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ORIGINAL ARTICLE

# Direct Effects of Carbon Dioxide-rich Water Bathing on Peripheral Blood Flow

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## ABSTRACT

**Introduction:** Several previous studies related to $CO_2$ water bathing noted improvement in blood flow. However, the molecular mechanism underlying this effect is as yet unclear. One of the possibilities was that the diffusion of $CO_2$ into the blood vessels, which is mediated by nitric oxide (NO) and endothelin-1 (ET1), increased the blood flow. Therefore, we investigated the dynamics of NO and ET-1 in increasing the blood flow by immersing legs in the $CO_2$-rich water bath. **Methods:** Thirteen healthy subjects (eight males and five females) participated in this study. Each participant immersed both their legs into $CO_2$-rich water bath. The peripheral blood flow was measured using pocket laser doppler flowmetry for 5 minutes before immersion, 10 minutes during immersion, and 5 minutes after withdrawing from immersion. The blood samples were taken from the median cubital vein, in the third minute before immersion, the seventh minute during immersion, and the third minute after immersion. The levels of NO and ET-1 were measured using Enzyme-Linked Immunosorbent Assay (ELISA). All the data were analysed using SPSS software. **Results:** The blood flow in the legs increased during immersion, and, in five minutes after withdrawing from immersion, the blood flow returned to a value close to the 'before immersion' value. There were no statistical differences of NO, ET-1, and the ratio of NO/ET-1 before, during, and after immersion. **Conclusion:** $CO_2$-rich water bathing improved the peripheral blood flow in healthy people through the direct effect of $CO_2$, which was not mediated by NO and ET-1.

**Keywords:** Carbon dioxide, Nitric oxide, Endothelin-1, Regional blood flow

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## INTRODUCTION

Carbon dioxide ($CO_2$) water bathing has been known to be useful as a treatment for cardiovascular diseases, peripheral arterial disease, hypertension, and heart disease (1–3). Carbon dioxide is one of the local metabolic factors that play a role in regulating the vascular smooth muscle cells (VSMCs) and is well-known as a potent vasodilator (2). Some previous studies on the topic of $CO_2$-rich water bathing had concluded that the improvement in blood flow depends on the cutaneous vasodilation effect and caused by high $CO_2$ content in the water causing $CO_2$ to diffuse into subcutaneous tissue through the skin layers (3,4). However, there are has not been much research to discover the mechanism that causes this effect, especially the molecular mechanism, which is still unclear (1,5). Moreover, the researchers are still debating whether $CO_2$ works directly or through mediators (6,7). Therefore, the possible role of endothelium as a mediator deserves consideration.

The endothelium plays a role in regulating the vessels, causing them to constrict or dilate to provide adequate organ perfusion pressure to the target organs by producing several biologically active substances (8,9). Endothelial cells enlarge the blood vessels by releasing nitric oxide (NO), which is the best-characterised endothelium-derived relaxing factor (EDRF) (10). On the other hand, to cause constriction of blood vessels, endothelial cells release endothelin-1 (ET-1), one of the endothelium-derived contracting factors (EDCF) (9). The dynamics of vasodilation and vasoconstriction are regulated by the dynamics of NO and ET-1 concentration.

The above background leads to the conclusion that $CO_2$-water bathing can improve peripheral blood flow through the mechanism of vasodilation. However, it is not known whether the $CO_2$ causes the vasodilation directly or indirectly through the substances secreted by the endothelial cells. Therefore, we investigate the possibility of mediation in the effect of $CO_2$-rich water bathing on peripheral blood flow by measuring the NO, ET-1 levels and the NO/ET-1 ratio.

## MATERIALS AND METHODS

### Ethical Approval

The research procedure was accepted and approved by the Medical Faculty Ethics Committee of Hasanuddin University, evidenced by the letter No. 387/H4.8.4.5.31/PP36-KOMETIK/2018. Also, before conducting the experiment, the informed consent was signed by each participant.

