Botulinum Toxin A, a Better Choice for Skeletal Muscle Block in a Comparative Study With Lidocaine in Rats

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Received May 11, 2022; accepted September 6, 2022

ABSTRACT

A positive response to scalene muscle block (SMB) is an important indication for the diagnosis of thoracic outlet syndrome. Lidocaine injection is commonly used in clinical practice in SMB, although there have been some cases of misdiagnosis. Botulinum toxin A (BTX-A) is one of the therapeutic agents in SMB, but whether it is also indicated for SMB diagnosis is controversial. To evaluate the muscle block efficiency of these two drugs, the contraction strength was repeatedly recorded on tibialis anterior muscle in rats. It was found that at a safe dosage, 2% lidocaine performed best at 40 µL, but it still exhibits an unsatisfactory partial blocking efficiency. Moreover, neither lidocaine injection in combination with epinephrine or dexamethasone nor multiple locations injection could improve the blocking efficiency. On the other hand, injections of 3, 6, and 12 U/kg BTX-A all showed almost complete muscle block. Gait analysis showed that antagonistic gastrocnemius muscle, responsible for heel rising, was paralyzed for nonspecific blockage in the 12 U/kg BTX-A group, but not in the 3 U/kg or 6 U/kg BTX-A group. Cleaved synaptosomal associated protein 25 (c-SNAP 25) was stained to test the transportation of BTX-A, and was additionally observed in the peripheral muscles in 6 and 12 U/kg groups. c-SNAP 25, however, was barely detectable in the spinal cord after BTX-A administration. Therefore, our results suggest that low dosage of BTX-A may be a promising option for the diagnostic SMB of thoracic outlet syndrome.

SIGNIFICANCE STATEMENT

Muscle block is important for the diagnosis and treatment of thoracic outlet syndrome and commonly performed with lidocaine. However, misdiagnosis was observed sometimes. Here, we found that intramuscular injection of optimal dosage lidocaine only partially blocked the muscle contraction in rats, whereas low-dosage botulinum toxin, barely used in diagnostic block, showed almost complete block without affecting the central nervous system. This study suggests that botulinum toxin might be more suitable for muscle block than lidocaine in clinical practice.

Introduction

Thoracic outlet syndrome (TOS) occurs when the brachial plexus or subclavian artery and vein are compressed by spasitic muscles, abnormal bones (Kuhn et al., 2015), or narrow space (Sanders and Roos, 1989). The incidence rate of TOS is 3% to 80% and the typical symptoms include pain, paresthesia, and weakness in the upper extremity. Generally speaking, cases of TOS can be classified as follows: neurogenic TOS (NTOS, 90% of all TOS cases) (Sanders et al., 2007), venous TOS, and arterial TOS (Freischlag and Orion, 2014).

NTOS is the most complicated TOS for diagnosis (Weaver and Lum, 2017; Wilbourn, 2000). The symptoms of NTOS are ambiguous and should be differentiated from cervical spondylosis, carpal tunnel syndrome, cubital tunnel syndrome, and many other neurologic disorders (Jones et al., 2019). Furthermore, the diagnosis of NTOS relies heavily on the exclusion methods (Feiler, 1997; Sanders et al., 2007; Bottros et al., 2017; Jones et al., 2019) as the validity of physical examination, imaging, and electrophysiological tests remains controversial (Sanders et al., 2008). Therefore, a positive response to scalene muscle block (SMB) has been reported to be one of the more useful methods to diagnose NTOS (Sanders et al., 2008) and predicts desired surgical outcomes (Braun et al., 2006). Successfully block by intramuscular injection results in a reduction of active muscle stiffness and relief of symptoms. To mimic the decompression effects of NTOS surgery, such as first costectomy or partial scalenectomy, the ideal outcomes of SMB should be close to zero force production (Supplementary Fig. 1).

SMB is usually performed with lidocaine, which inhibits action potential conduction by blocking Na+-voltage-gated
The results showed that 40 μm effort the TA muscle, and 40 SMB in TOS diagnosis. BTX-A was observed retrograde transported along the nerve, (6 and 12 U/kg) but not in the low dosage (3 U/kg). Although SNAP 25 cleaved by activated BTX-A, was observed in the sur- ticle strength in rats. By immunohistochemical staining, cleaved

effect (Monsivais and Monsivais, 1996; Jordan et al., 2000; Jordan et al., 2007a; Christo et al., 2010). It blocks the presynaptic acetylcholine release (Pamphlett, 1989; Jordan et al., 2000; Nakanishi et al., 2005) and triggers flaccid paralysis (Bomba-Warczak et al., 2016), which reduces hyperactivity and spasticity of focal muscles and leads to dystonia (Alessandrino and Balconi, 2013). In addition, BTX-A has been reported to be unlikely to block the brachial plexus in SMB, which makes it a promising substance for SMB (Jordan et al., 2000). However, lidocaine is still the preference for SMB in a clinical setting. Therefore, the reason for the unsatisfied SMB blocking efficiency of lidocaine in some cases and whether BTX-A may be a better choice for SMB require further exploration.

