



Original Article

Low-intensity treadmill exercise enhances fast recovery from bupivacaine-induced muscle injury in rats

Kijeong Kim^a, Tae-Won Jun^b, Hong Kim^c, Chang-Ju Kim^d, Wook Song^{b,*}

^a School of Exercise and Sport Science, College of Natural Sciences, University of Ulsan, Ulsan, Korea

^b Health and Exercise Science Laboratory, Institute of Sports Science, Seoul National University, Seoul, Korea

^c Department of Oriental Sports Medicine, College of Health and Therapy, Daegu Haany University, Gyeongsan, Korea

^d Department of Physiology, College of Medicine, Kyung Hee University, Seoul, Korea

ARTICLE INFO

Article history:

Received 1 September 2013

Received in revised form

17 September 2013

Accepted 17 September 2013

Available online 1 October 2013

Keywords:

caspase-3

heat-shock protein 70

inducible nitric oxide synthase

muscle injury

treadmill exercise

ABSTRACT

Background: Although bupivacaine has been used to study muscle degeneration and regeneration, the potential enhancement of muscle injury by exercise has not been well examined. The purpose of this study was to determine whether low-intensity treadmill exercise enhances fast recovery from bupivacaine-induced muscle injury and to examine concomitant changes in heat-shock protein 70 (HSP70) expression during regeneration process.

Methods: In this study, Sprague-Dawley rats were randomly divided into the following four groups: the control group (CON), the sham group (SHAM), the injury group (INJ), and the injury and exercise group (EX) ($n = 14$ in each group). Expressions of HSP70, inducible nitric oxide synthase (iNOS), and caspase-3 were determined at 1 and 7 days after bupivacaine-induced muscle injury in gastrocnemius.

Results: Results showed that bupivacaine-induced muscle injury (1 day) significantly increased the expressions of HSP70 and iNOS. At 7 days after the muscle injury, HSP70 expression was higher in the EX group compared with that in the INJ group and elevated level of HSP70 by exercise is concomitant with downregulation of iNOS and the decreased number of caspase-3-positive cells as a marker of apoptosis. Fewer necrosis of myofibers were also found in the EX group compared with the INJ group.

Conclusion: Our results suggest that low-intensity treadmill exercise may enhance fast recovery from bupivacaine-induced muscle injury in rat partly by HSP70 upregulation.

© 2013 Korea Institute of Oriental Medicine. Published by Elsevier. This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding author. Health and Exercise Science Laboratory, Institute of Sports Science, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-742, Korea.

E-mail address: songw3@snu.ac.kr (W. Song).

<http://dx.doi.org/10.1016/j.imr.2013.09.005>

2213-4220/© 2013 Korea Institute of Oriental Medicine. Published by Elsevier. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

For the study of muscle injury and its mechanisms, denervation, ischemia, and injection of neuromyotoxic agents have been adopted as indirect methods.^{1,2} Among them, local bupivacaine has been widely used to examine skeletal muscle degeneration and regeneration because even a single injection of this agent can rapidly induce necrosis in muscle fibers.¹ It thus appears that a bupivacaine injection is a suitable method to study histological and cytochemical changes associated with muscle injury.^{3,4}

Previous studies on the recovery process of muscle injury have been focused on the structural changes,^{5–7} whereas recent studies have focused on the identification and characterization of stress proteins, which play a pivotal role in cellular responses of the regeneration process. Recently, growing evidence showed that heat-shock proteins (HSPs) are involved in the recovery process of injured skeletal muscle.^{8–10} Because these molecular chaperones play a pivotal role in cytoprotection,^{11,12} HSPs are thought to be as one of the most important molecular factors in skeletal muscles response to injuries and subsequent regeneration.

Among the HSPs family, HSP70 is considered to be one of the most responsive molecular chaperones that plays a role in repairing folded peptides during physiological challenges in skeletal muscles.^{13,14} Recent reports showed that HSP70 has the capacity to suppress the injury process and apoptosis in response to a variety of stimuli including heat, DNA damage, and death receptor ligation,¹⁵ suggesting the potential survival-promoting effects of HSP70. Regarding the expression of HSP70 in skeletal muscle injury, previous studies reported a potential protective role of HSP70 in skeletal muscle injury models including ischemia reperfusion,⁸ denervation,¹⁰ and eccentric contraction.⁹ A recent study also strongly supported this notion by demonstrating that overexpression of HSP70 prevented the specific force deficit and protected against muscle damage.¹⁶ However, previous studies reported inconsistent expressions of HSP70 in the recovery process from muscle injury, and the reasons for such differences are mainly due to different muscle injury models, type of muscle fiber, and duration of injury period. Furthermore, as far as bupivacaine-induced muscle injury is concerned, currently there is no report demonstrating the potential protective role of HSP70 expression.

Skeletal muscle has a capability to adapt to a variety of stresses including contractile activity through induction of cytoprotective proteins such as HSP70. It is relatively well documented that exercise substantially induces production of HSP70 in the skeletal muscle and it may provide an insight into the underlying mechanisms by which regular exercise can protect against related and not-related stressors including muscle injury.^{17,18} Nonetheless, the effect of exercise-induced HSP70 expression on skeletal muscle injury and recovery has not been studied yet. Thus, in this study, the effect of low-intensity treadmill exercise on bupivacaine-induced muscle injury and the potential role of HSP70 expression in the recovery process were investigated.

