

Intramuscular Nerve Distribution in Mimetic Muscles: An Anatomical Study to Guide BoNT-A Injection

Distribución de Nervios Intramusculares en Músculos Mímicos: Un Estudio Anatómico para Guiar la Inyección de BoNT-A

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ZHANG, J.; MA, X. J.; XU, F. & YANG, S. B. Intramuscular nerve distribution in mimetic muscles: An anatomical study to guide BoNT-A injection. *Int. J. Morphol.*, 44(4):1122-1128, 2026.

SUMMARY: The precise targets of botulinum toxin type A (BoNT-A) for the treatment of hemifacial spasm (HFS) and facial-cervical wrinkle reduction remain unclear. This study aimed to elucidate the location and area of the intramuscular nerve dense region (INDR) in each mimetic muscle innervated by the facial nerve, and to use them as precise targets for BoNT-A injection. Mimetic muscles obtained from 12 formalin-fixed adult Chinese cadavers (male:female = 1:1) were subjected to modified Sihler's staining to demonstrate the whole-mount intramuscular nerve branching distribution pattern. The location and area of the INDRs were measured and identified for each muscle. The locations of the INDRs varied among the mimetic muscles: medial part of the middle muscle belly for the frontal belly of the occipitofrontalis; middle part of the upper and lower eyelids and lateral canthus level for the orbicularis oculi; middle muscle belly for the zygomaticus major, levator labii superioris, risorius, depressor anguli oris, depressor labii inferioris, and mentalis; middle-lower muscle belly for the zygomaticus minor; junction of the middle and upper thirds for the nasalis; junction of the middle and lower thirds for the levator labii superioris alaeque nasi; for the orbicularis oris, upper part at the midpoint of the oral commissure-philtrum line and lower part just below the oral commissure; and upper muscle belly for the platysma. The area of the INDR in the above-mentioned mimetic muscles varied in size, but no statistically significant differences were observed between the left and right sides or between males and females ($P > 0.05$). The delineation of the location and area of these INDRs can provide guidance for targeted BoNT-A injection, thereby ensuring therapeutic efficacy while reducing the risk of complications.

KEY WORDS: Mimetic muscles; Intramuscular nerve; Sihler's staining; Botulinum toxin A; Target localization.

INTRODUCTION

HFS is often secondary to stroke, tumor, or inflammation (e.g., facial neuritis) but may also be idiopathic, with an incidence of approximately 11/100,000 (Chaudhry *et al.*, 2015). Its typical clinical manifestation is paroxysmal, involuntary twitching of the muscles on one side of the face, often starting in the orbicularis oculi muscle and gradually spreading to the cheek and oral commissure. In severe cases, the platysma may also be involved, leading to social impairment and reduced quality of life (Tambasco *et al.*, 2021). BoNT-A inhibits muscle excitation by blocking presynaptic acetylcholine release at motor endplates and has become the first-line treatment for HFS. Its efficacy and safety are highly dependent on precise injection into motor endplate bands. The INDRs correspond to the distribution of motor endplate bands and represent the ideal injection

targets (Tang *et al.*, 2018). However, clinical practice lacks systematic knowledge of the intramuscular innervation of the mimetic muscles. Injections often rely on surface landmarks and empirical strategies, frequently resulting in suboptimal efficacy, short duration of effect, and complications such as ptosis and facial stiffness (Hong, 2023). Studies have shown that a 0.5 cm deviation of the injection point from the motor endplate band can reduce efficacy by 50 % (Parratte *et al.*, 2002). Therefore, identifying the INDRs of each mimetic muscle is critical for targeted injection. In addition to treating HFS, BoNT-A is widely used in aesthetic wrinkle reduction for glabellar lines, crow's feet, and platysmal bands, where precise injection also requires a clear map of intramuscular nerve distribution (Small, 2014; Yi *et al.*, 2026).

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FUNDING. This study was supported by the National Natural Science Foundation of China, Grant No. 32260217.

Traditional anatomical methods cannot fully visualize intramuscular nerves. The modified Sihler's staining technique clearly delineates these nerves and is the gold standard for studying their distribution. To date, this technique has been applied only to individual mimetic muscles (Han *et al.*, 2025; Piao *et al.*, 2026); no systematic study exists on the entire mimetic muscle group, and no nerve atlas covering both HFS treatment and aesthetic wrinkle reduction has been reported. Therefore, this study aimed to use modified Sihler's staining to comprehensively visualize the intramuscular nerves of the mimetic muscles, systematically localize each INDR, and quantify its area. The goal was to provide direct anatomical targets for precise BoNT-A injection, facilitate the shift from empirical to targeted injection practices, improve efficacy, reduce complication risks, and advance the precision medicine transformation of BoNT-A clinical applications.

