The effects of Onabotulinum Toxin A treatment on pediatric Sialorrhea patients: An ultrasound and histological evaluation

By

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Abstract

Background. Drooling, clinically known as sialorrhea, can cause many complications impeding the life of patients, most of who have neurological disabilities. Complications include constant wiping and bib changes, choking, skin irritation and aspiration pneumonia. Treatment can include oral motor therapy, medication, or surgical intervention. Onabotulinum Toxin A (OBTXA) injection is a novel treatment proven to be effective. As results are temporary, the patients must return every 3 to 6 months for another injection. There is currently no study that examined the trend in morphological changes and functionality or the histopathological effects of OBTXA on humans.

Objectives. To determine the effect of Onabotulinum toxin type A treatment by observing morphological and histopathological effects on the salivary glands.

Methods. Two studies were undertaken. The first study was a quantitative and qualitative study in which the effects on drooling patients (n=8) of repetitive OBTXA injections on the size and shape of submandibular glands, assessed using ultrasound, were analyzed.

The second study compared post gland excision histology of acinar cells in salivary glands of drooling patients (n=31). Fifteen of 31 glands examined were exposed to 3 or more OBTXA treatments prior to surgery.

Results. No significant difference was found in the pathology of the salivary glands when comparing the OBTXA group to the control. There were no significant morphological changes in the glands as well.

Conclusion. Although no significant physical or histological changes were found in this study, previous studies and clinical observations have demonstrated that repetitive use of OBTXA treatment has a positive outcome on many drooling patients and their caregivers. Further studies are required to relate clinical outcomes and associated histological changes.

Résumé

Introduction. La sialorrhée est une condition de salivation excessive qui peut provoquer de nombreuses complications de la qualité de vie des patients, la plupart handicapés neurologiques. Les complications comprennent, l'isolement social, essuyage et changements de bavette, l'étouffement, l'irritation de la peau et la pneumonie d'aspiration. Le traitement de la sialorrhée inclut la thérapie orale motrice, des médicaments par voie orale, et l'intervention chirurgicale. Onabotulinum Toxin A (OBTXA) est un nouveau traitement dont l'efficacité a été prouvée. Bien qu'il soit efficace, les résultats sont temporaires et les patients doivent retourner tous les 3 à 6 mois pour une injection. Il n'y a aucune étude qui a examiné l'évolution des changements morphologiques, de la fonction et les modifications histo-pathologiques induites par l'injection de OBTXA sur les glandes salivaires chez l'homme.

Objectif. Pour déterminer si l'effet du traitement par OBTXA s'accompagne de changements morphologiques et histopathologiques des glandes salivaires.

Méthodes. Deux études ont été soumises.

La première était une étude quantitative et qualitative ~~en~~ observant les effets du traitement répétitif OBTXA sur la taille et la forme des glandes sous-maxillaires, par échographies répétées des patients souffrant d'hypersialorrhée. (n = 8).

La deuxième étude décrit les modifications histologiques des glandes salivaires de patients souffrant de sialorrhée (n=31) et ayant finalement subi une exérèse chirurgicale.

Résultats. Aucune différence significative n'a été observée dans la pathologie des glandes salivaires lorsque l'on compare le groupe OBTXA au contrôle. De même, Il n'y avait pas de changements morphologiques significatifs notés en échographie.

Conclusions. Notre étude préliminaire montre que l'effet clinique de trois injections répétées Onabotulinum toxine A sur l'hypersialorrhée s'accompagne d'une amélioration de la qualité de vie de ces patients et leurs soignants, mais n'entraine pas de modifications précoces visibles en échographie ou en histopathologie des glandes salivaires. Autres études sont nécessaires pour établir un lien entre les modifications histologiques et les résultats cliniques associées.

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Preface

Contribution of Authors

This thesis is a manuscript-based thesis composed of two manuscripts.

The first manuscript (Chapter 3), "The Change in Salivary Gland Size, Shape and Texture over the course of three Onabotulinum Toxin A treatments: An Ultrasound Measurement" by Ashley Mosseri, Isabel Cardona, Christine Saint-Martin and Sam J. Daniel has been accepted for publication by *Laryngoscope.* Ashley Mosseri performed the data collection and wrote the initial draft for the manuscript. Dr. Isabel Cardona assisted in data collection and did a final revision for the manuscript. Dr. Christine Saint-Martin provided supervision, concept design, clinical expertise and final revision of the manuscripts. Dr. Sam J. Daniel provided supervision, guidance, concept design, expertise and review of manuscripts.

The second manuscript (Chapter 4), "The Histolopathological effects of Onabotulinum Toxin A treatment in Pediatric submandibular glands",has been accepted for publication in *Otolaryngology–Head and Neck Surgery*. Ashley Mosseri performed the data collection and wrote the initial draft of the manuscript. Dr. Isabel Cardona assisted in data collection and final revision for the manuscript. Dr. Miriam Blumenkrantz provided supervision, expertise, guidance and final revision of the manuscript. Dr. Sam J. Daniel provided supervision, guidance, concept design, expertise and review of the final manuscript.

Claim of originality

The first manuscript is the first study to observe the morphological effects of OBTXA from baseline of three injections on the salivary glands. It found that such injections did not cause significant early changes in sonographic appearance of the salivary glands despite a clinical improvement. The second manuscript is the first study to observe the histopathological effects of OBTXA on the submandibular glands of humans. It found that there was no significant change in number of acinar cells, indicating that changes in the number of acinar cells did not explain why OBTXA had an effect on the salivary glands.

Chapter 1 Introduction

1.1 Rationale

Sialorrhea, or drooling, is a common condition found in children with neurological diseases. Sialorrhea can be treated in various ways including oral and motor rehabilitation, medication and surgery [1]. The saliva clinic at the Montreal Children's Hospital offers Onabotulinum Toxin A injections (OBTXA) as a treatment option. This treatment reduces salivation for a short period of time (3 to 6 months). Depending on the severity of the drooling, OBTXA is injected in either one or both of major salivary glands (Submandibular and Parotid glands). The long-term positive outcomes have been observed at our clinic. However potential underlying physiological changes of the salivary glands after OBTXA injection treatments haven't been investigated in detail. Currently, there are no studies investigating the latter.

Significant differences in glandular size were not noted when comparing control patients to drooling patients in our clinic [2]. Other studies have found that repetitive OBTXA can cause atrophy to salivary glands [3, 4]. As the treatment option for sialorrhea with OBTXA is becoming more common, further studies are needed to determine the effects of OBTXA injections on the normalization of function of salivary glands.

1.2 Objectives

This thesis had two objectives:

1) Previous studies have demonstrated atrophy after repetitive use of OBTXA on the salivary glands. The first objective of this thesis was to determine if morphological changes occur in the submandibular glands starting as early as after three OBTXA injections.

2) Atrophy has been reported to occur after repetitive use of OBTXA. This suggests histological changes within the submandibular glands may be occurring. The second objective of this thesis was to determine the effect of repetitive OBTXA injections on acinar cells of the submandibular glands.

Chapter 2 Literature review

2.1 Saliva

2.1.1 Definition and Function of Saliva and Salivary Glands

Saliva is a fluid consisting of 99% water along with enzymes, glycoproteins, antibacterial agents, electrolytes and bacteria for immunity. Functions of saliva include lubrication, buffer functioning, swallowing initiation, and immunity of the oral cavity [5, 6]. Saliva aids the initial breakdown of dietary starches and fats with the help of its enzymes [7].

