SIHLER'S STAINING TECHNIQUE IN THE STUDY OF TONGUE INNERVATION IN THE RAT

SIHLER BOYAMA TEKNIĞI ILE RAT DIL INNERVASYONUNUN INCELENMESI

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ABSTRACT

Purpose: *The tongue is a complex muscular organ constituted of several intrinsic and extrinsic muscles, innervated by several cranial nerves.* ***Methods:*** *In this study, the nerves and their branches in the tongue were demonstrated by Sihler's stain technique.* ***Results:*** *The distributions of the hypoglossal nerve in the muscles of the tongue were visualized. The sensitive fiber contribution of the glossopharyngeal nerve to the posterior one-third of the tongue was followed. In addition, the course of the lingual nerve and its branchings in the corpus towards the tip were demonstrated.* ***Conclusion:*** *We think that this overlooked stain technique should be considered when studying the complex innervations of structures, especially in re-innervation experiments.*

Key Words: *Sihler's Staining, Rat, Tongue, Muscles, Lingual Nerve, Hypoglossal Nerve, Glossopharyngeal Nerve.*

ÖZET

Amaç: *Sinirleri ve dallanmalarını ayrıntılı bir şekilde gösteren Sihler boyama tekniğiyle farklı kraniyal sinirlerle innerve edilen dilin innervasyonu gösterilmiştir.* ***Metod:*** *Sıçan dilinin duyu ve motor sinir innervasyonunu göstermek amacıyla modifiye Sihler boyama tekniği kullanılmıştır.* ***Bulgular:*** *Dil kaslarında n.hypoglossus'un dağılımı gösterildi. Dilin 1/3 arka kısmına n.glossopharyngeus'un duyu liflerinin dağılımı izlendi. Ayrıca, n.lingualis ve dallarının corpus linguae'dan apex linguae'ya doğru olan seyri ve dallanması gösterildi.* ***Sonuçlar:*** *Uzun bir süredir gözardı edilmiş olan bu boyama tekniğinin yapılardaki karmaşık sinir dallanmalarını ve reinnervasyon deneylerinin sonuçlarını göstermesi bakımından önemli bir yöntem olacağı kanısındayız.*

Anahtar Kelimeler: *Sihler Boyama, Rat, Dil, Nervus Lingualis, Nervus Hypoglossus, Nervus Glossopharyngeus.*

INTRODUCTION

It has been 100 years since Charles Sihler first introduced his method for staining nerve spindles and more than 50 years since other workers reported modifications of this technique for identifying innervation in the kidney, uterus and ovary in humans and in the foot and leg in animals (1-4).

Sihler's stain renders specimens translucent while counterstaining the entire nerve supply; consequently, individual peripheral nerves become clearly visible in fine detail and traceable in their normal three-dimensional position (5). Modified Sihler's staining technique is highly useful in demonstrating neuromuscular organization in complex structures. Using this technique, Liu et al. successfully studied the distribution of intramuscular nerve branches in skeletal muscles (4). Drake et al., on the other hand, similarly applied Sihler's staining technique to define the exact branching pattern of nerves in the posterior cricoarytenoid muscles of canines, and claimed it was valuable both in understanding laryngeal function and in new therapies for dysfunction (6). Wu et al. processed 27 human hemi-larynges with Sihler's staining technique, and suggested that the communicating nerves supply motor innervation to the thyroarytenoid muscle as a second source, and sensory innervation to the subglottic area and cricoarytenoid joint (7).

There are two other techniques for studying intramuscular nerve supply, microdissection and serial histological sections. Microdissection is useful only in identification of the more proximal nerve branches as they enter the substance of the muscle. Tracing nerve branches deeper into a muscle is fairly difficult and differentiating fine branches from small blood vessels and connecting tissues could well be problematic. Preserving one branch often requires destruction of others. All of these factors may lead to errors in determining nerve branching patterns (6). Alternatively, the innervation pattern can be reconstructed from serial sections after staining of the nervous tissue using various histochemical techniques; however, this is time consuming and technically difficult as well as open to error in orienting in the sections (6,8).

