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HISTOPATHOLOGICAL EVALUATION OF CHOLECALCIFEROL OVERDOSE ON TONGUE STRUCTURE OF ALBINO RATS (IMMUNOHISTOCHEMICAL STUDY)

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ABSTRACT

Introduction: The risk of vitamin D toxicity is tremendously increased due to erroneous prescriptions along with prolonged and overconsumption of vitamin supplementation.

Aim: This study aimed to assess the histopathological changes caused by vitamin D_3 overdose on albino rats' tongue.

Material & Methods: Twenty adult male Wister rats were randomly divided into two groups (n=10): control group and experimental vitamin D_3 overdose treated group (200 IU of vitamin D_3) (1 ml/kg/day Cholecalciferol) for 30 days through oral gavage. Rat tongue was processed for histopathological assessment using routine hematoxylin and eosin and Alizarin red along with immunohistochemical assessment of TNF-α.

Results: Histological examination revealed loss of some filiform papillae of mucosal tongue epithelium, pyknotic nuclei of some cells of its basal layer, focal loss with rarefaction of cytoplasm of certain striated muscle fibers. Increased TNF-α immunoexpression has been detected along with increased calcification as revealed by alizarin red staining compared with controls.

Conclusion: Vitamin D_3 overdose in rats was associated with histological and immunohistochemical changes in the tongue tissues so vitamin D must be taken under medical supervision.

KEYWORDS: TNF-α, Calcification, Papillae, Atrophy, Alizarin red.

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INTRODUCTION

Vitamin D is generated biologically by the human body. Though, it can also be obtained from a variety of animal products and reinforced foods[1], vitamin D deficiency appears to be widespread throughout the world [2], leading to several diseases [3&4]. There are two known types of vitamin D; vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol), and both are necessary for human health[5,6&7]. Even though they are both referred to as "vitamin D," the two have significant important distinctions. Vitamin D_3 plays a role in controlling gene expression and boosting the immune system's capacity to combat bacteria and viruses[8].

Although vitamin D insufficiency is well known, hypervitaminosis D or toxicity is not an uncommon condition that is typically brought on by erroneous prescriptions or the overuse of over-the-counter pills that contain excessive amounts of vitamin D. Unlike insufficiency, toxicity has been linked more to aberrant calcifediol supplementation because excessive synthesis of inactive cholecalciferol in the skin is not comparable to excess calcifediol production. It can also occur less frequently as a result of poisoning from exposure to rodenticides that contain cholecalciferol[9&10].

Moreover, vitamin D toxicity have been linked to renal impairment, kidney stones, and cardiovascular illnesses. Hypercalcemia and its related signs and symptoms are caused by an imbalance in the regulation of bone metabolism[11]. Excessive vitamin D action might contribute to vascular calcification[12], that was identified in arterial calcification by the Red Alizarin stain in mice with defective adiponectin [13]. In addition, calcitriol, a vitamin D metabolite, was reported to cause dispersed calcification of soft tissues in rats [14].

Vitamin D stimulates the transcription of the tumor necrosis factor-alpha (TNF-α) gene, but other cofactors are necessary for TNF-α protein production[15]. TNF-α controls a variety of cell processes, as it modulates epidermal keratinocyte apoptosis, cell motility, cell cycle, immunological and inflammatory responses, and tissue remodeling. TNF-α enhances alkaline phosphate activity leading to vascular calcification[16].

Few histological studies[12&14] have dealt with the toxic histopathological effect of vitamin D on various tissues. Tongue is an enormously muscular organ which plays a role in food passage and sound production apart from its role in taste sensation[17&18]. Also, alterations in its color or texture can act as a mirror for the general health and hence it can be a diagnostic tool for many systemic diseases and underlying disorders. Thus, the aim of this study was to examine the histopathological effect of vitamin D_3 overdose on rat tongue structure.

MATERIALS AND METHODS

Experimental materials:

Cholecalciferol (Vitamin D_3): Cholecalciferol (Three 400 drops) (400 IU/0.5 ml) drops were purchased from Organix food supplements, Sadat city, for smart company, A.R.E.

