Antioxidant Status and Lipid Peroxidation in Diabetic Rats under Hyperbaric Oxygen Exposure

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Summary

Hyperglycemia is known to cause oxidative stress that leads mainly to enhanced production of mitochondrial reactive oxygen species (ROS). It has been demonstrated that hyperbaric oxygen (HBO) treatment also increases the formation of ROS. There are, however, no comprehensive evaluations of such oxidative effects in diabetes which requires HBO treatment. The purpose of this study is to investigate the influence of a clinically-recommended HBO treatment on glucose homeostasis and oxidative stress in rats with streptozotocin (STZ)-induced diabetes. Under the clinically-used HBO exposure protocol, the levels of blood glucose, thiobarbituric acid reactive substances (TBARS) as a lipid peroxidation marker, and the activity of superoxide dismutase (SOD) as an antioxidant enzyme marker were investigated in the erythrocytes, liver, pancreas, skeletal muscle, and brain of rats with STZ-induced diabetes. The levels of blood glucose and TBARS increased significantly ($p<0.05$), and the activity of SOD decreased significantly ($p<0.05$) in the erythrocytes and all organs of rats with diabetes subjected to HBO exposure. These results suggested that HBO exposure might boost glucose autoxidation and increase ROS production in STZ-induced diabetes as side-effects of administering HBO treatment for the first time.

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Introduction

Reactive oxygen species (ROS) which cause cellular damage by the oxidation ability have been implicated in the pathogenesis of diabetes mellitus (Brownlee 2001, Kowluru et al. 2007). During diabetes, persistent hyperglycemia increases the production of ROS through glucose autoxidation (Hunt et al. 1990, Wolff et al. 1991). The oxidative stress has also been associated with diabetic states in animals and humans (Kakkar et al. 1995, Ihara et al. 1999, Opara 2002, Davì et al. 2005). One of these studies using streptozotocin (STZ)-induced diabetic rats showed that levels of lipid peroxidation had increased, as indicated by thiobarbituric acid reactive substances (TBARS), which is one of oxidative stress markers suggesting the occurrence of oxidative stress (Kakkar et al. 1995). Moreover, diabetic patients have significant defects in antioxidant defense elements, and enhanced ROS generation is one of the major determinants of diabetic complications (Ihara et al. 1999, Opara 2002). Thus, diabetes closely relates to various oxidative activities.

Hyperbaric oxygen (HBO) therapy is achieved by exposing patients to breathing 100 % O_2 at barometric pressure higher than ambient (1 atmosphere absolute or 1 ATA). HBO therapy is a unique method used in the treatment of various illnesses and clinical conditions, such as carbon monoxide poisoning, decompression sickness, osteomyelitis, and even diabetic foot (Feldmeier 2003). The Undersea and Hyperbaric Medical Society (UHMS) has an approved list of clinical conditions where HBO treatment is indicated including the above-mentioned disorders

(Feldmeier 2003). However, it has been demonstrated that HBO also increases the formation of ROS that can cause cellular damage through the oxidation of lipids, proteins and DNA (Speit *et al.* 2002). It is also assumed that hyperoxic injury occurs *via* the metabolic production of oxygen radicals and related ROS, owing to the saturation of natural antioxidant defense mechanisms in the cells (Sies 1997).

HBO administration can also lead to unwelcome side-effects, such as oxidative stress and/or oxygen toxicity. Such side-effects have often been associated with the exposure to HBO levels much higher than those generally used for treating clinical conditions (Harbin *et al.* 1990, Torbati *et al.* 1992, Chavko *et al.* 1996, Speit *et al.* 2002). The clinically approved maximum pressure and duration of HBO exposure are 3 ATA and 120 min, respectively (Feldmeier 2003), although the most commonly-used protocol for standard therapeutic purposes is slightly lower (1.8-2.8 ATA for 60-90 min) (Benedetti *et al.* 2004). The oxidative effects of HBO have been investigated in animals and humans (Eken *et al.* 2005, Oter *et al.* 2005, Korkmaz *et al.* 2008). One study on the effects of HBO in rats revealed that, after 2 h of HBO exposure at 3 ATA, elevated levels of the oxidative stress markers, TBARS and superoxide dismutase (SOD), were found in the lung, brain and erythrocytes (Oter *et al.* 2005). Thus, HBO exposure seems to be an excellent model system for investigating oxidative stress.

