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탈신경화된 고령의 쥐에서 골격근 위축 방지에 대한 트레드밀 운동의 효과

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지도교수 방문석

이 논문을 의학박사학위논문으로 제출함

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이호준

이호준의 박사학위논문을 인준함

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The Effect of Treadmill Exercise on Denervation Induced Muscle Atrophy In Aged Rat

by

Ho Jun Lee

A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Rehabilitation Medicine) at Seoul National University College of Medicine

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Approved by Thesis Committee:

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Abstract

The Effect of Treadmill Exercise on Denervation Induced Muscle Atrophy In Aged Rat

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Introduction: Partial denervation in old people are common, for example peripheral polyneuropathy, motor neuron disease and lumbar or cervical radiculopathy including spinal stenosis. It is important to develop interventions to reduce or restore the muscle degradation in atrophy induced by denervation in addition to sarcopenia. Exercise has been reported to reduce denervation induced atrophy of skeletal muscle in young rats. However, the effect of exercise on denervated muscle atrophy in aged rat has not been well known. The purpose of this study is to investigate the effect of treadmill exercise on denervation induced muscle atrophy in aged rat.

Methods: Aged male Sprague-Dawley rats (22 months) were randomly assigned to three groups (normal control (NC, n=7), denervation

exercise (DE, n=8), denervation control (DC, n=8)). Partial denervation was performed by temporary compression of right sciatic nerve. From two weeks after denervation, treadmill resistance exercises with alternative concentric and eccentric mode were given to DE group for two weeks. Four weeks after the injury, muscle wet weight (MWW) ratio, myosin heavy chain (MHC) isoform composition from muscle homogenate and single muscle fibers and the expression of atrogene (Muscle RING Finger-1; MuRF1) and calpain-3 were measured in gastrocnemius and soleus muscles. Fiber cross sectional areas (FCSAs) were measured in gastrocnemius muscles.

Results: In denervation groups (DC and DC), MWW ratio of gastrocnemius and soleus and FCSA of gastrocnemius were significantly smaller than in NC and those were not significantly different between DC and DE. In gastrocnemius, the proportions of MHC types I and IIx isoforms in denervation (DC and DE) were significantly different compared with NC and no differences were found between DC and DE. The proportion of hybrid fiber (IIx/IIb) of single muscle fiber in gastrocnemius was greater in DE and the proportion of another hybrid fiber (IIx/I) was greater in DC. In soleus, there were no differences of the proportions of MHC isoforms among all groups. Calpain-3 expression of gastrocnemius had a tendency to increase in DC and decrease in DE but there was no significant difference. Calpain-3 expression of soleus was not significantly different among all groups. MuRF-1 expression of gastrocnemius was significantly higher in DC than NC and showed decreased tendency with no significant difference

in DE. MuRF-1 expression of soleus was higher in DC than NC and lower in DE than DC, which suggests denervation induced up-regulation of MuRF-1 and exercise after partial denervation suppressed MuRF-1 activation in gastrocnemius and soleus.

Conclusions: In aged rats with atrophied skeletal muscle caused by partial denervation, treadmill resistance exercise induced down-regulation of MuRF-1 with small features of muscle plasticity but failed to make significant morphologic changes. We suppose that treadmill exercise after partial denervation in aged rats can attenuate the degradation of muscle protein on the molecular level under the surface at 4 weeks. Further studies with longer training duration are necessary to identify gross changes following treadmill exercise in denervated old rats.

Keywords: Denervation, Muscle atrophy, Aging, Exercise, Sarcopenia

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Introduction

Recent extension of life years leads to increased population of old people. Aging is associated with a slow and progressive loss of muscle, which is called sarcopenia, leading to increasing frailty, weakness, and loss of functional independence.(1) With the increase of age, there is some loss of motor neurons, reduction of motor unit number, alterations in the neuromuscular junctions, and selective denervation of type II muscle fibers. Denervation atrophy of skeletal muscles has been studied as one of the mechanisms for muscle degeneration in old age.(2) Muscle wasting and weakness are also common in many diseasesand conditions including aging, cancer cachexia, sepsis, denervation, disuse, inactivity, chronic kidney or heart failure, unloading/microgravity and which are usually found in old people.(3) Skeletal muscle atrophy may influence to reduce patient recovery, independence and quality of life.(4)

Partial denervation such as peripheral polyneuropathy, motor neuron diseases and cervical or lumbar radiculopathy including spinal stenosis are common findings in old people. Major changes in skeletal muscles after denervation include muscle fiber atrophy, decreased muscle mass, and impaired function,(5-7) leading to rapid muscle wasting and weakness.(8,9) Long-term denervation was associated with decreased force producing capacity.(8) There was a significant shift in muscle phenotype towards a more ‘intermediate’ phenotype consisting of fast-slow hybrid fibers.(9)

Immobilization, aging, disuse and denervation are accompanied by

decreased protein synthesis and markedly increased protein degradation, called as catabolic process, which leads to changes to myofibrillar proteins and muscle atrophy.(10) It has been shown that myosin heavy chain (MHC) content decreases,(11) and the MHC isoform proportion, which are representative of muscle plasticity, changes in response to the disuse or denervation atrophy.(9,11,12)

Calpain-3 is thought to play a role in the protection of titin from ubiquitous calpains that have autocatalytic activity. Titin is a component of a sarcomere and involved with sarcomeric integrity.(13-18) In other words, calpain-3 activity correlates negatively with muscle degradation in contrast with the ubiquitous calpain which show a positive correlation.(19-20) Calpain-3 is able to cleave ubiquitous calpains that have been clearly implicated in the initial degradative events of the cytoskeleton observed in atrophic conditions. Therefore, the activity of calpain-3 is necessary for the homeostasis in skeletal muscles.(18)

Maintaining muscle mass is a balance between protein synthesis and protein degradation systems. “Atrogenes” or atrophy-related genes partly regulate the activation of the cell’s proteolytic systems such as the ubiquitin-proteasome system (UPS), which leads to acute muscle atrophy from many pathological conditions (cancer, denervation, spinal cord injury, immobilization). Among the known E3s (ubiquitin ligase), atrogin-1/MAFbx (Muscle atrophy F-Box) and MuRF1 (Muscle RING (Really Interesting New Gene) Finger-1) are both muscle-specific and up-regulated during muscle loss. MuRF1 was reported to interact with many muscle structural proteins, including troponin I, myosin heavy chains, actin, myosin binding protein C and myosin light chains 1 and 2.(4, 21)

Various interventions to reduce muscle atrophy including exercise have been reported with some beneficial results of chronic electrical stimulation and the potential medications related to myostatin inhibition. In the process of denervation atrophy and subsequent regeneration, the role of exercise has been studied but the results were quite inconsistent. Earlier functional recovery,(22) decreased type 2 muscle atrophy,(23,24) increased muscle reinnervation and regenerated myelinated axons,(25) and increased length of regenerated axons have been reported in terms of the exercise effects,(26) whereas there was an evidence of harmful effect of exercise.(22) The exact mechanisms need to be identified through the experimental studies including sarcomeric structural and regulatory components. However, the effect of exercise on myofibrillar proteins was not much studied except increased MHC content.(27) The effect of resistance exercise (slow-velocity resistance exercise: performing the concentric and eccentric phase of each muscle contraction in 2−3 s) on sarcopenia or skeletal muscle in aged is well known to induce muscle hypertrophy and increase strength.(28) but there were little reports about exercise effect on denervation induced muscle atrophy in aged skeletal muscle.Our hypothesis was that resistance exercise combined with concentric and eccentric mode, which is known beneficial to the elderly, may have also influence to reduce denervation induced muscle atrophy in aging skeletal muscles via promotion of protein synthesis or inhibition of protein degradation, The purpose of this study is to investigate the treadmill exercise effects on partial denervation induced atrophy of skeletal muscle in aging rat.

Materials and methods

1) Approval by Institutional Ethical Review Board (IRB)

This protocol was approved by Seoul National University Bundang Hospital Institutional Animal Care and Use Committee (SNUBH IACUC) (IACUC No. BA1009-090/060-01).

