Effects of Botulinum Toxin A on Histology and Ultrastructure of Submandibular Salivary Gland in Rats

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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: Eighteen 6-week-old male albino rats received 0.1 ml of either saline (sham group, n=9) or BoNTA (BoNTA group, n=9) injection in the right SSGs. Of 9 rats in each group, 3 were terminated at 2, 4 and 12 weeks after the injection. The harvested SSGs were embedded and sectioned at 6μm, and stained with H&E for histological study. Ultrathin sections (60-90nm) were cut from1 mm^3 pieces harvested from the center of SSGs, and mounted on copper grids for ultrastructural study using transmission electron microscope (TEM).

Results: All sham SSGs showed normal acinar cells with rounded nuclei and regular striated ducts (SD) with characteristic basal striations. By TEM, acinar cells exhibited rounded nuclei, mitochondria, and secretory granules at cytoplasm. Numerous mitochondria presented in SD. 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. TEM further revealed irregular nuclei of acinar cells and SD, and swollen mitochondria. In 4-week SSGs, some acini and ducts lost their spherical fashion and in some areas, these structures disappeared. Ruptured mitochondria were observed in acini and SD by TEM. However, all 12-week BoNTA-injected SSGs seemed to have similar structures to those of sham 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 sham counterparts. However, no significant score difference was found between 12-week BoNTA-injected and sham SSGs.

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

Keywords: Botulinum toxin A, Submandibular salivary glands, Histology, Ultrastructure, Rat.

1. 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 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*. 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

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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, and 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 and ultrastructural effects of BoNTA injection on submandibular salivary gland (SSG) over short, middle, and long terms in rats.

2. MATERIALS AND METHODS

Animals

Eighteen 6-week-old 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.

Application 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 (sham group, n=9) or 5 units of BoNTA (BoNTA group, n=9) (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. 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].

Tissue Harvest and Processing

Upon the termination, the right SSGs were harvested 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 6-7µm thickness were cut and mounted on glass slide and stained with hematoxylin and eosin. The specimens for transmission electron microscope examination (uranyl acetate & lead citrate) were prepared as follows: The specimens were first fixed in 4% glutaraldehyde in 0.2 ml sodium cacodylate buffer at PH 7.3 for 24 hours, and then post-fixed in 1% osmium tetroxide buffered for 1-2 hour. After 30' washing in the same buffer, dehydrated in ascending grades of ethanol (50 – 90%) for 15 minutes. Finally, they were placed in the absolute alcohol for 15 minutes and embedded into gelatin capsules by using fresh epoxy resin in an oven at 60^0 C for 24-36 hours. These capsules were trimmed, sectioned at 1µm, stained with toluidine blue, and examined by light microscopy. Ultrathin sections (60-90nm) were further cut by ultra-microtome, mounted on copper grids, and stained with uranyl acetate.

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 (2-4X) to locate the specific structures of the SSG, then each structure was further examined under higher magnification (40-60X) for descriptive histology.

For further histological analysis, a scoring system was designed to semi-quantify three major changes in SSG duct structures, i. e, basal striations, (BS), cytoplasmic vacuolization (CV), and stagnation of secretion in the lumen (SSL). The detailed definitions for each structural changes 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 rats and the total of 48 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 vacuolization(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%

The cellular organs and other ultrastructure of the glands were examined using Transmission Electron Microscope (TEM, JEOL Japan) and described qualitatively.

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. The Dahlberg errors (Dahlberg, 1940) for BS, CV and SSL were 0.18 ,0.03 and 0.10, respectively. 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 $p < 0.05$.

3. RESULTS

Histology

Few mucous acinar cells were identified in rats' SSGs, thus the effects of BoNTA on this type of acinar cells was not applicable. Only serous acinar cells were examined in the present study.

