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EPIDERMAL GROWTH FACTOR IMPROVES THE ULTRASTRUCTURAL PICTURE OF SUBMANDIBULAR SALIVARY GLANDS OF ALBINO RATS TREATED WITH A SINGLE BOTULINUM TOXIN INJECTION- A QUALITATIVE STUDY.

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ABSTRACT

Background*:* Botulinum toxin (BTX) is well known for its cosmetic as well as medical and dental uses. One of BTX main uses is the control of hypersalivation by intraglandular injection. However, some unwanted changes in the salivary gland elements have been detected**.** The aim of the current study was to explore if the effect of Epidermal growth factor was enough to heal the damage caused by botulinum toxin type A in submandibular salivary glands of rats. **Methods*:*** 21 adult albino rats were divided into three groups: Control Group, BTX Group B where rats received a single Botox® (BTX) injection subcutaneously, and EGF Group in which rats received intraperitoneal EGF daily for two months after the single BTX injection. After the experimental procedures, submandibular salivary glands were cut out and examined using transmission electron microscopy. **Results*:*** The BTX group showed severe signs of atrophy and damage which affected all glandular components. This was revealed in the form of degeneration, intracytoplasmic vacuolations and decrease in the number of desmosomal junctions. The EGF group showed marked improvement in acinar and ductal elements of the submandibular salivary glands. **Conclusions*:*** EGF restored the structural integrity of submandibular salivary glands in rats treated with a single BTX injection. Further quantitative studies were deemed necessary to evaluate the various degrees of damage by BTX and repair by EGF.

KEYWORDS*:* Submandibular salivary gland – Epidermal growth factor – Botulinum toxin – Clostridium – Ultrastructure – Electron microscope.

INTRODUCTION

Botulinum toxin also known as botox (BTX), is a powerful neurotoxin formed by anaerobic bacterium Clostridium botulinum and other few similar Clostridia.[1,2] BTXA is one of the most potent seven serotypes of BTX and is well known for its use as a cosmetic treatment.[3,4,5] Moreover, BTXA has been shown to be an effective neurotoxin to treat several medical conditions and to control muscle and glandular hyperactivity.[6,7,8,9] Moreover, BTX is also used as a treatment of other orofacial conditions such as myofascial pain, TMJ dislocation and bruxism.[10]

The BTXs exact mechanism of action is not fully known, but it inhibits the release of acetylcholine, a neurotransmitter, at the neuro-muscular junction which eventually causes relaxation of muscles. The same neurotransmitter, Acetylcholine, is also found in the postganglionic fibres of the autonomic nervous system. The parasympathetic division of the autonomic nervous system innervates many glands, such as the salivary glands.[11] Inhibition of acetylcholine release at the neuro-glandular junction results in temporary reduction in salivary flow rate.[12,13]

Intraglandular injection of BTXA into salivary glands has been shown to be effective and safe in controlling hypersalivation or drooling. Intraglandular injection of the toxin into the salivary glands diminishes the secretion of saliva by innervation disruption of the secreto-motor junction.[14]

However, some studies showed adverse effects on the salivary glands after BTX injection. Changes such as decrease in glandular volume as well as decrease in cell counts of acini and ducts have been described as among histological changes after intraglandular application of BTXA. Regueira et al, 2019[15] concluded in their study that chronic application of BXA can cause both

histological and cellular alterations in the submandibular glands of rats after as few as 12 days.

One of the major factors that are crucial for appropriate and efficient healing of wounds and tissue regeneration is Growth factors. The Epidermal growth factor (EGF) family, in particular EGF and transforming growth factor-alpha (TGF-α), plays a main part in wound healing and regenaration. EGF binds to its receptor, the EGFR, which is expressed on many types of cells and the activation of the EGF with its receptor leads to various biological and cellular actions, including migration, proliferation, cytoprotection, cellular differentiation, and apoptosis.[16]

An experiment was conducted on the acini of the parotid salivary glands of rats where apoptosis was induced in the acinar cells using an apoptotic agent with and without the addition of EGF. DNA extraction, immunoblotting and immunoprecipitation tests were performed on the cells. Results showed that cells treated with EGF showed suppression in apopotic cells in comparison to cells without any growth factors. These results suggested that EGF may be vital in regulating homeostasis of salivary glands. Moreover, EGF may have the potential to temporarily suppress apoptosis in salivary glands.[17]

Although many studies have been reported in the literature with respect to beneficial effects of BTX in the treatment of hypersalivation due to different causes,[12,13,18] few studies have evaluated the histological and ultrastructural effects of BTX on the salivary glands.[12,19-21] and to date, no clinical trials associated with epidermal growth factor (EGF) injection in BTX treated salivary glands have been reported.