Salivation is produced in the salivary glands. The three major glands are the parotid, the submandibular and the sublingual glans. The parotid gland is the largest of the salivary glands produces approximately 25% of total saliva production in the oral cavity and secretes saliva from the Stenson duct initiating the digestion of starches. The submandibular glands (SMG) are located below the jaw and they secrete saliva from Wharton duct. The submandibular glands produce 70% of saliva at rest, with the formation of a viscous and constant source of saliva [5, 8, 9].

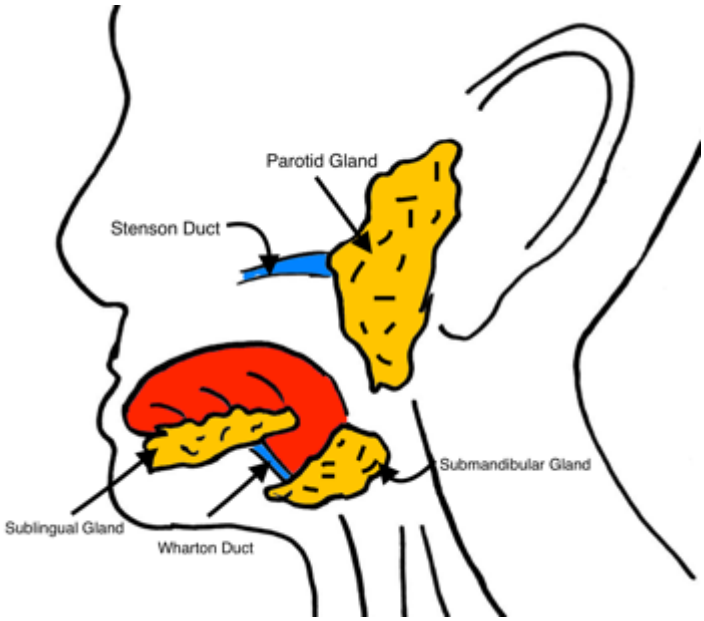

Figure 2.1: Main salivary glands (Parotid, Submandibular and Sublingual) with ducts

2.1.2 Mechanism of swallowing

Neuromuscular activity of swallowing consists of a coordinated sequence of movements among the several structures comprising the upper digestive system - including the oral cavity, pharynx, larynx and esophagus[10].

Normal swallowing is divided into 4 stages. The first and second stages are the oral and preparatory stages, which include mastication and transportation of the bolus from the anterior to the posterior portions of the oral cavity. The third phase is the pharyngeal phase consisting of laryngeal elevation and closing of the palate, propulsion of the bolus from the posterior oral cavity to the pharyngeal cavity. Finally, the fourth phase is the esophageal phage, in which the larynx closes, the crico-pharyngeal muscle releases and the initiation of esophageal peristalsis occurs [1, 10, 11].

Various muscles are involved in swallowing and mastication. Drooling can be a result of weak contraction or lack of coordination of the tongue and soft palate. This can initiate an early leakage into the pharynx. Sensory impairment can cause a similar effect, resulting in retention of food and/or liquids in the oral cavity after swallowing. Premature swallowing resulting from weak tongue contraction can also cause aspiration. This is commonly observed in serious cases of sialorrhea [12].

2.1.3 Neurophysiology of Saliva

Salivation is stimulated by the autonomic nervous system and can be by either direct or indirect innervation by the sympathetic or parasympathetic system [13]. First, the parasympathetic system is initiated via the cranial nerve where acetylcholine (ACh) is released. An increase in intracellular calcium ion concentration occurs when the ACh binds to the muscarinic receptors on the salivary acini cells. The increase in calcium allows the vesicles in the cells to fuse with the cell membrane leading to glandular secretion [7]. The sympathetic nervous system evokes the response in order to modulate the composition of saliva. Innervation of the blood vessels supplying the glands indirectly innervates the sympathetic nervous system [14-17].

2.1.4 Histology of Salivary Glands

Salivary glands are composed of secretory acinar cells and ducts. There are two types: serous acinar cells, which secrete a fluid containing proteins and mucous acinar forming a mucin lubricant. Submandibular glands consist of a mix of both mucous and serous acinar cells [18]. Parotid glands are mostly composed of serous acinar cells. Secretory units are composed of the acinar cells and merge into intercalated ducts, which turn into striated ducts where water resorption and ion secretion take place [18, 19].

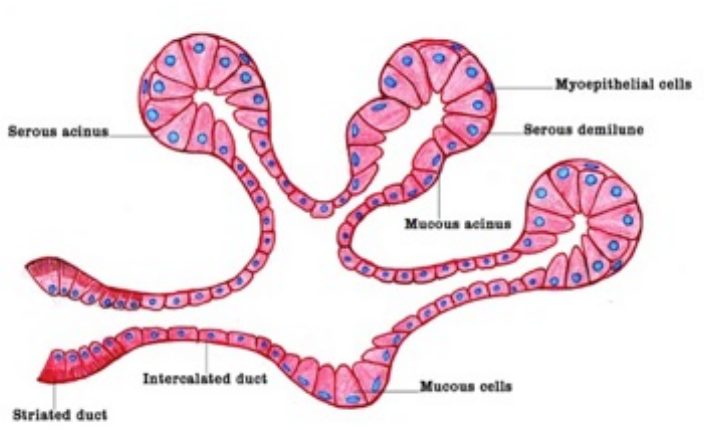

Figure 2.2: Acinar cells of Salivary Glands

2.2 Sialorrhea/Drooling

2.2.1 Definition

Sialorrhea is a chronic condition characterized by excessive drooling or by an increase of salivary flow in the oral cavity [20, 21]. Sialorrhea is commonly found in patients with poor oral-motor control [22] as a result of muscle incoordination [21]. Sialorrhea is present when the saliva chronically surpasses the labial border of the lower lip [22].

Sialorrhea can present in an anterior or posterior form. Anterior sialorrhea is when the saliva surpasses the oral cavity and descends the lower lip. This can results in constant wiping, bib changes, social embarrassment, and skin irritations [23, 24]. Posterior drooling results from erroneous swallowing mechanics.

2.2.2 Etiology

Sialorrhea occurs in patients with either poor oral-motor control [22] or increased production of saliva, which can be drug-induced or idiopathic [18, 21, 25-29]. Muscle incoordination disrupts the normal swallowing reflex, which can cause pooling of saliva in the mouth [21]. Various neurological disorders, infections, and Angelman syndrome can cause idiopathic sialorrhea.

Table 2.1: Causes and effects of anterior and posterior sialorrhea

	Causes	Effects
Anterior sialorrhea	• Epilepsy • Anti-epileptic drugs • Benzodiapen • Oral-pharyngeal infections and obstructions • Oral structural abnormalities • Impeded oral-motor control (e.g. facial palsy) • Poor coordination • Poor sensory awareness • Cognitive impairment	• Skin irritation • Constant wiping of mouth • Constant change of clothing, bibs and bed sheets • Social isolation • Bad breath
Posterior sialorrhea	• Poor head and trunk control • Poor coordination • Cognitive impairment • Attention deficit	• Feeding impairment • Aspiration pneumonia • Bad breath

2.2.3.a Idiopathic Paroxysmal sialorrhea

Idiopathic Paroxysmal Sialorrhea (IPS) occurs at high salivary flow rates, episodically with nausea and epigastric pain. The cause of IPS is unknown but it consists of an increase in salivary flow 1 to 2 times per week for a duration of 2 to 5 minutes [5, 30].

