Chronic effects of Onabotulinum Toxin A on the parotid glands: An animal model

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Dedication

I dedicate this work to my late mother and late sister. I also dedicate this work to my father, who has supported me throughout the process, and to my sisters, Anwar and Lama, and my brothers, Abdulrhaman, Mohammad, Abdulmalik, and Hamed, who have provided me with love and support. I am especially grateful to my loving fiancé, Mejd Alfalah, who has provided me with support and encouragement.

Abstract

Introduction: Intraglandular injection of botulinum toxin has become a very useful tool in treating sialorrhea through a transient denervation of the salivary glands. However, it is difficult to assess the long-term effects of treatment in patients. The purpose of the present study is to develop an animal model to study the longer-term effects of repetitive injections of botulinum toxin A focusing initially on changes in weight and volume of the parotid glands.

Methods: After evaluating various procedures, a new gerbil animal model was developed. This model was used to assess longer-term effects of botulinum treatment as follows: Fifteen gerbils were randomly assigned to 3 groups of 5 animals each. A small incision in the parotid area was performed to permit visualized injection into the chosen gland. 1.5 U botulinum toxin A, reconstituted with 0.03 mL physiologic saline, was injected into the right parotid gland and 0.03 mL of normal saline was injected into the left parotid gland. Group 1 received 3 injections, group 2 received 5 injections and group 3 received 7 injections, with 15-day intervals between each injection. Gland weights and volumes were measured after 45, 75 and 105 days respectively

Results: There was a 20% decrease in gland weight and 15% reduction in gland volume in parotid glands injected with onabotulinum toxin A as compared to parotid glands injected with saline (control). Overall, the weight and volume of the side treated with botulinum toxin A was less than the control side, especially in larger and older animals. However, these differences were not significantly different.

Conclusion: An animal model was developed to study long-term effects of Botulinum toxin.

Résumé

Introduction: L´injection intra-glandulaire de toxine botulinique est un outil important dans le traitement de la sialorrhée grâce à une dénervation transitoire de la glande salivaire. L'objectif de cette étude est de déterminer l´effet à long terme de l´administration répétée de la Toxine Botulinique A (Onabotulinum toxin A) sur le poids et le volume de la glande parotidienne des gerboises.

Méthode: Après nombreuses procédures, un modèle animal a été développé. Quinze gerboises ont été aléatoirement assignées à 3 groupes, chaque groupe étant constitué de 5 animaux. Le groupe 1 a reçu 3 injections, le groupe 2 a reçu 5 injections et le groupe 3 a reçu 7 injections avec un intervalle de quinze jours entre chaque injection. Une petite incision dans la région parotidienne a été effectuée à chaque fois avant l'injection. En total, 1,5 Unités de toxine Botulinique A reconstitué dans 0,3 ml de solution saline a été injecté dans la glande parotide droite et 0,03 ml de solution saline à la glande gauche comme groupe control.

Résultats: Une différence de 20% de poids et de 15% en volume des glandes a été trouvée entre les deux groupes (Botulinum toxin versus salin). Le poids et le volume des glandes traitées avec Toxine botulinique étaient toujours diminués comparé aux glandes contrôles. Toutefois, ces différences ne sont pas statistiquement significatives.

Conclusion: Un modèle animal a été développé pour étudier les effets à long terme de la toxine Botulinique A.

Preface

Contributions of authors

Dr. Abdullah A. Alarfaj was responsible for the literature review in its entirety, and for doing the experiments, data collection, and analysis of the results. Dr. Sam Daniel provided supervisory guidance; he conceived and designed the experiments, and he reviewed the entire thesis.

Claim of originality

This thesis developed the first animal model to study the longer-term effects of chronic use of Onabotulinum toxin type A (BTXA) The model was used to study changes in weight and volume of Gerbil parotid glands following multiple BTXA injections.

Acknowledgements

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Chapter One: Introduction

1.1 The problem and rationale for the thesis

Sialorrhea, also known as hypersalivation (drooling), is an involuntary overflow of saliva from the mouth. It causes a wide range of medical and social problems that negatively affects the quality of life of children and their families [1]. Chronic neurological diseases such as cerebral palsy, Parkinson's disease, amyotrophic lateral sclerosis, myasthenia gravis, and bilateral facial nerve palsy are the most common causes of drooling [2]. Therefore, a multidisciplinary approach is indispensable in the assessment and management of patients suffering from sialorrhea. Different surgical and non-surgical options, based on an individual's needs, are available for treating patients with sialorrhea. The treatment options available to decrease salivation include oral motor therapy, behavioral therapy, anticholinergic medication, surgeries, and recently, botulinum toxin injections into salivary glands [3]. There are currently 3 main botulinum toxin formulations used for clinical «off-label» treatment of sialorrhea: onabotulinumtoxinA (BOTOX®), abobotulinumtoxinA (Dysport®), and Rimabotulinum toxin B (Myobloc®). These should not be considered interchangeable. Onabotulinumtoxin A is currently the most commonly used agent in the treatment of drooling in children and adults and will be the medication tested in this thesis. Onabotulinum toxin A has been proven very useful tool in treating sialorrhea as well as many other devastating diseases, such focal dystonia, spasticity, and hyperhydrosis. Onabotulinum toxin A decreases salivation by inhibiting acetylcholine release from the neuromuscular junction. However, the effect of Onabotulinum toxin A fades after about 3-6 months, and thus requires frequent injections. This raises an important concern regarding the long-term effects of Onabotulinum toxin A. Many studies

have been conducted to assess the efficacy and safety of this drug long term; and a few animal studies have demonstrated the effect and histological changes after a single dose of Onabotulinum toxin A injection into salivary glands, but no study has characterized the effect and gland size changes after repetitive injections [4-7]. The need to develop a way to study such issues was the rationale of the current thesis.

1.2 Objectives

The objective of this thesis is to develop an animal model to study the long-term effects of repetitive injections of botulinum toxin A. Once a gerbil animal model is developed, the thesis will aim to use the model to characterize the effects of multiple doses of Onabotulinum toxin A on the weight and volume of gerbil parotid glands.

1.3 Thesis organization

The thesis consists of seven chapters. Chapter one will give the rationale and objective of the thesis. Chapter two, will outline the anatomy and physiology of the salivary glands, the history of Onabotulinum toxin A usage, the mechanisms of its action on salivary glands, and its use in treatment of sialorrhea,. Chapter three will give the methods used in this thesis. Chapter four will present the results of the thesis.. Chapter five will discuss the finding of the thesis and describe possible future studies. Chapter six will present the conclusions of the thesis.

Chapter Two: Background and Literature review

2.1 Basic anatomy and physiology of the salivary glands

There are three major pairs of salivary glands: the parotid, the submandibular and the sublingual glands (figure 1) [8]. Additionally, there are about 800 to 1000 minor salivary glands dispersed in the oral cavity. The largest gland is the parotid gland, which weighs, on average, 15–30 g. It is located in the preauricular region and along the posterior surface of the mandible. The second largest gland is the submandibular gland, which weighs 7–16 g. The sublingual is the smallest of the major salivary glands, weighing 2–4 g. It lies as a flat structure in the submucosal plane within the anterior floor of the mouth, superior to the mylohyoid muscle and deep in the sublingual folds, opposite the lingual frenulum. The sublingual gland is located in the submandibular triangle, which has a superior boundary formed by the inferior edge of the mandible, and inferior boundaries formed by the anterior and posterior bellies of the digastric muscle [8].

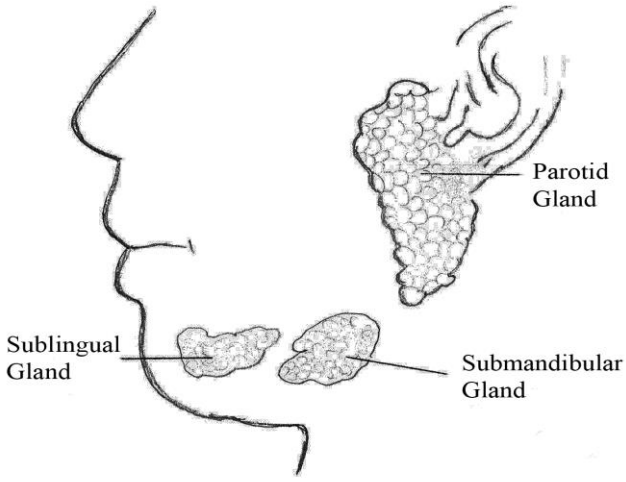

Figure 1. The three major salivary glands. McGill Auditory Sciences Laboratory.

The secretory unit of the salivary gland is referred to as the acinus (acinar cells) (figure 2), secretory tubules, which are involved in salt and water transport, and the collecting duct. There are three types of acinar cells. They are classified according to the secretory product being produced: serous, mucoserous and mucous. Both secretory tubules and collecting ducts are surrounded by myoepithelial cells leading to duct contraction and secretion of saliva [9].

