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Better Nerve Regeneration with Distally Based Fascicular Turnover Flap than Conventional Autologous Nerve Graft in a Rat Sciatic Nerve Defect Model

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이 논문을 박사학위 논문으로 제출함

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계 명 대 학 교 대 학 원

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감 사 문

먼저 좋은 주제로 논문을 완성하여 제출하기까지 도움을 주신 교수님들께 감사를 드리며, 특히나 바쁘신 가운데서도 아낌없이 보살펴 주시고 세세한 부분까지 지도해 주신 지도교수 김준형 교수님께 머리 숙여 깊이 감사를 드립니다. 그리고 자상하게 심사를 맡아주신 서성일 교수님, 조호찬, 정운혁, 그리고 김태곤 교수님께도 감사의 인사를 올립니다.

또한 많이 부족한 저를 위해 항상 사랑으로 보살펴 주시는 아버지, 어머니와 장모님, 그리고 제 삶의 이유이며 그 무엇보다 소중한 우리 락이, 욱이, 다솜이, 또한 결혼한 이후부터 지금까지 항상 변함없이 저에게 힘을 주는 인생의 활력소인 사랑하는 아내와 함께 박사가 된 기쁨을 나누고 싶습니다. 다시 한번 부족한 저를 이끌어 주신 모두에게 감사드립니다.

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김 진 한

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1. Introduction

Nerve defect should be repaired. Using the autologous nerve graft, nerve defect was expected to recover perfectly. For example, in patients with facial nerve damage that is responsible for facial expressions, if the defect was large, the autologous nerve graft using sural nerve can be used. Recently, autologous nerve graft had been carried out conventionally, however, many studies was done by Koshima et al, to eliminate the necessity of donor site and improve the results of nerve recovery.

Koshima et al. (1) proposed the 'fascicular turnover flap' as an alternative to autologous nerve grafts in nerve gap repair. A fascicular turnover flap is formed when fascicles split from the proximal or distal stump of the nerve gap are turned over the nerve gap to reach the contralateral nerve end. Because a part of the fascicular turnover flap is connected to the proximal or distal stump, axonal sprouting toward the distal direction through the communicating branches between the fascicles is possible, unlike with a free nerve graft, and vascularization of the fascicular turnover flap can be maintained by the rich microvascular networks around the fascicles (1). Therefore, the fascicular turnover flap can have many advantages, such as vascularization of the nerve flap, retention of the donor nerve, and shortened operation time due to the presence of only one site of microanastomosis. However, the fascicular turnover flap has not been widely used in nerve gap repair not only because there have been few preliminary clinical studies reporting favorable results (1-3) but also because there have been few studies comparing the fascicular turnover flap with the conventional nerve graft. Recently, Uehara et al. (4)

reported similar outcomes with the fascicular turnover flap as with autologous nerve grafts in a rat facial nerve defect model. However, their results are limited in that axonal regeneration was not evaluated.

I hypothesized that the fascicular turnover flap would achieve better nerve regeneration in nerve gap repair than the conventional nerve graft. In addition, nerve recovery is better after distal injuries than after proximal ones, as axons that need to travel only a short distance to reach their target tissue are more likely to reconnect (5,6). Therefore, I investigated whether the fascicular turnover flap could better facilitate nerve regeneration than the conventional nerve graft and whether the outcome depends on the donor site (proximal or distal stump) of the fascicular turnover flap.

2. Materials and Methods

2.1. Animals

Adult male Sprague-Dawley rats weighing from 300 to 325 g were purchased from ORIENT BIO (Seongnam, Republic of Korea) and maintained in the animal facility at Keimyung University. The university animal care committee for animal research at Keimyung University approved the study protocol (KM-2017-28). Animals were housed for at least 7 days prior to initiating the experiments in a well-ventilated and temperature-controlled environment with access to drinking water ad libitum. A total of 24 rats were randomly divided into an autologous nerve graft control group (nerve graft group, n=8), a proximal fascicular turnover flap group (proximal flap group, n=8), and a distal fascicular turnover flap group (distal flap group, n=8).

2.2. Surgical procedure

The rats were anesthetized with Zoletil 50® (10 mg/kg, intramuscular; Virbac Laboratories, Carros, France). The right sciatic nerve was exposed through a gluteal muscle-splitting incision. For animals in the proximal flap group, under a surgical microscope, 7 mm of the nerve located 1 cm below the inferior margin of the piriformis was resected to form the defect. Eight-millimeter-long fascicles involving 30% of the cross-sectional area of the sciatic nerve were split from the proximal stump of the nerve gap. The fascicular flap with a 1-mm-long

attachment to the proximal stump was turned over the nerve gap to reach the end of the distal stump, and the nerve was anastomosed between the end of the fascicular turnover flap and the end of the distal stump of the nerve gap using 10-0 nylon sutures without tension (Figure. 1). In the distal flap group, 7 mm of the nerve located 2 mm below the inferior margin of the piriformis was resected to form the defect. Eight-millimeter-long fascicles split from the distal stump of the nerve gap were turned over the nerve gap to reach the end of the proximal stump. In the nerve graft group, the nerve gap was bridged using the reversed nerve segment as an autologous nerve graft (Figure. 2). The skin incision was closed with nylon sutures (5-0). Postoperative analgesia was ensured by administering 5 mg/kg of ketoprofen subcutaneously. To prevent toe chewing, a bitter solution called Grannick's Bitter Apple was applied twice daily to the affected foot. Eight weeks after the surgery, the nerves were dissected, and biopsy at the midgraft and midflap levels was performed under anesthesia.

2.3. Walking footprint analysis

After 8 weeks, the rats were subjected to conditioning trials on a walking track (8.2 x 42 cm) darkened at one end. Their paws were soaked in Chinese ink, and the mice were walked multiple times on white paper to obtain measurable prints. The sciatic function index (SFI) was calculated using the formula proposed by Bain et al (7).