2. Methods

2.1. Animal model

A total of 56 male Sprague-Dawley rats, weighing 301.7 ± 3.4 g, were obtained from a commercial breeder (Orient Co., Seoul, Korea) for the experiments. All surgery and experimental procedures were performed in accordance with the animal care guidelines of the National Institutes of Health and the Korean Academy of Medical Sciences. The rats were housed in standard stainless steel wire-mesh cages in a room with a 12-hour light/dark cycle (light period: 7:00 AM–7:00 PM; temperature: 20 ± 2 °C). All animals had *ad libitum* access to food (rat chow) and water.

2.2. Experimental design

The animals were randomly divided into the following four groups: the control group (CON), the sham group (SHAM), the injury group (INJ), and the injury and exercise group (EX) ($n = 14$ in each group). The rats in the INJ group received bupivacaine into the left gastrocnemius muscle, whereas the rats in the SHAM group received an equivalent amount of NaCl.

Six hours after bupivacaine injection, two animals from each of the CON, SHAM, and INJ groups were killed to determine the level of tumor necrosis factor- α (TNF- α) as a marker of inflammation using reverse transcription-polymerase chain reaction (RT-PCR). Half of the animals in each group ($n = 6$) were killed at 24 hours after receiving a bupivacaine injection, whereas the others in each group ($n = 6$) were killed at 7 days after receiving a bupivacaine injection. Immediately after killing, the left hind limb was shaved and gastrocnemius muscles were carefully dissected. For the histological and immunohistochemical examinations, some parts of the gastrocnemius muscles were fixed in O.C.T. Compound (Sakura, Japan) and quickly frozen at -70 °C until analysis.

2.3. Muscle injury induced by bupivacaine injection

The animals were anesthetized by inhalation of diethyl ether, following which 0.1 mL of 1% bupivacaine hydrochloride prepared in 0.9% saline solution was injected six times into the left gastrocnemius muscles using a syringe equipped with a 26G needle (total injection volume: 0.6 mL).¹⁹ The sham group received 0.1 mL of 0.9% saline solution six times (total injection volume: 0.6 mL). The rats were allowed to recover in their cages in a warm environment.

2.4. Exercise protocol

The rats in EX were forced to walk 24 hours after receiving the bupivacaine injection on a motor-driven treadmill. Exercise was performed for 30 minutes either one time (1 day) only or once daily for 7 consecutive days to compare the acute effect and relatively short-term training effect. The exercise load for the EX consisted of walking at a speed of 8 m/minute, at 0 degree of inclination. This exercise regimen is a low-intensity treadmill exercise.²⁰ Electric shock to stimulate the animals to run was not used; however, uncooperative rats

were encouraged to run by occasional gentle manual brushing on their backs.

2.5. RNA isolation and RT-PCR

To identify expressions of TNF- α messenger RNA (mRNA), RT-PCR was performed. Total RNA was isolated from the gastrocnemius muscle using RNAzol B (TEL-TEST, Friendswood, TX, USA). Approximately 2 μ g of RNA and 2 μ L of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65 °C for 10 minutes. Approximately 1 μ L of avian myeloblastosis virus (AMV) RT (Promega), 5 μ L of 10 mM deoxyribonucleotide triphosphate (dNTP; Promega), 1 μ L of RNasin (Promega), and 5 μ L of 10 \times AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 μ L with diethyl pyrocarbonate-treated water. The reaction mixture was then incubated at 42 °C for 1 hour. PCR amplification was performed in a reaction volume of 40 μ L containing 1 μ L of the appropriate complementary DNA, 1 μ L of each set of primers at a concentration of 10 pM, 4 μ L of 10 \times RT buffer, 1 μ L of 2.5 mM dNTP, and 2 units of Taq DNA polymerase (Takara, Shiga, Japan). For rat TNF- α , the primer sequences were 5'-TAC TGA ACT TCG GGG TGA TT-3' (a 20-mer sense oligonucleotide) and 5'-CAG CCT TCT CCC TTG AAG AG-3' (a 20-mer antisense oligonucleotide). For glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the internal control used in the study, the primer sequences were 5'-TGG TGC TGA GTA TGT CGT CC-3' (a 20-mer sense oligonucleotide) and 5'-TTG TCA TTG AGA GCA ATG CC-3' (a 20-mer antisense oligonucleotide). The expected sizes of the PCR products were 295 bp for TNF- α and 650 bp for GAPDH. For TNF- α , the PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA). The final amount of RT-PCR product of the mRNA species was calculated densitometrically using Molecular Analyst version 1.4.1 (Bio-Rad, Hercules, CA, USA).

2.6. Hematoxylin and eosin staining

Approximately 10- μ m thick transverse sections were cut using a cryostat cooled to -20 °C and sections were stained with hematoxylin and eosin (HE) staining by routine protocol for general histological observation. In brief, the slides were dipped into Mayer's hematoxylin for 30 seconds, then rinsed with tap water until clear, and dipped in eosin for 30 seconds and again rinsed with water. The slides were air dried at room temperature and then they were dipped two times each in the following solutions: 95% ethanol, 100% ethanol, a solution of 50% ethanol and 50% xylene, and 100% xylene. The cover slips were finally mounted using Permount (Fisher Scientific, Fair Lawn, NJ, USA).

