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ORIGINAL PAPER

Increase in carbon dioxide accelerates the performance of endurance exercise in rats

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Abstract Endurance exercise generates CO_2 via aerobic metabolism; however, its role remains unclear. Exogenous CO_2 by transcutaneous delivery promotes muscle fibre-type switching to increase endurance power in skeletal muscles. Here we determined the performance of rats running in activity wheels with/without transcutaneous CO_2 exposure to clarify its effect on endurance exercise and recovery from muscle fatigue. Rats were randomised to control, training and CO_2 groups. Endurance exercise included activity-wheel running with/without transcutaneous CO_2 delivery. Running performance was measured after exercise initiation. We also analysed changes in muscle weight and muscle fibres in the tibialis anterior muscle. Running performance improved over the treatment period in the CO_2 group, with a concomitant switch in muscle fibres to slow-type. The mitochondrial DNA content and capillary density in the CO_2 group increased. CO_2 was beneficial for performance and muscle development during endurance exercise: it may enhance recovery from fatigue and support anabolic metabolism in skeletal muscles.

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Introduction

All animals generate energy by consuming oxygen (O_2) and releasing carbon dioxide (CO_2). Aerobic metabolism is performed with O_2 for energy production in the mitochondria [1, 2] using not only carbohydrates but also amino acids and fatty acids [1, 3]. CO_2 generated during aerobic metabolism is not merely a waste product but serves other important functions.

One role of CO_2 is vasodilation, with important implications in central nervous system function [4] and physiology [5]. CO_2 also exerts the Bohr effect on haemoglobin in red blood cells [6, 7], which have high levels of carbonic anhydrase that ionises CO_2 to carbonate and hydrogen ions [8]. Accumulating hydrogen ions dissociate O_2 from oxyhaemoglobin. Thus, tissues with increased CO_2 content can acquire necessary O_2 via the Bohr effect. Importantly, the Bohr effect can be achieved via exogenous CO_2 [9]. Moreover, we previously showed that transcutaneous CO_2 delivery to the hind limbs of rats upregulated peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) and vascular endothelial growth factor (VEGF) expression as well as the number of mitochondria. Furthermore, this approach promoted a change in muscle fibre type from IIB to IIA, similar to that observed in tibialis anterior (TA) muscles after aerobic exercise [10]. A recent study also suggested that carbonate ion-mediated PGC-1α upregulation might promote a fast-to-slow fibre-type shift in muscle cells [11].

Aerobic exercise is an actively performed exercise that utilises aerobic metabolism [2, 12]; endurance

exercise is typically referred to as aerobic exercise [12, 13]. Repeated performance of endurance exercise increases aerobic metabolism via a positive-feedback loop along with transformation of muscle fibres and subsequent muscle endurance [12]. Studies show that the upregulation of *PGC-1α* gene expression plays a major role in muscle fibre transformation [14–16]. PGC-1α in muscles not only increases the transcriptional activity of the mitochondrial transcriptional factor A (TFAM) but also induces capillary density by activating VEGF. As a result, slow-twitch muscle fibres shift, in order, to type IIB, IID, IIA and I fibres; consequently, muscle endurance is increased and endurance exercise performance is enhanced [17, 18].

The responses to exogenous CO_2 and endurance exercise are similar, and include changes, such as mitochondrial biogenesis, in muscle fibers following increase in PGC-1α, and increase in capillary density by activating VEGF. However, the relationship between endurance exercise and exogenous CO_2 remains unclear. Therefore, we hypothesised that the application of exogenous CO_2 may support exercise endurance and evaluated whether transcutaneous CO_2 application after endurance exercise increased endurance performance and aerobic capacity in rats. Supplementing CO_2 therapy during endurance exercise may be beneficial for long-distance runners as well as patients with reduced muscle capacity for endurance who might require postoperative rehabilitation.

Materials and methods

Animal care and experimental design

The use of animals was approved by the Animal Care and Use Committee of Kobe University Graduate School of Medicine (approval number, P100408). A total of 24 male Wistar rats that were 5 weeks old (CLEA Japan, Tokyo, Japan) were used for all experiments. Animals were fed ad libitum and housed in a thermostatic environment at 21 °C under a 12-h light/12-h dark cycle.

