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SCIENTIFIC RESEARCH ARTICLE

Transcutaneous application of carbon dioxide improves contractures after immobilization of rat knee joint

Shota INOUE, PT, BSc[1], Hideki MORIYAMA, PT, PhD[2], Yoshio WAKIMOTO, PT, MSc[1], Changxin LI, BSc[1], Junpei HATAKEYAMA, PT, BSc[1], Taisei WAKIGAWA, PT, BSc[1], Yoshitada SAKAI, MD, PhD[3] and Toshihiro AKISUE, MD, PhD[2]

[1)] *Department of Rehabilitation Science, Graduate School of Health Sciences, Kobe University*
[2)] *Life and Medical Sciences Area, Health Sciences Discipline, Kobe University*
[3)] *Division of Rehabilitation Medicine, Kobe University Graduate School of Medicine*

ABSTRACT. Objective: Joint contractures are a major complication following joint immobilization. However, no fully effective treatment has yet been found. Recently, carbon dioxide (CO_2) therapy was developed and verified this therapeutic application in various disorders. We aimed to verify the efficacy of transcutaneous CO_2 therapy for immobilization-induced joint contracture. Method: Twenty-two Wistar rats were randomly assigned to three groups: caged control, those untreated after joint immobilization, and those treated after joint immobilization. The rats were treated with CO_2 for 20 min once a daily either during immobilization, (prevention) or during remobilization after immobilization (treatment). Knee extension motion was measured with a goniometer, and the muscular and articular factors responsible for contractures were calculated. We evaluated muscle fibrosis, fibrosis-related genes (collagen Type 1α1 and TGF-β1) in muscles, synovial intima's length, and fibrosis-related proteins (Type I collagen and TGF-β1) in the joint capsules. Results: CO_2 therapy for prevention and treatment improved the knee extension motion. Muscular and articular factors decreased in rats of the treatment group. The muscular fibrosis of treated rats decreased in the treatment group. Although CO_2 therapy did not repress the increased expression of collagen Type 1α1, the therapy decreased the expression of TGF-β1 in the treatment group. CO_2 therapy for treatment improved the shortening of the synovial membrane after immobilization and decreased the immunolabeling of TGF-β1 in the joint capsules. Conclusions: CO_2 therapy may prevent and treat contractures after joint immobilization, and appears to be more effective as a treatment strategy for the deterioration of contractures during remobilization.

Key words: contracture, carbon dioxide, immobilization, remobilization, fibrosis

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Joint immobilization is widely used in the treatment of orthopaedic disorders, but simultaneously causes joint contractures[1)]. Joint contracture is characterized by reduction in the passive range of motion (ROM) resulting from structural changes in periarticular soft tissues[2)]. Contracture disturbs patients' activities of daily living in various aspects.

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Correspondence to: Hideki Moriyama, Life and Medical Sciences Area, Health Sciences Discipline, Kobe University, Hyogo 654-0142, JAPAN
e-mail: morihide@harbor.kobe-u.ac.jp
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In the clinical settings, positioning, stretching, and physical therapy are widely advocated as an effective means of preventing and treating contractures. The usefulness of these approaches has been verified by many clinical[3,4)] and animal studies[5-7)]. Nevertheless, we are often confronted with patients who have contractures with loss of functions. Therefore, novel therapeutic strategies are required to prevent and treat contractures as an alternative to the conventional treatments.

Recently, transcutaneous carbon dioxide (CO_2) therapy has been reported to be beneficial for improving various disorders and symptoms. Applications of CO_2 can lead to accelerated fracture healing[8)], increased muscle blood flow with hyperglycemia[9)], improved muscle atrophy after pe-

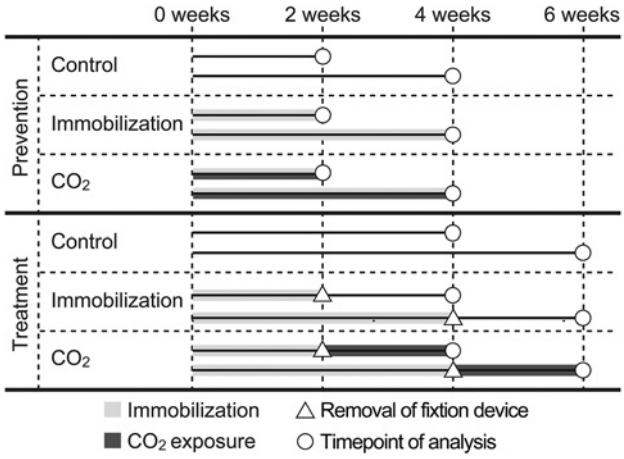

Fig. 1. A diagram of the experimental design is shown. The rats were randomly divided into the control group, IM group, and CO_2 group (n = 2 rats for each group per timepoint). The samples of the control group in the prevention group at 4 weeks and in the treatment group at 2 weeks were obtained from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

ripheral nerve injury[10], and suppressed tumor progression[11]. Additionally, it generates muscle fiber types switching, induces peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α), and increases mitochondria and angiogenesis in rat muscles[12]. These effects are similar to those of exercise[13], and therefore CO_2 therapy may be an alternative and complementary approaches of exercise.

