List of Publications Concerning Mild Hyperbaric Oxygen

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酸素カプセル（軽度高気圧酸素）に関する研究業績一覧

京都大学大学院人間・環境学研究科　教授　石原昭彦

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Hyperbaric exposure with high oxygen concentration enhances oxidative capacity of neuromuscular units

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Abstract

The effects of hyperbaric exposure with high oxygen concentration on spinal motoneurons and the skeletal muscle fibers that they innervate were investigated. Five-week-old male rats were exposed to a hyperbaric (1.25 atmospheric pressure) environment with a high oxygen concentration (35.0%) for 6 h daily. The number, cell body size, and oxidative enzyme activity of motoneurons innervating the soleus and plantaris muscles were examined after 8 weeks of hyperbaric exposure. In addition, the fiber type distribution, cell size, and oxidative enzyme activity of the slow soleus and fast plantaris muscles were examined. The oxidative enzyme activity of alpha motoneurons innervating the soleus and plantaris muscles increased after hyperbaric exposure, irrespective of their cell body sizes. The percentage of high-oxidative fibers in the soleus and plantaris muscles increased after hyperbaric exposure. The oxidative enzyme activity of all types of fibers in the soleus and plantaris muscles increased after hyperbaric exposure. It is concluded that hyperbaric exposure with high oxygen concentration enhances the oxidative capacity of neuromuscular units.

Keywords: Plantaris muscle; Retrograde neuronal labeling; Skeletal muscle fiber; Soleus muscle; Spinal motoneuron; Succinate dehydrogenase activity

1. Introduction

We have developed a specially designed hyperbaric chamber for animal experiments, which is an oxygen compression tank with an oxygen concentrator. The hyperbaric chamber is designed to automatically maintain increased levels of atmospheric pressure and oxygen concentration. The increased atmospheric pressure enhances the partial pressure of oxygen and dissolves more oxygen into the blood and plasma.

The neuromuscular unit is comprised of skeletal muscle fibers and spinal motoneurons associated with those muscle fibers. Motoneurons innervating the muscle fibers are located at the ventral horn of the spinal cord (McHanwell and Biscoe, 1981; Nicolopoulos-Stournaras and Iles, 1983; Peyronnard et al., 1986) and have specific morphological and metabolic properties (Burke et al., 1982; Sickles and McLendon, 1983; Ishihara et al., 1991, 1995). The phenotypic properties of motoneurons and muscle fibers in the neuromuscular unit are matched under normal conditions. Smaller motoneurons presumably innervating slow-twitch, high-oxidative fibers have higher oxidative enzyme activities than larger motoneurons presumably innervating fast-twitch, low-oxidative fibers.

It was anticipated that the increased availability of oxygen induced by hyperbaric exposure with high oxygen concentration would greatly increase the oxidative enzyme activity in the neuromuscular unit. In the present study, therefore, the effects of hyperbaric exposure with high

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oxygen concentration on the number, cell body size, and oxidative enzyme activity of motoneurons, which innervate the slow soleus and fast plantaris muscles in rats, were examined. In addition, the fiber type distribution, cell size, and oxidative enzyme activity of the soleus and plantaris muscles were examined after hyperbaric exposure with high oxygen concentration. Special attention was paid to investigating whether the effects of hyperbaric exposure with high oxygen concentration depend on the cell body size of motoneurons and the muscle fiber types that they innervate.

2. Materials and methods

2.1. Animals and treatments

All procedures were approved by the University Committee for the Care and Use of Animals for research purposes and followed the Guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals.

Ten 5-week-old male Wistar rats were randomly assigned into the control ($n = 5$) and hyperbaric ($n = 5$) groups. All rats were individually housed in cages of the same size (30 cm × 20 cm × 13 cm). The rats in the hyperbaric group were exposed to an atmospheric pressure of 1.25 with an oxygen concentration of 35.0% automatically maintained by a computer-assisted system. The hyperbaric chamber is 180 cm long, 70 cm in diameter. The size of the chamber allows multiple rats (up to 20 cages) to be housed simultaneously. The rats in the hyperbaric group were exposed to the hyperbaric environment for 6 h (from 10:00 to 16:00) in their inactive times daily for 8 weeks. Food and water were provided ad libitum for both groups. The rats were kept in a controlled environment with fixed 12:12 h light:dark cycles (lights off from 19:00 to 07:00) and room temperature maintained at 22 ± 2 °C.

2.2. Injection of neuronal tracer

Eight weeks later, the rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) for injection of the fluorescent tracer, nuclear yellow, into the right soleus and left plantaris muscles (Ishihara et al., 1997a). After the muscle was exposed, a 2% solution of the fluorescent tracer was injected into the muscle using a microsyringe. The fluorescent tracer was injected at multiple sites to ensure distribution throughout the muscle. Care was taken to inject the fluorescent tracer slowly and prevent leakage. After the fluorescent tracer had been injected into the muscle, the skin was sutured and the rats were allowed to recover from anaesthesia. The volumes of injected fluorescent tracer were 10 and 15 μl for the soleus and plantaris muscles, respectively. After 2 days of survival period, the rats were sacrificed by overdose of sodium pentobarbital.

2.3. Analyses

The lumbosacral enlargement of the spinal cord and the bilateral soleus and plantaris muscles were removed and frozen immediately in isopentane cooled with liquid nitrogen. Serial longitudinal sections of the lumbosacral enlargement, 10 μm thick, were cut in a cryostat set at −20 °C. The motoneurons innervating the soleus and plantaris muscles were identified by golden-yellow fluorescence of the nucleus with nuclear yellow on the untreated sections using a fluorescent microscope (Fig. 1A and B). The identified motoneurons were counted in the serial sections. The same sections used to identify the motoneurons were then stained for activity of succinate dehydrogenase (SDH), an oxidative marker enzyme, at room temperature for 10 min (Fig. 1C and D). The activity of SDH was rendered visible by incubating the sections in 0.1 M phosphate buffer (pH 7.6) containing 0.9 mM sodium azide, 0.9 mM 1-methoxyphenazine methylsulfate, 1.5 mM nitroblue tetrazolium, 5.6 mM EDTA-disodium salt, and 48 mM succinate disodium salt (Chalmers and Edgerton, 1989a,b). The reaction was terminated by multiple washings in distilled water, dehydration in graded ethanols, and passing through xylene. For histochemical controls, either the succinate disodium salt or the nitroblue tetrazolium was excluded from the incubation medium.

The cross-sectional areas of the identified motoneurons in which the nucleus was visible were measured from the SDH-stained sections using a computer-assisted image processing system (Neuroimaging System, Kyoto, Japan) (Ishihara et al., 1996). The sections were digitized as gray level images using a computer-assisted image processing system. Each pixel on a gray level image was quantified as one of 256 gray levels. A gray level value of zero was equivalent to 100% transmission of light and that of 255 was equivalent to 0% transmission. The mean optical density (OD) values of all pixels within the cytoplasm of each motoneuron were determined using a calibration tablet which has 21 steps of gradient density ranges and their diffused density values. The nucleus was excluded from measurements of staining intensity, because it is un-stained for the activity and would lower the OD values of small motoneurons to a greater extent than it would for large motoneurons.

Motoneurons with a cell body size greater than 500 μm^2 were presumed to be alpha motoneurons innervating extrafusal muscle fibers. Smaller motoneurons were presumed to be gamma motoneurons innervating intrafusal muscle fibers. The cut-off for the distribution between gamma and alpha motoneurons was primarily based on convention (Sickles and Oblak, 1984; Peyronnard et al., 1986; Swett et al., 1986).

Serial transverse sections of the left soleus and right plantaris muscles, 10 μm thick, were cut in a cryostat set at −20 °C. The sections were brought to room temperature, air-dried for 30 min and then stained for adenosine

