Topical Cutaneous CO₂ Application by Means of a Novel Hydrogel Accelerates Fracture Repair in Rats

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Background: We previously demonstrated that topical cutaneous application of CO₂, by means of a hydrogel in which the CO₂ readily dissolves, increases blood flow and oxygen dissociation from hemoglobin in the soft tissues surrounding bone. In the present study, we utilized a rat fracture model to test the hypothesis that application of this treatment to fractured limbs would accelerate fracture repair.

Methods: A closed femoral shaft fracture was created in each rat. Topical cutaneous application of CO₂ by means of a hydrogel was performed five times a week for up to four weeks in the CO₂/hydrogel group (n = 60). Sham treatments were performed in the control group (n = 60). Radiographic, histological, immunohistochemical, laser Doppler perfusion imaging, real-time polymerase chain reaction, and biomechanical assessments were performed.

Results: Radiographic fracture union was evident at week 3 in twelve (86%) of fourteen animals in the CO₂/hydrogel group compared with five (36%) of fourteen in the control group (p < 0.05; 95% CI [confidence interval] for the difference, 161 to 258 per mm²). Histological assessment revealed promotion of endochondral ossification in the CO₂/hydrogel group. Immunohistochemical assessment at week 2 showed significantly greater capillary density in the CO₂/hydrogel group (p < 0.05; 95% CI for the difference, 161.1 to 258.0 per mm²). Laser Doppler perfusion imaging demonstrated that the blood flow in the fractured limb was significantly greater at weeks 2 and 3 in the CO₂/hydrogel group (p < 0.05; 95% CI for the difference, 8.4% to 22.4% and 6.7% to 19.0%, respectively). Gene expression of chondrogenic, osteogenic, and angiogenic markers was significantly greater in the CO₂/hydrogel group than in the control group at several time points. Ultimate stress, extrinsic stiffness, and failure energy (relative to the contralateral limb) were significantly greater in the CO₂/hydrogel group at week 3 (p < 0.05; 95% CI for the difference, 24.8% to 67.5%, 4.0% to 22.7%, and 9.6% to 58.8%, respectively). There were no significant differences between the groups with respect to any outcome measure at week 4.

Conclusions: Topical cutaneous application of CO₂ by means of a hydrogel accelerated fracture repair in association with the promotion of angiogenesis, blood flow, and endochondral ossification.

Clinical Relevance: Topical cutaneous application of CO₂ by this method is a novel and potentially useful therapy for accelerating fracture repair.

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An estimated 7.9 million fractures occur in the United States annually, and 5% to 10% fail to heal normally, resulting in delayed union or nonunion. Tools such as low-intensity pulsed ultrasound and electrical stimulation have been developed to enhance fracture repair. However, clinically available tools are limited and none has been demonstrated to be efficacious in all cases.

Fracture-healing is a complex physiological repair process. Several factors such as blood flow, fracture site stability, and osteogenic activity can affect fracture repair. Angiogenesis...
and blood flow in the region of the fracture site are two of the critical components of fracture repair. During endochondral ossification, avascular cartilaginous tissue is invaded by blood vessels and transformed into vascular osseous tissue. Blood vessels also provide systemically circulating factors that modify fracture repair. Damage to the vascular system and impairment of blood flow disrupt the delivery of oxygen, inflammatory cells, and nutrients to the fracture site and are likely detrimental to fracture repair. In the present study, we focused on measurement of angiogenesis and blood flow at the fracture site.

