

EFFECT OF INTRA-MASSETERIC BOTULINUM NEUROTOXIN INJECTION ON RAT PAROTID GLAND: HISTOMORPHOMETRIC, HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDY

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ABSTRACT

Introduction: Botulinum neurotoxin “BoNT” is a potent toxin which causes muscle paralysis. However, it is widely used in management of muscle-related disorders such as masseter hypertrophy. Intra-masseteric BoNT injection may predispose certain adverse effects on the adjacent tissues including parotid gland. The exact mechanisms as well as the reversibility of these effects on parotid glands still have been not fully elucidated.

Purpose: To assess, histologically, histochemically and immunohistochemically, the effects of intra-masseteric botulinum neurotoxin injection on the parotid glands of albino rats.

Materials & Methods: Fifteen adult male albino rats were divided into 2 groups: control group (5 rats received intra-masseteric injection of sodium chloride), and BoNT group (10 rats received intra-masseteric injection of 2.5 units BoNT). The latter group was divided equally into 2 subgroups according to the time of rats' scarification (3 days and 14 days after injection). The parotid glands were studied by histomorphometric analysis, Periodic Acid Schiff reaction and immunohistochemical expression of neuron specific enolase marker.

Results: BoNT injected rats showed acinar vacuulations and decreased size, blood vessels congestion and mild fibrosis in both durations. 14 days subgroup displayed inflammatory cell infiltrate. BoNT group showed less intense reaction to PAS was observed in addition to under-expression of neuron specific enolase which was more severe in the rats sacrificed after 3 days.

Conclusion: BoNT has an adverse effect on parenchymal and connective tissue elements of parotid salivary gland which is slightly modified by time factor.

KEYWORDS: Botulinum toxin, Parotid gland, PAS, Neuron specific enolase.

INTRODUCTION

Botulinum neurotoxin (BoNT) is a potent neurotoxin produced by anaerobic bacterium, *Clostridium botulinum*.⁽¹⁾ It was first described in

1820 by Justinus Kerner to be causative factor of botulism, a fatal disease causing muscle paralysis mostly by inhibition of the release of acetylcholine (motor neurotransmitter).⁽²⁻⁴⁾ BoNTs set up a seven (A-G) serotypes with different antigenicity due

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to the difference in the *Clostridium botulinum* strain.⁽⁵⁾ There are 2 more serotypes mentioned by some authors, C2 Type⁽⁶⁾ and “H” type which possessed a hybrid-like structure with similarity to type A and F.⁽⁷⁾ From the known serotypes, BoNT-A & BoNT-B are used as therapeutics.⁽⁸⁾ They are used in the management of several medical conditions involving excessive sweat, sialorrhea, and muscle-related pain disorders. Moreover, BoNTs have been widely used in cosmetic applications.^(9, 10)

Botulinum neurotoxin type A was first proposed as a treatment modality for sialorrhea in 1997 through intra-glandular injection. Its action is based on the inhibition of acetylcholine release at the presynaptic level, by acting on the cholinergic nerve terminals (parasympathetic nerve terminals), causing local chemical blocking and the loss of neuronal activity in the target organ.⁽¹¹⁻¹⁴⁾

The existence of BoNT intra-glandular injection as a line of treatment of sialorrhea, necessitated the research work on the effects on salivary output, along with the structural changes in the glands following the BoNT injection. Clinical studies presented controversial results regarding the possible adverse effects of intra-glandular BoNT injection; some authors considered this injection to be safe for treatment of sialorrhea.^(11, 15) On the contrary, clinical complications were reported including pain, flu-like indicators, inflammatory reaction, xerostomia, dysphagia, facial palsy and vascular injuries.⁽¹⁶⁾ Furthermore, histologically, the results were inconsistent; slight increase in cell volume^(17, 18) or even maintained normal histology⁽¹⁹⁾ were reported. While other authors, on the contrary, observed acinar cellular atrophy, intracellular vacuole and interlobular spaces.^(20, 21)

As a muscular neurotoxin, BoNT intramuscular injection is widely used for treatment of muscular disorders including ocular synkinesis.⁽²²⁾ Besides, buccinator muscle was reported to be treated with BoNT in facial synkinesis.⁽²³⁾ Regarding the masticatory muscles, BoNT is used in the treatment of myofascial pain syndrome, Bruxism and temporomandibular joint disorders as well as chronic migraine headache.^(24, 25)