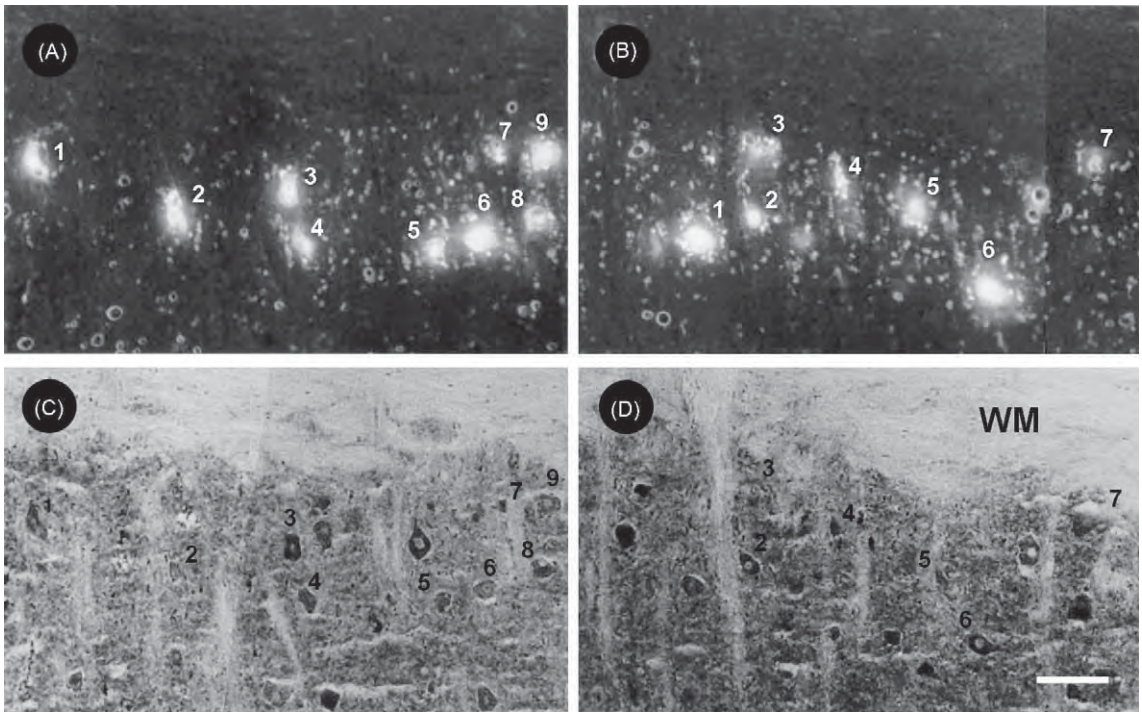

Fig. 1. Longitudinal sections of the spinal cord in the control (A and C) and hyperbaric exposed (B and D) rats. Nine and 7 motoneurons innervating the soleus muscle of the control (A) and hyperbaric exposed (B) rats are identified by retrograde neuronal labeling with nuclear yellow, respectively. C and D are the same sections as shown in A and B, but after processing for succinate dehydrogenase activity. WM: white matter. Scale bar = 100 μm.

triphosphate (ATPase) activity following acid (pH 4.3 and 4.5) and alkaline (pH 10.4) pre-incubation (Nakatani et al., 1999, 2000; Hirofuji et al., 2000). The muscle fibers were classified into type I (positive at pre-incubation pH 4.3 and 4.5, and negative at pre-incubation pH 10.4), type IIA (negative at pre-incubation pH 4.3 and 4.5, and positive at pre-incubation pH 10.4), type IIB (negative at pre-incubation pH 4.3, and negative at pre-incubation pH 4.5 and 10.4), and type IIC (positive at pre-incubation pH 4.3, 4.5, and 10.4) (Fig. 2). The fiber type distribution of the soleus muscle was determined from the entire transverse section of the muscle, while that of the plantaris muscle was determined from approximately 500 fibers in the middle region of the transverse section of the muscle.

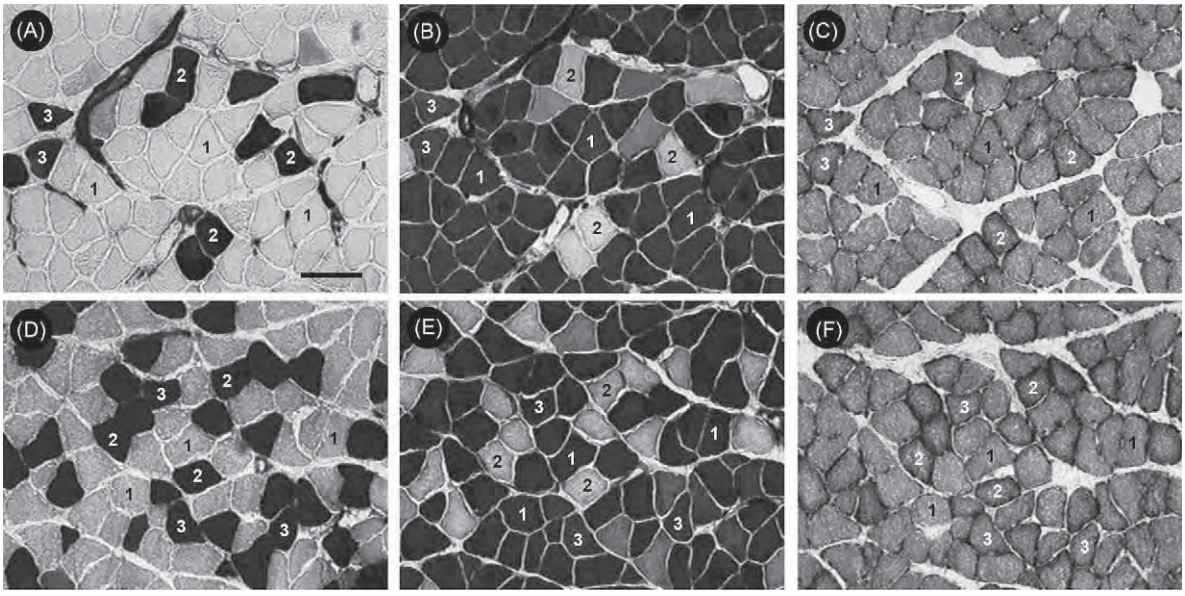

Fig. 2. Serial transverse sections of the soleus muscle in the control (A–C) and hyperbaric exposed (D–F) rats. A and D are stained for adenosine triphosphatase activity following pre-incubation at pH 10.4, while B and E are stained for adenosine triphosphatase activity following pre-incubation at pH 4.5. C and F are stained for succinate dehydrogenase activity. 1, type I; 2, type IIA; 3, type IIC. Scale bar = 100 μm.

The sections were also stained for SDH activity, an indicator of mitochondrial oxidative potential (Nakatani et al., 1999, 2000; Hirofuji et al., 2000). The SDH activities of approximately 500 fibers from each muscle were determined on digitized images of stained sections. The SDH staining intensity was measured using a computer-assisted image processing system (Neuroimaging System, Kyoto, Japan) (Ishihara et al., 1996).

2.4. Statistics

Means and standard deviations were calculated from individual values using standard procedures. The student's *t*-test was used to determine significant differences between the control and hyperbaric groups. Differences were considered significant at the 0.05, 0.01, or 0.001 level of confidence.

3. Results

3.1. Body and muscle weights

The body weights of the control and hyperbaric groups were 391 ± 15 g ($n = 5$) and 386 ± 18 g ($n = 5$), respectively. The soleus muscle weights of the control and hyperbaric groups were 141.5 ± 13.4 mg ($n = 5$) and 145.8 ± 14.0 mg ($n = 5$), respectively, and the plantaris muscle weights of the control and hyperbaric groups were 336.7 ± 20.3 mg ($n = 5$) and 339.5 ± 21.1 mg ($n = 5$), respectively. There were no differences in mean body or mean muscle weight between the control and hyperbaric groups.

3.2. Spinal motoneurons

There was no difference in the mean number, mean cell body size, or mean oxidative enzyme activity of gamma, alpha, or entire (gamma + alpha) motoneurons innervating the soleus or plantaris muscle between the control and hyperbaric groups (Table 1).

When examining the oxidative enzyme activity of motoneurons based on their cell body sizes, the oxidative enzyme activity of alpha, but not gamma, motoneurons of the hyperbaric group was higher than that of the control group, irrespective of their cell body sizes (Fig. 3).

3.3. Skeletal muscle fibers

The mean percentage of type I fibers was lower and those of type IIA and type IIC fibers were higher in the soleus muscle of the hyperbaric group than in the soleus muscle of the control group (Fig. 4). The mean percentage of type IIA fibers was higher and that of type IIB fibers was lower in the plantaris muscle of the hyperbaric group than in the plantaris muscle of the control group.

There were no differences in mean fiber cross-sectional area of the soleus or plantaris muscle between the control and hyperbaric groups, irrespective of the fiber types (Fig. 4).

The mean fiber oxidative enzyme activities in the soleus and plantaris muscles of the hyperbaric group were higher than those of the control group, irrespective of the fiber types (Fig. 4).

4. Discussion

Previous studies (Donselaar et al., 1986; Chalmers et al., 1991, 1992; Seburn et al., 1994; Ishihara et al., 1997b, 2004; Roy et al., 1999, 2001) showed no changes in morphological or metabolic properties in spinal motoneurons following chronic decrease (e.g., spinal cord transaction, spinal cord isolation, immobilization, or hindlimb un-loading) or increase (e.g., exercise, functional overload, electrical stimulation, or hypergravity) in the neuromuscular activity. Chalmers et al. (1992) observed no change in the cell body size or oxidative enzyme activity of motoneurons after spinal cord transection at a low thoracic level or spinal cord isolation at a low thoracic and a high sacral level plus bilateral deafferentation between the two transection sites in cats. Similarly, Seburn et al. (1994) showed no change in the cell body size or oxidative enzyme activity of motoneurons

Table 1
Numbers, cell body sizes, and succinate dehydrogenase activities of motoneurons innervating the soleus and plantaris muscles of the control and hyperbaric groups

	Number			Cell body size (μm^2)			SDH activity (OD)		
	Gamma	Alpha	Total	Gamma	Alpha	Total	Gamma	Alpha	Total
Soleus motoneurons									
Control	18 ± 3	45 ± 6	63 ± 8	328 ± 13	888 ± 12	727 ± 14	0.48 ± 0.03	0.38 ± 0.03	0.40 ± 0.03
Hyperbaric	17 ± 3	41 ± 5	59 ± 7	331 ± 15	900 ± 17	731 ± 18	0.49 ± 0.03	0.40 ± 0.03	0.42 ± 0.03
Plantaris motoneurons									
Control	23 ± 4	73 ± 9	96 ± 11	321 ± 17	922 ± 19	777 ± 22	0.48 ± 0.03	0.34 ± 0.03	0.38 ± 0.03
Hyperbaric	25 ± 4	74 ± 8	99 ± 9	322 ± 19	909 ± 22	759 ± 24	0.48 ± 0.03	0.37 ± 0.03	0.40 ± 0.03

Values are expressed as mean ± S.D. from 5 animals. Gamma and alpha motoneurons are divided by their cell body sizes at 500 μm^2. SDH, succinate dehydrogenase; OD, optical density.

