Enhancement of satellite cell differentiation and functional recovery in injured skeletal muscle by hyperbaric oxygen treatment

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Horie M, Enomoto M, Shimoda M, Okawa A, Miyakawa S, Yagishita K. Enhancement of satellite cell differentiation and functional recovery in injured skeletal muscle by hyperbaric oxygen treatment. *J Appl Physiol* 116: 149–155, 2014. First published December 12, 2013; doi:10.1152/japplphysiol.00235.2013.—Recently, the use of hyperbaric oxygen (HBO) treatments by elite athletes to accelerate recovery from muscle injuries has become increasingly popular. However, the mechanism of promoting muscle regeneration under HBO conditions has not yet been defined. In this study, we investigated whether HBO treatments promoted muscle regeneration and modulated muscle regulatory factor expression in a rat skeletal muscle injury model. Muscle injury was induced by injecting cardiotoxin (CTX) into the tibialis anterior (TA) muscles. As the HBO treatment, rats were placed in an animal chamber with 100% oxygen under 2.5 atmospheres absolute for 2 h/day, 5 days/wk for 2 wk. We then performed histological analyses, measured the maximum force-producing capacity of the regenerating muscle fibers, and performed quantitative RT-PCR analysis of muscle regulatory factor mRNAs. The cross-sectional areas and maximum force-producing capacity of the regenerating muscle fibers were increased by HBO treatment after injury. The mRNA expression of MyoD, myogenin, and IGF-1 increased significantly in the HBO group at 3 and 5 days after injury. The number of $Pax7^+/MyoD^+$, $Pax7^-/MyoD^+$, and $Pax7^+/BrdU^+$-positive nuclei was increased by HBO treatment. In this study, we demonstrated that HBO treatment accelerated satellite cell proliferation and myofiber maturation in rat muscle that was injured by a CTX injection. These results suggest that HBO treatment accelerates healing and functional recovery after muscle injury.

hyperbaric oxygen treatment; skeletal muscle injury; muscle regeneration

HYPERBARIC OXYGEN (HBO) treatment involves breathing pure oxygen at a high atmospheric pressure above sea level, and the worldwide standard HBO treatment is to apply 2.0–2.8 atmospheres absolute (ATA) for 60–90 min with pressurized air or oxygen inside the chamber (14). HBO treatment is thought to have complex effects on immunity, oxygen transport, and hemodynamics, resulting in favorable effects by reducing hypoxia and edema as well as by enabling normal host responses to infection and ischemia (12, 14). Therefore, HBO treatment is the method that is used for treating various illnesses and clinical conditions, such as carbon monoxide poisoning, decompression sickness, osteomyelitis, oxygen-deficient disorders, infectious diseases, and acute cerebral edema (12, 14).

Muscle injury presents a challenge in traumatology and commonly occurs in sports. The use of HBO treatments by elite athletes to accelerate recovery from tendon and muscle injuries began to gain popularity in the early 1980s (19). The application of HBO treatments for sports injuries has recently been suggested in the scientific literature as a therapy modality, i.e., a primary or an adjunct treatment (4, 6, 20, 21). However, published studies on the effects of HBO in models of muscle injury are relatively scarce (2, 3, 5, 13, 23, 29, 30), and the results of studies that have investigated the recovery of functional capacity have been inconsistent. Furthermore, the effect of HBO is unclear in terms of regeneration after muscle injury.

Skeletal muscles consist of bundles of contractile myofibers that contain hundreds of peripherally located postmitotic nuclei. However, these organs are plastic and can adapt to functional demands and regenerate following injury (28). Mononucleated satellite cells are responsible for providing myonuclei for postnatal growth or repair (7, 28, 34). After muscle injury, these satellite cells become activated and divide, which leads to the formation of new myofibers or to the repair of existing fibers. The myogenic lineage progression of satellite cells and their myoblast progeny is regulated by transcription factors, such as muscle-specific basic helix-loop-helix (bHLH) regulatory factors (34). The family of myogenic bHLH factors includes myogenic differentiation protein 1 (MyoD), myogenic factor 5 (Myf5), myogenin, and myogenic regulatory factor 4 (Mrf4). These factors contribute to the transcriptional activation of muscle genes during the commitment and differentiation of satellite cells. To begin satellite cell activation, the expression of Myf5 and MyoD is induced in the cells that coexpress paired box 7 (Pax7) (25). Myf5 and MyoD are involved in the process of muscle regeneration, during which their expression increases at either the protein or the mRNA levels (7, 27, 28, 34). The activation of satellite cells requires the timely, well-controlled upregulation of muscle transcription factors and muscle-specific genes. Some growth factors [e.g., basic-fibroblast growth factor (b-FGF), transforming growth factor-β (TGF-β) family, insulin-like growth factor (IGF), and hepatocyte growth factor (HGF)] are known key regulators of the proliferation and differentiation of satellite cells (17, 24, 28, 34).

The purpose of this study was to examine the effects of HBO treatment on the functional and structural properties of regenerating skeletal muscle after muscle-specific degeneration. In this study, we investigated whether HBO treatment promoted muscle regeneration and affected muscle regulatory factors after skeletal muscle injury induced by cardiotoxin (CTX).