2.7. Protein isolation and Western blot analysis

Gastrocnemius muscles were homogenized with a lysis buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM magnesium chloride hexahydrate, 1 mM ethylene glycol-bis-(β -aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 2 μ g/mL

leupeptin, 1 μ g/mL pepstatin, 1 mM sodium orthovanadate, and 100 mM sodium fluoride. Cellular debris was removed by microcentrifuging at 14,000 rpm, followed by quick freezing of the supernatant. The protein content was determined by Bradford method. Thirty micrograms of protein were separated on sodium dodecyl sulfate-polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany) in a prechilled transfer buffer. The blots were blocked for 1 hour with 5% nonfat milk reagent dissolved in 1 \times Tris-buffered saline with 0.05% Tween-20 (TBST). Mouse anti-actin, rabbit anti-iNOS, and mouse anti-HSP70 antibodies (1:1000; Santa Cruz Biotech, CA, USA) were used as primary antibodies. After further washing in TBST, horseradish peroxidase-conjugated anti-mouse antibody for actin and HSP70 and anti-rabbit antibody for inducible nitric oxide synthase (iNOS, 1:1000; Amersham Pharmacia Biotech GmbH, Freiburg, Germany) were used as the secondary antibody. The band detection was determined using enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech GmbH). To compare the relative expression of proteins, the detected bands were quantified by scanning densitometry (Gel Doc-2000, Bio-Rad, Hercules, CA, USA).

2.8. Caspase-3 immunohistochemistry

For visualization of caspase-3 expression, caspase-3 immunohistochemistry was performed as previously described.²¹ In brief, the sections were drawn from each muscle and incubated overnight on the gelatin-coated slides with mouse anti-caspase-3 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then for another 1 hour with biotinylated mouse secondary antibody. Bound secondary antibody was then amplified with Vector Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.02% 3,3'-diaminobenzidine. The slides were air dried overnight at room temperature, and cover slips were mounted using Permount (Fisher Scientific).

2.9. Data analysis

For HSP70 and iNOS, the percentage of difference in optical density scores was calculated according to a previous study.²² The number of caspase-3-positive cells was expressed as the mean number of cells per square millimeter of the cross-sectional area of the gastrocnemius muscle using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

All values were expressed as the means \pm standard error of the mean. For comparisons among groups, one-way analysis of variance with Duncan's *post hoc* test using the SPSS version 18.0 statistical package was used (SPSS Inc., Chicago, IL, USA). The differences were considered significant when $p < 0.05$.

3. Results

3.1. TNF- α mRNA expression in skeletal muscles

The RT-PCR analysis of the expression of TNF- α mRNA was performed to confirm whether bupivacaine injection induced

skeletal muscle injury through inflammatory processes or not. In the present results, the expression of TNF- α mRNA was markedly increased ($p < 0.05$) in the INJ group compared with the CON group (Fig. 1).

3.2. Morphological findings

Histological cross-sections of gastrocnemius muscles from 1 and 7 days after treatment with bupivacaine were stained with hematoxylin (blue) and eosin (purple) (Fig. 2). At 1 day after the muscle injury, the bupivacaine injection induced severe damage in gastrocnemius muscles, which is shown in both Fig. 2C and 2D, observed by the presence of hypercontracted fibers, inflammatory cell infiltration, and empty space between the cells, which clearly indicated myonecrosis, tissue disruption, and edema. Gastrocnemius muscles in the rats performing low-intensity treadmill exercise for 1 and 7 days following bupivacaine injection (Fig. 2D and 2H) demonstrated fewer necrosis of myofibers compared with rats in the INJ group (Fig. 2C and 2G). The gastrocnemius muscles in both the CON and SHAM groups did not show severe morphological alterations (Fig. 2A, 2E, 2B, and 2F).

3.3. HSP70 expression in gastrocnemius muscles

Western blot analysis of the expression of HSP70 protein was performed to provide an estimate of the relative levels of expression of this gene in each group (Fig. 3). The expression of HSP70 protein in CON was used as the control value of 1.00. At 1 day after the muscle injury, the expression of HSP70 protein was as follows: 1.00 (CON), 2.09 ± 0.22 (SHAM), 3.15 ± 0.42 (INJ), and 1.29 ± 0.31 (EX). The expression of HSP70 protein in the INJ group was significantly higher than that in the CON group ($p < 0.05$). Interestingly, the expression of HSP70 protein was significantly decreased ($p < 0.05$) in the EX group compared with the INJ group (Fig. 3A). At 7 days after the muscle injury, the expression of HSP70 protein is as follows: 1.00 (CON), 1.16 ± 0.02 (SHAM), 1.85 ± 0.14 (INJ), and 2.18 ± 0.27 (EX). The expressions of HSP70 protein in both the INJ and EX groups were significantly higher than that in the CON group ($p < 0.05$), whereas there was no difference between the INJ and EX groups (Fig. 3B).

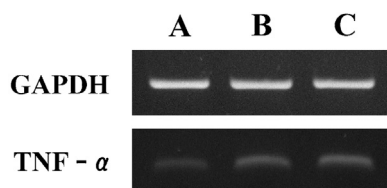


Fig. 1 – Tumor necrosis factor- α (TNF- α) messenger RNA (mRNA) expression in gastrocnemius after bupivacaine injection. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. Representative expressions of TNF- α mRNA and GAPDH mRNA in the gastrocnemius muscles after bupivacaine injection. (A) control group (CON); (B) sham group (SHAM); and (C) injury group (INJ).