2) Animals and experimental protocols

Experiments were conducted on aged (22 months) maleSpraugue-Dawley rats. They were housed in pathogen-free conditions at around 20℃ and exposed to a reverse light condition of 12:12-h of light/darkness each day. Aged rats were classified into 3 groups: Normal control (NC) group with no denervation and no exercise, denervation control (DC) group with partial denervation and no exercise and denervation exercise (DE) group with partial denervation and resistance exercise. Sciatic nerve injury (partial denervation) were given to DC and DE group rats, and treadmill exercises were taken to DE group and no exercises were taken to the DC group as control. At 4 weeks after injury we sacrificed all of them and conducted various analyses on gastrocnemius and soleus muscles (Fig. 1,2).

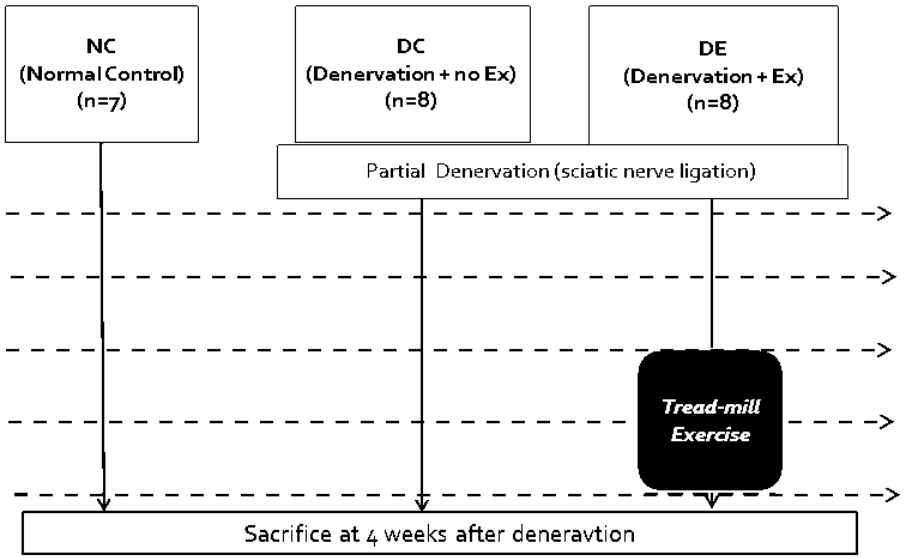

Figure 1. Overview of protocols

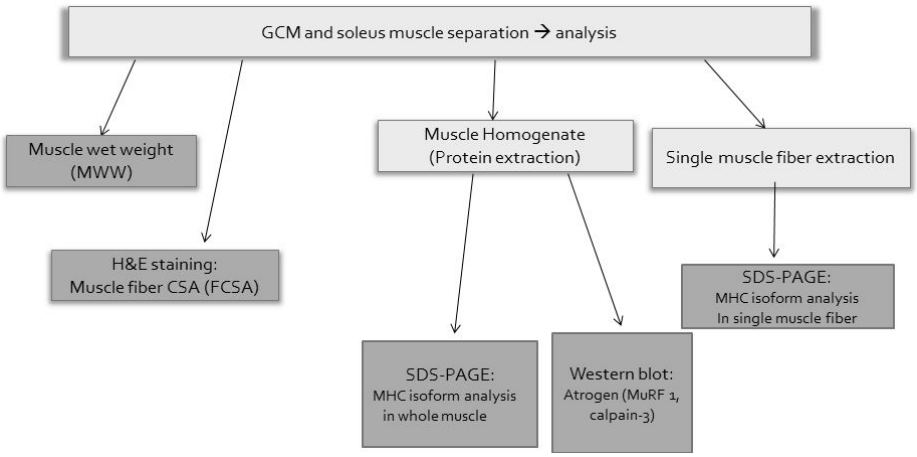

Figure 2. Overview of Analyses

3) Partial denervation model

Before nerve injury, general anesthesia was done with zoletil (zolazepam and tiletamine, 30 mg/kg body weight) and with xylazine (10 mg/kg body weight) through intraperitoneal injection. Under the sterile condition, a posterior thigh-split incision exposed the right sciatic nerve trunk. For partial denervation, the right sciatic nerve trunk 10 mm distal to the sciatic notch was crushed with a non-serrated hemostatic forceps for 90 seconds. To apply constant pressure to the nerve trunk, the compression force was controlled using the same level of locking forceps, and the inner margin of the nerve trunk was located 3 mm proximal to the end of the jaws between the arms of the forceps.(29) (Fig. 3) The muscle and skin were closed, and gentamycin 10 mg/kg was injected into gluteus muscle. The rats were then housed in a cage.

4) Exercise protocol

In exercise group, exercise on motor-driven treadmill (Dual-treadmill, Fine-S022K, EGR fine, Korea) began at 2 weeks after injury and continued for 2 weeks. Exercise was composed of warming up period (5 m/min, 5 min), uphill running for concentric exercise mode (15 m/min, 10 min, 15°inclination) and downhill running for eccentric exercise mode (15 m/min, 10 min, 15°inclination) and exercises were performed with moderate intensity and daily for 30 min and in 5 days a week. (Fig. 4) This exercise protocol was modified from other

studies.(30,31) The intensity of the exercise was moderate and downhill running (eccentric mode) was reported to be effective and safe,(31,32) and the treadmill exercise induced no damage to the muscle during reinnervation.(23)

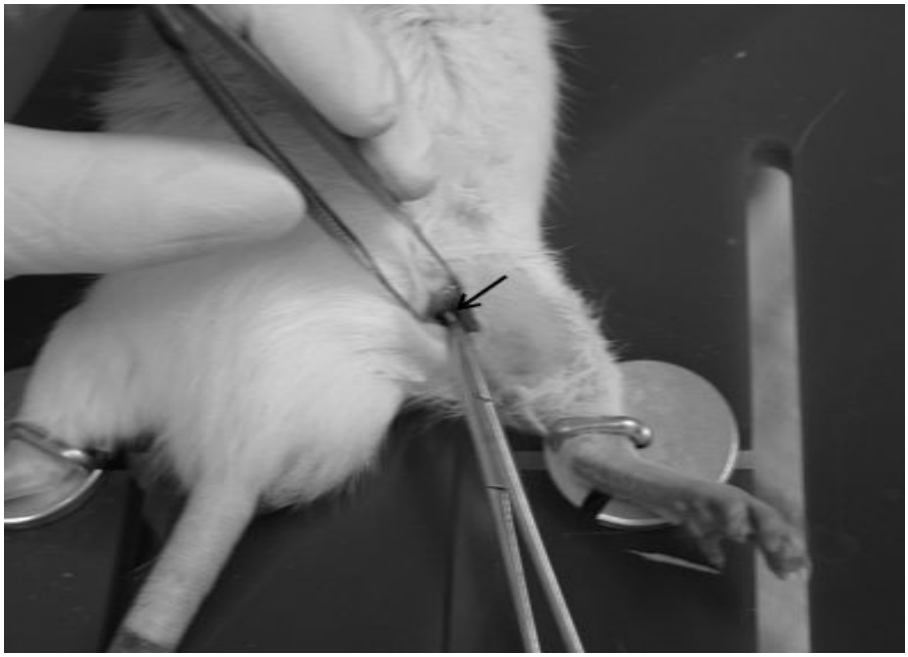

Figure 3. Partial denervation crush model. Using non-serrated hemostatic forceps, right sciatic nerve (arrow) was locked for 90 seconds

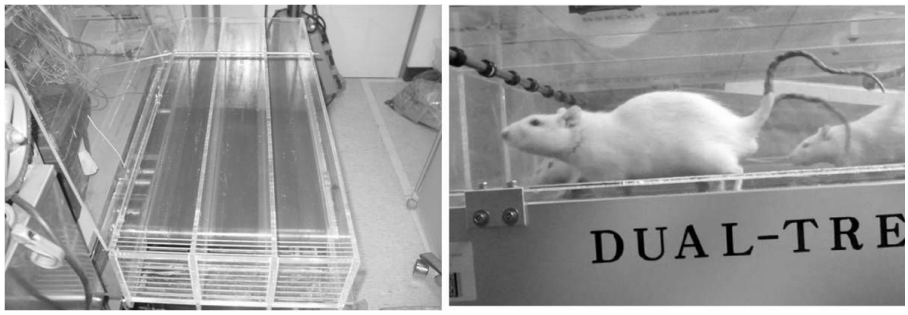

Figure 4. Motorized treadmill exercise with alternative concentric (up running) and eccentric (downhill running) modes.