An optimal drug for SMB should substantially inhibit muscle contraction by measuring muscle strength. Due to the difficulty of measuring scalene muscle strength in either patients or rats, we compared the block efficiency of lidocaine and BTX-A in the tibialis anterior (TA) muscles of rats, whose strength could be easily and repeatedly measured through ankle dorsiflexion. The results showed that 40 μL was the maximum injection volume for the TA muscle, and 40 μL of 2% lidocaine achieved the best muscle block effect among all lidocaine groups, reducing muscle strength by 61% ± 14%. However, lidocaine combined with epinephrine or dexamethasone or multiple locations injected (multipoint injection hereafter) showed similar blocking efficiency to that of lidocaine alone. It was also observed that 3, 6, and 12 U/kg of BTX-A caused virtually complete loss of muscle strength in rats. By immunohistochemical staining, cleaved synaptosomal associated protein 25 (c-SNAP 25), the residue of SNAP 25 cleaved by activated BTX-A, was observed in the sur- rounding muscles in high and medium BTX-A dosage groups (6 and 12 U/kg) but not in the low dosage (3 U/kg). Although BTX-A was observed retrograde transported along the nerve, c-SNAP 25 was not detected in the spinal cord. This study sug- gests that BTX-A has been identified as a promising choice for SMB in TOS diagnosis.

Materials and Methods

Animals. Male Sprague Dawley rats (n = 107, 265 ± 17 g) were obtained from Changchun Yisi Experimental Animal Technology CO. Ltd. (Changchun, China) and housed in a controlled environment (25 ± 1°C, 50 ± 10% humidity, and 12-hour light/dark cycle) with ad libitum access to food and water. The rats were randomly divided into groups for the subsequent experiments. Experimental procedures fol- lowed the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee of Jilin University, Changchun, China.

Drugs Administration. Lidocaine (0.5%, 1%, and 2%; Suicheng, China), epinephrine (1:200,000, 5 μg/mL; Suicheng, China), and dexamethasone (1.28 mg/mL; Suicheng, China) were prepared for experiment. Dried BTX-A complex (BOTOX, Allergan Pharmaceuticals, Ireland) was reconstituted with normal saline solution at concentra- tions of 3, 6, and 12 U/kg, while normal saline was adopted as the control. After anesthetized with isoflurane (1.8%–3.0% in oxygen, 2.0 L/min, small animal anesthesia apparatus; RWD, Shenzhen, Guangdong, China), the left legs of the rats were shaved and steril- ized. The needles were marked 3 mm from the tip to ensure that the injections were performed at the center of the target muscle and remained within there. As the neuromuscular junction of the TA muscle was distributed across 20% to 75% of the muscles’ proximal parts (Oswa, 2008), the drug was administered via intramuscular injec- tion into the middle part of the left TA muscle.

As isoflurane has been reported to have a postsynaptic potentiation effect on neuromuscular blockade (Kumar et al., 1996; Vanninathou et al., 1996; Sutcliffe et al., 2000), the blocking efficiency of BTX-A (3 U/kg) was compared under the anesthesia of isoflurane or ketamine (75 mg/kg, i.m.), with the latter only functioning on the central nervous system (Lee et al., 2010; Sun et al., 2020; Zhou and Guan, 2021), to determine whether isoflurane could enhance the effect of BTX-A.

Muscle Contraction Measurement. The rats were anesthetized and kept on a heated pad to maintain their body temperatures around 37°C. The surface temperature of the rat was measured with an infrared thermometer every 30 minutes. The room temperature was main- tained at ~26°C. The rats then were mounted in the experimental set-up (701C stimulator, 300D-305C force transducer, 806D test appa- ratus, Aurora Scientific, Canada). One TA muscle can be measured repeatedly in vivo by inserting one needle-like stimulating electrode percutaneously into the belly of the TA muscle and another one into the common peroneal nerve (CPN) near the fibular head (Fig. 6). Ankle movement torque, an indicator of TA muscle contraction, was measured with a pedal. Following two twitches (1 mA, 0.2 ms), the muscles were stimulated by a series of tetanic pulses (1 mA, 400 ms) at 20, 40, 50, 60, 80, and 100 Hz with an interval of 1 minute. Subse- quently, the muscles were rested for 2 minutes before being stimu- lated with three tetanic pulses (1 mA, 400 mA, 100 Hz) at 1-minute intervals on average. For the maximum injection volume of the TA muscle, all muscles were stimulated three times (1 mA, 400 ms, 100 Hz) with an interval of 1 minute after methylene blue injections.

