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The Effects of Venous Ensheatment on Facial Nerve Repair in Rat

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Abstract

Objective—To investigate the protective effect of autologous venous ensheathment on sutured rat facial nerve and to test whether the ensheathment could improve the functional recovery of repaired nerve and accuracy of axonal growth.

Study design—In vivo study.

Methods—Forty-six rats were examined, with 6 rats serving as normal controls and 40 receiving facial nerve transection and suture repair (SR) or transection and suture repair with an additional venous ensheathment (VE). Then they were subjected to functional testing, histological assessment of nerve specimens or retrograde tracing, respectively.

Results—At the postoperative day (POD) 60, the venous ensheathment showed no adhesion at the surrounding tissues. No significant difference in neuroma formation was found between the two surgical manipulations (SR and VE groups) ($p < 0.05$). Retrogradely-labeled motoneurons in facial nuclei were extremely disorganized after the facial nerve undertook surgical manipulation. In all manipulated groups, double retrogradely-labeled neurons, indicative of aberrant axonal branching during regeneration, could be observed after peripheral manipulation across all time points. With the two facial surgical manipulations, the average count of double-labeled neurons at POD 60 was significantly less than at POD 21 ($p < 0.05$).

Conclusions—Autologous venous ensheathment couldn't help with the functional recovery of facial nerve or improve the accuracy of axonal regeneration. Further studies are warranted to elucidate the effects of venous ensheathment in other motor and sensory nerve models.

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INTRODUCTION

Peripheral nerve injuries remain a challenge that confronts reconstructive surgeons, and the recovery from either motor, sensory, or mixed nerve damage is notoriously unpredictable. Peripheral nerve injuries are common, with its overall incidence standing at 6250 per 100,000 in the United States alone¹. Currently, research effort has been principally directed at improving functional outcome and regeneration accuracy of axons following neural anastomosis or grafting^{2–15}. Some studies suggested that isolation of the regenerating milieu, via ensheathment with autologous, biological, or synthetic materials, could promote axonal regeneration and improve functional outcome. Among the variables investigated, fascicular alignment, suture type, location, techniques, neurorrhaphy orientation² and tension³ have been exhaustively studied to determine the optimal neurorrhaphy methods^{2, 3–15}. Tensionless repair³, introduction of viable Schwann cells¹⁶, and delivery of certain neurotrophic agents only had modest recovery benefits^{17–20}. Further investigations on techniques that help improve axonal traversing of the neural suture site, and target accuracy, are warranted.

Over the past decades, isolating the neural regeneration milieu at the damaged site, to prevent axonal extravasation and misrouting has emerged as a potential method of improving functional recovery after nerve transection and repair^{21–24}. Histological investigation showed that autologous vein grafts out-performed allografts²³, and that venous ensheathment of nerve did not cause negative compression syndrome²⁴. Furthermore, some studies suggested that venous or biodegradable ensheathment was beneficial to neural regeneration across suture site^{21,22}. However, other studies suggested the otherwise²⁵.

Facial nerve anastomosis and the effects of different anastomosis strategies have long been subjects of active investigations on reconstruction of facial nerve^{3,7,12,14,26}. Animal models have been established to find alternatives of facial nerve suture, and studies exhibited insignificant difference in functional recovery of nerve between bio-absorbable sutures and their permanent counterparts¹³. Furthermore, the effect of using glue or entubulation on functional outcomes was negligible^{13,16,27–34}. More recently, highly quantitative methods, by using whisking analysis, have been employed for evaluating facial nerve recovery^{35–39}, and data have been generated regarding the architecture of the facial motor nucleus^{40–43}. Moreover, investigators used retrograde labeling to assess axonal target accuracy⁴⁴. The goal of this study was to use modern functional evaluation methods^{36,37} and retrograde labeling techniques^{40–44} to clarify, after facial nerve repair, whether the ensheathment with autologous vein across anastomosis site would benefit overall recovery of the nerve.

