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(Citation)
Journal of Orthopaedic Science,25(5):886-891

(Issue Date)
2020-09

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90007585

Effects of the duration of transcutaneous CO_2 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- CO_2 group, postoperative carbon dioxide treatment for 1 week; 2w- CO_2 group, postoperative carbon dioxide treatment for 2 weeks; 3w- CO_2 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- CO_2 group than in the control group ($p < 0.05$). Histological assessment revealed promotion of endochondral ossification in the 3w- CO_2 group than in the control group. In the biomechanical assessment, all evaluation items related to bone strength were significantly higher in the 3w- CO_2 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 (CO_2) 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 CO_2 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 CO_2 treatment that uses a novel hydrogel that is applied on the skin of a limb enhances CO_2 delivery into deep soft tissue [5, 6]. This system allows easy and noninvasive topical CO_2 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 CO_2 application acts on various processes including the inflammatory and reparative phases [7], the effect of the duration of CO_2 application on fracture repair is unknown.

The aim of this study was to investigate the effect of the duration of transcutaneous CO_2 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 CO_2 application

The animals were randomly divided into four groups (n=10 each): control group, sham treatment; 1w-CO_2 group, postoperative CO_2 treatment for 1 week; 2w- CO_2 group, postoperative CO_2 treatment for 2 weeks; 3w- CO_2 group, postoperative CO_2 treatment for 3 weeks. Transcutaneous CO_2 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% CO_2 for 20 minutes. Control animals received sham treatment, where the CO_2 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 CO_2 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℃ 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- CO_2 group, 60% of the animals in the 2w- CO_2 group, 30% of the animals in the 1w- CO_2 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- CO_2 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- CO_2 group. The degree of fracture repair as assessed by Allen's grading system at week 3 was significantly higher in the 3w- CO_2 group than in the control group (Fig. 2b). The cartilage area at week 3 was significantly smaller in the 3w- CO_2 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- CO_2 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- CO_2 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- CO_2 group than in the control group ($p < 0.05$) (Fig. 5). No significant difference was detected in any other group combinations.

157 **Discussion**

158 The fracture union rate was significantly higher in the 3w- CO_2 group than in the control group.
159 Histological assessment revealed better promotion of endochondral ossification in the 3w- CO_2 group
160 than in the control group. In the biomechanical assessment, all evaluation items related to bone strength
161 were significantly higher in the 3w- CO_2 group than in the control group. CO_2 treatment has been studied
162 for clinical application in various fields. There are some reports that CO_2 application is effective for
163 treating ischemic limbs and accelerating wound healing [12, 13]. In the field of dermatology, CO_2 facials
164 improve skin oxygenation through an artificial Bohr effect [14]. In the orthopedic field, expecting fracture
165 repair enhancement, we are investigating if clinical applications of CO_2 treatment to refractory fractures
166 such as non-union and delayed union is possible, and it is at the clinical trial stage targeting humans. It is
167 estimated that 5%-10% of all fractures fail to heal normally, resulting in delayed union or nonunion, but
168 this rate varies in patients with diverse medical histories [15, 16]. We believe that this inexpensive and
169 noninvasive treatment would help many patients with refractory fracture.
170
171 In the inflammatory phase of fracture repair, bleeding from broken blood vessels forms hematoma,
172 followed by necrosis of the edge of the fracture. The necrotic cells activate inflammatory cytokines.
173 Moreover, proliferation of MSCs and capillary neogenesis are observed. In the reparative phase, the bone
174 fracture ends connect by soft callus mainly composed of fibrous bone; thereafter, calcium is deposited in
175 this osteoid tissue to become hard callus. Differentiation of MSCs into chondrocytes and osteoblasts and
176 initiation of endochondral ossification occur in this phase. In the remodeling phase, fibrous bone is
177 replaced with stronger lamellar bone through repeated bone resorption and formation.
178
179 In this study, there was no significant difference in all assessments of fracture repair between the control
180 and 1w- CO_2 groups. In the rat fracture model, chondrogenesis and inflammatory response are seen at the
181 fracture site on the first week after the fracture [17, 18]. This fracture repair process supposedly
182 corresponds to the inflammatory phase. Therefore, this result suggested that CO_2 application only during
183 the inflammatory phase is insufficient for obtaining the effect of fracture repair enhancement.
184
185 Regarding bone union rate and biomechanical assessment in the 2w- CO_2 group, although there was no

