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Passive Length-tensile Properties of Extraocular Muscles Under Botulinum Toxin Type C

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Purpose: Passive length-tension of the extraocular muscle was measured after injection of botulinum neurotoxin type C (BoNT-C) to evaluate its chemo-denervation effect.

Methods: BoNT-C was injected unilaterally at 5.0 or 2.5 units in the superior rectus muscle of the albino rabbit. The muscle was separated several times at its original insertion between 3 days to 8 weeks after injection, and the passive length-tension produced by stretching the muscle from its physiologic length was measured. The length-tension curve was analyzed, and the passive load was determined from early components. The compliance was determined by approximating the entire ascending curve by an exponential function.

Results: In the 5.0-unit group, the passive load increased significantly 2 weeks after injection but decreased to the level of the control group after 4 weeks and remained at that level until after 8 weeks after injection. In the 2.5-unit group, also, there were changes similar to those in the 5.0-unit group, but the changes were not significant compared to the control group. No significant change was observed in the compliance in either group.

Conclusions: Persistent chemo-denervation effect was not observed in passive length-tension at the doses of BoNT-C used in this study. The increase in the passive load soon after injection was suggested to have been caused by the same mechanism as BoNT-A. **Jpn J Ophthalmol 2003;47:145–150**

Key Words: Botulinum toxin type C, compliance, extraocular muscle, passive length-tension curve, passive load.

Introduction

Clostridium botulinum neurotoxins (BoNTs) are proteases that cleave and decompose the group of specific proteins involved in the docking and fusion of acetylcholine vesicles to the presynaptic membrane by acting on the neuromuscular junction and, thus, inhibit neuro-exocytosis[1,2] and, because of these actions, are considered to cause the flaccid paralysis of muscles. BoNTs are used for the treatment of paralytic and nonparalytic strabismus,[3] hemifacial spasm, and spasmodic torticollis due to this chemo-denervation effect. BoNTs are classified into types A to G depending on the differences in their antigenicity. BoNT type A (BoNT-A) decomposes only SNAP-25, a member of the above group of proteins, but BoNT type C (BoNT-C) decomposes SNAP-25 and syntaxin.[4] The difference in the action mechanism between the two types is considered to be related to the characteristics in their chemo-denervation effects on the neuromuscular junction. Although the use of BoNT-A for the treatment of strabismus is not permitted in Japan,[5,6] it is in wide clinical use abroad. However, it has problems, such as the necessity of multiple administrations due to nerve sprouting[7–9] and antibody production due to its large molecular weight.[1,2]

BoNT-C has stronger neurotoxicity than BoNT-A. Kurokawa et al[10] reported swelling and loss of axons, degeneration of mitochondria of synaptic terminals, and appearance of membranous dense bodies and vesicles in primary neuron cultures of mouse embryos administered 4 μL of BoNT-C (concentration: $\geq 4 \times 10^2$ units) but no histological change in those administered BoNT-A or BoNT type E (BoNT-E)

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at the same concentration. Williamson et al[4] also reported degenerative changes similar to those described by Kurokawa et al in the mouse embryonic spinal cords treated with BoNT-C at 1×10^7 LD_{50}/mL for 5 days but no histological change in those treated with BoNT-A at 5×10^7 LD_{50}/mL. BoNT-C is, thus, considered to delay the recovery of synaptic functions by suppressing the outgrowth of axons and formation of synapses and inhibiting nerve sprouting at the neuromuscular junction. Therefore, BoNT-C may have a more durable chemo-denervation effect on skeletal muscles than BoNT-A,[11] and its characteristic actions are expected to be of clinical use. We, therefore, evaluated the effect of BoNT-C on the extraocular muscle by serially measuring passive length-tension after BoNT-C injection into the extraocular muscle, which was separated at its original insertion at each examination time, as described by Simonsz et al.[12]

Materials and Methods

Animals

For these experiments, adult Japanese albino rabbits weighing 2.4–3.9 kg were used.

