

TNF- α LOCALIZATION IN THE DENTAL PULP OF ALBINO RATS RECEIVING LONG TERM TREATMENT WITH TRAMADOL HYDROCHLORIDE

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ABSTRACT

Objectives: This investigation estimated the effect of Tramadol hydrochloride on the dental pulp of young male albino rats through histological examination and immune histochemical localization of TNF- α .

Materials and Methods: The study sample consisted of 30 young male albino rats which were divided into two groups; the control group (Group I) included 10 rats and the treatment group (Group II) included 20 rats and were given an oral dose consisting of 60 mg/kg body weight Amadol (tramadol hydrochloride) daily for a period of 4 months.

Results: The light microscopic examination of the dental pulp of the rats received Amadol revealed marked structural degenerative changes in the dental pulp. The immunohistochemical results of the dental pulp of Amadol group showed strongly positive **TNF- α** staining reactivity of the odontoblasts, fibroblasts, collagen fibers and blood vessels indicating the degenerating process.

Conclusion: long term use of Tramadol has destructive effects on dental pulp so, it must be prescribed and used with precautions.

Key words: Tramadol; dental pulp; TNF- α .

INTRODUCTION

The German company Grünenthal GmbH has launched Tramadol under the name of "Tramal" in 1977 and it was launched later after two decades in the United Kingdom, United States, and Australia¹. Under several names including Ultram, Zytram, Amadol and others, this medication is sold².

In addition, it is an opioid pain medication which is used for the treatment of pain ranging from moderate to moderately severe³. After taking this medication orally, it results in relieving the pain usually within an hour⁴. Further, the medication is used in combination with Paracetamol in order to increase its efficacy in relieving the pain⁵.

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The μ -opioid receptor to which it binds in order to achieve its desired results. Furthermore, Tramadol is considered a serotonin–norepinephrine reuptake inhibitor (SNRI)⁶ and it is classified in the class of benzenoid and as the desmetramadol is considered a more powerful opioid, the Tramadol is transformed to it in the body⁷.

When compared to the same dose of Morphine, the power of Tramadol reaches one tenth of that of morphine. Also, it is nearly considered as potent as pethidine and codeine⁸.

When it is used to treat moderately severe pain, it is considered as potent as morphine but in the type of severe pain, it is considered less potent than morphine as its analgesic effects can continue for about 6 hours⁹.

Moreover, the forms of Tramadol include liquids, syrups, drops, and effervescent tablets, powders which are used to be mixed with water, capsules, and tablets including extended release formulations, suppositories and injections¹⁰.

The drug shouldn't be used in case of pregnancy due to the fact that it could result in some reversible withdrawal effects in the newborn and it shouldn't be used during labor as its onset of action occurs within an hour¹¹.

In addition, the list of FDA includes that it shouldn't be used in case of those who are under 12 years old as they could suffer from the slow and difficulty of breathing¹².

In the USA, it was reported that the addition of Tramadol reached 21.5% in students of eighth grade and 39.8% in students of tenth grade¹³.

In Egypt, alcohol and drugs are illegal by religious conviction and law while tobacco is accessible even for adolescents¹⁴.

During the teen years, young people often suffer from emotional, societal, and somatic catastrophes, they become risky for drug abuse or even addiction¹⁵.

It was found that in 2014, the prevalence of substances abuse among young adolescent school students in Zagazig was 8.8% (tramadol 83.3%, cannabis 27.8%, and alcohol 16.7%)¹⁶.

Another Egyptian study reported that widespread use of tramadol (32.1%) among children and adolescents who presented to the Emergency Unit of the Poison Control Center of Ain Shams University Hospitals (PCC-ASU), Egypt, for toxicological assessment. The widespread of tramadol due to its availability without prescription, its easy illegal smuggling and its cheap prices¹⁷.

Teenagers who abuse drugs may have a higher risk of evolving an addiction when they became adults. Also, can have long-term cognitive and behavioral effects since the teenage brain is still developing¹⁷.

An increase in the risk of respiratory depression, falls, cognitive impairment and sedation is found in old-aged people and therefore it should be carefully used in patients with liver or kidney failure as it is metabolized in the liver and eliminated by the kidneys.¹⁸

Tramadol has side effects and the most common ones are nausea, dizziness, dry mouth, indigestion, abdominal pain, vertigo, vomiting, constipation, drowsiness and headache, and it was compared to other types of opioids, it was found that respiratory depression and constipation are less problematic with tramadol¹⁹.

