Research Article

Transcutaneous carbon dioxide application with hydrogel prevents muscle atrophy in a rat sciatic nerve crush model†

Running title: Transcutaneous CO_2 prevents muscle atrophy

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YS was responsible for the study conception and design. HN collected the data and performed the analysis and drafted the manuscript. AI and TU critically reviewed and contributed to the paper. All authors read and approved the final manuscript.

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Abstract

The acceleration of nerve regeneration remains a clinical challenge. We previously demonstrated that transcutaneous CO_2 application using a novel hydrogel increases the oxygen concentration in local tissue via an "artificial Bohr effect" with the potential to prevent muscle atrophy. In this study, we investigated the effect of transcutaneous CO_2 administration on limb function after peripheral nerve injury in a rat sciatic nerve injury model. In total, 73 Sprague–Dawley rats were divided into a sham group, a control group (crush injury to sciatic nerve and no treatment) or a CO_2 group (crush injury with transcutaneous CO_2 application). CO_2 was administered percutaneously for 20 min five times per week. Scores for the sciatic function index and pinprick test were significantly higher in the CO_2 group than control group. The muscle wet weight ratios of the tibialis anterior and soleus muscles were higher in the CO_2 group than control group. Electrophysiological examination showed that the CO_2 group had higher compound motor action potential amplitudes and shorter distal motor latency than the control group. Histological examination of the soleus muscle sections at postoperative week 2 showed shorter fiber diameter in the control group than in the CO_2 group. The mRNA expression of Atrogin-1 and MuRF-1 was lower, mRNA expression of VEGF and myogenin and MyoD was higher in CO_2 group at postoperative week 2 compared to the control group. *Clinical Significance*: Transcutaneous CO_2 application has the therapeutic potential to accelerate the recovery of muscle atrophy in peripheral nerve injury.

Keywords: Transcutaneous carbon dioxide application, peripheral nerve injury, Bohr effect, muscle atrophy

INTRODUCTION

Peripheral nerve injury is a serious health concern that sometimes results in restricted activity and lifelong disability[1]. Although microsurgical techniques for nerve injuries have improved during recent decades, the outcomes of peripheral nerve injury repair remain unsatisfactory[2]. Several adjunctive therapies, such as growth factors, stem cells, or synthetic materials, are used to promote peripheral nerve regeneration; however, the outcomes are not promising[3-5].

Carbon dioxide (CO_2) therapy in carbonated spas has been used historically in Europe as an effective treatment for cardiac diseases and skin lesions[6,7]. The administration of CO_2 can increase blood flow, initiate nitric oxide-dependent capillary formation, and partially increase oxygen pressure in local tissue, a phenomenon known as the Bohr effect[6,8]. Using a rat muscle injury model, we demonstrated that transcutaneous CO_2 therapy enhances muscle regeneration by inducing PGC-1a expression, which is an indicator of mitochondrial biogenesis and increasing the number of mitochondria in injured tissues[9]. This therapy also enhanced fracture healing in a rat model[10].

On the other hand, the effect of transcutaneous CO_2 administration on peripheral nerve injury has not determined yet. We hypothesized that the transcutaneous application of CO_2 enhances limb function recovery after peripheral nerve injury. In this study, we investigated the effect of transcutaneous CO_2 administration on limb function after a peripheral nerve injury using a rat sciatic nerve injury model.

METHODS

Animal Model and Surgical Procedure

The use of animals was approved by the Animal Care and Use Committee of the Kobe University School of Medicine. In total, 73 male 8-week-old Sprague–Dawley rats (CLEA Japan, Tokyo, Japan), each weighing 291.6±19.1g were used. General anesthesia was induced, and the right sciatic nerve was exposed at midthigh. The nerve was crushed with a hemoclip for 60 s at a point 6 mm proximal to the trifurcation of the nerve[11]. After the injury, the surgical wound was closed with 4-0 nylon sutures. In the sham surgery groups, the nerve was only exposed thereafter wound was closed in the same manner. In this model, nerve injury spontaneously recovers in 4-5 weeks[11-13]. The rats were randomly divided into three groups, a sham group, a control group (crush injury to sciatic nerve and no treatment) or a CO_2 group (crush injury with transcutaneous CO_2 application).