Masseter, one of the main masticatory muscles, may be subjected to hypertrophy.^(26, 27) There are two treatment modalities for masseter hypertrophy; one is the surgical partial muscle removal,⁽²⁸⁾ the second is the intra-masseteric BoNT injection which was first used in 1994.⁽²⁹⁾ This injection might predispose some shortcomings in the tissues adjacent to masseter via direct diffusion of the neurotoxin, while the distant tissues may be affected through neuro-axonal as well as hematogenous transport.⁽³⁰⁾ To minimize the toxin diffusion through the fascia surrounding the masseter, it is advisable to inject into the center of a muscle.⁽³¹⁾

Some studies have been performed to assess the effect of diffusion of botulinum neurotoxin to the parotid gland following the intra-masseteric injection. *Fietzek et al (2009)* conducted a study on 6 patients with spastic trismus and masseter hypertonia; the patients received BoNT injections into each masseter. The authors reported no significant changes in amount of saliva but they concluded that significant results may have been missed due to the small group size.⁽³²⁾ Another human study was done by *Kwon et al (2009)* on 34 patients received 25 units BoNT into each masseter muscle. The authors stated that no significant changes in salivary flow rate.⁽³³⁾ On the other hand, *Hu et al (2010)* reported decrease in saliva amount as well as thicker consistency of the saliva as a result of masseter BoNT injection. They proposed that these effects were attributed to excessive diffusion of the toxin to the parotid gland.⁽³⁴⁾

The previous work on the effects of intra-masseteric BoNT injection on parotid glands was mainly directed to human clinical studies. However, the precise evaluation of the structural changes of parotid glands requires animal studies. It seems that there is a scarceness of animal studies particularly the quantitative ones to achieve confident conclusion about the relation between intra-masseteric BoNT injection and parotid gland structure and function. **The present work aimed to** evaluate the effects of intra-masseteric BoNT injection on parotid gland via histomorphometric analysis, Periodic Acid Schiff reaction and immunohistochemical detection of neuron specific enolase.

MATERIALS AND METHODS

Fifteen adult male albino rats weighing around 250 grams were divided into 2 groups: control group (A) consisted of 5 rats received intra-masseteric injection of single dose 0.05 ml 0.9% sodium chloride solution. Group B (10 rats received single dose intra-masseteric injection of 2.5 units Botulinum neurotoxin (BoNT) type A “Neuronox®” made by Medytox, Korea. The BoNT powder was dissolved in 0.05 ml 0.9% sodium chloride solution. Group B was subdivided into 2 subgroups (B1 and B2) according to the time of rats’ scarification after injection (3 days and 14 days) respectively (table 1 summarizes the groups and subgroups). Care has been taken to perform the injection into center of masseter muscle. The experiment was performed in the animal house of Ain Shams University following the bioethical guidelines of the institution.

Table (1): Description of the groups and subgroups

Details \ Group	Group A (Control) (n= 5)	Group B (BoNT) (n= 10)	
		Subgroup B1	Subgroup B2
Injection	Sodium chloride (0.9%)	2.5 units BoNT type A	
Date of scarification	3 days after injection	3 days after injection	14 days after injection
Sample size	(n= 5)	(n= 5)	(n= 5)

Sample preparation for hematoxyline and eosin stain and PAS:

Parotid glands were dissected and placed in 10% buffered formaldehyde. The fixed samples were rinsed well and dehydrated by transferring through successively increasing concentrations of alcohol terminating by absolute alcohol. Specimens were then transferred to xylol and embedded in paraffin wax and sectioned by microtome (4–6 μ m thick). The sections were transferred in gradually decreasing concentrations of alcohol ended by distilled water. The prepared samples were then stained by hematoxylin and Eosin stain for histological examination and histomorphometric analysis. ⁽³⁵⁾ Another set of slides was used for

Periodic Acid Schiff (PAS) staining method to evaluate the glycoprotein content and secretory function of the gland.

Sample preparation for immunohistochemical examination:

For immunohistochemical detection of the neuronal and myoepithelial cells, *neuron specific enolase* (NSE) marker was used. The deparaffinized slides were washed two times in buffer and incubated in hydrogen peroxide block 10 minutes to reduce nonspecific background staining due to endogenous peroxidase. Samples were then washed 4 times in buffer and incubated 5 minutes at room temperature to block nonspecific background staining. The primary antibody was applied then samples were incubated and washed 4 times in buffer. Primary antibody enhancer was applied and the samples were incubated for 10 minutes at room temperature and washed 4 times in buffer. Horseradish peroxidase polymer was applied and samples were incubated for 15 minutes at room temperature and washed 4 times in buffer. One drop (40 microliter) Diaminobenzidine DAB Plus Chromogen was added to 2 ml of (DAB) Plus substrate, mixed by swirling and applied to the tissue then incubated for 5 minutes. Samples were washed in deionized water 4 times; the slides were stained by counter stain (hematoxyline) and finally over-slipped. ^(36,37)

Histomorphometric and immunohistomorphometric analysis:

Histomorphometric analysis was performed by examination of 5 fields in each slide. The sizes of five serous acini in each field were measured (i.e. total of 25 acini were measured in each slide). The average of acini sizes in each field was automatically calculated by the image analysis software then the average of acini sizes in each slide was calculated. Immunohistomorphometric analysis was done by measuring the area fraction (%) of the positive reaction to NSE marker in 6 different regions (3 in the connective tissue regions and 3 inside the lobules). The image analysis was done using ImageJ software version: 1.47 (Wayne Rasband, National Institute of Health, USA).

