Transcutaneous Carbon Dioxide Improves Contractures After Spinal Cord Injury in Rats

Inoue, Shota ; Moriyama, Hideki ; Yakuwa, Takumi ; Mizuno, Eriko ; Suzuki, Ryota ; Nomura, Masato ; Sakai, Yoshitada ; Akisue, Toshihiro

(Citation)
Clinical Orthopaedics and Related Research,477(8):1934-1946

(Issue Date)
2019-08

(Resource Type)
journal article

(Version)
Accepted Manuscript

Transcutaneous Carbon Dioxide Improves Contractures After Spinal Cord Injury in Rats

Running Title: Carbon Dioxide Therapy for Contracture

Shota Inoue BSc, Hideki Moriyama PhD, Takumi Yakuwa BSc, Eriko Mizuno BSc, Ryota Suzuki BSc, Masato Nomura MSc, Yoshitada Sakai MD, PhD, Toshihiro Akisue MD, PhD

S. Inoue, T. Yakuwa, E. Mizuno, R. Suzuki, M. Nomura
Department of Rehabilitation Science, Graduate School of Health Sciences, Kobe University, Kobe, Japan

H. Moriyama, T. Akisue
Life and Medical Sciences Area, Health Sciences Discipline, Kobe University, Kobe, Japan

Y. Sakai
Division of Rehabilitation Medicine, Kobe University Graduate School of Medicine, Kobe, Japan

Three of the authors (HM, YS, and TA) have received funding from the Japan Society for the Promotion of Science KAKENHI Grant No. 17K19908. Each author certifies that neither he, nor any member of his immediate family, has any commercial associations (consultancies, stock ownership, equity interest, patent/licensing arrangements, etc.) that might pose a conflict of interest in connection with the submitted article.

All ICMJE Conflict of Interest Forms for authors and *Clinical Orthopaedics and Related Research*® editors and board members are on file with the publication and can be viewed on request.

Each author certifies that his institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.

This work was performed at Kobe University, Kobe, Japan.

H. Moriyama ✉
Life and Medical Sciences Area, Health Sciences Discipline, Kobe University
Tomogaoka 7-10-2, Suma-ku, Kobe, Hyogo, 654-0142, Japan
Email: morihide@harbor.kobe-u.ac.jp

Abstract

Background Joint contractures are a major complication in patients with spinal cord injuries. Positioning, stretching, and physical therapy are advocated to prevent and treat contractures; however, many patients still develop them. Joint motion (exercise) is crucial to correct contractures. Transcutaneous carbon dioxide (CO_2) therapy was developed recently, and its effect is similar to that of exercise. This therapy may be an alternative or complementary approach to exercise.

Question/purposes Using an established model of spinal cord injury in rats with knee flexion contractures, we sought to clarify whether transcutaneous CO_2 altered (1) contracture, as measured by ROM; (2) muscular and articular factors contributing to the loss of ROM; (3) fibrosis and fibrosis-related gene expression in muscle; and (4) the morphology of and fibrosis-related protein expression in the joint capsule.

Methods Thirty-six Wistar rats were divided into three equal groups: caged control, those untreated after spinal cord injury, and those treated with CO_2 after spinal cord injury. The rats were treated with CO_2 from either the first day (prevention) or 15th day (treatment) after spinal cord injury for 2 or 4 weeks. The hindlimbs of rats in the treated group were exposed to CO_2 gas for 20 minutes once daily. Knee extension ROM was measured with a goniometer and was measured again after myotomy. We calculated the muscular and articular factors responsible for contractures by subtracting the post-myotomy ROM from that before myotomy. We also quantified histologic muscle fibrosis and evaluated fibrosis-related genes (collagen Type 1, α1 and transforming growth factor beta) in the biceps femoris muscle with real-time polymerase chain reaction. The synovial intima's length was measured, and the distribution of fibrosis-related proteins (Type I collagen and transforming growth factor beta) in the joint capsule was observed with immunohistochemistry. Knee flexion contractures developed in rats after spinal cord injuries at all timepoints.

Results CO_2 therapy improved limited-extension ROM in the prevention group at 2 weeks (22° ± 2°) and 4 weeks (29° ± 1°) and in the treatment group at 2 weeks (31° ± 1°) compared with untreated rats after spinal cord injuries (35° ± 2°, mean difference, 13°; 39° ± 1°, mean difference, 9°; and 38° ± 1°, mean difference, 7°, respectively) (95% CI, 10.50-14.86, 8.10-10.19, and 4.73-9.01, respectively; all $p < 0.001$). Muscular factors decreased in treated rats in the prevention group at 2 weeks (8° ± 2°) and 4 weeks (14 °± 1°) and in the treatment group at 2 weeks (14 ± 1°) compared with untreated rats (15° ± 1°, 4.85-9.42; 16° ± 1°, 1.24-3.86; and 17° ± 2°, 1.16-5.34, respectively; all $p < 0.05$). The therapy improved articular factors in the prevention group at 2 weeks (4° ± 1°) and 4 weeks (6° ± 1°) and in the treatment group at 2 weeks (8° ± 1°) compared with untreated rats (10° ± 1°, 4.05-7.05; 12° ± 1°, 5.18-8.02; and 11° ± 2°, 1.73-5.50, respectively; all $p < 0.05$). CO_2 therapy decreased muscle fibrosis in the prevention group at 2 weeks ($p < 0.001$). The expression of collagen Type 1, α1 mRNA in the biceps femoris decreased in treated rats in the prevention group at 2 and 4 weeks compared with untreated rat ($p = 0.002$ and $p = 0.008$, respectively), although there was little difference in the expression of transforming growth factor beta ($p > 0.05$). CO_2 therapy did not improve the shortening of synovial intima at all time-points (all $p > 0.05$). CO_2 therapy decreased transforming growth factor beta immunolabeling in joint capsules in the rats in the prevention group at 2 weeks. The staining intensity and Type I collagen pattern showed no differences among all groups at all timepoints.

Conclusion CO_2 therapy may be useful in preventing and treating contractures after spinal cord injuries. CO_2 therapy particularly appears to be more effective as a prevention and treatment strategy in early-stage contractures before irreversible degeneration occurs in a rat model.

Clinical Relevance Our findings support the idea that CO_2 therapy may be able to improve the loss of ROM after spinal cord injury.

Introduction

Joint contractures are a major complication in patients with central nervous system injuries [7, 8, 10]. They may result in decreased quality of life and ability to perform activities of daily living in patients with neurologic disorders. Contracture is characterized by limitations in passive ROM that result from structural changes in the periarticular soft tissues. Positioning, stretching, and physical therapy are advocated to prevent and treat contractures. The usefulness of these approaches has been validated in many clinical [16, 24] and animal studies [21, 32, 44] that determined their therapeutic efficacy. Nevertheless, we often see patients who have joints with contractures and no functional use of their limbs. Therefore, novel treatment strategies are needed to prevent and treat contractures.

Recently, transcutaneous carbon dioxide (CO_2) therapy has been developed, and it has been used in humans and a variety of animal models to evaluate its potential use as a treatment for a number of conditions. It can accelerate fracture healing [26], muscle injury repair [1], and tumor regression [38] in animals and improve lower-limb arteriopathy [40] and skin irregularities [6] in humans. Additionally, transcutaneous CO_2 may be therapeutic for recovery of muscular strength and improvement in muscle endurance [37]. It also generates muscle fiber type switching, leading to increased mitochondria and angiogenesis in the muscles of rats [37]. These effects are similar to that of exercise [20]; therefore, transcutaneous CO_2 therapy may be a suitable alternative or complementary approach to exercise in treating patients with contractures after spinal cord injuries.

We previously established a rat model of contracture after spinal cord injury as an animal model of central nervous system injury [31, 33–36] and showed that joint motion is crucial to prevent and treat contractures [21, 32]. Based on the findings of studies about the potential use of CO_2 as a treatment [1, 6, 26, 37, 38, 40], we believe that transcutaneous CO_2 therapy may be an alternative therapeutic strategy in patients with contractures, but to our knowledge,

this has not been evaluated. We therefore sought to evaluate the use of transcutaneous CO_2 to prevent and treat contractures after spinal cord injury in rats.

Using an established model of spinal cord injury in rats with knee flexion contractures, we sought to clarify whether transcutaneous CO_2 altered (1) contracture, as measured by ROM; (2) muscular and articular factors contributing to the loss of ROM; (3) fibrosis and fibrosis-related gene expression in muscle; and (4) the morphology of and fibrosis-related protein expression in the joint capsule.