We previously showed that joint motion is important for prevention and treatment of contractures[5,6], which leads to the suggestion that CO_2 therapy may be an alternative therapeutic strategy for contractures. Indeed, using a rat knee contracture model after spinal cord injuries, we demonstrated that the CO_2 therapy improved extension ROM by improving both the muscular and articular degeneration[14]. However, it remains unclear whether the CO_2 therapy is effective for immobilization-induced contractures, because the pathogenesis mechanism of contracture development after joint immobilization is distinct from the spinal cord injuries[2,15].

The purpose of this study was to verify the efficacy of transcutaneous CO_2 therapy for immobilization-induced joint contracture. For this purpose, we sought to clarify whether the CO_2 therapy can prevent contracture development during immobilization (prevention) and treat contracture during remobilization following immobilization (treatment).

Method

Experimental Design

All experimental procedures were approved by the Institutional Animal Care and Use Committee and performed according to the Kobe University Animal Experimentation Regulations (approval number: P160506). Twenty-two 10-week-old male Wistar rats weighting 320 to 340 g (Japan SLC Inc, Shizuoka, Japan) were used for this study. All rats were randomly divided into the following three groups (n = 2 rats for each group per timepoint): caged control (control group), those that were untreated after knee joint immobilization (IM group), and those that were treated after joint immobilization (CO_2 group). The rats were treated with CO_2 during immobilization (prevention, for 2 or 4weeks) from the first day after immobilization or during remobilization at either 2 or 4 weeks after immobilization (treatment, for 2 weeks) (Fig. 1). The right and left knee joint of each animal served as different samples (n = 4 limbs for each group per each 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 = 2 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[5,6]. Animals were housed in polycarbonate cages with bedding (cedar shavings), and were maintained under artificial conditions at 22 ± 1℃, with a constant humidity of 55 ± 5%, and a cycle of 12 h of light and 12 h dark; and allowed free access to standard food and water for 24 hours.

Joint Immobilization and Remobilization

The bilateral knee joints of rats in the IM and CO_2 groups were immobilized, according to the method of Nagai et al[16]. In brief, the rats were anesthetized by isoflurane with NARCOBIT-E II type (Natsume Seisakusyo Inc, Tokyo, Japan), Kirchner wires were screwed into the femur and the tibia. The Kirchner wires were then fixed with wire and resin (PROVINCE FAST; Matsukaze, Kyoto, Japan) to immobilize the knee joints at approximately 140° ± 5° flexion. These rats were subcutaneously administered buprenorphine 0.02 mg/kg as the analgesic every 12 hours for 3 days after immobilization to removal the postoperative pain as possible. After 2 or 4 weeks of this immobilization, the fixation device of the rats in the treatment group was removed and the knee joint was allowed to move freely.

CO_2 Treatment Protocol

Transcutaneous CO_2 absorption-enhancing hydrogel was provided by NeoChemir Inc. (Kobe, Japan), as previously described[17]. Briefly, the bilateral hindlimbs of the rats in the CO_2 groups were shaved and the hydrogel was applied, which promote absorption of the CO_2 to the lower limbs. A CO_2 adaptor was attached to the limbs and sealed,

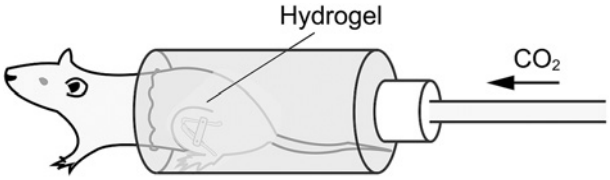

Fig. 2. The CO_2 therapy for the limbs of rats is shown.

and the entire limbs was exposed to diluted 100% CO_2 gas that was absorbed percutaneously (Fig. 2). This therapy was applied daily for 20 minutes. We took great care to ensure that the rat' knee joints were not moved during the therapy. CO_2 gas was flowed in the CO_2 adaptor to absorb it sufficiently, and the hair on the lower limb was shaved once a week. In the rats in the IM group, as a sham intervention, we applied only the hydrogel sealed with the CO_2 adaptor.

