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Effects of the duration of transcutaneous CO₂ application on the facilitatory effect in rat fracture

repair

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Abstract

Background

Carbon dioxide therapy has been reported to be effective in treating certain cardiac diseases and skin problems. Although a previous study suggested that transcutaneous carbon dioxide application accelerated fracture repair in association with promotion of angiogenesis, blood flow, and endochondral ossification, the influence of the duration of carbon dioxide application on fracture repair is unknown. The aim of this study was to investigate the effect of the duration of transcutaneous carbon dioxide application on rat fracture repair.

Methods

A closed femoral shaft fracture was created in each rat. Animals were randomly divided into four groups: the control group; 1w- CO₂ group, postoperative carbon dioxide treatment for 1 week; 2w- CO₂ group, postoperative carbon dioxide treatment for 2 weeks; 3w- CO₂ group, postoperative carbon dioxide treatment for 3 weeks. Transcutaneous carbon dioxide application was performed five times a week in the carbon dioxide groups. Sham treatment, where the carbon dioxide was replaced with air, was performed for the control group. Radiographic, histological, and biomechanical assessments were performed at 3 weeks after fracture.

Results

The fracture union rate was significantly higher in the 3w- CO₂ group than in the control group ($p < 0.05$). Histological assessment revealed promotion of endochondral ossification in the 3w- CO₂ group than in the control group. In the biomechanical assessment, all evaluation items related to bone strength were significantly higher in the 3w- CO₂ group than in the control group ($p < 0.05$).

Conclusions

The present study, conducted using an animal model, demonstrated that continuous carbon dioxide application throughout the process of fracture repair was effective in enhancing fracture healing.

Introduction

Carbon dioxide (CO₂) therapy is applied to certain cardiovascular diseases and skin problems such as limb ischemia and skin irregularity with adiposity [1-3]. The therapeutic effect of CO₂ is supposedly caused by increased blood flow, microcirculation, and nitric oxide-dependent neocapillary formation as well as increase in oxygen partial pressure in the local tissue known as the Bohr effect [4]. A system for transcutaneous CO₂ treatment that uses a novel hydrogel that is applied on the skin of a limb enhances CO₂ delivery into deep soft tissue [5, 6]. This system allows easy and noninvasive topical CO₂ application to the limbs. A recent study reported that this treatment accelerated fracture repair in a rat femoral fracture model in association with the promotion of angiogenesis, blood flow, and endochondral ossification [7]. The fracture repair process can be divided into three basic stages: inflammatory, reparative, and remodeling [8, 9]. In the inflammatory phase, various inflammatory cytokines are secreted to the fracture site. These cytokines recruit mesenchymal stem cells (MSCs) from various locations to the fracture site. Endochondral ossification, which is one of the most important processes for fracture repair, starts in the reparative phase. This is followed by the remodeling phase, during which fracture repair is completed. Although transcutaneous CO₂ application acts on various processes including the inflammatory and reparative phases [7], the effect of the duration of CO₂ application on fracture repair is unknown. The aim of this study was to investigate the effect of the duration of transcutaneous CO₂ application on rat fracture repair.

Material and Methods

Animal model

Twelve-week-old male Sprague-Dawley rats (CLEA, Tokyo, Japan) with a mean weight (and standard deviation) of 410.9 ± 9.2 g were used in this study. All experiments were performed under the approval and guidance of the Animal Care and Use Committee of Kobe University Graduate School of Medicine (approval no. P110601). We created a standard closed fracture in the shaft of the right femur of 40 rats, as described previously [10]. Briefly, under anesthesia induced by intraperitoneal injection with sodium pentobarbital, a 1.2-mm-diameter Kirschner wire was inserted into the femoral medullary canal, and a

closed transverse femoral shaft fracture was created with a three-point bending apparatus with a drop weight. Unprotected weight bearing was allowed postoperatively. Euthanasia through pentobarbital overdose was performed before assessments.

Transcutaneous CO₂ application

The animals were randomly divided into four groups (n=10 each): control group, sham treatment; 1w-CO₂ group, postoperative CO₂ treatment for 1 week; 2w-CO₂ group, postoperative CO₂ treatment for 2 weeks; 3w-CO₂ group, postoperative CO₂ treatment for 3 weeks. Transcutaneous CO₂ application to the fractured lower limbs of rats was performed, as previously described [7]. Briefly, both limbs were sealed with a polyethylene bag, which was filled with 100% CO₂ for 20 minutes. Control animals received sham treatment, where the CO₂ was replaced with air. After sedation was induced with a minimum dose of ether in a dark environment, the hair of the fractured limb was shaved, and the hydrogel, which enhances CO₂ absorption, was applied. The hydrogel (pH 5.5) consisted of carbomer, glycerin, sodium hydroxide, sodium alginate, sodium dihydrogen phosphate, methylparaben, and deionized water.

