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ORIGINAL ARTICLE

Impact of Electronic Cigarette Vaping on the Cytoarchitecture of the Tongue in the Adult male Albino Rat

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ABSTRACT

Background: Electronic cigarettes (E-cigs) are marketed as an alternative and replacement domain to the traditional cigarette smoking in many societies for the rescue of smoking cessation. Nevertheless, the vape of E-cigs produce different harmful chemical compounds and carcinogens. The aim of this study is to clarify the histopathological changes that may take place in rat's tongue after exposure to E-cigs and to assess the possible amelioration of such changes after giving up vaping.

Methods: Thirty adult male albino rats were divided into three equal groups. Control group: exposed to fresh air for 4 weeks. Exposed group: exposed to smoke vapor of 1ml burnt E-liquid for 1 hour, 5 consecutive days/ week for 4 weeks. Withdrawal group: exposed to the same dose and duration of smoke vapor like group 2 then left without exposure for another 4 weeks. By the end of the experiment, animals were weighted and the tongue was excised and processed for hematoxylin & eosin and Alcian blue/periodic acid Schiff's staining and for proliferating cell nuclear antigen (PCNA) immunohistochemical study. Also, serum malondialdehyde and superoxide dismutase activity were determined.

Results: E-cigs exposure hampered the cytoarchitecture of the tongue with loss of some papillae and hyperkeratosis of the others along with increased PCNA immunoreactivity and its area percentage. Also, E-cigs exposure led to redox imbalance. Obviously, stoppage of E-cigs exposure resulted in partial amelioration of histological and biochemical changes.

Conclusion: E-cigs vaping produced proliferative changes that impose a metaplasia in the tongue and their withdrawal revealed some degree of improvement.

Keywords: Electronic cigarettes, Vaping, Rat's Tongue, Withdrawal, Electronic cigarette liquid.

INTRODUCTION

Electronic cigarettes (E-cigs) are devices designed to deliver nicotine and other constituents by burning an electronic cigarette liquid (E-liquid) via vaping rather than smoke and without combustion of tobacco. They are considered as a safer alternative to conventional cigarettes and now they are marketed as lifestyle-choice consumables [1].

E-cigs contain a tank filled with E-liquid that contained different types of chemical compounds [2]. These chemical compounds involve nicotine, propylene glycol (PG), and vegetable glycerin (VG), together with distilled water and some flavors [3,4]. E-cigs vapor contains the above-motioned chemical compounds and their burred metabolites like formaldehyde, nitrosamines, metals, carbonyls, volatile organic compounds, and polycyclic aromatic hydrocarbons that are potentially toxic or carcinogenic [5].

Pathologically, smoking is highly linked to some oral diseases such as periodontal diseases and leukoplakia which is the most common potentially precancerous oral lesion [6,7]. Particularly, the nicotine constituent of E-cigs is highly related to the precancerous and cancerous oral lesions [8].

To the best of our knowledge from the previous available literature that almost no studies have

been conducted to assess the histological and immunohistochemical effects of E-Cigs vaping on the tongue in experimental animals or in humans. This was an impetus to designate this study in which the effect of exposure to E-cigs vaping and the effects of their giving up on the cytoarchitecture of the tongue in adult male albino rats.

METHODS

Electronic cigarette liquid (E-Liquid):

(Dollar blends company, Eg): Each 1ml of E-Liquid contains vegetable glycerin, propylene glycol (PG), natural flavorings, artificial flavorings, and nicotine (18 mg/ml).

Portable electric Incense Burner: (Home electric comp., China).

Experimental design

This study was carried out on 30 adult male albino rats weighting 200-250 gm. The rats were obtained from the Animal House of Faculty of Medicine, Zagazig University. All animals were supplemented standard food and tap water ad libitum. They were housed in ventilated wide polypropylene cages (38 × 26× 19 cm). Temperature was maintained at 23±2°C. For two weeks before being experimented, the rats were accommodated to the laboratory conditions. All rats were handled according to the standard guide for the care and use of laboratory animals. This study was approved by The Institutional Animal Care and Use Committee Zagazig University (ZU-IACUC) with approval number (ZU-IACUC/3/F/10/2020).

