Master of Science

Decellularized neuromuscular scaffold 를 이용한 근육결손부 재건 연구

A study on recovery of muscle defect using decellularized neuromuscular scaffold

울산대학교대학원

의 학 과

구 정 귀

A study on recovery of muscle defect using decellularized neuromuscular scaffold

지도교수 이 부 규

이 논문을 의학석사 학위 논문으로 제출함

2017년 11월

울산대학교 대학원
의 학 과
구 정 귀

구정귀의 의학석사학위 논문을 인준함

심사위원 김영균 (인)

심사위원 이부규 인

심사위원 김병수 인

울 산 대 학 교 대 학 원

2017 년 11 월

A study on recovery of muscle defect using decellularized neuromuscular scaffold

Jeong-Kui Ku, DDS

Directed by Bu-Kyu Lee, D.D.S., M.S.D., PhD

Departmant of Dentistry, Graduate school of Medicine, University of Ulsan

Introduction

It is known that there is more effective results to transplant the neuromuscular tissue than nerve alone for regeneration of damaged neuromuscular function. Therefore it would be necessary to acellular neuromuscular graft for recovery of effective myofunction. However the study for decellularization of skeletal muscle with motor nerve is rare. This study aims to establish a basis for attempting homologous transplantation from acellular neuromuscular scaffold of rats.

Methods

8-weeks male Sprague rats (SD rat, SPF) were operated to hervest some part of extensor digitorum longus (EDL) and tibialis anterior (TA) muscle with peroneal nerve. Acellular neuromuscular scaffolds were designed by three decellularization protocols which using trypsin and triton X-100 (protocol I), using Sulfobetaine(SB)-10, SB-16 and and triton X-100 (protocol II) and using SB-10, trypsin and triton X-100 (protocol III). The degree of decellularization of the produced scaffold is evaluated and transplanted into the neuromuscular defect which was made on the homologous rat. After clinical evaluation of peroneal nerve function in rats at 2 weeks after transplantation, the rats were sacrificed for histological analysis.

Result

In this study, the protocol III using SB-10, Trypsin, and Triton X-100, which are presented in this study, seems to be decellularized at an appropriate level which removed DNA of neuromuscular tissue and showed proper network structure. In addition, it showed no immunologic response in allografts and good biocompatibility with peripheral muscles. The acellular neuromuscular scaffold produced by the protocol III was found to be successful result in histologic and clinical evaluation at 2 weeks after allograft.

In order to use more clinical applications, further study for more specimens, population and long-term follow-up should be necessary to confirm the nerve reinnervation after transplantation of acellular neuromuscular scaffold with electromyography, histologic-chemical and histologic-enzymal staining.

Key words : acellular scaffold, tissue engineering, neuromuscular, peroneal nerve

Index

List of table

List of figure

Introduction

The skeletal muscle is a highly organized complex tissue with contractile elements and connective tissue, including nerve fibers and blood vessels. If more than 20% of muscle volume loss had occur due to trauma or surgery, regeneration would not be successful and the defect would be filled with scar tissue resulting functional problems.[1, 2] If serious damage or functional loss from a large amount of muscle defect, it could be considered to autologous muscle transplantation or muscular flap operation. But damage of donor tissue was inevitable in the case of autotransplantation.[3, 4] Therefore, many researches were tried to using the extracellular matrix (ECM) as a biological scaffold for repair of damaged or missing tissue. The ECM of skeletal muscle could function as a cell attachment, migration, storage and releasing of growth factors, cell activity and differentiation, which play an important role in the maintenance and regeneration of skeletal muscle.[5, 6] The ECM is composed of structural proteins such as collagen and elastic fiver, heparin, proteoglycan and unstructured molecules such as growth factors which help in remodeling and myogenesis. And it is well known to be good environment for formation of skeletal muscle.[1, 7-12]

Recent studies have been developed decellularization methods to make various extracellular matrix.[13] A decellularization protocol is divided into physical, chemical, enzymatic, protease inhibitor method and using antibiotics for manufacturing biomechanical ECM scaffolds.[13, 14] Inadequate decellularization would be result in the remnants of cells which were leading to a pro-inflammatory response and overexposure, conversely, could lead to denaturation of a necessary growth factors and structural proteins.[15] For clinical application of ECM scaffold of skeletal muscle, it should be need to adequate decellularization and at the same time maintain structural strength without physical damage. Depending on decellularization protocol, there could be changed that the degree of decellularization and characteristic of remained ECM. For clinical result, it is a necessary to develop decellularization protocol that can preserve biological and

physical function.

Many studies have developed a decellularization protocol through combination of various agents and evaluated the degree of decellularization. The most spotlight study is enzymatic method using trypsin (proteolytic enzyme) and Triton X-100 (detergent) for skeletal muscle decellularization.[16] Furthermore Zhang et al.[15] have published a study for decellularization and transplantation of skeletal muscle with feeding vessels, as leading to further advance the functional and anatomical regeneration.

If peripheral nerve does not recover after damage to muscle and nerve due to trauma, it could be lead to muscle function loss, incomplete sensation or neuropathic pain.[17] It was showed most ideal result that re-anastomosis of the damaged nerve but it could not be always operated. In that case, a nerve graft could be second options however it cannot be avoided to result the donor site damage in autologous graft as well as muscular graft. For these reason, many studies have to produce acellular nerve for transplantation.[18] it is known that there is more effective results to transplant the neuromuscular tissue than nerve alone for regeneration of damaged neuromuscular function.[19] Therefore it would be necessary that not only acellular nerve graft but also acellular neuromuscular graft for recovery of effective myofunction. However the study for decellularization of skeletal muscle with motor nerve is rare. Therefore, this study aims to establish a basis for attempting homologous transplantation from acellular neuromuscular scaffold of rat.

Material & Method

1. Harvesting neuromuscular tissue

8-weeks male Sprague rats (SD rat, SPF) were purchased for decellularization of skeletal muscle from Orientbio© (Seongnam, Korea). The rats were anesthetized with tiletamline and zolazepam hydrocholoride (Zoletil 50®)과 Xylazine (Rompun®) injected intraperitoneally 20mg per kg of body weight. The primary surgery was operated with removal of right thigh hair and disinfected with

povidone solution on prone position. Incision was made about 2cm to expose the peroneal nerve above the knee to posterior femur. The peroneal nerve branches from sciatic nerve were resected with a portion of the extensor digiroum longus (EDL) muscle and tibialis anterior (TA) muscle to a size of 10 mm x 6 mm x 2 mm (length x width x depth).(Fig. 1)

2. Decellularization of neuromuscular tissue

Harvested neuromuscular tissue were treated 4 % RPMI 1640 solution (Gibco®, MA, USA) with 1% antibiotic Antibiotic-Antimycotic (Gibco®, MA, USA; penicillin 10000 units/mL and streptomycin 10000 μg/mL and Fungizone® (amphotericin B) 25 μg/mL) for 24 hours. It was washed 3 times for 5 minutes with Phosphate buffer saline (PBS) after removal of connective tissue and fat from muscle and epineurium. And it was treated with protocol Ⅰ (Trypsin, Triton X-100), Ⅱ (sulfobetaine (SB)-10, sulfobetaine (SB)-16, Triton X-200), Ⅲ (sulfobetaine (SB)-10, Trypsin, Triton X-100)

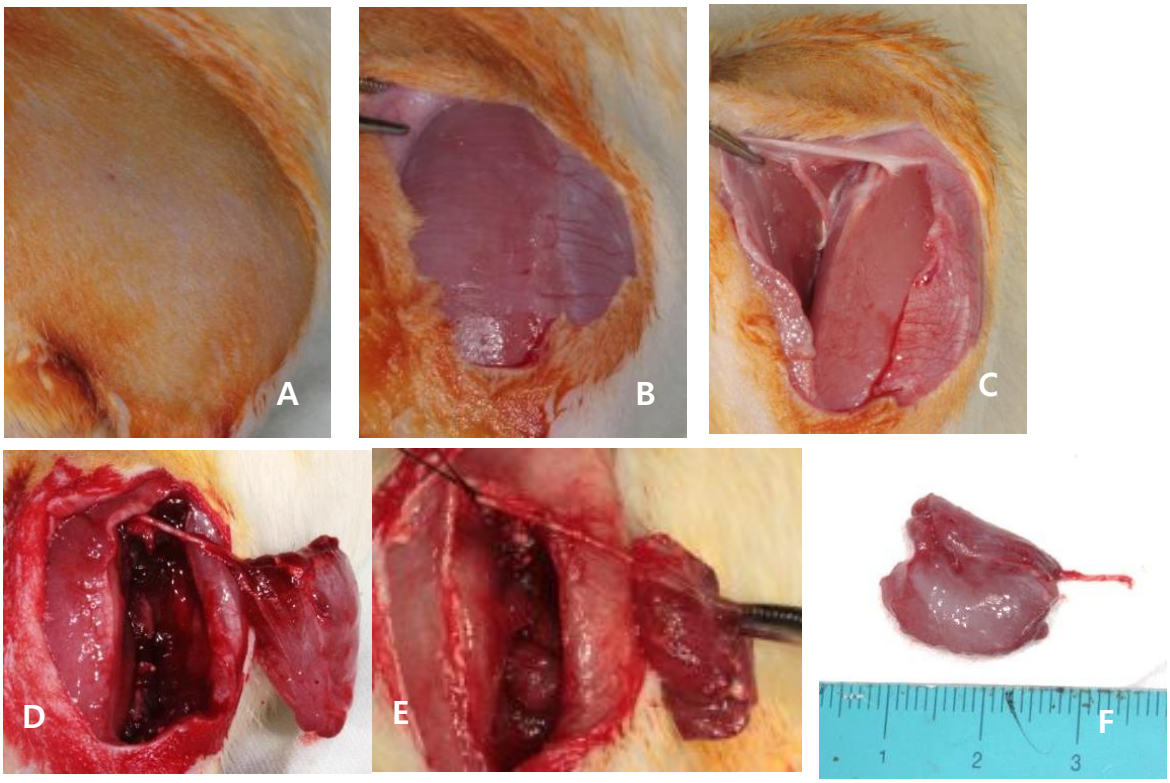
Control : No treatment after harvesting

Protocol-Ⅰ : It was washed with PBS after incubation for 1 hour in PBS at 37°C in 0.05% Trypsin and 0.02% EDTA. After agitation for 72 hours in 4°C in 2% Triton X-100-0.8% NH_4OH solution, it was washed with distilled water for 48 hours and rinsed 3 times with PBS.[14)]

Protocol-Ⅱ : It was agitated with 125mM SB-10 for 15 hours and washed with PBS. After agitation for 72 hours in 2% Triton X-100-0.6mM SB-16 solution, it was washed PBS 3 times for 5 minutes. After agitation for 7 hours at SB-10, it washed once with PBS and agitate for 15 hours with Triton X-200-SB-16. It was wash for 24 hours with distilled water and rinsed 3 times with PBS.[18)]

Protocol-Ⅲ : It was agitated with 125mM SB-10 for 24 hours and washed with PBS. It was washed with PBS after incubation for 1 hour in PBS at 37°C in 0.05% Trypsin and 0.02% EDTA. After agitation for 72 hours in 4°C in 2% Triton X-100-0.8% NH_4OH solution, it was washed with distilled water for 24 hours and rinsed 3 times with PBS

Figure 1. Harvesting gastrocnemius & soleus muscle with tibial nerve. A. preparation. B. skin incision. C. detecting peroneal nerve. D. myotomy of extensor digitorum longus (EDL) muscle and tibialis anterior (TA) muscle about 10mm× 6mm×2mm. E. tagging of peroneal nerve for preventing neuroma. F. Harvested neuromuscular flap.