Radiographic assessment

Rats were fixed in the supine position with the limbs fully extended under anesthesia, and radiographs of the fractured limbs were taken 3 weeks after the fracture (n=10 per group). Fracture union was identified by the presence of bridging callus formation on four cortices on the anteroposterior and lateral views.

Histological assessment

Histological assessment was performed with Safranin-O staining at 3 weeks after the fracture (n=5 per group). The degree of fracture repair was assessed on a five-point scale (grade 0-4) according to Allen's grading system [11]. To assess the progression of endochondral ossification, the total cartilage area was calculated as the sum of the areas of cartilage using NIH ImageJ software.

Immunohistochemistry for VEGF

Immunohistochemical assessment was performed at 3 weeks after the fracture. The sections were

incubated overnight at 4°C with anti-VEGF primary antibody (1:100 dilution, ab1316, Abcam, Cambridge, MA, USA) and subsequently treated with peroxidase-labeled anti-mouse immunoglobulin (Histofine Simplestain max PO (M), Nichirei Bioscience, Tokyo, Japan) at room temperature for 60 minutes. The signal was developed as a brown reaction product using the peroxidase substrate 3,3'-diaminobenzidine (Histofine Simplestain DAB Solution, Nichirei Bioscience). The sections were counterstained with hematoxylin.

Assessment of angiogenesis

Assessment of angiogenesis was performed at 3 weeks after the fracture. To evaluate cross-sectional capillary density, immunohistochemical staining of endothelial cells was performed with fluorescein-labeled isolectin B4 (Vector Laboratories, Burlingame, CA, USA). Nuclear staining was performed with DAPI solution (4',6-diamidino-2-phenylindole, Nacalai Tesque, Kyoto, Japan).

Biomechanical assessment

A standardized three-point bending test was performed with use of a load torsion and bending tester at 3 weeks after the fracture (n=5). The bending force was applied with the crosshead at a speed of 2 mm/min until rupture occurred. The ultimate stress (N), extrinsic stiffness (N/mm), and failure energy (N·mm) were measured. For each parameter, the ratio of the value in the fractured femur to that in the intact femur in the same animal was calculated.

Statistical analysis

Fisher's exact test was used for the radiographic assessments. To assess histological (degree of fracture repair and size of the cartilage areas) and biomechanical results, Kruskal-Wallis test was performed with Bonferroni corrected post hoc Mann-Whitney U-test. A p-value <0.05 was defined as statistically significant. Columns and error bars indicate means and standard deviations, respectively.

Results

Radiographic assessment of fracture repair

At 3 weeks after the fracture, fracture union with bridging callus formation was achieved in 90% of the animals in the 3w- CO₂ group, 60% of the animals in the 2w- CO₂ group, 30% of the animals in the 1w- CO₂ group, and 20% of the animals in the control group (Fig. 1; representative radiographs at week 3). The fracture union rate at week 3 was significantly higher in the 3w- CO₂ group than in the control group ($p < 0.05$). No significant difference was detected in any other group combinations.

Histological assessment of fracture sites

As shown in Fig. 2a, a thick cartilage area remained between the woven bones in the control group, whereas bony union was almost complete and only a small amount of cartilage was noted in the 3w- CO₂ group. The degree of fracture repair as assessed by Allen's grading system at week 3 was significantly higher in the 3w- CO₂ group than in the control group (Fig. 2b). The cartilage area at week 3 was significantly smaller in the 3w- CO₂ group than in the control group (Fig. 2c). No significant difference in either Allen's grading or the cartilage area was detected in any other group combinations.

Immunohistochemistry for VEGF

At 3 weeks after fracture, the immunoreactivity of VEGF was detected in osteoblasts lined on the trabecular bone in both the control and 3w- CO₂ groups (Fig. 3). There was no difference in the staining pattern of both groups.

Assessment of angiogenesis

Fluorescent vascular staining with isolectin B4 in histological sections collected at week 3 after fracture demonstrated greater angiogenesis surrounding the endochondral ossification region in the 3w- CO₂ group than in the control group (Fig. 4).