HBO treatment is used for the treatment of diabetic patients with unhealing foot ulcers (Feldmeier 2003). However, the side-effects of HBO treatment in diabetic patients have been poorly investigated. Previously, we have reported that a clinically-recommended HBO treatment caused a significant increase of glucose toxicity and decrease of insulin immunoreactive β-cells in STZ-induced diabetic rats (Matsunami *et al.* 2008). There are, however, no comprehensive articles describing the evaluation of such oxidative effects in diabetes with HBO treatment.

To evaluate the oxidative stress of HBO in STZ-induced diabetes, we examined levels of TBARS, the activity of antioxidant enzymes, and the physiological parameters associated with glucose homeostasis under HBO treatment, using pressure levels and duration of exposure to HBO typical of those used in the clinical setting.

Materials and methods

Animals

Twenty-four adult (12-week-old) male Sprague-Dawley rats (body weight 300-350 g), bred in our laboratory, were used for the experiment. They were housed at 23-25 °C with light from 7:00 AM to 7:00 PM and free access to water at all times. All rats were fed a commercial diet during the experiment. All study procedures were implemented in accordance with the Institutional Guidelines for Animal Experiments at the College of Bioresource Sciences, Nihon University under the permission of the Committee of Experimental Animal in our college.

Study design

The rats (*n*=24) were allowed to acclimatize for one week, prior to treatment and randomly distributed into four groups (6 rats per group): non-diabetic induction in the non-HBO group (Control), non-diabetic induction in the HBO group (HBO), diabetic induction in the non-HBO group (DM), and diabetic induction in the HBO group (DM + HBO), respectively. The animals were kept for 9 days from the first treatment for diabetic induction. In this study, diabetes occurred on the third day after diabetic induction, and then HBO exposure has been conducted once daily for 7 days in DM + HBO group. The mean body weight of the animals in all groups was measured at the onset and end of the study period.

Induction of diabetes

Sprague-Dawley rats were injected intraperito-neally (i.p.) with 80 mg/kg of streptozotocin (STZ, Wako Pure Chemical Industries, Ltd. Japan), dissolved in citrate buffer (pH 4.5), to induce diabetes as described previously (Like *et al.* 1976). Control and HBO rats were treated with equal volume and concentration of STZ injection vehicle, citrate buffer. Blood glucose levels in the auricular veins were measured 3, 6 and 9 days after STZ injection by an automated glucose measurement instrument, the G meter® (Sanofi-Aventis K.K. Japan). Diabetes was considered to have been induced when the blood glucose level reached at least 15.6 mmol/l (Matkovics *et al.* 1997).

HBO exposure

For all experiments referring to the clinically-used setting, HBO was applied at a pressure of 2.8 ATA for 2 h, once daily for 7 days, in a hyperbaric chamber for

small animals (Nakamura Tekko-sho K.K., Tokyo, Japan). The ventilation rate was 4-5 l/min. Each exposure was started at the same hour in the morning (10 AM) to exclude any confounding issues associated with the changes in biological rhythm.