5) Outcome evaluations

(1) Muscle separation and measurement of muscle wet weight

Whole gastrocnemius muscles were resected from just below the origin site of the gastrocnemius proximaly to just above the calcaneal insertion of triceps surae distally in both legs. Thereafter, soleus muscles were taken apart from the whole triceps surae muscles by detaching the small longitudinal muscles lying underneath the gastrocnemius. The weight of the gastrocnemius and soleus muscles was measured, and the ratio of denervated muscle weight to that of the intact side was calculated for each specimen.

(2) Morphologic analysis (cross sectional area of whole muscle of gastrocnemius)

A block of muscle taken from the mid-belly of gastrocnemius muscle was fixed in 4% paraformaldehyde. The fixed muscle specimens were washed in water, dehydrated in a gradedethanol series, cleared in xylene, embedded in paraffin and cut into 7-lm-thick sections. Haematoxylin & Eosin (HE) staining was then applied to the sections, and images were observed under light microscopy. Photographs were taken from five random fields (x200) chosen randomly in the central region of one cross-section of each gastrocnemius muscle with the aid of a AxioCam HR digital camera (Carl Zeiss, Göttingen, Germany). Images were digitalized into an Axiovision imaging software (Carl Zeiss, Göttingen, Germany), followed by analyses with a Image J 1.44a

software (Wayne Rasband, National Institutes of Health, USA, http://rsb.knfo.ih.gove/ij) to measure the cross-sectional area (CSA) of the muscle fibers. Muscle section was made from one animal per group, and a minimum of 150 fibers was measured per an animal.

(3) MHC isoform of whole muscle (homogenate) (SDS-PAGE)

Muscle samples for protein analysis taken from gastrocnemius and soleus were frozen by liquid nitrate and were stored at -80°C. To make muscle homogenate, a section of 100 mg of muscle tissue was cut and homogenized in 200 μl lysis buffer containing EDTA, pepstatin A, leupeptin and aprotinin. Sonification and centrifuge were repeated three times and the supernant were separated. The total protein contents were measured with an Absorbance Assay (280 nm) using Nanodrop, and the concentration was adjusted to 10 mg/ml with washing buffer. Subsequently, myofibrils were solubilized with an equal volume of 2 μl SDS sample buffer. The SDS samples were heated at 50°C for 20 min and stored at 4°C before analysis.

A step gradient minigel with an ambiguous interface, originally proposed to quantify the contents of MHC,(33) was used with some modifications. The glass plates (with 1.5mm spacers) and combs (15-well) were used to make gels. The lower 12% resolving gel was made with solutions (4 ml) containing 375mM Tris (pH 8.6), 0.1% SDS, 12% acrylamide/bisacrylamide solution (37.5:1), 0.05% APS, and 0.05% TEMED. The upper 4% resolving gel was made with solution (4.5 ml) containing 375mM Tris (pH 8.6), 0.1% SDS, 4%

acrylamide/bisacrylamide solution (37.5:1), 0.05% APS, and 0.05% TEMED. Stacking gel contained 2.4% acrylamide/bisacrylamide. Gel electrophoresis was performed at a constant current of 6.5mA/plate for 2 h at room temperature and at a constant 100V in succession until the bromophenol blue reached approximately 5mm above the bottom of the gel. After electrophoresis, the gel slabs were stained with Coomassie brilliant blue. The optical density (OD) of MHC was determined with a Photo-Print Digital Imaging System (IP-008-SD; Vilber Lourmat, France) and analytic software (Bio-1D Light, Vilber Lauret, French). The OD results of all lanes were plotted vs. their loadings.

(4) MHC isoform of single muscle fiber (SDS-PAGE)

MHC isoform composition was analyzed using two samples; protein homogenates and single muscle fibers more than 20 per muscles. To prepare single muscle fibers, bundles of fibers were chemically skinned for 24 h in relaxing solution containing 50% (v/v) glycerol at 4°C and stored at −20°C.(13, 23) Each single fiber was extracted under stereomicroscope, and dissolved in lysis buffer. The MHC composition was determined by 6% SDS-PAGE. The acrylamide concentration was 4% (w/v) in the stacking gel and 6% in the separating gel, and the gel matrix included 30% glycerol. SDS PAGE was run at a constant voltage of 90V for 30 min and 140V for 5.5 hours.(34) Protein homogenate mixture of gastrocnemius and soleus was used as an MHC standard. Protein bands in the gels loaded with muscle homogenates were visualized with Coomassie Brilliant Blue. Densitometry was

performed using analytic software (Bio-1D Light, Vilber Lauret, French) to measure relative proportions of MHC isoforms. Gels loaded with single muscle fiber were subsequently stained with silver to identify MHC isoform of each single fiber and the relative proportion of hybrid fibers in each muscle was determined.

(5) The expression of MuRF-1 and calpain-3 protein (Western blot)

Muscle protein (20 ug/lane) was separated on a 10% SDS-PAGE gel at 90V for 2 hours. Gel was cut at the point of 140 kDa marker; upper gel was stained with Coumassie blue R to visualize MHC and the lower gel was transferred onto nitrocellulose membranes to perform western blot for each MuRF-1 and calpain-3. Transferred membranes were exposed for calpain-3 to mouse anti-calpain-3 (1 in 250; Novocastra monoclonal 12A2, Newcastle, UK), after which donkey anti-goat IgG- horseradish-peroxidase (1 in 5000; sc-2020, Santa Cruz Biotechnology Inc., Santa Cruz, CA) or goat anti-mouse IgG peroxidase conjugated (1:20,000 dilution; Chemicon International, Temecula, CA) as a secondary antibody was added to the membranes. Calpain-3 was observed as a 94-kDa protein that autolyzed to proteins of 60, 58, and 56 kDa when activated.(35) Transferred membranes were exposed for MuRF-1 to a rabbit polyclonal anti-MuRF-1 (1 in 200; sc-32920, Santa Cruz Biotechnology Inc., Santa Cruz, CA) as primary antibody, after which goat anti-rabbit IgG horseradish peroxidase-conjugated (1 in 5000; sc-2004, Santa Cruz Biotechnology Inc., Santa Cruz, CA)

(dilution range: 1:2000-1:100,000) as a secondary antibody was added to the membranes.(36) MuRF-1 was observed as a 44-kDa protein band. Bands were visualized using ECL plus Western blotting detection reagents (RPN2132; AmershamTM GE healthcare, UK) and molecular imager system (ChemiDoc XRS; Bio-Rad, UK) and densitometry performed using Quantity One software (Bio-Rad, UK). Glyceraldehye-3-phophate dehydrogenase (GAPDH) served as a loading control (the housekeeping gene). After measuring band density of GAPDH, calpain-3 and MuRF-1 normalized values were obtained with the ratio of density in MuRF-1 or calpain-3 by that of GAPDH. These protocols were performed with gastrocnemius and soleus muscle of NC, right side of DC, right side (denervated side) of DE and left side (not denervated side) of DE because the comparison with NC and left side of DE was necessary to identify and rule out the effect of exercise on cytokines in normal muscle.

6) Statistical Analysis

The values for all three groups were analyzed with Krunskal-Wallis test or Analysis of Variance (ANOVA), if the data were normal distribution, to compare the differences of exercise effects among three aged groups with post hoc analysis with Tukey HSD if necessary. Analyses were performed using statistical software (SPSS Korean version 20.0 for Windows; SPSS, Inc., Chicago, Illinois). A P value less than 0.05 indicated the presence of a statistically significant difference.