3. Evaluation of decellularization of neuromuscular scaffold

The neuromuscular scaffolds made by methods described above were histologically examined to evaluate the degree of decellularization and maintenance of ECM structure

DNA quantification

Hematoxylin-eosin staining is standard method used primarily for histological evaluation and allows the observation of muscle fibers and nuclei. [20] In addition, another method of residual DNA quantification is that 20mg of neuromuscular scaffold and native sample were treated with 10% proteinase K (Qiagen, USA) in lysis buffer solution overnight. DNeasy kit (Qiagen, USA) was used for purifying DNA of each sample with manufacturer's protocol. Optical densities from 260/280 ratios were used to estimate the purity and yield of nuclei acids, which were quantified by spectrophotometer at 260 nm. And measured DNA quantity was normalized to the initial dry weight of the tissue.[21]

3D structure evaluation

For geometric evaluation of neuromuscular scaffold, 4% paraformaldehyde was treated at room temperature for 45 min and dehydrated using ethanol. The specimens were frozen in liquid nitrogen and then cut into 2 mm thick pieces. The specimens were then sputter coated and analyzed using a Scanning Electron Microscope at an accelerating voltage of 20 kV at 10 cm working distance.[22]

4. Homologous transplantation

The neuromuscular scaffold were transplanted into four groups of defects which was made on the same species.

group 0 : no treatment (negative control)

group 1 : transplantation of D-protocol Ⅰ

group 2 : transplantation of D-protocol Ⅱ

group 3 : transplantation of D-protocol Ⅲ

In the second surgery, 6 weeks rats were operated to make a neuromuscular defect of the same size and site as the first operation. The muscle part was

sutured with 5-0 vicryl and the nerve ending was anastomosed using 10-0 vicryl with a 10x magnification microscope. In each rat, right hind leg was used for operation and the other leg was used as a normal control group. The rats were provided a stable environment for 2 weeks and did not specifically treat any medications. After nerve re-innervation clinical test (walking track analysis, toe-spread test), each group was sacrificed at 2 weeks after transplantation [23] for histological evaluation with Nembutal® for 75mg per kg of body weight [22, 24]

Walking track analysis

Walking track analysis was performed at 2 weeks after surgery.[23] Front planters of rats were stained a waterproof blue ink and hind plantar were used black ink. The rats allowed to walk down darkened corridor (5x7.5x42cm^3), leaving their footprints on none-slip paper.[25].

Recordings were obtained with the lateral view of the corridor with video camera which is available to slow motion function. They were measured that angulation of tibial tuberosity to calcaneus and tarsal to 2nd digit during walking. (+; dorsiflexion, -; plantar flexion)

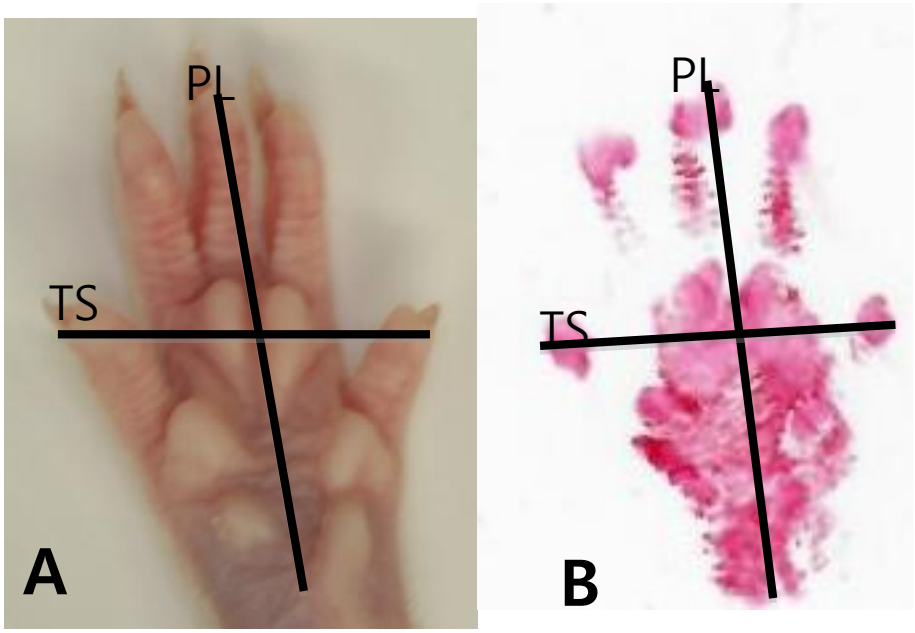
PFI was obtained based on multiple regression analysis in the walking track of rat with peroneal nerve damage. Factors affecting PFI were Print length factor (PLF) and toe-spread factor (TSF). In addition, the print length (PL) and toe-spread (distance between the 1st and fifth toes) were obtained from the normal control group (N). (Fig. 2)

PFI=174.9*(E.PL－N.PL)/N.PL +80.3*(E.TS－N.TS)/N.TS －13.4 [23]

Toe-spreading test

There is toe-spreading response when the rats raised by the tail lifting the hind plantar off the ground. Toe-spreading test using this response [26, 27] evaluated whether toes were maintaining closed together because of sciatic nerve damage which was leading to dorsiflexion failure. This reflex could be evaluated as present or absent, therefore it could be easily detected when the nerve became re-innervation to peri-around muscle.[28] It was evaluated by applying

Figure 2. Measuring the print length (PL) and toe spread (TS) between 1st and fifth toes for walking track analysis.



Gutmann[29]'s scale to degree of progressive toe spread reflex: degree 1, minimal spreading of one toe; degree 2, slight spreading of all toes; degree 3, spreading of all toes less forcefully than normal; degree 4, full and normal spreading of all toes.

Histological evaluation

Immediately after sacrifice, grafts were removed from each rats and grafted neuromuscular tissue was immediately frozen in isopentane cooled to -160 to -170C. Frozen grafts were firstly taken with a freezing section (-20C), and then continuous sections with a thickness of 5 to 8 μm were made on the longitudinal and transverse sections, respectively, followed by haematoxylin-eosin staining which is the standard method used for tissue examination.

Result

A total of 18 neuromuscular samples were obtained and decellullarized for one week according to protocol Ⅰ (Trypsin, Triton X-100), protocol Ⅱ (SB-10, SB-16, Triton X- 200), and protocol Ⅲ (SB-10, Trypsin, Triton X-100) with appropriate allocation of solution and time in protocols Ⅰ and Ⅱ. Six samples were produced per each protocol. There were observed transparently change on D-protocol Ⅰ and Ⅲ, however no color change was observed on D-protocol Ⅱ.(Fig. 3)

DNA quantification

Cellular content was significantly reduced from 162.5 ± 17.33 μg/mg in untreated control tissue (n=4) to 25.75 ± 12.45 μg/mg in protocol Ⅰ (n=4) and 35.25 ± 6.55 μg/mg in protocol Ⅲ (n=4), but not enough to reduce in protocol Ⅱ (143.25 ± 41.65 μg/mg, n=4).(Fig. 4) These results showed that DNA components had successfully removed in protocol Ⅰ and Ⅲ.

Histological analysis

Fig. 5 showed typical H&E staining of muscle part of neuromuscular scaffold after decellularization protocol. The image showed cell nuclei present in D-protocol Ⅱ but absent in D-protocol Ⅰ and Ⅲ. Tissue structure was relatively maintained in D-protocol Ⅱ, followed by D-protocol Ⅲ and Ⅰ.

Figure 3. After decellularization of neuromuscular scaffold. A: D-protocol Ⅰ (Trypsin, Triton X-100). B: D-protocol Ⅱ (SB-10, SB-16, Triton X-200), C: D-protocol Ⅲ (SB-10, Trypsin, Triton X-100).

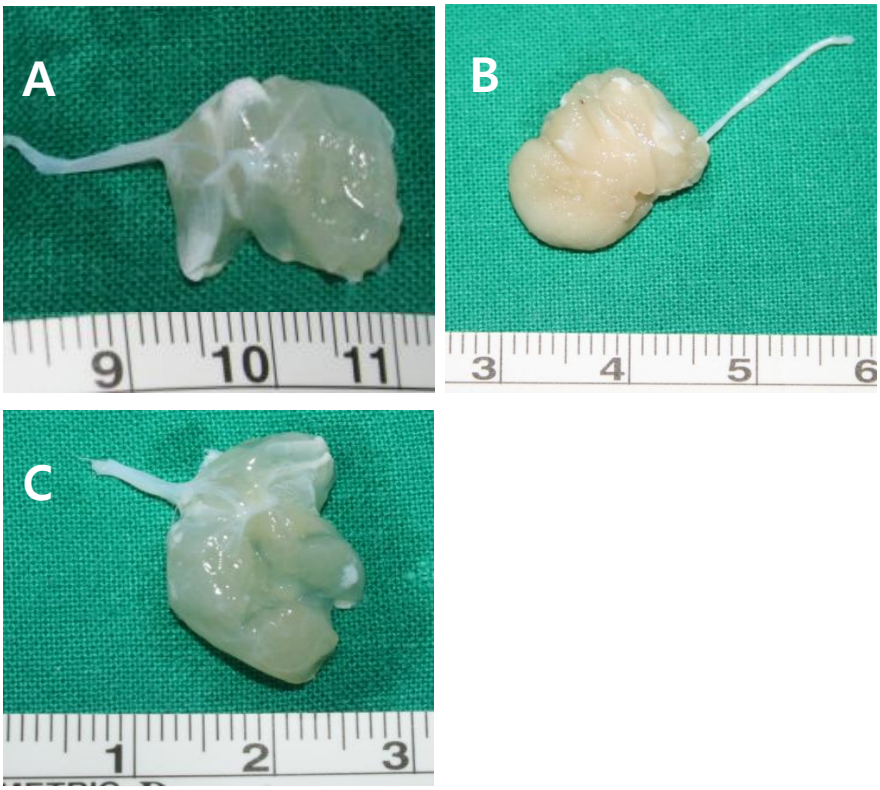


Figure 4. DNA contents in dry weight samples.