Procedure for Transcutaneous CO_2 Treatment

To enhance CO_2 administration to local tissue, we used a hydrogel that allows CO_2 absorption. The CO_2 hydrogel was composed of carbomer (0.65%), glycerin (5.00%), sodium hydroxide (0.18%), sodium alginate (0.15%), sodium dihydrogen phosphate (0.15%), methylparaben (0.10%), and deionized water (balance) and had a pH of 5.5 (International patent application number: WO2004/002393). The hind limb was shaved and hydrogel was applied to all the skin surface. This method is used in clinical research [9,10]. The skin area covered with the hydrogel was sealed with an

adaptor using a plastic bag, and 100% CO_2 gas was allowed to flow into the bag. CO_2 was administered for 20 min five times per week as described in a previous report[14].

Wet Muscle Weight and Histological Analysis

The tibialis anterior (TA) and soleus muscles were dissected and harvested at postoperative weeks 2 and 4. The wet muscle weight was measured on the injured and non-injured sides, and the ratio was calculated. Then, the muscle was frozen, and axial sections (10 μm thick) were cut and stained with hematoxylin and eosin. The myofiber cross-sectional area and the interstitial area between myofibers were calculated using ImageJ software and a light microscope.

Behavioral Evaluation

Sciatic Function Index (SFI)

Hind paw prints were recorded on prepared paper strips as follows. After the hind paws were dyed with blue ink, the animals walked on paper strips placed on a walking track, leaving three prints for each paw. The parameters measured in the prints of both the normal (N) and experimental (E) paws were print length (PL, or the longitudinal distance between the tip of the longest toe and the heel), toe spread (TS, or the transverse distance between the first and fifth toes), and intermediate toe spread (IT, or the transverse distance between the second and fourth toes) [15,16].

SFI was calculated as follows: SFI = –38.3[(EPL – NPL)/NPL] + 109.5[(ETS – NTS)/NTS] + 13.3[(EIT–NIT)/NIT] – 8.8. The prints were recorded and analyzed before surgery and at 2 and 4 weeks postoperatively. The same footprints were evaluated three times, and a mean value was

obtained for each animal for each period of observation. The walking track analysis clearly demonstrated individual hind limb muscle function [17, 18].

Paw Withdrawal Pinprick Score

To evaluate muscle power of ankle dorsiflexion, the hind paws were stimulated with a 27-G needle and the reaction was scored as follows: no response = 0, nonaversive response = 1, mild aversive response = 2, strong aversive response = 3, prolonged aversive behavior = 4. These scores were assigned by at least two observers[19].

Nerve Conduction Study

The animals were anesthetized, and serial motor nerve conductions studies were performed using an integrated evoked potential recording system (Neuropack Four Mini MEB-5304; Nihon-Koden, Tokyo, Japan). Subdermal recording electrodes were placed in the middle portion of the TA muscle, and a reference electrode was placed on the ipsilateral foot. A bipolar electrode used as a stimulating electrode was placed at a point 2 cm proximal to the trifurcation of the nerve. The voltage was kept constant, and the current was systematically increased to a maximum of 10 mA until distal motor latency (DML) and compound motor action potential (CMAP) amplitudes were calculated. The contralateral limb of each animal served as an internal control[20].

Analysis of Messenger RNA (mRNA) Expression

Total RNA was extracted from 10 mg thinly sliced tissue with 500 μl reagent (TRIzol; Invitrogen, Carlsbad, CA, USA) and purified with a purification kit (RNeasy Mini Kit; QIAGEN Science,

Germantown, MD, USA). Oligo(dT)-primed first-strand complementary DNA (cDNA) was synthesized (50 ng total RNA) using a transcription kit (High Capacity cDNA Transcription Kit; Applied Biosystems, Foster City, CA, USA). The quantification of mRNA transcription was performed with a real-time polymerase chain reaction (PCR) system (StepOne™; Applied Biosystems). Real-Time PCR reactions (20 μl) contained 0.5 μM forward primer, 0.5 μM reverse primer, and 1 μl of the cDNA template from the RT reaction, and 10 μl 10× master mix (Power SYBER green master mix; Applied Biosystems). The reaction conditions included 10 min at 95°C, followed by 40 cycles at 95°C (15 s) and 60°C (1 min). The level of each target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase. β-glucuronidase levels were expressed relative to the levels of the control group (ΔΔCT methods; Applied Biosystems) [9]. GAP-43 and S-100 were analyzed as nerve regeneration markers. For muscle regeneration analysis, VEGF, myogenin, MyoD, Atrogin-1, and MuRF-1 were analyzed.

