Original Article

Toxic Effects of Tramadol Abuse and Withdrawal on Histopathological Structure of Tongue in Sprague Dawley Rats

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ABSTRACT:

Background: Taste disturbance among tramadol abusers has been reported in the last few years. **Aim**: To assess the toxic effects of tramadol abuse on tongue structure including taste buds and reversibility of these changes on withdrawal. In addition, assess the effect of tramadol on Ki-67 as general marker for cellular proliferation. **Methods**: Forty-two adult male *Sprague Dawley* rats were divided into six groups: group I received normal saline, groups II and III received tramadol hydrochloride 40mg/kg for 20 and 30 days respectively orally, groups IV, V and VI received tramadol hydrochloride 40mg/kg for 30 days orally then received normal saline only for 10, 20, 30 days respectively. Rats were sacrificed and their tongues were dissected out. **Results:** On histological examination, the covering epithelium, fungiform and filiform papillae, taste buds and muscle layer were distorted in group II. In group III more damage occurred with complete disappearance of taste buds. On tramadol stoppage, gradual restoration of epithelium, papillae, taste buds and muscle fibers structure began in groups IV and V. In group VI, most histological features were restored but did not return to normal. Regarding Ki-67 immunostaining, the reaction area in tongue tissue increased with tramadol administration then began to decrease on its cessation but did not return to normal up to 30 days after withdrawal. **Conclusion:** Tramadol can disrupt the histological structure of the tongue tissue, destroy taste buds, and these changes are reversible on withdrawal. In addition, these toxic effects are not dependent on Ki-67 pathway.

KEYWORDS: Tramadol abuse; Ki-67; Taste buds.

I. INTRODUCTION:

Taste sensation is complex process. Taste involves peripheral perception then sensations are transmitted to the central nervous system where olfactory input and past experiences merge to give an emotional, sensory, and physiological response. The main organ of taste is classically considered the tongue and the primary structure that house the taste buds are the papillae (Gravina et al., 2013). Mammalian taste buds include taste receptor cells, supporting cells, and immature basal cells. Taste receptor cells are responsible for taste perception and transmission. The average life span of taste cells is about 10 days but some of them can survive for up to 3 weeks. To maintain structural stability and cell type equilibrium, taste progenitor cells give rise to newborn cells, which enter taste buds and differentiate into different types of mature taste bud cells. This group of progenitor cells express different cell proliferation markers and one of them is Ki-67 which is expressed in all active stages of the cell cycle (Cohn et al., 2010).

Tramadol is centrally acting synthetic opioid analgesic that is commonly used for management of moderate to severe pain. In addition, it is one of the highly reported abused drug in many countries and classified now as scheduled drug (Barbosa et al., 2017; Ibrahim and Salah-Eldin, 2019). Tramadol is metabolized in the liver into five metabolites. M1 metabolite is pharmacologically active one and about 200 times more potent on μ receptors than the parent drug. As regard its elimination, it is mainly eliminated by the kidney. Tramadol half-life is dose dependent. The half-life of M1 metabolite is longer than the parent drug (Nakhaee et al., 2021). Tramadol and its metabolites can be secreted in different body fluids with tramadol concentration in saliva is about 7- to 8- folds higher than that of plasma (Grond and Sablotzki, 2004; Ardakani and Rouini, 2007; Ardakani and Rouini, 2007).

In cases clinically presented with taste disturbance, the cause was attributed to prolonged use of different drugs in 22-28% of cases. Drugs may affect taste through different mechanisms as dryness of saliva with failure to transport tastings to taste buds, drugs acting directly on taste buds affecting their turnover, negative effect on calcium channels and disturbance of taste impulse and zinc deficiency. Taste disturbance among opiate abusers including tramadol abusers had been reported. Reported taste disturbance in tramadol abusers was in the form of Ageusia and/or Hypogeusia (Giudice, 2006; Schiffman, 2018). Tramadol induced xerostomia was previously reported. It decreases the salivary secretions by about 75% and this could be a possible explanation for reported dysgeusia

among tramadol abusers (Elhindawy and Ali, 2019). However, and according to our knowledge, no previous studies evaluated the exact mechanism of taste disturbance among tramadol abusers.

The aim of the present work was to assess the toxic effects of tramadol abuse on tongue structure including taste buds and reversibility of these changes on withdrawal. In addition, assess the effect of tramadol on Ki-67 as general marker for cellular proliferation.