Biomechanical assessment of fracture repair

All three evaluation items for biomechanical assessment (ultimate stress, extrinsic stiffness, and failure energy) were significantly higher in the 3w- CO₂ group than in the control group ($p < 0.05$) (Fig. 5). No significant difference was detected in any other group combinations.

Discussion

The fracture union rate was significantly higher in the 3w- CO₂ group than in the control group. Histological assessment revealed better promotion of endochondral ossification in the 3w- CO₂ group than in the control group. In the biomechanical assessment, all evaluation items related to bone strength were significantly higher in the 3w- CO₂ group than in the control group. CO₂ treatment has been studied for clinical application in various fields. There are some reports that CO₂ application is effective for treating ischemic limbs and accelerating wound healing [12, 13]. In the field of dermatology, CO₂ facials improve skin oxygenation through an artificial Bohr effect [14]. In the orthopedic field, expecting fracture repair enhancement, we are investigating if clinical applications of CO₂ treatment to refractory fractures such as non-union and delayed union is possible, and it is at the clinical trial stage targeting humans. It is estimated that 5%-10% of all fractures fail to heal normally, resulting in delayed union or nonunion, but this rate varies in patients with diverse medical histories [15, 16]. We believe that this inexpensive and noninvasive treatment would help many patients with refractory fracture.

In the inflammatory phase of fracture repair, bleeding from broken blood vessels forms hematoma, followed by necrosis of the edge of the fracture. The necrotic cells activate inflammatory cytokines. Moreover, proliferation of MSCs and capillary neogenesis are observed. In the reparative phase, the bone fracture ends connect by soft callus mainly composed of fibrous bone; thereafter, calcium is deposited in this osteoid tissue to become hard callus. Differentiation of MSCs into chondrocytes and osteoblasts and initiation of endochondral ossification occur in this phase. In the remodeling phase, fibrous bone is replaced with stronger lamellar bone through repeated bone resorption and formation.

In this study, there was no significant difference in all assessments of fracture repair between the control and 1w- CO₂ groups. In the rat fracture model, chondrogenesis and inflammatory response are seen at the fracture site on the first week after the fracture [17, 18]. This fracture repair process supposedly corresponds to the inflammatory phase. Therefore, this result suggested that CO₂ application only during the inflammatory phase is insufficient for obtaining the effect of fracture repair enhancement.

Regarding bone union rate and biomechanical assessment in the 2w- CO₂ group, although there was no

significant difference compared with the control group, a tendency for fracture repair promotion was seen. Studies reported that initiation of endochondral ossification is observed at some cartilages on the second week after fracture in the rat fracture model [17, 18]. This process almost corresponds to the reparative phase. The fracture repair in the 3w- CO₂ group was significantly enhanced compared with that in the control group. Histological assessment showed that the cartilage area at week 3 was significantly smaller in the 3w- CO₂ group than in the control group, suggesting that endochondral ossification was promoted by CO₂ application. These results indicated that the effect of enhancing fracture repair by CO₂ application was demonstrated through promotion of endochondral ossification in the reparative phase.

Various growth factors and cytokines are reported to be involved in the fracture repair process. It is reported that, in inflammatory phase, transforming growth factor β , platelet-derived growth factor, vascular endothelial growth factor (VEGF), interleukins 1 and 6, and tumor necrosis factor α coordinate the inflammatory cell response at the fracture site, which is necessary for fracture repair [17, 19]. Fibroblast growth factor 1, insulin like growth factor, VEGF, and the bone morphogenetic protein family plays an important role in soft and hard callus formation during the reparative phase [20, 21].

A study on rats demonstrated that the amount of VEGF expression in newly generated callus under CO₂ application is highest at 3 weeks after bone fracture [7]. VEGF is one of the most important factors involved in angiogenesis and is known to play a crucial role in angiogenesis at the fracture site [22-24]. Hu and Olsen [25] reported that VEGF stimulates vessel invasion and recruitment of chondroblasts into the hypertrophic cartilage and promotes endochondral ossification. In this study, we demonstrated the presence of cells stained with VEGF immunochemical staining in the newly generated callus and that angiogenesis is promoted in the 3w- CO₂ group. Considering these evidences, it is speculated that the fracture healing process of the 3w- CO₂ group was accelerated through the promotion of endochondral ossification by the increasing expression of VEGF in the newly generated callus. Based on the results of the present study, continuous CO₂ application throughout the process of fracture repair was demonstrated to be effective in the enhancement of fracture healing.

