Biorheology 40 (2003) 605–612
IOS Press

The influence of hyperbaric oxygen on hemorheological parameters in diabetic rats

Der-Zen Liu [a,*], Shu-Chen Chien [b], Li-Ping Tseng [a] and Charng-Bin Yang [c]

[a] *Graduate Institute of Biomedical Materials, Taipei Medical University, Taipei, Taiwan, R.O.C.*
[b] *College of Pharmacy, Taipei Medical University, Taipei, Taiwan, R.O.C.*
[c] *Taipei Municipal Chung-Hsin Hospital, Taipei, Taiwan, R.O.C.*

Received 7 January 2003
Accepted in revised form 27 June 2003

Abstract. The effect of hyperbaric oxygen (HBO_2) treatment on hemorheological parameters of diabetic rats was investigated. This study is a placebo-controlled, in vivo animal study. 30 streptozocin-induced diabetic rats were divided into two groups; one group received hyperbaric oxygen treatment while the other did not. Hematological and hemorheological parameters were tested with blood samples collected directly from the heart using surgical procedures. Student t-tests with a type I (α) error at 0.05 was used to test any significant difference between means of the hematologic and hemorheological parameters of the control (CON) and the HBO_2 groups. Compared with the placebo group, hyperbaric oxygen resulted in significant higher lipid peroxidation stress of the erythrocytes and resistance of erythrocytes to deformation in rats of the HBO_2 group. Whole blood viscosities measured at shear rates of 5, 150 and 400 s^{-1} were all higher for the rats in the HBO_2 group than those for rats in the control group. In addition, the oxygen delivery index was found to be significantly lower in rats of the HBO_2 group. Thus, our work demonstrates that hyperbaric oxygen treatment significantly changes the hemorheological parameters in diabetic rats.

Keywords: Hyperbaric oxygen, diabetic rats, blood viscosity, blood viscoelasticity, erythrocyte deformability

1. Introduction

Hyperbaric oxygen therapy (HBT), a therapy performed in an environment under 100% oxygen exposure of more than 1 atm (1 atm = 101.32 kPa) environment, has been practiced for more than 20 years. Although the basic mechanisms of action of HBO_2 are not clear, HBT has been widely practiced in treating wounds [11,13,27]. In diabetic patients, HBT was found to be effective in healing ulcers [16] and lesions on the foot [4,10].

However, HBT is not an ideal cure for all kinds of medical syndromes. Weaver and Churchill [36] found that HBO_2 was associated with the following syndromes: pulmonary edema caused by increasing left ventricular afterload; an increase in pressure when the left ventricular is undertaking a great loading, increase of oxidative myocardial stress, bradycardia along with left ventricular dysfunction, increasing pulmonary capillary permeability, and causing pulmonary oxygen toxicity. Furthermore, in terms of deep second burns, Shoshani et al. [26] confirmed that HBO_2 could cause a rise in tissue pO_2. These excessively high levels of tissue PO_2 might compromise the healing of burns.

* Address for correspondence: Dr. Der-Zen Liu, Graduate Institute of Biomedical Materials, Taipei Medical University, Taipei, Taiwan, R.O.C. Fax: +886 2 2739 0581; E-mail: tonyliu@tmu.edu.tw.

On the other hand, from a hemorheological point of view, in the rat model of Amin et al., they found that HBO_2 decreases erythrocyte deformability and produces a significant increase in fibrinogen concentration of plasma [1]. Both Pilgramm et al. and Amin et al. demonstrated that HBO_2 increases hematocrit (Hct) and blood viscosity [1,22]. As a result, HBT may have an affect on microcirculation since hemorheological behavior is closely related to microcirculation. Despite the fact that HBT is advantageous in healing wounds of diabetic mellitus patients, adverse effects such as elevated blood viscosity and possible decreased mal-peripheral circulation might limit its clinical utilization.

In order to learn more about the potential risks and benefits which HBT can cause with diabetes, an animal model was used in our research to study its effects on hemorheological parameters, including erythrocyte deformability, lipid peroxidation of erythrocyte membrane, blood viscosity and oxygen delivery index etc., as compared to those measured in non-exposed diabetic rats. These results may provide a useful reference for doctors for use in clinical treatment.

