ABSTRACT: Direct intramuscular injection of doxorubicin results in permanent myofiber loss. A previous phase I trial demonstrated that such injections could successfully treat blepharospasm and hemifacial spasm. Our previous in vitro study demonstrated that doxorubicin resulted in a dose-dependent reduction in isometric force generation in sternocleidomastoid muscle in rabbits. This present study examined alterations in force generation in these treated muscles in situ, i.e., with the blood and nerve supply intact. Two months after a single doxorubicin injection, functional changes in peak twitch, tetanic force generation, and fatigue rate were assessed in control and doxorubicin-treated sternocleidomastoid muscles in rabbits. Peak force measurements were reduced in the treated muscles. These reductions in muscle strength were significantly greater at tetanic peak amplitudes. Fatigue rate was not altered by doxorubicin treatment of the sternocleidomastoid muscles. These findings support the potential clinical use of doxorubicin chemomyectomy for the treatment of patients with cervical dystonia.


MUSCLE STRENGTH FOLLOWING DIRECT INJECTION OF DOXORUBICIN INTO RABBIT STERNOCLEIDOMASTOID MUSCLE IN SITU

JON H. FALKENBERG, PhD, 1 PAUL A. IAIZZO, PhD, 1,2 and LINDA K. McLOON, PhD 3,4

1 Department of Physiology, University of Minnesota, Minneapolis, Minnesota, USA
2 Department of Anesthesiology, University of Minnesota, Minneapolis, Minnesota, USA
3 Department of Ophthalmology, University of Minnesota, Room 374 LRB, 2001 6th Street SE, Minneapolis, Minnesota 55455, USA
4 Department of Neuroscience, University of Minnesota, Minneapolis, Minnesota, USA

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Cervical dystonia is difficult to treat due to the number and size of the muscles involved. Currently, injection of botulinum toxin A into the involved muscles is the preferred treatment. 8 This pharmacologic treatment is a safer alternative to the surgical treatments used previously. Although botulinum toxin A is extremely helpful in the initial treatment of most patients with cervical dystonia, the effects are temporary, and patients require reinjection of toxin every 3–4 months. The neck muscles are larger in size than the facial muscles, and this size difference requires higher doses of botulinum toxin to treat cervical dystonia compared with blepharospasm. Higher doses of botulinum toxin can result in muscle abnormalities distant from the injection site, including jitter 17 and atrophy 1 in muscles of the extremities. Although not clinically significant, they are still of concern. Patients can also produce antibodies to the botulinum toxin in response to higher doses, rendering the toxin ineffective. 2 Thus, despite the positive results that can be obtained with botulinum toxin treatment, a more permanent nonsurgical treatment of cervical dystonia is desirable.

A recently developed treatment for blepharospasm and hemifacial spasm involves direct injection of doxorubicin into the involved muscles. This results in permanent reduction of uncontrolled muscle spasms. 20,21 Doxorubicin is a myotoxin that results in permanent myofiber loss in both animals 12,13 and patients 13,21 when injected directly into a muscle. Results with blepharospasm suggest that even if full elimination of spasms is not achieved, the myofiber loss results in a substantial increase in the interval between requests for botulinum toxin injection by these patients. 21 We have been investigating the use of doxorubicin injection for the treatment of cervical dystonia.

Abbreviations: EMG, electromyography; PBS, phosphate-buffered saline
Key words: cervical muscles; doxorubicin; force assessment; myotoxin; skeletal muscle
Correspondence to: L. McLoon; e-mail: mcloo001@umn.edu

Our previous in vitro study demonstrated that injection of doxorubicin into rabbit sternocleidomastoid muscle results in a significant reduction in both muscle mass and muscle force in a dose-dependent manner. Muscles treated with doxorubicin showed alteration in twitch and tetanic force. Hence, doxorubicin may be useful for clinical treatment of patients with cervical dystonia. The previous in vitro study examined the muscles in the absence of normal nerve and blood supply. To better understand the effect of doxorubicin on muscle in a more normal physiological state, the effect of doxorubicin on sternocleidomastoid muscle has been examined in situ.

