Low-level laser irradiation promotes the recovery of atrophied gastrocnemius skeletal muscle in rat

Running Title:

Low-level laser irradiation in disuse muscle atrophy

Keyword:

Low-level laser; disuse muscle atrophy; satellite cell

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Abstract

Low-level laser (LLL) irradiation promotes proliferation of muscle satellite cells, angiogenesis and expression of growth factors.   Satellite cells, angiogenesis and growth factors play important roles in the regeneration of muscle.   The objective of this study was to examine the effect of LLL irradiation on rat gastrocnemius muscle recovering from disuse muscle atrophy.   Eight-week-old rats were subjected to hindlimb suspension for 2 weeks, after which they were released and recovered.   During the recovery period, rats underwent daily LLL irradiation (Ga-Al-As; 830 nm; 60 mW; total, 180 sec) to the right gastrocnemius muscle through the skin.   The untreated left gastrocnemius muscle served as the control.   In conjunction with LLL irradiation, 5-bromo-2'-deoxyuridine (BrdU) was injected subcutaneously for labeling of nuclei of proliferating cells.   After 2 weeks, myofiber diameters of irradiated muscle increased in comparison with those of untreated muscle, but did't recover back to normal levels. Additionally, in the superficial region of muscle, the number of capillaries and fibroblast growth factor levels in irradiated muscle exhibited meaningful elevation relative to those of untreated muscle.   In the deep region of muscle, BrdU-positive nuclei of satellite cells and / or myofibers of irradiated muscle increased significantly relative to that of the untreated muscle.   The results of this study suggested that LLL irradiation can promote recovery from disuse muscle atrophy in association with proliferation of satellite cells and angiogenesis.

Introduction

Skeletal muscle, which is a highly differentiated tissue comprised of multinucleated myofibers, exhibits a high capacity for morphological and functional adaptation. The number of myonuclei and the size of individual myofibers rapidly increase during postnatal growth as well as during periods of functional overload (Roy RR *et al.* 1999). On the other hand, myofiber atrophy and loss of myonuclei are induced during periods of decreased neuromuscular activity, e.g., in response to gravitational unloading during spaceflight, hindlimb unloading (Ohira Y *et al.* 1992) and prolonged bed rest (Ohira Y *et al.* 1999) in rats or humans. However, upon reloading, the muscle retains the ability to recover previously lost mass.

When skeletal muscle is challenged with increased mechanical (re)loading, various mechanisms are in operation. These mechanisms include activation of satellite cells (Grounds MD & Yablonka–Reuveni Z, 1993), expression of various growth factors (Adams GR *et al.* 1999) and angiogenesis (Hudlická O *et al.* 1992; Deveci D *et al.* 2002). Satellite cells, which are undifferentiated myogenic stem cells located between the myofiber plasma membrane and the basement membrane, are thought to serve as the source of new myofiber nuclei. The importance of satellite cells has been documented during normal muscle growth, regeneration, hypertrophy and recovery after atrophy (Grounds MD & Yablonka–Reuveni Z, 1993; Gallegly JC *et al.* 2004). Insulin-like growth factor I (IGF-I) plays a major role in skeletal muscle (re)growth in an autocrine / paracrine fashion (Adams GR *et al.* 1999). Downstream of IGF-I, the Akt pathway has been shown to be important in muscle (re)growth following atrophy (Rommel C *et al.* 2001). Fibroblast growth factor (FGF) functions as an activator and as a mediator of skeletal muscle hypertrophy (Mitchell P *et al.* 1999). Additionally, these growth factors accelerate the differentiation and proliferation of satellite cells and

induce angiogenesis in muscle (re)growth (Smith LE *et al.* 1999; Efthimiadou A *et al.* 2006). Angiogenesis is strongly correlated with muscle (re)growth (Hudlická O *et al.* 1992; Deveci D *et al.* 2002; Deveci D & Egginton S, 2002).

Low-level laser (LLL) irradiation modulates various processes in different biological systems. For example, in isolated mitochondria, He-Ne laser irradiation led to the elevation of membrane potential and ATP production (Passarella S *et al.* 1984), whereas in isolated fibroblasts following identical irradiation, an increase in collagen production was observed (Kovács IB *et al.* 1974). The effect of LLL irradiation on regeneration processes following trauma has been examined in muscle (Weiss N & Oron U, 1992; Bibikova A *et al.* 1994; Amaral AC *et al.* 2001), skin (Mester E *et al.* 1973) and the nervous system (Schwartz M *et al.* 1987). Furthermore, LLL irradiation has been shown to accelerate the healing process and growth of blood vessels in various tissues. Recent research has demonstrated the effect of LLL irradiation in detail. Saygun *et al.* (2008) revealed that LLL irradiation involving a diode laser (685 nm, 25 mW, 140 sec, 2.0 J / cm^2) elevates levels of basic FGF (bFGF) and IGF-I released by human fibroblast cells in vitro. A few reports documented bFGF induced by some type of LLL irradiation in vitro (Burd A *et al.* 2004; Golovneva ES, 2002; Poon VK *et al.* 2005). Moreover, Ben-Dov *et al.* (Ben-Dov N *et al.* 1999) and Shefer G *et al.* (Shefer G *et al.* 2001) suggested that LLL irradiation derived from a He-Ne laser (632.8 nm, 3 sec, 4.5 mW) induces muscle satellite cell proliferation in vitro.

The action of LLL irradiation and the mechanism of muscular re-growth in concert led us to hypothesize that LLL irradiation might be effective with respect to recovery from disuse muscle atrophy. If LLL irradiation can induce the release of bFGF and IGF-I, angiogenesis and muscle satellite cell differentiation and / or proliferation in muscle in vivo, then re-growth from disuse muscle atrophy would be accelerated. Therefore, this study examined the effects of LLL irradiation on muscle during recovery

from disuse muscle atrophy in a rat model.

Material and methods

Experimental procedure

Eight-week-old male Wistar rats were purchased from Kudo Laboratories, Japan and bred in our animal facility.   The rats, which were maintained in 30 x 40 x 20 cm cages, were exposed to a 12 : 12 h light-dark cycle with an ambient temperature of 24 °C. Food and water were available ad libitum.   In this investigation, 20 rats (220 ± 10 g) were utilized for experiments involving LLL irradiation, whereas one rat was employed in the pilot study for the evaluation of laser penetration.   Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

Disuse muscle atrophy was induced in ten rats via a hindlimb suspension (HS) method similar to that described by Morey (Morey ER *et al.* 1979); in addition, ten rats were maintained under normal conditions (control).   HS, which was initiated at the age of 8 weeks, was maintained for 2 weeks.   Briefly, rats were attached by the tail to a trolley system.   This apparatus permitted only the forelimbs to touch the cage floor; however, rats were able to rotate 360° to obtain food and water freely.   Upon completion of the 2-week HS, some HS-treated (HS group, n=5) and age-matched control animals (10-wk NC group, n=5) were sacrificed immediately for confirmation of disuse muscle atrophy.