### Research Design and Participants

This study was pre-experimental. A one-group pre-test-post-test design was followed in which all subjects are subjected to the same experiment. The subjects were healthy people over 18 years old who were ready to come to Hasanuddin University Hospital on the 6th floor to take part in the research. We excluded subjects who have a history of cardiovascular disease and wound at the foot that is known based on a questionnaire that had been distributed, blood pressure checks, and inspection. Thus, fifty-three subjects were obtained. Since we only use 1 reagent kit each for NO and ET-1 which only enough for fourteen samples in duplicate, therefore, from among fifty-three subjects, we randomly selected fourteen subjects belonging to age groups younger than 30 years or thirty years old and those older than 30 years. This means the subjects were selected from the entire age-range of the group. This may be stated differently as seven of the selected subjects were younger than 30 years of age, and seven others were above 30 years old. Of these, one male subject was dropped as his NO / ET-1 ratio measurement data was too high. Thus we had a group of thirteen healthy people of whom eight were males and five females for the analysis.

### Carbon Dioxide-Rich Water Bath and Blood Flow Measurement

Each subject immersed their legs into a mixture of water and $CO_2$ in 38ºC for 10 minutes. The water temperature was maintained at 38ºC with an electric water heater (Ariston, TI-SHAPE 15 OR 500 ID, Marche, Italy) which was connected to a gas cylinder containing $CO_2$. The water and $CO_2$ were mixed using highly concentrated hydrogen carbonate ($HCO_3$) ion spring (CREA, BC-2000, Nagoya, Japan). Twenty-four litres of water from this system were let into a plastic container through a tap. Then the temperature of the water was measured with a mercury thermometer to ensure that the temperature was 38ºC, and its pH was measured using a pH meter (Sato, SK-620PH II, China). The mixture of water and $CO_2$ was considered suitable for use when its pH ranged between 4.5–5.6. Blood flow was measured by laser doppler flowmetry (Pocket LDF Seri MBF-IIA, Hiroshima, Japan) and the value of the subcutaneous blood flow per second was directly recorded in a personal computer (Toshiba, Tokyo, Japan) with the help of a software package (LDF recorder, version 1.02, Japan).

This experiment was carried out in an air-conditioned room in which the temperature was maintained at approximately 26–27ºC. The protocol and setup of the experiment are shown in Fig.1 and Fig.2. The subjects were seated during the experiment. The pad sensor of LDF was attached to the dorsum of the left foot between the first (hallux) and second (pointer) toes. The peripheral blood flow was measured for five minutes before immersion, ten minutes during immersion, and five minutes after the legs were withdrawn from the bath. To eliminate the measurement of enhanced blood flow caused by movements while immersing the legs into and withdrawing them from the bath, the subject was allowed a two-minute break during which the LDF was paused.

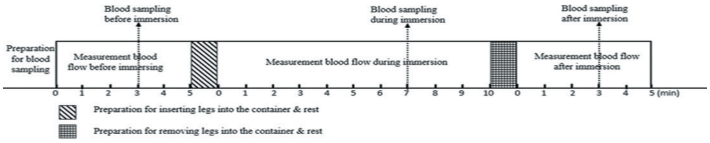

**Fig. 1 : The protocol for $CO_2$-rich water immersion.** Blood flow measurements were taken for five minutes before immersion, ten minutes during immersion, and five minutes after withdrawal of legs from the immersion. The blood samples were taken at the third minute for five minutes blood flow measurement before legs immersion, the seventh minute from total ten minutes blood flow measurement during immersion, and the third minute from total five minutes blood flow measurement after legs are taken out.

**Fig. 2 : Setup for immersing the legs and taking blood samples.** The blood sample is taken using a wing needle on the left arm's median cubital vein before, during, and after immersion. The LDF sensor pad is installed in the dorsal left foot continuously.

## Blood Sample Retrieval and Storage

About 3 ccs of blood were taken as a sample from the median cubital vein of each subject, respectively, at 1) the third minute during the five minutes of blood flow measurement before immersion of legs, 2) the seventh minute of total ten minutes blood flow measurement during immersion, and 3) the third minute from five minutes of blood flow measurement after legs were withdrawn from the bath. Based on our preliminary study, the blood flow values increased at the time of sampling. The times were chosen for collecting the blood samples with the expectation that the molecular effect would occur when the blood flow increase at those times. Each blood sample was labelled with the time of blood sampling. The samples were centrifuged and stored in a refrigerator at -80°C.

