**Effects of CO₂ water-bath treatment on blood flow and
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Effects of CO₂ water-bath treatment on blood flow and angiogenesis in ischemic hind limb of diabetic rat

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Short Title: Effects of CO₂ water-bath on peripheral disease

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Abstract

The effects of CO₂ water-bath therapy in the hind limb of diabetic animals with or without peripheral ischemia were examined. Diabetes was induced in rats by streptozotocin (65 mg/kg) and the animals were divided into three groups. After 4 weeks, peripheral ischemia was induced by ligation of the femoral artery for 2 weeks in two groups (Diabetic Ischemic) of diabetic rats, whereas the femoral artery was not occluded in the third group (Diabetic). All these animals were subjected to water-bath therapy (with or without CO₂ mixing; 20 min per day for 5 days per week) for a period of 4 weeks. Both peak and mean flows, unlike minimal flow, in diabetic ischemic limbs were increased by about 2 fold by CO₂ bath treatment. Morphological examination of hind limb tissue sections revealed about 2 fold increase in the small artery count in diabetic ischemic animals upon CO₂ bath treatment. These results indicate that CO₂ water-bath therapy augments the blood flow and development of angiogenesis in the skeletal muscle of diabetic ischemic animals and thus may be of some benefits for the treatment of peripheral arterial disease in diabetes.

Keywords: Skeletal muscle angiogenesis, peripheral blood flow, experimental diabetes, peripheral arterial disease, CO₂ water-bath therapy.

Introduction

It is now well known that peripheral vascular disease is commonly associated with reduction in blood flow and subsequent ischemia in the hind limb skeletal muscle (Carmeliet and Jain 2011; Dhalla et al. 2017; Krishna et al. 2015). Several medical and surgical interventions are available for the treatment of peripheral artery disease (Carmeliet 2003; Cooke and Losordo 2015; Dhalla et al. 2017; Kehler et al. 2013; Li et al. 1998; Potente et al. 2011); however, none of these therapies are satisfactory. Recently, both clinical and experimental studies have indicated the beneficial effects of CO₂ therapy in promoting blood flow in the ischemic limb in peripheral artery disease (Dhalla et al. 2017; Hartmann et al. 1997a; Hartmann et al. 1997b; Irie et al. 2005; Izumi et al. 2015; Pagourelas et al. 2011; Xu et al. 2017a). It should be mentioned that most of the experimental studies for developing CO₂ therapy have been carried out by employing different models of the hind limb ischemia in normal healthy subjects (Dhalla et al. 2017; Irie et al. 2005; Izumi et al. 2015; Xu et al. 2017a) and very little information in this regard has been derived from pathologically induced peripheral disease animals. Although diabetes is one of the major risk factor for the pathogenesis of peripheral artery disease (Dhalla et al. 2017; Dhalla et al. 1985; Muntner et al. 2005; Saweda and Arany 2017; Selvin et al. 2004; Krishna et al. 2015), virtually nothing is known about the effects of CO₂ on the peripheral blood flow in diabetic animals with hind limb ischemia. It is therefore the purpose of this study to investigate the action of CO₂-water bath therapy on the hind limb blood flow in the experimentally-induced diabetes with or without ischemia. Some experiments will also be carried to understand the mechanisms associated with the development of angiogenesis as well as modification of blood flow in the ischemic limb.

Methods

The experimental design and protocol 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 Use of Animals.

Induction of diabetes and skeletal muscle ischemia: Male Sprague Dawley rats weighing 175-200 g were injected with streptozotocin (65 mg/kg body weight) through tail vein for the induction of diabetes according to the procedure used in our laboratory (Ganguly et al. 1986; Ganguly et al. 1987; Taira et al. 1988). Four weeks after, these animals were anesthetized with 1% - 5% isoflurane in oxygen at a flow rate of 2L/min and divided into three groups as shown in Fig. 1. In two groups, the femoral artery was ligated for inducing the hind limb ischemia according to the method used previously (Xu et al. 2017a). Briefly, the femoral artery of the left thigh was ligated with 3-0 surgical silk approximately 5mm distal to the inguinal ligament in the anesthetized animals. The artery was not occluded in the sham-operated animals.