The results of this investigation provide useful information on the application of this system in the clinical

215 setting. However, many problems remain to be resolved before the clinical application of this treatment.
216 This study is directed at rats, and it is still unclear how long it should be continued when clinically
217 applied to humans. The reparative phase in humans is reported to be completed from 6 to 8 weeks after
218 fracture.
219 Although the treatment is only 20 minutes a day, performing the treatment every day during entire
220 duration of the fracture repair process is expected to be a considerable burden to the patient. Furthermore,
221 hospitalization during treatment or daily hospital visits might be necessary since it might be dangerous to
222 use high-concentration CO₂ gas at home. For clinical application, a method to carry out this treatment
223 more conveniently and safely should be developed, and it is considered to be our future work.

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286

Figure Captions

Fig. 1 Radiographic assessment at 3 weeks after fracture

(a, b) In the control and 1w- CO₂ groups, a wide radiolucent area was detected between the fracture callus (arrows). (c, d) With prolonged CO₂ application, the radiolucent area between the fracture callus decreased.

Fig. 2 Histological assessment at 3 weeks after fracture

(a) Representative histological sections stained with Safranin-O/fast green. Red staining represents cartilage areas. (b) Mean Allen's grading score (and standard deviations). (c) Mean area of the cartilage regions (and standard deviations).

Fig. 3 Immunohistochemistry for the callus of fracture sites at 3 weeks after fracture

Immunohistochemical analysis showing VEGF expression (brown staining) in the callus of fracture sites in the control and 3w-CO₂ groups as visualized using antibodies to VEGF at 3 weeks after fracture. Bars=100µm. Red arrows, osteoblasts lining the trabecular bone surface.

Fig. 4 Assessment of angiogenesis at 3 weeks after the fracture

The upper row: representative histological sections stained with Safranin- O/fast green in the control and 3w-CO₂ groups. The lower row: representative images of fluorescent vascular staining with isolectin B4 (ILB4; green) and 4',6-diamidino-2-phenylindole (DAPI; blue) in the Control and 3w-CO₂ groups. Bars=100 µm. The area surrounded by yellow squares in safranin-O/fast green staining indicates the region of interest observed using vascular staining.

Fig. 5 Biomechanical assessment with three-point bending test at 3 weeks after fracture

Values were normalized relative to the contralateral intact femur, and the mean value and standard deviations are shown. The control and 3w- CO₂ groups had significant differences.

