Carbon Dioxide Water-bath Treatment Augments Peripheral Blood Flow through the Development of Angiogenesis

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Canadian Journal of Physiology and Pharmacology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>cjpp-2017-0125.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>28-Mar-2017</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Xu, Yan-Jun; Institute of Cardiovascular Sciences, Elimban, Vijayan; Institute of Cardiovascular Sciences, Dhalla, Naranjan; Institute of Cardiovascular Sciences,</td>
</tr>
<tr>
<td>Is the invited manuscript for consideration in a Special Issue?:</td>
<td>IACS Sherbrooke 2016 special issue Part 1</td>
</tr>
<tr>
<td>Keyword:</td>
<td>CO$_2$ water-bath therapy, peripheral artery disease, angiogenesis, vascular blood flow, oxidative stress</td>
</tr>
</tbody>
</table>
Carbon Dioxide Water-bath Treatment Augments Peripheral Blood Flow through the Development of Angiogenesis

Yan-Jun Xu, Vijayan Elimban, and Naranjan S. Dhalla

Institute of Cardiovascular Sciences, St. Boniface Hospital Albrechtsen Research Centre,
Department of Physiology and Pathophysiology, Max Rady College of Medicine, Rady
Faculty of Health Sciences, University of Manitoba, Winnipeg, Manitoba, Canada

Short Title: CO\textsubscript{2}-induced Angiogenesis and Peripheral Blood Flow

Address for Correspondence

Dr. Vijayan Elimban
Institute of Cardiovascular Sciences
St. Boniface Hospital Albrechtsen Research Centre
351 Tache Avenue
Winnipeg, MB, R2H 2A6, Canada
Telephone: 204-235-3412
E-mail: velimban@sbrc.ca
Abstract

In this study, we investigated the effects of CO\textsubscript{2} water-bath therapy on blood flow and angiogenesis in the ischemic hind limb as well as some plasma angiogenic factors in peripheral ischemic model. The hind limb ischemia was induced by occluding the femoral artery for 2 weeks in rats and treated with or without CO\textsubscript{2} water-bath therapy at 37°C for 4 weeks (20 min treatment everyday for 5 days per week). The peak blood flow as well as minimal and mean blood flow in the ischemic skeletal muscle were markedly increased by the CO\textsubscript{2} water-bath therapy. This increase in blood flow was associated with development of angiogenesis in the muscle as well as reduction in the ischemia-induced increase in plasma malondialdehyde levels. Although plasma vascular endothelial growth factor and nitric oxide levels were increased in animals with peripheral ischemia, the changes in these biomarkers were not affected by CO\textsubscript{2} water-bath therapy. These results suggest that augmentation of blood flow in the ischemic hind limb by CO\textsubscript{2} water-bath therapy may be due to the development of angiogenesis and reduction in oxidative stress.

Keywords: CO\textsubscript{2} water-bath therapy; peripheral artery disease; angiogenesis; vascular blood flow; oxidative stress
**Introduction**

Peripheral artery disease, a major health hazard worldwide, is normally associated with obstruction of blood supply to the limb mainly as a consequence of atherosclerosis and thrombosis leading to the development of skeletal muscle ischemia (Dhalla et al. 2017; Kehler et al. 2013; Krishna et al. 2015; Norgren et al. 2007; Serano Hernando and Conejero 2007). Although a wide variety of defects for the production of different growth factors and various hormones, abnormalities in the function of endothelium and alterations in the vascular smooth muscle have been suggested to be intimately involved in the pathogenesis of peripheral artery disease (Carmaliet and Jain 2011; Dhalla et al. 2017; Krishna et al. 2015), the exact mechanisms of muscle dysfunction in the disease are not fully understood. Likewise, in spite of the availability of several surgical and medical interventions for the treatment of peripheral artery disease (Dhalla et al. 2017; Egginton et al. 2008; Li et al. 1998; Norgren et al. 2007; Potente et al. 2011; Serrano Hernando and Conjero 2007; Singla and Mehta 2013), none of the currently used therapies are satisfactory. Accordingly, it is of utmost importance that some new strategies be developed for improving the quality of life in patients suffering from peripheral artery disease.