II. MATERIAL AND METHODS:

II.1. Drug dosage:

Tramadol hydrochloride tablets (225 mg) were obtained from October Pharma Co. (Giza, Egypt). Tramadol dosage in human is about 50-100mg four times per day with maximum recommended daily dose 400 mg. Thus, the minimum recommended daily dose in average 60 kg-weighing human is (1.67mg/kg) and maximum recommended daily dose is (6.67mg/kg). Rat doses were calculated using the correction factor (K_m) for the conversion of the human dose into the animal equivalent dose (AED), through the following formula: Animal equivalent dose (AED) (mg/kg) = Human dose (mg/kg) x K_m ratio (K_m = 6.2) (Anroop B. and Jacob, 2016; Barbosa et al., 2017). From the previous data maximum daily dose for rats was calculated: 6.67x6.2=41.35 mg/kg.

II.2. Animals:

Forty-two adult male *Sprague Dawley* rats weighting (180-200gm) were purchased from Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura university. All experimental procedures were carried out in accordance with the laboratory guidelines of Ethics Committee of Faculty of Dentistry, Mansoura University, Egypt (code number: 0611/2/8). Rats were housed under conventional standard conditions (maximum five rats per cage at 22 ± 2°C, 60 ± 10% humidity, and a 12-h light/dark cycle, with free access to food and water). After acclimatization for two weeks, rats were randomly allocated into 6 groups:

1. Group I (control group): Ten rats received one ml of normal saline 0.9% orally by oro-gastric tube and two rats were sacrificed with each of experimental groups.
2. Group II: Six rats received tramadol hydrochloride 40mg/kg via oro-gastric tube for 20 days.
3. Group III: Six rats received tramadol hydrochloride 40mg/kg via oro-gastric tube for 30 days.
4. Group IV: Six rats received tramadol hydrochloride 40mg/kg via oro-gastric tube for 30 days then tramadol hydrochloride was stopped, and they received one ml of normal saline 0.9% by oro-gastric tube for 10 days.

5. Group V: Six rats received tramadol hydrochloride 40mg/kg via oro-gastric tube for 30 days then tramadol hydrochloride was stopped, and they received one ml of normal saline 0.9% by oro-gastric tube for 20 days.
6. Group VI: Six rats received tramadol hydrochloride 40mg/kg via oro-gastric tube for 30 days then tramadol hydrochloride was stopped, and they received one ml of normal saline 0.9% by oro-gastric tube for 30 days.

II.3. Histopathological analysis:

At the end of the experiment in each group and 24-h after the last tramadol hydrochloride or normal saline dose, the rats anesthetized with halothane and were sacrificed by decapitation. Their tongues were dissected out, cut longitudinally into two halves, fixed in 10% neutral buffered formalin, processed to be embedded in paraffin then stained with Hematoxylin & Eosin (H&E) (Obi and Nwoha, 2014).

II.4. Immunohistochemistry evaluation:

The sectioned heads were immune-stained using avidin-biotin technique. The sections were stained with rabbit anti-Ki-67 antiserum (Sigma- Aldrich, St Louis, USA) (Takkem et al., 2018).

The percentage of color density (brown) of the reaction was measured in the calibrated area using the color deconvolution plugin feature of the software Image J programme (Version 1.46i, NIH) to separate colors. The analysis was done at a magnification of ×200 so that positive reaction could be obvious. Six sections for each group were used and the mean value was then calculated.

Five non overlapped different fields from each slide were digitized using Olympus® digital camera installed on Olympus® microscope. The images were analysed on an Intel® Core I3® based computer using Video Test Morphology® software with a specific built-in routine for immune-stain quantification. The targeted positive immuno-stained areas were automatically selected and separated from the original image depending on the positive stain in range, the selected area was thresholder and defined as region of interest and then a 3D histogram was constructed, and integrated density of the area was calculated.

II.5. Statistical analysis:

Data were analysed using Graph Pad Prism Software (version 5, GraphPad Software Inc, San Diego, CA). Descriptive statistics were calculated in the form of mean ±Standard deviation (SD), student t-test and one way ANOVA were used to compare groups.

III. RESULTS:

III.1. Histopathological evaluation:

Examination of the dorsal surface of the tongue of the control group (group I) showed filiform papillae conical in shape

with tapering tips; the fungiform papillae were few, short, with broad apices and distributed in-between the filiform papillae, covered with stratified squamous epithelium. Fungiform papillae had the characteristic mushroom shape, normal keratinized epithelial covering, appeared elevated above the surface of the tongue with intra-epithelial normal barrel shape taste buds at its tips. Underlying lamina propria formed of connective tissue and appeared to merge with subjacent tongue muscles with numerous muscle bundles directed in different directions (Figure 1).