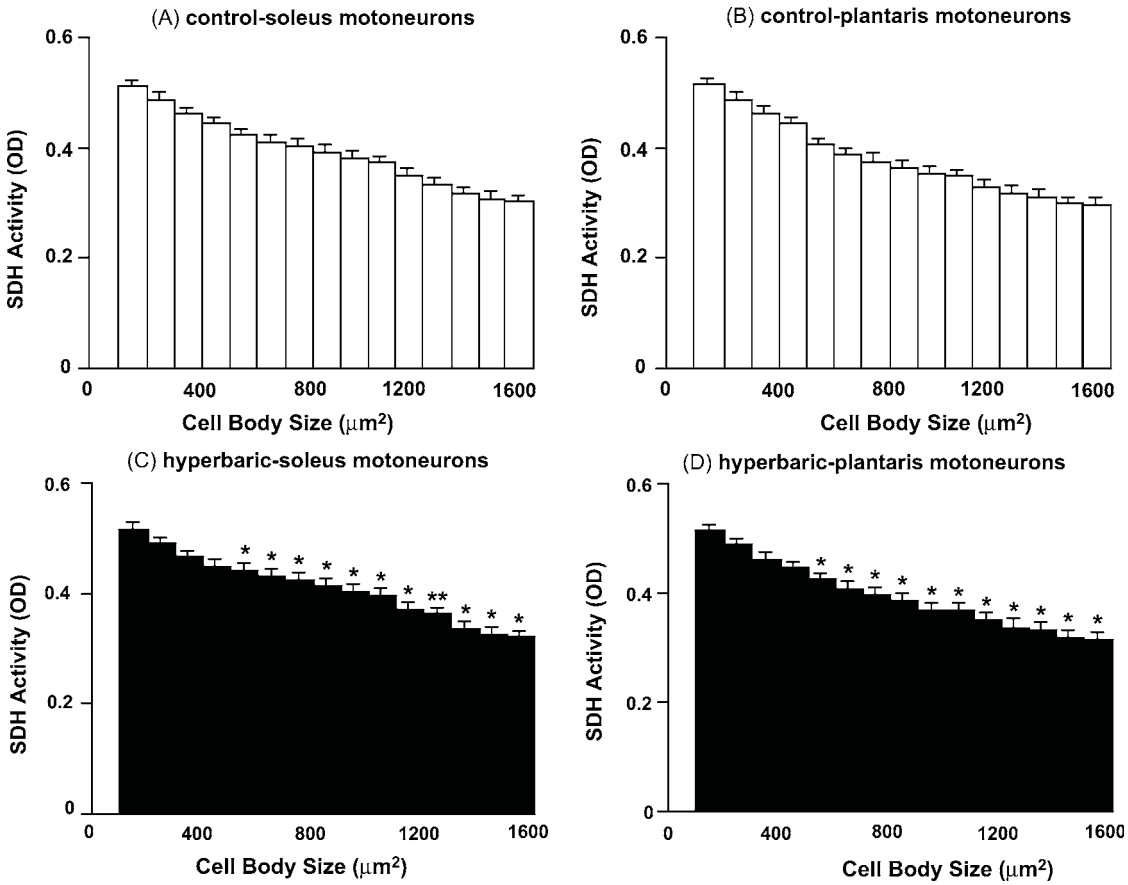

Fig. 3. Distributions of succinate dehydrogenase activities of motoneurons innervating the soleus (A and C) and plantaris (B and D) muscles of the control (A and B) and hyperbaric (C and D) groups. Values are expressed as mean and standard deviation from 5 animals, and grouped in 100 μm intervals of cell body size. SDH, succinate dehydrogenase; OD, optical density. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control value at the same cell body size.

after inactivity induced by chronic tetrodotoxin (a selective channel blocker) administration to the rat sciatic nerve. Donselaar et al. (1986) observed no change in the oxidative enzyme activity of motoneurons following anti-dromic electrical stimulation of the common peroneal nerve in cats. Furthermore, the cell body size and oxidative enzyme activity of motoneurons innervating the plantaris muscle in cats were unaffected following functional overload of the plantaris muscle by removal of the major synergists, gastrocnemius and soleus muscles (Chalmers et al., 1991).

In contrast, exposure to microgravity decreased oxidative enzyme activity of small-sized alpha motoneurons innervating the high-oxidative fibers in rat skeletal muscle, especially antigravity muscle (Ishihara et al., 1996, 2000b, 2002). The high-oxidative fibers in antigravity muscles, e.g., the soleus muscle, atrophied and type-shifted the most by exposure to microgravity. Hypobaric hypoxia (460 Torr, equivalent to an altitude of 4000 m) enhanced oxidative enzyme activity of small-sized alpha motoneurons innervating the high-oxidative fibers in the slow soleus and fast extensor digitorum longus muscles in rats (Ishihara et al., 1990; Taguchi et al., 1990). Hypoxia causes an increase in the capillary and mitochondrial densities, myoglobin concentration, and fiber oxidative enzyme activity of skeletal muscle (Ishihara et al., 2000a). It is considered that these changes in the neuromuscular unit are due to adaptations for maintenance of adequate levels of tissue oxygenation under hypoxic conditions.

Increased atmospheric pressure enhances the partial pressure of oxygen and dissolves more oxygen into the blood and plasma. Therefore, it is reasonable to expect that an increased delivery of oxygen and increased amount of oxygen induced by hyperbaric exposure with high oxygen concentration would greatly increase the oxidative enzyme activity in the neuromuscular unit. As expected, the present study showed that the oxidative enzyme activity of alpha motoneurons increased after hyperbaric exposure with high oxygen concentration, irrespective of their cell body sizes and the muscle fiber types that they innervate (Fig. 3) although the mean oxidative enzyme activities of entire motoneurons innervating the soleus and plantaris muscles were not changed by hyperbaric exposure with high oxygen concentration (Table 1). It is of great interest to note that the oxidative enzyme activity of alpha motoneurons increased after hyperbaric exposure with high oxygen concentration, irrespective of their cell body sizes, because previous studies (Ishihara et al., 1990; Taguchi et al., 1990) showed that the oxidative enzyme activity of only small-sized

