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The Effects of Systemıc Use of Nıcotıne on the Rat Nasal Mucosa: a Hıstopathologıc and Immunohıstochemıcal Study

Efectos del Uso Sistémico de Nicotina sobre la Mucosa Nasal de la Rata: un Estudio Histopatológico e Inmunohistoquímico

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SUMMARY: The objective of this study was to evaluate the histopathologic effects of systemic use of nicotine on the rat nasal mucosa. Twelve adult Sprague-Dawley rats weighing 180-220 g, were used as experimental animals. The rats were divided into Nicotine and control groups. The rats of Nicotine groups (n=6) were administered 2mg/kg Nicotine sulphate for 28 days. The rats of control group (n=6) were only administered 1,5 ml physiologic saline solution subcutaneously for 28 days. All animals were sacrified at the end of the study and nasal tissue samples were removed and prepared for histologic examination. The sections were stained with Hematoxylin and Eosin (H-E) and Periodic acid-Schiff (PAS) and Trichrome-Masson were observed under light microscope. E-cadherin immunreactivity of pseudostrafied epithelial cells of nasal mucosa was assessed by immunohistochemical staining. There were significant differences in average histopathological score between the groups treated and non-treated to nicotine. In nicotine group, degenerative change of epithelial cells and hypertrophy of goblet cells were observed. Leukocytes infiltration was observed in significant areas of connective tissue. E-cadherin expression was significantly decreased in epithelial cells of the nasal mucosa of Nicotine group.

KEY WORDS: Nicotine; Rat; Nasal mucosa; E-cadherin; Immunohistochemistry.

INTRODUCTION

Nicotine (NIC), tertiary amine composed of a pyridine and a pyrolidine ring, is a major active toxic component of tobacco (Tutka *et al.*, 2005). It is obtained from the dried leaves of the tobacco plant, Nicotiana tobacum (Isaac & Rand, 1969). NIC is commonly self-administered by the inhalation of tobacco smoke and by insufflation and chewing of tobacco and leads to addiction. Pharmaceutical NIC available as chewing gum, transdermal patches, or microtablets are used in the replacement therapy for stopping smoking. Nicotine and its metabolites are also being investigated and researched for the treatment of a number of disorders, including Alzheimer's disease, attention deficit disorder and Parkinson's disease (Hecht, 2003; Baron, 1996) NIC has been shown to be detrimental to wound healing, vascularization, organ acceptance and general health (Saldanha *et al*., 2004). Nasal cavity has a very large mucosal surface and lined with the same columnar epithelium that lines the airways of the lung. This large mucosal surface of nasal cavity covered with a rich vascular bed. Nasal surface may be affected from both inhalants in the air and agents in the systemic circulation. Therefore, nasal mucosa may be effected by nicotine either direct topical or indirect systemic way.

Topical effects of nicotine to nasal mucosa by smoking have been examined in earlier studies. However, to the best of our knowledge, there is not any study on histopathologic effects of systemic nicotine to nasal mucosa. The purpose of the present study, therefore, was to investigate the effects of nicotine subcutaneously given in rat nasal mucosa histopathologically.

MATERIAL AND METHOD

The study protocol was approved by the Animal Research Committee (DUHADEK) of Dicle University, Turkey. Twelve adult Sprague-Dawley rats, each weighing 180-220 g (±10 g) were used as experimental animals. The animals were group-housed (6 per cage) under standard

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conditions (21±2º C) in the Animal Health and Research Center of Dicle University (DUSAM). The animals were fed ad libitum with water and standard laboratory animal diet, under the care of trained wardens. The rats were divided into 2 groups as follows:

The rats of experimental group (n= 6) were nicotinized systemically with nicotine sulphate, (Sigma, Aldrich), 2mg/kg subcutaneously, daily in a period of 28 days. The rats of group control (n= 6) were used as control and only administered 1.5 ml physiologic saline solution subcutaneously for 28 days. The rats of control group were maintained in same environment and food as experimental group.