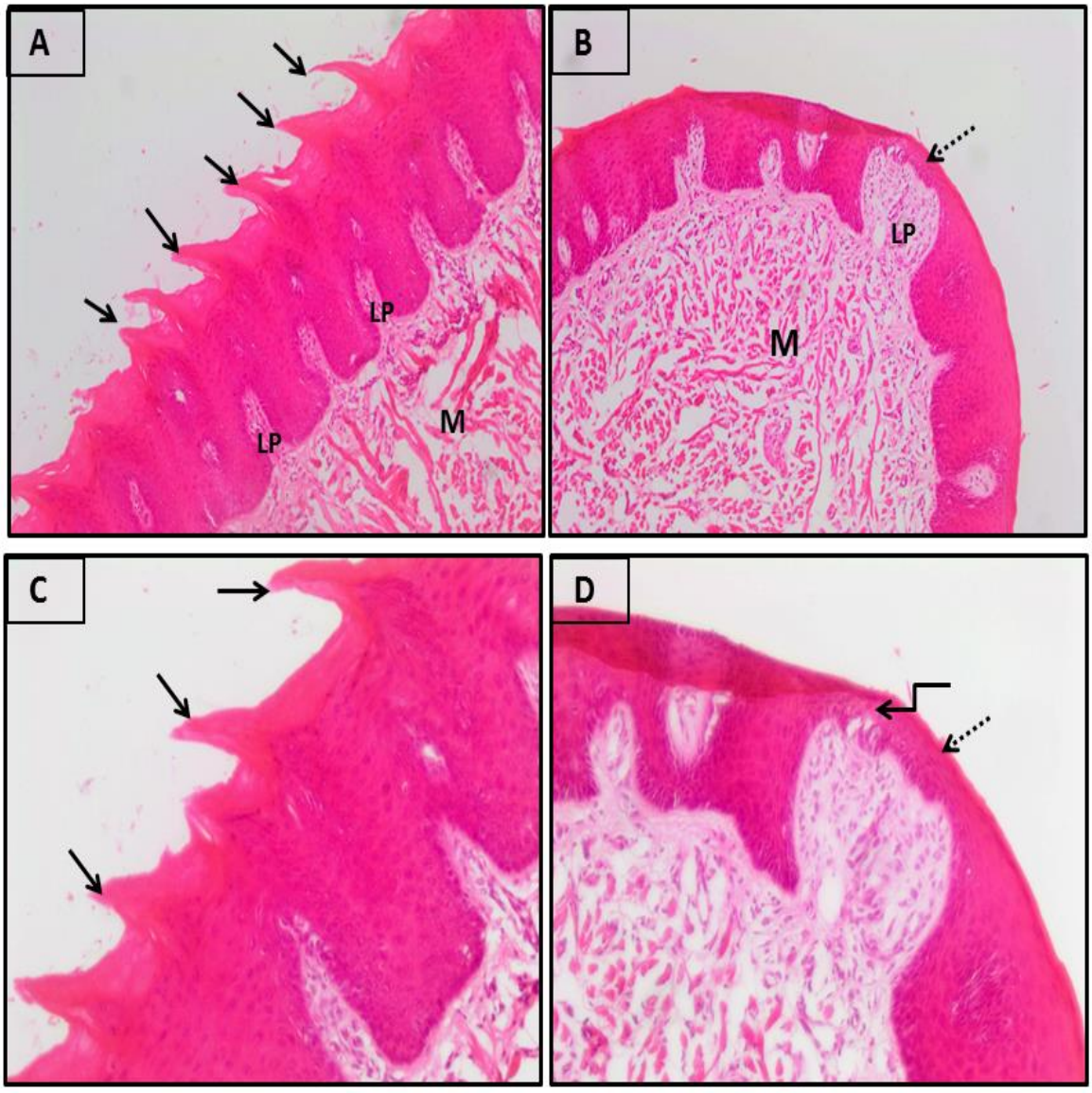

Figure 1: A photomicrograph of dorsal surface of the tongue of group I (control group) showing (**A)** Regularly oriented filiform papillae (arrows) with their thick epithelial covering. (**B**) The fungiform papilla (dotted arrow) with its thin epithelial covering. The underlying lamina propria was formed of connective tissue (LP).The lingual muscle fibers run in different directions (M). (**C**) The filiform papillae appeared conical in shape, covered by thick keratinized epithelium, and arranged in rows (arrows). (**D**) The fungiform papilla mushroom-shaped, deep red in color, covered by thin keratinized epithelium (dotted arrow). A barrel shaped taste bud could be seen on the superior surface of the papilla (curved arrow). **(H & E, A and B x 100, C and D x 200)**