Materials and Methods

Experimental Design

All experimental procedures were approved by our institutional animal care and use committee and performed according to the Kobe University Animal Experimentation Regulations (approval number: P160506). Thirty-six 10-week-old male Wistar rats weighing 320 to 340 g (Japan SLC Inc., Shizuoka, Japan) were used in this study.

The rats were randomly divided into the following three groups: normal caged rat group (control group, n = four rats per timepoint), those that were untreated after spinal cord injury (spinal cord injury group, n = three rats per timepoint), and those that were treated with CO_2 after spinal cord injury (CO_2 group, n = three rats per timepoint) (Fig. 1). The rats were evaluated at 2 or 4 weeks after the start of the experiment. As a preventive intervention, rats in the CO_2 group were treated with CO_2 from the first postoperative day. We previously reported that knee flexion contractures developed in rats with spinal cord injuries for the first 2 weeks after injury [35]. Therefore, as a treatment intervention, rats in the CO_2 group were treated with CO_2 from the 15th day postoperatively. The right and left knee joints of all rats in each group served as different samples (the control group, n = eight limbs per timepoint, the spinal cord injury and CO_2 groups, n = six limbs per timepoint). The samples of the

control group in the prevention group at 4 weeks and treatment group at 2 weeks were obtained from the same animals (n = four rats). The subgroup sample sizes were calculated with a power analysis based on pilot results detecting a 10° difference in ROM 19 of 20 times [21, 32]. Knee flexion contractures developed in rats after spinal cord injury at all timepoints. The animals were housed in polycarbonate cages with bedding and were maintained under artificial conditions at 22° ± 1 ℃, with constant humidity of 55% ± 5% and a 12-hour light-and-dark cycle. They were allowed free access to standard food and water 24 hours a day.

Surgical Procedure and Postoperative Care

Surgical procedures and postoperative care conformed to the protocol used in our previous studies [33–36]. The 24 rats in the spinal cord injury and CO_2 groups were administered NARCOBIT-EII-type isoflurane (Natsume Seisakusyo Inc., Tokyo, Japan). After the spinal cord was exposed by laminectomy of the T8 vertebra, it was completely transected at the level of T8. This procedure leads to the development of a knee joint flexion contracture, although the knee joint is not violated, as we have demonstrated previously [31]. After surgery, the rats received subcutaneous buprenorphine 0.02 mg/kg as an analgesic every 12 hours for 3 days after spinal cord injury. In addition, the bladders of all rats with spinal cord injuries were compressed manually twice daily throughout the experimental period. All animals were monitored for decubitus ulcers and self-injury every day throughout the experimental period.

CO_2 Therapy Protocol

Transcutaneous CO_2 absorption-enhancing hydrogel was provided by NeoChemir Inc. (Kobe, Japan) as previously described [39]. Briefly, the bilateral hindlimbs of the rats in the CO_2 groups after the 15th day were shaved and hydrogel was applied, which promoted absorption of CO_2 into the hindlimbs. A CO_2 adaptor was attached to the limbs and sealed, and the entire

limb was exposed to diluted 100% CO_2 gas that was absorbed percutaneously (Fig. 2). This therapy was applied daily for 20 minutes as described previously [43]. We took care to ensure that the rats' knee joints were not moved during the therapy. CO_2 gas was flowed in through the CO_2 adaptor to absorb it sufficiently, and the hair on the hindlimb was shaved once a week. In the rats in the spinal cord injury group, as a sham intervention, we applied only the hydrogel sealed with the CO_2 adaptor.

ROM Measurements and Determination of Muscular and Articular Factors

At the end of the experimental period, knee motion was measured as described in our previous studies [35, 36]. Briefly, knee motion in extension was measured with a goniometer, and we applied a standardized torque (0.06 Nm) with the rats under anesthesia with isoflurane. Earlier, we reported that the normal extension ROM of healthy rats is approximately 15° [35]. Two examiners (RS, NS) measured each limb five times, and the average of the 10 measurements was calculated. After ROM was measured, the animals were euthanized and exsanguinated while they were under anesthesia. Myotomy of the transarticular muscles was then performed and ROM was measured again. After myotomy, measurements were made within 15 minutes of the animals' deaths to minimize the possibility of postmortem rigidity. The muscular factor that contributes to contractures was defined as limited ROM in the tendons and fascia, and the articular factor was defined as limited ROM in the articular components (bone, cartilage, synovium, capsules, and ligaments). Per our previous method [32], we measured ROM before and after myotomy using the following formulas, which allowed us to isolate the muscular and articular factors that contribute to contractures: muscular factor = ROM without myotomy – ROM after myotomy (within each group); articular factor = ROM after myotomy in each group – ROM after myotomy in the control group.

Quantification of Fibrosis in Muscle Tissue

After ROM was measured, the biceps femoris of all animals was harvested, and the ratio of skeletal muscle weight to whole-body weight was calculated. The muscles were quickly frozen in isopentane at -75 ℃ and stored at -80 ℃ until they were analyzed. Cross-sections of 10 μm (three sections per sample) were prepared from frozen muscle samples with a cryostat (CM1860; Leica, Hessen, Germany). Six sections were randomly selected from each group at each timepoint. The sections were stained with picrosirius red. We quantified fibrosis in muscle tissue by slightly modifying the method of Hadi et al. [14]. We quantified fibrosis by identifying the yellow color of muscle cells and the red color of connective tissue using the threshold color plugin in ImageTool software (Image J 1.50b; National Institutes of Health, Bethesda, MD, USA). The area of each color was measured separately. We calculated the percentage of connective tissue area in the muscle tissue by dividing the connective tissue area by the total area, manually excluding the blood vessels and voids. Measurements were done by one investigator (SI) and the results were confirmed by a blinded observer (HM).

Real-time Polymerase Chain Reaction

We evaluated the mRNA expression levels of Type I collagen and transforming growth factor (TGF)-β1, a marker of fibrosis and tissue stiffness, in the biceps femoris. Six samples were randomly selected from each group at each timepoint. Total RNA of the frozen tissue was isolated with the RNeasy Plus Universal Mini kit (Quiagen, Hilden, Germany) according to the manufacturer's protocol. The purity and concentration of the isolated total RNA were measured with BioPhotometer D30 (Eppendorf, Hamburg, Germany). Reverse transcription was performed using total RNA and the TaqMan™ Fast Virus 1-Step Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA). mRNA was quantitatively analyzed with the StepOne real-time polymerase chain reaction system (Thermo Fisher Scientific Inc., Waltham, MA, USA) with TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA) for collagen Type I α 1 ([COL1A1]; Rn01463848_m1) mRNA, TGF-β1

(Rn00572010_m1) mRNA, and ribosomal protein S18 rRNA (Rn01428913_gH). All mRNA levels were calculated as ratios of the quantity of ribosomal protein S18 rRNA in the same cDNA sample.

Measurements of the Synovial Intima Length

The periarticular muscles were harvested to avoid damaging the soft tissue around the knee. The knee sections were prepared according to the protocols established by Kawamoto [25]. The entire knee from the distal femur to the proximal tibia was excised, frozen, and embedded with the SCEM embedding agent (8091140, Leica, Hessen, Germany) in isopentane at -75 ℃. Cross-sections of 5 μm (four sections per sample) at the medial meniscus level in the sagittal plane were prepared from non-demineralized frozen samples using a cryostat. Six sections were randomly selected from each group at each timepoint. We measured the posterior synovial intima's length to quantify adhesions to and atrophy in the joint capsule according to Ando et al.'s method [2]. The synovial lining's contour was traced on the histologic sections stained with hematoxylin and eosin, and its length was measured with ImageTool software (Image J; 1.50i; National Institutes of Health, Bethesda, MD, USA). The length of the superior and inferior subdivisions of the synovial intima in the posterior joint capsule were summed to provide the total length of the synovial intima. Measurements were done by one investigator (SI) and the results were confirmed by a blinded observer (HM).