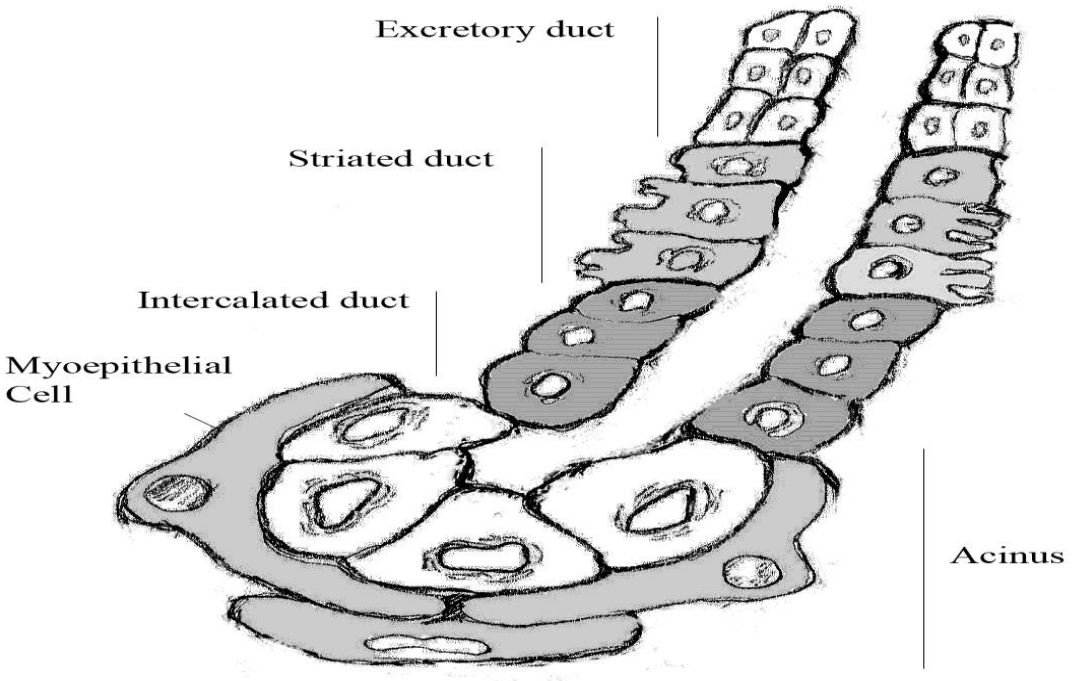

Figure 2. The histology of the functional secretory unit of the salivary glands. McGill Auditory Sciences Laboratory.

2.1.1 Autonomic Innervation

Parasympathetic Nervous System

Postganglionic parasympathetic fibers release acetylcholine in close proximity to the glands, and stimulation occurs by way of passive diffusion of a neurotransmitter; that is, no true synapse exists between the postganglionic nerves and the glands (figure 3). Acetylcholine receptors can be nicotinic or muscarinic, although only the latter appear to be involved in salivary gland stimulation [10]. Stimulation of the salivary glands by the PNS produces watery secretions.

Sympathetic Nervous System (SNS)

Norepinephrine is the major neurotransmitter of the SNS, and all synapses are adrenergic. Stimulation of the gland by the SNS produces a scant, viscous saliva rich in proteins and organic and inorganic solutes.

Both parasympathetic and sympathetic stimuli result in an increase in salivary gland secretions (figure 3) [10].

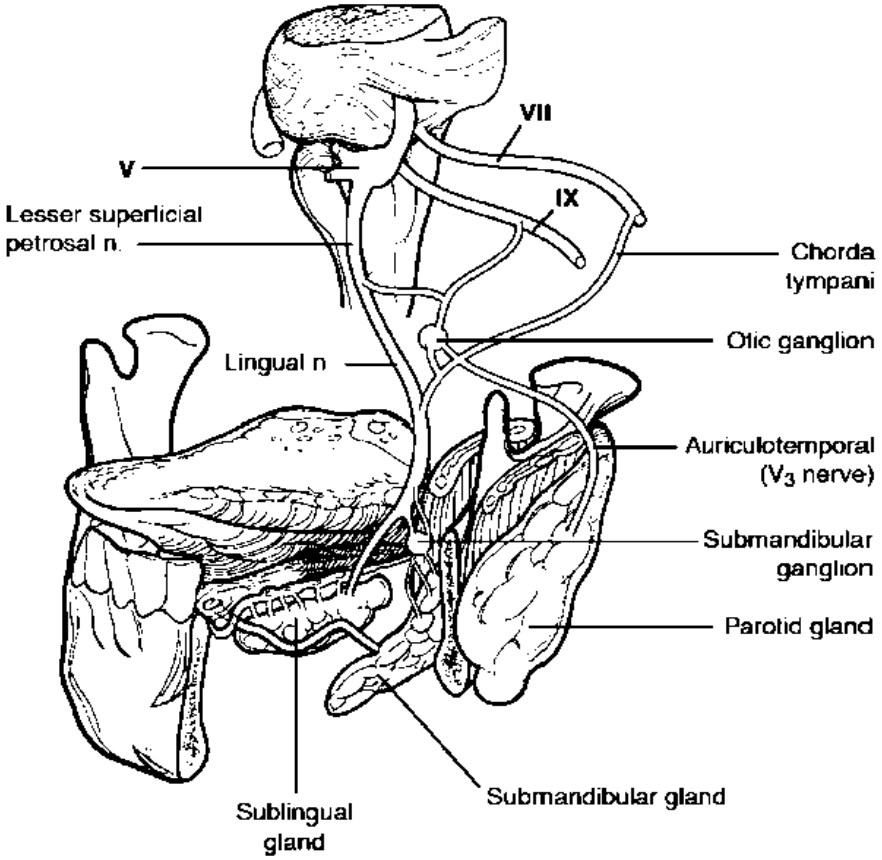

Figure 3. The nerve supply for the major salivary glands. Reproduced with Permission from Segal, et al. Operative Techniques in Otolaryngology-Head and Neck Surgery. Elsevier. December 1996. P333.

2.1.2 Salivary Flow Rates

In normal circumstances, during unstimulated conditions, the salivary flow rate is about 0.1 mL per minute, while the minimal stimulated flow rate is 0.2 mL per minute.

In unstimulated glands, salivary flow is primarily produced by the submandibular glands (65%), while the parotid and sublingual glands provide 20%, and 7% to 8% of the flow, respectively. Once stimulated, the relative contributions of the parotid and submandibular

glands are reversed, with the parotid gland supplying greater than 50% of the flow. The minor salivary glands, independent of stimulation, produce less than 10% of the total flow [11].

Comparably, rodent salivary glands used in animal experiments show a similarity in their anatomy and physiology when compared with human glands [12]. The parotid gerbil salivary gland is large and superficial, which, therefore, makes it an excellent animal model for this study.

2.2 History of Onabotulinum toxin A

In 1817, Justinus Kerner described the clinical symptoms of poisoning from botulinum toxin (BT) in spoiled sausage (the word botulism is derived from the Latin word botulus, which means sausage) [13]. Kerner not only established that death from poisoning by BT was due to paralysis of the muscles after spoiled sausage consumption, but was also the first to suggest possible therapeutic uses for this toxin. In 1895, a Belgian microbiologist, Emile-Pierre van Ermengem, first identified the bacterium, Clostridium botulinum. Seven different serotypes of BT (A through G) are produced by C botulinum. Although each of these serotypes produce muscle paralysis, each of them vary with respect to the onset of action, duration of action, degree of diffusion in the tissues, and other properties. Only types A and B are available for clinical use [14].

In the 1970s, Alan Scott tested BT type A (BT-A), a product developed by Edward Schantz, in animals and humans. In 1989, the first commercial preparation of BT-A, Botulinum toxin A (Allergan, Irvine, CA) was approved by the Food and Drug Administration

(FDA) as an orphan drug for clinical use in blepharospasm and strabismus. Myobloc, BT type B (BT-B) was introduced in the United States more recently, while outside the United States a second BT-A product, Dysport has also available for several years. Several serendipitous discoveries have led to a remarkable increase in the number of indications for BT, such as bladder dysfunction, blepharospasm, cervical dystonia, chronic migraine, spasticity, strabismus, hyperhidrosis, and sialorrhea [15, 16].

2.3 Onabotulinum toxin A mechanism of action

Botulinum toxin disrupts the normal function of the neuromuscular junction by inhibiting the release of acetylcholine from peripheral nerve cells to prevent muscular contraction [17]. Botulinum toxins are peptides that are composed of one heavy chain and one light chain. After the heavy chain of the injected toxin binds to receptors on the terminal ends of nerve cells, the peptide enters the cytoplasm through endocytosis. Once in the cytoplasm, the light chain cleaves components of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), a complex of proteins necessary for the exocytosis of acetylcholine. The sites of cleavage within the SNARE protein complex differ among the serotypes. Botulinum toxin types A, C1, and E cleave SNAP-25 (synaptosome-associated protein of 25 kd), while serotypes B, D, F, and G cleave VAMP (vesicle-associated membrane protein). VAMP is also known as synaptobrevin [18]. As a result of this cleavage, acetylcholine remains in the neuron, where it is unable to bind to receptors on muscle fibers and stimulate muscle contraction (chemo-denervation). The inhibitory effect of botulinum toxin is temporary; recovery of muscular function often becomes clinically evident

approximately three months after treatment as the neuromuscular junction begins to recover. The development of new collateral nerve ends may be partially responsible for recovery; however, these terminal buds appear to be transient, and recovery of the original nerve terminal eventually occurs [19].

2.4 General medical indications of Onabotulinum toxin A

Over the past 15 years, botulinum toxin has been shown to be useful in many conditions, especially strabismus and various movement disorders [13]. Encouraging clinical reports have generated an abundance of ideas for other uses, but many of these observations are anecdotal. Nevertheless, its potency, relative safety, and the reversibility of its effects have made botulinum toxin an attractive option for some chronic conditions that respond only partially to medical treatment. Sometimes it can be used as an alternative to surgical intervention. Many disorders have been treated with botulinum toxin, for example [20];

- Ophthalmological disorders: concomitant misalignment, primary or secondary esotropia or exotropia, paralytic strabismus, and restrictive or myogenic strabismus**.**
- Movement disorders: secondary dystonia, tic disorders, tremor, Parkinson's disease, cephalic tetanus, muscle stiffness, cramps, and spasms.
- Spasticity: multiple sclerosis, stroke, traumatic brain injury, cerebral palsy, spinal cord injury.
- Pain disorders: headaches, backaches, myofascial pain.
- Oromandibular disorders: masseter hypertrophy, temporomandibular joint dysfunction**.**

- Pharyngeal disorders: cricopharyngeal dysphagia, closure of larynx in chronic aspiration.
- Laryngeal disorders: vocal fold granuloma, ventricular dysphonia.
- Cosmetic applications: wrinkles, frown lines, rejuvenation of ageing neck**.**

Moreover, botulinum toxin has been tried in numerous other conditions. The list of possible new indications is rapidly expanding. It appears to be a promising alternative to sphincterotomy in patients with chronic anal fissures [21] and is effective in treating achalasia [22]. In addition, some autonomic disorders resulting in hypersecretion of glands, like excessive palmar hyperhidrosis, ptyalism, gustatory sweating, or drooling, respond well to botulinum toxin [23-25].

