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HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY ON THE EFFECT OF LONG TERM TREATMENT WITH AMADOL (TRAMADOL HYDROCHLORIDE) ON THE LINGUAL MUCOSA IN ALBINO RATS

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ABSTRACT

Objectives: This study aimed to evaluate the effect of Amadol (Tramadol hydrochloride) on the lingual mucosa of male albino rats through histological examination of their lingual mucosa to detect any structural changes, scanning electron microscopic examination of their lingual mucosa to detect any ultrastructural changes and immunohistochemical localization of proliferating cell nuclear antigen (PCNA).

Materials and methods: Thirty adult male albino rats divided into 2 group. Group I (10 animals) served as control. Group II (20 animals) received Amadol (tramadol hydrochloride) in daily oral dose of 60 mg/kg body weight using metallic curved oropharengeal tube for 4months. **Results**: The light microscopic examination of the tongue of the rats treated with Amadol drug showed degenerative changes that involved the surface epithelium and lamina propria of their lingual mucosa. The immunohistochemical results of the tongue of Amadol group showed weakly positive PCNA staining reactivity of the nuclei of the basal and parabasal cells of the surface epithelium. Scanning electron microscope results indicate the histological and imunohistochemical results, as showed marked degenerative changes of dorsal surfaces of the tongue.

Conclusion: Tramadol prescription, after weighing its risks and benefits, should be used with limits and under supervision**.**

KEY WORDS: Tramadol, lingual mucosa, PCNA

INTRODUCTION

Tramadol hydrochloride is a centrally acting synthetic opioid (morphine like drugs), acts through a dual mechanism of action [1] It stimulates the mu-opioid receptors or inhibits the serotonin and norepinephrine reuptake which explains its antidepressant effects[2]. Tramadol used for the treatment of moderate to severe pain[3,4]. When taken as an immediate-release oral formulation, the onset

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of pain relief usually occurs within about an hour and 2–4 hours to peak[5,6]**.** Tramadol effects similar to those of codeine and 10 times less than morphine[7].

Tramadol has lower susceptibility of addiction than morphine, it is commonly used for patients in the postsurgical period and also in patient with chronic pain syndromes like rheumatoid arthritis [8], renal colic pain [9], back pain [10], neuropathic pain and fibromyalgia [11].

Clinically active tramadol is a racemic mixture of two enantiomers that have two distinct but complementary mechanisms of action: the (+) tramadol is a selective agonist of mu-opioid receptor which preferentially inhibits serotonin reuptake and enhances serotonin efflux in the brain, whereas the (-) enantiomer mainly inhibits noradrenaline reuptake [12]. In fact the major metabolite of both enantiomers of tramadol (O-desmethyl Tramadol) is more potent to stimulate mu-opioid receptor than the parent compound [13,14]. In addition to its opioid actions, tramadol also inhibits the neuronal re-uptake of nor adrenaline (NA) and serotonin (5-HT)[15].

Tramadol was approved for marketing as a non-controlled analgesic in 1995 under the trade name of Ultram. Although the producing company claimed that this substance produced weak narcotic effects, researches demonstrated that opioid activity is one of the drug's pharmacological activity. Many physicians felt that this drug was safe to prescribe, because tramadol's products had inadequate labeling and established abuse potential. As a consequence, numerous reports of abuse, dependence and side effects had been received [16].

Opioid dependent drugs are recommended in severe dental and paradental pain as in cases of deep dental caries and periodontitis. They are central analgesics that act on the CNS (central nervous system) and PNS (preipheral nervous system). Opioids showed a significant increase in the level of anesthesia. The associated use of opioids improved anesthesia efficacy. This could lead to a new perspective in controlling dental pain**[17]**

Tramadol is an effective and well tolerated agent to reduce pain resulting from trauma, renal or biliary colic and labor, and also for the management of chronic pain of malignant or nonmalignant origin, particularly neuropathic pain[15].

Tramadol toxic effects should be kept in mind during long-term therapy especially in large doses. Wide-spread use of tramadol makes associated hepatotoxicity a clinically and economically important problem. Tramadol and its metabolites are excreted via kidneys, consequently the kidney is considered to be the primary target organ for tramadol toxicity[18]**.**

The most notable side effects of tramadol include serotonin syndrome/toxicity, seizures (including lowered seizure threshold), respiratory depression, and increased intracranial pressure and anaphylactic reactions.(15)

Although some evidence suggests there is less risk of respiratory depression with tramadol, the symptoms of tramadol overdose are similar to other centrally acting analgesics and include coma, convulsion, respiratory depression, respiratory arrest and cardiovascular collapse [19].

