

Heat Shock Protein 70 and Inducible Nitric Oxide Synthase Expression in Adenoid Tissue of Children Exposed to Passive Smoke

Pasif Sigaraya Maruz Kalmış Çocukların Adenoid Dokularında Isı Şok Proteini 70 ve İndüklenebilir Nitrik Oksit Sentaz Ekspresyonunun Araştırılması

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ABSTRACT Objective: We aimed to investigate the expression of heat shock protein 70 (HSP70) and inducible nitric oxide synthase (iNOS) in hypertrophic adenoid tissues of children with passive smoke exposure. **Material and Methods:** Fifteen children with passive smoke exposure (study group) and 15 children without passive smoke exposure (control group) who underwent surgical intervention due to adenoid hypertrophy were enrolled in this study. The expression of HSP70 and iNOS was investigated in the adenoid tissues obtained from both groups by immunohistochemical staining using anti-HSP70 and anti-iNOS antibodies. **Results:** The mean age of the 15 children who were exposed to passive smoke was 7.8 ± 2.5 (ranging from 4 to 13 years); 9 were boys and 6 were girls. Mean age of the control group, consisting of 8 boys and 7 girls was 6.9 ± 2.1 (ranging from 4 to 12 years). The groups had similar age and gender characteristics. HSP70 and iNOS expressions in the surface epithelium, lymphoid follicles and perifollicular lymphoid tissue of the adenoids in the study group were significantly higher than that of the control group ($p < 0.05$). **Conclusion:** Passive smoking induced HSP70 and iNOS overexpression in the adenoid tissue.

Key Words: Tobacco smoke pollution; adenoids; HSP70 heat-shock proteins

ÖZET Amaç: Pasif sigaraya maruz kalan çocukların hipertrofik adenoid dokularında ısı şok proteini 70 (İŞP70) ve indüklenebilir nitrik oksit sentaz (iNOS) ekspresyonunun immünohistokimyasal olarak araştırılması. **Gereç ve Yöntemler:** Adenoid hipertrofisi nedeni ile opere olan, 15'i pasif sigaraya maruz kalmış ve 15'i de pasif sigaraya maruz kalmamış toplam 30 çocuğun adenoid dokuları incelendi. Dokuda immünohistokimyasal boyama ile İŞP70 ve iNOS ekspresyonu araştırıldı. Sonuçlar istatistiksel olarak karşılaştırıldı. **Bulgular:** Pasif sigaraya maruz kalan 15 çocuğun yaş ortalaması $7,8 \pm 2,5$ (4-13), 9'u erkek ve 6'sı kız idi. Sekizi erkek ve 7'si kız olan kontrol grubunun yaş ortalaması ise $6,9 \pm 2,1$ (4-12) idi. Benzer yaş ve cinsiyet özelliklerine sahip gruplarda, adenoid yüzey epitelinde, lenfoid folikül ve perifoliküler dokuda İŞP70 ve iNOS ekspresyonu, pasif sigaraya maruz kalan grupta kontrol grubuna göre anlamlı derecede daha yüksek bulundu ($p < 0,05$). **Sonuç:** Pasif sigaraya maruz kalmanın, adenoid dokuda İŞP70 ve iNOS ekspresyonunu artırdığı saptandı.

Anahtar Kelimeler: Pasif sigaraya maruz kalma; adenoid; İŞP70 ısı şok proteini 70

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Passive smoke exposure (PSE) is one of the most common preventable health problems worldwide. In children, PSE has been shown to be associated with upper and lower respiratory tract infections, sudden infant death syndrome, asthma, behavioral and cognitive problems and physical growth retardation.^{1,2}

As a part of the Waldeyer's ring, the adenoid located in the nasopharynx has an important role in the development of immune processes.^{3,4} Adenoid tissue is continually exposed to antigens, such as bacteria, virus and allergens, and its lymphoid component is the reason of its enlargement.^{3,4} Nasopharyngeal adenoid hypertrophy (AH) is a common cause of nasal obstruction in the pediatric patients. Although adenoidectomy is the most common operation performed in childhood, etiopathogenetic mechanisms underlying the hypertrophy of adenoid tissue remains unclear.^{3,4}