Tissue preparation

After the final exposure to HBO, the rats were weighed and anesthetized with sodium pentobarbital (40 mg/kg i.p.). A 2-ml blood sample was collected by cardiac puncture and divided into three heparinized tubes, and various organs (brain, liver, pancreas, and skeletal muscle) were removed and washed with phosphate-buffered saline (PBS), pH 7.4, containing 0.16 mg/ml of heparin to remove any red blood cells (erythrocytes) and clots (Prasad *et al*. 1992). Brain, liver, pancreas, and skeletal muscle were homogenized with an ultrasonic homogenizer in cold phosphate buffer, pH 7.0 with ethylenediaminetetraacetic acid (EDTA), for TBARS measurement and cold 20 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.2, containing 1 mM ethyleneglycol-bis (2-amino-ethoxy)-tetraacetic acid (EGTA), 210 mM mannitol, and 70 mM sucrose for SOD measurement (Mattiazzi *et al*. 2002). The supernatant was put in separate tubes and stored at −70 °C. Two milliliters of blood was separated into plasma and erythrocytes by centrifugation at 10,000 × g for 10 min at 4 °C. The erythrocyte samples were washed three times with cold physiological saline (PS) and then hemolyzed by adding a fourfold volume of ice-cold high-performance liquid chromatography (HPLC)-grade water. Hemolyzed erythrocyte samples were centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was collected and stored at −70 °C for less than three months prior to the measurement of enzymatic activity. It is known that antioxidant enzymatic activity is stable in hemolysates stored under such conditions (Andersen *et al*. 1997).

Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation levels were measured by the thiobarbituric acid (TBA) reaction using the method of Ohkawa *et al*. (1979). This method was used to measure spectrophotometrically the color produced by the reaction of TBA with malondialdehyde (MDA) at 532 nm. For this purpose, TBARS levels were measured using a commercial assay as the NWLSS^{TM} Malondialdehyde Assay (NWLSS, WA, USA) according to the manufacturer's instructions. Tissue supernatant (50 μl) or

erythrocyte supernatant (50 μl) were added to test tubes containing 2 μl of butylated hydroxytoluene (BHT) in methanol. Next, 50 μl of acid reagent (1 M phosphoric acid) was added and finally 50 μl of TBA solution was added. The tubes were mixed vigorously and incubated for 60 min at 60 °C. The mixture was centrifuged at 10,000 × g for 3 min. The supernatant was put into wells on a microplate in aliquots of 75 μl, and its absorbance was measured with a BIO RAD Benchmark Plus plate reader at 532 nm (BIO RAD Laboratories, Inc., CA, USA). TBARS levels were expressed as nmol/mg protein in various organs (brain, liver, pancreas and skeletal muscle), and as nmol/g hemoglobin in erythrocyte hemolysates.

Superoxide dismutase (SOD)

SOD activity in the tissue supernatants or the blood was measured as previously described (Sun *et al*. 1988). For this purpose, SOD activity was measured using a commercially supplied assay kit of Superoxide Dismutase Assay Kit (Cayman Chemical, MI, USA) according to the manufacturer's instructions. The SOD assay consisted of a combination of the following reagents: 0.3 mM xanthine oxidase, 0.6 mM diethylenetriamine-penta acetic acid (DETAPAC), 150 μM nitroblue tetrazolium (NBT), 400 mM sodium carbonate (Na_2CO_3), and bovine serum albumin (1 g/l). The principle of the method is based on the inhibition of NBT reduction by superoxide radicals produced by the xanthine/xanthine oxidase system. The SOD stock solutions (0-200 μl) for SOD standard activities (0-0.25 U/ml) contained 20 μl of Cu/Zn-SOD from bovine erythrocyte dissolved in 1.98 ml of 50 mM Tris-HCl, pH 8.0; before being used in the assay, this solution was diluted by adding 18 ml of HPLC-grade water. For the assay, standard SOD solutions, tissue supernatant (10 μl) or erythrocyte supernatant (10 μl) were added to wells containing 200 μl of NBT solution that was diluted by adding 19.95 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 mM DETAPAC solution and 0.1 mM hypoxanthine. Finally, 20 μl of xanthine oxidase was added to the wells at an interval of 20 s. After incubation at 25 °C for 20 min, the reaction was terminated by the addition of 1 ml of 0.8 mM cupric chloride. The formazan was measured spectrophoto-metrically by reading the absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50 %. The calculated SOD activity was expressed as U/mg protein in various organs (brain,
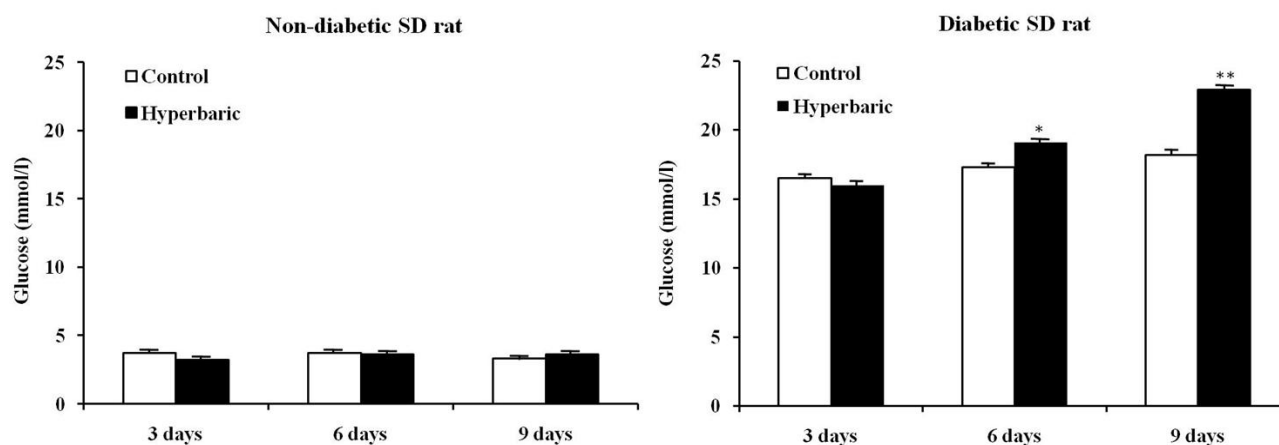
Fig. 1. Blood glucose levels in non-diabetic and diabetic Sprague-Dawley rats. Values are expressed as mean ± S.D. (*n*=6). * *p*<0.01, ** *p*<0.001 when compared with control value.

