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ORIGINAL ARTICLE

Dynamic muscle paralytic effects of a novel botulinum toxin A free of neurotoxin-associated proteins

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Abstract

Structurally, botulinum toxin type A (BTX-A) is composed of neurotoxin and nontoxic complexing proteins (CPs), and the neurotoxin has the function of blocking acetylcholine release from the neuromuscular junction and therefore paralyzing muscles. Nowadays, a novel botulinum toxin A free of CPs (chinbotulinumtoxin A, A/Chin) is produced, and the present study comprehensively evaluated the dynamic paralytic effect of A/Chin on the gastrocnemius muscle of rats. Different doses (0.01, 0.1, 0.5, 1, 2, and 4 U) of A/Chin and other BTX-As with and without CPs were administered to the gastrocnemius muscles of rats and muscle strength was measured and compared at different postinjection timepoints (from day 0 to 84). With the dose increased, time-to-peak paralytic effect of other BTX-As varied from day 3 to day 14, while A/Chin groups showed rapid and steady time to peak on day 3. At the lowest dose of 0.01 U, A/Chin showed significantly better peak paralytic effect than the others on day 3. When the dose increased to 0.5 U and more, A/Chin group also showed significant paralytic effect when the paralytic effect of other BTX-As was worn off. Moreover, the paralytic effect of A/Chin was confirmed as muscle atrophy while hematoxylin–eosin staining was performed. In conclusion, compared with other BTX-As, A/Chin showed rapid and steady time-to-peak paralytic effect and long-term paralytic efficacy at the same dose level. And it might lay a solid foundation for further wide application of A/Chin in both clinical and cosmetic areas.

KEYWORDS
botulinum toxin A, chinbotulinumtoxin A, complex proteins, muscle strength

Abbreviations: A/Abo, abobotulinumtoxinA; A/Chin, chinbotulinumtoxinA; A/Inco, incobotulinumtoxinA; A/Ona, onabotulinumtoxinA; BTX, botulinum toxin; CPs, complexing proteins; HA, hemagglutinin.

Wu-chao Liu and Jun-hui Su contributed equally to this work.

1 | INTRODUCTION

Botulinum toxin (BTX) is a biological toxin isolated from *Clostridium botulinum*. At present, there are seven different serotypes of BTXs (A, B, C1, D, E, F, G).[1] Among the BTX-A formulations, onabotulinumtoxinA (A/Ona) was first used by ophthalmologists to treat strabismus in 1973. Since the introduction of A/Ona, other BTX-A formulations such as abobotulinumtoxinA (A/Abo), incobotulinumtoxinA (A/Inco), and lanbotulinumtoxin (A/Lan) are being widely used in therapeutic and anesthetic areas.[2]

BTX-A consists of neurotoxin and nontoxic complexing proteins (CPs). The neurotoxin part comprises a heavy chain (HC, 100 kDa) and a light chain (LC, 50 kDa). The HC helps translocate the LC into the nerve terminal, while the LC acts on the soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE protein), leading to defect in releasing the acetylcholine from the presynaptic terminal.[3] The CPs consist of hemagglutinin (HA) and nontoxic non-hemagglutinin components (NTNHA). However, the function of CPs in clinical BTX-A use is still under exploration. According to varying degrees of CPs, A/Ona and A/Lan are formulated as an ~900 kDa BTX-A complex, A/Abo is formulated as an~400 kDa BTX-A complex, and A/Inco is the only BTX-A formulated as a core 150 kDa neurotoxin devoid of CPs.[4]

Compared to other BTX-A formulations, A/Inco has a longer shelf life (3 years compared to 2 years of other BTX-As) and fewer restrictions for storage temperature (25°C compared to 0°C of other BTX-As).[5] In clinical use, the efficacy of A/Inco is comparable to A/Ona in treatment of dystonia with the dose equivalence of 1:1.[6,7] Nowadays, another BTX-A free of CPs—chinbotulinumtoxinA (A/Chin) is successfully produced by the Lanzhou Institute of Biological Products, and a preliminary study found that A/Chin is superior in inducing muscle paralysis and inflammation stimulation.[8] However, more detailed paralytic effect of A/Chin and its difference from other BTX-As are still needed exploration. This study aimed to investigate the dynamic muscle paralytic effect of A/Chin and other BTX-As with and without CPs based on an assay of gastrocnemius muscle strength in rats.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Sprague Dawley rats (220–240 g) from B&K Universal Group Limited were used in this study. The animals were handled in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication NO 85–23, revised 1996) and the Policy of Animal Care and Use Committee of Tongji University. The rats were fed with chow and water at the Animal Center of Shanghai Tongji Hospital, Tongji University School of Medicine, under proper temperature (25°C) and a 12 h light/dark cycle. In addition, this study was approved by the Animal Care and Use Committee of Tongji University (approval number SYXK (hu) 2014–0026). This experiment was in accordance with the UK Animals (Scientific Procedures) Act 1986 and relevant guidelines.