Recently, some studies have been initiated for investigating the effects of stem cells and other cell therapy for improving blood flow in the ischemic limb (Botham et al. 2013; Cooke and Lasardo 2015; Raval and Losardo 2013). Furthermore, exercise training has been shown to induce collateral circulation for increasing the blood supply and maintaining the function of the ischemic muscle (Hoier et al. 2010; Kehler et al. 2013; Milkiewicz et al. 2005; Schirmer et al. 2015). In addition, both clinical and experimental investigations have indicated the beneficial effects of CO$_2$ therapy for promoting the blood flow to the ischemic limb in peripheral artery
disease (Dogliotti et al. 2011; Finzgar et al. 2015; Hartmann et al. 1997a, 1997b; Irie et al. 2005; Izumi et al. 2015; Nishimura et al. 2002; Pagourelias et al. 2011). However, extensive research work is needed to understand the mechanisms of CO$_2$-induced increase in blood flow in the ischemic skeletal muscle. In view of the development of angiogenesis in the skeletal muscle in response to ischemia (Carmeliet 2003; Carmeliet and Jain 2011; Hudlicka et al. 1992; Limbourg et al. 2009; Lloyd et al. 2001; Olfert et al. 2016), this study was undertaken to examine if the increase in blood flow in the ischemic limb due to CO$_2$ therapy is associated with the occurrence of angiogenesis. Furthermore, some of the angiogenic factors as well as plasma lipids were measured to test if any of these parameters could serve as biomarkers for identifying the beneficial effects of CO$_2$ therapy in peripheral artery disease.

**Methods:**

**Femoral artery ligation:** The protocol and experimental design for the use of animals in this study were approved by the University of Manitoba Animal Care Committee according to the guidelines of the Canadian Council on Animal Care and the Guide to the Care and Use of Laboratory Animals. Male rats weighing 200-250g were anesthetized with 1-5% isoflurane in oxygen at a flow rate of 2L/min. The surgical site was shaved and treated with a topical antiseptic, buprenorphine (Shao et al. 2005). Through a small incision on the medial aspect of the left thigh, femoral artery was isolated and completely occluded by ligation with 3-0 surgical silk approximately 5 mm distal to the inguinal ligament (Lloyd et al. 2001). The wound was closed with suture/clips, and the animals were allowed to recover in their cages. These animals with hind leg ischemia but with no infection at the surgical site after 2 weeks were used for experiments.

**CO$_2$-rich water-bath therapy:** Two weeks after the surgery, when the surgical wound was
completely healed, the ischemic animals were exposed to either CO₂-enriched water-bath or water-bath without CO₂ mixing at 37°C for 20 min everyday (5 days per week) for 4 weeks. It is pointed out that other investigators have also used 4 weeks duration for CO₂ water-bath therapy (Irie et al. 2005; Izumi et al. 2015). The CO₂-rich water was prepared by mixing CO₂ in tap water using Carbothera, a therapeutic footbath unit (Mitsubishi Rayon Engineering, Tokyo, Japan) containing a multi-layered composite hollow-fiber membrane (Nishimura et al. 2002). The CO₂ concentration was maintained at 1,000-12000 ppm (Figure 1A) throughout the bathing. Two groups of animals were kept in a Plexiglas plastic rat restrainer and the bottom portion including the hind limb was immersed in the water-bath at 37°C in a slant position. For one group (ischemic - CO₂), the animals were exposed to water-bath without mixing CO₂ whereas for the other group (ischemic + CO₂), the animals were exposed to water-bath enriched with CO₂. Two other groups of animals, one without and another with femoral artery ischemia were kept in cages with no water-bath treatment, served as controls. The experimental design for this study is depicted in Figure 1B. At the end of 4 week treatment, the animals were sacrificed under anesthesia (ketamine/xylazine; 90/9 mg/kg). Blood was collected from the abdominal aorta; serum was separated and used for biochemical analysis. The left leg skeletal muscle was collected and stored at -70°C for analysis.