For lidocaine treatment measurements, data were collected before and at 30 minutes; 1, 1.5, 2, 2.5, 3, 6, 12, and 18 hours; and 1 to 7 days after the drug injection. For BTX-A administration, measure- ments were performed before and at 30 minutes; 1, 6, 12, and 18 hours; and 1 to 7 days after the drug injection. Data were analyzed by the 611a Dynamic Muscle Analysis (Aurora Scientific, Canada). The effect of the drug on the maximum torque (maximum strength) and the area under the torque-time curve (integration) were evaluated.

Gait Analysis. Gait changes of the rats were tested by an MSI DigiGait Imaging System (Mouse Specifics, USA), as previously described (Sakuma et al., 2016; Xu et al., 2019). The DigiGait contains a transparent treadmill on which animals are restricted under a cover and forced to walk/run at a fixed velocity and gradient. Before the ex- experiment, animals were trained to run in three step-cycles constantly at a speed of at least 10 cm/s. Measurements were taken before and at 1 to 7 days after BTX-A injection or before and at 30 minutes and 1, 2, and 3 hours after the lidocaine injection. Each rat was placed on the treadmill repeatedly at intervals of at least 5 minutes to complete three uninterrupted runs (containing at least eight step-cycles per run) for further analyses. The velocity and gradient of the treadmill were set to 15 cm/s and 0°. Consecutive recording from the ventral direction provided the projected area. Gait images were analyzed using DigiGait Analyses software (Mouse Specifics, USA).
Immunohistochemical Staining. Rats were sacrificed 7 days after the injection. Before sacrifice, the rats were deeply anesthetized, perfused with 0.01 M PBS (pH 7.4) transcardially followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The left TA muscle, left extensor digitorum longus (EDL) muscle, left CPN, and the lumbar sacral spinal cord (L4–L5) were harvested for analysis. Tissues were postfixed for 12 hours in 4% paraformaldehyde and stored in a 10% to 30% sucrose gradient in 0.1 M phosphate buffer at 4°C. Afterward, the tissues were cut into slices of 10, 12, and 40 μm thickness, respectively. Tissue sections were washed and then incubated in PBS with 5% bovine serum albumin and 1% TritonX-100 for 1 hour at room temperature. Subsequently, the sections were incubated with primary antibodies diluted in PBS with 2% bovine serum albumin and 3% TritonX-100 overnight at 4°C. Specifically, the TA muscle and CPN sections were incubated with mouse polyclonal antibodies against BTX-A c-SNAP 25 (1:100; Genetex, GTX39119) and rabbit polyclonal antibodies against NeuN (1:500; Abcam, 243975). Bound antibodies were detected by Alexa Fluor546 donkey anti-rabbit antibodies (1:500; Invitrogen, A10400) and Alexa Fluor488 donkey anti-mouse antibodies (1:500; Invitrogen, Carlsbad, California, A21202) for 2 hours at room temperature. Sections were washed three times with PBST (0.5% Tween 20) and then mounted on coverslips. Images were captured with Nikon A1 laser scanning confocal microscope (Nikon, Tokyo, Japan). For the quantification of c-SNAP 25 staining, image stacks from both channels were thresholded to remove the background and preserve brighter pixels. Arbitrary fluorescence intensity was measured with Image J software (US National Institutes of Health). The presence of c-SNAP 25 was characterized by 40 μL of 2% lidocaine showed prominent block efficacy (P = 0.001, Kruskal–Wallis), with a reduction of 61% ± 14% in maximum strength and 72% ± 13% in integration, respectively. Even though the total amount of lidocaine was the same, the block efficacy of 1% lidocaine in 40 μL seemed to be greater than that of 20 μL 2% lidocaine (Fig. 1, C, D, and E), which might be the result of improved diffusion in a larger volume.

To improve the block efficiency of lidocaine on the TA muscle, the maximum dose that can be injected into the TA muscle was first determined. Previous literature has reported that the intramuscular injection of no more than 10% of the muscle volume does not result in extravasation (Hulst et al., 2014). The volume of the TA muscle was about 400 μL in the rat, so we administrated 40 or 50 μL of 1% methylene blue. After three tetanic stimulation sessions, extravasation was examined on the skin, subcutaneously, or intramuscularly (Fig. 1A). The results showed that a few cases (2/13) leaked in the 40 μL group; however, almost all the rat muscles leaked in the 50 μL group (12/13, P = 0.01, Fisher's exact test) (Fig. 1B). Therefore, in this study, 40 μL was considered to be the maximum injection volume for the TA muscle in the rat.

