Sihler's Staining of the Thoracic Cutaneous Nerve and its Significance

Tinción de Sihler del Nervio Cutáneo Torácico y su Relevancia

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SUMMARY: Our team has modified Sihler's intramuscular nerve staining method to allow for calculation of nerve density. Therefore, this study aimed to show the overall distribution pattern of the thoracic cutaneous nerves to provide a morphological basis for selecting and matching sensory reconstruction during skin flap transplantation. Twelve Chinese adult cadavers were dissected; the thoracic skin was removed, and the modified Sihler's staining method was performed. Centered around the nipple, the chest skin was divided into four regions: medial-superior, lateral-superior, lateral-inferior, and medial-inferior. The thoracic skin was not only innervated by the branches of the 1st to 7th intercostal and supraclavicular nerves, but also by a small number of nerves that directly reached the skin and passed through the pectoralis major muscle. There is a phenomenon of cross overlap between the branches of adjacent intercostal nerves were distributed in the breast, and the branches of the lateral and anterior cutaneous branches on both sides. The density of nerve distribution in the four regions of the chest was in the order of the medial-superior, lateral-inferior and medial-inferior region, respectively. These results may be used to map sensory regions when designing thoracic skin flaps for reconstruction surgery to obtain improved sensory recovery.

KEY WORDS: Thoracic cutaneous nerves; Distribution pattern; Sensory reconstruction; Sihler's stain.

INTRODUCTION

The thoracic cutaneous nerve primarily originates from the lateral and anterior cutaneous branches emanating from the intercostal nerve. These branches innervate the anterolateral and anteromedial skin of the chest wall, respectively (Miyawaki et al., 2006; Netter, 2014). In cases where skin defects are caused by radical mastectomy, chest wall trauma, or chronic ulcers, skin flap transplantation is an important repair method. Currently, surgeons have begun focusing on restoring the sensory function of flaps while ensuring the survival of the flap. The most commonly used method is to neuralize the skin flap. However, the available information regarding the distribution of the thoracic cutaneous nerve does not adequately meet current clinical needs. Recently, our team has modified Sihler's intramuscular nervestaining method, which has successfully shown the overall distribution pattern of cutaneous nerves that are not observable through gross anatomy studies of human skin in certain body regions. This modification also allows the calculation of nerve density (Li et al., 2019; Lai et al., 2021). On this basis, the present study aimed to use a modified Sihler's staining method to show the overall distribution

pattern of the thoracic cutaneous nerve and to enhance the understanding of its branching distribution, providing a basis for morphological guidance in selecting and matching flap grafts for sensory reconstruction.

MATERIAL AND METHOD

Specimens and ethics. Twelve formalin-fixed adult cadavers (equally split between sexes) aged between 35 and 75 years (mean age: 60 ± 5.6 years) were included in the study. None of the cadavers had a history of skin disease, diabetes, or neurological disease. The collection and use of specimens were approved by the Ethics Committee of Zunyi Medical University (approval number: 2016-1-006).

Gross anatomy, zoning, and measurements. A transverse incision was made from the jugular notch, extending through the clavicle to the acromion. This incision was then directed anteriorly along the anterior margin of the deltoid muscle, descending to the mid-axillary line, followed by a fold and incision along the costal arch to the xiphoid process. The

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entire skin was carefully excised while applying pressure against the surface of the muscle in males and the surface of the breast in females. To facilitate the analysis of the density of the cutaneous nerves in various regions, a horizontal and vertical line was drawn around the nipple on each side, dividing it into four areas: medial-superior, medial-inferior, lateral-superior, and lateral-inferior. During dissection, attention was paid to observing the source and passage of the cutaneous nerve. Additionally, the length, width, and thickness of the excised specimen were measured using a Vernier caliper.

Modified Sihler's staining. The specimens were first degreased in absolute ethanol for approximately three days, followed by hydrolyzation in 0.25 % collagenase for another three days. Subsequently, the specimens were subjected to Sihler's nerve staining. In summary, the specimens were first immersed in 0.2 % hydrogen peroxide and 3 % potassium hydroxide solution for 4-5 weeks, followed by Sihler's I solution (comprising one-part glacial acetic acid, two-part glycerin, and 12 parts 1 % hydrated trichloroacetaldehyde) for another 4-5 weeks to decalcify them. Specimens were then stained in Sihler's II solution for four weeks (comprising one-part Ehrlich hematoxylin solution, two parts glycerin, and 12 parts 1 % hydrated trichloroacetaldehyde). They were then re-immersed into Sihler's I solution for 2–10 hours. neutralized in a 0.05 % lithium carbonate solution for 2 hours, and finally treated with gradient glycerol (40, 60, 80, and 100 %) for 1 week.