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MATERIALS AND METHODS

Animals and experimental procedures. Male 7-wk-old Wistar rats weighing 200–250 g were kept in standard cages at a constant temperature and given water and food ad libitum throughout the experimental period. All animal experiments were performed under approved protocols and in accordance with the recommendations of the Committee for Animal Welfare at Tokyo Medical and Dental University for the proper care and use of laboratory animals. The animals were anesthetized with an intraperitoneal injection of chloral hydrate (280 mg/kg). Regeneration was induced by the injection of CTX (cardiotoxin from *Naja mossambica mossambica*; Sigma-Aldrich, MO). Up to 500 μl of 10 μM CTX was injected into the tibialis anterior (TA) muscles. Subsequently, the rats were divided into four groups: the nontreatment (NT) group, the hyperbaric oxygen treatment (HBO) group, the normobaric oxygen treatment (NBO) group, and the hyperbaric air treatment (HBA) group. After the CTX injection, the treatment groups underwent HBO, NBO, or HBA. Animals in the HBO group were placed in a hyperbaric experimental chamber in which 100% oxygen was administered at 2.5 ATA pressure for 2 h. The NBO group underwent 100% oxygen administration at 1 ATA pressure for 2 h. The HBA group underwent air administration at 2.5 ATA pressure for 2 h. These treatments started the day after the CTX injections and were performed once a day for 5 days, repeating the next week at the same protocol (10 sessions in total). The animals were killed for TA muscle sampling at *days 1, 3, 5, 8,* and *15* after the CTX injection. All rats were killed using a high dose of chloral hydrate. The TA muscles were removed and quickly frozen in liquid nitrogen-cooled 2-methylbutane. The muscles were stored at −80°C until further analysis.

Histology of the TA muscle. Transverse sections of the TA muscles were cut at 20 μm with a cryostat (CM 300; Leica Japan, Tokyo, Japan) and kept at −25°C. The sections were stained with hematoxylin and eosin (H&E). The slides were evaluated under light microscopy, and microphotographs were taken with a digital camera (Olympus AX70; Olympus, Tokyo, Japan) attached to a microscope (Olympus BX51; Olympus). The digitally captured images were processed and analyzed using image analysis software (ImageJ; National Institutes of Health, Bethesda, MD). Myofibers with centrally located nuclei were considered as regenerating after the injury. We randomly selected 250 myofibers with regenerating myofibers and measured the cross-sectional areas (CSA) using the ImageJ software.

Measurement of muscle isometric tensile strength. Rats in the NT or HBO group were anesthetized 8 days after the CTX injection and set in the supine position. The left hindlimb was fixed tightly on the leg holder. To evaluate TA muscle function, the isometric tensile strength produced by stimulating the common peroneal nerve was measured with a transducer (TB-653T; Nihon Koden, Tokyo, Japan) and recorded with a sensor interface (Power lab; AD Instruments Japan, Nagoya, Japan) and software (Power lab software; AD Instruments Japan). The common peroneal nerve was stimulated with an electrostimulator (Neuropack μ; Nihon Koden) at 1 Hz (twitch) or 50 Hz (tetanus), and the maximum strength was recorded (see Fig. 2A). Stimulation pulse was the minimum voltage that visibly contracted the TA muscle. The nerve was then stimulated by a measured voltage, and the maximum isometric tensile strength produced by the TA muscle was measured [R_f and L_f; maximum twitch and tetanic isometric tensile strengths of the right (non-injured) and left (injured) legs]. The strength ratio of the right muscle to the left muscle (ratio of L_f to R_f) was also calculated. This experiment was performed in the NT and HBO treatment group.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). TA muscles were snap-frozen in liquid nitrogen and stored at −80°C at each time point after the injury. Total RNA including the RNA fraction was isolated by lysis in 1 ml of Qiazol Total RNA Isolation Reagent (Qiagen, Hilden, Germany) using the RNeasy Mini Kit (Qiagen). One microgram of total RNA was reverse transcribed using the PrimeScript RT reagent Kit (TAKARA, Tokyo, Japan). Quantitative PCR was performed using GoTaq qPCR Master Mix (Promega), the qPCR System (Mx3000P; Agilent Technologies), 20 ng of cDNA, and specific primers according to the manufacturer's instructions.

The primer sequences are as follows: Pax7, 5′-GATTAGCCGAGT-GCTCAGAATCAAG-3′ (forward) and 5′-GTCGGGTTCTGATTC-CACGTC-3′ (reverse); MyoD, 5′-CTACAGCGGCGACTCAGACG-3′ (forward) and 5′-TTGGGGCCGGATGTAGGA-3′ (reverse); myogenin, 5′-GGCAATGCACTGGAGTTTGG-3′ (forward) and 5′-CAATCT-CAGTTGGGCATGGTTTC-3′ (reverse); bFGF, 5′-CGAACCGGTAC-CTGGCTATGA-3′ (forward) and 5′-GTATTTCCGTGACCGGTA-AGTGTTG-3′ (reverse); HGF, 5′-AGCATGACATCACTCCCGAG-AAC-3′ (forward) and 5′-TGGGAATTTGAGAGCAGTAAC-CAAC-3′ (reverse); IGF-1, 5′-GCACTCTGCTTGCTCAC-CTTTA-3′ (forward) and 5′-TCCGAATGCTGGAGCCATA-3′ (reverse); and TBP, 5′-TGCACAGGAGCCAAGA-3′ (forward) and 5′-CACATCACAGCTCCCC-3′ (reverse).