Increased bacterial load in the adenoids-especially *H. influenzae* and group A streptococci-respiratory syncytial virus infections, increased oxidative stress and up-regulated glucocorticoid receptor-alpha expression were reported as etiological factors for AH.^{3,5-15} In addition, allergy and PSE were also accused.¹⁶⁻²⁰ Finkelstein et al mentioned a different kind of lymphoid hypertrophy mediated by cytotoxic T lymphocytes due to harmful effects of smoking and they named this entity "smoking-induced nasopharyngeal lymphoid hyperplasia".¹⁹ Torre et al suggested that the histological and ultrastructural damages in the tonsillar lymphoid tissue increased as the duration of smoking increased.²¹

Nitric oxide (NO) plays an important role in many biochemical processes, such as the regulation of blood vessel dilatation and immune response. It also acts as a neurotransmitter. Large concentrations of locally produced NO have several cytotoxic effects, including reaction with proteins and nucleic acids, suppression of mitochondrial respiratory chain or induction of cell necrosis and apoptosis.^{13,22} iNOS, an enzyme that synthesizes NO from L-arginine, is mainly present in macrophages. Various reports suggest that tobacco smoke and inflammatory cytokines contribute to the increase of iNOS induction and NO production in different tissues.²³⁻²⁵

The ability of cells to express heat shock proteins (HSPs) in response to stress conditions, including exposure to heat shock, inflammatory stimuli, infection, and oxidative stress is believed to play a critical protective role against environmental and physiologic stresses. The synthesis of

stress-inducible HSP70 serves as a repairing mechanism involving increased amounts of unfolded or denatured proteins.^{14,26} Previous studies reported that in vitro and in vivo exposure to tobacco smoke resulted in induction of HSP70 overexpression in various tissues.^{27,28} To our knowledge, this has not been studied in adenoid tissue.

In this study, we aimed to determine the HSP70 and iNOS expression by immunohistochemistry in the adenoid tissues of children who were exposed to tobacco compared to that in children who were not exposed to tobacco, and to investigate the possible role of tobacco on adenoid hypertrophy.

MATERIAL AND METHODS

The study group consisted of 15 children with adenoid hypertrophy who had a history of passive smoke exposure. The control group consisted of 15 children of similar ages and gender, with adenoid hypertrophy without a history of PSE. All patients had snoring and mouth breathing as reported by their parents. A complete otolaryngological examination was performed by nasopharyngeal rigid endoscopy (2.7 mm 0°, Karl Storz, Germany) in all cases. All patients underwent adenoidectomy and/or adenotonsillectomy under general anesthesia with curettage and cold dissection methods. Children with a history of allergy, head and neck malformations, identified syndromes, neurological or any other systemic diseases and recent or ongoing upper respiratory tract infection were excluded.

Parents of all children were questioned for smoking status. Parents who were smokers were also questioned for smoking amount per day and whether they smoked near their children or not.

Passive smoke exposure was defined as exposure to smoke for at least 1 hour per day for more than 3 years.¹⁷

IMMUNOHISTOCHEMISTRY

Analysis of HSP70 and iNOS

Expression of HSP70 and iNOS in adenoid tissue was evaluated immunohistochemically using the avidin-biotinylated horseradish peroxidase comp-

lex. Formalin-fixed, paraffin embedded tissue sections were processed for microwave antigen retrieval in 0.01 M sodium citrate buffer (pH 6.0) for 10 min at maximum power. After blocking of non-specific binding sites, the sections were incubated with the primary anti-HSP70 (Ab-3) polyclonal antibody (diluted at 1:80) overnight at 4-8°C and with anti-iNOS monoclonal antibody (diluted at 1:70) for 30 minutes at 4-8°C, (Neomarkers, LabVision Coop., CA, U.S.A.). The slides were subsequently treated with biotinylated secondary antibody and avidin-biotin peroxidase complex (LabVision Coop., CA, U.S.A.) according to the manufacturer's instructions. Aminoethylcarbazol (AEC) was used to visualize the immunoreaction. Then, sections were counterstained with Mayer's

TABLE 1: Demographic features and smoke exposure states of the patients.

	PSE positive	PSE negative	p value
Age, mean±SD	7.8±2.5	6.9±2.1	>0.05
Gender			
Male	9	8	>0.05
Female	6	7	
Smoking state of parents			
Only father	6	-	
Only mother	1		
Both parents	6		
Other relatives	2		
Number of cigarettes smoked by parents, mean±SD years	11.7 ± 3.6	-	
Daily number of cigarettes children were exposed to, mean±SD	7.2 ± 2.0	-	

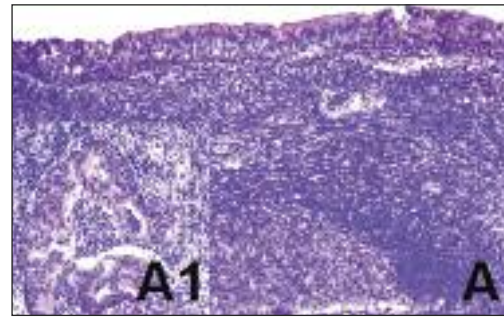
PSE: Passive smoke exposure.

