Antioxidant status and lipid peroxidation in diabetic rats under hyperbaric oxygen exposure

Tokio Matsunami¹, Yukita Sato¹, Takuya Sato¹, Masayoshi Yukawa¹

¹ Laboratory of Biomedical Science, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, Fujisawa 252-8510, Japan.

Corresponding author:
Yukita Sato, Laboratory of Biomedical Science, Department of Veterinary Medicine, College of Bioresource Sciences, Nihon University, Fujisawa 252-8510, Japan.

Phone and Fax: +81-466-84-3445

e-mail address: sato.yukita@nihon-u.ac.jp

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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 both HBO exposure and diabetes. 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.

Key words: hyperbaric oxygen, oxidative stress, diabetic rats, lipid peroxidation,
antioxidant enzyme

INTRODUCTION

Reactive oxygen species (ROS) which cause cellular damages 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 been also 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, indicated by thiobarbituric acid reactive substances (TBARS), 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 generation of ROS 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 a patient breathing 100% O2 to a 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
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, among others, the above-mentioned disorders (Feldmeier 2003). It has been demonstrated, however, that HBO also increases the formation of ROS that can cause cellular damage through the oxidation of lipid, protein, 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 saturation of the 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 exposure to levels of HBO 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 in 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.

In this study, to evaluate the oxidative stress of HBO in 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, in a STZ-induced diabetic model.

**MATERIALS AND METHODS**

**Animals and the usage policy:**
Twenty-four adult, 12-week-old, male Sprague–Dawley rats (weight range, 300–350 g), bred in our laboratory, were used for the experiment. They were housed at 23 to 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 1 week, prior to treatment and randomly distributed into four groups, 6 rats per group:- one group was 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 for 9 days from the first treatment for diabetic induction. In this study, diabetes was 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 initial and final during the study period.

**Induction of diabetes:**
Sprague–Dawley rats were injected intraperitoneally (i.p.) with 80mg/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 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-aminoethoxy)-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 3 months pending measurement of enzymatic activity. It is known that antioxidant enzymatic activity is stable in hemolysates stored in such cold 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.* (Ohkawa *et al.* 1979). This method was used to obtain a spectrophotometric measurement of the color produced during the reaction of TBA with malondialdehyde (MDA) at 532nm. For this purpose, TBARS levels were measured using a commercial assay as the NWLSS™ Malondialdehyde Assay (NWLSS, WA, USA) according to the manufacturer’s instructions, and indeed tissue supernatant (50 μL) and 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 minutes 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 are 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 and 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) solution, 150 μM nitroblue tetrazolium (NBT) solution, 400 mM sodium carbonate (Na₂CO₃), 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 sec. 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 spectrophotometrically by reading the absorbance at 560 nm. One unit (U) of SOD is defined as amount of protein that inhibits the rate of NBT
reduction by 50%. The calculated SOD activity is expressed as U/mg protein in various organs (brain, 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 nonparametric test (Kruskal-Wallis) were used. Differences were considered statistically significant if \( p < 0.05 \).

**RESULTS**

The baseline 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 (mean group weights ranged from 353 to 354 g). However diabetic rats presented with weight loss, and the body weight of DM + HBO rats (299 ± 8 g; mean ± S.D.), in particular, decreased significantly \( (p < 0.05) \), when compared with the rats in the other 3 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 (Figure 1). No significant differences were observed between any other groups (control and HBO groups) or experimental periods. Thus, HBO treatment resulted in a significant enhancement in the blood glucose levels of diabetes with hyperglycemia.

TBARS levels and SOD activity in the erythrocytes and all organs examined are presented in Figure 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 the 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 of all samples examined in HBO, DM, and DM + HBO groups was significantly decreased ($p < 0.001 - 0.05$), as compared with control group. Moreover, the SOD activity in the erythrocytes and organs (pancreas, skeletal muscle, and brain) were 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 has demonstrated for the first time that HBO significantly causes
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 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 contributory factor in 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 dissolved oxygen in the
blood, resulting in improvement in 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 pressures and duration, and investigated blood levels of glucose, in addition to levels of TBARS and the activity of SOD in the 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 stationary as well as 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 in conditions of continuous HBO exposure than in the 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 (i.e., no 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 T2DM, 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 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 T1DM state. 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.

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FIGURE LEGENDS

Figure 1: Blood glucose levels in non-diabetic and diabetic Sprague–Dawley rats. Values are expressed as mean ± standard deviation (n = 6). * p < 0.01, ** p < 0.001 compared with control value.

Figure 2: Lipid peroxidation content (TBARS) in the erythrocytes and organs (liver, pancreas, skeletal muscle, and brain) of all groups. Control (Non-diabetic rats in the non-HBO), HBO (Non-diabetic rats in the HBO), DM (diabetic rats in the non-HBO), and DM + HBO (diabetic rats in the HBO) groups. TBARS values are indicated as nmol/g hemoglobin in erythrocytes and as nmol/mg protein in the various organs. Values are expressed as mean ± standard deviation (n = 6). Statistical analysis was done by a one-way ANOVA or by a Kruskal-Wallis test (for comparison among HBO, DM, and DM + HBO groups). (* p < 0.05, ** p < 0.01, *** p < 0.001 when compared with HBO and DM groups).

Figure 3: Antioxidant enzyme (SOD) activity in the erythrocytes and organs (liver, pancreas, skeletal muscle, and brain) of all groups. Control (Non-diabetic rats in the non-HBO), HBO (Non-diabetic rats in the HBO), DM (diabetic rats in the non-HBO),
and DM + HBO (diabetic rats in the HBO) groups. SOD values are indicated as U/g hemoglobin in erythrocytes and as U/mg protein in the various organs. Values are expressed as mean ± standard deviation (n = 6). Statistical analysis was done by a one-way ANOVA or by a Kruskal-Wallis test (for comparison among HBO, DM, and DM + HBO groups). (* p < 0.05, ** p < 0.01, *** p < 0.001 when compared with HBO and DM groups).
Figure 1

[Graph showing glucose levels in non-diabetic and diabetic SD rats over 3, 6, and 9 days.]

Non-diabetic SD rat
- Control
- Hyperbaric

Diabetic SD rat
- Control
- Hyperbaric

Glucose (mmol/L)
Figure 2

Erythrocyte (nmol/g hemoglobin)

Liver (nmol/mg protein)

Pancreas (nmol/mg protein)

Skeletal muscle (nmol/mg protein)

Brain (nmol/mg protein)

Control
HBO
DM
DM + HBO