THERAPEUTIC BOTULINUM TOXIN: HISTOLOGIC
EFFECTS AND DIFFUSION PROPERTIES

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INTRODUCTION

Since the introduction of botulinum toxin into therapeutic medicine in 1978,¹,² the use of this drug has been expanded to other indications including blepharospasm, adult onset spasmodic torticollis, spasmodic dysphonia, occupational hand disease and jaw dystonia. Application of this therapy to other disorders is on the horizon and is further contributing to the driving force for expansion of clinical and basic research. However, despite the success obtained with botulinum toxin for treatment of blepharospasm and other focal and segmental movement disorders, its application is limited by the following properties of the therapy:

1. Repeated injections are required indefinitely when treating chronic disease.
2. Untoward spread of toxin to other muscles not targeted for injection.
3. Antibody formation with resistance to the therapeutic action of the toxin subsequent to repeated injections.
4. The consistency of biologic activity contained within the labeled vials.
5. Lack of standardization of the injections sites for the treatment of each syndrome.
6. Placement of the therapeutic toxin preparation into the correct anatomic position when access to the muscles is difficult requiring teflon coated electromyographic assistance (particularly for treatment of occupational hand disorders)
7. Adequate understanding of long term effects of repeated treatment with the therapeutic preparation.

This chapter will address the clinical importance of diffusion model to the efficacious administration of this drug and histologic changes which occur with therapeutic injections. Issues of efficacy as well as safety can be addressed by the diffusion model. The rabbit longissimus dorsi muscle model provides a method to study the diffusion properties of intramuscular injections of therapeutic botulinum toxin.
Strabismus

Strabismus is an ophthalmic term which describes the pathologic misalignment of the eye. The term is very general and is inclusive of paralytic disorders of the extraocular muscles from muscle and cranial nerve diseases, congenital defects within the extraocular muscles, or muscle imbalances associated with optical errors, visual sensory deficits or disorders of the image fusion coordinating centers within the central nervous system. In the past, therapy has included spectacles lenses and surgical procedures used to tighten and loosen various muscles to achieve better alignment. Botulinum toxin has been advocated by Scott as an alternative to conventional extraocular muscle surgery because of the increased simplicity of the procedure avoiding a more complex surgical procedure. Unfortunately therapy offered by the toxin injection is temporary, and this injection needs to be repeated in a majority of cases to maintain ocular alignment. The ability of the clinician to exactly target the desired muscle is limited by botulinum toxin diffusion away from the site of injection. Treatment usually involves injection of the medial rectus or lateral rectus muscles (horizontal rectus muscles) to treat horizontal deviations of the eyes. Diffusion of botulinum toxin into the vertical rectus muscles resulting in the complications is common. Induced vertical deviation of the eye when horizontal deviations are being treated is clearly a limiting factor occurring in approximately 15% of cases.

Blepharospasm and Meige Syndrome

Over the course of the past 12 years the therapy has expanded to treatment of focal and segmental dystonias. These conditions involve involuntary contractions of a group of muscles in a region of body causing debilitating symptoms. Neurologic imaging and autopsy usually do not reveal structural lesions within the brain. There is general agreement that these conditions occur as a result of a disorder within the central nervous system. The chapter in this volume by Dr. Mitch Bin will more comprehensively review the clinical details of these syndromes.

The first dystonia botulinum toxin therapeutic technology had been applied was benign essential blepharospasm and Meige Disease. This form of neurologic blepharospasm is associated with blinding involuntary eyelid closure which leads to debilitation and desperation. Driving an automobile becomes impossible as the condition progresses and in worse cases situations, the patients become unable to independently ambulate. In the past, therapy had included the use of neuroleptic medications which were usually ineffective. Surgical therapy involving transecting the facial nerve or removing the orbicularis oculi in surgical stripping procedures was tried, but in many patients this approach is only partially effective and occasionally associated with undesirable complications.

A distinctive form of blepharospasm occurs with hemifacial spasm and aberrant regeneration of the seventh cranial nerve. In each of these conditions, there is an involuntary movement in muscles only on one side of the face. Hemifacial spasm is characterized by intermittent synchronous involuntary contractions in all muscles supplied by one facial nerve. This disorder is thought to result from damage to the intracranial portion of the facial nerve caused by tortuous blood vessels at the base of the brain. Aberrant regeneration of the facial nerve causes constant involuntary eyelid closure during active facial expressions.

Botulinum toxin has become the only consistent therapy for the treatment of neurologic forms of blepharospasm. The eyelids are injected with small quantities of botulinum toxin (15-75 IU) producing an effective weakening of the orbicular muscle of eyelid closure, the orbicularis oculi. This muscle must be injected every three months in the case of essential blepharospasm and Meige syndrome and every 5 months in blepharospasm associated with hemifacial spasm and aberrant regeneration of the seventh cranial nerve. As there were very
few alternative therapies for these conditions, the considerable degree of efficacy obtained for these diseases was a therapeutic advance.