Experimental animals

The rats were divided equally into 3 groups, 10 rats each.

Group I: **(control group);** was exposed to fresh air for 4weeks.

Group II: (exposed group);

was exposed to smoke vapor of burnt 1ml/day of E-liquid for 1 hour for 5 consecutive days/week for 4 weeks [1].

Group III: (Withdrawal group);

as exposed to smoke vapor of burnt 1ml/day of E-liquid for 1 hour for 5 consecutive days/ week for 4 weeks and then the rats were left without exposure in a fresh air for another 4 weeks.

Protocol of exposure to E-liquid smoke

Exposure of the rats to the E-liquid smoke vapor was conducted for 1 hour for 5 consecutive days/ week for 4 weeks using Portable electric Incense.

Burner that put inside an inhalation chamber (38 × 26 × 19 cm) that contained a hole for entry of E-liquid smoke vapor and another hole for fresh airAfter exposure, the animals were transferred to a chamber of fresh air.

Necropsy, blood and tissue sampling:

By the end of the experiment, 24 hours after the last E-cigs exposure, the animals were weighed and anesthetized by intraperitoneal injection of thiopental (75mg/kg).Then, venous blood samples were obtained from the retro-bulbar plexus and left to clot at room temperature and were centrifuged to separate the serum. The serum samples were maintained at -20°C for further estimation of malondialdehyde(MDA) and superoxide dismutase (SOD). Thereafter, the tongue was excised out of the oral cavity and was processed for histopathological, immunohistochemical and morphometrical studies.

Histological study:

Tongue specimens were fixed overnight in 10% neutral buffered formalin, dehydrated in ascending grades of ethanol, cleared with xylene and embedded in paraffin wax.

Paraffin sections of 5 µm thick were cut and stained with Hematoxylin and Eosin (H&E) (to study the general histological architecture of the tongue) and stained also with Alcian blue/periodic acid Schiff's stains (AB⁄PAS) (to assess the keratin content).

Finally, the stained sections were examined under a light microscope (Leica Microsystems, Schweiz, AG, Heerbrugg, CH-9435, Switzerland) and photographed using a digital camera coupled to that microscope in the Department of Anatomy, Faculty of Medicine, Zagazig University, Egypt.

Immunohistochemical study

Sections of 4 µm thick from the paraffin blocks of tongue specimens were mounted on

positively charged slides, deparaffinized, rehydrated and incubated with hydrogen peroxide to block the activity of endogenous peroxidase. Blocking of non-specific antigens and antigen retrieval were conducted. Afterwards, the sections were incubated with rabbit monoclonal anti-PCNA primary antibodies (1:400, ab92552, Abcam, USA). The slides with primary anti-PCNA were incubated with biotinylated goat anti-polyvalent secondary antibody. Afterwards, a diaminobenzidine (DAB, Sigma-Aldrich Chemical Co., St. Louis, USA) chromogen solution was added to the slides. Finally, the sections were counterstained with Mayer's Hematoxylin. Immunostained sections were imaged using Leica DM 500, Microsystems, AG, Heerbrugg, CH-9435, Switzerland in the Anatomy Department, Faculty of Medicine, Zagazig University, Egypt.

Histmorphometric studies

The area percentage of PCNA in immunostained sections was measured using the public domain image-processing software "Image J 1.49v/Java 1.6.0_244"(National Institutes of Health, USA).Calibration of the image analyzer was done for measurements before use by automatically converting the image pixels into actual micrometer units and the presentation of data was expressed as mean± SD (Standard deviation).