Results

1) Muscle wet weight (MWW)

Body weights before denervation were not statistically different among all three groups. Therefore, body weights representative of general condition just before denervation were similar among NC group and denervation groups. After 4 weeks from denervation muscle weights of gastrocnemius (GCM) and soleus of DC and DE groups were significantly decreased in comparition with those of NC group, however there were no significant difference in muscle weights between DE group and only denervation group. Similar findings were found in the relative muscle weight: the muscle weight ratio of right side (denervated limb) versus left side (not denervated limb) and the ratio of muscle versus in body weight (Table 1) (Fig. 5).

Table 1. Body Weights and Muscle Wet Weights Including Ratios of Gastrocnemius and Soleus Muscles

	Pre BW (g)	MWW (g)		MWW ratio (D/N) (%)		MWW/BW (%)	
		GCM	Soleus	GCM	Soleus	GCM	Soleus
NC	812.98 ± 113.34	3.87 ± 0.49	0.34 ± 0.02	102.44 ± 4.50	99.19 ± 15.44	0.472 ± 0.062	0.037 ± 0.010
DC	860.62 ± 133.52	1.29 ± 0.15[a)]	0.13 ± 0.02[a)]	37.69 ± 5.38[a)]	52.42 ± 13.63[a)]	0.155 ± 0.021[a)]	0.016 ± 0.003[a)]
DE	823.70 ± 182.25	1.22 ± 0.19[b)]	0.15 ± 0.04[b)]	39.05 ± 6.92[b)]	52.86 ± 6.66[b)]	0.157 ± 0.031[b)]	0.019 ± 0.005[b)]

Values are presented as mean ± standard deviations

MWW: Muscle Wet Weight

Pre Bwt: body weight before denervation

GCM: gastrocnemius

D/N: denervated/ non-denervated

NC: normal control

DC: denervation without exercise group

DE: denervation with exercise

a) $p < 0.05$, between NC and DC (Krunskal Wallis test with post hoc analysis: Tukey HSD)

b) $p < 0.05$, between NC and DE (Krunskal Wallis test with post hoc analysis: Tukey HSD)

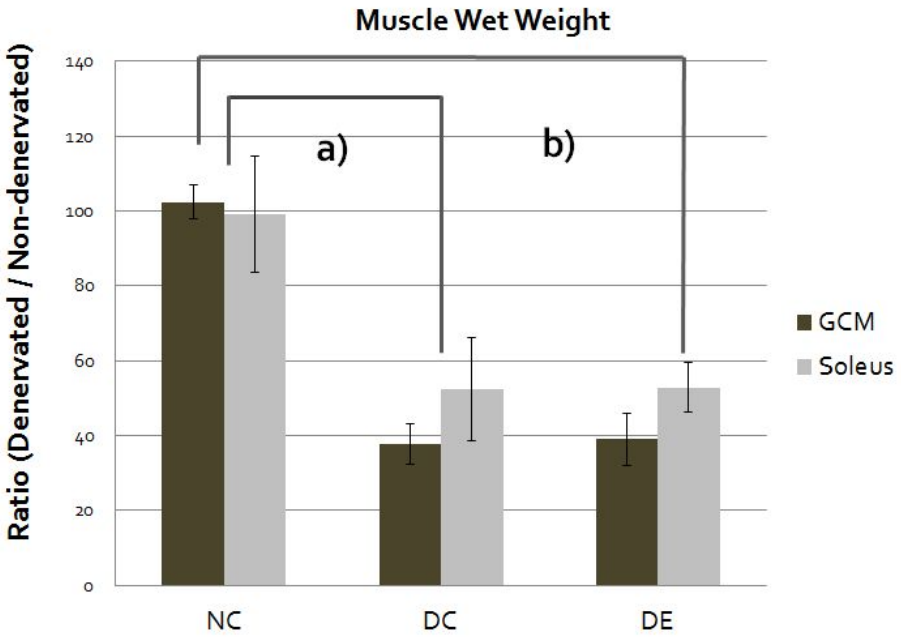

Figure 5. The graph shows the ratio of muscle wet weight of three groups. The ratio is that of denervated side over non denervated side. There are significant differences between NC and NC (a) and between NC and DE (b). However there was no significant difference between NC and DE. (NC: Normal Control, DC: Denervation Control, DC: Denervation Exercise)

2) Morphologic analysis (fiber cross sectional area)

H & E stains of both denervation groups showed smaller muscle fibers than normal group, which are typical findings of neurogenic change of muscle fiber (Fig. 6). The average fiber cross sectional areas (FCSA) of gastrocnemius muscle fibers of DE (1006.1 ± 103.1 μm^2) and DC (881.2 ± 290.9 μm^2) were respectively significantly smaller than those of NC (1877.9 ± 418.0 μm^2, p=0.001). FCSA of DE group seemed to be larger than those of DC group, but there was no significant difference between two groups. There was also no significant difference in FCSA ratio of right side (denervated limb) versus left side (non denervated limb) between DE and DC group (p=0.201) (Table 2) (Fig. 7).

The muscle weight and the average FCSA significantly decreased after denervation but the treadmill exercise could not help to restore the muscle weight and FCSA at 4 weeks after denervation in aged rat.

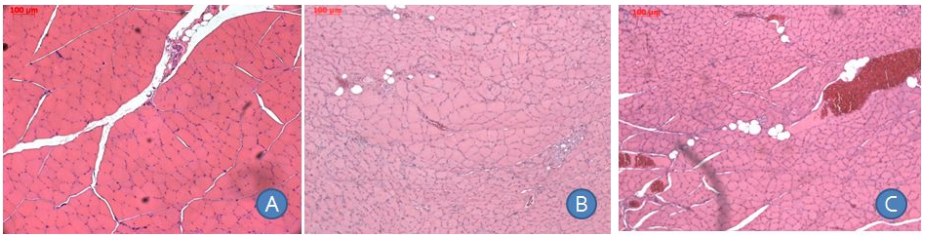

Figure 6. H & E stains of gastrocnemius muscle in NC (A), DC (B) and DE (C) group. Stains of both denervation groups show small, angulated muscle fibers and grouped atrophy, which are typical findings of neurogenic changes of muscle fiber. (NC: Normal Control, DC: Denervation Control, DE: Denervation Exercise)

Table 2. Fiber Cross Sectional Area (FCSA) of Gastrocnemius Muscle

	FCSA (Rt GCM) (μm^2)	FCSA ratio (D/N; GCM)
NC	1877.9 ± 418.0	
DC	881.2 ± 290.9 [a)]	50.77 ± 15.89
DE	1006.1 ± 103.1[b)]	60.68 ±14.23

Values are presented as mean ± standard deviations
NC: normal control
DC: denervation control
DE: denervation exercise
FCSA: average fiber cross sectional area
D/N: denervated/ non-denervated
a) $p < 0.05$, between NC and DC (Krunskal Wallis test with post hoc analysis: Tukey HSD)
b) $p < 0.05$, between NC and DE (Krunskal Wallis test with post hoc analysis: Tukey HSD)

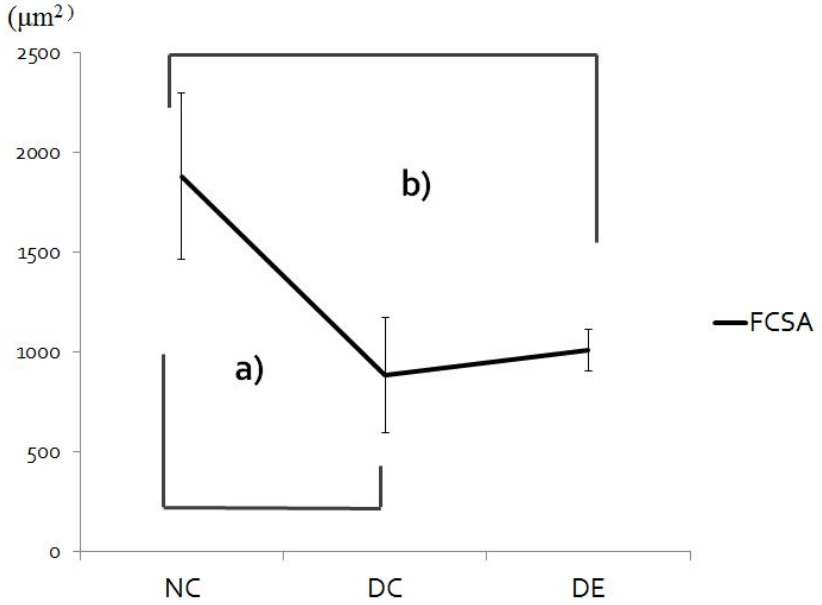

Figure 7. FCSA (Fiber Cross-Sectional Area) of right gastrocnemius (GCM). The graph shows significantly decreased FCSA in each DC and DE in comparison with NC. However, there is no significant difference between DC and DE. (NC: Normal Control, DC: Denervation Control, DE: Denervation Exercise)