Statistical Analysis

The mean values of serous acini sizes as well as the immuno-positive area fraction in the lobular and connective tissue regions were calculated for each group and subgroup. Results were tabulated and statistically analyzed using one way ANOVA test to compare between groups followed by Post Hoc test (pairwise comparison with Bonferroni adjustment of P value) to compare between each 2 groups.

RESULTS

Hematoxyline and eosin results:

The parotid glands of the control group showed dark densely packed serous acini with distinct spherical nuclei. The striated ducts were observed with columnar cellular lining and spherical to oval nuclei with associated blood vessel (fig. 1.a). The

interlobular septa revealed intact excretory ducts surrounded by fibrocellular connective tissue with minimal inflammatory cell infiltrate. Variable sized non-congested blood vessels were observed enclosing red blood corpuscles (RBCs) (fig. 1.b). Examination of parotid glands of group B rats (subjected to BoNT) displayed certain changes in both parenchymal as well as connective tissue elements. Regarding subgroup (B1), the rats of which were killed 3 days after BoNT injection, the serous acini showed many vacuoles in their cells. Moreover, the cells appeared to be less intensely stained in comparison to the control group (fig. 2). The most noticeable findings in the interlobular connective tissue were the fibrosis surrounding the excretory ducts and the relatively dilated blood vessels which were slightly congested RBCs (fig. 2b).

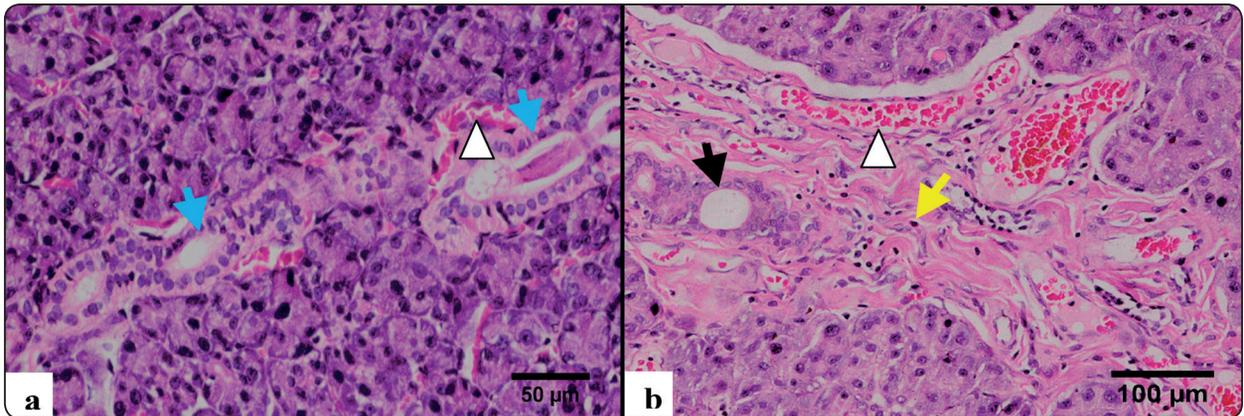


Fig. (1) Photomicrographs of control group. (a): intensely stained serous acini and clear striated ducts (arrow) associated with blood vessel (arrow head). (b): Connective tissue septum with excretory ducts (black arrow), minimal inflammatory cells (yellow arrow) and normal blood vessels (arrow head) (H&E. a: x400, b: x200).