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Conflicts of interest: None

Figure.1

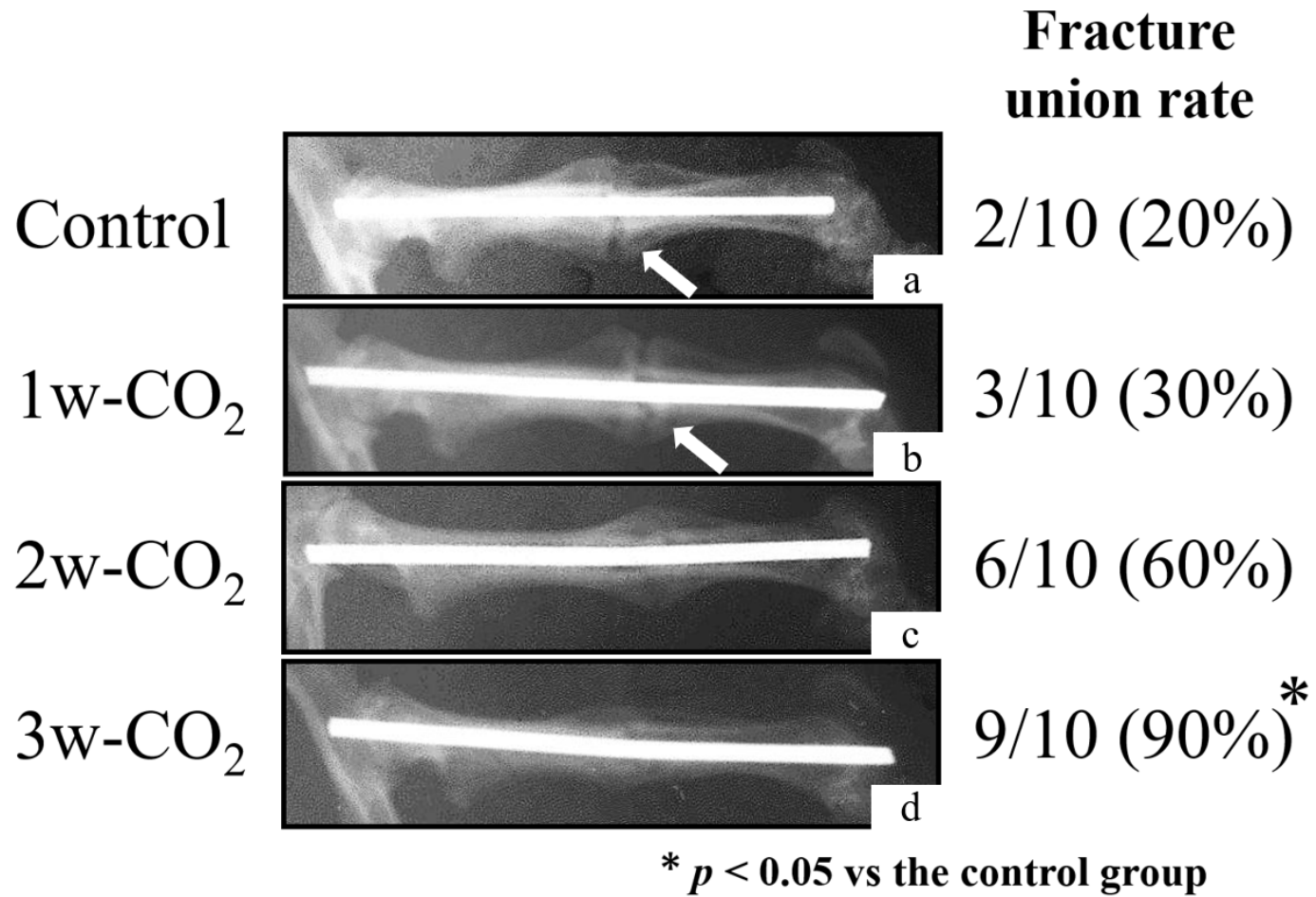
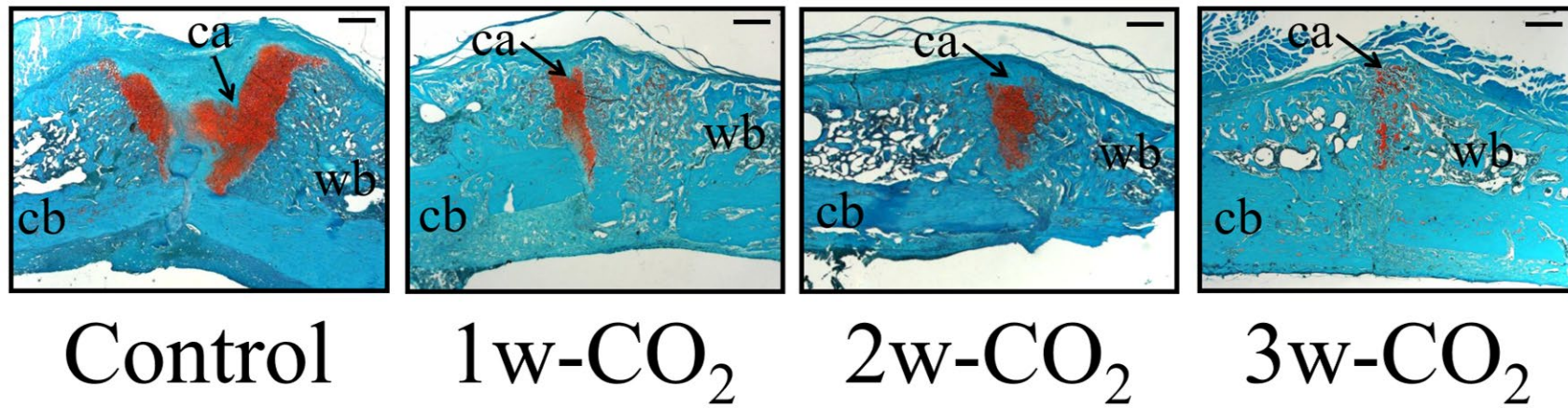


Figure. 2a



(cb: cortical bone, ca: cartilage, wb: woven bone, Bar= 500 μm)