3) MHC isoform of muscle homogenates

In gastrocnemius, the content of type I was higher (p=0.049) and type IIx lower (p=0.016) in DE and DC groups than NC group. At 4 weeks after denervation data showed significantly more MHC type I (13.60 ± 15.14 vs 1.27 ± 3.12, p=0.047) and significantly less MHC type IIx (37.20 ± 12.30 vs 61.92 ± 16.2, p=0.007) in DC group than NC group. In DE group similar results were found in type I (15.96 ± 14.79 vs 1.27 ± 3.12, p=0.054) and type IIx (40.67 ± 12.15 vs 61.92 ± 16.2, p=0.040). No significant differences were found between DE group and DC group. At 4 weeks after denervation, there was a tendency of transition from MHC isoform type IIx to slower MHC isoform type (I, IIa) (Table 3) (Fig. 8,9).

In soleus there was no statistical differences in MHC isoform composition between NC group and denervation groups. MHC type I isoform was composed mostly in all groups (NC: 94.53 ± 6.83, DC: 92.43 ± 4.56, DE: 95.19 ± 4.37) (Table 4). Despite non significant difference more MHC type IIx isoforms were shown in DC and DE group than those in control group (Fig. 10). Densitometric analysis showed small multiple bands, which means MHC type II a or II x isoforms, in DC group and DE group in comparison with NC group (Fig. 11). This findings suggested a little transformation occurred from type I to type IIa or IIb in soleus after denervation, however exercise appeared to have no influence to denervation.

Table 3. Myosin Heavy Chain (MHC) Isoform Proportions of Gastrocnemius Homogenate Muscle Tissue

	MHC isoform type (Gastrocnemius)			
	I	IIa	IIx	IIb
NC	1.09 ± 2.89	0.00	61.92 ± 16.2	36.81 ± 15.58
DC	13.60 ± 15.14[a)]	11.56 ± 18.70	37.20 ± 12.30[a)]	41.53 ± 24.60
DE	15.96 ± 14.79[b)]	9.43 ± 13.91	40.67 ± 12.15[b]	32.56 ± 21.13

Values are presented as mean ± standard deviations

NC: normal control

DC: denervation control

DE: denervation exercise

a) $p < 0.05$, between NC and DC (Krunskal Wallis test with post hoc analysis: Tukey HSD)

b) $p < 0.05$, between NC and DE (Krunskal Wallis test with post hoc analysis: Tukey HSD)

Table 4. Myosin Heavy Chain (MHC) Isoform Proportions of Soleus Homogenate Muscle Tissue

	MHC isoform type (Soleus)			
	I	IIa	IIx	IIb
NC	94.53 ± 6.83	5.29 ± 6.89	0.18 ± 0.48	0.00
DC	92.43 ± 4.56	5.17 ± 5.35	2.17 ± 2.55	0.23 ± 0.65
DE	95.19 ± 4.37	3.22 ± 2.83	1.60 ± 2.47	0.00

Values are presented as mean ± standard deviations.

NC: normal control

DC: denervation control

DE: denervation exercise

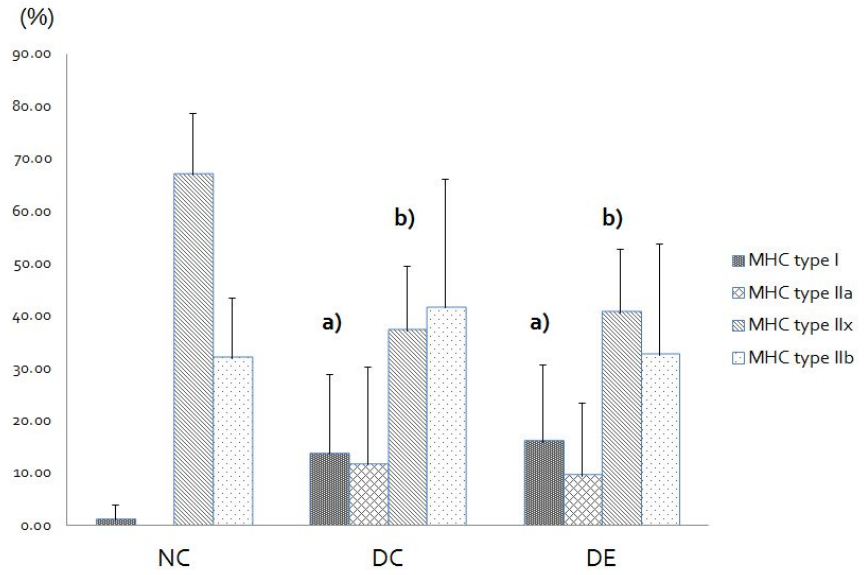

Figure 8. Myosin Heavy Chain (MHC) isoform proportions of gastrocnemius homogenate muscle tissue. There are significant differences in MHC type I and type IIx between DC and NC and between DE and NC. The graph shows more MHC type I and less MHC type IIx in DC and DE in comparison with NC. However there is no significant difference between DC and DE. a): significant difference between DC and NC and between DE and NC in type I, b): significant difference between DC and NC and between DE and NC in type IIx, (NC: Normal Control, DC: Denervation Control, DE: Denervation Exercise) Values are presented as the mean ± standard deviations.

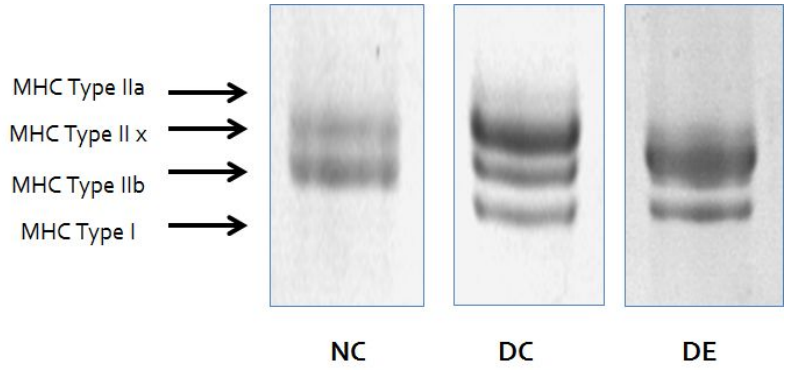

Figure 9. Myosin Heavy Chain (MHC) isoforms of gastrocnemius muscle are separated with different bands on SDS-PAGE. More MHC type I isoforms are present in DC and DE group.