MATERIAL AND METHOD

Specimens and ethics. Twelve formalin-fixed Chinese adult cadavers (six males, six females) without neuromuscular disease or facial deformities, aged 35–75 years (mean 57.7 ± 11.5 years), were collected. This study followed the ethical principles of the Declaration of Helsinki. The specimens were from voluntary donors, and their collection and use were approved by the Ethics Committee of Zunyi Medical University (#2022-1-003).

Gross anatomy. The specimens were placed in a supine position with the head slightly turned to the opposite side. The epicranial aponeurosis was transected at the hairline as the upper boundary. An incision was made from the anterior auricular fold at the front of the tragus downward to the earlobe, curving around the earlobe to the mastoid tip, and then extending along the inferior border of the mandible to a point 2 cm lateral to the oral commissure. The skin and subcutaneous tissue were incised, and the flap was reflected anterior to the parotid gland and masseter muscle to expose the superficial musculoaponeurotic system (SMAS), which continued downward as the platysma muscle. The deep surface of the SMAS was the parotideomasseteric fascia. Below the inferior border of the mandible, the platysma muscle was separated from the overlying subcutaneous tissue, its lower edge was transected, and the muscle was reflected upward. From the anterior border of the parotid gland, the facial nerve branches (temporal, zygomatic, buccal, marginal mandibular, and cervical) were carefully separated by blunt dissection, and each branch entered the corresponding mimetic muscle in a fan-shaped manner. The origins of the zygomaticus major, zygomaticus minor, and other muscles were detached along the inferior border of the zygomatic bone; the depressor anguli oris and depressor labii inferioris were detached along the

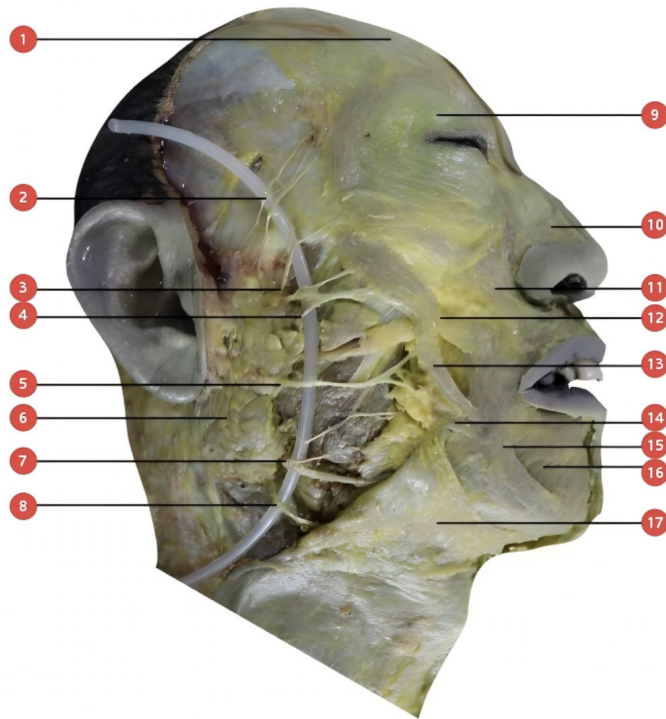
inferior border of the mandible. The mimetic muscles were sharply separated from the superficial surface of the masseter muscle. The frontal belly of the occipitofrontalis, orbicularis oculi, zygomaticus major and minor, perioral muscles, and depressor anguli oris were lifted from the bony surface and parotid gland and connected with the reflected platysma to form one intact specimen.