2.2.3.b Drug-induced

Many drugs can induce overproduction of saliva. These medications include Cholinergic medication, clozapine, lithium, anticholinesterase and nitrazepam [5, 31]. Over 70 medications including antibiotics, vitamin C, and anti-inflammatories (non-steroidal) have been associated with mucosal damage to the esophagus. It has been reported that Sialorrhea often occurs with dysphagia and/or odynophagia[5].

2.3 Prevalence of Sialorrhea

2.3.1 Management

Drooling can be treated in various ways including oral and motor rehabilitation, medication, or surgery on the salivary glands[32-34]. Oral and motor rehabilitation might be effective, but was proven not effective in some patients with neurological deficiencies, since compliance is required [35]. Although medication may be effective in reducing salivation, it is often accompanied by side effects such as blurred vision and urinary retention. Surgery, when it consists in surgical excision of the salivary glands, can also be a permanent solution to sialorrhea. However, most of these patients present with high-risk anesthesia due to co-morbidities, limiting surgery indications [1, 6, 36].

Anticholinergic medication includes a Hyoscine patch (Scopolamine) used for long-term management. However, this can cause skin irritation if an allergic reaction occurs [37]. Other medications include: Glycopyronnium bromide, Trihexyphenidy-benzhexol, Benztropine, Ipratropium bromide[37-39].

Onabotulinum Toxin A (OBTXA) treatment reduces salivation temporarily. Although not a permanent treatment, extensive clinical evidence has demonstrated beneficial effects in children [40-43].

Table 2.2: Pro and cons of sialorrhea treatment modalities

Treatment	Oral-Motor Therapy	Anticholinergic Medication	Onabotulinum Toxin Type A	Surgery
Pros	- Non invasive - No side effects	- Level B Evidence (probably effective) [21]	- Level A Evidence (effective and established) [21] - Localized - Minimally invasive	- Permanent
Cons	- Challenging for patients with neurological deficiencies and poor swallowing mechanism. - Requires cooperation from the patient. - Family must be committed to long term support in order for success[37]	- blurred vision - constipation - urinary retention - Absolute contra-indicating use for patients with Myasthenia Gravis and Glaucoma [37]	- Not permanent. Requires treatment every 3-6 months depending on the severity of the drooling.	- Requires patient to undergo anesthesia

2.4 Onabotulinum Toxin Type A

2.4.1 Definition and pharmacology

OBTXA is a type of protein and a potent neurotoxin [44]. When in contact with SNAP-25, a cytoplasmic protein that assists in the fusion of synaptic vesicle to the presynaptic membrane, it blocks the receptors at the synaptic vesicles from releasing acetyl choline (ACh) and disrupts the secretory pathway [20, 21, 45]. This blockage can possibly cause a permanent change to the salivary glands after several OBTXA injection treatments.

2.4.2 Onabotulinum Toxin A for sialorrhea

The mechanism of OBTXA on the salivary glands is not entirely delineated. As stated in the previous section, OBTXA is a potent neurotoxin that blocks the receptor of the pre synaptic vesicle allowing the release of acetylcholine (ACh). Without that release, the secretion of saliva is inhibited, thus limiting salivation.

2.4.3 Clinical implication of study

The studies described in the present thesis helped further knowledge to the scientific community on the effects of OBTXA treatment on the salivary glands. Ultimately, the clinical implications of the studies are to allow clinicians to opt for the best management for patients to better their general well being. The studies implicated in the thesis furthered scientific evidence on the physical and histological changes of salivary glands after repetitive use of OBTXA treatment. Last, the well being of patients and their caregivers was investigated.

2.4.4 Saliva Management Clinic

The saliva management clinic at our institution offers OBTXA injections as an option for the treatment of sialorrhea at a standard dose of 4U/kg [1]. Patients are followed and treated by a single otolaryngologist (SJD) following a standardized protocol established over 10 years ago. Depending on the severity of drooling, the otolaryngologist may opt for OBTXA injections in both or either the submandibular and parotid glands. As OBTXA is not a permanent treatment option, patients must return every 3 to 6 months for follow up and/or repeat treatment [1].

2.5 Linking Statement to first manuscript

The physiological effects of OBTXA on salivary glands are still unknown. It is uncertain

whether the treatment has permanent effects pertaining to the atrophy found in past studies[3]. The study described in Chapter 3 investigates the potential change of the size of the treated glands. Measurement prior to any treatment are used for baseline references and compared to OBTXA injected glands after three or more treatments at various time points. The study analyzes the size, shape and echotexture of the salivary glands. To our knowledge, it is the first study examining the potential early physical effects of OBTXA on the salivary glands by using ultrasound.

Chapter 3 The Change in Salivary Gland Size, Shape and Texture over the course of three Onabotulinum Toxin A treatments: An Ultrasound Measurement

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3.1 Abstract

Background. Onabotulinum toxin A (OBTXA) is a well-known treatment for patients with drooling. Its transient effects necessitate repeated injections every 3 to 6 months depending on the drooling severity. Previously , our group demonstrated salivary gland atrophy after repeated OBTXA injections[3].

Objective. The objective of this pilot study was to determine if morphological changes would occur in the salivary glands over the course of 3 OBTXA injections.

Methods. A cohort study observing the size, shape, and echotexture of the salivary glands of drooling patients who underwent three repeated OBTXA injections were rated in a blinded fashion. All patients were followed for a minimum of 12 months and underwent ultrasound of their submandibular glands prior to each therapeutic injection.

Results: 8 Patients were included (median age 13.5 years, 5 males). Changes in submandibular gland shape, size and echotexture were observed but not significant. A relationship was however observed between the non-significant morphological changes in salivary glands and improvements in drooling functionality. No post injection complications occurred.

Conclusion: Morphological changes were observed, however not significant, from the initiation of OBTXA treatment. Results from this study encourage further investigation as to when permanent significant morphological changes occur on the salivary glands in relation to the clinical outcomes of the treatment.

Key Words: Salivary Glands, Onabotulinum Toxin A, morphology, ultrasound, sialorrhea

3.2 Introduction

When children experience drooling after age of 4 years old, the condition is known as Sialorrhea[21]. The injection of Onabotulinum Toxin A (OBTXA) into the salivary glands is an effective management solution for patients with sialorrhea. OBTXA is a protein derived from *Clostridium botulinum*. When injected into the salivary glands, it inhibits the cholinergic pathway at the neuromuscular junction within the salivary glands[46]. This polypeptide binds to the protein receptor (SNARE), which is responsible for the release of acetylcholine from the synaptic vesicles in the neuromuscular junction[3, 41, 47-55]. The inhibition of the release of acetylcholine impedes the secretory action of the acinar cells within the salivary glands[18, 19, 29, 56, 57]. OBTXA is a minimally invasive procedure as an alternative therapeutic option to surgery. The complications are minor with low risk rates [46].

Previous studies have analyzed the change in size of the salivary glands due to OBTXA treatment[3]. These studies have shown that with chronic application of OBTXA on the salivary glands, atrophy occurs. A significant decrease in size of the submandibular gland was found, to be possibly of resting saliva, with subsequent improvement of drooling[3]. Other studies have demonstrated with duct obstruction, resulting in impairment of the gland's function may result in the gland atrophy[58]. For example, a 48 hour duct ligation, resulted in glandular atrophy along with secretory cell degranulation. There was a relationship observed between the structural and physical changes within the salivary glands, with regards of the treatment and positive clinical outcomes [58] .

