Quantitative Analysis of Muscle Histologic Method in Rodent Facial Nerve Injury

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Objective: To describe denervation features of facial musculature following facial nerve injury in a rodent model.

Methods: Six Wistar-Hannover rats underwent unilateral transection and immediate repair of the facial nerve. After 8 weeks, muscular bundles consisting of dilator naris and levator labii superioris from both sides were analyzed for mean muscle cell diameter and the percentage of muscle cell cross-sectional area using image processing software. The atrophic features of facial muscles were quantified and compared with the contralateral, healthy side of the face.

Results: Weekly postoperative whisking assessment demonstrated the anticipated course of recovery. We observed significant differences between the normal side and the manipulated side, respectively, in the percentage of muscle specimen cross-sectional area attributable to muscle cell profiles (57% vs 29%; \( P = .006 \)) and total fiber counts (1346 vs 794; \( P = .02 \)). The mean cross-sectional area of individual muscle fibers was higher on the normal side (1129 vs 928 \( \mu \text{m}^2 \); \( P = .39 \)); however, this difference was statistically nonsignificant.

Conclusion: The objective, quantitative measures of muscle microstructure used in this report provide a valuable point of comparison for whisking function and electrophysiologic measures and can be used in future studies to assess muscle atrophic features associated with facial nerve injury and repair techniques.


Facial nerve injury leads to devastating consequences for patients; in many cases, recovery may be prolonged or incomplete.\(^1\)\(^-\)\(^4\) These consequences include corneal exposure, epiphora, and brow ptosis, as well as external nasal valve collapse, oral incompetence, and loss of smile ability. Unlike in rodents, peripheral motor nerve regeneration and functional recovery in humans is not robust. The injured human nerve undergoes a cascade of molecular events during the period of denervation that may inhibit neuronal regeneration.\(^3\) There are 3 requirements for successful recovery of function following nerve injury: first, survival of the injured neuron; second, presence of neurotrophic factors to promote axonal extension; and third, survival of the appropriate target muscle and reestablishment of functional synapses.

The rodent facial nerve injury model has been developed to understand these mechanisms and test functional interventions that affect recovery following facial nerve injury.\(^6\)\(^-\)\(^7\) Functional recovery in the rodent can be assessed using video-based motion analysis of whisking behavior or, more recently, through laser micrometer-based whisking detection providing precise recording of various kinematic factors in real time.\(^8\)\(^-\)\(^11\) Although this latter technique has contributed to significant advances in understanding functional outcome following facial nerve injury, a method to analyze histologic features of recovering facial muscles would complement studies of functional outcomes by enhancing our understanding of structure/function relationships.

Herein we describe a relatively easy, reliable, and quantitative technique, using histologic and computational analyses, to evaluate specific microscopic changes in rodent facial musculature following denervation and repair.

Methods

Head Fixation, Conditioning, and Whisking Function Measurement

Six adult female Wistar-Hannover rats (Charles River Laboratories) weighing 250
to 300 g were studied in accordance with an animal use protocol approved by the Massachusetts Eye and Ear Infirmary institutional review board and Institutional Animal Care and Use Committee. Following 1 week of daily handling, head fixation devices were surgically implanted. After the animals were anesthetized with an intramuscular injection of ketamine hydrochloride, 50 mg/kg (Fort Dodge Animal Health), and medetomidine hydrochloride, 0.5 mg/kg (Orion Corporation), lightweight titanium head implants with 4 external attachment points for rigid head fixation were secured to the calvarium using screws.6,9 After recovery, the animals underwent approximately 2 weeks of acclimation to the restraint apparatus with a daily conditioning regimen.

Whisking was measured during weekly, 5-minute recording sessions in restrained rats. The animals were placed in a body restraint device, and their C-1 whiskers were marked with lightweight (3-4 mg) polyamide tubes. The horizontal movements of these tubes were tracked using 2 pairs of laser micrometers positioned 1 cm lateral to the right and left face surface. Software provided by Bermejo et al11 was used to calculate whisking amplitude, in which the 3 largest amplitude whisks were used to calculate the mean amplitude for each session.

FACIAL NERVE TRANSECTION AND REPAIR

Following induction of general anesthesia as described in the first paragraph of the “Methods” section, all animals underwent left facial nerve exposure through a preauricular incision. The parotid gland was removed and the main trunk of the facial nerve was widely exposed, then completely transected and immediately repaired with 2 or three 10-0 nylon epineurial sutures. The incision was closed and anesthesia was reversed with atipamezole hydrochloride, 0.5 mg/kg.

HISTOCHEMICAL STAIN AND IMAGE ANALYSIS

After 8 weeks, the animals were killed and the bundle consisting of dilator naris muscle (DNM) and levator labii superioris (LLS) was harvested from both sides of the face. Specimens were placed in 10% sucrose overnight and then permounted in preparation for cryosection.

The frozen sections were cut at 10-µm thickness. The slides were stained with Masson trichrome stain (MTS) (American Master Tech Scientific Inc). Digital photographs of the slides were taken at ×20 and ×400 magnification.

**Figure 1.** After Masson trichrome staining (MTS) of tissue sections containing dilator naris muscle and levator labii superioris muscles, a sequential color deconvolution algorithm was applied, using Image J software, to measure muscle content of the specimens, fiber surface area, and fiber counts. A through C, This example of a control specimen at ×20 magnification after MTS demonstrated distinct 3-colored staining patterns. B, The entire specimen was then outlined with the Image J software. C, The muscle area (red stain) was selectively outlined through color-based image thresholding. D, The same specimen is shown at ×400 magnification. E, Individual muscle fiber staining is selectively outlined using the same thresholding approach.

