



## Expression of Ki67 in submandibular salivary glands of rabbits after BTX injection: Histological and immunohistochemical study

A.A. Ahmed<sup>1</sup> and J.K. Mammdoh<sup>2</sup> 

<sup>1</sup>Nineveh Health Directorate, <sup>2</sup>Department of Dental Basic Science, College of Dentistry, University of Mosul, Mosul, Iraq

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#### Correspondence:

J.K. Mammdoh

[jawnaakhalid@uomosul.edu.iq](mailto:jawnaakhalid@uomosul.edu.iq)

### Abstract

This study aimed to examine the possible histological effects of local injections of BTX in rabbits submandibular SGs and to find the dose-dependent and time relationship between injections and study immunohistochemistry expression of Ki67. Thirty male rabbits randomly divided into 3 groups (10 rabbits for Each) 1st group: control (without treatment), 2<sup>nd</sup> group treated with 5U of BTX and 3<sup>rd</sup> group treated with 10U of BTX, five animals of each group were sacrificed in 1st week of treatment and another five animals sacrificed in 4<sup>th</sup> week of treatment. The rabbit was anesthetized then injected with the BTX in the gland. The histopathological changes in Group 5, 10 Unit BTX (1<sup>st</sup> week) were vacuole degeneration of mucous acini cells, degeneration of serous acini cells, while the lesions showed hyperplasia and necrosis of epithelial cells lining striated ducts, necrosis of serous acini epithelium. The Diameter of mucous acini were found to be significantly increased in 10 Unit BTX groups. During the 1<sup>st</sup> and 4<sup>th</sup> weeks, the surface area of the striated ducts in the 5- and 10-unit BTX groups increased significantly, and the number of striated ducts in the 10 Unit BTX group decreased significantly when compared to the 1st week period of the same group. BTX groups revealed moderate to weak positive cytoplasmic reactivity for Ki67 protein in the parenchymal tissue of the glands. We conclude that BTX causes histological changes in the salivary gland as well as affecting Ki67. This data could be used in a future study to investigate the usage of BTX in cancer treatment.

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### Introduction

Salivary glands (SGs) are crucial for dental health as well as overall health and well-being. While the major salivary glands only release fluid when stimulated, the minor mucous glands serve to protect the oral tissues (1,2). Botulinum toxin (BTX) is made by anaerobic fermentation of *Clostridium botulinum* bacteria, which produces eight immunologically unique serotypes (types A to H), with serotypes A and B being developed for human clinical use (3). Botulinum toxin A was initially licensed by the US Food and Drug Administration in 1989 for the treatment of blepharospasm and strabismus (4). Both sympathetic and parasympathetic

autonomic nerves govern salivary production. The action of acetylcholine on muscarinic and noradrenaline receptors on adrenergic receptors stimulates fluid and electrolyte secretion from the salivary glands, whereas isoproterenol on B-adrenergic receptors stimulates protein secretion primarily after activation of muscarinic cholinergic receptors (5). Oral locomotor therapy, behavioral therapy, anticholinergic medications, surgical methods, and most recently, BTX injections directly in the tissue of salivary glands are all used to reduce salivation (6), BTX has been widely used in a variety of clinical settings, including intra-salivary gland applications for the treatment of drooling disorders in which saliva overflow from the mouth creates physical and

psychological issues. It's also been used to address drooling caused by swallowing problems after surgery to remove upper gastrointestinal tumors especially in cases of salivary fistulas following salivary gland excision or surgery for oropharyngeal cancer, where the gland's secretion must be temporarily stopped to encourage healing. Botulinum peptide enters the cytoplasm via endocytosis after being injected into receptors on the terminal ends of neurons (3). Ki67 is a crucial cell division protein because anti-deposition nucleotides of Ki67 inhibit mitosis, which is required for ribosome production the fact that Ki67 immune expression is linked to protein production and ribosome activity backs up this theory (7). The localization of numerous nucleolar proteins to the per chromosomal layer (PCL) is reportedly reliant on pre-ribosomal RNAs and the indicators of proliferation, Ki67, which is a major PCL-localized protein (8). To the best of our knowledge, a few studies are reporting the histological analysis of the effect of botulinum toxin in submandibular salivary gland tissues. This study aims to examine the possible histological effects of local injections of BTX at different doses (5U and 10U) in rabbits submandibular SGs to find the dose-dependent and time relationship and study the Immunohistochemical expression of Ki67 in rabbits submandibular SGs.

## **Materials and methods**

### **Ethical approve**

All experimental procedures were done in approval by the Ethic committee of college of Dentistry, University of Mosul for the care and use of laboratory animals at UoM.Dent/A.L.36/21.

### **The experimental animals**

Thirty male Albino rabbits with an average body weight of 1.5 kg were used in the experiment, were kept in a standardized animal house conditions with a room temperature of 25- 21 degrees. Their ages were ranged between 6-8 months. Rabbits were randomly divided into 3 groups (10 rabbits for each) and were housed in steel cages with a 12 hours' day-night cycle. Every experimental procedure was done in agreement with the guiding principles of the University of Mosul, College of Dentistry for the laboratory animals' care and use. The rabbits were allowed free access to food and water before the experiment and the care were taken to avoid any unnecessary stress and clean the cages once per two days. The animals were kept for at least 15 days for adaptation before beginning the experiment.

### **The study designs**

Animals are divided into three groups each consist of 10 animals. First group considered as control (without treatment). Second group treated with 5U of botulinum toxin A. Third group treated with 10U of botulinum toxin A. Five

animals of each group were sacrificed in 1st week of treatment and another five animals were sacrificed in 4th week of treatment.

### **Preparation of BTX toxin-A (Xeomin)**

The dilutions of botulinum toxins (Xeomin, Merz, and Germany) were done for the 100 units of xeomin with 2 ml of normal saline 0.9% injected in the vacuumed vial, so the volume of 5U BTX-A is 0.1 ml and volume of 10U BTX-A is 0.2 ml (9).

