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Quantitative Histologic Analysis of Muscle Micro-architecture Following Facial Nerve Injury in a Rodent Model

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Abstract

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

Methods—Six female 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 harvested. The specimens were fixed, cryo-cut, and stained with Masson's trichrome stain. Tissue sections were analyzed for average muscle cell diameter and the percentage of muscle specimen attributable to muscle cell cross-sectional area using Image J image processing software. The atrophic features of facial muscles ipsilateral to nerve transection and repair were quantified and compared to the contralateral, healthy side of the face.

Results—Weekly post-operative whisking assessment demonstrated the anticipated time course of whisking recovery, with all animals demonstrating the initiation of recovered movement by post-repair day 17, and progressing to approximately 25% recovered whisking amplitude (repaired side / healthy side) by the end of the 8 week survival period. We observed significant differences in the percentage of muscle specimen cross-sectional area (including connective tissues) attributable to muscle cell profiles (57% vs 29%; $p=0.01$), and total fiber counts (1,346 vs 794; $p=0.02$) for the normal side and the manipulated side, respectively. While the average cross-

sectional area of individual muscle fibers was higher on the normal side ($1,129\mu\text{m}^2$ vs $928\mu\text{m}^2$; $p=0.39$), this difference was not statistically significant.

Conclusion—Although reinnervation of rat facial muscles begins within three weeks after facial nerve transection and suture repair, after an 8-week survival period whisking remain substantially impaired and rats experience a substantial loss (approximately 40%) of muscle cells and a roughly parallel loss of muscle cell surface area (approximately 49%) in two facial muscles associated with the whisker pad and snout. The objective, quantitative measures of muscle microstructure used in this report provide a valuable point of comparison for whisking function and electrophysiological measures, and can be used in future studies to assess muscle atrophic features associated with facial nerve injury and repair techniques.

Introduction

Facial nerve injury leads to devastating consequences for patients, and in many cases, recovery may be prolonged or incomplete^(1–4). Patients develop 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⁽⁵⁾. There are three 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 parameters in real time^(8–11). While this later technique has contributed to significant advances in understanding functional outcome following facial nerve injury, a methodology to analyze histologic features of recovering facial muscles would complement studies of functional outcomes by enhancing our understanding of structure/function relationship.

Herein we describe a novel technique, employing histologic and computational analyses, to evaluate specific microscopic changes in rodent facial musculature following denervation and repair.

Materials and Methods

Head Fixation, Conditioning, and whisking function measurement

Six adult female Wistar-Hannover rats (Charles River Laboratories, Wilmington, MA) weighing 250 to 300 grams were studied in accordance with an animal use protocol approved by the Massachusetts Eye and Ear Infirmary IRB and IACUC. Following one week of daily handling, head fixation devices were surgically implanted. After animals were anesthetized with an intramuscular injection of ketamine (50mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and medetomidine hydrochloride (0.5mg/kg, Orion Corporation,

Espoo, Finland), lightweight titanium head implants with 4 external attachment points for rigid head fixation were secured to the calvarium using screws^(6,9). After recovery, 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. Animals were placed in a body restraint device, and their C-1 whiskers were marked with lightweight (3–4 mg) polyimide tubes. The horizontal movements of these tubes were tracked using two pairs of laser micrometers positioned 1 cm lateral to the right and left face surface. Software provided by Bermejo et al was used to calculate whisking amplitude, in which the 3 largest amplitude whisks were averaged for each session⁽¹¹⁾.

Facial Nerve Transection and Repair

Following induction of general anesthesia as described above, 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 two or three 10-0 nylon epineurial sutures. The incision was closed, and anesthesia reversed with Atipamezole (0.5mg/kg, Pfizer, Cambridge, MA).

Histochemical Stain and Image Analysis

After 8 weeks, animals were sacrificed 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, then permounted in preparation for cryosection.

The frozen sections were cut at 10µm thickness. The slides were stained with Masson's trichrome stain (MTS) (American Master Tech Scientific Inc, Lodi, CA). Digital photographs of the slides were taken at 20× and 400× magnification using an inverted-light microscope (Nikon Eclipse TS100, Nikon Digital Sight DS-Fi1 mounted-digital camera).

Images were analyzed using Image J software¹². The investigators used the software to outline the area of interest, then counted pixels and converted the results to metric units (Figure 1). To ensure measurement reliability, two investigators independently performed image analysis and compared the results which were within 2% error margin. The average cross-sectional area of muscle fibers, total fiber count, and the ratio of muscle to connective tissue for DNM/LLS bundles on each side of the face were obtained using Image J software¹². Areas of interest were outlined in the software, and pixel counts were converted to metric units in relation to a ruled slide digitized at the magnification used for tissue sample measurement. Two investigators independently analyzed XX% of the tissue samples, which produced values varying approximately 2%.

by outlining areas of interest and obtaining metric units in relation to Statistical analysis software was used to perform two-tailed Student's T tests with assumption of heteroscedasticity to compare each histologic parameter between the experimental and control side.