## Measurement of NO and ET-1

Before, during, and after immersion, the concentrations of plasma NO and ET-1 were measured using the ELISA technique. The reagent-kit used was Parameter™ Total Nitric Oxide and Quantikine® ELISA Endothelin-1 (R&D Systems, Inc., Minneapolis, USA). The measurements were taken in duplicate. The instrument used to measure the NO level was Microplate Reader Biorad model 680 with Microplate Manager software version 5.2.1 (Bio-rad Laboratories Inc., CA, USA), while the ET-1 level was measured using Microplate Reader with a wavelength of 450 nm (Bio-rad Laboratories Inc., CA, USA).

## Statistical Analysis

To compare the blood flow and the ratio of NO/ET-1 before, during, and after immersion, we used the Friedman test. Then to compare NO and ET-1 levels before, during, and after immersion, we used the Repeated ANOVA test. The data were shown as mean ± standard deviation, and the value was considered significant at $P < 0.05$.

## RESULTS

The sample consisted of thirteen subjects (61.5% males and 38.5% females) of a mean age of 38 years. They were randomly selected out of 53 healthy subjects who had enrolled in this study. Of these 13 subjects, those below and above 30 years of age were 46.2% and 53.8%, respectively. Furthermore, the mean values of body-mass index (BMI), systolic blood pressure (SBP), and diastolic blood pressure (DBP) were 23.09 ± 3.980 kg/m2, 119.69 ± 12.744 mmHg, and 76.46 ± 10.195 mmHg, respectively. Overall, statistically, these mean parameters of the thirteen subjects agree with the mean parameters of fifty-three volunteers thereby indicate that 13 subjects could represent 53 participants in this study. A summary of the subject characteristics is shown in Table I.

**Table I : Subject Characteristics**

| Parameters | Frequency (n=53) | Frequency (n=13) | P-Value |
|---|---|---|---|
| [a,b] Age (years) | 34.68±14.421 | 38±7.378 | 0.583 |
| ≤ 30 | 26 (49.1) | 6 (46.2) | 0.430 |
| > 30 | 27 (50.9) | 7 (53.8) | |
| [b] Gender | | | |
| Male | 30 (56.6) | 8 (61.5) | 0.747 |
| Female | 23 (43.4) | 5 (38.5) | |
| [a] Body Mass Index (BMI, kg/m$^2$) | 24.44±4 .931 | 23.09±3.980 | 0.429 |
| [a] Systolic blood pressure (SBP, mmHg) | 115.49±12.783 | 119.69±12.744 | 0.333 |
| [a] Diastolic blood pressure (DBP, mmHg) | 75.43±9.099 | 76.46±10.195 | 0.745 |

n = Number of Sample

[a] For parameters with numerical data, values are expressed as mean±SD

[b] For parameters with categorical data, values are expressed as n (%)

The blood flow values that were measured, namely, NO levels, ET-1 levels, and the ratio of NO/ET-1 before, during, and after immersion in the $CO_2$-rich water bath are shown in Table II. The blood flow values are presented as the average blood flow measurements for five minutes before, ten minutes during, and five minutes after the immersion. The mean values of peripheral blood flow were 5.63 ±3.792; 9.19 ± 4.392; and 6.38 ± 3.359 mL/min, respectively. Thus, the $CO_2$-rich water bath influences the peripheral blood flow significantly (P= 0.000). This result is also shown in Figure 3 where the average value of peripheral blood flow per minute during immersion increased from the baseline values. The mean peripheral blood flow begins to drop again when the legs are withdrawn from a $CO_2$-rich water bath, but the values were not lower than the baseline values (Fig. 3).

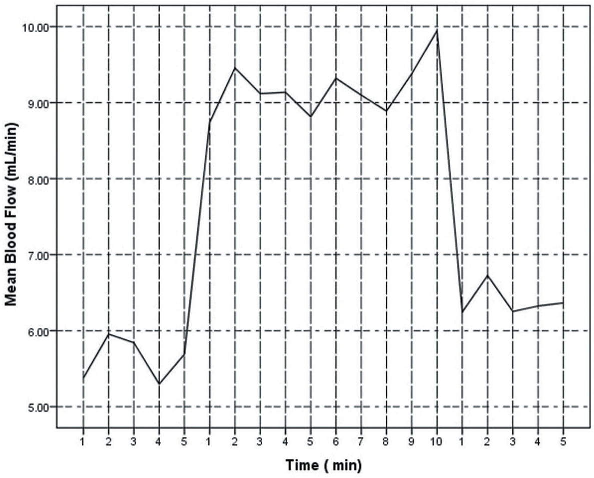

**Fig. 3 : Mean value of blood flow per minute in 13 healthy subjects.** Blood flow increases during immersion, and then it decreases after the legs are withdrawn from the bath, but the value blood flow does not fall lower its pre-immersion value.