Animals and study design:

Twenty adult male Wister rats, with an average body weight of 200-250 grams, were purchased from histology department, faculty of medicine, in Tanta University and were left for one week before use for acclimatization in the standard conditioned animal houses under controlled temperature, a 12-hour alternating light/dark cycle. They were fed a standard diet and tap water throughout the experimental period. Rats were randomly divided into two equal groups; control and experimental group each consisted of ten rats (n= 10). Sample size calculation was performed with G Power (version 3.1.9.4, Germany). Rats in the experimental group were treated with 1 ml/kg/day Cholecalciferol (200 IU of vitamin D_3) for 30 days through oral gavage, while rats in the control group received distilled water through oral gavage for the same experimental period [19].

This study was approved by the Ethical Committee of Faculty of Dentistry, Tanta university (#R-OB-12-23-3083) and were conducted in accordance with the guidelines laid down by the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines for conducting animal research.

Rats' euthanasia and sample processing:

At the end of experimental period, rats were anesthetized before scarification with ketamine chloride (Ketalar, 40 mg/kg body weight), Ketalar (par pharmaceutical companies, Inc. suffern, NY, USA). Then they were euthanized by cervical dislocation to minimize pain and discomfort. Tongue was dissected and immediately fixed in buffered formaldehyde (pH: 7.4) for 48 h.

Histological and immunohistochemical study:

After fixation, tongue samples were washed in tap water over night and then dehydrated in ascending grades of alcohol, cleared in xylol, and embedded in paraffin wax (56–58°C melting point). Sections of (5μm) were cut using a rotary microtome (Leica) and then stained with hematoxylin and eosin and Alizarin red[20] for light microscopic examination (LM).

Immunohistochemistry:

Sections were deparaffinized, rehydrated and washed in phosphate-buffered saline (PBS). The specimens were preheated at 100 °C for 10 minutes in citrate buffer using a microwave oven for antigen retrieval (Arçelik MD 554; Arçelik, Istanbul, Turkey). Then endogenous peroxidase activity was blocked via incubation with 3% aqueous hydrogen peroxide. The sections were incubated in blocking solution (10% normal goat serum in PBS) (Thermo Scientific/Lab Vision, Fremont, CA) to block nonspecific staining. Sections then were incubated overnight at 4°C with primary antibodies to TNF-α (Santa Cruz Biotechnology, USA) diluted 1: 100, and then were washed in PBS plus 1% BSA and incubated at room temperature for 45 minutes with biotinylated secondary antibody. After incubation with antibodies, sections were incubated with biotinylated secondary antibody sections. Then were incubated with a horseradish peroxidase-avidin biotin complex; AB complex (Vector Laboratories, USA) for 45 minutes. Sections were washed again, and the reaction was revealed by DAB (Sigma-Aldrich, USA) and finally counterstained with Mayer's hematoxylin. Sections were gradually dehydrated and mounted with coverslips and examined under light microscope by blinded examiners[21&22]. Images were imported to ImageJ software and 5 fields of X40 magnification from each rat tongue were analyzed to calculate the average percentage of TNF-α immunostaining for each rat.

Statistical analysis

The quantitative numerical data that were collected from the measurement of the area percentage of TNF-α immunostaining, were tabulated and presented as mean ± standard deviation (SD). Normality of data was checked using the Shapiro-Wilk test. Comparison of the mean difference of the area percentage between the groups was done by unpaired t-test. The significance level was set as P value ≤ 0.05 is significant. Statistical analysis was performed using statistical package for social sciences (SPSS) version 24, IBM, Armonk, NY, United States of America.