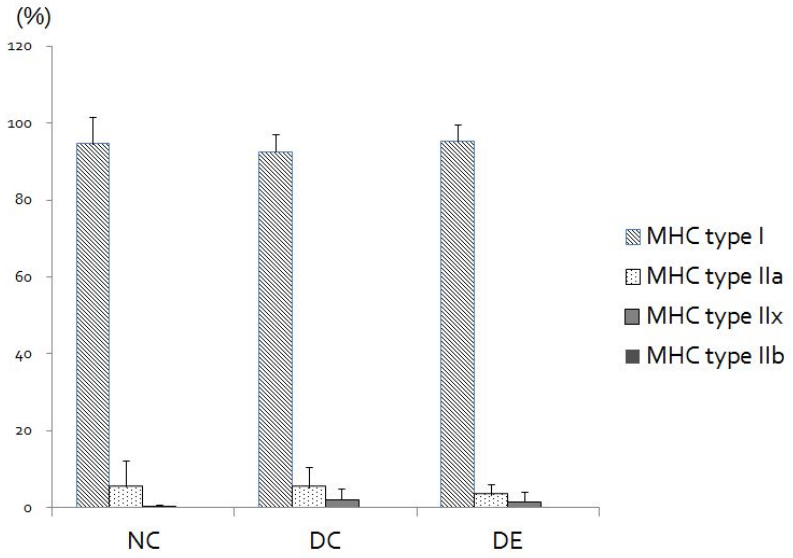

Figure 10. Myosin Heavy Chain (MHC) isoform proportions of soleus homogenate muscle tissue. Values are presented as the mean ± standard deviations. (NC: Normal Control, DC: Denervation Control, DE: Denervation Exercise)

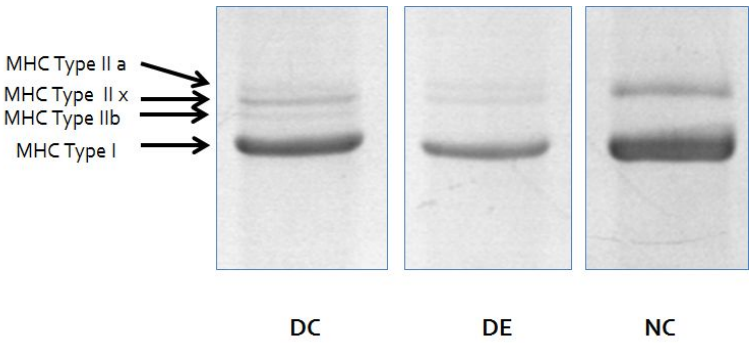

Figure 11. Myosin Heavy Chain (MHC) Isoforms of soleus muscle are separated with different bands on SDS-PAGE. Densitometry showed small multiple bands, which means MHC type IIa or IIx isoforms, in DC group and DE group in comparison with NC group.

4) MHC isoforms of single muscle fibers

In gastrocnemius, MHC isoform proportions showed different features among three groups. In normal control, type IIb and IIx muscle fibers were relatively high, but, in DC, slow twitch hybrid type IIx/I became most prevalent. In DE, fast twitch hybrid fiber type IIx/IIb was found with a high proportion (Fig. 12). In soleus muscle, the proportions of MHC isoforms was shown similar to those of muscle homogenates. Predominance of type I muscle fibers was not changed among three groups (Fig. 13). Because of the difficulty with the extraction of single muscle fiber small numbers of gastrocnemius and soleus fibers were analyzed and the statistical analysis was not performed.

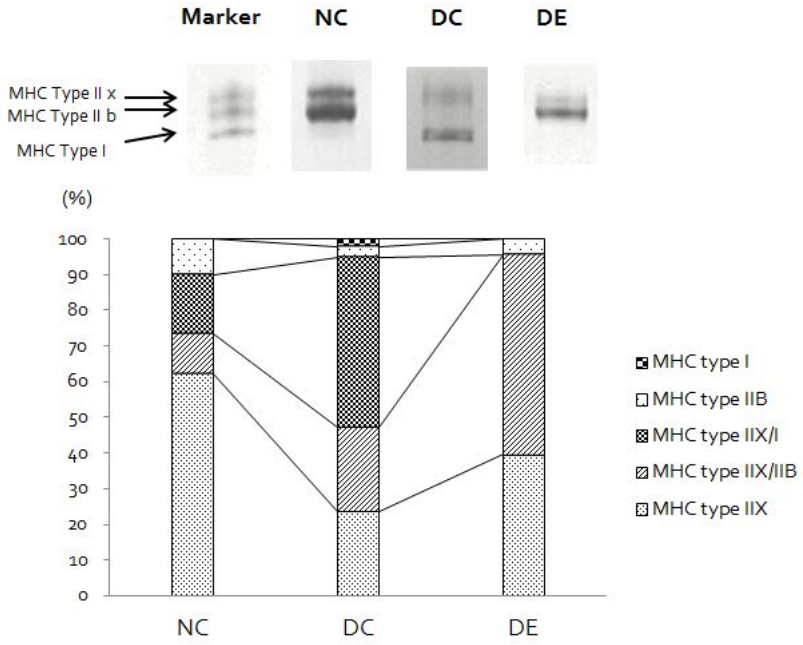

Figure 12. Myosin Heavy Chain (MHC) Isoform proportions of gastrocnemius single muscle fiber. MHC isoforms are separated on SDS-PAGE (above) and proportions are shown in graph (below). More type IIx/I hybrid fibers are found in DC than NC and DE. More type IIx/IIb hybrid fibers are present in DE than other groups.(NC: Normal Control, DC: Denervation Control, DE: Denervation Exercise)

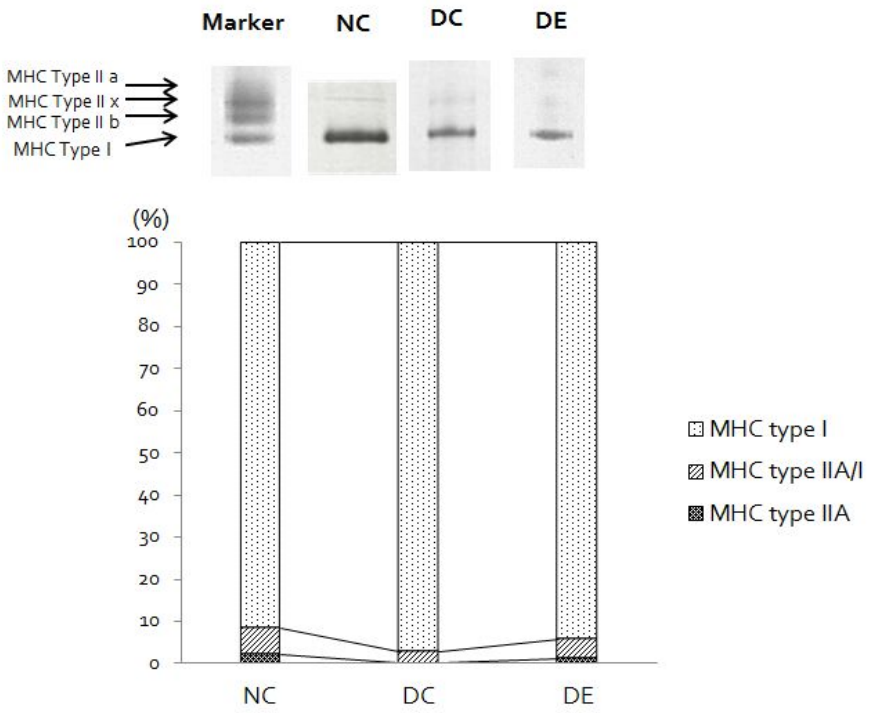

Figure 13. Myosin Heavy Chain (MHC) isoform proportions of soleus single muscle fibers. MHC isoforms are separated on SDS-PAGE (above) and proportions are shown in graph (below). Similar to MHC isoform proportions of homogenated muscle, most proportions are composed of MHC type I in all groups. (NC: Normal Control, DC: Denervation Control, DE: Denervation Exercise)

5) Western blot: Calpain-3, MuRF-1

The normalized values of calpain-3 of right gastrocnemius muscle in DC group was likely to be higher than other groups, but there were not significantly different among NC (2.84 ± 0.64), DC (3.87 ± 0.29) and DE (2.79 ± 1.00). There was no significant difference between NC group and normal exercise group (left gastrocnemius) (2.19 ± 0.53). There was significant difference only between DC group and normal exercise group (left gastrocnemius) (p=0.003). In soleus muscle there were no significant differences among all groups (p=0.466) (Fig.15-B). These findings suggest that exercise had a little effect on regulation of calpain-3 at the time of 4 weeks after partial denervation in gastrocnemius and soleus (Fig.14-A).

MuRF-1 expression of gastrocnemius and soleus were significantly higher in DC group than NC (gastrocnemius: 50.1 ± 5.6 vs 32.1 ± 8.5 9, p=0.002) (soleus: 58.1 ± 6.5 vs 32.9 ± 1.7, p=0.000), which means that denervation induced up-regulation of MuRF-1. In gastrocnemius exercise after denervation showed a tendency to attenuate the expression of MuRF-1 but there was no statistical significance between DC group (50.1 ± 5.6) and DE group (41.0 ± 3.8) and significant difference between NC group and normal exercise group (46.8 ± 4.5) (p=0.013). On the contrary exercise in contralateral normal muscle seemed to induce up regulation of MuRF-1. In soleus MuRF-1 expression of DE group and normal exercise group (38.3 ± 5.6) were significantly lower than that of DC group (Fig. 14-B). These findings suggest that in the soleus the treadmill exercise after partial denervation had an effect of

suppressing muscle proteolysis involved in muscle atrophy and exercise itself had little effect on MuRF-1 expression in contralateral normal muscle.

