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

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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 injury. 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).
transcribed using the PrimeScript RT reagent Kit (TAKARA, Tokyo, Japan). Quantitative PCR was performed using GoTag 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'-GATTACCGCCGAT- 
GCTCAGAAATCAAG-3' (forward) and 5'-GTCGTTCTGTAGTC- 
CACGTC-3' (reverse); MyoD, 5'-CTACAGCGCGCTACGAGC-3' 
(reverse) and 5'-TTGGGCGCGATGGAGA-3' (forward); myogenin, 
5'-GGCAATGCTAGGATGTGTTG-3' (forward) and 5'-CAATCT- 
CAGTGGGCATGTTCCT-3' (reverse); bFGF, 5'-CGAACCCTGAC- 
CTGGCTATGA-3' (forward) and 5'-GTATCCCCGTGCAGGTA- 
GTTGTTG-3' (forward); HGF, 5'-AGCATGACATCATTGCGAC- 
AAC-3' (forward) and 5'-TGGGAATTTTGAGAGCAGTAAAC- 
CAC-3' (reverse); IGF-1, 5'-GCACTCTGTTCTGTCAC- 
CTTTA-3' (forward) and 5'-TCCGAATGCTGAGCCATA-3' (re- 
verse); and TBP, 5'-TGACACAGGACCAAG-3' (forward) and 5'-CACATCACAGTCCC-3' (reverse).

The expression of the target mRNAs was normalized to TATA- 
binding protein (TBP) mRNA. Relative quantification of gene expres- 
sion was calculated based on the comparative CT (threshold cycle 
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 (ΔΔCT). This 
experiment was performed in the NT, HBO, NBO, and HBA treat- 
ment 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 incubated for over 3 h with 
the primary antibody (MyoD, mouse monoclonal antibody; Dako 
Japan, Tokyo, Japan; Pax7, mouse monoclonal antibody, R&D Sys-
tems; 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) for 1:500 in PBS for 1 h. The sections were incubated 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, Yo-
kohama, 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 over-
night, 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 ± 
the 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. 1A. 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. 1B. 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 ± 10.8 μm²; HBO, 322.3 ± 10.9 μm²; NBO, 250.3 ± 8.2 μm²; HBA, 307.8 ± 8.6 μm²), day 8 (NT, 490.8 ± 32.2 μm²; HBO, 616.4 ± 36.1 μm²; NBO, 483.3 ± 12.4 μm²; HBA, 545.8 ± 32.2 μm²) and in the NT, NBO, or HBO group at day 15 (NT, 866.5 ± 35.5 μm²; HBO, 1,091.6 ± 45.2 μm²; NBO, 823.3 ± 45.0 μm²; HBA, 863.8 ± 21.9 μm²) 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 muscle to noninjured legs (L/t) were shown in Fig. 2B. The twitch muscle isometric tensile strength in the HBO group was stronger than in the NT group (NT, 41.5 ± 3.9%; HBO, 49.4 ± 5.5%). The tetanic muscle isometric tensile strength was significantly stronger in the HBO group than in the NT group (NT, 41.7 ± 1.5%; HBO, 53.0 ± 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. 3A). 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. 3B). 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. 4A). 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. 4B). 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. 4C). 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;
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. 4D). 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. 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. Lc, muscle isometric tensile strength of left leg (injured); Rn, 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).

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).
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+ 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 regeneration of 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. 4D). 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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No conflicts of interest, financial or otherwise, are declared by the author(s).

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