The expression of the target mRNAs was normalized to TATA-binding protein (TBP) mRNA. Relative quantification of gene expression was calculated based on the comparative CT (threshold cycle value) method (ΔCT = CT gene of target − CT TBP gene). A comparison of gene expression in different samples was performed based on the differences in ΔCT of individual samples ($\Delta\Delta$CT). This experiment was performed in the NT, HBO, NBO, and HBA treatment groups.

Immunohistochemistry. Transverse sections of the TA muscles were cut at 20 μm with a cryostat (CM 300; Leica) and kept at −25°C. The slides were fixed in 2% paraformaldehyde for 5 min and then washed in PBS for 5 min. After washing, the slides were immersed in blocking solution (5% normal goat serum in PBS with 0.5% Triton X-100) for 30 min and then incubated for over 3 h with the primary antibody (MyoD, mouse monoclonal antibody; Dako Japan, Tokyo, Japan; Pax7, mouse monoclonal antibody, R&D Systems; or rabbit polyclonal antibody, Abcam, Cambridge, UK; laminin, rabbit polyclonal antibody; Sigma-Aldrich) diluted 1:100 in PBS. The sections were washed for 5 min in PBS and then incubated with the secondary antibodies (goat anti-mouse IgG-Alexa Fluor 594, goat anti-rabbit IgG-Alexa Fluor 488; Life Technologies Japan, Tokyo, Japan) diluted 1:500 in PBS for 1 h. The sections were washed three times for 5 min in PBS. Finally, the sections were incubated with DAPI (Life Technologies) for 1 min, washed in PBS, and mounted in mounting solution (PermaFluor; Thermo Fisher Scientific Japan, Yokohama, Japan). PAX7$^+$/MyoD$^-$, PAX7$^+$/MyoD$^+$, PAX7$^-$/MyoD$^+$ positive cells were counted in 20 high-power fields (HPF) of 25 sections prepared from 5 rats for each group. This experiment was performed in the NT, HBO, NBO, and HBA treatment groups.

5-Bromo-2-deoxyuridine (BrdU) incorporation and detection. To assess cell proliferation, animals were given water that contained BrdU (0.8 mg/ml) for 3 days after CTX injection. The animals were killed for TA muscle sampling 3 days after the injury. Fixed sections were incubated with 2 N HCl for 20 min. After several washes, the sections were incubated with mouse anti-BrdU monoclonal antibody (1:200; BD) and anti-Pax7 polyclonal antibody (1:200) at 4°C overnight, followed by Alexa 594-conjugated goat anti-mouse IgG (1:500) and Alexa 488-conjugated goat anti-rabbit IgG (1:500). Finally, the sections were incubated with DAPI (Life Technologies Japan) for 1 min, washed in PBS, and mounted in mounting solution (Thermo Fisher Scientific Japan). The number of positive cells was counted in 20 high-power fields (HPFs) of 25 sections that were prepared from 5 rats from the NT and HBO treatment groups. We showed the relative number of Pax7$^+$/BrdU$^+$-positive cells in the HPF.

Statistical analysis. The data are presented as the means ± standard error of the mean (SE). Differences were analyzed by Student's *t*-test or by a one-way or two-way ANOVA, which was followed by the Bonferroni test. $P < 0.05$ was considered statistically significant.

RESULTS

Histology of regenerating muscle and measurement of the cross-sectional areas (CSAs) of regenerating myofibers. H&E-stained injured TA muscles were observed at *days 1, 3, 5, 8,* and *15* after CTX injection in the NT and HBO groups, as shown in Fig. 1*A*. On the first day after the CTX injection, muscle fiber degeneration and the presence of cellular infiltration were evident. On *day 3*, necrotic muscle fibers were observed, and sporadic islets of preserved muscle fibers were dispersed throughout the cross section. On *day 5*, the number of necrotic muscle cells decreased, and regenerating myofibers with central nuclei began to appear. On *day 15*, a further maturation of regenerating fibers was observed.

The mean CSAs of the regenerating myofibers are shown in Fig. 1*B*. In the HBO group, the CSA values of the regenerating myofibers were larger than those in the NT or NBO group at *day 5* (NT, $258.9 \pm 10.8 \ \mu m^2$; HBO, $322.3 \pm 10.9 \ \mu m^2$; NBO, $250.3 \pm 8.2 \ \mu m^2$; HBA, $307.8 \pm 8.6 \ \mu m^2$), *day 8* (NT, $490.8 \pm 32.2 \ \mu m^2$; HBO, $616.4 \pm 36.1 \ \mu m^2$; NBO, $483.3 \pm 12.4 \ \mu m^2$; HBA, $545.8 \pm 32.2 \ \mu m^2$) and in the NT, NBO, or HBO group at *day 15* (NT, $866.5 \pm 35.5 \ \mu m^2$; HBO, $1,091.6 \pm 45.2 \ \mu m^2$; NBO, $823.3 \pm 45.0 \ \mu m^2$; HBA, $863.8 \pm$

$21.9 \ \mu m^2$) after the CTX injection. The quantitative analysis revealed that the HBO treatment increased myofiber maturation at an early phase after injury compared with the NT, NBO, or HBA treatment.

Measurement of muscle isometric tensile strength. In the functional analysis, we measured the muscle isometric tensile strength of the injured muscle at *day 8* after the CTX injection. The ratios of the twitch and tetanic muscle isometric tensile strength of the injured to noninjured legs (L_f/R_f) are shown in Fig. 2*B*. The twitch muscle isometric tensile strength in the HBO group was stronger than in the NT group (NT, $41.5 \pm 3.9\%$; HBO, $49.4 \pm 5.5\%$). The tetanic muscle isometric tensile strength was significantly stronger in the HBO group than in the NT group (NT, $41.7 \pm 1.5\%$; HBO, $53.0 \pm 4.9\%$).