**Blood flow measurements:** Blood flow to the left leg skeletal muscle was measured before and immediately after femoral artery ligation as well as at 4 weeks after CO₂-rich water-bath treatment using a Pulse Wave Doppler System (Indus Instruments, Webster, Texas, USA) (Hartley et al. 2011; Reddy et al. 2009). Rats were anesthetized with inhaled isoflurane (1% in O₂) and fixed in a supine position on the temperature-controlled ECG board (THM100, Indus Instruments, Webster, Texas, USA). Body temperature was maintained at 37°C. Blood flow
velocity was recorded using a 20-MHz pulsed Doppler probe at the level of femoral artery. Data were analyzed using an Indus Instruments Doppler Signal Processing Workstation. Heart rate and R-R interval were calculated using the Doppler spectrogram analysis software.

**Tissue staining and angiogenesis:** A cross section of skeletal muscle tissue just above the femoral artery ligation was fixed with 10% formalin for 24-48 h and processed after paraffin embedding using standard protocol (Feldman and Wolfe 2014; Fischer et al. 2008; Limbourg et al. 2009). About 10-15 sections of 5 µm thickness each were taken using a Shandon, Finesse 325 microtome (Thermo Electron Corporation, Hampshire, UK). All tissue sections were stained with hematoxylin and eosin and observed under light microscope. Capillaries were visualized from images taken using Image ProPlus Digital System (Media Cybernetics, Rockville, MD, USA) (Garg and Mehrotra 2014). The quantitative analysis of angiogenesis was performed in a blinded manner. Although the area examined in all four groups was not the same, the data have been presented per tissue section.

**Biochemical analysis:** Serum analysis was performed on the Roche Cobas 6000 analyzer (Roche Diagnostics GmbH, Mannheim, Germany) using original Roche reagents. Cholesterol, triglycerides, and high density lipoproteins (HDL) were enzymatic-colorimetric assays. Glucose was run on a hexokinase UV method. Creatine kinase (CK) was determined using N-acetyl cysteine activated UV assay. Vascular endothelial growth factor (VEGF) (Sigma-Aldrich, St. Louis, MO, USA), tumor necrosis factor-alpha (TNF-α) (R&D Systems, Minneapolis, MN, USA), nitric oxide (NO) (Enzo Life Science, Farmingdale, NY, USA), and malondialdehyde (MDA) (ZeptoMetrix, Buffalo, NY, USA) were measured using appropriate ELISA kits.

**Statistical analysis:** All values are expressed as mean ± SE. The statistical analysis was carried out by using the Microcal Origin Version 6 (Microcal Software Inc., Northampton, MA, USA).
One-way ANOVA analysis was used; the comparison of mean values of two groups was performed by Student’s t-test and P values less than 0.05 were considered to be significantly different.

Results

As shown in Figure 1 for the experimental protocol, ischemia in the hind leg of normal rats was induced by ligating the femoral artery for 2 weeks and thereafter these animals were subjected to water-bath treatment for 4 weeks with (ischemic + CO\textsubscript{2} group) or without (ischemic - CO\textsubscript{2} group) mixing CO\textsubscript{2}. No water-bath treatment was carried out for normal rats with (ischemic control group) or without (control group) peripheral ischemia. The electrocardiographic assessment of animals showed that for both heart rates and R-R intervals there were no significant differences among control, ischemia control, ischemic - CO\textsubscript{2} group, and ischemic + CO\textsubscript{2} groups (Table 1). Furthermore, values for plasma creatine kinase level (Table 1) as well as for plasma cholesterol, high density lipoproteins and glucose levels (Table 2) were similar among all four groups. Although plasma triglyceride level in ischemic control and ischemic - CO\textsubscript{2} groups was significantly lower than in the control groups, these values were not different from that in the ischemic + CO\textsubscript{2} group (Table 2).