2.4. Wet muscle weight and muscle histology

After 8 weeks, the animals were euthanized, and the soleus and

gastrocnemius muscles of both hind limbs were harvested and weighed immediately using a digital scale. The muscle weight ratio (operated side/nonoperated side) was recorded for analysis. Then, the harvested muscle specimens were fixed in formalin, embedded in paraffin, and sectioned at 5 μm. The sections were stained with hematoxylin and eosin (H&E). The muscle fiber diameter in 3 high-power (400 X magnification) fields was measured in areas of normal and atrophic muscle, and the average was calculated by a pathologist blinded to the study conditions.

2.5. Immunohistochemical analysis

To evaluate the microcirculation in the nerve graft and fascicular turnover flaps, double-fluorescence immunohistochemistry was performed with anti-CD34 and anti-α-smooth muscle actin (SMA) antibodies. To detect nerve fibers and Schwann cells, fluorescence immunohistochemistry was performed with anti-neurofilament (NF)-200 and anti-S-100 antibodies. Immunohistochemistry was conducted using standard methods with the following antibodies: CD34 (1:100; Sigma Chemical Co., St. Louis, Mo, USA), α-SMA (1:100; Cell Signaling Technology, Danvers, MA, USA), NF-200 (1:100; Abcam, Cambridge, UK), and S-100 (1:100; Abcam, Cambridge, UK). A pathologist blinded to the study conditions counted the newly formed capillaries and mature capillaries in 5 high-power (200 X magnification) fields and calculated the average. Newly formed capillaries have a small vessel diameter ($\leq$ 10 μm) and consist of only CD34-positive cells or CD34 (internal layer)- and α-SMA (external layer)-positive cells. Mature capillaries have a

vessel diameter > 10 μm and consist of CD34- and α-SMA-positive cells (8). ZEN 2 blue edition software (Carl Zeiss AG, Oberkochen, Germany) was used to quantify the fluorescence intensity of NF-200- and S-100-positive areas at 400 X magnification.

2.6. Transmission electron microscopy (TEM)

The harvested nerve specimens were fixed by immersion in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for overnight at 4 °C. The specimens were postfixed in 1% OsO4 in 0.1 M phosphate buffer (pH 7.4) for 3-4 h at 4 °C, dehydrated with an ascending series of ethanol, longitudinally oriented, and embedded in epoxy resin. Ultrathin (70-90 nm) nerve cross-sections were stained with uranyl acetate and lead citrate for examination using an electron microscope (Hitachi H-7100, Hitachi Co. Tokyo, Japan).

2.7. Statistical analysis

The results are presented as the mean ± standard error of the mean (SEM). All of categorical variables were analyzed using the nonparametric Kruskal-Wallis test with the Bonferroni-corrected Mann-Whitney U post hoc test in SPSS version 21.0 (IBM Corp., Armonk, NY, USA). All error bars represent the SEM. Differences with a $p < 0.05$ were considered statistically significant.

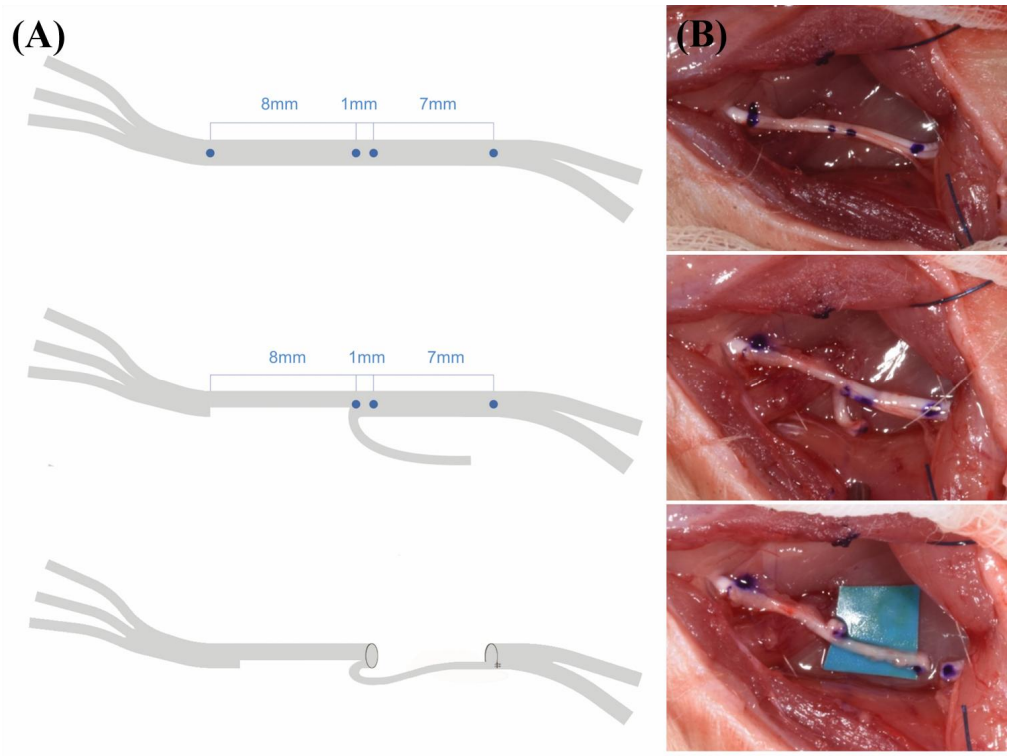

Figure 1. The proximal fascicular turnover flap. (A) Schematic illustrations. Seven millimeters of the nerve located 1 cm below the inferior margin of the piriformis were resected to create the defects. Eight-millimeter-long fascicles involving 30% of the cross-sectional area of the sciatic nerve were split from the proximal stump of the nerve gap. The fascicular flap with a 1-mm-long attachment to the proximal stump was turned over the nerve gap to reach the end of the distal stump. (B) Intraoperative photos.