The remaining HS-treated rats were released from the apparatus, after which they were allowed to recover for 2 weeks in a normal breeding environment (HS recovery group, n=5).   The remaining control rats were subsequently age-matched with the animals of the HS recovery group (12-wk NC group, n=5).   During the recovery period, all rats in the HS recovery and 12-wk NC groups received LLL irradiation daily

to the right gastrocnemius muscles (irradiated muscle).   Contralateral gastrocnemius muscles received identical handling and treatment, except that the placebo treatments were conducted with the laser device applied albeit in the un-switched position (untreated muscle).

LLL irradiation

In earlier studies regarding the biological action of LLL, the He-Ne laser (wavelength, 630 nm) was utilized frequently (Weiss N & Oron U, 1992; Ben-Dov N *et al*. 1999; Amaral AC *et al*. 2001; Schwartz M *et al*. 2008,); however, the low-level Ga-Al-As laser (wavelength, 830 nm; 60 mW; 0.3 cm^2 spot-size) was selected for the current investigation.   Ga-Al-As is not absorbed by water; as a result, it penetrates deep tissue (King PR, 1989).   A laser generated at around 850 nm can provide greater penetration depths in mammalian tissues than can a laser displaying a wavelength of approximately 630 nm (Wan S *et al*.1981; King PR, 1989).   We concluded that the Ga-Al-As laser is more suitable for muscular laser treatment than is the He-Ne laser in this study.

Prior to the initiation of the experiment and every day before irradiation of rats, the laser output was confirmed with a power monitor attached to the laser equipment. Additionally, using the same Ga-Al-As laser equipment and LLL monitor, a test was developed to evaluate laser penetration.   Briefly, one rat (10 weeks of age, male, 240 g) was anaesthetized with pentobarbital sodium (40 mg / Kg); subsequently, skin tissue of the facies posterior cruris and gastrocnemius muscle was removed.   The skin tissue and muscle were placed immediately between the laser probe and the laser sensor. Next, LLL irradiation was delivered under conditions identical to those of the experiment.

Prior to administration of LLL irradiation, rats were anaesthetized with

pentobarbital sodium (40 mg / Kg).   Knee and ankle joints were maintained in maximal

extension and maximal flexion, respectively.   LLL irradiation was applied daily to the

right gastrocnemius muscle at the same time on 14 consecutive days.   The laser beam

was applied directly to the previously shaved skin of the entire gastrocnemius muscle

region.   LLL irradiation (0.6 J / cm^2) of 3-second duration was repeated 60 times (total

of 180 sec); the irradiation site was changed each time.   Doses were selected based on a

previous study, which demonstrated that the most effective LLL irradiation with

respect to satellite cell proliferation in vitro involved doses of 3 sec (Ben-Dov N, *et al*.

1999); furthermore, 60 sites were necessary in order to cover the entire region of the

gastrocnemius muscle.   The beam incidence angle was maintained perpendicular (90°)

to the irradiation surface.   The procedure was performed in an identical manner on the

left side, but without the LLL irradiation emission.

Nuclear labeling

Mitotically active cells incorporate the thymidine analogue,

5'-bromo-2'-deoxyuridine (BrdU), into DNA.   Myonuclei, which are post-mitotic, do not

incorporate the BrdU label.   BrdU labeling has been shown to be a reliable technique

for distinguishing myonuclei from satellite cells (Carson JA & Always SE, 1996).   Rats

of the HS recovery and 12-wk NC groups received BrdU (Sigma, St. Louis, MO) (45 mg /

kg) via intraperitoneal injection 1 h prior to LLL irradiation.   BrdU injection was

performed each time before LLL irradiation treatment.

Tissue sampling and preparation

On the last day of the experimental periods, all rats were anaesthetized with

pentobarbital sodium (40 mg / Kg); subsequently, bilateral muscles of the gastrocnemius

medialis were extracted and weighed.   Proximal half of the muscles were embedded in

tragacanth gum, after which the samples were frozen in isopentane cooled by liquid nitrogen and stored in a deep freezer (–80 °C).   Serial frozen cross-sections of muscle, 7 μm in thickness, prepared on a cryostat were mounted on glass slides for histological and immunohistochemical analysis.   The distal half muscles were immediately cut into 50mg tissue samples comprised of each of the superficial and deep regions.   The superficial region of gastrocnemius muscle is organized with fast-twitch fibers almost exclusively; however, the deep region includes slow- and fast-twitch fibers (Mozdziak PE *et al.* 2001).   Tissues were homogenized in 0.01 M phosphate buffer (PBS, pH 7.4). Homogenates were centrifuged at 4 °C at 10,000 revolutions / second for 10 minutes; the supernatant solution were stored in a deep freezer (–80 °C).   Supernatant solutions were used for ELISA; the amount of protein in each supernatant of muscle was determined with a BCA Protein Assay Kit (Pierce).

Histochemical analysis of myofiber and capillary

Cross-sections were evaluated with an optical microscope linked to a video print system and a Macintosh personal computer.   Some cross-sections of muscle were stained for hematoxylin-eosin, myosin ATPase activity after acid pre-incubations (pH 4.3) and alkali phosphatase activity.   Hematoxylin-eosin staining identified myofiber morphological characteristics and signs of previous muscle injury such as centralized nuclei.   The myosin ATPase reaction served to identify myofiber type (Lind A & Kernell D, 1991).   Myofiber diameter was determined on at least 200 fibers per major fiber type in superficial (type IIb) and deep (type I, IIa and IIb) regions with an image analysis computer program (NIH Image software program).   The alkali phosphatase reaction served to visualize the location of capillaries employing an indoxyl-tetrazolium method (Ziada AM *et al*, 1984).   Capillary supply was evaluated as numerical density of caplillary and capillary-to-myofiber ratio (Deveci D & Egginton S, 2002).   In brief,

capillaries and myofibers were counted in five unbiased photographs (x100; 0.58 mm^2) covering the entire area of superficial and deep regions of the muscle.   In each photograph, the density and the capillary-to-myofiber ratio were given as the number of capillary per unit area (mm^{-2}) and the number of capillary per one muscle fiber respectively.

Immunohistochemical analysis of BrdU

Activated satellite cells and satellite cell-derived myonuclei exhibit incorporated BrdU in their DNA.   Fibroblasts and other mitotically active cells in the interstitium will also uptake the BrdU label; however, these cells would reside outside of the myofiber.   Next, the BrdU label was localized through double immunostaining with anti-BrdU (1:500 dilution, Santa Cruz Biotechnology) and anti-laminin (1:500 dilution, Progen Biotechnik) antibodies.   The anti-laminin antibody served to identify the basal lamina and to ascertain whether BrdU-positive nuclei were located within the basal lamina of the parent fiber.   BrdU-positive myonuclei and myofibers were counted involving five unbiased photographs (x100) covering the entire area of the superficial and deep regions of muscle; moreover, the number of BrdU-positive nuclei per 100 myofibers were calculated.