ROM Measurements and Determination Muscular/Articular Factors

At the end of the experimental period, knee motion was measured for extension as described by our published studies[15,18]. Briefly, knee motion was measured for extension with a goniometer applying a standardized torque (0.06 Nm) and was measured again after myotomy. The muscular factors were defined as ROM limitation by muscles including tendons and fascia, and the joint factors were defined as ROM limitation by articular components (bone, cartilage, synovium, capsule, and ligament). According to our previous method[6], the formulas were used to allow isolation of the muscular and articular factors contributing to contractures by measuring ROM before and after the myotomies and are as follows: muscular factors = ROM without myotomy - ROM after myotomy (within each group); articular factors = ROM after myotomy in each group - ROM after myotomy in the control group.

Quantification of Fibrosis in Muscle Tissue

After ROM measurements, biceps femoris of all animals was removed and fat and connective tissue of them were removed. Cross-sections of 10 μm then were prepared from unfixed frozen muscle samples with cryostat (CM1860; Leica, Hessen, Germany). The sections were stained with picrosirius red. We evaluated quantification of muscular fibrosis in muscle tissue by a slight modification of the method according to Hadi et al[19]. Fibrosis was quantified by identifying the yellow color of muscle cells and the red color of connective tissue using threshold color plugin of ImageTool software (Image J 1.50b; National Institutes of Health, Bethesda, MD, USA). The area of each color was measured separately, and we calculated the percentage of connective tissue area in the muscle tissue.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA of the frozen tissue was isolated from the biceps femoris with the RNeasy Plus Universal Mini kit (Quiagen, Hilden, Germany) according to manufacturer's protocol. Reverse transcription was performed using total RNA and the TaqMan™ Fast Virus 1-Step Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA). Quantitative analysis of mRNA was performed with the StepOne real-time PCR Cystem (Thermo Fisher Scientific Inc.) with Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for Type I collagen (COL1 A1; 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 18s rRNA in the same cDNA sample.

Measurements of Synovial Intima Length

The knee joint sections were prepared according to the protocols established by Kawamoto[20]. The entire knee joint from the distal femur to the proximal tibia was excised and frozen and embedded in isopentane at -75℃ and embedding agent SCEM (8091140, Leica). Cross-sections of 5 μm at the medial meniscus level in the sagittal plane were then prepared from undecalcified frozen samples with cryostat. We measured posterior synovial intima length to quantify adhesions and atrophy in the joint capsule according to the method of Ando et al[21]. The synovial lining 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 a total synovial intima length.

Immunohistochemistry Analysis

Immunohistochemistry was conducted following protocols established in our laboratory[6]. Briefly, the frozen sections of the knee joints were incubated with mouse monoclonal anti-Type I collagen (diluted 1:4000; C2456, Sigma-Aldrich, St. Louise, MO, USA) and anti-TGF-β1 (diluted 1: 50; ab64715, Abcam, Cambridge, UK) antibodies at 4℃ overnight. A subsequent reaction was made by the streptavidin-biotin-peroxidaze complex technique using Elite ABC kit (diluted 1:50; PK-6100; Vector Laboratories) for 30 min. Immunoreactivity was observed with 3,30-diaminobenzidine tetrahydrochloride (K3466; Dako Japan, Tokyo Japan). Finally, the sections were counterstained hematoxylin. All sections were stained for each primary antibody in one session on the same day with same reagents and same protocol. The staining intensity and the pattern of each antibody binding were qualitatively assessed in random order by one blinded observer (SI).

Statistical Analysis

Results for ROM, percentage of connective tissue area in muscle tissue, synovial intima length, and the expression levels of the Type I collagen and TGF-β1 mRNA 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)[22]. First, 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 with ANOVA followed by Tukey's honestly significant difference test. An alpha less than 0.05 was chosen as the significance level for these statistical analyses. Statistical analyses of muscular and articular factors in contractures were performed with Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). We calculated the standard deviations (SD) for the mean differences among the groups and 95% confidence intervals (CI) were estimated. A post hoc power analysis was used to confirm that sufficient number of animals had been used. All graphs are shown as mean ± SD.

Results

Improvements of Limitation in ROM

The limitation in ROM decreased in rats treated with CO_2 in the prevention group at 4 weeks and in the treatment group at 4 weeks, when compared with rats in the IM group at each timepoint ($P = 0.003$ and $P < 0.001$, respectively) (Fig. 3A). However, knee motion of all CO_2 groups did not recover to the same range as that in the control group (all $P < 0.001$, power = 1.00).

Improvements in the Muscular and Articular Factors

The muscular factor decreased in rats treated with CO_2 only in the treatment group at 2 weeks compared with rats in the IM group ($P < 0.05$, power = 0.94) (Fig. 3B). For the articular factor, the CO_2 group only in the treatment group at 4 weeks was smaller than the IM group ($P < 0.05$, power = 0.99) (Fig. 3C).