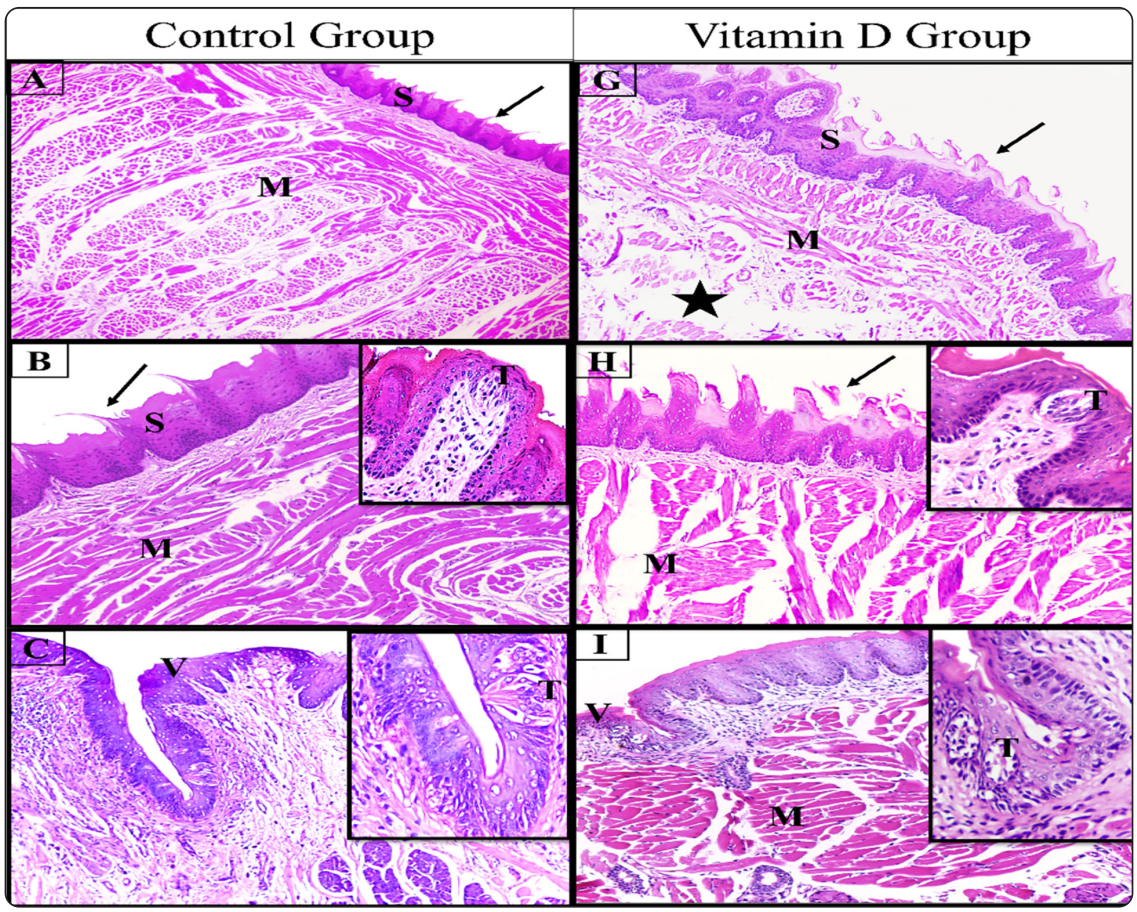
RESULTS

Histological and immunohistochemical results:

Hematoxylin and eosin-stained sections of the dorsal surface of control rat tongue revealed filiform papillae covered by keratinized stratified squamous epithelium with underlying lamina propria. In addition, bundles of striated muscle fibers were arranged in 3 different planes (longitudinal, transverse, and vertical) and were separated by little connective tissue containing normal blood

capillary in-between these fibers. The ventral surface of the tongue were covered with keratinized stratified squamous epithelium. While tongue of the experimental group revealed increased keratinization of stratified squamous epithelium covering dorsal surface with focal loss of filiform papillae in certain sites and atrophy of epithelium over fungiform papilla. Moreover, the basal cell layer of epithelium was disrupted with pyknotic nuclei and perinuclear halo. Striated muscle fibers showed focal loss with rarefaction of cytoplasm in some of them while others revealed atrophy and irregularity with widening of connective tissue in between. Furthermore, there was cuboidal metaplasia of blood capillary with thickening of its wall together with continuous basophilic line on its luminal side. The epithelium of the ventral surface of the tongue appeared thinner, containing hyperchromatic nuclei of basal and parabasal cells. The underlying lamina propria were less fibrous containing more loose delicate fibers along with cuboidal metaplasia of blood capillary. Moreover, degeneration of taste buds of fungiform and circumvallate papillae were detected (Figure. 1).