As botulinum toxin treatment for this disease has proven to be effective,\textsuperscript{1,7,17,18} it has also been limited by several complications, including ptosis, exposure keratopathy, diplopia (double vision) and epiphora (tearing). Ptosis is defined as a drooping of the upper eyelid causing encroachment of the upper lid on the visual axis and effectively decreasing vision. This complication is generally transient and disappears as the denervative effect of the botulinum toxin wears off. It occurs approximately in 10\% of patients treated and is particularly prone to occur when the injections of botulinum toxin are made close to the superior sulcus (Figure 1). In that patients already have eyelid disease causing obstruction of vision, the ptosis further aggravates their visual function. Understanding the cause of this complication is vital to the effective application of the therapy. The occurrence of ptosis is thought to relate to diffusion of the toxin from injected orbicularis oculi into the superior orbit effectively weakening the retractor of the upper eyelid, the \textit{levator palpebrae superioris} muscle (Figure 2). Weakening of the muscular portion of the levator causes dropping of the upper lid margin. Given that the control of the upper eyelid movement is primarily a balance between the \textit{orbicularis muscle} and the \textit{levator palpebrae superioris} muscle, effective application of botulinum toxin for this condition involves containing biologic effect of the toxin only to the \textit{orbicularis} muscle, the protagonist muscle causing the abnormal movement. The anatomic configuration of the muscles of the upper eyelid allow selective weakening of the orbicularis muscle and therefore effective therapy with botulinum. The long tendon of the upper eyelid retractor, the \textit{levator aponeurosis}, extends along the undersurface of the orbicularis muscle into the tarsal plate. In that the muscular portions of the levator palpebrae superioris muscle is remote to the antagonist (orbicularis), there is little opportunity for the botulinum toxin to diffuse into the levator muscle of the eyelid. This anatomic distance between eyelid pretarsal eyelid orbicularis

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Ptosis complicating botulinum therapy. Ptosis is defined as drooping of the upper eyelids. The patient shown here suffered ptosis for a period of 3 weeks following botulinum toxin injection for the treatment of hemifacial spasm. The injection sites in this patient were close to the superior sulcus and specifically not close to the lash line along the lateral and medial extent. This complication results from diffusion of toxin into the orbit causing weakening of the lid retractor musculature.}
\end{figure}
Fig 2. Importance of eyelid anatomy to the beneficial clinical effect of injectable botulinum toxin. This photograph represents a surgical field demonstrating the levator palpebrae superioris muscle. Note that the upper lid retractor extends only as a tendon underneath the orbicularis muscle throughout the upper lid. The actual muscular portion of the upper lid retractor is deep in the orbit, distant from superficial upper lid structures. Such an anatomical arrangement allows botulinum toxin to specifically affect the antagonist of eyelid closure, the orbicularis muscle, which is targeted in the treatment of blepharospasm. The anatomic space between the upper lid retractor and the orbicularis muscle allows for a selective denervation of the orbicularis muscle without involvement of the antagonist levator muscle. Anatomic distances between muscles are important in the administration of botulinum toxin because its potential to diffuse.

(pretarsal orbicularis) and levator muscle provides an anatomic explanation for therapeutic success in selectively targeting the orbicularis in treating blepharospasm. This explanation also provides insight into the cause of this complication and a method to mitigate against its occurrence (Figure 3).

Another complication associated with therapeutic injections of botulinum toxin into the eyelids is diplopia. This occurs less commonly (<5% patients) and is transient, lasting several days to several weeks. Nelson and her co-workers have linked this complication to injections in the lower lid, particularly the inner aspect of the lower lid. The reason for this complication appears to relate to the anatomic proximity of the inferior oblique muscle to the inner portion of the lower lid. The inferior oblique muscle arises in the very anterior portions of the medial orbit and penetrates the major fascia of the lower lid (capsulopalpebral fascia) very close to the cutaneous surface of the medial lower lid. Injections into the inner aspects of the lower lid bring the toxin within several millimeters of this muscle so that the toxin can readily diffuse into this region resulting in this complication. Avoiding medial lower lid injections reduces the incidence of this complication.

