

## Effect of Nicotine on the Rat's Tongue and the Possible Protective Role of Royal Jelly Light and Scanning Electron Microscopic Study

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### ABSTRACT

**Background:** nicotine (NIC) is an active substance in tobacco, the excessive use of tobacco products has been associated with various lesions in the oral cavity. Royal jelly (RJ) believed by many authors to be an anti-oxidant that protects different organs. **Aim of the work:** this study aimed to detect the effect of nicotine on the structure of the tongue and the possible protective effect of royal jelly. **Materials and methods:** 30 adult male rats were equally categorized into 3 groups. Group I: 10 rats used as the control group; Group II: 10 rats received nicotine sulphate 2 mg/kg subcutaneously daily for 4 weeks; Group III: 10 rats received nicotine as the previous group and royal jelly (100 mg/kg by intra-gastric tube daily) for 4 weeks. After 24 hrs of the last dose, the animals were dissected. Tongues were processed for the histological and scanning electron microscopic study. **Results:** group II revealed that the lingual dorsal surface was formed of irregularly and destructed papillae. Most of these papillae were thin with blunted or absent tips. Fungiform papillae showed destruction of their epithelium and ill-defined pores. Lamina propria showed depletion of the collagen fibers, skeletal muscle fibers revealed separations and destruction. Group III revealed restoration of the normal appearance at some regions and presence of nicotine effects at the others within light microscope and apparent restoration of the normal shape as seen within the electron microscope. **Conclusion** nicotine has harmful effects on the tongue and the addition of RJ can decrease these harmful effects, but not prevent it.

**Key Words:** Nicotine, Royal Jelly, Tongue.

### INTRODUCTION

The nicotine, the main component of tobacco, stimulates the central nervous system, addicting firmly and fast. The nicotine changes the metabolism of proteins and nucleic acids and disorders functioning of defense-and repair system. The tobacco smoke provides also physical damages because of increases temperature inside the mouth and burns tissues<sup>(1)</sup>. Tobacco use affects the surface epithelium, resulting in changes in the appearance of the tissues. The changes may range from an increase in pigmentation to thickening of the epithelium (White lesion)<sup>(2)</sup>. Nicotine has been recognized as capable of inducing changes in taste functionality in conditions of chronic exposure. The mechanisms underlying these sensory alterations, however, are currently unknown<sup>(3)</sup>. Royal jelly (RJ) as the most important beehive product is secreted from the salivary glands of worker honeybees and serves as a primary super food for the queen and larvae during their first three days of life. The RJ includes several important compounds such as sugars, free amino acids, fatty acids, minerals (e.g., calcium) and vitamins with biological activities<sup>(4)</sup>. Royal jelly was believed to have beneficial protective effects in the experimental animals with anti-oxidant<sup>(5)</sup>, anti-inflammatory and anti-cancerous properties<sup>(6)</sup>. The RJ regulated the balance between the pro-oxidative and anti-oxidative effectors and has antioxidant property; therefore, administration of RJ

created interference with excessive oxidative cascade and production of free radicals<sup>(7,8)</sup>.

**Aim of the work:** this study aimed to investigate the histological changes in the tongue following nicotine toxicity in experimental rats and to assess the possible protective effects of Royal jelly using light microscopy (LM) and scanning electron microscopy (SEM).

### MATERIALS AND METHODS

#### Drugs:

**Nicotine:** it was used as a powder and obtained from El-Nasr Company for Pharmaceutical Industries, Egypt.

**Royal jelly:** RJ 1000 mg tablet soft gelatin capsules produced by Pharco- Pharmaceuticals, Alexandria, Egypt.

**Animals:** thirty adult male Albino rats, aging 4 - 6 months and weighing 200 - 250 g, obtained from Assiut Faculty of Medicine. They were housed in Animal Facility at Faculty of Medicine, Sohag University, Egypt. All rats were given access for rodent chow diet and water.

#### Ethical approval:

**The experiment was performed according to the "Guide for the Care and Use of Laboratory Animals" (Institutes of laboratory Animal Research)<sup>(9)</sup> and in accordance with the guidelines**



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of the Sohag University Animal Ethics and approved by Research Ethics Committee considering care and use of laboratory animals.

**Experimental design** (The experiment was done through February 2020)

After a 7-day acclimatization period animals were equally categorized into three groups as follows:

**Group I (The control group):** it was composed of 10 adult male Albino rats. They were not subjected to any treatment.

**Group II (The nicotine group):** it included 10 adult male rats subjected to nicotine sulphate 2 mg/kg subcutaneously dissolved in distilled water daily<sup>(10)</sup> for 28 days.

**Group III (Nicotine+Royaljellygroup):** included 10 adult male rats subjected to the same dose of nicotine +Royal jelly(The capsules were dissolved in corn oil for oral administration). Rats were administered 100 mg/kg royal jelly<sup>(11)</sup> for 28 days.

## Methods

After 24 hours from the last dose, rats were anesthetized using ether inhalation, sacrificed, carefully dissected and the anterior 2/3 of the tongues were removed.

**Preparation of the tongues for light microscopic examination:** perfusion fixation was used and the specimens were fixed in 10% neutral buffered formalin and processed for light microscopic study. Paraffin sections of 5µm thickness were obtained for hematoxylin and eosin and Masson's trichrome stain<sup>(12)</sup>.

## Scanning Electron Microscopic Preparation:

Tongue specimens were fixed in 5% gluteraldehyde. Following fixation, the specimens were washed several times with cold cacodylate buffer and post-fixed in 1% osmium tetroxide. They were dehydrated in a graded ethanol series, exposed to liquid CO<sub>2</sub> in a drying apparatus and coated with a thin layer of gold (10-15 µm) deposited over the surface in vacuum evaporator<sup>(13)</sup>. Then examined by a Jeol-JSM-5400 LV scanning electron microscope in Assiut University Center.

## Morphometric study and statistical analysis

Collagen quantification; semi-automated image analysis was applied from each Masson's trichrome stained section, 5 random fields for each group were selected and imaged using an objective lens magnification of 10x. Image J software (version 1.51k, Wayne Rasband, National Institutes of Health, USA) was used for the analysis. Variables were represented by mean ± Sd (Mean ± standard deviation of mean). The SPSS program version 16 was used to analyze the differences among all groups in all the data parameters by one-way analysis of variance and

a post-hoc test was used to find the statistical difference between the groups when ANOVA was statistically significant (P value ≤0.05)<sup>(14)</sup>.