CO2 therapy has been reported to be effective in treating certain cardiac diseases and skin problems. For example, bathing in CO2-enriched water reportedly improves symptoms of limb ischemia, and subcutaneous injection of CO2 has been used in plastic surgery to treat skin irregularity caused by liposuction. These therapeutic effects of CO2 are thought to be caused by increases in blood flow, microcirculation, and nitric oxide-dependent formation of new capillaries as well as by an increase in the oxygen partial pressure in the local tissue (the Bohr effect). We previously designed a system for topical cutaneous application of CO2 by means of a novel hydrogel, and the present study was designed to evaluate the beneficial effects of such CO2 therapy. This hydrogel, in which CO2 readily dissolves, is applied to the skin of a limb to enhance CO2 delivery; the limb is then covered with a polyethylene bag, and the bag is filled with 100% CO2 gas. The effects of the CO2 application (including increased blood flow and oxygen dissociation from hemoglobin in the soft tissues surrounding bone) occur only in the limb to which the hydrogel is applied, and it continues during the treatment. This system thus allows easy and noninvasive topical application of CO2 to limbs. Use of this CO2 application system results in an “artificial Bohr effect” in the treated limb, causing increased blood flow and tissue oxygenation in that limb. Moreover, our previous study demonstrated that topical cutaneous application of CO2 significantly increased the expression of the VEGF (vascular endothelial growth factor) gene in rat muscle. Because VEGF is a critical factor for angiogenesis, it is possible that the topical cutaneous application of CO2 may promote angiogenesis at the site of a fracture.

We are aware of no previous reports on the effect of CO2 treatment on fracture repair. We therefore utilized a rat fracture model to test our hypothesis that the topical cutaneous application of CO2 to a fractured limb by means of our novel hydrogel system would accelerate fracture repair.

Materials and Methods

Femoral Fracture in the 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, which was approved by the Animal Care and Use Committee of the Kobe University Graduate School of Medicine. We created a standard closed fracture in the shaft of the right femur of 120 rats as described previously. Briefly, anesthesia was induced with pentobarbital, a 1.2-mm-diameter Kirschner wire was inserted into the right femoral medullary canal, and a closed transverse femoral shaft fracture was created with use of a three-point bending apparatus with a drop weight. Hypodermic injection of buprenorphine provided postoperative analgesia. Unprotected weight-bearing was allowed postoperatively. Euthanasia by means of a pentobarbital overdose was performed prior to assessment.

CO2/Hydrogel Treatment

The animals were randomly assigned to receive either the CO2/hydrogel treatment (international patent application number WO2004/002393) or the sham treatment; the treatment and control groups each contained sixty animals. After sedation was induced with use of a minimum dose of ether in a dark environment, the hair of the fractured limb was shaved and the hydrogel was applied. The hydrogel (pH 5.5) consisted of carbomer, glycerin, sodium hyaluronate, sodium alginate, sodium dihydrogen phosphate, methylparaben, and deionized water. A polyethylene bag was used to seal the body surface and retain the gas inside; this was attached to the lower limbs, sealed, and filled with 100% CO2 for twenty minutes (Fig. 2). This treatment...
was performed five times a week for four weeks. Control animals received a sham treatment in which the CO2 was replaced with air.

**Radiographic Assessment of Fracture Repair**

At weeks 1, 2, 3, and 4 after fracture, fourteen animals in each group were anesthetized and fixed in the supine position with the limbs fully extended, and radiographs of the fractured limb were made. Fracture union was identified by the presence of bridging callus on four cortices on the anteroposterior and lateral views. The radiographs of each animal were examined by three observers blinded to the treatment.

**Histological Assessment of Fracture Repair**

At weeks 1, 2, 3, and 4 after fracture, the fractured femur was harvested from five animals in each group. The femur was fixed in 4% paraformaldehyde, decalcified with EDTA (ethylenediaminetetraacetic acid), and embedded in paraffin wax. Sagittal sections (6 μm thick) were prepared and stained with safranin-O/fast green for histological assessment. The degree of fracture repair was assessed with use of the Allen grading system, which utilizes a five-point scale (grades 0 through 4).

**Assessment of Angiogenesis**

At week 2 after fracture, angiogenesis was evaluated in five animals in each group. Immunohistochemical staining of endothelial cells was performed with fluorescein-labeled isocitric B4 (Vector Laboratories, Burlingame, California). Nuclear staining was performed with DAPI (4',6-diamidino-2-phenylindole) solution. Capillaries were then examined by fluorescence microscopy; the capillaries in five randomly selected fields in granulation tissue in the vicinity of the fracture were counted, and the mean was calculated. All morphometric studies were performed by three blinded examiners.