The immunostaining protocol was as follows.   Some cross-sections were air-dried and fixed in ice-cold ether for 10 min.   To inhibit endogenous peroxidase, these sections were then incubated with 0.3% H$_2$O$_2$ in methanol for 20 min at RT.   The sections were blocked with 10% bovine albumin in PBS for 20 min.   For the first immunostaining, the primary rabbit polyclonal anti-laminin antibody was applied to the sections overnight at 4 ºC.   The sections were rinsed in PBS for 15 min, after which biotinylated goat anti-rabbit IgG (1:500 dilution, Vector Lab.) was applied for 30 min at RT, followed by rinsing in PBS.   After rinsing in PBS, the sections were allowed to react with

avidin-biotin peroxidase complex (VECTASTAIN® Elite kit; Vector Lab.) for 30 min at RT. HRP-binding sites were visualized with 0.05% 3, 3'-diaminobenzidine (DAB) (the reaction products were dark brown) and 0.01% H_2O_2 in 0.5 mol / L Tris-HCl buffer at RT. Next, the sections were washed thoroughly in PBS and the second immunostaining was implemented. The sections were then treated with 1 N HCl for 60 min at RT for DNA denaturation, followed by washing in PBS. The primary mouse monoclonal anti-BrdU antibody was applied to the sections overnight at 4 °C. The sections were rinsed in PBS for 15 min, after which biotinylated goat anti-mouse IgG (1:500 dilution, Vector Lab.) was applied for 30 min at RT, followed by rinsing in PBS. Immunoreactivity was visualized with 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution (TrueBlue, KPL Inc.; the reaction products were blue).

Enzyme-linked immunosorbent assay for IGF-I and bFGF

The levels of IGF-I and bFGF in muscles were measured with enzyme-linked immunosorbent assay (ELISA) kits (Quantikine®, R&D System) according to the instructions of the manufacturer. Briefly, the supernatants of muscle were incubated on pre-coated microplates with IGF-I or bFGF for 2 h at RT. After incubation, microplates were washed and incubated with IGF-I or bFGF conjugated HRP for 2 h at RT. Subsequently, microplates were washed and incubated with substrate solution for 30 min at RT in the dark. The reaction was terminated upon the addition of sulfuric acid. Color development was monitored at 450 nm employing a microplate reader (Biotec, JAPAN); concentrations were calculated based on the standards curve, as per protein (pg / mg).

Statistical analysis

All data are presented as mean ± SD. Differences between groups were assessed

utilizing one-way analysis of variance (ANOVA) followed by Fisher's PLSD *post hoc* test.

Differences were considered significant at $p < 0.05$.

Results

Penetration of LLL

In a pilot study for the evaluation of laser irradiation with respect to laser penetration, a 60-mW Ga-Al-As laser was weakened to 20 mW and 5 mW by rat skin penetration and rat skin plus gastrocnemius muscle penetration, respectively (Fig.1). Thus, rat gastrocnemius muscle tissue was irradiated with about 20 mW in the superficial region and more than 5 mW in the deep region in this investigation. However, intravital blood flow cannot be ignored; in actuality, the irradiation might have been slightly weaker.

Muscle wet weight and myofiber diameter

The mean final body weight and the mean muscle wet weight of each group are presented in **table 1**.　The muscle wet weight of the HS group was significantly lower than that of the 10-wk NC group.　These data reveal that disuse muscle atrophy arose as a result of HS for 2 weeks.　In a comparison between untreated and irradiated muscle in the 12-wk NC group, the muscle wet weight of irradiated muscle exhibited a high level; however, the difference was not significant.　In the HS recovery group, the muscle wet weight of irradiated muscle displayed a high level, which differed significantly from that of untreated muscle.

Little myofiber necrosis was evident and tissue edema was not detected in muscle of all groups.　However, myofibers with central nuclei, which appeared to be regeneration fibers, were sometimes observed in bilateral muscles of the HS recovery group (data not shown).

In the superficial region of muscle, myofibers consisted of only type IIb fibers (Fig.2-A); furthermore, myofiber diameter of type IIb fibers in the HS group decreased

significantly in comparison with the 10-wk NC group (Fig.3-A).　The decreased

myofiber diameter in the HS recovery group persisted following a 2-week recovery from

HS.　Comparative evaluation of untreated and irradiated muscle in the HS recovery

group revealed that irradiated muscle was significantly greater (Fig.4-A).　No

difference was observed between untreated muscle and irradiated muscle in the 12-wk

NC group.　In contrast, type I, type IIa and type IIb fibers were detected in the deep

region of muscle (Fig.2-B).　In terms of myofiber diameter in the deep region of muscle,

the results of each fiber type were similar to the result for the type IIb fiber of the

superficial region of muscle (Fig.3-B, 4-B).

BrdU-labeled nuclei

　　　In the superficial region of muscle, a small number of BrdU-positive nuclei (Fig.5)

were observed in the 12-wk NC group; this value was less than three per 100 fibers.

The number of BrdU-positive nuclei in the HS recovery group exceeded that of the

12-wk NC group by more than two times.　However, no difference between untreated

muscle and irradiated muscle was apparent in either group (Fig.6-A).　In the deep

region of muscle, the number of BrdU-positive nuclei of untreated muscle was similar to

that of the superficial region of muscle.　In contrast, in irradiated muscle of the HS

recovery group, the number of BrdU-positive nuclei was significantly greater than that

of untreated muscle.　This tendency was observed in the 12-wk NC group as well;

however, it was not meaningful (Fig.6-B).

Capillary

　　　The capillary network in gastrocnemius muscle displayed more substantial

distribution over the deep region relative to the superficial region (Fig.7).　This

situation was similar in all groups.　In the HS group, the capillary-to-myofiber ratio in

both the superficial and deep regions in the HS group was lower than that of the age-matched control rats, however the density of capillary was not changed. In untreated the muscle, the capillary-to-myofiber ratio in both the superficial and deep regions in the HS recovery group were lower than that of the age-matched control rats.

In the superficial region, significantly higher densities and ratios were evident in irradiated muscle of the 12-wk NC and HS recovery groups, as compared to the untreated side in the same group. Additionally, the density of capillary in the irradiated muscle of the HS recovery group was higher rather than that of the untreated muscle of the 12-wk NC group. In the deep region, however, no difference was detected between the untreated and irradiated muscles in either group (Table 2).

Levels of bFGF and IGF-I

In the superficial region, bFGF levels increased markedly in irradiated muscle in comparison with untreated muscle in the 12-wk NC and HS recovery groups. In contrast, in the deep region, no difference was observed between the irradiated and untreated muscles in either group (Fig.8-A , -B). Greater IGF-I levels were detected in the HS recovery group relative to the 12-wk NC group; however, any difference attributable to laser irradiation was not disclosed (Fig.8-C, -D)

Discussion

Muscle atrophy induced by HS

HS induces hypokinesia and hypodynamia of the hindlimb muscles.   These conditions, especially hypodynamia, are thought to be important contributing factors with respect to the development of muscle atrophy.   In comparison to age-matched normal rats and the 10-wk NC group, the gastrocnemius muscle wet weight of the HS group was significantly lower.   The mean diameter of type IIb myofibers in the superficial region declined 26.9%, whereas the mean diameter of type I, type IIa and type IIb myofibers in the deep region decreased 23.5%, 23.9% and 22.4%, respectively. Muscle atrophy induced by HS in the HS group was clear; moreover, atrophic response was uniform in all myofiber types in both the superficial and the deep regions.