With respect to the issue of efficacy in the treatment of blepharospasm, it appears that diffusion may again play a role. The location of injection sites used today was empirically derived and based on apparent efficacy as well as a strategy that limited complications. Because multiple injection points are needed, this has added to the discomfort of the application procedure. We initially studied the effects of injecting only electrically determined motor points of the orbicularis oculi. The motor points of orbicularis oculi are essentially in two
Figure 3. Anatomy and injection sites. The usual injection sites for the treatment of blepharospasm are outlined by cross marks. Note that upper lid injections should be close to the lash line and at the lateral and medial extreme positions. These anatomic positions maximize the distance between injected orbicularis and the antagonistic muscular portion of the levator palpebrae superioris. Maximizing this distance allows a more specific effect on the orbicularis muscle and avoids the complication of ptosis as seen in Figure 1. Also, note that the inner aspects of the lower lid are avoided (circle of arrows). Avoiding the area marked helps prevent diplopia which can result from diffusion of the biologic effects of the toxin into the inferior oblique muscle which is directly deep to this area of the lid.

locations. The first is in the upper outer portion of the eyelid and the second motor point is in the medial portion of the eyelid. The motor point is defined as the area within muscle which has the lowest threshold for contraction to external electrical stimulation. Generally the motor point corresponds to points of major motor nerve branch penetration into the muscle proper. Comparisons of single motor injection to multiple point injections were done on a series of ten patients using the multiple point injection strategy on one eye and the motor point injection strategy on the other, each group receiving the same dose. Eight of the 10 patients found that the multiple point injected eye had a substantially better effect than the motor point injected. Two of the 10 patients noted no difference between the two eyes. The results of this clinical study suggested several points:

1. Dose independent variables are involved in determining efficacy in botulinum toxin treatment.
2. Spreading the toxin throughout the muscle may have increased the toxin diffusion throughout most of its innervation zone.
3. The results tend to negate the prevailing opinion that motor points could provide a superior injection location.

In an effort to explore the innervation zone, that is, the topographic distribution neuromuscular junctions within a muscle, strips of orbicularis oculi muscle taken from the pretarsal portions of the muscle taken during routine ptosis surgery were analyzed for concentration and distribution of the neuromuscular junctions using a standard
acetylcholinesterase stain. Although only the pretarsal portions of the muscle were analyzed in this study, however, this portion of the muscle is exactly over the lateral motor point of the upper lid.

The results indicated that the neuromuscular junctions were diffusely distributed throughout the entire upper portion of the orbicularis muscle. This finding clearly correlates with the large degree of facial nerve projection and ramification into the orbicularis muscle from temporal, zygomatic and buccal branches. This observation led to the hypothesis that multiple injection points were preferable because this injection technique tended to cover more of the innervation zone of the orbicularis muscle as compared to a single large dose point injection. For orbicularis muscle, this concept appears to be a plausible explanation for the superiority of the multiple point injection technique. It appears that diffusing the toxin along the muscle proper was preferable in this particular anatomic location. This study also disproved the notion that motor point of the muscle corresponds to the innervation zone (areas of concentrated motor end plates).

ADULT ONSET SPASMODIC TORTICOLLIS

Adult onset spasmodic torticollis is a segmental dystonia involving cervical muscles. The condition can occasionally be associated with Meige Syndrome or other forms of head and neck movement disorders. A thickened protruding sternomastoid muscle is the most characteristic appearance of these patients on physical examination (Figure 4A). Torticollis has variable expression with the most common being a distorted posture with head rotated to a side of shoulder elevation (type 1, type 2). Another variation involves the head being tilted toward the side of shoulder elevation (type 3). Another variation includes the head being tilted backwards involuntarily (retrocollis, type 4a) or being flexed toward the chest (antecollis, type 4b). Patients often develop pain early in the course of the disease which often becomes progressive. The disease is often associated with involuntary jerking movements of varying frequency and amplitude. The patients in addition to pain, develop substantial stiffness and eventually obvious hypertrophied muscles.

This disease is chronic and often unremitting and progressive. Functional disabilities including driving, maintaining gainful employment often detract from quality of life. Often the patients develop depression and futile attitude towards relief because of the constant nature of the symptoms and prior experience with suboptimal therapy.

Past therapy has included the use of neuroleptic medications, use of various forms of myectomy and denervating surgery and occasionally biofeedback. Unfortunately previous medical therapies have not been satisfactory. Surgical procedures are often associated with inconsistent results and occasionally disfiguring scarring and further impairment in posture.

The implementation of botulinum toxin for the treatment of this segmental disorder has been a great contribution to neurologic medicine. The application has proven to be the most efficacious therapy for torticollis and can be maintained over a period of years.

The major complication associated with the use of botulinum toxin for spasmodic torticollis has been dysphagia. Dysphagia is defined as difficulty swallowing which can occasionally lead to the misdirection of food into the upper airway. Such a misdirection can occasionally cause complete upper airway obstruction which is a medical emergency possibly leading to death. Upper airway obstruction has occurred in at least one patient involved in the North American clinical studies. This patient was immediately treated by the Heimlich maneuver successfully. Other complications have included weakness within the cervical muscle.