Injection of BoNT-C

After a small incision in the conjunctiva, 5.0 or 2.5 units of BoNT-C diluted in 0.1 mL of 0.02 M-phosphate buffer was injected into the superior rectus muscle (SR) unilaterally using a 30-gauge needle; then the conjunctival flap was sutured tightly with 8-0 silk. In the same manner, 0.1 mL of normal saline solution was injected into the opposite eye as a control. All rabbits developed blepharoptosis in the toxin-injected eye within 24 hours, which persisted for 2 to 4 weeks.

Measurement Procedure of Length-tension Curves

At 3 days and 1, 2, 4 and 8 weeks after the injection, rabbits were anesthetized with intravenous sodium pentobarbital (20 mg/kg) and the head and limbs were fixed in a prone position. After a fornix-based conjunctival flap procedure, and cautious exposure of the SR with a double-armed suture with 4-0 silk at the tip of the muscle, tenotomy was performed at the original insertion, bilaterally. A silk thread (length: 3.0 cm) was attached to the original length-tension strain gauge via a glass fiber twist

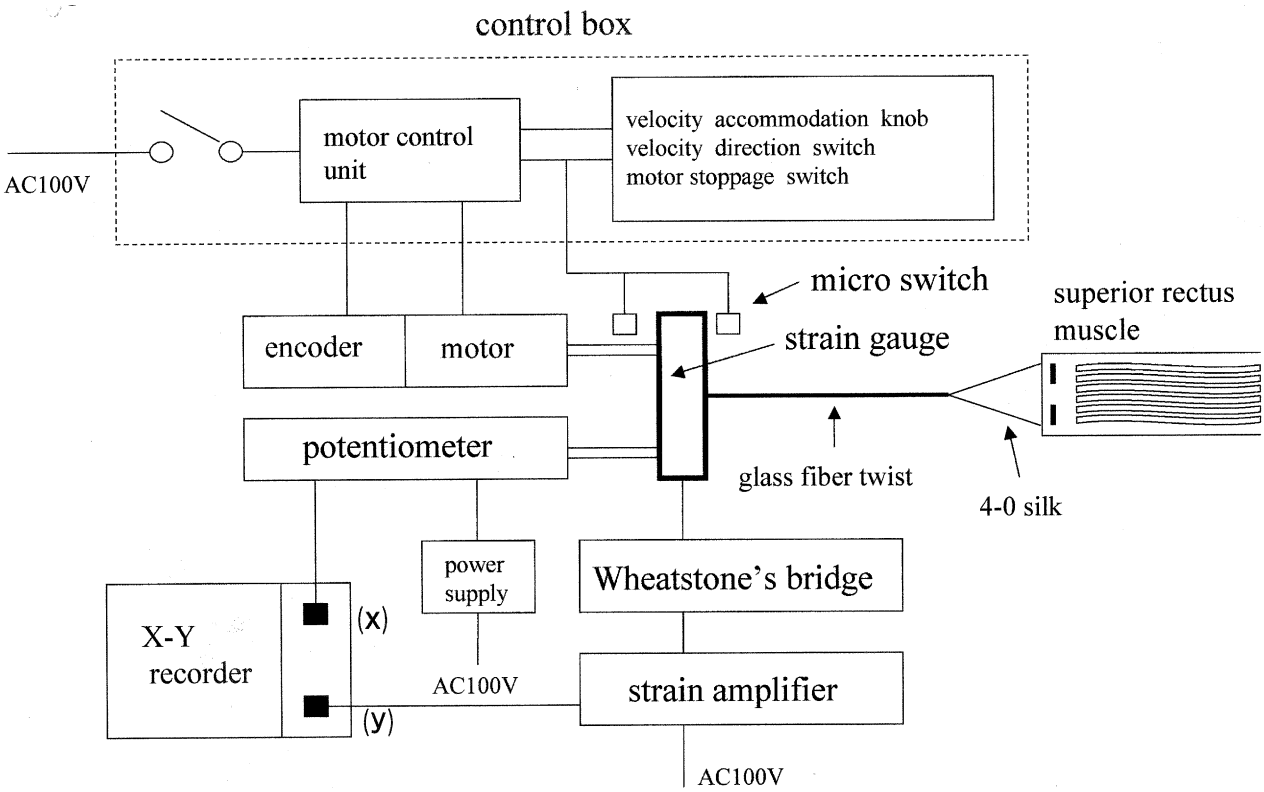

Figure 1. Schema showing the set-up of the extraocular muscle tensile force measuring apparatuses.