As lidocaine is typically given at the concentration of 0.5% to 2% (Jordan et al., 2000; Lum et al., 2012; Weaver et al., 2019), 0.5%, 1%, and 2% lidocaine at the volumes of 20 and 40 μL were selected in this study. Data obtained before injection served as the baseline, and the data collected after injection at each time point were converted into percentages by dividing them with the baseline. Compared with the control saline group, both the maximum strength (Fig. 1, C and D) and the integration (Fig. 1, C and E) dropped at 30 minutes after lidocaine administration, followed by a stable increase until fully returning to the normal level approximately 2 hours later. Moreover, higher lidocaine concentration was associated with a smaller maximum strength and smaller integration (R = 0.631, P < 0.05, and R = 0.658, P < 0.01, respectively; Pearson correlation coefficient). Particularly, 40 μL of 2% lidocaine showed prominent block efficacy (P = 0.001, Kruskal–Wallis), with a reduction of 61% ± 14% in maximum strength and 72% ± 13% in integration, respectively. Even though the total amount of lidocaine was the same, the block efficacy of 1% lidocaine in 40 μL seemed to be greater than that of 20 μL 2% lidocaine (Fig. 1, C, D, and E), which might be the result of improved diffusion in a larger volume.

The block efficacy of lidocaine (2%, 40 μL) was also assessed at different stimulation frequencies. The block efficacy under low-frequency stimulation was stronger and longer than that under high-frequency stimulation (Fig. 1F). Overall, lidocaine at a concentration of 2% in 40 μL was optimal in TA muscle block and was selected for further study.

Effects of Lidocaine Combined With Epinephrine or Dexamethasone or Multipoint Injection on Muscle Block. Lidocaine is usually applied in combination or at multiple points in clinical. To evaluate the efficacy of synergy, we set up several groups based on the administrated agents: lidocaine + epinephrine (1:200000, 5 μg/mL), epinephrine (1:200000, 5 μg/mL), lidocaine + dexamethasone (1.28 mg/mL), dexamethasone (1.28 mg/mL), and a multipoint injection group, with a total of 40 μL 2% lidocaine injected simultaneously at the middle point and the locations 5 mm away from that into the TA muscle. The injection of epinephrine led to a decrease in muscle strength (20% ± 7%), while the combination did not enhance the block efficacy of lidocaine (P = 0.504, two-way ANOVA) (Fig. 2, A and B, Fig. 3, C), even though the combination prolonged the recovery of muscle strength and integration (Fig. 2, A and B). Additionally, epinephrine administration resulted in pale skin around the injection site, which may be due to a reduction in blood supply to the muscles (Fig. 2C).

Injection of dexamethasone alone did not affect muscle contraction. However, when combined with lidocaine, dexamethasone attenuated the blocking efficacy (P = 0.006, two-way ANOVA) (Fig. 2, A and B, Fig. 3C), especially at 30 minutes after the injection. The multipoint injection was found to be less effective than a single injection when the total administrated volume of lidocaine was the same (P = 0.010, two-way ANOVA) (Fig. 2, A and B, Fig. 3C). Therefore, drug combination or multipoint injection has no contribution to enhancing the muscle block efficacy of lidocaine.

Effects of BTX-A and Its Comparation With Lidocaine on Muscle Block. BTX-A has been used clinically in SMB; however, there is no consensus on its outcomes. To characterize the performance of BTX-A on muscle blockage, 3, 6,
and 12 U/kg BTX-A were administrated in TA muscle, respectively. The results showed that the muscle was blocked gradually and dose-dependently. An obvious muscle block was observed as early as 6 hours after the drug injection. All three groups reached a stable blocking level at 1 day after the administration (Fig. 3, A and B) and continued for 7 days when the experiment was ended. Aside from the significant reduction of the maximum strengths (a reduction of 82% ± 16% in the 3 U/kg group, and almost complete loss in the other two groups) 1 day after injection (Fig. 3A), there was also a decrease in integration (Fig. 3B). However, no difference was observed among the three BTX-A groups in terms of maximum strength or integration (Fig. 3, C). In general, BTX-A administration almost completely blocked muscle contraction, even at low doses.