## RESULTS

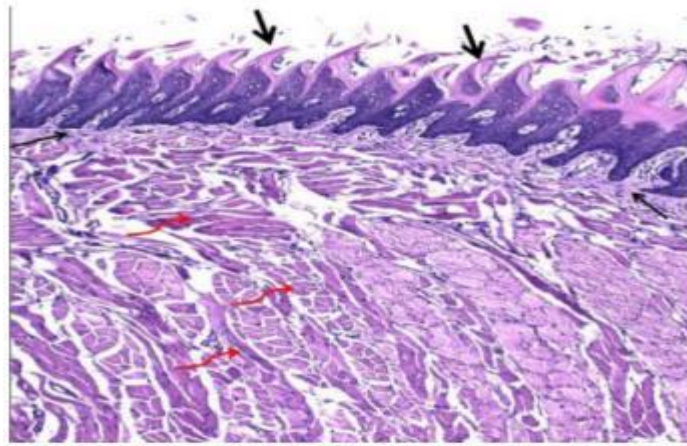
### A-Light microscopic study

**Group I:** hematoxylin and eosin sections revealed normal control tongue tissue which showed numerous, regularly-distributed tongue papillae covered with keratinized stratified squamous epithelium with regular orientation of the papillae. The filiform papillae (The most numerous) appeared conical in shape with tapering tips; the fungiform papillae were few, short, with broad apices and distributed in-between the filiform papillae, their taste buds appeared on the upper surface. The underlying lamina propria formed of connective tissue and appeared to merge with subjacent tongue muscles, muscles appeared formed of numerous bundles directed in different directions **Figs. 1-3**.

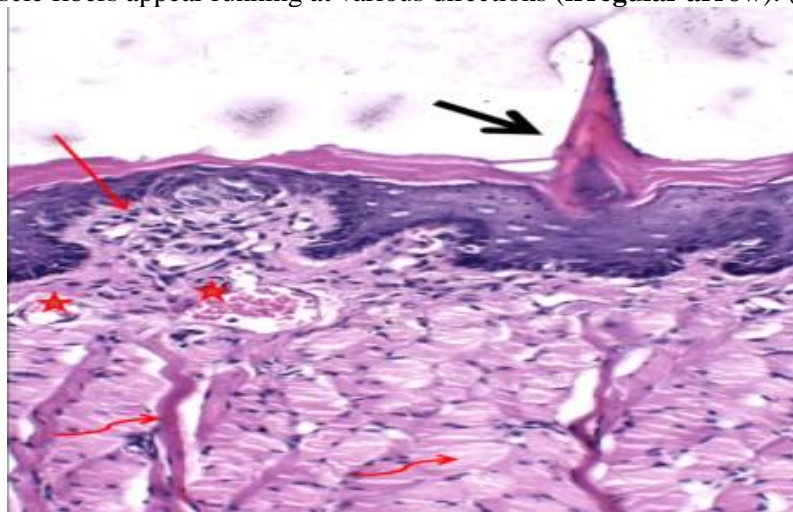
The collagen fibers revealed normal distribution with strongly positive staining reactivity to Masson's trichrome stain in the lamina propria and thin rims in-between muscle fibers **Fig. 10**.

**Group II:** the nicotine treated tongue showed noticeable atrophic and degenerative changes that involved the surface epithelium and lamina propria. The filiform papillae showed deformation, decrease in length and number, their epithelial covering was atrophic, areas devoid of the epithelial ridges and hyperkeratosis were found. Fungiform papillae appeared ballooning with signs of atrophy in their epithelial covering; their lamina propria and taste buds were completely lost. Inflammatory cell infiltrations were seen in-between the muscle fibers which also showed spacing and discontinuations **Figs. 4-6**. Using Masson's trichrome stain, the underlying lamina propria showed degeneration and dissociation of collagen fibers that revealed weakly positive staining reactivity to Masson's trichrome stain, on the other side thick bands of collagen appeared in-between the muscle fibers **Fig. 11**.

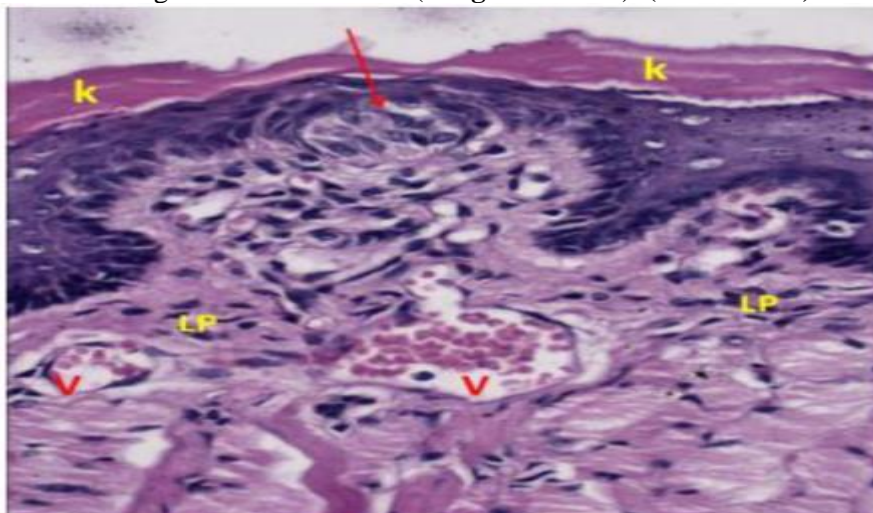
**Group III:** comparing with the previous group hematoxylin and eosin sections of this group revealed some improvement; most filiform papillae appeared with intact tapering tips but, still areas of flattening of the dorsal surface with atrophied filiform papillae were present. Fungiform papillae preserved their normal shape. Skeletal muscle fibers appeared within normal shape as seen in the control group; muscle separations still appeared within the muscle cell layers **Figs. 7-9**. With Masson's trichrome stain; the collagen fibers restore their normal distribution with strongly positive staining within the lamina propria and thin rims of collagen fibers in-between the muscle fibers **Fig. 12**.



