

Botulinum toxin A, a better choice for skeletal muscle block in a comparative study with lidocaine in rats

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Abbreviations used: BPB, brachial plexus block; BSA, bovine serum albumin; BTX-A, botulinum toxin A; CPN, common peroneal nerve; c-SNAP 25, cleaved synaptosomal associated protein 25; EDL, extensor digitorum longus; NTOS, neurogenic thoracic outlet syndrome; SMB, scalene muscle block; TA, tibialis anterior; TOS, thoracic outlet syndrome.

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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 μ L, 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 U/kg, 6 U/kg, 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 non-specific blockage in the 12 U/kg BTX-A group, but not in the 3 U/kg or 6 U/kg BTX-A group. c-SNAP 25 was stained to test the transportation of BTX-A, and was additionally observed in the peripheral muscles in 6 U/kg 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 spastic muscles, abnormal bones (Kuhn et al., 2015), or narrow space (Sanders and Roos, 1989). The incidence rate of TOS is 3 - 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.

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 neurological 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 predict 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 (Figure S1).

SMB is usually performed with lidocaine which inhibits action potential conduction by blocking Na^+ -voltage-gated channels. However, the rate of misdiagnosis, symptoms persist after surgery in patients with positive SMB, ranges from 38.46% to 86.67% and the rate of missed diagnosis which refers to the symptoms are relieved after surgery in patients with negative SMB varies from 8.14% to 12.82% (Jordan and Machleder, 1998; Jordan et al., 2007b; Lum et al., 2012; Gelabert et al., 2018). These data suggest that the application of lidocaine sometimes fails to predict the response to surgical intervention in SMB. On the other hand, extravasation of lidocaine has been observed, blocking the brachial plexus (Jordan et al., 2000), leading to a false-positive SMB (Sanders and Annest, 2017) and a compromised SMB value in NTOS diagnosis (Bottros et al., 2017). Botulinum toxin A (BTX-A) is another agent applied to block the scalene muscle with marked 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). Besides, BTX-A has also 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 clinical. 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 either in 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 μ L was the maximum injection volume for the TA muscle, and 40 μ L of 2% lidocaine achieved the best muscle block effect among all lidocaine groups, reducing muscle strength by 61% \pm 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 U/kg, 6 U/kg 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 surrounding muscles in high and medium BTX-A dosage groups (6 U/kg and 12 U/kg), but not in the low dosage (3 U/kg). Though BTX-A was observed retrograde transported along the nerve, c-SNAP 25 was not detected in the spinal cord. This study suggests 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, $50 \pm 10\%$ humidity, and 12-H light/dark cycle) with ad libitum access to food and water. The rats were randomly divided into groups for the subsequent experiments. Experimental procedures followed the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. 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 (1:200000, 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 concentrations of 3 U/kg, 6 U/kg, and 12 U/kg, while normal saline (NS) was adopted as the control. After anesthetized with isoflurane (1.8% to 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 sterilized. The needles were marked 3 mm from the tip to ensure that the injections were performed at the center of the target muscle remain within there. As the neuromuscular junction of the TA muscle was distributed across 20% -75% of

the muscles' proximal parts (Osawa, 2008), the drug was administered via intramuscular injection into the middle part of the left TA muscle.

As isoflurane had been reported to have a post-synaptic potentiation effect on neuromuscular blockade (Kumar et al., 1996; Vanlinthout 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, im), the latter one only functioned 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 maintained at ~26°C. The rats then were mounted in the experimental set-up (701C stimulator, 300D-305C force transducer, 806D test apparatus, 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 (Figure 6). Ankle movement torque, an indicator of TA muscle contraction, was measured with a pedal. Following 2 twitches (1 mA, 0.2 ms), the muscles were stimulated by a series of tetanic pulses (1 mA, 400 ms) at 20 Hz, 40 Hz, 50 Hz, 60 Hz, 80 Hz, and 100 Hz with an interval of 1 min. Subsequently, the muscles were rested for 2 min before being stimulated with 3 tetanic

pulses (1 mA, 400 ms, 100 Hz) at 1 min intervals on average. For the maximum injection volume of the TA muscle, all muscles were stimulated 3 times (1 mA, 400 ms, 100 Hz) with an interval of 1 min after methylene blue injections.

For lidocaine treatment measurements, data were collected before and at 30 min, 1 H, 1.5 H, 2 H, 2.5 H, 3 H, 6 H, 12 H, 18 H and 1-7 day after the drug injection. For BTX-A administration, measurements were performed before and at 30 min, 1 H, 6 H, 12 H, 18 H 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 experiment, animals were trained to run in 3 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 30min, 1H, 2H and 3H after the lidocaine injection. Each rat was placed on the treadmill repeatedly at intervals of at least 5 min to complete three uninterrupted runs (containing at least 8 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 phosphate-buffered saline (pH 7.4) (PBS) transcardially followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). The left TA muscle, left extensor digitorum longus (EDL) muscle, left CPN, and the lumbosacral spinal cord (L4-L5) were harvested for analysis. Tissues were post-fixed for 12 H in 4% PFA and stored in a 10%-30% sucrose gradient in 0.1 M phosphate buffer at 4°C. Afterward, the tissues were cut into slices of 10 µm, 12 µm, and 40 µm thickness, respectively. Tissue sections were washed and then incubated in PBS with 5% bovine serum albumin (BSA) and 1% TritonX-100 for 1 H at room temperature. Subsequently, the sections were incubated with primary antibodies diluted in PBS with 2% BSA and 3% TritonX-100 overnight at 4°C. Specifically, the TA muscle and CPN sections were incubated with mouse polyclonal antibodies against BTX-A-cleaved-SNAP 25 (c-SNAP 25, 1:100; GeneTex, GTX39119) and rabbit polyclonal antibodies against NF-L (1:100; Cell Signaling Technology, C28E10). The primary antibodies for the spinal cord section samples were mouse polyclonal antibodies against BTX-A-cleaved-SNAP 25 (c-SNAP 25, 1:100; GeneTex, GTX39119) and rabbit polyclonal antibodies against NeuN (1:500; Abcam, 24307S). Bound antibodies were detected by Alexa Fluor546 donkey anti-rabbit antibodies (1:500; Invitrogen, A10040) and Alexa