3.4. Inducible nitric oxide synthase expression in gastrocnemius muscle

Western blot analysis of the expression of iNOS protein was performed to provide an estimate of the relative level of expression of this gene in each group. In the present study, the expression of iNOS protein (130 kDa) in the CON group was used as the control value of 1.00. At 1 day after the muscle injury, the expression of iNOS protein is as follows: 1.00 (CON), 1.49 ± 0.09 (SHAM), 2.60 ± 0.25 (INJ), and 1.34 ± 0.13 (EX) (Fig. 4). The expression of iNOS in the INJ group was significantly higher than that in the CON group ($p < 0.05$). However, the expression of iNOS was markedly decreased in the EX group ($p < 0.05$) compared with the INJ group. At 7 days after the muscle injury, iNOS protein expression in all groups was not significantly different (Fig. 4).

3.5. Caspase-3 expression in skeletal muscle

At 1 day after the muscle injury, the expression of any caspase-3 expression was not observed (data not shown). At 7 days after the muscle injury, the number of caspase-3-positive cells is as follows: $23.00 \pm 5.01/\text{mm}^2$ (CON), $32.25 \pm 4.78/\text{mm}^2$ (SHAM), $106.20 \pm 11.23/\text{mm}^2$ (INJ), and $42.10 \pm 4.81/\text{mm}^2$ (EX) (Fig. 5). The expression of caspase-3 in the INJ group was significantly higher than that in the CON group ($p < 0.05$). However, caspase-3 expression was significantly decreased in the EX group ($p < 0.05$) compared with that in the INJ group (Fig. 5).

4. Discussion

A major finding of this study was that low-intensity treadmill exercise promotes fast muscle regeneration to recover from bupivacaine-induced muscle injury in rat gastrocnemius muscle and enhanced recovery is accompanied by upregulation of HSP70.

In the present study, the six injections of 0.1 mL of 1% bupivacaine (total injection volume: 0.6 mL) induced muscle injury, which is shown by an increased expression of TNF- α mRNA in the gastrocnemius muscle 6 hours later (Fig. 1); it has been well documented that TNF- α plays a central role in cellular interactions during muscle damage.^{22,23} Moreover, based on the histological analysis of transverse sections of skeletal muscle, the hypercontracted myofibrils, inflammatory cell infiltration, and empty spaces between cells, indicating myonecrosis and edema, were clearly observed in the INJ group 1 day after bupivacaine injection (Fig. 2C), which was consistent with a previous report,² although those phenomena were a little mitigated in a week (Fig. 2G).

By contrast, low-intensity treadmill exercise reduced the incidences of necrotic myofibrils, and more intact cells remained in the EX group compared with the INJ group at 1 day (Fig. 2D) and 7 days (Fig. 2H) after the bupivacaine injection. These results are the first to demonstrate the possibility that low-intensity treadmill exercise may have a therapeutic effect on skeletal muscle injury during inflammatory phase. Although the precise mechanisms underlying exercise-induced protective effect on skeletal muscle injury remain unknown, several studies have suggested that the protective

