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EVALUATION OF THE EFFECTS OF BOTULINUM NEURO TOXIN A (BONTA) ON SUBMANDIBULAR SALIVARY GLAND IN RATS BY HISTOLOGICAL EXAMINATION AND SEMI-QUANTITATIVE SCORING METHOD

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ABSTRACT

Introduction: Botulinum toxin A (BoNTA) has been used for treating hyperfunction of various glands such as sweat, lacrimal, and salivary glands. However, the long-term histological sequences are largely unknown.

Objectives: The present study is to evaluate the histological and ultrastructural effects of BoNTA on submandibular salivary gland (SSG).

Methods: Sixty male albino rats received 0.1 ml of either saline (control group) or BoNTA (BoNTA group) injection in the right SSGs. The rats were terminated at 2, 4 and 12 weeks after the injection. The harvested SSGs were embedded and sectioned at 4-5μm and stained with H&E for histological study.

Results: All control SSGs showed normal acinar cells with rounded nuclei and regular striated ducts (SD) with characteristic basal striations. Compared with these features, 2-week BoNTA-injected SSGs showed loss of spherical fashion and basal striations in serous acini and SD respectively, and the cell boundaries were not clear. In 4-week SSGs, some acini and ducts lost their spherical fashion and, in some areas, these structures disappeared. However, all 12-week BoNTA-injected SSGs seemed to have similar structures to those of control SSGs. By using scoring system for semi-quantifying the histological structural changes of BoNTA-injected SSGs, 2- and 4-week BoNTA-injected SSGs showed significantly higher scores as compared with their control counterparts. However, no significant score differences were found between 12-week BoNTA-injected and control SSGs.

Conclusions: Although application of BoNTA results in significant changes in histological structures of SSGs, these detrimental effects seems to be transient, and the major recovery occurs after 3 months. Thus, BoNTA can be used for treating SSG hyperfunction.

Keywords: Botulinum toxin A; Submandibular salivary glands; Histology; semi-quantitative scoring; Rats.

INTRODUCTION

Botulinum toxin is produced by anaerobic fermentation of the bacterium Clostridium botulinum which produce eight immunologically distinct serotypes (type A to H), and serotypes A and B have been developed for clinical applications in humans. The U.S. Food and Drug Administration (FDA) first approved Botulinumtoxin A in 1989 for treatment of blepharospasm and strabismus[1]. Since then, BoNTA has been widely applied in various clinical conditions, including intraglandular applications. BoNTA is also the most widely used agent

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approved by the US FDA for aesthetic purposes[2]. The BoNTA inhibits acetylcholine release at the neuroglandular junction, which acts similar to the chemical parasympathectomy, thus produces a distinct reduction in salivary flow [3]. Therefore, it has been used to treat sialorrhoea (drooling), defined as overflow of saliva from the mouth that causes physical and psychosocial problems [4]. For example, it was reported that a single-dose BoNTA injection in the submandibular glands has significant effect on drooling without serious side effects[5]. BoNTA has also been used for treating drooling caused by swallowing disorders due to the surgery to remove tumors in the upper aero digestive tract[6], and for the cases of salivary fistulas after sialadenectomy or oropharyngeal cancer surgery where temporary stopping of glandular secretory action is needed to promote healing[7]. Laing et al. in 2008 also reported that the use of BoNTA in glandular hypersecretion resulted in overall promising results with minimal side effects[8]. Intraglandular injection of BoNTA is considered a safe, minimally invasive treatment of sialorrhoea [9]. Bothwell et al., further demonstrated that BoNTA is a relatively effective treatment for some children with significant drooling without serious side effects[10].

Therefore, the application of BoNTA for the treatment of sialorrhea could replace the use of cholinergic drugs, which usually has undesirable adverse effects such as constipation, urinary retention, tiredness, irritability, drowsiness, and potential severe cardiac side effects [11,12]. However, the biological effect of intraglandular BoNTA injection and its long-term histological sequences are largely unknown. Therefore, the aim of the present study is to evaluate the histological effects of BoNTA injection on submandibular salivary gland (SSG) over time in rats using H&E staining and semi-quantitative scoring method.