186 significant difference compared with the control group, a tendency for fracture repair promotion was seen.
187 Studies reported that initiation of endochondral ossification is observed at some cartilages on the second
188 week after fracture in the rat fracture model [17, 18]. This process almost corresponds to the reparative
189 phase. The fracture repair in the 3w- CO_2 group was significantly enhanced compared with that in the
190 control group. Histological assessment showed that the cartilage area at week 3 was significantly smaller
191 in the 3w- CO_2 group than in the control group, suggesting that endochondral ossification was promoted
192 by CO_2 application. These results indicated that the effect of enhancing fracture repair by CO_2 application
193 was demonstrated through promotion of endochondral ossification in the reparative phase.
194
195 Various growth factors and cytokines are reported to be involved in the fracture repair process. It is
196 reported that, in inflammatory phase, transforming growth factor β, platelet-derived growth factor,
197 vascular endothelial growth factor (VEGF), interleukins 1 and 6, and tumor necrosis factor α coordinate
198 the inflammatory cell response at the fracture site, which is necessary for fracture repair [17, 19].
199 Fibroblast growth factor 1, insulin like growth factor, VEGF, and the bone morphogenetic protein family
200 plays an important role in soft and hard callus formation during the reparative phase [20, 21].
201
202 A study on rats demonstrated that the amount of VEGF expression in newly generated callus under CO_2
203 application is highest at 3 weeks after bone fracture [7]. VEGF is one of the most important factors
204 involved in angiogenesis and is known to plays a crucial role in angiogenesis at the fracture site [22-24].
205 Hu and Olsen [25] reported that VEGF stimulates vessel invasion and recruitment of chondroblasts into
206 the hypertrophic cartilage and promotes endochondral ossification. In this study, we demonstrated the
207 presence of cells stained with VEGF immunochemical staining in the newly generated callus and that
208 angiogenesis is promoted in the 3w- CO_2 group. Considering these evidences, it is speculated that the
209 fracture healing process of the 3w- CO_2 group was accelerated through the promotion of endochondral
210 ossification by the increasing expression of VEGF in the newly generated callus. Based on the results of
211 the present study, continuous CO_2 application throughout the process of fracture repair was demonstrated
212 to be effective in the enhancement of fracture healing.
213
214 The results of this investigation provide useful information on the application of this system in the clinical

setting. However, many problems remain to be resolved before the clinical application of this treatment. This study is directed at rats, and it is still unclear how long it should be continued when clinically applied to humans. The reparative phase in humans is reported to be completed from 6 to 8 weeks after fracture.

Although the treatment is only 20 minutes a day, performing the treatment every day during entire duration of the fracture repair process is expected to be a considerable burden to the patient. Furthermore, hospitalization during treatment or daily hospital visits might be necessary since it might be dangerous to use high-concentration CO_2 gas at home. For clinical application, a method to carry out this treatment more conveniently and safely should be developed, and it is considered to be our future work.

225 **References**

226 1. Hartmann BR, Bassenge E, Pittler M. Effect of carbon dioxide-enriched water and fresh water on
227 the cutaneous microcirculation and oxygen tension in the skin of the foot. Angiology
228 1997;48:337–43.
229 2. Pagourelias ED, Zorou PG, Tsaligopoulos M, Athyros VG, Karagiannis A, Efthimiadis GK.
230 Carbon dioxide balneotherapy and cardiovascular disease. Int J Biometeorol 2011;55:657–63.
231 3. Brandi C, D'Aniello C, Grimaldi L, Caiazzo E, Stanghellini E. Carbon dioxide therapy: effects on
232 skin irregularity and its use as a complement to liposuction. Aesthetic Plast Surg 2004;28:222–5.
233 4. Jensen FB. Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood
234 O2 and CO_2 transport. Acta Physiol Scand 2004;182:215–27.
235 5. Oe K, Ueha T, Sakai Y, Niikura T, Lee SY, Koh A, Hasegawa T, Tanaka M, Miwa M, Kurosaka M.
236 The effect of transcutaneous application of carbon dioxide (CO_2) on skeletal muscle. Biochem
237 Biophys Res Commun 2011;407:148–52.
238 6. Sakai Y, Miwa M, Oe K, Ueha T, Koh A, Niikura T, Iwakura T, Lee SY, Tanaka M, Kurosaka M.
239 A novel system for transcutaneous application of carbon dioxide causing an "artificial Bohr effect"
240 in the human body. PLoS One 2011;6:e24137.
241 7. Koga T, Niikura T, Lee SY, Okumachi E, Ueha T, Iwakura T, Sakai Y, Miwa M, Kuroda R,
242 Kurosaka M. Topical cutaneous CO_2 application by means of a novel hydrogel accelerates fracture
243 repair in rats. J Bone Joint Surg Am 2014;96:2077–84.
244 8. Claes L, Recknagel S, Ignatius A. Fracture healing under healthy and inflammatory conditions.
245 Nat Rev Rheumatol 2012;8:133–43.
246 9. Dimitriou R, Tsiridis E, Giannoudis PV. Current concepts of molecular aspects of bone healing.
247 Injury 2005;36:1392–404.
248 10. Bonnarens F, Einhorn TA. Production of a standard closed fracture in laboratory animal bone. J
249 Orthop Res 1984;2:97–101.
250 11. Allen HL, Wase A, Bear WT. Indomethacin and aspirin: effect of nonsteroidal anti-inflammatory
251 agents on the rate of fracture repair in the rat. Acta Orthop Scand 1980;51:595–600.