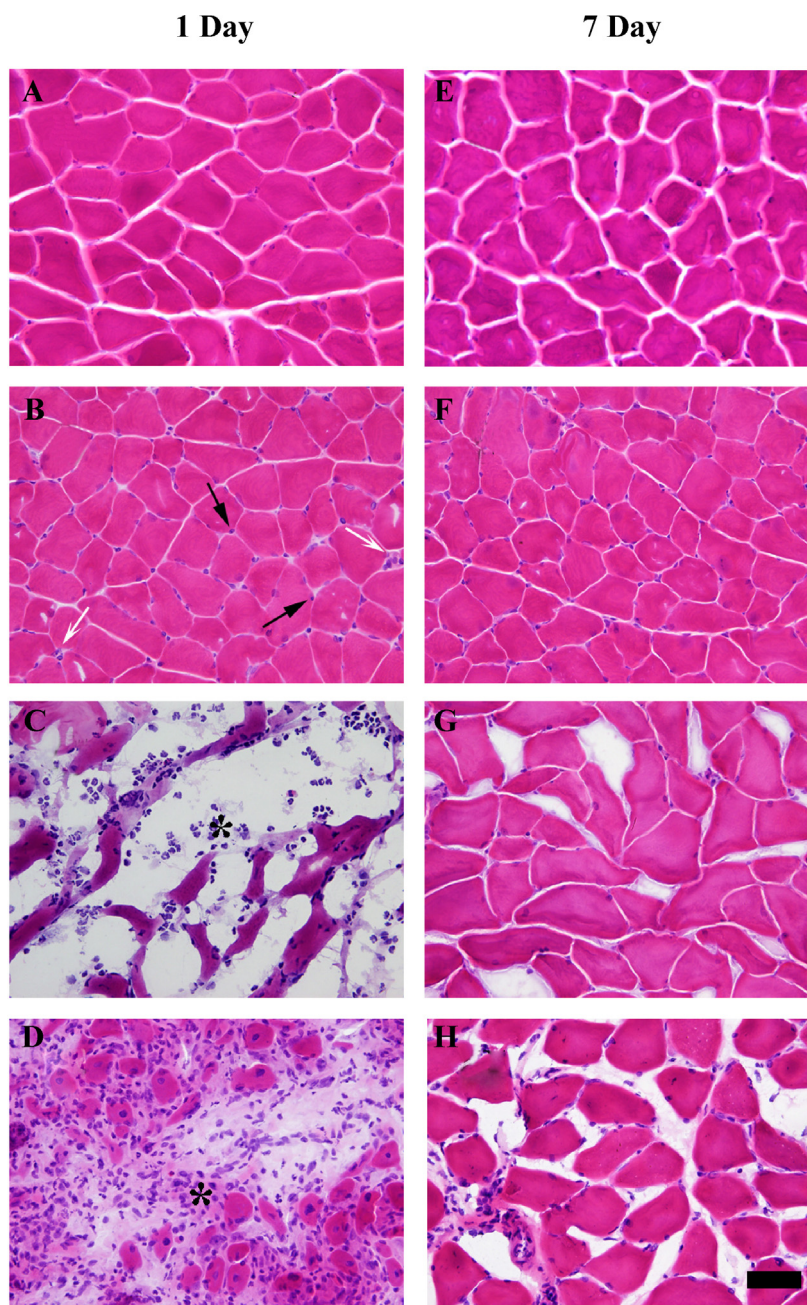


Fig. 2 – Photomicrographs of gastrocnemius muscles at 1 and 7 days after bupivacaine injection. Cross-sections of gastrocnemius muscles in each group were stained with hematoxylin (blue) and eosin (purple). The black arrows indicate nuclei, and white arrows indicate capillaries. Asterisks indicate inflammatory cell infiltration. (A and E): CON; (B and F): SHAM; (C and G) INJ; and (D and H) EX. The scale bar represents 25 μ m (original magnification: 400 \times).

effect of exercise is likely to be mediated by HSP70. HSP70 may mainly contribute to protect cellular function against a variety of stresses such as increased temperature, hypoxia, glucose deprivation, and cellular damage in skeletal muscle. Thus, we hypothesized that exercise would show some protective effect on skeletal muscle injury and enhance fast recovery from the injury during inflammation. Accordingly, our result showed that at 1 and 7 days after bupivacaine-induced muscle injury there was an increase in the expression of HSP70 compared with the CON group (Fig. 3A and 3B).

In general, it has been suggested that overexpression of HSP70 in skeletal muscles protects against muscle damage¹⁶ and that HSP has a complementary protection against oxidative stress induced by exercise.²⁴ However, the present results showed that highly expressed HSP70 was diminished by treadmill exercise at 1 day after skeletal muscle injury (Fig. 3A). One of the possible reasons for this discrepancy is the different experimental design. Most studies focused on the effect of exercise on HSP70 expression under normal skeletal muscles, whereas our study dealt with the effect of exercise under

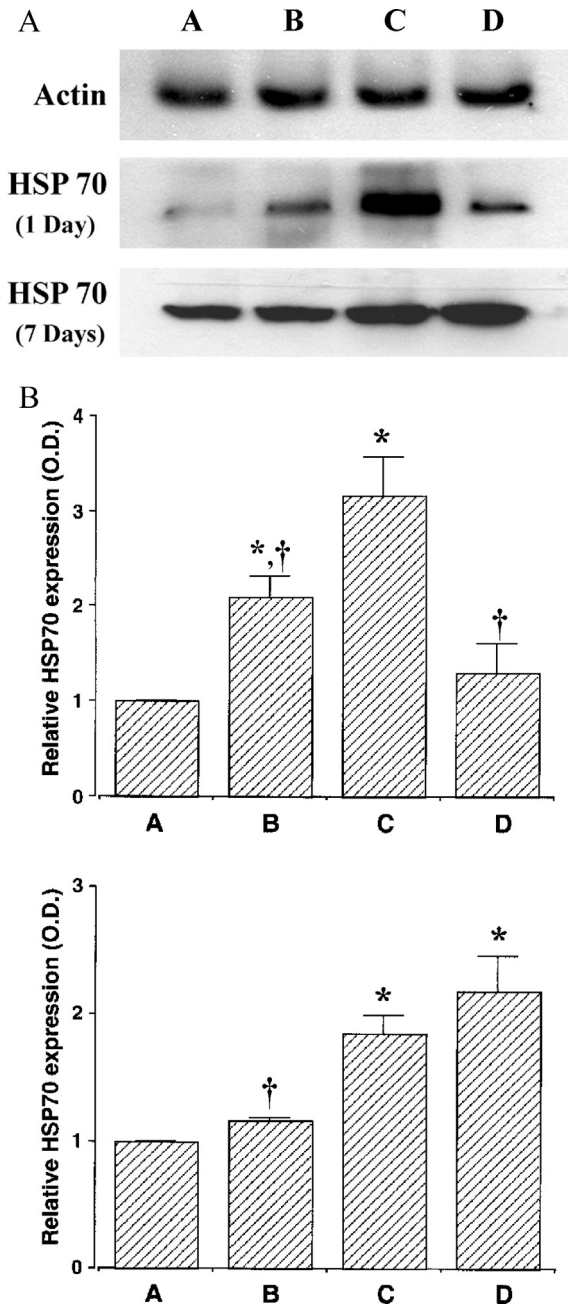


Fig. 3 – Changes in heat-shock protein 70 (HSP70) expression at 1 day (A) and 7 days (B) after the muscle injury. Equal amounts of proteins were electrophoresed and blots were probed with specific antibodies against HSP70 and actin. The representative Western blot photographs are shown. Images A and B indicate HSP70 expression at 1 and 7 days after the muscle injury, respectively. (A) CON; (B) SHAM; (C) INJ; and (D) EX. * $p < 0.05$ compared with CON. † $p < 0.05$ compared with INJ.

the muscle injury condition where HSP70 could be compensated by inflammation as indicated by a significant increase of iNOS expression (Fig. 4). Furthermore, a previous study showed that early exercise induced more rapid and intensive capillary growth in the injured area, better regeneration of