On beginning of tramadol administration, dorsal surface of the tongue in group II showed papillae smaller in size and shorter in length with areas of splitting of covering epithelium. Taste buds appeared smaller in size with distorted shape. Lamina propria appeared normal and vacuolation was seen in between the lingual muscle fibers (Figures 2A &2B). Examination of dorsal surface of a rat tongue in group III showed markedly atrophied papillae and completely lost in some area. The covering epithelium was thin with areas of splitting. No taste buds appeared. Lamina propria appeared normal. Empty spaces and a congested blood vessel were seen in between the lingual muscle fibres (Figure 2C).

On tramadol withdrawal, dorsal surface of a rat tongue in group IV showed partial restoration of the shape and length of the filiform papillae. There was marked thinning of the covering epithelium of the fungiform papillae. No taste buds appeared inside it. Some vacuolations between the muscle fibers were still seen (Figure 2D). Examination of the dorsal surface of a rat tongue in group V showed restoration in the shape, length, and epithelial covering of both filiform and fungiform papillae with appearance of taste buds. However, vacuolations were still present between the muscle fibers (Figure 2E). In group VI, examination of the dorsal surface of a rat tongue showed complete restoration of the shape, length and epithelial covering of the filiform papillae, the barrel shaped taste bud appeared on the superior surface of the fungiform papilla, normal histological features of the lamina propria and the muscle fibers. However, some areas of completely lost papillae were still present (Figure 2F).

III.2. Ki-67 staining and evaluation:

Examination of group I (control group) showed positive (brown dotted) reaction to Ki-67 in the papillae basal cells indicating proliferation of different cells (Figure 3A). On tramadol administration, positive reaction to Ki-67 was detected in papillae cells, and muscle fibers in group II (Figure 3B). The reaction was stronger than that of control with statistically significant difference (p value <0.0001). In, group III the positive reaction extended to be detected in the papillae cells in basal and parabasal layers, muscle tissue and connective tissue cells (Figure 3C). The reaction was stronger than that of control (p value <0.0001) and that of group II (p value <0.0001).

On tramadol withdrawal, Group IV showed positive reaction to Ki-67 in papillae cells at basal and first one or two layers of the epithelium (muscle and connective tissue cells (Fig. 3D). The reaction was stronger than that of control (p value <0.0001), but it was weaker than that of group III (p value <0.0001). Group V showed positive reaction to Ki-67 in epithelial cells and muscle fibers (Fig. 3E). The reaction was stronger than

that of control (p value <0.0001), but it was weaker than that of group IV (p value <0.0001). Group VI showed positive reaction to Ki-67 in papillae basal cells only (Fig. 3F). The reaction was stronger than that of control (p value <0.0001), but it was weaker than that of group V (p value 0.002). The means±SD of area percentage of positive reaction Ki-67 in different groups and statistical difference between them is shown in (Figure 4).