It has been reported that volatile anesthetics, such as isoflurane, could potentiate neuromuscular blockade via effects at the motor end plate (Raines et al., 1995; Sokoll et al., 1995; Paul et al., 2002; Zhou and Guan, 2021). To determine whether isoflurane could enhance the blocking efficacy of BTX-A, we compared it with ketamine (75 mg/kg, i.m.) in rats, which had an antagonist action at N-methyl-D-aspartate receptors noncompetitively throughout the central nervous system (Lee et al., 2010; Sun et al., 2020; Zhou and Guan, 2021). Results showed that no significant difference was observed between these two anesthetic drugs during the first day when the BTX-A reached a stable blocking effect (Supplementary Fig. 2).

Lidocaine is regarded as a more suitable drug than BTX-A for SMB, while others have suggested that a higher dosage of BTX-A may reverse the conclusion (Donahue et al., 2020). To investigate these two drugs on muscle contraction block, three dosages of BTX-A were compared with lidocaine (2%, 40 μL) alone or combined with epinephrine (1:200000, 5 μg/mL) by

**Fig. 1.** Effects of intramuscular injection of lidocaine on muscle contraction block. (A) Representative images of solution leakage or not after intramuscular injection of 1% methylene blue into the TA muscles. Scale bar, 1 cm. (B) Statistical analysis of leakage cases after intramuscular injection of 40 μL or 50 μL. Two out of 13 cases leaked in 40 μL, and 12 out of 13 cases leaked in 50 μL. For each group, n = 13. P = 0.01, Fisher’s exact test. (C) Representative tetanic curves of muscle stimulated at 100 Hz after the injection of 0.5%, 1%, and 2% lidocaine in the volume of 20 μL or 40 μL, respectively. (D) Statistical of the maximum strength related to the initial value. The percentage changed with time, and the maximum strength significantly decreased at 30 minutes and recovered at 2 hours. 2% lidocaine in 40 μL showed the most blockage efficiency. n = 3 in the saline group and n = 5 in other groups. (E) Statistical of the percentage of area under the strength-time curve (integration), which showed the same tendency as the maximum strength. (F) Statistical analysis of the block percentage of 2% lidocaine in 40 μL stimulated at frequencies of 20, 40, 50, 60, 80, and 100 Hz. The muscle block efficacy was greater and longer when the muscles were stimulated at the low frequency than the high ones. n = 5 per groups.
measuring muscle strength. The maximum strength was collected at 30 minutes after lidocaine injection and at 1 day after BTX-A injection (Fig. 3C). The BTX-A group showed a further decrease in maximum intensity compared with the optimal lidocaine dose (2%, 40 µL) used alone or in combination (Fig. 3C). In addition, the efficacy of lidocaine, either given alone or combined with epinephrine, could not last for 1 day after the injection (Fig. 3, A and B). These results suggested that BTX-A as low as 3 U/kg led to a higher muscle block efficiency than lidocaine at the optimal dosage.

**Gait Changes After the Drug Administrations.** To evaluate muscle block under physiologic conditions, the gait of the rats was recorded consistently. Parameters related to the gait changes were compared 30 minutes after injection of lidocaine (2%, 40 µL) and 1 day after the BTX-A injection. As shown in Fig. 3D, rats receiving lidocaine had comparable paw-to-ground contact to control rat (2.20 cm² vs. 2.45 cm² and 1.16 cm² vs. 1.21 cm², respectively) when fully placed (0.2 second) or lifting up (0.5 second). However, for 12 U/kg BTX-A injected rat, the areas were just about 1.71 cm² (Fig. 3D), which indicated a delayed heel rising (Fig. 3E). In addition, the left hind toes showed adduction (2.20 cm² vs. 1.79 cm²), and the left hind foot showed prolonged contact with the ground ($P = 0.017$, Kruskal–Wallis) (Fig. 3F). These results suggest that the gastrocnemius muscle, which is responsible for heel rising, was also paralyzed without BTX-A injection. However, such nonspecific blockage did not occur in the 3 or 6 U/kg BTX-A group (Fig. 3F). Therefore, BTX-A at the concentrations of 3 and 6 U/kg specifically blocked the injected muscle in rats.