The examination of peripheral blood flow by the Pulse Wave Doppler System in the hind legs of all animals revealed a marked depression for the peak blood flow, minimal blood flow and mean blood values in the ischemic control or ischemic - CO\textsubscript{2} group in comparison to the control group (Figure 2). However, the blood flow in the ischemic animals was significantly improved upon CO\textsubscript{2} water-bath therapy (ischemic + CO\textsubscript{2} group) (Figure 2). It should be mentioned that no blood flow was detected in the hind limb immediately after the femoral artery
ligation indicating that the degree of ischemia in different groups of animals was comparable. The histological evaluation revealed markedly higher magnitude of angiogenesis in the hind leg skeletal muscle upon CO$_2$ water-bath treatment (ischemic + CO$_2$ group) in comparison to other groups (Figure 3). It may also be noted that the peripheral ischemia with or without water-bath treatment did not produce any significant effect on the magnitude of angiogenesis in comparison to the control group (Figure 3).

In order to investigate if the improvement in peripheral blood by CO$_2$ water-bath therapy was associated with any changes in some biomarkers, the concentrations of different angiogenic factors in plasma from all the four groups of animals was measured. The data in Figure 4 indicate that, unlike TNF-α, the levels of plasma VEGF, NO and MDA were elevated in the ischemic control, ischemic - CO$_2$ and ischemic + CO$_2$ groups in comparison to the control group. The CO$_2$ water-bath treatment did not affect the elevated plasma levels of VEGF and NO in the ischemic - CO$_2$ group but the elevated plasma levels were significantly decreased in the ischemic + CO$_2$ group.

**Discussion**

In this study we have demonstrated that CO$_2$ water-bath therapy improves the peak blood flow, minimal blood flow and mean blood flow in the ischemic hind leg of rats. These observations are consistent with earlier reports showing increase in blood flow in the ischemic limb upon treatment with CO$_2$ (Finzgar et al. 2015; Hartmann et al. 1997a; Irie et al. 2005; Izumi et al. 2015; Nishimura et al. 2002). Although CO$_2$ water-bath therapy has been suggested to produce inhibition of the sympathetic activity or activation of the parasympathetic activity (Hashimoto and Yamamoto 2004; Sato et al. 2009), it is highly unlikely that the increased
peripheral blood flow observed in our study is due to the involvement of these mechanisms because no changes in heart rate, R-R intervals or plasma creatine kinase levels were observed upon therapy with CO₂ water-bath. Furthermore, alterations in blood viscosity, membrane permeability and muscle metabolism (Dhalla et al. 2017) cannot explain the improved blood in the limb because no significant changes in plasma cholesterol, low density lipoprotein, triglyceride and glucose levels were seen due to CO₂ water-bath treatment. On the other hand, since the elevated plasma level of MDA, a well known index of oxidative stress, in the ischemic animals was depressed by the CO₂ water-bath therapy, it is suggested that the observed increase in peripheral blood flow may be related to the reduction of oxidative stress in the CO₂-treated animals. It is pointed out that Dogliotti et al. (2011) have shown a depression of free radical release and elevation of the total antioxidant status upon immersion of lower legs of patients with peripheral occlusive disease in CO₂-enriched water. In fact, CO₂ has been found to be a potent inhibitor for the generation of reactive oxygen species by different types of cells (Bolevich et al. 2016).