The normal structure of SSG was seen in all three sham groups (2, 4 and 12 weeks), i.e., 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 (Figure **1A** and **C**). However, the round-shaped striated ducts showed characteristic basal striation, and were surrounded by blood vessels engorged with red blood cells (Figure **1A, B**, and **C**). The excretory ducts presented stratified squamous epithelium and were surrounded by connective tissue in addition to patent blood vessels (Figure **1B**).

Compared with those in sham 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 (Figure **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 (Figure **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 sham SSGs (compared Figure **1A, B** and **C** to **F**).

As shown in Figure **2**, while there was no significantly differences of scoring 3 structural changes of sham SSGs over 3 time points, significantly higher scores than those of shams 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 3 structural changes of BoNTA-injected SSGs returned to the levels even slightly lower than those of sham SSGs.

Ultrastructure

In sham SSGs, secretory granules (Figure **3A**) surrounded all nuclei. Moreover, at the basal third, a huge number of closed membranous sacs were seen, that is, the rough endoplasmic reticulum. In addition, Golgi apparatus was seen to locate apical to the nucleus, and the cytoplasm was dispersed with numerous rounded electron lucent secretory granules. The striated ducts showed characteristic basal striations of the plasma membrane, numerous mitochondria, and minute secretory granules (Figure **4A**).

For BoNTA-injected SSGs at 2 weeks, nucleus of the acinar cells was surrounded by degenerated