Immunohistochemistry Analysis

We qualitatively observed the distribution of Type I collagen and TGF-β1 in the knee joint capsules. Three sections were randomly selected from each group at each timepoint. The frozen sections of the knee joints were air-dried for 30 seconds, fixed in 100% ethanol for 2 minutes and 4% paraformaldehyde/0.01 M phosphate-buffered saline ([PBS], pH 7.4) for 2

minutes, and rehydrated in PBS for 5 minutes. The sections were treated with 0.5% hyaluronidase in PBS for 60 minutes as an antigenicity activation treatment. After rinsing the sections twice in PBS for 5 minutes each time, we inactivated endogenous peroxidase by incubating the sections in methanol containing 0.3% H_2O_2 for 20 minutes. We blocked nonspecific reactions by treating the sections with 1% normal horse serum (VEC S-2000, Vector Laboratories, Burlingame, CA, USA) for 60 minutes. After removing the blocking solution, we incubated the sections with mouse monoclonal anti-Type I collagen (diluted 1:4000; C2456, Sigma-Aldrich, St. Louis, MO, USA) and anti-TGF-β (diluted 1: 50; ab64715, Abcam, Cambridge, UK) antibodies at 4 ℃ overnight. The sections were rinsed in PBS and incubated with horse biotinylated anti-mouse immunoglobulin G (diluted 1:250; BA-2001, Vector Laboratories, Burlingame, CA, USA) for 60 minutes at room temperature (22 ℃). A subsequent reaction was made with the streptavidin-biotin-peroxidase complex technique using the Elite ABC kit (diluted 1:50; PK-6100; Vector Laboratories) for 30 minutes. Immunoreactivity was observed using 3,30-diaminobenzidine tetrahydrochloride (K3466; Dako Japan, Tokyo, Japan). Finally, we counterstained the sections with hematoxylin. One investigator (SI) was blinded to the observations and performed them in random order.

Outcomes of Interest

Our primary study outcome was the use of transcutaneous CO_2 to prevent and treat contractures after spinal cord injury in rats, which we measured with ROM. Our secondary study outcomes were its use in improving alterations in muscle and the joint capsule that contribute to contractures, which we measured with biomechanical, histomorphometric, biochemical, and immunohistochemical analyses.

Statistical Analysis

ROM results, the percentage of connective tissue area in the muscle tissue, synovial intima length, and the expression levels of Type I collagen and TGF-β1 mRNAs were analyzed statistically with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [23]. All results were checked for normality with the Shapiro-Wilk test. Normality was observed in all analyses; thus, the results were compared among all groups using ANOVA followed by Tukey's honestly significant difference test. An α value less than 0.05 was considered significant. Muscular and articular factors in rats with contractures were statistically analyzed with Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA). We calculated the SD and mean difference among the groups, and 95% CIs were estimated.

Results

Animals

All animals survived throughout the experimental period and appeared to be healthy, except for spinal cord-injured rats with functional deficits in the hindlimbs.

Limitation to ROM

Extension ROM decreased in rats treated with CO_2 in the prevention group at 2 weeks (22° ± 2°) and 4 weeks (29° ± 1°) and in the treatment group at 2 weeks (31° ± 1°) compared with rats in the spinal cord injury group at each timepoint (35° ± 2°, 39° ± 1°, and 38° ± 1°, respectively) (95% CI, 10.50-14.86, 8.10-10.19, and 4.73-9.01, respectively; all $p < 0.001$) (Fig. 3A). However, extension ROM in all CO_2 groups did not recover to the same range as that in the control group (control group versus CO_2 prevention group: 16° ± 1° versus 22° ± 2° at 2 weeks; 16° ± 1° versus 29° ± 1° at 4 weeks; control group versus CO_2 treatment group; 16° ± 1° versus 31° ± 1° at 2 weeks; 16° ± 1° versus 37° ± 1° at 4 weeks) (95% CI, 4.31-8.38, 12.62-14.57, 13.42-17.43, and 19.98-22.43, respectively; all $p < 0.001$).

Muscular and Articular Factors

Muscular factors decreased in rats treated with CO_2 in the prevention group at 2 weeks (8° ± 2°) and 4 weeks (14° ± 1°) and in the treatment group at 2 weeks (14° ± 1°) compared with rats in the spinal cord injury group at each timepoint (15° ± 1°, 16° ± 1°, and 17° ± 2°, respectively; $p < 0.05$), except for treatment at 4 weeks (CO_2 group, 16° ± 1°; spinal cord injury group, 15° ± 2°) (95% CI, 4.85-9.42, 1.24-3.86, 1.16-5.34, and -3.09 to 1.39, respectively) (Fig. 3B).

For the articular factors, the CO_2 groups in the prevention group at 2 weeks (4° ± 1°) and 4 weeks (6° ± 1°) and in the treatment group at 2 weeks (8° ± 1°) were smaller than those in the spinal cord injury group (10° ± 1°, 12° ± 2°, and 11° ± 2°, respectively; $p < 0.05$), except for the treatment group at 4 weeks (CO_2 group, 11° ± 1°; spinal cord injury group, 11° ± 1°) (95% CI, 4.05-7.05, 5.18-8.02, 1.73-5.50, and -1.96 to 1.26, respectively) (Fig. 3C).

Changes in Fibrosis and Fibrosis-related Gene Expression in Muscle

CO_2 therapy did not diminish the decrease in the ratio of the muscle wet weight to whole-body weight after spinal cord injury (Table 1). The percentage of muscular fibrosis was higher after spinal cord injury than it was before therapy. CO_2 therapy decreased muscular fibrosis after spinal cord injury based on a histological quantification of fibrosis in the biceps femoris when the untreated rats in the prevention group at 2 weeks (20% ± 2%) were compared with the CO_2-treated rats in the prevention group at 2 weeks (12% ± 3%; 95% CI, 4.07-12.62; $p < 0.001$) but not at 4 weeks (untreated, 22% ± 3% versus treated, 15% ± 6%; 95% CI, -0.14 to 12.66; $p = 0.06$), with the numbers available. With the numbers available, there was no change in the treatment group at either 2 weeks or 4 weeks (untreated versus treated: 16% ± 5% versus 17% ± 3%, 95% CI -1.10 to 4.53; $p = 0.87$ at 2 weeks; 20% ± 5% versus 16% ± 1%, 95% CI -0.92 to 9.26; $p = 0.11$ at 4 weeks) (Fig. 4A).

The expression of COL1A1 mRNA in the spinal cord injury groups was increased at 2 weeks (1.9 ± 0.4-fold compared with controls) and 4 weeks (2.0 ± 1.0-fold) after injury compared with the control group (all p = 0.04). Additionally, its expression was decreased in the CO_2 prevention group at 2 weeks (0.6 ± 0.2-fold; p = 0.002) and 4 weeks (0.8 ± 0.3-fold; p = 0.008) compared with that in the spinal cord injury group, but no differences were found between the CO_2 and spinal cord injury groups in treatment at 2 and 4 weeks (untreated versus treated: 2.0 ± 1.0-fold versus 1.5 ± 0.3-fold; p = 0.48 at 2 weeks; 0.7 ± 0.1-fold versus 1.0 ± 0.7-fold; p = 0.95 at 4 weeks) (Fig. 4B). The expression of TGF-β1 mRNA was decreased only after spinal cord injury in the treatment group at 4 weeks (0.7 ± 0.1-fold less than in control; p = 0.003) compared with the control group (Fig. 4C).

Changes in Morphology and Fibrosis-related Protein Expression in the Joint Capsule

CO_2 therapy did not alter shortening of the synovial membrane after spinal cord injury based on the measurement of the synovial intima's length when the untreated rats in the prevention group at 2 weeks (5.6 ± 2.2 mm) and 4 weeks (4.9 ± 2.2 mm) were compared with the CO_2-treated rats in the prevention group at 2 weeks (5.4 ± 1.9 mm) and 4 weeks (7.5 ± 1.8 mm) (95% CI, -2.44 to 2.78; p = 0.98 and 95% CI, -4.67 to 0.35; p = 0.11, respectively). The synovial intima's length in the CO_2 group (6.8 ± 1.8 mm) was shorter than that in the control group with treatment for 2 weeks (9.5 ± 0.6 mm) (95% CI, 0.56-4.94; p = 0.01), whereas no differences were found among the other groups (Fig. 5).

Immunolabeling of Type I collagen was qualitatively seen in all knee joint capsules and was uniformly distributed in them. The staining intensity of Type I collagen showed no differences among the groups at all timepoints (Fig. 6A-L).

Immunolabeling of TGF-β1 was seen in all observed knee joint capsules and was strongly distributed in the surface of the synovial intima. TGF-β1 immunostaining of the spinal cord

injury prevention group at 2 weeks was stronger than that of the control group (Fig. 7A and B). TGF-β1 immunolabeling of the CO_2 group in the prevention group at 2 weeks (Fig. 7C) was less than that in the spinal cord injury group. In the prevention group at 4 weeks (Fig. 7D-F) and in the treatment group at 2 weeks (Fig. G-I) and 4 weeks (Fig. 7J-L), immunolabeling of the spinal cord injury group was stronger than that of the control group, but did not differ from that of the CO_2 group.