Sections of tongue stained immunohistochemically by TNF-α showed mild localized nuclear and cytoplasmic reaction of stratified squamous epithelial cells and few connective tissue cells with moderate reaction of striated muscle fibers in control group. On the contrary, experimental group revealed strong generalized cytoplasmic reaction of stratified squamous epithelial cells and connective tissue cells with strong generalized cytoplasmic reaction of striated muscle fibers. Control stained sections of tongue by Alizarin red showed negative reaction of the wall of blood capillaries opposite to positive reaction in the wall of blood capillaries seen in the experimental group (Figure. 2)



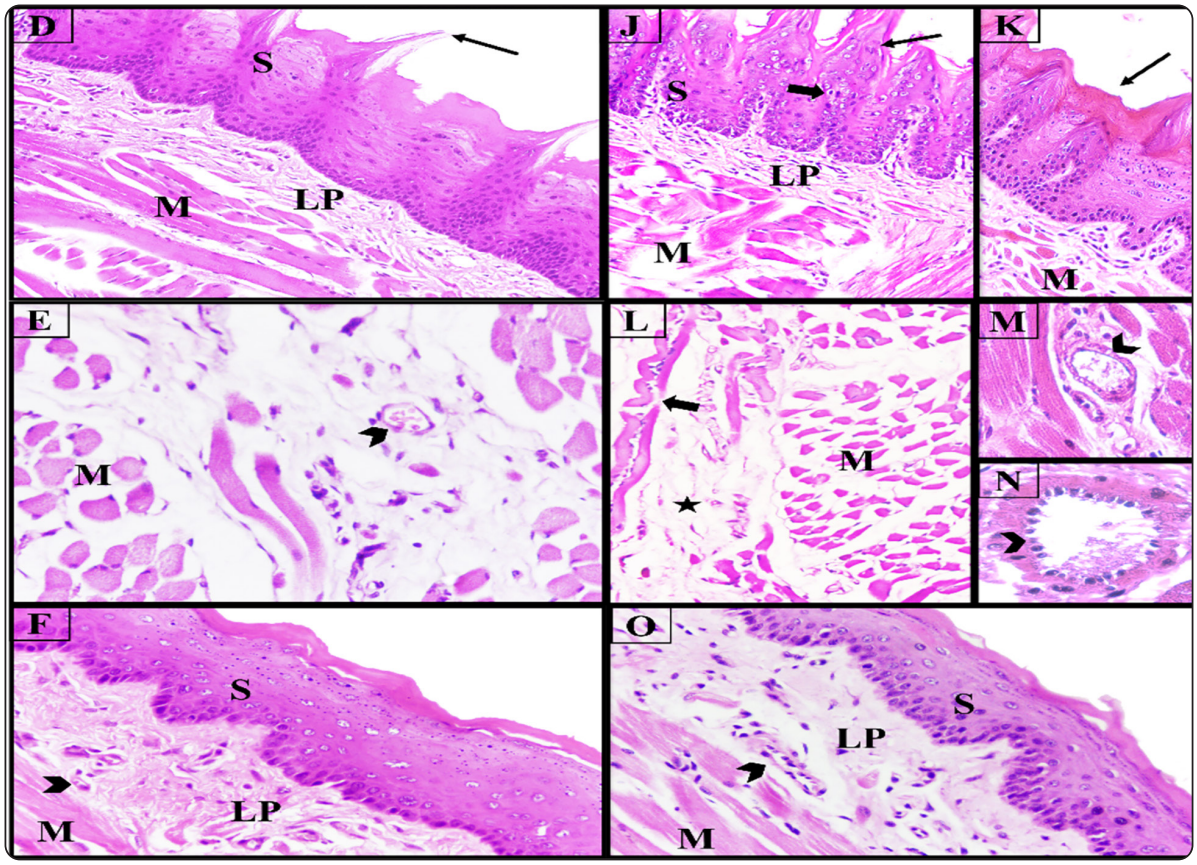


Fig. (1) A photomicrograph of sections of rat tongue of control group **(A,B,C,D,E&F)** showing keratinized stratified squamous epithelium **(S)** covering dorsal surface with filiform papillae **(thin arrows)**, circumvallate papillae **(V),** underlying loose connective tissue of lamina propria **(LP)**, bundles of striated muscle fibers **(M)** arranged in 3 different planes (longitudinal, transverse and vertical) separated by little connective tissue and normal blood capillaries lined by squamous endothelial cells **(arrowheads)** in-between bundles of striated muscle fibers. Small insets showed normal fungiform and trough of circumvallate papillae **(V)** with normal taste buds **(T)**. Normal mucous membrane lining the inferior surface of tongue consisting of keratinized stratified squamous epithelium **(S)**, lamina propria **(LP)** containing normal blood capillary **(arrowhead)**. Tongue of the experimental group **(G,H,I,J,k,L,M,N&O)** showing increased keratinization of stratified squamous epithelium **(S)** covering dorsal surface with focal loss of filiform papillae in certain sites **(thin arrows)**, disruption of its basal cell layer, pyknotic nuclei with perinuclear halo **(thick arrow)**, normal lamina propria beneath the epithelium **(LP)**, focal loss **(*)** with rarefaction of cytoplasm of certain striated muscle fibers **(M)**, atrophy and irregularity **(thick arrow)** of others with widening of connective tissue in between **(*)**, I): reveals atypical shape of circumvallate papilla **(V)**. **M):** reveals continuous basophilic line on its luminal side of blood capillary **(arrowhead)**. **N):** shows cuboidal metaplasia of blood capillary lined by cuboidal cells **(arrowhead)** with thickening of its wall. **Notice:** Small insets showing atrophy of epithelium overlying the fungiform papilla and degeneration of tastebuds **(T)** of fungiform and circumvallate papillae **(V)**. **O)** shows thinner epithelial covering, with hyperchromatic basal and parabasal cells nuclei. Underlying lamina propria are less fibrous and shows more loose delicate fibers along with cuboidal metaplasia of blood capillary **(arrowhead)**. (H&E, **A:** X 4 **- B,C,G,H &I:** X10 - **D,E,J,K&L:** X 20- **F,M,N,O & Insets:** X 40)