### **The injection of BTX in rabbit**

The rabbit was anesthetized with xylazine and ketamine, then the medium cervical incision was done by the surgical blade, the palpation was done for the gland and injected with the Xeomin.

### **Dissection of Animals**

All animals were survived for their end periods and then salvaged. The animals were dissected, and specimens for all groups were taken and immersed in neutral buffer formalin of 10% for 24h for fixation and histological technique.

### **Store specimen**

The gland was bisected in a midsagittal plane, after 1 week or 4 weeks for each group. Fixation of the specimens was made by using 10% isotonic formalin saline for 24-48 hours, then washed by tap water, and dried in ascending grades of ethyl alcohol, dunked in xylene and embedded later on in paraffin wax, and the sections were cut by 6 µm thickness using rotary microtome and placed on a glass slide to be stained with hematoxylin as well as eosin.

### **Morphometric analysis**

All parameters were measured using the color USB 2.0 digital image camera (Scope Image 9.0- China) which was afforded with image analysis software.

### **Immunohistochemistry for Ki67**

Immunohistochemistry for Ki67 protein measured by Anti-KI67 antibody kit and detection DAB staining kit (Sigma-Aldrich, Germany). The specificity and sensitivity of antigen detection is dependent on the specific primary antibody used.

### **Counting process of Ki67**

In general, there are two techniques for counting Ki67-marked cells: visually counting the cells within the field of highest staining, i.e., the hotspot, and automated digital analysis of the same field of view using computer-based software. Counting the total number of positive staining cells in each image at 40x, counting the total number of cells in each image to determine the Ki67 index, we used the Ki67 kit for analysis this kit from (Dako, Belgium).

### Statistical analysis

Statistical package of social science (SPSS 26) was used to conduct the histomorphometrically analysis. Data were measured by means  $\pm$  SE (Standard Error) and analyzed by using t-test for the independent two means and One-Way ANOVA for analysis of means in more than two groups, Duncan's test with significant level set on  $P < 0.05$ .

### Results

#### Group of 5 Unit BTX (1st week)

The histopathological changes of 5 UI test BTX group were vacuolar degeneration of mucous acini cells, presents of edema surrounding some striated ducts, degeneration of serous acini cells, and mucous acini (Figure. 1). Also, there are deposition of hyaline eosinophilic material, hyperplasia of epithelial cells lining striated duct, apoptosis, hobnail cell with bulbous nucleus (Figures 2 and 3).

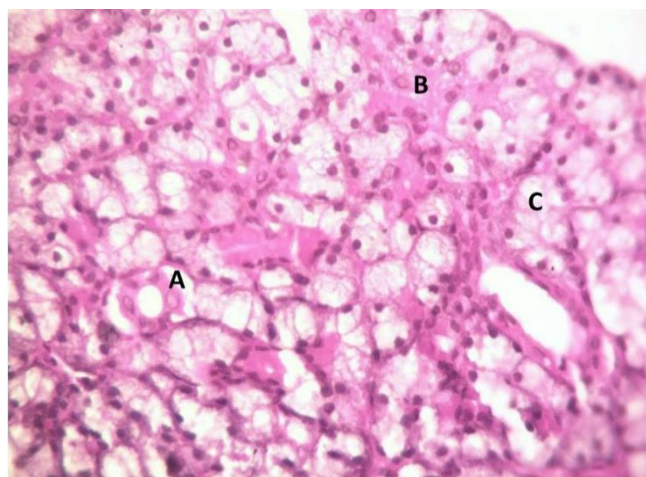


Figure 1: A photomicrograph of a submandibular salivary gland section of (BTX 5-unit group) shows presents of edema surrounding striated ducts (A), degeneration of serous acini cells (B) and of mucous acini (C). H&E stain. 400X.

#### Group of 10 Unit BTX (1st week)

This group revealed vacuolar degeneration (cell swelling) of mucous acini cells, hyperplasia of epithelial cells lining striated ducts, congestion of blood vessels, (Figure 4) and necrosis of epithelial cells lining striated ducts, necrosis of mucous acini cells, and detachment of basement membrane (Figure 5).

#### Group of 5 Unit BTX (4<sup>th</sup> week)

The submandibular gland of the rabbits of this group showed hyperplasia and necrosis of epithelial cells lining striated ducts, necrosis of serous acini epithelium and congestion of blood vessels and apoptosis (Figure 6). Also,

there are hydropic degeneration of mucous acini epithelium and infiltration of inflammatory cells, infiltration of inflammatory cells, congestion of blood vessel, detachment of basement membrane and apoptosis (Figure 7).

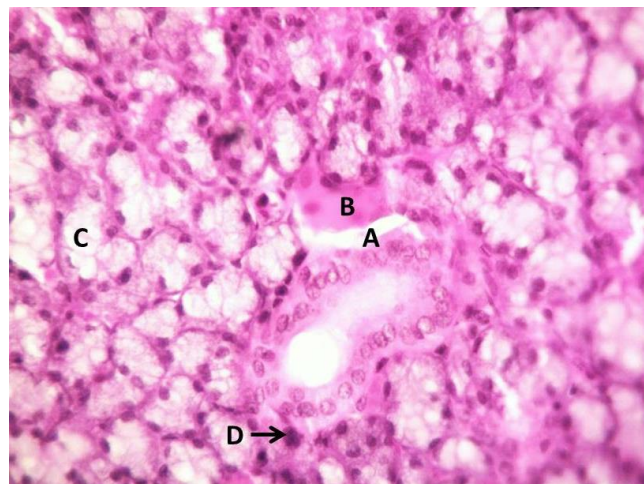


Figure 2: A photomicrograph of a submandibular salivary gland section of (BTX 5 unit group) shows presents of edema surrounding striated ducts (A), deposition of hyaline eosinophilic material(B), vacuolar degeneration of mucous acini cells (C) and apoptosis (D). H&E stain. 400X.