2.5 Sialorrhea

Sialorrhea (drooling or excessive salivation) is an involuntary overflow of saliva from the mouth due to the inability to control oral secretions [26]. This condition is usually not due to true hypersalivation, but is rather a poorly coordinated control mechanism of orofacial and pharyngeal musculature [3]. Even patients who produce less saliva can suffer from drooling, and hypersalivation does not necessarily lead to drooling. This condition is normal in infants but usually stops by 15 to 18 months of age. Sialorrhea after four years of age is generally considered to be pathologic [27]. Its physical and psychosocial complications can range from mild and inconvenient symptoms to severe problems that can have a significant negative impact on quality of life. The most common cause of sialorrhea is neuromuscular dysfunction. It affects about 28% of children with neurological impairments such as cerebral palsy and mental retardation [28]. It also affects 10% of patients with chronic neurological diseases such

as Parkinson's disease, amyotrophic lateral sclerosis (motor neurone disease), and post-traumatic encephalopathy [2]. Other causes include medication side effects (tranquilizers, anticonvulsants), gastroesophageal reflux, and anatomic problems (macroglossia, oral incompetence, dental malocclusion).

The management of drooling remains a problem. Despite effective treatment modalities to diminish saliva production, drooling may still persist. Different invasive (surgical and non-surgical) and non-invasive options, based on the individual's needs, are available for treating patients with sialorrhea. Conservative therapy includes: oral motor therapy, behavioral modification via biofeedback, and orofacial regulation therapy. If sialorrhea continues to interfere with the patient's health and quality of life after non-invasive therapy, other measures can be tried such as anticholinergic medication, radiation, and surgical therapy [3]. However, many studies show that minimally invasive methods for treating drooling problems, such as botulinum toxin injections into salivary glands, are very effective and useful clinically [3, 25, 29, 30].

2.6 Usage of Onabotulinum toxin A in salivary glands

As outlined in section 2.4, botulinum toxin (BTX) is widely used in the treatment of different neuromuscular and hypersecretory disorders [31]. Some examples include dystonia, spasticity, and other conditions characterized by focal muscle hyperactivity. Additionally, it has been reported that BTX is both safe and effective in the treatment of focal hyperhidrosis in sweat glands, which have a similar physiology to salivary glands. [32]. In the late 1990s, Bushara proposed anatomical landmark intraparotid injections of BTX for treatment of

sialorrhea; this was the first trial of intraglandular BTX injection [33]. Subsequently, two other preliminary studies used intraparotid BTX injections using the same technique [25, 34]. Following that, many studies and clinical trials have been performed using different techniques (blind, ultrasound guided or electromyography guided) to inject Onabotulinum toxin A into the salivary glands. All of them reported no, or minimal, side effects such as xerostomia, dysphagia and chewing difficulties [29]. Recently, many studies have recommended intragladular Onabotulinum toxin A injections as a first line treatment for drooling, after failure of conservative management [1, 2, 30, 35-47]. Because of the reversible action of Onabotulinum toxin A, repeated injections are mandatory in order to take care of drooling for the long term.

Although, there are few animal studies showing changes in gland weight, volume, and histology after a single intraglandular dose injection of Onabotulinum toxin A, to date, no studies have studied the effects of repetitive injections. [4, 6, 7].

2.7 Animal models that have been used to assess effects of multiple BTXA treatments

The previous section has outlined the need to study effects of multiple BTXA injection. However, it is uncertain which animal model would be most suitable to study this issue. In the literature, most of the studies performing BTX injection into the salivary glands used rats or rabbits [5, 6]. Nevertheless, these animal species have been used only for single-injection studies. The rat parotid gland is slightly deep and located behind and below the ear. It is embedded in subcutaneous adipose tissues and having the extra-orbital lacrimal gland located anterior to the parotid gland[48]. Thus, this model would make the identification of the parotid

gland difficult especially after multiple injections due to fibrosis and scaring. Moreover, the submandibular glands are located in the anterior neck spaces, very close and in between the submandibular lymph nodes [12] (figure 4), thus, rendering the blind injection to the gland unreliable due to the anatomical relation with the lymph nodes.

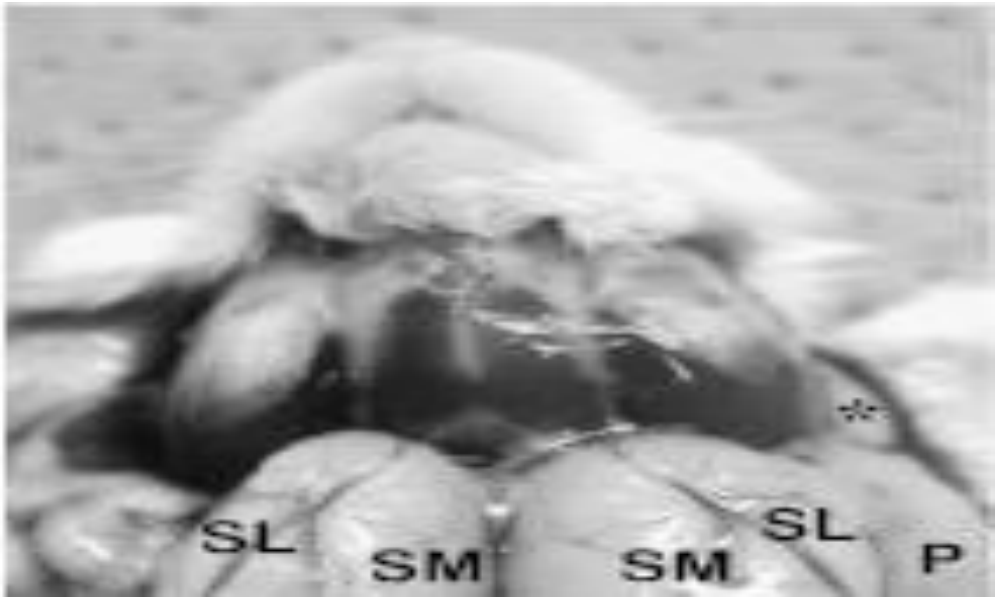

Figure 4. Rat anatomy view of the floor of the mouth. SL: submandibular lymph; SM: submandibular glands; P: parotid gland. Reproduced with Permission from Amano, et al. Anatomy and Histology of Rodent and Human Major Salivary Glands. Acta Histochem Cytochem. 2012 Oct 31; 45(5): 241–250

Regarding the histology, both human and rats parotid glands are composed of pure serous acini. The main difference is that the human parotid gland has numerous intralobular adipose tissues, whereas the adipocytes are not prominent in the rat's parotid gland. However, the submandibular gland in human is a mixed of both serous and mucous acinar cells, whereas the rats submandibular gland has only the serous type [12]. Therefore, the rat salivary glands would

not be a good option for multiple injection studies due to deep location of parotid gland, very small submandibular glands, and histological dissimilarities.

The rabbit salivary glands are similar to rat salivary glands anatomically and histologically, except that rabbit salivary glands are bigger in size. Nevertheless, the gerbil salivary gland has more histological similarities with the human salivary gland [49].

Chapter Three: Methods

3.0: Development of an animal model

There is a need to study the effects of multiple salivary gland BTXA injections in patients, but currently there is no way to do this. For example, detailed studies on how BTXA works could only be done if changes in salivary glands (e.g., size, histology) were characterized over the course of such multiple injections. Such studies could not be done on human subjects, but they could be performed in animals. However, no such animal studies have been done. Therefore, a new animal model was required in order to evaluate the effects of multiple injections. This thesis set out to develop such a model with pilot studies to determine (a) a suitable animal to use, and (b) to evaluate the new model by trying to characterize the effects of multiple BTXA injections. This chapter will first describe the pilot experiments to develop an animal model (section 3.1), and then describe a set of experiments using the new animal model to evaluate how salivary glands change during multiple BTXA injections (section 3.2).

3.1: Preliminary pilot experiments:

3.1.1 Evaluation of a Blind injection technique in a chinchilla and a guinea pig model

First a study was done to determine if the salivary glands could be injected in a blind fashion to avoid multiples skin incision, which would also cause scaring in analogous

treatments in patients. This procedure was evaluated in 7 chinchillas, 3 guinea pigs and in 4 gerbils (bilateral glands) by injecting methylene blue using manual palpation and external anatomical landmarks. This was done after macroscopic anatomical dissection of the gland of 2 chinchillas, 2 guinea pigs and 2 gerbils.

After identifying gland position using suitable landmarks, 0.05 of diluted methylene blue (figure 5) was blindly injected into the parotid gland, using a 29 gauge fine needle. Then, a small skin incision was made to determine if the methylene blue had been injected into the gland, or if there had been leakage, or spread, of the methylene blue dye into surrounding tissue. It was found that the dye was outside of the target parotid gland in 5 of 7 chinchillas, in 2 of 3 guinea pigs and in 2 of 4 gerbils (e.g. See Figure 6).. Clearly, the procedure lacked precision, indicating that the gland needed to be visualized before injection.

