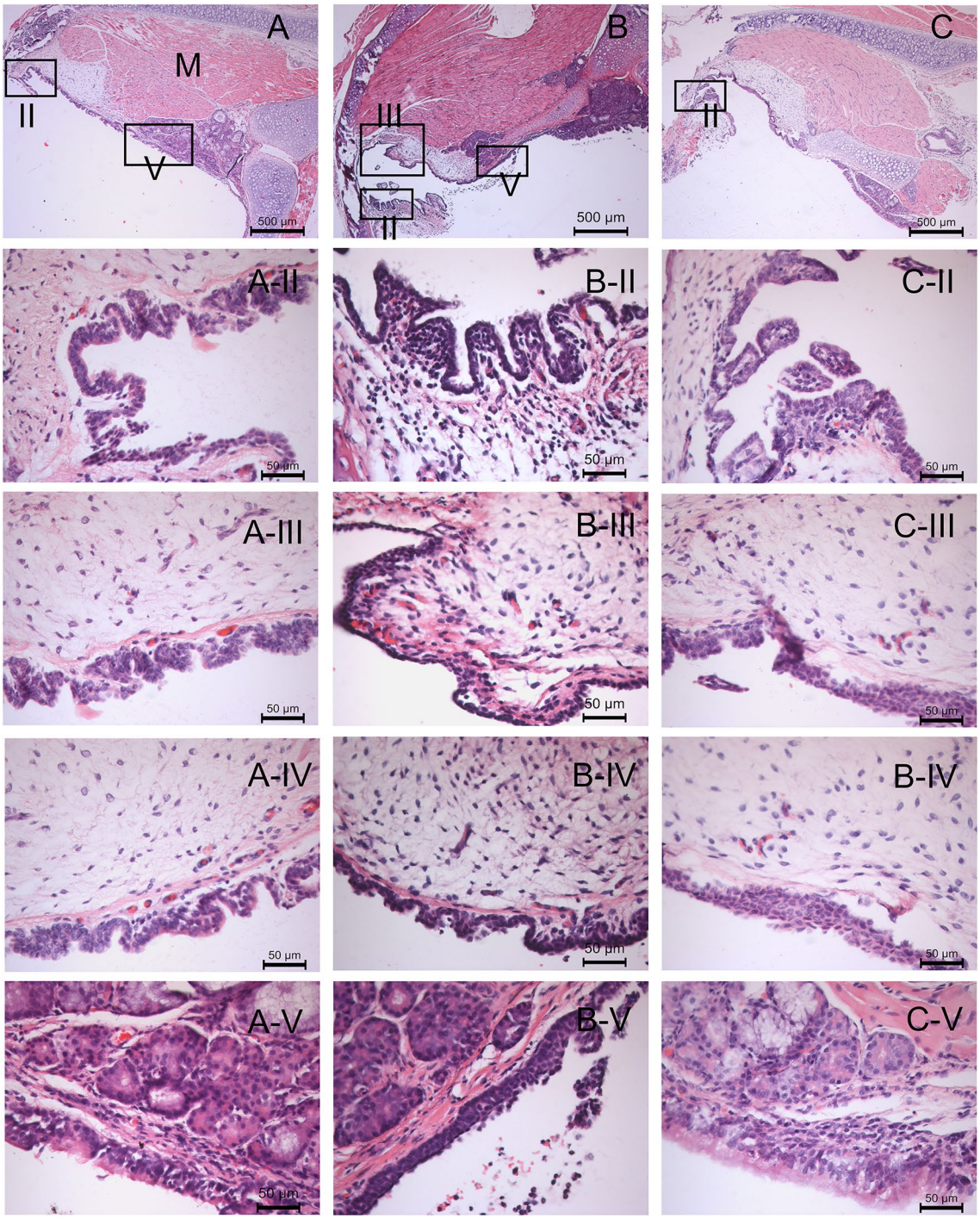

Fig. 1 A single epithelial cell layer, edema and severe inflammatory cell infiltration in vocal folds of smoking group. Stratified squamous epithelium in false vocal folds in smoking group. Mild edema and less inflammatory cell infiltration in the lamina propria of exercise group. *M* Vocal cord muscle, *A* control, *B* smoking, *C* exercise (Hematoksilen–eosin X50, II, III, IV, V X400)

Expression profiles of GSTA1, CYP1A1, and CYP2E1

In the control group, the vocal folds were characterized by low expression levels of CYP2E1 and negative staining for CYP1A1 but comparatively high expression levels of GSTA1 (Table 2).