The diffusion model has proven to be important in finding a solution to the dysphagia problem. In retrospective studies, dysphagia appeared to be linked to the dose of botulinum toxin dose injected to the sternomastoid muscle (see Figure 4B). This retrospective data
Figure 4. A. Dystonic sternomastoid muscle seen in adult onset spasmodic torticollis. Adult onset spasmodic torticollis is characterized by the presence of a large thickened inflamed muscle as seen in this patient. The involuntary impulses coming from the central nervous system causes increased resting tone, frequent spasmodic contractions which leads to hypertrophy of this muscle. B. Dysphagia and the treatment of torticollis. Dysphagia (difficulty swallowing) can result from diffusion of botulinum toxin into the pharyngeal musculature. This complication particularly occurs when high doses of botulinum toxin is given to the sternomastoid muscle which directly overlays the pharyngeal muscles. This complication occurs as a result of direct spread of biologic effect of from injected sternomastoid muscle into the deeper structures of the neck. Table 1 and Table 2 outline the results of the clinical study linking dysphagia to the dose of botulinum toxin given over the sternomastoid muscle.
Table 1. Comparison of botulinum toxin dose and injection strategies in patients who later experienced dysphagia and those who did not.

<table>
<thead>
<tr>
<th></th>
<th>Dysphagia</th>
<th>No dysphagia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sternocleidomastoid Dose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. Injection</td>
<td>7</td>
<td>42</td>
</tr>
<tr>
<td>Median dose</td>
<td>150</td>
<td>100</td>
</tr>
<tr>
<td>Interquartile Range</td>
<td>10</td>
<td>150</td>
</tr>
<tr>
<td>Wilcoxon test</td>
<td>Z=2.22</td>
<td>p=0.026</td>
</tr>
<tr>
<td><strong>Total Dose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. Injection</td>
<td>7</td>
<td>42</td>
</tr>
<tr>
<td>Median dose</td>
<td>160</td>
<td>150</td>
</tr>
<tr>
<td>Interquartile range</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Wilcoxon test</td>
<td>Z=0.75</td>
<td>p=0.45</td>
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</table>

Table 2. Influence of changing the injection protocol limiting the dose to the sternocleidomastoid muscle to 100 IU or less per injection: Prospective analysis.

<table>
<thead>
<tr>
<th></th>
<th>Dose not limited</th>
<th>Dose limited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysphagia</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>No Dysphagia</td>
<td>42</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>31</td>
</tr>
</tbody>
</table>

Numbers refer to injections. Fisher's exact test (1-tailed), p = 0.027.

indicated that doses in excess of 100 IU injected to the sternomastoid muscle was associated with the complication (see Table 1). Prospective data analysis limiting the dose to the sternomastoid muscle was shown to markedly decrease the incidence of this complication (see Table 2). The complication rate reported initially in our studies was comparable to those noted in other studies, approximately 15%. Limiting the sternomastoid dose to less than 100 IU during an injection session reduced the incidence of this complication to less than 2% (see Table 2). Plausible explanation for these findings was that diffusion of toxin from the sternomastoid into the periharyngeal musculature resulted in weakening the muscles involved in the swallowing reflex. The sternomastoid muscle lies directly over the periharyngeal musculature while the other muscles usually injected in the treatment of spasmodic torticollis (levator scapulae, posterior scalene, trapezius, splenius capitis, splenius cervicis as well as others) are more posterior located and remote to the pharyngeal muscles.

As it appears that dysphagia was secondary to toxin spread, that is, toxin spread and diffusion from the sternomastoid muscle, limiting this complication can be accomplished by reducing the dose over this muscle (see Figure 4B). Such an explanation for the results of these clinical studies suggests a dose dependent diffusion phenomenon over the sternomastoid muscle.

Efficacy in the treatment of adult onset spasmodic torticollis may also be dependent on diffusion of the biologic effects of the toxin from the injection sites. As this disease is definitely associated with multiple muscle group involvement in remote areas of the neck, injection of the entire complex of muscles involved with the posture disfigurement is necessary to achieve the most beneficial result.
Table 3. Response rates comparing multiple-single point injections.

<table>
<thead>
<tr>
<th></th>
<th>Single point</th>
<th>Multiple point</th>
<th>Mean dose of favorable response (IU)</th>
<th>Mean dose No response (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain</td>
<td>15/31</td>
<td>27/31*</td>
<td>165.71</td>
<td>147.7</td>
</tr>
<tr>
<td>Posture deformity</td>
<td>13/42</td>
<td>33/44*</td>
<td>162.7</td>
<td>148.3</td>
</tr>
<tr>
<td>Range of motion</td>
<td>15/39</td>
<td>33/44*</td>
<td>156.4</td>
<td>144.3</td>
</tr>
<tr>
<td>Activity</td>
<td>13/39</td>
<td>29/38*</td>
<td>187.5</td>
<td>145.6</td>
</tr>
<tr>
<td>Hypertrophy</td>
<td>27/39</td>
<td>34/44</td>
<td>161.4</td>
<td>154.6</td>
</tr>
<tr>
<td>Tremor</td>
<td>4/17</td>
<td>9/17</td>
<td>163.5</td>
<td>146.9</td>
</tr>
</tbody>
</table>

*p <0.002 by Chi square.