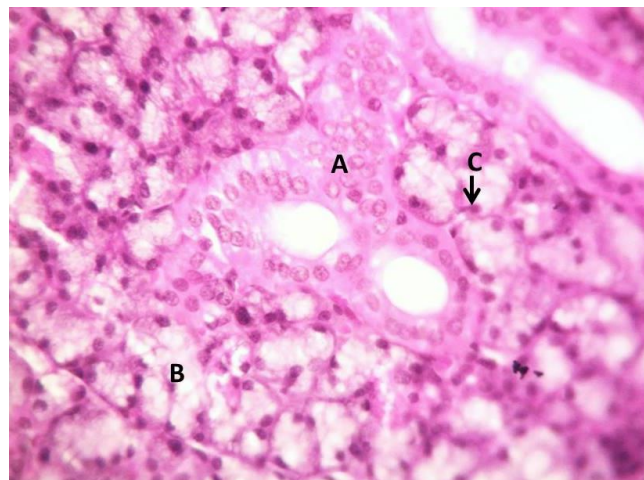


Figure 3: A photomicrograph of a submandibular salivary gland section of (BTX 5-unit group) shows presents of hyperplasia of epithelial cells lining striated ducts (A) and vacuolar degeneration of mucous acini cells (B) and hobnail cell with bulbous nucleus (C). H&E stain. 400X.

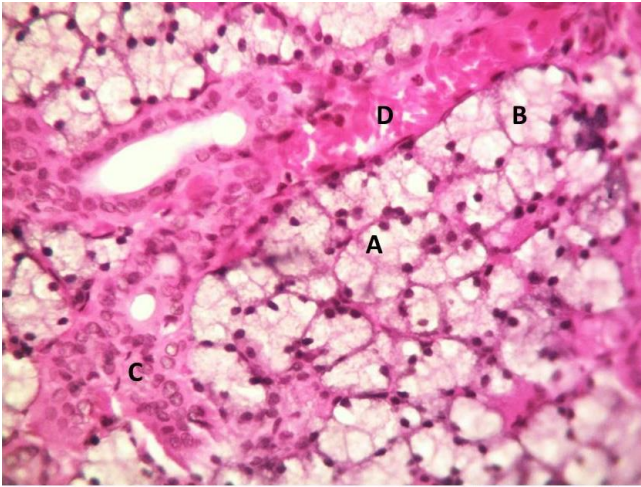


Figure 4: A photomicrograph of a submandibular salivary gland section of (BTX 10-unit group) shows vacuolar degeneration (A) and necrosis (B) of mucous acini cells, hyperplasia of epithelial cells lining striated ducts (C) and congestion of blood vessels (D). H&E stain. 400X.

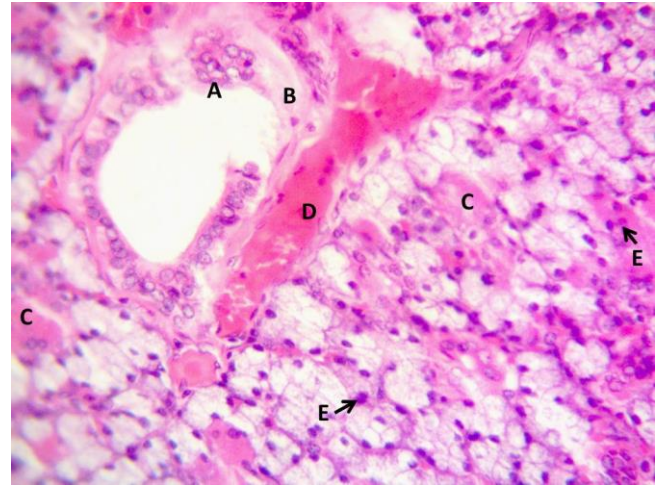


Figure 6: A photomicrograph of a submandibular salivary gland section of (BTX 5-unit group, 4<sup>th</sup> week) shows hyperplasia (A) and necrosis (B) of epithelial cells lining striated ducts, necrosis of serous acini epithelium (C) and congestion of blood vessel (D) and apoptosis (E). H&E stain. 400X.

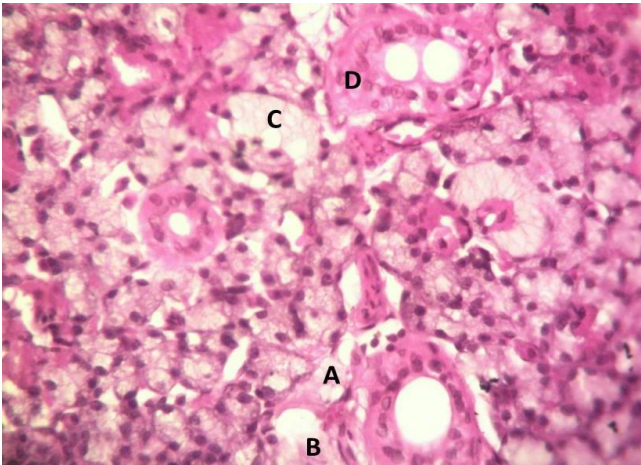


Figure 5: A photomicrograph of a submandibular salivary gland section of (BTX 10-unit group) shows degeneration (A) and necrosis (B) of mucous acini cells (A), severe necrosis of epithelial cells lining striated ducts (C) and hyperplasia of others (D), detachment of basement membrane (E). H&E stain. 400X.

**Group of 10 Unit BTX (4<sup>th</sup> week)**

This group showed necrosis of epithelial cells lining striated ducts and serous acini, atrophy of serous and mucous acini, hyperplasia of epithelial cells lining striated ducts and congestion of blood vessels (Figures 8 and 9).