**Assessment of Blood Flow**

Immediately after fracture and at weeks 1, 2, 3, and 4, blood flow in each hind limb was assessed in four animals in each group by means of laser Doppler perfusion imaging (Moor Instruments, Wilmington, Delaware). This permitted determination of the effect of the CO2/hydrogel treatment on blood flow over the course of the healing process. In addition, blood flow was measured immediately prior to an individual treatment and immediately after the twenty-minute treatment to demonstrate the instantaneous effect of the treatment. All measurements were made with the animal under ether sedation. The blood flow in the fractured limb and that in the intact, contralateral limb of the same animal were used to calculate the ratio of flow in the fractured (right) femur to that in the intact (left) femur (thus compensating for variations among individuals and for the influence of the sedation).

**Assessment of Gene Expression**

At weeks 1, 2, 3, and 4 after fracture, gene expression was measured in six animals in each group by means of real-time PCR (polymerase chain reaction). Newly generated callus tissue was harvested. Total RNA was extracted from the tissue with use of an RNeasy Mini Kit (Qiagen, Valencia, California) and reverse-transcribed into single-stranded DNA with use of a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, California). Real-time PCR was performed in duplicate on the cDNA with use of an ABI PRISM 7700 Sequence Detection System and SYBR Green reagent (Applied Biosystems). We examined the expression of genes for type-II collagen, type-X collagen, and MMP-13 (matrix metalloproteinase-13) to evaluate chondrogenic differentiation; alkaline phosphatase, runx2 (run-related transcription factor 2), and osteonectin to evaluate osteogenic differentiation; and VEGF to evaluate angiogenesis (see Appendix). The expression level of each gene was first normalized with respect to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which served as an internal control, and all results are presented as the fold change relative to the control group at week 1, which was normalized to a value of 1 (ΔΔCt method).

**Biomechanical Assessment of Fracture Repair**

At week 3 after fracture, biomechanical assessment was performed in four animals in each group. Briefly, the fractured femur and the contralateral, intact femur were harvested and the intramedullary fixation pin was removed. A standardized three-point bending test was performed with use of a load torsion and bending tester (MZ-500S; Maruto Instrument Company, Tokyo, Japan). The bending force was applied with the crosshead at a speed of 2 mm/min until rupture occurred. The ultimate stress (in N), extrinsic stiffness (in N/mm), and failure energy (in N-mm) were assessed. 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**

We wished to be able to detect the effect of a twofold increase in each type of analysis. An a priori power calculation based on a postulated standard deviation equal to one-fourth of the mean (resulting in an effect size [d] of 2.53) indicated that a minimum sample size of four would be needed to provide 80% power to detect such a difference at a significance level of 0.05 (two-tailed). However, we used five rats per group for the histological and immunohistochemical assessment and six rats per group for the real-time PCR, and almost all of the rats (n = 14 per group) underwent the radiographic assessment.

The chi-square test was used to compare the radiographic results between the groups at each time point. The Mann-Whitney U test was used to compare the histological, immunohistochemical, and biomechanical results between the groups at each time point. For the comparisons of blood flow results between the groups, the Mann-Whitney U test was used for the weekly results and the Wilcoxon signed-rank test was used for the changes in instantaneous blood flow between the start and end of an individual treatment. The Kruskal-Wallis test and a subsequent post hoc Bonferroni-corrected Mann-Whitney U test were used for the real-time PCR results. A p value of <0.05 was considered significant.

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**Results**

**Radiographic Assessment of Fracture Repair**

At week 1, anchoring callus was already observed in the CO2/hydrogel group but not in the control group (Fig. 3). At week 2, anchoring callus was observed in the control group and enlargement of the callus was observed in the CO2/hydrogel group. At week 3, twelve (86%) of the animals in the CO2/hydrogel group had achieved fracture union compared with only five (36%) in the control group (p < 0.05; 95% confidence interval [CI] for the difference in the union rate, 2.26% to 99.64%). At week 4, bridging callus was observed in the remaining animals in the control group and remodeling was observed in the CO2/hydrogel group.