The effect of doxorubicin treatment on the sory nerve in situ. Twitch and tetanic force was measured by direct stimulation of the spinoaccessory nerve in situ. Muscle contraction was generated by direct stimulation of the spinoaccessory nerve in situ. Hence, doxorubicin may be useful for clinical treatment of patients with cervical dystonia. The previous in vitro study examined the muscles in the absence of normal nerve and blood supply. To better understand the effect of doxorubicin on muscle in a more normal physiological state, the effect of doxorubicin on sternocleidomastoid muscle has been examined in situ.

We examined the effect of direct injection of doxorubicin into sternocleidomastoid muscle on the development of fatigue. Twitch and tetanic force was measured in the control and doxorubicin-treated muscles. The effect of doxorubicin injection on the development of fatigue was also examined.

METHODS

All animal studies were approved by the University of Minnesota Animal Care and Use Committee and followed the published guidelines of the National Institutes of Health for use of animals in research. Adult New Zealand white rabbits (at least 4 months of age) were obtained through Birchwood Valley Farms (Red Wing, Minnesota) and housed with Research Animal Resources at the University of Minnesota.

Rabbits were anesthetized with an intramuscular injection of 1:1 xylazine:ketamine at doses of 2 mg/kg; 10 mg/kg, respectively. The ventral side of the neck was shaved. A skin incision was made, and the sternocleidomastoid muscles were visualized within the neck. A total dose of 2 mg doxorubicin HCl (Adriamycin; Pharmacia, Kalamazoo, Michigan) in 1 ml sterile isotonic saline was injected into one sternocleidomastoid muscle in eight rabbits. The doxorubicin was injected in two doses using a 30-gauge needle. First, the needle was inserted at the center of the muscle belly and directed towards the proximal attachment; then the needle was inserted at the center of the muscle belly and directed towards the distal attachment. The doxorubicin solution was slowly injected into the muscle over the course of approximately 60 s. Due to the red color of doxorubicin, the drug could easily be visualized within the muscle epimysial sheath. We verified during the injection procedure that the doxorubicin was injected along the full length of the muscle. The needle was left in place for 30 s after injection, and leakage was either not seen or very minimal. Any leakage out of the epimysium sheath was immediately irrigated with sterile saline. For each animal, the contralateral muscle served as a control, and received either an injection of sterile saline in a comparable volume (sham control) or no injection. A second set comprised four control animals that were examined but did not receive any doxorubicin in either muscle; these muscles were either injected with saline or received no injection, in order to test for possible effects of unilateral weakening of one sternocleidomastoid muscle on force generation of the muscle on the opposite side. The animals were checked daily for changes in head position and changes in the skin adjacent to the injection site. No noticeable change in head posture was observed in any of the rabbits.

Two months after the doxorubicin injections, the rabbits were deeply anesthetized with ketamine and xylazine and prepared for surgical isolation of the paired sternocleidomastoid muscles. A time period of 2 months was chosen so the data could be directly compared with our previous in vitro study. Previous histological studies indicated that muscle loss was largely completed within the first week after treatment. The mean weight of the rabbits was 4.04 kg ± 0.17 (SEM). The fur was removed from the ventral aspect of the neck. A large skin incision was made (6–8 cm) exposing the full length of the sternocleidomastoid muscle. Two head-pins were tightened to the bone of the rabbit’s head to immobilize it, and the chin was held tightly in place. Immediately after tracheal intubation, the lungs were ventilated mechanically, and anesthesia maintained with 2.5% isoflurane in 95% O2 and 5% CO2. The isoflurane was monitored continuously by use of a gas analyzer. Heart rate, intramuscular temperature, and rectal temperature were monitored. Temperature was maintained at 38°C, normal rabbit body temperature, using a heating pad and heat lamp.

A small piece of the sternum was cut to free the sternocleidomastoid muscle. A suture was tied to the bone, which in turn was connected to a force transducer. Electromyographic (EMG) electrodes were inserted 0.5 cm apart from each other and were embedded approximately halfway into the muscle. The left and right spinal accessory nerves were allowed to rest on a pair of stimulating electrodes using fine nichrome wire electrodes with the 0.051-mm wire bent to a small barb and 1 mm of the Teflon coating removed (California Fine Wire, Grover City, California). In addition to stimulating the nerve, the muscle was stimulated directly using two separate stimulat-
ing electrodes (2 cm long) in four rabbits. Mineral oil was used to keep the nerve, muscle, and exposed tissue from drying. Additionally, no detectable force of either sternocleidomastoid muscle was measured after surface stimulation of the omohyoid muscle, nor when the opposite sternocleidomastoid muscle was stimulated. To ensure that all the nerve fibers were stimulated sufficiently, a supramaximal stimulation intensity was ensured by increasing the voltage until maximal contraction was achieved using square-wave pulses of 0.3 ms duration; the voltage was then increased by an additional 10–30%. The sternocleidomastoid muscles examined were all approximately 9 cm in length. Optimal length for maximal force production was obtained by incrementally stretching the muscles 0.2 cm after every three twitches. When the optimal length was determined, the muscle was allowed to rest for 10 minutes. The muscles were stimulated with 0.3-ms pulses at tetanic frequencies of 10 Hz, 20 Hz, 40 Hz, 60 Hz, and 100 Hz for 1 s. The muscles were allowed to rest for 4 min between successive stimuli.