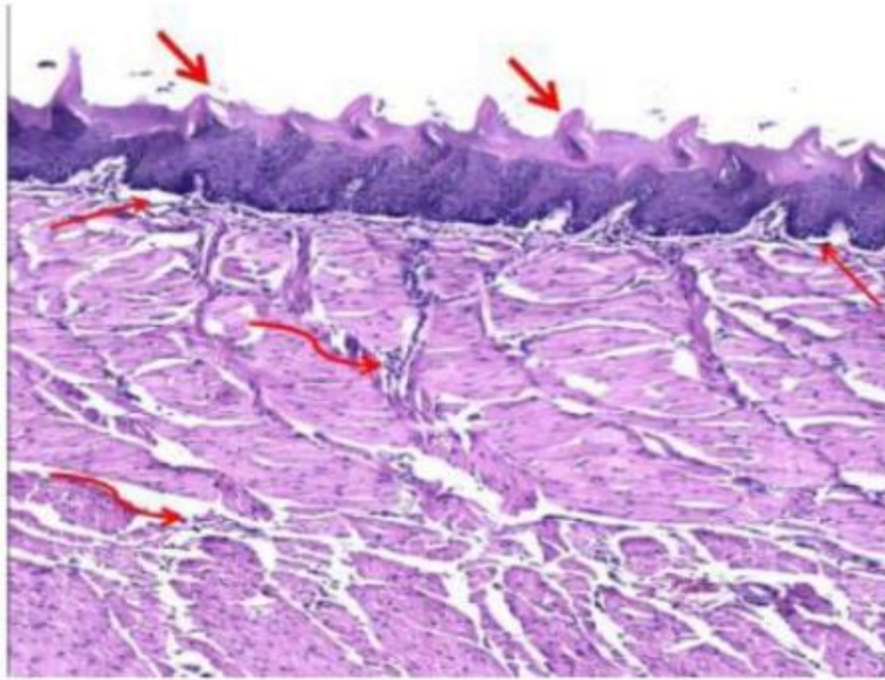
**Fig 1:** a photomicrograph of the dorsal surface of tongue of the control group showing regular orientation of the papillae which are covered by keratinized epithelium (**thick arrow**). Lamina propria appears formed of connective tissue (**thin arrow**). Muscle fibers appear running at various directions (**irregular arrow**). (**H&E X 100**).



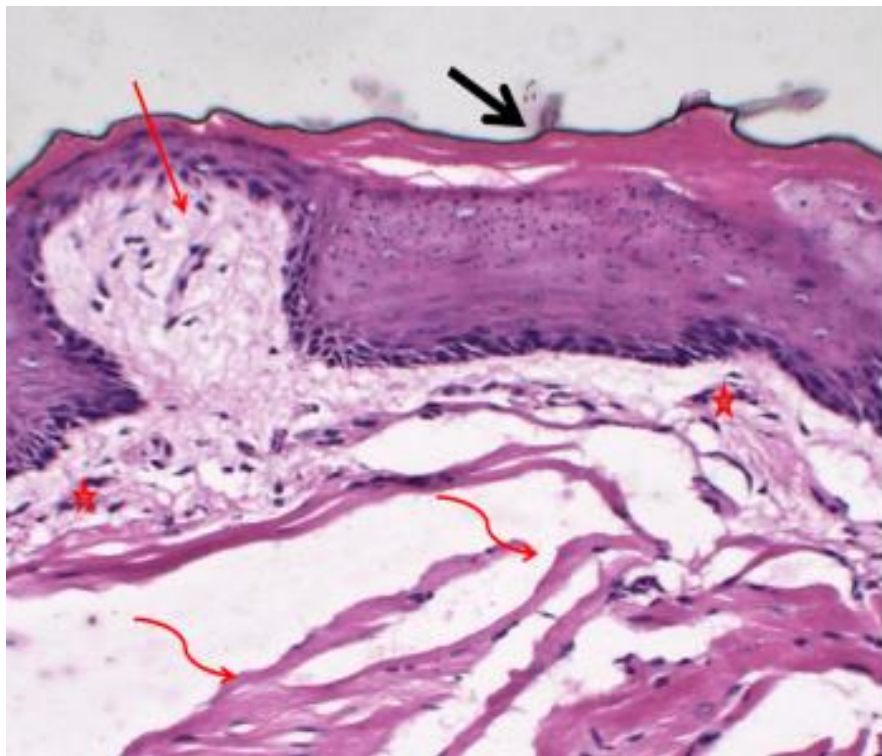
**Fig 2:** a photomicrograph of the dorsal surface of tongue of the control group showing: a-sharp conical projections of filiform papillae with thin smooth keratinized epithelial covering (**thick arrow**). A fungiform papilla showing normal barrel like intra-epithelial taste bud (**thin arrow**). Lamina propria showing well prominent blood vessels (**stars**). Skeletal muscles running at various directions(**irregular arrow**). (**H&E X 200**).



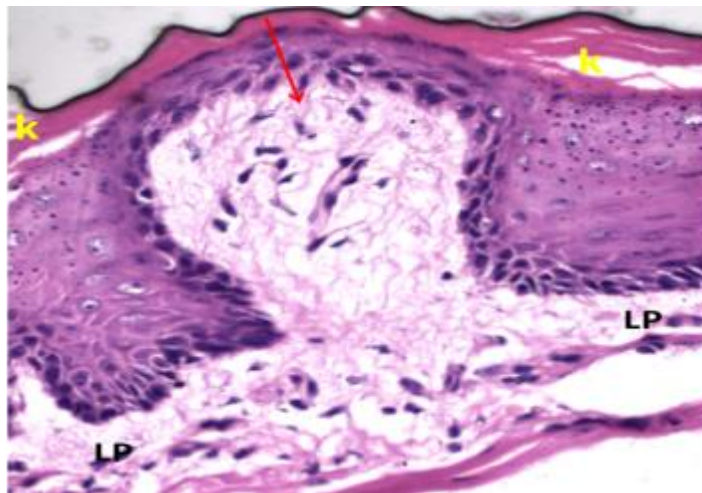
**Fig 3:** magnification of the previous fungiform papilla showing the characteristic mushroom-shape which appears elevated above the surface of the tongue and covered by keratinized stratified squamous epithelium. A thin uniform layer of keratin could be seen on the most superficial epithelial layer (**K**). A single well-defined barrel-shaped taste bud was observed on the dorsal surface (**arrow**). The lamina propria(**LP**) formed of well-defined connective tissue papillae and blood vessels (**V**)(**H&E X400**).