It is well known that CO₂ is a powerful vasodilator and has been shown to increase blood flow (Ito et al. 1989; Nishimura et al. 2002); however, it is difficult to assess the contribution of the direct vasodilatory effect in promoting the increase in blood flow under chronic conditions of CO₂ water-bath therapy. On the other hand, this study has shown that the magnitude of angiogenesis in the hind limb skeletal muscle was markedly increased due to CO₂ water-bath therapy. Such an increase in the degree of angiogenesis can be taken to suggest that the observed increase in peripheral blood flow in the ischemic limb upon CO₂ water-bath therapy may be mainly due to the development of angiogenesis. In this regard, it is pointed out that exercise training has also been reported to promote blood flow and induce angiogenesis in the peripheral
artery disease (Kehler et al. 2013; Schirmer et al. 2015). Since the development of angiogenesis is considered to have different therapeutic implications by promoting blood supply in several diseased organs (Carmeliet and Jain 2011; Potente et al. 2011), the induction of angiogenesis by \( \text{CO}_2 \) water-bath treatment can be seen to form the basis of therapy for the peripheral artery disease. However, the involvement of several angiogenic factors in \( \text{CO}_2 \)-induced angiogenesis in the ischemic limb is not readily apparent because the plasma levels of VEGF, TNF-\( \alpha \) and NO, which are known to promote angiogenesis (Dhalla et al. 2017; Hoier et al. 2010; Lloyd et al. 2001; Milkiewicz et al. 2005) were not augmented by the \( \text{CO}_2 \) water-bath therapy. Such results may be due to the fact that these angiogenic factors were already elevated to maximal levels in animals with peripheral ischemia at the time of determination or these markers became increased during the early periods of \( \text{CO}_2 \) water-bath therapy. Nonetheless, from the results presented in this study, it is evident that the improvement in blood flow in the ischemic limb may be due to the development of angiogenesis in the skeletal muscle. Our preliminary experiments have revealed no increase in blood flow or magnitude of angiogenesis in hind limb of control animals upon treatment with \( \text{CO}_2 \)-enriched water-bath.

Appearance of collateral blood vessels upon the obstruction of blood flow in the hind limb is considered to be an adaptive response to ischemia/hypoxia for maintaining the skeletal muscle function (Dhalla et al. 2017; Irie et al. 2005; Krishna et al. 2015; Serrano Hernando and Conejero 2007; Yang et al. 2009). Such a response has been shown to be a consequence mainly due to the production of hypoxia-inducible factor and formation of VEGF, NO, cytokines and other growth factors in the ischemic limb (Carmeliet and Jain 2011; Dhalla et al. 2017; Irie et al. 2005; Lloyd et al. 2001). In this study, we have observed that ligation of the femoral artery resulted in no blood flow immediately but thereafter in 6 weeks only a small amount of blood
flow was detected in the hind limb. This was not associated with any increase in the magnitude of angiogenesis in the ischemic limb; however, a marked increase in the plasma levels of both VEGF and NO, unlike TNF-α, was evident in animals with peripheral ischemia. Thus it appears that a small amount of blood flow in the ischemic limb may not be sufficient to maintain the skeletal muscle function leading to the development of peripheral artery disease. It is pointed out that we did not determine the time-course of changes in plasma TNF-α levels upon the induction of peripheral ischemia or during early periods of starting CO₂ water-bath therapy. Thus some caution should be exercised while interpreting the data regarding the effect of CO₂ therapy on this angiogenic factor.

The marked depression in blood flow in the ischemic limb may also be due to the occurrence of oxidative stress in the skeletal muscle because the plasma level of MDA was markedly elevated in the animals with peripheral ischemia. This view is consistent with marked increase in the magnitude of angiogenesis and blood flow as well as a reduction in the plasma MDA level upon CO₂ water-bath treatment of patients with peripheral arterial disease (Dogliotti et al. 2011). Furthermore, the plasma level of Ox-LDL, another marker of oxidative stress, was also found to be reduced in ischemic diabetic rats upon CO₂ water-bath therapy (Dhalla and Elimban 2017). Thus reduction in the occurrence of oxidative stress and increase in the development of angiogenesis by CO₂ water-bath treatment may be important mechanisms for its beneficial effects in the peripheral artery disease. It is pointed out that oxidative stress at low levels or during the early period of its development is considered to exert beneficial effects through the activation of redox sensitive alterations in the cell whereas excessive or chronic oxidative stress is known to produce cellular damage (Tappia et al. 2006). It is likely that reduction in the development of oxidative stress by CO₂ water-bath therapy may also increase
the peripheral blood flow through the activation of some redox-sensitive signal transduction mechanisms in the ischemic limb. These events involving the reduction in oxidative stress and the development of angiogenesis leading to increased blood flow in the ischemic limb upon CO$_2$ water-bath therapy are illustrated in Figure 5.