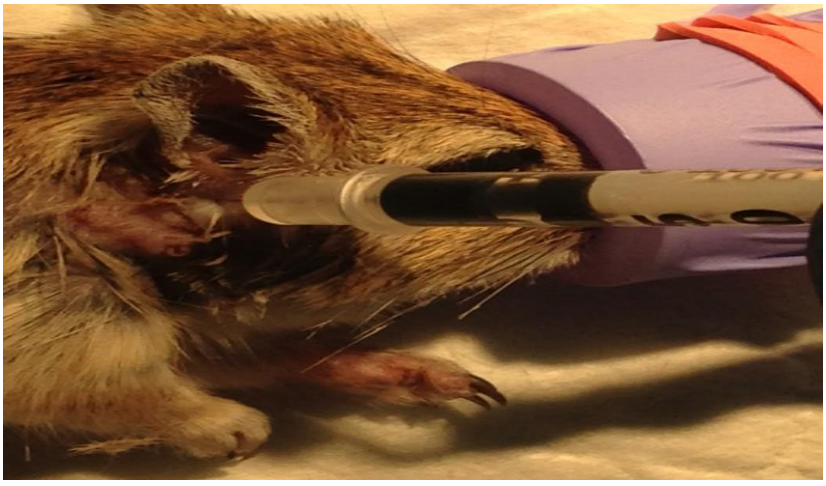

Figure 5.Blind injection of methylene blue into the parotid gland .

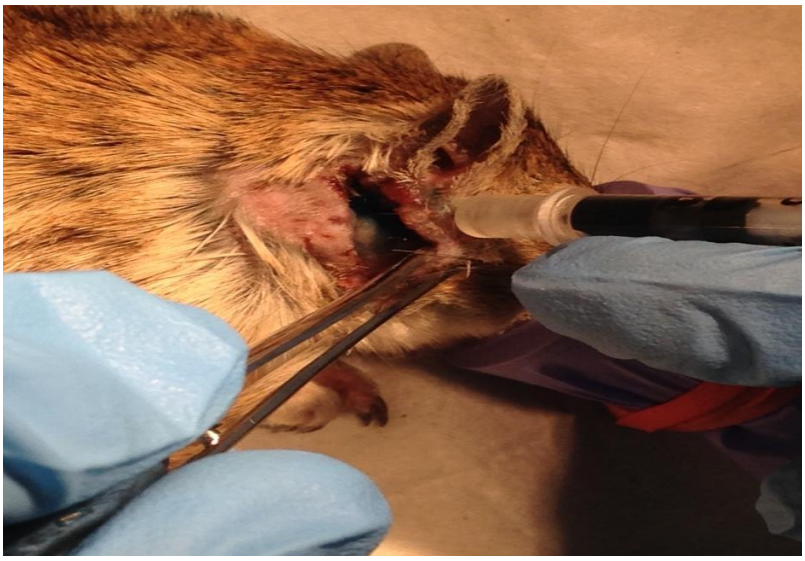

Figure 6. The methylene blue leak outside the parotid gland.

It was found that injections could not be reliably delivered to the target gland. This showed that an open technique would be required. However even if an open technique were used, these 2 species (chinchilla and guinea pig) would be inappropriate as an animal model because the target glands would be difficult to identify due to their deep location, thicker animal skins, and due to animal cost compared to the gerbil. In conclusion, using a "blind injection technique" employing manual palpation and clinical landmarks was neither reliable nor precise in targeting exclusively the parotid glands.

To develop an alternative "open-surgical" technique, several animal species were considered. The suitability of rats as an animal model was rejected due to their deep parotid glands, their very-small submandibular glands which attached to the submandibular lymph node, as well as their histological differences from humans, as was outlined in the literature review (section 2.7 above). Instead, the suitability of rabbits or gerbils was evaluated using an open-surgical technique, which is described in the following section.

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3.1.2: Open surgical technique

The open surgical technique used a small incision to allow visualisation of the gland so that the injection, under direct vision, could provide greater accuracy and precision rather than using the blind technique. The technique was evaluated using rabbits and gerbils.

3.1.3: Consideration of rat and rabbit as an animal model

It was uncertain which animal species might be best for an animal model. As a result of the study described in Section 3.1, both the chinchilla and guinea pig were found to be unsuitable as an animal model candidate. After reviewing the literature (see section 2.7 and section 3.1.1 above), usage of a rat animal model was rejected. However, the literature review suggested that rabbits might be suitable. Because rabbit salivary glands are bigger in size and seemed similar to humans, they were considered for the animal model.

3.1.4: Consideration of rabbit & gerbil animal models using surgical visualization

Four rabbits were dissected. The rabbit parotid gland was found to be deep and difficult to locate while injecting under direct visualization and the submandibular gland was attached to the lymph nodes (figure 7).

Three gerbils were dissected with the open approach. The gerbil parotid gland was found to be superficial with an acceptable size. It was easy to locate and visualize.

The parotid glands of the gerbils and the submandibular glands of the rabbits were excised and sent for histopathological examination. The histology of the gerbil parotid gland was found to be more similar to the human than the rabbit submandibular gland.

Based on (a) the ease and reliability of injection of the gerbil parotid gland, (b) the lack of morbidity and mortality in this survivorship experiment despite multiple procedures, and (c) the histological similarity with humans, it was decided to use the gerbil as an animal model.

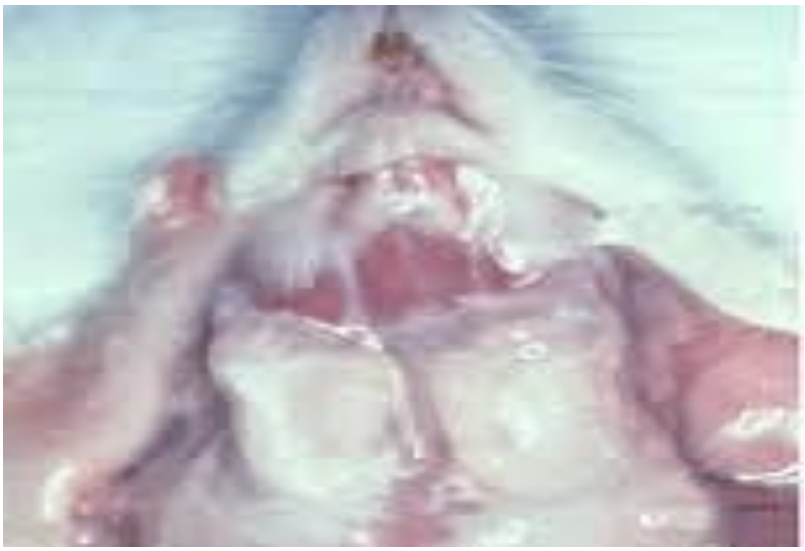

Figure 7. Rabbit submandibular gland and lymph nodes.

3.2 Using the animal model to assess changes during multiple treatments

3.2.0 Introduction

This study was performed on six to eight months old Mongolian Gerbils (60 to 80g) purchased from Charles River Laboratories (Wilmington, Mass, U.S). Mongolian gerbils

(Meriones unguiculatus) were chosen as experimental animals based on the anatomical and histological similarity of their salivary glands to humans'. They have been widely used as experimental rodent models that have large and superficial parotid glands (figure 8). The animals were kept in the animal care research facility of The Montreal Children's Hospital Research Institute in standard housing at 22°C ± 4°C ambient temperature with a 12-hour light/dark cycle. The animals had free access to food and water and were examined daily for signs of pain or weight loss. The study was approved by the McGill University Animal Care Committee.

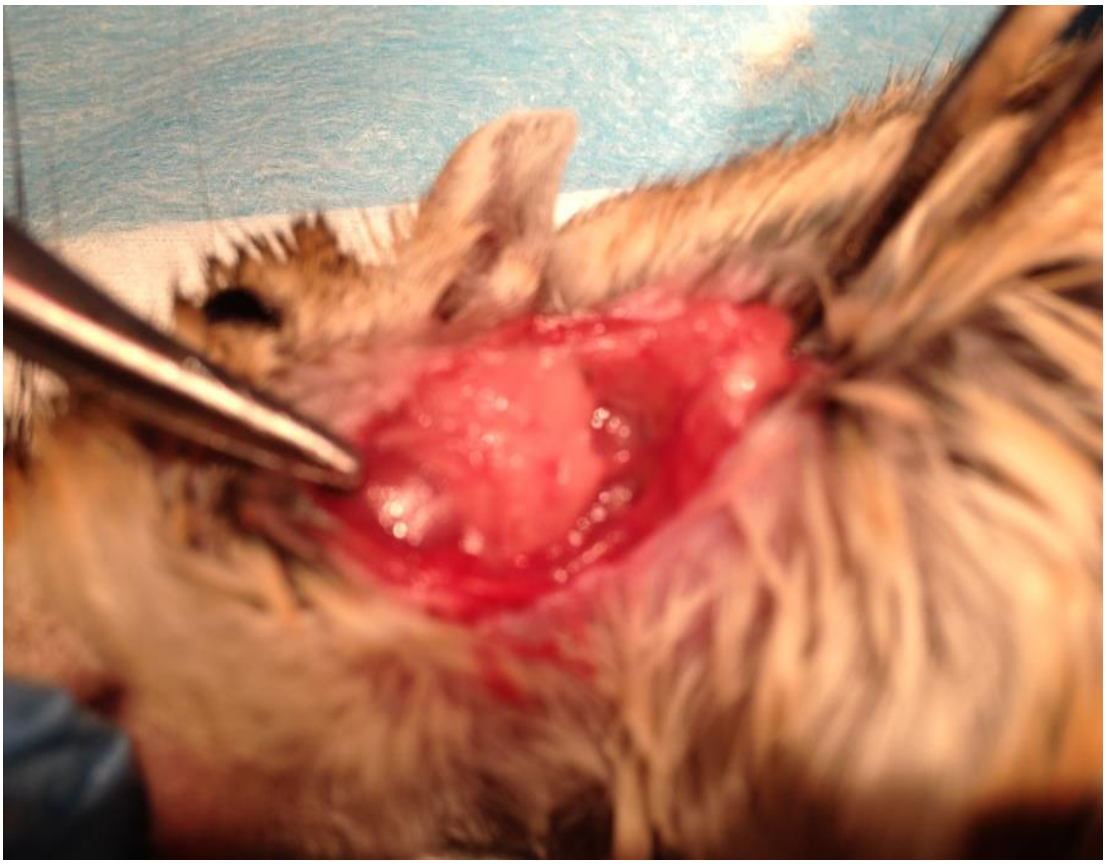

Figure 8. The gerbil parotid gland after skin incision demonstrating how it is superficial.