liver, pancreas and skeletal muscle), and as U/g hemoglobin in erythrocyte hemolysates.

Statistical analysis

The collected and calculated data are expressed as mean ± standard deviation (S.D.). Student's *t*-test was used to determine significant differences between the control and hyperbaric groups. For the analysis of TBARS levels and SOD activity data, analysis of variance (ANOVA) and a non-parametric test (Kruskal-Wallis) were used. Differences were considered statistically significant if *p*<0.05.

Results

The body weight of the rats at beginning of the study was similar in all groups. At the end of the treatment (after 9 days), there was no difference in body weight between control and HBO rats. However, diabetic rats had a considerable weight loss, and the body weight of DM + HBO rats (299±8 g) decreased significantly (*p*<0.05), when compared with the rats in other three groups (control 354±11 g; HBO 353±5 g; DM 320±8 g).

The blood glucose levels of DM + HBO group were significantly higher at 6 and 9 days after diabetes induction (*p*<0.05) as compared with DM group (Fig. 1). No significant differences were observed between control and HBO groups or experimental periods. Thus, HBO treatment resulted in a significant enhancement of blood glucose levels in diabetes with hyperglycemia.

TBARS levels and SOD activity in erythrocytes and all organs examined are presented in Figures 2 and 3. TBARS levels in HBO, DM, and DM + HBO groups were significantly increased (*p*<0.001-0.05), as compared

with control group. In addition, TBARS levels in erythrocytes and all organs were higher in HBO group than in DM group (*p*<0.01-0.05). Furthermore, HBO treatment was associated with a significant increase in TBARS levels (*p*<0.001; DM + HBO group vs. HBO and DM groups). The SOD activity in all samples examined in HBO, DM, and DM + HBO groups was significantly decreased (*p*<0.001-0.05), as compared with control group. Moreover, SOD activity in erythrocytes and organs (pancreas, skeletal muscle, and brain) was lower in HBO group than in DM group (*p*<0.001-0.05). Furthermore, HBO treatment was associated with a significant decrease in SOD activity (*p*<0.001; DM + HBO group vs. HBO and DM groups).