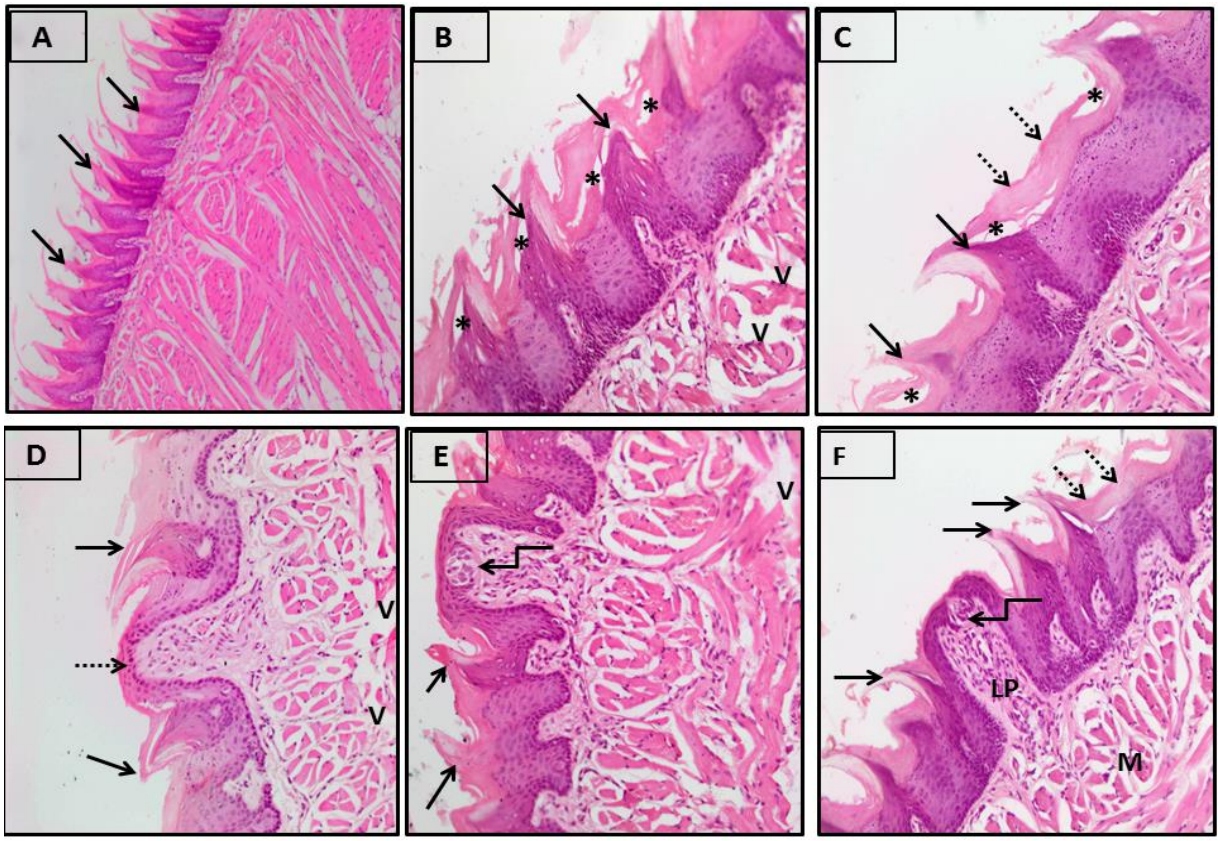

Figure 2: A photomicrograph of dorsal surface of a rat tongue from (**A**) Group II showing atrophied filiform papillae (arrows) **(B)** Filiform papillae appeared shorter in length when compared with the control group (arrows), areas of splitting within the keratinous covering (*) and vacuolations were seen between the muscle fibers (V). **(C)** Group III showing marked atrophy of the filiform papillae (arrows). There was atrophy of the covering epithelium with areas of splitting within it (*). The filiform papillae were completely lost in some areas (dotted arrows). (**D**) Group IV showing partial restoration of the shape and length of the filiform papillae (arrows). Marked thinning of the covering epithelium of the fungiform papillae (dotted arrow). No taste buds appeared inside it. Some vacuolations between the muscle fibers were seen (V). (**E**) Group V showing more restoration in the shape, length, and epithelial covering of the filiform papillae (arrows). Fungiform papilla appeared with its mushroom shape having a barrel shaped taste bud on its superior surface (curved arrow). Vacuolations seen between the muscle fibers (V). (**F**) Group VI showing complete restoration of the shape, length, and epithelial covering of the filiform papillae (arrows. The barrel shaped taste bud appeared on the superior surface of the fungiform papilla (curved arrow). Normal histological features of the lamina propria (LP) and the muscle fibers (M). However, some areas of completely lost papillae were still present. **(H & E, A x 100, B, C, D, E and F x 200).**