12. Yamaguchi T, Yamazaki T, Nakamura Y, Shiota M, Shimada K, Miura K, Iwao H, Yoshiyama M, Izumi Y. Percutaneous carbon dioxide mist treatment has protective effects in experimental myocardial infarction. J Pharmacol Sci 2015;127:474–80.

13. Li WP, Su CH, Wang SJ, Tsai FJ, Chang CT, Liao MC, Yu CC, Vi Tran TT, Lee CN, Chiu WT, Wong TW, Yeh CS. CO_2 delivery to accelerate incisional wound healing following single irradiation of near-infrared lamp on the coordinated colloids. ACS Nano 2017;11:5826–35.

14. Seidel R, Moy R. Effect of carbon dioxide facial therapy on skin oxygenation. J Drugs Dermatol 2015;14:976–80.

15. Marsh D. Concepts of fracture union, delayed union, and nonunion. Clin Orthop Relat Res 1998;355 Suppl:S22–30.

16. Hak DJ, Fitzpatrick D, Bishop JA, Marsh JL, Tilp S, Schnettler R Simpson H, Alt V. Delayed union and nonunions: epidemiology, clinical issues, and financial aspects. Injury 2014;45:S3–7.

17. Einhorn TA. The cell and molecular biology of fracture healing. Clin Orthop Relat Res 1998;355:S7–21.

18. Einhorn TA. The science of fracture healing. J Orthop Trauma 2005;19:S4–6.

19. Bolander ME. Regulation of fracture repair by growth factors. Proc Soc Exp Biol Med 1992;200:165–70.

20. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 2003;88:873–84.

21. AI-Aql ZS, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA. Molecular mechanisms controlling bone formation during fracture healing and distraction. Osteogenesis J Dent Res 2008;87:107–18.

22. Street J, Bao M, deGuzman L, Bunting S, Peale FV Jr, Ferrara N, Steinmetz H, Hoeffel J, Cleland JL, Daugherty A, van Bruggen N, Redmond HP, Carano RA, Filvaroff EH. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci USA 2002;99:9656–61.

279 23. Hankenson KD, Dishowitz M, Gray C, Schenker M. Angiogenesis in bone regeneration. Injury
280 2011;42:556–61.
281 24. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic
282 cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med
283 1999;5:623–8.
284 25. Hu K, Olsen BR. The roles of vascular endothelial growth factor in bone repair and regeneration.
285 Bone 2016;91:30–8.
286

287 **Figure Captions**

288 **Fig. 1** Radiographic assessment at 3 weeks after fracture

289 (a, b) In the control and 1w- CO_2 groups, a wide radiolucent area was detected between the fracture callus

290 (arrows). (c, d) With prolonged CO_2 application, the radiolucent area between the fracture callus

291 decreased.

292

293 **Fig. 2** Histological assessment at 3 weeks after fracture

294 (a) Representative histological sections stained with Safranin-O/fast green. Red staining represents

295 cartilage areas. (b) Mean Allen's grading score (and standard deviations). (c) Mean area of the cartilage

296 regions (and standard deviations).

297

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

299 Immunohistochemical analysis showing VEGF expression (brown staining) in the callus of fracture sites

300 in the control and 3w-CO_2 groups as visualized using antibodies to VEGF at 3 weeks after fracture.

301 Bars=100μm. Red arrows, osteoblasts lining the trabecular bone surface.

302

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

304 The upper row: representative histological sections stained with Safranin- O/fast green in the control and

305 3w-CO_2 groups. The lower row: representative images of fluorescent vascular staining with isolectin B4

306 (ILB4; green) and 4',6-diamidino-2-phenylindole (DAPI; blue) in the Control and 3w-CO_2 groups.