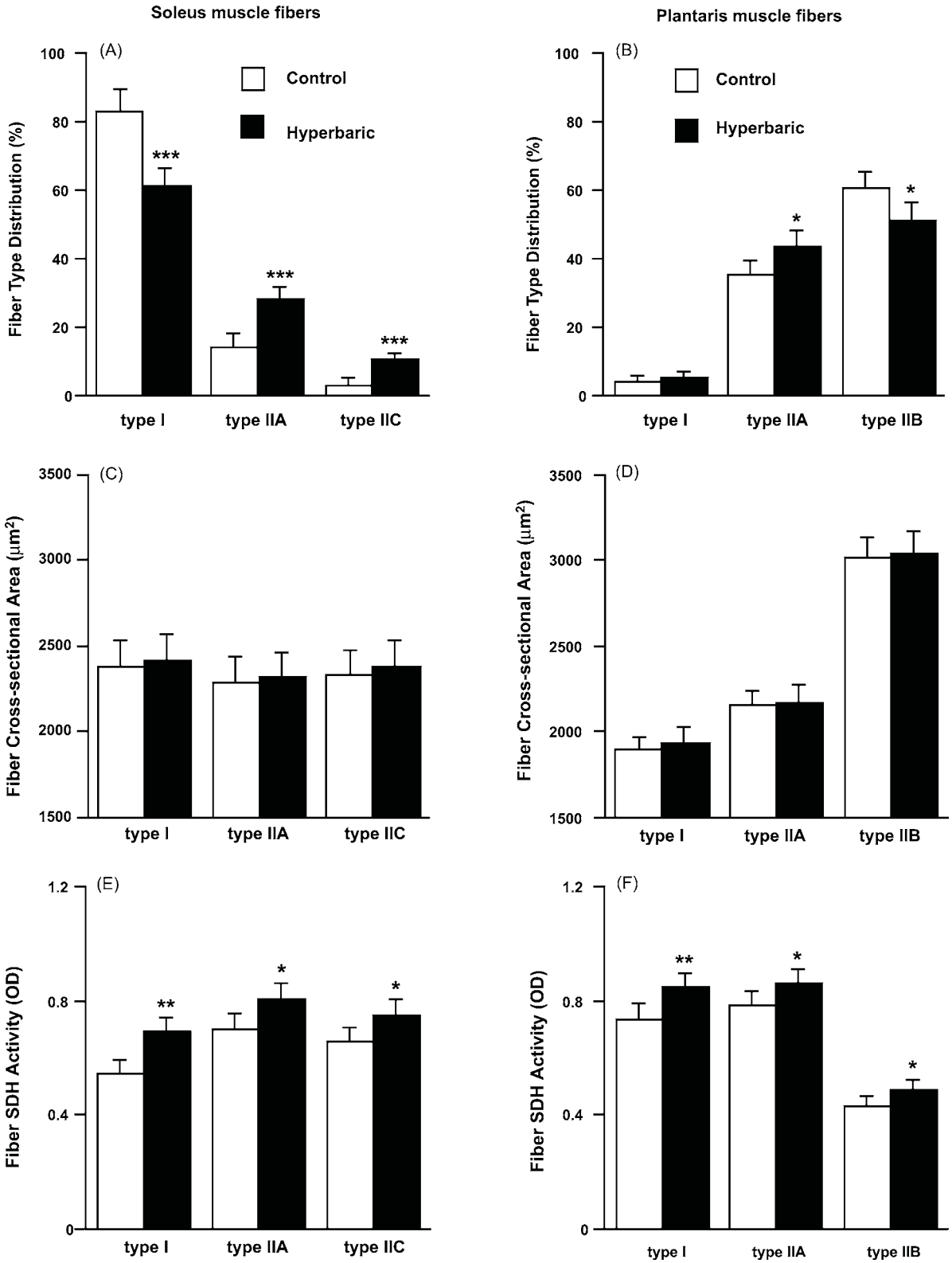

Fig. 4. Fiber type distributions (A and B), cross-sectional areas (C and D), and succinate dehydrogenase activities (E and F) of the soleus (A, C, and E) and plantaris (B, D, and F) muscles in the control and hyperbaric groups. Values are expressed as mean and standard deviation from 5 animals. SDH, succinate dehydrogenase; OD, optical density. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control value.

alpha motoneurons was increased by hypobaric hypoxia. When subjected to hypobaric hypoxia, the adaptations of motoneurons and muscle fibers in the neuromuscular unit were matched, i.e., the oxidative enzyme activities of slow-twitch, high-oxidative fibers and of small-sized alpha motoneurons innervating those fibers were increased by hypobaric hypoxia.

The mechanisms remain unclear for the increased oxidative enzyme activity of alpha motoneurons, irrespective of their cell body sizes, following hyperbaric exposure with high oxygen concentration. It is suggested that hyperbaric exposure with high oxygen concentration used in the present study was adequate to stimulate and enhance the oxidative enzyme activity of entire alpha motoneurons. The increased oxidative enzyme activities of all sizes of alpha motoneurons by hyperbaric exposure with high oxygen concentration corresponded with those observed in the muscle fibers that they innervate, i.e., the oxidative enzyme activities of all types of fibers in the soleus and plantaris muscles were increased by hyperbaric exposure with high oxygen concentration (Fig. 4).

In summary, an increase in the pressure of oxygen results in more oxygen being dissolved in the blood and plasma, making the oxygen available for diffusion into the tissues and offering an increased oxidative capacity in the neuromuscular unit. Therefore, the present study showed that hyperbaric exposure (1.25 atmospheric pressure) with high oxygen concentration (35.0%) induced an enhancement of the oxidative enzyme activity of alpha motoneurons and the muscle fibers that they innervate.

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Effects of Hyperbaric Exposure with High Oxygen Concentration on the Physical Activity of Developing Rats

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Key Words
High oxygen concentration · Hyperbaric exposure · Oxidative capacity · Physical activity of developing rat · Skeletal muscle fiber · Spinal motoneuron

Abstract
The effects of hyperbaric exposure with high oxygen concentration on the physical activity of developing male rats were investigated. Five-week-old male rats were exposed to an atmospheric pressure of 1.25 with an oxygen concentration of 36.0% for 12 h (7.00–19.00 h) and exercised voluntarily for 12 h (19.00–7.00 h) daily for 8 weeks. The voluntary running activities were compared with those in age-matched rats without hyperbaric exposure. In addition, the properties of the soleus and plantaris muscle fibers and their spinal motoneurons were examined. The voluntary running activities of rats with or without hyperbaric exposure increased during development. However, the mean voluntary running activities were higher in rats with hyperbaric exposure (7,104 m/day) than in those without hyperbaric exposure (4,932 m/day). The oxidative capacities of the soleus and plantaris muscle fibers and their spinal motoneurons increased following hyperbaric exposure. It is suggested that adaptations of neuromuscular units to hyperbaric exposure with high oxygen concentration enhance the metabolism, and thus, the function of neuromuscular units is promoted.

Introduction

We developed a specially designed hyperbaric chamber for animal experiments [Ishihara et al., 2005]. It is an oxygen compression tank with an oxygen concentrator. The hyperbaric chamber is designed to automatically maintain increased levels of atmospheric pressure and oxygen concentration. The increased atmospheric pressure enhances the partial pressure of oxygen and dissolves more oxygen into the blood and plasma. The increased oxygen enhances the metabolism in cells of peripheral tissues, e.g., enhanced oxidative enzyme activity in the mitochondria and increased energy expenditure in the cells. In addition, more carbon dioxide, which is increased by an enhancement in the cell metabolism, facilitates oxygen release from hemoglobin and dilates vessels and capillaries.

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Skeletal muscle fibers are classified into several types based on their histochemical properties [Hori et al., 1998]. In addition, spinal motoneurons innervating the skeletal muscle fibers have specific morphological and metabolic properties [Ishihara et al., 2000a]. The properties of muscle fibers and their spinal motoneurons in the neuromuscular units are matched under normal conditions. Small-sized, high-oxidative α-motoneurons presumably innervate slow-twitch, high-oxidative fibers, while large-sized, low-oxidative α-motoneurons innervate fast-twitch, low-oxidative fibers.

Our recent study [Ishihara et al., 2005] revealed that hyperbaric exposure (1.25 atm) with high oxygen concentration (35.0%) enhances the oxidative capacity of all types of muscle fibers and their spinal motoneurons. The present study was designed to assess whether or not the increased oxidative capacity of muscle fibers and their spinal motoneurons has a measurable effect on physical activity, since our previous study [Ishihara et al., 2005] showed that hyperbaric exposure with high oxygen concentration caused elevations in the oxidative capacity of neuromuscular units.

Materials and Methods

Animals and Treatments

All procedures were approved by the University Committee for the Care and Use of Animals for research purposes and followed the Guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals.

Five-week-old male Wistar rats were assigned to control groups with (C-Ex, n = 8) or without voluntary running exercise (C, n = 8) or to hyperbaric groups with (H-Ex, n = 8) or without voluntary running exercise (H, n = 8). The rats in the hyperbaric groups (H and H-Ex) were exposed to an atmospheric environment for 12 h (7.00–19.00 h) daily for 8 weeks, while the rats in the exercise groups (C-Ex and H-Ex) exercised for 12 h (19.00–7.00 h) daily for 8 weeks. All rats were individually housed in cages of the same size (30 × 20 × 13 cm). The rats in the hyperbaric groups were exposed to an atmospheric pressure of 1.25 with an oxygen concentration of 35.0%, automatically maintained by a computer-assisted system [Ishihara et al., 2005]. The hyperbaric chamber was 180 cm long and 70 cm in diameter. The size of the chamber allows multiple rats (up to 20 cages) to be housed simultaneously. The rats in the exercise groups exercised voluntarily on running wheels with no load, which we developed [Ishihara et al., 1998]. Food and water were provided ad libitum for all groups. The rats were kept in a controlled environment with fixed 12-hour light/12-hour dark cycles (lights off from 19.00 to 07.00 h) and a room temperature maintained at 22 ± 2°C.

Injection of Neuronal Tracer

Eight weeks later, the rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) for injection of the fluorescent tracer, nuclear yellow, into the right soleus and left plantaris muscles [Ishihara et al., 1991, 1995]. After the muscle was exposed, a 2% solution of the fluorescent tracer, nuclear yellow, was injected into the muscle using a microsyringe. The fluorescent tracer was injected at multiple sites to ensure distribution throughout the muscle. Care was taken to inject the fluorescent tracer slowly and prevent leakage. After the fluorescent tracer had been injected into the muscle, the skin was sutured and the rats were allowed to recover from anesthesia. The volumes of injected fluorescent tracer were 10 and 15 μl for the soleus and plantaris muscles, respectively. After 2 days, the rats were sacrificed by an overdose of sodium pentobarbital.