Statistical Analysis

Data are shown as mean values ± standard deviation. The statistical analysis was performed using a spreadsheet (Excel 2010; Microsoft Corporation, Redmond, WA, USA) with add-ins (Statcel2; OMS Publishing Inc., Tokorozawa, Japan). For walking track testing, pinprick testing, wet muscle weight, and electrophysiological examination were analyzed using repeated measured ANOVA, followed by Tukey's post hoc tests. The difference between two groups (histological examination and gene expression analysis) was determined with the two-sided Mann–Whitney *U* test. A p value of <0.05

was considered statistically significant.

RESULTS

Behavioral Examination

SFI Score

SFI scores improved in control and CO_2 groups over time. The sham group showed significantly the highest score throughout the examination period. The scores of CO_2 group were significantly higher than in the control group at postoperative weeks 2 and 4. (Figure 1a)

Paw Withdrawal Pinprick Score

Pinprick scores improved in control and CO_2 groups over time. Compared with the control, the CO_2 group had significantly higher scores at postoperative weeks 2 and 4. At postoperative 4 weeks, a statistical difference was not seen between the CO_2 group and sham surgery group.(Figure 1b).

Muscle Weight Ratio (Ratio = Crush/Control Side)

The wet weight of the TA and soleus muscles increased over time in control and CO_2 groups . The wet muscle weight ratio of CO2 group was significantly higher than in the control group, while the statistical difference was not seen compared to the sham group.(Figure 2a, 2b).

Nerve Conduction Study

The CMAP amplitude ratio (crush side/contralateral normal side) in each animal was calculated. Compared with the control group, the CO_2 group had a higher CMAP amplitude at each time point. Significant differences between the two groups were found at postoperative weeks 2 and 4 ($p < 0.05$;

Figure 3a). There were no significant differences between the CO_2 group and the sham group at postoperative weeks 2 and 4.

The DML ratio was calculated in the same manner. Significant differences between the control group and the CO_2 group were found at postoperative week 2 ($p < 0.05$) and 4 ($p < 0.01$). Compared with the control group, the CO_2 group had shorter DML at postoperative week 4. There were no significant differences between the CO_2 group and the sham group at postoperative weeks 2 and 4.(Figure 3b).

Histological Examination

At postoperative week 2, the fiber diameter of the soleus muscle was reduced in the control group (Figure 4a), whereas it was normal in the CO_2 group (Figure 4b). At postoperative week 4, no reduction in muscle fiber diameter had occurred in either group (Figure 4c, 4d). Myofiber cross-sectional area was statistically larger in CO_2 group at postoperative 2 weeks. (Figure 4e) The interstitial area between the myofibers was also analyzed (Figure 4f), and a significant difference was found between the control group and the CO_2 group at postoperative week 2. No significant difference was found between the groups at postoperative week 4.

Real-Time PCR Analysis

Compared with the control group, the CO_2 group showed greater gene expression of GAP-43 and S-100 as nerve regeneration markers, and a statistically significant difference was found in S-100 expression at postoperative week 4 (Figure 5a, 5b).

The expressions of VEGF and myogenic regulatory factor (myogenin and MyoD) were significantly higher in CO_2 group at postoperative 2 weeks. (Figure 5c, 5d, 5e).The gene expression of MuRF-1 as a muscle atrophy marker was significantly decreased in the CO_2 group at postoperative week 2. On the contrary, the expression of Atrogin-1 did not differ significantly between the groups (Figure 5f, 5g).

DISCUSSION

Prolonged motor dysfunction associated with muscle atrophy after peripheral nerve injury can have serious impacts on patient quality of life and is a societal health concern[21]. Although peripheral nerves can regenerate via axonal growth after appropriate initial treatment, in the majority of cases, muscle reinnervation is insufficient to regain muscle strength. The axotomy of peripheral nerves leads to a rapid decline in muscle mass, which is reversible if adequate muscle reinnervation occurs within approximately 2 months. In cases of sustained denervation, patients show progressive atrophy, myocyte death, and fibrosis with irreversible muscle impairment. At 6 months after injury, denervated muscle weighs 5%–10% of its original weight and is replaced primarily by non-contractile connective tissue[22-27]. Therefore, the prevention of muscle atrophy is crucial in the treatment of nerve injury.