CO₂ – rich water bath therapy: Two weeks after inducing ischemia, both diabetic ischemic animals as well as diabetic animals without femoral artery ligation were subjected to water-bath treatment at 37°C for 20 min everyday (5 days per week) for a period of 4 weeks (Xu et al. 2017a). While one group of animals (Diabetic Ischemic + CO₂) was treated with CO₂ - enriched water-bath whereas the other two groups (Diabetic – CO₂ and Diabetic ischemic – CO₂) were exposed to water-bath without mixing CO₂. The CO₂ - rich water was prepared by mixing CO₂ in tap water using Carbothera unit (Mitsubishi Rayon Engineering, Tokyo, Japan). The concentration of CO₂ in the water-bath was maintained at 1,000 – 1,200 ppm. The exposure of diabetic animals to water-bath with or without CO₂ was carried out exactly in the same manner as described for the normal animals (Xu et al. 2017a). At the end of 4 weeks, the animals were

sacrificed under anesthesia (ketamine/xylazine; 90/9 mg/kg). Blood was collected from the abdominal aorta in heparinized tubes; plasma was separated and used for biochemical analysis. The left leg skeletal muscle was collected and stored at -70°C for analysis.

Measurements of blood flow: The blood flow to the left leg skeletal muscle in all animals was measured at 4 weeks of water-bath treatment using Pulse Wave Doppler System (Indus Instruments, Webster, Texas, USA) according to the methods employed by Reddy et al. 2009 and Hartley et al. 2011. The blood flow velocity was recorded using 20-MHz pulsed Doppler probe at the level of femoral artery and the 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. These methods are same as those used previously (Xu et al. 2017a).

Evaluation of angiogenesis: The techniques of embedding the skeletal muscle as well as for processing and staining the tissue section ($5\text{ }\mu\text{m}$ thickness each) were same as used earlier (Xu et al. 2017a). The tissue sections stained with hematoxylin and eosin were observed under light microscope and capillaries were visualized from the images taken using image ProPlus Digital System (Media Cybernetics, Rockville, MD, USA) according to the method of Garg and Mehrotra (2014).

Biochemical plasma analysis: The Roche Cobas 6000 analyzer (Roche Diagnostics GmbH, Mannheim, Germany) and original Roche reagents were used for plasma analysis. Cholesterol, triglycerides, and high density lipoproteins (HDL) were measured by enzymatic-colorimetric assays. 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. Glucose was determined by the hexokinase UV method using N-acetylcysteine activated UV assay.

Statistical analysis: All values are expressed as mean $\pm$ S.E.M. 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 from two groups was performed by Student's t-test and P values less than 0.05 were considered to be significantly different.

Results

Effect of CO₂ water-bath on cardiac function and plasma biomarkers:

The data in Table 1 show that various parameters such as body weight, heart rate and R-R interval, as well as plasma CK and glucose levels were not different ($P > 0.05$) among diabetic – CO₂, diabetic ischemic – CO₂ and diabetic ischemic + CO₂ groups. The results in Table 2 indicate that the values for plasma cholesterol, HDL and MDA levels in these groups of diabetic rats were not different from each other except that triglyceride levels were markedly decreased in both diabetic ischemic – CO₂ and diabetic ischemic + CO₂ groups in comparison to the values in diabetic – CO₂ group. Although the MDA level in the diabetic ischemic + CO₂ group was about 25% lower than that in the diabetic ischemic – CO₂ group, this difference was not significant ($P > 0.05$).

The data in Table 3 show no difference ($P > 0.05$) in values for plasma NO and VEGF among diabetic – CO₂, diabetic ischemic – CO₂ and diabetic ischemic + CO₂ groups. On the other hand, the value for plasma TNF- α in diabetic ischemic – CO₂ group was significantly ($P < 0.05$) higher than that in the diabetic – CO₂ group and this increase was significantly depressed in the diabetic ischemic + CO₂ group.

Effects of CO₂ water-bath therapy on blood flow and angiogenesis

The effects of CO₂ water-bath therapy on blood flow and angiogenesis in diabetic-ischemic rat skeletal muscle were also examined. The results in Table 4 show that the values for peak blood flow, minimal blood flow and mean blood flow in diabetic ischemic – CO₂ group were markedly depressed in comparison to the diabetic – CO₂ group. Furthermore, the values for peak blood flow and mean blood flow, unlike the minimal blood flow, in diabetic ischemic + CO₂ group were about 2.1 fold higher in comparison to the diabetic ischemic – CO₂ group.