Observation and measurement after Sihler's staining. The stained specimens were placed on an X-ray lightbox for visual examination of the distribution of the nerve branches at all levels. Subsequently, they were photographed, and the observed patterns were drawn. As the specimens shrank during the decalcification process, it became necessary to reassess the length and width of the specimens with Vernier calipers. The scaling coefficient of the skin area was then calculated as follows: coefficient=specimens after staining/specimens before staining. The innervation range of each cutaneous nerve was measured using CAD software (Autodesk, Inc.).

Measurement of nerve branch densities. Images were analyzed using Photoshop 13.0 software (Adobe). For each image, a 1´1 cm grid of vertical and horizontal reference lines was created to enable quantification of the number of nerve branches per square centimeter. By dragging the reference line, the density of primary, secondary, and subsequent branches was counted in each region. The nerve branch density in the region was calculated using the formula=(total number of nerve branches in the region/area of specimens in the region)´scaling coefficient. Finally, the total nerve branch density was determined by summing the densities across all regions. Statistical analysis. Data were processed using SPSS 21.0 software (IBM Corp.). Comparisons between regions were analyzed by one-way analysis of variance, and if the variance was not homogeneous, the Games–Howell test was used. Statistical significance was determined at a threshold of P<0.05.

RESULTS

Gross anatomy. In addition to the easily visible lateral cutaneous branches of the 4th to 7th intercostal nerves and the trunks of the 1st to 7th anterior cutaneous branches, a few thin branches of the nerve passed through the pectoralis major muscle (in men) or the breast (in women) directly to the skin through the subcutaneous tissue.

Sihler's staining. After staining, the specimen showed mild shrinkage, revealing the overall distribution pattern of cutaneous nerve branches (Fig. 1). Small intradermal nerve branches were also observed to twist or knot with each other (Fig. 2).



Fig. 1. Sihler's staining showing the overall distribution of cutaneous nerves in the female chest.



Fig. 2. Intracutaneous twisted and knotted traveled nerve branches

The lateral cutaneous branches of the 4th to 7th intercostal nerves penetrate the subcutaneous area near the anterior axillary line, and then proceed medially, mostly in parallel, and in a few cases, obliquely forward and downward, gradually giving rise to arborized branches that interconnect. Among these, the main trunks of the lateral cutaneous branches originating from the 4th to 6th intercostal nerves extend toward the base of the female breast, subsequently branching out within the breast. The lateral cutaneous branch of the second intercostal nerve can be clearly seen descending obliquely anteriorly toward the nipple. Branches of the lateral cutaneous branch of the 1st to 4th intercostal nerves were distributed in the lateralsuperior region, while branches of the lateral cutaneous branch of the 5th to 7th intercostal nerves were distributed in the lateral-inferior region. Branches of the lateral cutaneous branch of the 2nd to 6th intercostal nerves were distributed to the breasts, and branches of the lateral cutaneous branch of the 3rd to 5th intercostal nerves extended to the areola and nipple.

In the upper part of the anterior pectoral region, branches of the supraclavicular nerve were observed to branch from the lateral-superior to the medial-inferior region, with the presence of recurrent branches. The anterior cutaneous branches of the 1st to 7th intercostal nerves travel more horizontally from medial to lateral after penetrating from the deep surface. The trunk of the 1st to 6th anterior cutaneous branches reaches the base of the breast, with denser branches around the nipple, forming a grid-like anastomosis with the branches of the lateral cutaneous branches. Branches of the 1st to 4th anterior cutaneous branches were distributed in the medial-superior region, and branches of the 5th to 7th anterior cutaneous branches were distributed in the medial-inferior region. Arborized branches of the supraclavicular nerve were seen in the lateral-superior and medial-superior regions below the clavicle. Scattered fine branches of the nerve penetrated the deep surface and directly reached the skin. There was cross-overlapping innervation between the branches of adjacent intercostal nerves, but there was no cross or overlap between the branches of the anterior cutaneous branches on both sides (Figs. 1 and 3).

