

Histological Analysis of Rabbit Lacrimal Gland in Response to Intraglandular Botulinum Toxin A Therapy

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Abstract

Background: Although surgical intervention is the therapeutic mainstay for epiphora secondary to nasolacrimal obstruction, off-label injection of onabotulinumtoxinA (BTX-A) into the lacrimal gland has emerged as an effective, minimally invasive alternative. We investigated the structural and molecular changes within the lacrimal gland of the New Zealand White rabbit model, following intraglandular BTX-A injections.

Methods: Experimental animal study. In a volume of 0.1 ml, 0.625-, 1.25-, or 2.5-units of BTX-A were injected into separate lacrimal glands under direct visualization. Lacrimal gland samples were obtained via surgical biopsies at 2- and 4-week intervals. Negative (untreated) and positive control rabbits (treated with 0.1 ml of 0.9% sodium chloride) were established. Tissue analysis was performed via histopathology, immunohistochemistry, and electron microscopy.

Results: At 2 and 4 weeks, mild intraglandular inflammation was noted in both low- and high-dose BTX-A treated lacrimal glands. Increased collagen and mild fibrosis were noted surrounding the acini, suggestive of minor gland destruction and regeneration. Electron microscopy demonstrated no distinct intracellular changes. Immunohistochemistry revealed upregulation of ATPase expression levels in the ducts, as well as upregulation of acinar and ductal AQP4 and AQP5 levels.

Conclusions: Our preliminary data shows that BTX-A induces dose-dependent inflammation and structural remodeling within lacrimal gland tissue that may contribute to its clinical efficacy. The immunohistochemistry profile quantifies an increase in ductal ATPase levels and concomitant increase in ductal/acinar AQP4 and AQP5 expression, implying a possible change in glandular energy demands as well as an alteration of the aqueous flow patterns within the gland following injections with BTX-A.

Keywords: Botulinum Toxin; Epiphora; Lacrimal Gland

Introduction

Epiphora is a common ocular complaint that may contribute to blurred vision and can understandably be quite distressing to patients. It often results from nasolacrimal duct obstruction related to aging, infection, or trauma. Less frequently, it may occur in the setting of gustatory lacrimation (colloquially referred to as “crocodile tears”) secondary to aberrant reinnervation of the facial nerve [1]. This rare synkinesis typically arises after a seventh cranial nerve palsy or from injury to the facial nucleus itself. Consequently, patients suffer from hyperlacrimation with gustatory stimulation, such as when chewing or speaking.

Although surgical intervention in the form of balloon dacryocystoplasty (DCP), dacryocystorhinostomy (DCR), or conjunctivodacryocystorhinostomy (CDCR) remains the therapeutic mainstay for nasolacrimal duct obstruction, it may not adequately treat neurogenic hyperlacrimation. Furthermore, some patients may not be appropriate surgical candidates. For this subset of patients, less invasive alternatives, such as onabotulinumtoxinA (BOTOX, Allergan Inc., Irvine, CA) injections into the lacrimal gland, may be clinically valuable.

Despite its demonstrated clinical efficacy [2] however, lacrimal gland treatment for epiphora remains an off-label use of onabotulinumtoxinA (BTX-A). This discrepancy may exist in part due to an incomplete understanding of the underlying physiologic mechanisms of action. Recently, studies described a reduction in tear production with treatment of BTX-A to rabbit lacrimal gland, in addition to an upregulation of epidermal growth factor expression [3,4]. We attempt to further elucidate this mechanism in a dose- and time-dependent fashion through histological analysis, including immunohistochemistry (IHC) and electron microscopy (EM).

Methods

Animals: Studies were performed using 5 adult New Zealand White (NZW) male rabbits, each weighing 8 to 9 pounds. Rabbits were sedated with 1-ml intramuscular injection of ketamine (40 mg/mL) and xylazine (10 mg/mL). All animals were handled in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research and according to the Ochsner Institutional Animal Care and Use Committee (IACUC) approved protocol.