To our knowledge, potential early morphological changes starting when OBTXA treatment is initiated have not yet been studied. The objective of this study was to analyze the effects of OBTXA injections on the sonographic features of the submandibular glands from zero (0) to after three (3) injections with regards to submandibular gland shape, size and echogenicity.

3.3 Methods

3.3.1 Study Design and population

A cohort study was conducted at the Montreal Children's hospital. Ethics approval was obtained from the Research Ethics Board of McGill University Health Center. Written informed consent was obtained from caregivers prior to study inception.

Inclusion criteria were pediatric patients with sialorrhea, aged 4 to 18 years, who did not

have any previous OBTXA injection, and underwent three (3) consecutive OBTXA treatments between September 2013 and August 2015, with a 3 to 6 months' interval between the injections.

The patients' medical records were reviewed to obtain demographic characteristics, medical diagnoses and number of OBTXA injections.. The ultrasound of the salivary glands was performed according to the protocol described in previous studies reported from our institution [3] with the same equipment (HDI 5000 SonoCT (Philips Healthcare, Andover, MA), VA 12 MHz probe with a 15 MHz frequency).(figure 3.1) Ultrasound evaluations were performed by a single observer (AM), all of the provided images were reviewed before selection and analysis by an experienced pediatric radiologist (CSM). Every patient included had an ultrasound performed before each of the OBTXA injection. Trend of the gland size was analyzed using Microsoft Excel 2011 for Mac.

In the first component of the study, analysis of the shape of the submandibular gland only was performed over time. A blinded analysis was performed by two observers based on a novel categorical scale ranging from 0 to 3, analyzed the contours of the gland and its irregularities (Figure 3.1). A shape criterion was analyzed before the size criteria to avoid bias in the picture analysis.

0	**1**	**2**	**3**
			
• Smooth contour • No groove	• No groove/ indentations • Start of irregular contour • Loss of convex contour	• Slight groove/ indentations • Start of irregular contour • Loss of convex contour	• Deep groove/ indentation • Irregular contour • Loss of convex contours

Figure 3.1 : Categorical Shape Scale for the Submandibular gland

The second component of the study was the analysis of the change in size of the glands from before to after 3 OBTXA injections. The Ultrasound measurements of the surface area, Anterior-Posterior (AP) and medial-lateral (ML) dimensions were done according to our

previously reported protocol[3]. ML was determined by the thickness of the gland in the coronal axis. The AP was maximum distance along the transverse axis. The images and measurements were evaluated and approved by the same individual blinded to the number of OBTXA injections the patient received.

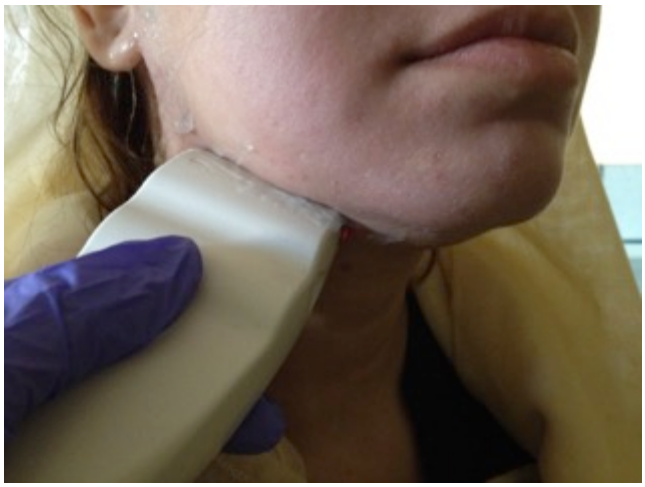

Figure 3.2: Ultrasound of the Submandibular gland: the probe is placed inferior to the mandibular angle and body.

Figure 3.3: . Ultrasound of the Parotid gland: the probe is placed horizontally inferior to the ear lobe and superior to the mandibular angle.

As a third component of the ultrasound evaluation, analysis of the echotexture of the

glands, was done using the program ImageJ© for Mac, which employs a selected JPEG image to produce a histogram of the gray scales appearing within a specific area chosen of the gland. The size of the box used on the imaging program for grey scale analysis was consistent throughout the study (Figure 3.4). The baseline and post-treatment images were displayed side by side in order to compare the same approximate position of the analysis area within the gland.

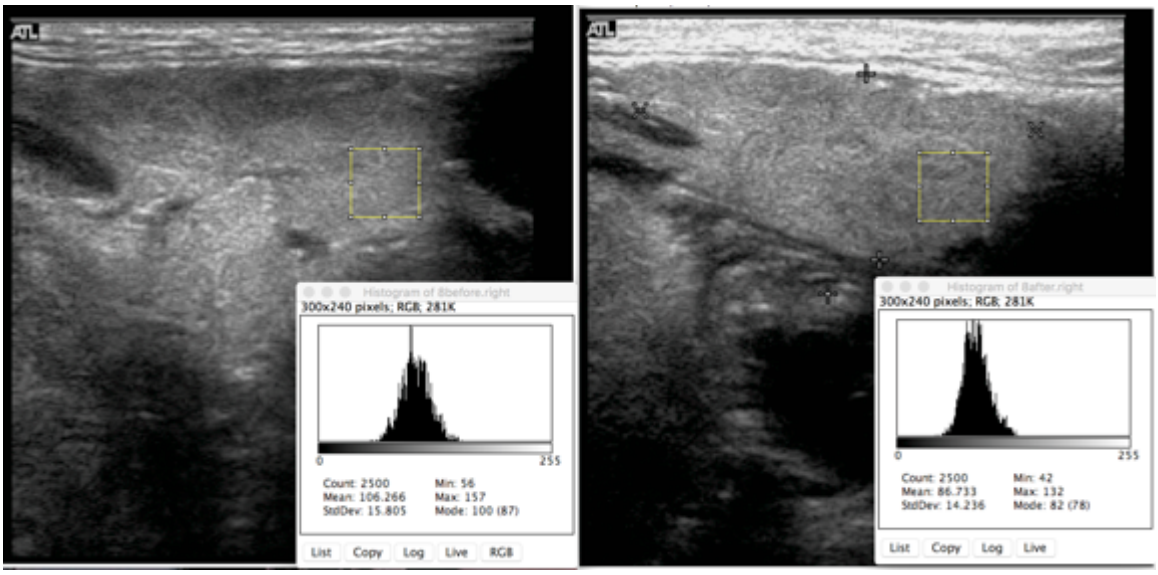

Figure 3.4: Methods of texture analysis of Submandibular glands. The greyscale has 256 levels from 0 (black or anechoic texture) to 255 (white or hyperechoic texture)

In order to determine whether morphological changes were correlated with drooling functionality, the sonographic data were compared to the scores of validated drooling questionnaire completed by the caregivers that consisted of three questions on (i) drooling frequency, (ii) severity and (iii) number of bib changes (Fig. 3.5; see [25, 39, 59]) – all assessed at baseline and after 3 OBTXA injections.