The overdose of tramadol has risk factors including depression, addiction and seizures and only Naloxone can partially result in reversing the toxic effects of its overuse but it may result in an increase in seizure risk²⁰.

The administration of chronic opioid can result in inducing the tolerance of immunity despite the enhancement of immune function by Tramadol, unlike other types of opioids.²¹

Long term exposure to tramadol hydrochloride also resulted in devastating histological, immunohistochemical, and ultrastructural changes in the dental pulp which get partially improved after 4 weeks recovery period²².

TNF- α increased expression is usually associated with degenerating conditions, as those encountered with long term exposure to tramadol hydrochloride. Also its expression alone can produce inflammation similar to pulpitis and osteitis²³.

Tramadol does not affect osteoclastic activity and bone resorption and does not cause to change the resulted root resorption either if used in therapeutic doses, but with higher doses can reduce orthodontic tooth movement in rats²⁴.

The type of cells which signal protein (cytokine) involved in systemic inflammation include Tumor necrosis factor (TNF) which is also considered one of the cytokines forming the acute phase reaction. Although the Tumor necrosis factor (TNF) is generally produced by activated macrophages, CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons also produce it²⁵.

The TNF plays a primary role in regulating the cells of immunity and as an endogenous pyrogen, it can result in inducing fever, apoptotic cell death and inflammation. Moreover, it has the ability to prevent tumorigenesis and viral replication and via IL1 & IL6 producing cells, it responds to sepsis. Its production dysregulation is included in set of human diseases such as Alzheimer, cancer²⁶, major depression²⁷, psoriasis²⁸ and inflammatory bowel disease (IBD).²⁹ However, studies linked depression and IBD to the levels of TNF³⁰. In the context of malignancy and parathyroid hormone, the production of TNF could result in secondary hypercalcemia and cancers which are associated to its overproduction²⁶.

Tramadol is the most commonly abused substances among teenagers in Egypt¹⁶, so we

should assume that some of them may be drug abusers.

This research was a pioneer and innovative one in investigating the relation between long term abuse and dramatic changes encountered in dental pulp in young adolescents.

The null hypothesis was that tramadol has no effect on dental pulp.

MATERIAL AND METHODS

A total thirty young male albino rats were used in this study. In order to obtain sufficient sample size, Using alpha (α) level of 0.05 and Beta (β) level of 0.05, the size of effect was 1.24 which means that its power – 95% and the calculation of sample size was carried out using the version 3.1.9.2 of G*Power statistical analysis software³¹.

Those rats divided as follows:

Group I (control): consisted of 10 young male albino rats.

Group II (experimental): consisted of 20 young male albino rats, they received Amadol (tramadol hydrochloride) in daily oral dose of 60 mg/kg body weight³² using metallic curved oropharyngeal tube for 4 months.

Inclusion criteria³³:

1. Young male albino rats (3 months old).
2. Body weight ranges from 180-200 grams.
3. Good overall health with normal physical examination of rats.

Exclusion criteria:

1. Unable to meet the inclusion criteria.
2. Current active infection or acute illness of any kind.
3. No medications taking either before or during the experiment.
4. Evidence of malignancy.

The animals were kept in specially designed cages, 5 animals per cage and labelled according to their group. They fed natural diet and given drinking water adlibitum. They kept under proper condition of temperature and ventilation in the animal house at Faculty of Dentistry Suez Canal University, Ismailia, Egypt.

The percentage of mortality for each group were calculated and recorded, gross examination of head and neck of each animal was carried out through the whole experimental period.

At the end of the experiment the rats of the different groups sacrificed by cervical dislocation. Their mandibles dissected out, cut into two halves, fixed in 10% neutral buffered formalin, for 4 hours and washed in the buffer for 24 hours at 4°C then the specimens decalcified in 10% ethylene diamine tetra acetic acid (EDTA) PH 7-7.4.

After complete decalcification, jaw specimens washed in the phosphate buffer and prepared for light microscopic examination. They dehydrated in alcohol, cleared in xylene, embedded in paraffin, sectioned and subjected to:

- 1- Hematoxylin and eosin for histological examination.
- 2- Masson's trichrome for collagen examination.
- 3- Immunohistochemical localization of TNF- α .

Two thirds of the specimens were processed for histological examination and stained with hematoxylin and eosin (H&E) and Masson's trichrome stain for structural examination to find out any possible microscopic changes.

The remaining third of the specimens were stained with Immuno-peroxidase avidin/biotin staining for immunohistochemical localization of TNF-alpha IHC Antibody in the dental pulp incubated by primary polyclonal rabbit anti TNF-alpha IHC antibody and color developed by DAB.