Intraglandular Injection of BTX-A: A limited superior-temporal transconjunctival orbitotomy was performed through an upper-forniceal incision in each rabbit eye, using a wire speculum and inferonasal globe rotation for adequate exposure. Careful blunt dissection was carried out in the superior orbital space to expose the lacrimal gland (Figure 1). Under direct visualization, intraglandular injections were performed with a 30-gauge needle. Topical moxifloxacin was instilled onto the ocular surface upon the conclusion of each biopsy.



Figure 1: Intra-operative photograph. Upper-forniceal, transconjunctival orbitotomy incision created, depicting exposure of lacrimal gland (in between curved tips of hemostat clamp).

In a volume of 0.1 ml of 0.9% sodium chloride solution, 0.625-, 1.25-, or 2.5-U BTX-A was injected directly into six distinct lacrimal glands (N = 2). A separate positive control rabbit was treated with 0.1 ml of 0.9% sodium chloride. At two and four weeks following treatment, the lacrimal glands were biopsied to obtain approximately 3-mm x 3-mm specimens from all rabbits, including an untreated, negative control rabbit. A total of five rabbits were utilized.

Lacrimal Gland Histology: The biopsy specimens were fixed in 10% formaldehyde and embedded in paraffin. Five-micron sections were stained with hematoxylin and eosin (H and E) and then observed under an optical microscope.

Immunohistochemistry: Immunohistochemistry staining was used to assess for localized changes in protein expression levels within the glandular acini, ductules, and myoepithelium. A standard protocol with internal controls was followed for the immunohistochemistry experiments. The antibodies used were goat anti-NKCC1 (N-16) polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), at a dilution of 1:75; goat anti-AQP4 (C-19) polyclonal antibody (Santa Cruz), dilution 1:400; goat anti-AQP5 (C-19) polyclonal antibody (Santa Cruz), dilution 1:200; mouse anti-ATPase (464.6) monoclonal antibody (Novus Biologicals, Littleton, CO), dilution 1:100; mouse anti-SMA (RTU, ready-to-use) monoclonal antibody (Leica Microsystems, Buffalo Grove, IL); mouse anti-CK5 (RTU) monoclonal antibody (Leica Microsystems). Specimens were stained using the Leica BOND-III machine (Leica Microsystems) and visualized using the Bond Polymer Refine Detection program (Leica Biosystems). Slides were examined with a laser scanning confocal microscope (LSM510; Carl Zeiss Meditec, Dublin, CA) and the images analyzed with image-editing software (PhotoShop; Adobe Systems, Mountain View, CA).

Electron Microscopy: The tissue samples were immediately placed in 3% glutaraldehyde (prepared in 0.1 M phosphate buffer, pH 7.4) for 24 hours at 4°C as a prefixation step. They were then rinsed twice with 0.1 M phosphate buffer (pH 7.4), postfixed using 1.5% osmium tetroxide for 1 hour at 10°C, and finally rinsed twice with 0.1 M phosphate buffer. Next, the specimens were dehydrated using graduated concentrations of ethyl alcohol (50%, 70%, and 95%) for 15 to 30 minutes each, followed by three changes of absolute alcohol for 30 minutes each. The tissues were infiltrated with Spurr’s epoxy and embedded into plastic capsules. Polymerization of the embedded tissue took place in a 75 to 90°C vacuum oven for approximately 24 hours. After polymerization, the epoxy tissue blocks were sectioned at 0.3 microns using an ultra-microtome. Sections were mounted onto copper grids. Grids were stained with uranyl acetate followed by lead citrate. The grids were allowed to dry at 45°C for approximately 30 minutes. Finally, each specimen (grid) was examined with a transmission electron microscope (CM10; Philips; San Francisco, CA). Several areas of each sample were systematically scanned.

Results

At two and four weeks, mild interstitial inflammation was noted in both the low- and high-dose BTX-A treated lacrimal glands, compared to the controls (Figure 2). Additionally, increased collagen and mild fibrosis were noted surrounding the acini, suggestive of minor gland destruction and regeneration.