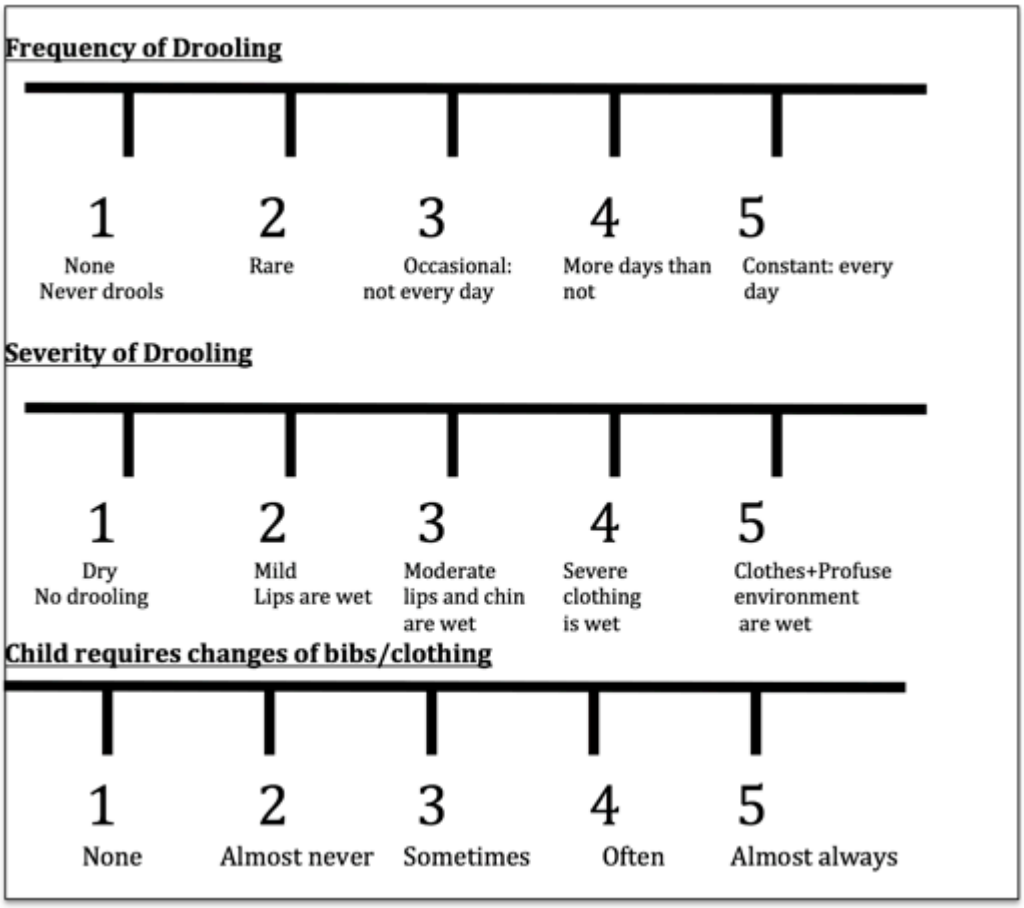

Figure 3.5: Questionnaire measuring improvements in drooling functionality

3.3.3 Statistical Analysis

The median and standard deviation (SD) were obtained for continuous variables (e.g. age, BMI, echogenicity of glands, and size).

A descriptive analysis was performed for all variables. A Mann-Whitney Exact test was done to compare the size and echogenicity of the glands at baseline (time=0), OBTXA dosage 1 (time=1), OBTXA dosage 2 (time=2) and after 3 OBTXA treatments (time=3). A comparison was made between median size at baseline and at time=3. The Statistical Package for Social Science (version 16.0 for Windows; SPSS Inc, Chicago, IL USA) was used. A P-value of <0.05 was considered the threshold for statistical significance.

3.4 Results

3.4.1 Quantitative Results

Between October 2013 and December 2015, eight (8) patients met the inclusion criteria of our study (median age of 13.5 years, five (5) male). The patients' median weight was 14.35 kg. The baseline characteristics of patients are described in Table I. Included patients had various neurological diseases, such as Global Developmental Delay, cerebral palsy, encephalopathy, and autism.

Table 3.1- Baseline characteristics of subjects

Variable	**N=8**
Gender	
Male	5 (63%)
Female	3 (38%)
Age (yr.)	13.5
Weight (kg)	14.35
Diagnosis	
Global Delay Disorder	3 (37.5%)
Cerebral Palsy	3 (37.5%)
Epilepsy	1 (12.5%)
Rett Syndrome	1 (12.5%)
Spastic Quadriperisis	1 (12.5%)
Cerebral malformation	1 (12.5%)

*Quantitative variables are presented in Md (median) and Categorical variables are presented in N (%).

3.4.2 Gland Shape

Based on the Gland Shape scale, the samples analyzed did not show a significant change in shape of the salivary glands ($P > 0.05$) during with treatment after only 3 injections. As seen in Figure 3.3, the submandibular glands tended to show a difference in shape at 3 OBTXA injections. Half of the submandibular glands had a baseline rating of 0 (according to Gland Shape Grading Scale) and more than half of patients resulted with a categorical rating of 2 after three OBTXA injections.

Table 3.2: Dual blinded rating results of gland shape before and after 3 OBTXA injections

Gland	1L	1R	2L	2R	3L	3R	4L	4R	5L	5R	5L	6R	7L	7R	8L	8R
Rater 1																
Before	0	1	1	1	0	0	1	0	1	0	1	0	0	0	0	0
After	2	2	2	3	3	2	2	2	3	2	3	3	2	3	1	1
Rater 2																
Before	0	1	1	0	0	0	2	1	1	0	2	1	0	0	1	1
After	2	3	1	0	2	2	2	3	2	2	3	2	2	3	2	2

*L=Left, R=Right

3.4.3 Gland Size

Table 3.3 shows the area, AP, and ML dimensions of the submandibular glands at baseline (0 OBTXA injections) and at 3 OBTXA injections. Median baseline size was 2.88cm^2 and at 3 injections 2.87cm^2. Median baseline for AP dimension was 2.94cm and 2 cm after three OBTXA injections. Median baseline for ML dimension was 1.45 and 1.45 cm after three OBTXA injections. No significant difference in size between baseline and three OBTXA injections was found. Differences were not statistically significant (P>0.05).

Table 3.3- Median values for area, AP and ML dimensions

Variable	Baseline	3 OBTXA Injections*
Area	2.88	2.82
AP	2.94	2
ML	1.45	1.45

AP= Anterior-Posterior dimension,
ML= Medial-Lateral dimension

3.4.4 Echotexture of salivary glands

The median echotexture before any injection was 81.4615 Gray units (Gy) and 86.508 Gy after the 3 injections. Over time, with 3 OBTXA injections, the grey scale tended towards more minimal-hyperechoic levels in comparison to the baseline. Subjectively, the glands appeared to increase in heterogeneity as well. There was an increase in the median value of grey scale, but this was not statistically significant (P> 0.05).