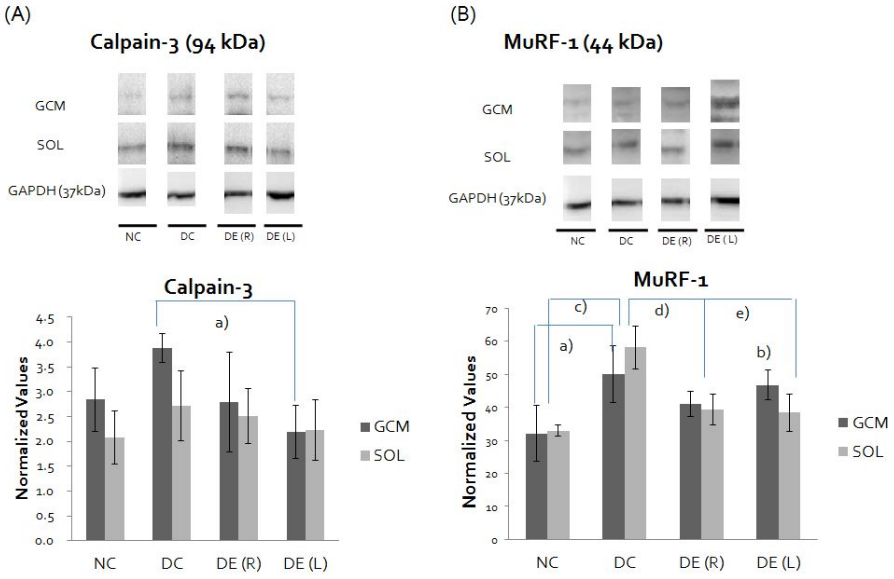

Figure 14. (A) The bands of Calpain-3 (94kDa) are shown in the western blot. The graph of normalized values to GAPDH shows no statistically significant differences among NC, DC and DE in both gastrocnemius and soleus. There is statistical significant difference a): between DC and DE (L). (B) The bands of MuRF-1 (44kDa) are shown in the western blot. The graph of normalized values to GAPDH shows up-regulation of MuRF-1 after denervation and down-regulation in exercise after denervation in soleus and up-regulation after denervation and the tendency of down-regulation in gastrocnemius. Values are presented as the mean ± standard deviations. Protein expression was quantified by densitometry and normalized to GAPDH. There is statistical significant difference a): between NC and DC, b): NC and DE (L), c):NC and DC, d):DC and DE (L), e):DC and DE (L). (NC: Normal Control, DC: Denervation Control, DE(R): Denervation Exercise (Right side; denervated side), DE(L): Denervation Exericse (Left side; non-denervated normal side))

Discussion

This study was performed to identify the effects of treadmill resistance exercise on atrophied skeletal muscles after denervation in aged rats. MWW and FCSA were significantly decreased after denervation however there were no significant differences between DC and DE, which suggest exercise after partial denervation had no effects on the MWW and FCSA. MHC isoform compositions in gastrocnemius were significantly different in both DC and DE in comparison with NC and more type I and IIx, with relatively slow twitch characteristics, in both denervtion groups than NC. There was also no significant difference between DC and DE. The proportion of hybrid fiber (IIx/IIb), characteristics of tonic contraction, of single muscle fiber in gastrocnemius was greater in DE and the proportion of another hybrid fiber (IIx/I), characteristics of tonic contraction was greater in DC. Treadmill resistance exercise would have an influence muscle plasticity caused by reinnervation without gross change after partial denervation. In soleus, there were no differences of the proportions of MHC isoforms among all groups. Calpain-3 expressions in gastrocnemius and soleus were not significantly different among all groups but with a little tendency to increase after denervation and decrease after exercise. MuRF-1 expressions were higher, which suggest to accelerate protein degradation, after denervation and lower, which suggest to attenuate protein degradation, after exercise following denervation in only soleus, as expected.

If the situation of partial denervation occurs in the elderly, the

combination of denervation and aging would have more influence on muscle atrophy than young age because of additional effect of sarcopenia. There are conflicting reports concerning the degree of denervation-induced atrophy as a function of age, with some studies reporting that the degree of atrophy is larger, similar, or even attenuated with age.(37) Recent studies reported similar time course of decreased muscle mass between young adult and aged rats after complete denervation in soleus muscle(37) and gastrocnemius muscle.(38) Although there are still controversies about the effect of aging on denervation, loss of muscle mass called as sarcopenia is evident and its effects on muscle power and motor function can not be neglected. Therefore, exercise intervention which is believed to be effective for both sarcopenic and denervated muscles is essential to maintain muscle mass or to prevent the progression of muscle atrophy. At 4 weeks after denervation, treadmill exercise was proved to be effective for suppression of proteolysis process in soleus and possibly in gastrocnemius and it made significant hybrid isoform changes of single muscle fiber indicating muscle fiber plasticity in gastrocnemius. After denervation, muscle wet weight (MWW) and fiber cross sectional area (FCSA) were significantly lower than NC, therefore muscle atrophy was identified. However exercise after partial denervation had no effects on the MWW and FCSA , which was similar in young rats after partial denervation.(39) The findings on molecular level were not reflected to the gross changes such as muscle wet weight and fiber cross sectional area. Therefore we suggested the process of exercise

induced beneficial effects (the attenuation of the process of muscle atrophy and restoration of muscle fiber type composition) occurs slowly and exercise duration of two weeks may be not enough to overpass the natural reinnervation after partial denervation.

This study found MuRF-1 up-regulation after denervation in gastrocnemius and soleus, which is consistent with the known reports. Moreover in exercise after denervation group MuRF-1 expression was significantly lower in soleus and had a tendency to be lower in gastrocnemius, which was expected. However MuRF-1 expression of gastrocnemius was higher in normal exercise (left gastrocnemius, not denervated, in DE group) than in NC group, which suggests the possibility that moderated intensity resistance exercise in normal aged rat can induce proteolysis in type II dominant and fast twitch gastrocnemius. This can be caused by an effect of eccentric exercise or by exercised induced increase of remodeling of type II fibers, which is known to be much decreased in sarcopenia. MuRF-1 is one of representative atrogenes involving UPS (ubiquitin-proteasome system) as noted in the introduction and may target MHC related proteins to affect overall protein degradation and atrophy. The involvement of the UPS in human muscle wasting was documented in a number of noninflammatory (spinal cord injury, immobilization) and inflammatory conditions (cancer, sepsis, chronic obstructive pulmonary disease, amyotrophic lateral sclerosis).(40) MuRF-1 is increasingly being implicated in muscle remodelling. Denervation causes a breakdown of myofibrillar proteins and MuRF-1 is reported to be up-regulated in many situations such as spinal cord injury, denervation and motor neuron diseases in human and rats.(4) Sarcopenia, age-related muscle atrophy, is linked to elevated rates of protein degradation, reduced regenerative capacity of satellite cells and inefficient activation of protein synthesis pathways. However in animal and

human studies atrogene including MuRF-1 and atrogin-1 expression was reported to be controversial. Most studies reported an increase of MuRF-1 and not consistent results of atrogin-1.(41) However there is no reports about the treadmill resistance exercise effects, especially of atrogenes, on muscle atrophy in aged denervated situation. The effect of exercise in muscle atrophy such as denervation,(42) hindlimb suspension(43) and congestive heart failure(44) is reported to be beneficial and induced suppression of MuRF-1 expression, which was up-regulated in the situation of muscle atrophy. Exercise in unaccustomed healthy condition, such as resistance exercise and eccentric exercise, is known to induce MuRF-1 up-regulation and controversies of atrogin-1 up-regulation, which is suggested that UPS including MuRF-1 involved in muscle remodeling after muscle damage from intense eccentric exercise and increase in muscle protein breakdown simultaneously with more enhanced muscle protein synthesis leading to an overall positive accumulation of muscle protein contents, that is imbalance between anabolic and catabolic process, after resistance exercise.(45) Following a single bout of resistance exercise, for both adult and old subjects there was an increase in MuRF-1, but only the old subjects had an increase in atrogin-1.(46) These reports suggest MuRF-1 can be involved in the regulation of myofibril replacement and turnover. Recently a single bout of treadmill exercise induced autophasic related protein expression including MuRF-1 and the authors suggest a single bout of treadmill exercise attenuates the autophagic response, which are essential for myofiber maintenance and for the clearance of damaged proteins and altered organells, in murine skeletal muscle.(47)