2. Subjects and methods

2.1. The animals

Female diabetic Sprague-Dawley rats weighing 220 to 240 gm were given 55 mg/kg streptozocin, dissolved in citrate buffer (pH = 4.5), intravenously to induce diabetes. Tail vein blood glucose concentrations were measured 3 days after the injection. Any rats with plama glucose concentrations less than 14 mM were excluded from the study. 30 female diabetic rats were randomly divided into two groups; the HBO_2 group was exposed to HBO_2 and the other (CON group) was not. After HBO_2 exposure at pressure of 2.8 atm for 2 h daily for 7 days, the 15 rats of HBO_2 group were allowed to recover for 24 h in room air.

2.2. Hyperbaric oxygen exposure

The diabetic rats were exposed to HBO_2 in a 27-ft^3 animal chamber with three plexiglass windows. The chamber was placed in an air-conditioned room, and the temperature in the chamber was maintained at 25°C. Maximally, 3 diabetic rats were placed in the chamber and simultaneously exposed to HBO_2. Water was freely accessible to these diabetic rats during the exposure. One hundred percent of oxygen was used to fill the chamber prior to compression. Compression and decompression of the chamber were performed gradually at the rate of 1 kPa/h with the pressure monitored by a precision gauge. The oxygen concentration of the chamber was checked hourly with a calibrated oxygen analyzer.

2.3. Collection of blood samples

Before being sacrificed, all experimental subjects were weighed and anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). Blood samples of the heart were collected by a surgical procedure and divided into three tubes. Heparin was added to the first tube for the measurement of hematocrit (Hct), blood viscosity, elasticity, and erythrocyte deformability. EDTA was added to the second tube to measure Hb_{A1C}. Sodium citrate was added to the third tube for the determination of fibrinogen concentration.

2.4. Hematological measurements

Hematocrit levels were measured using an automatic cell counter (SYSMEX NE-800, TOA Medical Electronic Co., Kobe, Japan). Plasma was separated from whole blood by centrifugation at $1500 \times g$ for 10 minutes. Plasma fibrinogen was determined by the thrombin clot technique [23]. Hb_{A1C}, an index of mean blood glucose level, was measured with Glyc-affinity columns, which quantitate total Hb_{A1c} by the blood glucose oxidase method.

2.5. Hemorheological parameter measurements

Plasma and blood viscosity were measured using a Rheostress 1 double cone viscometer (HAAKE Mess-Technik, Karlsruhe, Germany), with a cone angle of 1° at 37°C. The viscosities of whole blood at different shear rates were continuously measured by computer-controlled testing programs. In the experiment, we provide data measured at shear rates of 400, 150, and 5 s^{-1}, reflecting high, medium, and low shear rates. In terms of the viscoelastic properties of whole blood, the viscoelasticity of whole blood was tested under a constant oscillatory shear strain of 5% and at frequencies ranging from 0.1 to 0.3 Hz.

The concentration of malondialdehyde (MDA), a product of lipid peroxidation was derived by measuring the quantities of the MDA-TBA (thiobarbituric acid) complex at 532 nm with a spectrophotometer (Hitachi U2000, Hitachi Corp. Japan) to determine the oxidation stress of erythrocyte membranes [28]. The detailed preparation procedures are described elsewhere [14]. Concentrations of MDA presented in the results are expressed as the moles of MDA per 10^{10} erythrocytes.

To prepare the erythrocyte suspensions for erythrocyte deformability, we used constant flow rate filtration methods [6]. After separating from plasma by centrifuging the whole blood sample at $1500 \times g$ for 10 minutes, the erythrocytes were washed three times in PBS. After preparation, the erythrocyte suspensions with a 5% hematocrit, and leukocyte concentrations less than 100 cells mm^{-3}, were filtered through Nuclepore membrane, which had a pore size of 5-μm, a disc diameter of 13 mm and an effective area of 0.8 cm^2 at a constant flow rate of 1.6 ml min^{-1}.

Pressure-time data were measured with a pressure transducer (Model DP45, Validyne Engineering Corp, Northridge, USA) connected to a Validyne digital transducer indicator (Model CD-23). The continuous data output of the indicator was digitized and recorded on a computer. Recorded data were played back off-line, and P_o values for Ringer solutions and P_i values for erythrocyte suspensions were determined as reported previously [7]. The values of β were calculated using the P_i/P_o data and were indexed as the resistance of erythrocytes when flowing through the pores. The level of $1/\beta$ was defined as an index of erythrocyte deformability. Erythrocyte rigidity (T_K) was calculated at a shear rate of 400 s^{-1}, 150 s^{-1} and 5 s^{-1} using the equation of Dintenfass [9]. Furthermore, the oxygen transport efficiency (T_E) of the blood was calculated as the ratio of the Hct to blood viscosity at a fixed shear rate [5].