Several approaches have been used to treat denervated muscle. Schaakxs D et al. transplanted injected adipose-derived stem cells or differentiated Schwann cells into the gastrocnemius muscle after sciatic nerve injury in a rat model and showed that walking track test scores and muscle

morphology were superior in the Schwann cell treatment group[28]. The injection of muscle satellite cells into denervated muscle has also resulted in improved muscle morphology at early time points after injury. However, the use of stem cells is costly and time-consuming, and it is not widely accepted clinically[29]. Another approach is the use of mechanical muscle stimulation. Electrical stimulation (ES) is widely used clinically. Gigo-Benato et al. reported that the application of ES to denervated rat muscle every 48 h suppressed the mRNA expression of muscle atrophy markers. However, no significant difference in muscle fiber diameter was found between the experimental group and controls[30,31]. Some clinicians consider ES to have only limited effects. Moreover, one study reported that ES can delay the innervation of muscle[32].

The benefits of carbonated spa use (CO_2 therapy) have long been acknowledged in Europe in the treatment of cardiac diseases and skin problems[7]. Artificial CO_2 enriched water baths have also been clinically applied to improve ischemic limb symptoms. These therapeutic effects of CO_2 are due to an increase in blood flow and microcirculation, nitric oxide-dependent capillary formation, and a partial increase in oxygen pressure in local tissues (the Bohr effect) [8]. We have reported that the transcutaneous application of CO_2 using a novel hydrogel has therapeutic effects and enhances muscle regeneration and fracture healing[10,33].

The present study analyzed the effect of transcutaneous CO_2 application on nerve injury using an axonotmesis model, which mimics clinical situations such as traction or compression injuries. In the axonotmesis model, spontaneous recovery of limb function can be expected. However, in a clinical

situation, muscle weakness can be prolonged in some cases even though regenerated nerve reaches to muscle after the injury. This is because the numbers of neuro-muscular junction are reduced before the nerve reaches the target. Administration of transcutaneous CO_2 potencially prevent this problem since the results showed shorter DML and higher nerve regeneration markers in the CO_2 treatment group. Better outcomes in the behavioral assessments (walking track analysis and paw withdrawal test) occurred in the CO_2 group, such as higher wet weight muscle ratios, higher CMAP values in nerve conduction analysis, and larger fiber diameter in CO_2 administration group. In gene expression analysis lower gene expression of muscle atrophy markers and higher expression of myogenic markers and VEGF were seen, particularly at earlier time points after injury.

Our previous report demonstrated the upregulation of VEGF expression in rat muscle after transcutaneous CO_2 application[33]. The upregulation of VEGF expression increase vascularization in the muscle. Expressions levels of MyoD and myogenin were increased at postoperative weeks 2 after nerve crush in the CO_2 group, which may be considered a developmental stage of the early regeneration process where MyoD and myogenin enhanced muscle-cell differentiation[33]. The present study also showed the upregulation of VEGF and MyoD and myogenin which might accelerate muscle regeneration and prevent muscle atrophy after nerve crush by the transcutaneous CO_2 application.

The present report has several limitations. The time and frequency of CO_2 application were determined according to the previous reports[9,10,33]. We did not optimize these conditions in the

current model due to the number of animals. The mechanism of the CO_2 effect might be the same as that reported previously; however, we did not assess it in the same manner. The model used in the study is axonotmesis model which shows spontaneous recovery in several weeks. To assess the effect of CO_2 over a longer period, more severe injury model such as neurotmesis model should be assessed.

In conclusion, the transcutaneous application of CO_2 using hydrogel appears to have therapeutic potential for accelerating the recovery of muscle atrophy in peripheral nerve injury.

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Conflict of interest: One of the authors of this paper is a board member of NeoChemir Inc. and has a financial relationship with the organization that could influence the content of the paper.

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Figure Legends

Figure 1.

Behavioral examination.