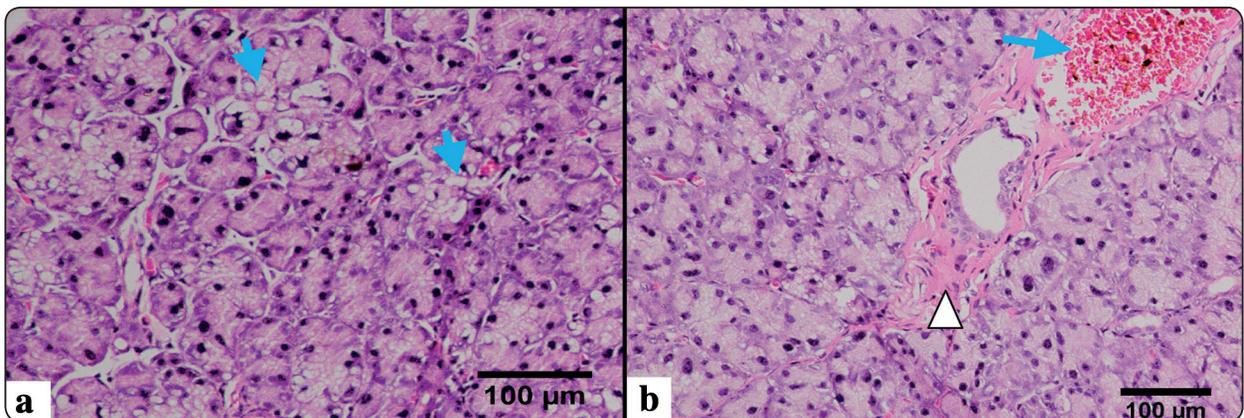


Fig. (2) Photomicrographs of subgroup B1 (3 days). (a): Pale stained serous acini with cytoplasmic vacuoles (arrows). (b): dilated and slightly congested blood vessel (arrow) and fibrosis surrounding the excretory duct (arrow head) (H&E. x 200).

The subgroup B2 subjected to BoNT injection and sacrificed 14 days after injection showed almost similar results observed in the subgroup B1 regarding the fibrosis surrounding the excretory ducts (fig. 3.a) and the congested dilated blood vessels (fig. 3.b). Moreover, there were apparent aggregations of the inflammatory cells in some acinar areas (fig. 3.b) as well as in connective tissue stroma (fig. 3.c). Rarely, more obvious degenerative areas in the serous acini were observed (fig. 3.d).

Histomorphometric results:

The average size of serous acini was measured in five regions in each sample. The mean and standard deviation of each group were calculated and data were statistically analyzed. The control group showed the highest value followed by subgroup B1 and the least value was of subgroup B2 (table "2" and figure "4"). One way ANOVA test revealed significant difference between the groups ($P < 0.05$). While in comparison between each 2 groups, and subgroups the difference was statistically nonsignificant.