Therefore, in our present study we examine the ultrastructural changes in rat submandibular salivary glands treated with Botox® and EGF concurrently.

Aim of the study

The aim of the current study was to investigate whether EGF can reverse the adverse effects of BTXA on the submandibular salivary gland of adult Albino rats or not. Transmission electron microscopy was used for ultrastructural investigation of the salivary gland components.

MATERIALS AND METHODS

This study design and the animal care procedures as well as ethical approval was granted by the Suez Canal University Research Ethics committee (SUEZ-REC 35/2014). The current study used 21 adult female Albino rats, 3 months old and weighing about 200-220 gm. The rats were housed in separate cages and kept in a controlled environment with temperature (23-25°C), and photoperiod of alternating 12:12 hour light-dark cycle. The animals had free access to food and water and were fed natural diet and drinking water adlibitum throughout the whole experimental period. The rats were acclimatized for one week before the launch of the experiment.

The rats were then randomly divided into three groups (n=7) as follows:

Control group: rats were subjected to subcutaneous injection of 0.1 ml saline in the region of submandibular salivary glands.

BTX group: rats were subjected to single dose of subcutaneous injection of 2.5 units of BTXA (Botox® 100 units Allergen), reconstituted in 0.1 ml of physiologic saline in the region of submandibular salivary glands.[22]

EGF group: Rats were treated with a single BTX injection as in the previuos group and on the following day they were exposed to daily intra peritoneal injection of EGF, provided by Sigma-Aldrich, Inc. in a dose of 10 µg/Kg body weight[23] for 60 days.

After the experimental procedures, the animals of the whole groups were sacrificed by euthanization and their submandibular salivary glands were dissected out. The specimens were used for Ultrastructural investigation where they were examined by transmission electron microscope JEOL (JEM-1400 TEM) at different magnifications and images were captured using Lica ICC50 HD camera at Cairo University Research Park.

RESULTS

Control group

The control group showed normal ultrastructural structures of acini and ducts of submandibular salivary gland (Fig 1).

Botox® (BTX) group

The serous acini appeared shrunken with many signs of degeneration. Cells of the salivary gland acini showed degenerated amd pyknotic nuclei with variation in size and shape. They were darkly stained with loss of continuity of the nuclear membrane which was often irregular and nuclear vacuolization was observed. The cytoplasm of the serous acinar cells revealed numerous large vacuolations. RER was dilated and mitochondria showed signs of degeneration manifested as loss of their cristae and even total rupture. Decrease in desmosomal junctions between cells was encountered in many areas. A lot of myelin like figures were seen in the connective tiisue beneath the acinar cells (Figs. 2a, 2b, 2c, 2d).