Modified Sihler's staining: The harvested intact muscle specimens underwent the modified Sihler's staining procedure (Ji *et al.*, 2025). They were first rinsed under running tap water for 30 min to remove the formalin. They were then placed in a maceration solution containing a mixture of KOH and H₂O₂ for 2-3 weeks; during this process, the thinner parts were supported to prevent over-maceration and damage. Once the entire muscle specimens became semi-translucent, they were rinsed under running water for 30 min. The specimens were then decalcified in Sihler's I solution (1 part glacial acetic acid, 2 parts glycerin, 12 parts chloral hydrate) for 2-3 weeks. After the shrunken muscle blocks had relaxed and expanded again, they were rinsed under running water for 30 min and then placed in Sihler's II solution (1 part Ehrlich's hematoxylin, 2 parts glycerin, 12 parts chloral hydrate) for staining for 4 weeks. When the specimens had completely sunk in the staining container and appeared uniformly dark blue-black, they were placed back in Sihler's I solution for destaining for 2-18 h. The ideal destaining endpoint was when the nerve branches were black and the muscles were light purple. After destaining, the specimens were neutralized in 0.05 % lithium carbonate solution for 2 hours and finally cleared in graded glycerin (40 %, 60 %, 80 %, and 100 %) for 4 weeks. Finally, the intramuscular nerve distribution was observed on an X-ray film viewer, and schematic diagrams were drawn.

Statistical analysis. Data were processed using SPSS 29.0 software (IBM Corp., Armonk, NY, USA) and expressed as mean ± standard deviation ($\bar{x} \pm s$). Because the data conformed to a normal distribution, comparisons between left and right sides were performed using a paired *t*-test, and comparisons between males and females were performed using an independent samples *t*-test. A *P*-value < 0.05 was considered statistically significant.

RESULTS

Gross anatomical observations. After emerging from the parotid gland, the facial nerve clearly divided into branches: the temporal branch innervating the frontal belly of the occipitofrontalis and the upper part of the orbicularis oculi; the zygomatic branch innervating the lower part of the orbicularis oculi and the zygomaticus major; the buccal branch, which had the most subdivisions, innervating the



zygomaticus major, zygomaticus minor, risorius, and the perioral muscles; the marginal mandibular branch innervating the depressor anguli oris, depressor labii inferioris, and mentalis; and the cervical branch innervating the platysma (Fig. 1).

Sihler's staining findings. The intramuscular nerves of the mimetic muscles exhibited a non-uniform distribution. The location, number, and area of the INDRs varied among muscles. The specific locations and areas were shown in Fig. 2 and Table I. Comparisons between left and right sides and between males and females showed no statistically significant differences ($P > 0.05$; Table II).

Fig. 1. Gross anatomy of the mimetic muscles and the facial nerve. 1 = frontal belly of the occipitofrontalis muscle; 2 = temporal branch; 3 = zygomatic branch; 4 = superior buccal branch; 5 = inferior buccal branch; 6 = parotid gland; 7 = marginal mandibular branch; 8 = cervical branch; 9 = orbicularis oculi muscle; 10 = nasalis muscle; 11 = levator labii superioris alaeque nasi muscle; 12 = zygomaticus minor muscle; 13 = zygomaticus major muscle; 14 = risorius muscle; 15 = depressor anguli oris muscle; 16 = depressor labii inferioris muscle; 17 = platysma muscle.