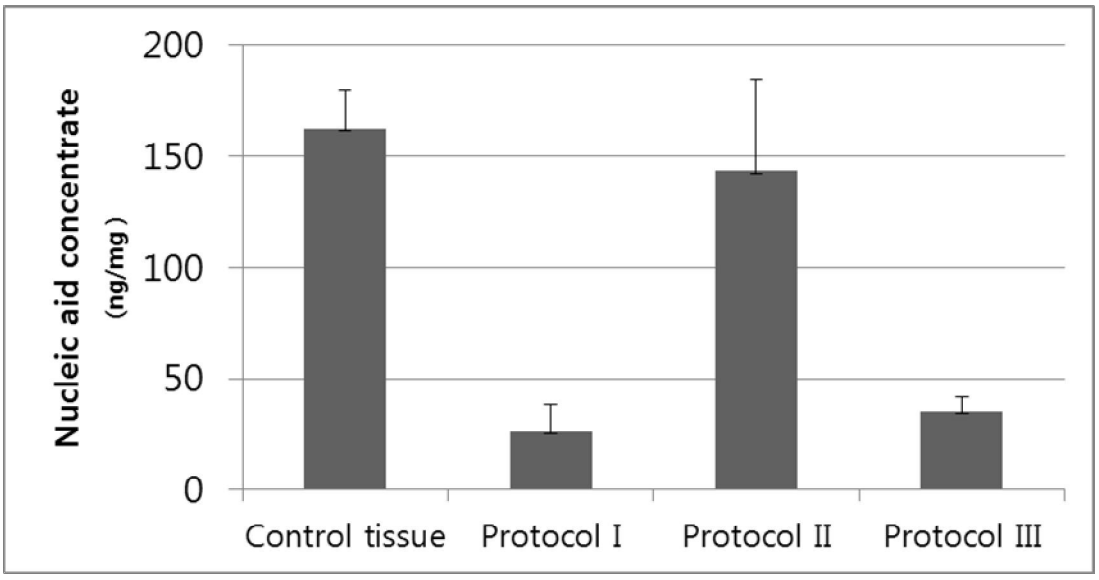
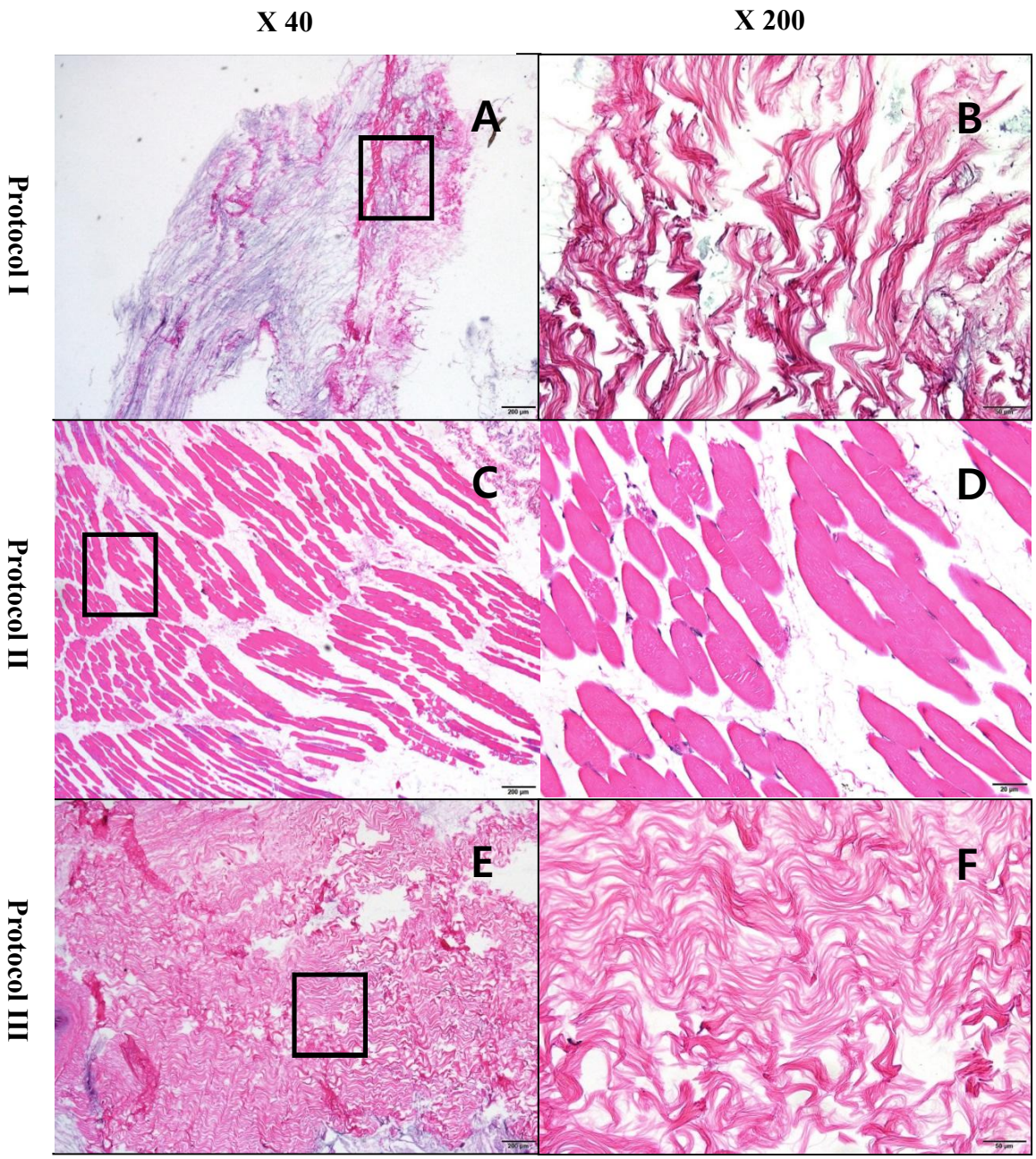


Figure 5. Hematoxylin-eosin stained sections of decellularized neuromuscular scaffold with protocol Ⅰ (Trypsin, Triton X-100), Ⅱ (SB-10, SB-16, Triton X-200), Ⅲ (SB-10, Trypsin, Triton X-100). Protocol Ⅰ (A), Ⅱ (C), Ⅲ (E) (Scale bar: 200μm) and protocol Ⅰ (B), Ⅱ (D), Ⅲ (F) (Scale bar: 50 μm).



3-D structure evaluation

SEM was used to evaluate the 3D structure of muscle part in untreated control tissue and neuromuscular scaffold after protocol Ⅰ and Ⅱ, Ⅲ. Nanofibrous structure was observed in D-protocol Ⅱ (Fig. 6 (C)) similar to control tissue (Fig. 6 (A)). It was also observed in D-protocol Ⅲ (Fig. 6 (D)) with a little damage and in D-protocol I (Fig.6 (B)) with significant alter ECM structures.

Transplantation

All neuromuscular scaffolds constructed according to three decellularization protocols showed enough structural strength to suture with 5-0 vicryl in muscle part and 10-0 vicryl in nerve part. (Fig. 7) immunoreactivity and infection symptoms were not observed in all groups for 2 weeks, and it was observed that the continuity of peroneal nerve and neuromuscular scaffold was maintained in all groups. (Fig. 8 (B, D, H)) there was moderate recovery and continuity of D-protocol I and remained EDL and TA without volumetric loss.(Fig. 8 (A)) Although adhesion aspect with around muscle was observed, poor recovery of D-protocol Ⅱ and remained EDL and TA was detected with volumetric loss of implanted D-protocol Ⅱ. (Fig. 8 (C)) however, there was observed that fibrous tissue grew above D-protocol Ⅲ covered with fascia. (Fig. 8 (E, F)) In addition, it showed relatively significant recovery and continuity of D-protocol Ⅲ and remained EDL and TA without volumetric loss. (Fig. 8 (F)

Walking track analysis

In the walking track analysis performed 2 weeks after transplantation, group 0, 1 and 2 were observed to walk on the instep but the rat of group 3 walked with rotation. There was observed that negative angulation of ankle plantar at dorsiflexion in group 0, 1 and 2. But it showed positive angulation in group 3, though the planter was not completely extended as control rat. (Fig. 9, 10) In the foot print, groups 0, 1 and 2 showed a similar pattern, while group 3 showed different aspect of rotation. (Fig. 10) In the PFI measured using foot print, the groups 0, 1, 2 and 3 were calculated to -163.24, -152.15, -150.69 and -21.6 in order. (Table 1)

Figure 6. SEM images of decellularized neuromuscular scaffold. A. untreated control tissue B. protocol Ⅰ (Trypsin, Triton X-100) C. protocol Ⅱ (SB-10, SB-16, Triton X-200) D. protocol Ⅲ (SB-10, Trypsin, Triton X-100). (Scale bar = 500μm)