Discussion

The present study demonstrated for the first time that HBO significantly causes the enhancement of ROS production in STZ-induced diabetic rats, a recognized model of type 1 diabetic mellitus (T1DM). In addition to oxidative parameters, we have clearly demonstrated the changes in blood glucose levels. Hyperglycemia caused by diabetes is known to be a cause of oxidative stress that leads mainly to enhanced production of mitochondrial ROS (Brownlee 2001, Jay *et al.* 2006, Kowluru *et al.* 2007). Furthermore, oxidative stress induced by hyperglycemia leads to the activation of stress-sensitive signaling pathways, which worsen both insulin secretion and action, and promote the development of type 2 diabetic mellitus (T2DM) (Evans *et al.* 2002). Similarly, oxidative stress and damage to the tissues and the blood in STZ-induced diabetic rats enhance glucose autoxidation, and may be a factor contributing to
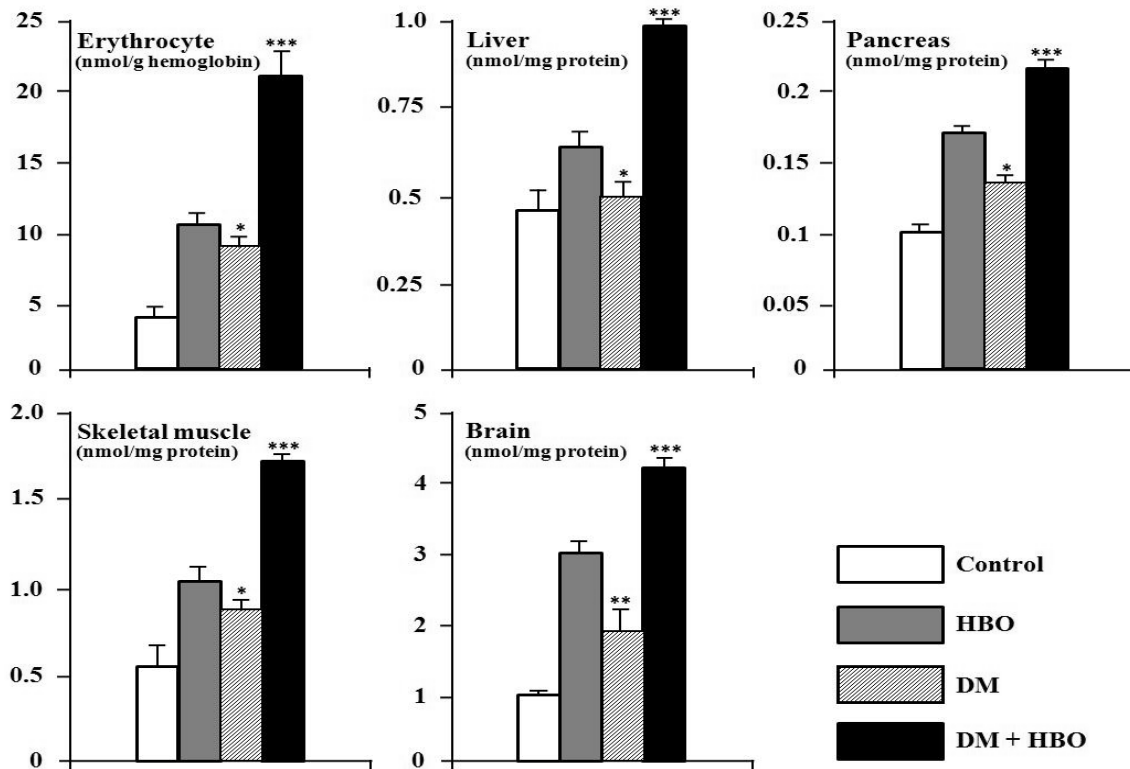
Fig. 2. Lipid peroxidation content (TBARS) in the erythrocytes and organs (liver, pancreas, skeletal muscle, brain) of all groups. Control (non-diabetic rats without HBO), HBO (non-diabetic rats with HBO), DM (diabetic rats without HBO), and DM + HBO (diabetic rats with HBO) groups. TBARS values are indicated as nmol/g hemoglobin in erythrocytes and as nmol/mg protein in various organs. Values are expressed as mean ± S.D. (n=6). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ when compared with HBO and DM groups.
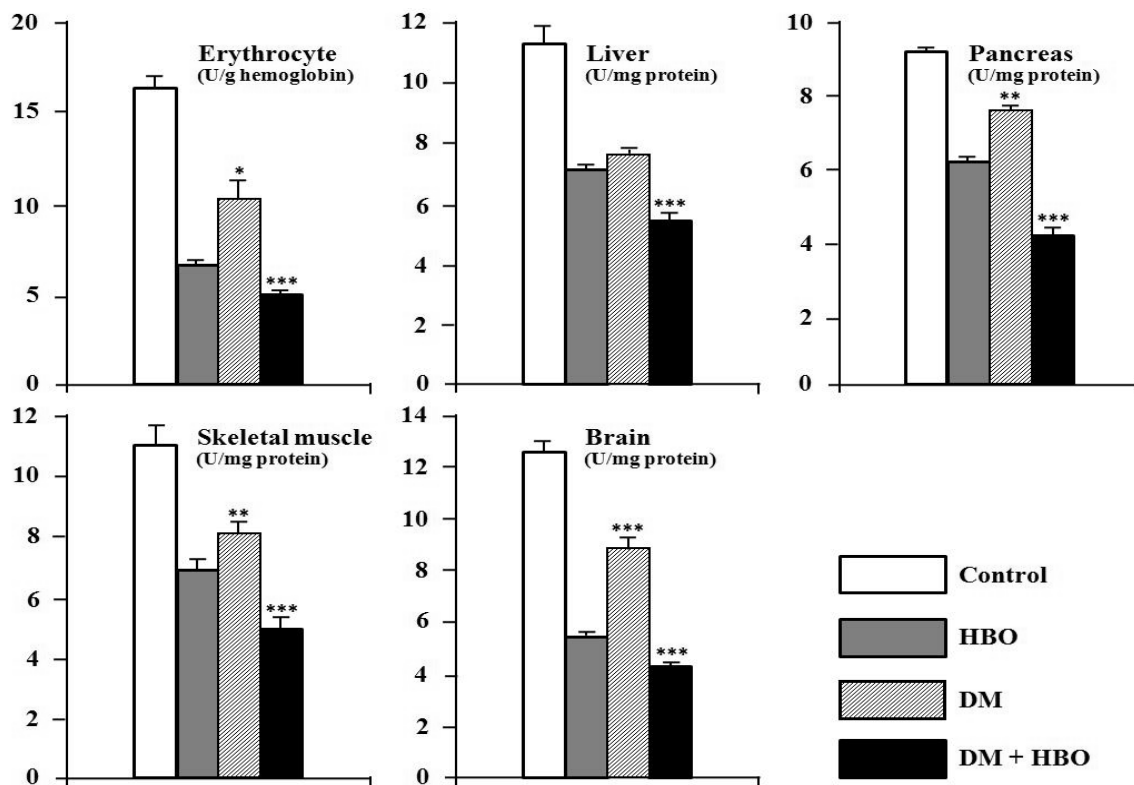
Fig. 3. Antioxidant enzyme (SOD) activity in the erythrocytes and organs (liver, pancreas, skeletal muscle, brain) of all groups. For other legend see Fig. 2.