**Figure 2.** Mean maximal horizontal whisking amplitude at postrepair day 28. Recovery was significantly greater for the control side (58.5°) compared with the experimental side (17.1°). Limit lines indicate 95% confidence intervals.
using an inverted-light microscope (Nikon Eclipse TS100, Nikon Digital Sight DS-Fi1 mounted digital camera; Nikon Corporation).

Images were analyzed using Image J software. The software was used to outline the area of interest and then pixels were counted and converted to metric units (Figure 1). The mean cross-sectional area of muscle fibers, the total fiber count, and the ratio of muscle to connective tissue associated with the DNM/LLS bundle for each side were obtained from an investigator blinded to the experimental condition (S.W.K.). Measurement reliability was assessed by having 2 blinded investigators (S.W.K. and J.S.W.) independently perform muscle area analysis for all tissue sections, which resulted in values that were a mean of 98.7% in agreement. Statistical analysis software (Minitab, version 16; Minitab Inc) was used to perform 2-tailed, unpaired heteroscedasticity t tests with assumption of heteroscedasticity to compare each histologic variable between the experimental and control side.

RESULTS

DEGREE OF FUNCTIONAL RECOVERY

Following transection and repair, all animals demonstrated total loss of whisking movement on the experimental side. By postrepair day 17, all animals demonstrated some recovery of whisking amplitude on the experimental side, indicative of reinnervation. In all animals, the recovery of whisking, as demonstrated by amplitude of whisk movement, reached its plateau by postrepair day 28 (Figure 2). This was consistent with previously published results. Mean whisking amplitude was 17.1° for the experimental side and 58.5° for the control side.

QUALITATIVE HISTOLOGIC ASSESSMENT OF DNM/LLS USING MTS

Marked qualitative differences were noted in the muscle architecture of DNM/LLS between the control and experimental sides (Figure 3). Notable bulk asymmetry was observed in every animal during specimen harvest. Under the light microscope at ×20 and ×400 magnifications, the experimental side displayed fewer muscle fibers distributed among a relatively larger proportion of collagen and adipose tissues, consistent with degenerative changes.
Following motor nerve injury, skeletal muscle becomes atrophic and fibrotic, resulting in progressive impairment of function.14 Denervated muscle may lose up to 85% of its mass and a loss of tensile strength.15 Histologically, there is an increase in volume of intramuscular connective tissues, ranging from 50% to 700%, both endomysially and perimysially. There is also a decrease in cross-sectional area of the muscle fibers and reduced capillary density within the muscle tissue.16,17 These structural changes in both the muscle fibers and the surrounding connective tissues contribute to poor functional outcome following prolonged denervation and immobility.18-21

Extensive investigation in both basic science and clinical arenas has focused on the kinetics and structural aspects of muscle atrophy, along with quantitative measurements of motor unit force.12,22 Development of a measurement tool for quantifying whisking movement in the rodent has allowed investigators to examine vibrissal motor recovery following various therapeutic interventions after facial nerve injury.10,11 These interventions have included pharmacologic treatments, mechanical or electrical stimulation, and repair techniques.7,13,24-29 To our knowledge, no other studies have used quantitative analysis of the denervated vibrissal musculature concurrently to correlate quantitative functional outcomes and histologic changes in the muscle architecture.

Herein we describe a method for quantitative analysis of rodent facial musculature following denervation and repair using image processing software. This technique determines total muscle fiber count, cross-sectional area of individual fibers, and the ratio of fiber content to surrounding connective tissue in a digitized, precise fashion. Our method is modeled after the color deconvolution algorithm that was first described by Ruifrok and Johnston.30 This histochemical analysis technique quantitatively characterizes multiple stains, using public domain National Institutes of Health Image J software, and permits comparative quantification by calculating the contribution of each stain-specific red-green-blue absorption.

Masson trichrome stain is an ideal histochemical adjunct for color deconvolution analysis because of its 3-colored stain pattern; it produces red for keratin and muscle fibers, blue for collagen, and dark brown for cell nuclei.31 Its distinct color scheme has been extensively used in computerized quantitative analysis in research and clinical applications.12,32 Although we have observed minor variations in the intensity of trichrome hue among different specimens, we were able to perform consistent calculation of image pixel counts because our quantitative image analysis technique depends on the absolute color contrast and not hue intensity.
The 3 key features of atrophy in muscle architecture are total muscle fiber count, cross-sectional area of individual fibers, and the ratio of fiber content to surrounding connective tissue. Numerous anatomy and physiology studies have confirmed these features as reliable and consistent markers of atrophic changes in denervated muscle, and these features also have been shown to correlate with impaired muscle function.\textsuperscript{17-19,34-36} Despite the small sample size in our series, the differences in the total muscle fiber count and the ratio of fiber content to surrounding connective tissue between manipulated and un-manipulated sides were statistically significant and consistent across animals.

CONCLUSIONS

Herein we describe a relatively easy, reliable, and quantitative method of analyzing histologic changes in MTS-stained DNM/LLS muscle bundle specimens through image processing software. Using this technique, we observed statistically significant atrophic changes using both muscle fiber count and muscle fiber area (as a proportion of total muscle area) following facial nerve transection and repair. Our approach may serve as a powerful tool that complements the existing functional outcome paradigm, permitting quantitative analysis of histologic changes in the rat facial nerve injury model. In the future, quantitative analysis of the atrophic features of denervated muscle in conjunction with measurement of functional recovery will allow us to examine whether specific patterns of vibrissal muscle changes are predictive of long-term functional outcomes following facial nerve injury.

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REFERENCES