Sections that were stained with H&E, and sections that were stained with Immuno-peroxidase

avidin/biotin staining for immunohistochemical localization of TNF-alpha were selected, examined and photographed with Leica DM 1000 light microscopy and Camera using Leica Application suite-LAS software in the centre of excellence of molecular and cellular medicine (CEMCM), Suez Canal University.

The intensity of the immunohistochemical staining results from different groups were histometrically analyzed using Image J (1.46 a, NIH, USA) computer system (1.46 a, NIH, USA).

Images were manually corrected for brightness and contrast. Color thresholding was then performed automatically after which pictures were converted to RGB stack type. Masking of the brown TNF-alpha, immuno-stain was performed by red color where any brown stain of any intensity was considered positive whereas the background grey stain was considered negative. Area fraction was then calculated automatically representing the area percentage of immune positive cells to the total area of the microscopic field.

All data obtained from histomorphometric analysis was statistically described.

Ethics:

The present study approved from the Research Ethics Committee, Faculty of Dentistry, Suez Canal University code (175/2019).

RESULTS

Light microscopic results:

A) Heamatoxylin and Eosin results:

1-Group I: Control group:-

The pulp tissue was formed of loose vascular connective tissue bound with a layer of specialized cells, the odontoblasts. Odontoblasts were formed of a layer of cells arranged at the periphery of the pulp core and had processes extending to dentin.

Their crowding gave them pseudo stratified appearance. Active odontoblasts appeared long with intensely basophilic cytoplasm, and large basal nucleus. The pulp core showed a uniform arrangement of its cellular constituent. The fibroblasts constituted the major population of pulpal cells. They were large star shape cells with long processes, and large amount of basophilic cytoplasm. Their nuclei contained one or more prominent nucleoli. Collagen fibers embedded in an amorphous ground substance were seen. (Figure 1, A).

Defensive cells as histiocytes, lymphoid cells, plasma cells and mast cells were sometimes found scattered irregularly throughout the pulp. The pulp also contains vascular and neural elements.

2-Group II: Experimental group:

The pulp revealed granulation tissue formation, vasodilatation and marked inflammatory cell infiltration (Figure 1 B, C). Separation of the odontoblastic layer from the dentin was frequently seen (Figure 1, D). Marked pulpal necrosis in the form of massive odontoblastic degeneration, loss of organization of the remaining degenerated cells with marked narrowing of pulp space. Complete absence of pulp tissue could be seen in some sections. (Figure 1, E).

B) Masson's Trichrome stain Results:

1-Group I: Control group:

The pulp tissue examination revealed normal distribution of the collagen fibers of the pulp with strongly positive staining reactivity. (Figure 2 A).

2-Group II: Experimental group:

The pulp tissues examination revealed that the collagen fibers was weakly to moderately positive staining with Masson's trichrome stain. (Figure 2 B, C).

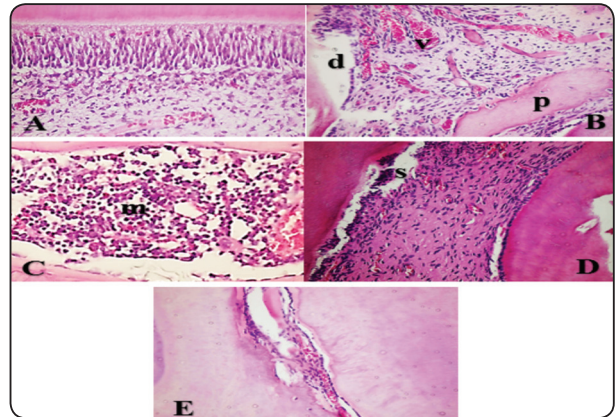


Fig. (1): (A): A photomicrograph of the pulp of the control group showing normal histological structure. (B): The pulp of a rat incisor from the experimental group showing marked vasodilatation, RBCs stagnation & congestion (v), massive odontoblastic degeneration and loss of organization (d) & area of dystrophic calcification (false denticle or pulp stone) (p). (C): Dental pulp of the experimental group showing marked inflammatory cell infiltrate (m). (D): A photomicrograph of the pulp of a rat from the experimental group showing separation of the odontoblastic layer (s). (E): The pulp of from the experimental group showing narrowing of pulp space with generalized pulpal necrosis. (H&E.orig.mag: A, C, E 200 B, D 400).