2.2 | Study design and injections

Rats were gently anesthetized using an intraperitoneal injection of pentobarbital (30 mg/kg) and randomly administered either A/Chin (Lanzhou Institute of Biological Products; $n = 30$), A/Ona (Allergan, Irvine, California; $n = 30$), A/Lan (Lanzhou Institute of Biological Products; $n = 30$), A/Inco (Merz Pharmaceuticals GmbH; $n = 30$), or normal saline (NS, $n = 5$) via injection into the right gastrocnemius muscle. Rats receiving the BTX-A injection were randomly allocated into six groups with different doses (0.01 U, 0.1 U, 0.5 U, 1 U, 2 U, and 4 U), with five rats in each group. Different doses of BTX-A were reconstituted in 100 μl 0.9% NS, thus, the final concentrations were 0.01 U/100 μl, 0.1 U/100 μl, 0.5 U/100 μl, 1 U/100 μl, 2 U/100 μl, and 4 U/100 μl. Each rat in the BTX-A group received 100 μl of the BTX-A injection into the right gastrocnemius muscle, whereas rats in the NS group were injected with an equivalent volume of 0.9% NS.

2.3 | Muscle strength measurement

The paralytic effect of the BTX-As on right gastrocnemius muscle of the rats was measured by a survey system (CN102599921A) involving fixing equipment, sensing means and data handling software as previously described.[8] Under sciatic nerve stimulation (28 V over 0.4 ms), the gastrocnemius contraction that caused plantar flexion and footboard rotation was converted to electrical signals through muscular tension energy transducer. Because the sensing means we developed were designed according to the principle of the scale, gram (g) was used as the unit. The reduction of muscle strength induced by 0.1μg BTX-A could be detected with this equipment. Using a software (MFLab3.01, Shanghai Jia Long Educational Instrument Factory), the muscle strength of the right gastrocnemius muscle was measured at day 0 (baseline), 3, 7, 14, 21, 28, 42, 56, 70, and 84 postinjection. And researcher carrying out the muscle measurements was blinded for the injected dose and kind of BTX-A.

2.4 | Hematoxylin–eosin (HE) staining

After 2 U BTX-As or NS injection for the 28 days, rats were sacrificed by cervical dislocation. The right gastrocnemius muscle was harvested for atrophy evaluation. The muscles were fixed in a 4% formalin solution for 1 day and dehydrated in 20% sucrose for 1 day and 30% sucrose for 2 days. Then, they were frozen in liquid nitrogen-cooled isopentane and cut into sections with a cryotome. Thereafter, the frozen-sectioned muscle tissues were dyed in a Harris hematoxylin solution for 15 min and gently washed with ddH_2O for 10 min. The muscles were dyed in eosin solution for

10 min, and then the floating color was removed gently with ddH_2O. Next, gradient alcohol was used for dehydration, and the muscles were dipped in xylene I for 15 min and then xylene II for another 15 min. At last, slides containing muscle tissues were mounted with neutral gum and observed.

2.5 | Statistical analysis

Normally distributed data were expressed as the mean value ± standard error (SE). Analysis of variance was used to compare the muscle strength at each timepoint. Tukey test was used for multiple comparison of the lowest muscle strength among different doses of A/Chin groups on day 3. In addition, at each dose level, the difference of muscle strength between different BTX-A groups and NS group, the difference of muscle strength between A/Chin group and other BTX-A groups were compared through Dunnett test when the variances were homogenous. And the Games–Howell test was used when the variances were not homogenous. All data analyses were performed using SPSS version 21.0 (SPSS Inc.). And p value <.05 was considered for statistical significance.