The mRNA expression of myogenic regulatory factors (MRFs) and growth factors (GFs) in injured muscle. The expression patterns of Pax7, MyoD, myogenin (MRFs), HGF, b-FGF, and IGF-1 (GFs) mRNAs are shown in Fig. 3. The expression levels of MyoD mRNA at 3 and 5 days post-CTX injection and IGF-1 mRNA 5 days post-CTX injection were higher in the HBO group compared with levels of the NT group (Fig. 3*A*). However, the expression levels of other factors were not significantly different between the NT and HBO groups. Furthermore, we performed a comparative analysis of MyoD, myogenin and IGF-1 mRNA expression in all groups, including the NBO and HBA groups, at *day 3* or *day 5* (Fig. 3*B*). MyoD mRNA expression in the HBO group was significantly higher than in the other groups (HBO, 3.45 ± 0.53; NBO, 1.95 ± 0.29; HBA, 1.09 ± 0.11) at *day 3* after the CTX injection. Myogenin mRNA expression in the HBO group was significantly higher than in the other groups (HBO, 1.62 ± 0.12; NBO, 1.10 ± 0.14; HBA, 0.57 ± 0.053) at *day 5* after the CTX injection. However, IGF-1 mRNA expression was not significantly different among the HBO, NBO, and HBA groups at *day 5* after the CTX injection (HBO, 1.65 ± 0.17; NBO, 1.50 ± 0.24; HBA, 1.17 ± 0.17).

The expression of Pax7 and MyoD protein in injured muscle. Pax7- or MyoD-positive nuclei were observed beside the basement membrane of injured muscles at *day 5* after injury (Fig. 4*A*). The quantitative analysis revealed that the number of MyoD positive nuclei increased in the HBO group at *days 3* and *5* (data not shown). To distinguish between proliferating and differentiated cells, the number of Pax7$^+$/MyoD$^-$, Pax7$^+$/MyoD$^+$, or Pax7$^-$/MyoD$^+$ nuclei were analyzed in the injured TA muscle of the HBO and NT groups (Fig. 4*B*). The number of Pax7$^+$/MyoD$^+$ nuclei increased significantly at *day 3* in the HBO group (NT, 19.8 ± 2.5/HPF; HBO, 24.0 ± 3.2/HPF) and at *day 8* in the NT group (NT, 12.0 ± 3.2/HPF; HBO, 8.36 ± 1.4/HPF). The number of Pax7$^-$/MyoD$^+$ nuclei increased significantly at *day 5* in the HBO group (NT, 25.7 ± 5.3/HPF; HBO, 41.6 ± 6.6/HPF) and at *day 8* in the NT group (NT, 28.1 ± 4.1/HPF; HBO, 17.5 ± 3.4/HPF). In contrast, there was no difference between the number of Pax7$^+$/MyoD$^-$ nuclei in the NT and HBO groups at each time point. To examine the differentiation effect in the NBO or HBA condition, we compared the number of these positive nuclei in each treatment group at *day 5* (Fig. 4*C*). There was no difference in the number of Pax7$^+$/MyoD$^-$ and MyoD$^+$/Pax7$^+$ cells among the groups; however, the number of Pax7$^-$/MyoD$^+$ nuclei increased significantly in the HBO group compared with the NT and HBA groups (NT, 25.7 ± 5.3/HPF; HBO, 41.6 ± 6.6/HPF;
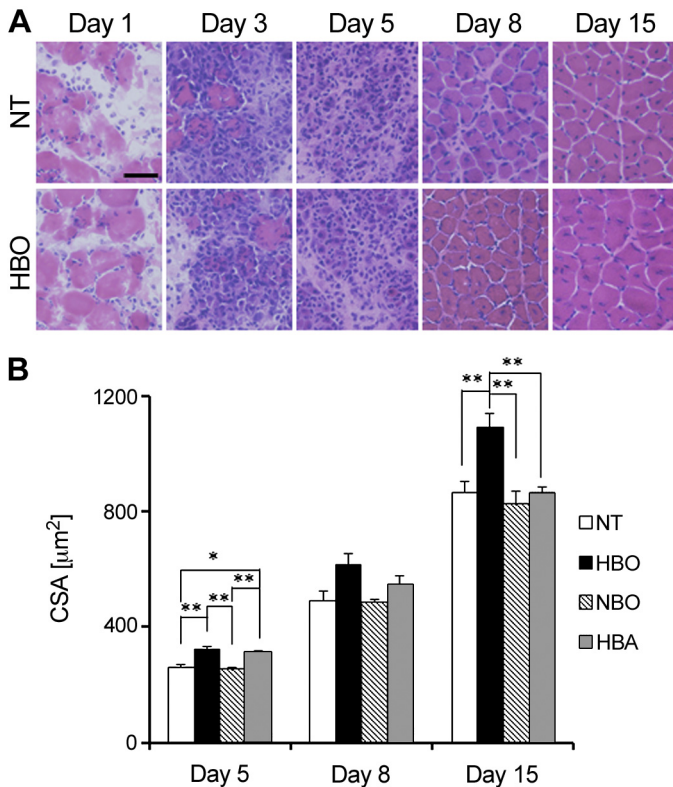