(a) SFI scores improved in control and CO_2 groups over time. The sham group showed significantly the highest score throughout the examination period. The scores of CO_2 group were significantly higher than in the control group at postoperative weeks 2 and 4. *; $p < 0.05$ vs control and $p < 0.01$ vs. sham, **; $p < 0.01$ vs. control and sham

(b) Pinprick scores (stimulated by pain induced with a 27-G needle) improved in control and CO_2 groups over time. Compared with the control, the CO_2 group had significantly higher scores at postoperative weeks 2 and 4. At postoperative 4 weeks, a statistical difference was not seen between the CO_2 group and sham surgery group. W, weeks. *; $p < 0.05$, **; $p < 0.01$

Figure 2.

Muscle weight ratio (ratio = crush/control side).

In the (a) tibialis anterior (TA) and (b) soleus (SOL) muscles, wet weight of the TA and soleus muscles increased over time in control and CO_2 groups. The wet muscle weight ratio of CO_2 group was significantly higher than in the control group, while statistical difference was not seen compared to the sham group. W, weeks. *; $p < 0.05$, **; $p < 0.01$

Figure 3.

Nerve conduction study.

(a) The CMAP amplitude ratio (crush side/contralateral normal side) . Compared with the control group, the CO_2 group had a higher CMAP amplitude at each time point. Significant differences between the two groups were found at postoperative weeks 2 and 4 ($p < 0.05$). There were no significant differences between the CO_2 group and the sham group at postoperative weeks 2 and 4. W, weeks. *; $p < 0.05$, **; $p < 0.01$

(b) Distal motor latency ratio (crush side/contralateral normal side). Significant differences between the control group and the CO_2 group were found at postoperative week 2 ($p < 0.05$) and 4 ($p < 0.01$). Compared with the control group, the CO_2 group had shorter DML at postoperative week 4. There were no significant differences between the CO_2 group and the sham group at postoperative weeks 2 and 4. W, weeks. *; $p < 0.05$, **; $p < 0.01$

Figure 4.

Histological examination (hematoxylin and eosin staining).

The fiber diameter of the soleus muscle was reduced in the control group (a) and normal in the CO_2+ group (b) at postoperative week 2. No reduction in muscle fiber diameter was found in the control (c) or CO_2+ group (d) at postoperative week 4. A significant difference in myofiber cross-sectional area was found between the groups at postoperative week 2 (e). A significant difference in interstitial area between myofibers was found between the groups at postoperative week 2 (f). W, weeks. *; $p < 0.05$, **; $p < 0.01$

Figure 5.

Real-time polymerase chain reaction.

The gene expression of the nerve regeneration markers GAP-43 (a) and S-100 (b) in the CO_2 group was higher than that in the control group, and a statistically significant difference was found in S-100 expression at postoperative week 4 ($p < 0.01$). The expressions of VEGF (c) and myogenic regulatory factor (myogenin (d) and MyoD (e)) were significantly higher in CO_2 group at postoperative 2 weeks. The gene expression of the muscle atrophy marker Atrogin-1 (f) did not differ significantly between the groups. On the contrary, the gene expression of another muscle atrophy marker, MuRF-1 (g), was significantly lower in the CO_2 group at postoperative week 2 ($p < 0.01$). mRNA, messenger RNA; W, weeks. *; $p < 0.05$, **; $p < 0.01$