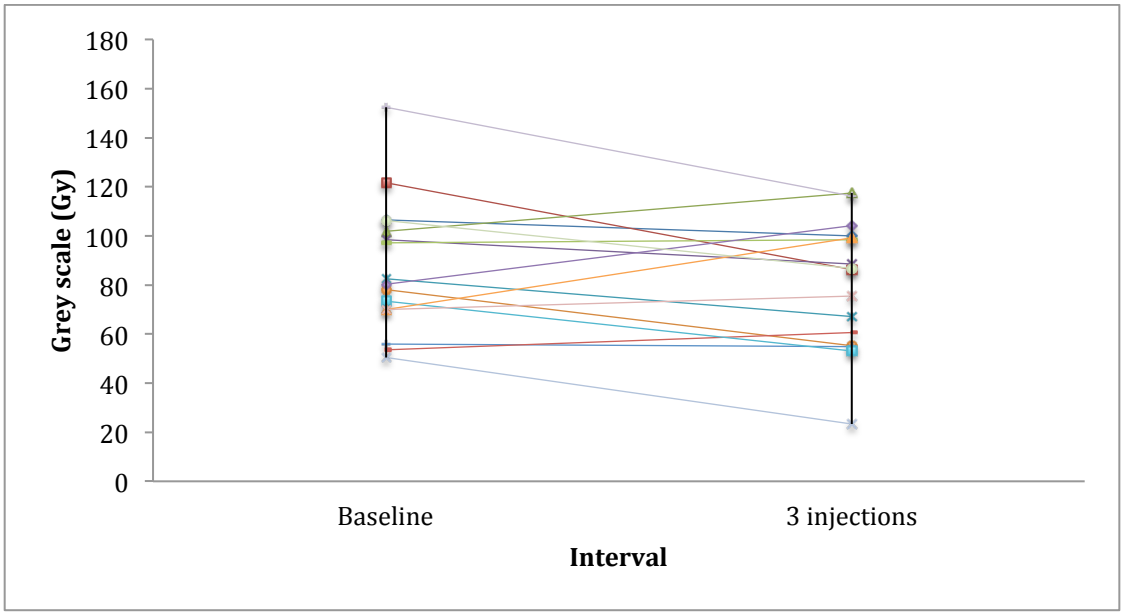

Figure 3.6: Change in Submandibular Gland echotexture from baseline to 3 OBTXA injections- measured in grey scale (Gy)

3.4.5 Drooling Functionality

After OBTXA treatment, some subjects who showed a change in gland shape, although not statistically significant, also had an improvement on the validated frequency and severity drooling scales (Fig. 3.5). Specifically, 12.5% of patients with morphological changes in the submandibular glands had an improved (lower) Frequency-of-Drooling classification (top scale Fig 3.5); 37.5% of patients with morphological changes had an improved Severity-of-Drooling classification (middle scale, Fig 3.5); over half (56%) of patients with morphological changes in the glands had an improved Changes-of-Bibs/clothing classification (bottom, scale Fig 3.5). Further studies are required to determine if there might be a statistically significant association between the observed morphological changes and the improved classification scores, which would indicate a reduction of drooling.

3.5 Discussion

The purpose of this study was to determine if there were any significant morphological changes in the submandibular glands, comparing them at baseline, and after 3 OBTXA injections. A previous study has reported that the submandibular glands atrophy with chronic use of OBTXA treatment [3]. Another study found that there was no difference in size of the salivary glands between control and drooling patients [2]. Evolution of the shape and size of the salivary glands has never been studied, starting from a baseline of zero OBTXA injections.

Regarding the shape of the gland, early morphological changes were noted, however with no statistical significance. A normal submandibular gland resembles a triangle from the longitudinal and transverse section[60]. In our study, we found that the majority of the glands rated 0 at baseline, and then received a score of 2 after three OBTXA injections - mainly due to irregularity and loss of convexity of the contours. We hypothesize that such early changes could be related to loss of acinar cells since there was a clinical improvement observed. However, the changes observed might also be due to post-traumatic scaring after repeated needle injections [3, 61].

We tried to compare changes in surface area, medial-lateral dimensions, and anterior-posterior dimensions starting from a baseline of 0 OBTXA injections, and increasing up to 3 injections. None of our findings demonstrated significant differences ($P>>0.05$). This may be because more than 3 OBTXA injections may be required for significant changes to appear. Continuing the study with more subjects may show that changes may occur that are statistically significant, even before 3 injections are administered.

The difference in the gland echotexture between baseline and the third OBTXA injection was not statistically significant; however, a trend towards increased echogenicity of the submandibular glands compared to adjacent muscles as well as to baseline was noted. Echogenicity of the normal major salivary glands is usually homogenous and hyperechoic compared to proximal neck muscles[60]. There was a subjective increase in heterogeneity as well. It may be too early to observe a significant change after only three OBTXA injections; a significant change might be observed after more (>3) OBTXA injections, which might parallel the previously observed decrease in gland size[47].

In our study, we did not use semi-quantitative or quantitative methods to assess salivary gland function, by using, for instance, salivary gland scintigraphy or direct duct cannulation [9, 62, 63]. Echogenicity using gray-scale might be useful in future studies. With the changes in shape, some measures of drooling improved in some patients. For example, over 50% of patients with morphological changes of their submandibular glands, also showed a decrease in the number of bib changes after 3 OBTXA injections. Further studies are required to determine if such associations are statistically significant.

3.5.1 Limitations of Study

The main limitation of our study was our small sample size, with subsequent limited statistical analysis power. As well, there was a technical limitation associated with the operator-dependent ultrasound technique, although this was partially addressed by using a sonographic imaging protocol already used in larger cohorts [2,3]. Finally, another limitation was the absence of a pathological analysis of the salivary glands, which might have better identified any early morphological changes.

3.6 Conclusion

Although there were limitations to this study, analysis of the physical effect of OBTXA on submandibular glands beginning from baseline of 0 OBTXA injections to 3 injections has never been previously reported. Although the morphological changes were not statistically significant, trends were observed in changes of gland shape (loss of smooth convex contours), gland size (decreasing size) and echotexture (increasing echogenicity). The results of our preliminary study encourage further studies with a larger cohort of patients, who are monitored from baseline as they receive more treatment, while assessing gland changes, sonographic changes, as well as changes of various measures of drooling in order to better understand when and how the response to OXBTA injection takes place. Also, the relationship with the pathology of the salivary glands might clarify the non-treatment response or poor-treatment response of some patients.

3.7 Acknowledgements

We would like to thank the Montreal Children's Hospital Library, Ms. Elena Guadagno and Mr. Alex Amar, for their help in finding background information pertaining to this study.

3.8 Conflict of Interest

The authors have no conflicts of interest.

3.9 Discussion and linking statements

Linking statement

The above first manuscript described the effects of OBTXA on the size and shape of the gland using ultrasound. This is the first study to compare salivary glands before and after 3 OBTXA injections. However, it is still unknown when significant changes in shape and size of the salivary glands occur. Previous findings have claimed that there is atrophy in the salivary glands after repetitive OBTXA injections (>3 injections)[3]. Studies employing salivary-gland duct ligation, have reported both atrophy as well as secretory cell degranulation[58]. To our knowledge, no human study has observed salivary glands post OBTXA injections on a histological level. The study in the following chapter will try to determine if there are histopathological changes in the gland associated with repeated OBTXA treatment.

Implications

Indications of permanent changes resulting in decreased functionality of the salivary glands are being observed. Analyzing different aspects pertaining to the functionality of the glands should help understand the implications as to why the salivary glands decrease in long-term saliva production. Unfortunately, these results are not being seen in the above study due to the limitations of our study. A general conclusion for the total drooling population cannot be drawn, and thus further investigations must proceed.

Chapter 4 The Histolopathological effects of Onabotulinum Toxin A treatment in Pediatric submandibular glands

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4.1 Abstract

Background. Onabotulinum toxin A (OBTXA) is an effective treatment for drooling. There have been no human studies reporting the effect of recurrent OBTXA injections on acinar cells numbers.

Objective. To determine if there are histological changes in the submandibular glands (SMG) after multiple OBTXA injections.