Tissue Preparation for Light Microscopy. At the end of the study, animals were sacrified by decapitation. The skin, as well as all the soft tissues surrounding the nasal cavity, was removed. Then, the bony-framework of the nasal cavity including nasal septum were nibbled out by bone-nibbler. The samples were fixed with neutral buffered 10% formalin solution and decalcified with 5% EDTA (Ethylenedaiminetetraacetic acid). After preservation, nasal samples were directly dehydrated in a graded series of ethanol and embedded into paraffin wax. Five mm sections were cut with microtome (Rotatory Microtome, Leica, RM 2265, Germany) and mounted on the coated slides. The sections were stained with Haematoxylin and Eosin (H-E), and Periodic acid-Schiff (PAS) and Trichrome-Masson in order to be observed under light microscope (Eclipse 80i, Nikon, Japan). All morphological changes including inflammatory leukocyte infiltration and cellular hyperplasia, goblet cell hypertrophy and epithelial degeneration were noted. Semiquantitative scaling of inflammatory leukocyte infiltration, cellular hyperplasia, goblet cell hypertrophy and epithelial degeneration were carried out. The intensity of these changes were graded from 0 to 3 (0: no change, 1: low, 2: moderate, 3: intense).

Immunohistochemical staining. Antigen retrieval process was performed in citrate buffer solution (pH:6.0) two times first 7 minutes, later 5 minutes boiled in microwave oven at 700 W. They were allowed to cool to room temperature for 30 minutes and washed in distilled water for 5 minutes two times. Endogenous peroxidase activity was blocked in % 0.1 Hydrogen peroxide for 15 minutes. Ultra V block (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 10 minutes prior to the application of primary antibodies (E-cad antibody, mouse monoclonal, 1/200, Santa Cruz) for overnight. Secondary antibody (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 20 minutes. Slides then were exposed to streptavidin-peroxidase for 20 minutes. Diaminobenzidine (DAB, Invitrogen, Carlsbad) was used as a chromogen. Control slides were prepared as mentioned above but omitting the primary antibodies. After counterstaining with Hematoxylin, washing in tap water for 5 minutes and in distilled water for 2 x 5 minutes, the slides were mounted. Immunoreactivity of pseudostrafied epithelial cells of nasal mucosa was assessed. Semiquantitative scaling of immunoreactivity was carried out. The intensity of staining was graded from 0 to 3 (0: no staining, 1: faint staining, 2: moderate staining, 3: intense staining).

Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences for Windows (version 15.0, SPSS Inc.,Chicago, IL, USA). The Mann–Whitney U test was used for the statistics as indicated, test and results were expressed as mean ±SD. P values below 0.05 were considered to indicate statistical significance.

RESULTS

Histopathological. The histopathological results of the present study were evaluated under light microscope. There were no any histopathological changes in control group sections of nasal mucosa. Normal characteristic features of nasal mucosa were viewed in control group, whereas in experimental group, some changes were observed in nasal mucosa (Table I). One of the most conspicuous histopathological finding was cellular hyperplasia. It was highly increased in nicotine treated group, as compared to the non-treated group.

Table I. Comparison of Nicotine and control groups by means of E-cad (E-cadherin) expression and histopathological features.

	Control (n=6) (Mean±SD)	NIC (n=6) (Mean±SD)	P
E-cadherin	2.33±0.51	0.33±0.51	<0.01
Hyperplasia	0.16±0.40	2.66±0.51	<0.01
Goblet Hypertrophy	0.0±0.0	3.0±0.0	≤0.001
Leukocyte infiltration	0.33±0.51	2.83±0.40	<0.01
Epithelial Degeneration	0.0±0.0	2.83±0.40	≤0.001

In addition to this finding, the hypertrophy of goblet cells were also marked. Hypertrophy of goblet cells were increased in nicotine treated group sections. Degenerative changes in epithelium were observed in sections of nicotine-treated group. A number of inflammatory cells were also observed in nicotine-treated group (Fig. 1a-e).

Immunohistochemical. With respect to E-cadherin expression, there was a remarkable difference between nicotine treated and non-treated group (Fig. 2a-b) (Table I). While the intensity of E-cadherin expression in non-treated group was very high, it was at low level in nicotine treated group.