(length: 27.0 cm). The torque generated by a motor was slowly (0.2 mm/s) increased and decreased, exerting a tension that ranged between 0 g and 40 g. The trace of the length-tension curve was recorded two times to estimate the repeatability.

Measurement Apparatuses

The system consists of a measuring unit, a motor control box (HS-230-05; Harmonic Drive Systems, Tokyo), a fixation device for the muscle specimen, and a 2-channel X-Y recorder (WX 1000; Graphtec, Osaka) (Figure 1).[13,14] The rotation of the gear built into the DC servomotor (RH-5 5002; Harmonic Drive Systems), causes a thin plate of phosphorus bronze attached to the tip of the unit to shift forward and backward. This tugs the detached extraocular muscle via the glass fiber twist. Two pairs of precision strain gauges are also installed on the strain gauge plate and the tension generated can be detected accurately. The length of the extended muscle is inputted to the X-recorder through a potentiometer connected to the metallic plate. The digital signal from the strain gauges is amplified (Strain Amplifier, DPM611A; Kyowa Electronic Instruments, Chofu) and converted into an analogue signal, and fed into a Y-recorder. This method for the measurement of detached muscles has the advantage of excluding the influence of connective tissues around the muscle as much as possible.

Analysis of the Recorded Measurements

All length-tension diagrams consisted of an upward curve (the pulling phase) and a downward curve (the return phase) (Figure 2). The data of the length-tension curve were processed by a personal computer to determine the coordinates and calculate the regression curve. Using the characteristic upward curve we determined the following two data.

Passive load. The early phase of the upward curve between 0 g and 5 g was approximated to be linear, so we regarded the property as elastic tissue according to Hooke's law and showed the following simple equation, $Y = k * X$. We defined the constant k (spring constants), expressed in unit gram per mm, as the passive load.

Compliance. The whole diagram of the pulling phase was regressed into an exponential equation,[12] which would read: *Tension* (g) = a * {exp (b * *length change*$_{(mm)}$) −1}. We considered b a constant factor, which characterized the whole diagram, so we defined the reciprocal, that is $1/b$, expressed in unit mm per g, as the compliance.

Statistical Analysis

The data was analyzed statistically by analysis of variance and the Mann–Whitney U-test to evaluate the difference in results between the BoNT-C injected eyes and control eyes. A P-value of .05 or less was considered significant. All experiments were