MATERIALS AND METHODS

Animals

Sixty male albino rats with average body weight of 250-300g were obtained from Veterinary Research Institute of Faculty of Medicine, Cairo University, where they were kept in laboratory animal house under standard conditions of controlled environment. The room temperature was adjusted within the range of 20-25˚C and food and water were available *ad libitum*. Institutional Animal Care and Use committee of Cairo University, Egypt approved all procedures.

Injection of BoNTA:

Anesthesia was induced by an intramuscular injection of 100mg/kg ketamine in combination with 5mg/kg xylazine. The right SSG was exposed via submandibular incision, then either 0.1 ml saline (control group) or 5 units of BoNTA (BoNTA group) (Botox®, Allergan Inc., Irvine, CA, USA) reconstituted in 0.1 ml saline was injected at the center of the SSG. The regular housing and feeding regime remained and each group of rats were euthanized with over dosage of inhaled ether at the following time points after the injection: short term: 2 weeks, middle term: 4 weeks; and long term: 12 weeks.

Tissue harvest and processing

Upon the termination of the experimental periods, the right SSGs were surgically removed then fixed immediately in 10% calcium formol for 72 hours, washed by tap water, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and embedded in paraffin wax. Sections of 4-5 μm thickness were cut and mounted on glass slide and stained with hematoxylin and eosin.

Image examination and semi-quantitative scoring

By using a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan), the entire H&E section was first screened under low magnification (20-40X) to locate the specific structures of the SSG, then

each structure was further examined under higher magnification (400-600X) for descriptive histology.

For further histological analysis, a unique scoring system was designed by Professor. Zi Jun Liu and Professor. Suzan Herring at school of dentistry, University of Washington to semi-quantify three major changes in SSG structures, i. e, basal striations, (BS), cytoplasmic vacuolization (CV) and stagnation of secretion in the lumen (SSL). The detailed definitions for each structural change and score criteria are listed in **Table 1**. The intra- and inter-examiner reliabilities of the scoring system (method errors) were assessed by analyzing the difference between duplicate measurements taken at 10 days apart by the same observer (MA), and by two observers (MA and MD) on randomly selected H&E sections from 5 r ats and the microscopic fields were scored.

TABLE (1) Scoring System for Histological Evaluation.

Structural changes	Definitions	Score 0	Score 1	Score 2	Score 3
Basal striations (BS)	% loss of basal striation	No loss	<25%	25-50%	>50%
Cytoplasmic Nacuolization (CV)	% of vacuolization in the area of ducts	No vacuolization	<25%	25-50%	>50%
Stagnation of secretion in the lumen (SSL)	% of secretion stagnation in the area of lumens	No stagnation	<25%	25-50%	>50%

Statistical analysis

Nonparametric independent-sample median test was performed for inter-investigator's agreement, and the result indicated that the score medians of BS, CV, and SSL were the same between the two investigators. These results demonstrated the reliability of the proposed scoring system for semi-quantitative analysis of histology.

While the major data analyses were descriptive, the scoring results were examined using Kruskal-Wallis test across the 3-time points and further examined using Mann-Whitney test to detect the differences between the two groups at each time point. The significant level was set as $p < 0.05$.

RESULTS

A- Histological results

The normal structure of SSG was seen in all three control groups (2, 4 and 12 weeks), i.e., serous acini with pyramidal shaped cells containing rounded or ovoid deeply basophilic nuclei were located in the basal third of the cell and the granular convoluted tubules were consisted of cuboidal cells with nuclei located at the basal third (**Plate. 1A, B** and **C**). However, the round-shaped striated ducts showed characteristic basal striation and were surrounded by blood vessels engorged with red blood cells (**Plate. 1A,** and **C**). The excretory ducts presented stratified squamous epithelium and were surrounded by connective tissue in addition to patent blood vessels (**Plate. 1B**). Compared with those in control SSGs, serous acinar cells presented the following changes at 2 weeks after the application of BoNTA: loss of their spherical fashion, cytoplasmic vacuoles, and certain degenerative changes including loss of basal striations and defective borders in striated ducts (**Plate. 1D**). After 4 weeks, these acinar cells showed mitotic nuclei and further loss of their spherical fashion. Degeneration appeared to be severe, featured by the loss of basal striations and extended cytoplasmic vacuolization. Excretory ducts with interrupted outline and stagnated secretion into their lumen were observed (**Plate. 1E**). After 12 weeks, serous acinar cells appeared to show regular spherical fashion again, along with characteristic basal striations and clear cellular borders in striated ducts surrounded by patent blood vessels as seen in control SSGs (compared **Plate. 1A, B** and **C** to **F**).

