The Effects of the Duration of Transcutaneous CO\textsubscript{2} Application on the Facilitatory Effect in Rat Fracture Healing

Takashi Iwakura, M.D., Ph.D.\textsuperscript{1}, Takahiro Niikura, M.D., Ph.D.\textsuperscript{1}, Sang Yang Lee, M.D., Ph.D.\textsuperscript{1}, Etsuko Okumachi, M.D.\textsuperscript{1}, Takahiro Waki, M.D.\textsuperscript{1}, Shunsuke Takahara, M.D.\textsuperscript{1}, Michio Arakura, M.D.\textsuperscript{1}, Yoshitada Sakai, M.D., Ph.D.\textsuperscript{2}, Ryosuke Kuroda, M.D., Ph.D.\textsuperscript{1}, Masahiro Kurosaka, M.D., Ph.D.\textsuperscript{1}.
\textsuperscript{1}Department of Orthopaedic Surgery, Kobe University Graduate School of Medicine, Kobe, Japan, \textsuperscript{2}Division of Rehabilitation Medicine, Kobe University Graduate School of Medicine, Kobe, Japan.


Introduction: Carbon dioxide (CO\textsubscript{2}) therapy has been reported to be effective in treating selected cardiac diseases and skin problems. These therapeutic effects of CO\textsubscript{2} are thought to be caused by increased blood flow, microcirculation, and nitric oxide-dependent neocapillary formation, as well as a partial increase of oxygen pressure in the local tissue known as the Bohr effect [1]. The fracture repair process can be divided into three basic stages: the inflammatory, reparative, and remodeling phases. We recently reported that transcutaneous application of CO\textsubscript{2} using a hydrogel accelerated fracture repair in association with the promotion of angiogenesis, blood flow, and endochondral ossification [2]. Although it is suggested that the transcutaneous application of CO\textsubscript{2} acts on various processes such as inflammatory phase or reparative phase, it is unknown that how the duration of the application of CO\textsubscript{2} influence on the fracture repair. In this study, we investigated the effect of the duration of transcutaneous CO\textsubscript{2} application on the rat fracture repair.

Methods: Femoral fracture in an animal model
Forty twelve-week-old male Sprague-Dawley rats were used under a research protocol approved by the institutional ethical committee. To create a standard femoral fracture model in rats, a 1.2-mm-diameter k-wire was inserted retrograde into the right femoral intramedullary canal, and a closed transverse femoral shaft fracture was produced using a three-point bending apparatus with a drop weight [3].

Procedure for the transcutaneous application of CO\textsubscript{2}
Animals were randomly divided into 4 groups; the CO\textsubscript{2} treatment for 1 week (1-week CO\textsubscript{2} group), 2 weeks (2-week CO\textsubscript{2} group), and 3 weeks (3-week CO\textsubscript{2} group) after fracture, and the sham treatment group (Control group). Transcutaneous application of CO\textsubscript{2} to the lower fractured limbs of rats was performed as previously described [4]. Briefly, we applied the CO\textsubscript{2} absorption enhancing hydrogel on fractured limb. A polyethylene bag, used to seal the body surface and retain the gas inside, was attached to the limbs, sealed, and filled with 100% CO\textsubscript{2} for 20 minutes. Control animals were treated by sham treatment, where the CO\textsubscript{2} was replaced with air.

Radiographic assessment of fracture repair
Rats were fixed in the supine position with the limbs fully extended under anesthesia, and radiographs of the fractured limbs were taken at week 3 after fracture (n = 10 in each group). Fracture union was identified by the presence of bridging callus formation on four cortices on the anteroposterior and lateral views.
Histological assessment of fracture sites
Histological assessment was performed with Safranin-O staining at week 3 (n = 6 in each group). The degree of fracture repair was assessed using five-point scale (grade 0-4) by Allen’s grading system [5]. 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.

Statistical analysis
Fisher’s exact test was used for the radiographic assessments. In the assessment of the degree of fracture repair and the size of the cartilage areas, Kruskal-Wallis tests performed with Bonferroni corrected Mann-Whitney U post hoc tests. A p-value of < 0.05 was defined as statistically significant. Columns and error bars indicate means and standard deviations.

Results: Radiographic assessment of fracture repair
At week 3, the fracture union with bridging callus formation was achieved in 80% of animals in 3w-CO₂ group, 60% of animals in 2w-CO₂ group, 40% of animals in 1w-CO₂ group, and 30% of 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).

Histological assessment of fracture sites
As shown in Fig. 2, a thick cartilage area remained between woven bones in the control group, while 3w-CO₂ group showed only a small amount of cartilage and bony union was almost complete. The degree of fracture repair as assessed by Allen’s grading system at week 3 was significantly higher in 3w-CO₂ group than in the control group (Fig. 3a). The cartilage area at week 3 was significantly smaller in 3w-CO₂ group than in the control group (Fig. 3b).

Discussion: This study showed that fracture repair in 3w-CO₂ group was significantly enhanced compared to the control group. Histological assessment eavealed that the cartilage area at week 3 was significantly smaller in 3w-CO₂ group than in the control group, which suggested that the facilitatory effect of transcutaneous application of CO₂ was mediated by the promotion of endochondral ossification. In addition, our results suggested that continuous CO₂ application from the occurrence of the fracture to the bony union would be effective in the enhancement of fracture repair.

Significance: The present study demonstrated that continuous CO₂ application throughout the process of fracture repair was effective in the enhancement of fracture repair. The results of this investigation would provide useful information on the application of this system in the clinical setting.
Figure 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fracture Union Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3/10 (30%)</td>
</tr>
<tr>
<td>1w-CO₂</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>2w-CO₂</td>
<td>6/10 (60%)</td>
</tr>
<tr>
<td>3w-CO₂</td>
<td>8/10 (80%)</td>
</tr>
</tbody>
</table>

* : p < 0.05 vs the control group

Figure 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1w</th>
<th>2w</th>
<th>3w</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(cb: cortical bone, ca: cartilage, wb: woven bone, bar: 500 μm)
Figure 3

(a)

Score

Control 1w 2w 3w

CO₂

$P < 0.05$

(b)

Cartilage area (cm²)

Control 1w 2w 3w

CO₂

$P < 0.05$

ORS 2015 Annual Meeting
Poster No: 0648