A fatigue test was performed as follows: a tetanic stimulus was delivered every 10 s and consisted of a train of pulses with a duration of 250 ms and an interpulse frequency of 40 Hz. This stimulation was performed for a total duration of 120 min. The expectation of muscle fatigue was to produce a reduction of maximal force over time. The amplitudes of the contractions were measured in grams and converted to Newtons that were read both from digital meters calibrated in grams and from a LabView program interfaced with a computer that stored the data. To enable comparison between muscles with different lengths and masses, the force was normalized to force per cross-sectional area (N/cm²). After testing, the muscle was removed completely from the animal. The area of the muscle was calculated by dividing the weight of the muscle by the product of the known density of muscle (1.056 g/cm³) and the muscle length. Student’s t-tests were used for statistical analysis. Values were considered statistically significant when \( P < 0.05 \). Error bars are displayed as standard error of the mean.

An additional four rabbits received injections of 2 mg/ml doxorubicin in one sternocleidomastoid and were sacrificed after 6 months. Both sternocleidomastoid muscles were dissected free in each rabbit. Proximal, middle, and distal regions of each sternocleidomastoid were removed along the length of both control and doxorubicin-treated muscles. Muscle specimens were obtained from the sternohyroid and the sternothyroid muscles medial to both sternocleidomastoid muscles and from the anterior scalene muscles lateral and posterior to the sternocleidomastoid muscles. All muscle specimens were embedded in tragacanth gum, frozen in methylbutane that was chilled to a slurry on liquid nitrogen, and sectioned at 12 µm on a cryostat. Tissue sections were stained with the Gomori trichrome protocol. Sections were also stained immunohistochemically for the presence of the fast myosin heavy chain isoform and for collagen type I. Cross-sections were rinsed in phosphate-buffered saline (PBS), pH 7.4, incubated in normal horse serum for 15 min, followed by incubation in the appropriate primary antibody for 1 h at room temperature. Tissue sections were incubated with either an antibody to collagen type I at a dilution of 1:100 (Oncogene Labs., Cambridge, Massachusetts) or with an antibody to fast myosin heavy chain isoform at a dilution of 1:40 (NovoCastra, Vector Labs., Burlingame, California). The sections were rinsed in PBS and incubated using the Vectastain ABC peroxidase kit (Vector Labs.). The peroxidase was visualized by incubation with diaminobenzidine and heavy metals.

Total muscle cross-sectional areas were determined by techniques previously described. Briefly, cross-sections through the entire muscle were traced using the Bioquant Nova morphometry program (R and D Biometrics, Nashville, Tennessee). Comparisons were made with the aid of the Graphpad Prism program (Graphpad, San Diego, California) using the Student’s t-test.

RESULTS

Two months after injection of doxorubicin or saline, the cross-sectional areas of the injected muscles were determined morphometrically. Compared with controls, the mean cross-sectional area of the sternocleidomastoid muscle injected with doxorubicin was reduced by 71 ± 3% (control = 39 ± 2 mm²; doxorubicin-treated = 11 ± 2 mm²). The average weight of the injected muscle was 0.67 ± 0.07 (SEM), which was significantly less than the average weight of 1.49 ± 0.07 of the contralateral control muscle. No fibrosis was observed histologically within the treated muscles. This indicates that myofibers killed by the treatment were not replaced by connective tissue. Neither degenerating myofibers nor reduction in the cross-sectional areas was observed in muscles neighboring the doxorubicin-injected sternocleidomastoid muscle, indicating that the effect was restricted to the injected muscle. These results show that a direct injection of doxorubicin into the sternocleidomastoid muscle in vivo causes a significant reduction in muscle mass by 2 months.