Discussion

Contractures are a major complication in patients with spinal cord injuries. Transcutaneous CO_2 therapy has been used in humans and a variety of animal models to evaluate its potential use as a treatment for a number of conditions; however, the use of CO_2 therapy to prevent and treat contractures after spinal cord injury has not been evaluated. Using either a prevention or treatment approach, we found in a rat model that transcutaneous CO_2 can improve knee extension ROM and both muscular and articular factors that lead to joint contractures, although the knee joint was left untouched. CO_2 therapy for prevention (2 and 4 weeks) and treatment (2 weeks) improved limitations to ROM after spinal cord injury in rats. On the other hand, the therapy did not improve limitations to ROM at the 4-week timepoint in the treatment group; this may be because of irreversible degeneration after spinal cord injury that cannot be restored by CO_2 therapy. Furthermore, based on the histologic and biochemical analyses, CO_2 therapy appeared to effectively prevent fibrosis in both the muscle and joint capsule after spinal cord injury.

This study had several limitations. First, and most importantly, we used a small animal model. The adolescent male Wistar rats used this study cannot fully reflect the variability found in humans (such as variations in age, race, sex, genetic variation, and lifestyle); therefore, it is unlikely that our results can be directly translated to patients with spinal cord injuries. However, small animal models are preferred for preliminary screening, and the

model of rats with spinal cord injury appears to closely reflect the outcome in humans in terms of histopathology and function [27]. In addition, our primary outcome in the spinal cord injury group demonstrated similar results to those of our previous study in rats [36], although sensitivity to neuropathic pain in rats differs between males and females [11]. A second limitation is that the question remains as to what contributes to the improvement in muscular and articular factors responsible for contractures because of CO_2 therapy. CO_2 therapy increases blood flow by inducing vasodilation and oxygen tension (Bohr effect) in peripheral tissues as reported in humans and rats [22, 30, 39], and the cause and effect of CO_2 needs further investigation. In addition, rats were exposed to CO_2 for a limited time (20 minutes per day) and duration (2 and 4 weeks). Ueha et al. [43] reported that the anti-tumor effects of CO_2 application show dose dependency, and its optimal treatment time was 20 minutes. We may need to determine the optimal conditions for treating contractures. Finally, we used the right and the left knees as different samples. The use of both joints has the advantages of minimizing the number of experimental animals needed and providing equivalent sample sizes for statistical purposes. However, its use cannot preclude chance findings attributable to intra-animal and inter-animal variation.

Central nervous system injuries, including spinal cord injuries, cause various types of muscle degeneration, including atrophy of skeletal muscle and increases in connective and adipose tissue [4, 9, 13]. In our study, atrophy of the biceps femoris was observed after spinal cord injury; however, CO_2 therapy did not improve it (Table 1). A recent report [37] has shown that CO_2 exposure tends to induce muscle hypertrophy in the tibial anterior muscles of healthy rats. Unlike in the normal rat, in rats with hypertrophy, CO_2 therapy might not be sufficient to slow the progression of muscle atrophy after spinal cord injury. In our study, we observed muscular fibrosis after spinal cord injury, and CO_2 therapy in the prevention group at 2 weeks improved fibrosis. Muscle extensibility is defined by the fascia and muscle fiber,

and the fascia is strongly associated with contractures [12, 46]. A decrease in fascia extensibility is caused by fibrosis with elevated collagen content, and Type I collagen is especially associated with stiffness and marked increases in fibrotic tissue [4, 17]. Similarly, in the current study, the mRNA expression of Type I collagen increased at 2 and 4 weeks after spinal cord injury when muscle fibrosis developed, and CO_2 therapy in the prevention group decreased its expression. These results suggest that CO_2 may improve muscular fibrosis because of the reduced gene expression of Type I collagen, thereby improving the muscular factor. However, muscular fibrosis occurring after contractures develop did not improve with CO_2 therapy in the treatment group. In a rat model of immobilization-induced contractures, the mRNA expression of Type I collagen increased during the first week of the early stage of contracture development [19]. Type I collagen is deposited during this period, and in our study, CO_2 therapy in the treatment group may have been unable to inhibit muscular fibrosis. In addition, TGF-β1 increases in the early stage of muscular fibrosis and promotes the production of collagen [3], and the mRNA levels of Type I collagen and TGF-β1 increased in a rat soleus after immobilization [19]. Per our results, TGF-β1 did not increase but rather decreased at the 4-week timepoint in the spinal cord injury groups in the treatment group, although the mRNA levels of Type I collagen increase in the prevention group at 2 and 4 weeks and in the treatment group at 2 weeks. In the spinal cord injury groups, muscular contractions related to spasticity (involuntary movements) that were different from the movements of intact muscles allowed voluntary isometric contractions to occur in the immobilized joints. Therefore, muscular fibrosis in contractures may develop after spinal cord injury without increasing the levels of TGF-β1.

Based on the results of our histologic analysis, the posterior synovial intima's length was shorter after spinal cord injury, and CO_2 therapy did not lengthen the posterior synovial intima. We reported that joint motion is essential to prevent shortening of the synovial intima

[32]. Shortening of the synovial membrane is caused by adhesion between the synovial fold and synovial membrane because of joint immobility [42]. Joint motion stretches the synovial membrane and may prevent adhesion by releasing the synovial fold. In CO_2 therapy, because the knee joint was left untouched, this therapy did not affect synovial intima shortening.

Contrary to the results regarding the synovial intima's length, CO_2 therapy improved the articular factor in rats with contractures, except for the treatment group at 4 weeks. This may be related to fibrosis of the joint capsules. The joint capsules are lined with the synovial membrane and consist of Types I and III collagen. Joint immobilization leads to distortion of the collagen sequence and increased collagen density; therefore, the elasticity of the synovial membrane is decreased [45]. There were qualitatively no differences in the staining intensity and pattern of Type I collagen among the groups at all timepoints in our study. Thus, changes in the articular factor may be independent of the composition of Type I collagen in the joint capsule. In addition, these results in the joint capsule are different from the relationship between Type I collagen and fibrosis in muscle, as mentioned above. Although it is well-accepted that Type I collagen increases in fibrotic muscles [29, 41], after joint immobilization, the changes in Type I collagen in the joint capsule are still controversial. The underlying pathogenesis of fibrosis after spinal cord injury may differ between muscles and joint capsules. However, TGF-β1 is associated with tissue fibrosis [5, 28], and its protein level was found to increase in the joint capsule of rats with immobilized knees [15, 18]. We found that TGF-β1 protein in the joint capsule increased after spinal cord injury, and CO_2 therapy reduced this protein, but only in the prevention group at 2 weeks. In another study, TGF-β1 in the synovial membrane increased from 3 days to 2 weeks after immobilization, and its staining intensity increased at the first 2 weeks and was stronger from 4 to 16 weeks [15]. Thus, in our study, CO_2 therapy in the prevention group at 2 weeks may have improved fibrosis in the joint capsules after spinal cord injury by suppressing the production of TGF-

β1. Additionally, the TGF-β1 protein did not change with CO_2 therapy at 4 weeks after spinal cord injury; this is likely because the increase of TGF-β1 exceeded the efficacy of CO_2 therapy. These results suggest that CO_2 therapy improves fibrosis in the joint capsules in the early stage of contracture development; therefore, the articular factor of contractures is restored.

Our findings indicate that CO_2 therapy may be useful in preventing and treating contractures after spinal cord injury in rats by improving knee extension ROM and both the muscular and articular factors that are responsible for contractures. CO_2 therapy particularly appears to be more effective as a preventive and treatment strategy in the early stage of contractures before irreversible degeneration occurs. Surprisingly, although CO_2 therapy is an external treatment absorbed through the rat's skin, it improved the articular factors, which are located in the deep tissue. We speculate that that CO_2 therapy, combined with conventional treatments such as positioning, stretching, and physical therapy, may be a better therapeutic strategy in the early stage of contracture development after spinal cord injury; future studies should explore this approach.

Acknowledgments

We thank Naoyoshi Sakitani, Shoko Takano, Rui Miyamoto, and Shin Ogasawara for their skilled technical assistance.

References

1. Akahane S, Sakai Y, Ueha T, Nishimoto H, Inoue M, Niikura T, Kuroda R. Transcutaneous carbon dioxide application accelerates muscle injury repair in rat models. *Int Orthop*. 2017;41:1007–1015.

2. Ando A, Hagiwara Y, Onoda Y, Hatori K, Suda H, Chimoto E, Itoi E. Distribution of type A and B synoviocytes in the adhesive and shortened synovial membrane during immobilization of the knee joint in rats. *Tohoku J Exp Med.* 2010;221:161–168.

3. Bernasconi P, Torchiana E, Confalonieri P, Brugnoni R, Barresi R, Mora M, Cornelio F, Morandi L, Mantegazza R. Expression of transforming growth factor-beta 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine. *J Clin Invest*. 1995;96:1137–1144.