3.2.1 Sample size

This study aimed to assess anatomical changes in gerbil parotid glands after multiple intraglandular Onabotulinum toxin A injections. Based on prior similar research studies that assessed gland changes after a single injection of Onabotulinum toxin A, a sample size of five or more animals per group was judged to be satisfactory [4-6].

3.2.3 Experimental protocol

***Experimental groups**:*

Fifteen gerbils were randomly assigned to three groups; group A (N=5) received three injections, group B (N=5) received five injections, and group C (N=5) received seven injections. All animals received onabotulinum toxin A to the right parotid gland and normal saline to the left parotid gland.

***Outcome measures**:*

1) Gland weight:

Gland weight was measured using a calibrated electronic microbalance scale. The measurement was done immediately after the extraction of the parotid gland, 15 days after the last injection.

2) Gland volume:

The size of the gland was measured using a millimeter ruler. The length, width, and depth were measured immediately after the extraction of the parotid gland 15 days after the last injection.

3.2.3: Onabotulinum toxin A dose and surgical technique

The BTXA was reconstituted using 2 mL of 0.9% saline per 100 U, which meant that the concentration of BTXA solution was 50 U per mL Thus, the total volume of 1.5U BTXA was 0.03 mL and was injected into the gland using 29 gauge insulin needles. Group A was injected on days 0, 15 and 30. Group B was injected on days 0, 15, 30, 45, and 60. Group C was injected on days 0, 15, 30, 45, 60, 75, and 90. All procedures were conducted using inhalational anesthesia with isofluorane. The parotid area was shaved using an electronic shaver (figure 9). Then, Chlorhexidine was applied on the parotid area on both sides as an antiseptic measure. A small incision (< 0.5 cm) under each ear was made with a scalpel taking care not to damage the gland (figure 10)**.** Then, 0.03 mL of BTXA was injected into right parotid gland and 0.03 mL of normal saline in the left gland, under direct vision. The wound was closed using a 4-0 absorbable suture. Then, the animal was monitored closely until complete recovery.

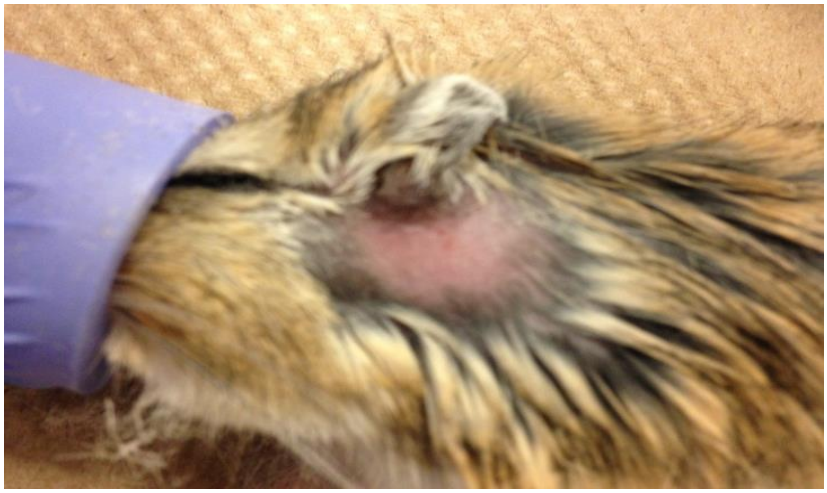

Figure 9. Shaved parotid area.

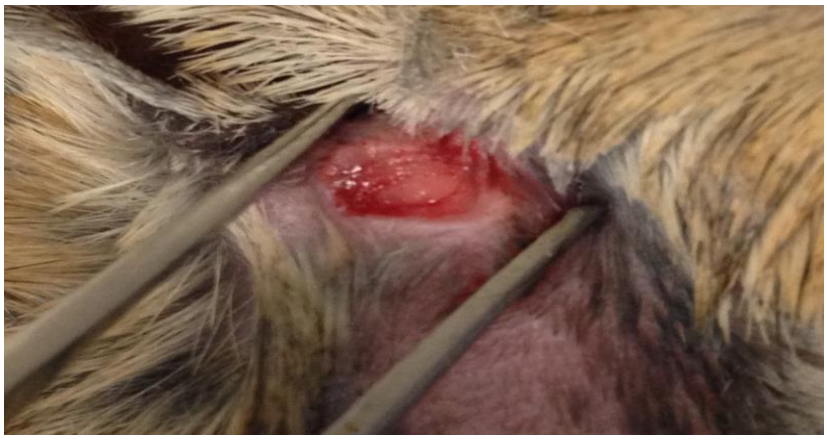

Figure 10. Small 0.5 cm skin incision to provide access to inject Onabotulinum toxin A or saline into the parotid gland under direct vision.

Parotid Gland extraction

Fifteen days after the last injection, induction of anesthesia was done using isofluorane; and then both right and left parotid glands were dissected from the surrounding connective tissue and extracted (figure 11). After that, the animals were euthanized using an overdose isoflurane and bilateral pneumothoraxes.

Figure 11. Partially dissected parotid gland

3.2.4 Statistical analysis

The Mann–Whitney test was used to verify significant differences between the Onabotulinum toxin A and saline injected glands accounting for non-normal distribution of the data. Differences between the subgroups (A, B and C) were compared with the Mann–Whitney test and P-values of 0.05 were considered to indicate statistical significance.

Chapter Four: Results

4.1 Animal behaviour and weight

The animals were monitored closely after surgery and examined daily for signs of pain, weight loss or head tilt in order to assess toxicity or side effects from Onabotulinum toxin A. Three animals lost 2-5 grams after the third day of surgery but they recovered shortly thereafter. No complications occurred during the entire experimental period and there were no deaths.

4.2 Gland weight

Figure 12 compares parotid gland weights of experimental groups (onabotulinum toxin A, BTXA on the left of the figure) to control groups (saline on the right of the figure), for all animals studies (N=15). Error Bars show the standard error of the mean (SEM). The Onabotulinum toxin A injected glands had a mean weight of 0.22 $\pm$0.14 g. The control saline-injected glands had a mean weight of 0.28g $\pm$ 0.19 g. The total difference was 20% in gland weight between the two groups, with the Onabotulinum toxin A injected group having a lower weight than the control group.

Mann–Whitney tests were used to determine if the differences in the weights between the 2 groups were significant. It was found that the differences were not significantly different ($p = 0.1313$).

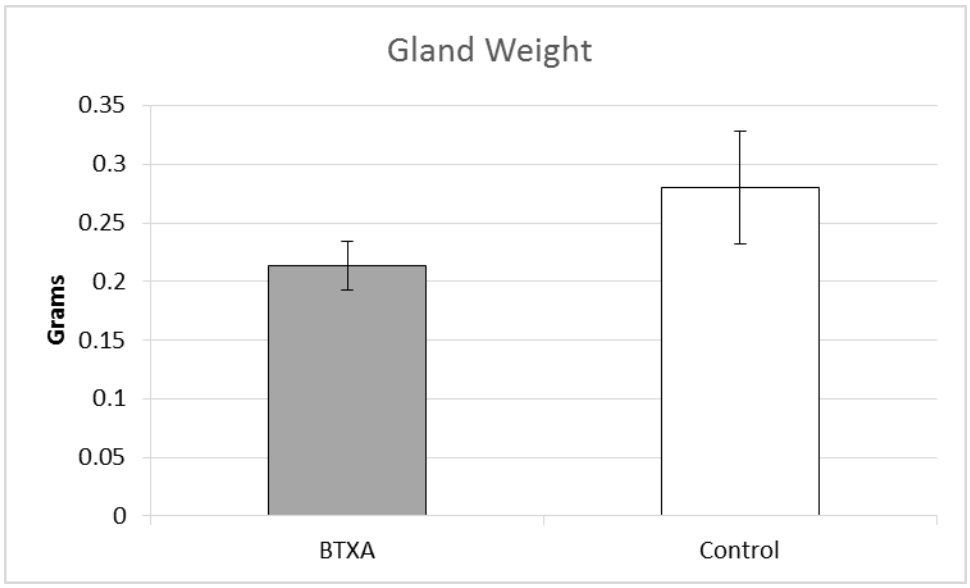

Figure 12. Parotid gland weights of experimental (onabotulinum toxin A, BTXA) groups versus control group. Error Bars represent standard error of the mean (SEM). Number of animals, N=15

Then, the weight of the glands in the experimental and control groups was analyzed by subgroups according to the number of doses that the animals received. In all the subgroups, there was a trend of lower weight in those glands treated with botulinum toxin.

Three-injection group:

The left side of figure 13 compares parotid gland weights of the experimental group (onabotulinum toxin A, BTXA on the left of the figure) to the control group (saline on the right of the figure), for the 5 animals that received 3 injections each. Onabotulinum toxin A injected glands had a mean of 0.1 $\pm$0.14 g, while saline injected glands had a mean of 0.14 $\pm$ 0.05g. The weights of the glands post removal were not significantly different ($p = 0.173$).