Changes in the Fibrosis in Muscles

The muscular fibrosis in rats treated with CO_2 decreased in the treatment group at 2 weeks and 4 weeks compared with rats in the IM group at each timepoint ($P = 0.03$, power = 0.46 and $P = 0.02$, power = 0.95, respectively), but not in the prevention group (Fig. 4A).

The expression of COL1A1 mRNA was higher in the IM prevention group at 4 weeks and in the treatment group at 2 weeks and 4 weeks than that of the control group ($P = 0.03$, $P = 0.02$, and $P = 0.05$, respectively), but no significant differences were found between the CO_2 and IM groups at all-timepoints ($P = 0.99$, power = 0.37; $P = 0.82$, power = 0.72; $P = 0.34$, power = 0.70; and $P = 0.77$, power = 0.51; respectively) (Fig. 4B). The expression of TGF-β1 mRNA in the IM group was increased at all-timepoint compared with that in the control group. Its expression was decreased in the CO_2 treatment group at 2 weeks and 4 weeks compared with that in the IM group ($P = 0.008$, power = 1.00 and $P < 0.001$, power = 1.00, respectively), but not in the prevention group ($P = 0.83$, power = 0.96 and $P = 0.09$, power = 1.00 at 4 weeks, respectively) (Fig. 4C).

Changes in Joint Capsules

The synovial intima length became shorter in the IM groups at all-timepoints, and the length increased only in the CO_2 treatment for 4 weeks compared with the IM group ($P = 0.05$, power = 1.00) (Fig. 5).

The staining intensity and pattern of type I collagen showed no differences among the groups at all-timepoints (data not shown).

The TGF-β1 immunolabeling was seen in all observed joint capsules and was mainly distributed in the lining layer of the synovium. The immunostaining of the IM groups at all-timepoints was stronger than that of the control groups (Fig. 6B-I). The immunostaining of the CO_2 treatment groups at 2 and 4 weeks showed attenuated expression when compared with that of the IM groups, but not in the prevention group (Fig. 6J-M).

Discussion

Our results indicate CO_2 therapy can improve the limitation in knee extension ROM after joint immobilization. The CO_2 application can also restore either muscular or articular factors responsible for contractures only when the spontaneous joint movements are allowed. In addition, from histopathologic and biochemical findings, the CO_2 application appeared to be more effective for improving immobilization-induced contracture during remobilization rather than during immobilization.

The limitation in knee extension ROM after immobilization begin at the first week and progress rapidly thereafter until reaching a plateau at 8 weeks[2,16,23] as observed in the present study. The CO_2 therapy for the prevention for 4 weeks increased knee extension ROM, suggesting that the CO_2 therapy suppress the progression of ROM restrictions. On the other hand, the CO_2 application for the prevention for 2 weeks had no observable effect on ROM. Trudel et al.[2] reported that the limitation in ROM progressed rapidly for the first 8 weeks in a rat immobilized knee. Therefore, the CO_2 application as a preventive strategy for contractures may require at least 4 weeks of exposure. Knee flexion contractures improve spontaneously following remobilization, however it is not fully recovery when joint immobilization is prolonged (more than 2 weeks)[24]. In this study, the CO_2 therapy for the treatment led to a greater recovery of the limitation in ROM than that of the untreated group, imply-