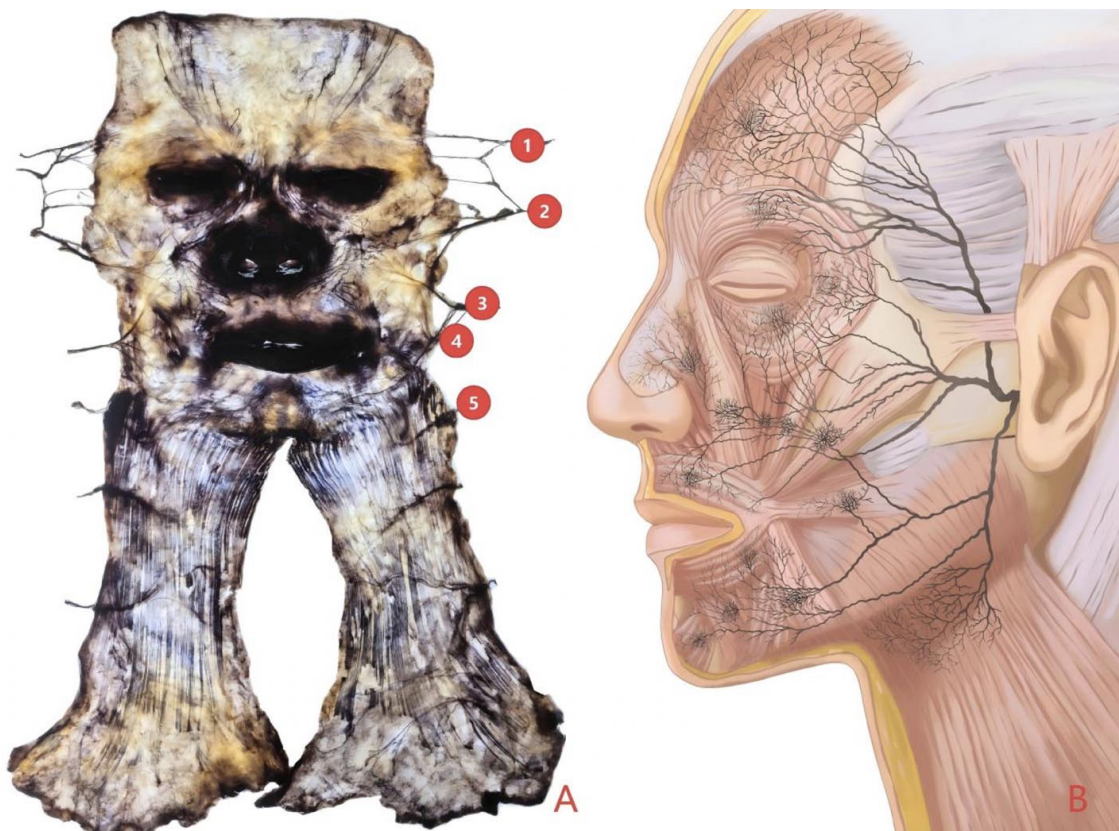


Fig. 2. Sihler's staining of the mimetic muscles in the facial nerve territory and the intramuscular locations of the INDRs. A: Sihler's staining showing: 1 = temporal branch; 2 = zygomatic branch; 3 = buccal branch; 4 = marginal mandibular branch; 5 = cervical branch. B: Schematic diagram of the intramuscular positions of the INDRs.

Table I. Location and area of INDR in each muscle.

INDR in muscle	Location	Area (cm ²)
Frontal belly of occipitofrontalis	Medial part of the middle muscle belly	0.88±0.11
Upper part of orbicularis oculi	Middle part of the upper eyelid	0.39±0.05
Lower part of orbicularis oculi (1)	Middle part of the lower eyelid	0.40±0.05
Lower part of orbicularis oculi (2)	Lateral canthus level	0.70±0.08
Zygomaticus major	Middle muscle belly	0.25±0.03
Zygomaticus minor	Middle-lower muscle belly	0.10±0.01
Levator labii superioris	Middle muscle belly	0.17±0.02
Levator labii superioris alaeque nasi	Junction of the middle and lower thirds of the muscle belly	0.23±0.02
Risorius	Middle muscle belly	0.17±0.02
Nasalis	Junction of the middle and upper thirds of the muscle belly	0.15±0.01
Upper part of orbicularis oris	Midpoint of the oral commissure-philtrum line	0.21±0.02
Lower part of orbicularis oris	Inferior to the oral commissure	0.35±0.03
Depressor anguli oris	Middle muscle belly	0.44±0.05
Depressor labii inferioris	Middle muscle belly	0.29±0.03
Mentalis	Middle muscle belly	0.13±0.01
Platysma	Upper part of muscle belly	2.03±0.18

DISCUSSION

Both HFS and facial dynamic wrinkles are caused by overactivity of the mimetic muscles. BoNT-A, by blocking presynaptic acetylcholine release at the neuromuscular junction, has become the first-line treatment for HFS and a preferred method for aesthetic wrinkle reduction (Nittari *et al.*, 2025). Studies have reported a total effective rate as high as 95 % (Singh, 2013), but the incidence of adverse reactions is approximately 16 %–18 %, including common complications such as ptosis, deviation of the oral commissure, and facial stiffness (Khatkova *et al.*, 2026). The core problem is imprecise injection targeting. Although the motor endplate band is theoretically the optimal target, its specific location in each mimetic muscle remains unclear, and clinical practice has long relied on surface landmarks for empirical injection (Vartanian & Dayan, 2004). Therefore, based on the correspondence between the INDRs and the motor endplate bands, establishing an anatomical framework for precise injection using INDR has clear clinical value. By using modified Sihler's staining, this study systematically located the INDRs in each mimetic muscle, providing a direct basis for targeted injection.