Analyses

The bilateral soleus and plantaris muscles and the lumbosacral enlargement of the spinal cord were removed and frozen immediately in isopentane cooled with liquid nitrogen. Serial transverse sections of the left soleus and right plantaris muscles, 10 μm thick, were cut in a cryostat set at –20°C. The sections were brought to room temperature, air-dried for 30 min, and then stained for adenosine triphosphate (ATPase) activity following acid (pH 4.3 and 4.5) and alkaline (pH 10.4) preincubation [Hirofuji et al., 2000; Nakatani et al., 1999, 2000]. The muscle fibers were classified into type I (positive at preincubation, pH 4.3 and 4.5, and negative at preincubation, pH 10.4), type IIA (negative at preincubation, pH 4.3 and 4.5, and positive at preincubation, pH 10.4), type IIB (negative at preincubation, pH 4.3, and positive at preincubation, pH 4.5 and 10.4), and type IIC (positive at preincubation, pH 4.3, 4.5 and 10.4). The fiber type distribution of the soleus muscle was determined from the entire transverse section of the muscle, while that of the plantaris muscle was determined from approximately 500 fibers in the middle region of the transverse section of the muscle.

The sections were also stained for succinate dehydrogenase (SDH) activity, an indicator of mitochondrial oxidative potential [Hirofuji et al., 2000; Nakatani et al., 1999, 2000]. The SDH activities of approximately 500 fibers from each muscle were determined on digitized images of stained sections. The SDH staining intensities of individual muscle fibers were measured using a computer-assisted image processing system (Neuroimaging System, Kyoto, Japan).

Serial longitudinal sections of the lumbosacral enlargement, 10 μm thick, were cut in a cryostat set at –20°C. The motoneurons innervating the soleus and plantaris muscles were identified by golden-yellow fluorescence of the nucleus with nuclear yellow on the untreated sections using a fluorescent microscope. The identified motoneurons were counted in the serial sections. The same sections used to identify the motoneurons were then stained for activity of SDH, an oxidative marker enzyme, at room temperature for 10 min [Chalmers and Edgerton, 1989a and b].

The cross-sectional areas of the identified motoneurons in which the nucleus was visible were measured from the SDH-stained sections using a computer-assisted image processing system (Neuroimaging System) [Ishihara et al., 1997a, 2002b]. Motoneurons with a cell body size greater than 500 μm^2 were presumed to be α-motoneurons innervating extrafusal muscle fibers. Smaller motoneurons were presumed to be γ-motoneurons innervating intrafusal muscle fibers. The cutoff for the distribution between γ- and α-motoneurons was primarily based on convention [Peyronnard et al., 1986; Sickles and McLendon, 1983; Sickles and Oblak, 1984; Swett et al., 1986].

Statistics

Means and standard deviations were calculated from individual values using standard procedures. Student's t test was used to determine significant differences between the control and hyperbaric groups. Differences were considered significant at the 0.05 level of confidence.

Results

Voluntary Running Distances

The voluntary running distances of the C-Ex and H-Ex groups increased during development (fig. 1). The mean voluntary running distances during an 8-week running exercise period were higher in the H-Ex group (7,104 ± 285 m/day, n = 8; $p < 0.05$) than in the C-Ex group (4,932 ± 169 m/day, n = 8).

Body and Muscle Weights

There were no differences in the mean body weight among the C (392 ± 16 g, n = 8), C-Ex (379 ± 18 g, n = 8), H (387 ± 17 g, n = 8), or H-Ex groups (378 ± 19, n = 8). Similarly, there were no differences in the mean soleus or plantaris muscle weight among the C, C-Ex, H, or H-Ex groups (fig. 2). The mean soleus and plantaris muscle weights per unit body weight were higher in the C-Ex and H-Ex groups than in the C and H groups, while there were no differences in the mean soleus or plantaris muscle weight per unit body weight between the C and H groups or between the C-Ex and H-Ex groups.

Skeletal Muscle Fibers

In the soleus muscle, the mean percentage of type I fibers was lower and those of type IIA and type IIC fibers were higher in the H and H-Ex groups than in the C and C-Ex groups (fig. 3). There were no differences in the mean fiber type distribution of the muscle between the C and C-Ex groups or between the H and H-Ex groups.

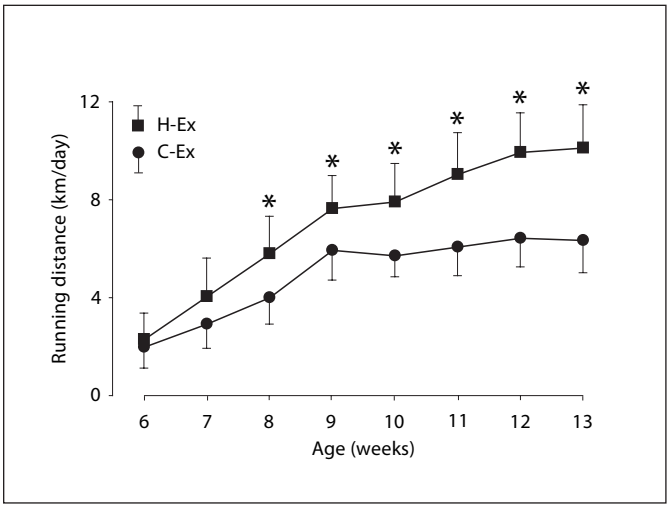

Fig. 1. Voluntary running distances on wheels of rats with (H-Ex) or without (C-Ex) hyperbaric exposure. Values are expressed as mean and standard deviation from 8 animals. * $p < 0.05$, compared with the values of the C-Ex group.

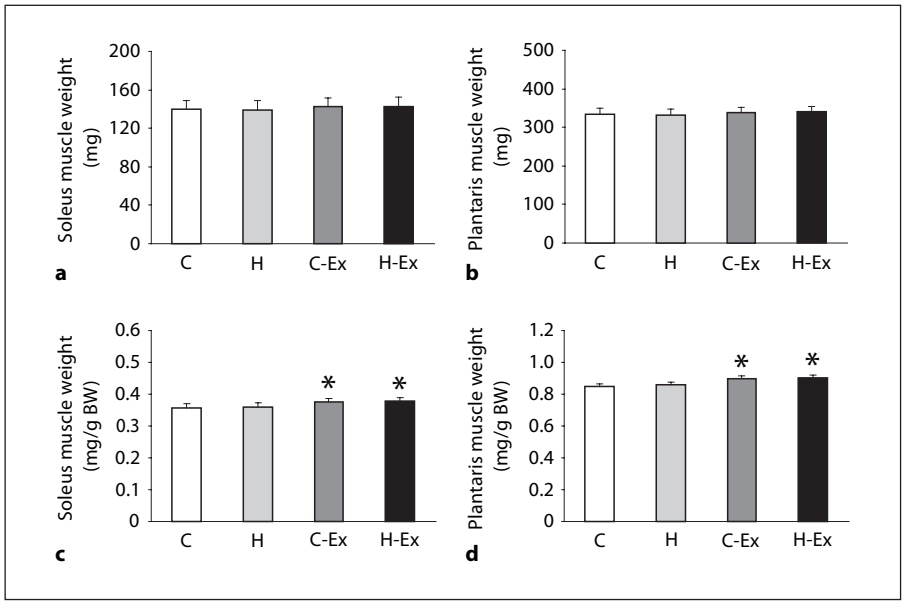

Fig. 2. Soleus (**a**, **c**) and plantaris (**b**, **d**) muscle weights. BW = Body weight. Values are expressed as mean and standard deviation from 8 animals. * $p < 0.05$, compared with the values of the C and H groups.