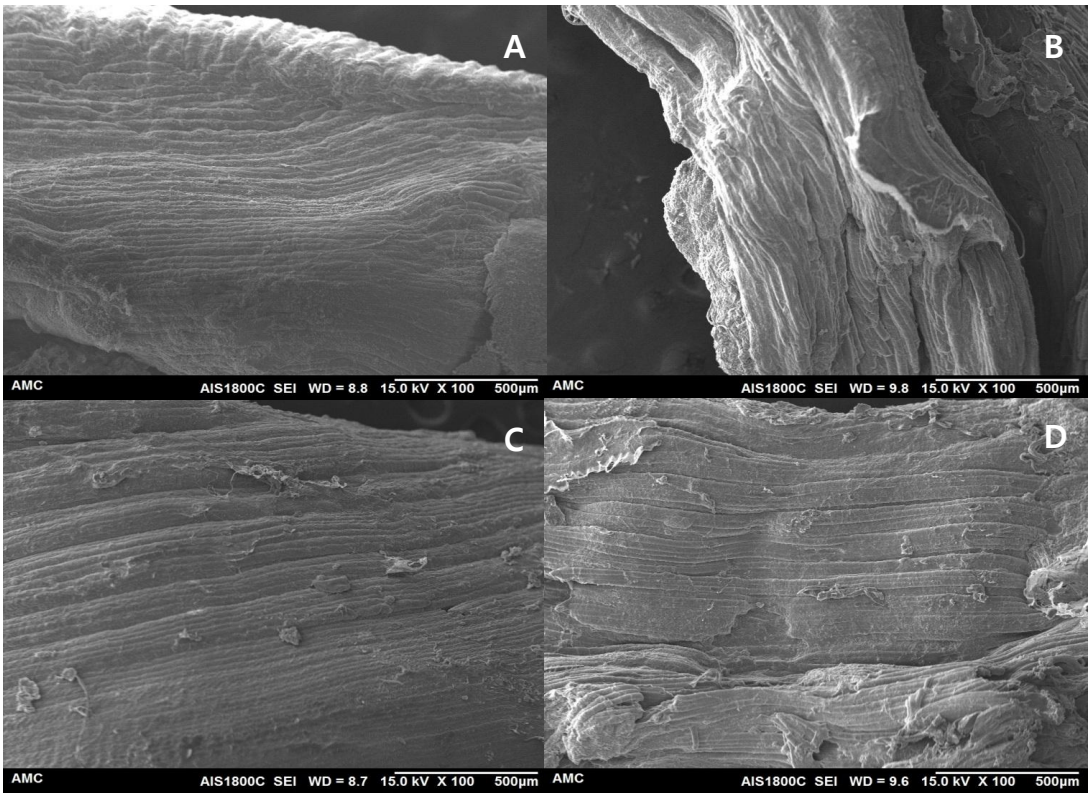


Figure 7. Transplantation of neuromuscular scaffold. A: preparation of neuromuscular defect. B: adjustment of neuromuscular scaffold for muscular scaffold trimming. C: anastomosis of peroneal nerve and neuromuscular scaffold with 10-0 vicryl. D: intermuscular suture with 5-0 vicryl.

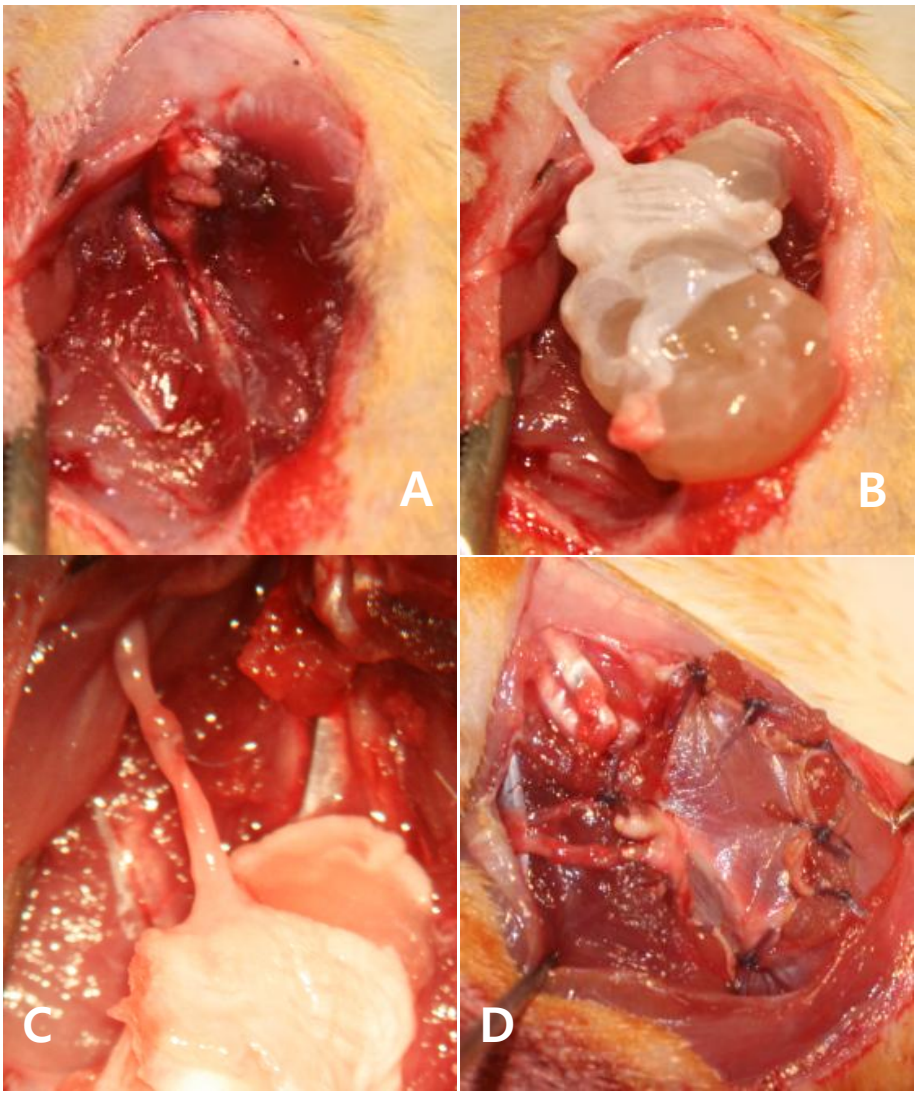
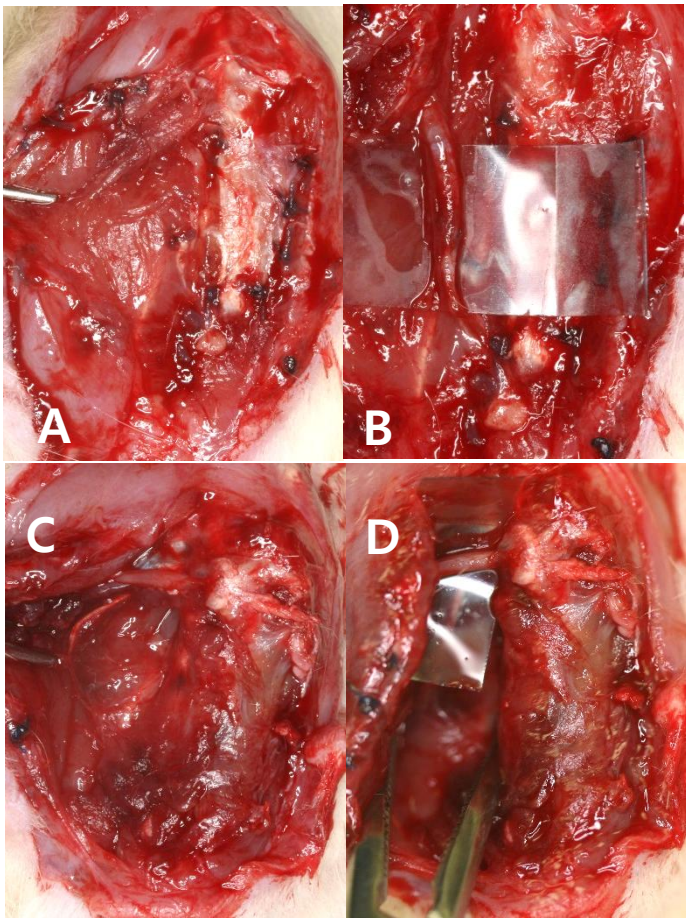


Figure 8. 2 weeks after transplantation. A: moderate recovery and continuity of D-protocol Ⅰ and remained EDL and TA without volumetric loss. B: maintaining the continuity of peroneal nerve and D-protocol Ⅰ. C: poor recovery of D-protocol Ⅱ and remained EDL and TA with volumetric loss. D: maintaining the continuity of peroneal nerve and D-protocol Ⅱ. E: removal of fascia covering D-protocol Ⅲ. F: engrowth of fibrous tissue above D-protocol Ⅲ. G: relatively significant recovery and continuity of D-protocol Ⅲ and remained EDL and TA without volumetric loss. H: maintaining the continuity of peroneal nerve and D-protocol Ⅲ.



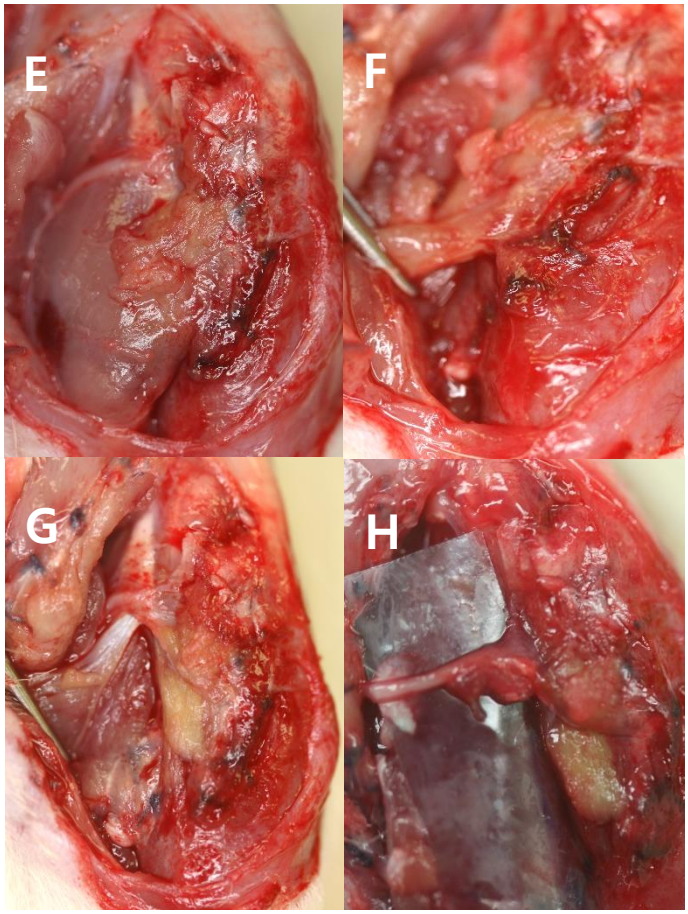

E
F
G
H

Figure 9. Measuring angulation of ankle plantar at dorsiflexion. 2D digital video analysis of the ankle (tibial tuberosity to calcaneus) & toe angles (tarsal to second digit) at toe-off (TO): A. control B. group 0, 1 and 2 at 2 weeks after surgery. C. group 3 at 2 weeks after surgery.