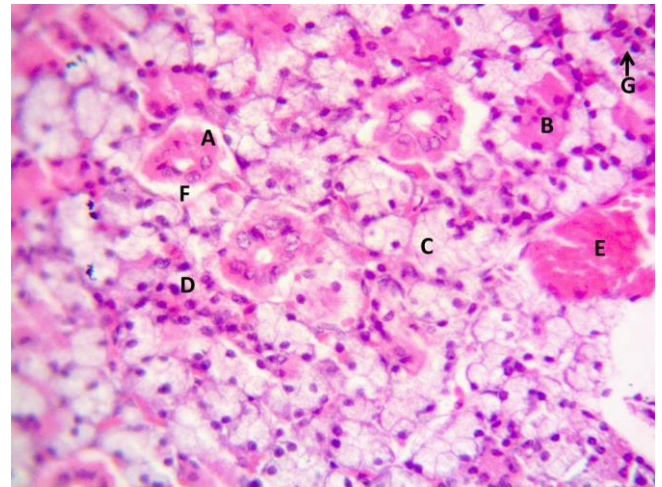


Figure 7: A photomicrograph of a submandibular salivary gland section of (BTX 5-unit group, 4<sup>th</sup> week) shows necrosis of epithelial cells lining striated ducts (A) and serous acini (B), degeneration of mucous acini epithelium (B), infiltration of inflammatory cells (D) congestion of blood vessel (E), detachment of basement membrane (F) and apoptosis (G). H&E stain. 400X.

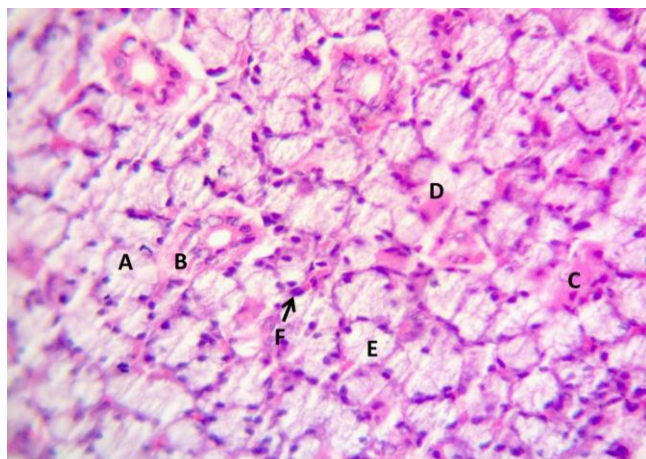


Figure 8: A photomicrograph of a submandibular salivary gland section of (BTX 10-unit group, 4<sup>th</sup> week) shows degeneration of mucous acini (A), necrosis of epithelial cells lining striated ducts (B) and serous acini (C) and atrophy of serous (D) and mucous acini (E) and apoptosis (F). H&E stain. 400X.

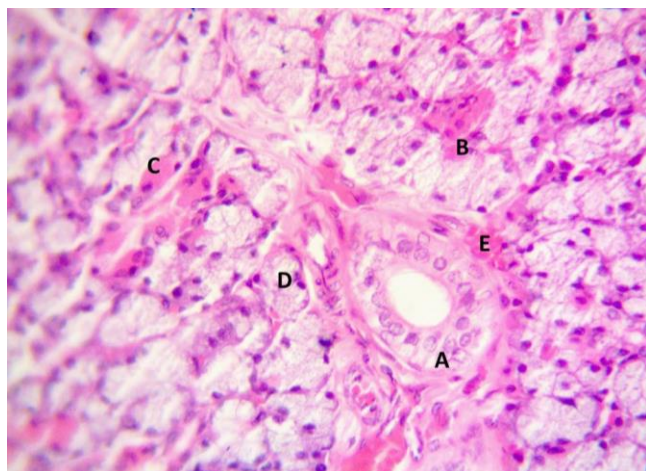


Figure 9: A photomicrograph of a submandibular salivary gland section of (BTX 10-unit group, 4 weeks) shows hyperplasia of epithelial cells lining striated ducts (A), necrosis of serous acini epithelium (B), atrophy of serous (C) and mucous (D) acini and congestion of blood vessel (E) and apoptosis (F). H&E stain. 400X.

#### **Histomorphometric measurement of the groups**

Measurement of 5 Unit BTX groups revealed there was a significant decrease in the diameters of mucous and serous acini and height of mucous acini epithelium at the 4th week compared with these parameters at the 1st week, but there are no significant differences in the height of serous acini and striated ducts diameter and epithelium between two periods 1<sup>st</sup> and 4<sup>th</sup> week. The measurement of 10 Unit BTX groups

revealed there was a significant decrease in the diameters of mucous and serous acini and striated ducts. Also, there is a significant decrease in the height of mucous acini and striated ducts epithelium at the 4th week comparing with it at the 1st week, but there is no significant difference in the height of serous acini epithelium between two periods 1st and 4th week (Table 1).

#### **Histomorphometric measurement of the striated ducts surface area of all groups**

The measurements revealed there was a significant increase in the surface area of the striated ducts in the 5- and 10-unit BTX group comparing the control group during the 1st and 4th week period (Table 2).

#### **Histomorphometrics counting of numbers of striated ducts of all groups**

There was a significant decrease in the numbers of striated ducts of the 10 Unit BTX group in the 4th week period compared with the 1st week period of the same group (Table 3).

#### **IHC at first week period**

The immunohistochemistry sections of the control group showed strong positive cytoplasmic immunoreactivity for Ki67 protein in the parenchymal tissue of the glands, which appeared more distinctive in the duct system. Scattered nuclear reactivates were identified for the protein antigen. A few localized focal areas in the secretory acini, as well as the endothelial cells of blood vessels, expressed the proliferation antigen at a higher intensity. This group had observed 37.8% positive cells of Ki67 per five fields taken by a light microscope (Figure 10). Five Unit BTX group revealed moderate positive cytoplasmic immunoreactivity for Ki67 protein which was observed in 18.6% positive cells of Ki67 (Figure 11). Ten Unit BTX group revealed mild positive cytoplasmic immunoreactivity for Ki67 protein which was observed in 11.2 % positive cells of Ki67 (Figure 12) (Table 4).