B- Semi-Quantitative Scoring results:

As shown in **Figure 2**, while there were no significant differences of scoring 3 structural changes of control SSGs over three-time points, significantly higher scores than those of controls were identified for all these structures in 2- and 4-week BoNTA-injected SSGs, and the highest scores were all in 4-week BoNTA-injected SSGs. At 12 weeks, these three structural changes of BoNTA-injected SSGs returned to the levels even slightly lower than those of controls SSGs.

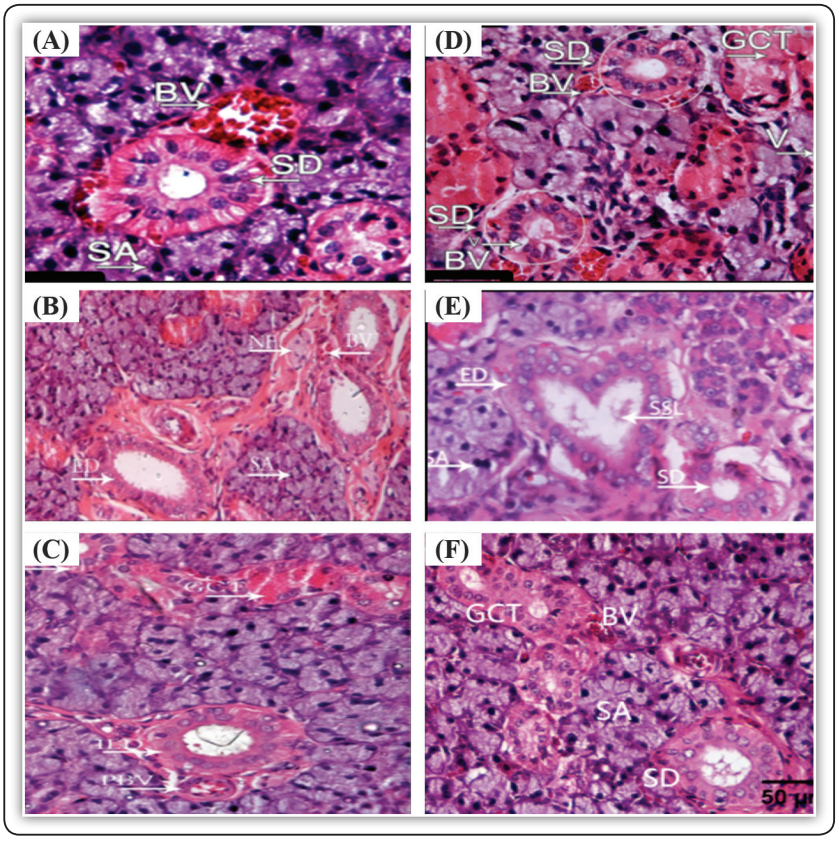

Plate 1: Microscopic presentations of control (left column) and BoNTA-injected (right column) SSGs.

Top (A&D): 2 weeks;

Middle(B&E): 4 weeks;

Bottom (C&F): 12 weeks.

SA: serous acini; SD: striated duct; GCT: granular convoluted tubules; ED: excretory duct; NF:nerve fascicles; CT: connective tissue; BV: blood vessels; V: cytoplasmic vacuolization.