**Table II : Comparison of Measured Parameters between Before, During, and After Immersing the Legs into $CO_2$-Rich Water Bath**

| Variable | n | Condition | | | P-value |
|---|---|---|---|---|---|
| | | Before immersing | During immersing | After immersing | |
| Blood Flow (mL/min) | 13 | 5.63±3.790 | 9.19±4.392 | 6.38±3.359 | 0.000$^{@}$* |
| NO (µmol/L) | 13 | 134.66±9.261 | 130.41±12.320 | 134.02±15.808 | 0.651^ |
| ET-1 Pg/mL | 13 | 0.41±0.202 | 0.45±0.207 | 0.41±0.234 | 0.335^ |
| Ratio NO/ET-1 | 13 | 421.80±230.528 | 340.12±137.95 | 411.72±203.033 | 0.926$^{@}$ |

NO = Nitric Oxide; ET-1 = Endothelin-1
* = Significant, P<0.05
^ = Repeated Anova Test; $^{@}$ = Friedman Test
n = Number of Samples

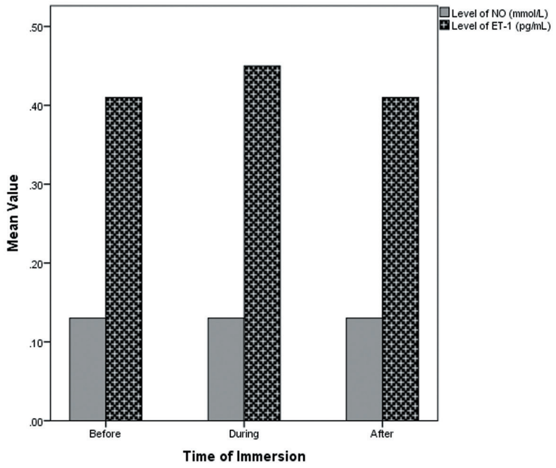

**Fig. 4 : Comparison of NO and ET-1 measured at the points of time during the experiment.** In this graph, NO levels are presented in units of mmol/L so that the visual difference between NO and ET-1 levels is not excessive.
In contrast to the values of NO level which did not change at any point in time, before, during, or after immersion, ET-1 level increased during immersion, although the increase was not statistically significant.

In contrast to the fluctuations in the values of peripheral blood flow, no changes were observed in the average levels of NO and ET-1 before, during, and after the immersion of legs in $CO_2$-rich water (P = 0.651 and P = 0.335, respectively for NO and ET-1). Likewise, there was no significant difference among the NO/ET-1 ratios before, during, and after the immersion legs in the $CO_2$-rich water bath (P = 0.926). This result is also shown in Fig. 4.

## DISCUSSION

Carbon dioxide ($CO_2$) balneotherapy, a kind of medicine with beneficial effects, has been known and used since the Middle Ages (1,11). The studies related to $CO_2$ balneotherapy had developed since Piderit and Beneke described the major direct effects such as warm sensation and skin flushing for the first time in 1836 and 1859 as cited by Schmidt (12). Several previous studies showed that immersion in $CO_2$ water baths improves the blood flow either in the hind limb ischemic of male rats or in the legs of healthy men (2,5). The studies have continued, and the $CO_2$ water bath is found as a useful treatment and a therapy for both peripheral arterial disease and cardiovascular disease (3,13). Likewise, the results of this research in healthy people showed an improvement in peripheral blood flow during immersion of the legs in a $CO_2$-rich water bath even though blood flow gradually decreased after the legs were withdrawn from the immersion. In this study, the mean blood flow after immersion did not dip below the basal mean blood flow. This observation may support the supposition that a $CO_2$-rich water bath is clinically useful for improving microcirculation, particularly if it is applied repeatedly as a therapy.