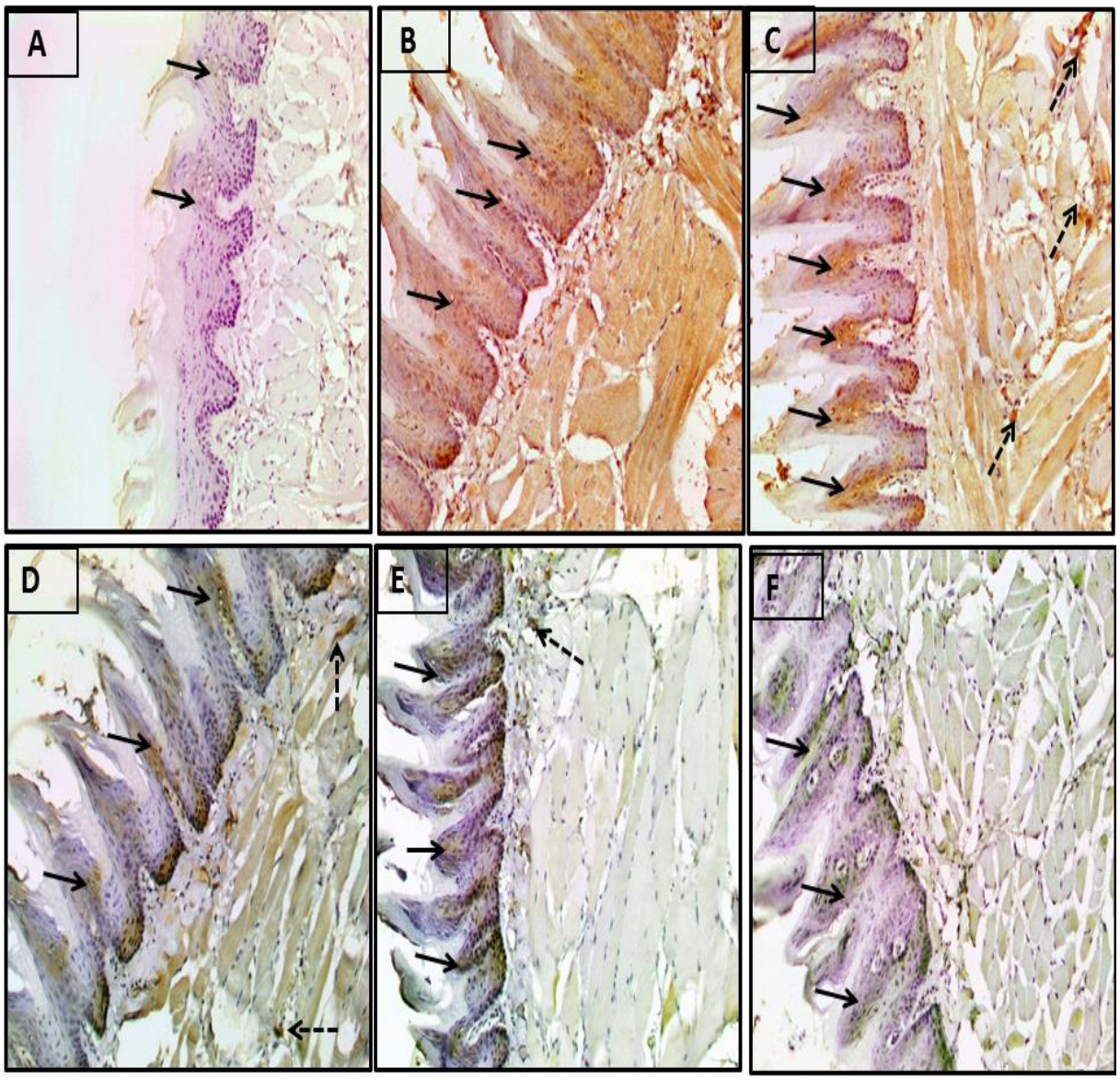

Figure 3: A photomicrograph of dorsal surface of a rat tongue**: (A)** Group I showing weak positive reaction to Ki-67 in the papillae cells (arrows). **(B)** Group II showing an increase in Ki-67 immunoreaction more than the control group in the papillae basal and parabasal layers cells (arrows) with detected reaction in the muscle tissue and connective tissue cells. **(C):** Group III showing strong positive reaction to Ki-67 in the papillae cells nearly all epithelium layers (arrows) as well as muscle and connective tissue cells (dotted arrows). **(D)** Group IV showing a reduction in Ki-67 immunoreaction less than group III in the papillae cells at basal and first one or two layers of the epithelium (arrows) and weak reaction in muscle and connective tissue cells (dotted arrows)**. (E)** Group V showing weak positive reaction to Ki-67 in the papillae epithelial cells (arrows) and muscle tissue (dotted arrows)**. (F)** Group VI showing weak positive reaction to Ki-67 in the papillae cells (arrow) at basal cells only **(x 200).**