MATERIALS AND METHODS

Animals

Forty-six female Wistar Hannover rats (Charles River Laboratories, Wilmington, Massachusetts), weighing 250–300g, were obtained and treated in strict accordance with Massachusetts Eye and Ear Infirmary guidelines for animal care and use. Six rats, serving as normal group, were used for fluorescence tracer labeling. The remaining 40 animals received nerve manipulation at a site proximal to the *pes anserinus* at the midpoint between the main trunk trifurcation and origin of the post auricular branch. The nerve was subjected to either transection plus suture repair (SR)(n=20), or transection and suture repair with an additional venous ensheathment of the nerve (VE)(n=20). In 16 rats head-fixation device was implanted for the collection of functional whisking data (n=8 per SR or VE group). The animals were head-fixed and subjected to conditioning before operation. The other 24 rats underwent retrograde tracer analysis at two time points: the postoperative day (POD) 21 or 60 (n=6 per group pre timepoint). An equal number of animals from each SR and VE groups were used for functional testing or retrograde tracing analyses. Additionally, 2 animals were added to the functional testing group to account for possible attrition³⁶.

Surgical Procedures

Rats were anesthetized by intramuscular injection of ketamine hydrochloride (50 mg/kg) (Fort Dodge Animal Health, Fort Dodge, Iowa) and medetomidine hydrochloride (0.5 mg/kg) (Orion Corp., Espoo, Finland). All surgical procedures were performed by using of Leica Wild m651 Operating Microscope.

Head Fixation Implantation

Animals undergoing head fixation were anesthetically treated as previously described. Briefly, following induction of anesthesia, a midline incision was made in the scalp with the scalp exposed and temporalis muscles elevated off the cranium. The sterile implant was affixed to the calvarium with surgical titanium screws (1.3 by 4 mm; Synthes CMF, Needham MA), and the muscle was replaced and the skin closed. Animals were monitored on daily for infection, implant extrusion, unusual cage behaviors, and behavioral changes during handling. Following a two-week recovery period, all animals underwent a 10–14 day period of conditioning, to allow the animals to acclimatize to the rigid restraint device. Once animals responded favorably to behavioral conditioning, (no signs of struggle), the animals were subjected to facial nerve manipulation and then functional testing was conducted.

Facial Nerve Manipulation

In all animals, the left facial nerve was microsurgically exposed. The main trunk of the facial nerve was identified and dissected close to the *pes anserinus*. Transection was performed at the main trunk distal to the origin of the posterior auricular branch (Figure 1), obliquely to help prevent rotation during repair⁴⁰. In the SR group, standard epineurial suture was done with two 10-0 nylon sutures. In the VE group, an 8-mm segment of the retromandibular vein was harvested, the proximal nerve stump was passed through the venous segment, and the

same epineurial suture was performed. Finally, the venous segment was approximated to cover the repair site to complete the suture (Figure 2).

Functional testing

Functional whisking data were collected on weekly basis for 15 weeks, using a previously described testing apparatus^{35–37}. Briefly, once animals were placed in the body restraint and head fixation apparatus, polyimide tubes (SWPT-045, SWPT-008, Small Parts Inc, Miami Lakes, FL) were threaded onto the C-1 whisker. Whisking behavior was monitored by employing laser micrometers (MetraLight, Santa Mateo, CA) interfaced to a data acquisition computer. Each data collection period lasted 5 minutes.

Retrograde labeling and observation

Application of retrograde tracers—Three days prior to sacrifice (either POD day 18 or 57), rats were anesthetized as previously described. The distal branches of the posterior auricular, zygomatic, buccal, and mandibular branches of the facial nerve were exposed through four small non-connecting incisions to prevent unwanted tracer spread. Once the nerves were separately exposed, they were injected with fluorescent retrograde neuronal tracers as previously described⁴⁰. Five microliters of 3% Fluoro-Emerald (FE) (Sigma Chemical Co., St. Louis, MO), 2% Fluoro-Gold (FG) (Fluorochrome LLC, Denver, CO) and 4% Fluoro-Ruby (FR) (Molecular Probes, Eugene, OR) were injected by using a Hamilton syringe, into the posterior auricular branch, the zygomatic branch, and the buccal/mandibular branches, respectively (Figure 1).