3 | RESULTS

3.1 | Dynamic paralytic effects of different doses of A/Chin on gastrocnemius muscle strength

A dose–time–effect curve was plotted according to the muscle strength at different timepoints to visualize the trend of dynamic paralytic effects of different doses of A/Chin on the right gastrocnemius muscles of the rats (Figure 1). At baseline, there was no significant difference in muscle strength between different A/Chin groups and the NS group ($p > .05$). On day 3, the gastrocnemius muscle strength was significantly reduced to the lowest value in all A/Chin groups, and it was significantly lower that the NS group ($p < .05$). The decrease in the muscle strength plateaued when the dose was increased to 0.5 U and more, with significantly decreased muscle strength was still noted on day 70 compared with NS group ($p < .05$). Until day 56, the muscle strength of rats injected with 0.01 U A/Chin recovered to the NS level ($p > .05$), whereas the 4 U group still showed a significant reduction (66.9% of the NS group) on day 84 after A/Chin injection ($p < .01$) (Figure 1).

3.2 | Paralytic effect of different BTX-A formulations on gastrocnemius muscle strength

The paralytic effect of different BTX-A formulations on muscle strength was then explored according to different doses (0.01 U, 0.1 U, 0.5 U, 1 U, 2 U, and 4 U) (Table 1, Table S1–S5). With the dose increased, time-to-peak paralytic effect of other BTX-A groups varied from day 3 to day 14, while all doses of A/Chin showed rapid time-to-peak paralytic effect on day 3. For the regular dose of 1 U, the lowest muscle strength was noted only in the A/Chin group on day 3, whereas the others were on day 7 or day 14. Subsequently, muscle strength started to increase. On day 70, all BTX-A groups still had reduced muscle strength compared with NS group, but A/Chin group showed significantly lower muscle strength than other BTX-A groups ($p < .05$). Thereafter, on day 84, while other BTX-A groups had their muscle strength recovered to the NS group level, only A/Chin group had relatively lower muscle strength which was significantly lower than that of A/Lan and A/Inco group ($p < .05$) (Table 1).

At the lowest dose of 0.01 U, all BTX-A groups had lowest muscle strength on day 3, and A/Chin showed significantly lower values than the other three BTX-A groups ($p < .001$). Muscle strength started to increase thereafter. On day 42, only the A/Chin and A/Inco groups showed significant changes in terms of paralytic effects compared to the NS group ($p < .05$), and all BTX-A groups had recovered to the NS group level at the next timepoint (Figure 2).

At the highest dose of 4 U, the lowest muscle strength was noted only in the A/Chin group on day 3, whereas it was noted in the other three BTX-A groups on day 7. From day 7 to day 70, muscle strength was significantly reduced in all BTX-A groups ($p < .05$), and no

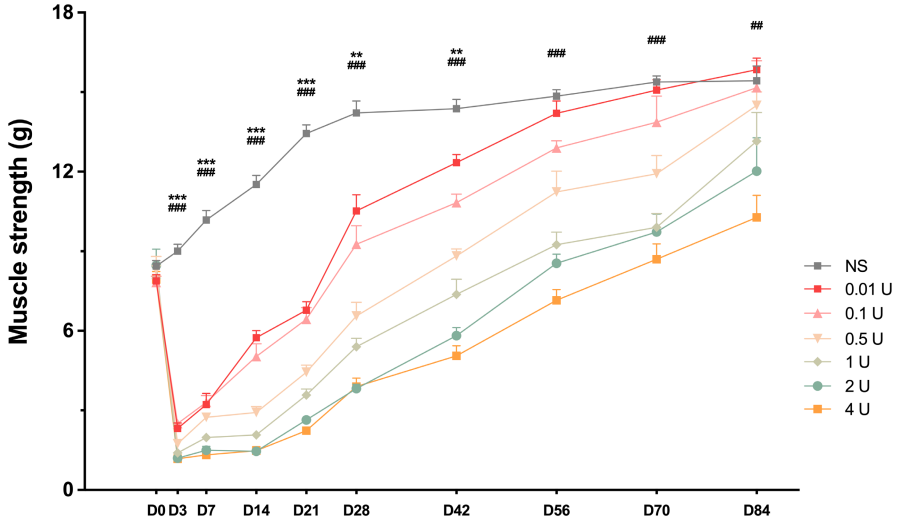

FIGURE 1 Dynamic paralytic effects of different doses of A/Chin on the gastrocnemius muscle strength of rats. $^{**}p < .01$ and $^{***}p < .001$ for 0.01 U A/Chin versus the NS group. $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$ for 4 U A/Chin versus the NS group. All data points represent mean ± SE

TABLE 1 Comparison of right gastrocnemius muscle strength of rats after different BTX-As injection at a dose of 1 U