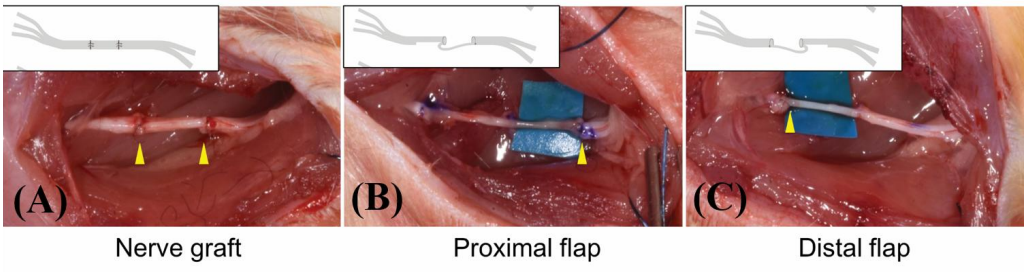

Figure 2. Schematic illustrations and intraoperative photos of the three experimental groups. The yellow arrowheads indicate the sites of microanastomosis. (A) Nerve defect was recovered with autologous nerve graft. (B) Nerve defect was recovered with proximally based nerve fascicular turnover flap. (C) Nerve defect was recovered with distally based nerve fascicular turnover flap.

3. Results

At 8 weeks after the surgery, I estimated the functional nerve recovery of the rats using a walking footprint analysis. The SFI in the nerve graft group was significantly higher than that in the proximal flap group ($p < 0.05$), but there was no significant difference between the nerve graft and distal flap groups (Figure 3). When the wound was reopened for gross examination of the sciatic nerve at 8 weeks after the surgery, the nerves were moderately adhered to the adjacent muscle. Neuroma-like tissue was not found in the site of anastomosis or the fascicle donor area (Figure 4). Next, I assessed muscle atrophy. Macroscopically, all animals showed significant muscle atrophy on the operated side. The average muscle weight ratio of the soleus muscle and gastrocnemius muscle (operated side/nonoperated side) in the nerve graft group, the proximal flap group, and the distal flap group were 41.7 ± 3.2%, 37.9 ± 3.1%, and 44.9 ± 1.9%, respectively (Figure 5). There were no significant differences among the groups ($p > 0.05$). Under light microscopy, there were no significant differences in the muscle fiber diameter among the groups (Figure 6).

I investigated axonal regeneration and Schwann cell regeneration by staining for NF-200 and S-100. In the cross-sections, the S-100 staining intensity was higher in the distal flap group than in the nerve graft group ($p < 0.05$), but no significant difference in the NF-200 staining intensity was observed (Figure 7). In the longitudinal sections, the NF-200 staining intensity was higher in the distal flap group than in the nerve graft group ($p < 0.01$) and the proximal flap group ($p < 0.01$), but no significant difference in the S-100 staining intensity was observed (Figure 8). I also assessed the microvessel density and extent

of neovascularization. More mature capillaries (vessel diameter > 10 μm, consisting of CD34- and α-SMA-positive cells) were observed in the proximal ($p < 0.001$) and distal ($p < 0.05$) flap groups than in the nerve graft group. No significant difference was observed between the proximal and distal flap groups. There were no significant differences in the density of newly formed capillaries among the groups (Figure 9).

Finally, I performed a TEM examination. In the distal flap group, a compact, regular myelin sheath was observed around the myelinated nerve fibers. However, in the other groups, abnormal myelination, such as irregular thickening, splitting, invagination, and evagination of the myelin sheath, was commonly observed (Figure 10).

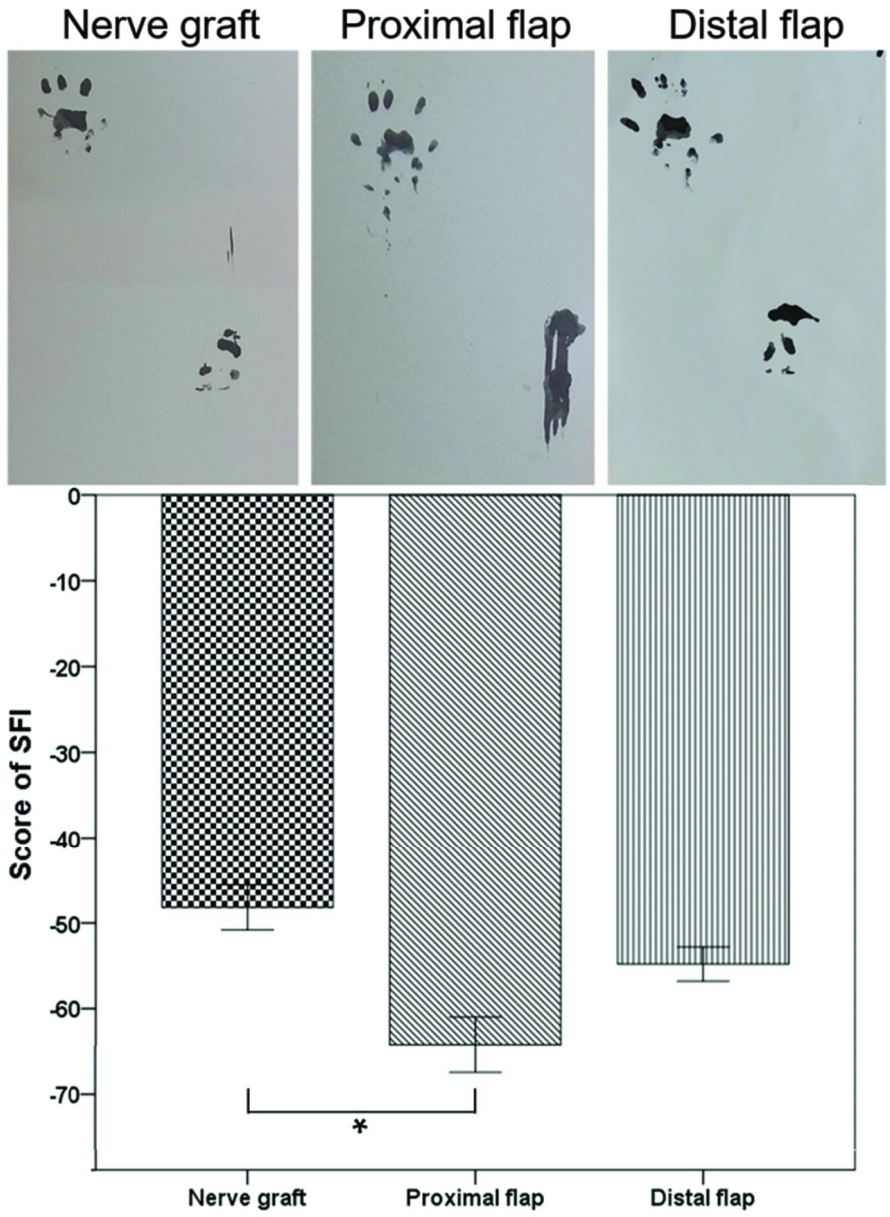

Figure 3. Walking footprint analysis to obtain the sciatic function index (SFI). *: $p < 0.05$.