In the smoking group, CYP1A1 expression was greater ($p=0.01$) and that of GSTA1 was significantly lower in the

Table 1 Scores for histopathological changes in vocal folds

	Edema	Vascular congestion	Inflammation
Control group ($n=8$)	0 ± 0	0 ± 0	2 ± 0
Smoking group ($n=8$)	2.25 ± 0.70	2.62 ± 0.51	2.62 ± 0.51
Exercise group ($n=8$)	1.25 ± 1.03*	2.37 ± 0.51	1.62 ± 1.06*

Mean ± standard deviation

*$p<0.05$, compared to smoking and exercise groups

Table 2 Scores for immunohistochemical stainings of vocal folds

	GSTA1	CYP1A1	CYP2E1
Control group ($n=8$)	1.25 ± 0.88	0 ± 0	0.75 ± 0.88
Smoking group ($n=8$)	0.12 ± 0.35	0.5 ± 0.53	0.5 ± 0.53
Exercise group ($n=8$)	0.87 ± 0.35*	0 ± 0*	0.5 ± 0.53

Mean ± standard deviation

*$p<0.05$, compared to smoking and exercise groups

vocal cord epithelium ($p=0.03$), as compared with the control group, whereas there was no significant difference in CYP2E1 expression levels ($p=0.741$) (Table 2).

Among the studied proteins, GSTA1 expression was significantly higher in the exercise group than in the smoking group ($p=0.047$) with no statistically significant difference in comparison with the control group, whereas CYP2E1 expression was similar between the smoking and exercise groups, and CYP1A1 was not expressed in the vocal folds of the exercise group ($p=0.01$) (Table 2).

Discussion

Although cigarette smoking is harmful to public health and is restricted by law in most places, it is still a very common and socially accepted addiction. Quitting this addiction is both physically and mentally challenging for most smokers. Not only smokers but also those who live with them are affected from the harmful effects of passive cigarette smoke. It would be desirable to be free of exposure to cigarette smoke and other environmental toxic agents, but this goal is not feasible. Therefore, an alternative option is the use of agents to protect against diseases related to cigarette smoke exposure. Although several agents, such as melatonin, vitamin E, and isothiocyanates, are known to reduce the detrimental effects of cigarette smoke on various tissues [6–8], further studies are needed. In this context, the aim of the present study was to evaluate the protective role of exercise against cigarette smoke in laryngeal tissues.

The results of the present study showed a statistically significant increase in the incidence of edema ($p<0.001$), congestion ($p=0.02$), and inflammatory cell infiltration ($p<0.001$) of the vocal folds of the smoking group. In addition metaplasia was observed only in the smoking group. Although the period of exposure was sufficiently long for comparisons with the results of previous studies that revealed dysplastic lesions in the tissues of the smoking groups [9–11], in the present study, there was no significant difference in the extent of keratinization of the tissues in the smoking group and no dysplastic lesions due to cigarette smoke exposure were observed. Leao et al. [12] also reported moderate focal inflammation of the laryngeal and tracheal mucosa, but no hyperplastic, metaplastic, or dysplastic lesions. Differences in the cigarette