An in situ physiological analysis was used to de-
determine whether the reduction in muscle mass following doxorubicin treatment was accompanied by a reduction in muscle strength. Two months after doxorubicin injection, force was determined in both the control and doxorubicin-injected muscles after a twitch stimulus. Stimulation of control sternocleidomastoid muscle resulted in a mean twitch force of 0.44 ± 0.06N, compared with 0.36 ± 0.04N for the doxorubicin-treated muscle (Figs. 1 and 2A). This difference was not statistically significant. No significant differences were seen in the twitch-force measurements between the right and left sternocleidomastoid muscles in the uninjected or sham injected controls.

We examined whether the reduced muscle mass caused by doxorubicin was accompanied by reduction in muscle force produced by tetanic stimulation. The peak force generated by tetanic stimulation was significantly reduced in doxorubicin-treated muscles (Figs. 1 and 2A). There was a 50% and 65% reduction in total force when the muscles were stimulated at 10 Hz and 100 Hz, respectively, compared with contralateral controls (Fig. 2A). Thus, at the higher stimulation frequencies, the difference between the contralateral control muscle and doxorubicin-injected muscle became more pronounced.

As injection of the sternocleidomastoid muscle with doxorubicin resulted in a pronounced reduction in muscle mass, the peak force data was analyzed to determine the relative force per cross-sectional area of each muscle. Although the total force at all stimulation frequencies was reduced after doxorubicin treatment, the force per cross-sectional area was not statistically different from that of the contralateral control muscles for any of the stimulation frequencies tested (Fig. 2B). The muscle force in response to direct surface stimulation at 10 Hz, 20 Hz, 40 Hz, 60 Hz, and 100 Hz was not statistically different from muscle force in response to nerve stimulation (Fig. 3).

Previous studies indicated that doxorubicin might alter the individual twitch responses of the sternocleidomastoid myofibers. Multiple contractile properties of individual twitch responses were compared for the doxorubicin-treated and untreated contralateral sternocleidomastoid muscles (Table 1). No significant differences were seen in peak rates of development and decay of twitch tension.

The reduction in muscle mass caused by doxorubicin injection into the sternocleidomastoid muscle may result in changes in the rate of muscle fatigue. The doxorubicin-treated and control sternocleidomastoid muscles were tested for rate of muscle fatigue using repetitive stimulation. Repetitive firing initially potentiated muscle force for control and doxorubicin-injected sternocleidomastoid muscles, but this was followed by a decrease in force (Fig. 4). It took an average of 37 min of repetitive stimulation for the control muscle to reach its maximal force and 23 min for the injected muscle to reach its maximum force level. At the end of the fatigue test, the control and doxorubicin-injected muscles had fatigued to approximately 15% and 16% of their initial force and to approximately 52% and 47% of their maximal force after potentiation, respectively.

**DISCUSSION**

Studying the sternocleidomastoid muscles of rabbits using an in situ protocol in which the nerve and blood supply were intact, we found that doxorubicin treatment resulted in decreased muscle mass and decreased muscle force following tetanic stimulation.

The overall results of the in situ and in vitro analyses were similar, but distinct differences were evident when the results from each experiment were compared. Total muscle strength of the control sternocleidomastoid muscle was larger using the in situ preparation than the in vitro preparation at each stimulation intensity. An intact nerve and vascular supply appeared to help maintain the muscles in a more normal physiological state, allowing the sternocleidomastoid muscle to produce greater contractile forces. Total muscle strength of the doxorubicin-injected muscles was also different with the two methods. The decrease in force was 50% of control for the muscles tested in situ and 67% for the muscles tested in vitro. It may be that the doxorubicin-treated muscles were more susceptible to loss of blood and nerve supply than were the uninjured, control muscles. Regardless, the significant reduction in force in the treated sternocleidomastoid muscle demonstrates the therapeutic potential for direct injection of doxorubicin into the muscle for the treatment of cervical dystonia.

Distinct differences were also evident when rate of fatigue using the two methods was compared. In doxorubicin-treated muscle examined in vitro, fatigue did not occur after 120 min of stimulation. In the present study, the fatigue rate of the doxorubicin-treated muscle was similar to control muscle. This supports the need to use multiple experimental methods to get a clear picture of physiological changes caused by muscle injury.