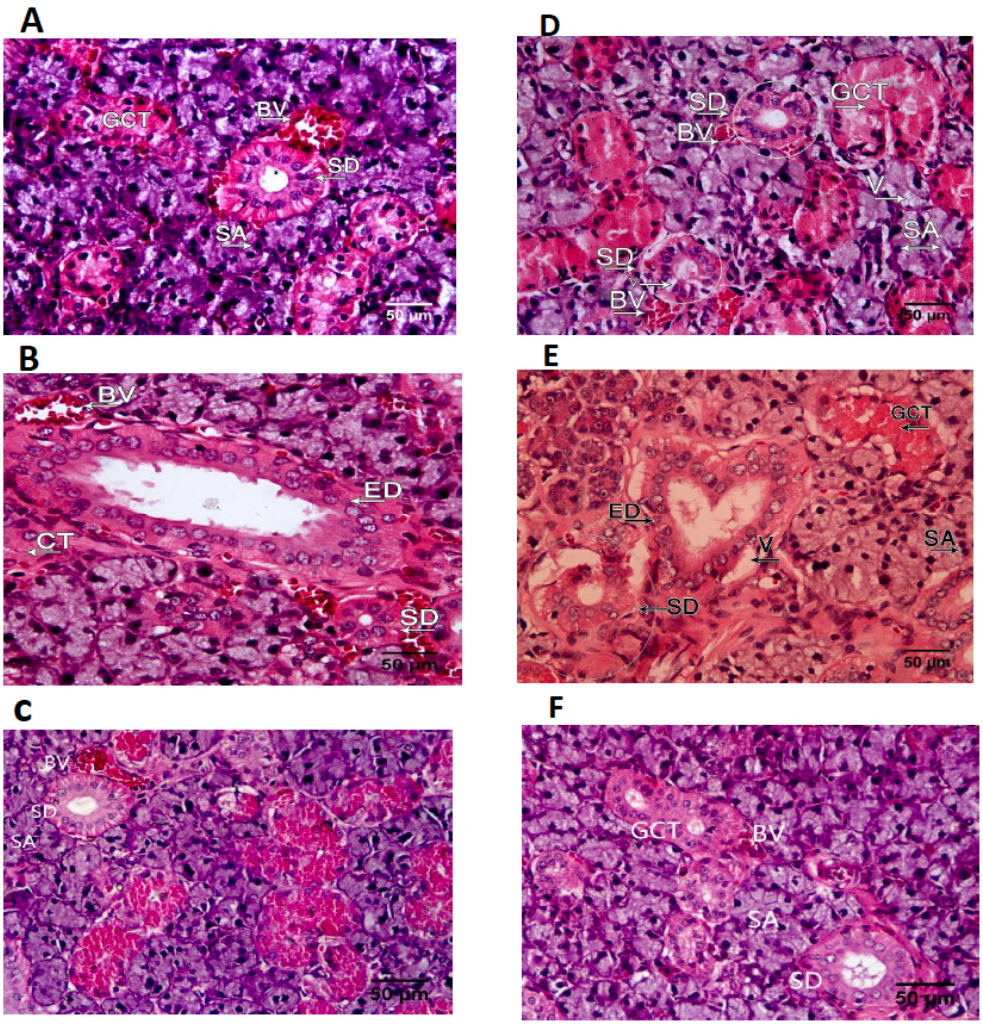

Figure 1: Microscopic presentations of sham (left column) and BoNTA-injected (right column) SSGs. **Top:** 2 weeks; **Middle:** 4 weeks; **Bottom**: 12 weeks. **SA:** serous acini; **SD:** striated duct; **GCT:** granular convoluted tubules; **ED:** excretory duct; **CT:** connective tissue; **BV:** blood vessels; **V:** cytoplasmic vacuolization.

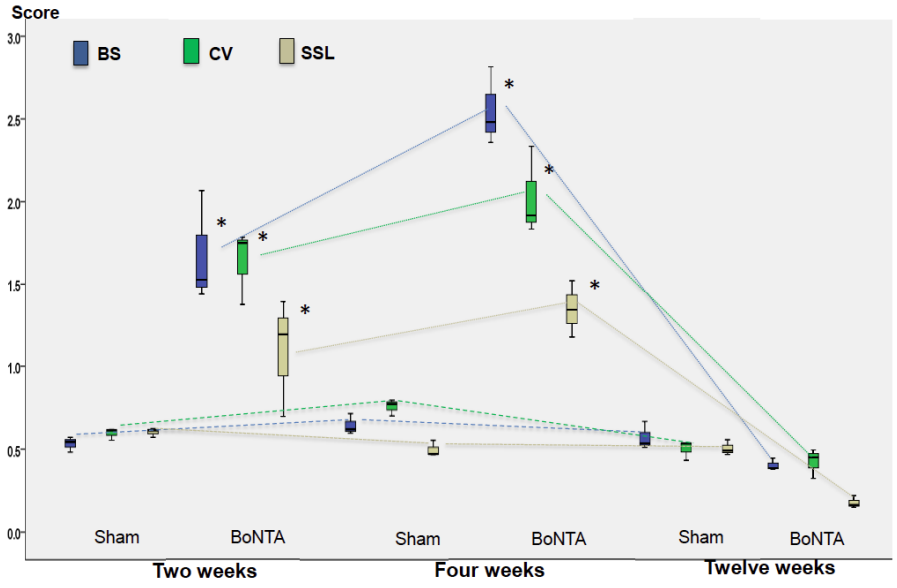

Figure 2: Results of scoring BS, CV and SSL. Upper and lower limits of box represent 75th and 25th percentiles of each structure, respectively. Horizontal line in each box represents the median of each score. Dashed and dotted lines represent the time-course curves in sham and BoNTA SSGs, respectively.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