**Acknowledgements**

The infrastructural support was provided by the St. Boniface Hospital Research Foundation, Winnipeg, Canada.

**Conflict of Interest**

The financial support for this study was provided by a grant from the Mitsubishi Rayon Cleansui Co., Ltd., Tokyo, Japan. This is to declare that Y.-J. Xu, V. Elimban and N.S. Dhall had no conflict of interest.

**References**


Cooke, J.P., and Losardo, D.W. 2015. Modulating the vascular response to limb ischemia:


Dhalla, N.S. and Elimban, V. 2017. CO₂ water bath therapy promotes blood flow and angiogenesis in diabetes with peripheral ischemia. In Program of the 9th International Conference of the International Academy of Cardiovascular Sciences, Delhi, pp. 33


Legends to Figures

Figure 1. Concentration of CO$_2$ in water-bath during and after mixing (A) and the experimental design of study (B). Assessment of each animal immediately after the ligation of femoral artery did not show any blood flow in the hind limb. It should be noted that animals in both control and ischemic control groups were not subjected to water-bath whereas rats in ischemic + CO$_2$ group and ischemic – CO$_2$ group were subjected to water-bath with or without CO$_2$ mixing, respectively.

Figure 2. Effect of CO$_2$ water-bath therapy on peak blood flow (A), minimal blood flow (B), and mean blood flow (C) in the rat hind leg skeletal muscle. Normal rats with 2 weeks femoral artery ligation were subjected to water-bath at 37°C with or without CO$_2$ for 4 weeks and were labelled as ischemic + CO$_2$ and ischemic – CO$_2$ groups, respectively. Normal control rats and ischemic control rats were not subjected to water-bath. Values are mean ± SE of 6 animals in each group. *-$P<0.05$ vs control, #-$P<0.05$ vs ischemic – CO$_2$ group. Since the values for each parameter of blood flow in ischemic control group were not significantly ($P>0.05$) different from the respective value in the control group, the data for the ischemic control group are not shown in this figure. It should be noted that both control group and ischemic control group were not subjected to water-bath treatment.

Figure 3. Representative histological alterations in the skeletal muscle sections for control (A), ischemic control (B), ischemic – CO$_2$ (C) and ischemic + CO$_2$ (D) groups. Quantitative analysis of the number of blood vessels per tissue section in each group is shown in Panel E and the values are mean ± SE of 6 animals in each group. *-$P<0.05$ vs control, #-$P<0.05$ vs ischemic + CO$_2$ group. It should be noted that animals
in both control and ischemic control groups were not subjected to water-bath whereas those in ischemic + CO\(_2\) group and ischemic – CO\(_2\) group were subjected to water-bath with or without CO\(_2\) mixing, respectively.

**Figure 4.** Plasma VEGF, NO, TNF-\(\alpha\) and MDA levels with or without CO\(_2\) water-bath treatment in rats subjected to femoral artery ligation. Each value is a mean \(\pm\) SE of 6 animals in each group. VEGF – vascular endothelial growth factor, NO – nitric oxide, TNF-\(\alpha\) – tissue necrosis factor-\(\alpha\), and MDA – malondialdehyde. *\(P<0.05\) vs control, *\(P<0.05\) vs ischemic + CO\(_2\) group. Since the values for VEGF, NO, TNF-\(\alpha\), and MDA levels in ischemic control group were not significantly \((P>0.05)\) different from the respective values in control group, the data for the ischemic control group are not shown in this figure. Animals in both control and ischemic control groups were not subject to water-bath.