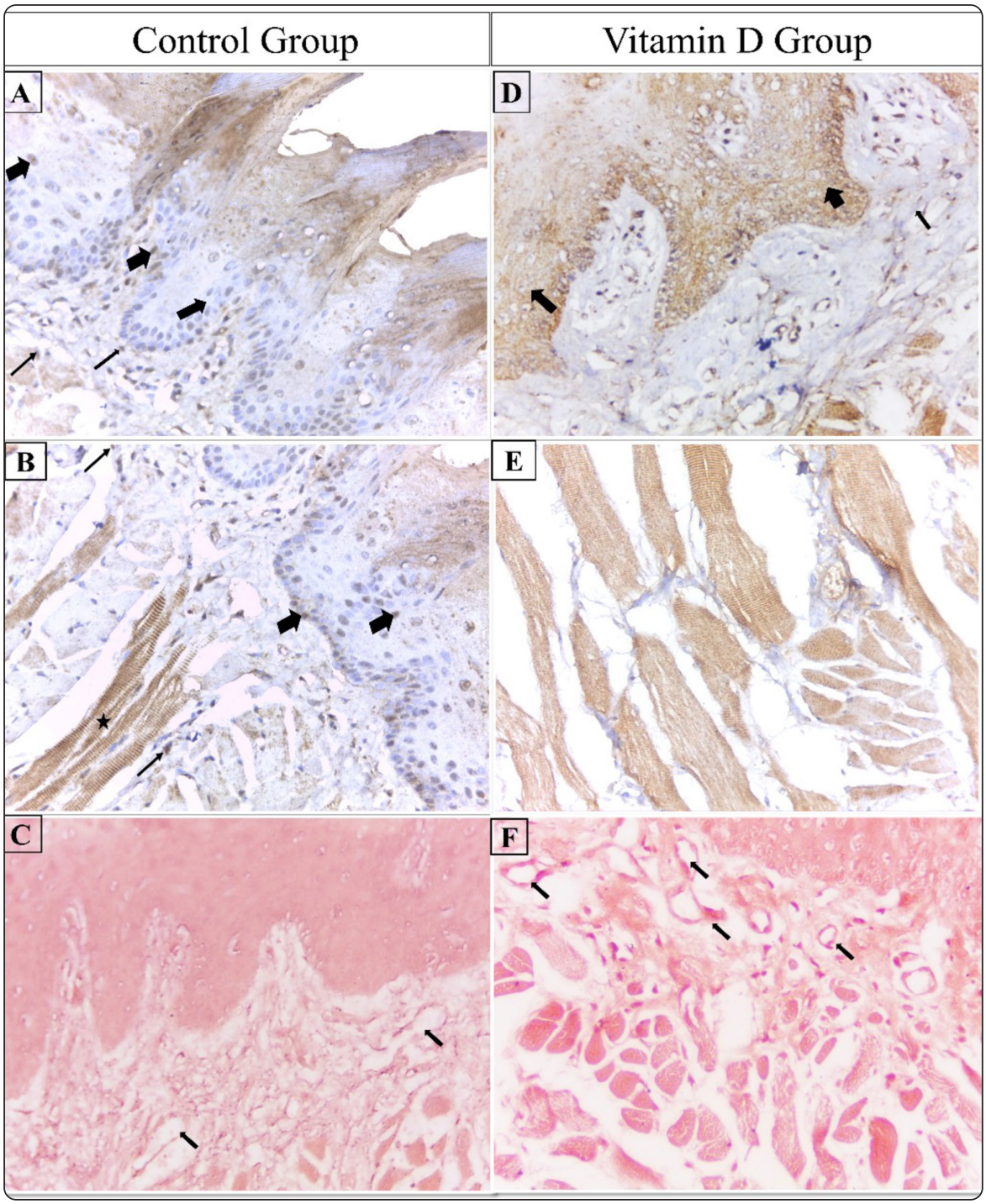


Fig. (2) : A photomicrograph of sections of tongue stained by TNF-α and red stain showing **(A&B):** control group showing mild localized nuclear and cytoplasmic reaction of stratified squamous epithelial cells (thick arrows), few connective tissue cells (thin arrow) and moderate reaction of striated muscle fibers (*). **C)** control group of red stain showing negative reaction of the wall of blood capillaries (thin arrows). **(D&E):** Experimental group showing strong generalized cytoplasmic reaction of stratified squamous epithelial cells (thick arrows), connective tissue cells (thin arrow) and strong generalized cytoplasmic reaction of striated muscle fibers. F) Experimental group of red stain showing positive reaction in the wall of blood capillaries (thin arrows). (TNF-α (A,B,D&E) and red stain (C&F) X 40).

Statistical results:

The experimental group showed statistically highly significant difference in the mean area percentage of TNF-α compared with control group (table 1& figure 3).

Table (1): The mean area percentage of TNF-α in studied groups

HPF	Control group	Experimental group
1	14.26	182.63
2	20.21	164.83
3	16.34	175.55
4	12.24	174.32
5	13.78	167.69
6	14.22	187.32
7	11.35	171.24
8	15.27	168.22
9	13.65	172.01
10	17.45	166.85
Mean ± SD	**14.88 ± 2.59**	**173.07 ± 7.19**