4. Booth CM, Cortina-Borja MJ, Theologis TN. Collagen accumulation in muscles of children with cerebral palsy and correlation with severity of spasticity. *Dev Med Child Neurol*. 2001;43:314–320.

5. Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. *N Engl J Med*. 1994;331:1286–1292.

6. Brandi C, D'Aniello C, Grimaldi L, Caiazzo E, Stanghellini E. Carbon dioxide therapy: Effects on skin irregularity and its use as a complement to liposuction. *Aesthetic Plast Surg*. 2004;28:222–225.

7. Diong J, Harvey LA, Kwah LK, Eyles J, Ling MJ, Ben M, Herbert RD. Incidence and predictors of contracture after spinal cord injury--a prospective cohort study. *Spinal Cord*. 2012;50:579–584.

8. Fergusson D, Hutton B, Drodge A. The epidemiology of major joint contractures: a systematic review of the literature. *Clin Orthop Relat Res*. 2007;456:22–29.

9. Foran JRH, Steinman S, Barash I, Chambers HG, Lieber RL. Structural and mechanical alterations in spastic skeletal muscle. *Dev Med Child Neurol*. 2005;47:713–717.

10. Fox P, Richardson J, McInnes B, Tait D, Bedard M. Effectiveness of a bed positioning program for treating older adults with knee contractures who are institutionalized. *Phys Ther*. 2000;80:363–372.

11. Gaudet AD, Ayala MT, Schleicher WE, Smith EJ, Bateman EM, Maier SF, Watkins LR. Exploring acute-to-chronic neuropathic pain in rats after contusion spinal cord injury. *Exp Neurol*. 2017;295:46–54.

12. Gillies AR, Lieber RL. Structure and function of the skeletal muscle extracellular matrix. *Muscle Nerve*. 2011;44:318–331.

13. Gracies J. Pathophysiology of spastic paresis. I: Paresis and soft tissue changes. *Muscle Nerve*. 2005;31:535–551.

14. Hadi AM, Mouchaers KTB, Schalij I, Grunberg K, Meijer GA, Vonk-Noordegraaf A, van der Laarse WJ, Beliën JAM. Rapid quantification of myocardial fibrosis: a new macro-based automated analysis. *Cell Oncol (Dordr)*. 2011;34:343–354.

15. Hagiwara Y, Chimoto E, Takahashi I, Ando A, Sasano Y, Itoi E. Expression of transforming growth factor-beta1 and connective tissue growth factor in the capsule in a rat immobilized knee model. *Ups J Med Sci*. 2008;113:221–234.

16. Harvey LA, Herbert RD. Muscle stretching for treatment and prevention of contracture in people with spinal cord injury. *Spinal Cord*. 2002;40:1–9.

17. Hibino I, Okita M, Inoue T, Banno Y, Hoso M. Effect of Immobilization on Insoluble Collagen Concentration and Type I and Type III Collagen Isoforms of Rat Soleus Muscle. *J Jpn Phys Ther Assoc*. 2008;11:1–6.

18. Hildebrand KA, Zhang M, Hart DA. Myofibroblast upregulators are elevated in joint capsules in posttraumatic contractures. *Clin Orthop Relat Res*. 2007;456:85–91.

19. Honda Y, Sakamoto J, Nakano J, Kataoka H, Sasabe R, Goto K, Tanaka M, Origuchi T, Yoshimura T, Okita M. Upregulation of interleukin-1β/transforming growth factor-β1 and hypoxia relate to molecular mechanisms underlying immobilization-induced muscle contracture. *Muscle Nerve*. 2015;52:419–427.

20. Iwabu M, Yamauchi T, Okada-Iwabu M, Sato K, Nakagawa T, Funata M, Yamaguchi M, Namiki S, Nakayama R, Tabata M, Ogata H, Kubota N, Takamoto I, Hayashi YK, Yamauchi N, Waki H, Fukayama M, Nishino I, Tokuyama K, Ueki K, Oike Y, Ishii S, Hirose K, Shimizu T, Touhara K, Kadowaki T. Adiponectin and AdipoR1 regulate PGC-1alpha and mitochondria by Ca(2+) and AMPK/SIRT1. *Nature*. 2010;464:1313–1319.

21. Iwasawa H, Nomura M, Sakitani N, Watanabe K, Watanabe D, Moriyama H. Stretching After Heat But Not After Cold Decreases Contractures After Spinal Cord Injury in Rats. *Clin Orthop Relat Res*. 2016;474:2692–2701.

22. Izumi Y, Yamaguchi T, Yamazaki T, Yamashita N, Nakamura Y, Shiota M, Tanaka M, Sano S, Osada-Oka M, Shimada K, Wanibuchi H, Miura K, Yoshiyama M, Iwao H. Percutaneous Carbon Dioxide Treatment using a Gas Mist Generator Enhances the Collateral Blood Flow in the Ischemic Hindlimb. *J Atheroscler Thromb*. 2015;22:38–51.

23. Kanda Y. Investigation of the freely available easy-to-use software "EZR" for medical statistics. *Bone Marrow Transplant*. 2013;48:452–458.

24. Katalinic OM, Harvey LA, Herbert RD. Effectiveness of stretch for the treatment and prevention of contractures in people with neurological conditions: a systematic review. *Phys Ther*. 2011;91:11–24.

25. Kawamoto T. Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. *Arch Histol Cytol*. 2003;66:123–143.

26. Koga T, Niikura T, Lee SY, Okumachi E, Ueha T, Iwakura T, Sakai Y, Miwa M, Kuroda R, Kurosaka M. Topical Cutaneous CO application by means of a novel hydrogel accelerates fracture repair in rats. *J Bone Joint Surg Am*. 2014;96:2077–2084.

27. Kuhn PL, Wrathall JR. A mouse model of graded contusive spinal cord injury. *J Neurotrauma*. 1998;15:125–140.

28. Leask A, Abraham DJ. TGF-beta signaling and the fibrotic response. *FASEB J*. 2004;18:816–827.

29. Matsumoto F, Trudel G, Uhthoff HK. High collagen type I and low collagen type III levels in knee joint contracture: an immunohistochemical study with histological correlate. *Acta Orthop Scand*. 2002;73:335–343.

30. Matsumoto T, Tanaka M, Ikeji T, Maeshige N, Sakai Y, Akisue T, Kondo H, Ishihara A, Fujino H. Application of transcutaneous carbon dioxide improves capillary regression of skeletal muscle in hyperglycemia. *J Physiol Sci*. 2018;69:317-326.

31. Moriyama H, Nishihara K, Hosoda M, Saka Y, Kanemura N, Takayanagi K, Yoshimura O, Tobimatsu Y. Contrasting alteration patterns of different cartilage plates in knee articular cartilage after spinal cord injury in rats. *Spinal Cord*. 2009;47:218–224.

32. Moriyama H, Tobimatsu Y, Ozawa J, Kito N, Tanaka R. Amount of torque and duration of stretching affects correction of knee contracture in a rat model of spinal cord injury. *Clin Orthop Relat Res*. 2013;471:3626–3636.

33. Moriyama H, Yoshimura O, Kawamata S, Takayanagi K, Kurose T, Kubota A, Hosoda M, Tobimatsu Y. Alteration in articular cartilage of rat knee joints after spinal cord injury. *Osteoarthritis Cartilage*. 2008;16:392–398.

34. Moriyama H, Yoshimura O, Kawamata S, Takemoto H, Saka Y, Tobimatsu Y. Alteration of knee joint connective tissues during contracture formation in spastic rats after an experimentally induced spinal cord injury. *Connect Tissue Res*. 2007;48:180–187.

35. Moriyama H, Yoshimura O, Sunahori H, Nitta H, Imakita H, Saka Y, Maejima H, Tobimatsu Y. Progression and direction of contractures of knee joints following spinal cord injury in the rat. *Tohoku J Exp Med*. 2004;204:37–44.

36. Moriyama H, Yoshimura O, Sunahori H, Tobimatsu Y. Comparison of muscular and articular factors in the progression of contractures after spinal cord injury in rats. *Spinal Cord*. 2006;44:174–181.

37. Oe K, Ueha T, Sakai Y, Niikura T, Lee SY, Koh A, Hasegawa T, Tanaka M, Miwa M, Kurosaka M. The effect of transcutaneous application of carbon dioxide (CO_2) on skeletal muscle. *Biochem Biophys Res Commun*. 2011;407:148–152.

38. Onishi Y, Kawamoto T, Ueha T, Kishimoto K, Hara H, Fukase N, Toda M, Harada R, Minoda M, Sakai Y, Miwa M, Kurosaka M, Akisue T. Transcutaneous Application of Carbon Dioxide (CO2) Induces Mitochondrial Apoptosis in Human Malignant Fibrous Histiocytoma In Vivo. *PLoS One*. 2012;7:1–8.