2.6. Statistics

Calculated group data are presented as the mean $\pm$ SD. All data were normally distributed. *Student* t-tests with a type I (α) error at 0.05 were used to test for any significant difference between the CON and the HBO_2 group. All statistics were analyzed using the SigmaStat Statistical Software (Jandel Scientific, San Rafael, CA, USA).

3. Results

Table 1 shows that mean Hct level (HBO_2: $47.5 \pm 0.6\%$; CON: $43.2 \pm 0.6\%$, $P < 0.01$) and the mean fibrinogen concentration of plasma (HBO_2: 259.8 ± 32.3 mg/l; CON: 187.4 ± 26.3 mg/l, $P < 0.01$) in rats of the HBO_2 group were significantly higher than those of the CON group. In addition, Table 2 also gives the hemorheological parameters of the CON and HBO_2 groups. The experiments clearly showed that blood viscosities of the HBO_2 group were significantly higher than those of the CON group regardless of the shear rate, whether high, middle or low (HBO_2: 20.18 ± 1.12; CON: 13.03 ± 0.94, $\gamma = 5\ s^{-1}$ $P < 0.01$; HBO_2: 8.36 ± 0.34; CON: 6.31 ± 0.16, $\gamma = 150\ s^{-1}$ $P < 0.01$; HBO_2: 6.43 ± 0.19; CON:

Table 1

Hematological data in diabetic rats of the CON and HBO_2 group ($n = 15$ in each group). Values are expressed as the mean $\pm$ SD

Parameter	CON group mean $\pm$ SD	HBO_2 group mean $\pm$ SD	Paired t-test P value
MCV (f1)	52.6 ± 0.9	53.2 ± 0.8	NS
MCHC (g/dl)	35.3 ± 0.8	34.8 ± 0.7	NS
Hct (%)	43.2 ± 0.6	47.5 ± 0.6	*
RBC (10^{12}/dl)	8.2 ± 0.3	8.9 ± 0.4	*
Fibrinogen (mg/l)	187.38 ± 26.29	259.78 ± 32.26	**

* $P < 0.01$.

** $P < 0.005$.

Table 2

Hemorheological data and the MDA level of erythrocyte membranes in diabetic rats of the CON and HBO_2 groups ($n = 15$ in each group). Values are expressed as the mean $\pm$ SD

Parameter	CON group mean $\pm$ SD	HBO_2 group mean $\pm$ SD	Paired t-test P value
η plasma (cp)	1.61 ± 0.02	1.62 ± 0.16	NS
η blood (cp)[a] ($\gamma = 400\ s^{-1}$)	5.13 ± 0.19	6.43 ± 0.19	**
η blood (cp)[a] ($\gamma = 150\ s^{-1}$)	6.31 ± 0.16	8.36 ± 0.34	**
η blood (cp)[a] ($\gamma = 5\ s^{-1}$)	13.03 ± 0.94	20.18 ± 1.12	**
η' blood (cp)[b]	14.15 ± 0.56	22.52 ± 1.05	**
η'' blood (cp)[b]	4.14 ± 0.20	5.08 ± 1.21	**
β	8.92 ± 0.49	19.18 ± 1.29	**
T_K ($\gamma = 400\ s^{-1}$)	0.86 ± 0.03	0.89 ± 0.01	**
T_K ($\gamma = 150\ s^{-1}$)	0.97 ± 0.01	1.01 ± 0.01	**
T_K ($\gamma = 5\ s^{-1}$)	1.31 ± 0.04	1.34 ± 0.01	*
T_E ($\gamma = 400\ s^{-1}$)	8.43 ± 0.37	7.39 ± 0.19	**
T_E ($\gamma = 150\ s^{-1}$)	6.85 ± 0.10	5.69 ± 0.18	**
T_E ($\gamma = 5\ s^{-1}$)	3.33 ± 0.27	2.36 ± 0.11	**
MDA ($\times 10^{10}$ mol/cell)	5.46 ± 0.27	9.03 ± 0.39	**

* $P < 0.01$.

** $P < 0.005$.

[a] steady flow model of whole blood.

[b] oscillatory flow model of whole blood (0.1 Hz).

η' whole blood dynamic viscosity. η'' whole blood elasticity viscosity. γ shear rate.