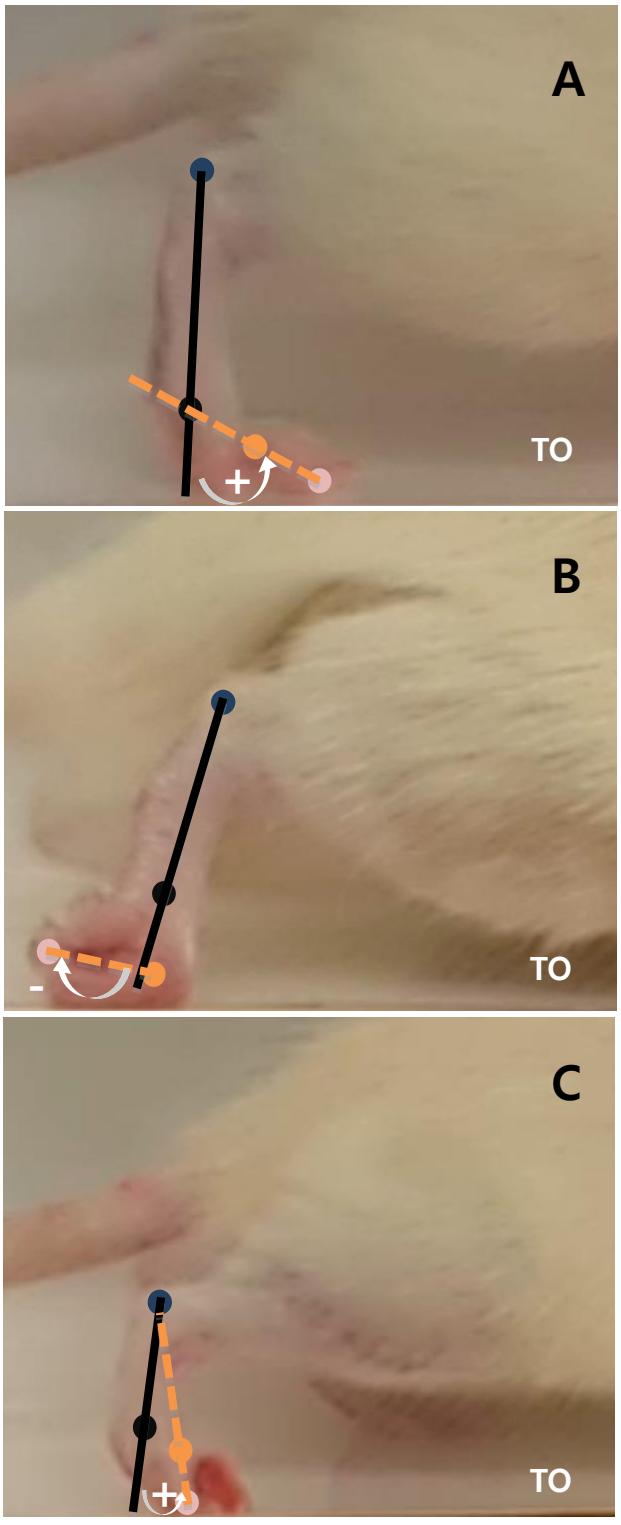


Figure 10. Walking track analysis. A: foot print of group 0 (negative control), B: foot print of group 1 (D-protocol Ⅰ), C: foot print of group 2 (D-protocol Ⅱ), D: foot print of group 3 (D-protocol Ⅲ). (blue color : front plantar, black color : hind plantar, red arrow : treated right hind feet)

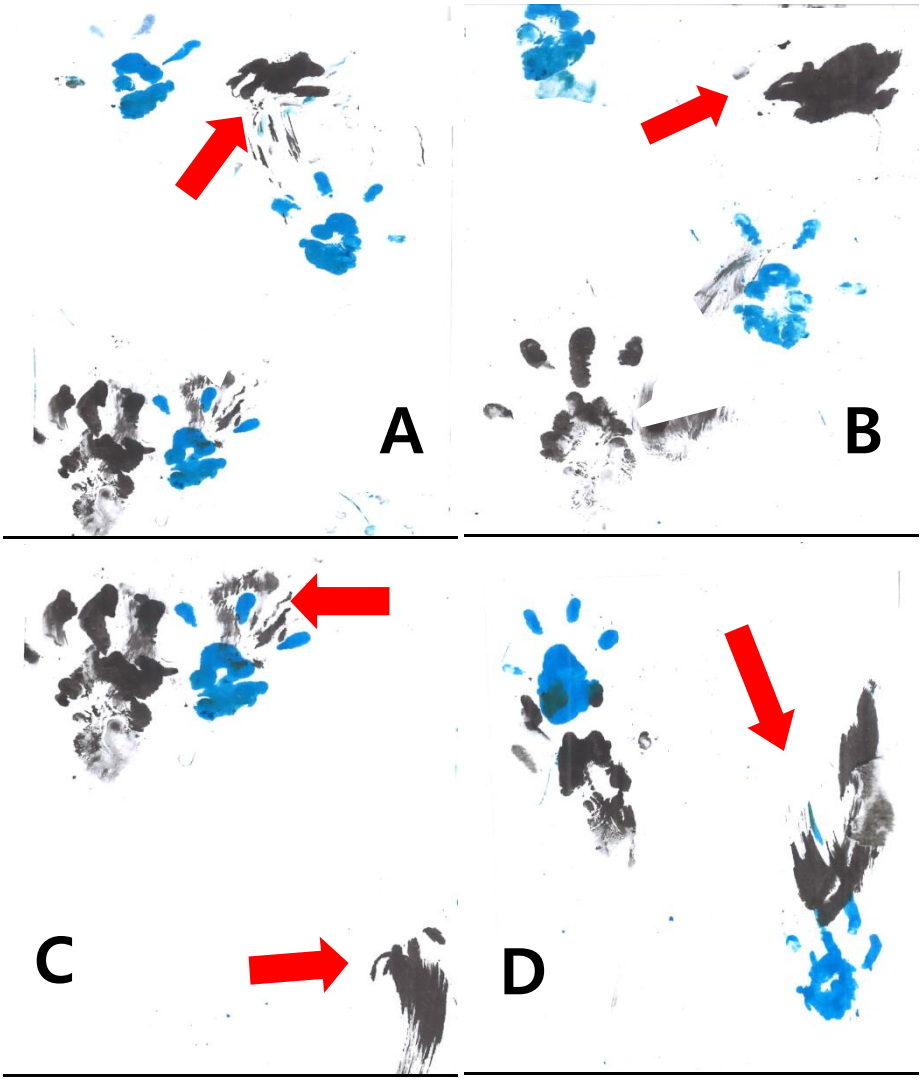


Table 1. Peroneal functional index (PFI) on each group

	Peroneal functional index (PFI)
Group 0 (negative control)	-163.24
Group 1 (D-protocol I)	-152.15
Group 2 (D-protocol II)	-150.69
Group 3 (D-protocol III)	-21.6

Toe-spreading test

Toe-spreading response was showed in group 3 that was slightly spreading of all toes as Gutmann's spread reflex recovery degree 2. But toes of group 0, 1 and 2 were close together and couldn't be dorsiflexed at the ankle. (Fig. 11)

Histological analysis

In H & E staining, group 1 showed that blood vessels grew into the scaffold. However, there were observed that little remaining ECM structure and lysis aspect without ingrowth of cells. (Fig. 12 (A)) In group 2, ECM structure was observed clear patterns but generally lysed without blood vessel infiltration. (Fig. 12 (B)) While in group 3, there was observed no lysis pattern and well maintained ECM structure with blood vessel and cellular engrowth. (Fig. 12 (C))

Discussion

In the rabbit study in which comparing three methods of neurorrhaphy, nerve graft and nerve regeneration though muscle repair, the motor nerve damaged muscle was evaluated that the neurorrhaphy was best effective as 73% of normal function on degree of muscular contraction after 6 months. However, in the case of nerve regeneration by muscle repair, the degree of nerve regeneration was confirmed to some extent by electromyography, but the degree of recovery was extremely low compared with neurorrhaphy, nerve graft.[30] Therefore, it is better to operate nerve graft or neuromuscular flap to operate neuromuscular anastomosis when there is a large muscle defect with myofunction disorder. Since the problem of donor defects could not be avoided when nerve graft or neuromuscular flap was used, the necessity of artificial nerve or neuromuscular scaffold has been emerging.

As development of tissue engineering, there are proposed many studies on the production of extracellular matrix (ECM), which contains regulating and regenerating functions of muscle fibers to regenerate damaged or missing tissue and decellularization protocol using various materials.[1, 7-12, 31] Among various materials, trypsin is a very specific enzyme that breaks down the linkage of

Figure 11. Toe-spreading test at 2 weeks after transplantation. A: normal control, B: negative control, C: group 1 (D-protocol Ⅰ), D: group 2 (D-protocol Ⅱ), E: group 3 (D-protocol Ⅲ).