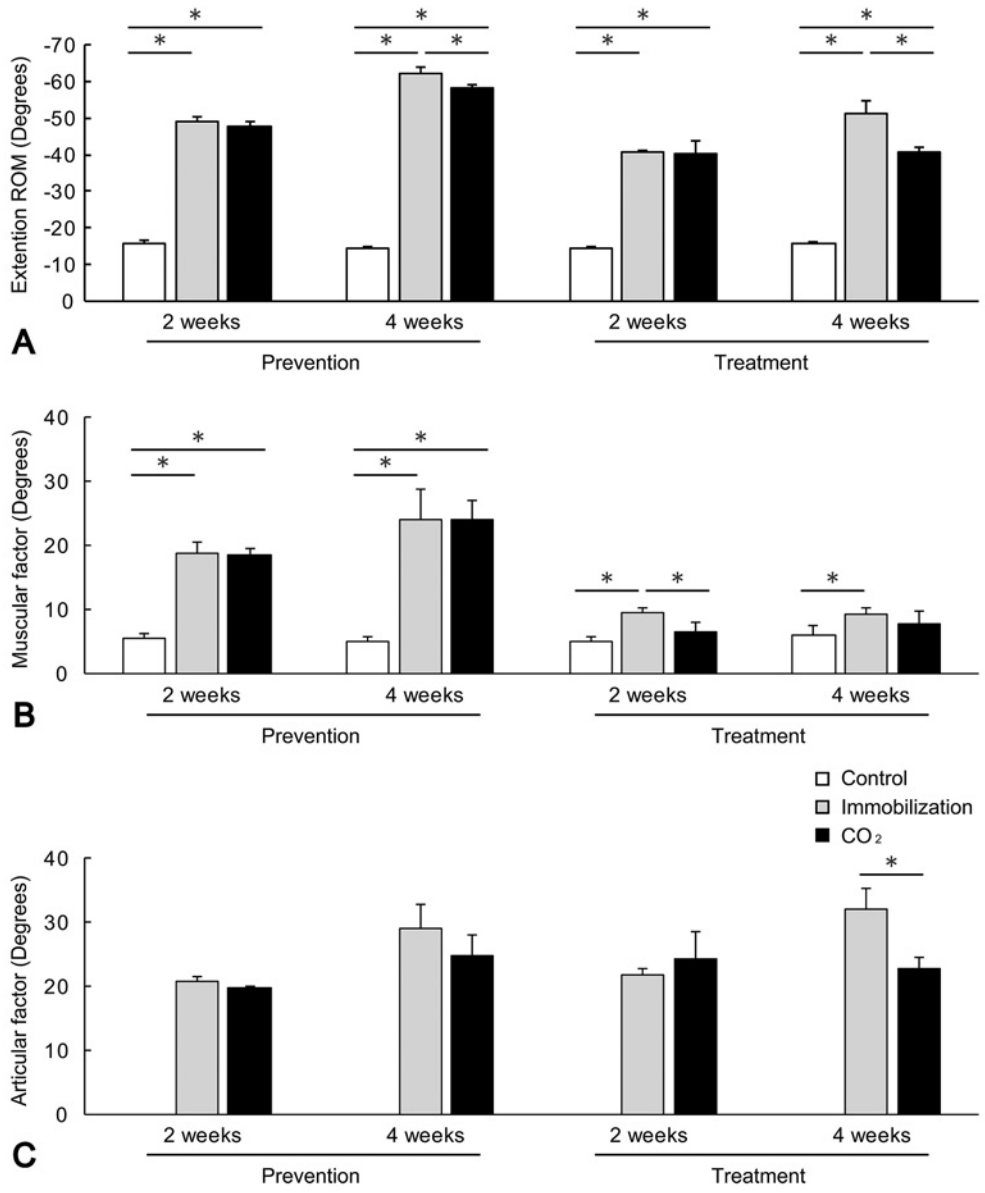

Fig. 3. Recovery of knee motion, muscular factor, and articular factors. (A) Extension ROM which was measured for extension with a goniometer. *$P < 0.01$. (B) Muscular factors and (C) articular factors responsible for contractures. Two approaches to CO_2 therapy were evaluated: in the prevention approach, CO_2 therapy was provided beginning on the first day after immobilization, and in the treatment approach, CO_2 therapy was given after the removal of the fixation device following either 2 or 4 weeks immobilization. Four limbs from all groups were evaluated at each timepoint. Data are means ± SD. * There were significant differences among the groups.

ing that the CO_2 therapy accelerates spontaneous recovery after remobilization. An unexpected finding was that the improvement in ROM was observed in the treatment group at 4 weeks rather than 2 weeks, although immobilization was longer. In short-term (within 2 weeks) immobilization, the joint contracture is largely myogenic, whereas in long-term (more than 4 weeks), arthrogenic contracture becomes the primary determinant of the contracture[2,16,24]. Additionally, although the myogenic contracture is resolved by remobilization, the arthrogenic contracture does not improve spontaneously[24]. An another study showed that remobilization after 3 weeks of immobilization recovers overall ROM, but develops the arthrogenic contracture[25]. Our data suggest the CO_2 application for the treatment for 4 weeks contributes to a restoration in the articular factor more than natural recovery, thereby improving the overall joint ROM. Unlike the articular factor, the CO_2 therapy improved the muscular factor in the treatment group at 2 weeks but not the overall ROM. Presumably, this is because the muscular factor after remobilization recover spontaneously, and then the im-