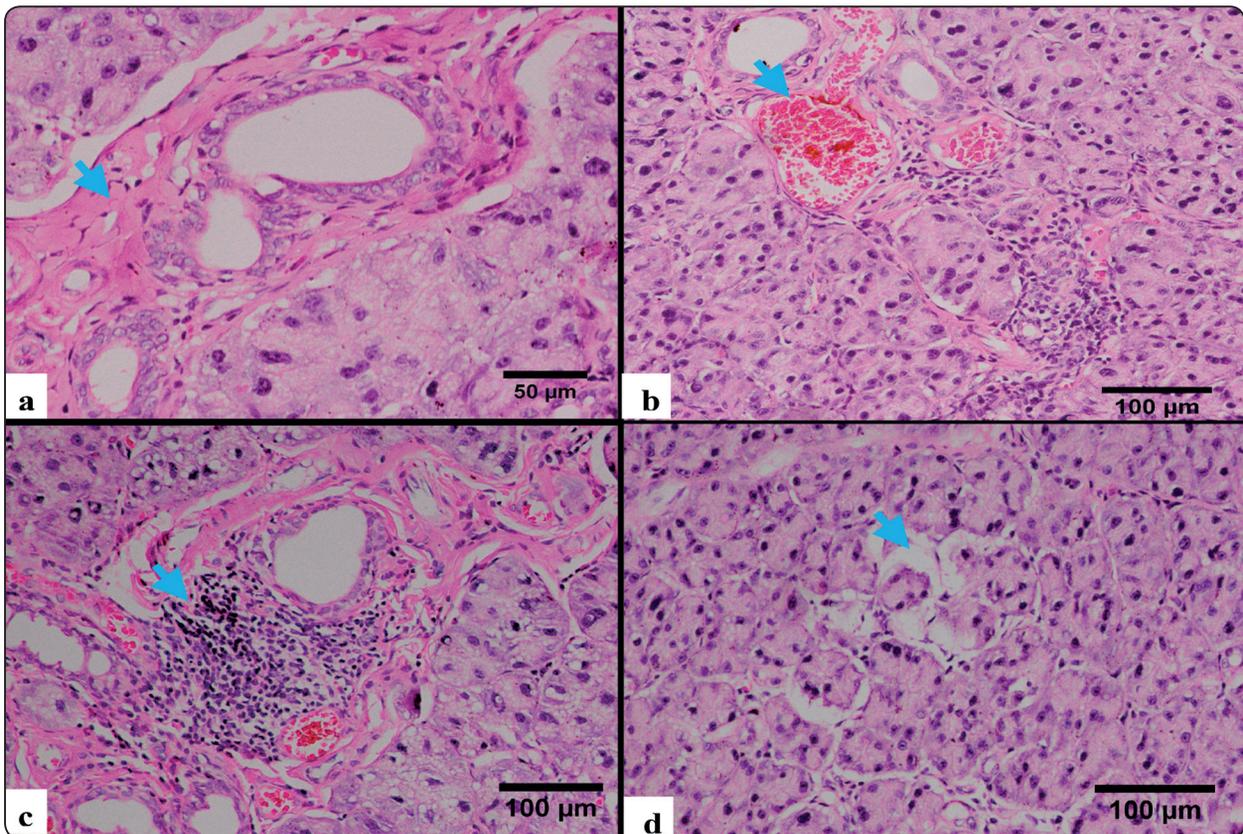


Fig. (3) Subgroup B2 (14 days). (a): fibrosis surrounding the excretory ducts (arrow) (b): congested dilated blood vessels (arrow) and inflammatory cells in some acinar areas (arrow head). (c): inflammatory cells in connective tissue stroma (arrow). (d): areas of acinar degenerations (arrow) (H&E. a: x400. b, c and d: x200).

TABLE (2): The mean values and standard deviation of serous acini size in the different groups.

Group	Group A (control)	Group B	
		Subgroup B1	Subgroup B2
Mean (square micrometers)	3326.8	2994.5	2468.2
Standard deviation	384.9	399	623.1

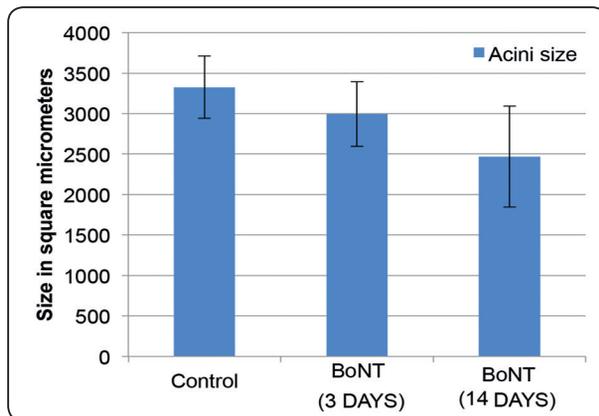


Fig. (4) Change in the mean values of serous acini size in the groups.