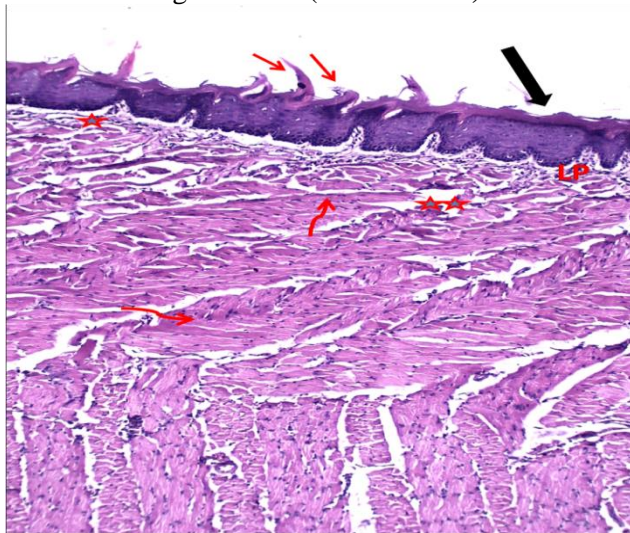
**Fig 4:** a photomicrograph of the dorsal surface of the nicotine treated tongue(group II) showing: atrophied filiform papillae (**thick arrow**),thinning of the underlying lamina propria (**thin arrow**). Infiltration of inflammatory cells is well seen in-between the lingual muscle fibers (**irregular arrow**). (H&E X 100).



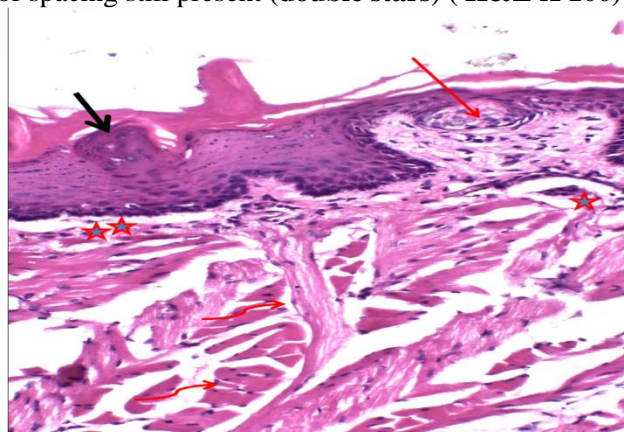
**Fig 5:** a photomicrograph of the dorsal surface of the tongue of nicotine treated rats (group II)showing focal area of hyperkeratosis and flattening of the dorsal surface of the tongue with atrophied filiform papillae (**thick arrow**),destroyed fungiform papilla with degenerated intraepithelial taste bud(**thin arrow**). The connective tissue of the lamina propria shows loose appearance and degenerations (**stars**), muscle fibers appear atrophied with increased spacing (**irregular arrow**) (H&E X 200).



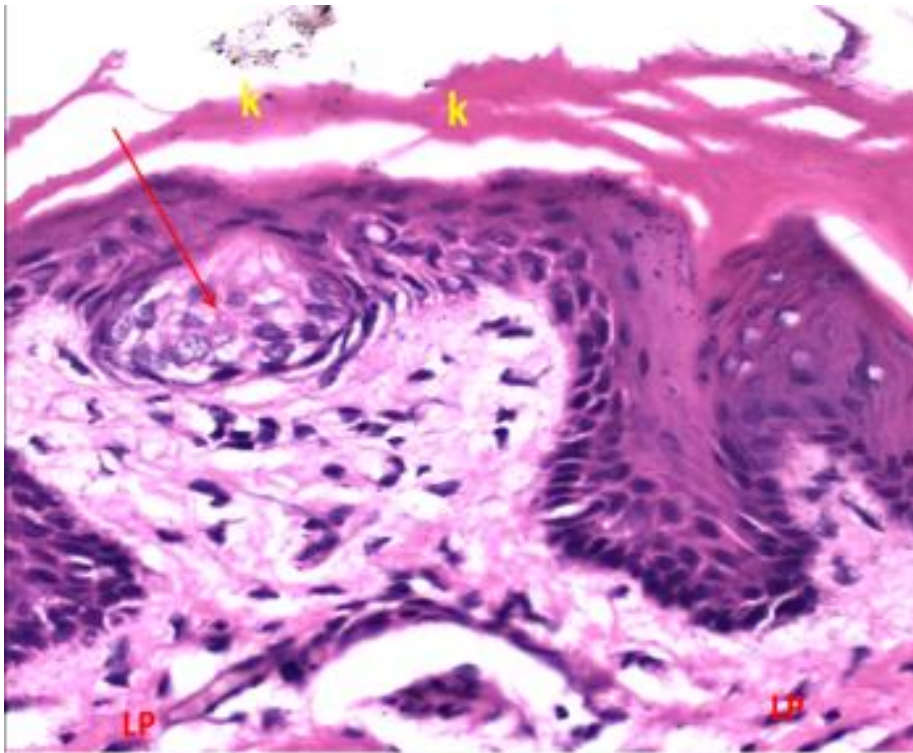
**Fig 6:** magnification of the previous fungiform papilla showing swollen fungiform papilla which appears distorted with atrophied taste bud on its dorsal surface(**arrow**), Keratin layer is separated (**K**), the lamina propria(**LP**) shows loose appearance and degenerations( **H&E X 400**).



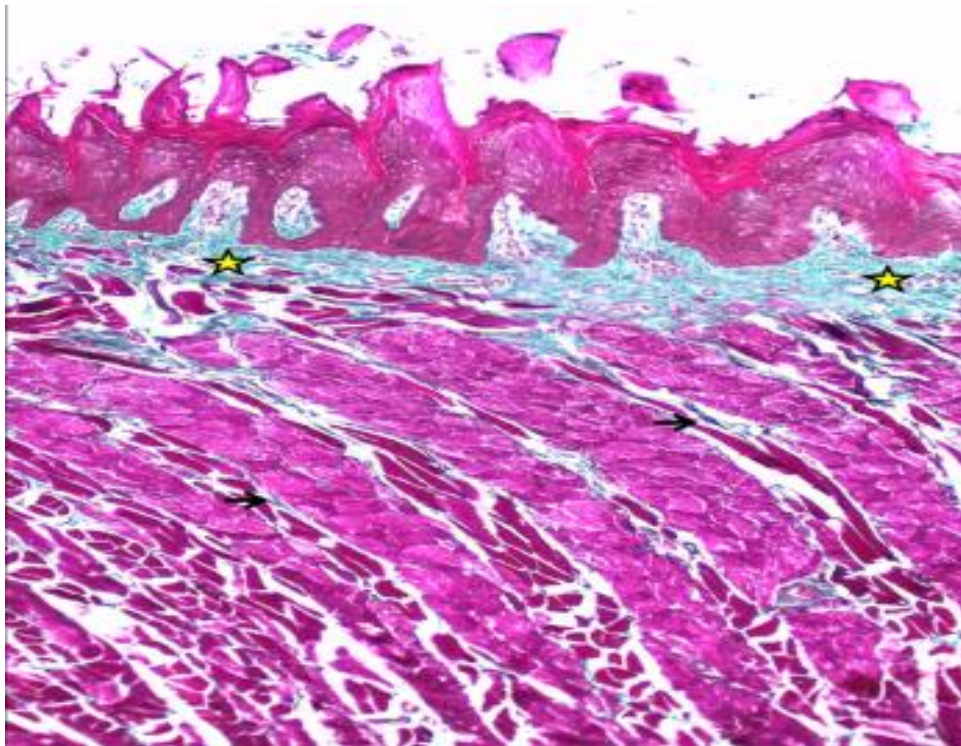
**Fig 7:** a photomicrograph of the dorsal surface of the nicotine +Royal jelly treated tongue(group III) showing; presence of normal filiform papillae(**thin red arrow**), still focal area of flattening of the dorsal surface with atrophied filiform papillae present (**thick black arrow**). The lamina propria(**LP**) shows normal appearance, still area of loose connective tissue present (**star**), normal appearance of muscles of different directions appears (**irregular red arrow**), area of spacing still present (**double stars**) ( **H&E X 100**)