Tramadol is metabolized to its principal active metabolite M1, through O-demethylation by cytochrome p450 (CYP) isoenzyme 2D6 (CYP2D6). Poor metabolites of medicines via CYP2D6 may therefore get less benefit from tramadol use due to reduced formation of the active metabolite [20].

Common adverse reactions include nausea, vomiting, constipation, dizziness, autonomic nervous effects (mainly dry mouth, perspiration), headache, sedation, asthenia (weakness) and fatigue [15].

The administration of tramadol as an atypical opioid analgesic in a single dose or multiple doses impairs the memory in rat. The observed effects can

be justified according to the unique properties of tramadol in the opioid family. It shows an inhibitory effect on a wide range of different neurotransmitters and receptors including muscarinic, NMDA, AMPA as well as some second messenger like cAMP and cGMP or its stimulatory effect on the opioid, GABA, dopamine or serotonin in the brain. More studies are requires to discover the main mechanisms involved in the inhibitory effect of tramadol on the memory[21].

Numerous studies have indicated the effect of opioids on the metabolism of bone Opioids exert their effect through opioid receptors that are located on the cells of the central nervous system and other tissues [22]. The effect of tramadol hydrochloride on orthodontic tooth movements (OTM) depends on the dosage used, high doses of the drug reduce the extent of OTM significantly [23]. Tramadol induced oral dryness results in saliva with preserved protein concentration but with decreased IgA concentration [24] .tramadol exerts its principal xerogenic effect by activating inhibitory pathways in the central nervous system and has no anticholinergic effect on the salivary glands at dosages that may be clinically relevant. Furthermore, the tramadol-induced increase of the acetylcholine-evoked secretion occurred at a glandular level and depended most likely on a release of noradrenaline from glandular nerve terminals [25].

MATERIALS AND METHODS

Thirty adult male albino rats, 3 months old with body weight range from 180-200 gram were used in this investigation. They divided as follows:-

Group I: consisted of 10 animals and served as controls.

Group II : consisted of 20 rats and served as the experimental group, they received Amadol (tramadol hydrochloride) in daily oral dose of 60 mg/kg body weight[26] using metallic curved oropharengeal tube for 4months.

The animals were kept in specially designed cages, 5 animals per cage. They were fed natural diet and given drinking water adlibitum. They were kept under proper condition of temperature and ventilation.

At the end of the experiment the animals of the different groups were sacrificed by cervical dislocation. Their tongues were dissected out, cut longitudinally into two halves, fixed in 10% neutral buffered formalin, processed to be embedded in paraffin. Six microns thick sections were cut to be stained with:

1- Hematoxylin and eosin for histological examination.

2- Immunohistochemical localization of PCNA.

Representative tongue specimens from the different groups were chosen to be prepared to be examined with scanning electron microscope, using SEM Model Quanta 250 FEG (Field Emission Gun) attached with EDX Unit (Energy Dispersive X-ray Analyses), FEI company- Netherlands, in the central laboratories sector the of the Egyptian mineral resources authority, Gezah – Egypt.

RESULTS

I-Light microscopic results:

A - Haematoxylin and Eosin results:

Control group (Group I): The histological examination of the mucous membrane of the tongue of the control rats showed the normal histological features of surface epithelium and underlying lamina propria. The dorsal surface of the tongue showed the different types of papillae including the filiform, fungiform papillae and circumvallate papillae, **Fig. 1 (A, B, C).**

Group II animals: Light microscopic examination of the tongue of group II rats treated with Amadol presented severe atrophic and degenerative changes that involved the surface

epithelium and lamina propria of both the dorsal and ventral surfaces of the tongue as well as the lingual glands.