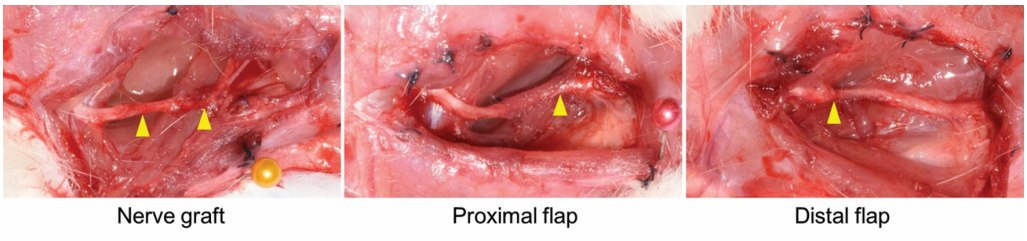

Figure 4. Gross appearance of the sciatic nerve 8 weeks after the surgery. All of the group showed that neuroma-like tissue was not found in the site of anastomosis or the fascicle donor area.

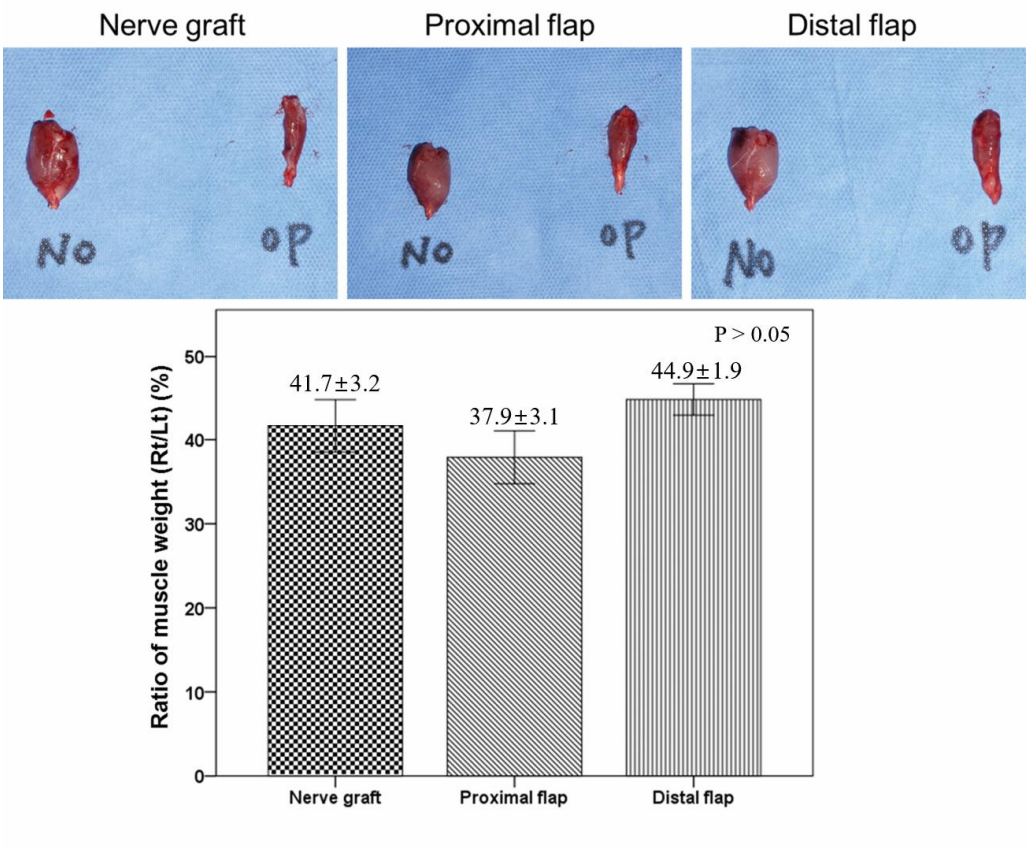

Figure 5. The change in the wet muscle (soleus and gastrocnemius muscles) weight 8 weeks after the surgery. There were no significant differences among the groups ($p > 0.05$). No: nonoperated side, Op: operated side.

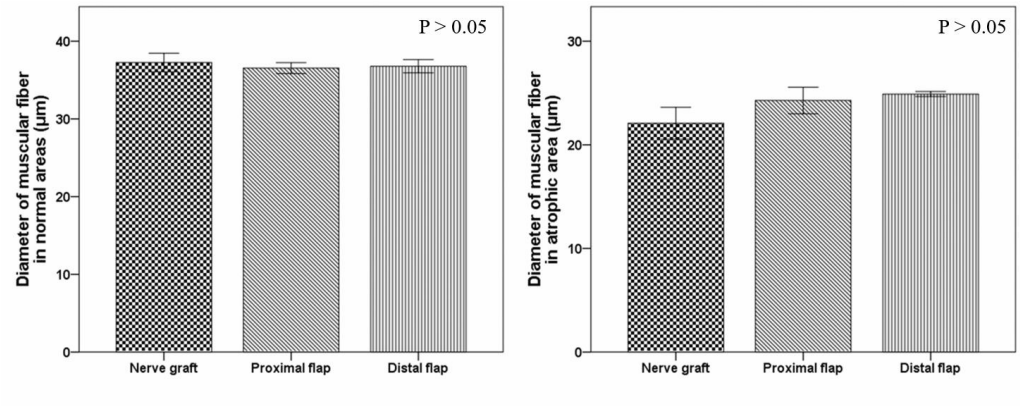

Figure 6. The change in the muscle fiber size 8 weeks after the surgery. There were no significant differences in the muscle fiber diameter among the groups ($p > 0.05$).