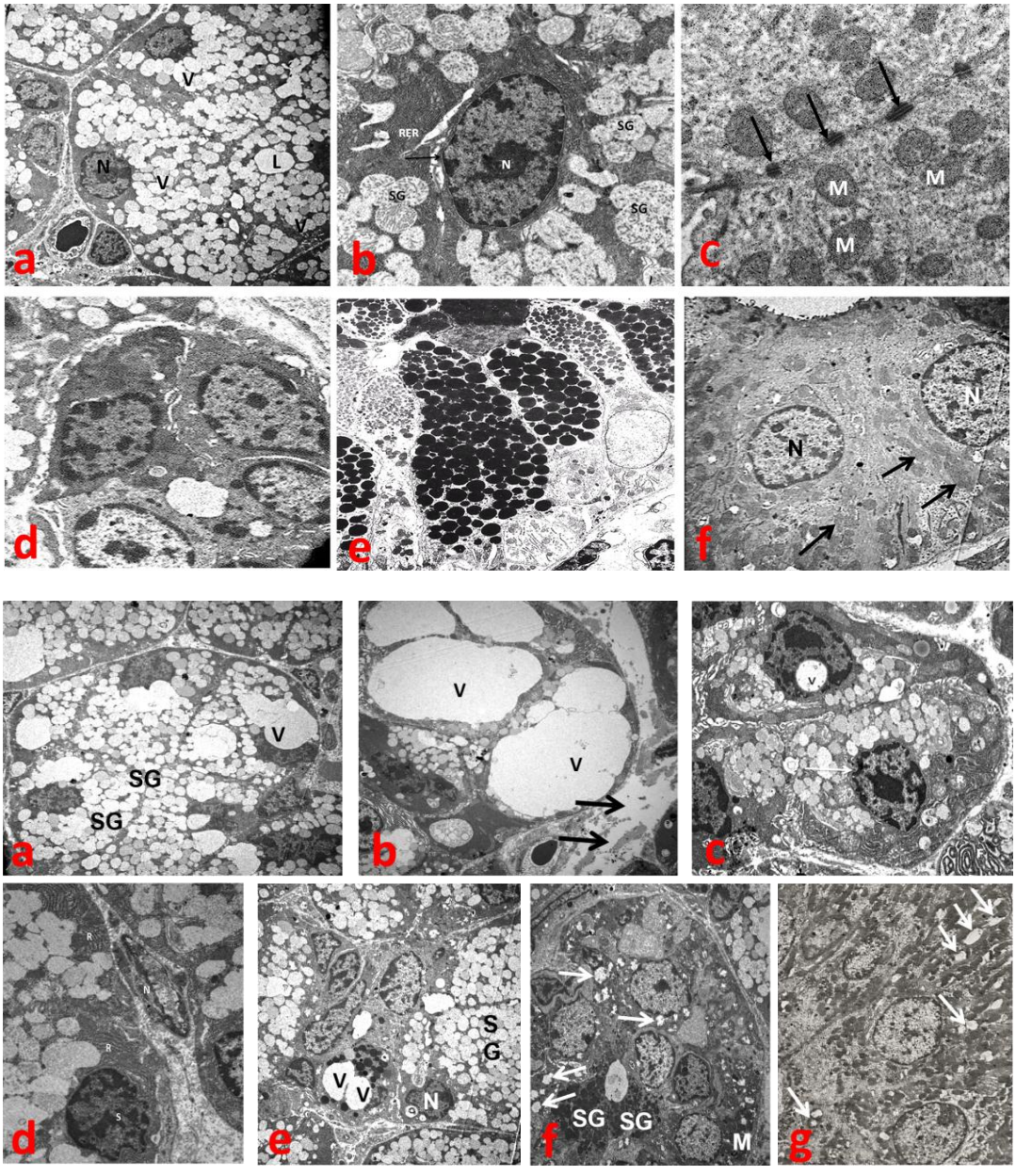
Myoepithelial cells appeared to have abnormal distribution of nuclear chromatin (Fig. 2d). The intercalated ducts appeared distorted with cells showing cytoplasmic vacuolations. The granular convulated tubules demonstrated marked reduction in the size and number of their granules, degenerated mitochondria and degenerative vacuolization in their cytoplasm (Figs. 2e, 2f).