	Gastrocnemius muscle strength, Mean ± SE, g								
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28	Day 42	Day 56	Day 70	Day 84
A/Chin	8.0 ± 0.2	1.4 ± 0.1***	2.0 ± 0.1***	2.1 ± 0.1***	3.6 ± 0.2***	5.4 ± 0.3***	7.4 ± 0.6***	9.3 ± 0.5***	9.9 ± 0.5***	13.2 ± 1.0
A/Lan	7.1 ± 0.4*	1.9 ± 0.3***	1.6 ± 0.1***	1.8 ± 0.2***	2.5 ± 0.1$^{***,\#}$	4.4 ± 0.4***	7.7 ± 0.4***	9.2 ± 0.5***	12.2 ± 0.2$^{**,\#}$	15.9 ± 0.2$^{\#}$
A/Ona	8.2 ± 0.4	2.5 ± 0.3$^{***,\#}$	2.1 ± 0.1***	2.0 ± 0.1***	3.1 ± 0.2***	4.7 ± 0.2***	6.7 ± 0.4***	9.2 ± 0.5***	12.2 ± 0.4$^{**,\#}$	15.5 ± 0.2
A/Inco	8.3 ± 0.4	2.7 ± 0.2$^{***,\#\#}$	2.3 ± 0.1***	2.1 ± 0.2***	3.4 ± 0.2***	4.3 ± 0.2***	7.2 ± 0.4***	9.5 ± 0.2***	12.6 ± 1.0$^{**,\#\#}$	15.8 ± 0.5$^{\#}$
NS	8.4 ± 0.2	9.0 ± 0.3$^{\#\#\#}$	10.2 ± 0.4$^{\#\#\#}$	11.5 ± 0.3$^{\#\#\#}$	13.4 ± 0.3$^{\#\#\#}$	14.2 ± 0.4$^{\#\#\#}$	14.4 ± 0.3$^{\#\#\#}$	14.9 ± 0.2$^{\#\#\#}$	15.4 ± 0.2$^{\#\#\#}$	15.4 ± 0.6

Abbreviations: BTX-A, botulinum toxin type A; NS, normal saline; SE, standard error.
$^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus the NS group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ versus the A/Chin group.

difference was found between the A/Chin and other BTX-A groups ($p > .05$). On day 84, only the A/Chin group showed significant paralytic effects on muscle strength compared to the NS group ($p < .01$). Moreover, A/Chin group showed significantly lower muscle strength than other BTX-As groups (Figure 2).

3.3 | Muscle atrophy of gastrocnemius muscle after injection of A/Chin and other BTX-As

In addition to the muscle strength measurement of rats under different BTX-As injection, muscle atrophy was also evaluated to show the effect of A/Chin on the muscular morphology. Based on the former findings of the present study that the longest time to peak paralytic time for all BTX-As was day 14 and the decrease in the muscle strength of A/Chin plateaued when the dose was increased to 0.5 U and more, the dose of 2 U and the evaluation day of 28 were chosen for muscle atrophy evaluation. On day 28, HE staining showed that all BTX-As displayed obviously widened space between muscle fibers, decreased volume, and increased nuclei of muscle fibers (Figure 3). Moreover, A/Chin showed similar extent muscle atrophy as the other BTX-As, and it was in accordance with the muscle strength findings of 2 U BTX-As on day 28.

4 | DISCUSSION

This study explored the dynamic paralytic effects of different doses of A/Chin and other BTX-A formulations. After an intramuscular injection to the right gastrocnemius muscle, a significant reduction in muscle strength was noted in all BTX-A groups. A/Chin showed rapid and steady time-to-peak paralytic effect and long-term paralytic efficacy at the same dose level compared to the others. Moreover, the muscle paralytic effect of A/Chin and other BTX-As was further confirmed as muscle atrophy through HE staining.