#### **IHC at fourth week period**

The immunohistochemistry sections of the control group showed strong positive cytoplasmic immunoreactivity for Ki67 protein which reached 36.6 % positive cells of Ki67 appear in the five fields taken by light microscope (Figure 13). Five Unit BTX group revealed moderate positive cytoplasmic immunoreactivity for Ki67 protein which reached 16.6% positive cells of Ki67 (Figure 14). Ten Unit BTX group revealed mild positive cytoplasmic immunoreactivity for Ki67 protein which reached 9.6 % positive cells of Ki67 (Figure 15). The measurement revealed that there was a significant change in the ki67 in different doses and the period intervals shown in (Table 4) and (Figure 16).

Table 1: Histomorphometrics measurement of the control and treated groups

Group s	Mucous acini diameter/ $\mu\text{m}$		Height of mucous acini epithelium/ $\mu\text{m}$		Serous acini diameter/ $\mu\text{m}$		Height of serous acini epithelium/ $\mu\text{m}$		Striated ducts diameter/ $\mu\text{m}$		Height of striated ducts epithelium/ $\mu\text{m}$	
	1st	4th	1st	4th	1st	4th	1st	4th	1st	4th	1st	4th
	Week	Week	Week	Week	Week	Week	Week	Week	Week	Week	Week	Week
Contro	29.77 $\pm$	21.96 $\pm$	17.322 $\pm$	18.16 $\pm$	32.14 $\pm$	25.18 $\pm$	16.58 $\pm$	12.66 $\pm$	46.782 $\pm$	40.96 $\pm$	13.66 $\pm$	14.14 $\pm$
1	2.91aA	1.056bA	1.61 aA	1.413aA	1.12aA	1.844bA	1.09 aA	0.68bA	3.24 aA	1.839aA	0.764aA	1.839aA
5 unit	47.94 $\pm$	24.18 $\pm$	21.67 $\pm$	13.22 $\pm$	31.96 $\pm$	27.72 $\pm$	15.232 $\pm$	13.76 $\pm$	57.142 $\pm$	53.18 $\pm$	16.12 $\pm$	14.24 $\pm$
	5.7 aB	0.42 bA	0.6aB	0.62 bB	1.7 aA	0.49 bA	0.7 aA	1.43aA	6.0 aA	2.87aB	1.2 aAB	0.93aA
10 unit	45.28 $\pm$	22.1 $\pm$	18.96 $\pm$	13.38 $\pm$	33 $\pm$	23.96 $\pm$	12.2 $\pm$	11.14 $\pm$	47.89 $\pm$	37.94 $\pm$	18.94 $\pm$	11.12 $\pm$
	3.1aB	0.9 bA	1.2 aAB	0.3 bB	2.4 aA	2.3 bA	1.04 aB	0.4 aA	1.2 aA	1.2 bA	0.7aB	0.7bB

The different small letters (horizontal) mean the significant difference at  $P \leq 0.05$ . The similar small letters (horizontal) mean there is no significant difference at  $P \leq 0.05$ . The similar large letters (vertical) mean there is no significant difference at  $P \leq 0.05$ . The different large letters (vertical) mean there is a significant difference at  $P \leq 0.05$ .

Table 2: Comparison the morphometric measurements of the surface area of the striated ducts for the control, 5 and 10 Unit BTX groups between 1<sup>st</sup> and 4<sup>th</sup> week

Area of striated ducts/ $\mu\text{m}^2$	Control	5 unit	10 unit	F test	P value
1 <sup>st</sup> week	a 1862.48 $\pm$ 111.2	ab 2778.42 $\pm$ 545.5	b 3610.62 $\pm$ 504.8	4.06	0.045*
4 <sup>th</sup> week	a 1448.32 $\pm$ 153.3	ab 2537.89 $\pm$ 334.6	b 3753.50 $\pm$ 894.9	4.26	0.040*

\* Just significant  $P \leq 0.05$ . \*\* Highly significant  $P \leq 0.01$ . \*\*\* Very highly significant  $P \leq 0.001$ . The different letters mean the significant difference from the control group at  $P \leq 0.05$ . The similar letters mean there is a no significant difference at  $P \leq 0.05$ .

Table 3: Comparison of counting of numbers of striated ducts/40x for the control, 5 and 10 Unit BTX groups at 1<sup>st</sup> and 4<sup>th</sup> week

Numbers of striated ducts	1 <sup>st</sup> week	4 <sup>th</sup> week	Independent t test	P value
Control	2.50 $\pm$ 0.33	3.00 $\pm$ 0.28	1.155	0.281
5 unit	5.37 $\pm$ 0.26	4.14 $\pm$ 0.27	3.281	0.011***
10 unit	5.78 $\pm$ 0.26	3.89 $\pm$ 0.26	5.140	0.000***

\* Just significant  $P \leq 0.05$ . \*\* Highly significant  $P \leq 0.01$ . \*\*\* Very highly significant  $P \leq 0.001$ .