Fluor488 donkey anti-mouse antibodies (1:500; Invitrogen, Carlsbad, Calif., 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 (NIH, USA). The presence of c-SNAP 25 was indicated by the ratio of the intensity of c-SNAP 25 normalized to that of NF-L.

Statistical analysis

All experimental values were presented as mean \pm SD. Fisher's exact test was used to identify differences in the maximum injection volume experiments. The Kruskal-Wallis test was used to evaluate the differences in the lidocaine block groups and the contact times between the left hind paw and the glass. Pearson correlation analysis was implemented to analyze the impacts of concentration and volume of lidocaine injection on its potency. Two-way ANOVA was used to compare the percentage decline in each group when maximum efficacy was achieved and to analyze the muscle wet weights as well as the pawprint areas. One-way ANOVA was used to compare the differences in the intensity of c-SNAP 25 staining of TA muscles and CPN in the BTX-A groups, and Tamhane's T2 test for EDL muscles. For all the tests and analyses herein, $P < 0.05$ was considered statistically significant. All statistical analysis were performed using SPSS (IBM®, Ver. 23).

Results

Effects of different concentrations and volumes of lidocaine on muscle block

To improve the block efficiency of lidocaine on the TA muscle, the maximum dose that can be injected into the TA muscle was firstly 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 μL or 50 μL of 1% methylene blue. After three tetanic stimulation sessions, extravasation was examined on the skin, subcutaneously or intramuscularly (Figure 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, Figure 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%-2% (Jordan et al., 2000; Lum et al., 2012; Weaver et al., 2019), 0.5%, 1% and 2% lidocaine at the volumes of 20 μL 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 (Figure 1C, D) and the integration (Figure 1C, E) dropped at 30 min after lidocaine administration, followed by a stable increase until fully returning to the normal level approximately 2 H 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 $61\% \pm 14\%$ in maximum strength and $72\% \pm 13\%$ 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 (Figure 1C-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 (Figure 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 $\mu\text{g}/\text{mL}$), epinephrine (1:200000, 5 $\mu\text{g}/\text{mL}$), 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. As shown, injection of epinephrine could lead to a decrease in muscle strength ($20\% \pm 7\%$), while the combination did not enhance the

block efficacy of lidocaine ($P = 0.504$, Two-way ANOVA, Figure 2A, B, 3C), even though the combination prolonged the recovery of muscle strength and integration (Figure 2A, 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 (Figure 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, Figure 2A, B, 3C), especially at 30 min after the injection. The multipoint injection was found to be less effective than a single injection when the total administered volume of lidocaine was the same ($P = 0.010$, Two-way, Figure 2A, B, 3C). Therefore, drug combination or multipoint injection has no contribution to enhancing the muscle block efficacy of lidocaine.

Effects of BTX-A and its comparison 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 U/kg, 6 U/kg and 12 U/kg BTX-A was administered 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 H after the drug injection. All three groups reached a stable blocking level at 1 day after the administration (Figure 3A, B) and continued for 7 days when the experiment was ended. Aside from the significant reduction of the maximum strengths (a reduction of $82\% \pm 16\%$ in the 3 U/kg group, and almost complete loss in the other two groups) 1 day after injection

(Figure 3A), there was also a decrease in integration (Figure 3B). However, no difference was observed among the three BTX-A groups in terms of maximum strength or integration (Figure 3C). 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, im) in rats, which had an antagonist action at N-methyl-D-aspartate (NMDA) 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 (Figure S2).

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 μ L) alone or combined with epinephrine (1:200000, 5 μ g/mL) by measuring muscle strength. The maximum strength was collected at 30 min after lidocaine injection, while at 1 day for BTX-A (Figure 3C). The BTX-A group showed a further decrease in maximum intensity compared to the optimal lidocaine dose (2%, 40 μ L)

used alone or in combination (Figure 3C). In addition, the efficacy of lidocaine, either given alone or combined with epinephrine, could not last for 1 day after the injection (Figure 3 A, 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

Then, to evaluate muscle block under physiological conditions, the gait of the rats was recorded consistently. Parameters related to the gait changes were compared 30 min after injection of lidocaine (2%, 40 μ L) and 1 day after the BTX-A injection. As shown in Figure 3D, rats receiving lidocaine had comparable paw-to-ground contact to control rat (2.20 cm^2 vs 2.45 cm^2 , 1.16 cm^2 vs 1.21 cm^2 , respectively) when fully placed (0.2 s) or lifting up (0.5 s). However, for 12 U/kg BTX-A injected rat, the areas were just about 1.71 cm^2 (Figure 3D), which indicated a delayed heel rising (Figure 3E). Besides, the left hind toes showed adduction (2.20 cm^2 vs 1.79 cm^2) and the left hind foot showed prolonged contact with the ground ($P = 0.017$, Kruskal-Wallis, Figure 3F). These results suggest that the gastrocnemius muscle, which is responsible for heel rising, was also paralyzed without BTX-A injection. However, such non-specific blockage did not occur in the 3 U/kg or 6 U/kg BTX-A group (Figure 3F). Therefore, BTX-A at the concentrations of 3 U/kg 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 (Figure 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, Figure 4B). Furthermore, the weights of the TA muscles in 6 U/kg and 3 U/kg were all decreased, and no difference was observed among the three groups (Figure 4B). However, BXT-A did not significantly affect the wet weights of the EDL muscles in these groups (Figure 4B).