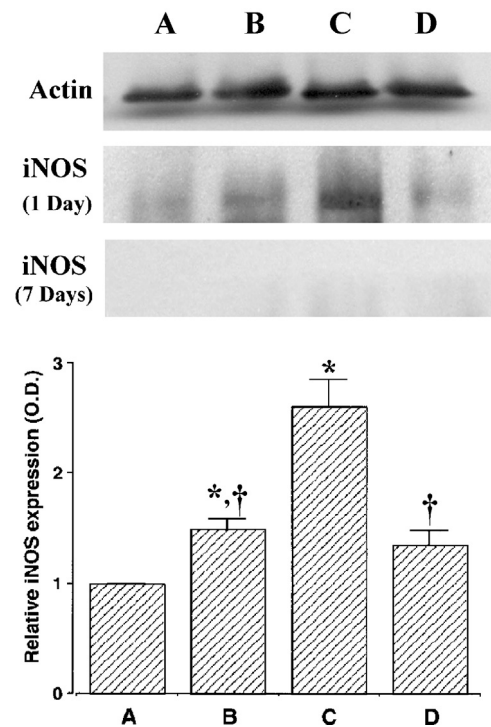


Fig. 4 – Changes in iNOS expression at 1 and 7 days after the muscle injury. Equal amounts of proteins were electrophoresed and blots were probed with specific antibodies against iNOS and actin. The representative Western blot photographs are shown. Bar graph represents iNOS expression at 1 day after the muscle injury. (A) CON; (B) SHAM; (C) INJ; and (D) EX. * $p < 0.05$ compared with CON. † $p < 0.05$ compared with INJ. iNOS, inducible nitric oxide synthase.

muscle fiber, and more parallel orientation of the regenerating myofibrils.²⁵ Therefore, it can be speculated that our results demonstrating protective effect of exercise on muscle injury could be caused by other factors, not primarily mediated by HSP70 directly.

At 7 days after the muscle injury, there was no difference in HSP70 expression between the INJ and EX groups (Fig. 3B) and compared with the result of 1 day after the muscle injury, increased HSP70 expression in the EX group was comparable to that of the INJ group. This phenomenon can be ascribed partly to the adaptation of exercise as regular exercise is shown to increase the level of HSP70 and exerts a protective influence on skeletal muscle, which was injured by various stressors.^{17,18} In the same context, a recent study well documented the notion that overexpression of HSP70 in skeletal muscle protects against muscle damage¹⁶ and supports the current finding of this study that fast recovery from the bupivacaine-induced muscle injury is likely to be mediated partly by exercise-induced HSP70 expression.

The inducible isoform of NOS is known to produce large amounts of nitric oxide as a defense mechanism as it may play an important role in the response of the cell to inflammation caused by infection, tumor growth, and many diseases. Regarding the association of iNOS with skeletal muscle injury,