Quantitative analysis of the histological sections revealed a marked increase (about 2 fold) in the number of small blood vessels (artery counts/section) in the diabetic ischemic + CO₂ group skeletal muscle sections in comparison to the number of blood vessels in the diabetic ischemic – CO₂ group (Fig. 2). Furthermore, the artery count in diabetic ischemic – CO₂ group was not different ($P > 0.05$) from the diabetic – CO₂.

Discussion

In this study, CO₂ water-bath therapy of diabetic ischemic rats was observed to augment the peak blood flow and mean blood flow, without any changes in the minimal blood flow, by about 2 fold in the hind limb skeletal muscle. It is pointed out that the values for peak blood flow in diabetic animals (Table 4) were not different from those from the corresponding values for the normal rats (Xu et al. 2017a). Although similar observations have been reported in normal rats, the increase in both peak and mean blood flow rates was 5 to 7 fold by CO₂ water-bath therapy whereas the minimal blood flow was increased by about 4 fold under similar experimental conditions (Xu et al. 2017a). Such differences in the effects of CO₂ water-bath therapy between normal ischemic and diabetic ischemic skeletal muscle blood flow rates were not due to any differences in the base-line values for blood flow rates between normal and diabetic animals. On

the other hand, the attenuated responses to CO₂ water-bath therapy in diabetic ischemic group may be due to differences in the modification by CO₂-therapy of both extrinsic and intrinsic factors which are known to regulate the peripheral blood flow (Dhalla et al. 2017; Kehler et al. 2013; Krishna et al. 2015). In fact, the observed results are consistent with the view that diabetes is a major risk factor for the development of peripheral artery disease (Muntner et al. 2005; Sawada and Arany 2017; Selvin et al. 2004), which is invariably associated with reduction in blood flow in the hind limb skeletal muscle (Dhalla et al. 2017; Krishna et al. 2015).

The increase in both peak and mean blood flow rates by CO₂ water-bath therapy was found to be associated with 2 fold increase in the development of angiogenesis in the diabetic ischemic skeletal muscle. Since angiogenesis is considered to play a critical role in promoting the peripheral blood flow (Carmeliet and Jain 2011; Cooke and Losardo 2015; Potente et al. 2011), it is evident that the observed increase in blood flow by CO₂ water-bath therapy in the diabetic ischemic group may be due to the occurrence angiogenesis. An association of angiogenesis and increased blood flow due to CO₂ water-bath therapy was also seen in the normal ischemic skeletal muscle (Xu et al. 2017a). It should be pointed out that the value for artery counts/section for the diabetic animals (Fig. 2) was not different from the values for the artery counts in the skeletal muscles of the normal rats under similar conditions (Xu et al. 2017a).

Although CO₂ is a powerful vasodilator (Finzgar et al. 2015; Ito et al. 1989), it is highly unlikely that the observed increase in blood flow by CO₂ water-bath therapy in diabetic ischemic group under the experimental conditions of this study is due to any direct effect of CO₂ on blood vessels. Furthermore, alternation in blood viscosity cannot be considered to explain the improvement in blood flow (Dhalla et al. 2017) in the diabetic ischemic group because the plasma levels of cholesterol triglyceride, HDL, and glucose were not affected by the CO₂ water-

bath therapy. Although inhibition of the sympathetic activity due to CO₂ water-bath therapy has been suggested to account for the increased peripheral blood flow (Hashimoto and Yamamoto 2004; Sato et al. 2009), this mechanism may not be involved in promoting blood flow because various indices of the sympathetic activity such as heart rate, R-R interval and plasma CK levels, in the diabetic ischemic animals were not altered by the CO₂ water-bath therapy. It is pointed out that the values for body weight and plasma glucose levels, unlike those for heart rate, R-R interval and CK levels, in the diabetic animals (Table 1) were 35% lower and 2 fold higher in comparison to the corresponding values in the normal rats under the exactly similar conditions, respectively (Xu et al. 2017a). Thus, it appears that the development of angiogenesis in the diabetic ischemic skeletal muscle may be the major mechanism for the observed increase in blood flow by the CO₂ water-bath therapy.