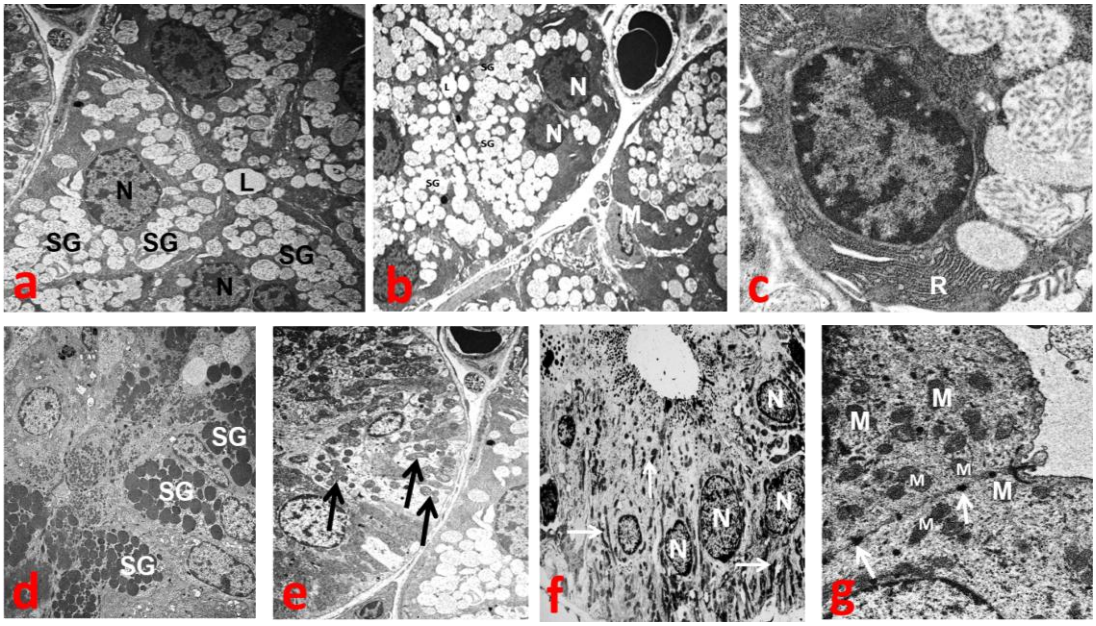
Striated duct cells showed signs of degeneration manifested as irregular nuclear membrane with degenerated organelles specially the mitochondria which appeared swollen and degenerated in comparison to control group. Dilated RER were observed in the duct cells and the cytoplasm contained some vacuolations. Loss of the infoldings of the basal plasma membrane was a constant finding (Fig. 2g). The excretory ducts showed marked reduction in the height of their cells which also revealed signs of degeneration manifested as cytoplasmic vacuolizations and the mitochondria lost their cristae and appeared almost empty. The connective tissue stroma was presented with degeneration and dissociation of their collagen fibers as well as fibroblastic degeneration (Fig.2b).

EGF group

Ultrastructural investigation of submandibular glands of rats treated with both BTX and injected with EGF revealed that the glands almost regained their normal architecture. The acinar cells appeared pyramidal in shape with rounded basally situated nuclei. Nearly normal RER and mitochondria were observed. Very few cytoplasmic vacuolations were found. Normal desmosomal junctions between cells were present (Figs. 3a, 3b, 3c).

Myoepithelial cells appeared normal with elongated nucleus and normal chromatin distribution (Fig. 3b). Granular convulated tubules were lined by tall columnar cells with basally situated nuclei and many electron dense secretory granules of different sizes (Fig. 3d). Striated ducts regained their normal features with columnar cells that had rounded normally appearing nuclei, numerous elongated basal infoldings and radially arranged mitochondria (Figs. 3e, 3f, 3g).





DISCUSSION

In this study, EGF was used to investigate its role in the gland recovery after BTX injection in the region of the submandibular salivary gland, as EGF has emerged as a powerful regulator of stem cells in different tissues such as salivary glands which are essential in tissue regeneration in addition to its major role in the healing process and regeneration in various tissues.[24,25]

In the present study, the ultrastructural changes observed, after injection of BTXA in the region of the submandibular salivary gland, were consistent with the histological examinations. The serous acinar cells suffered from loss of normal shape, the nuclei often had an irregular shape with irregular nuclear membrane and sometimes irregular chromatin distribution. Highly morphologic variations in RER and degenerated mitochondria were frequently observed. Moreover, marked intracytoplasmic vacuolations were present in both acinar and ductal cells. The striated duct cells presented degenerated mitochondria and loss of basal infoldings of their plasma membrane. The GCT showed marked reduction in their granularity, with total or subtotal degeneration of their mitochondria.