As depicted in Fig. 1a, rats were initially divided into two groups, exercise ($n = 16$) and no exercise (control group; $n = 8$), and further divided according to average body weight. All rats were pre-trained using a 5-day programme that comprised activity-wheel running for 30 min every day. On the 6th day, rats in the exercise group were then randomly assigned to one of the two experimental groups: CO_2 treatment (CO_2 group, $n = 8$) and no treatment (training group, $n = 8$). Both experimental groups were subdivided according to average running distance and body weight.

Measurement of running distance

We measured the total running distance during exercise every day to analyse running performance. Data for the running distance were expressed as a percentage of running distance during pre-training.

To evaluate time-dependent change, we recorded the distance run at 5-min intervals on the final day (day 28). Data were presented as average in the CO_2 group compared with those in the training group.

Activity wheels

Activity wheels were purchased from Hoei Kinzoku Kogyo (Aichi, Japan), as previously described [19, 20]. The wheels measured 1 m in circumference and 14 cm in depth. We measured the initial torques using torque-measuring equipment (Sugisaki Meter Co., Ltd, Ibaraki, Japan); the initial torque was less than 1 mN (Fig. 1b). The wheels had a magnetic counting system that recorded the number of rotations to determine the running distance.

CO₂ treatment

Transcutaneous CO_2 absorption-enhancing hydrogel was provided by NeoChemir Inc. (Kobe, Japan) as previously described [9, 10, 21]. Briefly, all animals in the three groups were anaesthetised with diethyl ether (Wako Pure Chemical Industries, Osaka, Japan), hair on their hind limbs was shaved, and CO_2 hydrogel was applied to their hind limbs. The area of skin covered with the CO_2 hydrogel was sealed with an adaptor. In only the CO_2 group rats, 100% CO_2 gas (Kobe Sanso KK, Kobe, Japan) was flowed into the area for 10 min, as previously described [10, 21]. The CO_2 group underwent this treatment every time after the training.

The expected mechanism of the transcutaneous CO_2 application was as follows. Because of its relatively high solubility, CO_2 gas dissolves in the hydrogel. The dissolved CO_2 then permeates the skin, moving down its concentration gradient when the maximum solubility in the hydrogel is reached. Our previous study showed that CO_2 penetrated human tissue by this system [9].

Muscle isolation and preparation

Animals were sacrificed with an overdose of the anaesthetic pentobarbital at 24 h after final exercise and/or treatment, followed by decapitation and dissection of intact TA muscles in order to analyse changes in muscle fibre types. Muscles were then weighed with an electronic balance (A&D, Japan) after removing excess connective tissue, immediately frozen in isopentane precooled with
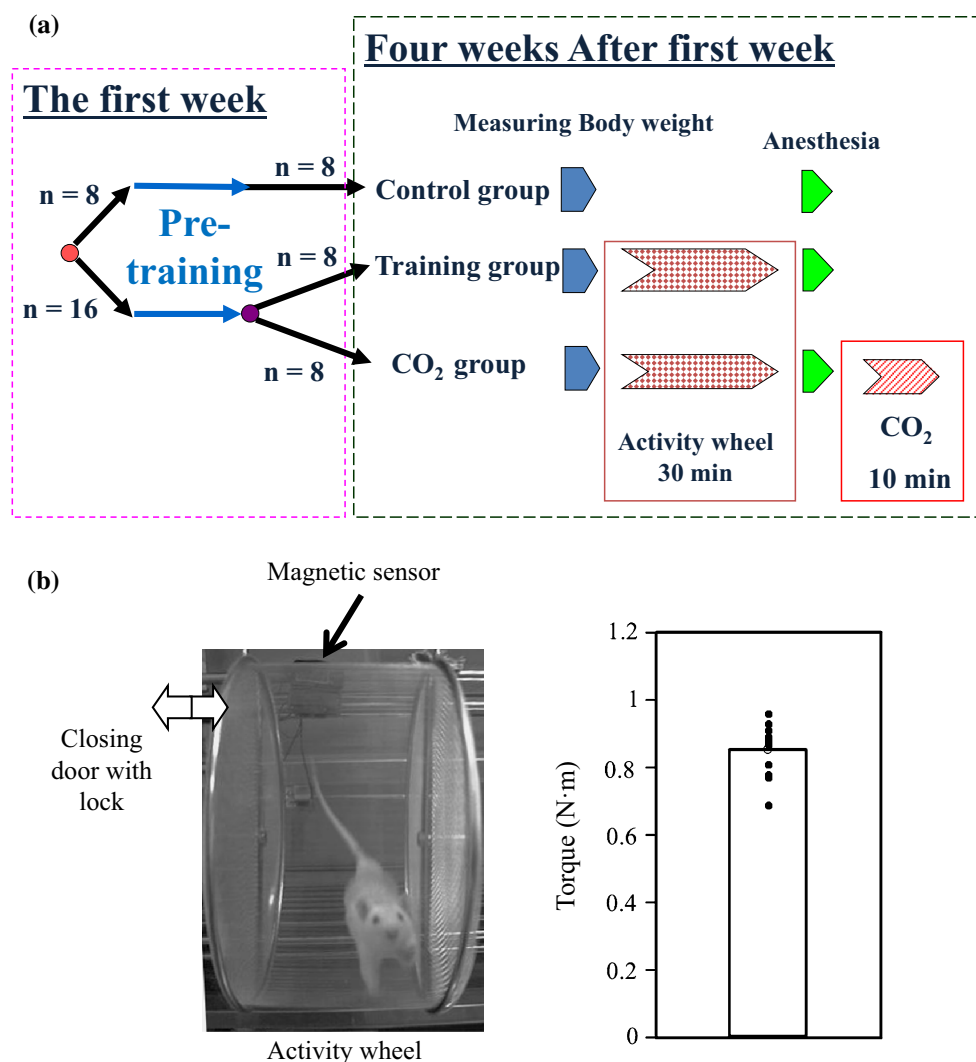
Fig. 1 a Experimental design to test the impact of transcutaneous CO_2 delivery on endurance exercise performance. **b** Photograph of a male Wistar rat running in an activity wheel equipped with a magnetic sensor (left). The initial torques measured by using torque-measuring equipment (right)