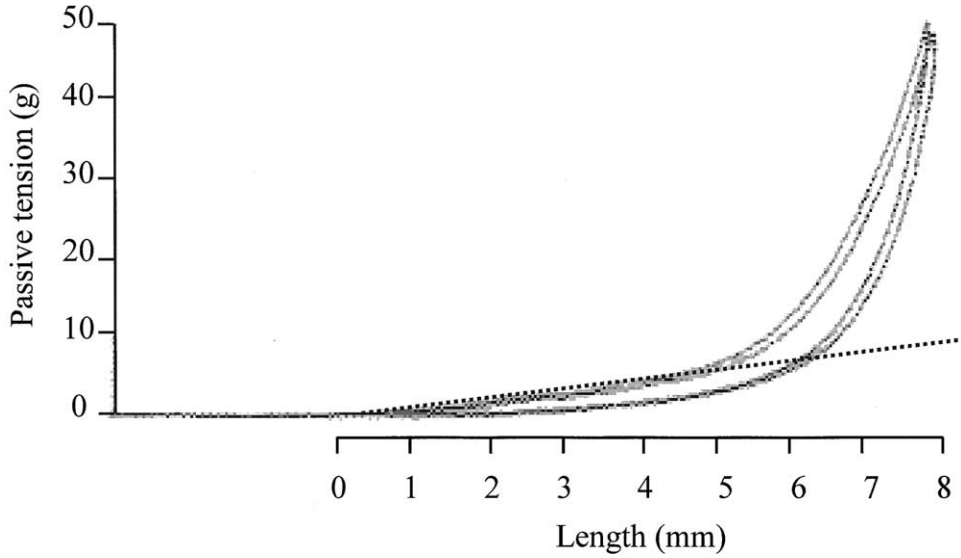

Figure 2. Stretched amount of superior rectus muscle and passive length-tension curve. It was possible to approximate the early phase (from 0 to 5 g of passive tension) of the ascending curve with linear function [$Y = k * X$. k: passive load (g/mm)].

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carried out in accordance with the ARVO statement on the use of animals in ophthalmic and vision research.

Results

Passive Load

The measurements of the passive load in the 5.0 units of BoNT-C injected group were 0.82 ± 0.12 g/mm (mean ± SD) at 3 days after the administration (n = 4); 0.77 ± 0.11 g/mm at 1 week after the administration (n = 5); 1.22 ± 0.22 g/mm at 2 weeks after the administration (n = 5); 0.94 ± 0.36 g/mm at 4 weeks after the administration (n = 6); and 0.92 ± 0.39 g/mm at 8 weeks after the administration (n = 5) (Figure 3). The measurements of the passive load in the 2.5 units of BoNT-C injected group were 0.92 ± 0.17 g/mm at 3 days after the administration (n = 4); 0.85 ± 0.37 g/mm at 1 week after the administration (n = 6); 1.26 ± 0.64 g/mm at 2 weeks after the administration (n = 5); 1.00 ± 0.32 g/mm at 4 weeks after the administration (n = 4); and 0.96 ± 0.29 g/mm at 8 weeks after the administration (n = 5) (Figure 4). In the 5.0-unit group, the mean passive load level showed a tendency to decrease in comparison with that in the control group at 1 week after the administration, to increase significantly at 2 weeks after the administration, and to return to the control values after 4 weeks (Mann–Whitney *U*-test; at 2 weeks after the administration, $P = .0472$). In the 2.5-unit group, the mean passive load level showed a tendency similar to that in the 5.0-unit group, but there were no significant changes in comparison with those of the control group through all time points.

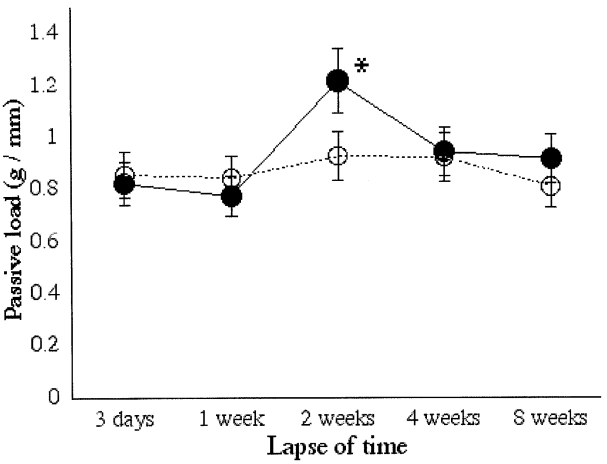

Figure 3. The mean passive load after 5.0 units of botulinum neurotoxin type C (BoNT-C) injection. • 5.0 units of BoNT-C group, ○ control group. Error bars are ±SD. Passive load increased significantly on postoperative week 2. *Mann–Whitney *U*-test, $P = .0472$.