*not statistically significant (Wilcoxon).

Given the superior clinical results obtained with multiple point injections used to treat blepharospasm, a study group was designed to test multiple point versus single point injection per muscle for the treatment of torticollis patients. Using typical efficacy criteria for spasmotic torticollis, that is, the effectiveness with respect to pain, posture deformity, range of motion of the cervical spine, hypertrophy, activity limitation, there was clearly better clinical results achieved within the multiple point injection group (see Table 3, Figure 5). There was no significant differences in the total dose given to each of these groups. These findings suggested that the administration technique within individual large muscles was important to efficacy. It is unknown whether the innervation zone to these muscles are diffusely spread throughout the muscle proper rather than focally distributed. However, the results of the clinical study tend to suggest a diffuse innervation of most muscles involved with the syndrome.

A DEPICTION OF THE DIFFUSION DENERVATION FIELD IN A PATIENT

An excellent example of the denervation field produced by a point injection of botulinum toxin is the regional depression of the vertical furrowing lines produced by the contraction of the corrugator muscles. These lines are associated with aging. Another application demonstrating the denervation field is to directly inject the frontalis muscle in patients with expressionistic overcorrection after ptosis surgery by frontalis sling procedure. For instance, the patient shown in Figure 6 had undergone a frontalis sling to correct a total ptosis using a tendon graft taken from her leg. During the period of high amplitude facial movements naturally occurring during expression, the upper lid would become overcorrected. The contractility of her frontalis muscle was reduced on both sides for symmetry with point injections of botulinum toxin (see Figure 6). This resulted in the blunting of the transverse creases of the forehead over a circular area. The transverse creases are generally produced by the insertion of the frontalis muscle into the dermis of the forehead skin. With high amplitude contractions and high tone in the muscles, these creases are accentuated. The circular area of blunting of creases indicates decreased tone in the frontalis muscle over a defined region within this muscle. Of note, this patient was injected with 10 IU to each location and has produced a denervation field of approximately 1.5 mm radius.

The denervation field may be a phenomena that may vary within various muscle fiber arrangements, after repetitive injections and different preparations of the toxin. Further evaluations with respect to these variables are currently under study.
Figure 5A-D. Efficacy and injection technique in the treatment of spasmodic torticollis. In clinical studies, the method of administration is as important to the beneficial results as is the dose of botulinum administered. Figures 5A-D depict two types of injection strategies to large dystonic muscles along the anterior and posterior cervical muscles. The multiple point injection strategy per muscle produces a superior clinical benefit compared to single injection points per muscle or injection points just along a single area. The reason for these clinical may be that a multiple point injection strategy allows a more homogeneous diffusion of the biologic effect of the toxin along the targeted muscles (see Table 3).

HISTOLOGIC CHANGES AFTER BOTULINUM TOXIN INJECTION

The first apparent histologic effects appear to be clustering of vesicles at the presynaptic membrane indicating an impairment of release of acetylcholine. Shortly within 7 to 10 days of injection, collateral axonal sprouting appears to occur at the terminal axon or occasionally from the distal node of Ranvier. Within 10 to 14 days, the muscle fibers appear to undergo substantial atrophy. The atrophy continues to develop over a 4 to 6 week period. Figure 7 shows the fiber atrophy achieved in an albino rabbit longissimus dorsi 4 weeks after injection of 10 IU of botulinum toxin compared to a saline injected control specimen. The collateral axonal sprouts reestablish proximity to neuromuscular junction as demonstrated by Alldersen.

During a period of 3 to 4 weeks after injection, there is considerable spread of acetylcholinesterase staining activity on muscle fibers. This diffuse acetylcholinesterase staining characteristic persists until the 12th to 16th week after injection after which there is considerable

632
Figure 6. Depiction of denervation field in a patient. The forehead creases are generated by attachments to the frontalis muscle to the skin dermis. This patient was injected along the forehead. Note that the creases are blunted over a circular area after a point injection of botulinum toxin. The blunting of these creases indicates the field of effect of botulinum toxin in this clinical situation.

contraction into the neuromuscular junction. The fiber atrophy measured as fiber size diameter variability on cross sectional analysis is also a reversible phenomenon with recovery over a 4 to 6 month period. Spread of acetylcholinesterase on human muscle fibers 5 weeks after botulinum injection to the orbicularis oculi muscle is seen on Figure 8A. Figure 8B demonstrates the correlation between fiber size (diameter) variability and cholinesterase staining pattern 5 weeks after injection in albino rabbit muscle.