307 Bars=100 μm. The area surrounded by yellow squares in safranin-O/fast green staining indicates the

308 region of interest observed using vascular staining.

309

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

311 Values were normalized relative to the contralateral intact femur, and the mean value and standard

312 deviations are shown. The control and 3w- CO_2 groups had significant differences.

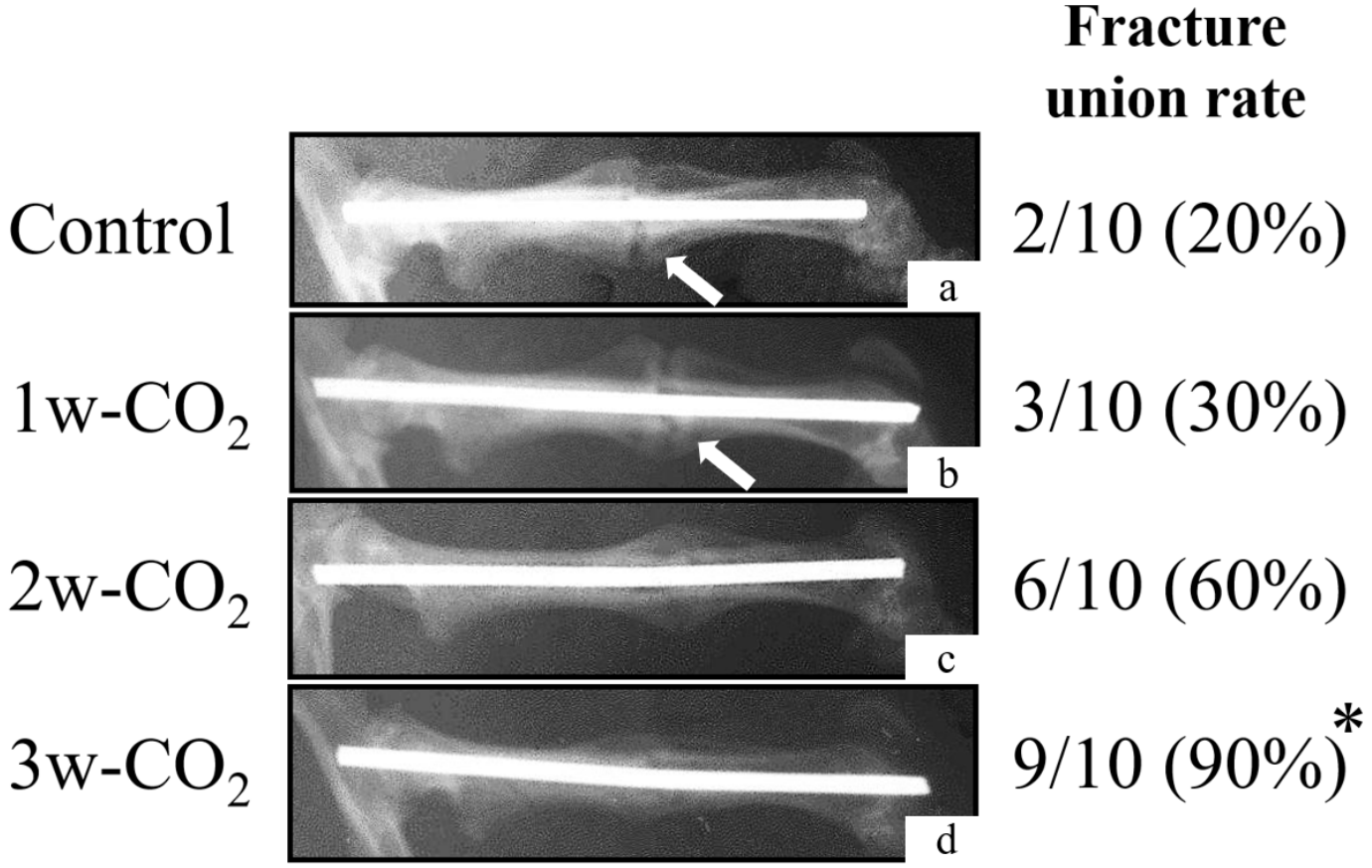
313

Acknowledgments: The authors thank Ms. K. Tanaka, Ms. M. Nagata, and Ms. M. Yasuda (Department of Orthopedic Surgery, Kobe University Graduate School of Medicine) for their excellent technical assistance.