39. Sakai Y, Miwa M, Oe K, Ueha T, Koh A, Niikura T, Iwakura T, Lee SY, Tanaka M, Kurosaka M. A novel system for transcutaneous application of carbon dioxide causing an “artificial Bohr effect” in the human body. *PLoS One*. 2011;6.

40. Savin E, Bailliart O, Bonnin P, Bedu M, Cheynel J, Coudert J, Martineaud JP. Vasomotor effects of transcutaneous CO2 in stage II peripheral occlusive arterial disease. *Angiology*. 1995;46:785–791.

41. Schollmeier G, Sarkar K, Fukuhara K, Uhthoff HK. Structural and functional changes in the canine shoulder after cessation of immobilization. *Clin Orthop Relat Res*. 1996:310–315.

42. Trudel G, Uhthoff HK. Contractures secondary to immobility: Is the restriction articular or muscular? An experimental longitudinal study in the rat knee. *Arch Phys Med Rehabil*. 2000;81:6–13.

43. Ueha T, Kawamoto T, Onishi Y, Harada R, Minoda M, Toda M, Hara H, Fukase N, Kurosaka M, Kuroda R, Akisue T, Sakai Y. Optimization of antitumor treatment conditions for transcutaneous CO2 application: An in vivo study. *Oncol Rep*. 2017;37:3688–3694.

44. Usuba M, Miyanaga Y, Miyakawa S, Maeshima T, Shirasaki Y. Effect of heat in increasing the range of knee motion after the development of a joint contracture: an experiment with an animal model. *Arch Phys Med Rehabil*. 2006;87:247–253.

45. Watanabe M, Hoso M, Kojima S, Matsuzaki T, Hibino I. Histopathological Changes in Joint Components in a Rat Knee Joint Contracture Model Following Mobilization. *J Phys Ther Sci*. 2012;24:1199–1203.

46. Williams P, Kyberd P, Simpson H, Kenwright J, Goldspink G. The morphological basis of increased stiffness of rabbit tibialis anterior muscles during surgical limb-lengthening. *J Anat*. 1998;193:131–138.

Legends

Fig. 1 A diagram of the experimental design is shown. The rats were randomly divided into the control group (n = four rats per timepoint), spinal cord injury group (n = three rats per timepoint), and CO_2 group (n = three rats per timepoint). The samples of the control group in the prevention at 4 weeks group and the treatment at 2 weeks groups were obtained from the same animals (n = four rats). The right and left knees of all rats in each group were regarded as different samples (the control group, n = eight limbs per timepoint; the spinal cord injury and CO_2 groups, n = six limbs per timepoint).

Fig. 2 CO_2 therapy for the hindlimbs of rats is shown.

Fig. 3 The graphs show (A) knee motion and (B) muscular and (C) articular factors responsible for contractures that were measured for extension with a goniometer. Two approaches to CO_2 therapy were evaluated: in the prevention approach, CO_2 therapy was provided beginning on the first day postoperatively, and in the treatment approach, CO_2 therapy was given on the 15th day postoperatively. Data are presented as the mean ± SD. * Indicates a difference among the groups at the $p < 0.05$ level. Eight limbs from control group and six limbs from spinal cord injury and CO_2 group were evaluated at each timepoint.

Fig. 4 The graphs show (A) quantification of muscular fibrosis in the biceps femoris and the relative expression of (B) COL1A1 and (C) TGF-β1 in the biceps femoris of rats in each group. Data are shown as the mean ± SD. *$p < 0.05$; **$p < 0.01$. Six samples from each group were evaluated at each timepoint.

Fig. 5 The graph shows the posterior synovial intima's length in rats in each group. Data are presented as the mean ± SD. *$p < 0.05$; **$p < 0.01$. Six samples from each group were evaluated at each timepoint.

Fig. 6 Representative photomicrographs show the distribution of Type I collagen in the posterior joint capsule of the (A) control, (B) spinal cord injury, and (C) CO_2 groups at 2 weeks in the prevention test condition; the (D) control, (E) spinal cord injury, and (F) CO_2 groups in the prevention test condition at 4 weeks; the (G) control, (H) spinal cord injury, and (I) CO_2 groups in the treatment test condition at 2 weeks; and the (J) control, (K) spinal cord injury, and (L) CO_2 groups in the treatment test condition at 4 weeks. Scale bars = 100 μm.

Fig. 7 Representative photomicrographs of the distribution of TGF-β1 in the posterior joint capsule of the (A) control, (B) spinal cord injury, and (C) CO_2 groups in the prevention test condition at 2 weeks; the (D) control, (E) spinal cord injury, and (F) CO_2 groups in the prevention test condition at 4 weeks; the (G) control, (H) spinal cord injury, and (I) CO_2 groups in the treatment test condition at 2 weeks; and the (J) control, (K) spinal cord injury, and (L) CO_2 groups in the treatment test condition at 4 weeks are shown. The immunolabeling to TGF-β1 is qualitatively stronger in the spinal cord injury group of the prevention condition at 2 weeks than that in the control group; however, the immunolabeling of the CO_2 group appears weaker than that of the spinal cord injury group. Scale bars = 100 μm.

Figure

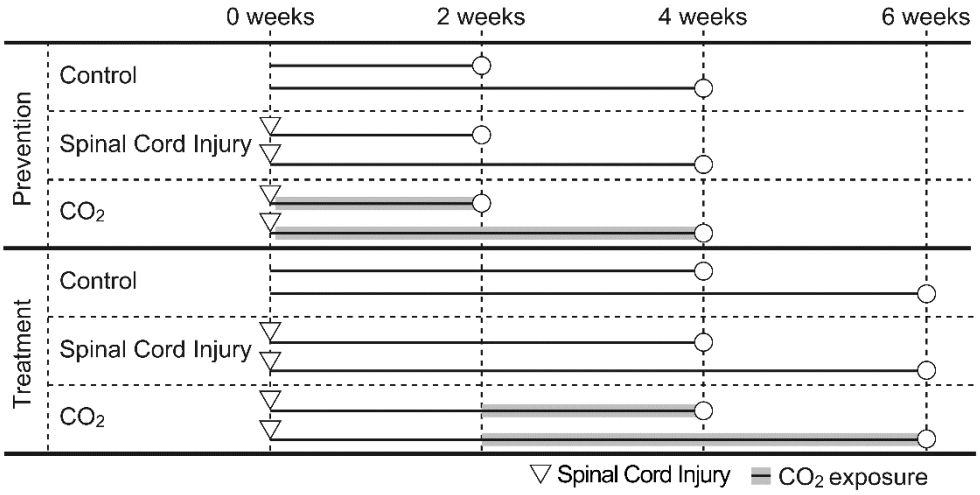
0 weeks
2 weeks
4 weeks
6 weeks
Prevention
Control
Spinal Cord Injury
CO2
Treatment
Control
Spinal Cord Injury
CO2
Spinal Cord Injury
CO2 exposure