The cycle of histologic changes at the neuromuscular junction was first described by Duchen and co-workers. Similar spread of cholinesterase staining activity and collateral axonal sprouting has been demonstrated on human muscle fibers. Seventeen orbicularis oculi muscle specimens taken from patients during ptosis and myectomy surgery were evaluated for cholinesterase staining characteristics and fiber variability. It appears that cholinesterase staining characteristics generally started at weeks 3 to 4 and maintained a diffuse staining pattern through 3 to 4 months after injection. In all patients studied after 6 months, the cholinesterase staining pattern could not be distinguished from controlled specimens. It is also of note that fiber size variability appeared to correlate temporally with cholinesterase staining pattern. Fiber size variability appeared to be transient lasting 3 to 12 weeks after injection (see Figure 9). These temporal relationships between these histologic changes closely correlates with the duration of action achieved with therapeutic doses of botulinum toxin.

Another important goal in evaluating human muscles specimens after the injection of botulinum toxin is assessing long term effects. If human specimens were not injected within 6 months of biopsy, there appeared to be no difference in fiber morphometric analysis or cholinesterase staining pattern compared to the controls. As there was no substantial differences in fiber size variability, cholinesterase staining pattern compared to controls in patients who had received multiple injections over several years, it appears that chronic denervation does not occur with repetitive use of the toxin. However, other authors have
Figure 7 A,B. Muscle fiber response after botulinum toxin injection. Intense fiber atrophy occurs several
weeks after botulinum toxin is administered. B. This photograph demonstrates large degree of fiber atrophy
after 10 IU of botulinum toxin injected into the longissimus dorsi muscle of an albino rabbit back. A is a control
injected with saline.

demonstrated possible permanent changes within the myoneural junction such as increased
number of preterminal axon sprouts and projections into the myoneural junctions. Although
changes may be present at the neuromuscular junction with respect to sprouting, there was no
substantial residual atrophy after multiple injections of botulinum toxin. Such data appears to
indicate that the reneration process is nearly complete after the botulinum toxin is no longer
administered and there are no residual atrophic effects.
Figure 8. A. Acetylcholinesterase staining in human orbicularis muscle. Spread of acetylcholinesterase is seen on human muscle fibers 5 weeks after injection of botulinum toxin. This is a similar effect that was described in animal muscle fibers in the past. B. Correlation between fiber size variability and cholinesterase spreading characteristic. A direct correlation between fiber size variability and acetylcholinesterase spread characteristics on muscle biopsies evaluated from the animal study.

ATP-ase staining characteristics were also evaluated in animal muscle tissues injected with botulinum toxin. There was a slight increase in the proportion of type I fibers at a pH of 9.4. However, type I muscle fiber grouping appears greater after the injection and was the most significant finding with this stain (see Figure 10). The grouping of Type I fibers is also associated with some fiber atrophy and increased fiber size variability. These changes appear to be quite subtle compared to the massive spread of cholinesterase seen in animal tissues 3 to 5 weeks after injection.
Figure 10. ATP-ase stain at pH 9.4. The amount of Type I fiber grouping appears to be greater after injection of botulinum toxin compared to controls. However, the total number of Type I and Type II fibers did not seem to be substantially different over controls. This appearance is consistent with denervation.

Botulinum A toxin was obtained from Oculinum Incorporated (Oculinum R). It is prepared in a lyophilized form and stabilized with human serum albumin. It is reconstituted in sodium chloride 9% for injection and was diluted to 1 IU per .1 ml, 2.5 IU per .1 ml, 5 IU per .1 ml in 10 IU per .1 ml. These dilutions were chosen because these concentrations are often used in clinical practice. Control injections were made with 9% sodium chloride diluent.

After injection, five weeks were allowed to elapse in order to provide an adequate interval for optimum muscle fiber atrophy and acetylcholinesterase spread. After five weeks, the animals are sacrificed by lethal injection of Nembutol.

Dissection was taken over the dorsal spine removing the latissimus dorsi muscle and exposing the longissimus dorsi muscle down its entire length to the caudal end of the lumbar spine.

Biopsies were taken at 15mm intervals and processed with liquid nitrogen cooled isopentane -140°C (Figure 11A). Tissue specimens were subsequently stored at -80°C until stained using enzyme histochemistry for acetylcholinesterase and hematoxylin and eosin. Cut sections were made at 15 microns on the cryostat.

Histologic measurements were made with the bioquant II computer system for data collection and statistical analysis with fiber size variation comparisons were conducted using standard deviation and variance from measuring at least 200 fibers. Statistical variations in fiber diameter were compared with F ratio analysis.

The hematoxylin and eosin stained specimens were used to analyze fiber size and fiber size variability because of higher contrast obtained with this stain. Fiber diameter and diameter variance analysis were done in each specimen beginning at the injection site, and at 15mm intervals to 45mm from the injection point.