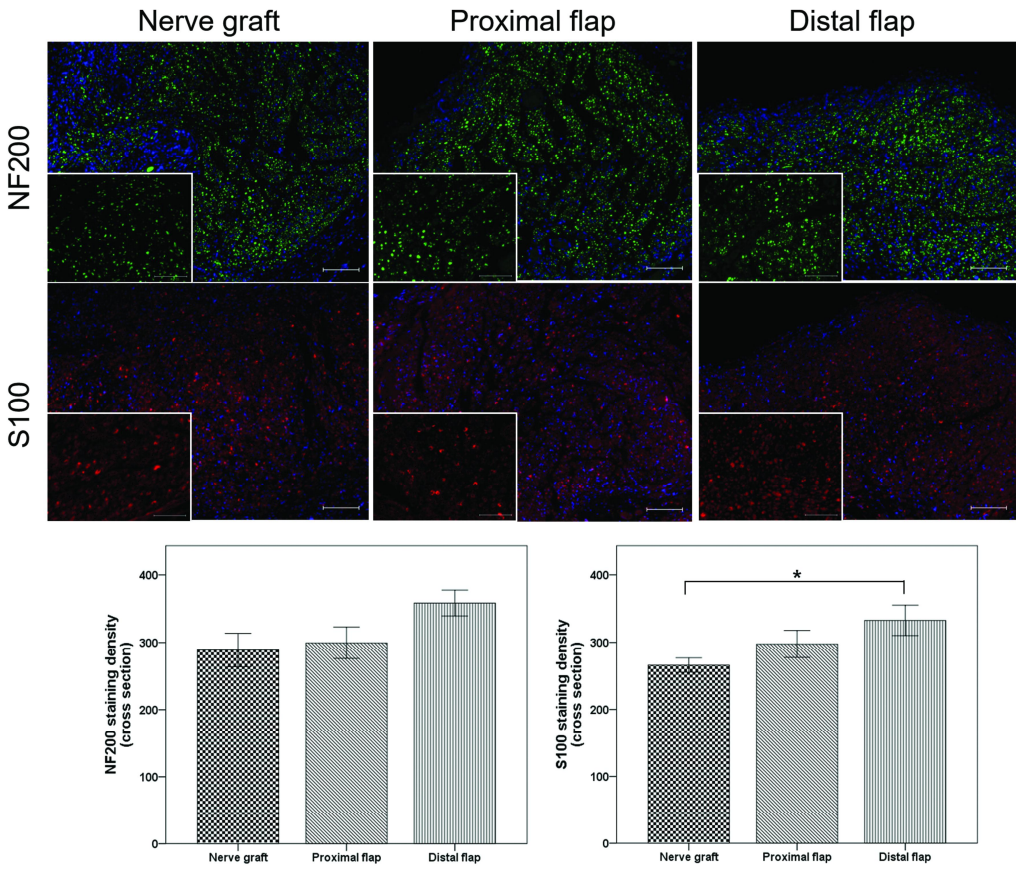

Figure 7. Immunofluorescence staining for NF-200 and S-100 in cross-sections 8 weeks after the surgery. The staining intensity for S-100 compared was higher in the distal flap group than in the nerve graft group ($p < 0.05$). *: $p < 0.05$. Scale bar = 100 μm. Scale bar of inset = 50 μm.

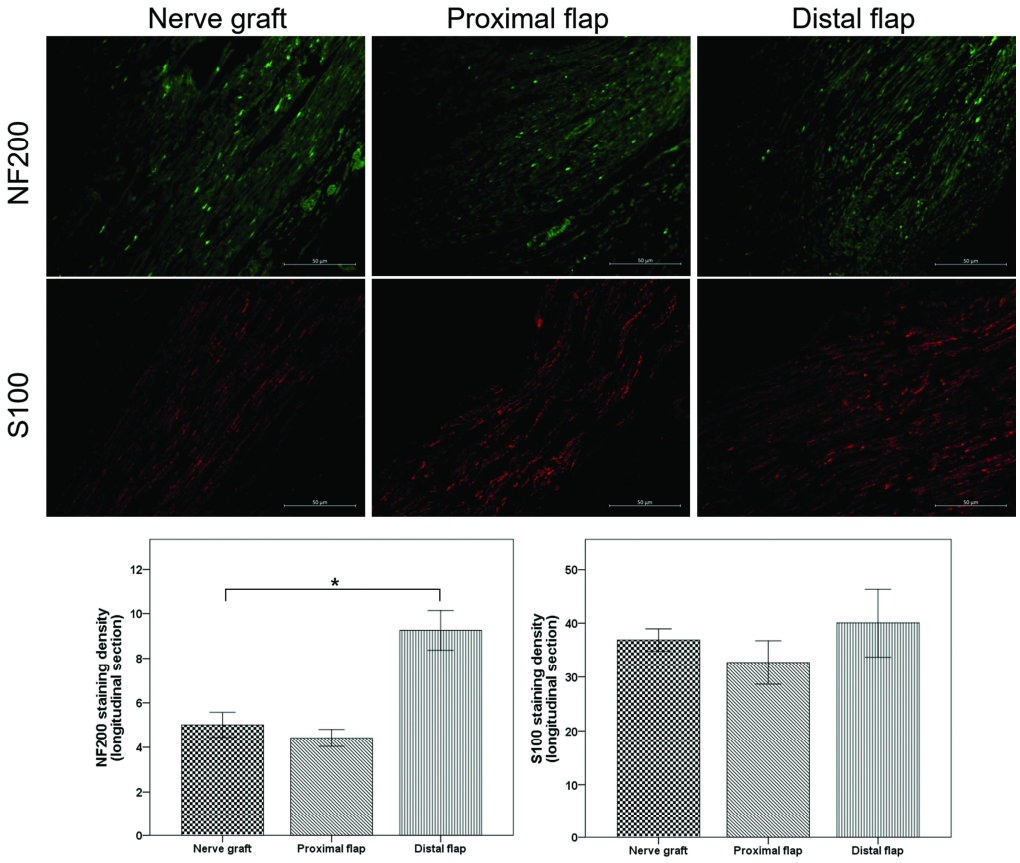

Figure 8. Immunofluorescence staining for NF-200 and S-100 in longitudinal sections 8 weeks after the autologous nerve graft, proximally and distally based nerve fascicular turnover flap. *: $p < 0.05$.