Vasodilatation plays an essential role in the mechanisms underlying the beneficial effects of $CO_2$ (11). Vasodilation caused by immersion in the $CO_2$ water bath is initiated by the difference between the gradients of $CO_2$ in the bathwater and the body. This difference triggers $CO_2$ to diffuse into the subcutaneous tissue (1,3,4). Carbon dioxide ($CO_2$) in high concentration dissolved in water will form carbonic acid ($H_2CO_3$) and rapidly will dissociate from the acid (14,15). However, in water, only a small amount of $CO_2$ is bounding chemically as carbonic acid, while another physically dissolved in water (7). Thus, the amount of $CO_2$ is possible to diffuse through the skin layer into the subcutaneous tissue.

Carbon dioxide has been known as a potent vasodilator and has been proven to improve blood flow, although no change in the systemic partial pressure of $CO_2$ ($PCO_2$) is noticed. This is because $CO_2$ is rapidly exhaled through venous and pulmonary blood flow (2,16). This situation is related to the Bohr Effect mechanism (17). The transfer of $CO_2$ to the skin increases blood flow through local vasomotor effects without triggering the haemodynamic system (18). In addition to $CO_2$

absorption, the transcutaneous system also causes a Bohr Effect which is shown by the raised oxyhaemoglobin level and lowered deoxyhaemoglobin level (17,18).

It is difficult to prove the direct vasodilation effect of $CO_2$ water bathing as the trigger for blood flow improvement (2). To the best of our knowledge, the research that measures EDRF and EDCF related to $CO_2$-rich water bathing on the mechanism of vasodilation in human subjects have not been investigated, especially in healthy people. A previous study still estimates the involvement of these factors in the response of $CO_2$ in the mechanism of vasodilation. According to Ogoh et al. (5), the improvement of endothelial-mediated vasodilator function related to $CO_2$-rich warm water in healthy men may be correlated with the changes in NO secretion. Another study has attempted to explain NO involvement in local vasodilation through experiments using experimental animals as the subjects. Xu et al. (2) noted an increase in NO levels related to $CO_2$ water bathing among experimental animals with ischemia, while such a change in NO levels did not occur among healthy experimental animals without ischemia subjected to $CO_2$ water bathing, even though, the blood samples were taken locally. The fact that we obtained the same results in this study from blood samples taken from the arms of the subjects, not locally from their legs, dispelled our doubts over our method of taking the blood samples.

That vasodilation as an effect of $CO_2$ involves NO levels in ischemic rats was also demonstrated by Dogaru et al. (19). In contrast to other studies that use ischemic animals as experimental subjects, we did not find any changes in NO or ET-1 levels as the response to CO2 water bathing among healthy people. The results of this study indicate that NO was not a causative factor for the increased peripheral blood flow, which is a response associated with $CO_2$ water bathing among healthy subjects. Thus, the increase of NO level by $CO_2$ water baths is a consequence of the production of hypoxia-induced factors in ischemic extremities and related to the development of angiogenesis (20).

Vasodilation as the response of $CO_2$ can be mediated either indirectly through NO or directly through the activation of potassium channels (1,21). Meanwhile, the results in this study indicate that $CO_2$-induced vasodilation among healthy people is through a direct effect. The mechanism that can explain this result is that $CO_2$ acts as the first messenger to the VSMCs through extracellular acidosis that can open the potassium ($K^+$) channels and the resulting hyperpolarisation. Furthermore, the activity of voltage-dependent calcium ($Ca^{2+}$) channels and also intracellular Ca decrease leading to vasodilatation (4,5,21).

Finally, it must be mentioned that this study has certain limitations, such as lacked some reagent kit to examine all 54 samples, inhomogeneous sample based on gender and age perspectives, and there are no control subjects. Further research is needed using more reagent kits in order to obtain a larger sample size and use a case-control design.

## CONCLUSION

This study shows an increase in peripheral arterial blood flow during $CO_2$ water immersion, while there is no change in the ratio NO/ET before, during, and after immersion. So, the conclusion drawn is that the improvement of peripheral blood flow during a $CO_2$ rich bath is not caused by NO or ET-1 but might be caused by the direct effect of $CO_2$. Further research is needed using more reagent kits in order to obtain a larger sample size and use a case-control design.

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