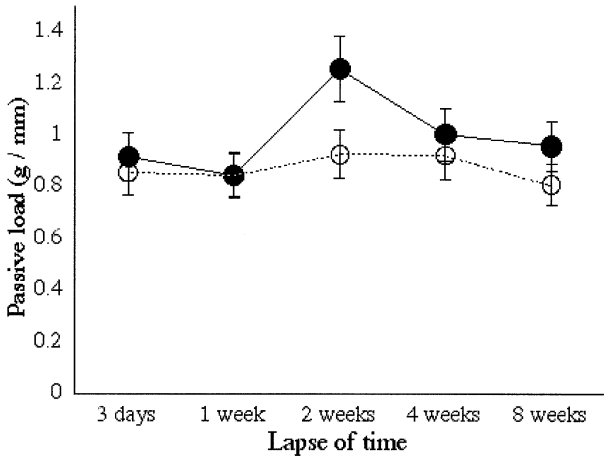

Figure 4. The mean passive load after 2.5 units of botulinum neurotoxin type C (BoNT-C) injection. • 2.5 units of BoNT-C group, ○ control group. Error bars are ±SD.

Compliance

The measurements of compliance in the group receiving 5.0 units of BoNT-C injected was 1.66 ± 0.22 g/mm (mean ± SD) at 3 days after the administration (n = 4); 1.96 ± 0.25 g/mm at 1 week after the administration (n = 5); 1.92 ± 0.32 g/mm at 2 weeks after the administration (n = 5); 1.70 ± 0.49 g/mm at 4 weeks after the administration (n = 6); and 1.62 ± 0.09 g/mm at 8 weeks after the administration (n = 5) (Figure 5). The measurements of compliance in the group receiving 2.5 units of BoNT-C was 1.90 ± 0.49 g/mm (mean ± SD) at 3 days after the administration (n = 4); 1.73 ± 0.38 g/mm at 1 week after the administration (n = 6); 2.02 ± 1.01 g/mm at 2 weeks after the administration (n = 5); 1.89 ± 0.54 g/mm at 4 weeks after the administration (n = 4); and 1.96 ± 0.43 g/mm at 8 weeks after the administration (n = 5) (Figure 6). During follow-up, there were no significant differences in the mean compliance level in either group, compared with that in the control group.

Discussion

Passive length-tension of the extraocular muscle of the albino rabbit was measured after BoNT-C injection. The passive load increased ($P < .05$ in the 5-unit group; the increase was not significant compared to the control group in the 2.5-unit group) 2 weeks after the administration, and returned to a level similar to the control group after 4 weeks. Okano[14] also reported

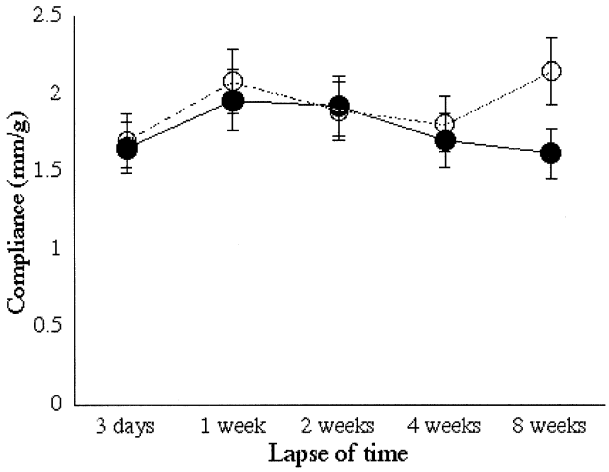

Figure 5. The mean compliance after 5.0 units of botulinum neurotoxin type C (BoNT-C) injection. • 5.0 units of BoNT-C group, ○ control group. Error bars are ±SD.