**Figure 5.** Proposed mechanisms of increase in blood flow in the ischemic limb due to CO\(_2\) water-bath therapy through the development of angiogenesis as well as modification of redox-sensitive signal transduction in the skeletal muscle. It should be mentioned that redox state represents a balance between the formation of oxyradicals and the removal of oxyradicals in the tissue.
Table 1. Heart rate, R-R interval and plasma creatine kinase level with or without CO₂ water-bath treatment in rats subjected to femoral artery ligation.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ischemic – CO₂</th>
<th>Ischemic + CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt (g)</td>
<td>541± 11</td>
<td>518 ± 13</td>
<td>534 ± 13</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>316 ± 5</td>
<td>322 ± 8</td>
<td>327 ± 11</td>
</tr>
<tr>
<td>R-R interval (msec)</td>
<td>186 ± 4</td>
<td>193 ± 7</td>
<td>192 ± 7</td>
</tr>
<tr>
<td>Creatine kinase (U/L)</td>
<td>585 ± 99</td>
<td>543 ± 92</td>
<td>466 ± 75</td>
</tr>
</tbody>
</table>

Each value is a mean ± SE of 6 animals in each group. Since the values for each parameter for the ischemic control group were not significantly ($P>0.05$) different from the respective values for the control group, the data for the ischemic control group are not shown in this table. It is pointed out that both control and ischemic control animals were not subjected to water-bath.
Table 2. Plasma lipids and glucose levels with or without 4 weeks CO$_2$ water-bath treatment in rats subjected to femoral artery ligation.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ischemic – CO$_2$</th>
<th>Ischemic + CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>1.62 ± 0.11</td>
<td>1.50 ± 0.14</td>
<td>1.70 ± 0.13</td>
</tr>
<tr>
<td>High density lipoprotein (mmol/L)</td>
<td>0.93 ± 0.08</td>
<td>1.01 ± 0.12</td>
<td>1.12 ± 0.14</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>2.63 ± 0.37</td>
<td>1.41 ± 0.18*</td>
<td>1.76 ± 0.77</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>17.2 ± 1.10</td>
<td>16.3 ± 1.40</td>
<td>15.3 ± 0.8</td>
</tr>
</tbody>
</table>

Each value is a mean ± SE of 6 animals in each group. *$P<0.05$ compared with control value. Since the values for each parameter for the ischemic control group were not significantly ($P>0.05$) different from the respective values for the control group, the data for the ischemic control group are not shown in this table. Both control and ischemic control animals were not subjected to water-bath.
A. CO$_2$ Concentration

![Graph showing CO$_2$ concentration over time]

During CO$_2$ mixing (min) | After CO$_2$ mixing (min)
---|---
1 | 3 | 5 | 10 | 20 | 30 | 60

B. Experimental Design

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Left femoral artery ligation</strong></td>
<td><strong>Ischemic control</strong></td>
</tr>
<tr>
<td><strong>Left femoral artery ligation</strong></td>
<td><strong>37°C water bath therapy without CO$_2$ (ischemic – CO$_2$)</strong></td>
</tr>
<tr>
<td><strong>Left femoral artery ligation</strong></td>
<td><strong>37°C water bath therapy with CO$_2$ (ischemic + CO$_2$)</strong></td>
</tr>
<tr>
<td><strong>2 Weeks</strong></td>
<td><strong>4 Weeks</strong></td>
</tr>
</tbody>
</table>

Xu et al – Figure 1
A. Peak blood flow

B. Minimal blood flow

C. Mean blood flow

Blood flow (cm/sec)

Control  Ischemic -CO₂  Ischemic +CO₂

Xu et al – Figure 2

https://mc06.manuscriptcentral.com/cjpp-pubs
A. Control

B. Ischemic Control

C. Ischemic – CO₂

D. Ischemic + CO₂

E. Blood vessel count

![Graph showing blood vessel count across different conditions.](https://mc06.manuscriptcentral.com/cjpp-pubs)
A. VEGF

B. NO

C. TNFα

D. MDA

Xu et al – Figure 4
CO₂ Water Bath Therapy

Exposure of Skeletal Muscle to CO₂

Modification of Redox-sensitive Signal Transduction

Angiogenesis

Increased Blood Flow

Xu et al – Figure 5