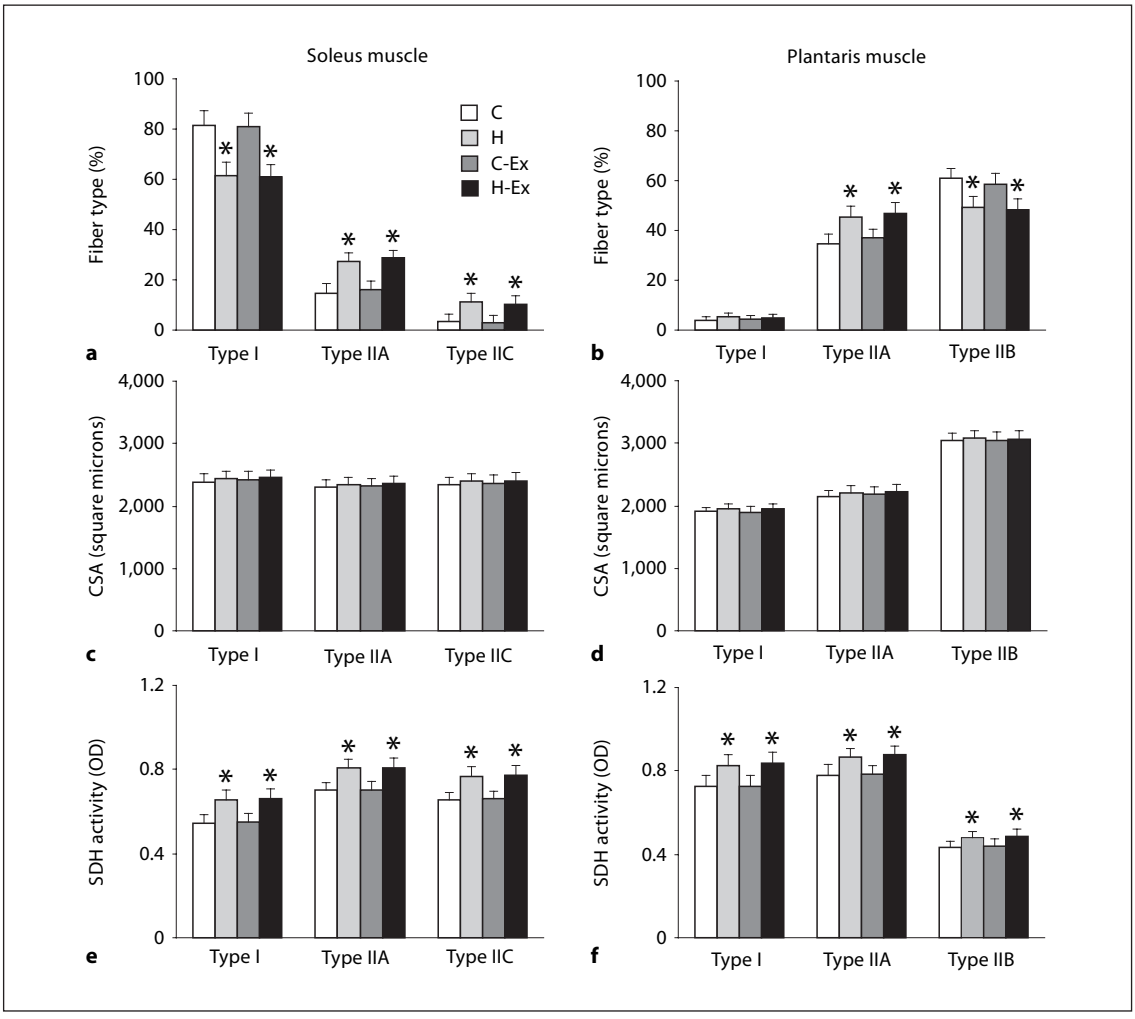

Fig. 3. Fiber type distributions (**a**, **b**), cross-sectional areas (**c**, **d**), and SDH activities (**e**, **f**) of the soleus and plantaris muscles. CSA = Cross-sectional area; OD = optical density. Values are expressed as mean and standard deviation from 8 animals. * $p < 0.05$, compared with the values of the C and C-Ex groups.

In the plantaris muscle, the mean percentage of type IIA fibers was higher and that of type IIB fibers was lower in the H and H-Ex groups than in the C and C-Ex groups (fig. 3). There were no differences in the mean fiber type distribution of the muscle between the C and C-Ex groups or between the H and H-Ex groups.

There were no differences in the mean fiber cross-sectional area of the soleus or plantaris muscle among the C, C-Ex, H, or H-Ex groups (fig. 3).

The mean fiber oxidative enzyme activities in the soleus and plantaris muscles were higher in the H and H-Ex groups than in the C and C-Ex groups, irrespective of the fiber types (fig. 3). There were no differences in the mean fiber oxidative enzyme activity of the soleus or plantaris muscle between the C and C-Ex groups or between the H and H-Ex groups.

Spinal Motoneurons

There were no differences in the mean number or cell body size of γ, α, or entire (γ + α) motoneurons innervating the soleus or plantaris muscle among the C, C-Ex, H, or H-Ex groups (fig. 4). There were no differences in the mean oxidative enzyme activity of γ or entire (γ + α) motoneurons innervating the soleus or plantaris muscle among the C, C-Ex, H, or H-Ex groups. The mean oxidative enzyme activities of α-motoneurons innervating the

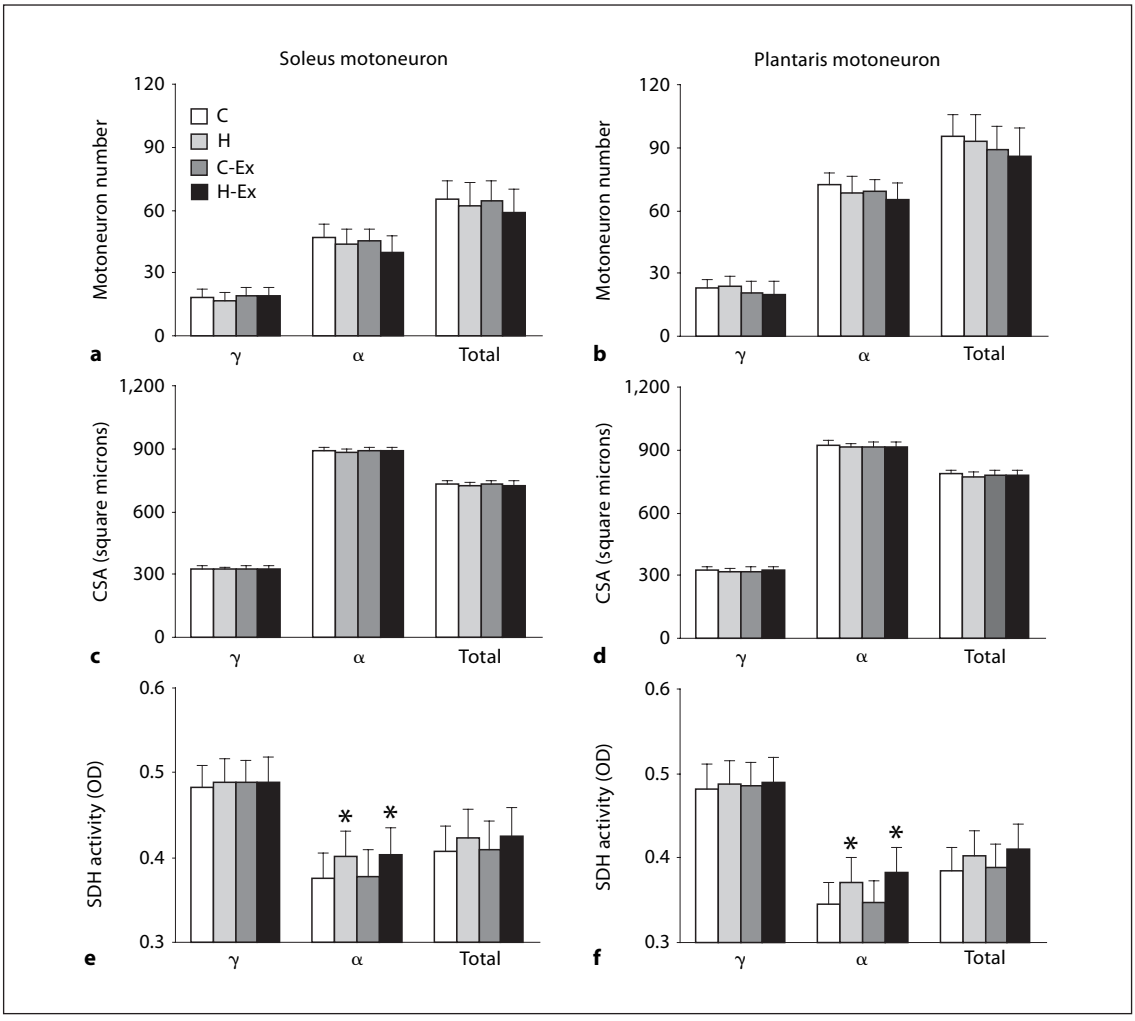

Fig. 4. Motoneuron numbers (**a**, **b**), cell body sizes (**c**, **d**), and SDH activities (**e**, **f**) of γ, α, and entire (γ + α) motoneurons innervating the soleus and plantaris muscles. CSA = Cross-sectional area; OD = optical density. Values are expressed as mean and standard deviation from 8 animals. * $p < 0.05$, compared with the values of the C and C-Ex groups.

soleus and plantaris muscles were higher in the H and H-Ex groups than in the C group, while there were no differences in the mean oxidative enzyme activity of α-motoneurons innervating the soleus or plantaris muscle between the C and C-Ex groups or between the H and H-Ex groups.

Discussion

Rats are a highly active species and run spontaneously on wheels at their own pace if given the opportunity [Mondon et al., 1985; Munoz et al., 1994; Rodnick et al., 1989; Russell et al., 1987; Shyu et al., 1984]. These studies indicate that rats spontaneously run distances greater than normally performed during forced treadmill exercise. In addition, voluntary running has no physical or psychological stress, i.e. no electrical shock, loud sound, air blasts, or food deprivation and reward to keep rats running at a constant level. Voluntary running results primarily in aerobic adaptations in the skeletal muscles [Lambert and Noakes, 1990; Rodnick et al., 1989; Sexton, 1995], but some hypertrophy has been reported in the slow soleus muscles of male [Rodnick et al., 1990a] and female rats [Munoz et al., 1994]. The fast plantaris muscle appears to induce hypertrophy in female [Munoz et al.,

1994], but not in male [Rodnick et al., 1989, 1990a, b] rats in response to voluntary running. In the present study, the voluntary running distances of both the C-Ex and H-Ex groups increased during development. These results are consistent with those in a previous study [Ishihara et al., 1998]. However, no exercise effects of voluntary running on the neuromuscular properties were observed because there were no differences in the properties of muscle fibers or spinal motoneurons between the nonexercised C and exercised C-Ex groups or between the nonexercised H and exercised H-Ex groups. The increased voluntary running distances of the H-Ex group compared with the C-Ex group are considered to be due to the effects of hyperbaric exposure with high oxygen concentration. Therefore, it is concluded that the increased running distance in the H-Ex group was an indirect indicator of elevated neuromuscular endurance capacity by the hyperbaric exposure with high oxygen concentration.