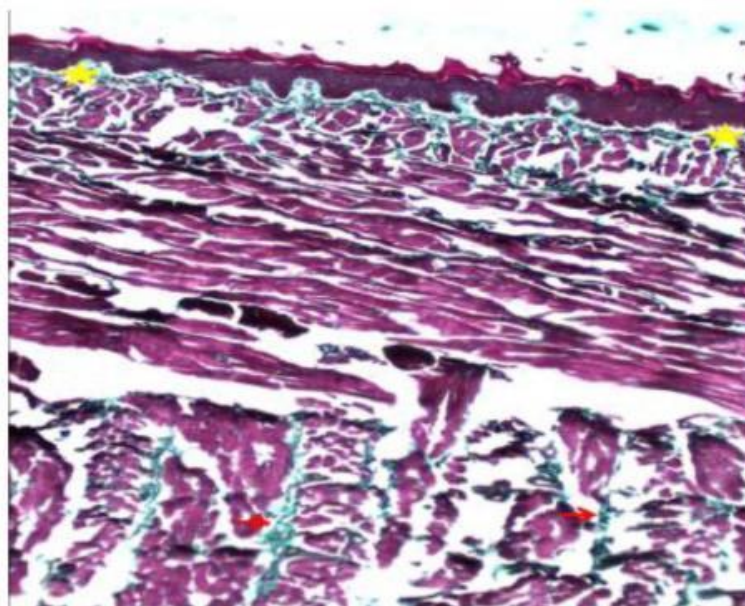
**Fig 8:** a photomicrograph of the dorsal surface of the nicotine +Royal jelly treated tongue(group III) showing; presence of shortly appeared filiform papilla(**thick black arrow**),normal appearance of the fungiform papilla (**thin red arrow**). The lamina propria shows normal appearance of blood vessels(**star**), still area of loose connective tissue is present( **double stars**),normal appearance of muscles of different directions also present (**irregular red arrow**). **H&E X 200**



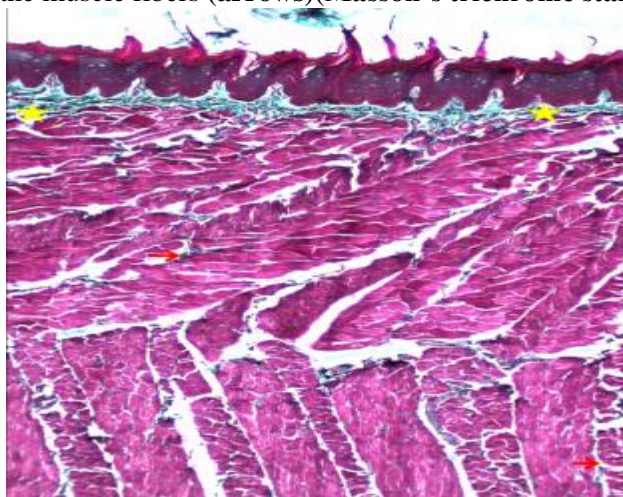
**Fig 9:** magnification of the previous fungiform papilla showing normal appearance, well-defined barrel-shaped on the dorsal surface (**arrow**).Keratin layer is still separated (**K**) in some areas. The lamina propria (**LP**) shows loose appearance of the connective tissue (**H&E X400**).



**Fig 10:** a photomicrograph of the tongue tissue of group I showing normal distribution the collagen fibers with strong staining affinity to Masson's trichrome stain mainly in the lamina propria(**stars**) and thin rims inbetween the muscle fibers (**arrows**). (**Masson's trichrome stainX100**)



**Fig 11** :a photomicrograph of the tongue tissue of group II showing the underlying lamina propria with degeneration and dissociation of collagen that revealed weakly positive staining affinity (stars) with thick bands of collagen appear in-between the muscle fibers (arrows)(Masson's trichrome stainX100).



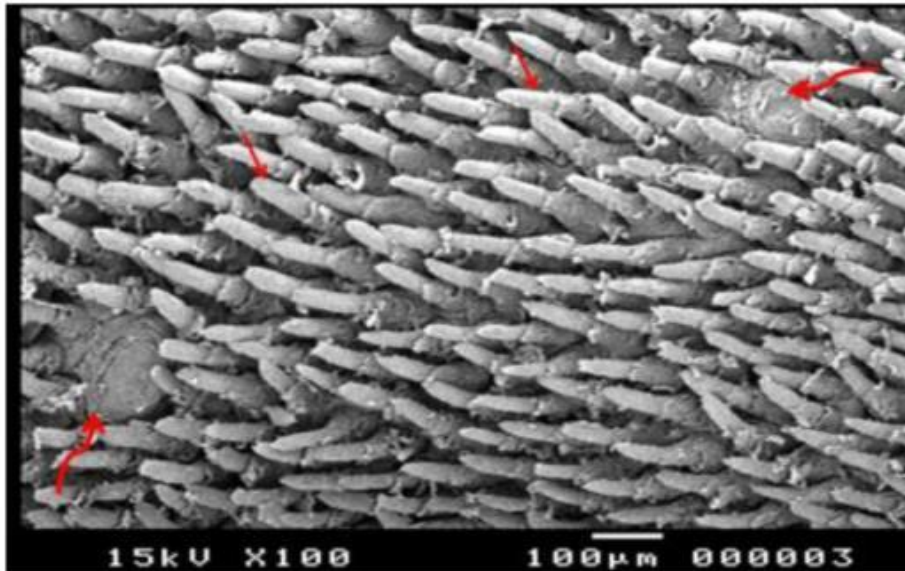
**Fig 12** :a photomicrograph of the tongue tissue of group III showing positive staining affinity to Masson's trichrome in the lamina propria(stars)and thin rims of collagen fibers inbetween the muscle fibers (arrows) . (Masson's trichrome stainX100).