Biochemical assays:

The serum levels of MDA and SOD were assessed using enzyme-linked immuno-sorbent assay (ELISA) kits. MDA kits (Cat No: MBS268427) and SOD kits (Catalog No: CSB-E08555r)

Statistical analysis

The collected data were computerized and statistically analyzed using the SPSS program (Statistical Package for Social Science, version 19.0. Inc,Chicago, IL, USA). Quantitative data were expressed as mean± SD. Analysis of variance (ANOVA) was used to calculate the difference between quantitative variables in more than two groups in normally distributed data followed by least significant difference (LSD) multiple comparison post hoc test to find the significance difference between two studied groups. The results were considered statistically significant when P value was less than 0.05.

RESULTS

Body weight results

There was no statistical significant difference (p=0.53) among the studied groups in the initial body weight, but there was a highly statistical significant difference (p <0.001) among them regarding their final body weight. Using LSD post hoc it was found that in Group II (exposed) there was a highly statistical decrease in the final body weight (p <0.001) compared with Group I (control). Group III (withdrawal) showed statistical increase (p=0.02) compared to group II but a significant decrease (p=0.03) compared to
Group I (control) (Table 1).

H&E stain results

H&E stained sections of the control group showed the dorsal surface of the tongue was covered by a mucous membrane. The anterior part of the dorsal surface of the tongue was covered by numerous mucosal elevations "lingual papillae". The filiform papillae were distributed over the entire anterior dorsal surface of the tongue. These papillae were conical in shape with their tips pointing backward. They were composed of a core of connective tissue covered by keratinized stratified squamous epithelium that did not contain taste buds. The epithelial rete pegs appeared regular (Fig. 1A**).** The core of the tongue was formed of skeletal muscle fibers that constituted the main bulk of the tongue; they were composed of interlacing bundles that were running in longitudinal, transverse, and vertical directions (Fig. 2A**).**

Sections of the dorsal surface of the tongue in the E-cigs exposed group showed an apparent shortening of the lingual papillae. The filiform papillae appeared very short, broad, and had blunt apex with loss of their common regular orientation and their

characteristic conical shape. These papillae were covered by thick keratin. The basal cell layer showed some disrupted areas with proliferation at the medial margins of two adjacent rete pegs allowing their fusion with sequestration of the connective tissue core of the papillae. Additionally, focal separation of the keratin layer from underlying epithelial cells was detected (Fig. 1B**).** The muscle fibers appeared atrophied and lost their regular organization. Highly increased spaces between muscle fibers were also demonstrated. Increased spaces in connective tissue with mild mononuclear cellular infiltration and dilated congested blood vessels were encountered. Moreover, there was a loss of normal appearance of these filiform papillae with alternating with areas of hyperkeratosis (Fig. 2B**).**

Sections of the dorsal surface of the tongue of the withdrawal group showed some degree of improvement. There were some normal filiform papillae alternating with persistent areas of hyperkeratosis. The basal cell layer showed some areas of disruption with proliferation at the medial margins of two adjacent rete pegs allowing their fusion with sequestration of the connective tissue core of the papillae (Fig. 1C).Some muscle fibers still appeared atrophied. Some spaces among muscle fibers were also detected. Some congested blood vessels still appeared (Fig. 2C).

AB/PAS Stains Results

AB-PAS stained sections of the control group revealed a normal appearance of the tongue papillae with stratified squamous epithelial layers that appeared blue and the keratin layer appeared purple (Fig. 3A). Sections of the exposed group revealed loss of normal appearance of tongue papillae with hyperkeratosis. There was a marked proliferation of stratified squamous epithelial layers that appeared deep blue with increased thickness of keratin layer that appeared deep purple (Fig. 3B). Sections of the withdrawal group revealed normal appearance of some tongue papillae alternating with persistent areas of hyperkeratosis. There was a moderate proliferation of stratified squamous epithelial layers that appeared deep blue and increased thickness of keratin layer that appeared deep purple (Fig. 3C).