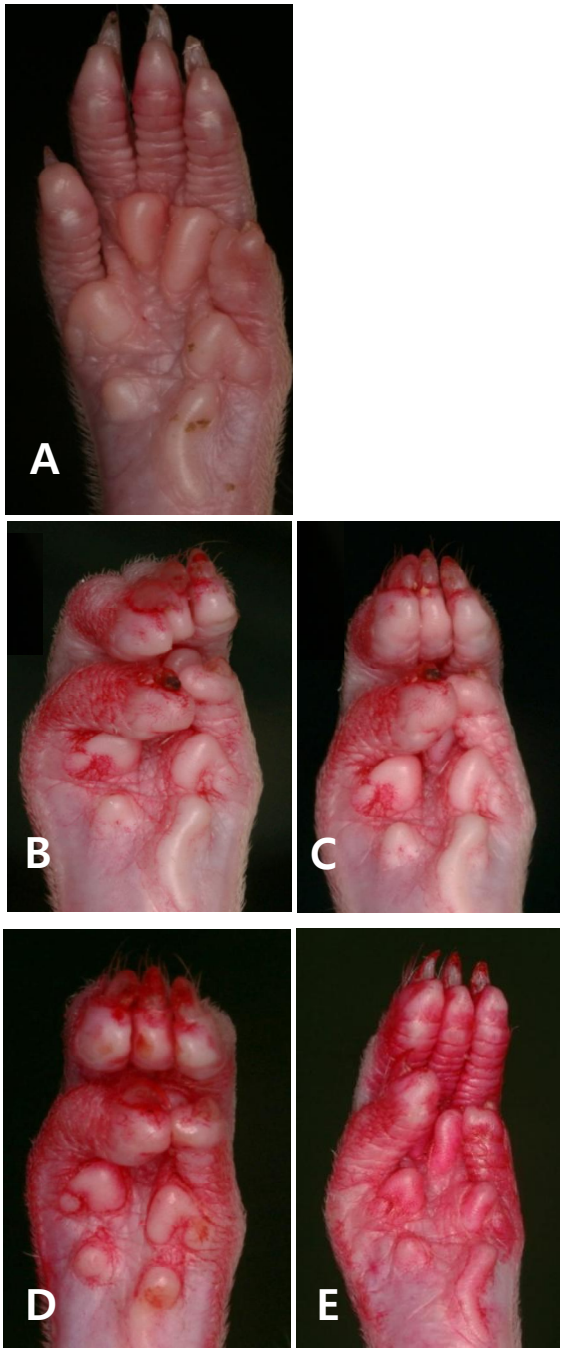
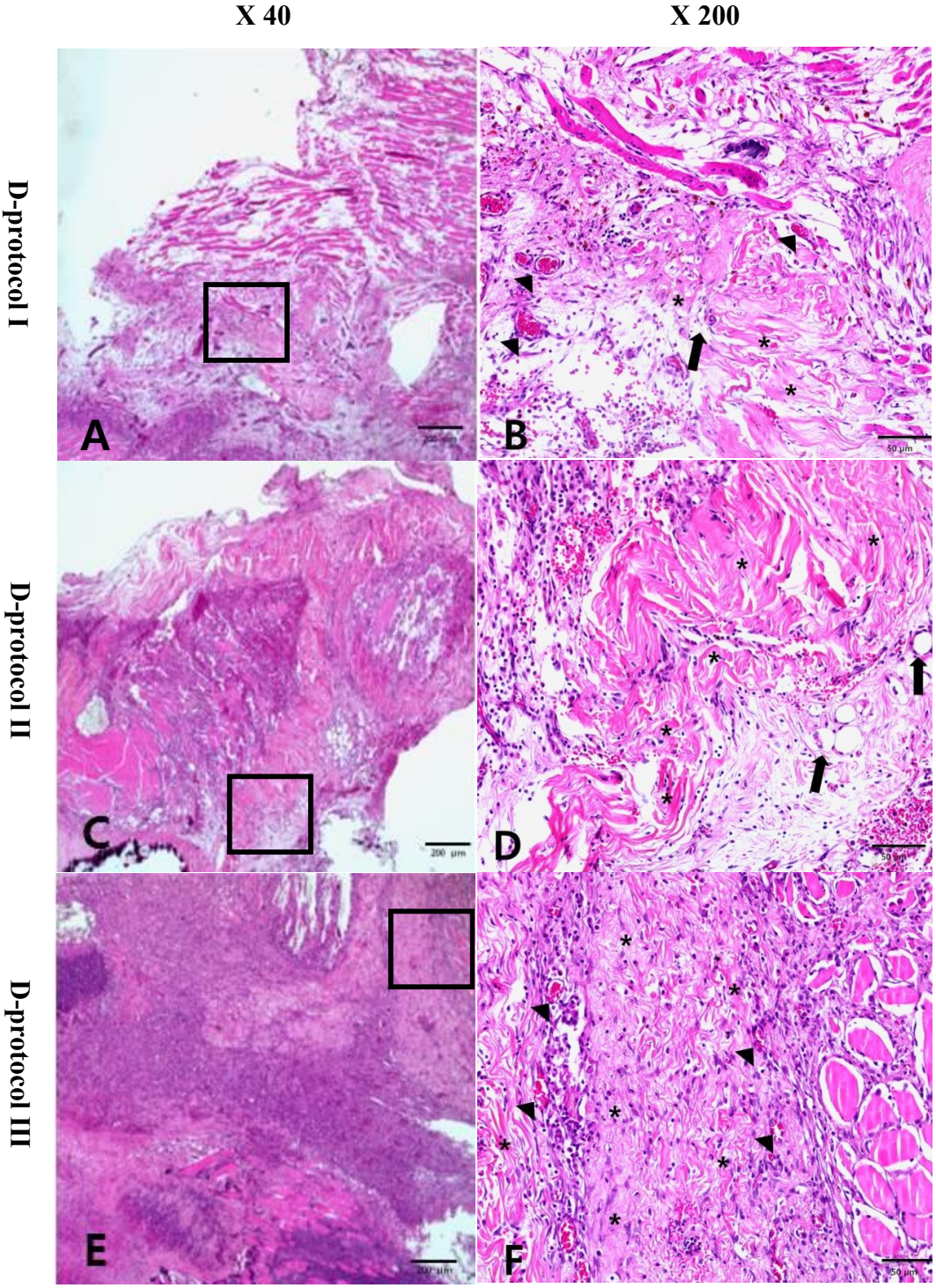


Figure 12. Hematoxylin-eosin stained. Sections at 2 weeks after transplantation of neuromuscular scaffold. D-protocol Ⅰ (A), Ⅱ (C), Ⅲ (E) (Scale bar: 200μm) and D-protocol Ⅰ (B), Ⅱ (D), Ⅲ (F). (arrow: lysis, arrowhead: blood vessel, *: ECM structure, Scale bar: 50μm).



arginine and lysine and is most active at 37 ° C in a solution at pH 8.0.[13)]

In many studies, trypsin has been demonstrated to be successfully decellularized, but, it is observed that cytoplasmic debris in muscle tissue treatment. Tripton has been reported to reduce collagen density [31)] and to change the molecular structure of the elastic fiber, which has resistance to damage, or to damage the fiber shape that leads to destruction of the fiber.[32)] Through Alcian Blue staining, it was observed that tripton eliminated a considerable amount of GAGs present in skeletal muscle.[32)] This is a disadvantageous result because GAGs help maintain the mechanical resistance of the scaffold.[33)] Therefore, in order to function as a neuromuscular scaffold for myofunction regeneration, a protocol that can maintain the mechanical structure while suppressing the immune response through decellularization is needed.

In the meantime, studies have been reported on acellular nerve grafts to obtain scaffolds for restoring dysfunction caused by nerve damage.[18)] Although immediate neural anastomosis is the most ideal result for neural innervation, it has been reported that neuromuscular transplantation is more effective than nerve graft alone if it could not be immediately operated.[19)] As a muscle defect caused to myofunctional disorder, artificial acellular neuromuscular scaffold could be considered as a more effective graft than decellularizing skeletal muscle. Recently, there have been reported to suggest a protocol to decellularization of vascularized muscle tissue[15)], but the study of decellularization protocol of neuromuscular tissue is rare. In this study, the new decellularization protocol (protocol Ⅲ) for neuromuscular tissue was designed that combination of muscular decellularization protocol [14)] which was reported excellent structural stability with deficient decellularization for skeletal muscle and neural decellularization protocol which was successfully reported by Hudson et al. [18)]

The purpose of decellularization is minimization of damage to the extracellular matrix while maintaining the mechanical and structural strength of skeletal muscular connective tissue. The successful decellularization is the absence of visible nuclei stained with 4',6-diamidino-2-phenylindole (DAPI) and below 200

base pairs in remaining DNA fragments or below 50ng dsDNA/mg ECM dry weight.[34-36] while D-protocol Ⅱ was calculated more than 50ng dsDNA/mg, D-protocol Ⅰ and Ⅲ was successfully produced acellular neuromuscular scaffold less than 50ng dsDNA/mg ECM dry weight. In addition H-E staining revealed that D-protocol Ⅰ and Ⅲ were muscle / neuromuscular acellular matrix. In the ECM structure, D-protocol Ⅱ was observed to maintain almost a native muscular nanofibrous structure. However, it was observed that many muscle fibers were loosened in D-protocol Ⅰ. D-protocol Ⅲ showed better matrix structure than D-protocol Ⅰ, but it was observed to be damaged in comparison with D-protocol Ⅱ. This neuromuscular scaffold mostly consisted of muscle tissue, and protocol Ⅱ which is suitable for nerve was ineffectively decellularized to muscle. In this reason, D-protocol Ⅱ was observed remaining amount of DNA and maintaining ECM structure. On the other hand, protocol Ⅰ showed to have the effect of producing acellular muscle matrix, while damage ECM structure. It was estimated that protocol Ⅲ was better for decellularization of muscle than protocol Ⅱ and more suitable than protocol Ⅰ for maintaining the nanofibrous structure.

It is also important to maintain a stable physical structure in order to function as a proper scaffold. The elastic fibers are arranged to form a three-dimensional network and are important in resisting the physical stress of the fabricated scaffold because it plays a role in restricting the expansion force of the tissue while preventing excessive elongation.[32] In this study, it was observed that D-protocol Ⅰ, Ⅱ and Ⅲ maintained the structural strength to enduring sutures in both nerve and muscle. In the SEM, the nanofibers were found to be damaged in the D-protocol Ⅰ like the histological findings and the D-protocol Ⅱ are observed almost similar to the native muscle. However, D-protocol Ⅲ was showed actually less damage to the ECM structure on the SEM than observed on H & E staining. Therefore, it can be considered as a basis to support the excellence of the protocol Ⅲ in the neuromuscular scaffold preparation.

Walking-track analysis is used in many studies because it can clinically assess the degree of rehabilitation of muscles by observing the actual voluntary

movement.[25, 37] In particular, the hind limb function by the sciatic nerve and branches is quantifiable, reliable, and useful for evaluation through foot print.[37] It is known to be more useful to use less slippery paper than thinner radiation films to track footprints.[38] Hasegawa et al.[39] reported a method of evaluating functional recovery after sciatic nerve crushing using the distance between the 1st and fifth toes and the distance between the 2nd and fourth toes in the hind limb of the rats.

If there is severe damage to the sciatic nerve, it is difficult to determine only the width of the toes in the walking track. For this case, De medinaceli et al[40] suggested a calculation using a random value of 6 mm.