#### **B-Scanning electron microscopic study**

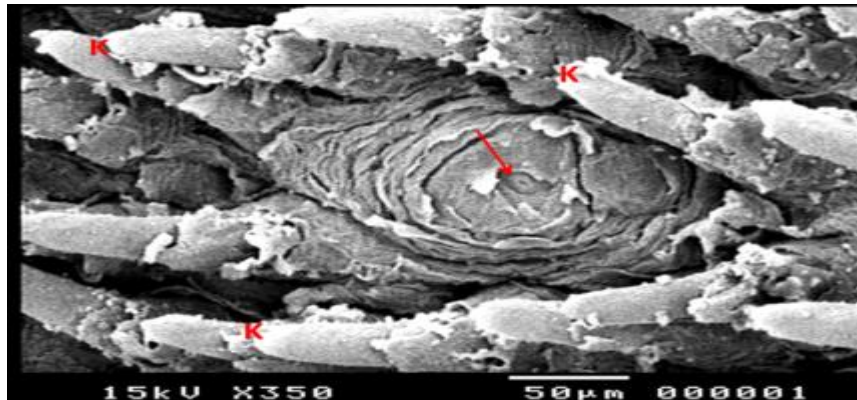
**Group I:** the tongue dorsal surface appeared formed of elongated, numerous, conical shaped-filiform papillae with intact, slightly curved, keratinized tapering tips that pointed into one direction. Fungiform papillae appeared sporadically in-between the filiform papillae. They were short, dome-like shaped and had flattened smooth upper surface with keratinized tips, they were traversed by taste pores in the center **Figs. 13- 15.**

**Group II:** nicotine treated group showed disorganized distribution of the filiform papillae with thin, atrophied, blunted edge and destructed picture. Fungiform papilla appeared with wrinkled keratinized epithelial covering. The gustatory pore appeared irregular and ill-defined **Figs. 16- 18.**

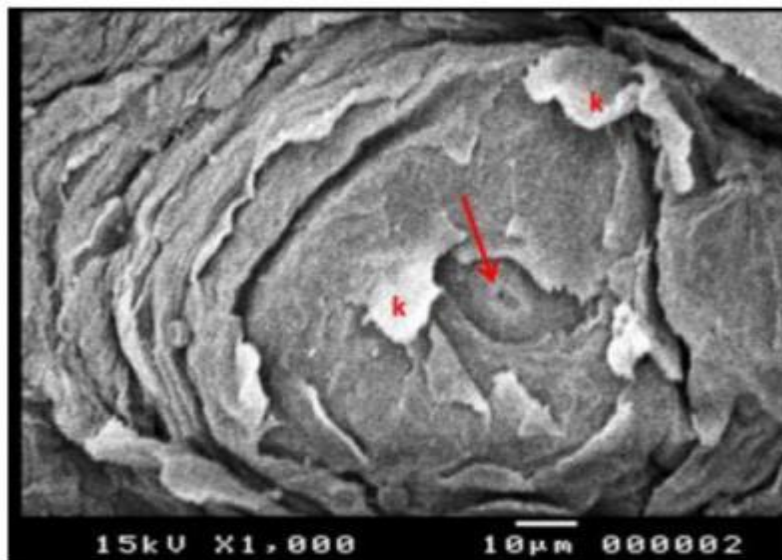
**Group III:** after addition of RJ tongue showed normal appearance of the filiform papillae with regular orientation. They appeared as long, conical shaped and mostly with intact tapering tips, some destructed cells still present. The fungiform papillae showed apparently normal picture with intact smooth surfaces and well defined taste pores **Figs. 19-21.**



**Fig 13 :** a scanning electron micrograph of a control rat's tongue (dorsal surface) showing filiform papillae (thread-like shape) which appear regular in size, shape and orientation with tapering ends (**arrow**) and normal inter-papillary distances and the dome-like shaped fungiform papillae (**irregular arrow**) in-between the filiform papillae. (X 100)

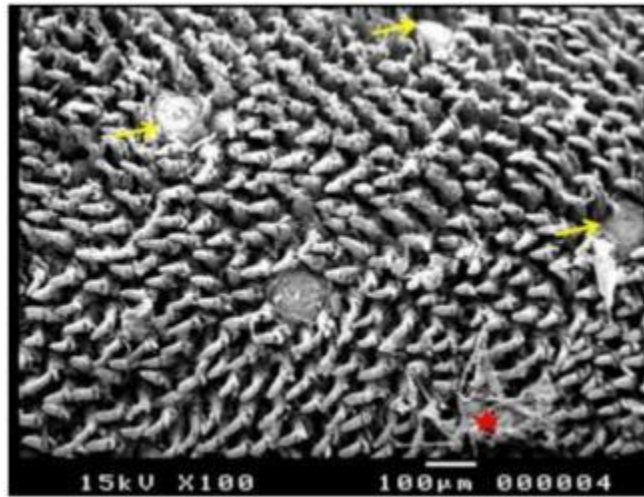


**Fig 14:** high magnification of the previous picture showing normal appearance of filiform papillae with keratinized tips (**K**) surrounding a Mushroom-like fungiform papillae with a central gustatory pore (**arrow**). (X 350)

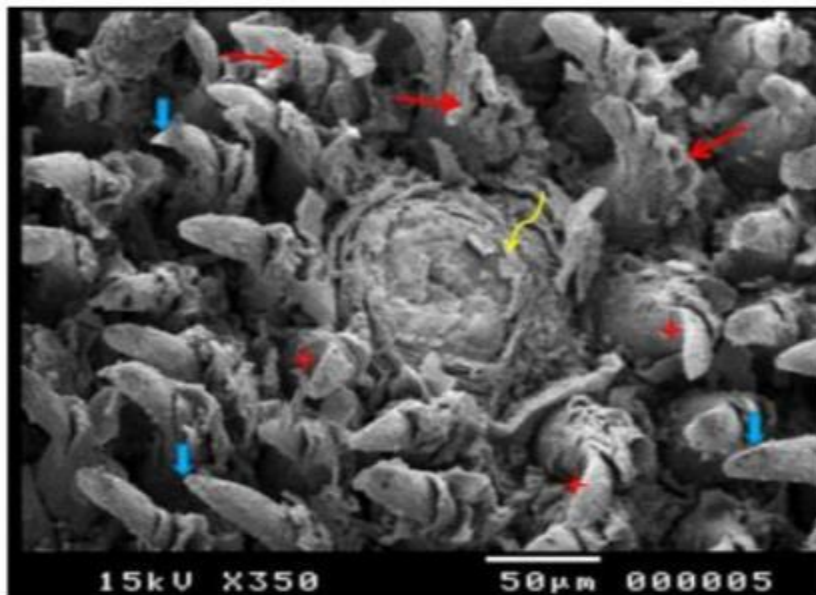


**Fig 15:** high magnification of the previous fungiform papilla covered by several regular epithelial cells. Some of these cells show keratinization (**k**). A well-defined regular gustatory pore surrounded by a shallow indentation in its center is also seen (**arrow**). (X1000)