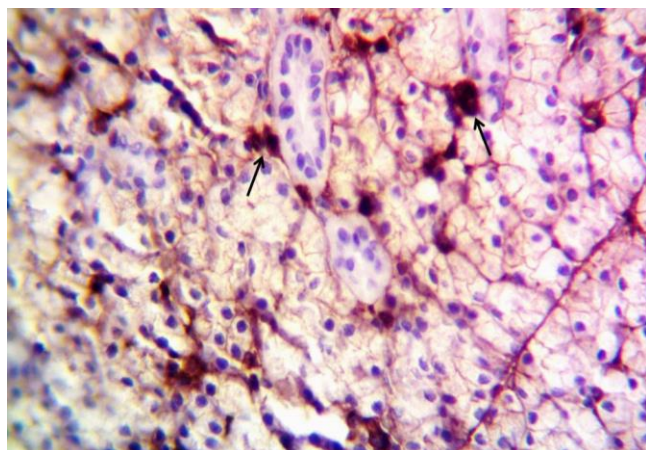


Figure 10: A photomicrograph of a submandibular salivary gland section of the control group (1st week) shows a strong positive reaction (+++) of Ki67 to immunohistochemistry in the proliferative cells. IHC for Ki67, 400X.

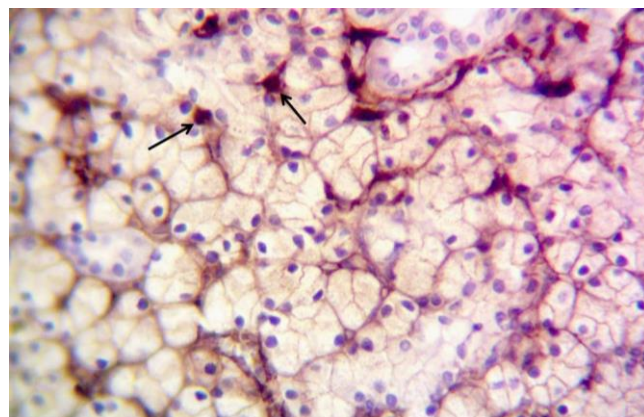


Figure 11: A photomicrograph of a submandibular salivary gland section of 5 Unit BTX group (1<sup>st</sup> week) shows a moderate positive reaction (++) of Ki67 to immunohistochemistry in the proliferative cells. IHC for Ki67, 400X.

Table 4: Comparison of immune-reactivity of ki67 between 1<sup>st</sup> and 4<sup>th</sup> week intervals for different groups

Ki67/ 40x fields	Control	5 unit	10 unit	F test	P value
1 <sup>st</sup> week	A 37.8±0.86	B 18.6±0.93	C 11.2±0.58	291.35	0.000***
4 <sup>th</sup> week	A 36.6±0.92	B 16.6±0.68	C 9.6±0.51	375.42	0.000***

\*Just significant  $P \leq 0.05$ . \*\* high significant  $P \leq 0.01$ . \*\*\*very high significant  $P \leq 0.00$ . The different letters mean the significant difference from the control group at  $P \leq 0.05$ . The similar letters mean there is a no significant difference at  $P \leq 0.05$ .

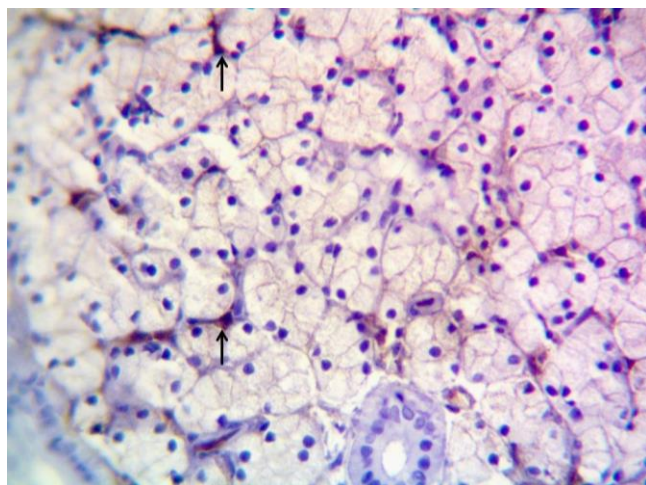


Figure 12: A photomicrograph of a submandibular salivary gland section of 10 Unit BTX group (1<sup>st</sup> week) shows a weak positive reaction (+) of Ki67 to immunohistochemistry in the proliferative cells. IHC for Ki67, 400X.

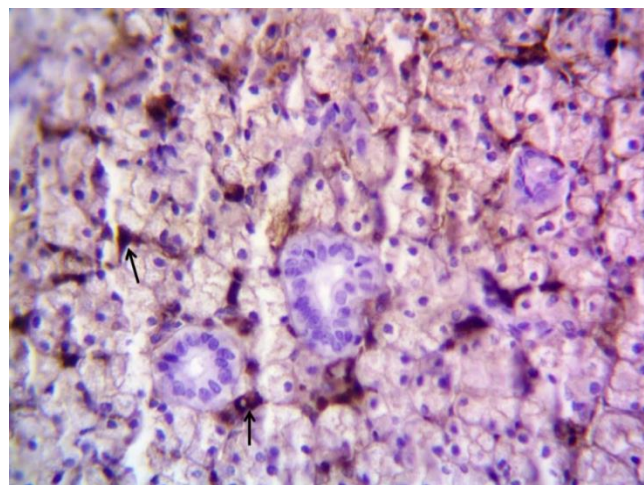


Figure 14: A photomicrograph of a submandibular salivary gland section of 5 Unit BTX group (4<sup>th</sup> week) shows a moderate positive reaction (++) of Ki67 to immunohistochemistry in the proliferative cells. IHC for Ki67, 400X.