c-SNAP 25, a product after processing with activated BTX-A (Antonucci et al., 2008; Caleo et al., 2009; Koizumi et al., 2014) 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 U/kg and 12 U/kg BTX-A groups, but negative in the EDL muscle in the 3U/kg group (Figure 4C, D). Considering the long distance of transportation, the common peroneal nerve and the spinal cord (L4, 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 (Figure 5A, B). However, no c-SNAP 25 was observed in spinal cord segments (Figure 5C). Altogether, these results suggest that 3 U/kg BTX-A administration for muscle block does not affect EDL muscle or spinal cord.

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 the retrograde transport of BTX-A along the nerves. Compared with lidocaine, BTX-A showed a higher muscle block efficacy and longer blockage duration than lidocaine.

Currently, there are no available animal models to mimic the pathological process of TOS. However, a quantification of the scalene 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, the symptoms relieve in SMB should be achieved through muscle relaxation, but not analgesic effects of 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 made 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 scalene muscle, or because of lidocaine leaking from the scalene 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., 2012; 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 non-polarizing muscle relaxants, such as mivacurium, pancuronium and atracurium (Kumar et al., 1996; Vanlinthout et al., 1996; Sutcliffe 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 to these clearly post-synaptic effects, BTX exerts its effect at the presynaptic terminal, which might 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) (Figure S1). 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 μ L) 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 almost completely been blocked at 6 U/kg or 12 U/kg (Figure 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, though 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 μ L) 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, CT guidance or other 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) though 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, so did the multipoint injection. To sum up, lidocaine can only partially block muscle contraction and cannot increase the blocking effect by combined use or multipoint injection. All of the above findings contributed to the reason why the diagnostic value of lidocaine for SMB was compromised in some clinical cases (Figure 6).

Lidocaine induced muscle block from 30 min and lasted for the first few hours after the injection in rats, consistent with the clinical report of 30 min after the administration (Sanders and Anest, 2017). On the other hand, 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 move on to check the muscle contraction after that, as in this study, we aimed to compare the blocking efficiency 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; Hulst 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 (Antonucci et al., 2008; Matak et al., 2011; Ramachandran et al., 2015) or the fascia (Shaari et al., 1991), or via endocytosis (Bomba-Warczak et al., 2016) from the injection site to the surrounding tissues (Yucesoy et al., 2012; Ateş and Yucesoy, 2014; Ateş and Yucesoy, 2018; Yucesoy and Ateş, 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 might not achieve the goal of SMB. Also, since 6 U/kg 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 U/kg 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. Besides, 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, it has been shown in our experiments that lidocaine can partially block muscle contraction, which might be the reason for the compromised diagnostic performance in SMB sometimes. Compared with lidocaine, BTX-A has higher block efficacy, 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 for SMB in TOS patients than lidocaine.

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None.

Authorship Contributions

Participated in research design: Xu K., Cao R. and Cui S.

Conducted experiments: Xu K., Zhang Z., Li Y.Y., Song L., Gou J., Sun C., and Li J.Y.

Contributed new reagents or analytic tools: Xu K., Cao R. and Cui S.

Performed data analysis: Xu K. and Du S.

Wrote or contributed to the writing of the manuscript: Xu K., Cao R. and Cui S.

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Footnotes

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Reprint requests

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Keywords: lidocaine, botulinum toxin A, muscle block, thoracic outlet syndrome, diagnosis

Figure legends

Figure 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) Statistic analysis of leakage cases after intramuscular injection of 40 μ L or 50 μ L. 2 out of 13 cases leaked in 40 μ L, and 12 out of 13 cases in 50 μ L. 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 μ L or 40 μ L, respectively. (D) Statistic of the maximum strength related to the initial value. The percentage changed with time, and the maximum strength significantly decreased at 30 min, and recovered at 2 H. 2% lidocaine in 40 μ L showed the most blockage efficiency. $n = 3$ in the saline group and $n = 5$ in other groups. (E) Statistic of the percentage of area under the strength-time curve (integration), which showed the same tendency as the maximum strength. (F) Statistic analysis of the block percentage of 2% lidocaine in 40 μ L stimulated at frequencies of 20 Hz, 40 Hz, 50 Hz, 60 Hz 80 Hz 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.

Figure 2. Effects of lidocaine in combination or multipoint injection on muscle contraction block.

(A) Statistic analysis of muscle strength blocking after lidocaine injected alone, combined with epinephrine (E, 1: 200,000, 5 μ g/mL) or dexamethasone (DXM, 1.28 mg/mL), or multipoint injection at the indicated time. The muscle was stimulated at 100 Hz

after the administration. $n = 5$. (B) Statistic of strength-time curve area (integration) of muscle stimulated at 100 Hz in each group. (C) Images of the hind limb after the injection of lidocaine alone, combined with dexamethasone or epinephrine. White circles, the ischemic areas lacking blood supply. Scale bar, 1 cm.