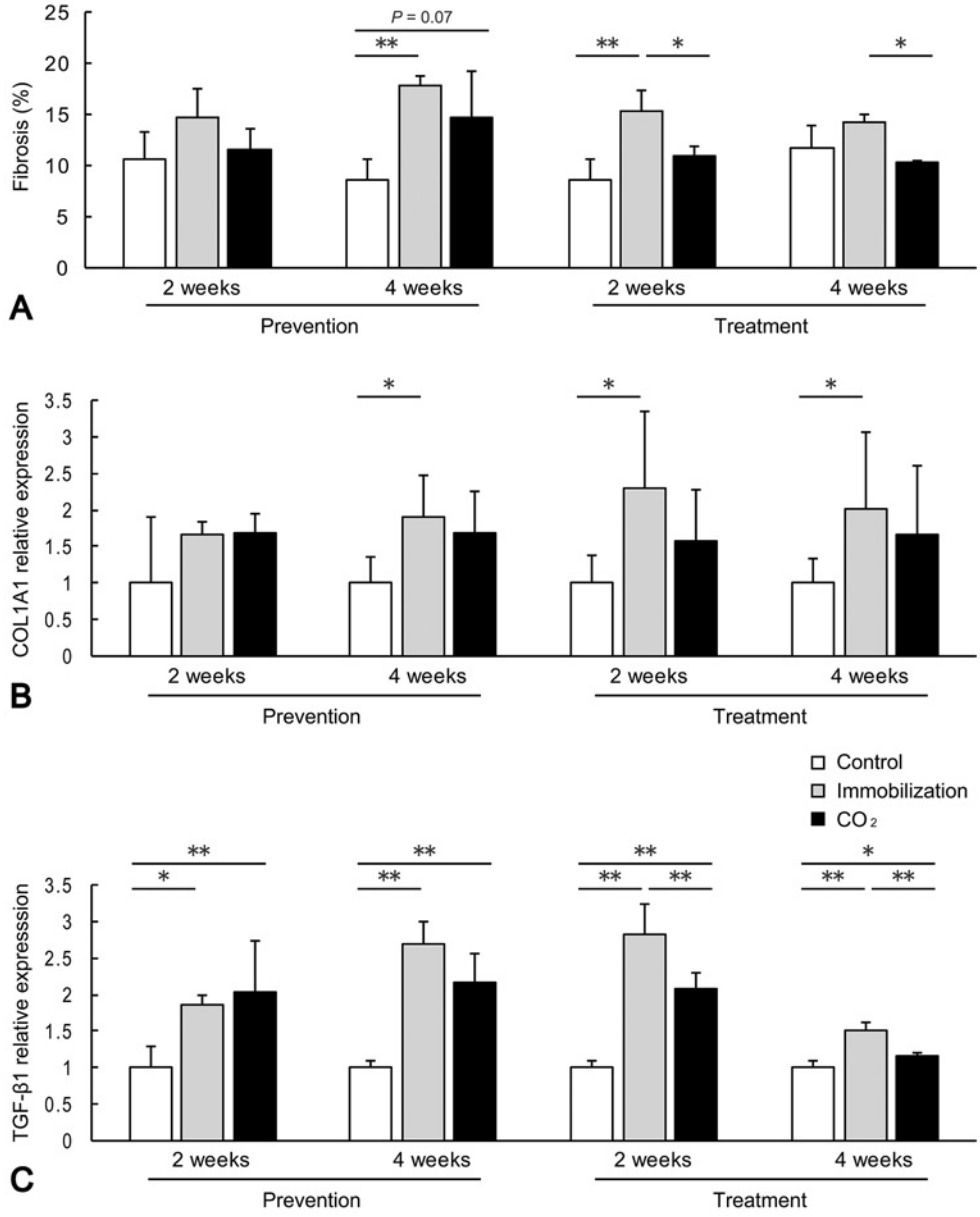

Fig. 4. The graphs show the muscular fibrosis and the expression of fibrosis-related genes in muscle. (A) The percentage of connective tissue area which was quantified by histology. (B) The gene expression of COL1A1 and (C) TGF-β1 in the biceps femoris which were quantified by real-time PCR. Data are means ± SD. Four samples from all groups were evaluated at each timepoint. *$P < 0.05$; **$P < 0.01$.

provement in the muscular factor by the CO_2 application only slightly affect the overall ROM.

Joint immobilization causes various muscle degenerations; skeletal muscle atrophy, muscle fibrosis, and an increase in adipose tissue[26-28]. Similarly, in this study, the muscle fibrosis was observed in the IM groups, and further the CO_2 therapy in the treatment group improved it. The muscle fibrosis with elevated collagen content decreases fascia extensibility and is strongly associated with myogenic contractures[29,30]. Generally, Type I collagen is associated with stiffness and markedly increases in the fibrosis tissue[31]. Similarly, in our model, mRNA expression of Type I collagen increased after immobilization when muscle fibrosis developed. However, there were no differences in gene expression of Type I collagen between the control and the CO_2 groups in the prevention group at 4 weeks and in the treatment group at 2 and 4 weeks, indicating that the CO_2 therapy suppressed the increase in its expression. Honda et al.[32] reported that TGF-β1 is greatly associated with muscle fibrosis in rats. This result supports that mRNA expression of TGF-β1 in the biceps femoris increased after immobilization in this study, and further the therapeutic application of CO_2 reduced it. However, the CO_2 therapy for the prevention had no beneficial effects on