This study demonstrated an increase in the muscle wet weight and the diameter of all myofiber types in LLL irradiated muscle in comparison with untreated muscle in the HS recovery group.   These results indicated that LLL irradiation of atrophied muscle during the process of recovery accelerates muscle re-growth.   Although myofibers of the HS recovery group should have atrophied in response to acute hindlimb unloading for 2 weeks in a manner similar to that of the HS group, they gradually recovered when rats were maintained on a solid surface consistent with previous studies (Mozdziak PE *et al.* 2001; Ishihara A *et al.* 2004).   However, the mean diameter of all myofiber types in both superficial and deep regions in untreated muscle in the HS recovery group was less than that of the 12-wk NC group; thus, reloading for 2 weeks did not lead to complete recovery in the atrophied muscle.   We noted that LLL irradiation advanced recovery in muscle wet weight and myofiber size in irradiated muscle in the HS recovery group.   This effect was evident in all myofiber types in both superficial and deep regions; it was remarkable in the deep region in which the myofiber diameter

recovered to normal.

In contrast, despite the laser effects on recovering muscle per the above, no changes were apparent in the muscle wet weight and myofiber diameter of irradiated muscle in 12-wk NC rats.   Two possible explanations for the differing laser effect between the recovering and the normal muscle may be considered: Firstly, even if some type of event were occurring in muscle, the effect of LLL irradiation could be insufficient in terms of inducing histologic and morphologic changes in normal muscle. With respect to the assessment of the intervention effect per LLL irradiation or other stimulations, histological evidence may be more difficult to obtain in normal muscle than in atrophied muscle, especially in young animals (McNulty AL *et al.* 1992; Kasper CE, 1999).   Secondly, special events occurring in recovering atrophied muscle could be influenced by LLL irradiation; as a result, re-growth of myofibers may be promoted. The recovery of atrophied muscle involves various events, e.g., activation of satellite cells (Grounds MD & Yablonka–Reuveni Z, 1993; Gallegly JC *et al.* 2004), expression of growth factor (Adams GR *et al.* 1999) and angiogenesis (Hudlická O *et al.* 1992; Deveci D *et al.* 2002).   If these events are promoted by LLL irradiation, a distinct laser effect between a recovering atrophied muscle and a normal muscle might be explicable.

Proliferation of satellite cells / myoblasts by LLL

In skeletal muscle of rats and toads, He-Ne laser irradiation delivered to the injured site enhanced regeneration by two- and eight-fold, respectively, relative to non-irradiated controls (Weiss N & Oron U, 1992; Bibikova A & Oron U, 1993).   The injured site in LLL irradiation-treated animals features many young myofibers, which suggests that the major irradiation-responsive candidate is the satellite cell (Weiss N & Oron U, 1992).   This concept is supported by studies of the effect of LLL irradiation on primary rat satellite cell cultures, which revealed the induction of cell cycle regulatory

protein expression, increased satellite cell proliferation and inhibition of cell

differentiation (Ben-Dov N *et al.* 1999).   In the search for a mechanism via which LLL

irradiation manifests these cell responses, Shefer *et al.* (Shefer G *et al.* 2001)

demonstrated that LLL irradiation specifically activates the mitogen-activated protein

kinase / extracellular signal-regulated protein kinase (MAPK / ERK) pathway.

Additionally, they reported that LLL irradiation induces de novo protein synthesis by

modulating the key enzymes that regulate capped mRNAs translation (Shefer G *et al.*

2003).

In this study, the number of BrdU-positive nuclei was more than twice that

observed in the HS recovery group in comparison with the 12-wk NC group in the

superficial and deep regions of gastrocnemius muscle.   These results indicated that

satellite cells are activated, followed by differentiation to myoblasts, proliferation and

subsequent fusion with myofibers when atrophied muscle is reloaded during the

recovery period (Schmalbruch H & Hellhammer U, 1977; Gallegly JC *et al.* 2004).

BrdU-positive nuclei only in the deep region of irradiated muscle increased significantly

in comparison with untreated muscle in the HS recovery group.   This increase in the

number of BrdU-positive nuclei in irradiated muscle was not evident in normal muscle.

Thus, LLL irradiation may promote the proliferation of myoblasts, which likely arose

from satellite cells; furthermore, promotion of myoblast proliferation is reflective of the

hypertrophy detected in the irradiated muscle in the HS recovery group.

However, it is reasonable to question why myoblast proliferation in the deep

region of irradiated muscle is promoted by LLL irradiation.   One possibility is that the

intensity of the laser beam incident at the superficial and deep regions of the muscle

differed.   In the current investigation, the superficial and deep regions of the

gastrocnemius muscle were irradiated with intensities of approximately 20 mW and 5

mW, respectively (Fig.1).   No reports appear in the literature documenting the variable

reaction of satellite cells / myoblasts consequent to laser intensity.   However, the effect of He-Ne laser irradiation (632.8 nm, 4.5 mW, 1.8 mm beam diameter) at various irradiation times (2, 3, 5 and 10 sec) on satellite cell proliferation was examined (Ben-Dov *et al*. 1999). This result was that 3-sec irradiation was most effective, whereas 10-sec irradiation inhibited cell proliferation.   Similar results were observed in an in vivo study (Amaral *et al*. 2001), which demonstrated that He-Ne laser (632.8 nm, 2.6 mW) irradiation for 7 sec (2.6 J / cm^2) enhanced muscle regeneration, whereas irradiation for 22 sec (8.4 J / cm^2) and 67 sec (72 J / cm^2) was not effective.   Therefore, it is possible that the reaction of satellite cells / myoblasts differs due to different exposure doses of LLL; moreover, excessive irradiation might not influence satellite cells/ myoblasts in the superficial region of gastrocnemius muscle in this study.   Another possibility is a difference of the potential function of the satellite cell in the superficial and deep regions of the gastrocnemius muscle.   The satellite cells in fast or slow muscles are intrinsically different in regenerating muscle (Dolenc I *et al*. 1994). Indeed, the satellite cell isolated from a slow muscle proliferates more actively than the satellite cell from a fast muscle in vitro (Lagord C *et al*. 1998).   The superficial region of gastrocnemius muscle was simple fast muscle consisting of type IIb muscle fiber; whereas, the deep region was intermediate muscle containing type I muscle fiber.   The sensitivity or reaction pattern to LLL, therefore, might be different in these two gastrocnemius regions.

Angiogenesis induced by LLL

The number of capillaries is altered by adaptation to increases or decreases in muscle activity.   Chronic decreases in muscle activity due to HS induced a decrease in the capillary-to-fiber ratio in muscle (Saltin B & Gollnick PD, 1983), which is indicative of a reduction in the absolute number of capillaries.   Additionally, when muscle activity

is recovered via reloading after HS, angiogenesis occurs in conjunction with re-growth of myofibers (Desplanches D *et al.* 1987).

In the current investigation, the capillary-to-myofiber ratio in untreated muscle of the HS group decreased in comparison with normal muscle in both the superficial and deep regions. The density of capillary of the HS group was not changed compared with the age matched normal rats, but as for this result, it was thought as an effect of the decrease of muscle fiber diameter. The number of capillaries, which decrease as a consequence of HS, did not completely recover after 2 weeks of reloading in the HS recovery group. However, the capillary-to-myofiber ratio in irradiated muscle increased relative to untreated muscle in the superficial region; this change was observed in the HS recovery and the 12-wk NC groups. These results suggested that LLL irradiation induces angiogenesis in muscle, which is consistent with the findings of previous reports (Smith LE *et al.* 1999; Bibikova A *et al.* 1994; , Templeton GH *et al.* 1984).