Figure. 2b

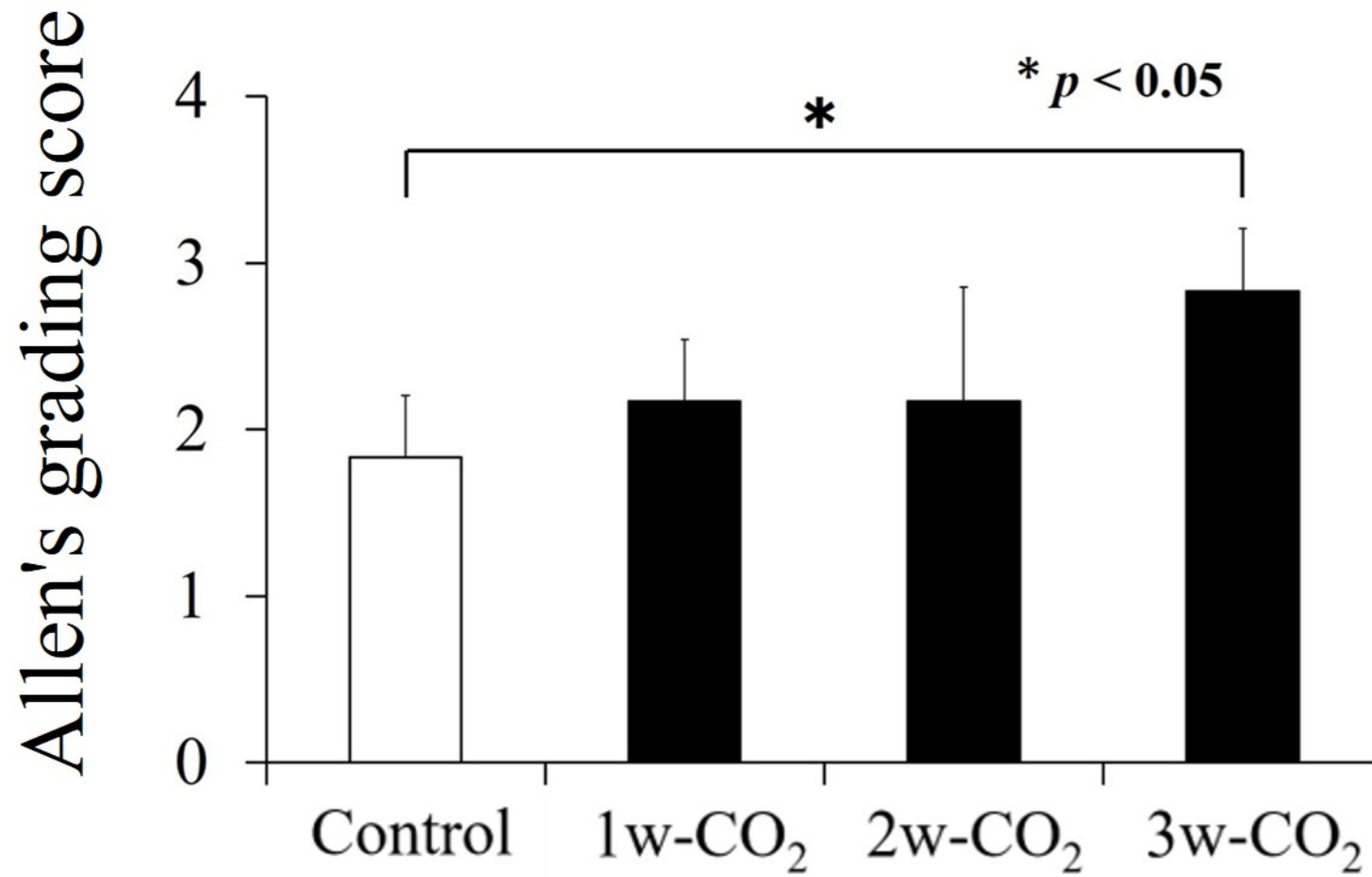


Figure. 2c