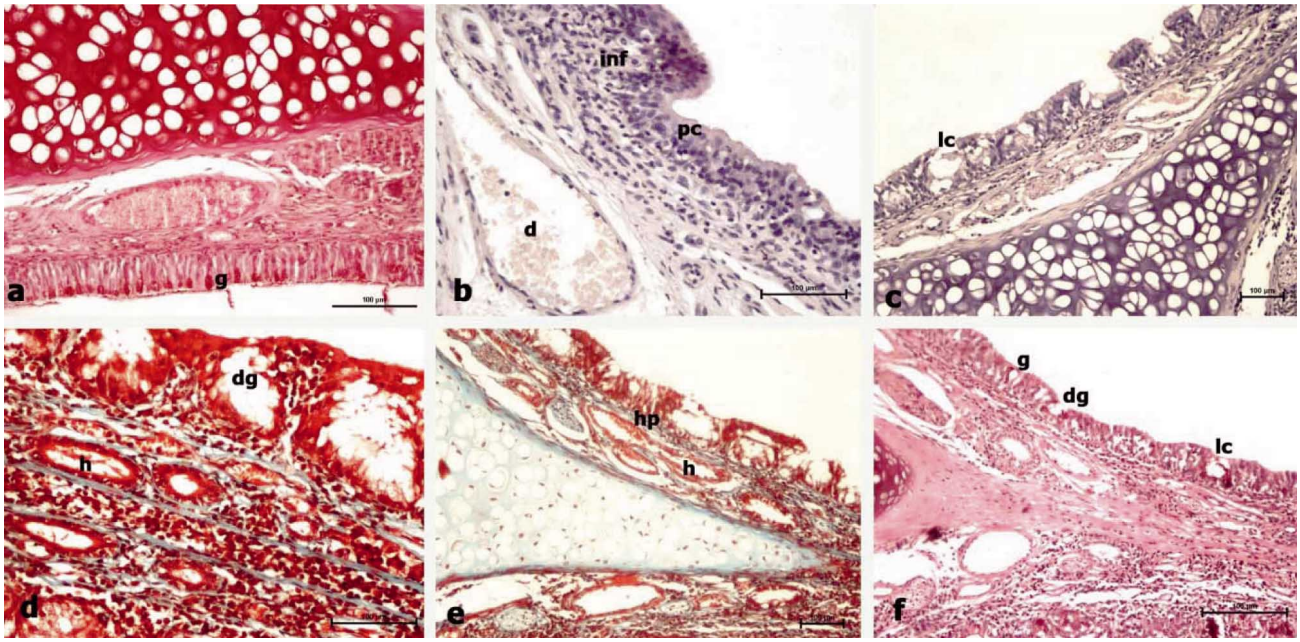

Fig. 1. Epithelial and goblet cells (g) and connective tissue of nasal mucosa appear normal in non-treated group sections (a). Accumulation of cells in the epithelial layer exvaginated towards outward with hypertrophic goblet cells. In addition, hyperplastic cells with picnotic nuclei (pc), dilatation of blood vessels (d) and leukocytes infiltration (inf) are seen from place to place (b). Loss of cilia (lc) and degeneration in some epithelial cells are viewed (c). A marked degeneration (dg) in the structure of respiratory epithelium is observed. In addition to hyperplastic cells, breakdown of the basement membrane is also seen. Leukocytes infiltration, hemorrhage (h) and dilation of blood vessels are observed (d). Epithelial degeneration and hyperplasie (hp) and dilation of blood vessels, hemorrhage (h) and leukocytes infiltration are evident (e). Degeneration(dg) of the cells which remained at the top of the epithelial layer as well as shortening and losing cilia (lc) are observed (f)

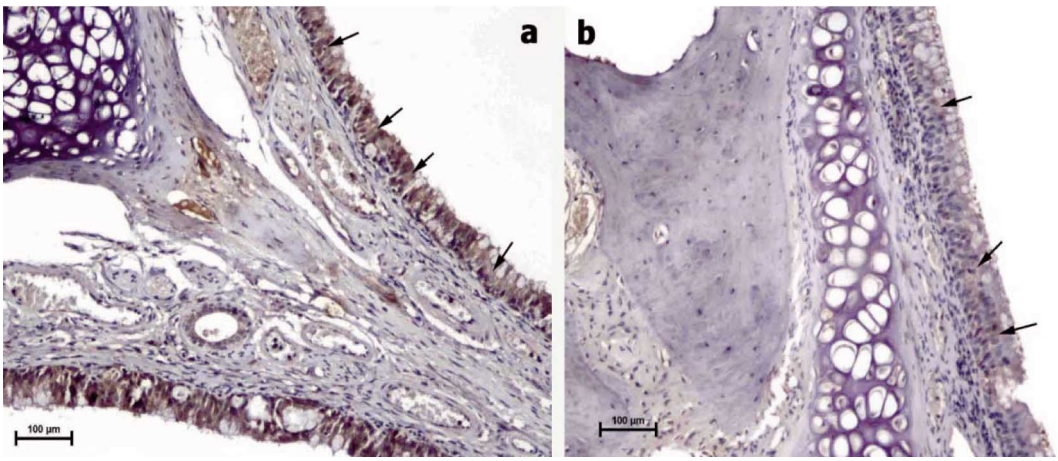

Fig. 2. Immunohistochemical expression of E-cadherin is detected in nasal mucosa. The epithelial cells of non-treated group nasal mucosa exhibit intense immunoreactivity (a), while low immunreactivity of the epithelial cells was observed in nicotine-treated group (b). Arrows: Expression of E-cadherin.