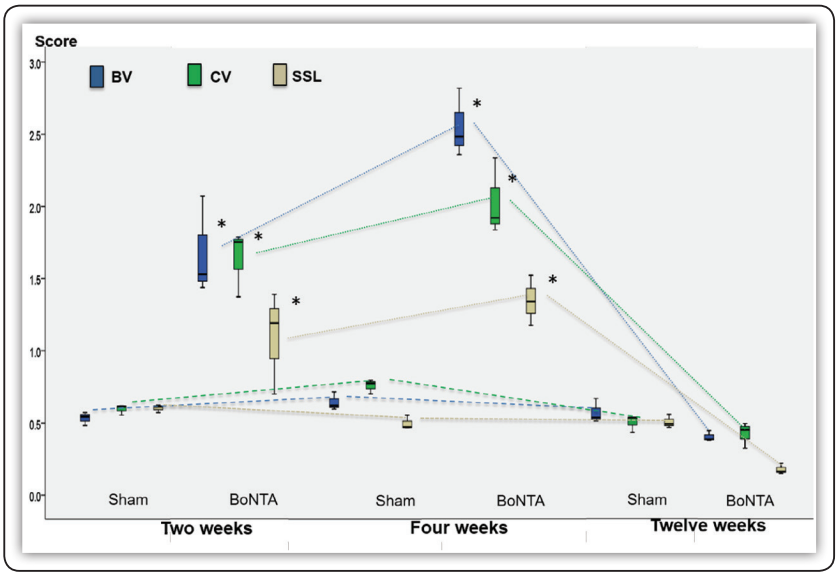

FIG (2) Results of scoring BS, CV and SSL. Upper and lower limits of box represent 75th and 25th percentiles of eachstructure, respectively. Horizontal line in each box represents the median of each score. Dashed and dotted lines represent the time-course curves in sham and BoNTA SSGs, respectively. Asterisks indicate significance, $p < 0.05$.

DISCUSSION

Hypersalivation accompanying various diseases can be a cause for Signiant discomfort in addition to physical and psychosocial problems to affected patients. The disadvantages of these approaches are the great distress for the patient and the need to use an irreversible treatment to control a temporary period of sialorrhea**.** Therefore, BoNTA injection into salivary glands has been used to treat sialorrhea and considered a safe, minimally invasive treatment approach. Moreover, it may be accidentally injected into the submandibular salivary glands during cosmetic treatment of facial lines at platysma or under the chin, therefore it was important to study the biological effect of BoNTA on submandibular salivary glands two, four and twelve weeks after intraglandular injection.

These time points were chosen because clinical effects of BoNTA application begins within 24-48 hours, peaks at 2-3 weeks and lasts for 3-4 months[13]; also, because the current treatment protocol suggests 12-week separation between each BoNTA application[14]. Considering the possibility of antibody production with resulting immuno-resistance with the use of BoNTA, it has been recommended that treatment session should be repeated not less than monthly intervals[15]. Ellies et al., also reported that salivary flow was reduced in most cases about 3 months after BoNTA application[6].

BoNTA has been a new therapeutic approach to treat various disorders including salivary glands. However, the short- and long-term biological effects of BoNTA on the function and histology of salivary glands have not been well studied. The present study provided some new findings of these effects as discussed below.

Changes in Histological structures

In the present study, the control SSGs showed normal histological structures of serous acini and duct system without significant histological differences at 2, 4 and 12 weeks, indicating the growth effects on these structures are not significant. In a study on rats' SSGs by Teymoortash et al. in 2007, the histological findings after 2 weeks of intragranular saline injection are similar to the present findings[16]. Similar findings were also reported in a study by Shan et al. who found regular acinar and ductal cells without any morphological changes with in rabbits SSGs injected with normal saline [17].

In the present study, significant histological structural and ultrastructural alterations were observed in BoNTA-injected SSGs in 2 weeks, and these changes became more obvious and aggravated in 4 weeks. Nonetheless, these alterations were transient, and these atrophic changes were recovered to normal histological structure in 12 weeks. These results indicate that BoNTA has a reversible biological effect on the histology of SSGs.