This study also examined some mechanisms for increase in angiogenesis in skeletal muscle from diabetic ischemic animals by CO₂ water-bath therapy. In the view of the angiogenic role of NO, VEGF and TNF- α (Dhalla et al. 2017; Hoeir et al. 2010; Lloyd et al. 2001; Milkiewicz et al. 2005), plasma levels of NO, VEGF and TNF- α were monitored in diabetic ischemic with or without CO₂ water-bath therapy. Since the plasma TNF- α levels, unlike the VEGF levels, were significantly depressed and increased in diabetic ischemic group upon water-bath therapy with or without CO₂ mixing, changes in the levels of TNF- α and VEGF cannot be taken to indicate the increase in angiogenesis in diabetic ischemic skeletal muscle upon CO₂ water-bath therapy. It is also pointed out that the values for NO and VEGF were about 1.5 fold higher in diabetic animals (Table 3) in comparison to the corresponding values for the normal rats under similar conditions (Xu et al. 2017a). Although the plasma levels for NO in the diabetic ischemic group were increased by 33% by CO₂ therapy, this change was not significant ($P>0.05$). Thus the increase

in angiogenesis due to CO₂ therapy may partially be due to an appreciable increase (though statistically insignificant) in the level of plasma NO. It should also be mentioned that plasma MDA levels, a well known index for oxidative stress (Dogliotti et al. 2011), was elevated 25% by CO₂ water-bath therapy in the ischemic diabetic group but this change was not statistically significant ($P>0.05$). Therefore, an appreciable decrease in oxidative stress in our study can be seen to partly promote the occurrence of angiogenesis. In fact, Dogliotti et al. 2011 have reported reduction in the development of oxidative stress in peripheral arterial occlusive disease upon exposure to CO₂-enriched water. Furthermore, CO₂ has been demonstrated to play a protective role for the generation of reactive oxygen species and thus reduce oxidative stress (Bolevich et al. 2016). CO₂ has also been shown to stimulate the redox sensitive signal transduction system for the development of angiogenesis (Xu et al. 2017*b*). It is pointed out that the values for cholesterol, triglyceride and MDA, unlike those for HDL, in diabetic animals (Table 2) were markedly higher than the corresponding values in normal rats under similar conditions (Xu et al. 2017*a*). Accordingly, seems that CO₂ water-bath therapy of the diabetic ischemic group may increase angiogenesis and subsequent peripheral blood flow as a consequence of reduction in oxidative stress and changes in some signal transduction mechanisms.

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Conflict of Interest

The financial support for this study was provided to the St. Boniface Hospital by Mitsubishi

Rayon Cleansui, Tokyo, Japan. Drs. Y-J Xu, V. Elimban, S. K. Bhullar and N. S. Dhalla had no conflict of interest to declare.

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Legends to Figures

Figure 1 Experimental design of the study showing the time periods for inducing ischemia and treatment with or without CO₂ water-bath in 4 week diabetic rats.

Figure 2. Representative hind limb skeletal muscle sections of Diabetic - CO₂ (A), Diabetic Ischemic - CO₂ (B), and Diabetes Ischemic + CO₂ (C), rats with or without CO₂ water-bath at 37°. Bar graphs in panel (D) show artery counts/section for Diabetic - CO₂, Diabetic Ischemic - CO₂, and Diabetic Ischemic + CO₂ groups. Values are mean $\pm$ S.E. of 6 animals in each group. *P<0.05 vs Diabetic - CO₂ group. Arrows indicate the capillaries and small vessels in the tissue sections. The value for artery counts/section for diabetic animals (Diabetic-CO₂ group) was not different from that for the corresponding value for control animals reported earlier (Xu et al. 2017a)

Table 1. Effects of 4 weeks CO₂ water-bath treatment on body weight (BW), heart rate (HR) and R-R interval (R-R) as well as plasma creatine kinase (CK) and glucose levels in 4 weeks diabetic rats with or without 2 weeks of peripheral ischemia (Ischemic).

Group	BW (g)	HR (beats/min)	R-R interval (msec)	CK (U/L)	Glucose (mmol/L)
A. Diabetic - CO₂:					
	406 ± 34	305 ± 11	199 ± 7	424 ± 65	37.4 ± 2.2
B. Diabetic Ischemic - CO₂:					
	408 ± 11	317 ± 30	203 ± 6	328 ± 17	36.7 ± 1.8
C. Diabetic Ischemic + CO₂:					
	387 ± 20	302 ± 13	177 ± 9	465 ± 87	38.8 ± 2.4

Each value is a mean ± S.E. of 6 animals in each group. The values of BW in diabetic animals (Diabetic-CO₂ group) were about 35% lower and those for plasma glucose were about 2 fold higher in comparison to the corresponding values for control animals reported earlier (Xu et al. 2017a). The values for HR, R-R interval and plasma CK in diabetic animals were not different from the control values.