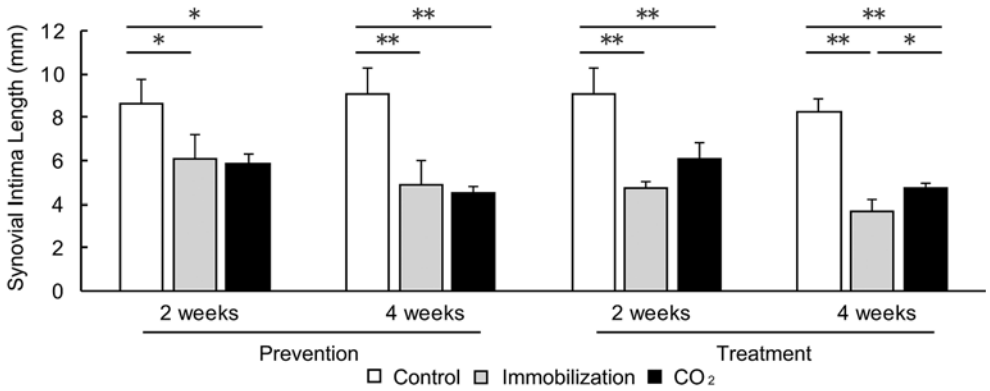

Fig. 5. The graphs show quantification of the synovial intima length. Four samples from all groups were evaluated at each timepoint. Data are means ± SD. $*P < 0.05$; $**P < 0.01$.

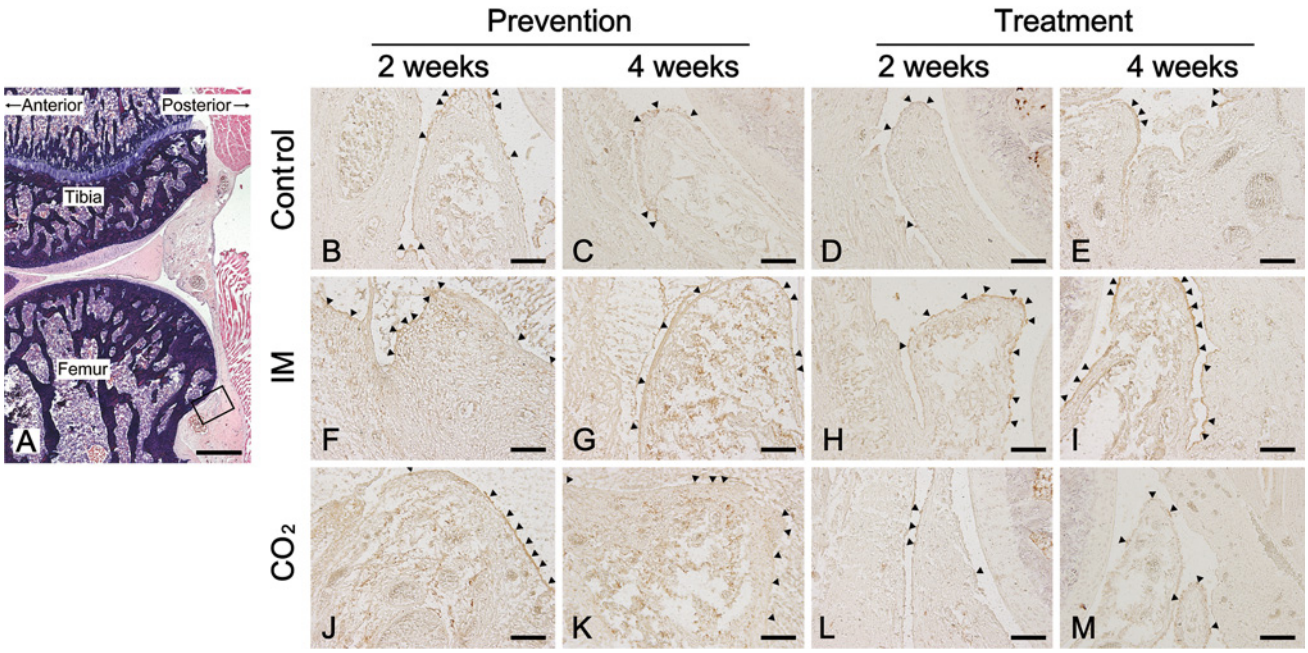

Fig. 6. Representative photomicrographs show the distribution of TGF-β1 in joint capsules. (A) Immunohistochemistry was assessed in the posterior joint capsule (box). Bar = 1 mm. The control group, in the prevention group at (B) 2 weeks and (C) 4 weeks and in the treatment group at (D) 2 weeks and (E) 4 weeks, the IM groups, in the prevention group at (F) 2 weeks and (G) 4 weeks and in the treatment group at (H) 2 weeks and (I) 4 weeks, and the CO_2 group, in the prevention group at (J) 2 weeks and (K) 4 weeks and in the treatment group at (L) 2 weeks and (M) 4 weeks, respectively. The TGF-β1 immunolabeling was mainly observed in the synovial membrane surface (arrowheads). Three samples from all groups were evaluated at each timepoint. Bars = 100 μm.