Angiogenesis is strongly related to muscle (re)growth (Hudlická O *et al.* 1992; Deveci D *et al.* 2002; Deveci D & Egginton S, 2002). Angiogenesis in skeletal muscle is thought to occur in parallel with muscle (re)growth (Plyley MJ *et al.* 1998). Myofiber size may affect capillary growth; it is possible that angiogenesis influences myofiber hypertrophy adversely. Indeed, Tirziu *et al.* (2007) demonstrated that stimulation of vascular growth via injection of angiogenic growth factor in normal adult mouse heart leads to induction of myocardial hypertrophy. Deveci *et al.* (2002) showed that myofiber hypertrophy occurs in the presence of angiogenesis induced by chronic hypoxia. Additionally, enhancement of muscle regeneration effects, including myofiber re-growth, by LLL irradiation is well known, which effect is associated with angiogenesis promotion (Bibikova A *et al.* 1994). Therefore, in this investigation, angiogenesis induced by LLL irradiation might have contributed to the promotion of myofiber

re-growth in the superficial region of gastrocnemius muscle in the HS recovery group.

Expression of growth factors by LLL

Expression of IGF-I is induced by load; furthermore, IGF-I is a major factor in muscle re-growth during recovery from muscle atrophy.   IGF-I may enhance skeletal muscle growth in terms of both satellite cell proliferation (Schmalbruch H & Hellhammer U,1977) and myofiber hypertrophy secondary to changes in protein synthesis (Bark TH *et al.* 1998).   IGF-I levels of untreated muscle in the HS recovery group tended toward a larger increase in comparison to the 12-wk NC group, which may be a natural phenomenon.   We expected elevated IGF-I levels in irradiated muscle of the HS recovery group and that this situation would influence myofiber re-growth as Sygan *et al.* (2008) reported that LLL irradiation (685 nm, 25 mW, 2.0 J / cm^2) induces the release of IGF-I and bFGF in vitro.   However, an effect on IGF-I by LLL irradiation was not evident in this study; the reason remains unclear.   On the other hand, the increase in bFGF was detected in the superficial region of irradiated muscle in the HS recovery and the 12-wk NC groups.   The domain in which increased bFGF occurred is consistent with angiogenesis enhanced by laser radiation.   bFGF is a key regulator of angiogenesis; therefore, angiogenesis observed in irradiated muscle is associated with expression of bFGF in this investigation, which is in agreement with the findings of previous reports (Golovneva ES, 2002; Efthimiadou A *et al.* 2006).

Basic FGF also functions as a paracrine and as an autocrine regulator of satellite cell / myoblast activity (Hawke TJ & Garry DJ, 2001).   The increase in bFGF observed in the superficial region of irradiated muscle may elevate the number of BrdU-positive nuclei, and thus, proliferation of satellite cells/ myoblasts; however, this result was not evident in the HS recovery group.   bFGF operates as an accelerator in satellite cells / myoblasts in terms of proliferation; however, bFGF inhibits satellite cell differentiation

to myofiber (Haugk KL *et al.* 1995; Bark TH *et al.* 1998). Additionally, proliferation and differentiation of satellite cells / myoblasts are controlled by various factors, e.g., hepatocyto growth factor, IGF-II, transforming growth factor-β, interleukin-6 cytokines, platelet-derived growth factor and macrophages, which act as promoters or inhibitors, respectively (Hawke TJ & Garry DJ, 2001). We hypothesized that even if bFGF alone is increased by LLL irradiation, the fusion of satellite cells / myoblasts to myofibers or subsequent proliferation may not be promoted in vivo.

Although the BrdU incorporation and histological capillary measurements represent cumulative changes in the recovery muscles, the growth factor expression data was unique to the 2-week recovery point in the recovery point. Significant laser effects may have been missed at an earlier time point in the recovery period in this study.

Conclusion

Our results suggested that LLL irradiation could promote recovery from disuse muscle atrophy in rat. Additionally, the effects of LLL irradiation appear to differ in the deep and superficial regions of the muscle. The promotion of re-growth of muscle fiber in the deep and superficial regions might be related to the proliferation of muscle satellite cells and angiogenesis associated with bFGF, respectively. However, the difference with respect to the reaction may depend on the conditions of LLL irradiation. Future studies are necessary in order to examine the effect of LLL irradiation under various conditions and periods in various muscles.

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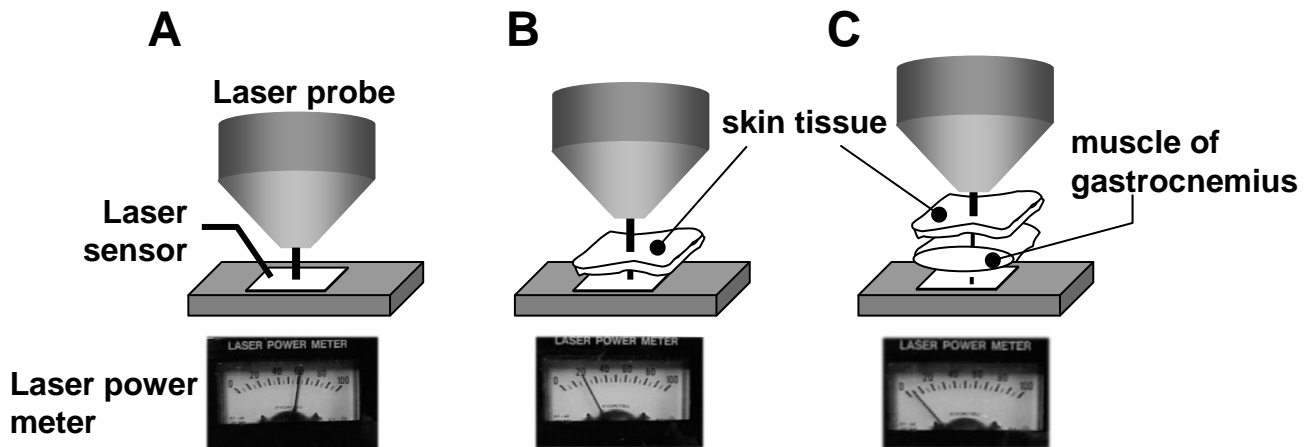
Fig.1. Pilot study: evaluation of laser irradiation on penetration. The power meter reading was 60 mW when the sensor was unobstructed (A). When skin tissue was placed immediately between the laser probe and the sensor, the power meter reading was approximately 20 mW (B). Furthermore, when skin tissue and muscle tissue were placed between the probe and the sensor, the power meter reading was approximately 5 mW (C).
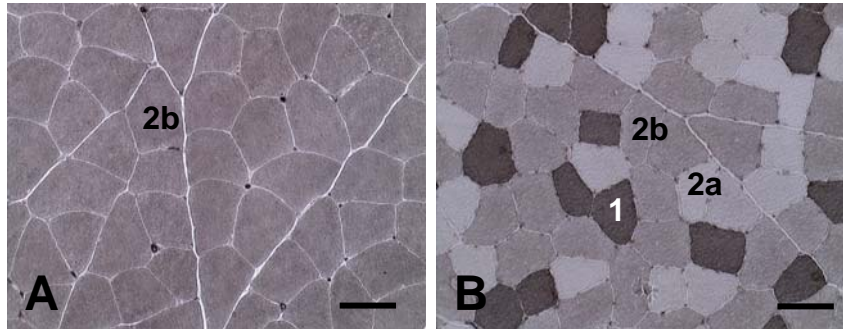
Fig.2. Representative photographs of the cross-section stained for myosin ATPase activity (pH 4.3) in superficial (A) and deep regions (D) of the gastrocnemius muscle (x200). Fibers labeled with 1, 2a and 2b are type I (dark), type IIa (light) and type IIb (intermediate), respectively. Bar is 50 μm.