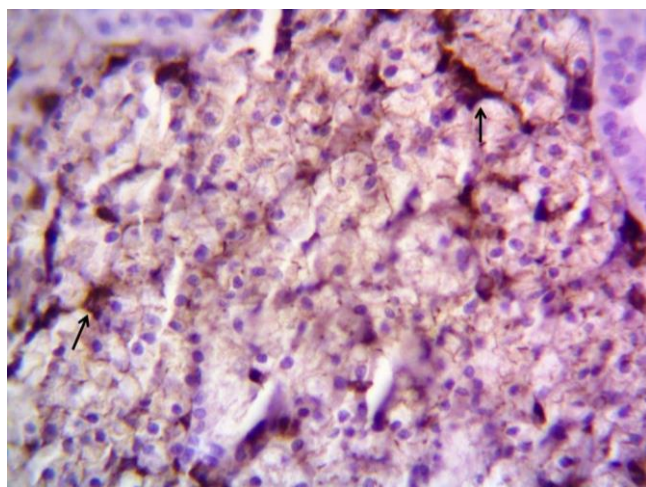


Figure 13: A photomicrograph of a submandibular salivary gland section of the control group (4<sup>th</sup> week) shows a strong positive reaction (+++) of Ki67 to immunohistochemistry in the proliferative cells. IHC for Ki67, 400X.

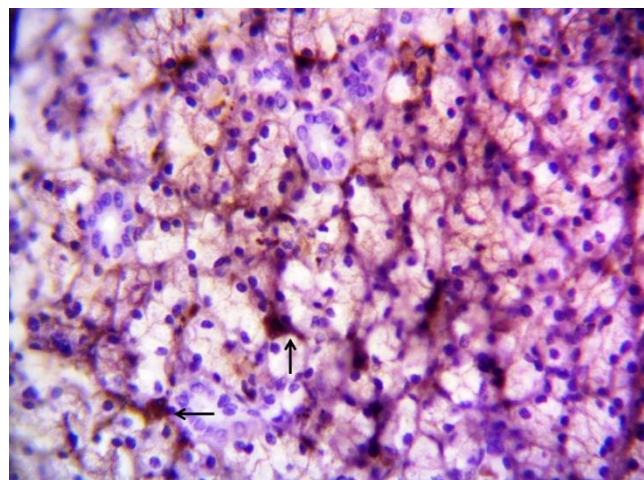


Figure 15: A photomicrograph of a submandibular salivary gland of 10 Unit BTX group (4<sup>th</sup> week) shows a weak positive reaction (+) of Ki67 to immunohistochemistry in the proliferative cells. IHC for Ki67, 400X.

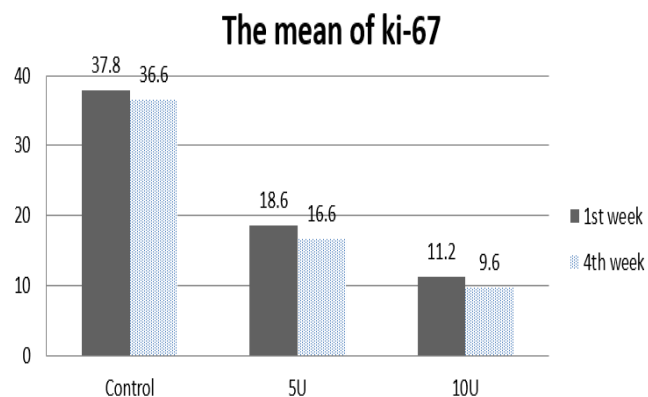


Figure 16: Histogram representing comparison the mean of ki67 among the control, 5 and 10 Unit BTX groups at 1st and 4th week at indicated time intervals

## Discussion

The current findings showed that BTX has a biological effect on tissues of the submandibular glands, and changes in the histological structure after a week of 5 and 10 units of BTX injection, our findings were congruent with the finding of, Younis *et al.* (10), which injected the BTX-A in the parotid gland of rats and noticed less secretory granules of acini with extensive and abrasive cytoplasmic vacuoles, an increased number of degenerate mitochondria and wider interlobular spaces. The changes which were explained by the reaction of toxin happen in the tissues of the gland.

According to one study, the influence of toxins on mitochondria causes a disturbance in energy production, which weakens the cell's activity and capacity to complete its role appropriately (11). The ultra-structural results of our findings were similar regarding the study of Teymoortash *et al.* (12) which explained that BTX inhibits the cholinergic parasympathetic innervation at the neuroglandular junction, specifically the inhibition of the SNAREs of the acini induced by, appears to affect the morphological features, as well as, secretory roles of the SGs.

BTX affects the normal function of the neuromuscular conductor by preventing muscle contraction by reducing the production of acetylcholine from peripheral nerve cells. Chemical denervation occurs when substances are found on muscle fibers and induce muscular contraction (13). According to Pérez-Legaspi *et al.* (14), SNAP-25 protein plays a crucial role in neuroexocytosis and transmitting a nerve impulse. Moreover, BTX inhibits acetylcholine release from the cholinergic nerve's end, resulting in the inactivity of the innervated muscles or glands (15).

The current study's findings, supported by histological evidence suggest that BTX injection causes chemical denervation, which then returns after some time by creating

new axons and synapses (16), with the duration ranging from weeks to months. The present study detected a histological alteration in the cells of the striated ducts of the submandibular salivary glands treated with BTX injections of 10 units compared to the control after one and four weeks of injection, with an improvement in visible signs and the number of ducts after four weeks of treatment with the injection of 5 units in comparing to the control. These results were similar to other investigations in rabbit submandibular glands after 2 weeks of BNTA injection, as well as a study on the parotid glands of mice treated with BTX 4 weeks later, which have produced similar results (17).

The histological abnormalities in the epithelium of the striated ducts and mucous acini improved after injecting 10 units of BTX into SMG after four weeks, and the acini diameter recovered normal. Atrophy and damaged sections of the gland are replaced. These findings were following one study when injected 5 units of BTX into the submandibular glands reduced saliva output in rabbits at rest and during breastfeeding without causing side effects such as dysphagia, and the gland function was partially restored after 4 weeks, showing that BTX had a prolonged but the effect on salivary gland secretion was reversible (18).