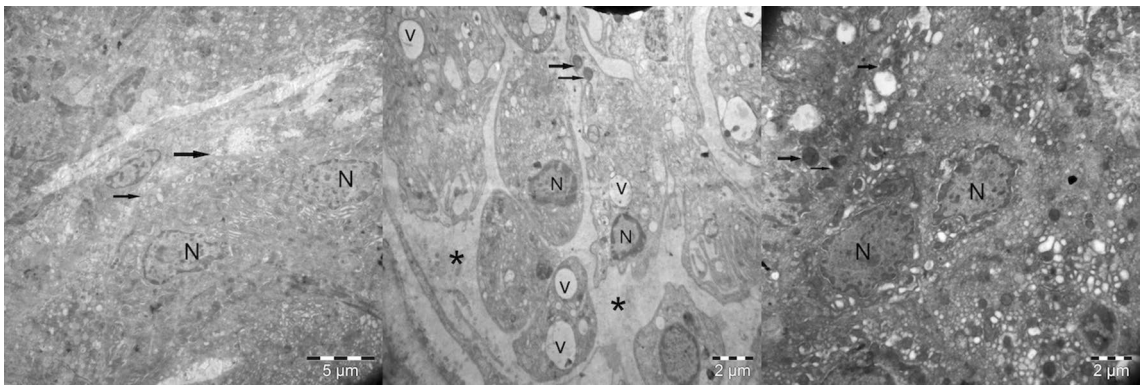

Fig. 2 Left: (control) normal nuclei (*N*) and cellular borders (arrows) are seen (scale bar: 5 micron). Middle: (smoking) the squamous cells had very large vacuoles (*v*) inside of their cytoplasm. Swollen mitochondria (arrows) and edematous areas (asterisk) were present. In nuclei (*N*) of these cells the chromatin material was condensed in the periphery of the nucleus (scale bar: 2 µm). Right: (exercise) The small size vacuoles inside of the cytoplasm were seen. Swollen mitochondria (arrows) and nuclear irregularities were observed in this group (scale bar: 2 µm)

brands or cigarette smoke cabins may explain these discrepancies. Thus, our results were compared between groups without evaluation of dysplastic lesions, as it was not possible to assess the efficiency of aerobic exercise in terms of this parameter. Ultrastructural evaluation with an electron microscope revealed prominent changes in the vocal folds of the smoking group, which contained comparatively large cytoplasmic vacuoles, edematous areas, swollen mitochondria, and condensed chromatin material in the periphery of the nucleus, as in previous studies [6]. The light microscopic and electron microscopic evaluations of the vocal cord tissues indicated improvement in terms of edema, inflammation, cytoplasmic vacuoles, swollen mitochondria, and condensed chromatin material in the exercise group. Although acute exercise induces production of reactive oxygen species with systemic adaption to oxidative stress, regular exercise conveys a protective role against inflammation and oxidative stress by various mechanisms and its antioxidant and anti-inflammatory effects [13, 14]. Various studies have reported the benefits of exercise on the functions of the brain, kidney, liver, and heart [15–19]. In addition, evidence suggests that regular physical activity lowers the risk of cancer [20]. Our findings also support the beneficial effects of exercise on laryngeal tissues exposed to cigarette smoke.

To the best of our knowledge, no previous study has reported the protein expression profiles of GSTA1, CYP1A1, and CYP2E1 in vocal folds tissue. In the present study, expression of GSTA1 was decreased and that of CYP1A1 was increased in the smoking group, whereas the expression of GSTA1 was increased and that of CYP1A1 was decreased in the exercise group. These findings demonstrate that exercise conveys a protective effect by activating isoenzymes that metabolize xenobiotics. CYPs are important for the detoxification of xenobiotics as well as the conversion of potential procarcinogens into toxic metabolites. Thus, overexpression of CYPs is linked to the development of various types of tumors [21–23]. GSTs are an important family of enzymes involved in the detoxification of several xenobiotics to protect tissues from the harmful effects of oxidative stress and chemically induced tissue damage [24].

This is the first animal study showing the protective effect of aerobic exercise on vocal fold mucosa against the harmful effects of exposure to passive cigarette smoke. A major limitation to this study is that no dysplastic lesion or keratinization was found in the vocal folds of rats in the smoking group; thus, the effects of aerobic exercise on these lesions remain unknown. Hence, future animal studies and, more importantly, clinical trials are needed to support or disapprove these results.

Conclusions

The effects of cigarette smoke on the larynx vary among individuals. Causes of these differences may involve genetic, lifestyle, and nutritional factors. Aerobic exercise is known to improve health via antioxidative and/or curative effects in different tissues. Our results indicate that aerobic exercise has a protective role on the larynx against the damaging effect of cigarette smoke. Smokers who exercise regularly may be at a lower risk of cigarette smoke-related laryngeal diseases, as compared with those who do not exercise.

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Compliance with ethical standards

Conflict of interest The authors declared no conflict of interest.

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