Figure 3. Comparison of the effects of lidocaine and BTX-A on muscle contraction block. (A, B) Statistic of percentages of maximum strength (A) and integration (B) after the administration of BTX-A at the concentration of 3 U/kg, 6 U/kg, 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 H 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 7th day, the end of the experiment. (C) Statistical analysis of the maximum reduction of strength or integration between 2% lidocaine in 40 μ L and 3 U/kg BTX-A, also 6 U/kg and 12 U/kg. The block efficacy of 3 U/kg BTX-A was significantly higher than 2% lidocaine in 40 μ 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 s) or lift up (lower panels, 0.5 s) (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).

Figure 4. Effects of BTX-A on surrounding muscles. (A) Images of the TA and the EDL muscle in normal saline, 2% lidocaine in 40 μ L, 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 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 μ m. (D) Quantification of c-SNAP 25 staining in TA muscle and EDL muscle ($**P = 0.004$, $***P < 0.001$, Tamhane's T2 test, $n = 4$)

Figure 5. Effects of BTX-A on innervating nerve and corresponding spinal cords. (A) Representative images of the longitudinal sections of the common peroneal nerve (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.

Figure 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.

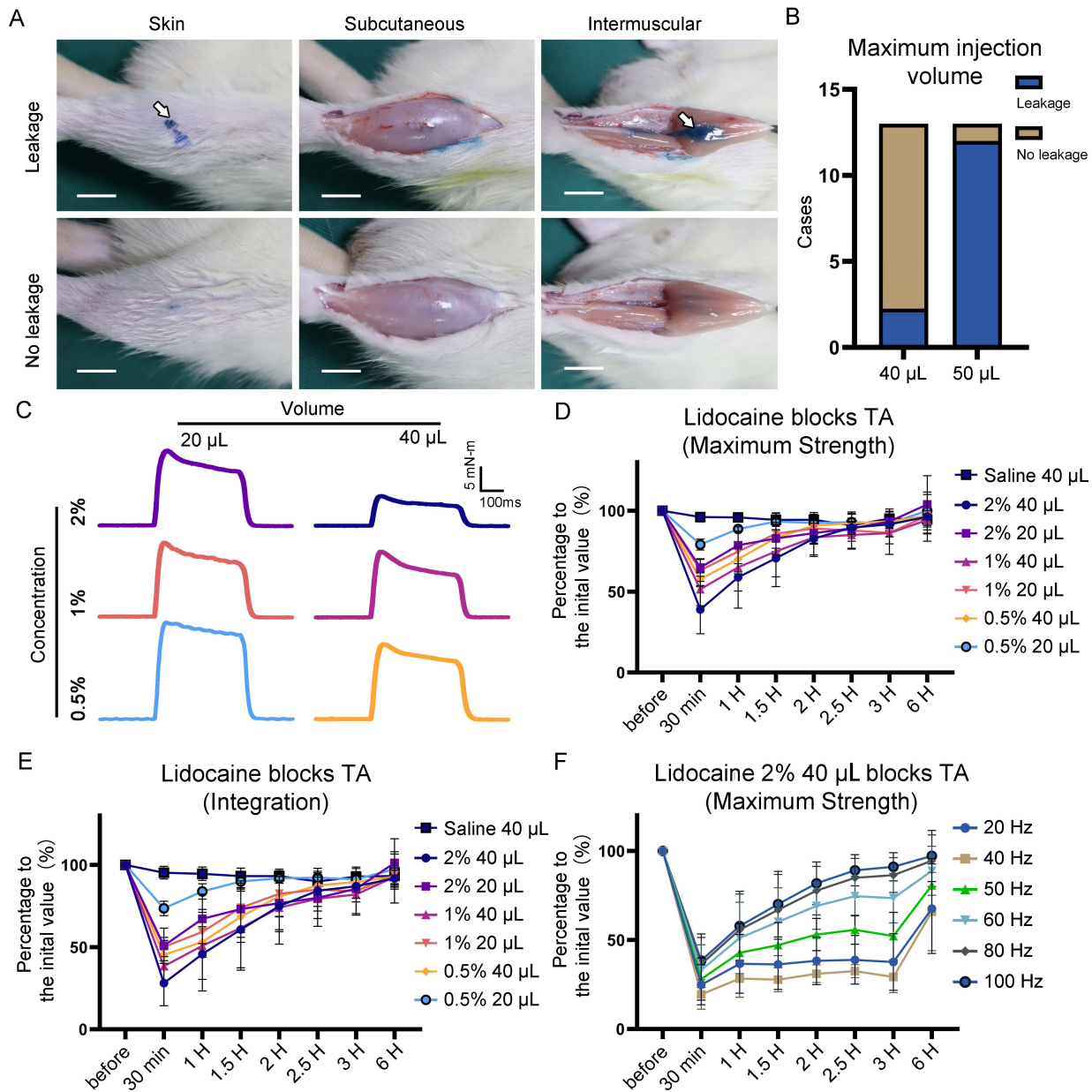


Figure 1

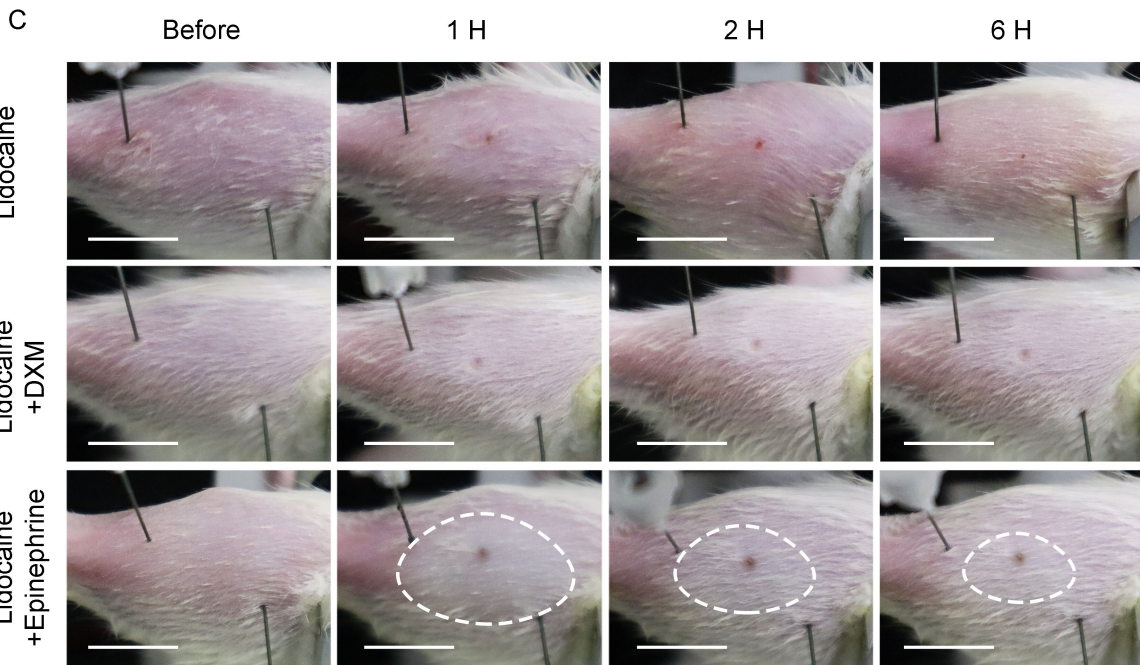
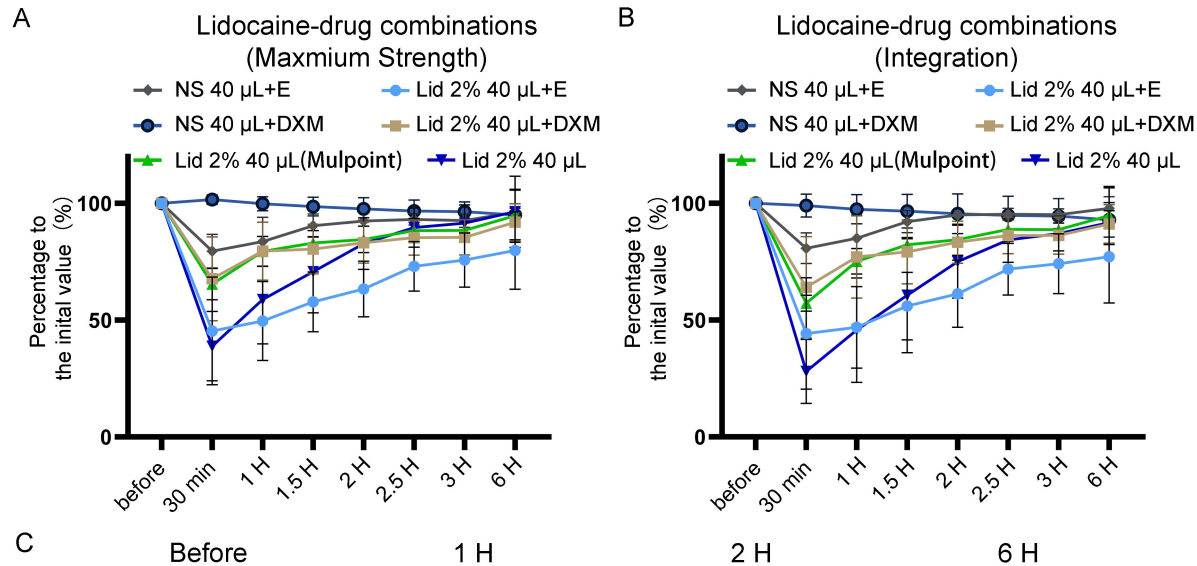