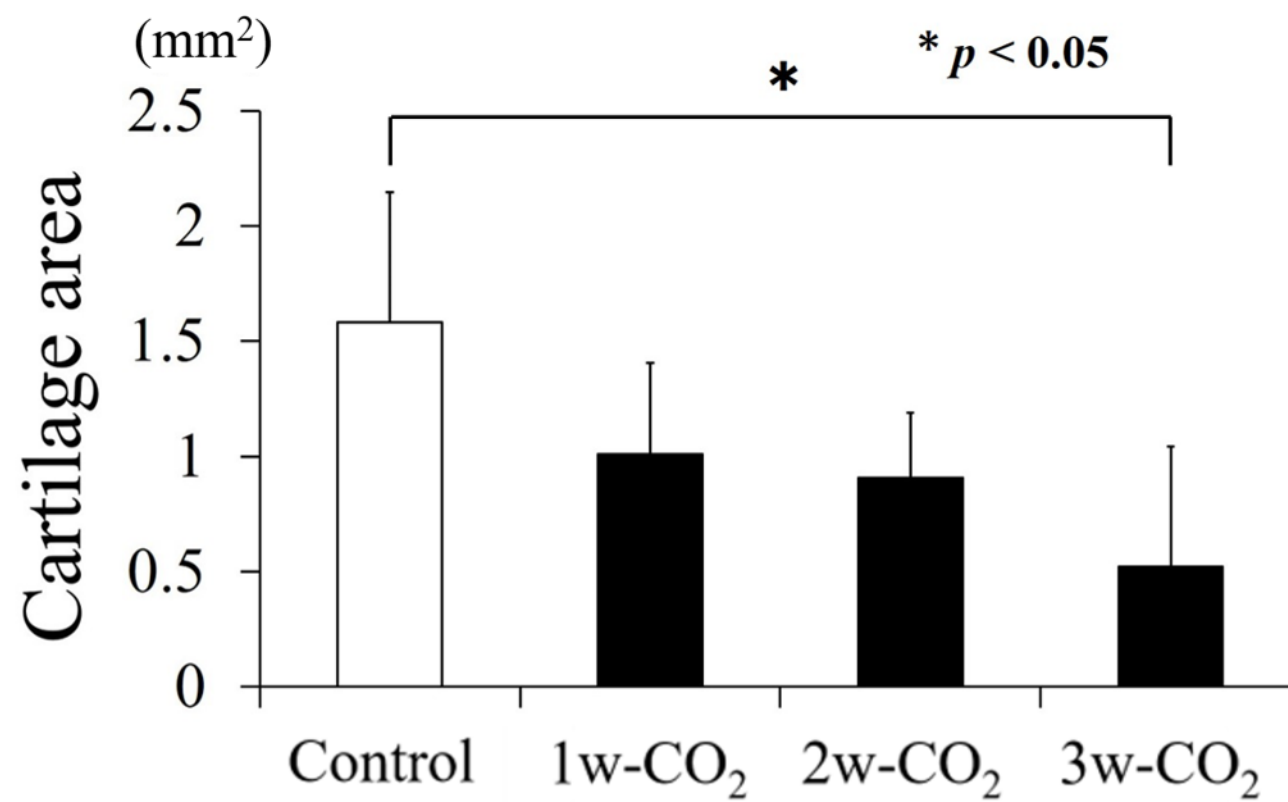
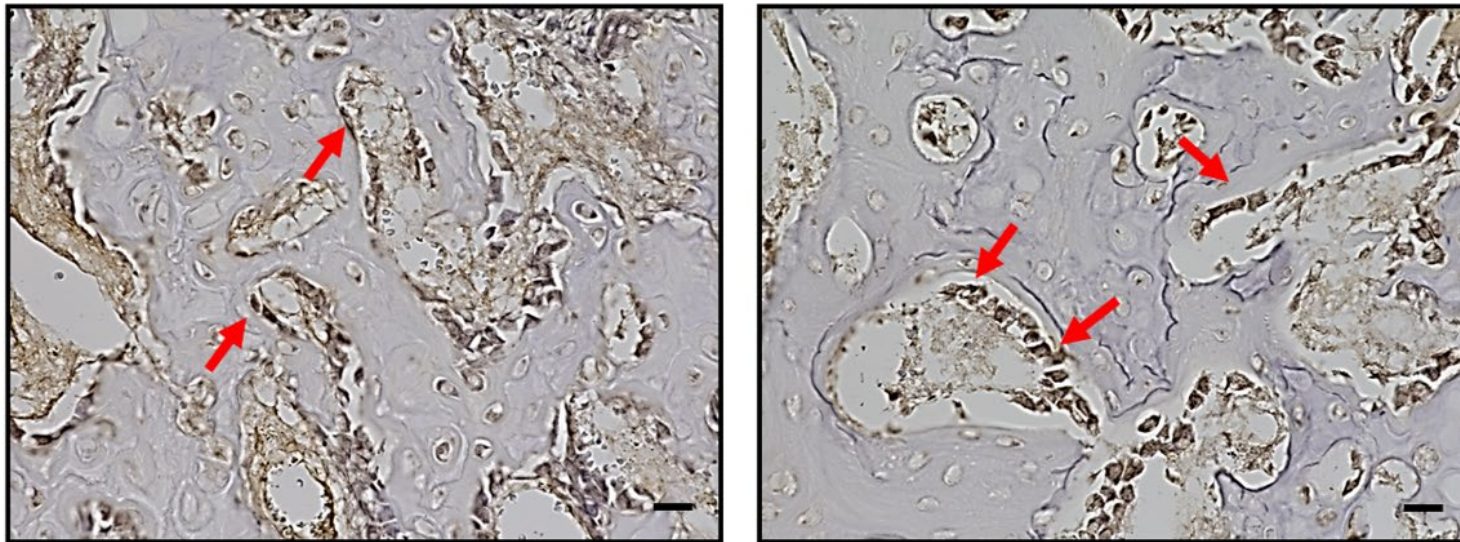