The muscles for which injection sites align with commonly used clinical targets include the frontal belly of the occipitofrontalis, zygomaticus minor, levator labii superioris, levator labii superioris alaeque nasi, risorius, depressor anguli oris, depressor labii inferioris, mentalis, and platysma (Carruthers *et al.*, 2013; Costa *et al.*, 2022; Hong, 2023; Hwang *et al.*, 2009; Maas *et al.*, 2012), allowing

traditional positioning methods to continue being used. The muscles requiring adjustment are as follows:

Orbicularis oculi – Clinically, it is typically injected at a single point 1.5–2.0 cm lateral to the lateral canthus (Flynn *et al.*, 2001). However, the results of this study indicate that the INDRs of the orbicularis oculi are located not only at the level of the lateral canthus but also in the middle regions of the upper and lower eyelids. Therefore, a single-point injection may yield insufficient efficacy. If the dose is excessive or the injection is administered too superiorly, it may easily diffuse to the levator palpebrae superioris, resulting in ptosis (Basar & Arici, 2016). It is recommended to inject at the middle of the upper eyelid, the middle of the lower eyelid, and the level of the lateral canthus, respectively, while strictly controlling the dose.

Zygomaticus major – Clinically, it is often injected into the upper-middle portion of the muscle belly (Maytharakcheep *et al.*, 2024). The results of this study reveal that the INDR is solely located in the middle portion of the muscle belly, with minimal distribution in the upper part. Therefore, it is recommended to target the middle portion of the muscle belly beneath the zygomatic body.

Nasalis – Clinically, it is frequently injected in the middle of the muscle belly (de Maio *et al.*, 2017). The results of this study demonstrate that the INDR is situated at the junction of the middle and upper thirds of the muscle belly, suggesting that the center of the INDR should be located above the lateral crus of the alar cartilage.

Table II. Comparison of INDR areas between left and right sides and between males and females.

INDR in muscle	INDR Areas (cm ²)					
	Left(n=12)	Right(n=12)	t	P	Male(n=6)	Female(n=6)
Frontal belly of occipitofrontalis	0.88±0.12	0.89±0.11	1.21	0.25	0.90±0.12	0.87±0.11
Upper part of orbicularis oculi	0.38±0.06	0.39±0.05	-0.49	0.63	0.39±0.05	0.38±0.06
Lower part of orbicularis oculi (1)	0.39±0.06	0.40±0.05	-0.47	0.66	0.41±0.05	0.39±0.06
Lower part of orbicularis oculi (2)	0.70±0.09	0.70±0.08	-0.52	0.62	0.71±0.10	0.69±0.08
Zygomaticus major	0.25±0.03	0.25±0.02	-1.58	0.18	0.25±0.03	0.24±0.02
Zygomaticus minor	0.11±0.01	0.10±0.01	0.55	0.60	0.11±0.01	0.10±0.01
Levator labii superioris	0.17±0.02	0.17±0.01	0.39	0.70	0.17±0.02	0.16±0.01
Levator labii superioris alaeque nasi	0.23±0.02	0.23±0.02	1.12	0.32	0.23±0.02	0.22±0.02
Risorius	0.17±0.02	0.17±0.01	0.56	0.59	0.18±0.02	0.17±0.02
Nasalis	0.15±0.02	0.15±0.01	-0.67	0.53	0.15±0.02	0.14±0.01
Upper part of orbicularis oris	0.21±0.02	0.21±0.01	0.58	0.57	0.21±0.03	0.20±0.02
Lower part of orbicularis oris	0.35±0.04	0.35±0.03	0.79	0.45	0.36±0.03	0.34±0.03
Depressor anguli oris	0.44±0.05	0.44±0.04	0.62	0.55	0.46±0.05	0.43±0.04
Depressor labii inferioris	0.29±0.03	0.28±0.02	0.84	0.42	0.30±0.03	0.28±0.03
Mentalis	0.13±0.02	0.13±0.01	0.31	0.76	0.14±0.02	0.13±0.01
Platysma	2.04±0.20	2.02±0.17	0.67	0.51	2.07±0.19	1.99±0.17

Orbicularis oris – Clinically, the orbicularis oris is often injected at the upper and lower edges lateral to the oral commissure (Kongsaengdao *et al.*, 2021). The results of this study indicate that the INDRs are located at the midpoint of the line connecting the oral commissure and the philtrum (upper lip) and below the oral commissure (lower lip), implying that the injection should be positioned at the upper and lower lips, respectively.