The tongue is a highly complex muscular organ constituted of several intrinsic and extrinsic muscles, innervated by several cranial nerves and involved in several important physiological tasks such as mastication, swallowing, taste, respiration and human speech (9-14). Four nerves supply innervation to the tongue. The hypoglossal nerve supplies motor innervation to all tongue muscles (10). The lingual nerve, a branch of the mandibular division of the trigeminal nerve, provides both gustatory and general sensory fibers to the tongue. The former component is contributed to by the chorda tympani branch of the facial nerve, which connects with the lingual nerve. Finally, the glossopharyngeal nerve terminates in the mucosa and the vallate papillae at the posterior third of the tongue to supply general sensation and taste (15,16). These classical descriptions of the innervation of the tongue are mainly based on findings obtained by gross dissection, nerve degeneration, neural tracing techniques, and electrophysiological methods (15-20).

A number of different techniques are available to demonstrate the sensory and motor innervations of the tongue; however, without disturbing the muscular structure, the demonstration of nerve distribution to the level of the finest branches requires a special technique and attention.

For this purpose, we used our modified Sihler's staining technique to show the tongue's nerve supply, both sensory and motor.

MATERIALS AND METHODS

Six adult Wistar albino rats weighing 400-500 g were sacrificed by i.v. sodium pentobarbital. The tongues were dissected out from the radix linguae, and processed by modified Sihler's staining technique. The Local Ethical Committee for Animal Studies approved the experimental protocol. Sigma and Merck Chemical Co. supplied the chemicals.

Tissue preparation

The specimens were fixed by immersion in 10% unbuffered formaldehyde for 1 month. The fixed specimens were washed under running water for 1 h, and then macerated in 3% KOH solution (3 drops of 3% H_2O_2 added to each 100 ml of 3% KOH for at least 3 weeks). The KOH solution was changed daily. The process was considered complete when the specimen appeared transparent or translucent.

The macerated specimen was decalcified in Sihler's solution I (1:1:6 glacial acetic acid: glycerin: 1% aqueous chloral hydrate) for 2 weeks. The solution was changed each week. The specimen became soft at the end of this stage.

Staining

In the present study, the staining method initially designed by Charles Sihler and recently modified by Liem and Van Willigen, Wu et al. and then Gozil et al. was used to investigate the distribution of the intramuscular nerve distribution of the tongues of rats (1,5,7,21).

Photography

Glycerin-preserved specimens were trimmed carefully using a binocular microscope to show the nerves clearly for photography. Specimens were photographed at x100 magnification.

RESULTS

The hypoglossal nerve was seen bifurcating near the base of tongue and then continuing to ramify within the body of the tongue. The lingual branch of the trigeminal nerve and the glossopharyngeal nerve were also identified ramifying within the tongue (note the merging of the hypoglossal (motor) and trigeminal (sensory) nerve fibers within the body of the tongue) (Fig. 1).

The lingual nerve, which is sensory to the tongue mucosa, appeared to enter near the sulcus terminalis and divided into anterior and posterior divisions. The anterior division runs forward along with the hypoglossal nerve to the tip of the tongue. During its course this division gives fine branches forming a grid-like network. The main trunk of the lingual nerve appeared to give communicating branches in the form of loops to the hypoglossal nerve just proximal to its anterior division (Fig. 1-3). The posterior division was brush-like, and finer and shorter in form than the anterior, and spreads in the mucosa of posterior 1/3 of the tongue (Fig. 1-3).

Hypoglossal nerves were seen to enter the radix linguae bilaterally as thick trunks (Fig. 1), and after a short course each divided into medial (thicker) and lateral (finer) branches near the sulcus terminalis. Both run along the corpus linguae to the apex, giving fine short branches.

The lateral branch of the hypoglossal nerve was visible, dividing dorsal and ventral divisions in the sagittal section, and innervating the superior longitudinal and inferior longitudinal muscles respectively (Fig. 2,3). The medial branch on the other hand appeared to give several fine branches to the transversus linguae and verticalis linguae muscles along its course to the apex (Fig. 2,3).