Fig.1

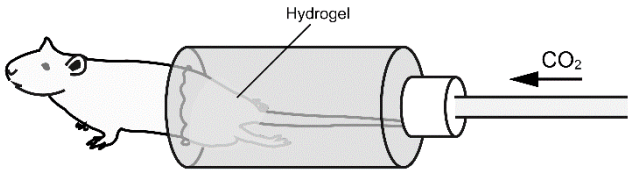

Fig.2

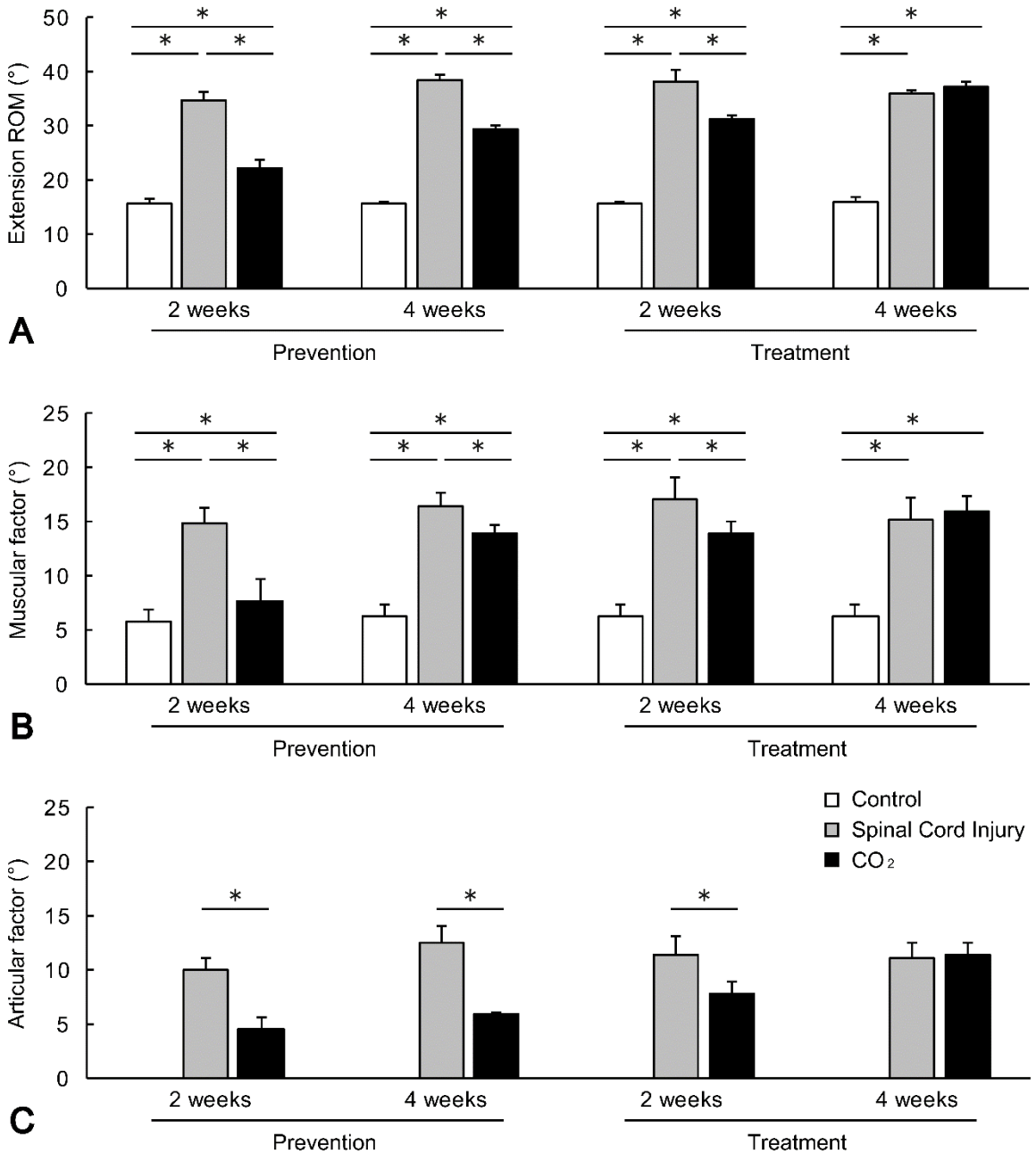
Extension ROM (°)
50
40
30
20
10
0
2 weeks
4 weeks
2 weeks
4 weeks
A
Prevention
Treatment
Muscular factor (°)
25
20
15
10
5
0
2 weeks
4 weeks
2 weeks
4 weeks
B
Prevention
Treatment
Articular factor (°)
25
20
15
10
5
0
2 weeks
4 weeks
2 weeks
4 weeks
C
Prevention
Treatment
Control
Spinal Cord Injury
CO2

Fig.3

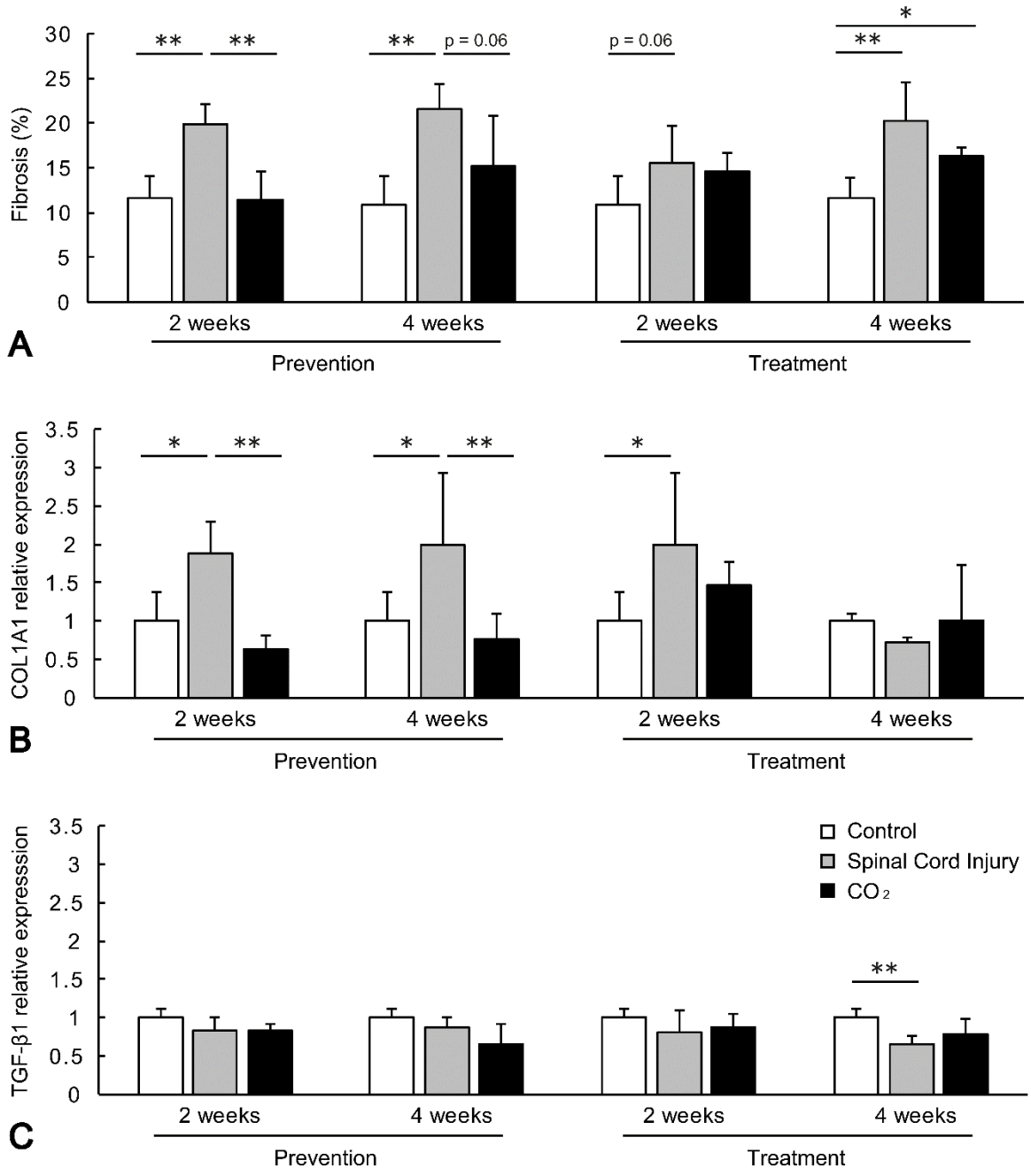
Fibrosis (%)
30
25
20
15
10
5
0
**
**
**
p = 0.06
p = 0.06
*
**
2 weeks
4 weeks
2 weeks
4 weeks
Prevention
Treatment
A
COL1A1 relative expression
3.5
3
2.5
2
1.5
1
0.5
0
*
**
*
**
*
2 weeks
4 weeks
2 weeks
4 weeks
Prevention
Treatment
B
TGF-β1 relative expresssion
3.5
3
2.5
2
1.5
1
0.5
0
Control
Spinal Cord Injury
CO2
**
2 weeks
4 weeks
2 weeks
4 weeks
Prevention
Treatment
C