**Muscle Atrophy and Transportation of BTX-A.** The atrophy of muscle could also be beneficial to a positive response of SMB by increasing thoracic outlet space. The wet weights of the TA and EDL muscle, an adjacent muscle to TA, were analyzed at day 7 (Fig. 4A). Compared with the control, neither the TA muscles nor the EDL muscle in the lidocaine group showed any atrophy, but the weight of TA muscle was reduced in rats receiving 12 U/kg BTX-A injection ($P = 0.003$, two-way ANOVA) (Fig. 4B). Furthermore, the weights of the TA muscles in 6 and 3 U/kg were all decreased, and no difference was observed between the three groups (Fig. 4B). However, BTX-A did not significantly affect the wet weights of the EDL muscles in these groups (Fig. 4B).
Fig. 3. Comparison of the effects of lidocaine and BTX-A on muscle contraction block. (A and B) Statistic of percentages of maximum strength (A) and integration (B) after the administration of BTX-A at the concentration of 3, 6, and 12 U/kg by tetanic stimulation at 100 Hz (n = 5). Lidocaine and lidocaine with epinephrine were served as controls (n = 4). The muscles were blocked gradually and dose-dependently, and an obvious muscle block was observed as early as 6 hours after the drug injection. All three groups reached a stable blocking level at 1 day after the administration. BTX-A completely blocked the contraction of skeletal muscles and showed no recovery until the seventh day, the end of the experiment. (C) Statistical analysis of the maximum reduction of strength or integration between 2% lidocaine in 40 L and 3 U/kg BTX-A, as well as 6 and 12 U/kg. The block efficacy of 3 U/kg BTX-A was significantly higher than 2% lidocaine in 40 L (P < 0.038, two-way ANOVA), but similar among the three BTX-A groups (P > 0.05, two-way ANOVA). Epinephrine could not affect lidocaine efficacy. Multipoint injections and a combination with steroids decreased the blockage efficacy of lidocaine (maximum strength, *P = 0.010, **P = 0.006, two-way ANOVA; integration, **P = 0.003, ***P < 0.001, two-way ANOVA). (D) Representative images of rats in each group, with the left hind paw fully on the ground (upper panels, 0.2 second) or lifted up (lower panels, 0.5 second) (others not shown). The toes adducted and the heel rising delayed in the 12 U/kg BTX-A group. Scale bar, 1 cm. (E) Representative curves of left hind paw area changed with time, from totally placed on the glass to lift up, in maximum blocking situation of two drugs (others not shown). (F) Statistic analysis of the area change or time for the paw fully placed on the glass (P = 0.017, n = 5; Kruskal-Wallis test).
Fig. 4. Effects of BTX-A on surrounding muscles. (A) Images of the TA and the EDL muscle in normal saline, 2% lidocaine in 40 μL, and 12 U/kg BTX-A groups. Scale bar, 1 cm. (B) Statistic of the wet weight of the injected TA muscle and EDL muscles in each group. The wet weight of the TA muscle was decreased in the BTX-A groups. (*P = 0.003, two-way ANOVA, n = 5). (C) Representative images of immunohistochemistry
c-SNAP 25, a product after processing with activated BTX-A (Antonucci et al., 2008; Caleo et al., 2009; Koizumi et al., 2014) that peaked at day 7 (Whelchel et al., 2004), was employed to evaluate the transportation of BTX-A. Specifically, c-SNAP 25 was stained in the TA and the EDL muscle. The results showed that c-SNAP 25 was positive in both the TA and the EDL muscle in the 6 and 12 U/kg BTX-A groups but negative in the EDL muscle in the 3 U/kg group (Fig. 4, C and D).

Considering the long distance of transportation, the common peroneal nerve and the spinal cord (L4 and L5) were also checked. c-SNAP 25 could be detected in the common peroneal nerves of all three BTX-A groups, indicating that the bioactive BTX-A was retrograde transported (Fig. 5, A and B). However, no c-SNAP 25 was observed in spinal cord segments (Fig. 5C). Altogether, these results suggest that 3 U/kg BTX-A administration for muscle block does not affect EDL muscle or spinal cord.

Fig. 5. Effects of BTX-A on innervating nerve and corresponding spinal cords. (A) Representative images of the longitudinal sections of the CPN stained with c-SNAP 25 (red) and NF-L (green) in each group. Square, images were enlarged. Scale bar, 100 μm. (B) Quantification of c-SNAP 25 staining in CPN. (C) Immunohistochemical images of L4 and L5 stained by NeuN (green) and c-SNAP 25 (red). No c-SNAP 25 was detected in NeuN positive neurons. Scale bar, 1 mm.

- c-SNAP 25 staining of c-SNAP 25 and NF-L on the TA muscle and the EDL muscle. NF-L (green) labeling presynapse of the neuromuscular junction, c-SNAP 25 (red) is the residue of SNAP 25 processed by activated BTX-A. Square, images were enlarged. c-SNAP 25 was negative in the EDL of the 3 U/kg group. Scale bar, 100 μm. (D) Quantification of c-SNAP 25 staining in TA muscle and EDL muscle (**P = 0.004, ***P < 0.001, Tamhane’s T2 test, n = 4).
Discussion

In this study, we compared the muscle block efficiency of lidocaine and BTX-A with the TA muscle in rats. The lidocaine blocked muscle contraction partially. Epinephrine could significantly prolong the effect duration of lidocaine but did not increase efficacy. Multipoint injections and a combination of steroids attenuated the blocking efficacy of lidocaine. BTX-A can block muscle contraction almost completely, but high doses of BTX-A also blocked the surrounding muscles. No c-SNAP 25 was detected in the central nervous system despite doses of BTX-A also blocked the surrounding muscles. No can block muscle contraction almost completely, but high increase efficac y and longer blockage duration than lidocaine.