liquid nitrogen and stored at -80 °C. Serial 10-μm-thick transverse sections were prepared from each block. Muscle weight ratio was calculated by the following equation: muscle weight ratio = muscle weight/body weight × 100.

ATPase staining

Unfixed frozen TA muscles were sectioned into 10-μm-thick slices with a cryostat, and ATPase immunostaining at pH 4.6 was performed as previously described [10, 22].

Isolation of myosin heavy chain

Protein was isolated from 10-mg transverse slices of the mid-belly of TA muscles using urea buffer, and 5–20 μl of homogenates were analysed for the expression of myosin heavy chain (MHC) isoforms by two-dimensional electrophoresis, as previously described [10, 22, 23]. After electrophoresis, gels were stained using the Wako Silver Stain Kit (Wako Pure Chemical Industries), and images of MHC bands were captured with a LAS-3000 imager to quantify band intensities using ImageJ software (https://imagej.nih.gov/ij/). Changes in MHC isoform expression among the three groups were presented as percent changes from pre-training values.

Mitochondrial DNA copy number

Genomic DNA was isolated from 10-mg transverse slices of mid-belly TA muscles using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Mitochondrial DNA (mtDNA) content relative to the β-actin gene copy number was measured using real-time PCR, as previously described [10, 23].

Fig. 2 **a** Percent change in running distance among the groups by week. $*p < 0.05$ **b** Changes in running distances on the final day in the CO_2 and training groups. $*p < 0.05$
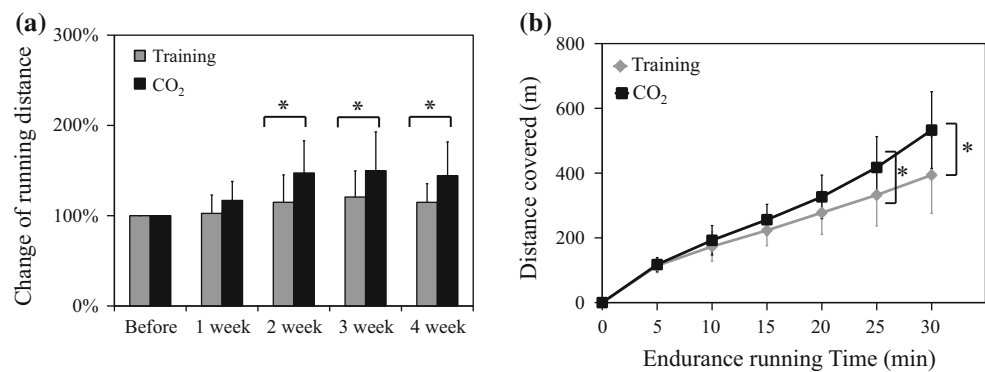
Quantitative real-time PCR