**Histological Assessment of Fracture Repair**

At week 1, the CO2/hydrogel group had a greater extent of woven bone formation (Fig. 4-A; see Appendix) as well as abundant cartilage formation. At week 2, cartilage formation was observed in the control group, whereas cartilaginous union had already completed in the CO2/hydrogel group. At week 3, only a small amount of cartilage was observed in the CO2/hydrogel group, indicating progression of endochondral ossification; in
contrast, thick cartilage remained between the woven bone in the control group. At weeks 2 and 3, the degree of fracture repair as assessed by the Allen grading system was significantly greater in the CO2/hydrogel group (p < 0.05; 95% CI for the difference, 8.4% to 22.4% and 6.7% to 19.0%, respectively)

At week 2, the instantaneous blood flow in the fractured limb was significantly greater immediately after the twenty-minute CO2/hydrogel treatment compared with immediately before the treatment (p < 0.05; 95% CI for the difference, 79% to 112%) (Figs. 6-B and 6-C). A similar significant increase in

**Angiogenesis**
Visualization of capillaries (by immunohistochemical isolectin B4 staining) in tissue samples collected at week 2 demonstrated greater angiogenesis surrounding the endochondral ossification region in the CO2/hydrogel group (Fig. 5-A). The capillary density was significantly greater in the CO2/hydrogel group (p < 0.05; 95% CI for the difference, 161 to 258 per mm²) (Fig. 5-B).

**Blood Flow**
Laser Doppler perfusion imaging demonstrated that blood flow in the fractured limb increased with time until week 2 and then decreased in both groups (Fig. 6-A). The blood flow was sig-
blood flow was also observed immediately after fracture and at weeks 1, 3, and 4.

**Gene Expression**

Real-time PCR (Fig. 7) revealed that gene expression of collagen II in the CO$_2$/hydrogel group was significantly greater than that in the control group at week 2 ($p < 0.05$; 95% CI for the difference, 0.32 to 4.90-fold). Peak expression of collagen II occurred at week 2 in the CO$_2$/hydrogel group but at week 3 in the control group. Gene expression levels for collagen X and MMP-13 were significantly greater in the CO$_2$/hydrogel group at week 3 ($p < 0.05$; 95% CI for the difference, 7.06 to 166.39 and 10.41 to 610.80-fold, respectively). Gene expression of alkaline phosphatase was significantly greater in the CO$_2$/hydrogel group at weeks 1, 2, and 3 ($p < 0.05$; 95% CI for the difference, 0.65 to 1.89, 0.080 to 10.78, and 10.98 to 36.31-fold, respectively).

Gene expression levels for runx2 and osterix were significantly greater in the CO$_2$/hydrogel group at week 3 ($p < 0.05$; 95% CI for the difference, 18.06 to 244.00 and 16.08 to 72,279-fold, respectively). Gene expression of VEGF was significantly greater in the CO$_2$/hydrogel group at weeks 1, 2, and 3 ($p < 0.05$; 95% CI for the difference, 18.06 to 244.00 and 16.08 to 72,279-fold, respectively).
CI for the difference, 0.31 to 1.90, 0.42 to 15.0, and 12,562 to 70,025-fold, respectively).

**Biomechanical Assessment of Fracture Repair**

At week 3, the ultimate stress, extrinsic stiffness, and failure energy measurements for the fractured femora (expressed as a percentage of the value in the intact femora) were significantly greater in the CO$_2$/hydrogel group than in the control group ($p < 0.05$; 95% CI for the difference, 24.8% to 67.5%, 4.0% to 22.7%, and 9.6% to 58.8%, respectively) (Fig. 8).

**Discussion**

Angiogenesis and blood flow at the site of a fracture are critical components of fracture repair, and VEGF plays a
crucial role in angiogenesis in this region. Therefore, tools for enhancement of angiogenesis and/or blood flow at fracture sites have been an active area of investigation; these tools include gene therapy, administration of VEGF protein, and cell transplantation. The evidence that these therapies promote angiogenesis, blood flow, and bone regeneration at the fracture site supports the hypothesis of our study. The importance of angiogenesis is further supported by the observations that addition of VEGF promotes fracture repair whereas blockage of VEGF receptors inhibits vascular ingrowth and delays or disrupts the regenerative process.