Currently, there are no available animal models to mimic the pathologic process of TOS. However, a quantification of the scalen muscle is required to compare the muscle-blocking efficiency of drugs, which is difficult to perform in humans. However, the strength of the rat’s TA muscle contraction could be measured through ankle dorsiflexion repeatedly in vivo. In clinical practice, symptom relief in SMB should be achieved through muscle relaxation, but not analgesic effects of the sensory block. Lidocaine used in SMB could probably lead to brachial plexus block and result in false-positive results (Torriani et al., 2009; Benzon et al., 2012), which makes it difficult to distinguish pain relief from nerve compression release or brachial plexus block. This is either because of direct blocking of the brachial plexus (Harry et al., 1997; Natsis et al., 2006) through the anterior scalen muscle or because of lidocaine leaking from the scalen muscle (Winnie, 1970; Benzon et al., 2012). To mimic the avoidance of lidocaine extravasation, the maximum injection volume of the TA muscle in rats was studied. Besides, a wide range of lidocaine concentrations, from 0.5% to 4%, has been clinically used for SMB (Jordan and Machleder, 1998; Jordan et al., 2007b; Lum et al., 2013; Braun et al., 2015; Bottros et al., 2017; Aktas et al., 2020). Since a high concentration of lidocaine would produce irreversible neurotoxicity (Bainton and Strichartz, 1994; Schneider et al., 1994), 4% was not discussed in this study. The BTX-A experiments were grouped by dose because the pharmacological effects of BTX-A are dose-dependent (Cichon et al., 1995; Dodd et al., 2005). There are reports that isoflurane could enhance neuromuscular blockade. However, we did not observe an obvious difference between isoflurane and ketamine on BTX-A in muscle block. Synergistic effects of isoflurane are observed in the combination of nonpolarizing muscle relaxants, such as mivacurium, pancuronium, and atracurium (Kumar et al., 1996; Vanlithout et al., 1996; Sutcliff et al., 2000), by enhancing the competitive binding of these drugs to acetylcholine receptors at the post-endplate membrane (Raines et al., 1995; Sokoll et al., 1995; Paul et al., 2002). In comparison with these clearly postsynaptic effects, BTX exerts its effect at the presynaptic terminal, which may not be significantly affected by isoflurane.

Abnormalities in the scalene muscles are explicit causes of NTOS, and the scalene space expansion through muscle relaxation (Weaver et al., 2019) or muscle atrophy would be beneficial in relieving the pressure on the nerves and vessels (Braun et al., 2015) (Supplementary Fig. 1). Therefore, compared with electrophysiological examinations, a positive response to SMB on muscle contraction blocking is a more accurate diagnostic predictor for NTOS (Jordan and Machleder, 1998; Gelabert et al., 2018). However, lidocaine injection could only lengthen the scalene muscle by 0.8% (Weaver et al., 2019), implying a limited block efficacy. Consistent with this, the optimal dosage of lidocaine (2%, 40 μL) resulted in an approximately 60% reduction in muscle strength in rats. In contrast, a low dose of BTX-A (3 U/kg) blocked more than 80% of muscle contraction, which were almost completely blocked at 6 or 12 U/kg (Fig. 6). Additionally, the application of BTX-A led to significant muscle atrophy in our study, consistent with earlier clinical reports (Dodd et al., 2005; Stone et al., 2011). Therefore, BTX-A might release more scalene space by muscle relaxation or atrophy.

The administration of lidocaine in SMB, such as dosage, concentration, and combination, varies in previous reports. Also, the blocking effect of lidocaine administrated intramuscularly has not been well investigated. According to our data, although the total amount of lidocaine is the same, low concentrations (1%) of lidocaine seem to block more at larger volumes than higher concentrations (2%) at smaller volumes, suggesting that the distribution of lidocaine in muscle affected the blocking efficacy. Specifically, our results showed that the maximum concentration (2% lidocaine) in a large volume (40 μL) led to a prominent muscle block (P = 0.001). Therefore, for the transformation of lidocaine dosage in clinic and to obtain the best block outcome, our study suggests a 2% concentration at a maximum volume that the target tissue could hold without any leakage, which might be determined by ultrasonic guidance, computed tomography guidance, or other.