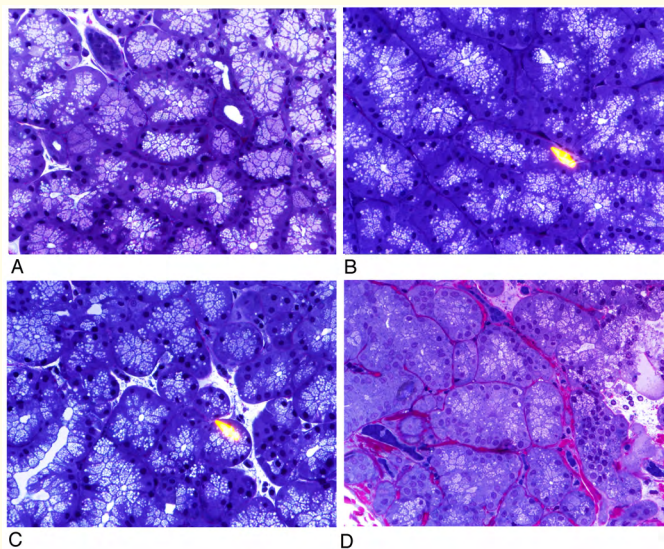


Figure 2: Plastic sections of rabbit lacrimal glands. (A) untreated control, (B) saline injection, (C) low-dose injection, and (D) high-dose injection. A) No inflammation and only a minimal scaffolding amount of connective tissue (staining red) surrounding the acini. B-C) Minimal amount of scaffolding connective tissue (arrows) surrounding the acini. In C, behind the arrow, are a few chronic inflammatory cells. D) Significantly increased amount of collagen between the acini, compared to A-C. More interstitial chronic inflammation than in C. (Toluidine blue and basic fuchsin, 400X).

At four weeks, EM demonstrated no distinct intracellular changes, but confirmed the presence of increased interacinar fibrosis, most notably within the high-dose treatment specimen (Figure 3).

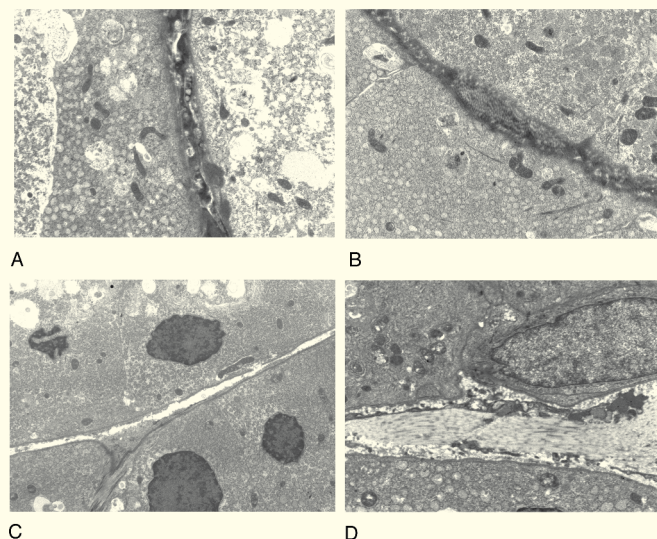


Figure 3: Electron microscopy. Untreated control (A), saline injection (B), and low-dose injection (C) demonstrate similar amounts of collagen between the acini. In the high-dose rabbit (D), the interacinar space is expanded by an increased amount of collagen. (Uranyl acetate and lead citrate, A, B and D, 5200X; C, 2950X).

IHC with intensity grading revealed an acute 3-4 fold upregulation of ATPase expression levels in the ducts within both low- and high-dose treatment specimens (Figure 4). It also showed a 2-3 fold upregulation of AQP4 and a 3-4 fold upregulation of AQP5 levels in both the acini and ducts. The changes in AQP4 and AQP5 expression were observed between two and four weeks following treatment and equivalently at both low- and high-dose treatments (Figure 5).