the progression of muscle fibrosis and an increase in the expression of TGF-β1. mRNA expression of TGF-β1 in the rat soleus muscle increases at the first weeks of immobilization[32]. Therefore, the CO_2 therapy for the prevention may be unable to inhibit this rapid early increase in gene expression of TGF-β1, thus leading to the resultant muscle fibrosis. Taken together, these results suggest that the CO_2 therapy improves muscular fibrosis due to reduced fibrosis-related gene (Type I collagen and TGF-β1) expression, thereby improving the muscular factor.

The adhesions and shortening of the synovial membrane due to joint immobility is closely associated with the progression of joint contractures[21,33]. Based on the results of histological analysis, posterior synovial intima length was shorter after joint immobilization. The shortening of synovial intima does not improve spontaneously with remobilization after immobilization for more than 2 weeks[34], and rather remobilization reduces the synovial length[25]. Meanwhile, the CO_2 exposure for the treatment has improve the shortening of synovial membrane. The joint motion stretches the synovial membrane and is able to prevent the adhesion by releasing the adhesion of synovial folds. CO_2 applications promote vascularization and increase blood flow in the hindlimb muscles and bone of rats[9,12]. There-

fore, we speculate that the improvement in the shortening of synovial membrane in the treatment group may be due to the synergistic effects of joint movements and the CO_2 application. Hence, the CO_2 therapy may improve irreversible synovial intima shortening following joint immobilization. A fibrosis of the joint capsules, in addition to the shortening of the synovial membrane, is a major cause of the progression of joint contractures[35,36]. The collagen content is associated with stiffness in connective tissues of knee; and immobilization leads to distortion of collagen sequence and increasing collagen density, whereby an elasticity of synovial membrane dwindles[37]. In the current study, there were no differences in the staining intensity and pattern of Type I collagen among groups at all-timepoints. The changes of Type I collagen in joint capsules after immobilization are still controversial[38,39]. On the other hand, TGF-β1 plays a key role in generating tissue fibrosis[40], and its protein level increases in capsules of a rat immobilized knee[41,42]. Joint immobilization also increased TGF-β1 protein level in our study. Furthermore, Kaneguchi et al.[25] showed that remobilization after 3-weeks immobilization increased TGF-β1 expression in the capsule. In our study, the CO_2 therapy in the treatment reduced the TGF-β1 protein in joint capsules associated with fibrosis. Fukui et al.[43] reported that suppression of TGF-β1 pathway attenuated the adhesion of joint capsules and improved the limitation in ROM. Therefore, the CO_2 therapy may suppress the increase in TGF-β1, improve the fibrosis of capsules, and consequently the articular factor of contractures is restored.

Our study has several limitations. First, we used a small animal model, which cannot fully reflect the variability found in humans. However, small animal models are preferred for preliminary screening, and the rat immobilized knee model used this study is applied to many studies of contracture[2,16,24]. The second limitation is that we used the right and the left knee joint served as different samples. The use of both joints has the advantages of minimizing the number of experimental animals needed for ethical reasons and providing equivalency of sample size for statistical purposes. However, its use cannot preclude chance attributable to intra-animal and inter-animal variation. Moreover, the statistical power of this study was partly limited due to the small sample size. Consequences of low statistical power are likely to generate/produce false-negative findings, necessitating larger sample size in future studies. Finally, we did not examine the underlying mechanism of the improvement in muscular and articular fibrosis after contracture development. The connective tissues are broken down primarily by extracellular proteolytic systems such as matrix metalloproteinases[44]; however it is unclear whether CO_2 therapy affects these systems. Additionally, the fibrosis of the joint capsule after remobilization is enhanced by inflammation[25] and is inhibited by anti-inflammatory drug[45]. Although topical application of CO_2 induces PGC-1α[12], which induces anti-inflammatory cytokines[46], in muscles, its anti-inflammatory effects on the joint capsule remains unexplained. Therefore, the cause of the improvement in the fibrosis and effect of CO_2 need further investigation.

Conclusion

Our findings indicate that CO_2 therapy can prevent and treat contractures after joint immobilization by improving ROM restriction and deterioration both muscular and articular factors responsible for contractures. This therapy increases the extensibility of the periarticular structures, and we propose that the combination with conventional treatments (eg, positioning or stretching) may be better therapeutic strategies for contractures after immobilization.

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Conflict of Interest: One of the authors (YS) holds shares in CO2BE Medical Engineering Inc.

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