Harvest and sectioning of brainstem and suture site of nerve—After 3 days, the animals were profoundly anesthetized with ketamine hydrochloride (50 mg/kg) (Fort Dodge Animal Health, Fort Dodge, Iowa) and medetomidine hydrochloride (0.5 mg/kg) (Orion Corp., Espoo, Finland), and perfused with 200 ml phosphate-buffered saline (0.1 M, pH 7) followed by 200 ml 4% paraformaldehyde. On the 60th post-operative day, the suture site of facial nerve was exposed and harvested. Two pathologists blind to the grouping identified the presence of traumatic neuroma by histologically assessing the longitudinal sections of each transected nerve with 0.1% Luxol Fast Blue staining⁴⁵. Neuromas were identified mainly against the following criteria: (1) the regenerating axons of a classic neuroma were relatively disorganized; (2) the funicular architecture was lost and (3) the intraneural fibrosis developed⁴⁶.

Subsequently, the brainstem, containing the facial motor nuclei, was removed and immersed into 30% sucrose at 4°C for cryoprotection⁴⁰. Then, the specimens were serially cut into coronal sections of 20 µm on a freezing microtome (Leica 100 Cryostat; Leica Microsystems, Wetzlar, Germany). For each five serial slices, the fourth and fifth sections in this series were selected, each section being separately assigned to two sets. The first set was stained with 1% toluidine blue for the Nissl-stained facial neurons to confirm that the second set contained facial nuclei. The second set was observed for neurons labeled by fluorescence tracer under an Olympus IX-70 fluorescence microscope (Olympus, Tokyo, Japan).

Counting of labeled neurons—The neurons labeled by FE, FR, and FG in the second set were counted, and only large neurons with a clear, visible contour at $\times 40$ magnification were included in the counts. The ratio between FR-labeled neurons and FE-labeled neurons, and the ratio between FG-labeled neurons and FE-labeled neuron were used to normalize the data before intergroup comparison. Double (FG/FR)-labeled neurons in merged images were counted and the count served as a measure of aberrant axonal branching during regeneration. To minimize inaccuracy in counting, the average number of double-labeled neurons of each slice in a set of facial nuclei slices was reported and the average counts of double-labeled neurons were used to reflect the aberrant axon regeneration following nerve manipulation.

Statistical Analysis

Whisking—Data were analyzed by utilizing a whisking software package developed by Bermejo *et al*^{38,39} and the program computes amplitude, velocity, and acceleration for every vibrissal excursion in 300 seconds. Following data collection, the three whisks with the greatest amplitude were identified for each animal, and the mean recorded. An independent two-sample, two-tailed Student's *t* test was used for data analysis, with a $p < 0.05$ being considered statistically significant. Daily behavioral variation in whisking effort among rats was normalized by obtaining the ratio between the whisk amplitude of the manipulated side and the value of the control side. This ratio was termed the “relative recovery”^{35–37}.

Histological observation—The numbers of neuromas were statistically analyzed by employing the Chi-square test, with a $p < 0.05$ being considered to be statistically significant.

Retrograde labeling—The mean and standard deviation of the normalized neuron counts after retrograde labeling in each of the four groups were compiled and compared to the normal group as well as to one another. Comparison was made among POD21SR, POD21VE, POD60SR and POD60VE in terms of the average counts of double-labeled neurons by using the F test. Statistical analysis was done by using the SPSS 13.0. P values less than 0.05 were considered to be statistically significant.

RESULTS

All animals uneventfully underwent facial nerve manipulations. Post-operatively, animals in all groups showed normal cage behaviors, weight gain, and social interactions. None of the animals in the retrograde labeling groups had to be excluded for any reason. One animal in the functional testing group was removed from the study due to head fixation failure, with the attrition rate being 6.25%, well below the accepted and reported rates^{36,47,48}.

Whisking analysis revealed a good recovery along a predicted time course, with no difference found between the SR and the VE groups and between the two groups and historical controls^{27,36,48} (Figure 3). Animals achieved approximately a 20% of normal whisking amplitude, which was consistent with previously reported values^{27,36,48,49}.

Under a surgical microscope, the venous ensheathment showed no adhesion at the surroundings in the POD60VE group while there was adhesion in POD60SR group (Figure 4). Histological examination revealed that, in POD60SR group, 5 of 6 sutured site of facial

nerve developed neuromas, and 4 of 6 had neuroma formation in the POD60VE group (Figure 4). Nonetheless, no statistically significant difference was found between the two groups ($p=0.566971$; Chi square test).