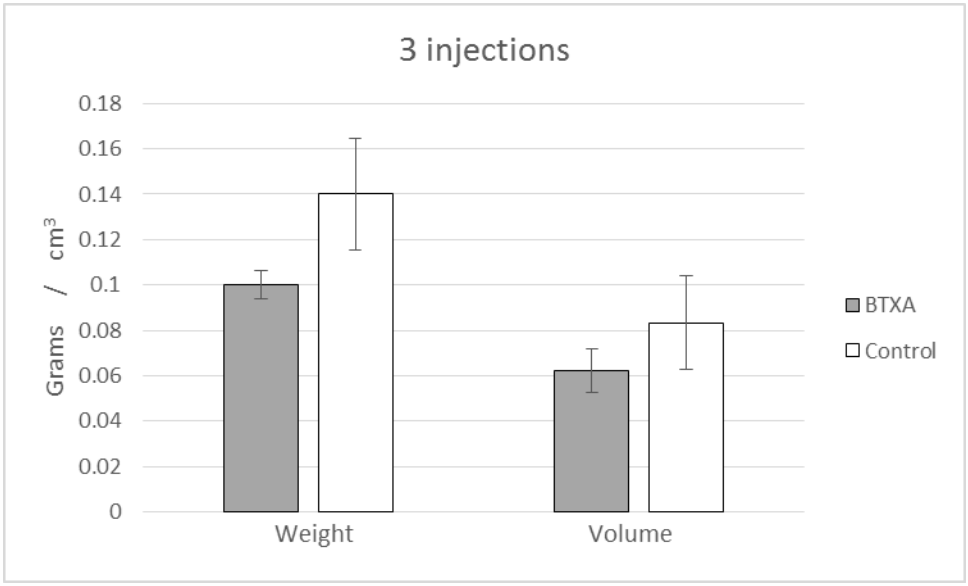

Figure 13. Comparison in Weight and Volume in the group treated with 3 injections of Botulinum Toxin. Error Bars represent SEM. Five animals.

Five-injection group:

The left side of figure 14 compares parotid gland weights of the experimental group (onabotulinum toxin A, BTXA on the left of the figure) to the control group (saline on the right of the figure), for the 5 animals that received 5 injections each. Onabotulinum toxin A injected

glands had a mean of 0.26± 0.05 g while saline injected glands had a mean of 0.32± 0.08 g. The weights of the glands were not significantly different (p=0.08692).

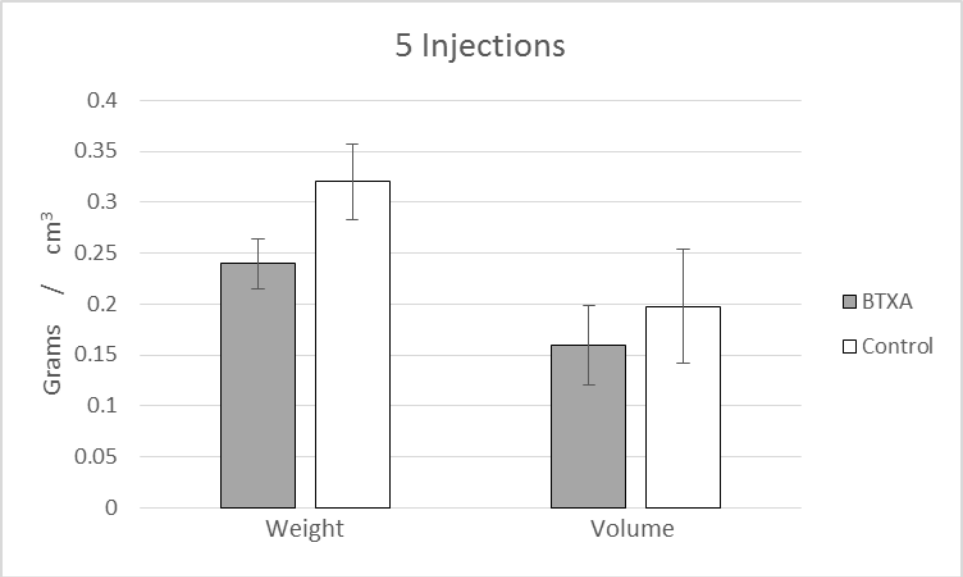

Figure 14. Comparison in Weight and Volume in the group treated with 5 injections of Botulinum Toxin. Error Bars represent SEM. Five animals.

Seven-injection Group:

The left side of figure 15 compares parotid gland weights of the experimental group (onabotulinum toxin A, BTXA on the left of the figure) to the control group (saline on the right of the figure), for the 5 animals that received 7 injections each. Onabotulinum toxin A injected glands had a mean of 0.3 ± 0.2 g while saline injected glands had a mean of 0.38± 0.26 g. The weights of the glands post removal were not significantly different (p = 0.33724).

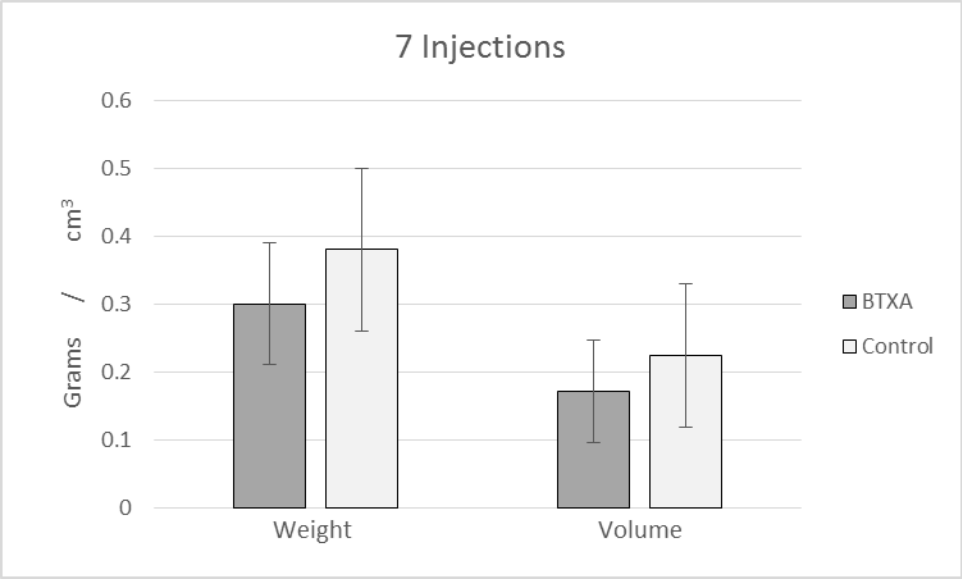

Figure 15. Comparison in Weight and Volume in the group treated with 7 injections of Botulinum Toxin. Error Bars represent SEM. Five animals.

4.3 Gland volume

Figure 16 compares parotid gland volume of all experimental groups (onabotulinum toxin A, BTXA on the left of the figure) to all control groups (saline on the right of the figure), for all animals studies (N=15). Error Bars show the standard error of the mean (SEM). Onabotulinum toxin A injected glands had a mean size of 0.13 ± 0.11 $cm^{3,}$ while saline injected glands had a mean of 0.17 ± 0.16 cm^3. The total difference was 15% in gland volume between the two groups, with the Onabotulinum toxin A injected group presenting, on average, a lower volume than the control group.

The Mann–Whitney test analyzed the differences in gland sizes separated into groups: saline injected and Onabotulinum toxin A injected glands. The sizes of the glands post removal were not significantly different ($p = 0.24825$);

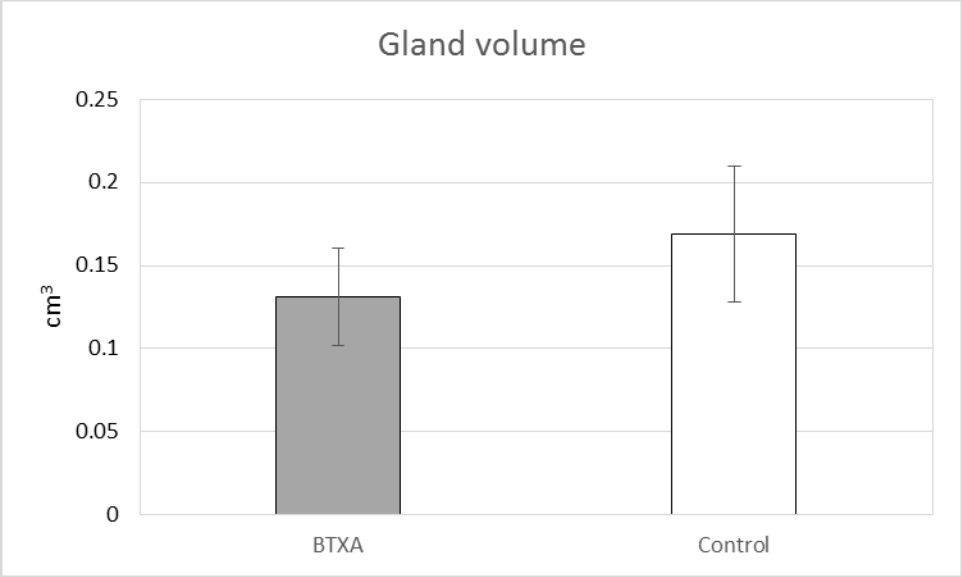

Figure 16. Parotid gland volume of experimental groups (BTXA) versus control groups. Error Bars represent SEM. Number of animals, N=15

Next as above, the volume of the glands in the experimental groups versus the control groups was analyzed by subgroups according to the number of doses that the animals received. In all the subgroups, there was a trend to a lower volume in the glands treated with botulinum toxin.

Three-injection group:

The right side of figure 13 (above) compares parotid gland volumes of the experimental group (onabotulinum toxin A, BTXA on the left of the figure) to the control group (saline on the right of the figure), for the 5 animals that received 3 injections each. Onabotulinum toxin A

injected glands had a mean size of 0.06 $\pm$0.02 cm^3 while saline injected glands had a mean size of 0.08$\pm$0.04 cm^3 . The sizes of the glands post removal were not significantly different (p = 0.41683).

Five-injection group:

The right side of figure 14 compares parotid gland volumes of the experimental group (onabotulinum toxin A, BTXA on the left of the figure) to the control group (saline on the right of the figure), for the 5 animals that received 5 injections each. Onabotulinum toxin A injected glands had a mean volume size of 0.16cm3 +0.08) cm3 while saline injected glands had a mean volume of 0.19cm3 +0.12) cm3. The sizes of the glands post removal were not significantly different (p = 0.46017).