Immunohistochemical Results

The control group specimens of the tongue showed normal expression of PCNA. Immunostaining of the nuclei of the epithelial cells was confined to the basal layer, while the upper epithelial layer displayed negative nuclear immunoreactivity. Some connective tissue cells showed positive immunoreactivity (Fig. 4A). On the other hand, the dorsal surface of the anterior part of the tongue in the exposed group revealed more expression of PCNA. There was positive nuclear immunoreactivity in the epithelial cells of the upper and basal layers. The connective tissue cells showed positive immunoreactivity to PCNA (Fig. 4B). In the withdrawal group, the dorsal surface of the tongue revealed moderate expression of PCNA. Some normal lingual papillae were alternating with persistent areas of hyperkeratosis. There was positive nuclear immunoreactivity in the epithelial cells of the basal and upper layer. Some connective tissue cells showed positive immunoreactivity to PCNA (Fig. 4C).

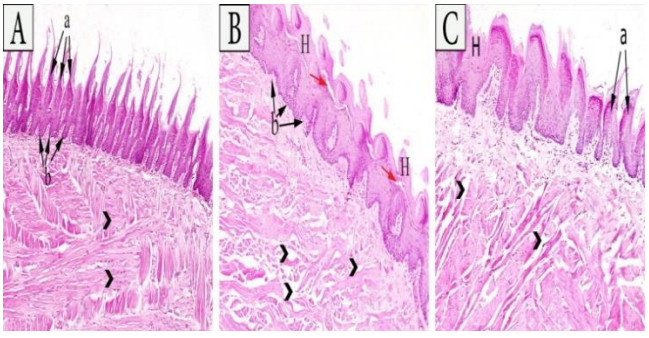

Fig. (1): Photomicrographs of adult male albino rat tongue sections of the different

groups. **(A)** Control group: showing normal regular filiform papillae covered by a thin keratin layer (a). Regular epithelial rete pegs (b), and organized muscle fibers run in different directions (black arrowheads). **(B)** Exposed group: showing hyperkeratosis (H), some focal separation of the keratin layer from underlying epithelial cells (red arrows), proliferation of the basal cell layer toward the connective tissue core of the papillae & loss of regular rete pegs (b), and highly increased spaces between muscle fibers (black arrowheads). **(C)** Withdrawal group: showing some normal filiform papilla (a), with persistent areas of hyperkeratosis (H), and some spaces among muscle fibers (black arrowheads). [H&E x 100]

Histomorphometric Results

There was a highly statistical significant difference ($p<0.001$) among the studied group. It was found that the mean area percentage (%) of PCNA in Group II (exposed) was statistically higher than ($p<0.001$) Group I (control), and Group III (withdrawal) was statistically lower than ($p<0.001$) group II and higher than ($p=0.003$) Group I (control) (Fig.4D).

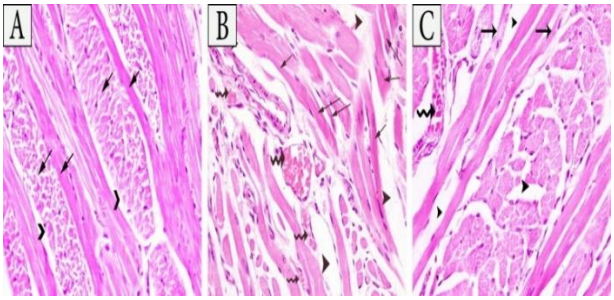

Fig. (2): Photomicrographs of adult male albino rat tongue sections of the different groups. **(A)** Control group showing organized muscle fibers run in different directions (black arrows), and normal spaces between the muscle fibers (black arrowheads). **(B)** Exposed group showing some atrophied muscle (black arrows), with highly increased spaces between the muscle fibers (black arrowheads), and dilated congested blood vessels (zigzag arrows). **(C)** Withdrawal group showing some atrophied muscle fibers (black arrows), with some spaces among the muscle fibers (arrow heads), and dilated congested blood vessels (zigzag arrow). [H&E x 400]

Biochemical Results

In Group II (exposed group) there was a highly statistical increase in MDA ($p<0.001$) compared with Group I (control). Group III (withdrawal) showed a statistical decrease compared to group II ($p<0.001$) and a statistical increase compared to group I ($p<0.001$) (Table 2). Also, in Group II (exposed group) there was a highly statistical decrease in SOD ($p<0.001$) compared with the control group. Group III (withdrawal) showed a statistical increase compared to group II ($p<0.001$) and a statistical decrease compared to group I ($p=0.002$) (Table 2).