Funding: This work was supported by the Grants-in-Aid for Scientific Research of Japan Society for the Promotion of Science, Grant Number JP26462266. The funder sources had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

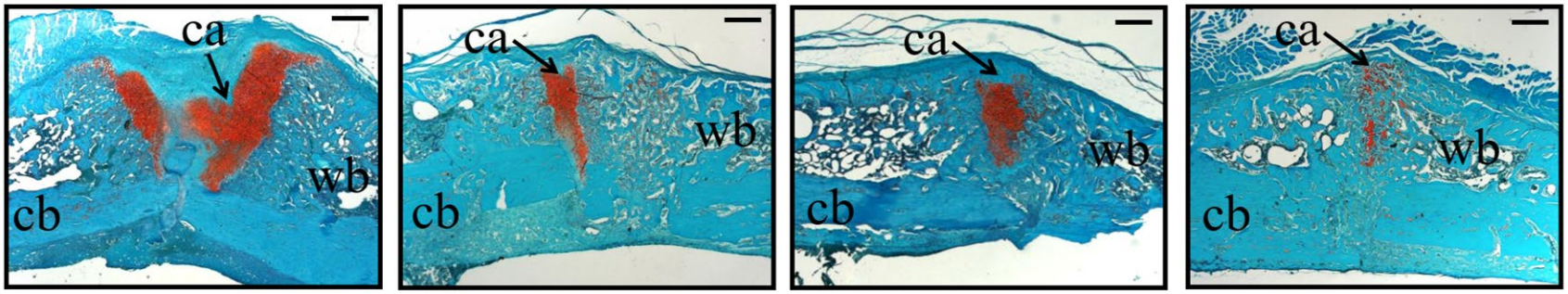