It has been reported that 1 U of BoNT-A can infiltrate an area of 1.5–3 cm² (Li *et al.*, 2021). Combined with the INDR areas of each muscle provided in this study (Table I), the “minimum effective dose” can be calculated. For example, the INDR area of the upper part of the orbicularis oculi is only 0.39 cm², giving a recommended dose of 0.15–0.30 U, which is far lower than the traditional 2.5 U per point (Johnson & Chen, 2023), reflecting a dose-sparing advantage and reduced cost. Recommended doses for other muscles can be calculated individually according to the infiltration pattern of BoNT-A and the INDR areas provided in this paper. The comparison of INDR areas between left and right sides and between males and females showed no statistically significant differences, indicating that target locations and baseline doses do not need to be adjusted for laterality or sex, which is conducive to standardized promotion.

This study employed an en bloc, mask-like gross dissection combined with modified Sihler’s staining, which completely preserved the anatomical structure of the “SMAS–mimetic muscle–facial nerve complex”. The INDR map generated from this study can not only guide BoNT-A injection but also provide neural navigation for procedures such as rhytidectomy and parotid surgery. Limitations of this study include the fact that the specimens were formalin-fixed, and tissue shrinkage may have affected the measurement of INDR areas; in addition, the sample size was relatively small. Nevertheless, overall, this study provides a clinically applicable anatomical basis for targeted BoNT-A injection.

CONCLUSION

In summary, the INDR locations and areas of the facial-nerve-innervated mimetic muscles delineated in this study can provide morphological guidance for achieving targeted BoNT-A injection, ensuring efficacy while reducing the risk of complications.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China, Grant No. 32260217. The authors sincerely thank all body donors and their families

who donated their bodies for anatomical research. Their selfless dedication was fundamental to the completion of this study. Findings from anatomical research based on body donation will continue to enrich the global medical knowledge system and ultimately benefit patients worldwide.

ZHANG, J.; MA, X. J.; XU, F. & YANG, S. B. Distribución de nervios intramusculares en músculos mímicos: Un estudio anatómico para guiar la inyección de BoNT-A. *Int. J. Morphol.*, 44(4):1122-1128, 2026.

RESUMEN: Los objetivos precisos de la toxina botulínica tipo A (BoNT-A) para el tratamiento del espasmo hemifacial (EHF) y la reducción de arrugas faciales y cervicales aún no están claros. Este estudio tuvo como objetivo dilucidar la ubicación y el área de la región densa de nervios intramusculares (RDNI) en cada músculo mímico inervado por el nervio facial, y utilizarlas como objetivos precisos para la inyección de BoNT-A. Se sometieron músculos mímicos obtenidos de 12 cadáveres adultos chinos fijados en formalina (hombre:mujer = 1:1) a la tinción de Sihler modificada para demostrar el patrón de distribución de ramificación de los nervios intramusculares en la preparación completa. Se midió e identificó la ubicación y el área de los INDR para cada músculo. Las ubicaciones de los INDR variaron entre los músculos mímicos: parte medial del vientre muscular medio para el vientre frontal del músculo occipitofrontal; parte media de los párpados superior e inferior y nivel del canto lateral para el orbicular del ojo; vientre muscular medio para el los músculos cigomático mayor, levantador del labio superior, risorio, depresor del ángulo oral, depresor del labio inferior y mental; vientre muscular medio-inferior para el músculo cigomático menor; unión de los tercios medio y superior para el músculo nasal; unión de los tercios medio e inferior para el levantador del labio superior y el ala de la nariz; para el músculo orbicular oral, parte superior en el punto medio de la línea comisura oral-filtrum y parte inferior justo debajo de la comisura oral; y vientre muscular superior para el platisma. El área del INDR en los músculos mímicos mencionados varió de tamaño, pero no se observaron diferencias estadísticamente significativas entre ambos lados izquierdo y derecho tampoco entre hombres y mujeres ($P > 0,05$). La delimitación de la ubicación y el área de estos INDR puede servir de guía para la inyección dirigida de BoNT-A, garantizando así la eficacia terapéutica y reduciendo el riesgo de complicaciones.

PALABRAS CLAVE: Músculos mímicos; Nervio intramuscular; Tinción de Sihler; Toxina botulínica A; Localización del objetivo.

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