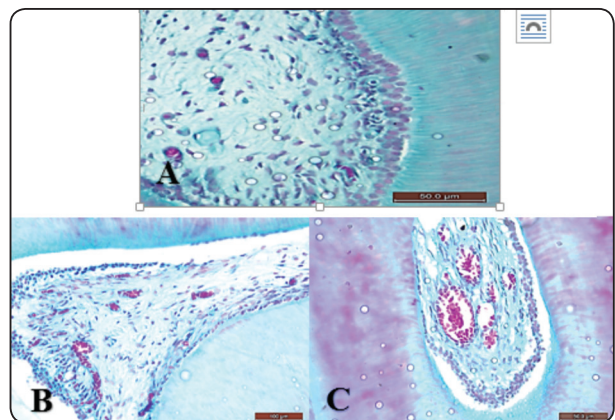


Fig. (2): (A): A photomicrograph of the pulp tissue taken from control group, and stained with Masson's trichrome stain revealed normal distribution of the collagen fibers with strongly positive staining reactivity. (B, C): The pulp tissues of group II collagen fibers showed weakly to moderately positive staining with Masson's trichrome stain. (Masson's trichrome, Origin. Mag. B 125, A, C 250).

II- Immunohistochemical Results:

1-Group I: Control group:

By examining the sections which were taken from the dental pulp of rats in this group, it is found that the **TNF-α** staining reactivity of the cells and fibers was weakly positive. (Fig.3 A, B).

2-Group II: Experimental group:

By examining the dental pulp of rats, using a light microscope, it is found that **TNF-α** staining reactivity of the odontoblasts, fibroblasts, collagen fibers and blood vessels was strongly positive indicating that the process of degeneration. (Fig.3 C, D).

Data collected from photomicrographs of sections taken from dental pulp of groups I & II and stained with **TNF-α**, **their stain intensity mean values were calculated using J image program** using Mann-Whitney (U) Test was employed to perform the analysis with Statistical Package for Social Sciences 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

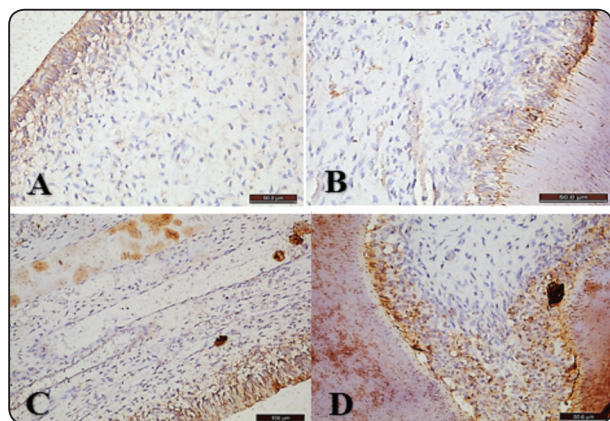


Fig. (3): (A, B): photomicrographs of sections taken from dental pulp of group I showed weakly positive **TNF-α** staining reactivity of the different cells & fibers. (**TNF-α** origin mag. A, B 250). (C, D): photomicrographs of sections taken from dental pulp of group II showed strongly positive **TNF-α** staining reactivity of the odontoblasts, fibroblasts, collagen fibers and blood vessels. (**TNF-α** origin mag. C 125, D 250).

Results in table (1) showed highly significant difference between two groups using Mann-Whitney test, the experimental group had the highest value for **TNF-α** (198.63 ± 5.91) comparing with control group (170.63 ± 11.78).

TABLE (1): Showed the mean, standard deviation and range of the **TNF-α** expression quantification values between control and experimental groups calculated using J image program.

	Control group	Experimental group	Mann-Whitney Test	
			U	p- value
Mean	170.63	198.63	-7.8425	0.00**
SD (±)	11.78	5.91		
Range	0-255	0-255		

******, means significance between groups at ($P < 0.01$)

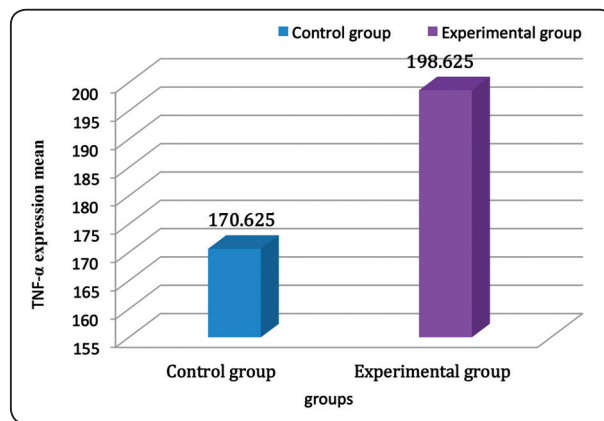


Fig. (4): A bar graph shows the quantification of the **TNF-α** expression mean values between control and experimental groups.