Quantification of mtDNA was performed using a StepOne[TM] Real-Time PCR System (Applied Biosystems). Real-time PCR reactions (20 µl) contained 0.5 µM each of forward and reverse primers, 1 µl genomic DNA template from the reverse transcription reaction and 10 µl Power SYBER Green PCR Master Mix (Applied Biosystems). Reaction conditions were 10 min at 95 °C, followed by 40 cycles at 95 °C (15 s) and 60 °C (1 min). mtDNA copy numbers were normalised to nuclear DNA copy numbers and compared among groups (ΔΔCt method; Applied Biosystems), as previously described [10, 24]. The following forward and reverse primers were used to determine mtDNA copy numbers: 5′-ACA CCA AAA GGA CGA ACC TG-3′ and 5′-ATG GGG AAG AAG CCC TAG AA-3′, respectively. Nuclear DNA (β-actin) forward and reverse primers were 5′-GCG GTG ACC ATA GCC CTC TTT-3′ and 5′-TGC CAC TCC CAA AGT AAA GGG TCA-3′, respectively. Primers were purchased from Invitrogen (Carlsbad, CA, USA).

Morphometric evaluation of capillary density

Skeletal muscle tissues cut into slices along the axial plane were washed in phosphate-buffered saline and incubated in MitoTracker Deep Red FM (Molecular Probes, Thermo Fisher Scientific, Carlsbad, CA, USA) and isolectin B4 (Vector Laboratories, Burlingame, CA, USA) at 37 °C for 1 h according to the manufacturer's protocol. Images from stained slices were captured using a BZ-8100 microscope (Keyence, Osaka, Japan), as previously described [25, 26]. All morphometric studies were performed by an examiner blind to the treatments. Capillary density was measured by quantification of isolectin B4 staining (green fluorescence) in randomly selected fields (250 × 250 µm) of muscle specimens using Image J software, as previously described [25, 26].

Statistical analysis

The level of statistical significance was set at $p < 0.05$. Data were expressed as mean ± standard deviation. Significance was determined via analysis of variance (ANOVA) and the two-tailed Mann–Whitney U test.

Results

Running distance

As shown in Fig. 2a, although the running distance was longer in the CO_2 group than in the training group after 1 week, the difference was not statistically significant. However, rats that received transcutaneous CO_2 ran significantly longer distances than those in the training group at 2, 3 and 4 weeks after treatment ($p < 0.05$, $n = 8$ in each group).

Figure 2b shows the running distances and speeds at 5-min intervals over 30 min on the final day. In the first stage (0–10 min), the running performance did not differ between the training and CO_2 groups. However, the CO_2 group had progressively increased speed and ran a longer distance, and the running performance of this group was significantly higher than that of the training group in the last stage (25–30 min, $p < 0.05$, $n = 8$ in each group).

Muscle weight

The muscle weight and muscle weight ratio tended to be higher in both exercise groups than those in the control group. After the 4 weeks of training, the TA muscle weight and muscle weight ratio in the CO_2, training and control groups were as follows: 0.464 ± 0.026 g and 0.171 ± 0.007, 0.464 ± 0.056 g and 0.173 ± 0.008, 0.443 ± 0.016 g and 0.161 ± 0.005, respectively. Therefore, activity-wheel running exercise for 30 min for 5 days/

Fig. 3 **a** ATPase staining in the deep layer of the tibialis anterior muscles of the control, training, and CO_2 groups