Table 1. Body and muscle weight

Groups (n)	body weight (g)	untreated / irradiated	muscle wet weight (mg)
10-wk NC (n=5)	321.3 ± 24.6	—	732.0 ± 56.2
HS (n=5)	230.0 ± 11.5	—	523.8 ± 13.7 #
12-wk NC (n=5)	367.0 ± 47.0	untreated irradiated	977.4 ± 93.6 1043.1 ± 60.4
HS recovery (n=5)	236.1 ± 49.3 *	untreated irradiated	690.9 ± 30.9 #, † 770.3 ± 56.8 #, †,*

Data are means ± SD; # significant difference (p<0.05) compared with age-matched NC group. †significant difference (p<0.05) compared with the HS group. * significant difference (p<0.05) compared with untreated side in same group.
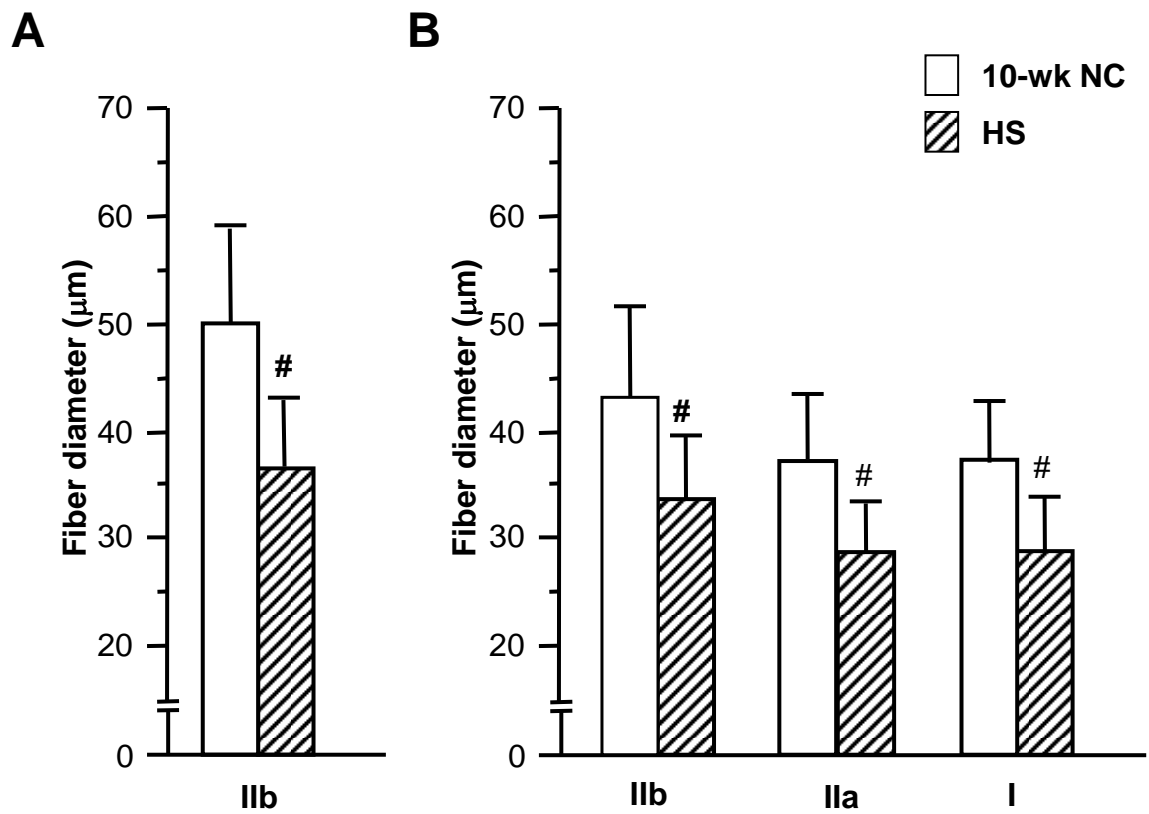
Fig.3. Fiber diameter in superficial region (A) and deep region (B) of gastrocnemius muscle in the HS and 10-wk NC groups. Data are means $\pm$ SD; # significant difference ($p<0.05$) compared with the 10-wk NC group.
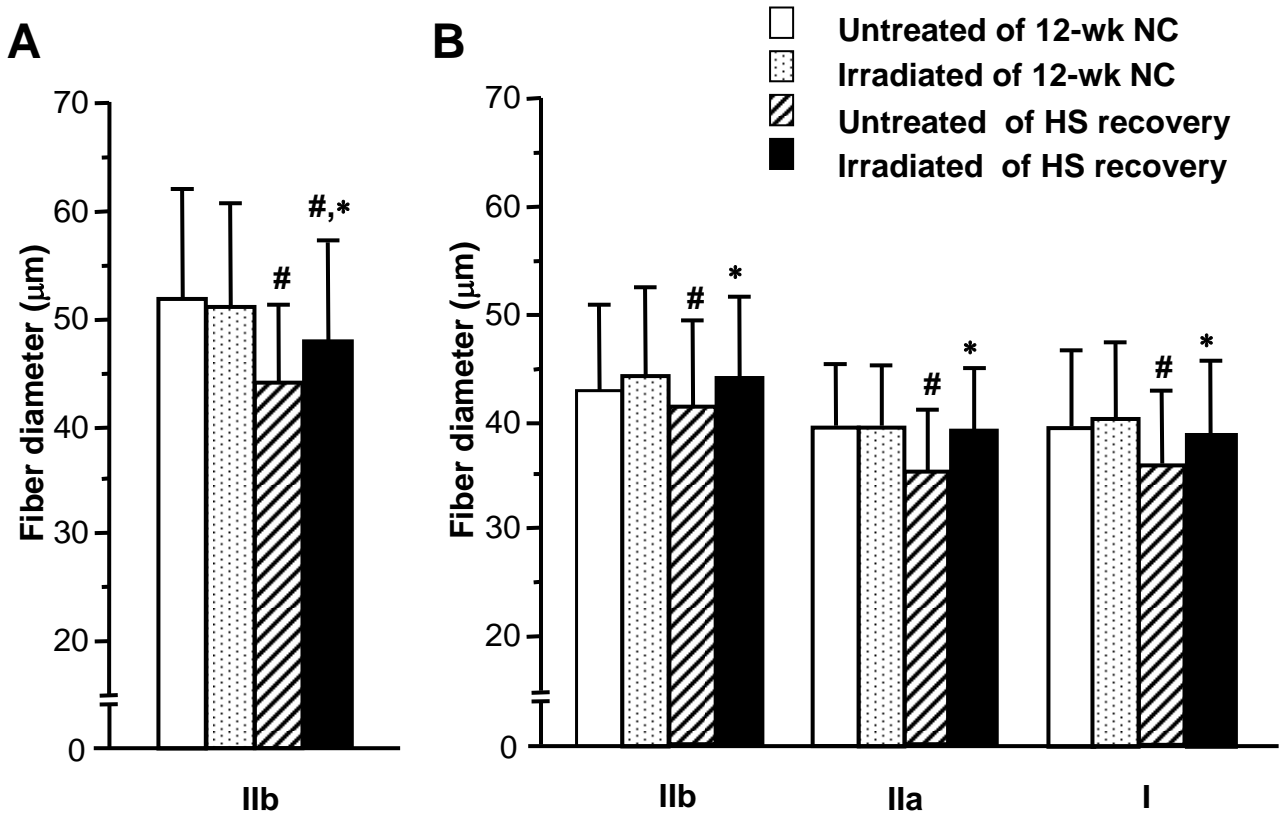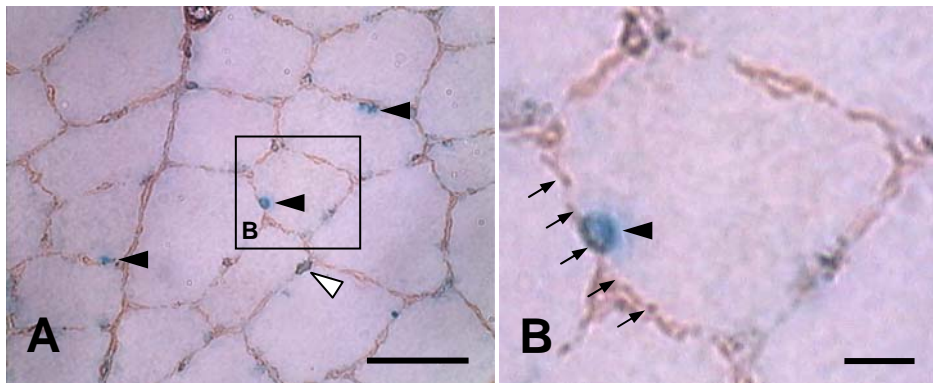
Fig.4. Fiber diameter in superficial region (A) and deep region (B) of gastrocnemius muscle in the HS recovery and 12-wk NC groups. Data are means $\pm$ SD; # significant difference ($p<0.05$) compared with the 12-wk NC group. $*$ significant difference ($p<0.05$) compared with untreated side in same group.