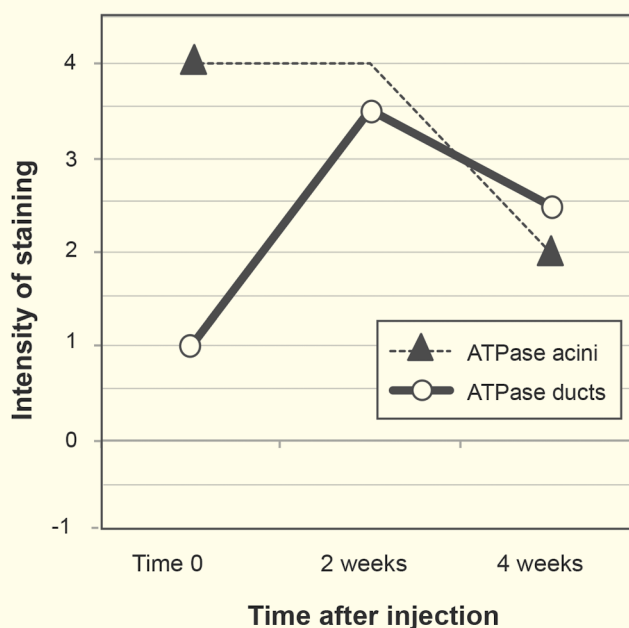


Figure 4: Immunohistochemistry. Within the first 2 weeks of intraglandular injection, both low- and high-dose treatment specimens demonstrated a 3- to 4-fold increase in ductal ATPase expression. (ATP = adenosine triphosphate).

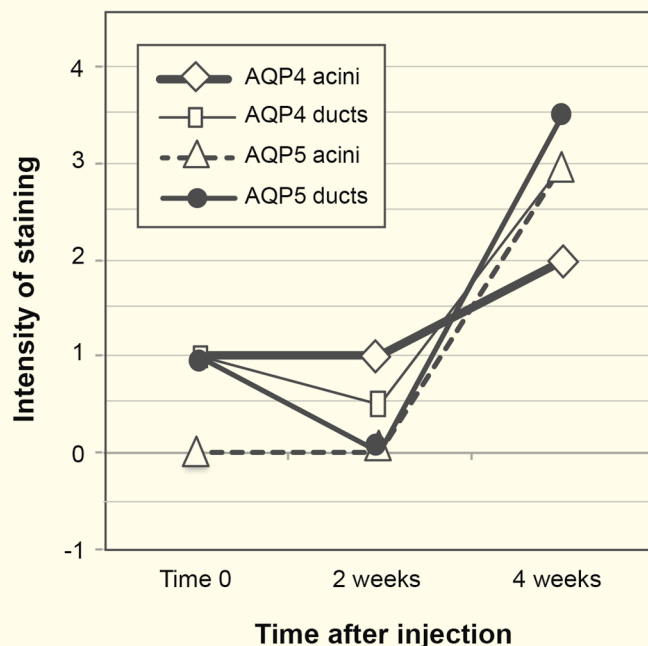


Figure 5: Immunohistochemistry. Between 2 and 4 weeks following low- and high-dose treatment, the glandular acini and ducts showed a 2-3 fold upregulation of AQP4 and a 3-4 fold upregulation of AQP5 levels. (AQP4 = aquaporin 4; AQP5 = aquaporin 5).

Discussion

The history of botulinum toxin dates back to 1895, when *Clostridium botulinum* was first identified as a lethal toxin-producing bacterium in the setting of food poisoning [5]. By the early 1900s, the toxin had been isolated and purified, resulting in the eventual discovery of seven different serotypes labeled A through G [6]. Its clinical applications have ranged from strabismus correction and treatment of facial dystonia to the modification of cosmetically unfavorable skin wrinkles. Pharmacologically, botulinum neurotoxin consists of polypeptide chains that become active upon bacterial lysis into heavy and light chains. Following internalization by cholinergic receptor-mediated endocytosis, the neurotoxin cleaves specific synaptosomal proteins and thus interferes with the presynaptic release of acetylcholine. Paresis of the innervated muscle or gland gradually reverses with collateral sprouting of new nerve terminals and restoration of the original functional endplate [7].

Past studies have highlighted the beneficial effects of BTX-A on gustatory sweating and skin erythema that occur in Frey’s syndrome and other autonomic disorders [8]. Frey’s syndrome is a condition characterized by excessive facial perspiration while eating. It typically develops from surgical trauma to the parotid gland and the adjacent sympathetic innervation to sweat glands, followed by a misdirected regeneration of parasympathetic salivary gland fibers to facial sweat glands. In 1994, Drobik and Laskawi demonstrated a profound reduction in Frey’s syndrome-related hyperhidrosis with intracutaneous injections of BTX-A to affected areas [9]. The effect persisted indefinitely in many patients with just one to two injections, theorized to be a result of sweat gland atrophy from chronic denervation.