Figure 2

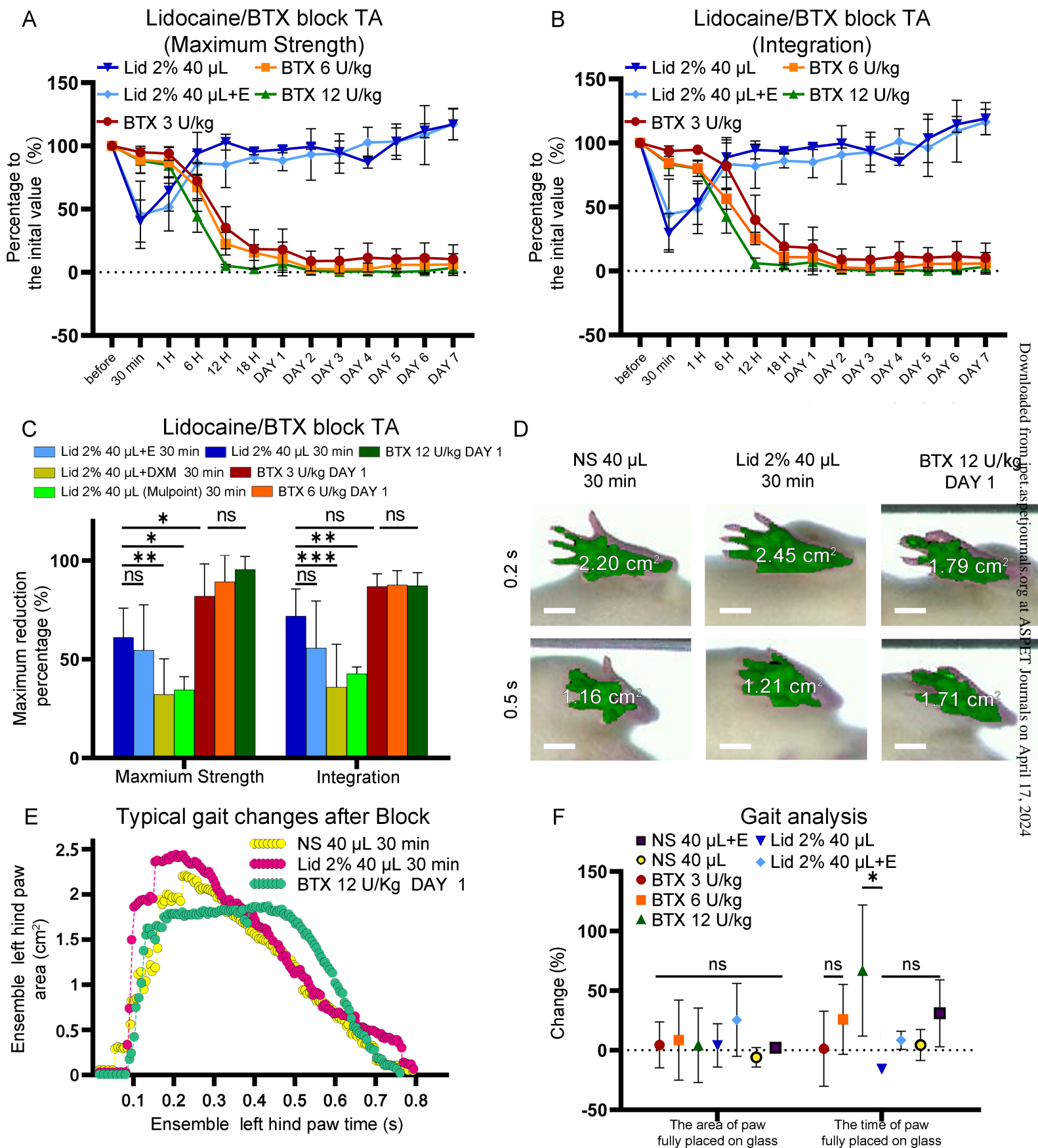


Figure 3

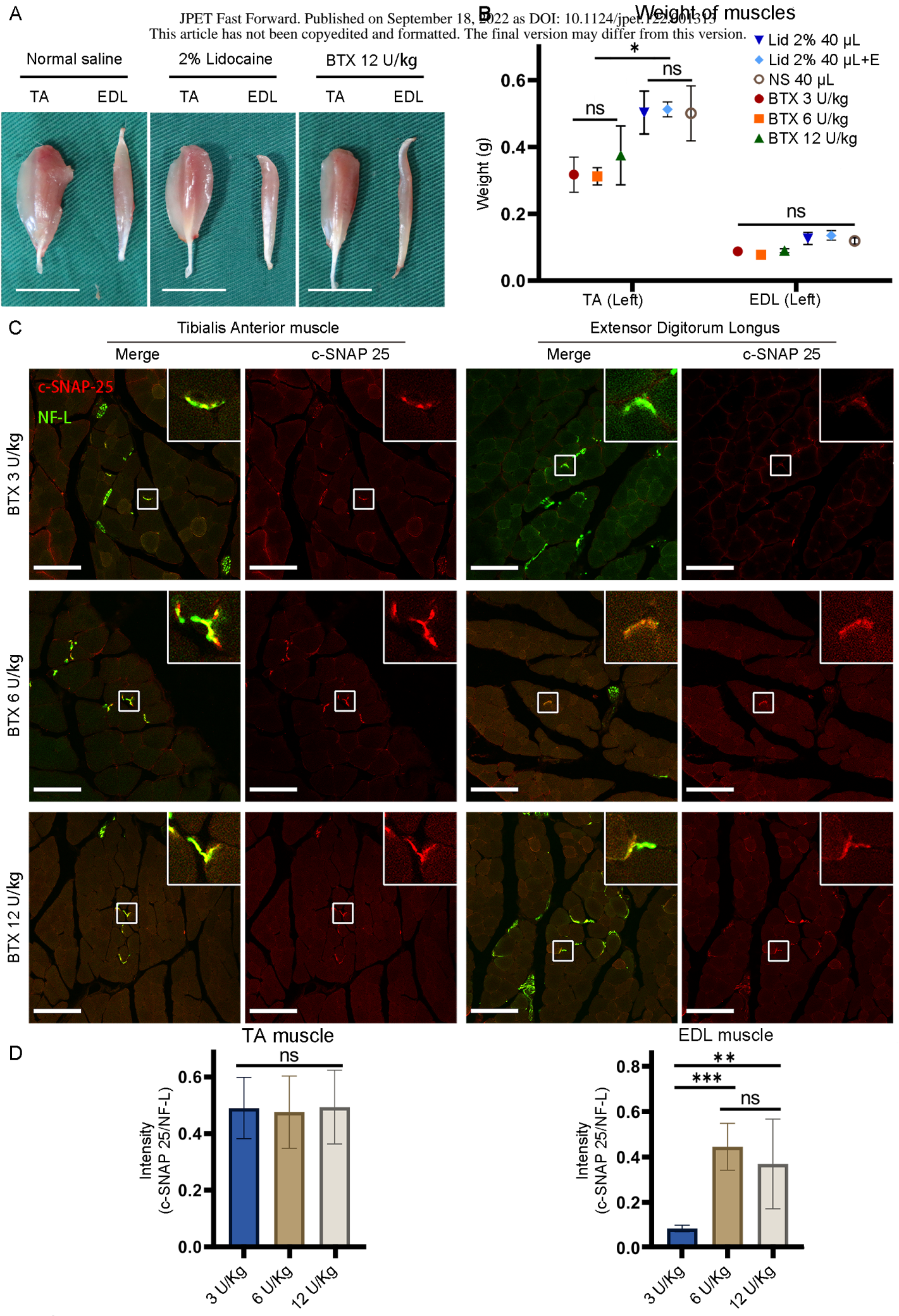


Figure 4

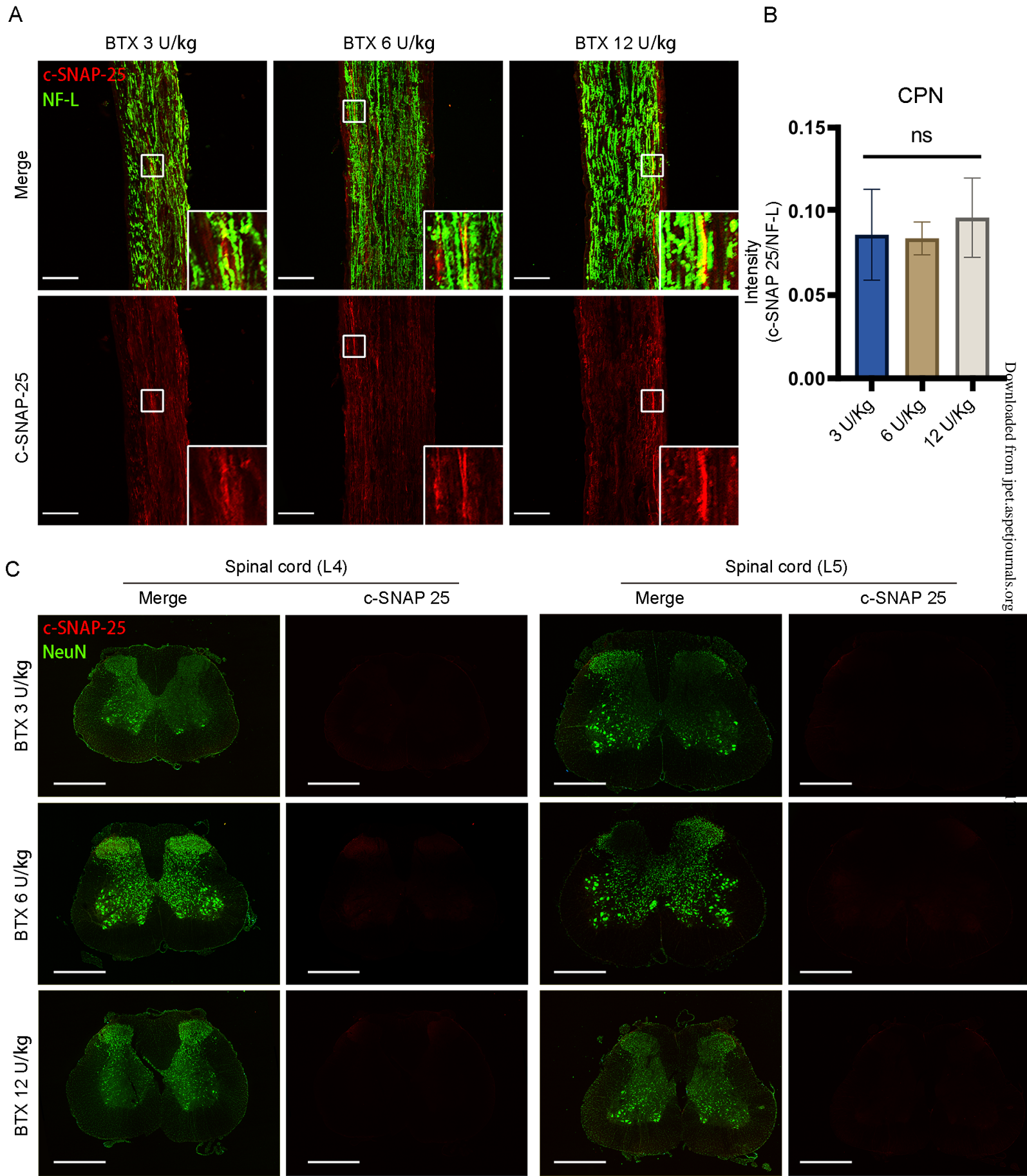


Figure 5

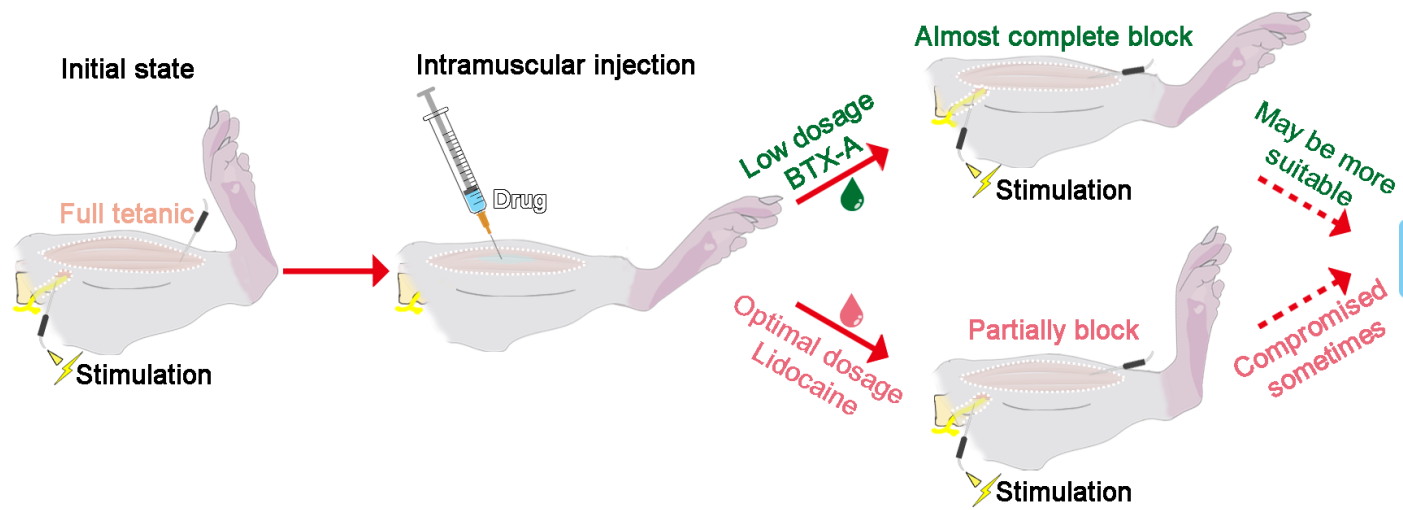


Figure 6

Supplemental Data

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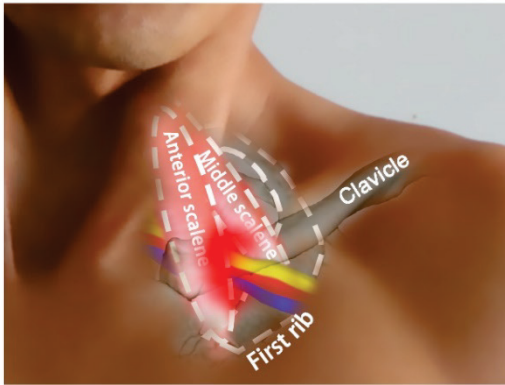
JPET-AR-2022-001313

**Botulinum toxin A, a better choice for skeletal muscle block in a
comparative study with lidocaine in rats**