In the present study, we confirmed that the topical cutaneous CO2/hydrogel treatment increased gene expression of VEGF in newly generated callus tissue. Xu et al. reported transcriptional activation of the VEGF gene by low pH in human glioblastoma cells. In our previous study, we demonstrated that the topical cutaneous CO2/hydrogel treatment significantly lowered the intracellular pH of human muscle. On the basis of these findings, a possible mechanism for the observed increase in VEGF in the present study is triggering of the transcriptional activation of VEGF by the low intracellular pH at the fracture site induced by the topical cutaneous CO2/hydrogel treatment. The capillary density at the fracture site was also increased in association with the increased expression of VEGF. At the same time, blood flow in the fractured limb was increased by the topical cutaneous CO2/hydrogel treatment, as demonstrated by laser Doppler perfusion imaging. Taken together, the results of the present study suggest that the topical cutaneous CO2/hydrogel treatment accelerated fracture repair in association with the promotion of angiogenesis and blood flow.

During the endochondral ossification stage of fracture repair, mesenchymal stem cells are induced to differentiate into chondrocytes that deposit a collagen-II-rich extracellular matrix. Our results revealed that collagen-II gene expression was significantly increased by the topical cutaneous CO2/hydrogel treatment at week 2. Peak expression of collagen II occurred at week 2 in the CO2/hydrogel group but at week 3 in the control group. This is consistent with the histological finding of accelerated cartilage formation in the CO2/hydrogel group (see Appendix). After the cartilage matrix is produced, chondrocytes differentiate into a hypertrophic state and begin to deposit an extracellular matrix composed of collagen X. The extracellular matrix mineralizes, and a transition from mineralized cartilage to bone then occurs, initiated by the resorption of mineralized cartilage. MMP-13 is required for chondrocyte differentiation into the hypertrophic state prior to invasion of blood vessels and osteoclasts during endochondral ossification. In this study, gene expression of collagen X and MMP-13 at week 3 was significantly greater in the CO2/hydrogel group, indicating that differentiation of chondrocytes and resorption of cartilage were accelerated by the topical cutaneous CO2/hydrogel treatment. Overall, endochondral ossification was promoted by the topical cutaneous CO2/hydrogel treatment.

The fracture repair process can be divided into three basic phases: inflammatory, reparative, and remodeling. The increased angiogenesis and blood flow induced by the topical cutaneous CO2/hydrogel treatment may have enhanced the supply of oxygen, nutrients, and inflammatory cells to the fracture site during the inflammatory phase. During the reparative phase, blood vessel invasion and highly metabolically active regeneration, such as transformation of the avascular cartilaginous matrix into vascularized osseous tissue, may be promoted by increased angiogenesis and blood flow induced by the topical cutaneous CO2/hydrogel treatment.

The present results may have important clinical implications. Our findings suggest that the topical cutaneous CO2/hydrogel treatment is a promising strategy for accelerating fracture repair. In the clinical setting, our topical cutaneous CO2 application system is minimally invasive and can be used at relatively low cost. Furthermore, our novel topical cutaneous CO2 application system may be beneficial in the treatment of particularly difficult fracture cases such as open fractures and fractures in patients with ischemic disease or diabetes mellitus. However, additional in vivo experiments are needed to investigate whether our topical cutaneous CO2 application system can accelerate fracture repair in larger limbs such as those in humans as well as to determine the optimal timing, frequency, and duration of the topical cutaneous CO2/hydrogel treatment.

One limitation of this study is that the change in CO2 concentration in the tissue during the CO2/hydrogel treatment was not measured directly. Previously, we demonstrated that topical cutaneous CO2 treatment using the hydrogel decreased the intramuscular pH. The change in pH at the fracture site caused by the CO2/hydrogel treatment may be one of the mechanisms leading to the promotion of angiogenesis and blood flow. Further investigation is required to clarify whether CO2 is actually absorbed into the tissue at the fracture site and whether the promotion of angiogenesis, blood flow, and endochondral ossification by the CO2/hydrogel treatment is directly induced by the CO2 itself or by a secondary reaction to the topical cutaneous CO2/hydrogel treatment.

Appendix

Figures showing representative histological sections and measurements of cartilage area as well as a table listing the PCR primers are available with the online version of this article as a data supplement at jbjs.org.

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