similar time-related changes in the passive load in an experiment using BoNT-A injection. When he injected 5 units of BoNT-A at 0.1 mL into the superior rectus muscle of albino rabbits, the passive load decreased after 1 week, increased after 2–3 weeks, and became comparable to the control level after 4 weeks. Okano quoted the Spencer thesis,[15] and speculated about these results. Spencer et al,[15] who reported a 24.75% thickening of singly innervated orbital muscle fibers, a 61.35% decrease in the vascular density, and a 77.98% decrease in the vascular lumen compared with normal fibers 2 weeks after injection of 10 units of BoNT-A (Oculinum) into the monkey medial rectus muscle. They also reported that mitochondria were swollen and displaced to peripheries. Okano speculated that the increase in the passive load at 2–3 weeks after administration was due to the influence of these histological changes caused by BoNT-A. From these results, BoNT-C is also considered to have caused histological changes similar to BoNT-A. The increase in the passive load 2 weeks after BoNT-C injection appears to have been caused as follows: As neuromuscular transmission is blocked by the toxin, the oxygen consumption and enzyme activity of the oxidization of muscle fibers are reduced. This causes a decrease in muscle fiber activity and associated shrinking of the capillary network related to the muscle fibers, inducing ischemia and consequent swelling of the tissue.

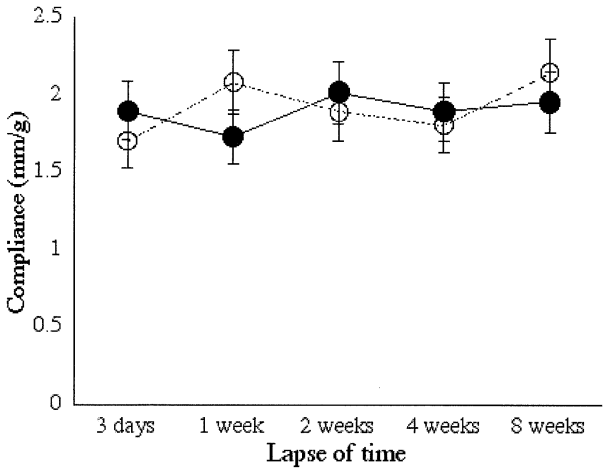

Figure 6. The mean compliance after 2.5 units of botulinum neurotoxin type C (BoNT-C) injection. • 2.5 units of BoNT-C group; ○ control group. Error bars are ±SD.

The ascending curve obtained by slowly stretching the skeletal muscle from its physiologic length can be approximated by an exponential function using the nonlinearity optimization method. The compliance (mm/g), one of its parameters, appears to be an index that represents characteristics of the entire ascending curve of the extraocular muscle tone from the viewpoint of soft tissue rheology. In this study, no serial change was observed in the compliance after injection of type C toxin at 5 or 2.5 units. These results suggest that BoNT-C clearly affects the passive load, which is an early component of the passive length-tension of the extraocular muscle, but that its effect is transient, and that it does not cause sustained changes in passive length-tension. These findings may be explained by differences in the sensitivity to botulinum neurotoxins among species[2] or the concentration of the toxin administration.[4,10] The degree and duration of the chemo-denervation effect of the toxin may also vary in the same species according to the type of muscle fibers, such as the orbital layer and global layer.[16–18]

In this study, BoNT-C was administered at 5 or 2.5 units, which are clinical doses of BoNT-A for ophthalmologic treatment. However, these doses may need reevaluation because the changes in the early component of passive length-tension were transient. Also, the length-tension curve obtained in this study represents the total tension of all muscle fibers of the extraocular muscle, and whether it accurately reflects the tension of singly innervated orbital muscle fibers, which are considered to be a primary target of chemo-denervation by botulinum toxin, remains unclear. Further evaluation is needed of the appropriate concentration of BoNT-C for maintaining its neurotoxic effect and to determine whether the effect of the toxin is attenuated by neutralization due to antibody production on repeated administration.

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Moreover, it is also necessary to consider the creation of a system for the evaluation of the effects of BoNT-C on the active force of the extraocular muscle, the clarification of differences in the effect of the toxin among muscle fiber types such as twitch fibers and tonic fibers for future quantification of the corrective effect of the toxin, and the evaluation of how such differences affect the ocular position and ocular movements.

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