Seven-injection group:

The right side of figure 15compares parotid gland volumes of the experimental group (onabotulinum toxin A, BTXA on the left of the figure) to the control group (saline on the right of the figure), for the 5 animals that received 7 injections each. Onabotulinum toxin A injected glands had a mean size of 0.16 $\pm$0.08 cm^3 while saline injected glands had a mean of 0.22 $\pm$0.23 cm^3. The volumes of the glands post removal were not significantly different (p = 0.30153).

4.4 Relationship between animal-weight and either gland-weight or gland-volume

Animal weight is strongly affected by animal growth, animal weight and animal age. Animal weight, gland weight and gland volume would all be expected to increase as an animal grows. This section will describe the relationship between (a) animal weight and gland weight or (b) animal weight and gland volume that was observed in the above study. Note that at the start of the study, each animal had a different weight, partly because they were of different ages (6-8 months), and partly due to individual variations. Then during the experiment, which could last from 1 to 3 months, all animals increased in weight as they aged.

Figure 17 shows the relationship between animal weight (x axis) and gland weight (y-axis) for all animals. The filled circles show the weight of the gland treated with BXTA, the open circles show the weight of the side injected with saline. Usually, the weight of the BTXA side was less than the control side, especially in larger and older animals.

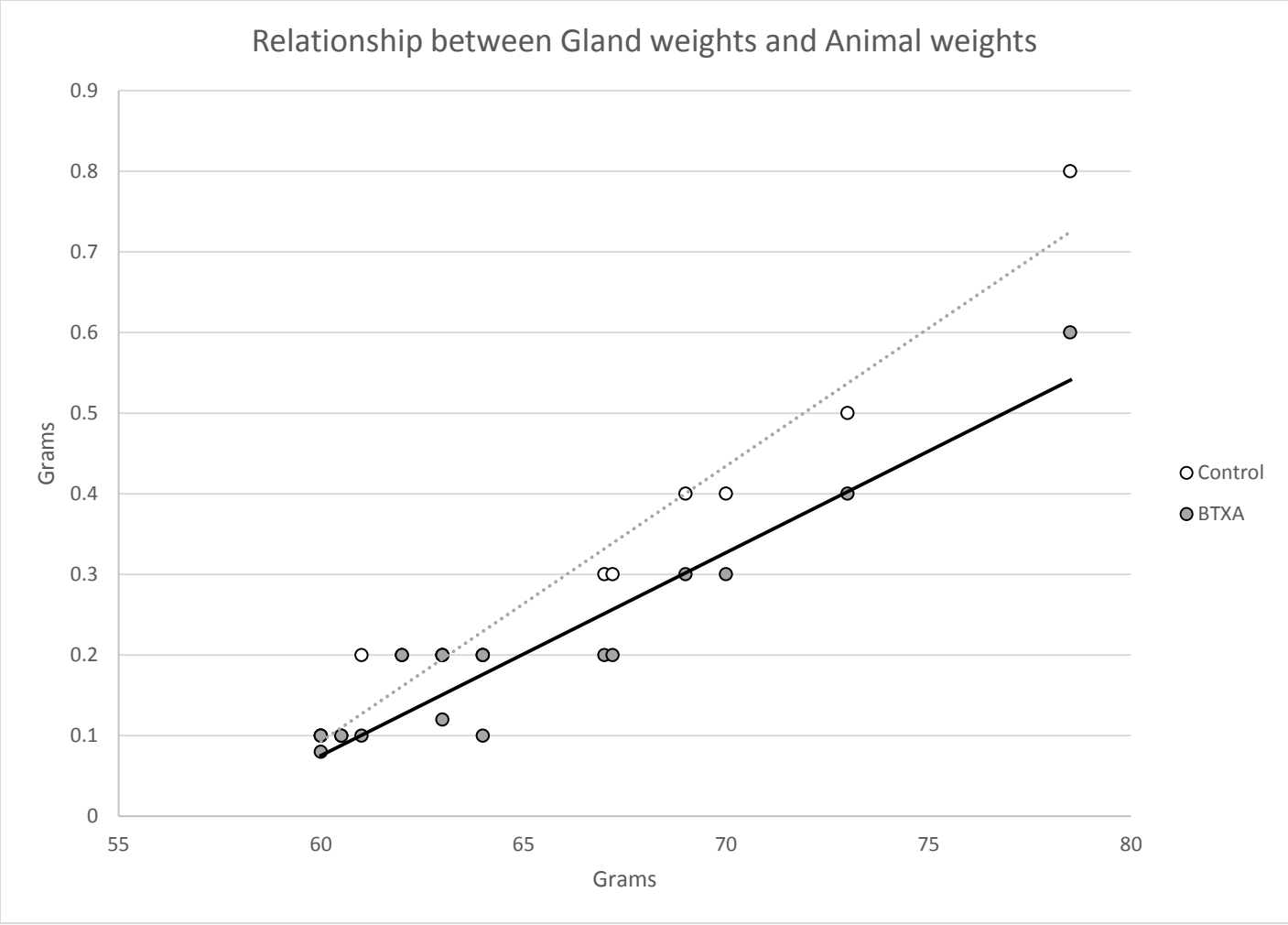

Figure 17. Relationship between gland weights in BTXA group (filled circles) and control groups (open circles) in different weight animals. Number of animals, N=15

Animals who weighed more had higher changes in salivary gland weight compared to animals that weighed less, in the glands treated with saline. The glands treated with botulinum toxin presented with less weight increase per gram of animal weight compared to the control group. The differences between both groups were more marked as the animal gained weight. This could indicate that there may be changes caused by botulinum toxin in normal gland growth, which may suggest that the effects of botulinum toxin injections on gland anatomy can

be observed more clearly in animals of a greater age versus younger or lighter animals (figure 17).

Figure 18 shows the relationship between animal weight (x axis) and gland volume (y-axis) for all animals. The filled circles show the volume of the gland treated with BTXA, the open circles show the volume of the side injected with saline. Usually, the volume of the BTXA side was less than the control side, especially in larger and older animals.

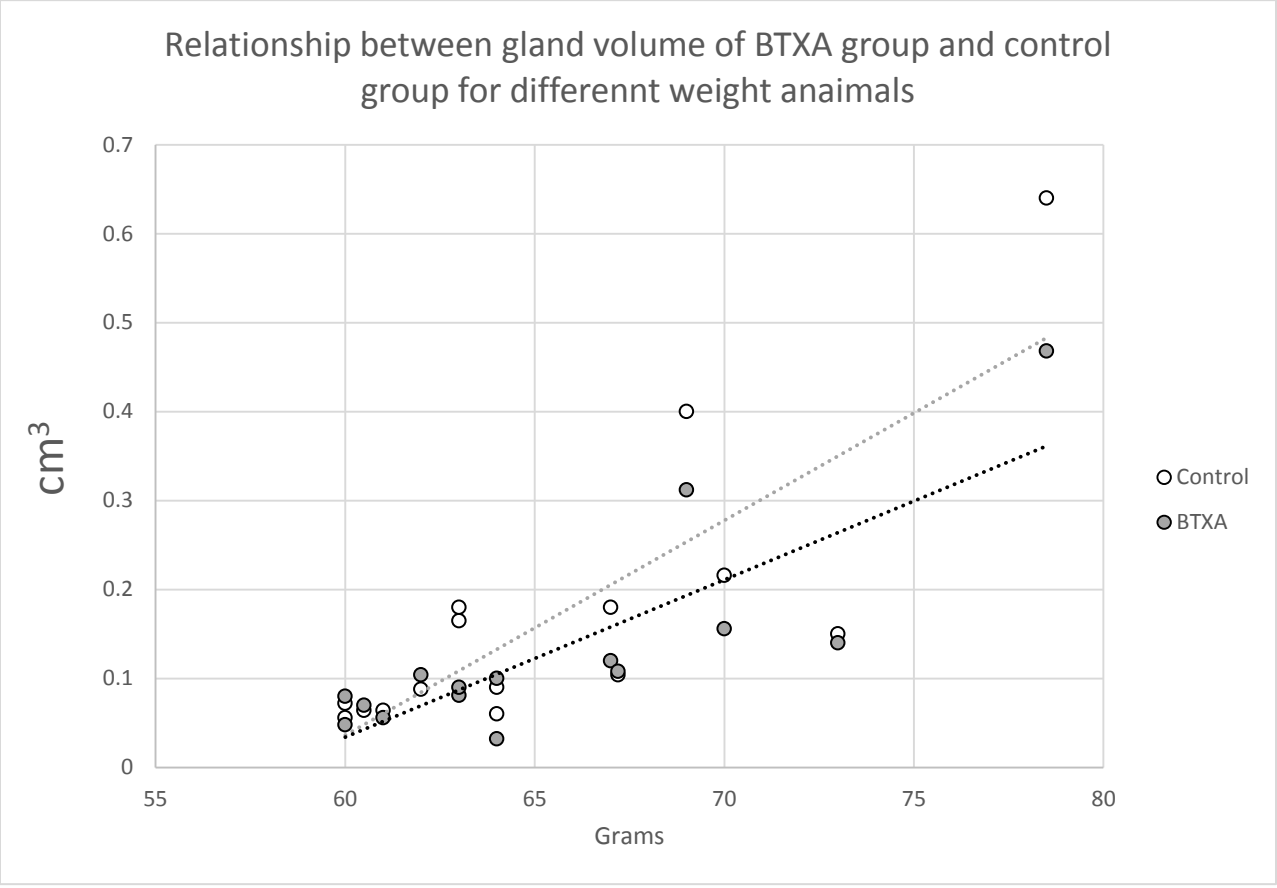

Figure 18. Relationship between gland volumes in BTXA group (filled circles) and control groups (open circles) in different weight animals. Number of animals, N=15.