Fig.5. Double immunostaining for BrdU and Laminin in gastrocnemius muscle (x400). The anti-laminin antibody was used for demonstrating the basal lamina (arrow) of muscle fiber and BrdU-positive nuclei located inside the basal laminin (dark arrowhead) was counted. B shows a regional enlarged view surrounded with a square in A. Note that BrdU-positive nuclei located outside the basal laminin (light arrowhead) was excepted from analysis. Bar in A is 50 μm. Bar in B is 10μm.
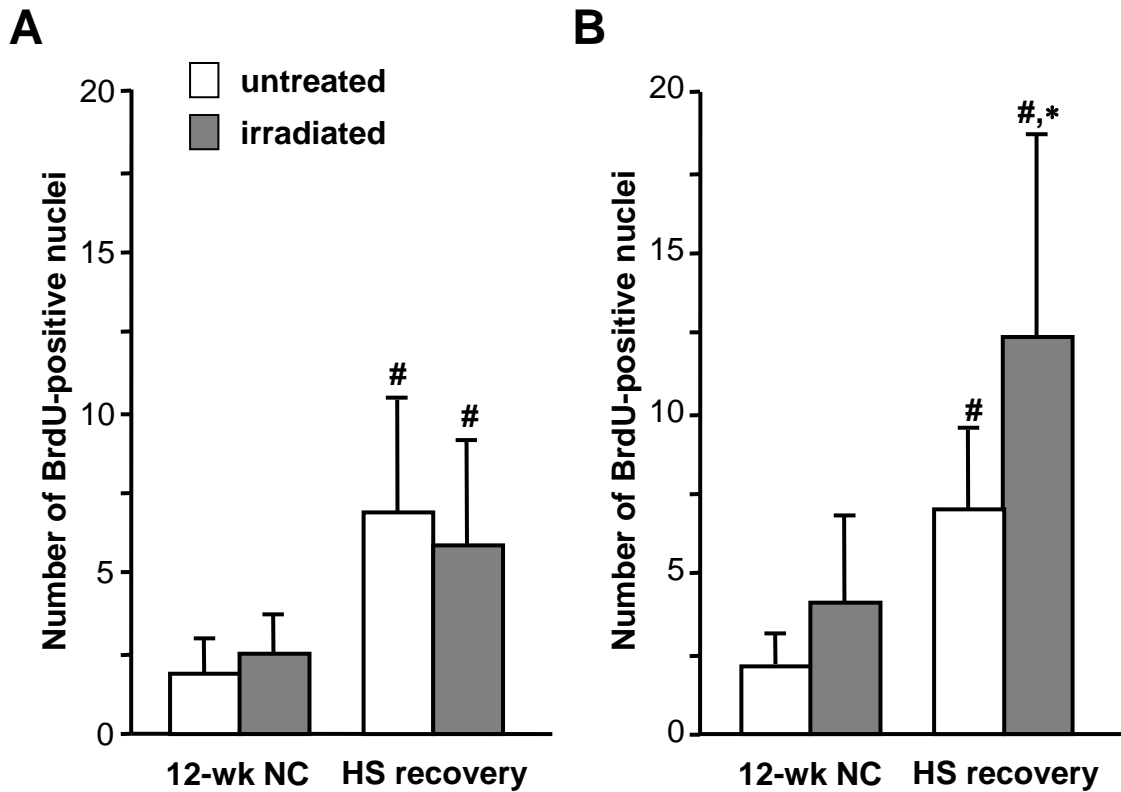
Fig.6. Number of BrdU-positive nuclei in superficial region (A) and deep region (B) of gastrocnemius muscle in HS recovery and the 12-wk NC groups. The numbers of BrdU-positive nuclei in 100 myofibers are displayed. Data are means ± SD; # significant difference (p<0.05) compared with untreated side of the 12-wk NC group. * significant difference (p<0.05) compared with untreated side in same group.
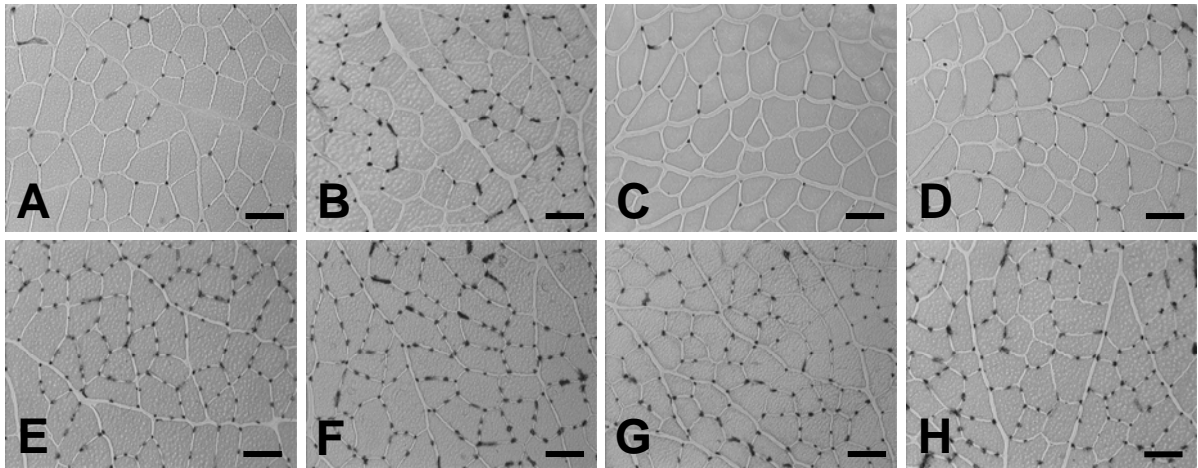
Fig.7. Photographs of the cross-section stained for alkali phosphatase activity (pH 4.3) in superficial (A-D) and deep regions (E-H) of the gastrocnemius muscle (x200). Capillary around myofiber is dyed. (A and E) untreated muscle of the 12-wk NC group; (B and F) irradiated muscle of the 12-wk NC group; (C and G) untreated muscle of the HS recovery group; (D and H) irradiated muscle of the HS recovery group. Bar is 50 μm.