Fig. 1. Histology of regenerating muscle and measurements of the cross-sectional area (CSA) of regenerating myofibers. *A*: representative images of the typical regenerative processes in injured tibialis anterior (TA) muscles. Tissue sections that were obtained at each time point were stained with hematoxylin and eosin (H&E). Scale bar, 50 μm. *B*: CSA of regenerating myofibers of TA muscle at *days 5, 8,* and *15* after cardiotoxin (CTX) injection. The sample number for each group was $n = 5-6$. NT, nontreatment group; HBO, hyperbaric oxygen treatment group; NBO, normobaric oxygen treatment group; HBA, hyperbaric air treatment group. Statistical analysis was performed using a one-way ANOVA, which was followed by Bonferroni test. Asterisks denote significance (*$P < 0.05$, **$P < 0.01$).
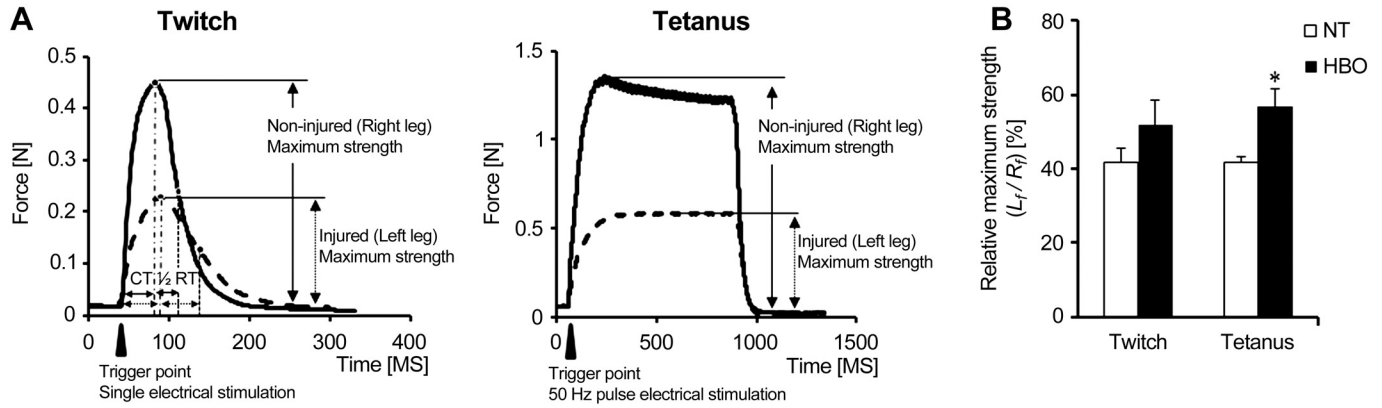
Fig. 2. Measurement of the isometric tensile strength of the TA muscle. *A*: twitch and tetanic forces that were evoked by electrical stimulation. The maximum strength was recorded and the ratio of left leg force (injured) and right leg force (noninjured) was calculated. Shown under the curves are contraction time (CT) and half-relaxation time (1/2 RT) of the noninjured (solid two-headed arrow) and injured (dashed two-headed arrow) legs. *B*: ratio of muscle isometric tensile strength at *day 8* after the CTX injection in the NT and HBO groups. L_f, muscle isometric tensile strength of left leg (injured); R_f, muscle isometric tensile strength of right leg (noninjured). Statistical analysis was performed by unpaired Student's *t*-test. The sample number for each group was $n = 6$. Asterisks denote significance (*$P < 0.05$ compared with NT).

NBO, 32.7 ± 6.4/HPF; HBA, 23.12 ± 4.7/HPF). The total number of either Pax7- or MyoD-positive nuclei increased in the HBO group compared with the other groups (NT, 45.0 ± 4.4/HPF; HBO, 61.7 ± 5.4/HPF; NBO, 48.4 ± 5.6/HPF; HBA, 35.5 ± 3.8/HPF).

In addition, the number of BrdU-labeled cells was analyzed in injured muscles of the HBO and NT groups 3 days after CTX injury (Fig. 4*D*). The number of Pax7$^+$/BrdU$^+$ nuclei increased significantly in the HBO group (NT, 32.4 ± 3.3/HPF; HBO, 40.2 ± 4.6/HPF).