Control Training CO_2

100 μm
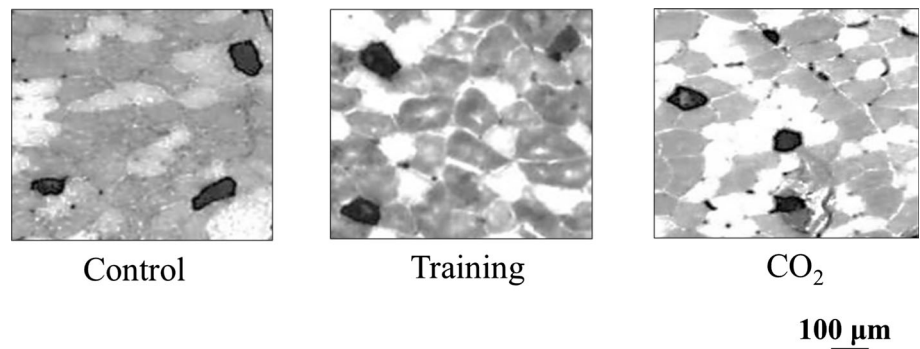
week for 4 weeks led to increased muscle weight; however, there were no significant differences between the groups ($p = 0.08$, $n = 8$ in each group).

Muscle fibre type

The mean percentage of type IIB fibres decreased while that of type IID and/or IIA fibres increased in both the CO_2 and training groups. There were significant differences in the percentages of IIA, IID and IIB fibres between the control and CO_2 groups (Figs. 3, 4a, b; $p < 0.01$, $n = 6$ in each group). These results indicated that in comparison with non-transcutaneous CO_2 delivery, transcutaneous CO_2 delivery following pre-training led to the change of a significantly higher percentage of muscle fibres from type IIB to type IIA.
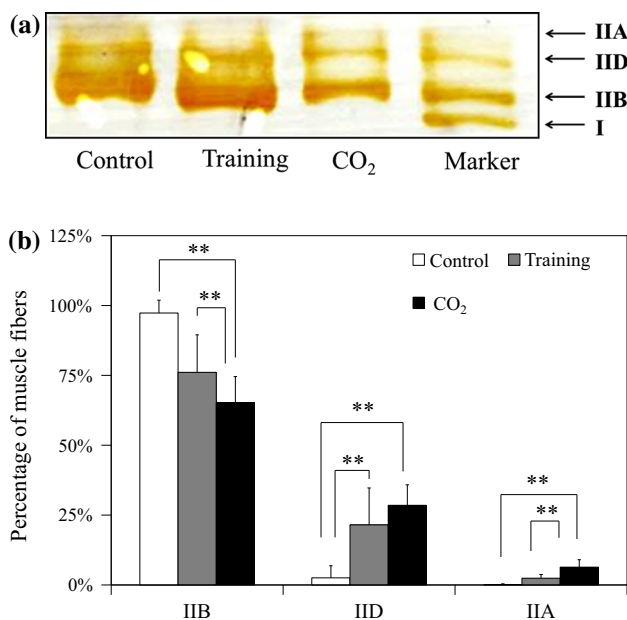
Capillary density and mitochondrial number in muscle

The mean capillary density in the CO_2 group was significantly higher than that in the control group (Fig. 5a, b; $p < 0.05$, $n = 4$ in each group). Moreover, the amount of mitochondrial DNA in the CO_2 group was significantly higher than that in the control group, as determined by real-time PCR (Fig. 6; $p < 0.05$, $n = 4$ in each group). The results showed that although there were differences in the number of muscle mitochondria and the capillary density between the training and the control groups as well as
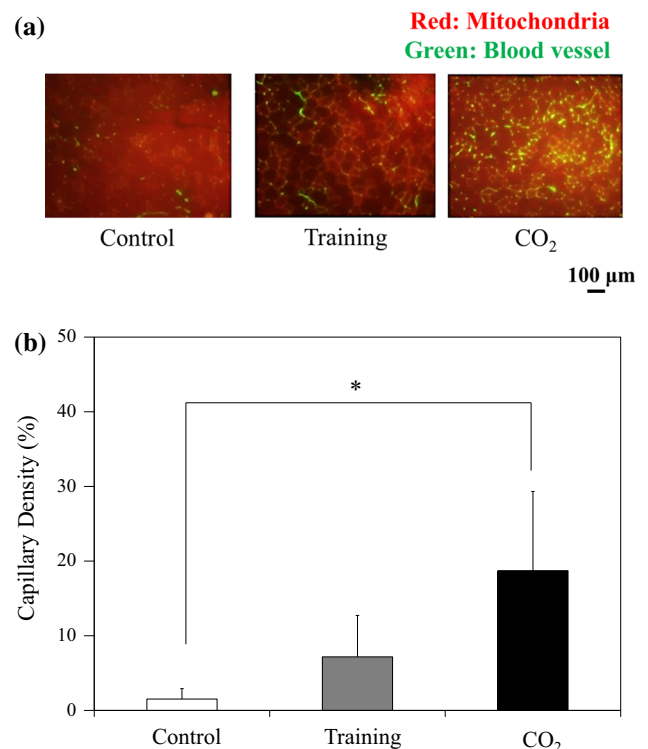
(a)