DISCUSSION

The detrimental effects of tobacco smoke exposure on the respiratory mucosa are well described. Cunningham *et al.* (2001) showed the erosion, inflammation and metaplastic transformation in the directly tobacco smoke exposed tissues of the respiratory tract. However cigarette contains a lot of toxigenic, mutagenic and even carcinogenic compounds (Mori *et al*., 1995). We studied only nicotine in this study in order to establish nasal mucosal effects of systemic NIC. Animal models, such as the rat are being used to understand cellular sequelae from nicotine (Shoaib & Stolerman, 1999). Therefore, rats were selected for this study.

Nicotine is a ganglionic stimulant agent, stimulating airway sensory innervation to produce secretion, bronchoconstriction, and cough (Greiff *et al*., 1993). NIC may have effects on the nasal mucosa in either topical or systemic ways. Nicotine that is applied to the nasal cavity can activate the olfactory sensory system (Thuerauf *et al.*, 1999). In a study on healthy subjects, Greiff *et al*. demonstrated that nasal challenge with nicotine produces intense nasal pain and dose-dependent secretion of mucin, but fails to produce any mucosal exudation of plasma. Systemic NIC reaches many organs and tissues and undergoes extensive metabolism. It is well known that systemic NIC exerts a number of cardiovascular and behavioural effects (Tutka *et al.*). The main pharmacological action of nicotine is the activation of the sympathetic nervous system. Nicotine in tobacco products causes peripheral vasoconstriction and tissue ischemia and decreases oxygen tension (Selcuk *et al*., 2012). Intravenous nicotine produces dose-related increases in heart-rate and blood pressure.

Therefore, blood supply is primarily affected by nicotine. In the study, we observed that nicotine leads to degenerative changes. Hyperplasia of epithelial cells, leukocyte infiltration and breakdown of basement membrane underlying epithelium were observed. We suggest that these effects may be due to altered blood supply and toxic effects of NIC. Cell-cell junctions are important to maintain cell and tissue polarity and integrity. E-cadherin (E-cad) is a cell-cell adhesion transmembrane molecule. It plays important roles in cell adhesion and morphogenesis (Guarino *et al*., 2007). In addition, in the wound re-epithelialization mechanims, the involvement of E-cadherin especially in controlling cellular polarity (Acloque *et al.*, 2009), differentiation, growth and migration is crucial (Thomas & Speight, 2001). In our study we observed that E-cadherin expression was significantly decreased in nicotine group, as compared to non-treated group. We concluded that NIC might affect the cellular junctions in the nasal mucosa.

In conclusion, the present study is the first investigation that examined the effect of systemic nicotine on rat nasal mucosa. We demonstrated the effects of systemic nicotine exposure on nasal mucosa histopathologically. It can be suggested that a systemic NIC may affect nasal mucosa. However, applying animal data directly to humans requires restrain. Therefore, additional extensive studies are needed to confirm the deleterious effects of subcutaneously administered. nicotine in nasal mucosa.

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RESUMEN: El objetivo de este estudio fue evaluar los efectos histopatológicos del uso sistémico de nicotina sobre la mucosa nasal de la rata. Se utilizaron como animales de experimentación 12 ratas Sprague-Dawley adultas, entre 180-220 g, divididas en grupos de nicotina y control. Al grupo de nicotina (n = 6) se le administró sulfato de nicotina 2mg/kg durante 28 días. Al grupo control (n = 6) se les administró sólo 1,5 ml de solución salina fisiológica por vía subcutánea durante 28 días. Todos los animales fueron sacrificados al final del estudio. Se tomaron muestras del tejido nasal y se examinaron histológicamente. Las secciones fueron teñidas con H-E, ácido periódico de Schiff (PAS) y tricrómico de Masson, observándose bajo microscopía de luz. Además, se evaluó la inmunoreactividad a E-cadherina de las células del epitelio pseudoestraficado de la mucosa nasal. Hubo diferencias significativas en la puntuación histopatológica media entre los grupos tratados y no tratados con nicotina. En el grupo de nicotina, se observaron cambios degenerativos de las células epiteliales e hipertrofia de las células caliciformes. Se observó una infiltración significativa de leucocitos en diferentes áreas del tejido conectivo. La E-cadherina se redujo significativamente en las células epiteliales de la mucosa nasal del grupo nicotina.

PALABRAS CLAVE: Nicotina; Rata; Mucosa nasal; E-cadherina; Inmunohistoquímica.

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