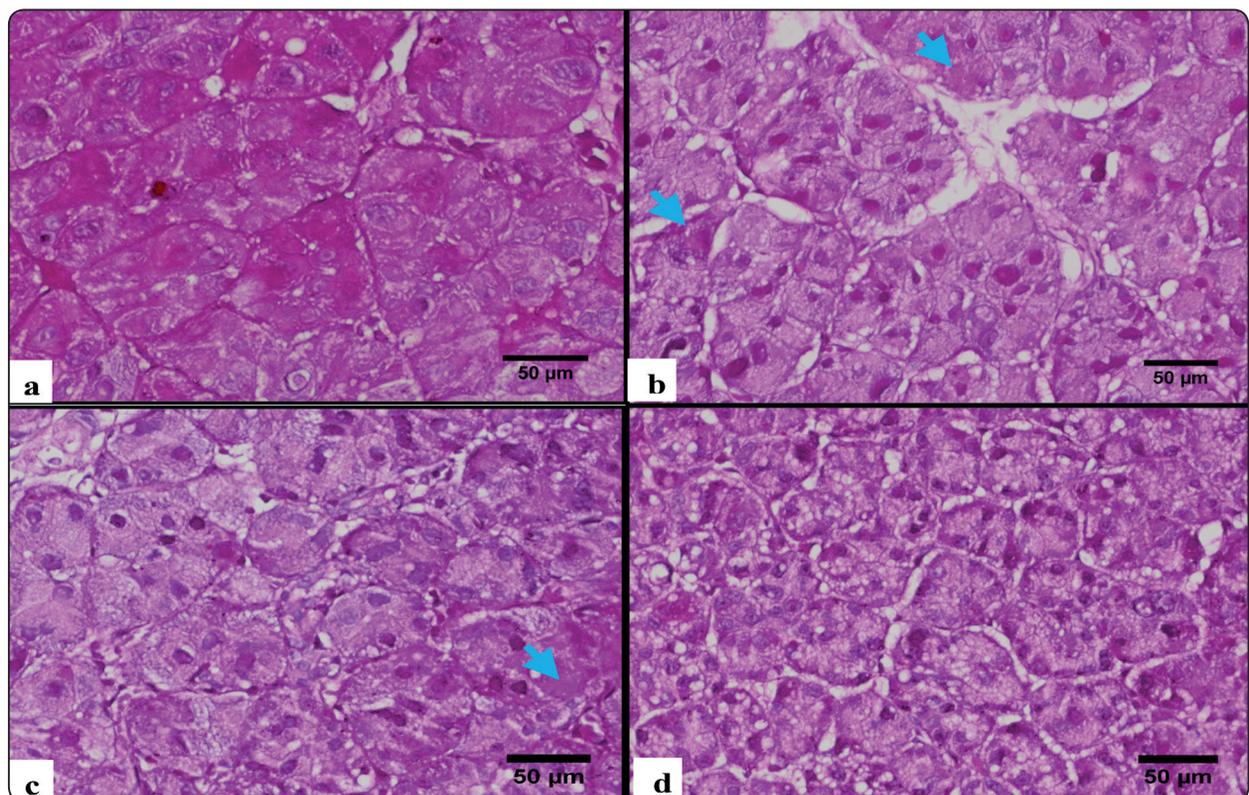


Fig. (5) Photomicrographs of samples stained with PAS. (a): Group A: showing intensely positive reaction in almost all of the serous acini. (b): Subgroup B1 with relatively pale stained cytoplasm spotty positive reactions (arrows). (c): Subgroup B2 showed spotty positive reactions (arrows). (d): B2 with foamy appearance (PAS. x400).

Immunohistochemical results:

The expression of neuron specific enolase was observed as brown staining in nuclei and cytoplasm with different degree of intensity and area fraction occupied. The serous acini of both groups showed scattered positive reaction with stellate or crescent

Results of PAS reaction:

The samples of control group stained with PAS showed intensely positive reaction in almost all of the serous acini. On the other hand, Subgroup B1 revealed a relatively pale stained cytoplasm except minimal spotty positive reactions in discrete acini. Subgroup B2 showed similar result as that of subgroup B1 in some regions and foamy heterogenous appearance in other regions (fig. 5).

shaped areas bordering the acini. But the reaction was apparently stronger in group A and subgroup B2 (fig. 6 a, c, e). The connective tissue regions showed different sized masses of positive reaction which was strongest in subgroup B2 while it was moderately positive in group A and subgroup B1 (fig. 6 b, d, f).