Fig. 3. mRNA expression analyses of muscle regulatory factors (MRFs) and growth factors (GFs) in the injured muscle. *A*: expression patterns of MRFs and GFs mRNA in injured muscle. The target mRNA was normalized to TBP mRNA, and the *y*-axis was presented as the relative ratio compared with the value of *day 0*. The statistical analysis was performed by a two-way ANOVA, which was followed by Bonferroni test. The sample number for each group was $n = 5$–6. Asterisks denote significance (*$P < 0.05$ compared with NT). *B*: comparative analysis of all groups. MyoD is at *day 3*, myogenin is at *day 5*, and IGF-1 is at *day 5*. The target mRNA was normalized to TBP mRNA, and the *y*-axis was presented as a relative ratio for the value of NT expression. Statistical analysis was performed by a one-way ANOVA, which was followed by Bonferroni test. The sample number for each group was $n = 5$–6. Asterisks denote significance (*$P < 0.05$, **$P < 0.01$).
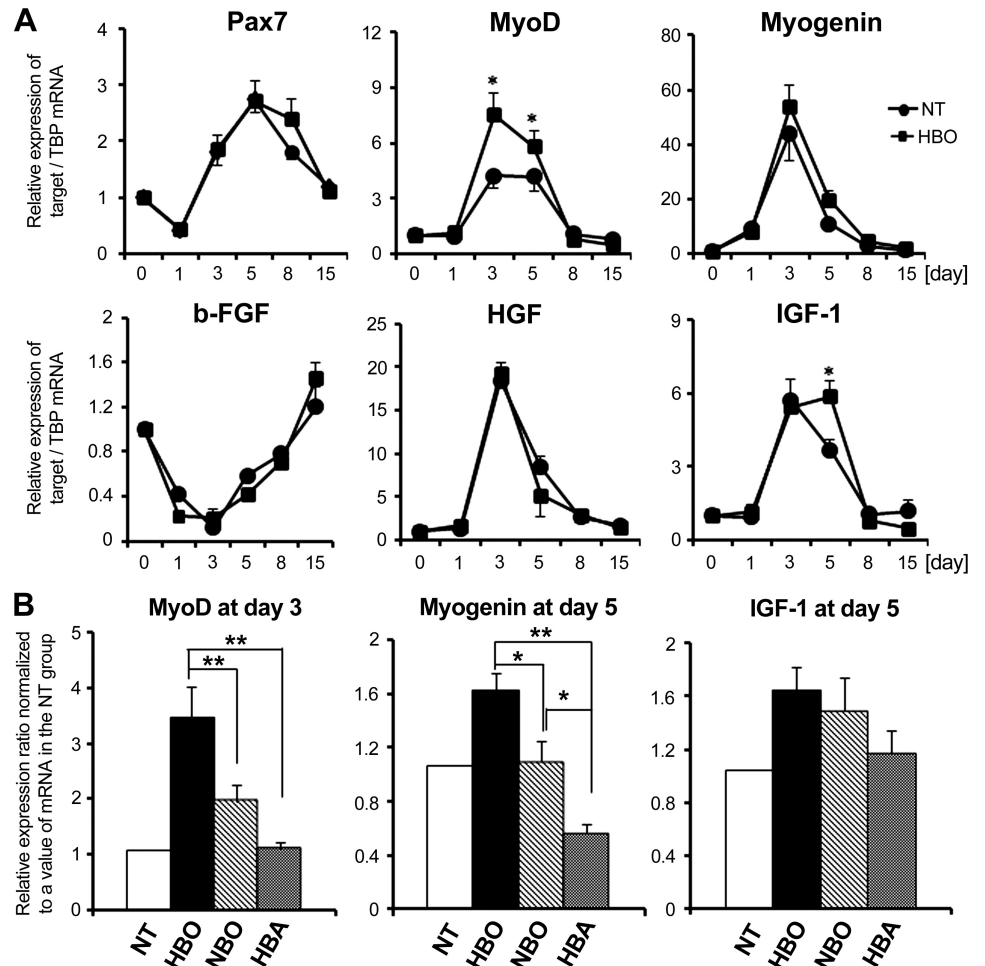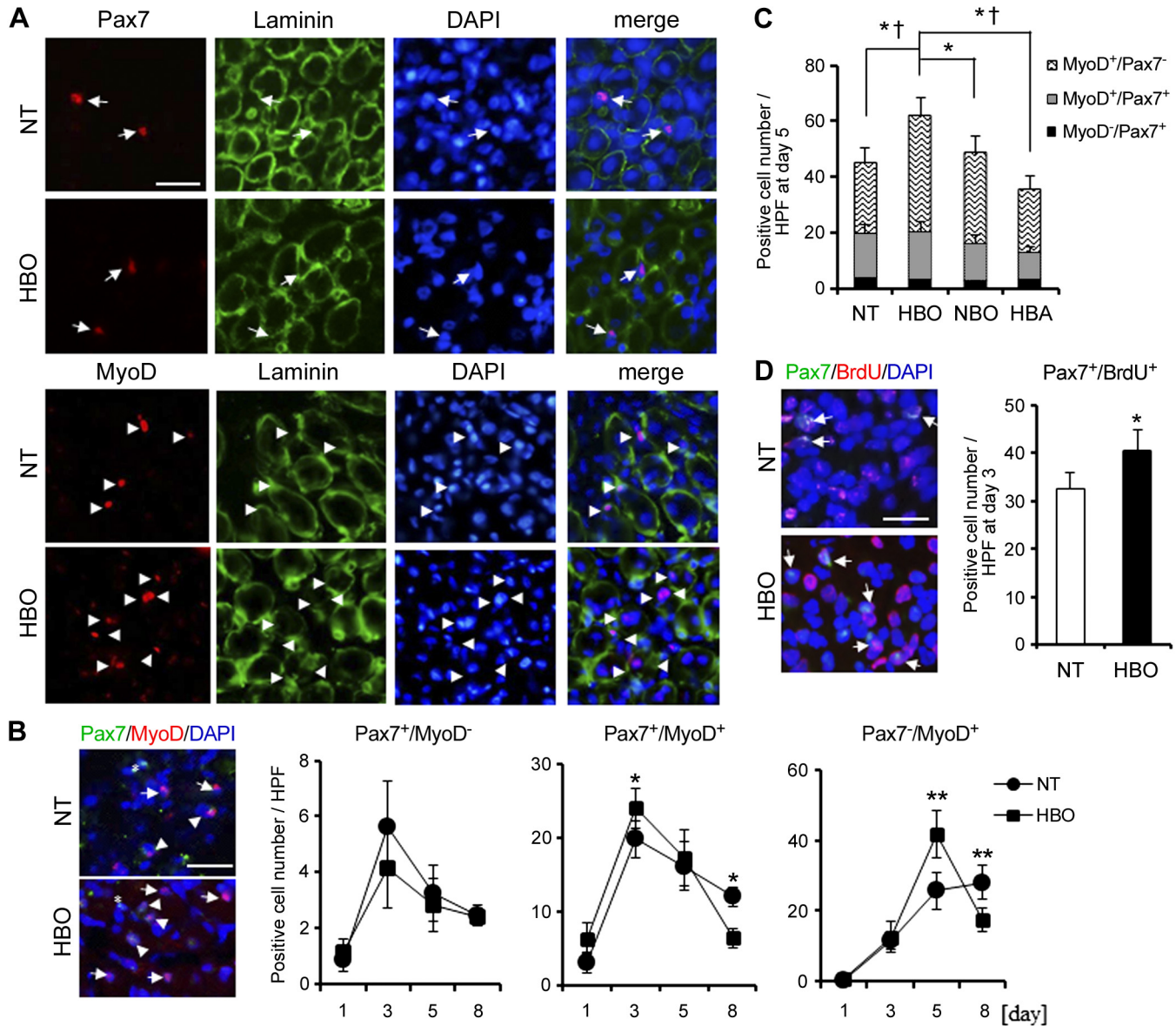
Fig. 4. The expression of Pax7 and MyoD proteins in the injured TA muscle. *A*: representative images of Pax7 or MyoD immunofluorescence staining with laminin in injured muscle at *day 5* after CTX injection. Double positive nuclei were indicated with arrows (Pax7$^+$/DAPI$^+$) or arrowheads (MyoD$^+$/DAPI$^+$). Scale bar, 20 μm. *B*: representative merged images of Pax7 (green), MyoD (red), and DAPI (blue) immunofluorescence staining in injured muscle at *day 5* after CTX injection. Pax7$^+$/DAPI$^+$ nuclei are indicated with asterisks. Pax7$^+$/MyoD$^+$/DAPI$^+$ nuclei were indicated with arrowheads. MyoD$^+$/DAPI$^+$ nuclei are indicated with arrows. Scale bar, 20 μm. The temporal pattern of Pax7$^+$/MyoD$^-$, Pax7$^+$/MyoD$^+$ and Pax7$^-$/MyoD$^+$-positive cell numbers in injured muscle is shown. The number of each immunopositive nuclei/HPF is shown. Statistical analysis was performed by a two-way ANOVA, which was followed by Bonferroni test. The sample number for each group was $n = 5$. Asterisks denote significance (*$P < 0.01$, **$P < 0.001$ compared with NT). *C*: comparative analysis among all groups at *day 5* after the CTX injection. Statistical analysis was performed by a one-way ANOVA, which was followed by Bonferroni test. The number of immunopositive nuclei/HPF is shown. The sample number for each group was $n = 5$. Asterisks denote the significance of total immunopositive nuclei (*$P < 0.001$). Daggers denote the significance of Pax7$^-$/MyoD$^+$-positive nuclei (†$P < 0.001$). *D*: representative merged images of Pax7 (green), BrdU (red), and DAPI (blue) immunofluorescence staining in injured muscle at *day 3* after CTX injection. Triple positive nuclei are indicated with arrows (Pax7$^+$/BrdU$^+$/DAPI$^+$). Scale bar, 20 μm. The number of Pax7$^+$/BrdU$^+$/HPF nuclei at *day 3* after CTX injection. Statistical analysis was performed by Student's *t*-test. The sample number for each group was $n = 5$. Asterisks denote significance (*$P < 0.01$).