The glossopharyngeal nerve was identified as fine fibers spread out in the posterior to the sulcus terminalis (Fig. 1-3).

DISCUSSION

Since its first introduction in 1895, Charles Sihler's staining technique has been modified and used by several investigators (1,2,4-8,20).

Compared with procedures described in the literature (5,21-26), our modifications have mainly concerned fixation, maceration and decalcification. Table 1 gives a summary of the major modifications of Sihler's original technique, including ours.

Some investigators made changes in the stages of fixation or maceration-pigmentation, and others in the decalcification stage (1). Wharton used Sihler's staining technique directly without fixing the tissues; however, others, adding some chemicals in 10% formalin, fixed the tissues in varying periods from 1 week to 1 month (2). Although Sihler and Wharton used no special chemicals during the stages of maceration and depigmentation, many researchers applied a 3% KOH + 3% H_2O_2 combination in varying ratios (1,2). On the other hand, glacial acetic acid was also used in decalcification in various time periods.

In this study, we applied Gözil et al.'s modified staining technique (5). Accordingly, the tissues were kept for 3 weeks in 3% KOH + 3 drops 3% H_2O_2 during the stages of maceration and depigmentation, but the staining stage was modified by extending the time to 5 weeks.

Investigators especially choose muscles that are innervated by several nerves in various animal models, and visualized the intramuscular branching.

Kierner et al. clearly demonstrated the contributions of the cervical plexus and spinal accessory nerve to the innervation of the trapezius muscle and emphasized the importance of varying innervations in neck surgery (27).

The branching patterns of innervations of extraocular muscles and masticatory muscles in rabbits were finely detailed using modified Sihler's staining technique (5).

Abd-El-Malek and Hellstrand reported that the hypoglossal nerve in humans and cats innervates the tongue by dividing into a lateral and a medial division (17,18). Mu and Sanders, in a detailed study, presented the neuromuscular organization of the three layers of the canine tongue, namely the inferior layer (supplied by the lateral division), and the middle and superior layers (supplied by the medial division) of the hypoglossal nerve (14).

McClung and Goldberg, in an extensive study using modified Sihler's staining technique, further demonstrated the course of the lateral and medial divisions of the hypoglossal nerve within the body of the tongue and showed the intramuscular course of the nerve within the styloglossus, hyoglossus and genioglossus muscles (28). The branches to the styloglossus and hyoglossus can be seen leaving the lateral division of the hypoglossal nerve near the bifurcation of the hypoglossal nerve trunk at the base of the tongue. The lateral division of the hypoglossal nerve first continued into the substance of the tongue, where it divided into branches to the superior as well as the inferior aspect of the body of the tongue. Similar to the lateral branch, the rest of the medial branch of the hypoglossal nerve can be seen to continue into the body of the tongue and then to give off multiple small nerve branches as it run through the length of the tongue. These numerous branches innervate the alternating sheets of transversus and verticalis linguae muscle fibers throughout the body of the tongue. It is obvious that the largest portion of the lateral and medial divisions of the hypoglossal nerve continue into the body of the tongue to innervate intrinsic muscles.

In our study, the lateral and medial branches of the hypoglossal nerve were clearly identifiable; the superior longitudinalis, inferior longitudinalis and styloglossus muscles were innervated by the lateral branch and the transversus linguae, verticalis linguae and genioglossus muscles by the medial branch. On the other hand, it was visible that the lingual nerve branched out anterior to the sulcus terminalis, whereas the glossopharyngeal nerve branched out to the posterior. The communicating branches were noticeable between the hypoglossal and lingual nerves.

We think that this long overlooked stain technique should be considered in the study of complex innervations of structures, especially in re-innervation experiments.

Clinically, determining the exact branching patterns of the nerves to the tongue will be helpful for designing neurosurgical procedures to restore sensory or motor function to the paralyzed or non-sensate tongue.

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