Furthermore, our findings revealed substantial atrophy with degenerative alterations in rabbits injected with 10 units compared to rabbits that were injected with 5 units and degenerative changes could also be observed in other ducts types. This comes to an agreement with the above study who demonstrated apoptosis in acinar and ductal cells rabbits 'submandibular glands injected with 5 U and 10U of BTX According to the morphometric analysis of the submandibular glands of the control group, the results reported morphological changes in gland after 4thweek of treatment interval comparing with 1st week. These changes were supported by (19,20) which explained that aging caused disturbing effects on the histological structure of submandibular and sublingual salivary glands.

Ultra-structural features support the evident atrophy of the acini caused by chemical denervation mediated by BTX, such as morphological variations of rough endoplasmic reticulum, mitochondrial degeneration, secretory vacuoles varying in size, shape in salivary glands treated with BTX in a single dose. Based on previous studies, it can be supposed that the recovery occurring three months after BTX injection is due to the release of acetylcholine following a semi-temporary sympathetic cessation of supply to the epithelial cells of the salivary gland, where these cells are located in the epithelium (LRC) after months of continued growth, it divides and maintains the DNA copy (21,22)

Ki67 is a 395 kDa heavy protein that is a proliferation marker that is extensively expressed in many cancers. Ki67 is controlled by proteolytic pathways and shares critical features with other cell cycle regulators (23). Our findings in the Department of Immunohistochemistry revealed strong



Ki67-positive visceral immunoreactivity in the control group, which reached 36.6 % of Ki67-positive cells, and moderately positive Ki67 visceral immunoreactivity in the 5-unit BTX group after 1 and 4 weeks, which reached 16.6-18.6 % of Ki67-positive cells, while the 10-unit BTX group showed mild positive cytokine immunoreactivity. These results were consistent with one study that demonstrated that Ki67 was found to promote heterochromatin organization in proliferating cells. Studying Ki67 expression control, we have found that the cell regulation cycle accounts for Ki67 variability levels in human normal cells, proliferating tissues in mice, human cancer cell lines, and cancer patients (24,25). Moreover, that Ki67 depletion can protect mice from intestinal carcinogenesis in experimental models used.

The findings of the current study revealed for the first point, the other side of the effect of BTX injections into rabbits' submandibular salivary glands, as BTX injections resulted in a reduction in the percentage of active phases within the cell division cycle proportional to the amount of injected dose in direct proportion, and this finding can be used in future research to study the likelihood of BTX injections in the submandibular salivary glands. The latest research found that BTXA inhibits the generation of Ki67 protein, which could explain why BTX induces apoptosis, as numerous studies have shown. However, to counteract this effect, the cells boost mRNA production, resulting in more protein. Finally, the cell's protein production and BTXA's induction of death achieve a state of balance, resulting in a lack of protein change following BTXA injection into rabbits (21).

## Conclusion

While BTX appears to cause short- to medium-term damage to SSG tissue structures and cellular organelles, these side effects were only temporary and dose-dependent, with partial recovery occurring after four weeks of therapy. The effect of BTX injection into the submandibular SGs on Ki67 protein was recorded for the first time, as BTX administration induced a reduction in the proportion of active phases in the cell division cycle. This finding can be applied to future study into the usage of BTX in cancer treatment.

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## Conflict of interest

There is no conflict of interest.

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## التعبير عن Ki67 في الغدة اللعابية تحت الفكية للأرانب بعد حقن سم البوتيلينيوم: دراسة نسيجية ونسجية مناعية

عبدالله عبد الموجود احمد<sup>1</sup> و جوائز خالد ممدوح<sup>2</sup>

<sup>1</sup>دائرة صحة نينوى، فرع علوم الأسنان الأساسية، كلية طب الأسنان، جامعة الموصل، الموصل، العراق

### الخلاصة

هدفت هذه الدراسة إلى فحص التغييرات والقياسات النسيجية للحقن الموضوعي لسم البوتيلينيوم في الغدة اللعابية تحت الفكية للأرانب وإيجاد العلاقة المعتمدة على الجرعة والوقت في الحقن مع النشاط المناعي لـ Ki67. وزع ثلاثين ذكراً من الأرنب بشكل عشوائي إلى 3 مجاميع (10 أرانب لكل مجموعة) المجموعة الأولى: مجموعة السيطرة (بدون علاج)، المجموعة الثانية عوملت بخمس وحدات من سم البوتيلينيوم والمجموعة الثالثة عوملت بعشر وحدات من سم البوتيلينيوم، تم التضحية بخمسة حيوانات من كل مجموعة في الأسبوع الأول من العلاج ثم ضحي بالخمس الباقية في الأسبوع الرابع من العلاج. خدرت الأرانب ثم حقنت باستخدام سم البوتيلينيوم في الغدة. أظهرت المجاميع المعاملة بكل من 5 وحدات و 10 وحدات في الأسبوع الأول كانت تنكس فجوي لخلايا أسيني المخاطية، بينما أظهرت الأفات تضخماً ونخراً في الخلايا الظهارية المبطنة للقنوات المخططة، ونخر في القنوات المخططة. كما وجد أن قطر الوحدات الإفرازية المخاطية قد زاد بشكل ملحوظ في مجموعة 10 وحدات من سم البوتيلينيوم خلال الأسبوعين الأول والرابع كما ازدادت مساحة سطح القنوات المخططة بشكل ملحوظ في مجموعتي 5 وحدات و 10 وحدات من سم البوتيلينيوم وانخفض عدد القنوات المخططة في مجموعة 10 وحدات من سم البوتيلينيوم بشكل ملحوظ مقارنة بفترة الأسبوع الأول من نفس المجموعة. كشفت مجاميع سم البوتيلينيوم عن تفاعل إيجابي متوسط إلى ضعيف لبروتين Ki67 في النسيج المتني للغدة. نستنتج ان البوتنكس يسبب تغييرات نسيجية في الغدة اللعابية فضلاً عن التأثير على Ki67، يمكن استخدام هذه البيانات في دراسة مستقبلية للتحقيق في استخدام سم البوتيلينيوم في علاج السرطان.