The filliform papillae were markedly atrophic, their number and length were apparently much decreased when compared with those of the control animals. Their epithelial covering was atrophic; areas devoid of epithelial ridges were found. The fungiform and circumvallate papillae showed signs of atrophy in their epithelial covering, their lamina propria and taste buds. The later were shrunken, degenerated or completely lost**.** The underling lamina propria showed degeneration and dissociation of collagen fibers with inflammatory cell infiltration. The lingual glands also suffered from marked degenerative changes in the acini and ducts of both the serous and mucous glands. The serous acinar cells presented marked intracytoplasumic vacuolization**.** The mucous acinar cells were also seriously affected with cystic transformations**.** The ducts of both serous and mucous glands were atrophic with marked flattening of their epithelial lining and collection of stagnant secretions forming cyst-like structures. The ventral surface of the tongue showed marked atrophic and degenerative changes in its mucous membrane. The surface epithelium was so thin, atrophic with intracellular cytoplasmic vacuolization. The collagen fibers of the lamina propria showed dissociation with dilatation of blood vessels engorged with blood and inflammatory cell infiltration**. Fig. 1 (D, E, H, I).**

B- Immunohistochemical Results:

Immunohistochemical localization of PCNA:

Control group (Group I): Examination of sections taken from tongue of control rats revealed strongly positive PCNA staining reactivity of the nuclei of the basal and parabasal cells of the surface epithelium indicating normal proliferationof the cells of the dorsal and ventral surfaces of the tongue (Fig.2 A, B).

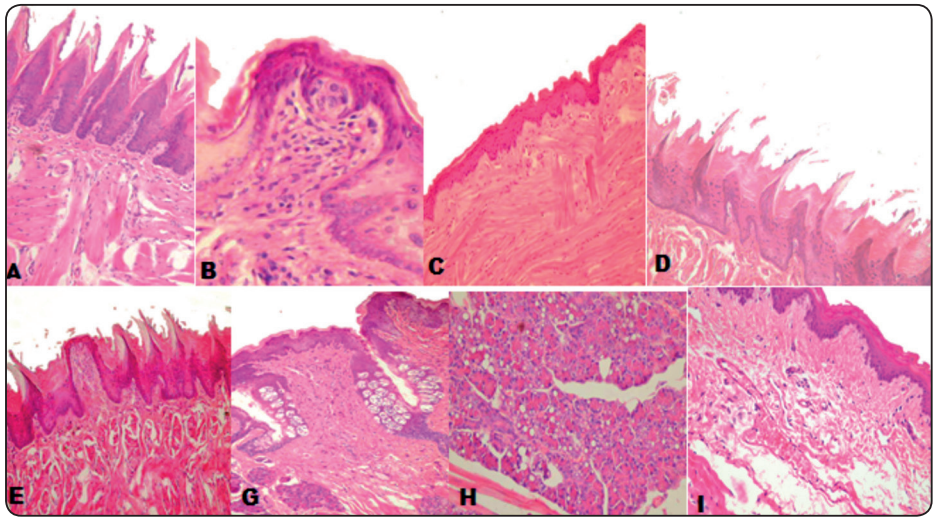

Fig. (1) A photomicrograph of tongue of a control rat showing (A) dorsal surface of the tongue of control with filliform papillae (B) fungiform papilla with taste bud at its superior position (C) the mucous membrane of the ventral surfaces {H&E, orig. mag. 200, 400, 200}. A photomicrograph of group II rats showing (D) atrophied filliform papillae with hyperkeratosis and areas of degeneration of underlying collagen fibers (E) atrophied fungi and filliform papillae (G) showed circumvallate papillae with degenerated taste buds (H) serous acinar cells with cytoplasmic vacuolization (I) ventral surfaces of tongue showing degenerated epithelial surface with disassociation and degeneration of under laying collagen fibers of lamina propria {H&E, orig. mag. 200, 200, 400, 200, 200}