TABLE 2: HSP70 and iNOS intensity and distribution of the surface epithelium in the adenoid tissue.

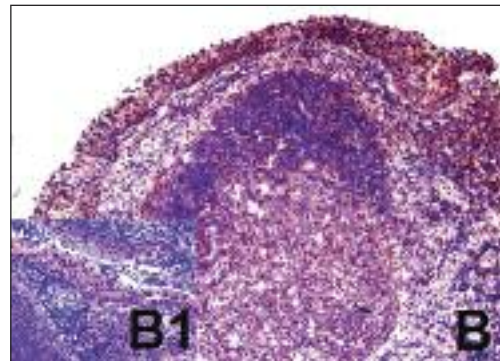
	Surface epithelium Median (Min-Max)		p value
	PSE positive	PSE negative	
HSP70	6 (4-6)	3 (2-5)	<0.001
iNOS	5 (4-6)	3 (1-5)	<0.001

PSE: Passive smoke exposure.

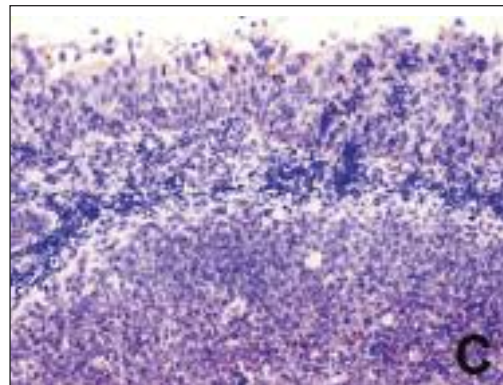
Cells in each section, the values of intensity and diffusion 0, no changes; 1, minimal; 2, low; 3, moderate; 4, strong; 5, heavy; and 6, most heavy



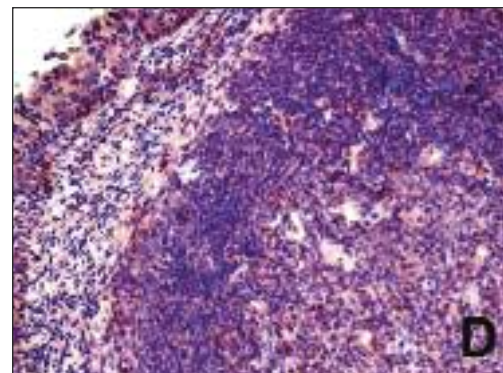
A-A1; Control, (A: x100-A1: x200).



B-B1; Passive smoking; immunostained cells are increased in the surface epithelium in all adenoid tissues, (B: x100, B1: x200).



C; Control; only a few positive cells are observed in the lymphoid follicles and perifollicular lymphoid tissue, (x200).



D; Passive smoking; increased number of positive cells in the lymphoid follicles and perifollicular lymphoid tissue, (x200).

FIGURE 1: Photomicrographs of the iNOS analysis in the adenoid tissue, surface epithelium, lymphoid follicles and perifollicular areas in all adenoid structures (as detected by immunohistochemical staining).

hematoxylin. The slides were examined under a light microscope (Olympus BX51; Olympus Corp.; Tokyo, Japan). The staining of cytoplasmic HSP70 in the surface epithelium and crypt epithelium was defined as follows: Intensity and distribution of staining were separately rated from 0 to 3+ semi-quantitatively. Following this, the counted values were summed in each section, recombining the values of intensity and diffusion as follows: 0, no changes; 1, minimal; 2, low; 3, moderate; 4, strong; 5, heavy; and 6, most heavy.²⁹ The number of staining lymphocytes in lymphoid follicles and perfollicular lymphoid tissue was evaluated and the results were determined by counting the number of cells stained positive for HSP70 and iNOS in 25 fields of 0.2 mm². Results (number of cells per 0.2 mm² field) were recorded as follows: 0: none; 1: 1-10 (mild); 2: 11-25 (moderate) and 3: more than 25 (strong) positive cells, as defined previously.³⁰

STATISTICAL ANALYSIS

Mann-Whitney-U test was used for statistical analysis of iNOS and HSP70 intensity and distribution in the adenoid tissue. P values < 0.05 were considered significant.

