**Transcutaneous application of CO₂ accelerates fracture repair in Rat**

**INTRODUCTION**

Transcutaneous application of CO₂ therapy for cardiac disease and skin trouble by spa or CO₂-enriched water bathing has been used for hundreds of years. Previously, we demonstrated that transcutaneous application of CO₂ up-regulated O₂ pressure in the local tissue in human [1]. Additionally, we revealed that transcutaneous CO₂ application to skeletal muscle in rat under the non-exercise conditions led similar effects to the aerobic exercise [2].

To date, there has been no report investigating the effect of the carbonated therapy on fracture repair. We hypothesized that the transcutaneous application of CO₂ to fracture site would accelerate fracture repair. In this study, we transcutaneously applied CO₂ to the lower fractured limbs of rats, and investigated if fracture repair would be promoted.

**MATERIALS and METHODS**

**Femoral Fracture in animal model:** Ten-weeks old male S-D rats were used under a research protocol approved by the institutional ethical committee. A 1.2-mm diameter K-wire was retrograde into the right femoral intramedullary canal and a closed transverse femoral shaft fracture was produced in all animals using a three-point bending apparatus with a drop weight [3]. For each analysis, six animals were euthanized at the following time points: post-fracture weeks 1, 2, 3 and 4.

**Procedure for CO₂ treatment:** CO₂ was administrated as described previously [2]. Briefly, transcutaneous CO₂ absorption enhancing hydrogel and CO₂ adaptor that seals the body surface and retains the gas inside were used. The skin of the limbs with the hydrogel was sealed up, and 100% CO₂ gas was flowed into the area. Rats without CO₂ treatment served as a control (CO₂- group).

**Radiographical Assessment of the Fracture Healing:** Radiographs of the fractured legs were taken at weeks 1, 2, 3, and 4. Fracture union was defined by the presence of bridging callus on four cortices.

**Histological Analysis:** Histological evaluation was performed with Safranin-O staining to address the process of endochondral ossification at weeks 1, 2, 3, and 4.

**Real-time PCR Analysis:** The expression of alkaline phosphatase (ALP), Runx2, osterix (OSX), Collagen-II -X, matrix metalloproteinase-13 (MMP-13), vascular endothelial growth factor (VEGF), and GAPDH was measured by real-time PCR. The level of each target gene was normalized to GAPDH levels and expressed relative to the levels of CO₂- group at week 1 (ΔΔCT methods).

**RESULTS**

**Radiographical Assessment of the Fracture Healing:** The callus was not seen in both groups at week 1. Although the anchoring callus was seen in both groups, the bridging callus was not seen in all rats at week 2. At week 3, although the bridging callus was seen in 36.3% of CO₂- group (Fig. 1A), and in 85.7% of CO₂+ group (Fig. 1B). Union rate at week 3 in CO₂+ group was significantly higher compared to CO₂- group. The bridging callus was seen in all rats at week 4.

**Histological Analysis:** Histological evaluation with Safranin-O demonstrated that the callus was seen only in the small range and union was almost complete at week 3 in CO₂+ group (Fig. 2B). In contrast, the thick cartilage was still observed and the fracture did not unite yet at in CO₂- group (Fig. 2A).

**DISCUSSION**

Our radiographic assessment revealed an acceleration of fracture repair by transcutaneous application of CO₂. The histological analysis suggested that the acceleration of fracture repair was due to an accerelation of endochondral ossification. The up-regulation of mRNA expression of ALP, Runx2, and OSX by CO₂ application confirmed the molecular basis of ossification.

By CO₂ application, Collagen-II mRNA was up-regulated at the earlier time point compared to CO₂- group. In addition, at week 3, CO₂ application induced higher expression levels of MMP-13 and VEGF mRNA. These findings suggest that the process of endochondral ossification and vascularization was promoted by transcutaneous application of CO₂. Our study indicated that transcutaneous application of CO₂ could accelerate fracture repair.

**SIGNIFICANCE**

Our findings suggest that transcutaneous application of CO₂ may become a new and useful therapy for promoting fracture repair.