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Schematic of TOS



Schematic of SMB

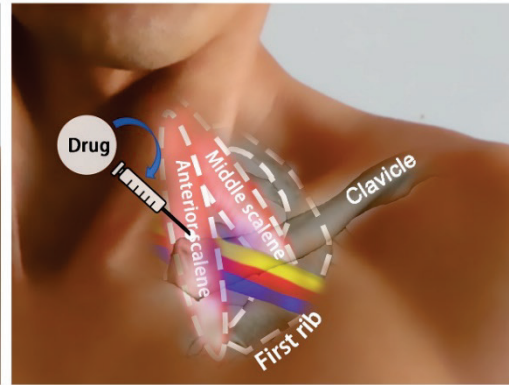


Figure S1. The schematic of SMB. The drug injected into the scalene muscle relaxes the affected muscle and the first rib sinks, which frees the compressed nerves and vessels. The diagnosis of thoracic outlet syndrome could be confirmed by the relief of symptoms in the ipsilateral upper limb.

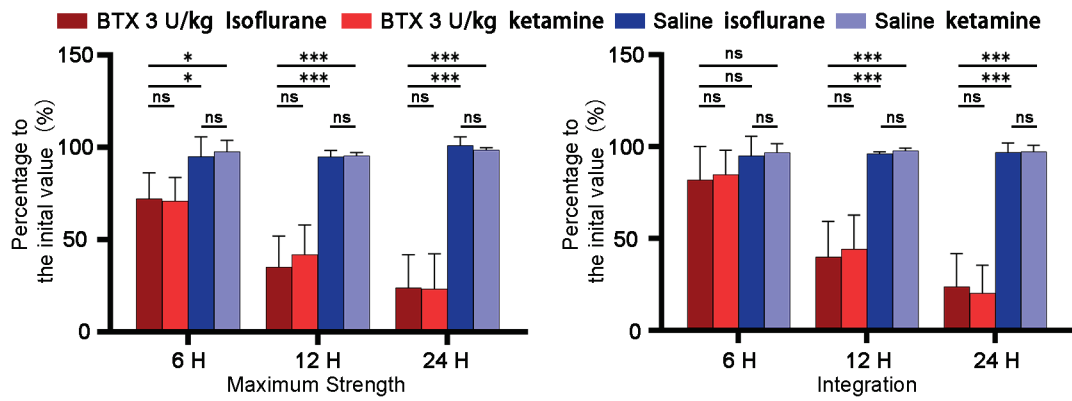


Figure S2. Effects of isoflurane and ketamine on BTX-A in muscle contraction blocking. Statistical analysis of the maximum reduction of strength or integration after BTX-A (3 U/kg) administration under the anesthesia of isoflurane or ketamine. The saline was performed as control. *P < 0.05, *** P < 0.001, One-way ANOVA. n = 5.