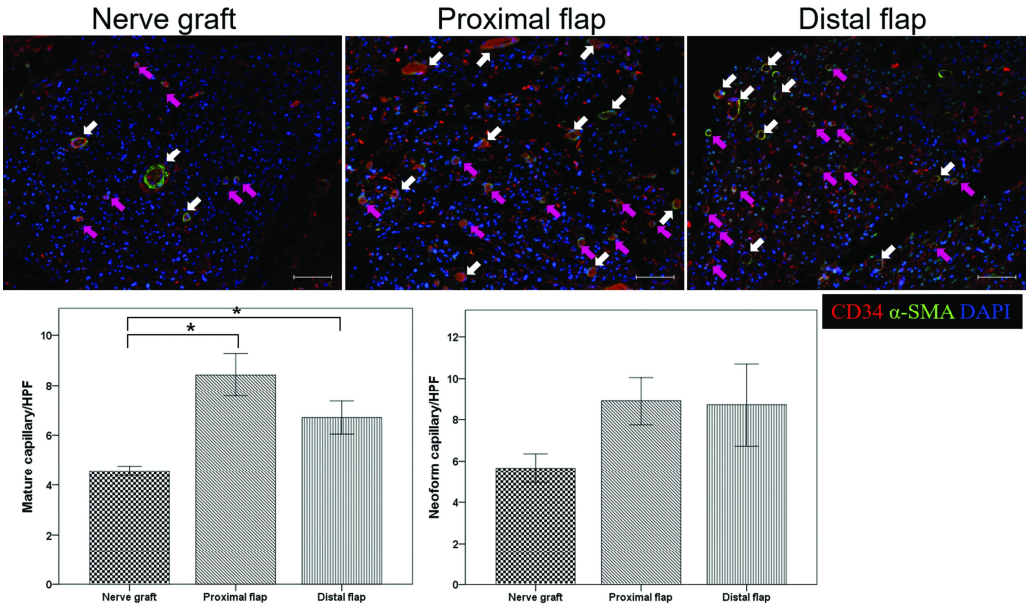

Figure 9. Immunofluorescence staining for CD34 and α-SMA 8 weeks after the surgery. More mature capillaries (vessel diameter > 10 μm, consisting of CD34- and α-SMA-positive cells) were observed in the proximal ($p < 0.001$) and distal ($p < 0.05$) flap groups than in the nerve graft group. There was no significant difference among the groups in the density of newly formed capillaries (small diameter (≤ 10 μm), consisting only of CD34-positive cells or CD34 (internal layer)- and α-SMA (external layer)-positive cells. The white and pink arrows indicate mature capillaries and new capillaries, respectively. *: $p < 0.05$. Scale bar = 50 μm.

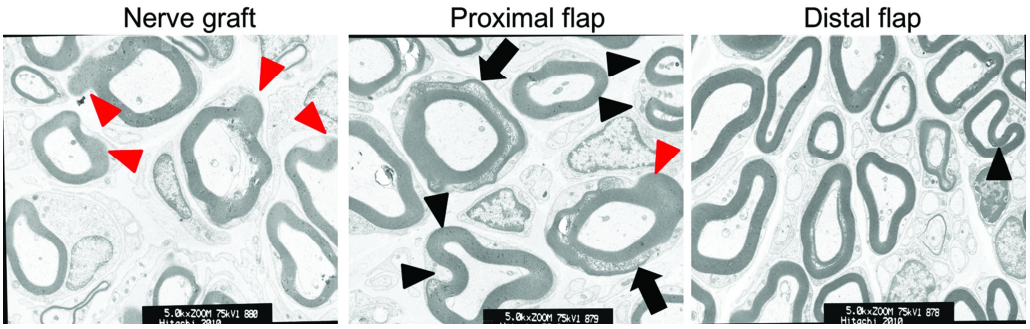

Figure 10. TEM showed a compact, regular myelin sheath around the myelinated nerve fibers in the distal flap group. However, in the other groups, abnormal myelination, such as irregular thickening, splitting, invagination, and evagination of the myelin sheath, was commonly observed. The black arrowheads indicate invagination and evagination of the myelin sheath. The red arrowheads and black arrows indicate irregular thickening and splitting of the myelin sheath, respectively.

4. Discussion

The fascicular turnover flap maintains vascularization via the rich microvascular networks around the fascicles connected to the nerve stump (1). In our study, more capillaries were observed in the proximal and distal flap groups than in the nerve graft group. Because the fascicular turnover flap has several advantages in nerve regeneration, such as the presence of one site of microanastomosis and nerve graft vascularization, I predicted that better nerve regeneration in nerve gap repair could be achieved with proximal and distal fascicular turnover flaps than with the conventional nerve graft. Our results show similar improvement in the SFI in the distal flap and nerve graft groups, with less improvement in the SFI in the proximal flap group than in the nerve graft group. Histology showed significant improvement in axonal regeneration and Schwann cell regeneration in the distal flap group compared with the nerve graft group but similar axonal regeneration and Schwann cell regeneration in the proximal flap and nerve graft groups. Therefore, the distal fascicular turnover flap might achieve better nerve regeneration in nerve gap repair than the conventional nerve graft, while the proximal fascicular turnover flap did not exhibit better results than the conventional nerve graft in nerve regeneration. These findings may be because division of the proximal stump for proximal fascicular flap elevation increases the extent of retrograde degeneration. Retrograde degeneration and Wallerian degeneration occur in the proximal and distal nerve stump, respectively. Unlike Wallerian degeneration, which occurs in the entire nerve segment distal to the injury site, retrograde degeneration occurs to a limited extent in the proximal stump, as far as to the first node of Ranvier (9). The degree

of degeneration of the nerve proximal to the site of anastomosis has a significant effect on nerve regeneration following nerve repair (10,11). Therefore, because of the increase in retrograde degeneration due to the length of the fascicular flap, the proximal turnover flap may achieve less favorable results in nerve regeneration than the distal turnover flap. In accordance with our findings, Uehara et al. (4) noted that the proximal turnover flap achieved results similar to those of an autologous nerve graft in facial nerve gap reconstruction.