Intensity of acetylcholinesterase staining was estimated using reference photographs representing gradations of spread and intensity of staining (see Figure 11B–E). These gradations were rated 0-4. The spread and intensity of acetylcholinesterase staining activity for each biopsy was matched to the closest reference photograph. Four biopsies were developed from each
Figure 11. A. Animal model for quantization of diffusion. Animal model for evaluating diffusion of botulinum toxin down longissimus dorsi involves taking multiple biopsies along this muscle. The contralateral muscle is also used for assessment of extramuscular spread. Other contiguous muscles can also be used. B–E. Diffusion down the longissimus dorsi muscle can be monitored using the Acetyl-cholinesterase staining characteristic. Note that at the injection site after 1.25 IU of botulinum toxin, there is diffuse spread of cholinesterase. Over 45 mm, there is gradual decrease in cholinesterase spreading until the stain become concentrated only at the neuromuscular junctions. The photographs used in this illustration provided reference standards by which tissue at varying distances were used to evaluate diffusion of biologic effect at varying doses (see Figures 12, 16).
Muscle including controls and 2-3 animals were used for each dose determination in the histochemical analysis as well as fiber diameter variability and size analysis.

The muscle fiber average diameter was determined from summation of counts on four biopsies taken at 15 mm intervals over a linear distance of 4.5 cm from the injection site. The average muscle fiber diameter appeared to correlate to the dose of botulinum toxin administered (Figure 12). The average muscle fiber diameter after a 10 IU injection was 26.7 microns (s=14.8) (n=1600), average diameter at 5 IU was 31.7 microns (s=14.6) (n=1600), the average diameter at 2.5 IU was 30.4 microns (s=14.0) (n=1600), the average diameter at 1 IU was 30.7 microns (s=11.1) (n=2400). Control fiber diameter was 35.4 microns (s=9.2) (n=1600).

Fiber size variability also correlated directly with the dose administered at the injection site (Figure 13) and throughout the entire muscle.

The field of biologic activity within the injected muscle was assessed using fiber size variability and acetyl cholinesterase staining characteristic. The diffusion of biologic activity within the injected muscle correlated with dose administered. Fiber size variability at 1 IU became insignificant compared to controls at 15 mm (F ratio < 1.4 based on 200 fiber counts per specimen). At 2.5, 5, 10 IU the biologic effect reflected by fiber size variability was sustained throughout a 45 mm length along the muscle strip (see Figure 14). Fiber size variation was significantly different from controls at all higher doses down the entire muscle strip (F ratio > 1.4). Spread and intensity of acetylcholinesterase staining confirmed the biologic effect substantially diminished at 15 mm for the 1 IU dose. The acetylcholinesterase staining characteristic suggested higher doses (2.5-10 IU) produced a biologic effect throughout the 45 mm length of the muscle strip (see Figure 15).

In order to assess extramuscular diffusion properties of botulinum A toxin, fiber diameter variations and fiber diameter size were determined on the contralateral longissimus dorsi muscle at 45 mm from the injection site at each dose. Fiber size variation was significantly greater in the injected muscle at 45 mm than 45 mm at the extramuscular location for 10 IU (F=2.25, p<0.01), and 5 IU (F=1.7, p<0.01). For 2.5 IU and 1 IU the fiber variation comparing the intramuscular injection and the extramuscular injection was not significant. This data indicated that linear spread of biologic effect may be greater within the injected muscle than in a remote muscle at an equivalent distance from the point injection of botulinum toxin although the biologic effect did spread to contiguous muscles when larger doses were used.

![Fiber Atrophy after Varying Doses of Botulinum Toxin](image)

**Figure 12.** Dose-dependent muscle fiber responses. Fiber atrophy is seen after botulinum toxin injections and the muscle response appears to be dose related.
Figure 13. Dose-dependent muscle fiber responses. A, at injection point; B, for entire muscle. Fiber size variability correlated directly with the dose at the injection site, as well as the average fiber variability throughout the entire muscle.

The acetylcholinesterase staining characteristic and the fiber variability pattern confirmed the presence of a dose dependent field of action for botulinum A toxin.

As diffusion of toxin away from targeted muscles appears to cause complications ("toxin jump"),
24 therefore it is useful to attempt to quantify diffusion of biologic activity from a point injection at various doses used in clinical practice. The findings indicate that the degree of fiber atrophy, fiber size variability as well as intensity of acetylcholinesterase staining at the point of injection are directly related to dose administered. Furthermore, the diffusion of biologic effects from the point of injection within the longissimus dorsi muscle is dose dependent. Animals given 2.5 to 10 IU showed substantial diffusion of the toxin's biologic effects over a linear distance of 45 mm within this individual large muscle. In contrast, animals given 1 IU demonstrated a graduated biologic effect inversely related to the distance from the point injection. There appeared to be collapse of biologic effect using fiber size variation and acetylcholinesterase staining characteristics between 15-30 mm from the point injection of 1 IU within the injected muscle.
Figure 14. Dose-dependent diffusion, denervation gradient after botulinum A. The biologic effect within longissimus dorsi muscle appears to be dose related. The larger the dose the more homogeneous the effect is throughout the muscle strip evaluated. Fiber variations were compared using F-ratios.