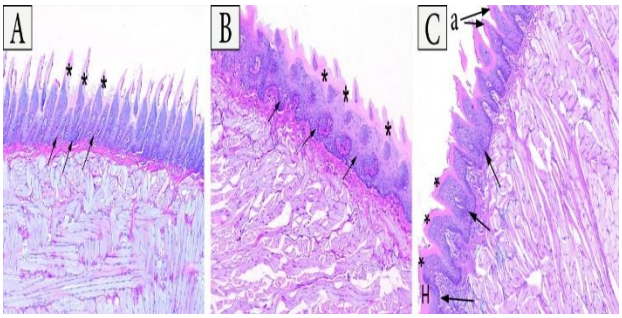

Fig. (3): Photomicrographs of adult male albino rat tongue sections of the different groups**.** (A) Control group: showing normal regular filiform papillae with its stratified squamous epithelium that stained blue (black arrows), covered by thin keratin layer that stained purple (*). **(B)** Exposed group: showing hyperkeratosis with a proliferation of stratified squamous epithelium that stained deep blue (black arrows) covered with thick keratin layer that stained deep purple (*). **(C)** Withdrawal group: showing some normal filiform papillae (a), with persistent areas of hyperkeratosis (H), and proliferation of stratified squamous epithelium that stained deep blue (black arrows) covered with moderately thick keratin layer that stained deep purple (*). [AB/PAS x 100]

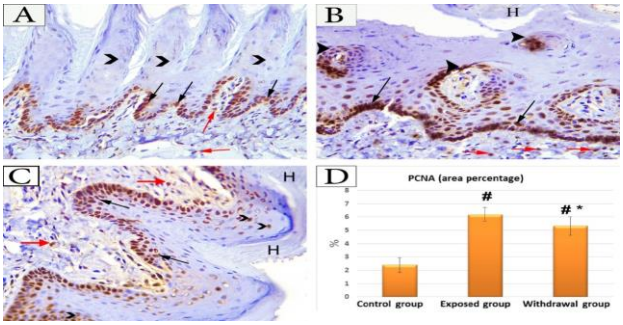

Fig. (4): Photomicrographs of adult male albino rat tongue sections of the different groups. **(A)** Control group: less expression of PCNA. Immunostaining of epithelial cells nuclei (black arrows), upper epithelial layer (black arrowheads), and connective tissue cells (red arrows). **(B)** Exposed group: more expression of PCNA. Hyperkeratosis with a positive nuclear immunoreactivity (black arrows), upper layer (black arrowheads), and the connective tissue cells (red arrows). **(C)** Withdrawal group: moderate expression of PCNA. Hyper-keratosis (black arrows) and upper layer (black arrowheads), and some connective tissue cells showed positive immunoreactivity (red arrows). **(D)** mean area of PCNA among the studied groups using one way ANOVA, followed by LSD post hoc test.

Table 1: Comparison of the mean initial and the final body weight among the different study groups (Mean± SD)

	Group I (Control)	Group II (Exposed)	Group III (Withdrawal)	F	P-value	LSD
Initial BW (gm)	241 ± 5.46	239.8 ± 4.05	238.1 ± 7.13	0.66	0.53 NS	-----
Final BW (gm)	277.2 ± 3.12	259.9 ± 2.6	269.1 ± 2.51	19.8	<0.001*	<0.001a 0.03b 0.02c

Data are expressed as mean±SD of 10 rats. Statistical analysis was carried out by One-way ANOVA (F-test) followed by LSD test. a: significant P value in Group I versus Group II . b: significant P value in Group I versus Group III. c: significant P value in Group II versus Group III. *: Significant (P<0.05). NS: Non significant (P>0.05)

Table 2: Comparison of the mean MDA and the SOD among the different study groups (Mean± SD)