Methods. In this cohort study, SMG of drooling children that underwent SMG removal post 4 or more OBTXA injections, were compared to a control group who had SMG removal without previous OBTXA treatment. The number of acinar cells was counted in histopathological samples.

Results. 31 glands were analyzed (14 non-exposed, 17 exposed). There was no significant difference in the number acinar cells per surface area in the control group as compared to the OBTXA group (1.35 cells/μm versus 1.36 cells/μm respectively).

Conclusion. In this first human study, we found no significant change in the number of acinar cells in SMG following multiple OBTXA injections.

Keywords: Sialorrhea, salivary gland, acinar cells, structural changes, histology, Onabotulinum toxin type A.

4.2 Introduction

Salivation is a process within the exocrine system. It occurs as secretion from the acinar cells within the salivary glands into the oral cavity[16]. Due to several causes, including poor oral-motor control, swallowing problems, esophageal atresia, overproduction of saliva due to medication, patients could experience hypersalivation, also known as sialorrhea. This is a common condition seen in patients with neurological diseases such as cerebral palsy and epilepsy.

Onabotulinum toxin A (OBTXA) injection is an effective option for sialorrhea management, including challenging conditions such as in Familial Dysautonomia[41]. OBTXA is a potent neurotoxin that binds with SNAP-25, a cytoplasmic protein that assists in the fusion of the synaptic vesicle with the presynaptic membrane. OBTXA temporarily blocks the secretory pathway by inhibiting the release of acetylcholine (ACh) from the synaptic vesicles.

It is known that multiple injections of OBTXA into human salivary glands causes gland atrophy to occur [3]. Previous studies in rat have reported decreased acinar cell size and a reduction in the number of nuclei proximal to the site of OTXA injection [64]. Other studies in rabbits have reported that apoptotic acinar cells recovered 12 weeks after OBTXA injection, thereby demonstrating the temporary effect of the treatment[19]. However, it is unknown whether similar histopathological changes in the salivary glands of patients may also occur after multiple injections of OBTXA into human salivary glands.

OBTXA has a temporary effect in patients, requiring repeated intraglandular injection treatments every 3-6 months[9, 44, 65]. The objectives of the current study is to determine (a) if there is a decrease in gland size subsequent to repetitive use of Onabotulinum toxin A for the treatment of sialorrhea in the pediatric population, and (b) if there are histologic changes in the salivary glands and particularly if there is a decrease in the number of acinar cells.

4.3 Materials and Methods

In this cohort study, we compared (i) the submandibular glands (SMG) of drooling children who received 4 or more intraglandular OBTXA injections prior to surgical removal of the glands with (ii) the SMG of drooling children who underwent surgery without any previous OBTXA treatments. This study received ethical approval from the Research Ethics Board of the

McGill University Health Center. Written informed consent was obtained from caregivers prior to study enrollment.

4.3.1 Inclusion/Exclusion Criteria

Retrospectively, patients were enrolled in the study using medical records information on the inclusion/exclusion criterion.

Inclusion criteria for the study included: Both exposed and non-exposed were patients with sialorrhea referred to a Saliva Management Clinic as described elsewhere[1]. Patients were between the ages of 4-18 years old. Exposed patients had received 4 or more OBTXA treatments prior to submandibular gland removal for salivary control. Non-exposed patients had not received any previous OBTXA injections prior to SMG removal. None of the salivary glands included had any malignant cells. All the OBTXA injections were done by the same Otolaryngologist, following a standardize protocol.

Exclusion criteria: patients undergoing SMG removal for reasons other than drooling. Patients with previous surgery of the salivary glands (duct ligation or other). Patients receiving pharmacotherapy for drooling.

4.3.2 Histopathological Analysis

After fixation in buffered 10% paraformaldehyde, salivary glands were processed for histological examination and stained with hematoxylin and eosin. These sections were examined and reviewed by light microscopy in a blinded fashion by the same pathologist to evaluate the presence or absence of fibrosis, inflammation, cellular/ductal damage, and apoptosis. The findings were compared between both treatment group and control.

Also, a comparison of the acinar cells was done in exposed and non-exposed patients. Slides were scanned onto Leica Biosystems®'s Aperio Pathology Imaging program. At a 17.8x zoom, cells were counted within a 200x200μm^2 quadrant of the slide. Ten 200x200μm^2 quadrants were counted per slide. The total acinar count was divided by the surface area of each quadrant (acinar count/ total count). The 10 quadrants in each slide were averaged to give one value per slide. Two counters conducted this measurement in a blinded fashion. A validation was performed by comparing both results and resolving differences.

4.3.3 Statistical Analysis

All measurements were reviewed and recorded quantitatively and qualitatively. The statistical analysis was conducted with the Statistical Package for Social Sciences (SPSS) for Windows Version 17.0 program. Results from both sample groups were tabulated in an excel spreadsheet and median values of cell counting were calculated. A Mann-Whitney Exact test was conducted using SPSS. $P< 0.05$ was considered statistically significant.

4.4 Results

Thirty-one (31) glands were analyzed; seventeen (17) glands from exposed patients who had received more than 4 OBTXA injections prior to SMG excision, and fourteen (14) glands from non-exposed patients who had received SMG excision for drooling without any previous OBTXA injections. Table 4.1 summarizes the characteristics of the two groups of patients.

A blinded histological comparison of tissue from exposed and non-exposed patients did not reveal any difference in the degree of fibrosis, inflammation, cellular ductal damage or apoptosis between the two groups of patients.

Two microscopic lobules were counted per histology slide. There was no statistical difference between the two counters ($P<0.05$). The results ranged from 295-492 cells. The median number of cells in the control group was 427 cells, and 370 cells in the case group, respectively.

The median number of acinar cells per unit area was 1.36 cells/μm in the glands of patients having undergone more than 4 OBTXA injections versus 1.35 cells/μm in the case group. There was no statistically difference between the two groups ($P=0.293>0.05$).

Table 4.1: Patient demographics

Variable	**N=15**
Age (years)	10.5(±4.10)
Gender	
Male	11 (69%)
Female	5 (31%)
Weight (kg)	29.5(±15.09)
Diagnosis	
Cerebral Palsy	7 (47%)
Epilepsy	3 (20%)
Global Developmental Delay	2 (13%)
Autism	1 (6.7%)
Mitochondrial Disorder	2 (13%)
OBTXA injections* **prior to surgery for experimental groups*	6(±3.83)
Average time of last injection before surgery (weeks)	17(±9.5)

Quantitative variables are presented in N (± St.Dev) and Categorical variables are presented in N (%).

4.5 Discussion

The purpose of this study was to determine if there were any changes in glandular histology after repetitive OBTXA injections in human submandibular glands. Patients who were injected with OBTXA injections were treated by the same physician, consistently using a standardized protocol [1]. To our knowledge, this is the first study in humans characterizing the histological effects of multiple salivary gland injections of OBTXA.

Acinar cells are the secretory cells of the salivary glands. The mechanism leading to decreased saliva production after OBTXA injection remains uncertain [21]. With regards to OBTXA, it is a potent protein that inhibits the secretory function of the acinar cells by blocking ACh release [9, 64]. It was hypothesized that the inhibitory effect of OBTXA on secretory acinar cells would be the result of apoptosis of these cells.