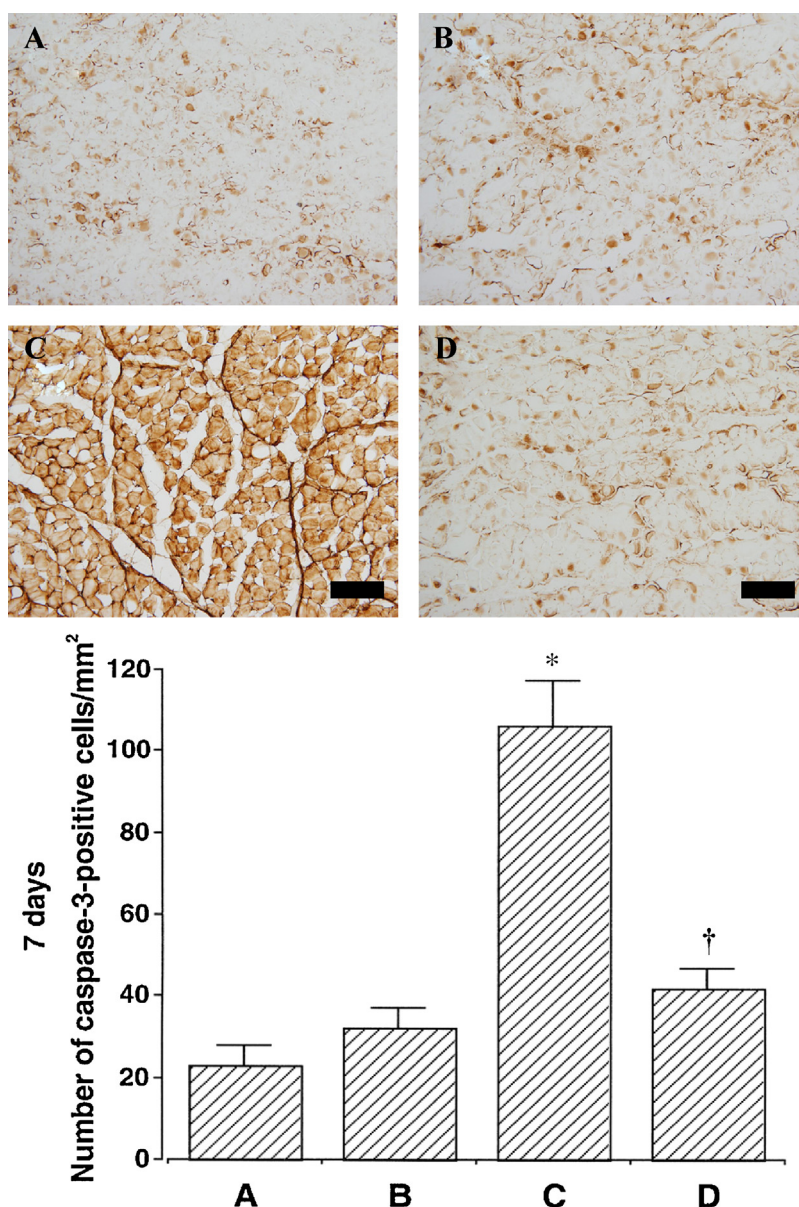


Fig. 5 – Caspase-3 expression at 7 days after the muscle injury. Caspase-3-positive cells in gastrocnemius muscles were stained and visualized by immunohistochemistry. Bar graph represents the number of caspase-3-positive cell indicating caspase-3 expression at 7 days after the muscle injury. (A) CON; (B) SHAM; (C) INJ; and (D) EX. * $p < 0.05$ compared with CON. † $p < 0.05$ compared with INJ. The scale bar represents 25 μm (original magnification: 400 \times).

reperfusion injury was shown to be alleviated in iNOS knockout mice whereas upregulation of iNOS exacerbated ischemia perfusion injury,¹⁴ suggesting a potential role of iNOS in skeletal muscle injury and recovery process. However, little is known about iNOS expression in the bupivacaine-induced muscle injury and the effect of exercise during recovery process. Interestingly, we found that bupivacaine-induced muscle injury increased the expression of iNOS, whereas enhanced expression of iNOS was reduced by treadmill exercise at 1 day after skeletal muscle injury (Fig. 4). A comparison of these results with previous reports supports the notion that iNOS appears to be a therapeutic target in protecting skeletal muscle against a certain type of injury. Further study investigating the upstream and downstream pathway of iNOS

in this regard would be required to elucidate the mechanisms by which skeletal muscle injury would be reduced and enhanced by low-intensity exercise by potentially exerting an anti-inflammatory effect, which has been well described in recent reviews.^{26,27}

After confirmation of the necrosis in the bupivacaine-induced muscle injury as shown in Fig. 2, it was determined subsequently whether apoptosis also occurred in this muscle injury model and low-intensity exercise possibly reduces apoptosis by measuring caspase-3 expression as a marker of apoptosis, as the activation of caspase-3 represents the consequence of a series of signaling events resulting from cell damage and is the culminating feature of a different apoptotic pathway. Caspase-3 immunohistochemical data showed that

at 7 days after the muscle injury, the expression of caspase-3 was significantly reduced in the EX group compared with that in the INJ group (Fig. 5), indicating potential antiapoptotic effect of low-intensity exercise in bupivacaine-induced muscle apoptosis. Although previous studies reported that apoptosis was induced in many different muscle injury models including crush injury,²⁸ pressure-induced injury,²⁹ burn injury,³⁰ and bupivacaine-induced injury,² our data was the first to show that low-intensity exercise has a potential to reduce muscle injury-induced apoptosis. The notion of the protective effect of physical exercise on skeletal muscle apoptotic process has been reported in aging,^{31,32} stress-induced, and disease models.³³ Many underlying mechanisms for this protective response have been proposed, including antiapoptotic protein expression, improved mitochondrial biogenesis, and reduced free radical generation³³; however, elucidation of these mechanisms would be an important theme of future study.

In conclusion, as a nonpharmacological intervention, low-intensity exercise and the associated upregulation of HSP70 is likely to provide potential protection against bupivacaine-induced muscle injury. Understanding the role of HSP70 in modulating cell-signaling pathways related to underlying mechanisms needs further investigation.

Conflicts of interest

There is no conflict of interest among authors.

Acknowledgments

This work was supported by a grant from National Research Foundation of Korea funded by the Korean Government (Grant No. NRF-2013S1A5A8023138).