Unlike the histological results, no differences in the calf muscle were found among the groups. Our study is different from other studies on nerve gap repair in that autologous grafts were compared in our study; in other studies, allografts or synthetic nerve conduits were compared with autologous nerve grafts (12-14). Sufficient time may be required to show differences in muscles among groups with similar autologous nerve grafts. Therefore, there might have been no significant difference in the calf muscle because the biopsy was performed too early (8 weeks) to observe such changes. Over time, significant changes in the calf muscle would occur.

There are several concerns regarding the fascicular turnover flap. First, a size discrepancy between the nerve stump and fascicular turnover flap in microanastomosis can negatively affect nerve regeneration. However, several authors have noted that in cable grafts, the results of nerve regeneration are not proportional to the cross-section of the grafted nerve. Seminow et al. (15) reported that single-fascicle nerve grafting achieved faster functional recovery and better morphometric outcomes than conventional nerve grafting. Okuyama et al. (16) reported that the outcomes of single- and multiple-fascicle nerve grafting were morphometrically and functionally similar. Tzou et al. (17) have noted that single-fascicle nerve grafting

has the potential for good functional nerve regeneration. Therefore, the size mismatch is not a weakness of the fascicular turnover flap. Second, the fascicular turnover flap can cause neuroma-in-continuity at the fascicle donor site. Significant nerve injury without disruption of the anatomical continuity of the nerve often causes the formation of neuroma-in-continuity (18). Several authors have reported neuroma-in-continuity models established by partial neurectomy of the nerve fascicle (19-21). Therefore, neuroma-in-continuity can occur at the fascicular turnover flap donor site. However, in our study, neuroma-like tissue was not found at the fascicular flap donor site. This may be because the diameter (70% of the entire nerve) of the remaining nerve following elevation of the fascicular turnover flap was larger than that in the other neuroma-in-continuity models, which was approximately 50% (19-21). The remaining nerve clearly provides guidance cues for axon regeneration toward the distal target and prevents neuroma formation (21). Therefore, the larger the diameter of the remaining nerve is after partial neurectomy, the less likely neuroma formation becomes. However, the capability for nerve regeneration and regrowth is lower in higher animals and humans than in lower species of animals, such as rats (19). Therefore, in the clinical setting, there is still a high probability of neuroma-in-continuity at the fascicular turnover flap donor site. Third, the fascicular turnover flap can cause functional loss to some extent due to sacrifice of the fascicle. Partial nerve lesions are associated with a varying degree of retained nerve function. Malushte et al. (20) observed incomplete functional recovery with a complementary degree of muscle atrophy after partial (50%) tibial neurectomy. In particular, large mixed fibers have a greater potential for inappropriate connections and poor functional recovery (20,22). Therefore, more favorable recovery of retained nerve function can be expected in

sensory nerves that carry a single (monofascicular) or very few (oligofascicular) fascicles. In a clinical study, Koshima et al. (1) reported favorable results of the fascicular turnover flap in the repair of facial nerve defects and digital nerve defects (monofascicular nerves). Ito et al. (3) reported favorable results in the repair of sural nerve defects (oligofascicular nerves) after sural nerve biopsy. I am currently conducting a clinical study on the usefulness of the fascicular turnover flap for sural nerve reconstruction after sural nerve biopsy. To date, the fascicular turnover flap has been applied for sural nerve reconstruction in 4 cases, with favorable results (Figure 11).

Autologous nerve grafting is the standard method for nerve gap repair, but it has the disadvantages of limited autologous donor nerve availability and donor site morbidities. However, the fascicular turnover flap does not require the sacrifice of additional nerves and has advantages in terms of nerve regeneration, such as the presence of one site of microanastomosis and nerve graft vascularization. In our study, the distal fascicular turnover flap achieved better nerve regeneration in nerve gap repair than did the conventional nerve graft. Although there is a probability of neuroma-in-continuity at the fascicular flap donor site and functional loss due to sacrifice of the fascicle, our findings suggest that the distal fascicular turnover flap can serve as an effective alternative to autologous nerve grafts. Therefore, further clinical studies comparing the fascicular turnover flap and conventional autologous nerve grafts are needed to investigate the efficacy of the fascicular turnover flap.

