Exploring the optimal treatment duration of transcutaneous CO₂ application for fracture repair

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INTRODUCTION
Transcutaneous CO₂ therapy has been shown to be effective in treating certain cardiac diseases and skin problems [1]. The benefits of therapy are attributed to increased blood flow, microcirculation, and formation of new capillaries due to higher nitric oxide levels and local tissue oxygenation (Bohr effect). We developed a system for topically applying CO₂ through a hydrogel, allowing non-invasive application to the limbs [2, 3]. This leads to an “artificial Bohr effect,” which increases the blood flow and tissue oxygenation in the treated limb. In our previous reports, the treatment duration was set at 20 min; however, this was only determined based on clinical convenience, and the optimization of the treatment duration remains unclear. This study aimed to investigate the dosage effect of CO₂ therapy on bone formation at two different treatment durations.

METHODS
This study was approved by our institution’s animal ethics committee.

Animal experiments: 12-week-old male Sprague Dawley rats (SLC Japan, Shizuoka, Japan) were used in this study. A standard stabilized closed femoral shaft fracture was induced according to a previously reported method [4]. Briefly, retrograde insertion of a 1.25-mm-diameter K-wire into the right femoral intramedullary canal was performed, followed by the induction of a closed transverse femoral shaft fracture using a three-point bending apparatus with a drop weight. After inducing the fracture, the patellar aponeurosis and skin were sutured using nylon sutures. Preoperatively, medetomidine (0.15 mg/kg), midazolam (2 mg/kg), and butorphanol (2.5 mg/kg) were administered intraperitoneally for anesthesia and sedation. Postoperatively, benzylpenicillin potassium (200000 units/kg) was administered intraperitoneally as an antibiotic agent. Unprotected weight bearing was allowed postoperatively. Rats were sacrificed using a pentobarbital overdose before assessment.

Transcutaneous application of CO₂: The animals were randomly divided into three groups: 60 min CO₂, 20 min CO₂, and control. Transcutaneous CO₂ was applied to the fractured lower limbs as previously reported [3]. Briefly, after sedation with a minimum dose of isoflurane, the fractured limb was shaved, and a hydrogel (META MEDILAB Inc., Osaka, Japan), which enhances CO₂ transcutaneous absorption, was applied. The hydrogel (pH 5.5) consisted of a carbomer, glycerin, sodium hydroxide, sodium alginate, sodium dihydrogen phosphate, methylparaben, and deionized water. Both limbs were sealed in a polyethylene bag filled with 100% CO₂ for 60 or 20 min. This treatment was performed 5 days a week. The control group received sham treatment in which CO₂ was replaced with air.

Radiographic assessment of fracture repair: At weeks 1, 2, 3, and 4 after the fracture, 10 animals in each group were anesthetized and fixed in the supine position with the limbs fully extended, and radiographs of the fractured limbs were obtained. The degree of fracture repair was assessed using the radiographic union score for tibial fractures (RUST) [5]. Radiographs of each animal were examined by three observers who were blinded to the treatment.

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

RESULTS
Radiographic evaluation: Representative radiographs of the three groups at each time point are shown in Fig. 1. At week 1, periosteal callus formation was not observed in either the CO₂ or control group. At week 2, bony calli were visible in the CO₂ group but not in the control group. Bony calli were observed at week 3 in the control group; however, the callus size was not sufficient to form a bridging callus even at week 4. At week 3, fracture union with bridging callus formation was achieved in some samples in the CO₂ group, while at week 4, bone union was achieved in most samples. The degree of fracture repair according to RUST was significantly higher in the 60 min CO₂ group than in the 20 min CO₂ and control groups at weeks 2 and 3 (Fig. 2). The fracture repair rate at week 4 was significantly higher in the 60 min CO₂ group than that in the control group.

Histological evaluation: At week 1, cartilage formation was observed between the woven bones in the CO₂ group, whereas almost no cartilage area was found in the control group. Cartilage formation in the control group was observed at week 2. At week 3, only a small amount of cartilage was observed between well-generated woven bones in the CO₂ group, whereas thick cartilage was observed in the control group. At week 4, bony union was already complete in the CO₂ group, whereas thick cartilage remained in the control group (Fig. 3). The degree of fracture repair according to Allen’s grading system was significantly higher in the 60 min CO₂ group than that in the 20 min CO₂ and control groups at week 2, and significantly higher in the 60 min and 20 min CO₂ groups than that in the control group at week 3 (Fig. 3).

DISCUSSION
The findings of the current study provide evidence that CO₂ therapy has a beneficial effect on bone formation at a rapid rate in a dose-dependent manner. Thus, the results suggest that a longer duration of CO₂ therapy (60 min) is more effective in enhancing fracture healing.

SIGNIFICANCE/CLINICAL RELEVANCE
The present study suggests for the first time a dose-dependent beneficial effect of CO₂ therapy on bone formation in fractures.

REFERENCES

Fig. 1. Representative radiographs of the fracture repair evaluated using RUST at each time point.

Fig. 2. The degree of fracture repair evaluated using RUST at each time point.

Fig. 3. Representative histological sections stained with safranin-O/fast green. The degree of fracture repair evaluated using Allen’s Grading Score at each time point.