The calpain-3 expression in aged rats after partial denervation was not significantly different among all groups in both gastrocnemius and

soleus despite some tendency of increase in DC in gastrocnemius. There was no significant difference between NC and normal exercise (non-denervated limb in DE group) and treadmill resistance exercise had little effect on calpain-3 expression at 4weeks. These findings suggest that the repair and remodelling of damaged and degradated muscle fibers by exercise may be slow not to represent at 4weeks after injury and moderate exercise did not induce muscle damage to need repair and remodelling process. Calpain-3 activation was reported to the result of the small, yet prolonged increased cytoplasmic calcium ion following eccentric exercise and more calpain-3 present in fast-twitch fiber dominant muscles such as extensor digitorum longus than slow-twitch fiber dominant muscle such as soleus. The role of calpain-3 was suggested as muscle repair and regeneration and sarcomere remodelling.(48) In young partial denervation rats the mRNA of calpain-3 was reported to be down-regulated and recovered after 2 weeks from injury(49) and exercise after partial denervation had a tendency to induce down-regulation of calpain-3 in gastrocnemius, but there was no significant difference as similar to this study.(39)

The MHC isoform analysis of homogenated gastrocnemius muscle revealed more type IIx than type IIb in NC, which is different from the results of young rats(39) and it may be associated with more atrophies of type II fiber in sarcopenia as known.(41) The analysis of gastrocnemius had a tendency of higher proportions of type I and type IIx in both DC and DE than in NC group, which means that more type IIb and IIx become atrophied or some portions of type IIb or IIa

might be transformed to type I or IIa in denervation groups but exercise might not reverse the process after denervation at 4 weeks after injury. However in analysis of single muscle fiber more hybrid fiber of type IIx/IIb was found in exercise group and more hybrid fiber of type IIx/I was found in DC group and we think the changes of molecular level by exercise might have an influence of reinnervation of gastrocnemius microscopically. In soleus MHC isoform analysis of homogenate showed no significant changes among groups with a small tendency of increases of type IIa and IIx in soleus after denervation and analysis of single muscle fiber showed a tendency of more hybrid fiber of type IIa/I in exercise group than DC group, which seemed to be similar to NC. However, type I muscle fiber was mostly occupied with all components of isoforms, the proportions of hybrid fibers or type IIa was not significantly different. We think these findings may be a small tendency of reinnervation toward recovery also in soleus muscle. In young rats with partial denervation, the effect of treadmill exercise on muscle atrophy were the enlargement of the fiber cross sectional area of gastrocnemius, reverse of denervation-induced change of MHC isoform proportion (the tendency of increased type IIx and decreased type IIb) and change of the proportion of hybrid muscle fibers.(39)

This study has some limitations. At first, there was the small numbers of animals in each group to perform the statistical analysis more powerfully. Second, lack of serial follow-up should be considered to inverstigate the changes after denervation. If the additional analyses

were performed at 2 weeks after denervation we would more clearly suggest the changes of findings, especially the changes of MHC and calpain-3, after denervation and the exercise effects on denervation in aged rats. The further study with larger sample size and additional follow-up to investigate serial changes is necessary to cleary identify the effect of exercise in denervated skeletal muscle of aged rats. The third limitation is no direct comparison of our data with those of young rats, especially,MuRF-1, which has not be known whether there is any differences of MuRF-1 expression between aged rats and young rats or not.

Conclusion

In aged rats with atrophied skeletal muscle caused by partial denervation treadmill exercise induced the down-regulation of MuRF-1 in soleus muscle, which was up-regulated in denervation, and a small features of muscle plasticity in gastrocnemius with no definite morphologic changes such as muscle wet weight and fiber cross sectional area. We s suppose that treadmill resistance exercise after partial denervation in aged rats can attenuate the protein degradation only on the molecular level in type I dominant muscle without resultant gross findings, under the surface at 4 weeks after injury. Further studies with longer training duration and more subjects are necessary to identify gross changes related with the changes of molecular level following treadmill exercise in denervated old rats.

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초 록

서론: 노인에서의 탈신경화는 말초성 다발신경병증, 운동원성 질환과 척추 협착증을 포함한 신경근병증 등에서 흔하다. 노화에 따른 근감소증 외에 이러한 탈신경화에 의한 근위축에서 근육의 퇴행을 감소시키거나 회복시키는 중재 방법의 개발이 중요하다. 운동은 젊은 쥐 연구에서 탈신경화에 의한 골격근 위축을 감소시키는 것으로 보고되고 있다. 본 연구의 목적은 노화 쥐에서 트레드밀 운동이 탈신경화에 의한 골격근 위축에 대하여 어떤 영향을 미치는지 알아보고자 한다.

방법: 22월령의 수컷 노화쥐를 정상 대조군(n=7), 탈신경화 이후 운동군(n=8), 탈신경화 대조군(n=8) 등 3가지로 분류하였다. 탈신경화 모델은 우측 좌골 신경의 90초간 일시적 압박으로 부분적 탈신경화를 유발하였다. 탈신경화 운동군은 탈신경화 2주후부터 구심성 수축 운동과 편심성 수축 운동을 번갈아 시행하는 트레드밀 운동을 2주 동안 받았으며, 탈신경화 대조군은 탈신경화이후 운동을 받지 않았다. 탈신경화 4주후 비복근과 가자미근을 분리하여 근육 무게를 측정하고, 조직 표본에서 근섬유 단면적을 측정하였다. 단백질을 추출하여 SDS-PAGE를 통해 myosin heavy chain (MHC) isoform의 비율을 측정하고, 단일근섬유를 분리하여 각각의 MHC type을 분석한 뒤 hybrid 근섬유의 비율을 구하였다. Western blot으로 calpain-3와 MuRF-1 발현을 측정하였다.

결과: 탈신경화군에서 정상군과 비교시 근육 무게와 평균 근섬유 단

면적이 의미 있게 감소하였으나, 탈신경화 운동군과 탈신경화 대조군에서 의미 있는 차이는 관찰되지 않았다. 비복근에서의 MHC isoform 비율은 탈신경화군이 정상대조군에 비하여 type I과 IIx 비율이 더 높았지만, 탈신경화 운동군과 탈신경화 대조군사이에 차이가 없었으며, 가지미근에서는 3군 사이에 차이가 없었다. calpain-3 발현은 비복근과 가자미근에서 3군 사이에 차이가 없었으며 MuRF-1 발현은 탈신경후 비복근과 가자미근에서 대조군에 비하여 증가된 상태이며 가자미 근에서는 운동 후 감소하였다. 운동에 의한 효과는 가자미 근에서 MuRF-1이 운동한 정상 근육에서 정상대조군보다 더 높게 나탔으며 비복근에선 차이가 없었다.

결론: 부분적 탈신경화에 의한 골격근 위축이 나타난 노화 쥐에서 2주간의 트레드밀 운동은 탈신경화 대조군에 비하여 근육 무게와 근섬유 단면적 등 형태적 차이는 뚜렷하지 않았지만 탈신경화 이후 증가한 MuRF-1의 감소와 근육 가소성의 일부 변화가 관찰되었다. 이러한 분자 단계의 변화와 형태적 변화의 연관성을 파악하기 위하여 장기간 훈련과 더 많은 개체수를 대상으로 한 추가 연구가 필요할 것으로 생각한다.

주요어: 탈신경화, 근위축, 노화, 운동, 근감소증

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