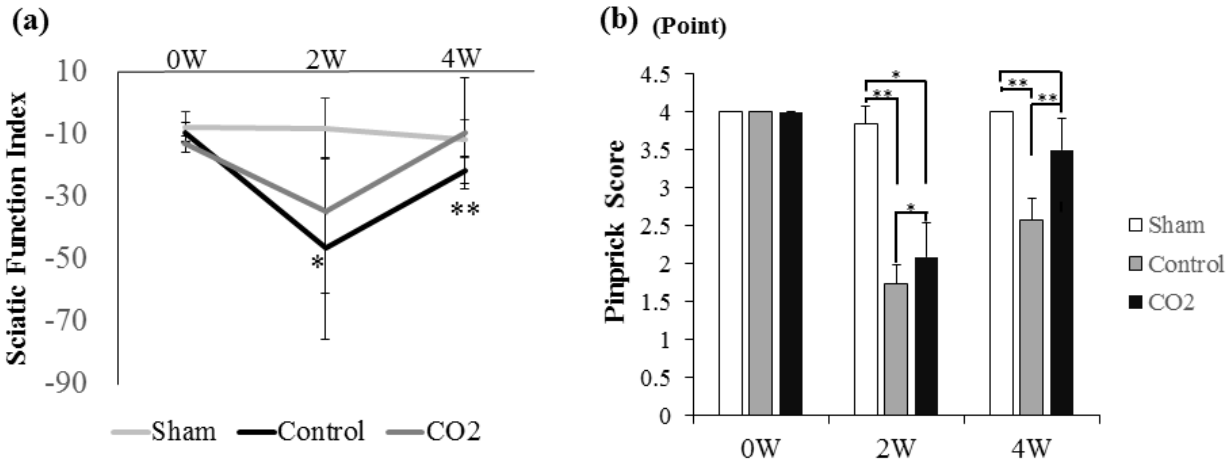
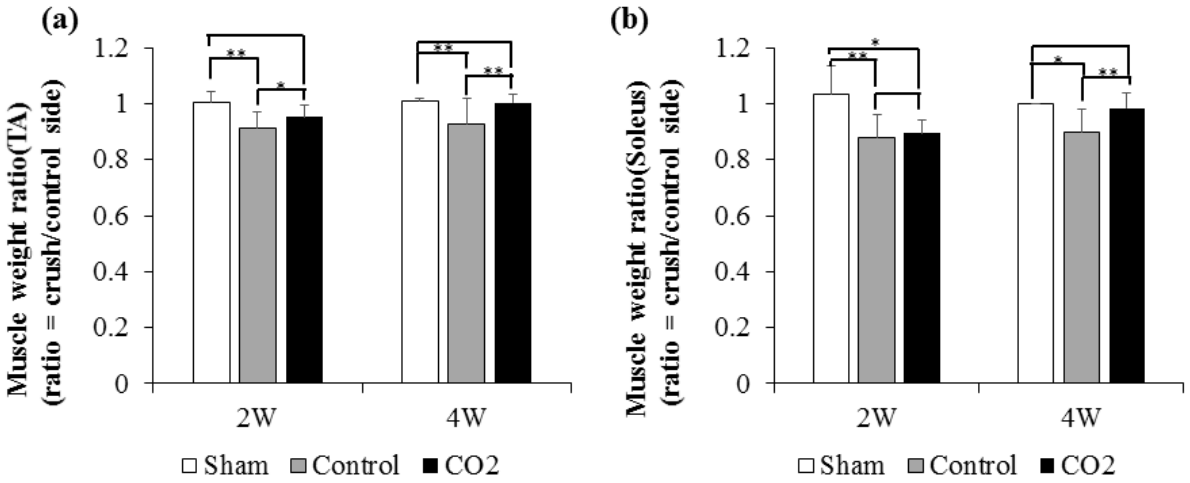


Figure 1



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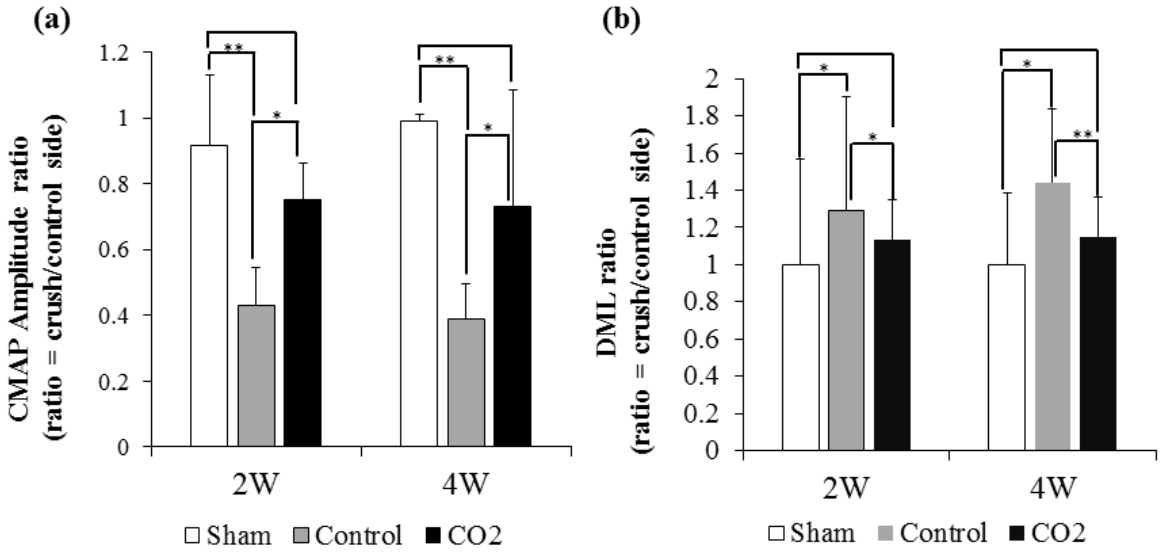