Figure. 3



Control

3w-CO₂

Figure. 4

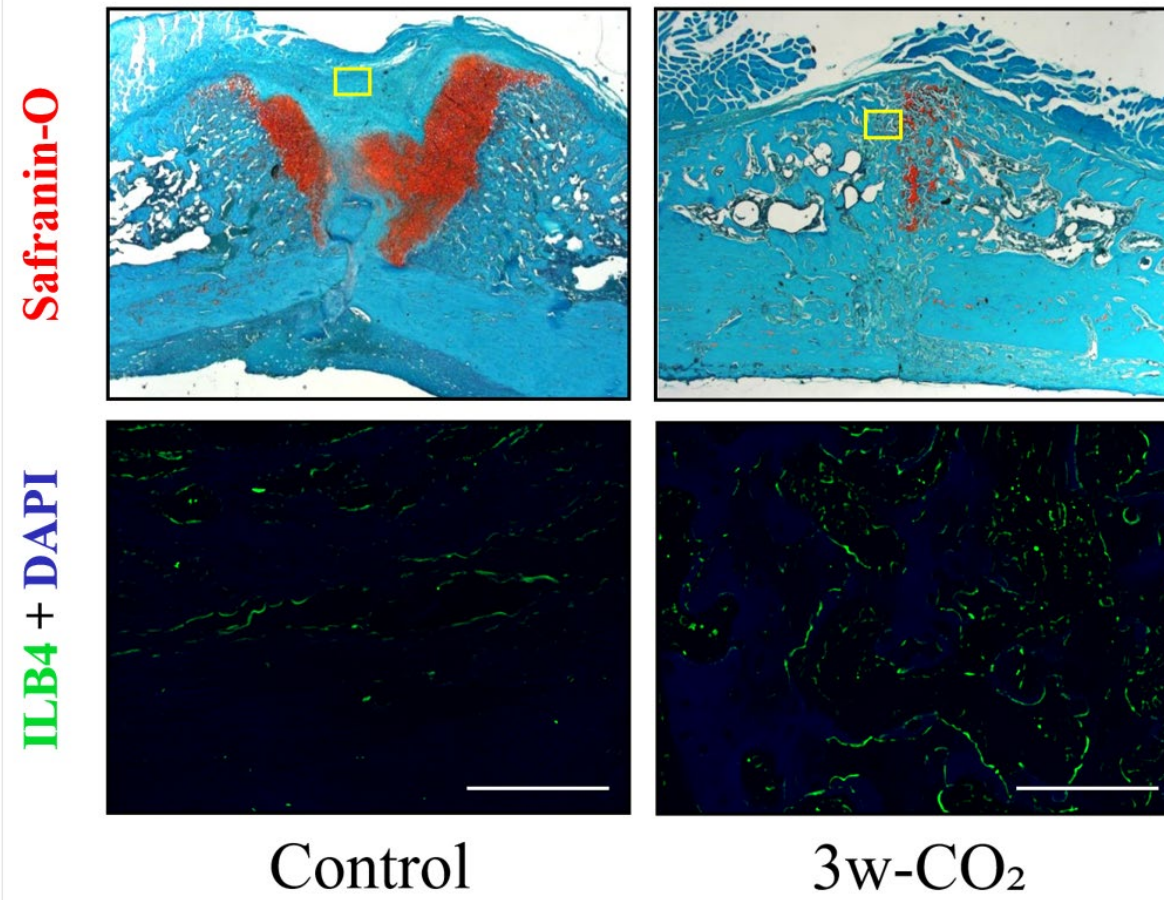


Figure. 5