complications associated with diabetes (Kakkar *et al.* 1995). A recent study has reported that TBARS levels are elevated and activities of antioxidant enzymes (SOD, glutathione peroxidase, and catalase) are significantly reduced in STZ-induced diabetic rats (Jin *et al.* 2008). Therefore, we further examined the oxidative effects of HBO in STZ-induced diabetic rats, and found that the TBARS levels and SOD activity of diabetic rats were significantly affected by HBO treatment.

HBO exposure leads to an increase in the amount of oxygen dissolved in the blood, resulting in the improvement of a variety of clinical conditions such as hypoxia, acute carbon monoxide intoxication, air embolism and diabetic lower limb wounds (Feldmeier 2003, Lipsky *et al.* 2004). The most commonly used doses of HBO for standard therapeutic purposes are 1.8-2.8 ATA for 60-90 min (Benedetti *et al.* 2004). Although the oxidative effects of HBO have been investigated in animals and humans (Chavko *et al.* 1996, Eken *et al.* 2005, Oter *et al.* 2005, Korkmaz *et al.* 2008), the HBO conditions applied in previous studies seemed to be higher than usual therapeutic levels. For example, one study in rats revealed that, after HBO exposure at 3 ATA, oxidative stress markers, including TBARS levels and SOD, glutathione peroxidase, and nitrate/nitrite (NOx) activity, were found to be elevated in the brain (Korkmaz *et al.* 2008). In the present study, we used an HBO exposure protocol based on clinically used oxygen pressure and exposure duration, and we investigated blood glucose levels, in addition to TBARS levels and SOD activity in erythrocytes, liver, pancreas, skeletal muscle, and brain of rats with STZ-induced diabetes. Our results showed that blood glucose levels were not improved in HBO-treated diabetic rats, suggesting that clinically used HBO administration might enhance the functional disorder of pancreatic cells. In addition, the levels of blood glucose in non-diabetic rats with HBO treatment remained stable also in our previous study (Matsunami *et al.* 2008). On the other hand, we showed that the levels of TBARS were significantly higher in non-diabetic rats with HBO treatment than in diabetic rats without HBO treatment. As TBARS levels are closely associated with lipid peroxidation (Ohkawa *et al.* 1976, Chavko *et al.* 1996, Opara 2002, Eken *et al.* 2005), these results suggest that lipid peroxidation is greater under the conditions of continuous HBO exposure than under DM condition. Previously, we have shown for the first time in STZ-diabetic rats that the increase in blood glucose levels started after the initiation of HBO treatment administered at a clinically relevant pressure level and duration (Matsunami *et al.* 2008). Furthermore, in the present study, we have shown that diabetic rats receiving HBO treatment had significantly increased TBARS levels and significantly decreased SOD activity as compared with the diabetic control group (without HBO treatment).

Interestingly, it has been shown that blood glucose levels are decreased by HBO treatment in Goto-Kakizaki (GK) rats, an animal model of spontaneous type 2 DM, suggesting that HBO could interfere with the activity of the natural anti-oxidative defense system and thus promote *de novo* generation of free radicals (Ihara *et al.* 1999, Maritim *et al.* 2003, Yasuda *et al.* 2007). Our results suggest that the mode of action of HBO on the glucose control system might be different in STZ- and GK-induced diabetic rats. The possible side-effects of HBO seen in this study – namely, the elevation of glucose levels in the peripheral blood – might occur as a result of the enhancement of STZ cytotoxicity by HBO administration as shown in our previous study (Matsunami *et al.* 2008). The probable cause of the enhancement of glucose toxicity or autoxidation might be oxidative stress as described previously (Hunt *et al.* 1990, Wolff *et al.* 1991, Ihara *et al.* 1999, Maritim *et al.* 2003). It is widely accepted that HBO administration increases superoxide and SOD activity, and there is no doubt that HBO causes lipid peroxidation (Oury *et al.* 2002). However, it has been demonstrated that the enhancement of SOD activity by HBO administration is due to intermittent or single HBO exposure, and SOD activity decreases with continuous HBO exposure (Harabin *et al.* 1990). Our study showed that HBO treatment significantly increased TBARS levels and decreased SOD activity, clearly demonstrating that HBO causes oxidative stress in type 1 DM. Thus, the synergistic elevation of blood glucose levels between STZ-induced diabetes and HBO treatment might lead to glucose autoxidation, which would increase the induction of ROS. Our experimental system, which uses STZ-induced diabetes with HBO treatment, could be applied for evaluating the manifold toxicities of insulin resistance and metabolic syndrome in DM, by monitoring the unusual increases in blood glucose levels and oxidative stress.

Conflict of Interest

There is no conflict of interest.

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