Red: Mitochondria
Green: Blood vessel

Control Training CO_2

100 μm

(b)

Fig. 5 **a** Staining for mitochondria and capillaries in the deep layer of the tibialis anterior muscles of the control, training, and CO_2 groups. **b** Neovascularisation in the CO_2 group, as determined by the quantification of capillary density, was significantly higher than that in the control group. *$p < 0.05$
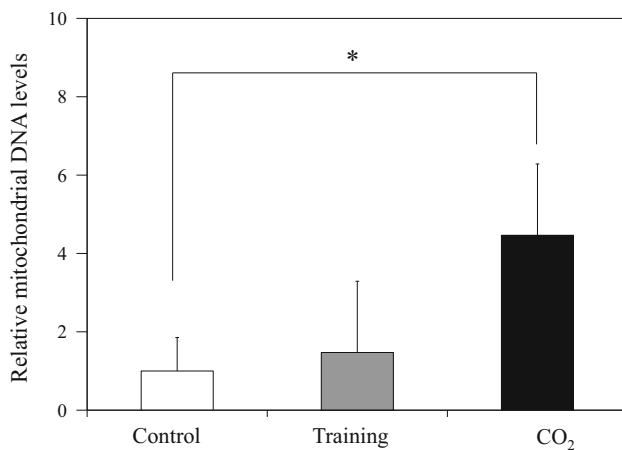
(a)

Control Training CO_2 Marker

(b)

Fig. 4 **a** Electrophoretic analysis of myosin heavy chain isoforms. **b** The control group primarily expresses IIB fibres, as determined by the semi-quantification of the band, whereas the CO_2 group mainly expresses IIB, IIA, and IID fibres. *$p < 0.05$; **$p < 0.01$

Fig. 6 Real-time PCR analysis of mitochondrial DNA copy number. The mitochondrial number was higher in the CO_2 group than in the control group. $*p < 0.05$

between the CO_2 and the training groups, these were not statistically significant.

Discussion

In the present study, running performance evaluated at 2, 3 and 4 weeks after intervention was significantly higher in rats that received transcutaneous CO_2 than that in rats that were trained but did not receive treatment. This difference might be due to an increase in muscle weight, change in muscle fibre type from IIB to IIA and/or IID and increase in capillary density and mitochondrial number within the muscle as a result of CO_2 treatment. Muscle fibres usually switch from type IIB to type IID and/or IIA fibres as a result of training [10]. However, in the present study, training after transcutaneous CO_2 treatment resulted in a higher percentage of TA muscle fibres changing to type IIA and/or IID when compared with animals that were trained without CO_2 application. Type IIA, IID and IIB fibres differ in ATP production, mitochondrial number and capillary density. In general, in skeletal muscles, type IIA and IID fibres have a higher capacity for ATP production and higher mitochondrial numbers and capillary density compared with type IIB fibres [22, 23]. Transcutaneous CO_2 delivery led to increased mitochondrial number and capillary density, providing evidence that this therapeutic approach might potentially aid in the performance of endurance exercise.

Gaseous molecules control various cellular signalling pathways in the body [27–29]. CO_2 is an important gaseous molecule with well-defined roles [6, 30], including those related to nitric oxide [30]. In addition, CO_2 therapies, including carbonate spa and artificial CO_2-enriched water, are used in many medical conditions, such as cardiac,

ischaemic diseases [31, 32], and Raynaud's phenomenon [33]. However, the mechanism and potential of CO_2 as a therapeutic modality are not well known. Moreover, CO_2 concentration of saturated CO_2-enriched water is only 0.1%, and there is little evidence of CO_2 absorption into the human body. Therefore, we previously used near-infrared spectroscopy to demonstrate that a novel transcutaneous application of CO_2 upregulated O_2 pressure in the local tissue and showed, for the first time, that our transcutaneous CO_2 system could cause absorption of CO_2 and the Bohr effect in the human body [9] and suggested that these results might be applicable in human cases.