The paralytic effect of different doses of A/Chin on the gastrocnemius muscle was demonstrated by a relatively intact dynamic dose–time–effect curve where all doses administered to the A/Chin group resulted in the lowest muscle strength at day 3, indicating a rapid time to peak. In a previous study,[9] the time to peak of 0.5 U A/Ona was seen at day 7, which was similar to our A/Ona group and longer than that of A/Chin. In addition, the significant paralytic effect of 0.5 U A/Ona was maintained till day 70, which to some extent was consistent with our results. In our study, at a dose of 0.5 U, A/Ona group also showed insignificant lower muscle strength than NS group, and only A/Chin showed a significant constant paralytic effect at day 70. In a recent study, the muscle strength at day 7 and 28 was significantly lower with A/Chin at a dose of 0.5 U than other BTX-As. And no significant difference was found at a dose of 2 U, indicating that the dose of 0.5 U could differentiate the real paralytic effect of different BTX-A formulations without the ceiling effect. In addition, the paralytic effect of different BTX-As (at doses of 0.5 and 2 U) returned to the control level at day 84.[8] In the present study, we

added more doses and evaluation timepoints. At the dose of 0.5 U, we did not find a difference on muscle strength between A/Chin and other BTX-A groups on day 7. However, we found that the time to peak was rapid and steady for A/Chin on day 3. And at a lower dose of 0.01 U, we found the paralytic effect was more robust for A/Chin compared with other BTX-As on day 3. In addition, we also found that the paralytic effect of all BTX-A groups with doses of 0.5 and 2 U returned to the normal level on day 84. However, when the dose increased to 4 U, only A/Chin showed constant paralytic effect on day 84. Combined these results, we might conclude that A/Chin had superior potency than the other BTX-As. And this might relate to its more robust effect on gene expression involved in neuromuscular junction stabilization and muscle genesis.[8]

Although both A/Chin and A/Inco are free of CPs, our study showed that A/Chin had shorter time to peak and longer efficacy than A/Inco. A recent study also compared the local paresis and chemodenervation efficacy of another novel BTX-A free of CPs with A/Ona and A/Inco. Through digit abduction sore (DAS) and compound muscle action potential (CMAP) assays, the results showed that the novel neurotoxin also had superior efficacy and duration of action

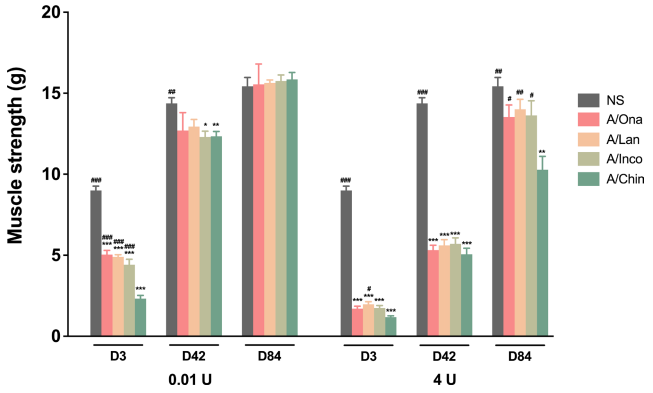

FIGURE 2 Right gastrocnemius muscle strength of rats after different doses of BTX-As injection at three key timepoints. $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ versus the NS group. $^{\#}p < .05$, $^{\#\#}p < .01$ and $^{\#\#\#}p < .001$ versus the 0.01 U A/Chin group. All columns represent mean ± SE

than A/Inco.[10] Since the introduction of A/Inco, the interchangeability of A/Ona and A/Inco is still under debate. A recent study found A/Ona had greater biological activity than A/Inco through both in vitro (light-chain activity high-performance liquid chromatography and cell-based potency assay) and in vivo (CMAP and DAS) assays.[4] And another study showed that under the detection assay of the A/Ona potency reference standard, the average potency of A/Inco only ranged from 69–78 U per vial, which was lower than the labeled 100 U per vial.[11] However, other studies have found that there were no differences between A/Ona and A/Inco potency with a 1:1 conversion ratio. In a preclinical study with the LD_{50} method, Dressler et al. found that the biological potency of A/Ona ranged from 96.6 to 111.0 U, while A/Inco ranged from 99.0 to 114.6 U.[12] In addition, in clinic, it is reported the efficacy of A/Inco is comparable to A/Ona in treatment of cervical dystonia and blepharospasm with the dose equivalence of 1:1.[6,7] However, the mouse LD_{50} method and manufacture processes for every BTX-A formulation are different, and the interchangeability of different BTX-As could not be clearly made. In our study, we used conversion ratio of 1:1 for these BTX-As, and this might be the reason for the different efficacy of A/Chin and other BTX-As.