RESULTS

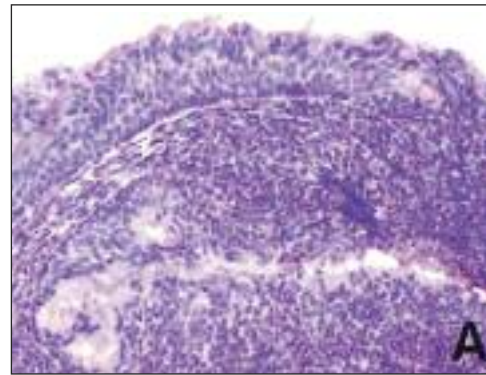
Demographic features and smoke exposure states of the patients were shown in Table 1. Mean age of the study and the control groups was 7.8 ± 2.5 (4-13) years and 6.9 ± 2.1 (4-12) years, respectively.

The distribution of iNOS and HSP70 expression in the surface epithelium of adenoid tissues of children according to PSE status was shown in

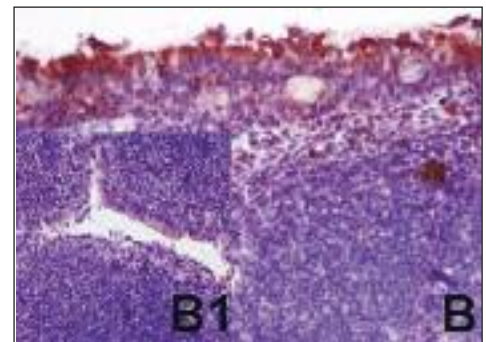
TABLE 3: Number of HSP70 and iNOS positive cells in the lymphoid follicles and perfollicular lymphoid tissue of the adenoid tissue.

Lymphoid follicles and perfollicular lymphoid tissue			
Median (Min-Max)			
	PSE positive	PSE negative	p value
iNOS	3 (2-3)	1 (0-2)	<0.001
HSP70	2 (2-3)	2 (0-3)	0.006

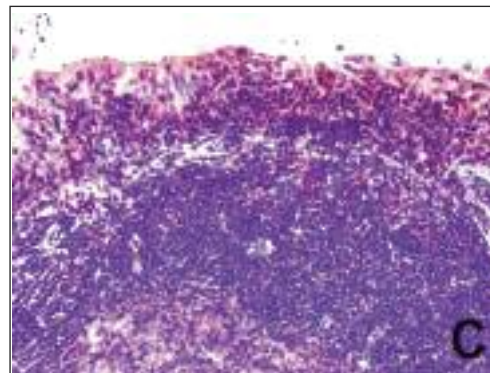
PSE: Passive smoke exposure. Cells per 25 fields of 0.2 mm². 0: none cells; 1: 1-10 cells; 2: 11-25 cells and 3: more than 25 positive cells.



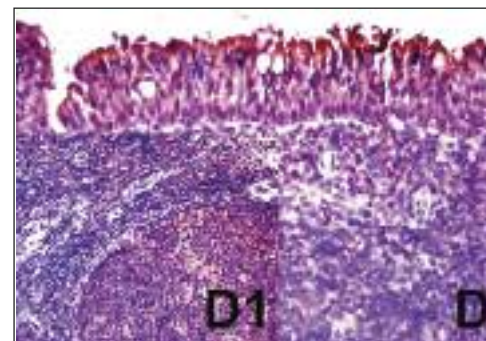
A; Control; (x100). B-B1; passive smoking, immunostained cells are increased in the surface epithelium in all adenoid tissues,



(B: x100-B1: x200).



C; Control; only a few positive cells are observed in the lymphoid follicles and perfollicular lymphoid tissue, (x100).



D-D1; Passive smoking; increased number of positive cells in the lymphoid follicles and perfollicular lymphoid tissue, (D: x100-D1: x200).

FIGURE 2: Photomicrographs of the HSP70 analysis in the adenoid tissue, surface epithelium, lymphoid follicles and perfollicular tissue in all adenoid structures (as detected by immunohistochemical staining).

Table 2. iNOS and HSP70 expression in the study group was significantly higher than in the control group ($p < 0.05$) (Figure 1A,1B; Figure 2A, 2B). iNOS and HSP70 expression in the lymphoid follicles and perifollicular lymphoid tissue of the adenoid tissue of children according to PSE status were shown in Table 3. Also, iNOS and HSP70 expression in the study group was significantly higher than in the control group ($p < 0.05$) (Figure 1C,1D; Figure 2C, 2D).