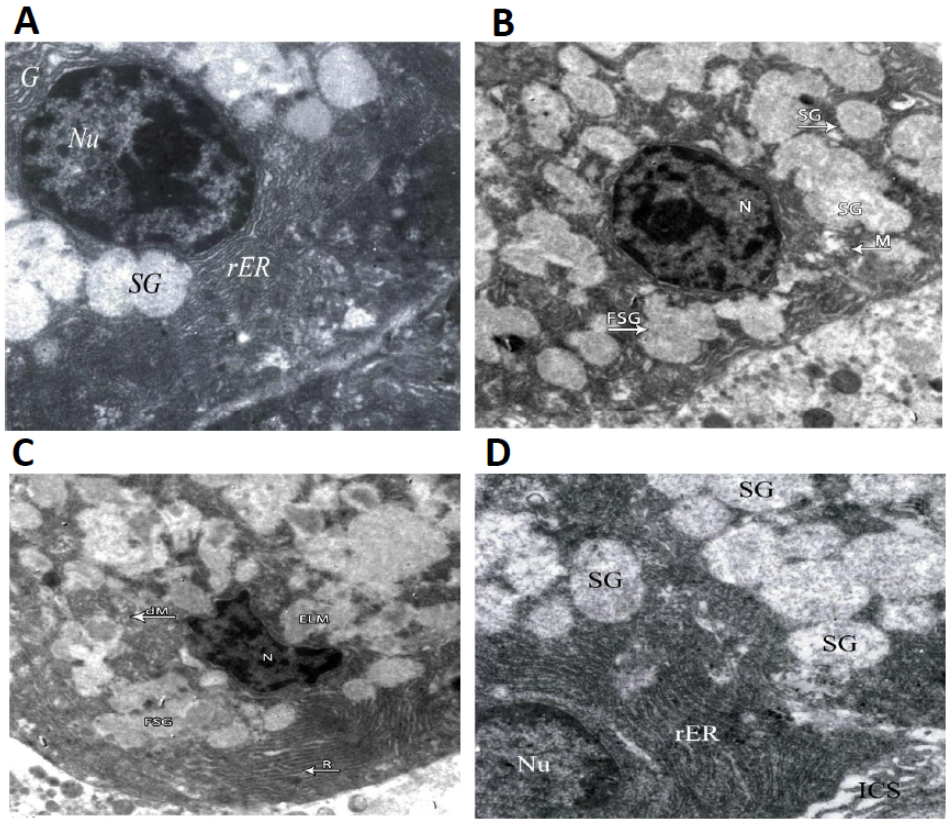

Figure 3: TEM presentation of serous acini of SSGs. **A:** sham at 4 weeks; **B:** BoNTA-injected at 2 weeks; **C:** BoNTA-injected at 4 weeks; **D:** BoNTA-injected at 12 weeks. **Nu:** nucleus; **rER:** rough endoplasmic reticulum; **SG:** secretory granules; **G:** Golgi apparatus; **FSG:** fused secretory granules; **ELM:** electron lucent material; **M:** mitochondria; **dM**: deformed mitochondria.