The reduction coefficient was 0.91 ± 0.11 , indicating a 9 % reduction after staining. The thickness, area, and nerve branch density in each pectoral region are shown in Table I. Interregional comparison of primary nerve branch density showed a statistically significant difference among regions (P<0.05), with the following order of magnitude: medial-superior region>lateral-superior region>medialinferior region>lateral-inferior region. Comparison of secondary and subsequent branch densities also showed a statistically significant difference (P<0.05), with the following order of magnitude: medial-superior region>lateral-superior region>lateral-superior region>lateral-inferior region>medial-inferior region. The total density of nerve branches in each region was in the following order: medial-superior region>lateral-superior region>lateral-superior region>lateral-inferior region. The comparison of total nerve branch density in the thoracic region between the left and right sides in males and females is shown in Table II. With a P-value >0.05, the difference was not statistically significant.



Fig. 3. Distribution pattern of the pectoral cutaneous nerve. SCN = supraclavicular nerve

DISCUSSION

Significance of the overall distribution pattern of the pectoral cutaneous nerve. In this study, the overall branching distribution pattern of the pectoral cutaneous nerve was successfully shown for the first time using modified Sihler's staining. This technique bridges the gap between gross anatomy and histology and holistically shows the branching distribution details inaccessible through gross anatomy and histology. This provides intuitive visual anatomical insights, offering a potential foundation for morphological guidelines in the selection and matching of flaps for sensory reconstruction.

Gross anatomical study of the pectoral cutaneous nerve. Jaspars *et al.* (1997), dissected the anterior and lateral cutaneous branches of the 1st to 7th intercostal nerves and measured the surface area percentage covered

Table I. Comparisor	of area, thickness, and	branch density of cuta	uneous nerve branches	in various pectoral regions (Mean±SD, n=24).	
Regions	Skin ar	ea (cm^2)	Thickness of the	Primary nerve branch	Density of secondary and	Total nerve branch
			specimen (cm)	Density (branches/cm ²)	subsequent branches	Density (branches/cm ²)
	Before	After			(branches/cm ²)	
	staining	staining				
medial-superior	149.58 ± 15.46	134.62±14.71	0.61 ± 0.21	0.08 ± 0.011	0.97 ± 0.15	1.05 ± 0.17
medial-inferior	$177.27\pm17.42^{*}$	$159.35\pm17.68*$	$0.73\pm\!0.21^{*}$	$0.05{\pm}0.008$ *	$0.78\pm0.12^{*}$	$0.83\pm0.13^{*}$
lateral-superior	$198.29{\pm}18.28^{*}$	$178.46\pm18.94*$	$0.68\pm0.19*$	$0.06{\pm}0.007^{*}$	$0.88{\pm}0.09^{*}$	$0.92\pm\!0.11^{*}$
lateral-inferior	$283.45\pm31.21^{*}$	$256.17\pm29.14*$	$0.78\pm0.23*$	$0.04{\pm}0.008^{*}$	$0.81 {\pm} 0.08^{*}$	$0.85\pm0.10^{*}$
F	165.768	146.048	2.772	26.176	12.771	14.475
Ρ	0.012	0.032	0.046	0.028	0.011	0.017
*Compared with the in	ner-superior region, P<0.(35 .				
	Tab. II Comparison	of nerve branch density	v of pectoral cutaneous	nerve between left and right si	ides in males and females (branche	s/cm ²).
	Regions	left	Rioht	t P Males	s females t	=

Tab. II Comparisoi	n of nerve branch dens	sity of pectoral cutan	sous nerve t	oetween le	ft and right sides if	n males and temales	(branche	s/cm ^t).
Regions	Left	Right	t	Ъ	Males	females	t	d d
medial-superior	1.085 ± 0.161	$1.015\pm0.186^{*}$	0.996	0.876	1.044 ± 0.209	1.056 ± 0.140	-0.16	0.29
medial-inferior	0.851 ± 0.145	$0.809 \pm 0.122^{*}$	-0.769	0.608	0.844 ± 0.161	0.816 ± 0.103	0.49	0.11
lateral-superior	0.944 ± 0.088	$0.896\pm0.132^{*}$	1.068	0.624	0.915 ± 0.144	0.925 ± 0.075	-0.21	0.23
lateral-inferior	0.871 ± 0.108	$0.829{\pm}0.09^{*}$	1.012	0.653	0.862 ± 0.132	0.838 ± 0.647	0.56	0.86
*Compared with I	.eft. P>0.05: →com	pared with males. P	>0.05.					