Table 3

Whole blood viscosity in CON and HBO_2 groups measured at different shear rates and expressed as the mean ± SD. In this data, a portion of erythrocytes in rats of the HBO_2 group ($n = 15$) was removed from the whole blood sample to make the level of Hct equivalent to that of the CON group ($n = 15$)

Parameter	CON group mean ± SD	HBO_2 group mean ± SD	Paired t-test P value
η blood (cp) ($\gamma = 400$ s^{-1})	5.13 ± 0.19	6.01 ± 0.19	**
η blood (cp) ($\gamma = 150$ s^{-1})	6.31 ± 0.16	7.19 ± 0.18	**
η blood (cp) ($\gamma = 5$ s^{-1})	13.03 ± 0.94	15.32 ± 0.98	**

** $P < 0.005$.

5.13 ± 0.19, $\gamma = 400$ s^{-1} $P < 0.01$). Moreover, both dynamic viscosity (η') and elasticity (η'') of whole blood were significantly higher than those of the CON group at 0.1 Hz (Table 2). In addition, as shown in Table 3, whole blood viscosities in the HBO_2 group were significantly higher than those in the CON group, even when measured after part of the erythrocytes had been removed to make the Hct level equivalent to that of the CON group (HBO_2: 6.01 ± 0.19 cP; CON: 5.13 ± 0.19 cP, $\gamma = 400$ s^{-1} $P < 0.01$; HBO_2: 7.19 ± 0.18 cP; CON: 6.31 ± 0.16 cP, $\gamma = 150$ s^{-1} $P < 0.01$; HBO_2: 15.32 ± 0.98 cP; CON: 13.03 ± 0.94 cP, $\gamma = 5$ s^{-1} $P < 0.01$). As regards the plasma viscosity, there was no statistical difference between the 2 groups (HBO_2: 1.62 ± 0.16 cP; CON: 1.61 ± 0.02 cP, $P > 0.05$) (Table 2).

Table 2 also shows that the mean MDA level in rats of the HBO_2 group, an index of lipid peroxidation of the erythrocyte membrane, was significantly higher than that of the CON group (in moles/10^{10} erythrocytes; HBO_2: 9.03 ± 0.39; CON: 5.46 ± 0.27, $P < 0.01$). Moreover, both flow resistance of the erythrocytes (β) (HBO_2: 19.18 ± 1.29; CON: 8.92 ± 0.49, $P < 0.01$) and erythrocyte rigidity (T_K) (HBO_2: 0.89 ± 0.01; CON: 0.86 ± 0.03, $\gamma = 400$ s^{-1} $P < 0.01$) (Table 2) in rats of the HBO_2 group were much higher than those of the CON group. And lastly, a significant decrease in oxygen delivery index (T_E) (HBO_2: 2.36 ± 0.11; CON: 3.33 ± 0.27, $\gamma = 5$ s^{-1} $P < 0.01$) was detected in rats of the HBO_2 group, as shown in Table 2.

4. Discussion

For more than 20 years, HBO_2 has been used for the treatment of various clinical conditions [17,19,30]. Little has been known, however, about the mechanism of its action, especially from the hemorheological point of view. Even through HBO_2 has been an effective treatment in healing diabetic wounds [13,31], the influence of HBO_2 on the hemorheological parameters of diabetic patients has not been fully elucidated. Since the hemorheological behavior is closely related to microcirculation, it is important to understand the influence of HBO_2 on the hemorheological parameters of diabetic patients. In our study, mature diabetic rats are used as an animal model to extrapolate the effect of HBO_2 on hemorheological parameters with diabetes, and two aspects of the influence of HBO_2 in diabetic rats are discussed in this work: firstly, how HBO_2 affects erythrocyte deformability; and second, the effects of HBO_2 on blood viscosity and viscoelasticity.

Concerning the effect of HBO_2 on lipid peroxidation of erythrocyte, Nikolaeva et al. performed HBO_2 research on patients with lung cancer, and found that HBO_2 resulted in increased lipid peroxidation of the plasma and erythrocyte [20]. In addition, Verrazzo et al. also reported that HBO_2 increased the plasma level of patients with peripheral occlusive arterial disease [34]. Based on their research, Nylander et al. [21] and Narkowicz et al. [18] proposed that HBO_2 might increase oxygen free radicals as well

as enhance lipid peroxidation. Moreover, Ansari et al. found that HBO_2 could enhance the erythrocyte antioxidant enzyme responsible for scavenging free radicals [3]. Nonetheless, one study done by Visona et al. [35] reached results inconsistent with previous findings. In patients with peripheral vascular disease, HBO_2 decreased the level of MDA in plasma [35]. Based on these studies, we postulate that HBO_2 not only increases the production of free radicals but also mediates the enzymes responsible for scavenging free radicals, and consequently the level of free radicals. In the present work, however, we detected that the mean MDA level in the erythrocyte membrane in the HBO_2 diabetic rats was significantly higher than that of the CON group rats.