There was a trend of higher changes in salivary gland volume that correlated with animal weight in the glands treated with saline. The glands treated with botulinum toxin presented less of a volume increase per gram of animal weight compared to the control group. The differences between both groups were more marked as the animal gained weight. This could indicate that there may be changes caused by botulinum toxin in normal gland growth. As in the weight case, this may suggest that the effect of botulinum toxin injections on gland volume might be observed more clearly in animals of a greater age versus younger or lighter animals. Overall, causing a reduction in size and weight may have functional consequences (figure 18).

Chapter Five: Discussion

5.1: General

Drooling is an unpleasant condition resulting in chronic irritation of the facial skin, which can lead to a decline in the quality of life and to social embarrassment. Anticholinergic medication, surgery and other therapies have all been used to reduce excessive secretion with various side effects. It is critical to find a safe and effective therapy to treat these symptoms. BTXA has been used to treat drooling with encouraging results. When injected in both submandibular and parotid glands, its efficacy rates can reach up to 95% [50]. Unfortunately, BTXA injections improve symptoms significantly but temporarily; patients usually need multiple injections to control drooling with efficacy varies from 6 weeks to 6 months [51, 52].

BTXA is a protein formed by anaerobic bacterium (Clostridium botulinum). It produces seven antigenically distinct neurotoxins, designated from A to G, where only type A and most recently B are approved for clinical use [1]. Type A is the most commonly used for therapeutic purposes. The mechanism underlying decreased salivation after a BTXA injection is thought to be ultrastructural and functional changes of salivary gland acinar cells [4]. These ultrastructural and functional changes lead to gland atrophy and a reduction in the weight and volume of the salivary glands due to inhibition of acetylcholine release at the neuromuscular junction.

The clinical use of BTXA to treat excessive drooling is now well established and rapidly growing, although the long-term safety and gland changes are still not well known or described [53]. There is scarce literature on size and histology changes of the salivary glands after Onabotulinum toxin A injection with sometimes conflicting histological results. However, most of these studies demonstrate gland changes after a single injection of BTXA. Only one

study, by our group, has shown changes in volume of the salivary glands after multiple injections of BTXA in humans , using an ultrasonography volume assessment technique[54]. However these results warrant further studies to corroborate the effect of the toxin on the salivary glands. To our knowledge, the research conducted in this thesis is the first animal study to measure weight and volume of the salivary gland after multiple injections of BTXA. The average reduction in salivary gland weight or volume varies in different studies.

The study lead by our group, in humans, was the first to demonstrate size changes in parotid and submandibular glands in pediatric population as measured by ultrasound after a minimum of three injections of BTXA [54]. We found an 11% to 17% reduction in the area of parotid and submandibular glands in comparison to the control group. We also reported shape and appearance changes seen on ultrasound in treated salivary glands. There was acorrelation between body weight and salivary gland size; the smaller the body weight, the smaller the salivary gland and vice versa. The results in our animal model are comparable to the results in our human study with the additional information of changes in the weight of the gland, which could not be measured in humans, for obvious reasons.

In another ultrasonography study, the Medial–lateral dimension and anteroposterior dimension of the rat's Submandibular gland was measured after a single Onabotulinum toxin A injection using US. They had two groups of 15 Wistar albino rats; group 1 (control group) was not given any substances, while group 2 was injected with 2.5 U of botulinum toxin A into their right gland, through a median cervical incision. Sonograms were obtained before the application, on the first day of the Onabotulinum toxin A application, on the 14th day, and again on 28th day [6] . Coskun et al concluded that the gland size was lower in the injected

group compared to the control group. Moreover, the gland size was lower on day 28 compared to day 14 after the Onabotulinum toxin A injection. However, they did not specify the percent of reduction after the Onabotulinum toxin A injection, though it was deemed statistically significant. Despite the changes in ultrasound, the authors did not find changes in the histological analysis (including apoptosis) after BTXA application. [6].

In both Schneyer and Hall's and Ekstrom and Reinhold's studies, the authors dissected the chorda tympani in rats in order to denervate the parotid gland. They found that parasympathetic denervation induced a 15% to 30% weight reduction of the parotid salivary gland [55, 56]. In another study Teymoortash et al. they found a 9.2% reduction in the weight of the submandibular gland 14 days after a single injection of BTXA. They also reported structural and functional changes at the acinar level in submandibular glands of rats [4].

There is evidence of size reduction in other glands due to apoptosis after BTXA application, as seen on nasal glands and prostatic glands [57, 58]. Doggweiler et al., found that Onabotulinum toxin A induced a generalized atrophy of prostatic glands with no evidence of inflammatory infiltrate. The reduction in prostatic weight was of 15%; and 57% after a single and four Onabotulinum toxin A injections respectively (with a one week interval between each injection in the latter) [58].

In our study with multiple BTXA injections, we found that the gerbils´ parotid glands decreased 20% and 15% in weight and volume respectively which is consistent with the findings of our group in pediatric patients subjected to multiple Onabotulinum toxin A injections. Although the reduction in weight and volume was not statically significant in our animal model, we think that this might be due to an insufficient sample size. Additionally, it

was found that the group that received three injections had a greater reduction in weight and volume than the groups that received five and seven injections, respectively. This result most likely was due to increasing in the animal weight during the experiment or it could be related to other factors like sample size, due to a development of antibodies against BTXA, or due to fibrosis of the gland. This may imply that Onabotulinum toxin A injection may be useful only at a certain number of doses. Further studies are needed to evaluate these hypotheses. Additional correlation with histological finding will be helpful to define the long term effect of BTXA and whether the decrease in size is related to atrophy, apoptosis, decrease in cell volume, or due to fibrosis. Moreover, it will allow for further understanding of BTXA mechanism at the cellular level. Eventually, it will help clarify whether the several BTXA injections will cause permanent or transient changes on salivary glands.

5.2: Future directions

Future studies are needed with pathological correlation to evaluate structural changes and histology of the salivary glands after multiple intra-glandular injections of Onabotulinum toxin A. These studies would help us to determine whether the changes in volume and weight of the glands are the result of atrophy or apoptosis or inflammation of the secretory units or the cells of the glands. Measurements that could be performed include:

5.2.1: Atrophy measurements:

(Morphometric analysis) By using the light microscope we could determine the quantity of atrophy in each group and which part of the gland are affected (acinar or ductal cells), total

acinar area and acinar cell number. Also we could determine the general morphology and percent or absence of fibrosis in both treated and control groups.

5.2.2: Apoptosis:

TUNEL assays used to investigate whether BTXA could induce apoptosis in the salivary glands. Especially their controversial results existed in the literature, regarding the BTXA if it will cause apoptosis or not, where they injected one dose of BTXA into salivary gland. So, we would like to know if BTXA will induce apoptosis or not?. Also it would be interesting to analyze if there are differences between multiple and single injections of BTXA in term of apoptosis.

5.2.3 Aquaporin-5 (AQP5):

Aquaporin-5 (AQP5) is a water channel protein. It plays a role in the generation of saliva, tears and pulmonary secretions. Activation of muscarinic receptors results in IP3 synthesis and subsequent increase in cytoplasmic calcium concentration via calcium release from IP3-sensitive intracellular stores. Increased cytoplasmic calcium activates Ca dependent K and Cl channels in the salivary gland, causing a translocation of aquaporin-5 (AQP5) from intracellular vesicles to the apical membrane. These events in turn allow water excretion into saliva. Therefore, decrease in the amount of aquaporin-5 will lead to reduce in salivary secretions. In support of this, it would be interesting to use knockout mice lacking AQP5, which show depressed rates of salivary secretion and hypertonic saliva [59]. There are two methods to identify or localized the AQP5 in salivary gland by using molecular or immunohistochemical techniques (Antibodies to AQP5). It would be interesting to analyze the effect of multiple injections of BTXA on these types of water channels.

5.2.4: M3-muscarinic receptor:

It remains unknown whether BTXA decreases the secretion of salivary glands by inhibiting the function of post synaptic muscarinic receptors. It would be interesting to analyze by immunohistochemistry whether multiple injections lead to down regulation of M3-muscarinic receptor and give much details on BTXA - muscarinic receptor relationship.

5.2.5: Antibody to BTXA:

Due to the need of multiple injections of Botox to control drooling, cases of resistance to Botox therapy may occur. We would like to identify whether there is any deposition of antibodies against BTXA in the salivary gland section after multiples injections.

5.2.6 Permanence of changes

It would also be interesting to use this animal model to analyze whether the changes in gland morphology are temporary or permanent.

5.3: Additional comments

The results presented in this thesis corroborate recent reports from our laboratory [54] aiming to assess quantitative salivary gland changes after the chronic use of intraglandular OBTXA for sialorrhea treatment in children. Using ultrasonographic measurements, our laboratory observed a significant decrease in the size dimensions (surface area and depth) of the salivary glands ($P < .05$). Results were also correlated with clinical outcomes.

The overall results of the current thesis should have a direct clinical impact as it would allow the prediction of: (1) the course of sialorrhea in children treated with multiple injections of onabotulinum toxin A, and (2) changes in their salivary gland histology and morphology.

Chapter Six: Conclusion

This thesis developed a new animal model, using gerbil parotid glands, to assess the effects of multiple intra-glandular injections of Onabotulinum toxin A. Then this model was used to characterize changes in weight and volume of the gerbil parotid glands after multiple intra-glandular Onabotulinum toxin A injections. It found a 20% decrease in gland weight and 15% reduction in gland volume in parotid glands injected with onabotulinum toxin A as compared to parotid glands injected with saline (control). However, these changes were not statistically significant. It was also found that the group that had three injections had a higher reduction in weight and volume than the groups that five or seven injections.

The results of this thesis are important because they report the first animal model that can be used to study the effects of chronic Onabotulinum toxin A salivary injection with minimal morbidity or mortality during a longer-term survivorship experiment. The thesis also successfully described changes in salivary gland weights post-injection, data that could not be easily obtained in humans.

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