Fig. 6. Illustration of the comparison of BTX-A and lidocaine in muscle block. A low dosage (3 U/kg) of BTX-A almost completely blocks the muscle contraction by intramuscular injecting, suggesting that it may be more suitable for SMB. However, the optimal dosage of lidocaine only partially blocks the muscle, indicating a possible reason for compromised blocking efficiency for SMB by lidocaine in some cases.
imaging methods (Mashayekh et al., 2011; Bottros et al., 2017; Donahue et al., 2020). Furthermore, the combination of lidocaine with vasoconstrictors (e.g., epinephrine) has been reported to have a longer duration (Collinsworth et al., 1975; Sinnott et al., 2000; Sinnott et al., 2003) although reducing nerve blood flow (Winnie et al., 1977; Myers and Heckman, 1989; Partridge, 1991) and slowing down the clearance of lidocaine from the nerves (Sinnott et al., 2003). Similarly, our experiments showed that the time of full recovery was significantly prolonged. However, there was no increase in the efficacy of combined use in blocking muscle contraction. Moreover, injection of steroid, either separately or in combination with lidocaine (2%), could also be implemented for SMB (Foley et al., 2012; Kim et al., 2016). In contrast, our experiments revealed that steroids did not affect muscle contraction. Surprisingly, the combination of lidocaine with dexamethasone attenuated the blocking efficacy, as did the multipoint injection. In sum, lidocaine can only partially block muscle contraction and cannot increase the blocking effect by combined use or multipoint injection. These findings contributed to the reason why the diagnostic value of lidocaine for SMB was compromised in some clinical cases (Fig. 6).

Lidocaine induced muscle block from 30 minutes and lasted for the first few hours after the injection in rats, consistent with the clinical report of 30 minutes after the administration (Sanders and Annest, 2017). In comparison, BTX-A showed a maximum blockade at 1 day after the injection and maintained for 7 days when the experiment was terminated. We did not check the muscle contraction after that, as in this study, we aimed to compare the blocking efficacy for SMB in diagnosis and find a suitable time point to evaluate the blocking outcome. There are reports that the overall effect of BTX-A could be maintained for 2 to 3 months (Cichon et al., 1995; Dodd et al., 2005).

If given at higher doses, BTX-A should have a better blocking efficiency than lidocaine (Donahue et al., 2020). Clinically, doses of BTX-A used for SMB range from 12 to 30 U (Jordan et al., 2000; Porta, 2000; Finlayson et al., 2011; Benzon et al., 2012; Hlust et al., 2014), and some even reached 80 U (Porta, 2000). Nevertheless, side effects have been reported with high doses of BTX-A, as it can be transported along the axon (Antonacci et al., 2008; Matak et al., 2011; Ramachandran et al., 2012) or the fascia (Shaari et al., 1991), or via endocytosis (Cichon et al., 2016) from the injection site to the surrounding tissues (Yucesoy et al., 2014; Ates and Yucesoy, 2018; Yucesoy and Ates, 2018) and the central nervous system (Yaraskavitch et al., 2008). Our data showed that low, medium, and high dosages of BTX-A all effectively blocked muscle contraction. Given that 3 U/kg BTX-A resulted in a reduction of more than 80% of muscle contraction, a further lower dosage may not achieve the goal of SMB. Also, since 6 and 12 U/kg almost completely blocked the muscle and 12 U/kg already showed gait changes, we did not explore a higher dosage group. Nevertheless, c-SNAP 25 was found to be positive in the surrounding synergistic muscle (EDL) only in the 6 and 12 U/kg groups. In addition, in all three BTX-A groups, BTX-A was found to be retrograde transported along the nerve but was not detected in the spinal cord. In addition, a significant gait change was observed in the 12 U/kg group. Therefore, a low-dose BXT-A is recommended as it has a better muscle blocking ability than lidocaine and without potential side effects, which suggests a new application of BTX-A in clinical transformation.

In summary, our experiments show that lidocaine can partially block muscle contraction, which may be the reason for the compromised diagnostic performance in SMB sometimes. Compared with lidocaine, BTX-A has higher block efficacy and a longer duration of muscle blockage and pharmacologic atrophy. A low dose of BTX-A is sufficient to achieve muscle blocking, and it may be a more appropriate option than lidocaine for SMB in TOS patients.

Authorship Contributions

Participated in research design: Xu, Cao, Cui.
Conducted experiments: Xu, Zhang, Y. Li, Song, Gou, Sun, J. Li.
Contributed new reagents or analytic tools: Xu, Cao, Cui.
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