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EFFECTS OF BOTULINUM TOXIN A (BOTOX) ON CELLULAR ORGANS OF SUBMANDIBULAR SALIVARY GLAND IN RATS (ULTRASTRUCTURAL STUDY)

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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 4-5 μm 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 control 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 basal striations. Examination by TEM 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 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 difference was found between 12-week BoNTA-injected and control 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 after 3 months. Thus, BoNTA can be used for treating SSG hyperfunction.

Keywords: Botulinum toxin A; Submandibular salivary glands; Histology; ultrastructures; Rat.

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INTRODUCTION

Botulinum toxin (Botox) is best known for its beneficial role in facial aesthetics but recently it is used in multiple non-cosmetic medical and surgical conditions. Botox is an exotoxin produced from Clostridium botulinum, so it is considered as bacterial toxin that could be used as a medicine and it appears to be as 'the poison that heals" [1]. 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. 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 and ultrastructural effects of BoNTA injection on submandibular salivary gland (SSG) over t in rats

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. 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.

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

Ultrastructural results

Ultrastructural examination of control group revealed that the acinar cells presented pyramidal cells with basally located rounded nuclei, rER and secretory granules. However, Golgi apparatus located apical and lateral to the nucleus. The cytoplasm was dispersed with numerous mitochondria. The granular convoluted tubules with electron lucent basally situated nucleus surrounded by electron dense secretory granules. The cells of the striated ducts showed characteristic basal infoldings of the plasma membrane and numerous mitochondria. Their nuclei surrounded by cisterna of rough endoplasmic reticulum, minute secretory granules and huge number of mitochondria which are scattered throughout the cytoplasm.

In controls SSGs, secretory granules surrounded all nuclei (**plate. 1A**). Moreover, at the basal third, a huge number of a 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 (**plate. 2A**).

For BoNTA-injected SSGs at 2 weeks, nucleus of the acinar cells was surrounded by degenerated mitochondria presented acinar cells with irregular nuclear pattern, degenerated mitochondria and surrounded by fused secretory granules(FSG) (**plate. 1B**). Striated ducts showed degenerated mitochondria and short basal striations (**plate. 2B**).

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 Golgi apparatus and thickened rough endoplasmic reticulum were also seen (**plate. 1C**). The striated ducts showed the loss of basal infolding, cristae-lost mitochondria, and some ruptured mitochondria, although some normal mitochondria were also noted (**plate. 2C**). At 12 weeks, the serous acinar cells were pyramidal in shape with centrally located nucleus, mitochondria, and rough endoplasmic reticulum still thickened adjacent to the nucleus, secretory granules of variable electron density. Normal mitochondria were observed but some slightly swollen mitochondria and some cytoplasmic vacuolization were also obser acinar cells were surrounded by regular rough endoplasmic reticulum and secretory granules of variable electron densities (**plate. 1D**). The striated ducts showed well-defined basal striations and infolding of the basal lamina along with many mitochondria that had normal internal cristae (**plate. 2D**).

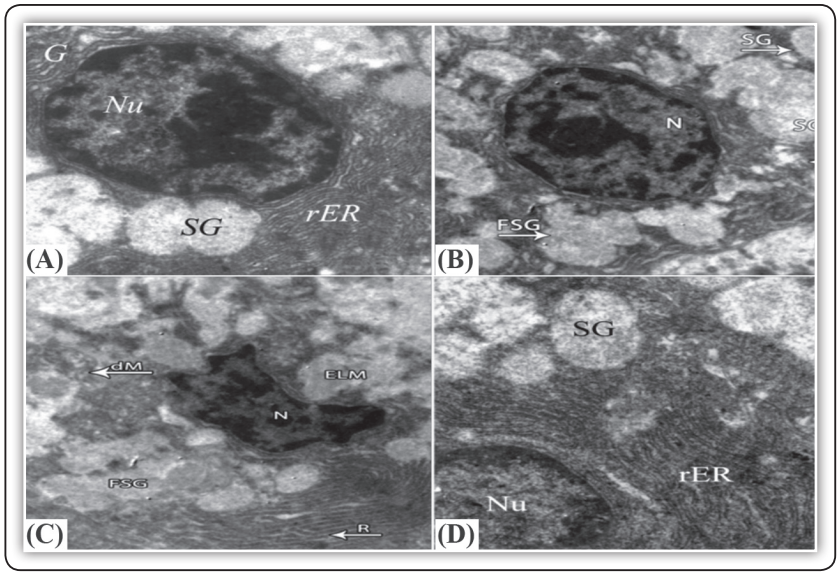

Plate 1: TEM presentation of serous acini of SSGs. A: sham at 4 weeks; B: BoNTA-injected at 2 weeks; C: BoNTA-injected at4 weeks; D: BoNTA-injected at 12 weeks. Nu: nucleus; rER: rough endoplasmic reticulum; SG: secretory granules; G: Golgi apparatus; FSG: fused secretory granules; ELM: electron lucent material; M: mitochondria; dM: deformed mitochondria.

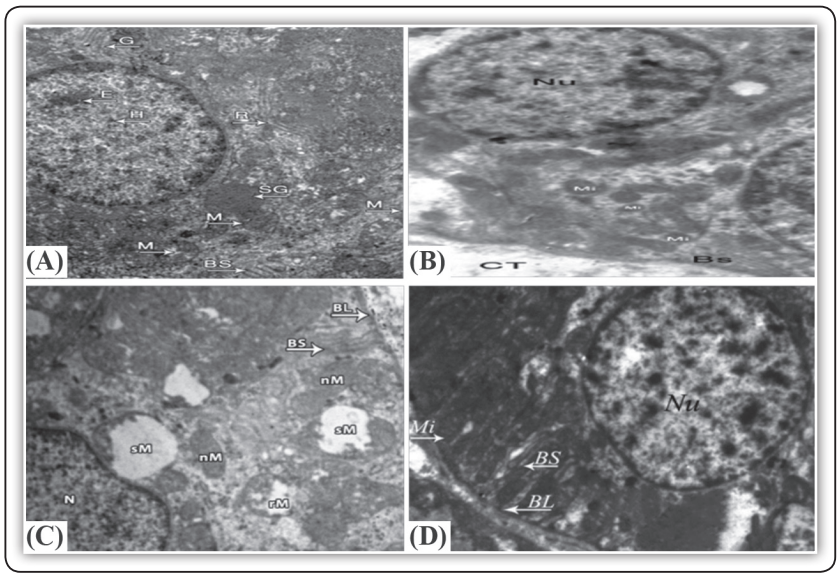

Plate 2: TEM presentations of striated ducts of SSGs. A: sham at 12 weeks; B: BoNTA-injected at 2 weeks; C: BoNTAinjected at 4 weeks; D: BoNTA-injected at 12 weeks; Nu:nucleus; Arrows: mitochondria lost internal cristae; SG: secretory granules; BS: basal striations; BL: basal lamina; CT: connective tissue;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

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.

In the present study, the control SSGs presented normal ultrastructural findings of serous acini and duct system. However, 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 returned to almost normal histological structure in 12 weeks. 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].

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].

Changes in ultrastructures

The ultrastructural results of the present study proved that most extensively affected cellular organelle was the mitochondria. Sharma et al. in 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 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].

CONCLUSIONS AND RECOMMENDATIONS

1. Although application of BoNTA results in obvios dmages of both histological structures and cellular organs of SSGs in short and middle terms, these detrimental effects were transient and major recovery occurs after 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 recommended due to the transient effects of BoNTA on SSGs.

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