Previous studies [Chalmers et al., 1991, 1992; Donselaar et al., 1986; Ishihara et al., 1997b, 2004; Roy et al., 1999, 2001; Seburn et al., 1994] showed no changes in morphological or metabolic properties in spinal motoneurons following chronic decrease (e.g., spinal cord transaction, spinal cord isolation, immobilization, or hindlimb unloading) or increase (e.g., exercise, functional overload, electrical stimulation, or hypergravity) in the neuromuscular activity, while fiber atrophy or hypertrophy and/or type shift of fibers in the skeletal muscles were observed following chronic decrease or increase in the neuromuscular activity. In contrast, the oxidative capacity of small-sized α-motoneurons innervating the high-oxidative fibers in the skeletal muscles, especially the antigravity muscles, decreased following exposure to microgravity [Ishihara et al., 1996, 2000a, b, 2006]. The high-oxidative fibers in the antigravity muscles, e.g., the soleus muscle, atrophied and type-shifted the most by exposure to microgravity. Similarly, hypobaric hypoxia (460 mmHg, equivalent to an altitude of 4,000 m) enhanced the oxidative capacity of small-sized α-motoneurons innervating the high-oxidative fibers in the slow soleus and fast extensor digitorum longus muscles [Ishihara et al., 1990; Taguchi et al., 1990]. The percentage of high-oxidative fibers in the skeletal muscles was increased by hypobaric hypoxia. It is considered that these changes in the neuromuscular units are due to adaptations for maintenance of adequate levels of tissue oxygenation under microgravity or hypoxic condition.

Increased atmospheric pressure enhances the partial pressure of oxygen and dissolves more oxygen into the blood and plasma (Henry's law). The increased oxygen (tissue hyperoxygenation) enhances the metabolism in cells and tissues. In addition, more carbon dioxide, which was increased by an enhancement in the cell metabolism, facilitates oxygen release from hemoglobin and enlarges vessels and capillaries (Bohr effect). Therefore, it was expected that the increased amount and delivery of oxygen induced by hyperbaric exposure with high oxygen concentration would greatly enhance the cell metabolism and therefore increase the oxidative capacity in the neuromuscular units. In the present study, the oxidative capacities in the slow soleus and fast plantaris muscle fibers and their spinal motoneurons increased following hyperbaric exposure with high oxygen concentration.

The mechanisms for the increased voluntary running activity of the H-Ex group remain unclear. A previous study [Harrison et al., 2002] observed that the muscle fiber type composition was related to the voluntary running activity level, because myosin heavy chain IIX or IIB null mice showed a lesser activity level than wild-type mice. In addition, a higher voluntary running activity level was observed in fast-twitch, high-oxidative fiber-dominant rats, which were developed by selective breeding until the 8th generation [Suwa et al., 2003]. These studies suggest that muscle fiber type composition affects the physical activity level.

It is suggested that the hyperbaric exposure with high oxygen concentration used in the present study was adequate to enhance the oxidative capacity in the neuromuscular units and that the increased oxidative capacity in neuromuscular units stimulated and enhanced the voluntary running activities of individual rats in the H-Ex group. Previous studies [Huang et al., 2005; Shirasawa et al., 2003] yielded similar results using model mice that carry low-affinity hemoglobin with Titusville mutation in the α-globin gene or Presbyterian mutation in the β-globin gene. These mutant mice had a higher percentage of high-oxidative fibers in the fast tibialis anterior muscle and an increased oxidative capacity of spinal motoneurons innervating the tibialis anterior muscle. In addition, the mutant mice spontaneously ran twice as far as controls (wild-type mice). These data suggest that, as the result of adaptation to the tissue hyperoxygenation, energy metabolism in the neuromuscular units of the mutant mice is augmented, and thus, the function of the neuromuscular units is promoted.

In summary, it is concluded that hyperbaric exposure (1.25 atm) with high oxygen concentration (36.0%) induces an enhancement of the oxidative capacity in α-motoneurons and the muscle fibers they innervate. It is suggested that voluntary running activities of rats exposed to hyperbaric environment with high oxygen concentration are promoted by the increased oxidative capacity in the neuromuscular units.

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ABSTRACT: The effects of hyperbaric exposure with high oxygen concentration on glucose and insulin levels and skeletal muscle-fiber properties were investigated in type 2 diabetic Goto-Kakizaki rats. Five-week-old rats were exposed to a hyperbaric environment (1.25 atmospheric pressure) with a high oxygen concentration (36%) for 6 h daily. Glucose and insulin levels and properties including fiber-type distribution, cross-sectional area, and oxidative enzyme activity in the soleus muscle were examined after hyperbaric exposure for 4 weeks. The growth-related increase in glucose level was inhibited by hyperbaric exposure, and insulin also showed lower levels compared with control rats. The percentage of low-oxidative type I fibers in the muscle decreased and high-oxidative type IIA and type IIC fibers, which were not detected in the muscle of control rats, were observed after hyperbaric exposure. The oxidative enzyme activity of type I fibers in the muscle increased after hyperbaric exposure. Hyperbaric exposure with high oxygen concentration might therefore provide a new approach to improve the glucose tolerance, insulin resistance, and altered skeletal muscle metabolism that are caused by diabetes mellitus.

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EFFECTS OF HYPERBARIC EXPOSURE WITH HIGH OXYGEN CONCENTRATION ON GLUCOSE AND INSULIN LEVELS AND SKELETAL MUSCLE-FIBER PROPERTIES IN DIABETIC RATS

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Skeletal muscles are comprised of heterogeneous types of fibers that have different functional, morphological, and metabolic properties.[7,20] Patients with type 2 diabetes mellitus have altered patterns of fiber types in the skeletal muscles, i.e., a decreased percentage of high-oxidative fibers in the skeletal muscles.[5,7,22] Our previous studies[29,30] revealed that Otsuka Long–Evans Tokushima Fatty (OLETF) and Goto–Kakizaki (GK) rats, animal models of spontaneous type 2 diabetes mellitus that have hyperglycemia and insulin resistance, have a lower percentage of high-oxidative fibers in the skeletal muscles than age-matched nondiabetic rats. Skeletal muscle is a major target of insulin-stimulated glucose uptake. Therefore, altered patterns of fiber types in the skeletal muscles of patients and animal models with type 2 diabetes mellitus may be linked to glucose tolerance and insulin resistance.

We have designed a hyperbaric chamber for animal experiments, which is an oxygen tank with an oxygen concentrator and an air compressor[9] that automatically maintain the elevated atmospheric pressure and oxygen concentration. Increased atmospheric pressure enhances the partial pressure of oxygen and causes more oxygen to dissolve into the blood and plasma.

Our recent study[28] observed that hyperbaric exposure with high oxygen concentration inhibited a growth-related increase in the glucose level of GK

Abbreviations: ATPase, adenosine triphosphatase; GK, Goto–Kakizaki; GLUT, glucose transporter; IRI, immunoreactive insulin; OD, optical density; SDH, succinate dehydrogenase

Key words: glucose; high oxygen concentration; hyperbaric exposure; muscle oxidative capacity; type 2 diabetes mellitus

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rats. We postulated that the increased availability of oxygen induced by hyperbaric exposure might have a beneficial impact on the metabolism of skeletal muscles, for example, on oxidative enzyme activity, which might be related to improvements in glucose tolerance and insulin resistance. In the present study we tested this hypothesis by exposing GK rats to a hyperbaric environment with high oxygen concentration for a period of 4 weeks, and then determined glucose and insulin levels and closely examined the fiber-type distribution, cross-sectional area, and oxidative enzyme activity of fibers in the soleus muscle.

MATERIALS AND METHODS

Animals and Treatments. All procedures were approved by our institutional review committee and followed US national guidelines.

GK rats are animal models of type 2 diabetes mellitus, developed by selective breeding of an outbred colony of Wistar rats with high glucose levels as measured by the oral glucose tolerance test.[4] They were selected for the present study because they have elevated levels of glucose, but not of insulin, and they do not become obese.[1,11,23,24]

Five-week-old male Wistar ($n = 10$) or GK ($n = 10$) rats were randomly assigned to control ($n = 5$) or hyperbaric ($n = 5$) groups. All rats were individually housed in cages of the same size. The rats in the hyperbaric group were exposed to an atmospheric pressure of 1.25 with an oxygen concentration of 36% automatically maintained by a computer-assisted system. The chamber was 180 cm long and 70 cm in diameter, making it large enough to house a number of rats (up to 20 cages) simultaneously.[9] Rats in the hyperbaric group were exposed to the hyperbaric environment for 6 h (10:00 to 16:00) daily for 4 weeks. Food and water were provided ad libitum for both groups. All rats were kept in a controlled environment with fixed 12:12-h light:dark cycles (lights off from 19:00 to 07:00) and room temperature maintained at 22 ± 2°C. Food intake in a 24-h period was measured.