DISCUSSION

Chronic adenotonsillar hypertrophy that is characterized by chronic inflammation and hypoxia/reoxygenation episodes may lead to the formation of inflammatory cytokines, free nitrogen and oxygen radicals^{13,31} Also, oxidative stress and lipid peroxidation are the key participants in the pathogenesis of chronic adenotonsillar hypertrophy.⁹ Moreover, the levels of serum arginase and iNOS activities were reported significantly higher in children with adenotonsillar hypertrophy.¹³ Cigarette smoking manifests its harmful effects by the presence as well as generation of reactive oxygen and nitrogen species. These reactive metabolites react with most of the cell components leading to lipid peroxidation, protein oxidation and DNA damage.³² Exposure to cigarette smoke results in increased iNOS expression in the lungs and vascular tissues.^{23,24} Nicotine was also reported to potentiate lipopolysaccharide/interferon-gamma (LPS/IFN- γ)-induced cytotoxic effects by enhancing NO production via enhancing iNOS gene expression in RAW264.7, a monocyte-macrophage cell line.²⁵ We found that iNOS activity was significantly higher in the adenoid tissue of children exposed to cigarette smoke.

Induction of the HSPs by oxidants has long been used as a marker for cell and tissue injury. In vitro and in vivo experiments have shown that oxygen free radicals (hydroxyl, superoxide and nitric oxide) and cadmium are inducers of heat shock response.^{32,33} A correlation between smoking habits and expression of HSP70 was found in adenocarcinomas of the lung in smokers.²⁷ In addition, induction of HSP70 expression was observed in rat brain

upon chronic exposure to cigarette smoke. A similar induction of HSP70 by tobacco smoke has been reported in rodent and human cell lines.³² Aktepe et al reported expression of HSP70 in the hypertrophic adenoid tissues by an immunohistochemical study and they suggested that increased expression of HSP70 may play an important role in the pathogenesis of adenoid hypertrophy.¹⁴ We found that the HSP70 activity was significantly higher in the adenoid tissue of children exposed to cigarette smoke. It is clear that there is a relationship between HSP70 overexpression and apoptosis. HSP70 inhibits apoptosis and thereby increases the survival of cells exposed to a wide range of lethal stimuli.^{34,35} Nicotine, the principal addictive component of cigarette, plays a major role in the induction or inhibition of apoptosis.^{32,36,37}

Nitric oxide is reported to induce apoptotic death in many cell types, such as macrophages, pancreatic cells, and human tubular epithelial cells.³⁸ We determined increased iNOS activity in the adenoid tissue of children exposed to cigarette smoke. However, HSP70 protects cells from the apoptosis induced by various stresses and agents including NO, oxidative stress, tumor necrosis factor, anticancer drugs, ceramide and radiation. Moreover, HSP70 attenuates nitric oxide-induced apoptosis in mouse macrophages by preventing cytochrome C release.³⁹

Adenoid hypertrophy is a major health problem in preschool and school-aged children with significant social and physical morbidity by negatively influencing the nutrition, growth, development and social lives of children.⁴ As such, the economic burden of the medical and surgical treatment of this disease is tremendous. Despite its frequency and the significant morbidity it causes the pathogenesis of AH is still unclear.^{3,4}

Environmental tobacco smoke exposure is probably one of the most important public health hazards worldwide. In the present study, passive smoking was found to induce HSP70 and iNOS overexpression in the adenoid tissue. This overexpression may be a mechanism for smoking to cause adenoid hypertrophy. Our results supported the results of previous reports that suggested

the role of passive smoke in the development of adenoid hypertrophy.^{19,20} Thus, PSE should be questioned before medical or surgical interventions in children with adenoid hypertrophy, and in case of PSE, the family should be informed about the condition and the treatment should be planned accordingly.

A limitation of our study was that the presence of allergy and PSE in the children were based on the declaration of the family members instead of objective serum cotinin levels, and in vitro and in vivo allergic tests. Moreover, another limitation of the present study was that the socioeconomic levels were not identified definitely although all chil-

dren whose adenoid tissues were examined seemed to have similar regional and climatic features.

Although HSP70 and iNOS overexpression was shown in the hypertrophic adenoid tissue of children exposed to passive smoking, whether there is a relation with hypertrophy and HSP70 and iNOS overexpression is not clear yet. Further larger studies are needed to evaluate the relation between HSP70 and iNOS overexpression and adenoid hypertrophy.

CONCLUSION

Passive smoking was found to induce HSP70 and iNOS overexpression in the adenoid tissue.

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