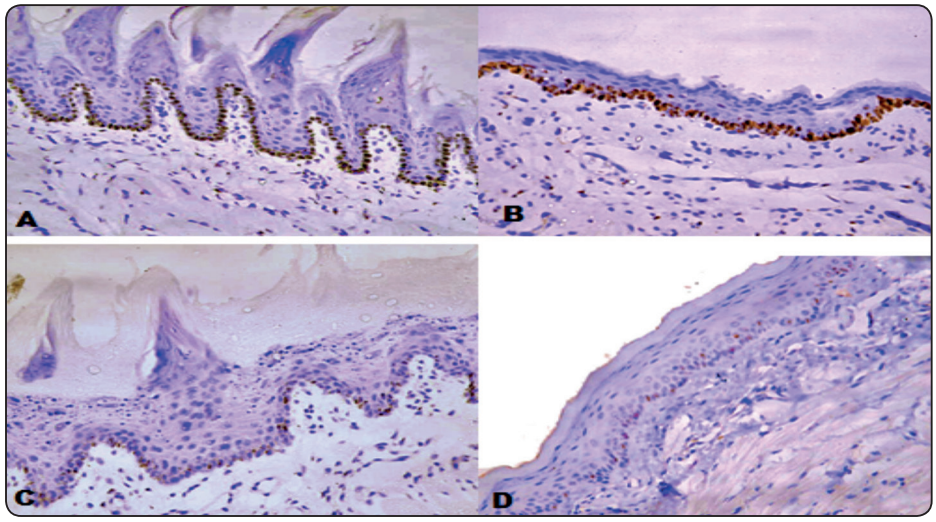

Fig. 2 (A) A photomicrograph of the dorsal surface of the tongue of a control rat showing strongly positive staining PCNA reactivity of nuclei of the basal and suprabasal cells.(B) ventral surface of the tongue showing strongly positive staining PCNA reactivity of its basal and suprabasal cells {Orig mag. 200,200}. A photomicrograph of the dorsal surface of the tongue of group II animal showing (C) weakly positive reaction of the surface epithelium to PCNA. (D) The ventral surface of the tongue showing weakly positive staining reaction of the basal and suprabasal cells to PCNA {Orig mag. 200,200}. .

Group II animals: Light microscopic examination of the tongue of the rats which subjected to Amadol in a dose of 60 mg/kg body weight showed weakly positive PCNA staining reactivity of the nuclei of the basal and parabasal cells of the surface epithelium (Fig.2 C, D)

TABLE (I) Illustrates the mean labeling index of PCNA in the surface epithelium of the lingual mucosa at the different groups:

Group	Leballing index of PCNA
Group I (Control group)	123.1 ±15.9
Group II (Amadol group)	39.8 ± 6.4

Statistical analysis

The significance of the results was assessed by determining the probability factor (P value) where P < or= 0.05 is considered significant.

There was significant decrease in PCNA labeling index of the surface epithelium of the gingiva, dorsal and ventral surfaces of the tongue of group II animals compared with the controls. The analysis of variance clarified a high significant differences ($p<0.01$) between Amadol group rats and control group

This means that the PCNA staining of the surface epithelium of the lingual mucosa was decreased after administration or amadol.

II- scanning electron microscope of the dorsal surface of the tongue:

Control group: The dorsal surface of the tongue of the control group showed four types of papillae: filiform, fungiform, circumvallate and foliate.The most numerous papillae were the filiform papilla, which covering the apex and body of the tongue. They were simple conical or branched, curved principallytowards the posterior region. The fungiform papillae projected among the filiform ones, they were dome-shaped or mushroom shaped and wider in diameter than the filiform ones. The epithelial cells appeared as scales on the surface with a depression in the center of their upper surface

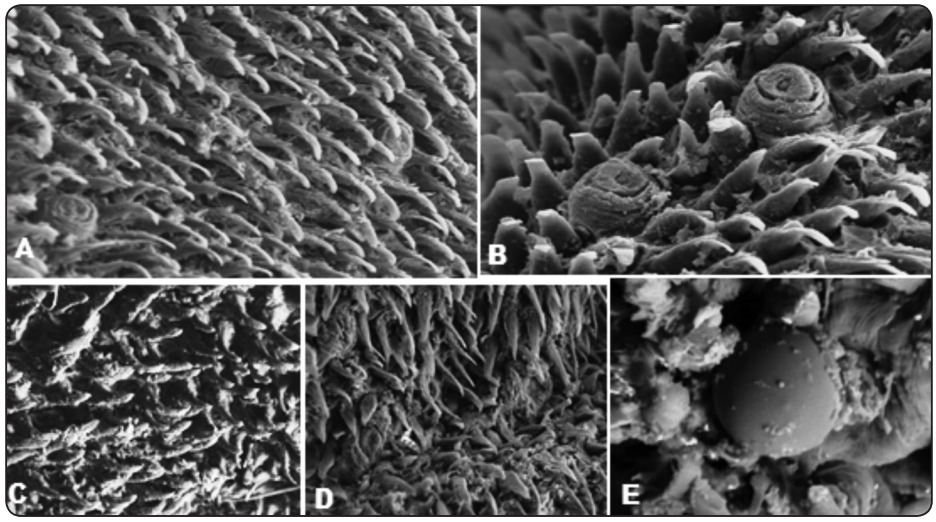

Fig. (3) Scanning electron micrograph of the tongue of control group showing (A) sharp and regularly parallel rows of long conical filiform papillae with keratinized tip and fungiform papillae in between (Mag. X130). (B) Showing fungiform papillae covered by several regular epithelial cells, well defined gustatory pore surrounded by shallow indentation in its center (Mag. X 250). Scanning electron micrograph of the tongue of group II rats showing (C) disorientation and desquamation of filliform papilla. The interpapillary ridges are irregular and heavily keratinized (Mag. X 150). (D) Shrunken circumvallate papilla (Mag. X 150). (E) desquamated fungiform papilla, losing its characteristic mushroom shape with depressed gustatory pore, Interpapillary ridges are heavily keratinized (Mag. X 600).