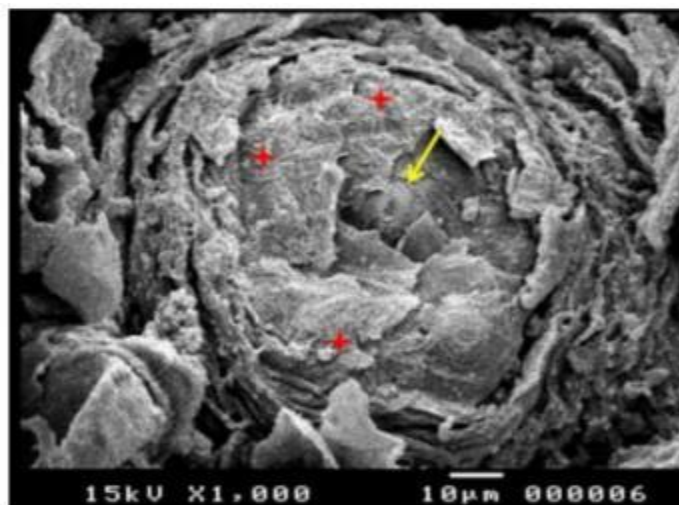




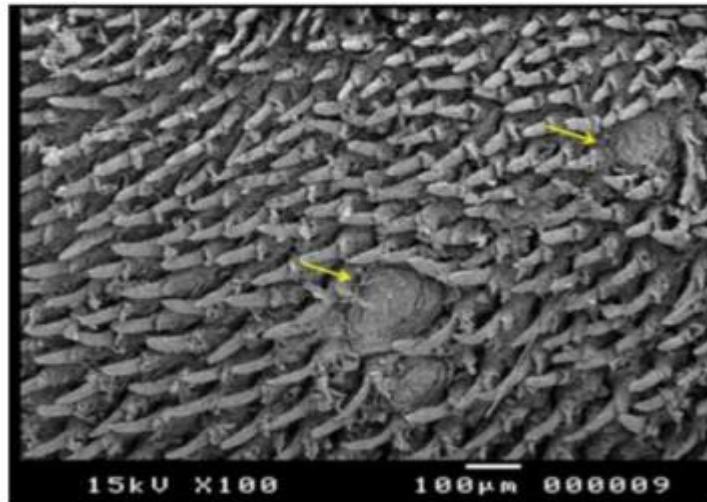
**Fig 16:** scanning electron micrograph of nicotine rat's tongue (dorsal surface) showing abnormal arrangement of the filiform papillae which appear destroyed with desquamation of their epithelial topping (**star**) and surrounding the abnormal fungiform papillae (**arrows**)(X 100).



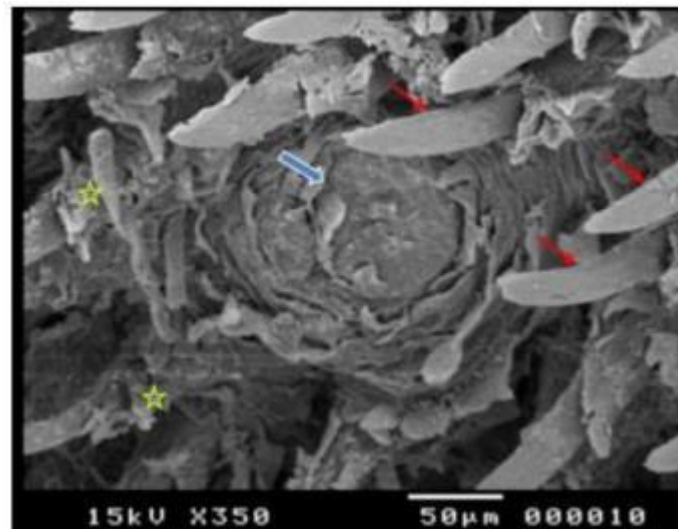
**Fig 17:**high magnification of the previous picture showing damaged filiform papillae; destroyed(**arrow**) thin (**thick arrow**) and blunted edge(**stars**) surrounding one fungiform papilla with ill-defined taste pore(**irregular arrow**) (X 350).



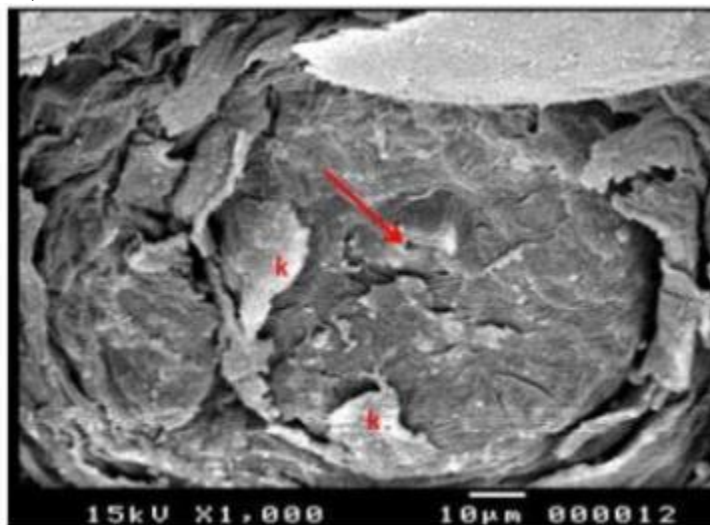
**Fig 18:** high magnification of the previous fungiform papilla which shows wrinkled keratinized epithelial surface (**stars**). The gustatory pore appears elevated and ill-defined (**arrow**)(X1000).



**Fig 19:** a scanning electron micrograph of nicotine +royal jelly treated tongue (dorsal surface) showing normal architecture of the filiform papillae and normal inter-papillary distances between them with normal appearance of fungiform papillae in-between them (↑). (X1000)



**Fig 20 :** high magnification of the previous picture showing that most of the filiform papillae appear normal with no thinning or bending of tips( **arrows**), others still distorted (**stars**),fungiform papilla appear nearly normal(**thick arrow**)(X 350).