DISCUSSION

In this study, we demonstrated that HBO treatment accelerated satellite cell proliferation and myofiber maturation in rat muscle that was injured by a CTX injection. The size and maximum force-producing capacity of the regenerating muscle fibers were increased by HBO treatment after injury. The mRNA expression levels of MyoD, myogenin, and IGF-1 were higher in the HBO group than in the other groups, and the number of Pax7$^+$/MyoD$^+$, Pax7$^-$/MyoD$^+$ and Pax7$^+$/BrdU$^+$-positive nuclei was increased in the HBO-treated muscles at the early phase after injury.

To study the process of muscle regeneration in a controlled and reproducible way, the development of an experimental animal model with a stable muscle injury was necessary. The

use of myotoxins, such as bupivacaine, CTX, and notexin, is the most popular method of inducing muscle regeneration, particularly compared with the muscle contusion model (10, 16). CTX, which is a peptide that is isolated from snake venom, is a protein kinase C-specific inhibitor that appears to induce the depolarization and contraction of muscle cells to disrupt membrane organization and lyse various cell types (34). CTX is specific to muscle fibers, thereby eliminating the complicating factors of damage to motor nerves and their associated blood vessels that occur with other models of muscle injury (10, 34). Specifically, the CTX-induced muscle injury model is a useful experimental model to analyze the proliferation and differentiation of satellite cells and is appropriate for evaluating whether HBO treatments promote muscle regeneration.