Fig.4

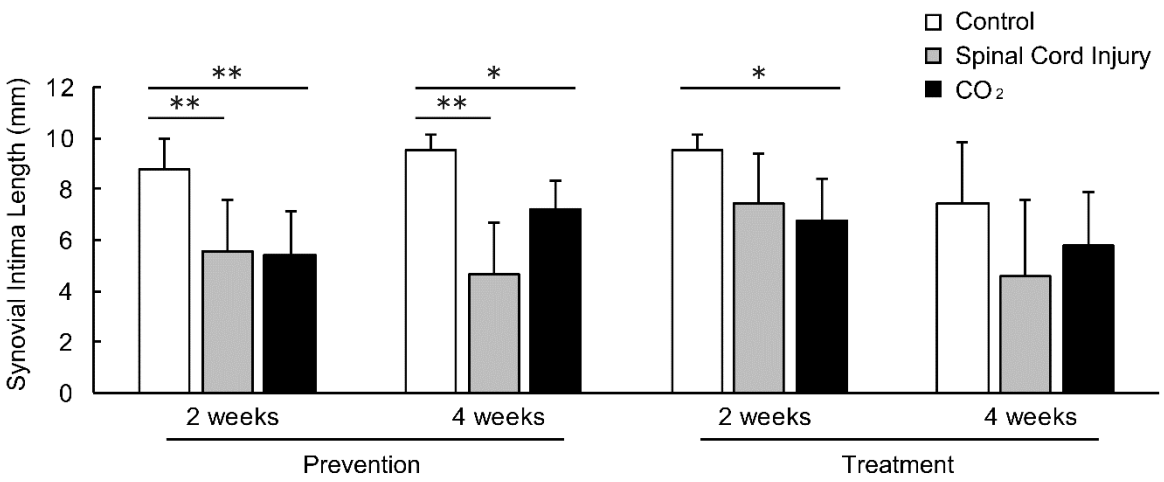
Control
Spinal Cord Injury
CO2
Synovial Intima Length (mm)
12
10
8
6
4
2
0
**
**
*
**
*
2 weeks
4 weeks
2 weeks
4 weeks
Prevention
Treatment

Fig.5

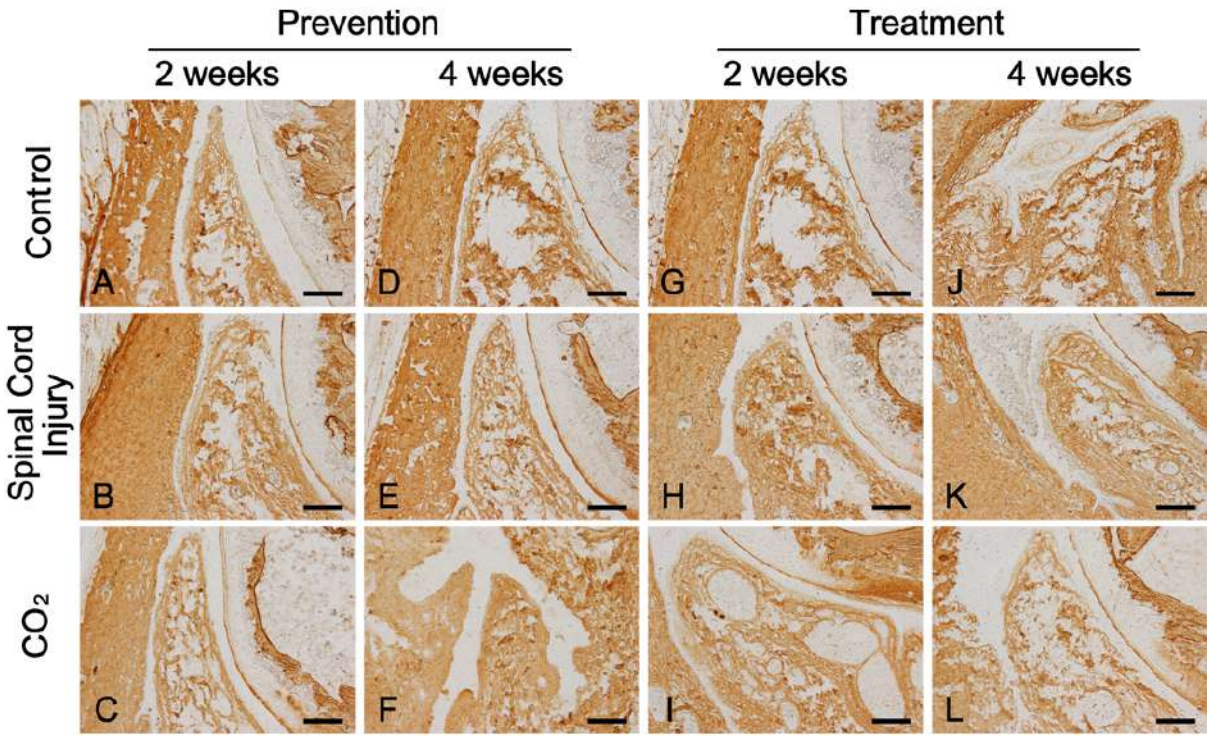
Prevention
Treatment
2 weeks
4 weeks
2 weeks
4 weeks
Control
Spinal Cord Injury
CO2
A
B
C
D
E
F
G
H
I
J
K
L

Fig.6

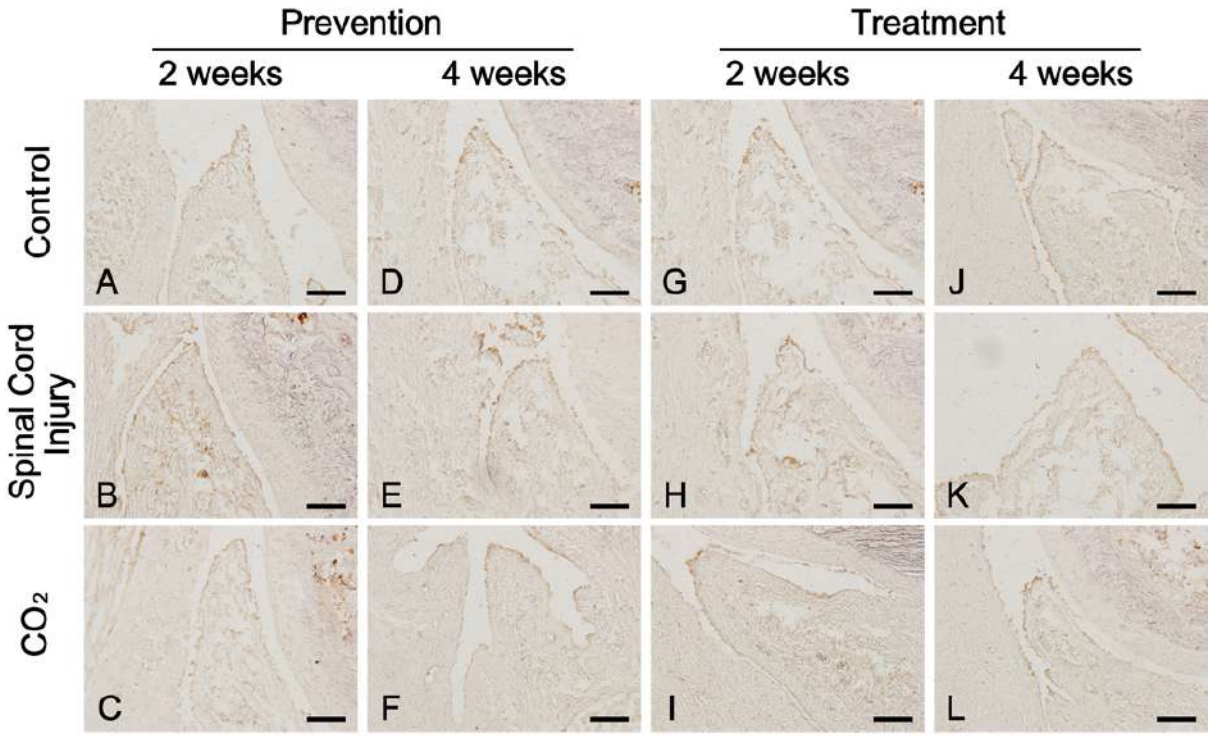
Prevention
Treatment
2 weeks
4 weeks
2 weeks
4 weeks
Control
Spinal Cord Injury
CO_2
A
D
G
J
B
E
H
K
C
F
I
L

Fig.7

Table 1. Ratios of biceps femoris weight to rat body weight

	Control	Spinal cord injury			Carbon dioxide		
	Mean (SD)	Mean (SD)	Mean difference compared with control (95 CI)	p	Mean (SD)	Mean difference compared with control (95 CI)	p
Prevention							
2 weeks	8.2 ± 0.8	6.8 ± 0.8	-1.4 (-2.4, -0.4)	0.007	7.4 ± 0.6	-0.8 (-1.9, 0.2)	0.13
4 weeks	8.1 ± 0.6	6.9 ± 0.9	-1.2 (-2.2, -0.3)	0.009	6.8 ± 0.5	-1.3 (-2.3, -0.4)	0.005
Treatment							
2 weeks	8.1 ± 0.6	7.2 ± 0.6	-0.9 (-1.8, -0.1)	0.02	6.9 ± 0.6	-1.2 (-2.0, -0.4)	0.005
4 weeks	8.1 ± 0.4	6.4 ± 0.2	-1.7 (-2.3, -1.2)	< 0.001	6.6 ± 0.5	-1.5 (-2.0, -0.9)	< 0.001

Displacement values are given as the mean ± SD mg/g.