	Group I (Control)	Group II (Exposed)	Group III (Withdrawal)	F	P-value	LSD
MDA (nmol/ml)	21 ± 3.53	76.6 ± 3.24	40.30 ± 4.57	545.4	<0.001	$<0.001^a$ $<0.001^b$ $<0.001^c$
SOD (U/ML)	197.7 ± 6.85	27.7 ± 8.86	127.2 ± 18.16	480.9	<0.001	$<0.001^a$ 0.002^b $<0.001^c$

Data are expressed as mean±SD of 10 rats. Statistical analysis was carried out by One-way ANOVA (F-test) followed by LSD (Least significant difference) test. a: significant P value in Group I versus Group II. b: significant P value in Group I versus Group III. c: significant P value in Group II versus Group III. *: Significant (P<0.05). NS: Non significant (P>0.05)

DISCUSSION

In the present work, regarding the final body weight, the E-cigs vaping exposed group exhibited a statistically lower weight gain when compared with the control group. However, there was an increase in weight gain of the

withdrawal group more than the E-cigs vaping exposed group. But, the final body weight was still lower than the control group. This result was in accordance with Shi et al. [9] who exposed the mice to E-cigs vaping (3 h/day for 14 days) and have found these mice displayed a lower weight gain compared to the age-matched mice exposed to the natural room air. Also, this result was in agreement with Phillips et al.[10] and Phillips et al. [11] who reported a significant decrease in the body weight gain in male rats upon exposure to E-cigs aerosols containing nicotine for 28-day and 90 days, respectively. Jo et al. [12] stated that the possible cause of such decline in the bodyweight upon exposure to E-cigs vaping is the nicotine content of the E-liquid that was reported to be an appetite suppressing component of smoke that causes a reduction in food consumption.

Actually, some neuropeptides in the hypothalamus are involved in regulating food intake and are modulated by circulating hormones such as leptin. It was reported that nicotinic receptors have been found in appetite-regulating regions of the hypothalamus, and their distribution overlaps with that of leptin receptors where the nicotine augmented the anorectic effects of leptin on the brain [12]. Another contributing factors have been proposed for the significant decrease of the body weight among E-cigs vaping exposed rats in this study as adopted by VanGaal et al. [13] might be related to the release of inflammatory markers and cytokines, enhancement of oxidative stress, direct effects on adipose tissue through lipolysis and a decrease in adiponectin in particular.

In the present study, examination of the tongue of the control rats stained with H&E revealed a normal histological architecture. The anterior part of the dorsal surface of the tongue showed numerous filiform papillae that were conical in shape with their tips pointing backward. They were composed of a core of connective tissue and a covering of keratinized stratified squamous epithelium. The underlying skeletal muscle fibers run in different directions. These normal histological features were similar to those described by Silva et al. [14] and by Ibrahim& Elwan [15].

On the other hand, exposure of the rats to E-cigs vapor 1 hour for 5consecutive days/week for 4 weeks) induced some histopathological changes in the tongue of the rats at H&E stained sections, particularly shortening of the filiform papillae with blunt apex alternating with areas of hyperkeratosis. Also, upon PCNA immunohistochemical staining of the tongue sections of E-cigs vaping exposed group, there was positive nuclear immunoreactivity in the epithelial and connective tissue cells together with increased area percentage of PCNA immunoreactions of the tongue compared to the control group. A result that denoting a pathological proliferation of the epithelial cells of the tongue upon E-cigs exposure was in line with Silva et al. [14].

These findings were in agreement with Ouies [16] who documented that nicotine sulfate (2 mg/kg subcutaneously daily for 4 weeks) caused cellular proliferative changes in the mucosa of the tongue of the rats with leukoplakia. Also, these proliferative changes were in harmony with Salturk et al. [17] who concluded that exposure to E-cigs for 4 weeks induced hyperplasia and metaplasia of the laryngeal mucosa in rats.