(gustatory pore). On the root of the tongue, oval or rounded circumvallate papillae were found, they were situated symmetrically and obliquely to the median line of the tongue. The body of these papillae was limited by a deep circular sulcus andthis area was surrounded by less numerous filiform papillae (Fig. 3A&B).

Group (II): The dorsal surface of the tongue of the rats of group II (Amadol group) showed loss of the normal appearance of filiform papillae as they appeared severely destructed with apparent decrease in their thickness, blunt eroded tips of some filiform papillae were also observed. They were randomly distributed, irregularly arranged. The fungiform papillae showed marked decrease in their size, lost their characteristic mushroom shape and revealed ill-defined taste pores. The circumvallate papillae appeared shrunken with marked reduction of their size (Fig. 3 A,B,C,D.E).

DISCUSSION

The current study was carried out on tramadol as an opioid analgesic drug that is increasingly used or abused for several purposes in a wide range of people. Tramadol belongs to the opioid analgesic drugs acting with different mechanism of action [27,28] and is classified as a Class II drug for the treatment of moderate intensity pain according to the WHO recommendation [29] .

The histological results of the present investigation demonstrated that tongue of group II rats that received Amadol (tramadol hydrochloride) in daily oral dose of 60 mg/kg body weight showed severe atrophic and degenerative changes in the oral mucosa of male albino rats due to disturbance in cellular homeostasis resulting in adverse changes in the structure of lingual mucosae. These degenerative changes manifested as marked decrease in the thickness of keratinized stratified squamous epithelium, the epithelial ridges were too

short, scanty and in most cases lost their normal pattern with possible total absence in multiple areas. The dorsal surface of the tongue revealed marked decrease in number, shape and height of their papillae. Taste buds in the fungiform papillae and circumvallet papillae were atrophied and showed degeneration. Hyperkeratosis of the keratin layer was observed.

Li et al., (2005)[30] reported that normal terminal differentiation is associated with increased keratohyaline granules and a reduction in nuclei in superficial layer cells. The amount of keratohyaline granules determines the extent of the keratinization process. The author added that the decrease or absence of keratohyaline granules is associated with abnormal terminal differentiation (parakeratinization)

According to the previous research hyperkeratosis of present study may be due to disturbance in the rate of epithelial turnover as a result of present degeneration that may affect the keratohyaline granules leading to abnormal desquamation and renewal of the surface epithelium causing the present hyperkeratosis.

Scanning electron microscopic examination of the tongue of the hypothyroid animals revealed disoriented filliform papillae which appeared less in number and thickness compared with those of the controls, also the fungiform papillae lost their mushroom like shape and the taste buds appeared ill-defined or even completely lost. . The circumvallate papillae appeared shrunken with marked reduction of their size these finding confirm the present histological results.

The histological alterations of tissues under investigation indicate that their surface epithelium suffered cellular failure as a result of administration of tramadol hydrochloride.

The subepithelial lamina propria showed marked degeneration and dissociation of collagen fibers which revealed destruction of the underlying connective tissue fibers. This may be due to degenerative changes that occurred in the fibroblasts, causing failure or defective collagen synthesis. In addition to collagen destruction there was inflammatory cell infiltration in connective tissue which may be a response to products released upon the death of fibroblast cells. The hypoxia and ischemia of the tissue cannot be neglected, since the blood vessels showed dilatation and engorgement with RBCs resulting in possible stasis and decrease in blood flow.

Lingual salivary gland showed marked degeneration in both serous and mucous acini with a lot of intracytoplasmic vacuoles. These vacuoles that appeared in secretory cells as well as duct system may be due to intracytoplasmic degeneration of organelles leaving empty spaces, or accumulation of fat leaving empty spaces on processing[31]. Cystic transformation of some ducts and mucous acini was obvious.