T test	**65.436**	
P value	**0.001***	

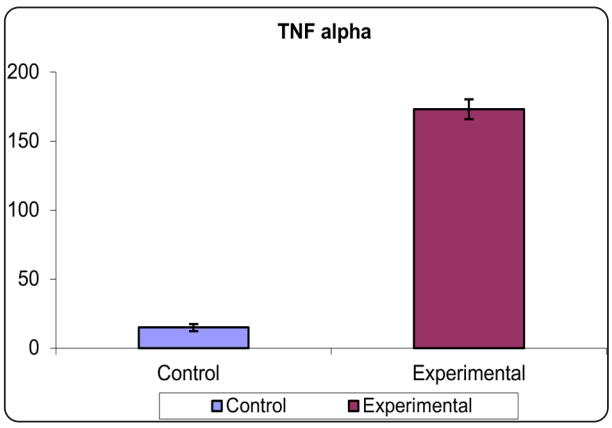


Fig. (3): Bar chart of the mean area percentage of TNF-α in control and experimental groups.

DISCUSSION

Being one of the fat-soluble vitamins, hypervitaminosis D possess a pronounced effect since it gets accumulated within the body tissues leading to a toxic condition with a threaten impact on the body in cases of administration of high doses for a prolonged time[23]**.** The action of vitamin D is not limited to calcium/ phosphate metabolism and bone remodeling, however, an extraosseous effect of vitamin D has been reported in various organs resulting in immunological, antimicrobial, and anti-inflammatory action. Such action is mediated via vitamin D receptors that have been localized in numerous types of cells such as macrophages, monocytes, dendritic cells, osteoblasts, smooth muscle cells, epithelial cells of the gingival attachment and cells of the placenta, parathyroid gland and prostate [24&25]. Thus, the aim of the current study was to evaluate the effect of overdose of vitamin D on tongue structure. Since any variation in color or texture of the tongue can act as a mirror for the general health and hence, tongue can act as a diagnostic tool for many systemic diseases and underlying disorders.