Conflicts of interest: None

Figure.1



*** $p < 0.05$ vs the control group**

Figure. 2a



Control 1w-CO_2 2w-CO_2 3w-CO_2

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

Figure. 2b

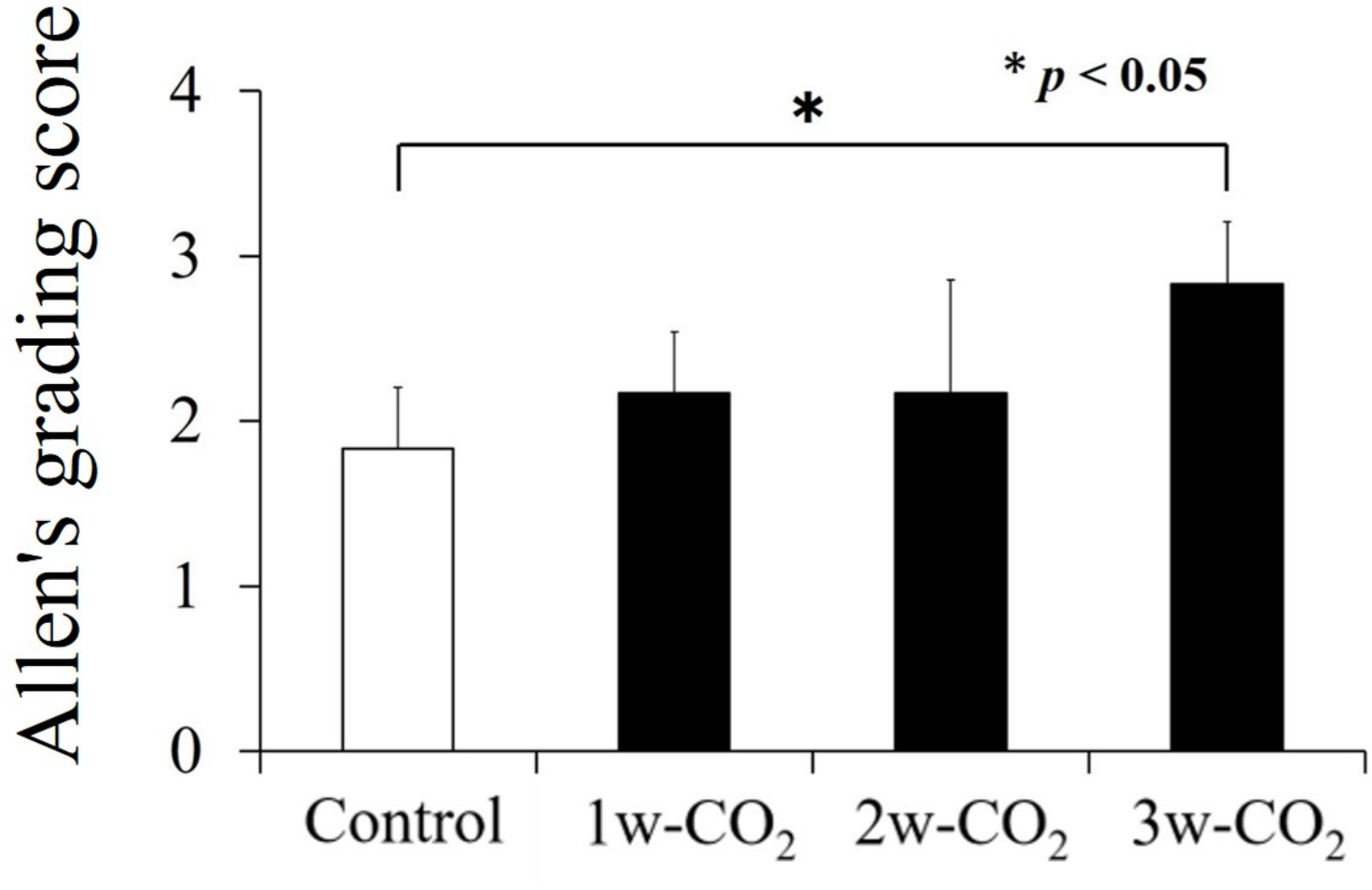


Figure. 2c