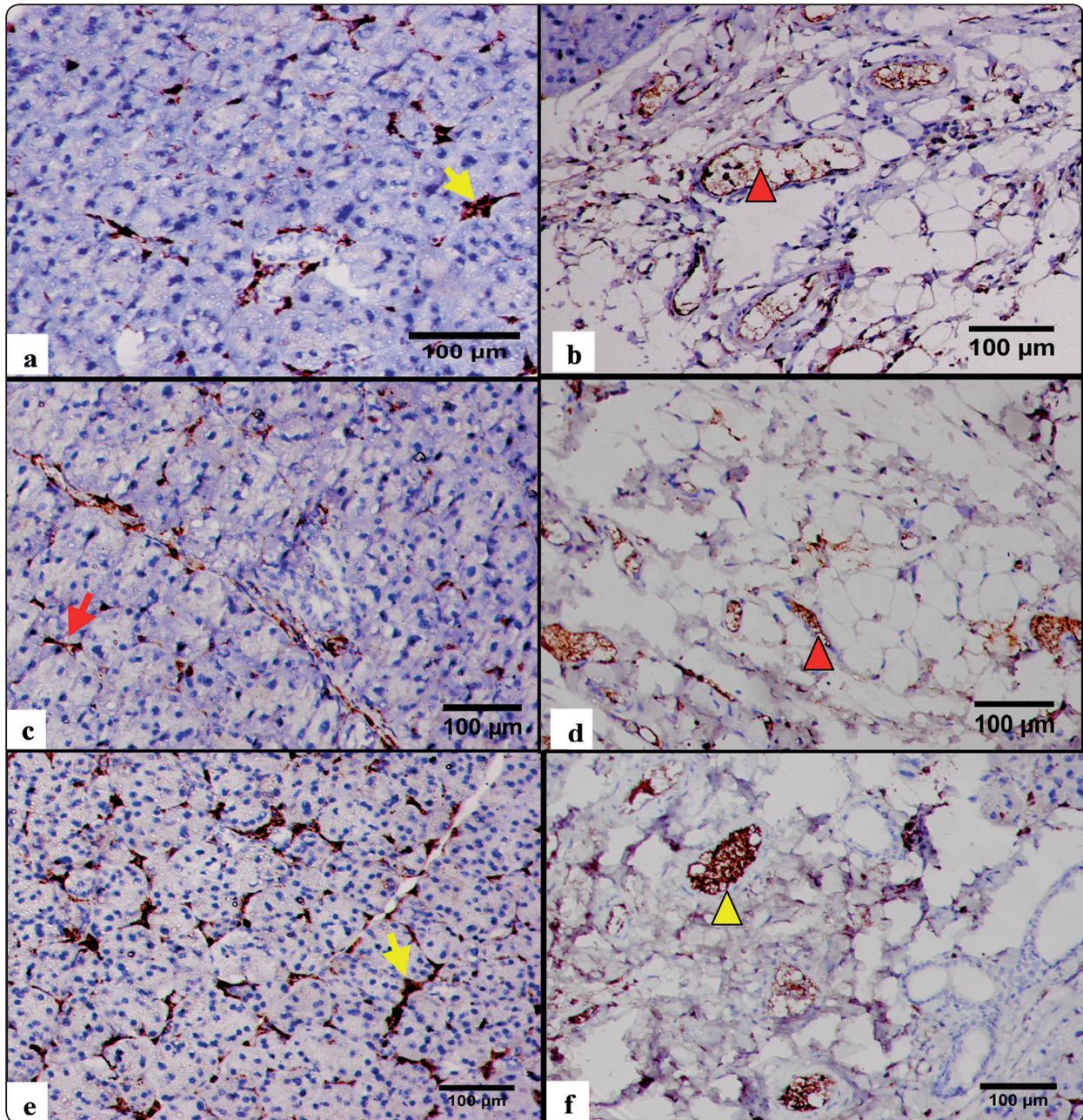


Fig. (6) Photomicrographs of samples stained with NSE. Strong positive reaction is seen in the acini of group A and subgroup B2 (a, e) (yellow arrows) in addition to connective tissue of subgroup B2 (f) (yellow arrow head). Moderate positive reactions are seen in acini and connective tissue of subgroup B1 (c, d) (red arrow and arrow head). In addition to the connective tissue of group A (b) (red arrow head) (Neuron specific enolase “NSE”. x200).

Immunohistomorphometric results:

The area fractions of the immuno-positive expression were measured in 6 regions in each slide (3 in lobules and 3 in the connective tissue). 2 Averages for each slide were calculated: 1st for the area fraction of the lobular area, 2nd for the connective tissues regions. Regarding the connective tissue region area fraction, the highest mean value was that of group A followed by B2 then B1. While in the

lobular region, the lowest mean value was of control group followed by subgroup B1 then the highest was subgroup B2 (table 3 and figure 7). One way ANOVA test revealed significant difference between the 3 groups in both the connective tissue and lobular regions. However, in comparison between each 2 groups, and subgroups the difference was statistically non-significant.

TABLE (3): The mean & standard deviation values of the area fraction for the immuno-positive expression in control and BoNT groups.

	Lobular region			Connective tissue		
	Group A (control)	Group B (BoNT)		Group A (control)	Group B (BoNT)	
		Subgroup B1 (3 days)	Subgroup B2 (14 days)		Subgroup B1 (3 days)	Subgroup B2 (14 days)
Mean	1.09	1.48	2.42	11.80	5.11	10.08
Standard deviation	0.14	0.40	0.50	0.50	2.35	3.86

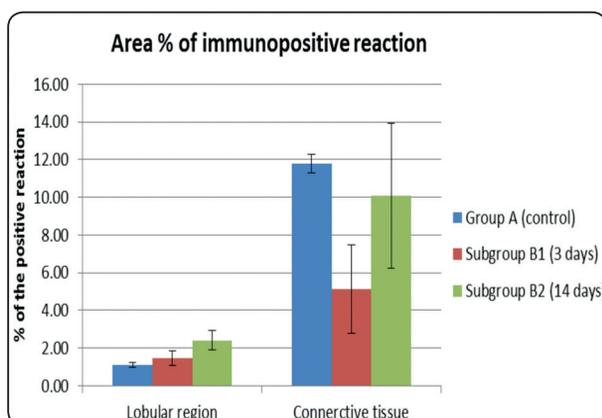


Fig. 7: Change in the mean values of the area % of the immuno-positive expression of NSE.