Another finding in this study was the presence of a lot of myelin like figures around the acinar cells. Myelin like figures are thought to be a sign of reversible cell injury and they are derived from plasma or organelles' membrane enclosing water and dissociated lipoproteins between the lamellae of injured membranes. The presence of myelin figures has been suggested to be characteristic of cells undergoing degeneration.[26] This is an indication that the glandular cells of the female rats injected with BTX had, with all the presented ultrastructural alterations, already lost most of their functions.

The present findings accord with several studies which reported that injecting BTX into the submandibular and parotid salivary glands exhibited morphological and ultrastructural changes in the cell organelles and secretory granules. Clear atrophy of the acini of glands injected with the BTXA were detected and the acinar cells revealed nuclei which appeared either flattened or pyknotic denoting signs of degeneration induced by the pressure exerted by the intracytoplasmic vacuolations and the accumulation of secretory products inside the cells.[12,19-22]

The ultrastructural examination of the present study revealed that the most extensively affected cellular organelle was the mitochondria which appeared vacuolated, losing their cristae, swollen or totally ruptured. It is documented that in early stages of cell degeneration, the mitochondria were the first cell organelles that become affected leading to disturbance in the energy production, affecting the activity of the cell and its ability to perform its function properly.[27] Recent studies showed similar ultrastructural findings were observed in rats treated with streptozotocin[28] and doxorubicin.[29]

The mitochondria which were the most extensively affected organelle in this study could be the cause of the intracytoplasmic vacuolations observed frequently in acinar and ductal cells, since mitochondria are very susceptible to toxic agents. When damaged, mitochondrial cellular metabolism fails and this results in increased intracellular accumulation of sodium ions. This osmotic effect within the damaged cell causes the appearance of cytoplasmic vacuoles. The intracytoplasmic vacuolations could also be explained by intracytoplasmic degeneration of other cell organelles, like the golgi apparatus, that was mostly of fatty nature thus appearing as empty spaces.[30]

In the current study, BTXA can be considered as vacuolization inducers. Cytoplasmic vacuolization is generally considered to be an early form of degeneration and several researches indicates that cytoplasmic vacuolization is a morphological phenomenon commonly observed during exposure to various bacterial and viral pathogens as well as during exposure to clinically used drugs and other compounds. Vacuolization in cells is considered as an adaptive physiological protective reaction, most likely for damage limitation.[31]

The observed dilatation of RER in this study might be a sign of cell injury. Dilation of RER might be due to impaired secretory mechanism of the acinar cells so the secretory material, which is protein in nature, becomes entrapped within the arrays of the RER which eventually causes their dilatation.[31]

The structural and ultrastructural effects of BTX injection found in the present study may be attributed to glandular denervation caused by the toxin which inhibits acetylcholine release at the neuroglandular junction which temporary causes chemical denervation of the salivary gland.

This explanation is supported by several authors who found that parasympathetic denervation of the injected gland leads to distension in acinar cell volume and a decrease in the number of nuclei as well as degenerative changes in the organelles of acini and ducts of the affected glands. Such alterations might ultimately lead to decrease of functional capacity of these tissues necessary for their secretion and thus lead to change in flow rate of saliva[14,33,34]

In another experiment, BTXA was injected intra-glandularly into the parotid gland of Albino rats where morphological and ultrastructural analyses revealed a clear atrophy of the glandular acini injected with BTXA. Acinar cells also showed highly morphologic changes of rough endoplasmic reticulum and degenerated mitochondria.[19]

On the other hand, ultrastructural examination of the EGF group revealed that the glandular elements regained their normal features. These results come in agreement with several studies that showed that EGF activation after binding with its receptor on the salivary gland cells may represent an influential way of regulating the regeneration of salivary gland cells by stimulating proliferation of all parenchymal cell types. In addition to this role, EGF activation might impact the biological cellular activities of cells of both acini and ducts such as secretion or absorption.[35, 36]

The results obtained from the present study encourages our research team to investigate the effect of multiple subsequent BTX injections on the histology, ultrastructure and immunohistochemical expression of different markers in the rat submandibular salivary gland. The effect of EGF on counteracting and reversing the damage caused by multiple subsequent BTX injections should also be investigated.

CONCLUSION

Daily intraperitoneal injection of EGF for 60 days resulted in regaining normal features of the submandibular salivary glands parenchymal and stromal elements after BTXA injection. This was confirmed by ultrastructural examination using transmission electron microscope.