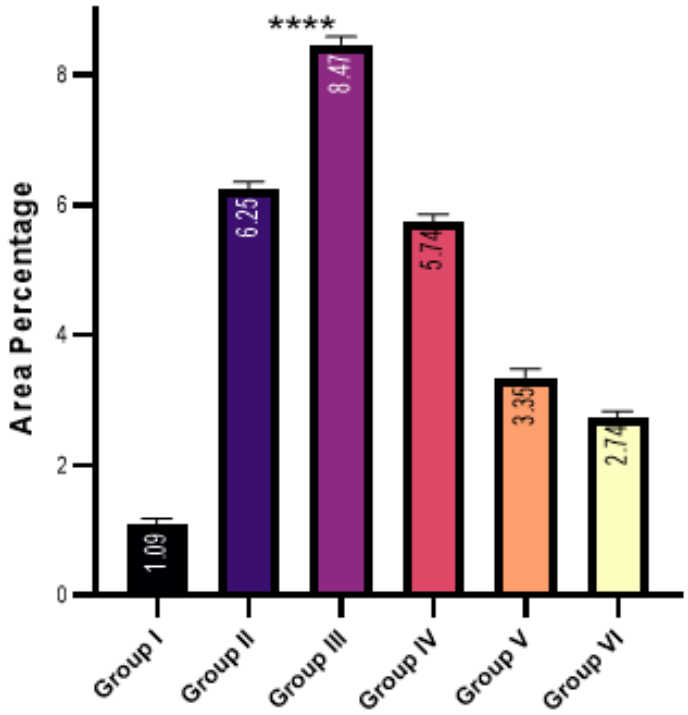

Figure (4): Graph demonstrating area percentage of positive reaction to Ki-67 immunostaining in rat tongues of different groups. Group I: control group; Group II: rats received tramadol hydrochloride for 20 days; Group III: rats received tramadol hydrochloride for 30 days (****P value < 0.0001). Group IV: rats received tramadol hydrochloride for 30 days followed by normal saline only for 10 days; group V: rats received tramadol hydrochloride for 30 days followed by normal saline only for 20 days; Group VI: rats received tramadol hydrochloride for 30 days followed by normal saline only for 30 days; (****P value < 0.0001). $p < 0.05$ is significant.

IV. DISCUSSION:

In the last few years, the Egyptian community demonstrated high rate of tramadol abuse although it has been scheduled (Yassa and Badea, 2019). In addition, decrease in taste sensations among tramadol abusers was reported (Schiffman, 2018). The aim of the present work was to assess the toxic effects of tramadol abuse on tongue structure including taste buds and reversibility of these changes on withdrawal. In addition, assess the effect of tramadol on Ki-67 as a general marker for cellular proliferation.

The results showed that tramadol abuse produced thinning in covering epithelium of the tongue, distorted shape, and size of both fungiform and filiform papillae, vacuolations in between the muscle layers with no alteration in the underlying lamina propria. These changes are in accordance with previously reported changes in rats' tongue exposed to tramadol hydrochloride at dose 60mg/kg for four months (Taha, 2017). As regard taste organs, the taste buds in fungiform papillae had

misshaped morphology with marked time dependent distorted shape and size till complete disappearance. These toxic effects may be direct toxicity from the drug or as a result of tramadol induced xerostomia (Camacho-Alonso et al., 2012).

On tramadol hydrochloride stoppage, partial restoration of covering epithelium, shape, and size of lingual papillae 10 days after stoppage occurred but the taste buds still completely absent. Twenty days after withdrawal, taste buds began to appear, but they restored most of its histological structure 30 days after withdrawal. The reversibly of tramadol induced pathological changes on different body organs as liver, testicular function and salivary glands was previously reported. However, although of reported improvement, the pathological changes did not return to normal levels up to 8 weeks after complete withdrawal (Nna et al., 2015; Elhindawy and Ali, 2019; Ibrahim and Salah-Eldin, 2019).

To gain an overview of the effects of tramadol on cell proliferation, tissue sections were immunoreacted with Ki-67 antibody, a general marker of cell cycle activity. The antigen Ki-67 is a ubiquitous human nuclear protein expressed in G1-, S-, and G2-phases of the cell cycle but not in the G0-phase. It is expressed in basal and progenitor cells of lingual papillae but not in intragemmal cells. The Ki-67 is a preferable marker for assessment of proliferative activity in both human and rats tissue (Muskhelishvili et al., 2003; Miura and Barlow, 2010). The Ki-67 protein level often indicates cell proliferation potency but does not determine if cells expressing Ki-67 will certainly divide (Lazarczyk et al., 2010).

In the present study, on immunostaining with Ki-67, the area gained positive reactions increased in time dependent manner on tramadol exposure. After tramadol withdrawal, the reaction decreased but did not return to normal up to 30 days after withdrawal. The effect of opiates including tramadol on Ki-67 expression is still controversial. Özgürbüz et al. (2019) assessed the effect of tramadol on Ki-67 in different cancer colon cell lines and reported that tramadol decreased Ki-67 expression in Colo320 CD133(+) cells, HCT116 CD133(+) and CD133(−) cells and increased it in Colo320 CD133(−) but all these changes were not significantly different from the control. Regarding other opioids, morphine decreased Ki-67 expression in human glioblastoma T98G cell in some doses only, but on prolonged exposure, it increased cellular proliferation despite of the effect on Ki-67 (Lazarczyk et al., 2010). Methadone has no effect on Ki-67 in rats' hippocampal tissue (Sankararaman et al., 2012). However, on assessing solitary μ receptors stimulation, it

increased Ki-67 activity up to 350% in intestinal tissue (Goldsmith et al., 2011).