DISCUSSION

Tramadol is a narcotic-like opioid pain reliever that is used to treat moderate to severe pain, even though, it should not be taken if patient suffers from severe breathing problems¹, stomach or intestines blockage, or current usage of sedatives, tranquilizers or narcotic medication².

When Tramadol is misused, it could result in addiction or even death, particularly in case of children or other people who might use it without prescription. In addition, its use in pregnant women could result in the withdrawal symptoms threatening the lives of newborn.¹¹

According to this study, microscopic examination of dental pulp sections obtained from group II (experimental group), revealed granulation tissue formation, vasodilatation and marked inflammatory cell infiltration. Separation of the odontoblastic layer from the dentin was frequently seen marked pulpal necrosis in the form of massive odontoblastic degeneration, loss of organization of the remaining degenerated cells with marked narrowing of pulp space were obvious. Complete absence of pulp tissue could be seen in some sections.

Vasodilatation may be due to the prostaglandin stimulating effect of tramadol.

In terms of flushing and flushing-like symptoms (i.e., diaphoresis and vasodilation) which were found in the administration of Tramadol as related to the dose, these results are considered consistent. However, such symptoms can be reduced using aspirin 30 minutes before tramadol as it inhibits the effect of prostaglandin.³⁴

Some reviews^{35,36} have been discussed the anti-inflammatory and immunomodulatory properties of opioids, specifically tramadol which appears to have immunostimulatory effects. A temporary increase in airway macrophage numbers in mice that received tramadol was also noted³⁷.

Moreover, these findings imply that the effects on chemotaxis are not only drug and dose-specific but may also depend on the anatomic location and the degree of injury as well as the type of inflammatory cell of interest.

These reviews concur with our study which revealed marked inflammatory cell infiltration in group II (experimental group) that received tramadol.

Dilatation of blood vessels and their engorgement with RBCs which results in stasis and decrease in the blood flow are probably the possible causes of hypoxia and ischemia of the tissues that trigger the degenerative effects of tramadol long term use on dental pulp. This finally results in marked pulpal necrosis, odontoblastic degeneration and even complete absence of pulp tissue in some sections.

The TNF α is considered a strong pro-inflammatory agent as it regulates the function of inflammatory cells in several ways. In addition, after trauma or infection, the release of TNF α is fast and it is one of several mediators which are released early in inflamed tissues³⁷. The TNF α plays a primary role in the regulation of production of pro-inflammatory cytokine cascade. Accordingly, it is considered the master regulator of production of pro-inflammatory cytokine³⁸. Moreover, it results in an increase in the mediators of lipid signal transduction including prostaglandins and platelet activating factor³⁹. Considering such roles, it is presented as the master of activation and recruitment of inflammatory cell. Finally, it is suggested to have a fundamental role in developing several chronic inflammatory diseases.⁴⁰

These findings strongly agree with the immunohistochemical localization of TNF- α in the dental pulp group II animals which are subjected to Amadol in a dose of 60 mg/kg body weight for 4 months that showed strongly positive TNF- α staining reactivity of the odontoblasts, fibroblasts,

collagen fibers and blood vessels. Also the highly significant difference between two groups at ($P < 0.01$), as the experimental group had the highest value for TNF- α comparing with control group, indicating the degenerating process in the experimental group.

The results of our investigation also concur with those of another study that held on forty rats receiving a daily oral dose of tramadol Hcl suspended in saline solution equal to 22.5 mg/kg/day for nine weeks (therapeutic dose group), and forty rats that received oral dose of tramadol Hcl suspended in saline solution at doses of 30, 60 and 90 mg/kg/day on the first, second and third three weeks of the study, respectively for nine weeks (over dose group). The obtained results showed significant increase in serum marker (TNF- α) levels in both tramadol therapeutic and overdose groups. These results showed that, chronic tramadol administration induced inflammatory stress in rats as well as degenerating endogenous antioxidant defense system mechanisms⁴¹.

These findings are of great importance to be considered in dental treatment of patients who use tramadol in long term condition, also highly beneficial in the treatment of homeless kids, juvenile detentions center and rehabilitation center kids as those are highly suspected to drug addictions as tramadol.

CONCLUSION

Long term use of Tramadol has destructive effects on dental pulp so, it must be prescribed and used with precautions.

RECOMMENDATION

Researches on dental pulp of extracted premolars for orthodontic reasons in patients who previously or under long term tramadol

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