With regard to the function of CPs on paralytic efficacy, CPs consist of HA which can compromise the E-cadherin-mediated intercellular barrier and facilitate the paracellular absorption of BTX-A from the digestive tract,[13] and NTNHA which can regulate a complex assembly depending on the pH level and can protect the complex in the gastrointestinal tract.[14,15] However, when BTX-A is injected into the targeted tissue, CPs do not seem to be necessary because in a study, a 150 kDa neurotoxin was rapidly released from the complex with a half-life of <1 min at pH >7.0.[16] Except from this, CPs played a vital role in the antibody-induced failure of the BTX therapy, probably via increasing the BTX-A antigenicity.[17] A study also showed that the independent risk factors for neutralizing the antibody-induced complete secondary treatment failure included switching between A/Ona and other BTX-A formulations (except A/Inco), indicating A/Inco might have less chance of secondary treatment failure.[18] Moreover, a recent study found significant long-lasting improvement after switch to A/Inco for cervical dystonia patients with

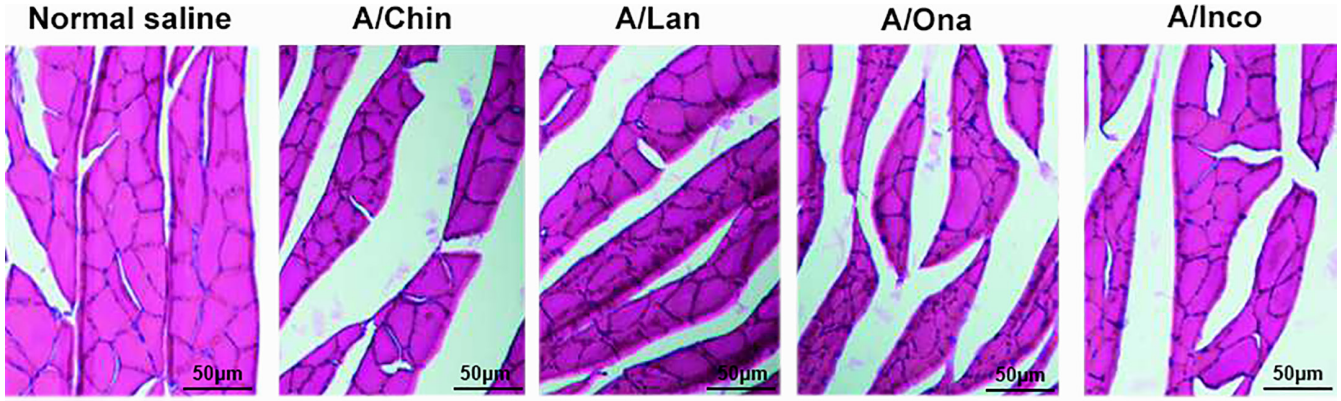

FIGURE 3 Hematoxylin–eosin staining of gastrocnemius muscle after injection of 2 U A/Chin and other BTX-As for 28 days

secondary treatment failure.[19] In addtion to the CPs, the excipients in different BTX-As might be the reason for the enhancement of paralytic efficacy, and studies had reported that the stabilizer such as human serum albumin could result in enhanced BTX-A activity in the mLD50 assay.[12,20,21]

There are some limations in this study. Firstly, although we tested a wide range of doses from 0.01 to 4 U (approximately between 0.04 U/kg and 18 U/kg body weight), due to the high sensitivity of the survey system for muscle strength, the median effective dose of BTX-As was not acertained in our study. Secondly, there was an incremental increase in muscle strength observed in the NS group during the first four weeks, which indicated that the target muscles were still actively developing. Although the paralytic effect of BTX-A could be explained as more robust even with the muscle strength naturally increasing, the actual effectivity of BTX-As could still be affected, and study should be designed with the muscle strength consistent in the NS group throuthgout the evaluation period in the future.

5 | CONCLUSION

Our study clarified the dynamic paralytic effect of the novel A/Chin free of CPs and provided evidence for neurotoxic potency, thus laying a solid foundation for its wide application in both clinical and cosmetic usage in the near future.

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DISCLOSURE

The authors have declared that no any conflicts interest exists.

ETHICAL APPROVAL

This study was approved by the Animal Care and Use Committee of Tongji University (approval number SYXK (hu) 2014-0026). And this experiment was in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

AUTHOR CONTRIBUTIONS

WCL: Study design, data collection, drafting of the article; JHS: Data collection and analysis, drafting of the article; YF: Data collection; XRX, LZP, YL, LM, ZYN: Revising the article for scientific and intellectual content; XPZ: Idea initiation, study design, article revising; LJJ: Idea initiation, study design, article revising, final approval of the version to be published. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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