Analogous to Frey’s syndrome, gustatory lacrimation arises from misdirected salivary gland parasympathetic nerve fibers innervating the lacrimal gland, following an insult to the facial nucleus or nerve. In the neurology literature, Boroojerdi *et al.* was among the first to

propose the use of BTX-A in treating hyperlacrimation associated with facial nerve palsy, specifically with injections to the lacrimal gland [10]. Patients enrolled in the study received 20 mouse units of BTX-A to the palpebral lobe of the lacrimal gland. All subjects experienced a moderate to complete recovery of hyperlacrimation, as determined by Schirmer testing. Furthermore, the authors noted an appreciable clinical improvement in hyperlacrimation even in those patients who were injected only in orbicularis oculi, an effect presumably attributed to diffusion of BTX-A.

Clinically, BTX-A injections to the lacrimal gland demonstrate great potential in the treatment of hyperlacrimation. Nava-Castañeda, *et al.* presented a statistically significant improvement in Schirmer test values in each patient with a history of gustatory epiphora that received a single 2.5-U BTX-A injection into the palpebral lobe of the lacrimal gland [11]. The effect lasted approximately six months. Only a minority of patients experienced mild transitory ptosis that soon resolved.

In the largest retrospective series to date, Dr. Wojno analyzed clinical outcomes in 46 patients treated with 2.5-U BTX-A injections to the lacrimal gland for symptomatic epiphora from multiple etiologies, including nasolacrimal obstruction and gustatory tearing [2]. The study reported a remarkably high patient satisfaction rate of 74%, i.e. those patients who felt that their epiphora significantly improved with BTX-A injections. The clinical effect lasted four months on average, and nearly 70% of patients returned for repeat injections.

The exact physiologic mechanisms of action for BTX-A on lacrimal gland tissue remain unclear. Demetriades, *et al.* described a reduction in tear production from injections of BTX-A into rabbit lacrimal gland [3]. Moreover, the authors reported maximal clinical benefit with 1.25-U BTX-A and suggested possible glandular saturation at higher doses. Histologically, no evidence of inflammation or hemorrhage was detected within glandular tissue at one week following treatment. More recently, Baek, *et al.* also demonstrated decreased tear volume and increased epidermal growth factor expression in rabbit lacrimal gland treated with 2.5-U BTX-A [4]. The tear volume was reduced by half within two weeks of treatment. The authors observed no histological or structural changes to the lacrimal gland itself.

Our preliminary histopathology data shows that BTX-A induces dose-dependent inflammation and structural remodeling within lacrimal gland tissue that may contribute to its clinical efficacy. This effect was observed most predominantly in the high-dose treatment specimens at four weeks from initial treatment, however, it was also evident at two weeks within the low-dose treatment groups. Notably, EM did not show any intracellular changes, but confirmed the presence of increased interacinar fibrosis. In contrast to the above studies, we observed histological inflammatory changes that were not a result of needle trauma (given the positive saline control glands) and seemed to occur in a dose-dependent fashion that was not previously analyzed in other studies.

Simultaneously, the IHC profile quantified a relatively acute increase in ductal ATPase levels and subsequent increase in ductal/acinar AQP4 and AQP5 expression, implying a possible change in glandular energy demands as well as an alteration of the aqueous flow patterns within the gland following injections with BTX-A.

Although not statistically significant due to low sample size, our data suggest a trend towards increased intraglandular tissue fibrosis and a concurrent rise in cellular ATPase and aquaporin channel expression levels. We theorize that BTX-A causes specific stress-induced responses within treated lacrimal gland, including mild glandular remodeling and compensatory modifications in aqueous flow. These collective findings indicate changes on a structural and molecular level beyond disruption of cholinergic neurotransmission, and likely contribute to the perceived effectiveness of BTX-A in the treatment of epiphora. Further histochemical analysis with an expanded sample set will be necessary to expound upon these initial findings.

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