In the normal group, neurons, as expected, were FE-, FG-, and FR-labeled in their respective sub-nuclear regions, and the results were consistent with our previous investigations and findings made by other researchers^{40–44}. FE-labeled neurons innervating the posterior auricular branch were located only within medial ventral subnuclei, FR-labeled neurons innervating the buccal and marginal branches were in the intermediate and lateral subnuclei (the distribution of these three subnuclei assumed an “L” shape). The FG-labeled neurons innervating the zygomatic branch lied in the lateral dorsal and medial dorsal subnuclei (Figure 5).

In the four surgically manipulated groups (POD21SR, POD21VE, POD60SR and POD60VE), only FE-labeled neurons, as expected, remained located in their normal anatomical area. The FR- and FG-labeled neurons demonstrated extreme disorganization at POD 21 and POD 60 (Figure 5). F test showed no significant differences in the ratio between FR-labeled neurons and FE-labeled neurons, and in the ratio between FG-labeled neurons and FE-labeled neurons among all groups respectively (Figure 6).

In the four manipulated groups (POD21SR, POD21VE, POD60SR and POD60VE), double (FR/FG)-labeled neurons could be observed (Figure 5). ANOVA revealed that there existed differences in the average counts of double-labeled neurons among the four groups. Moreover, Student–Newman–Keuls test showed that the average count of double-labeled neurons at POD 60 was significantly less than at at POD 21 in both SR and VE groups (Figure 7).

DISCUSSION

Great effort has been made to improve neural anastomosis techniques. Many studies showed that it is possible to improve neural recovery by isolating the surgical bed by ensheathment with various materials^{50–55}. The venous materials can cover both ends of the suture site, thus avoiding mushrooming due to size mismatch, and can allow for better regeneration by reducing neuroma formation. Galeano M *et al* reported that vein was a promising ensheathing material that could isolate regenerating neural tissues from the surrounding environment⁵⁶, thereby decreasing inflammatory response and reducing the possibility of neuroma development at the anastomotic site. Other studies yielded similar results and demonstrated that vein could be effectively used for ensheathment^{23,57}.

In this study, we, by employing a quantitative technique for measuring the recovery of whisking^{27,35–37,48,49}, in combination with triple-retrograde labeling of nerves, observed the results of two neural suture methods and found that facial nerve suture with venous ensheathment failed to apparently improve the quality of regenerated axons, in terms of functions and morphology. Our study, to some extent, provided more information that might help to resolve the contradictions with respect to venous ensheathment.

The loss of somatotopic organization of the facial motor nuclei has been described by other investigators, and had been employed to evaluate the accuracy of axonal regeneration^{40,41,44}. For example, Angelov, Chen and Choi *et al* reported similar findings in regard to application of retrograde tracer to facial nerve evaluation^{40,41,44}. All the investigators agreed that the distribution of sub-nuclei of the facial nucleus possessed certain fixed patterns, the arrangement might be disturbed and double-labeled neurons, an indication of axonal branching, might appear after peripheral injury. This study confirmed that aberrant axonal branching following nerve insults and regeneration is inevitable, which was consistent with the findings reported by Chen and Angelov *et al*^{40,58,59}. Angelov *et al* suggested that excessive axonal branching is a major factor contributing to post-injury synkinetic, hypertonic and/or incomplete function recovery and our study supported the notion^{40,58,59}.

As expected, this study demonstrated that the neurons retrogradely labeled by FE, when injected into the posterior auricular branches, were distributed in certain subnuclear regions, independent of nerve manipulation. This finding can be explained by the fact that the posterior auricular branch leaves the main trunk of the facial nerve before the transection site. Therefore, the neurons in the posterior auricular subnucleus were undisturbed by the transection of the main trunk facial nerve and repair. The relative stable count of FE-labeled neurons can be used to normalize the counts of neurons labeled by FG or FR. By such normalization, we found that the counts of FG- or FR-labeled neurons didn't experience substantial changes despite the fact that the distribution of the labeled neurons was disturbed due to axonal misrouting during regeneration, which might lead to the poor functional recovery of the facial nerve.