In the study of Hoogeveen et al.,[41] 30% toe spreading could be seen as 100% motor function loss, and 65% toe spreading can be seen as 50% motor function loss. Bervar et al.[42] suggested that a simple ratio of the distance between the 1st and fifth toes of the injured per the unaffected hind leg is more advantageous than the traditional walking track analysis. In general, PFI measurement is known to be suitable for short and narrow foot prints due to lack of dorsal flexion of toe and ankle due to complete injury of peroneal nerve.[43] In this study, the sole of group 3 was rotated at walking and the lateral surface of sole was trod on the ground. While other groups trod on instep. As a result, PFI was seen as a significant improvement in group 3 at 2 weeks after transplantation but there is need to more experimentation for statistical significance.

Among passive measures of measuring the degree to which a toe is stretched, [24, 44] Toe-spreading test is a simple and useful method for evaluating reinnervation after sciatic nerve damage.[26, 27] Rats hold toe-spreading reflexes when lifted by the tail. If there is damage to the sciatic nerve, the toe is held together because the dorsiflexion fails. This reflex can be assessed as present or absent, even though the nerve is not fully recovered.[28] A study has shown that the rats of crushed peroneal nerve showed a positive response to toe-spreading test at two weeks.[23] In the present study, D-protocol Ⅲ showed a positive signal in the toe-spreading test after 2 weeks of transplantation. According to

Gutmann's scale[29], group 3 was degree 2 and the others were degree 0. Toe spreading requires reinnervation of the extensor digitorum muscle. Therefore, it could be clinically effective to transplantation D-protocol Ⅲ, but a larger number and long-term follow-up are required for accurate evaluation. To evaluate the peroneal nerve recovery of the neuromuscular scaffold transplanted with the PFI values and toe-spreading test of this study, however it is difficult to obtain statistical significance due to the small sample size and short follow-up period. And further studies on neuronal regeneration may be needed.

The reliable methods for evaluation of reinnervation are the electromyographic examination (nerve conduction and electromyography test), the muscle contraction test and the axonal transport study using horseradish peroxidase (HRP). Among these, the most reliable test for the evaluation of nerve reinnervation is to observe muscle contraction and actual voluntary movement.[45] The electromyographic test consists of test using motor nerve conduction and a needle electrode test. The motor nerve conduction test was performed by attaching the electrode to the examined muscle and taking the maximal electrical stimulation to the control nerve to detect latency from the muscle to the origin of the complex muscle activity potential. The latency of the complex muscle activity potential recorded in the muscle, the nerve conduction velocity using this latency, and the amplitude of this complex muscle activity potential are used as a measure of nerve conduction study. The needle electrode test is a clinically useful diagnostic assistant that can be widely used to differentiate between neurogenic lesions and myogenic lesions and to evaluate the conduction function of neuromuscular junctions. The insertion activity and the spontaneous activity of the muscle in the relaxed state and the motor unit action potential of the motor unit observed when the needle electrode is inserted. In addition, we use the shape and recruitment pattern of the exercise unit activity potential as a minimum contraction of the muscles.[46].

For histological and chemical staining of muscles, there is modified Gomori's trichrome staining for verifying myofibers and connective tissues, Verhoeff van

Gieson staining, Periodic acid-Schiff (PAS) staining for confirmation of glycogen in muscle fibers, red O or Sudan black B staining for fat. In Modified Gomori's trichrome staining, the muscle fibers are stained blue-green, the collagen fibers are light blue-green, and the nucleus, the nerves and the mitochondria are dyed with red dots. The Verhoeff-van Gieson stain is known to be suitable for observing the proliferation of collagen fibers because the muscle fibers are yellow and the collagen fibers are stained with pink.[47] There are ATPase, NADH-TR, succinate dehydrogenase(SDH), lactic acid dehydrogenase(LDH) for enzymatic histologic-chemical staining for muscles. In ATPase (pH 9.4) staining, muscle fibers can be classified as weakly stained type 1 and dark stained type 2, and in ATPase (pH 4.3) staining, muscle fibers are strongly stained as type 1 and very weakly stained as type 2. According to NADH-TR staining, type 1 muscle fibers are the most intense, type 2A is the middle type, and type 2B is the most dyed.[47] On the other hand, the histologic-chemical staining of nerve does not reveal all components of cells when they are stained by one method. Therefore, each component should be stained and observed and then synthesized.[48] Methods for staining neural tissue are staining with basic aniline dye, toluidine blue, methylene blue, thionine, and cresyl violet. Myelin staining is proceeding with potassium dichromate, followed by hematoxylin -toxylin (Weigert method), or Luxol fast blue staining. Reduced silver stain and golgi method are typical staining examination of nerve axon.[48]

Zheng et al. [49] reported that a reliable test for nerve reinnervation is motor nerve ending test which is to measure the number of motor nerve endings. If the reinnervation went successful, there is constantly increased in the number of motor nerve ending. Therefore, as in the previous study[22], it seems necessary to follow-up study for redistribution of nerve using –NF-200 antibody or to count motor nerve neurons using combined silver acetylcho-linesterase. In order to confirm nerve reinnervation, it is necessary to collect tissue specimens at the time of recovery of nerve and compare with control group. Because there are studies to observe the neuromuscular acellular scaffold for up to 180 days after

the neurorrhaphy[50], a study for the recovery time of the neuromuscular acellular scaffold through walking track analysis should be need to the long term observation.

Conclusion

In this study, a successful neuromuscular decellularization protocol was proposed. Through detailed characterization, it was confirmed that the important three dimensional structure of the acellular scaffold fabricated through this protocol, which is expected to maintain important ECM component and mechanical strength.

The protocol Ⅲ using SB-10, Trypsin, and Triton X-100, which are presented in this study, seems to be decellularized at an appropriate level in H & E staining and DNA quantification with proper network structure on SEM. In addition, it showed no immunologic response in allografts and good biocompatibility with peripheral muscles.

In order to use more clinical applications, further study for more specimens, population and long-term follow-up should be necessary to confirm the nerve reinnervation after transplantation of acellular neuromuscular scaffold with electromyography, histologic-chemical and histologic-enzymal staining.

Reference

1. Sicari, B.M., V. Agrawal, B.F. Siu, C.J. Medberry, C.L. Dearth, N.J. Turner, et al., *A murine model of volumetric muscle loss and a regenerative medicine approach for tissue replacement.* Tissue Eng Part A, 2012. **18**(19-20): p. 1941-8.
2. Turner, N.J. and S.F. Badylak, *Regeneration of skeletal muscle.* Cell Tissue Res, 2012. **347**(3): p. 759-74.
3. Edward K. Merritt, D.W.H., Matthew Tierney, Laura J. Suggs, Thomas J. Walters, Roger P. Farrar. , *Functional Assessment of Skeletal Muscle Regeneration Utilizing Homologous Extracellular Matrix as Scaffolding.* Tissue Eng Part A, 2010. **16**(4): p. 1395-1405.

4. Bach AD, B.J., Stern-Staeter J, Horch RE, *Skeletal muscle tissue engineering.* J Cell Mol Med, 2004. **8**(4): p. 413-22.
5. Flaumenhaft R, M.D., Saksela O, Rifkin DB., *Role of extracellular matrix in the action of basic fibroblast growth factor: matrix as a source of growth factor for long-term stimulation of plasminogen activator production and DNA synthesis.* J Cell Physiol, 1989. **140**(1): p. 75-81.
6. Cornelison, D.D., M.S. Filla, H.M. Stanley, A.C. Rapraeger, and B.B. Olwin, *Syndecan-3 and syndecan-4 specifically mark skeletal muscle satellite cells and are implicated in satellite cell maintenance and muscle regeneration.* Dev Biol, 2001. **239**(1): p. 79-94.
7. Ma, J., S. Sahoo, A.R. Baker, and K.A. Derwin, *Investigating muscle regeneration with a dermis/small intestinal submucosa scaffold in a rat full-thickness abdominal wall defect model.* J Biomed Mater Res B Appl Biomater, 2015. **103**(2): p. 355-64.
8. Wolf, M.T., C.L. Dearth, S.B. Sonnenberg, E.G. Loboa, and S.F. Badylak, *Naturally derived and synthetic scaffolds for skeletal muscle reconstruction.* Advanced drug delivery reviews, 2015. **84**: p. 208-221.
9. Heine, J., A. Schmiedl, S. Cebotari, H. Mertsching, M. Karck, A. Haverich, et al., *Preclinical assessment of a tissue-engineered vasomotive human small-calibered vessel based on a decellularized xenogenic matrix: histological and functional characterization.* Tissue Eng Part A, 2011. **17**(9-10): p. 1253-61.
10. Zhang, J., G.Y. Wang, Y.P. Xiao, L.Y. Fan, and Q. Wang, *The biomechanical behavior and host response to porcine-derived small intestine submucosa, pericardium and dermal matrix acellular grafts in a rat abdominal defect model.* Biomaterials, 2011. **32**(29): p. 7086-95.
11. Perniconi, B., A. Costa, P. Aulino, L. Teodori, S. Adamo, and D. Coletti, *The pro-myogenic environment provided by whole organ scale acellular scaffolds from skeletal muscle.* Biomaterials, 2011. **32**(31): p. 7870-82.
12. Wolf, M.T., K.A. Daly, J.E. Reing, and S.F. Badylak, *Biologic scaffold composed of skeletal muscle extracellular matrix.* Biomaterials, 2012. **33**(10): p. 2916-25.

13. Gilbert, T.W., T.L. Sellaro, and S.F. Badylak, *Decellularization of tissues and organs.* Biomaterials, 2006. **27**(19): p. 3675-83.

14. Lu, H., T. Hoshiba, N. Kawazoe, and G. Chen, *Comparison of decellularization techniques for preparation of extracellular matrix scaffolds derived from three-dimensional cell culture.* J Biomed Mater Res A, 2012. **100**(9): p. 2507-16.

15. Zhang, J., Z.Q. Hu, N.J. Turner, S.F. Teng, W.Y. Cheng, H.Y. Zhou, et al., *Perfusion-decellularized skeletal muscle as a three-dimensional scaffold with a vascular network template.* Biomaterials, 2016. **89**: p. 114-26.

16. Stern, M.M., R.L. Myers, N. Hammam, K.A. Stern, D. Eberli, S.B. Kritchevsky, et al., *The influence of extracellular matrix derived from skeletal muscle tissue on the proliferation and differentiation of myogenic progenitor cells ex vivo.* Biomaterials, 2009. **30**(12): p. 2393-9.

17. Evans, G.R., K. Brandt, M.S. Widmer, L. Lu, R.K. Meszlenyi, P.K. Gupta, et al., *In vivo evaluation of poly(L-lactic acid) porous conduits for peripheral nerve regeneration.* Biomaterials, 1999. **20**(12): p. 1109-15.