To increase muscle healing, various HBO pressure conditions or treatment times should be tested. In a previous study, the application of a 3 ATA HBO treatment was reported to improve the contractile capacity and fiber size of rat extensor digitorum longus muscles that were injured by bupivacaine injection (13). This study also demonstrated that the HBO treatment accelerated the regeneration of muscle fibers after injury. However, in clinical usage, HBO application at a pressure of 2.5–2.8 ATA is more common than 3 ATA, as has been previously reported. Therefore, we investigated the effect of muscle regeneration processes under 2.5 ATA hyperbaric conditions, which has not been previously studied. It has been reported that HBO treatment at a pressure of 3 ATA for 60 min increased the expression of Myf5 mRNA of the limb muscle in the ischemia injury model (2). However, HBO treatment at a pressure of 2.2 ATA for 60 min did not affect the expression of MyoD or myogenin in rat muscle injuries that were induced by bupivacaine (3). In light of these previous reports, a long exposure time should be required to promote muscle regeneration; therefore, we adopted a pressure of 2.5 ATA for 120 min.

Satellite cells first maintain their normal quiescent state until they initiate proliferation during muscle regeneration. After several rounds of proliferation, the majority of satellite cells differentiate and fuse to form new myofibers or to repair damaged myofibers. As satellite cell activation is not restricted to the damaged site, once injury occurs at one end of a muscle fiber, the satellite cells actively proliferate along the length of the fiber and migrate to the damaged site, leading to the regeneration of injured muscle (27, 34). To begin satellite cell activation, Myf5 and MyoD, which make up myogenic regulatory factors, are induced in the cells coexpressing Pax7. After the downregulation of Pax7, MyoD and its target, myogenin, stimulate terminal differentiation through the activation of genes that are expressed in mature muscle, such as the myosin heavy chain gene family (28, 34). In this study, the most important finding is that the expression of both MyoD and myogenin was increased at the early phase of myogenesis by HBO treatment, which resulted in the promotion of regenerating muscle fibers. In addition, the number of proliferating cells expressing $Pax7^+/MyoD^+$ nuclei increased significantly at *day 3*, and differentiated cells expressing $Pax7^-/MyoD^+$ nuclei increased significantly at *day 5* in the HBO group compared with the NT group (Fig. 4, *B* and *C*). The number of $Pax7^+/BrdU^+$ positive nuclei also increased significantly at *day 3* in HBO (Fig. 4*D*). These results indicate that the HBO treatment

could advance the muscle regeneration process faster than the other groups. In the present study, we conclude that the proliferation and differentiation of satellite cells are accelerated by HBO treatment at the early phase after CTX muscle injury.

After muscle injury, an increase in vascular permeability due to inflammatory cells induces edema, and the reduction of oxygen partial pressure is attributed to hypoxia in injured muscles (31). Recent reports indicate that oxygen may serve as a developmental trigger in these contexts; low-oxygen conditions maintain skeletal myoblasts in an undifferentiated state in vitro (11, 15, 22, 26, 33). In the hypoxic environment of developing or regenerating skeletal muscle, oxygen-dependent pathways may constrain progenitor differentiation until there is an ample blood supply, thereby conserving the stem/progenitor pool until conditions become appropriate for growth (22). Therefore, HBO treatment has a beneficial effect that immediately improves hypoxia and that maintains high oxygen concentrations in regenerating muscle tissue after injury, together with the acceleration of both the proliferation and differentiation of muscle satellite cells. Studies remain to be done to investigate the oxygen concentrations in injured muscle tissues and to establish a protocol for appropriate HBO treatments for muscle injury.

Growth factors, which are produced in the muscles, motor nerves, or inflammatory cells, are important elements for muscle regeneration. In this study, we demonstrated an increase in IGF-1 mRNA and in the promotion of the muscle regeneration at *day 5* in the HBO group. IGF-1 has long been known to regulate the proliferation and differentiation of satellite cells. More recently, the paracrine and/or autocrine regulation of IGF-1 and its activity during skeletal muscle development and repair have become apparent (1). In in vitro studies, IGF-1 is able to alter the expression of MRFs, such as MyoD, and to promote both the proliferation and differentiation/fusion of myoblasts (1, 8, 32). Meanwhile, a hypoxic environment has been reported to reduce the expression and activity of IGF-1 receptors (22). Therefore, HBO may have stimulated IGF-1 secretion to improve the hypoxic environment of the injured muscle condition. However, the precise molecular mechanisms underlying these effects cannot be elucidated from the data in this study alone. Studies remain to be done to investigate growth hormone concentrations and the relation of oxygen concentrations in the muscle tissue.

In this study, we used an experimental muscle injury model with CTX. Therefore, the effect of HBO on the CTX model is not fully representative of its effects on clinical cases, such as strains, contusions, or lacerations of muscles. Muscle contusions damage not only the muscle fiber but also the motor nerves and peripheral blood vessels (9, 18). It is possible that HBO treatments also have favorable effects on neuronal or vascular parameters other than muscle regeneration, with the promotion of satellite cell proliferation and differentiation. Further research is required to determine the molecular mechanisms through which HBO exerts its beneficial effects on muscle regeneration under clinically relevant muscle injuries.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.H., M.E., and K.Y. conception and design of research; M.H. and M.S. performed experiments; M.H. and M.E. analyzed data; M.H. and M.E. interpreted results of experiments; M.H. prepared figures; M.H. drafted manuscript; M.H., M.E., M.S., A.O., S.M., and K.Y. edited and revised manuscript; M.E., M.S., A.O., S.M., and K.Y. approved final version of manuscript.

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