Transcutaneous Application Of CO2 Accelerates Muscle Injury Repair In Rat Models

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Disclosures: S. Akahane: None. Y. Sakai: None. T. Ueha: 3A; I was a full-time employee of NeoChemir Inc.. H. Nishimoto: None. K. Oe: None. T. Niikura: None. R. Kuroda: None. M. Kurosaka: None.

Introduction: A muscle injury is common injury in sports medicine. Although there are various causes of muscle injury, previous reports showed that the healing process of this was the same in any causes. However, we have no established treatment for muscle injury. Structural and functional problems sometime remain after treatments, such as muscle atrophy, pain, and limited joint range of motion. For these reasons, development a novel of therapy for early recovery from muscle injury is expected. Carbon dioxide (CO2) therapy like as the carbonated spa known in Europe refers to the transcutaneous administration of CO2 for therapeutic purpose. Recently, we designed a novel transcutaneous CO2 application system using 100% CO2 gas, a transcutaneous CO2 absorption-enhancing hydrogel. We showed that our transcutaneous CO2 system could cause the absorption of CO2, and the O2 dissociation of hemoglobin by the Bohr effect in the human body [1]. In addition, we have previously demonstrated that transcutaneous application of CO2 works effectively on delayed-onset muscle soreness (DOMS) after exercise in human. Thus, we hypothesized that the CO2 therapy may accelerate tibialis anterior muscle injury repair. In this study, we investigated the effects of transcutaneous CO2 application for injured tibialis anterior muscle in rat.

Methods: Animal models: Animal care: The use of animals was approved by the Animal Care and Use Committee of Kobe University Graduate School of Medicine. Animals were fed ad libitum and kept in a thermostatic environment a 21 ºC with a 12h light / 12h dark cycle. Eighteen 8 weeks old male Sprague Dawley rats were randomly divided into two groups; CO2 treatment group(CO2 Group; N=9; 18 legs), and no treatment group (NT Group; N=9; 18 legs). At the first day, all rats are injured tibialis anterior muscle by bupivacaine. In the CO2 treatment group, CO2 treatment was performed to both legs twice a week. We sacrificed three rats of each group 4, 6, and 8 weeks after muscle injured.

Transcutaneous application of CO2 was performed as previously described[2]. CO2 treatment was performed to both legs. Body weight of rats was monitored the last day. Muscle preparation: Rats of each group were sacrificed 4, 6, and 8 weeks after muscle injured. All rats were sacrificed by an overdose pentobarbital anesthesia followed by decapitation. The muscles were weighed after connective tissues, were removed and then immediately frozen in isopentane precooled by liquid nitrogen and stored at -80 ºC.

Gene expression: Quantification of mRNA transcription (induplicate) was performed in Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems, FosterCity, CA, USA). We previously described [2].
immunohistochemistry: Immunofluorescence staining was performed on paraffin-embedded coronal section as described previously. Sections were incubated with the primary antibodies (anti-PGC-1, anti-VEGF, anti-Myo-D, anti-NRF-1, anti-Tfam, anti-Laminin and anti-Dystrophin), then the secondary antibodies (anti-mouse immunoglobulin Alexa Fluor 488, anti-goat immunoglobulin Alexa Fluor 488 and anti-rabbit immunoglobulin Alexa Fluor 594) were for 60 minutes at room temperature. The nucleus were stained with DAPI. The images were obtained using a BZ-X700 microscope.

Statistics analysis: Data are shown as the mean values ± S.E. The results of the 3 groups were analyzed using Mann-Whitney U test or repeated measure ANOVA. The level of statistical significance was set at p < 0.05.

Results: The muscle weights of tibialis anterior muscle at the sacrificed day were significantly increased in the CO2 group compared with the NT group at 4, 6, and 8 weeks after muscle injury. (Fig.1) As shown in Fig.2(A), we compared cross-sections of tibialis anterior muscles stained with hematoxylin-eosin of the NT group to that of CO2 group at 4, 6 weeks after muscle injury. In the NT group, cross-sections were showed muscle fiber with irregular shape and spread stroma at 4 weeks after muscle injury. And central nucleis and small fibers were observed at 6 weeks in the NT group.

On the other hand, in CO2 group, central nucleis of muscle fiber were observed at 4 weeks after muscle injury. We considered that injured muscles of the NT group were completely repaired from injury for about 8 weeks, and that of CO2 group were completely repaired for about 6 weeks.

As shown in Fig.2(B), immunohistochemistry for Laminin and Dystrophin revealed that basement membrane was positively stained in the muscle tissue. There was irregular staining of basement membranes at 4 weeks in the NT group after muscle injury. On the other hand, there were basement membranes were repaired at 6 weeks in the NT group and at 4 weeks in the CO2 group after muscle injury. The results of immunohistochemistry for Laminin and Dystrophin showed that the repair of basement membrane after muscle injury in the CO2 group was earlier than in the NT group.

Moreover, as shown in Fig. 3, in real-time PCR analysis, the gene expressions of PGC-1a, VEGF, and Tfam in the CO2 group were increased larger than, those in the NT group at 6 weeks after muscle injury. The expression of PGC-1a, VEGF and Tfam in the CO2 group had the tendency of increase compared with those of the NT group(not significant). However, the expression of MyoD had no significant difference between the CO2 group and the NT group.

Discussion: We have previously demonstrated that transcutaneous application of CO2 could induce oxygen release from red blood cells and may induce oxygenation in the treated tissue. [1] Body weight of the CO2 group was almost equal to that of the NT group at each age. Nevertheless the muscle weight of the CO2 group was significantly increased to that of the NT group at each age in this muscle injury model. We consider CO2 therapy gives muscle hypertrophy. Histological findings, at 6 weeks after muscle injury in the NT group were quite similar to that at 4 weeks after muscle injury in the CO2 group. The reason might be that the Bohr effect by the CO2 therapy caused increasing local blood flow, the gene expression of vascular endothelial and mitochondrial were increased, and the proteins of muscle synthesis were increased. Therefore, consequently transcutaneous application of CO2 twice a week contributed to acceleration of muscle repair after injury. This study may imply that a transcutaneous CO2 application can bring a therapeutic breakthrough for promotion of injured muscle repair.
Significance: A novel transcutaneous CO2 therapy accelerates repairs for injured muscle. These results suggest that a transcutaneous CO2 therapy may be one of novel therapeutic strategies for sports medicine and rehabilitation.

Fig. 1

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<tr>
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<th>Muscle Weight(mg)/Body Weight(g) average</th>
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<tr>
<td></td>
<td>NT</td>
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<tr>
<td>4 weeks</td>
<td>0.1886±0.0031</td>
</tr>
<tr>
<td>6 weeks</td>
<td>0.1960±0.0021</td>
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<tr>
<td>8 weeks</td>
<td>0.2307±0.0053</td>
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The muscle weight ratios of tibialis anterior muscle at 6 weeks after muscle injury were significantly increased in the CO2 group, compared with the NT group.

Fig. 2

(A) Cross-sections stained with hematoxylin-eosin were showed central nuclei and spread stroma at 6 weeks after muscle injury in the NT group. Those were observed at 4 weeks after muscle injury in the CO2 group.

(B) Immunohistochemistry for Laminin(red) and Dystrophin(green) revealed that basement membrane were positively stained in the muscle tissue. The basement membrane were repaired in the NT group at 6 weeks after muscle injury, and in the CO2 group at 4 weeks after muscle injury.
In real-time PCR analysis, at 6 weeks after muscle injury the gene expression of PGC-1a, VEGF, and Tfam in the CO2 group were increased larger than, those in the NT group at 6 weeks after muscle injury.