Contiguous muscles were evaluated for biologic effect using primarily the longissimus dorsi muscle contralateral to the injection. At lower doses, the toxin did not disseminate to 45 mm on a muscle remote from the injected muscle. In a previous study, the biologic activity of botulinum toxin has been shown to cross fascial planes and cause histochemical changes within non-injected muscles within lesser distances (<2 cm.). The explanation for the extension of biologic effect at a greater distance within the injected longissimus muscle than extramuscular locations may be that the toxin’s activity diffuses to a greater degree within an individual muscle. Alternatively, the linear distance over which chemodenervation occurs may be exaggerated in muscles with very long parallel muscle fibers such as the longissimus

Figure 15. Dose-dependent diffusion, denervation field determined by cholinesterase stain. Acetylcholinesterase staining characteristic suggested that higher dose produced a biologic effect throughout the entire strip. Whereas, the smaller dose produced a gradient down the length of the strip studied.
dorsi. At higher doses there appeared to be dissemination of biologic activity down the contralateral muscle, although not to the same degree as the injected muscle.

In that containment of biologic activity within a targeted area of the body represents a desirable goal for botulinum toxin injection therapy, this scientific data offers insight into the intensity and diffusion of biologic activity within the injected muscle and at muscles remote from the injection site. Diffusion of biologic activity within the muscle appears to be a function of dose and can be graduated. The denervation field can be defined as a linear distance from the point injection over which botulinum toxin causes a denervation effect. The degree of denervation as indicated by fiber size atrophy and fiber size variation is at any distance from the point of injection a function of dose. The size of the denervation field is also a function of dose as indicated by the homogeneous effects the larger injection doses produced down the long muscle strip. As denervation field size and degree of neurogenic fiber atrophy are both dose dependent, larger doses of botulinum toxin can be expected to produce more weakness and fiber atrophy but with greater spread of the toxin from the injection sites (Figure 14). As complications in clinical practice are often related to undesirable toxin spread ("toxin jump"), the field of denervation must be considered in the clinical use of botulinum toxin.

As muscle atrophy has been noted to qualitatively occur after injection in the clinical setting on gross inspection, it is of interest to quantitate the degree of muscle fiber atrophy in this experimental model. Using cross sectional muscle fiber diameter changes after botulinum injection, it appears that a fiber atrophy of 25% was possible. This analysis is consistent with clinical observations in the sternomastoid muscle in patients treated for torticollis.

In that the diffusion of biologic activity away from a point injection is dose related and measurable, it may be possible to calculate reasonable diffusion fields away from a site of a given dose of botulinum toxin in clinical protocols. Diffusion fields can be established within injected muscles, within contiguous muscles, and within muscles of various fiber orientations. The results of this study underscore the importance of the clinician being knowledgeable of the anatomic distances between important muscles within the area being injected as well as the action of muscle groups in which the botulinum toxin is administered. Such information may provide a scientific approach to determining distances between injection sites at various doses. Furthermore, the minimum dose necessary to produce a homogeneous denervation effect down a long muscle would be ideal if just that muscle was being targeted for injection. Doses in excess of the minimum dose for homogeneous denervation would be more prone to spread outside the fascial planes of the muscle and into contiguous muscle groups potentially causing complications.

**SUMMARY**

1. Diffusion of therapeutic botulinum toxin from points of intramuscular injection appears to be a dose dependent phenomena.
2. Diffusion of the denervative effect outside the muscles targeted for injection is responsible for side effects associated with the clinical use of botulinum toxin ("toxin jump").
3. Limiting the dose of botulinum toxin in critical anatomic areas can be helpful in preventing complications (e.g. limiting the sternomastoid dose to prevent dysphagia in spasmodic torticollis patients).
4. Multiple point injections within targeted muscles have produced the most desirable clinical effects in patients with blepharospasm and adult onset spasmodic torticollis. This injection approach may be more beneficial because it diffuses the biologic effect of the toxin more evenly throughout the innervation zone of the muscle.
5. Muscle fiber size variability and spread of acetylcholinesterase on muscle fibers are consistent histologic markers for therapeutic botulinum toxin in effect. A dose dependent gradient
of biologic activity can be demonstrated both within muscles injected and within adjacent muscles.

6. Long term and repetitive therapeutic injections does not seem to produce permanent denervating effects using fiber size variation and cholinesterase staining characteristics in the evaluation of human muscle specimens.

REFERENCES


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