Additionally, our findings were consistent with Werley et al. [18] who observed mild mucus cell hyperplasia in the anterior nasal sections in a 90-day E-cigs vaping exposed rats.

Alharbi & Rouabhia [19] and Geng et al. [20] have reported that the process of proliferation enhancement is one of pathological hyperplasia and the subsequent involvement of keratin cytoskeleton protein that facilitates hyperkeratosis.

Similar to our findings, Bardellini et al. [21] whose study was undertaken on former human cigarettes smokers (at least 100 cigarettes in their lifetime) and current E-cigs users (smoking E-cigs for at least 6 months) noticed a hairy tongue among E-cigs consumers more than in former smokers and no differences in precancerous oral mucosal lesion leukoplakia

(hyperkeratosis, dysplasia) were found between the two groups. Similarly, Lee et al. [22] reported that E-cigs exposure promotes DNA damage and impairs DNA repair in the human lung and bladder cells, suggesting susceptibility of these cells to oncogenic transformation and carcinogenesis following E-cigs exposure.

Hyperkeratosis and hyperplasia observed in this study were in agreement with Ayuningtyas et al. [23] where the rats were exposed to cigarette smoke for 4 & 8 weeks explained that the nicotine in cigarette smoke contains tobacco-specific N-nitrosamines (TSNA) derivatives induce epidermal growth factor receptor (EGFR) which in turn causes an increase in epithelial cell proliferation.

Also, hyperkeratosis of the tongue following E-cigs may be caused by the release of reactive oxygen species (ROS) as was encountered in this study, where serum MDA" a lipid peroxidation product" was increased and SOD "antioxidant enzyme" was decreased. This redox imbalance was reported to trigger epithelial cell proliferation and hyperplasia through various signaling pathways. In a nearly similar pathological manner to the hyperkeratosis and hyperplasia obtained in our results, Fuller et al. [24] stated that E-cigs users are more vulnerable to develop bladder carcinoma due to the high content of the carcinogenic aromatic amines of E-cigs.

In the present work, hyperkeratosis of tongue epithelium was confirmed upon staining of the tongue sections with AB-PAS that revealed focal loss of the normal appearance of the tongue papillae alternating with areas of hyperplasia and hyperkeratosis. Histologically, Rao et al. [25] demonstrated that AB-PAS is a special stain for keratin. Our results were close to Ye et al. [26] who showed that long-term cigarette smoking for 3 and 6 months caused hyperplasia in the rat mucosa. AB-PAS stained sections of the withdrawal group revealed some normal appearance of tongue papillae alternating with persistent areas of hyperplasia and hyperkeratosis.

In this study, the congested blood vessels and inflammatory cell infiltration in the tongue tissue obtained in our results in the E-cigs exposed group were consistent with Eratilla et al. [27] who observed inflammatory leukocyte infiltration, blood vessel dilation, and hemorrhage in the tongue of rats after administration of nicotine sulfate 2 mg/kg subcutaneously daily for 28 days. Bardellini et al. [21] have documented nicotine stomatitis more in former human cigarettes smokers (at least 100 cigarettes in their life time) than the current E-cigs users (smoking E-cigs for at least 6 months).Also, Leigh et al. [28] explained that nicotine stomatitis in E-cigs consumers may depend not only on the exposure of the palatal mucosa to nicotine but also due to some cytotoxic flavored products chemical compounds in the E-liquid.

In the current work, upon giving up E-cigs vaping in the withdrawal group, H&E stained-sections showed some degree of improvement compared to the exposed group, particularly hyperplasia, hyperkeratosis, together with partial restoration of PCNA immunoreactivity, area percentage of PCNA and redox balance following the withdrawal regimen.

CONCLUSION

E-cigs produces histopathological changes in the structure of the tongue with an imminent proliferation that may be precancerous of adult male albino rats. Stoppage of exposure (withdrawal) revealed some degree of improvement.

Conflict of Interest

The authors of this manuscript declare no relevant conflicts of interest, and no relationships with any companies, whose products or services may be related to the subject matter of the article.

Financial Disclosures

None.

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