Figure Legends

Fig 1: An electron photomicrograph of submandibular salivary gland of the control group showing normal ultrastructural features: (a): Serous acinus that is spherical in shape with narrow lumen (L), pyramidal acinar cells with rounded basally situated nuclei (N) and many secretory vesicles (V) with variable electron densities (Uranyl acetate & lead citrate x3000). (b): Serous acinus with normal appearance of the nucleus (N), regular nuclear membrane (arrow), numerous secretory granules (SG) and arrays of rough endoplasmic reticulum (RER) (Uranyl acetate & lead citrate x8000). (c): Normal desmosomal junctions between acinar cells (arrow) and also normal mitochondria (M) (Uranyl acetate & lead citrate x20000). (d): Normal intercalated duct with cuboidal cell lining surrounding narrow lumen (Uranyl acetate & lead citrate x8000). (e): Granular convulated tubule with columnar cells and numerous electron dense granules (Uranyl acetate & lead citrate x8000). (f): Striated duct with columnar cell lining having rounded nuclei (N), numerous basal infoldings and radially arranged mitochondria (arrow) (Uranyl acetate & lead citrate x8000).

Fig 2: An electron photomicrograph of submandibular salivary gland of the BTX group showing signs of degeneration. (a): Degenerated serous acinus with pyknotic nuclei and irregular nuclear membrane (N), accumulation of numerous secretory granules (SG) and numerous large cytoplasmic vacuoles (V) (Uranyl acetate & lead citrate x3000). (b): Degenerated serous acinus with large intracytoplasmic vacuolations (V). Notice the degeneration of collagen fibers within the connective tissue stroma around the acini (arrow) (Uranyl acetate & lead citrate x3000). (c): Degenerated acini. Beading in nuclear membrane (arrow), large nuclear vacuolization (V) and dilated RER (R). A lot of myelin like figures are seen in the connective tissue beneath the acinar cells (F) (Uranyl acetate & lead citrate x3000). (d): Serous secretory cells with irregular nuclear membrane (S), dilated RER (R) and myoepithelial cell with abnormal distribution of its nuclear chromatin (N) (Uranyl acetate & lead citrate x8000). (e): Part of the granular convulated tubule containing cytoplasmic vaculations (V) and serous acinar cell with the nucleus having an irregular nuclear membrane (N) and numerous secretory granules (SG) (Uranyl acetate & lead citrate x3000). (f): Degenerated granular convulated tubule with

constricted lumen, degenerated mitochondria (M), cytoplasmic vacuoles (arrow) and a marked reduction of their electron dense secretory granules (SG) (Uranyl acetate & lead citrate x3000). (g): Part of striated duct with numerous intracytoplasmic vacuolations (arrow) (Uranyl acetate & lead citrate x3000).

Fig 3: An electron photomicrograph of submandibular salivary gland of the EGF group showing signs of regaining normal architecture. (a): serous acinus with narrow lumen (L), normal pyramidal cells, normal nucleus (N) and numerous secretory granules (SG) (Uranyl acetate & lead citrate x5000). (b): Serous acinus with narrow lumen (L), normal pyramidal cells, normal nuclei (N) and numerous secretory granules (SG) and normal myoepithelial cell (M) (Uranyl acetate & lead citrate x3000). (c): serous acinar cell with almost normal nucleus and RER (R) with parallel arrays (Uranyl acetate & lead citrate x8000). (d): Granular convulated tubule containing numerous secretory granules (SG) and basally situated nuclei (N) (Uranyl acetate & lead citrate x2000). (e): Striated duct with high columnar cells with basal infoldings and numerous rod shaped mitochondria (arrows) (Uranyl acetate & lead citrate x2000). (f): Striated duct with high columnar cells having rounded nuclei (N), numerous basal infoldings and radially arranged mitochondria (arrow) (Uranyl acetate & lead citrate x2000). (g): Striated duct cells with normal mitochondria (M) and desmosomal junctions (arrow) (Uranyl acetate & lead citrate x8000).

CONFLICTS OF INTEREST: None declared

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