Opiates effect on cell growth and proliferation is controlled by various pathways as it may either interfere with normal cell cycle or interfere with mechanisms controlling cell survival. Opiates were found to affect MAPK/ERK pathway that regulate cell cycle and division. In addition, they regulate cellular apoptosis, autophagy and affect anagenesis (Szczepaniak et al., 2020). As possible explanation for increased Ki-67 immunoreactivity in the present study, previous studies reported that tramadol can induce ERK phosphorylation in some body tissues which is known to be able to increase Ki-67 expression (Wang et al., 2006; Sadek et al., 2018). However, this pathway needs to be further assessed.

V. CONCLUSION:

In conclusion, tramadol abuse related taste abnormalities may be attributed to its local effects on tongue structure. These changes are reversible, but it may take relatively long time to return to its baseline characteristics. However, these toxic changes were associated with increase in the proliferative marker Ki-67 indicating that these atrophic changes are non-Ki-67 dependent.

VI. RECOMMENDATION:

It is recommended to study other possible pathways that can mediate the illustrated pathological changes as MAPK/ERK pathway. In addition, assess the taste functions in tramadol abusers at the level of peripheral perception.

VII. FUNDING:

"This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors".

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الملخص العربي

التأثير السمي لإدمان ترامادول هيدروكلوريد وانسحابه على البنية النسيجية المرضية للّسان في جرذان سبراج داولي

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الملخص العربي للبحث

مقدمة البحث: يعد اضطراب التذوق بين متعاطي ترامادول هيدروكلوريد في السنوات القليلة الماضية من الأعراض الشائعة. **الهدف من البحث:** تهدف هذه الدراسة إلي تقييم التأثير السمي لتعاطي ترامادول هيدروكلوريد على التكوين الخلوي للّسان بما في ذلك براعم التذوق ورجوع هذه التغييرات لطبيعتها بعد الإيقاف. بالإضافة إلى دراسة التأثير على Ki-67 كعلامة عامة للتكاثر الخلوي. **طريقة البحث:** تم تقسيم اثنين وأربعين ذكر بالغًا من جرذان سبراج داولي إلى ست مجموعات، المجموعة الأولى تلقت محلول ملحي طبيعي، المجموعات الثانية والثالثة تلقت ترامادول هيدروكلوريد ٤٠ مجم / كجم عن طريق الفم لمدة ٢٠ و٣٠ يومًا على التوالي، المجموعات الرابعة والخامسة والسادسة تلقت ترامادول هيدروكلوريد ٤٠ مجم / كيلوغرام عن طريق الفم لمدة ٣٠ يوما ثم تلقت المحلول الملحي العادي فقط لمدة ١٠، ٢٠، ٣٠ يوما على التوالي. تم ذبح الجرذان وتم تشريح ألسنتهم. **النتائج:** في الفحص النسيجي، تبين تشويه الظهارة المغطاة والحليمات الفطرية والخيطية وبراعم التذوق وطبقة العضلات في المجموعة الثانية. في المجموعة الثالثة، يحدث المزيد من الضرر مع الاختفاء التام لبراعم التذوق. عند التوقف عن ترامادول هيدروكلوريد، بدأت الاستعادة التدريجية للظهارة والحليمات وبراعم التذوق وبنية الألياف العضلية في المجموعات الرابعة والخامسة. في المجموعة السادسة، تمت استعادة معظم السمات النسيجية، ولكنها لم تعد إلى وضعها الطبيعي. فيما يتعلق بالصبغة المناعية Ki-67، زادت منطقة التفاعل في أنسجة اللسان مع التعرض للترامادول هيدروكلوريد ثم بدأت في الانخفاض عند توقفها، ولكنها لم تعد إلى وضعها الطبيعي لمدة تصل إلى ٣٠ يومًا بعد الانسحاب. **الخلاصة:** نستخلص مما سبق أن ترامادول هيدروكلوريد يتسبب في تغير شكل أنسجة اللسان وبراعم التذوق، وهذه التغييرات قابلة للرجوع لطبيعتها عند الانسحاب. بالإضافة إلى ذلك، لا تعتمد هذه التأثيرات السامة على مسار Ki-67.