Our findings suggest that CO_2 enhances the performance of endurance exercise in rats. Moreover, we previously demonstrated that CO_2 activated *PGC-1α* gene expression and increased the endurance potential in skeletal muscles [10]. Together with the results presented herein, these findings strongly suggest that CO_2 therapy might improve endurance. Moreover, several studies demonstrated that endurance exercise induced *PGC-1α* gene expression via Ca^{2+} influx and AMPK pathway activation [11, 34]. Based on our previous study showing that transcutaneous CO_2 delivery induced Ca^{2+} influx into cells, these results altogether suggest that endurance exercise improves performance by CO_2-mediated *PGC-1α* upregulation and Ca^{2+} influx.

Aerobic metabolism occurs in mitochondria [1]. The number of mitochondria increases as a result of upregulation of *PGC-1α* and skeletal muscle activity during aerobic exercise or recovery from muscle injury [35, 36]. Skeletal muscle includes fast (white) muscle fibres that provide instant power and slow (red) muscle fibres that provide endurance. Exercise training was extensively shown to increase the mitochondrial content of skeletal muscles [11, 35, 36]; however, the underlying mechanisms directing this adaptive response are incompletely understood. Increases in mitochondrial function require complex coordination of genes encoded by nuclear and mitochondrial DNA [37, 38]. PGC-1α appears to play an integral role in regulating the transcription of mitochondrial and nuclear genes [37, 38]. Our previous results suggested that transcutaneous CO_2 application increased nuclear PGC-1α protein and induced mitochondrial biogenesis [10, 38]. Therefore, an increase in mitochondrial number by transcutaneous CO_2 delivery might lead to a significant increase in aerobic capacity.

CO_2 therapy is supported by some scientific evidence. One is that CO_2 increases blood flow, and our transcutaneous CO_2 application has shown increased blood flow in the deep tissue [9, 21]. There is a relationship between blood flow volume and muscle fatigue, and increased blood flow reduced muscle fatigue [39]. Therefore, there may be a possibility that transcutaneous CO_2 application might

increase the performance of endurance exercise via the reduction of muscle fatigue. Another piece of evidence is that this model of CO_2 therapy accelerates muscle injury repair in rat models [40]. This research was able to explain that this form of CO_2 therapy may increase next-day performance via muscle injury repair.

This study includes several limitations. First, our experimental design did not include a group that received CO_2 but no training. However, in our previous study, we demonstrated that transcutaneous CO_2 in the absence of exercise training upregulated *PGC-1α* gene expression and promoted muscle fibre changing to increase endurance in skeletal muscles [10]. Therefore, the present study focused on whether CO_2 enhanced endurance exercise performance. Second, only TA muscle was analysed at the end of the 4-week experiment. Most studies investigating endurance exercise in rat models use longer durations such as 45 days [41] and 10 weeks [42]. Because the duration of our study was shorter than those of previous studies, there was little change in muscle fibres of the training group. However, this research aimed to assess the role of the CO_2 effect. Compared with the control group, the CO_2 group showed the most transformation in muscle fibres, followed by the training group. We could not show effects on human performance in this study. Ishida et al. have reported the lack of an effect of CO_2 on human exercise performance [43]. However, another study did find an effect [44]. Overall, the CO_2 effect on exercise performance is not well understood. We have previously described CO_2 permeation of rat skin [9] and that the Bohr effect increased and pH decreased following transcutaneous CO_2 application in humans [9]. Therefore, we propose further study of transcutaneous CO_2 application in animals and humans.

Based on the findings of the current study, we suggest that CO_2 regulation might enhance performance in endurance exercise. Moreover, transcutaneous CO_2 delivery should be considered as a potential therapeutic approach in patients recovering from injury or in those with damaged tissue that requires O_2 and energy.

Author Contributions YS and MM conducted all experiments. TU, KO and TH contributed to the animal experiments. TU, KO, AK and HN contributed to the biological and histological analyses. YS and SYL analysed the data. TN, RK and MK supervised all aspects of this study. TU, KO and YS wrote the manuscript.

Compliance with ethical standards

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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