The Effects Of A Transcutaneous CO2 Application On Tibialis Anterior (TA) Muscle In Short Period.

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Disclosures:
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Introduction: Carbon dioxide (CO2) therapy refers to the transcutaneous administration of CO2 for therapeutic purpose. The benefits of the carbonated spa have long been known in Europe. These therapeutic effects of CO2 are caused by an increase in blood flow and microcirculation, nitric oxide-dependent neocapillary formation, and a partial increase of O2 pressure in the local tissue known as the Bohr effect.
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 transcutaneously applied CO2 to the lower limbs of rats(Figure1),and investigated the effect on tibialis anterior (TA) muscle for a few weeks and months. The transcutaneous CO2 application caused: (1) the gene expression of PPAR gamma co-activator 1-alpha (PGC-1alpha), silent mating type information regulation 2 homologs 1 (SIRT1) and vascular endothelial growth factor (VEGF),and increased the number of mitochondria, as proven by real-time PCR and immunohistochemistry. [2] However, those studies applied CO2 for a few weeks and months, and the effect of transcutaneous CO2 application in short period such as a few days was still unknown. In addition, we did not investigate the frequency of CO2 application to obtain optimal effect in a week. Thus, in this study, we investigated the effects of transcutaneous CO2 application in short period on tibialis anterior (TA) muscle in vivo.

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.
Experiment:17 Male SD rat, aged 8 weeks were randomly divided into three groups; CO2 treatment group, and no treatment group (Control Group; n=7). The CO2 treatment group was divided into two groups;DAY1 group (n=5)was applied CO2 once and sacrificed after 24 hours, DAY5 group (n=5) was applied CO2 once a day for 5 days and sacrificed after 24 hours from last application,Transcutaneous application of CO2 was performed as previously described. CO2 treatment was performed to both leg for 20 minutes. Body weight of rats was monitored on the last day.
Muscle preparation: All rats were sacrificed by an overdose pentobarbital anesthesia followed by decapitation, and intact tibialis
anterior (TA) muscle was dissected. The muscles were weighed after excessing connective tissue, was removed and then immediately frozen in isopentane precooled by liquid nitrogen and stored at -80ºC.
Real-time PCR: Quantification of mRNA transcription (induplicate) was performed in Applied Biosystems StepOne™ Real-Time PCR System (Applied Biosystems,FosterCity, CA, USA). Real-Time PCR reactions (20 µl) contained 1 µM forward primer, 1 µM reverse primer, and 1 µl of cDNA template from RT reaction, and 10 µl 10 × master mix for Power SYBER green master mix(Applied Biosystems,FosterCity, CA, USA). Reaction conditions included 10 min at 95 ℃,followed by 40 cycles at 95 ℃ (15 s) and 60 ℃ (1 min). The level of each target gene was normalized to GUSB(b-glucuronidase) levels and expressed relative to the levels of the control group (DDCT methods; Applied Biosystems).
Immunohistochemistry:
Immunofluorescence staining was performed on paraffin-embedded coronal section as described previously. Sections were incubated with the primary antibodies anti-PGC-1alpha (monoclonal antibody, Taiwan), anti-VEGF(polyclonal antibody,England),then the secondary antibodies used were goat anti-rabbit immunoglobulin Alexa Fluor 488 (Life Technologies, Carlsbad, CA USA) and goat anti-goat immunoglobulin Alexa Fluor 555 (Life Technologies) for 60 minutes at room temperature. The nucleus was stained with DAPI. The images were obtained using a BZ-8000 confocal microscope (Keyence).
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 $p < 0.05$ (*, $p < 0.05$).

Results: Experiment: The muscle weights of tibialis anterior muscle were not significantly increased in the CO2 groups compared with control group. (Table 1) Moreover, in real-time PCR analysis, the gene expression of PGC-1alpha, VEGF and MyoD in the CO2 groups was increased compared to control group(Control:n=7, DAY1 and DAY5: n=5). The average PGC-1alpha in the CO2, DAY5 group was significantly increased the average of the control group (P=0.024). The expression of VEGF and MyoD in the CO2, DAY5 group was increased, however, the significance have not been appeared (P=0.050, P=0.086) As shown in Fig. 3, immunohistochemistry for PGC-1alpha and VEGF revealed that many nuclear were positively stained in the muscle tissue of the CO2 group. However, there was no positive staining for these proteins in the nuclear in the control group. The results of immunohistochemistry for VEGF showed that the positive staining area in the CO2 group was much larger,compared to the control group. The results were comparable with results of real-time PCR analysis.