Doxorubicin had a greater effect on muscle force generated at high stimulation frequencies than at stimulation frequencies that elicit unfused (twitch) contractile responses. Doxorubicin-injected muscles
FIGURE 1. Responses from a noninjected control muscle (left panels) and from a muscle treated with 2 mg doxorubicin 2 months previously (right panels) to electrical stimulation of the spinal accessory nerve.
showed a decrease in force compared with control muscles only at stimulation frequencies over 20 Hz. For patients, it may thus be that muscle function is not affected by doxorubicin treatment at the low levels of muscle activity needed to maintain normal posture, whereas forceful contractions or spasms are reduced.

There is a significant reduction in muscle mass as a result of doxorubicin treatment, but there is no difference between the force per cross-sectional area of the treated and control sternocleidomastoid muscles at any of the stimulation frequencies. Although there is a large overall reduction in force, there is also a large reduction in total number of myofibers and total muscle cross-sectional area.14 Despite this dramatic decrease in muscle mass after treatment, the remaining myofibers within the treated muscle are able to elicit normal contractile forces based on total muscle cross-sectional area.

### Table 1. Twitch properties of the injected and noninjected rabbit sternocleidomastoid muscles.

<table>
<thead>
<tr>
<th>Property</th>
<th>Noninjected</th>
<th>Injected</th>
</tr>
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<tbody>
<tr>
<td>Force (N)</td>
<td>0.44 ± 0.06</td>
<td>0.36 ± 0.12</td>
</tr>
<tr>
<td>Force/CSA (N/cm²)</td>
<td>3.21 ± 0.62</td>
<td>4.28 ± 1.09</td>
</tr>
<tr>
<td>Contraction time (ms)</td>
<td>21.70 ± 2.12</td>
<td>21.77 ± 2.22</td>
</tr>
<tr>
<td>Half-relaxation time (ms)</td>
<td>30.09 ± 3.83</td>
<td>25.95 ± 1.18</td>
</tr>
<tr>
<td>Peak rate of development (N/ms)</td>
<td>0.016 ± 0.003</td>
<td>0.010 ± 0.002</td>
</tr>
<tr>
<td>Peak rate of decay (N/ms)</td>
<td>−0.007 ± 0.001</td>
<td>−0.005 ± 0.001</td>
</tr>
<tr>
<td>Time to peak development (ms)</td>
<td>6.61 ± 1.13</td>
<td>5.95 ± 0.88</td>
</tr>
<tr>
<td>Time to peak decay (ms)</td>
<td>30.52 ± 2.71</td>
<td>31.45 ± 2.82</td>
</tr>
<tr>
<td>Torque latency (ms)</td>
<td>7.42 ± 1.67</td>
<td>7.18 ± 2.73</td>
</tr>
<tr>
<td>Halfmax value time (ms)</td>
<td>42.96 ± 4.95</td>
<td>38.68 ± 2.73</td>
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Values shown are mean ± SEM (n = 8).
in vitro study, no significant difference was seen in force generation of the sternocleidomastoid muscles at 2 and 6 months after doxorubicin injection. A small amount of regeneration occurs in treated muscles, but this is minimal. Even 2 years after treatment of patients with blepharospasm by doxorubicin injections, orbicularis oculi muscle had not returned. This is not particularly surprising, as doxorubicin is primarily used in cancer chemotherapy due to its powerful antimitotic effects.

Cervical dystonia can be recalcitrant to treatment, often as a result of the complexity of muscle involvement in individual patients. In a quantitative EMG study, there was no clear correlation between the clinical appraisal of neck muscle involvement in abnormal movements and abnormal neck muscle activity. In neck muscles that were thought to be unaffected clinically, EMG measurements demonstrated abnormal activity. Even in cervical dystonia patients treated unsuccessfully with botulinum toxin, the pattern of neck muscle activity changed substantially over time. It remains to be seen whether permanent weakening of a muscle or muscle groups by doxorubicin can subvert these effects and alter the general motor program for head position. A study on the effect of doxorubicin injection on muscle mass and muscle force generation in the dorsal neck musculature will be very important prior to work in humans with cervical dystonia, as it is critical to determine whether direct injection of doxorubicin into these more complex muscles will be equally effective in reducing muscle strength.

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