Changes in histological structure 2 weeks after BoNTA injection were presented in the form of loss of spherical fashion and cytoplasmic vacuoles of some serous acini. In addition, degenerative changes of striated ducts such as loss of basal striations and defective borders were observed. These findings were congruent with the findings of Teymoortash et al. where smaller acinar cells and wider lumen of striated ducts were noted in parotid glands treated with BoNTA as compared with the controls [16]. The present findings are also similar to what found in rabbit SSGs 2 weeks after BoNTA injection [17], and findings on 4-week BoNTA injected SSGs are similar to **a** rat study on parotid gland after BoNTA injection [18]. This study also reported clear-cut signs of atrophy and degeneration in the parotid glands. However, the current findings on 4-week BoNTA-injected SSGs are not in accordance with the findings by Shan et al. in 2013[17]. They observed partial recovery on rabbit SSGs 4 weeks after BoNTA application, but our findings demonstrated severer atrophy and degenerative changes at 4 weeks as compared with those at 2 weeks. Nevertheless, the current findings on 12-week BoNTA-injected SSGs agree with what reported by Shan et al, in which the structure of rabbits' SSGs returned to the normal shapes 12 weeks after BoNTA application [17].

Mechanism of BoNTA on salivary glands

BoNTA works by blocking the release of acetylcholine from the cholinergic nerve end plates thus leading to inactivity of the muscles or glands innervated [3]. In a study by Bhogal et al, histological evidence suggested that toxin injection is followed by a chemical denervation then re-sprouting of axon occurs [21]. The timing of axonal re-sprouting is variable over a period of weeks to months. Intramuscular injection of BoNTA results in local chemical denervation and the loss of neuronal activity in the target organ [21]. On the other hand, BoNTA acts to inhibit salivary production by binding to SNAP–25, a cytoplasmic protein involved in the fusion of synaptic vesicles with the presynaptic membrane. This ultimately disrupts the secretory pathway for acetylcholine and produces a chemo denervation [22]. Therefore, it is speculated that the recovery of SSGs to normal histological structure 12 weeks after BoNTA injection in the present study may be due to neural sprouting with re-innervation of the gland after chemo denervation.

Ferreira and Hoffman pointed out that increased sympathetic activity results in reduced progenitor cell self-renewal. They further concluded that epithelial organ repair or regeneration could occur after injury if parasympathetic innervation is maintained [23]. Moreover, Knox et al hypothesized that parasympathetic innervation maintained the epithelial progenitor cell function during salivary gland organogenesis [24]. They further demonstrated that acetylcholine signaling enhances epithelial proliferation and morphogenesis of the keratin 5-positive progenitor cells [24]. Hence, this mechanism could be applied for organ repair or regeneration. Based on these studies, it could be inferred that the major recovery that happen three months after BoNTA injection in the present study is due to release of acetylcholine after transient Para sympathectomy. Therefore, nerves may play an instructive role for submandibular salivary gland repair and regeneration.

The epithelial salivary gland stem/progenitor cells are located at the epithelial part of the gland and they are known as label-retaining cells (LRC) because they are slowly dividing cells that retains the DNA-label after months of continuous growth [25]. Studies done by Carpenter et al and Denny et al confirmed that LRCs are located in the intercalated ducts and play an important role in regeneration of submandibular salivary glands [26,27]. In the present study, the glandular architecture showed major recovery three month after BoNTA injection. This may be due to remittance of normal mitosis of the nuclei of serous acini and proliferation of intercalated ducts to replace the atrophied and degenerated part of the gland that occurred 4 weeks after BoNTA injection.

CONCLUSIONS AND RECOMMENDATIONS

1. Although application of BoNTA results in obvious damages of both histological structures and cellular organs of SSGs in short and middle terms, these detrimental effects were transient and major recovery occurs in 3 months.
2. Instead of surgical intervention or duct ligation, BoNTA can be used for treatment of SSG hyperfunction as a minimally invasive treatment modality. However, periodical applications with the separation of not less than 6 months is suggested due to the transient effects of BoNTA on SSGs.
3. Periodical clinical applications and injection of Botox with intervals of longer periods (not less than 6 months) is recommended due to the transient effects of BoNTA on submandibular salivary glands.

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