The histological examination of the tongue mucosa in our study depicted loss of filiform papillae in certain sites with disruption of some of its basal cell layer along with detection of pyknotic nuclei with perinuclear halo in other cells. Additionally, the lamina propria revealed focal loss with rarefaction of cytoplasm of certain striated muscle fibers along with atrophy and irregularity of others with widening of their intervening connective tissue. These histological changes coincided with **Singla and Kaur (2015)** who reported minor to moderate degenerative changes in almost all organs of rats subjected to toxic doses of vitamin D_3 together with separation, degeneration, and necrosis of their cardiac muscle fibers [26]. They attributed such degenerative changes to the disturbance of the calcium/ phosphorous

hemostasis that was accompanied with alternation of cell membrane permeability, the pump activity of calcium/ phosphorous with resulting reduction in cellular energy production and cellular necrosis [27].

Moreover, our histological examination revealed vascular calcification that was further confirmed with Alizarin staining for calcification. This effect agreed with **Tang et al. (2006)** who depicted vascular calcification mainly within the tunica media in the form of light blue color that was further confirmed by von Kossa staining for calcification together with the increase in both vascular calcium content and alkaline phosphatase activity and its mRNA expression in abdominal aorta which is a marker of osteogenic differentiation of osteoblastic cells [28]. Also, this calcification in the current study agreed with **Chavhan, et al. (2011)** who revealed deposits of calcification along with mild inflammatory infiltration within the lamina propria, among muscle fibers and the tunica intima of blood vessels in rats subjected to toxic level of vitamin D_3[29]. Noteworthy, vascular stiffness and disturbance of vascular tone has been suggested as a sequel of this induced vascular calcification. Remarkably, excess vitamin D represents one of the etiological factors of vascular calcification and this action could be mediated by its role in enhancing both intestinal calcium absorption together with bone resorption. Moreover, excess vitamin D may lead to hyperphosphatemia which may trigger transdifferentiation of vascular smooth muscles into osteoblast-like cells and subsequent calcification[30].

Cells of taste buds are regenerated through life by trophic interaction from underlying nerves[31]. In the current study, degeneration of taste buds were evident in vitamin D_3 overdose group. This may be explained by the reported reductive effect of vitamin D_3 on neurotrophic factors causing nerve degeneration[32].

TNF-α is one of the cytokines, which are vital for regulating several physiological processes, immunological responses, and inflammation[33,34]. Production of significantly elevated levels of TNF-α may be incorporated with necrosis, or even death. In this study, the experimental group showed strong generalized cytoplasmic reaction of epithelial cells. This finding could be in coincidence with **Ali et al., (2018)** who reported parenchymatous degeneration in the rat's liver after vitamin D_3 overdose. They related this to the enhanced action of the accumulated hydroxylated products of vitamin D in the cells[35]. The increased levels of TNF-α may also explain the observed increased calcifications within the capillaries in the current study as TNF- was reported to increase endothelial cell permeability and enhance adhesion of monocyte activating the differentiation of resident fibroblasts or quiescent valvular interstitial cells into myofibroblasts and osteoblast like cells with consequent calcification[36].

Our investigation revealed a strong generalized cytoplasmic reaction to TNF-α in the connective tissue of the experimental group. This agreed with that the TNF-α is produced by macrophages[37] and could induce resident cells and recruited inflammatory cells to produce lytic enzymes that lead to connective tissue destruction[38]. On the other hand, the current study revealed a moderate response of striated muscle fibers in the control group along with strong reaction in the experimental group to TNF-α. This may be explained by that TNF-α has been linked to physiological muscle regeneration[39] and its presence in both inflammatory and non-inflammatory myopathies at significant levels in damaged myofibers[40].

CONCLUSION

The effects of vitamin D overdose have possible therapeutic implications for inflammatory and immune-mediated diseases and these effects are dose-dependent.

RECOMMENDATIONS

It should be taken under medical supervision and individualized to each patient's needs in order to prevent negative side effects and toxicity.

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