by each cutaneous nerve branch in the breast. In their study, the skin of the breast area was innervated by the anterior and lateral cutaneous branches of the 1st to 7th intercostal nerve, with the anterior and lateral cutaneous branches of the 4th intercostal nerve having the highest percentage of surface area coverage on the skin of the breast. Additionally, there were no instances of cross-innervation between the anterior cutaneous branch and the contralateral side. The study by Ricbourg (1992), however, concluded that the lateral branch contributesmore to the cutaneous innervation of the breast than the anterior branch, although the evidence supporting this claim was not specified. Miyawaki et al. (2006), found through autopsy of 73 cadavers, that the cutaneous nerves in the 1st and 2nd intercostal spaces did not consistently originate from their respective intercostal nerves but had distinct distribution patterns. For example, the anterior branch of the 1st intercostal nerve has two distribution patterns: In the first pattern, the anterior cutaneous branch emerges at the anterior end of the first intercostal space, runs through the pectoralis major muscle, and extends within the first intercostal space. In the second pattern, another branch originating from the same location descends along the anterior surface of the second costal cartilage, passing deep to the pectoralis major muscle. It reaches the inferior edge of the second costal cartilage or the second intercostal space and then penetrates the pectoralis major muscle to extend toward the second rib or the second intercostal space. These studies were limited by traditional anatomical methods, which only allowed the observation of thick nerve branches without demonstrating the extent of innervation provided by each nerve. Bijkerk et al. (2020), used ultrasonography to localize the anterior cutaneous branch of the intercostal nerve followed by injection of a lidocaine block and then sensory testing to determine the distribution of the cutaneous nerve. They concluded that the anterior cutaneous branch of the 4th intercostal nerve had the widest distribution in the breast, with innervations in the inner superior and inner inferior quadrants of 9¥7.5 cm and 12¥8 cm, respectively. This study revealed the boundaries of innervation but did not provide details of the distribution of cutaneous nerve branches, thus serving as a limited guide for flap surgery. Further exploration is necessary to find the ideal flap recipient nerve. Knackstedt et al. (2019), through dissection, concluded that the lateral branch of the 4th intercostal nerve primarily courses beneath the 4th rib, averaging a distance of 13.1±1.3 cm from the sternum and 11.8±2.2 cm from the midline of the clavicle. They observed that it penetrated the lateral side of the pectoralis minor muscle or within 2cm of the lateral side, with a nerve cross-section of 2mm, suggesting its suitability as a receptor nerve. Mohan et al. (2021), found that the location of the lateral cutaneous branch of the 4th intercostal nerve

was unfavorable for surgery. However, they determined that the median lengths of the anterior cutaneous branches of the intercostal nerves from 2nd to 4th were 43 mm, 37.5 mm, and 37 mm, respectively. Immunohistochemical staining confirmed that the nerve bundles and axon counts of both the anterior and lateral branches were also comparable. Consequently, the anterior cutaneous branch could be prioritized as a suitable recipient nerve for the skin flap. Yang et al. (2001), in a female breast dissection study, found that the female breast is innervated by the anterior cortical and lateral branches of the 2nd to 6th intercostal nerves. Additionally, they noted that the nerves in the nipple and areola region are mainly innervated by the lateral and anterior cortical branches of the 3rd to 5th intercostal nerves, with the 4th intercostal nerve being the most predominant. Furthermore, they found that supraclavicular nerves do not branch into the nipple and areola region, which aligns with our results. The supraclavicular nerve contributes to the cutaneous innervation of the upper chest and does not extend to the nipple and areola area, making it unsuitable as a recipient nerve for flaps (Pandya & Moore, 2011; Lorsuwannarat & Jirangkul, 2023). It has been reported that the intercostal nerve does not always travel intradermally; it can also traverse deeper surfaces, such as the muscle, mammary tissue, and skin (Smeele et al., 2022). This phenomenon was also observed in our study. This explains why a high number of primary nerve branches or secondary and subsequent branches do not necessarily correlate with high total nerve branch density.