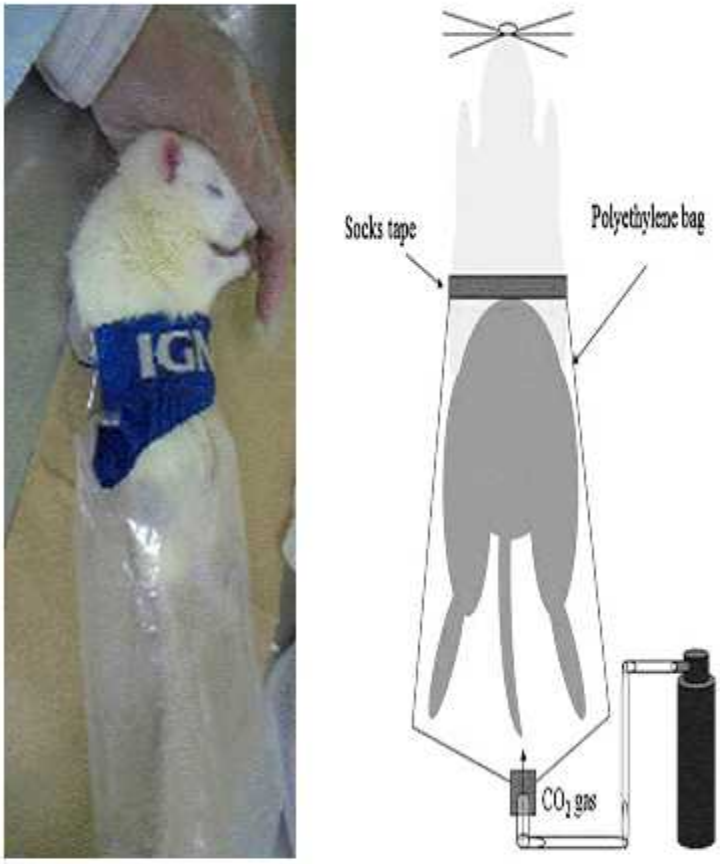
Discussion: In this study, we showed the effect of transcutaneous application of CO2 on the muscle in short period such as 1 day or 5 days application. The transcutaneous application of CO2 have some effects especially, mitochondrial and neovascular effects on muscle in short period. In addition, 1 day application has some effect, however, 5 days application has more effect of transcutaneous application of CO2. This result may contribute to the decision of the optimal frequency of transcutaneous application of CO2.

Significance: A novel transcutaneous CO2 therapy effect increased the expressions of PGC-1alpha,VEGF, and MyoD in vivo in short period. These results suggest that a transcutaneous CO2 therapy in short period may have positive effect on the muscle weakness, and may contribute the decision of the optimal frequency of transucutaneous application of CO2.

Acknowledgments:

References: [1] Sakai Y, Miwa M, Oe K et al.,PloS one, 2011
[2] Oe K., Ueha T., Sakai Y., et al.,BBRC, 2011

Figure1



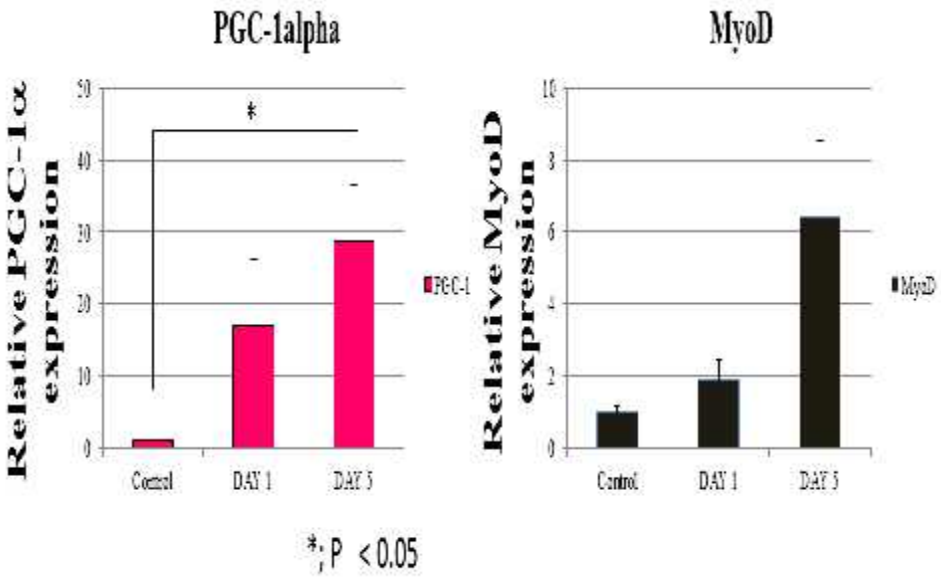
Shaved lower limbs of rats. Hydrogel was administered, sealed in a polyethylene bag, and CO2 gas was released into the polyethylene bag once or fifth times.