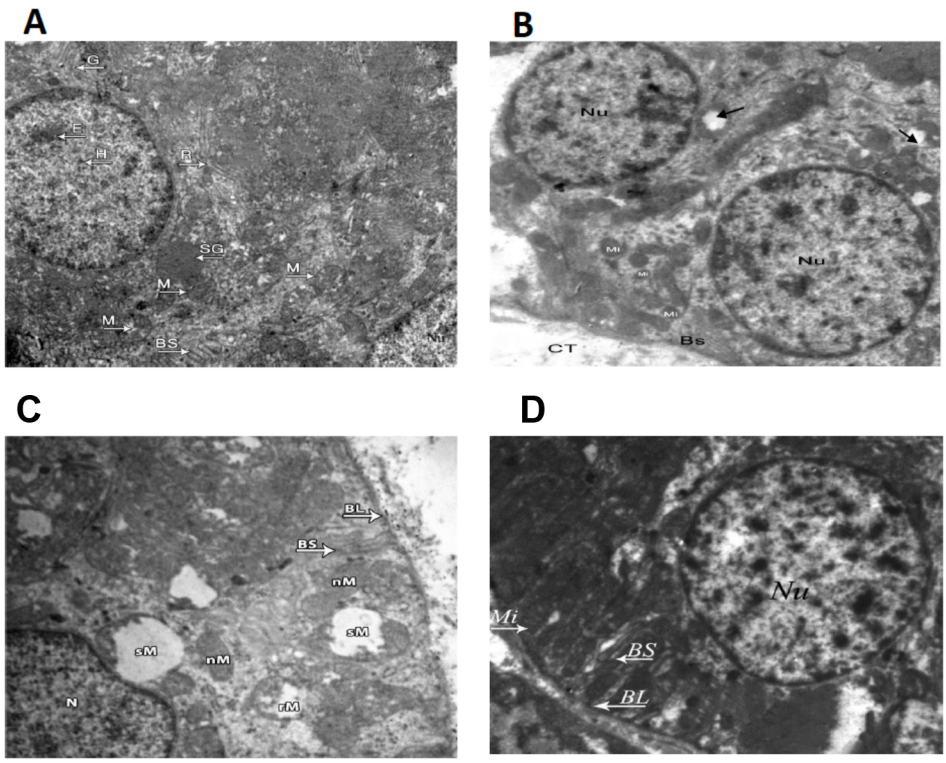

Figure 4: TEM presentations of striated ducts of SSGs. **A:** sham at 12 weeks; **B:** BoNTA-injected at 2 weeks; **C:** BoNTA-injected at 4 weeks; **D:** BoNTA-injected at 12 weeks; **Nu:** nucleus; **Arrows:** mitochondria with loss of internal cristae; **SG**: secretory granules; **BS**: basal striations; **BL:** basal lamina; **CT:** connective tissue; **M**: mitochondria; **nM**: normal mitochondria; **sM:** swollen mitochondria; **rM:** ruptured mitochondria.

mitochondria (Figure **3B**), and electron lucent secretory granules of different size around the nucleus were fused together (Figure **3B**). Striated ducts showed degenerated mitochondria and short basal striations (Figure **4B**). At 4 weeks, acinar cells showed more disfigured nucleus due to its compression by electron lucent material addition to pleomorphic secretory granules of variable size and electron density that fused together around the nucleus. Moreover, degenerated mitochondria, deformed mitochondria and thickened rough endoplasmic reticulum were also seen (Figure **3C**). The striated ducts showed the loss of basal infolding, cristae-lost mitochondria and some ruptured mitochondria, although some normal mitochondria were also noted (Figure **4C**). At 12 weeks, acinar cells were surrounded by regular rough endoplasmic reticulum and secretory granules of variable electron densities (Figure **3D**). The striated ducts showed well-defined basal striations and infolding of the basal lamina along with many mitochondria that had normal internal cristae (Figure **4D**).

4. DISCUSSION

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 sham 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*., 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 and ultrastructure 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.* [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].

Changes in Ultrastructures

The ultrastructural results of the present study prove that most extensively affected cellular organelle is the mitochondria. Sharma *et al.,* (2003) reported that the mitochondrial matrix contains many ribosomes that can carry out protein synthesis and fine circular threads of DNA [19]. Thus, any change in this composition leads to reduction in structural and enzymatic protein, which in turn reduces the capability of the cells to perform its function. Moreover, the major function of mitochondria is the production of adenosine triphosphate (ATP) which is used in various energy requiring activities [20]. Therefore, these alterations of mitochondria might lead to functional deteriorations, as any reduction in production of ATP affects the activity of the cell.

The main function of the rough endoplasmic reticulum (rER) is protein synthesis. RER shares the Golgi apparatus in lipids and phospholipid synthesis of all classes of lipids including cholesterol phospholipids, triglycerides, and steroid hormone. Therefore, degenerative changes of rER will affect cell function

[20]. Since distorted Golgi apparatus and cytoplasmic vacuolization were always detected in 2- and 4-week BoNTA-injected SSGs in the present study, these changes might lead to the cellular degeneration and functional affection in synthesis and secretion of saliva.

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 chemodenervation [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 chemodenervation.

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 month after BoNTA injection in the present study is due to release of acetylcholine after transient parasympathectomy. 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.

5. CONCLUSIONS

In conclusions, the present study suggests 1) although application of BoNTA results in significant 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 4-5 weeks is suggested due to the transient effects of BoNTA on SSGs.

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