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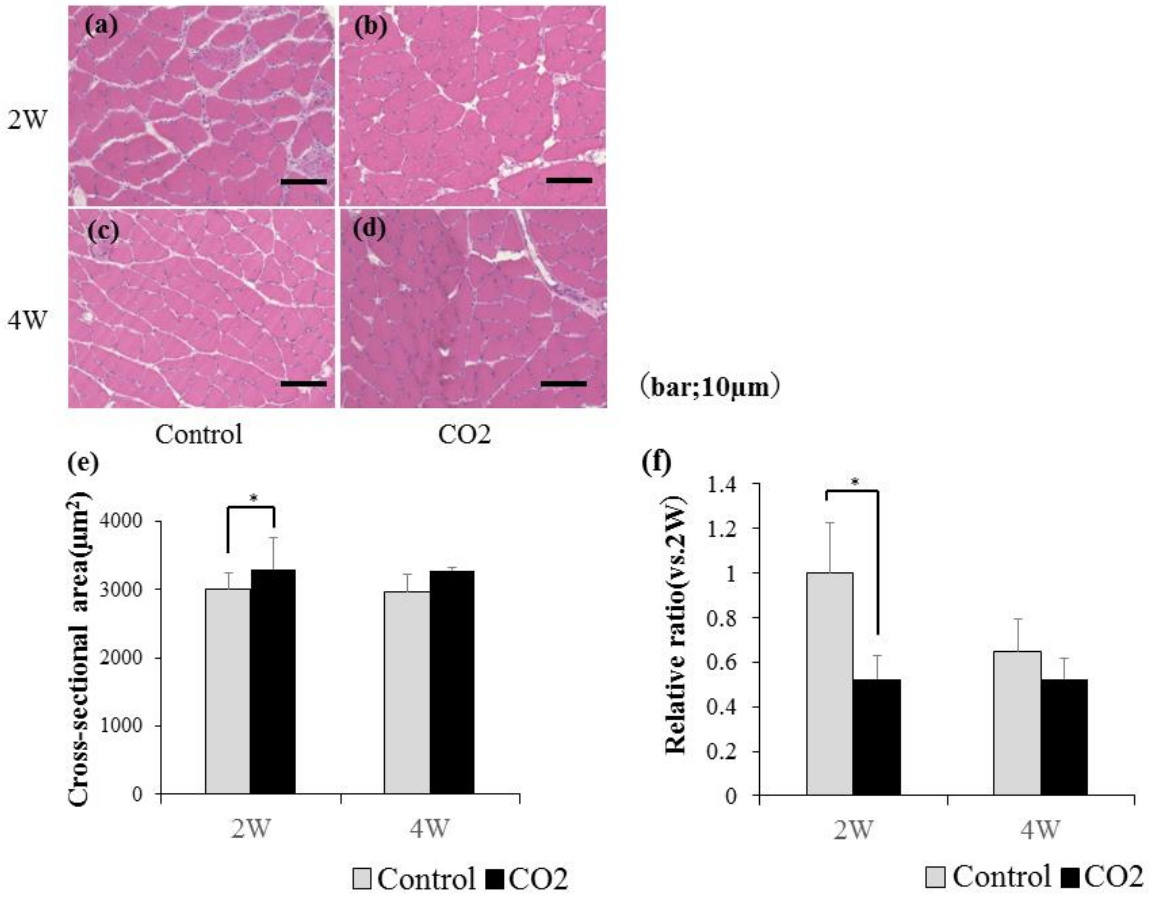