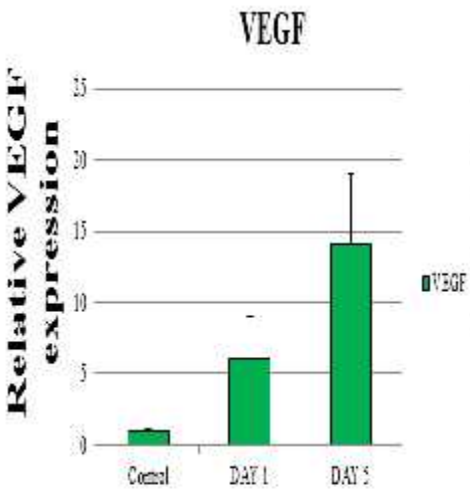
Table1

	Muscle Weight(mg) / Body Weight (g)
Control	0.1829±0.0040
DAY1	0.1782±0.0037
DAY5	0.1847±0.0035

The muscle weight ratios of tibialis anterior muscle were not significantly increased in the CO2 groups compared with control group.

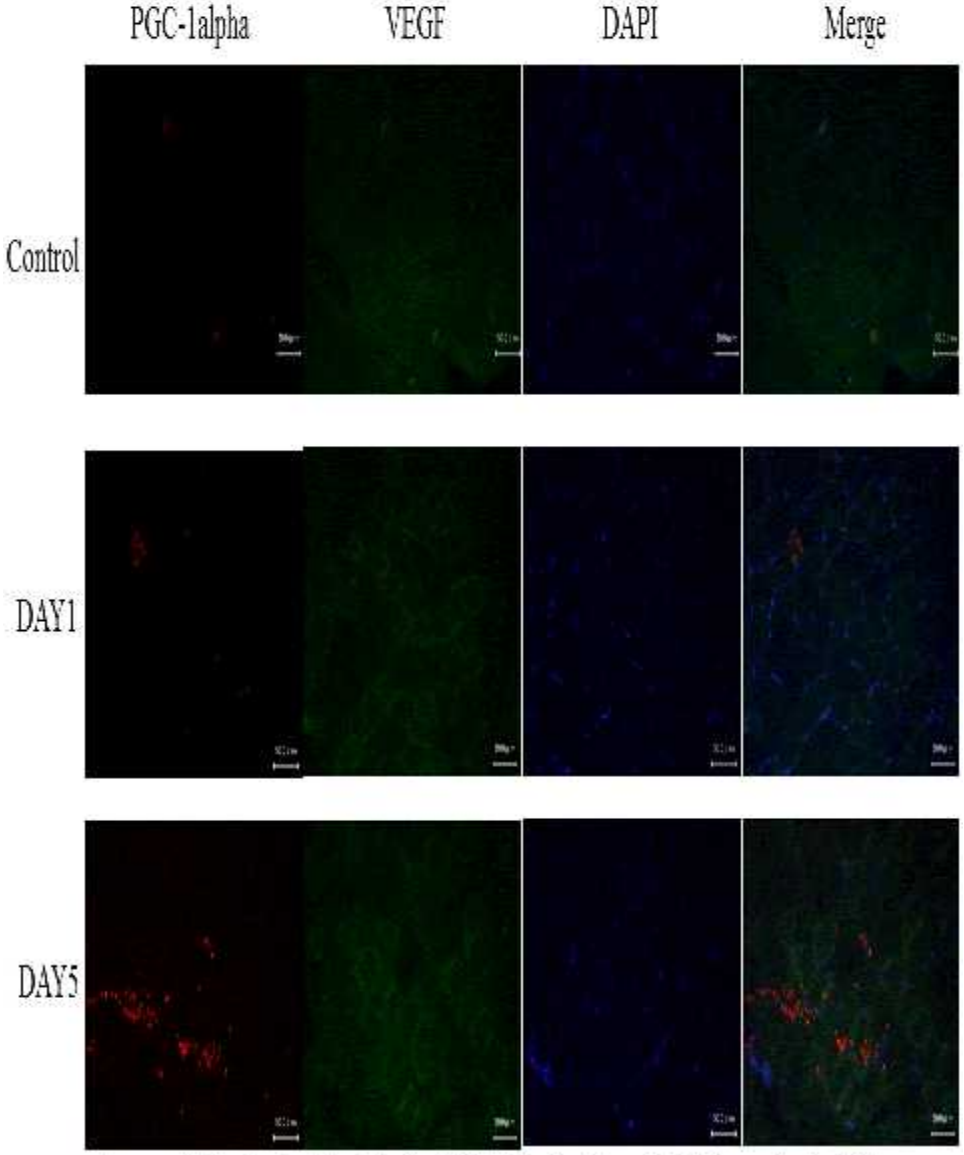
Figure 2





In real-time PCR analysis, the gene expression of PGC-1alpha, VEGF and MyoD in the CO2 groups was increased compared to control group (Control:n=7, 1 day and 5 days: n=5).

Figure 3



Immunohistochemical staining for PGC-1alpha (red), and VEGF (green) of middle section of TA muscle. PGC-1alpha and VEGF showed that the positive staining area in the CO2 group was much larger, compared to the control group.