However, contrary to what has been observed in animal studies, we observed no significant histological change at the level of the salivary glands after repetitive OBTXA injections. We found no significant fibrosis, or apoptosis; nor were there a difference between the number of acinar cells in the glands of OBTXA patients and those of control patients. This suggests that there was down-regulation of salivary gland activity secondary to OBTXA treatments that lead to physiological down regulation, and that this occurred without any direct glandular damage. This could explain the observed decrease in salivation.

In previous animal models, studies have shown functional and structural changes of the acinar cells with OBTXA injections. In rats, there was a decrease in acinar cell size as measured by a decrease in the number of nuclei proximal to the site of OBTXA injection[64]. In rabbits, 12 weeks after 5 units of OBTXA injections in the SMG, there was a significant decrease in salivary flow and acinar cell size proximal to the site of injection as well as presence of fibrous tissue. Acinar and ductal cells demonstrated apoptosis 2 weeks after OBTXA injections, however the number of apoptotic cells decreased 4 weeks later and nearly fully recovered 12 weeks after OBTXA injections. These changes parallelled changes in salivary secretion, which could explain the temporary effects of the OBTXA treatment [19]. Other studies in rats, found atrophy of the gland post intraglandular OBTXA injections but no apoptotic cell were observed[64]. [64]OBTXA decreases neuronal nitric oxide synthase in submandibular glands, which may have an influence on the decrease in salivary secretion[66].

There are several management strategies for sialorrhea, and the treatment is usually tailored to the child's needs[1]. Treatments include oral-motor therapy and pharmacological medication. In severe sialorrhea, more permanent and aggressive treatments are considered such as surgical removal of the submandibular glands and a salivary duct ligation[9, 44]. Onabotulinum toxin A (OBTXA) injection is an effective treatment for sialorrhea.

4.5.1 Limitations of this study

One limitation of our study includes the small sample size. Due to the sample size, the study does not have the ability to represent the whole drooling population. Also the mechanism behind OBTXA on the salivary glands is still unclear. In order to strengthen our understanding of the histopathological effects of OBTXA on the salivary glands, additional pathological staining and investigations are recommended such as immunohistochemical (IHC) staining for Acetyl Choline receptors to demonstrate down regulation.

4.6 Conclusion

To conclude, in this small study looking at the histological effects of Onabotulinum Toxin A on the submandibular glands, we did not find any fibrosis, inflammation, cellular or ductal damage in the acinar cells. Along with those findings, there was no significant difference in acinar cell count with 4 or more OBTXA treatments in comparison to glands without the OBTXA treatment in the same drooling population.

4.7 Acknowledgements

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Ms. Franziska Richter, Medical Student from Friedrich-Willhelms Universität in Bonn, Germany for her contribution to data measurements.

4.8 Conflict of Interest

The authors report no conflicts of interest.

Chapter 5 Overall Discussion

5.1 Discussion

The objective of this thesis was to determine if permanent effects occurred in the submandibular glands of patients with sialorrhea after repetitive OBTXA injections. Based on the previous literature, we hypothesized that changes in the salivary glands would occur after repetitive OBTXA injections for drooling treatment.

The first study of this thesis characterized the physical effects of OBTXA on the salivary glands after repetitive injections using ultrasound measurement. Previous studies suggested that OBTXA might cause permanent morphological changes to the salivary glands. In our study, when the salivary glands were measured prior to treatment and compared after 3 OBTXA injections, the shape, echogenicity and size appeared to be altered. However, analysis of results showed that such changes were not statistically significant – presumably due to the limitations of our study, despite observations that clinical improvements in saliva function were present. An association between the change in gland shape and a decrease in frequency and severity of drooling seemed present when analyzing the drooling scales of the patients. This result is encouraging for clinical reference, by seeming to indicate that there are long-term positive effects of OBTXA within a drooling population. As OBTXA is minimally invasive, it is clinically advantageous to treat these patients without performing invasive procedures such as surgery. The change in echotexture of the glands was not statistically significant but observations suggested that there was a slightly more hypoechoiec texture of the salivary gland post-injections, which might explain a decrease in activity of the glands. The primary limitation of this study was its sample size. which was too small to permit any significant conclusions. Although there were no significant findings, the results are encouraging as a preliminary study, suggesting the value of additional studies using ultrasound observation during multiple OBTXA injection of salivary glands.

The second study of this thesis characterized the histopathological effects of OBTXA on salivary glands of humans. Previous studies have only been tested on animals. This was done retrospectively by observing the pathology slides of submandibular glands that were excised. The control group had their SMG removed without any prior OBTXA treatment, while the

treated group received at least 4 OBTXA injections prior to surgery. The acinar cells (with specified criteria) were counted. No significant differences (e.g., no significant differences in number of acinar sells) were found during repetitive OBTXA injections of submandibular glands. These results support the hypothesis that there is down-regulation of salivary gland activity secondary to OBTXA treatments. In turn, this suggests that while there have been physiological atrophy, there was no direct glandular damage that might caused a decrease in salivation. The apparent lack of histopathological changes is reassuring since this suggests that there was no permanent glandular damage due to the OBTXA treatment.

Clinical observations were documented using a validated questionnaire for salivation functionality [26, 39, 67] (Fig. 3.5). As has been previously reported [26, 68, 69], the questionnaire used in Chapter 3 has been used to document that OBTXA treatment reduces drooling. The same questionnaire was also used to show that drooling was reduced for many of the patients in the current study.

5.2 Future Directions

Previous studies have provided evidence of atrophy of the salivary glands with repetitive OBTXA injections in human subjects [2]. Our study used similar subjects, but did not reveal any significant morphological changes in the treated glands (Chapter 3). Nonetheless, morphological changes seemed to have been observed. Studies with longer follow-up times, and using more subjects, are required.

Previous animal studies have described the histopathological effects of OBTXA on the salivary glands [18, 66, 70, 71]. To our knowledge, the study in Chapter 4 is the first study to describe the histopathological effects of OBTXA on salivary glands of human subjects. However, these results suggest that histopathological changes were not associated with clinical reports of drooling reductions, since there was no significant difference in number of the acinar cells within the salivary glands of the patients treated.

Future studies should use other forms of histopathological staining, such as immuno-histochemical (IHC) staining for acetylcholine receptors, to clarify possible down-regulation mechanisms. Such studies would improve understanding of any permanent effects OBTXA on salivary glands. Associations between any histological analyses with ultrasound measurements, using the same specimens, would be of interest.

Chapter 6 Final conclusion and summary

The studies described in the thesis did not support our hypothesis with regards to physical and histopathological changes in the salivary glands after repetitive OBTXA injections. The preliminary ultrasound study did not show significant results nor did the histopathological study; this was possibly due to the limitations of the studies. Although the results were not significant, they are encouraging for future observations. The effects of OBTXA on salivary glands were not proven permanent in this study, but clinical observations have demonstrated improvements in the patients' and their families' lives, which is a motive to continue research in this field to progress with more permanent solutions and findings.

Chapter 7 List of Abbreviations

ACh- acetylcholine

AP- Anterior-Posterior dimension (ultrasound measurement)

ALS- Amyotrophic Lateral Sclerosis

CP- Cerebral Palsy

CG- caregiver of drooling patient

FD- Familial Dysautonomia

IPS- Idiopathic Paroxysmal Sialorrhea

ML- Medial-lateral dimension (ultrasound measurement)

OBTXA- Onabotulinum Toxin A

QOL- Quality of Life

SA- Surface area (ultrasound measurement)

SMG- Submandibular Gland

US- Ultrasound

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