18. Hudson, T.W., S. Zawko, C. Deister, S. Lundy, C.Y. Hu, K. Lee, et al., *Optimized acellular nerve graft is immunologically tolerated and supports regeneration.* Tissue Eng, 2004. **10**(11-12): p. 1641-51.

19. Kwon, Y.J., J.Y. Kim, Y.I. Moon, I.W. Lee, B.J. Lee, H.J. Roh, et al., *Pattern of Reinnervation in Denervated Rat Gastrocnemius Muscle by Various Procedures of Reinnervation.* Korean J Otorhinolaryngol-Head Neck Surg, 2002. **45**(7): p. 693-705.

20. Crapo, P.M., T.W. Gilbert, and S.F. Badylak, *An overview of tissue and whole organ decellularization processes.* Biomaterials, 2011. **32**(12): p. 3233-43.

21. Qiagen, *DNeasy blood & tissue handbook.* 2006. **USA: Qiagen**.

22. Lee, B.K., Y.M. Ju, J.G. Cho, J.D. Jackson, S.J. Lee, A. Atala, et al., *End-to-side neurorrhaphy using an electrospun PCL/collagen nerve conduit for complex peripheral motor nerve regeneration.* Biomaterials, 2012. **33**(35): p. 9027-36.

23. Wong, K.H., M. Naidu, P. David, M.A. Abdulla, N. Abdullah, U.R. Kuppusamy, et al., *Peripheral Nerve Regeneration Following Crush Injury to Rat Peroneal Nerve by*

Aqueous Extract of Medicinal Mushroom Hericium erinaceus (Bull.: Fr) Pers. (Aphyllophoromycetideae). Evid Based Complement Alternat Med, 2011. **2011**: p. 580752.

24. Inserra, M.M., D.A. Bloch, and D.J. Terris, *Functional indices for sciatic, peroneal, and posterior tibial nerve lesions in the mouse.* Microsurgery, 1998. **18**(2): p. 119-24.
25. Varejao, A.S., M.F. Meek, A.J. Ferreira, J.A. Patricio, and A.M. Cabrita, *Functional evaluation of peripheral nerve regeneration in the rat: walking track analysis.* J Neurosci Methods, 2001. **108**(1): p. 1-9.
26. GUTMANN, E., L. GUTTMANN, P.B. MEDAWAR, and J.Z. YOUNG, *The Rate of Regeneration of Nerve.* Journal of Experimental Biology, 1942. **19**(1): p. 14-44.
27. Garcia, M.L., M.V. Rao, J. Fujimoto, V.B. Garcia, S.B. Shah, J. Crum, et al., *Phosphorylation of highly conserved neurofilament medium KSP repeats is not required for myelin-dependent radial axonal growth.* J Neurosci, 2009. **29**(5): p. 1277-84.
28. Navarro, X., *Functional evaluation of peripheral nerve regeneration and target reinnervation in animal models: a critical overview.* Eur J Neurosci, 2016. **43**(3): p. 271-86.
29. Gutmann, E. and L. Guttmann, *FACTORS AFFECTING RECOVERY OF SENSORY FUNCTION AFTER NERVE LESIONS.* J Neurol Psychiatry, 1942. **5**(3-4): p. 117-29.
30. Frey, M., H. Gruber, J. Holle, and G. Freilinger, *An experimental comparison of the different kinds of muscle reinnervation: nerve suture, nerve implantation, and muscular neurotization.* Plast Reconstr Surg, 1982. **69**(4): p. 656-69.
31. Grauss, R.W., M.G. Hazekamp, F. Oppenhuizen, C.J. van Munsteren, A.C. Gittenberger-de Groot, and M.C. DeRuiter, *Histological evaluation of decellularised porcine aortic valves: matrix changes due to different decellularisation methods☆.* European Journal of Cardio-Thoracic Surgery, 2005. **27**(4): p. 566-571.
32. Hrebikova, H., M. Vobornikova, M. Hetesova, and J. Mokry, *Histological Evaluation of Decellularized Skeletal Muscle Tissue Using Two Different Decellularization*

Agents. Acta Medica (Hradec Kralove), 2016. **59**(4): p. 107-112.

33. Du, L., X. Wu, K. Pang, and Y. Yang, *Histological evaluation and biomechanical characterisation of an acellular porcine cornea scaffold.* Br J Ophthalmol, 2011. **95**(3): p. 410-4.

34. Derwin, K.A., A.R. Baker, R.K. Spragg, D.R. Leigh, and J.P. Iannotti, *Commercial extracellular matrix scaffolds for rotator cuff tendon repair. Biomechanical, biochemical, and cellular properties.* J Bone Joint Surg Am, 2006. **88**(12): p. 2665-72.

35. Gilbert, T.W., J.M. Freund, and S.F. Badylak, *Quantification of DNA in biologic scaffold materials.* J Surg Res, 2009. **152**(1): p. 135-9.

36. Leyh, R.G., M. Wilhelmi, T. Walles, K. Kallenbach, P. Rebe, A. Oberbeck, et al., *Acellularized porcine heart valve scaffolds for heart valve tissue engineering and the risk of cross-species transmission of porcine endogenous retrovirus.* J Thorac Cardiovasc Surg, 2003. **126**(4): p. 1000-4.

37. Bain, J.R., S.E. Mackinnon, and D.A. Hunter, *Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat.* Plast Reconstr Surg, 1989. **83**(1): p. 129-38.

38. Johnston, R.B., L. Zachary, A.L. Dellon, W.A.t. Seiler, and D.M. Teplica, *Improved imaging of rat hindfoot prints for walking track analysis.* J Neurosci Methods, 1991. **38**(2-3): p. 111-4.

39. Hasegawa, K., *A new method of measuring functional recovery after crushing the peripheral nerves in unanesthetized and unrestrained rats.* Experientia, 1978. **34**(2): p. 272-273.

40. de Medinaceli, L., W.J. Freed, and R.J. Wyatt, *An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks.* Experimental Neurology, 1982. **77**(3): p. 634-643.

41. Hoogeveen, J.F., D. Troost, J. Wondergem, A.H.W. van der Kracht, and J. Haveman, *Hyperthermic injury versus crush injury in the rat sciatic nerve: A comparative functional, histopathological and morphometrical study.* Journal of the Neurological Sciences, 1992. **108**(1): p. 55-64.

42. Bervar, M., *Video analysis of standing — an alternative footprint analysis to assess functional loss following injury to the rat sciatic nerve.* Journal of Neuroscience Methods, 2000. **102**(2): p. 109-116.

43. Chen, B., Y. Song, and Z. Liu, *Promotion of nerve regeneration in peripheral nerve by short-course FK506 after end-to-side neurorrhaphy.* J Surg Res, 2009. **152**(2): p. 303-10.

44. Varejão, A.S.P., M.F. Meek, A.J.A. Ferreira, J.A.B. Patrı cio, and A.M.S. Cabrita, *Functional evaluation of peripheral nerve regeneration in the rat: walking track analysis.* Journal of Neuroscience Methods, 2001. **108**(1): p. 1-9.

45. Anonsen, C.K., H.C. Patterson, R.E. Trachy, A.M. Gordon, and C.W. Cummings, *Reinnervation of skeletal muscle with a neuromuscular pedicle.* Otolaryngol Head Neck Surg, 1985. **93**(1): p. 48-57.

46. Kim, H.-B., Y.-H. Park, and S.-S. Bae, *Clinical application of elactromyography and nerve conduction studty.* The Journal of Korean Society of Physical Therapy, 1998. **10**(1): p. 199-212.

47. Y, S.G., *Tumor of musculoskeletal and soft tissue.* Pathology part2, 1998. **6**: p. 1065-1126.

48. Lee, Y.T. and K.A. Park, *Staninig of nerve.* medical neuroanatomy part 2, 1996: p. 16-18.

49. Zheng, H., Z. Li, S. Zhou, Y. Cuan, W. Wen, and J. Lan, *Experimental study on reinnervation of vocal cord adductors with the ansa cervicalis.* Laryngoscope, 1996. **106**(12 Pt 1): p. 1516-21.

50. Hare, G.M., P.J. Evans, S.E. Mackinnon, T.J. Best, J.R. Bain, J.P. Szalai, et al., *Walking track analysis: a long-term assessment of peripheral nerve recovery.* Plast Reconstr Surg, 1992. **89**(2): p. 251-8.

국문요약

배경 및 목적

손상된 신경근 기능의 재생을 위해 신경 단독보다 신경근 조직을 이식하는 것이 더 효과적인 결과가 있다는 것이 알려져 있다. 따라서 효과적인 근육 기능 회복을 위한 탈세포화 신경편이 필요하다. 그러나 운동 신경을 이용한 골격근 탈 세포화 연구는 드물다. 이 연구는 쥐의 무 세포 신경 근육 발판에서 동종 이식을 시도하기 위한 기초를 확립하는 것을 목표로 한다.

방법

8 주 수컷 Sprague rats (SD rat, SPF)에서 비장근 (EDL)과 경골 근위 (TA) 근육의 일부를 비골 신경과 함께 채취한다. 채취한 근신경편은 Trypsin 과 triton X-100 을 이용한 protocol I, Sulfobetaine(SB)-10, SB-16 과 triton X-100 을 이용한 protocol II, 그리고 SB-10, trypsin, triton X-100 을 이용한 protocol III 의 3 가지 프로토콜에 따라 탈세포화를 진행한다. 제작된 scaffold 의 탈세포화 정도를 평가한 뒤 동종 쥐에 만든 근신경결손부에 이식한다. 2 주뒤 쥐의 비골신경 기능에 대한 임상적 평가를 한 뒤 희생하여 조직학적 분석을 한다.

결과

이 연구에서 SB-10, trypsin 그리고 triton X-100 을 이용한 프로토콜 III 은 근신경조직의 DNA 를 성공적으로 제거했으며, 구조적 강도가 유지되는 것이 관찰되었다. 이 프로토콜을 통해 제작한 탈세포화된 근신경조직을 동종 이식한 2 주 후에 시행한 조직학적, 임상적 평가에서 긍정적인 결과로 관찰되었다. 그러나 신경재지배 양상을 평가하기 위해서는 더 많은 개체와 장기간의 경과관찰을 통한 추가적인 연구가 필요할 것으로 보인다.

중심단어 : 탈세포화 비계, 조직공학, 근신경, 비골신경