Proliferating cell nuclear antigen (PCNA) is useful marker of proliferating cells because its expression and distribution correlate with cellular proliferating rate [32]. Immunohistochemical expression of PCNA increases during G1- phase, peaks at the S- phase and declines during the G2/M phase of the cell cycle. Anti- PCNA antibodies provide an appropriate method for clarifying all phases of the cell cycle of proliferating cells. PCNA being involved in DNA repair, suggesting that it may be expressed by cells that are not cycling [33].

From immunostaining with anti PCNA antibody; the PCNA labeling index was significantly decreased in group II animals that received Amadol. This suggests a possible delay in the rate of turnover and cell renewal that may result in thinning of the lingual mucosa of the rats.

Our result are agreed with **Youssef, 2015** [34] who studied the effect of long term tt with amadol on the gingiva and buccal mucosa in rats and revealed

histopathological changes manifested as atrophy and degeneration in the surface epithelium, lamina propria and buccal salivary gland of expermintal rats.

Taha, 2015 [35] also reported that long term exposure to Amadol hydrochloride resulted in marked degenerative changes in dental pulp of molars and growingincisors and demonstrated immunohistochemistry that there was decrease in the positivity of VEGF and fbronectin compared to control ones , upon the ultrastructural level the author demonstrated cytoplasmic vacolizationof fibrobalsts and abnormal homogenization of their nuclear chromatin, marked dilatation of blood vessels and destruction of collagen fiber. These results also come agreed with the results with present investigation

Tramadol is one of the synthetic opoids that has toxic effects at the cellular level by increasing lipid peroxidation that can be used as a marker of the reactive oxygen species (ROS)- induced cell damages[36]**.** Another study, indicated that exposure to opioid receptor agonists increases their liability to death by apoptotic mechanisms [37]. In addition, **Bodera et al., 2013**[38] have been revealed that chronic morphine (opoids) administration in rats is associated with significant changes in the principle proteins involved in the apoptosis signaling which collectively leads to induction of apoptosis.

According to previous researches we can suggested that long term administration of tramadol may cause a traumatic cell injury resulting in cell necrosis or initiating the cell apoptosis which responsible for noticeable degenerative changes in the lingual mucosa of the rats of present study. the limitation of the present study that we should use apoptosis marker to distinguish if the degeneration that occurred in the lingual mucosa as a result of initiation of apoptosis process or as result of necrosis .

Batta. (2016)[15] stated that most common adverse effect of tramadol include sadation, dizziness, nausea, constipation and dry mouth.

According to the previous study we can also suggested that tramadol administration may leading to dry mouth which limits the washing effect of the saliva on the oral cavity, consequently this lead to cracking and dryness of the oral mucosa including tongue that in turn by time causing the observed atrophy and degeneration of the tongue.

We should also consider the adverse direct toxic effect of the Amadol drug on the lingual mucosal tissue that may lead to the present degeneration.

The results of the present investigation are supported by various studies on other tisuues. **Atici et al.**[39] who observed renal tubular vacuolization, mononuclear cell infiltration, focal necrosis and hemorrhage as well as an increase in creatinine levels in rats receiving opioids. These observations can be considered as evidence of renal damage. Similar results were reported by **Elkhateeb et al.**[40], who found atrophied glomerulus with collapsed tuft, wide Bowman's space, degenerated tubules, cellular infiltration and hemorrhage in tramadol treated group. **Elmanama et al.**[41] suggests that the long-term use of tramadol has negative impacts on kidney functionality. Also **Saleem et al., 2014**[42] revealed that tramadol has damaging effects on liver tissues,

Azari et al, 2014 [43] reported that seminiferous tubules. Basement membrane was interrupted. The germinal epithelium of affected tubules appeared disorganized and spermatogenesis was reduced. The affected tubules showed necrotic spermatocytes with nuclear changes such as karyolysis and karyorrhexis and were absence of spermatids and spermatozoa. Germinal cells of some tubules were sloughed and scattered into the lumens. Intra-epithelial spaces were increased. Some sertoli cells had large cytoplasmic vacuoles.

According to the pervious opinions and investigations tramadol is a synthetic opioid analgesic, it is commonly prescribed for moderate to severe pain, becoming abused more popular among teens in most countries after weighing the possible risks and benefits, its use should be in limits and under supervision

CONCLUSION

Tramadol has a degenerative effect in the lingual mucosa of the tongue.

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