Table 2. Capillary supply in gastrocnemius muscle

Groups (n)	untreated / irradiated	superficial region		deep region	
		capillary-density (mm^{-2})	capillary-to-myofiber ratio	capillary-density (mm^{-2})	capillary-to-myofiber ratio
10-wk NC (n=5)	—	180.0± 46.6	0.82 ± 0.30	820.0± 54.6	2.1 ± 0.2
HS (n=5)	—	182.8 ± 48.5	0.42 ± 0.15 #	899.7 ± 219.7	1.46 ± 0.30 #
12-wk NC (n=5)	untreated	183.2 ± 44.6	0.84 ± 0.22	811.5 ± 156.4	2.18 ± 0.30
	irradiated	350.0 ± 88.4 *	1.31 ± 0.33 *	865.9 ± 138.2	2.17 ± 0.28
HS recovery (n=5)	untreated	146.6 ± 95.6	0.45 ± 0.25 #	674.2 ± 182.5 #	1.48± 0.48 #
	irradiated	253.0 ± 121.0 *,#	0.78 ± 0.32 *,†	760.9 ± 156.9	1.52 ±0.29 #

Data are means ± SD; * significant difference (p<0.05) compared with untreated side in same group. # significant difference (p<0.05) compared with age-matched NC group. †significant difference (p<0.05) compared with the HS group.
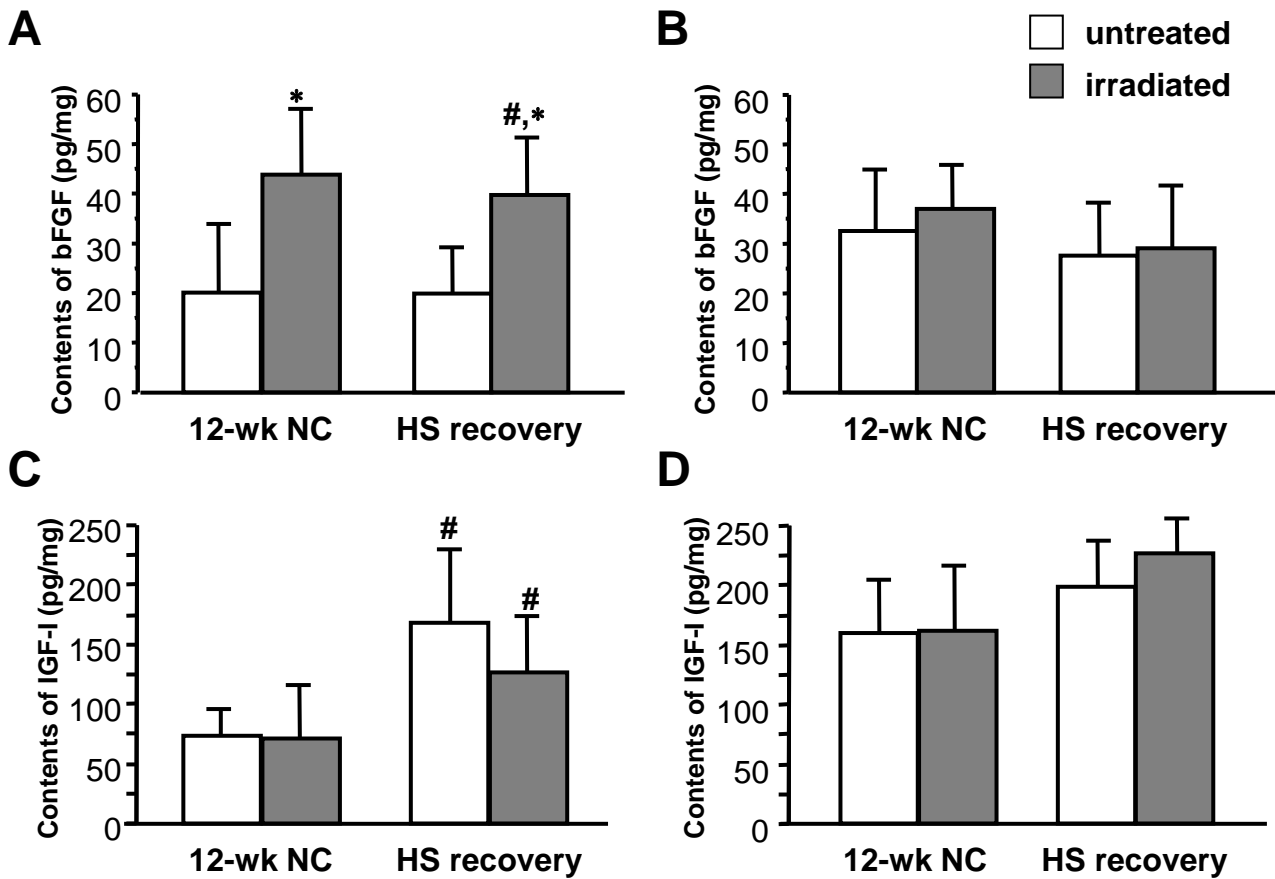
Fig.8. Levels of bFGF and IGF-I in superficial region (A and C) and deep region (B and D) of gastrocnemius muscle in HS recovery and 12-wk NC groups. Data are means ± SD; # significant difference (p<0.05) compared with untreated side of the 12-wk NC group. * significant difference (p<0.05) compared with untreated side in same group.