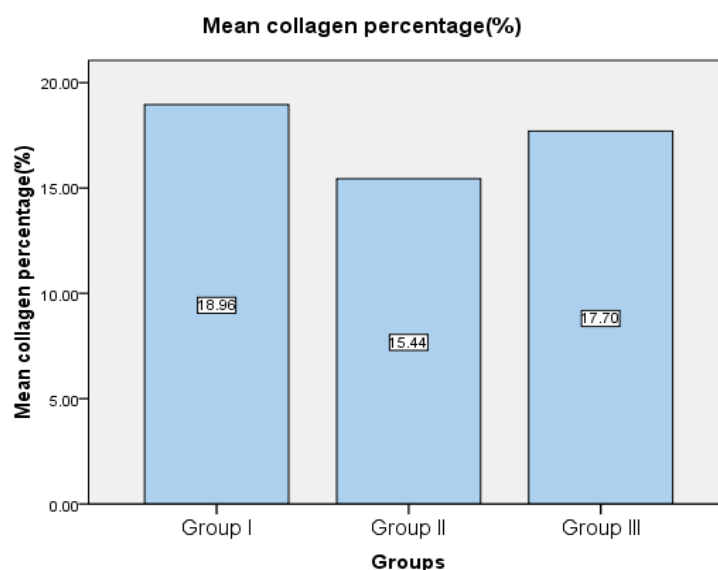
**Fig 21:** high magnification of the previous fungiform papilla which appears normal with keratinization (**k**) of its epithelial cells and normal picture of the gustatory pore with shallow indentation in its center (**arrow**) (X1000)

**Morphometric results:**

The mean area percentage occupied by collagen in the nicotine treated group (15.44±.32) was very highly significant decreased in comparison with the control group (19.35) p=0.000. The mean area percentage occupied by collagen in the nicotine +Royal treated group (17.7) was very highly significant decreased in comparison with the control group (19.35) p= 0.000. The mean area percentage occupied by collagen in the nicotine treated group was very highly significant decreased in comparison with the nicotine +Royal group p=0.000. (Table 1, Histogram 1).

Groups	Group I	Group II	Group III
Collagen Percentage (%)	19.35	15.44±.32***	17.7***

Table 1: showing the meanCollagen Percentage (%) in control and experimental groups. (\*\*\*) → Very high significant difference.



**Histogram 1: Comparison between the studied groups according to the percentage of the area of collagen(%)in the control and experimental groups.**

**DISCUSSION**

The dorsal surface of the tongue has four types of lingual papillae, the filiform, fungiform, circumvallate and foliatepapillae. The filiform papillae are more widely distributed and cells of these papillae have a high metabolic activity and easily affected by any disturbance before any other papillae (15).

Light microscopic study of nicotine treated rats revealed an obvious atrophy of the lingual papillae, hyperkeratosis, shrunkenof the lamina propria and destruction of the muscle contents of the tongue with reduction in the collagen contents, Similar studies (10,16,17) observed that nicotine caused morphological changes on rat tongue mucosa, including inflammatory leukocyte infiltration and cellular desquamation, blood vessel dilation, hemorrhage and epithelial degeneration. Nicotine

induces oxidative stress and apoptosis, apart from inducing proliferative signaling pathways in number of cell types and tissues(18).**Cecchi et al.** (19), studied the *in vitro* effects of low nicotine concentrations on human fibroblasts and noted a reduction in cell proliferation and inhibition of alkaline phosphatase and collagen production. Smokers exhibited significantly lower taste sensitivity than non-smokers, the higher the nicotine dependence (Fagerström scores), the lower the taste sensitivity (20).

Scanning electron microscopy (SEM)of nicotine treated rats in this study revealed disorganized distribution of the filiform papillae and destruction of the fungiform papillae, Similar results were observed by **Lobna et al.**(21) who found that exposure to passive smoking associated with epithelial pathological changes of the rat tongue epithelium in the form of desquamation with the disfigurement and adherence of the papillae was seen by SEM with numerous polymicrobial grouping of

Cocci intermingled with lymphocytes and macrophages.

At the same line, previous study used SEM to examine the surface of the tongue, pharynx and larynx of animals exposed to tobacco and found that the filiform tongue papillae of the tobacco group were irregularly displayed, flattened and adhered to each other<sup>(22)</sup>.

Histological examination of nicotine + Royal jelly treated rats revealed incomplete improvement of the mucosa in the form of regularly arranged filiform papillae and normal fungiform papillae with presence of destructed areas in both mucosa and muscles. Similar results were observed by **Güngörmüş and Erdem**<sup>(23)</sup> who demonstrated that royal jelly administrated by a certain procedure improved the signs and symptoms of oral mucositis in patients undergoing radiotherapy and chemotherapy and markedly shortened its healing time. This improvement may be as Royal jelly was found to protect tissue DNA against the oxidative damage. A previous study showed that after Royal jelly diet to mice for 16 weeks, the levels of 8-hydroxy-2-deoxyguanosine (an oxidative stress marker) were markedly reduced in kidney DNA and the average life span of the mice was increased through the mechanism of reducing the oxidative damage<sup>(24)</sup>.

Scanning electron microscopy (SEM) of nicotine + Royal jelly treated rats revealed marked improvement of the tongue mucosa in the form of regular orientation of the lingual filiform papillae and normal picture of the fungiform papillae, similar results were observed by a previous study that was carried out by SEM that royal jelly caused amelioration of the ethanol damaging effects on the lingual papillae<sup>(25)</sup>. Royal jelly was proved to have a potential repro-protective action against nicotine-induced sperm abnormalities and embryo-toxicity in mice and protects male mice against nicotine-induced reproductive failure<sup>(26)</sup>. On the other hand a previous study showed that honey caused marked regeneration of the lingual papillae observed by both light and SEM against the lead-induced lingual atrophy<sup>(27)</sup>. Bee products are considered to be a potential source of natural antioxidants such as flavonoids, phenolic acids, or terpenoids which are capable of counteracting the effects of oxidative stress underlying the pathogenesis of numerous diseases as well as negative effects of different harmful factors and drugs<sup>(8)</sup>.

## CONCLUSION

Nicotine caused cellular proliferative typical changes both in mucosa and muscles of the tongue which might subsequently develop into precancerous

and cancerous lesions. Royal Jelly is an important protective agent for these lesions.

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