Results

A degree of functional recovery was seen in all animals following injury and repair

Following transection and repair, all animals demonstrated total loss of whisking movement on the experimental side. By post-repair 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 post repair day 28 (Figure 2). This was consistent with our previously published results^(7,13). Mean whisking amplitude was 17.1 degrees for the experimental side. The mean whisking amplitude was 58.5 degrees for the control side.

Qualitative histologic assessment of DNM/LLS using MTS

We noted marked qualitative differences in muscle architecture of DNM/LLS between the control and experimental sides (Figure 3). Notable bulk asymmetry was appreciated 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 collagen and adipose tissues, consistent with degenerative changes.

Quantitative histologic analysis of DNM/LLS using MTS

A significant difference was noted on quantitative comparison of muscle architectures of DNM/LLS bundles between the two groups (Figure 4). Compared to the control side, the experimental side demonstrated, on average, 41% fewer fibers (1,346 vs 794; $p=0.02$). There was also an average of nearly 50% lower muscle content on the experimental side (57% vs 29%; $p=0.01$). The muscle fibers appeared to have been replaced by collagen and adipose tissue as described above. These differences between control and experimental muscles were present in each animal and were statistically significant ($p<0.02$) when comparing groups. The individual DNM/LLS muscle fibers on the experimental side had on average 17.8% smaller cross-sectional area than the control side ($1,129\mu\text{m}^2$ vs $928\mu\text{m}^2$; $p=0.39$). While the experimental muscles had, on average, smaller cross-sectional area than healthy contralateral muscle in all animals, this difference did not reach statistical significance.

Discussion

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^(22–23). 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). Previously, no other studies have utilized quantitative analysis of the denervated vibrissal musculature to compare quantitative whisking function with histologic changes in whisker pad muscle architecture after facial nerve transection and suture repair.

Herein, we describe a methodology for quantitative analysis of rodent facial musculature following denervation and repair, employing 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 which was first described by Ruifrok and Johnston⁽³⁰⁾. This histochemical analysis technique quantitatively characterizes multiple stains using public domain NIH Image J software, and permits comparative quantification by calculating the contribution of each stain-specific, red-green-blue absorption.

Masson's trichrome is an ideal histochemical adjunct for color deconvolution analysis, due to its three-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 utilized in computerized quantitative analysis in both research and clinical applications^(32–33). 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 three 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 also have been shown to correlate with impaired muscle function^(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 unmanipulated sides were statistically significant and consistent across animals.

Conclusion

Herein, we describe a reliable, quantitative method of analyzing histologic changes in MTS treated DNM/LLS muscle bundle specimen through image processing software. Using this technique, we report statistically significant atrophic changes using both muscle fiber count and muscle fiber volume 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 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.

Herein, we describe a relatively easy, reliable, and quantitative method of analyzing histologic changes in MTS stained DNM/LLS muscle bundle specimen through image processing software. Using this technique, we report 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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Figure 1.

Sequential color deconvolution algorithm of LLS/DNM in Masson's Trichrome stain.

A-C: 20x magnification of specimen after MTS (Control side).

B: Entire specimen is outlined with the image software.

C: Adjusting the threshold, only the muscle area with red stain is outlined.

D: Same specimen in 400x magnification

E: Individual muscle fiber with red stain is outlined.

Recovery of whisking as demonstrated by average whisking amplitude was obtained by tracking horizontal movement of C-1 whisker using laser micrometer.

Control: 58.5

Experimental: 17.1

P-value: <0.001

Figure 2.

Measured recovery of whisking

Recovery of whisking as demonstrated by average whisking amplitude by tracking horizontal movement of C-1 whisker using laser micrometer.

Control: 58.5

Experimental: 17.1

P-value: <0.001

20x (A, C) and 400x (B, D) magnification of specimen

A and B: Control side reveals healthy features of muscle fibers including polygonal shape with overall uniform fiber size.

C and D: Experimental side reveals decreased volume of muscle replaced by collagen (blue stain) and adipose tissue (white space with septation). Individual fibers display irregular, round appearance.

Figure 3.

Qualitative comparison of LLS/DNM histology

20x (A,C) and 400x (B,D) magnification of specimen

A and B: Control side reveals healthy features of muscle fibers including polygonal shape with overall uniform fiber size.

C and D: Experimental side reveals decreased volume of muscle replaced by collagen (blue stain) and adipose tissue (white space with septation). Individual fibers display irregular, round appearance.

Figure 4.
Quantitative histochemical analysis of LLS/DNM

A: Muscle content (%)
Control: 57% Experimental: 29%
P-value: 0.006

B: Muscle fiber count
Control: 1,346 Experimental: 794
P-value: 0.02

C: Individual fiber surface area
Control: 1,129 μm^2 Experimental: 928 μm^2
P-value: 0.39