REFERENCES

- Politi PK, Havaki S, Manta P, Lyritys G. Bupivacaine-induced regeneration of rat soleus muscle: ultrastructural and immunohistochemical aspects. *Ultrastruct Pathol* 2006;30:461–9.
- Zink W, Seif C, Bohl JR, Hacke N, Braun PM, Sinner B, et al. The acute myotoxic effects of bupivacaine and ropivacaine after continuous peripheral nerve blockades. *Anesth Analg* 2003;97:1173–9.
- Sakakima H, Kamizono T, Matsuda F, Izumo K, Ijiri K, Yoshida Y. Midkine and its receptor in regenerating rat skeletal muscle after bupivacaine injection. *Acta Histochem* 2006;108:357–64.
- Vignaud A, Hourd e C, Butler-Browne G, Ferry A. Differential recovery of neuromuscular function after nerve/muscle injury induced by crude venom from *Notechis scutatus*, cardiotoxin from *Naja atra* and bupivacaine treatments in mice. *Neurosci Res* 2007;58:317–23.
- Beitzel F, Gregorevic P, Ryall JG, Plant DR, Sillence MN, Lynch GS. Beta2-adrenoceptor agonist fenoterol enhances functional repair of regenerating rat skeletal muscle after injury. *J Appl Physiol* (1985) 2004;96:1385–92.
- Carlson BM, Shepard B, Komorowski TE. A histological study of local anesthetic-induced muscle degeneration and regeneration in the monkey. *J Orthop Res* 1990;8:485–94.
- Hwang JH, Ra YJ, Lee KM, Lee JY, Ghil SH. Therapeutic effect of passive mobilization exercise on improvement of muscle regeneration and prevention of fibrosis after laceration injury of rat. *Arch Phys Med Rehabil* 2006;87:20–6.
- Duguez S, Bihan MC, Gouttefangeas D, F easson L, Freyssenet D. Myogenic, nonmyogenic cells differentially express proteinases, Hsc/Hsp70, BAG-1 during skeletal muscle regeneration. *Am J. Physiol Endocrinol Metab* 2003;285: E206–15.
- Ingalls CP, Warren GL, Armstrong RB. Dissociation of force production from MHC and actin contents in muscles injured by eccentric contractions. *J Muscle Res Cell Motil* 1998;19:215–24.
- Tews DS, Goebel HH, Schneider I, Gunkel A, Stennert E, Neiss WF. Expression profile of stress proteins, intermediate filaments, and adhesion molecules in experimentally denervated and reinnervated rat facial muscle. *Exp Neurol* 1997;146:125–34.
- Benjamin IJ, McMillan DR. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. *Circ Res* 1998;83:117–32.
- Mestrlil R, Dillmann WH. Heat shock proteins and protection against myocardial ischemia. *J Mol Cell Cardiol* 1995;27:45–52.
- Pilon M, Schekman R. Protein translocation: how Hsp70 pulls it off. *Cell* 1999;97:679–82.
- Qi WN, Chen LE, Zhang L, Eu JP, Seaber AV, Urbaniak JR. Reperfusion injury in skeletal muscle is reduced in inducible nitric oxide synthase knockout mice. *J Appl Physiol* (1985) 2004;97:1323–8.
- Beere HM. The stress of dying”: the role of heat shock proteins in the regulation of apoptosis. *J Cell Sci* 2004;117:2641–51.
- McArdle A, Dillmann WH, Mestrlil R, Faulkner JA, Jackson MJ. Overexpression of HSP70 in mouse skeletal muscle protects against muscle damage and age-related muscle dysfunction. *FASEB J* 2004;18:355–7.
- Liu Y, Gampert L, Nething K, Steinacker JM. Response and function of skeletal muscle heat shock protein 70. *Front Biosci* 2006;11:2802–27.
- Morton JP, Kayani AC, McArdle A, Drust B. The exercise-induced stress response of skeletal muscle, with specific emphasis on humans. *Sports Med* 2009;39:643–62.
- Steer JH, Mastaglia FL, Papadimitriou JM, Van Bruggen I. Bupivacaine-induced muscle injury. The role of extracellular calcium. *J Neurol Sci* 1986;73:205–17.
- Kim YP, Kim HB, Jang MH, Lim BV, Kim YJ, Kim H, et al. Magnitude- and time-dependence of the effect of treadmill exercise on cell proliferation in the dentate gyrus of rats. *Int J Sports Med* 2003;24:114–7.
- Sim YJ, Kim SS, Kim JY, Shin MS, Kim CJ. Treadmill exercise improves short-term memory by suppressing ischemia-induced apoptosis of neuronal cells in gerbils. *Neurosci Lett* 2004;372:256–61.
- Armitage LL, Mohapel P, Jenkins EM, Hannesson DK, Corcoran ME. Dissociation between mossy fiber sprouting and rapid kindling with low-frequency stimulation of the amygdala. *Brain Res* 1998;781:37–44.
- Seekamp A, Warren JS, Remick DG, Till GO, Ward PA. Requirements for tumor necrosis factor-alpha and interleukin-1 in limb ischemia/reperfusion injury and associated lung injury. *Am J Pathol* 1993;143:453–63.
- Quadrilatero J, Alway SE, Dupont-Versteegden EE. Skeletal muscle apoptotic response to physical activity: potential mechanisms for protection. *Appl Physiol Nutr Metab* 2011;36:608–17.

25. Järvinen TA, Järvinen TL, Kääriäinen M, Kalimo H, Järvinen M, Muscle injuries: biology treatment, *Am J Sports Med* 2005;33:745–64.
26. Petersen AM, Pedersen BK. *The anti-inflammatory effect of exercise J Appl Physiol (1985)* 2005;98:1154–62.
27. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, et al. Position statement. *Part one: immune function and exercise Exerc Immunol Rev* 2011;17:6–63.
28. Stratos I, Li Z, Rotter R, Herlyn P, Mittlmeier T, Vollmar B. Inhibition of caspase mediated apoptosis restores muscle function after crush injury in rat skeletal muscle. *Apoptosis* 2012;17:269–77.
29. Siu PM, Tam EW, Teng BT, Pei XM, Ng JW, Benzie IF, et al. Muscle apoptosis is induced in pressure-induced deep tissue injury. *J Appl Physiol (1985)* 2009;107:1266–75.
30. Duan H, Chai J, Sheng Z, Yao Y, Yin H, Liang L, et al. Effect of burn injury on apoptosis and expression of apoptosis-related genes/proteins in skeletal muscles of rats. *Apoptosis* 2009;14:52–65.
31. Marzetti E, Lawler JM, Hiona A, Manini T, Seo AY, Leeuwenburgh C. Modulation of age-induced apoptotic signaling and cellular remodeling by exercise and calorie restriction in skeletal muscle. *Free Radic Biol Med* 2008;44:160–8.
32. Song W, Kwak HB, Lawler JM. Exercise training attenuates age-induced changes in apoptotic signaling in rat skeletal muscle. *Antioxid Redox Signal* 2006;8:517–28.
33. Smolka MB, Zoppi CC, Alves AA, Silveira LR, Marangoni S, Pereira-Da-Silva L, et al. HSP72 as a complementary protection against oxidative stress induced by exercise in the soleus muscle of rats. *Am J Physiol Regul Integr Comp Physiol* 2000;279:R1539–45.