Figure 4

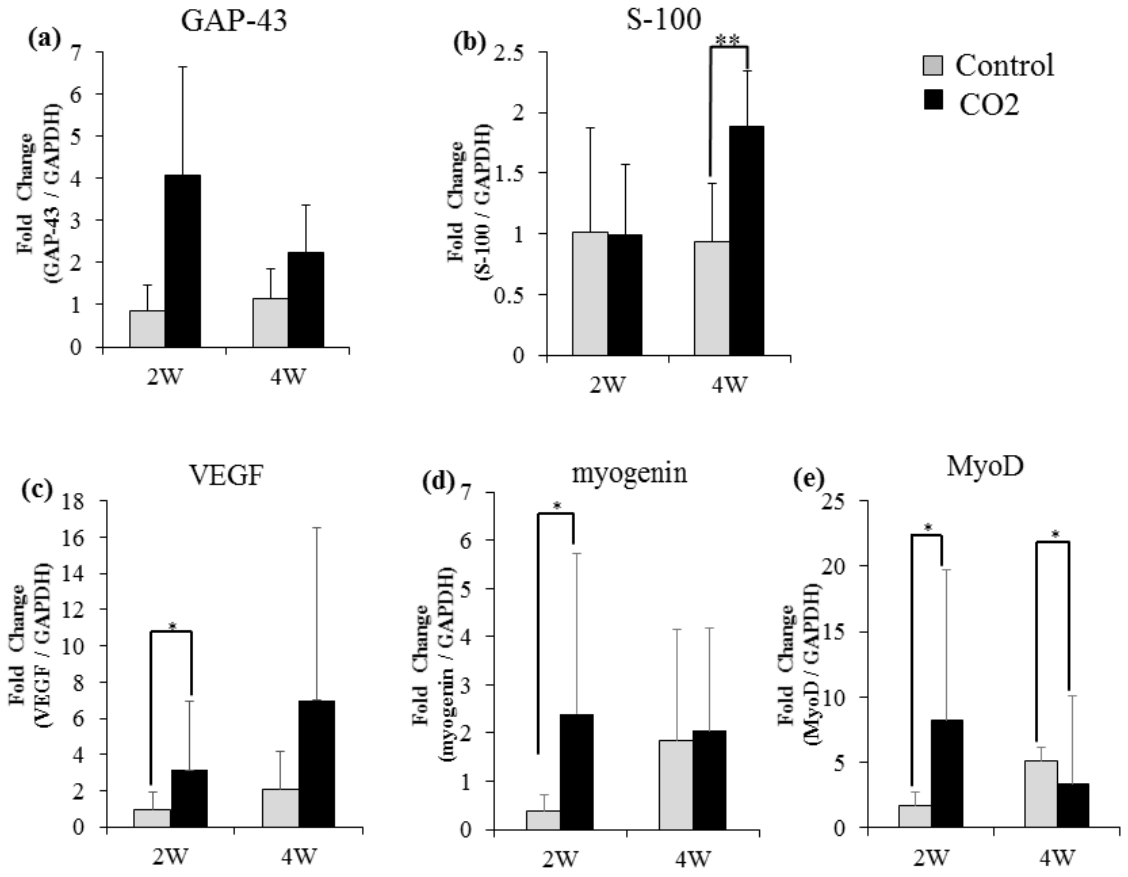

Figure 5

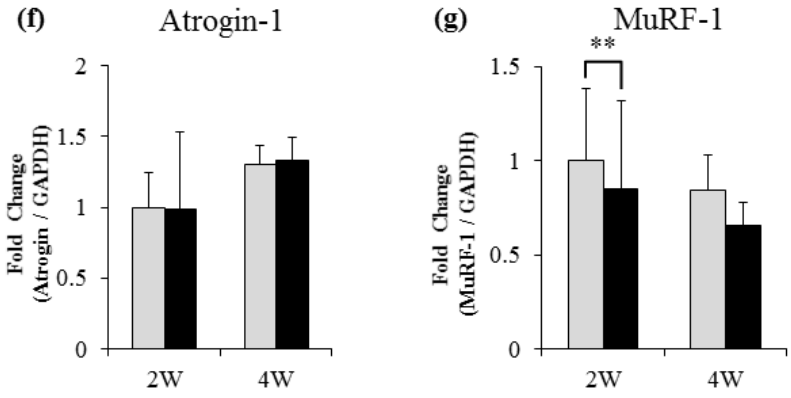

Figure 5