DISCUSSION

The present work was directed to evaluate possible effects of BoNT intra-masseteric injection on parotid glands. The recent increase in the frequency of using BoNT in the treatment of many muscular disorders including masseter⁽²⁹⁾ necessitates the clarification of the extent of the affection of neighboring tissues particularly parotid gland. Parotid glands have close anatomical relation to masseter muscle in rats⁽³⁸⁾ and human as well.⁽³⁹⁾ This might increase the susceptibility of parotid glands to be affected by diffusion of BoNT injection as per the suggestion proposed by *Kuwahara et al (2016)* who reported that adverse effects might occur in the tissues adjacent to masseter via direct diffusion of the neurotoxin.⁽³⁰⁾

In the present work, animal model was selected to perform histological sections to elucidate the structural changes in the glandular tissues. The selected dose was 2.5 units per rat. i.e (10 units per kilogram body weight as the rats in the present work were around 250 grams). This dose could be considered as a moderate dose, as the dose starting from 15 unit/ kg in human is considered high dose. ⁽⁴⁰⁾ Moreover, the experimental rats in the present study were sacrificed in 2 different dates (3 and 14 days after injection) in order to study the early and relatively late effects. Besides, the injection of BoNT in the present study was performed with small amount of solvent; this was to fit with the suggestion of *Salles et al (2015)* that the injection site and volume were reported that greatly affects the extent of diffusion of BoNT to the neighboring tissues. ⁽⁴¹⁾

Furthermore, care has been taken to ensure the injection into center of masseter muscle, as possible, in order to decrease the possibility of toxin diffusion through the fascia surrounding the masseter. ⁽³¹⁾ The evaluation tests in the present work in addition to routine histologic examination were the histomorphometric analysis to obtain numerical rather than descriptive data. PAS stain was used to assess the secretory activity of the cells. Finally Neuron specific enolase marker was used as it can be used to identify neuronal cells ⁽⁴²⁾ and myoepithelial cells ⁽⁴³⁾. This would be useful to assess the neural changes in the glands after BoNT injection.

In the present study, the histological examination revealed vacuoles in serous acini in both subgroups and pale staining of cells. This is in agreement with *Shan et al (2013)* ⁽⁴⁴⁾ who described apoptosis in acinar cells of salivary glands with BoNT injection. Also *Younis et al (2013)* specifically reported cellular vacuoles in parotid glands as well as decreased cytoplasmic vesicles and rough endoplasmic reticulum. ⁽²¹⁾ As these authors studied the intraglandular injection of BoNT, the coincidence of the results might support the suggestion of BoNT diffusion from masseter to parotid gland.

The connective tissue in the present study showed fibrosis. The increase in fibrous content with BoNT application was reported by *Byung et al (2009)* in the wound healing. ⁽⁴⁵⁾ Moreover, Dilated blood vessels with slightly congested RBCs in addition to aggregations of the inflammatory cells might be explained as a compensatory mechanism for the downregulatory effect of BoNT on the salivary gland function. On the contrary, *Yuan et al (2004)* didn't find inflammatory reaction in a study on submandibular glands. ⁽⁴⁶⁾ The mismatched results might be due to the difference of the gland physiological response between parotid and submandibular glands. Most of the previous work in the literature which studied the effect of intra-masseteric BoNT injection on parotid glands revealed no detectable effects on the salivary flow. ^(32, 33) This could be attributed to the different study design as these were human "clinical" work which might not reflect the internal microstructure of the glands particularly in the short term periods as those in the present work.

It could be suggested that the effect of botulinum toxin might be due to indirect mechanism through the effect of BoNT on the activity of masseter muscle causing their temporary weakness. ^(47,48) The change in bite forces could adversely affect the salivary glands as it was reported by *ElGhamrawy (2015)* that the diet consistency is a major effector on parotid gland; liquid diet caused cellular damages and apoptotic changes in parotid gland. Moreover this mechanism in the same study resulted in less PAS reaction of the parotid glands which coincides with the results of the present study. ⁽⁴⁹⁾

In the current work, the expression of neuron specific enolase was affected particularly in the subgroup B1 (3 days). This is in accordance to *Nozais et al (1999)* who correlated between neural injury such as denervation and the drop of enolase transcript levels. ⁽⁵⁰⁾ The increased expression of enolase in both of the experimental subgroups in the parenchymal region might be explained by that the activity of myoepithelial cells is increased as a defense mechanism to compensate the affected glandular

neurophysiology. Moreover, the reverse increase in the NSE expression in the 14 days subgroup connective tissue region might suggest some sort of reversibility of the effects of BoNT on the glands. However, the change in NSE expression could add a value in the explanation of the mechanism of action of intramasseteric BoNT injection on the structure of parotid gland which is more probably a multifactorial involving direct neuronal effects and indirect secondary effects from the muscular changes.

The present study could give a primary valuation of the intra-masseteric BoNT effects on parotid glands. However, further investigations might be needed with larger sample size and different doses to widen the range of confidence and reliability of the conclusion.

CONCLUSIONS

Within limitations of the present work, it could be concluded that intra-masseteric BoNT injection adversely affects parotid glands with mild effect of time factor.

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