In this study, we found that double-labeled neurons were decreased from POD 21 to POD 60 in both SR and VE group. We believe the reduced double-labeled neurons might be explained by a selective deletion or "pruning" of regenerating aberrant axons, a plastic process during the recovery period, though no functional improvement was achieved^{60–62}. Ito and Kudo *et al* also found that the number of double-labeled neurons dropped at a late stage of regeneration (12–13 weeks). They used different tracers for double-labeling in adult guinea pigs with facial nerve nerve subjected to 5-min crush injury using a microsurgical needle holder (0.5 mm in width)⁶³. Of note, Angelov *et al* showed that in adult female Wistar rats, the lesion of the contralateral infraorbital nerve reduced the branching (or enhanced the elimination of branches) of transected facial axons (as estimated by the number of double-labeled neurons), and even promoted the recovery of coordinated activity of the facial muscles⁵⁸. Nonetheless, some researchers reported different findings. For instance, Choi *et al* exhibited that in 38 adult female AS strain rats subjected to similar facial nerve transection and suture, doubled-labeled neurons by FR and Fluoro-Blue (FB) did not change significantly during a regeneration period ranging from 1 to 10 months⁴¹. Therefore, the inconsistency in the finding of the double-labeled neurons might be ascribed to differences in growth rate of nerve regeneration, injury, animal models, anatomical configuration, tracers and methods for tracer delivery^{41,58,60–65}. Asahara *et al* reported a conflicting finding that the injury and regeneration of one branch of facial nerve may affect the somatotopy of the intact postauricular branch⁶⁴, and the aforementioned factors might also account for the inconsistency.

This study had a limitation in that it failed to observe certain aspects of the sequela of facial paralysis, such as synkinesis as observed in Choi's study⁴¹, crocodile tears, hypertonicity and *etc.* Therefore, it is hard for us to definitely assess the effect of venous ensheathment on the development of such sequelae, though some studies in humans reported that pain syndrome following neural repair with venous ensheathment was relieved⁵⁷.

CONCLUSIONS

Our study exhibited that venous ensheathment failed to improve functional recovery after facial nerve transaction and suture repair compared to suture repair alone. We found that axonal branching was aberrant and accuracy of axonal regeneration was improved at the late stage of the regeneration after facial nerve suture in rats with or without venous ensheathment. Further studies are warranted to elucidate the effects of venous ensheathment in other motor and sensory models.

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Fig. 1. Illustration of the facial nerve anatomy with demarcation of repair site (x-suture) and the injection sites of the FE-labeled posterior auricular branch, FG-labeled zygomatic branch and FR-labeled buccal and submandibular branches.

Fig. 2. Facial nerve transected and suture repair (A and B), transected facial nerve and one segment of retromandibular vein (C) and sutured site of facial nerve with venous ensheathment (D). (Star indicates the proximal segment)

Fig. 3.

Whisking amplitude of suture repair (SR) and suture repair with venous ensheathment (VE) groups plotted in terms of the ratio between the amplitude of the recovering side and that of the healthy side (i.e. relative recovery) over time. A value of 1 represents symmetrical whisking amplitude. Error bars indicate ± 1 standard deviation.

Fig. 4.

Microphotograph exhibiting that there was epineural adhesion at the sutured site in SR group while no adhesion was found in VE group. Luxol fast blue staining shows the formation of neuroma in sutured site in both groups ($\times 200$). (The large black arrows in two upper photos show the sutured sites and two asterisks indicate the proximal ends of facial nerve). Two insets show the specimens from the sutured sites. Two lower photos show the histopathological features of neuromas (Luxol fast blue staining, $\times 200$). (The small black arrows show loss of the funicular architecture, the white arrows indicate development of intraneural fibrosis, the two triangles show the ensheathed venous wall and the round circle indicates the suture knot.)

Fig. 5.

Microphotographs showing the distribution of FE-, FG- and FR-labeled neurons in one brain slice at different time points (normal, POD21SR, POD21VE, POD60SR and POD60VE). The first photo shows the Nissl-stained facial nuclei in a normal rat. The white arrows indicate double (FG/FR)-labeled neurons.

Fig. 6.

The ratio between FR- and FG-labeled neurons and FE-labeled neurons were used for analysis (FR/FE & FG/FE). There were no significant differences in the ratio between FR-labeled neurons and FE-labeled ones, or in the ratio between FG-labeled neurons and FE-labeled neurons among all groups, respectively.

Fig. 7.

Double(FG/FR)-labeled neurons were counted in each of the four surgically manipulated groups (POD21SR, POD21VE, POD60SR and POD60VE). The average counts of double-labeled neurons of each slice in a set of facial nuclei slices were used for comparison.

* compared to POD 21 group subjected to identical surgical manipulation, $p < 0.05$.