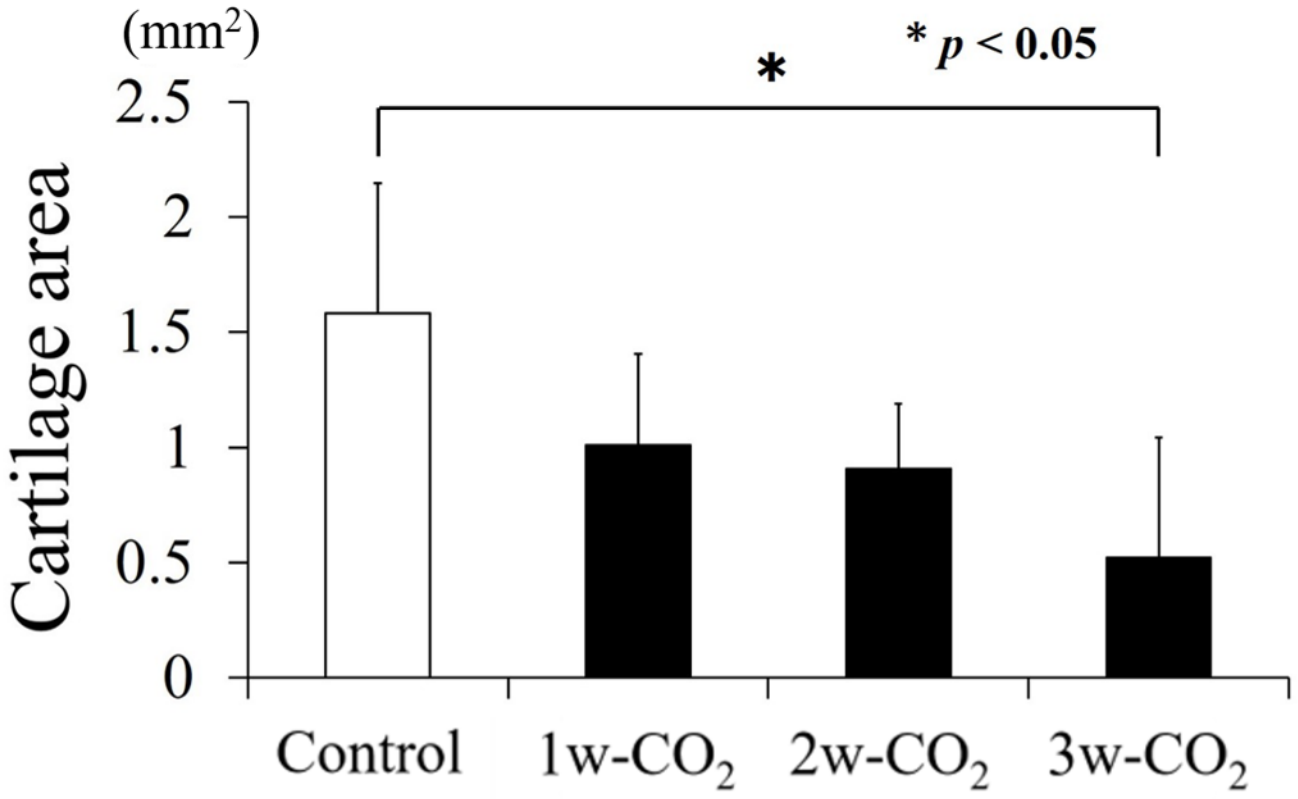

(mm²)
* p < 0.05
*
Cartilage area
2.5
2
1.5
1
0.5
0
Control
1w-CO2
2w-CO2
3w-CO2

Figure. 3

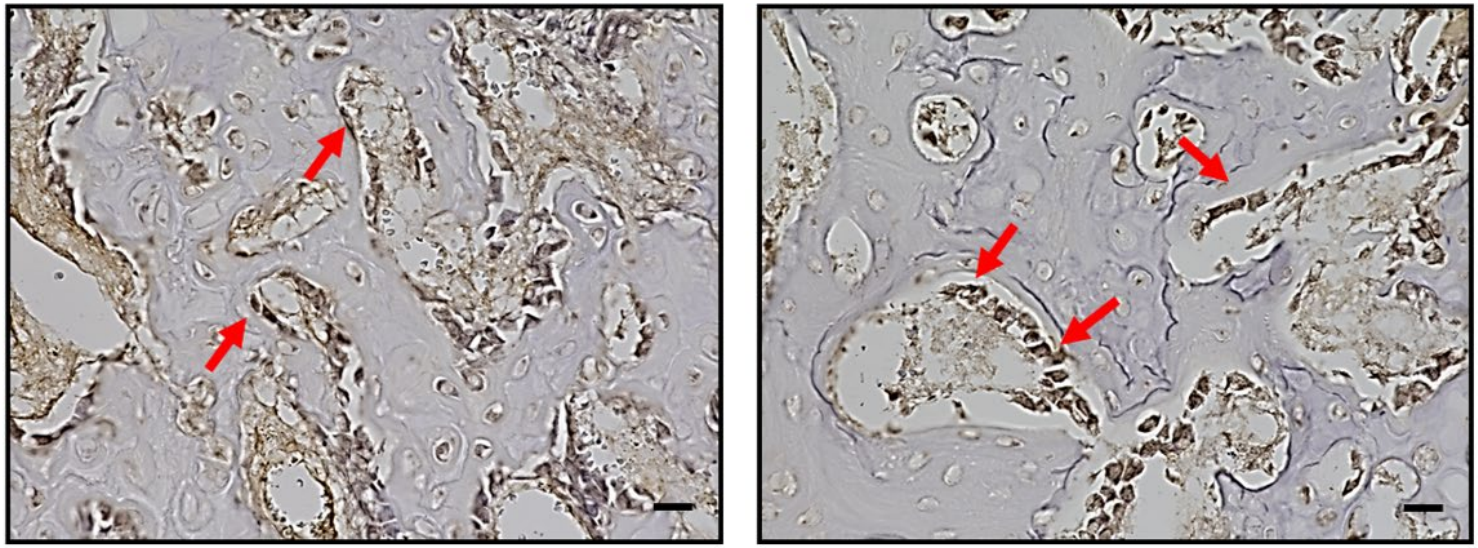


Figure. 4

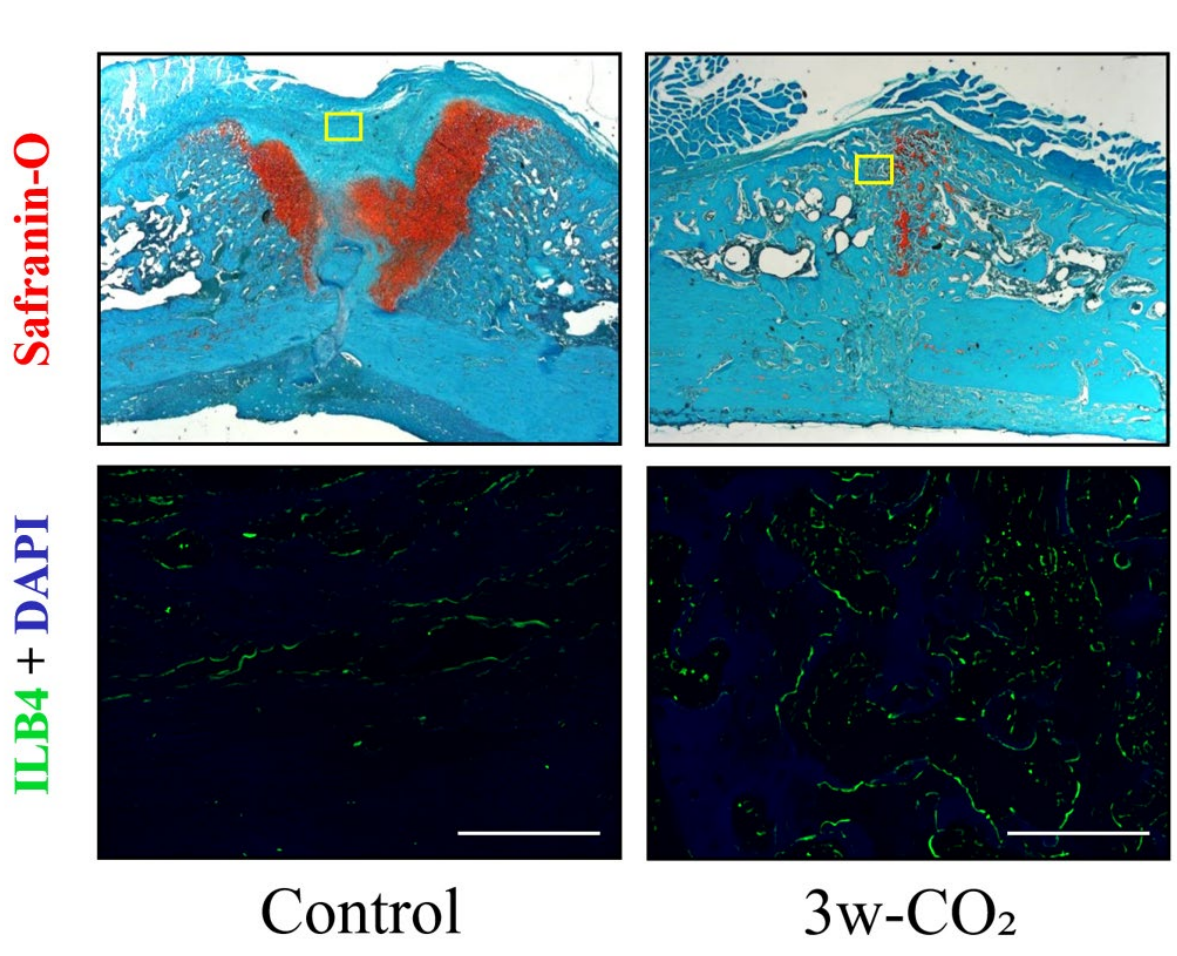
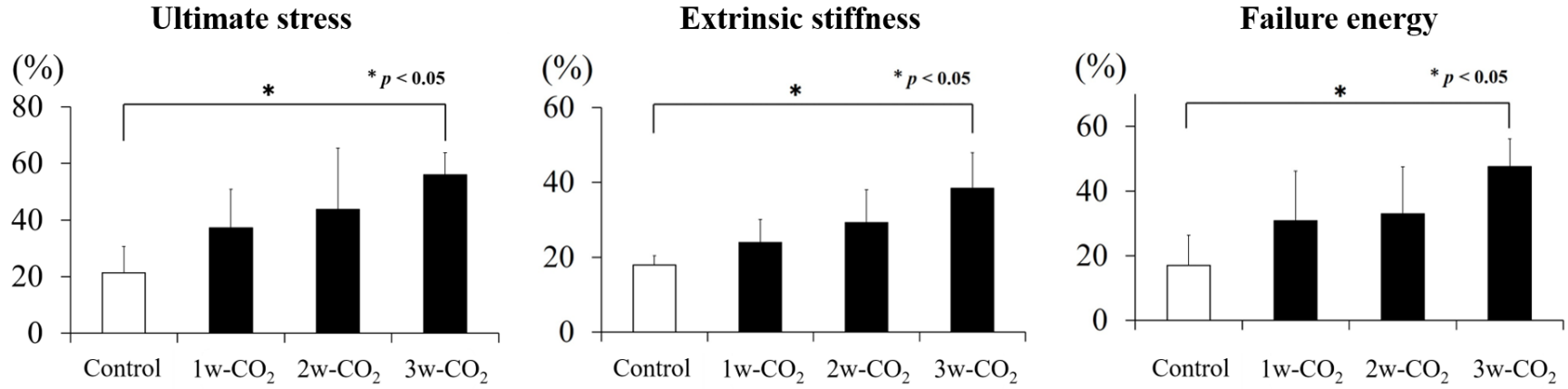


Figure. 5


Ultimate stress
(%)
80
60
40
20
0
*
* p < 0.05
Control
1w-CO2
2w-CO2
3w-CO2
Extrinsic stiffness
(%)
60
40
20
0
*
* p < 0.05
Control
1w-CO2
2w-CO2
3w-CO2
Failure energy
(%)
60
40
20
0
*
* p < 0.05
Control
1w-CO2
2w-CO2
3w-CO2