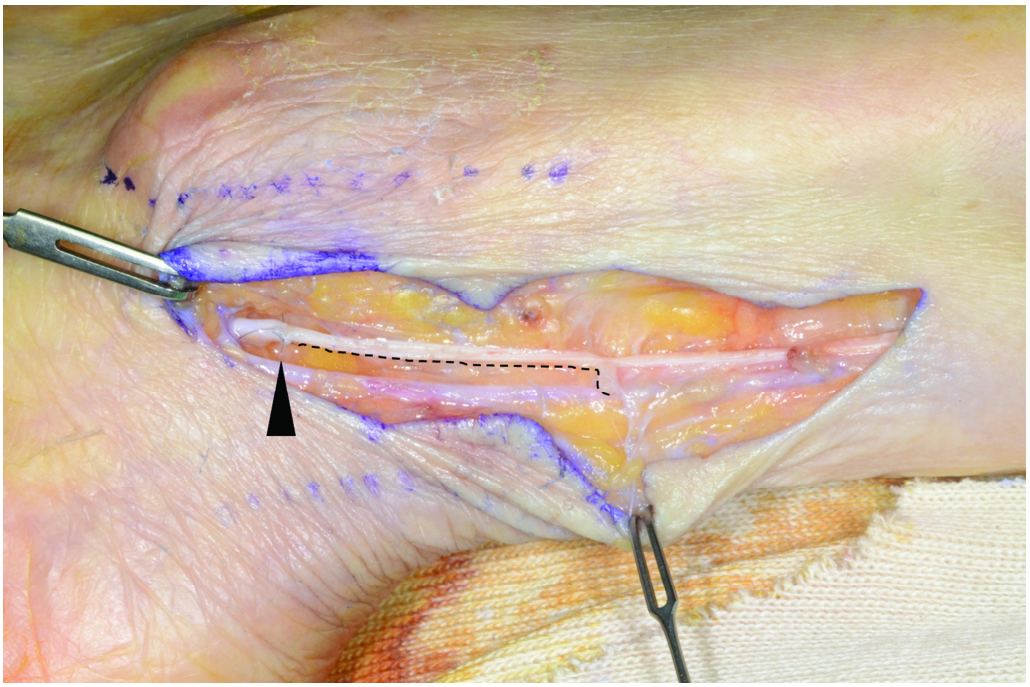

Figure 11. Reconstruction of sural nerve defect via the proximal fascicular turnover flap after sural nerve biopsy. The dotted lines and arrowhead indicate the proximal fascicular turnover flap and the site of anastomosis, respectively.

5. Summary

In a rat nerve defect model, neural reconnection was accomplished with autologous nerve graft, proximally and distally based fascicular turnover flap. Distally based fascicular turnover flap showed similar improvement of sciatic function index compared with autologous nerve graft, and it showed the higher staining intensity of NF-200 and S-100 than proximally based fascicular turnover flap and autologous nerve graft. In addition, Transmission electron microscopy showed a compact, regular myelin sheath in distally based fascicular turnover flap group. The distal fascicular turnover flap achieved better nerve regeneration without a probability of neuroma-in-continuity at the fascicular flap donor site and functional loss. This method can be an effective alternative to autologous nerve grafts. Further investigations are warranted to accumulate more data and to provide objective evidence of its clinical effectiveness in humans.

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Better Nerve Regeneration with Distally Based Fascicular Turnover Flap Than Conventional Autologous Nerve Graft in a Rat Sciatic Nerve Defect Model

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(Abstract)

This study was investgated that the fascicular turnover flap will achieve better nerve regeneration in nerve gap repair than the conventional nerve graft in a rat sciatic nerve defect model. Seven-millimeter-long sciatic nerve defects were repaired with an autologous nerve graft, a proximal fascicular turnover flap, or a distal fascicular turnover flap. Following walking footprint analysis at 8 weeks after the surgery, the gastrocnemius-soleus muscles of both hind limbs, the nerve graft and the flaps were harvested for a wet muscle weight assessment, immunohistochemistry, and transmission electron microscopy. The distal fascicular turnover flap exhibited a similar

improvement in the sciatic function index as did the autologous nerve graft. Histologically, cross-sections showed a higher staining intensity for S-100 in the distal fascicular turnover flap group than in the nerve graft group ($p < 0.05$). In the longitudinal sections, the staining intensity for NF-200 was higher in the distal fascicular turnover flap group than in the nerve graft ($p < 0.01$) and proximal fascicular turnover flap groups ($p < 0.01$). More mature capillaries were observed in the proximal ($p < 0.001$) and distal ($p < 0.05$) fascicular turnover flap groups than in the nerve graft group. Transmission electron microscopy showed a compact, regular myelin sheath around the myelinated nerve fibers in the distal fascicular turnover flap group, unlike in the nerve graft and proximal fascicular turnover flap groups. In conclusion, This study demonstrates better nerve regeneration in nerve gap repair with the distal fascicular turnover flap than the conventional nerve graft.

쥐 좌골신경 결손 모델에서 고식적 자가 신경 이식술에 비교한 원위 기반의 신경다발 전위 피판술의 신경재생의 우수성

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(초록)

본 연구에서 신경 다발 전위 피판이 쥐 좌골 신경 결함 모델에서 전통적인 신경 이식술보다 더 나은 신경 재생을 달성 할 것이라는 가설을 세웠다. 7 mm 길이의 좌골 신경 결손 모델에 자가 신경 이식편, 근위 신경다발 전위 피판, 또는 원위 신경다발 전위 피판을 시행하였다. 수술 후 8 주에 쥐의 걸음걸이를 분석 하였고, 근육 무게를 측정하고, 면역 조직 화학 및 투과 전자 현미경 검사를 위해 양측 뒷다리의 비복근-가자미 근육과 신경 이식편 및 피판을 채취하였다. 결과로, 원위 기반의 신경다발 전위 피판술은 자가 신경 이식술과 좌골 기능 지수에서 유사한 개선을 보였으며 조직학적으로 횡단면은 근위 대퇴골 회전판의 S-100에 대한 염색 강도가 자가 신경 이식술군보다 높았다($p < 0.05$). 그리고 원위부에서 NF-200의 염색 강도는 자가 신경 이식술군($p < 0.01$)과 근위 기반의 신경다발 전위 피판술군($p < 0.01$)보다 원위 기반의 신경다발 전위 피판술군에서 더 높았다($p < 0.01$).

또한, 자가 신경 이식술군에서보다 근위부($p < 0.001$) 및 원위부($p < 0.05$) 신경다발 전위 피판술군에서 더 성숙한 모세 혈관이 관찰되었다. 투과 전자현미경 검사는 자가 신경 이식편군과 근위 신경다발 피판술과는 달리 원위 기반의 신경다발 전위 피판술군에서 치밀하고, 규칙적인 신경말이집을 보였다. 결론적으로, 원위 기반의 신경다발 전위 피판술이 기존의 자가 신경 이식술보다 더 나은 신경 재생을 보여준다.

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