Table 2. Effects of 4 weeks CO₂ water-bath treatment on plasma total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) and malondialdehyde (MDA) levels in 4 weeks diabetic rats with or without 2 weeks peripheral ischemia (Ischemic).

Group	TC (nmol/L)	TG (nmol/L)	HDL (nmol/L)	MDA (nM)
A. Diabetic - CO₂:	3.20 ± 0.42	11.80 ± 2.18	1.22 ± 0.16	20.6 ± 2.9
B. Diabetic Ischemic - CO₂:	2.40 ± 0.49	3.12 ± 1.03*	1.27 ± 0.09	22.3 ± 2.5
C. Diabetic Ischemic + CO₂:	1.96 ± 0.54	3.55 ± 0.88*	1.29 ± 0.11	16.5 ± 2.0

Each value is a mean ± S.E. of 6 animals in each group. *P<0.05 vs corresponding value in Diabetic - CO₂ group. The values for plasma TC, TG, and MDA, unlike HDL, in diabetic animals (Diabetic - CO₂ group) were about 2 fold, 4.5 fold and 68 fold higher in comparison to the corresponding values for control animals reported earlier (Xu et al. 2017a).

Table 3. Effects of 4 weeks CO₂ water-bath treatment on plasma nitric oxide (NO), vascular endothelium groweighth factor (VEGF) and tumour necrosis factor-alpha (TNF- α) levels in 4 weeks diabetic rats with or without 2 weeks of peripheral ischemia (Ischemic).

Group	NO (μ M)	VEGF (pg/ml)	TNF- α (pg/ml)
A. Diabetic - CO₂:	2.8 $\pm$ 0.4	24.3 $\pm$ 3.8	12.2 $\pm$ 0.3
B. Diabetic Ischemic - CO₂:	2.7 $\pm$ 0.2	20.2 $\pm$ 2.2	13.5 $\pm$ 0.3*
C. Diabetic Ischemic + CO₂:	3.6 $\pm$ 0.3	22.1 $\pm$ 3.7	11.4 $\pm$ 0.2 [#]

Each value is a mean $\pm$ S.E. of 6 animals in each group. *P<0.05 vs corresponding value in Diabetic - CO₂ group. [#]P<0.05 vs corresponding values in Diabetic Ischemic - CO₂ group. The values for plasma NO and VEGF (unlike that for TNF- α) in diabetic animals (Diabetic -CO₂ group) were about 1.5 fold and 1.6 fold higher in comparison to the corresponding values for control animals reported earlier (Xu et al. 2017a).

Table 4. Effects of 4 weeks CO₂ water-bath treatment on hind limb skeletal muscle peak, minimal and mean blood flow in 4 weeks diabetic rats with or without 2 weeks peripheral ischemia (Ischemic).

Group	Peak Blood Flow (cm/sec)	Minimal Blood Flow (cm/sec)	Mean Blood Flow (cm/sec)
A. Diabetic - CO₂:	28 ± 1.2	0.11 ± 0.02	5.03 ± 0.85
B. Diabetic Ischemic - CO₂:	1.98 ± 0.9 [*]	0.02 ± 0.02 [*]	0.77 ± 0.07 [*]
C. Diabetic Ischemic + CO₂:	4.27 ± 0.7 [#]	0.02 ± 0.02	1.63 ± 0.13 [#]

Each value is a mean ± S.E. of 6 animals in each group. ^{*}P<0.05 vs corresponding value in Diabetic – CO₂ group. [#]P<0.05 vs corresponding value in Diabetes Ischemic - CO₂ group. The values for peak blood flow, minimal blood flow and mean blood flow in diabetic animals (diabetic - CO₂ group) were not different from the corresponding values for control animals reported earlier (Xu et al. 2017a).

Diabetic		– CO₂ water bath (37°)
Diabetic	Diabetic Ischemic	Diabetic Ischemic – CO₂ water bath (37°)
Diabetic	Diabetic Ischemic	Diabetic Ischemic + CO₂ water bath (37°)
4 Weeks	2 Weeks	4 Weeks

Xu et al - Figure 1

A. Diabetic - CO₂

B. Diabetic Ischemic -CO₂

C. Diabetic Ischemic + CO₂

D. Artery counts

Xu et al - Figure 2