Clinical application study of the thoracic cutaneous nerve. A study (Beugels et al., 2017) has demonstrated that sensory recovery begins earlier and is more comprehensive in innervated flaps than in non-innervated flaps. Furthermore, sensory improvement progresses gradually over time, increasing the likelihood of returning to normal sensation. Beugels et al. (2021), reconstructed the breast with a lateral thigh perforator flap by anastomosing a branch of the lateral femoral cutaneous nerve to the anterior cutaneous branch of the intercostal nerve. Patients who underwent this procedure exhibited significantly better postoperative sensory recovery than those without nerve anastomosis. Spiegel et al. (2013), used the deep inferior epigastric artery perforator flap, anastomosing the anterior branch of the 3rd intercostal nerve to repair breast defects. They found that this surgical procedure significantly increased sensory recovery in patients undergoing breast reconstruction. Additionally, they observed that the operative time could be shortened owing to easier dissection of the anterior branch of the 3rd intercostal nerve. Puonti et al. (2017), used a double nerve suture technique, anastomosing the 3rd to 4th intercostal

nerves or the 4th to 5th intercostal nerves as a receptor in patients undergoing thoracic reconstruction for breast cancer. This approach resulted in a better restoration of tactile and cold sensation than the traditional single-nerve anastomotic flap. Although the latissimus dorsi flap can also be used for breast reconstruction, it is a musculocutaneous flap with greater motor innervation than sensory innervation. Consequently, reconstruction using this flap may result in sensory dysfunction or even complete loss of sensation. However, sensory recovery can be improved to a certain extent by anastomosing the cutaneous branch of the intercostal nerve (Zhu et al., 2022). Neural regeneration can be facilitated by nerve tissue alignment, allowing Schwann cells at both ends to directly establish communication channels, enabling regenerating nerve fibers to grow smoothly into the distal neurotubules (Wang et al., 2017). Our results indicate that both the anterior cutaneous trunks of the 1st to 7th intercostal nerves and the lateral cutaneous trunks of the 4th to 7th intercostal nerves can be used as viable recipients for nerve anastomosis. Anastomosing multiple nerves can increase the likelihood of restoring breast sensation while ensuring the integrity of the flap. When repairing defects in the inner superior region, it is essential to select a donor area with a relatively high cutaneous nerve density. This is because better sensory recovery requires a higher nerve density in the donor area, facilitating increased nerve contact and enhancing sensory reconstruction. The present study's results may be used to map sensory regions when designing thoracic skin flaps for reconstruction surgery to obtain improved sensory recovery.

Strengths and limitations of this study. In this study, the modified Sihler's staining method was applied to reveal the overall branching pattern of the thoracic cutaneous nerve for the first time, bridging the gap in branching information that could not be demonstrated by gross anatomy alone. This method provides a morphological basis for the selection and design of flaps for sensory reconstruction. Its limitations are that it does not show intradermal nerve branches on tissue sections, has a relatively small sample size, and does not reveal whether there are differences between races.

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RESUMEN: Nuestro equipo ha modificado el método de tinción nerviosa intramuscular de Sihler para permitir el cálculo de la densidad nerviosa. Por lo tanto, este estudio tuvo como objetivo mostrar el patrón de distribución general de los nervios cutáneos torácicos proporcionando una base morfológica para seleccionar y combinar la reconstrucción sensorial durante el trasplante de colgajo de piel. Se diseccionaron 12 cadáveres de individuos adultos chinos. Se eliminó la piel torácica y se realizó el método de tinción de Sihler modificado, centrada alrededor del pezón, la piel del pecho se dividió en cuatro regiones: medialsuperior, lateral-superior, lateral-inferior y medial-inferior. La piel torácica no solo estaba inervada por los ramos de los nervios intercostal y supraclavicular 1º a 7º, sino también por un pequeño número de nervios que llegaban directamente a la piel y pasaban a través del músculo pectoral mayor. Existe un fenómeno de superposición cruzada entre los ramos de los nervios intercostales advacentes. Los ramos de los nervios intercostales 2º a 7º se distribuyeron en la mama, y los ramos de los ramos cutáneos lateral y anterior se distribuyeron densamente alrededor del pezón, formando una anastomosis en forma de rejilla. No hubo inervación cruzada entre los ramos cutáneos anteriores en ambos lados. La densidad de la distribución nerviosa en las cuatro regiones del tórax estaba en el orden de región medial-superior, lateral-superior, lateral-inferior y medial-inferior, respectivamente. Estos resultados pueden ser útiles para mapear regiones sensoriales al diseñar colgajos de piel torácicos para utilizarlos en cirugía de reconstrucción y obtener así una mejor recuperación sensorial.

PALABRAS CLAVE: Nervios cutáneos torácicos; Patrón de distribución; Reconstrucción sensorial; Tinción de Sihler.

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