The major determinants of erythrocyte deformability include cell geometry, the internal viscosity of erythrocyte, and the viscoelastic properties of the erythrocyte membrane. Amin et al. showed electron micrographs of normal and HBO_2 erythrocytes, and found that the echinocytic erythrocytes had an elevated level of the CON group [2]. In addition, the echinocytic erythrocytes were found to have an unusual geometrical shape with a consequent increase in their filtration resistance [24]. On the other hand, Corry and his colleagues found that exposure to oxidant stress leads to a significant increase in the rigidity of erythrocytes [8]. The results of our study showed that HBO_2 could decrease the erythrocyte deformability index ($1/\beta$) of diabetic rats. This might result from the oxidant stress applied, causing a subsequent significant increase in MDA of erythrocytes and erythrocyte rigidity (Table 2). A recent communication reported that hardened erythrocyte or poor deformability may hinder erythrocytes to pass through the micropore filters, which would subsequently increase the resistance to blood flow in the microcirculation [12].

Clinically, Hct is a relatively simple and useful measure to roughly estimate the oxygen-delivery capacity of the blood. Based on this study, even though HBO_2 increased the level of Hct (Table 1), the decreased erythrocyte deformability and increased whole blood viscosity might neutralize the effect and further compromise the oxygen transport efficiency to peripheral tissues in diabetic rats. In addition, both Kon et al. [15] and Vandegriff and Olson [33] reported that the increased level of echinocytic erythrocytes may result in less efficient release of oxygen to peripheral tissues.

Despite the significant increase in fibrinogen concentration in diabetic rats treated with HBO_2, no increase in the plasma viscosity was detected. This discrepancy could be attributed to the fact that the increased fibrinogen concentration was not high enough to induce a significant increase in plasma viscosity. Apart from it, we found HBO_2 could enhance whole blood viscosity under high and low shear stress. In general, an increase in Hct is associated with an increase in whole blood viscosity. More specifically, at a high shear rate, HBO_2 increased blood viscosity by decreasing erythrocyte deformability; while, at a low shear rates, HBO_2 increased whole blood viscosity by enhancing the aggregation of erythrocytes. However, when some of the erythrocytes were removed from the whole blood sample of diabetic rats in the HBO_2 group to make the Hct value equal to that of the CON group, the whole blood viscosity of the HBO_2 group was still higher than that of the CON group. This illustrates that, in addition to Hct, deformability of erythrocytes and their aggregation may be important factors in causing whole blood viscosity to increase.

Since information on oscillatory flow models of whole blood in the previous literature is scarce and limited [25,32], we designed an oscillatory flow model to provide a better simulation of blood circulation in vivo. From this model, we measured the whole blood viscoelasticity (dynamic viscosity and dynamic elasticity) in diabetic rats under a constant 5% shear strain and different frequencies ranging from 0.3 to 0.1 Hz. Generally speaking, at low shear rates the viscoelasticity of whole blood is primarily determined by the aggregation and de-aggregation of erythrocytes. In addition, the parameters η' (dynamic viscosity) of whole blood reflects the ability of erythrocytes to aggregate and adjust their shape while η'' (dynamic

elasticity) reflects the elastic properties of the erythrocytes as they aggregate. It is possible to obtain qualitative information on blood when it flows in large vessels in pulsation and on rouleaux formation of erythrocytes in microcirculation [29]. In our work, the results show that both η' and η'' increased after HBO_2 treatment in diabetic rats (Table 2), which indicates that there was increased erythrocyte aggregation, similar to that observed in the steady flow model. We postulate that the result is attributable to HBO_2 causing an increment in fibrinogen levels in the plasma, and enhancing the interaction between erythrocytes and fibrinogen in plasma, thereby further promoting erythrocyte aggregation. However, the decrease in cell deformability will tend to increase η' and η'' as well.

In conclusion, our work demonstrates that HBT changed the hemorheological properties in diabetic rats, producing increased erythrocyte rigidity, lipid peroxidation, whole blood viscosity and membrane viscoelasticity, and decreased erythrocyte deformability and oxygen delivery ability.

Acknowledgements

We would like to thank the National Science Council of the Republic of China for financially supporting this research under grant no. NSC- 90- 2213 – E -038 -008.

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