Tissue Preparation. The rats were weighed and anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The soleus muscle was removed, cleaned of excess fat and connective tissue, and wet-weighed. White adipose tissue including epididymal, omental, and retroperitonal fat was surgically removed and weighed. The total weight of these three types of tissues was taken to be the white adipose tissue weight.

Measurements of Fasting Plasma Glucose and Insulin. Plasma obtained by centrifugation was used for measurements of glucose and immunoreactive insulin

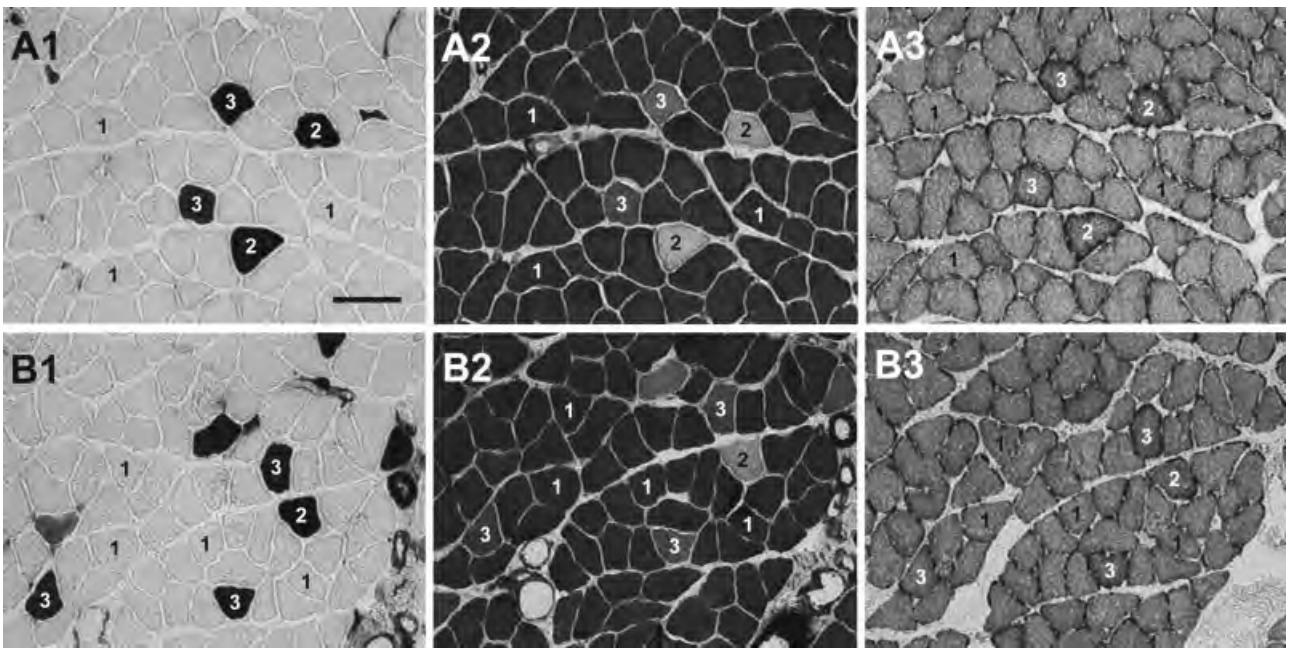

FIGURE 1. Transverse sections of the soleus muscle in nondiabetic Wistar rats under normobaric **(A1–A3)** and hyperbaric **(B1–B3)** conditions. **(A1,B1)** Stained for adenosine triphosphatase activity following preincubation at pH 10.4; **(A2,B2)** stained for adenosine triphosphatase activity following preincubation at pH 4.5; **(A3,B3)** stained for succinate dehydrogenase activity. 1, type 1; 2, type IIA; 3, type IIC. Scale bar, 100 μm.

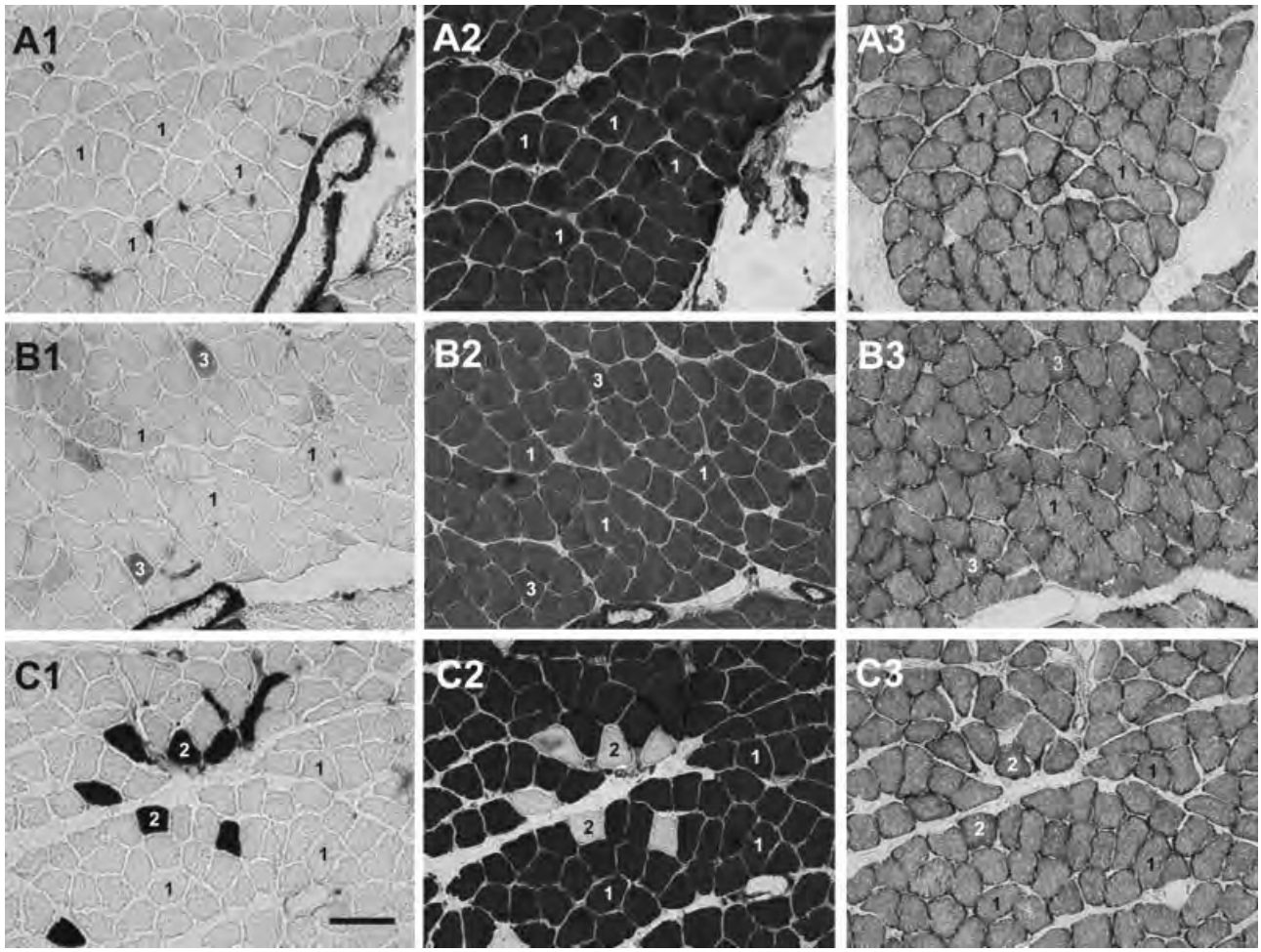

FIGURE 2. Transverse sections of the soleus muscle in diabetic Goto-Kakizaki rats under normobaric **(A1–A3)** and hyperbaric **(B1–B3,C1–C3)** conditions. **(A1,B1,C1)** Stained for adenosine triphosphatase activity following preincubation at pH 10.4; **(A2,B2,C2)** stained for adenosine triphosphatase activity following preincubation at pH 4.5; **(A3,B3,C3)** stained for succinate dehydrogenase activity. 1, type 1; 2, type IIA, 3, type IIC. Scale bar, 100 μm. All fibers in the muscle of GK rats under normobaric conditions were type I **(A1–A3)**. Two of five GK rats under hyperbaric conditions had only type I fibers in the muscle, whereas two GK rats had type I and type IIC fibers **(B1–B3)**. One rat under hyperbaric conditions had type I and type IIA fibers in the muscle **(C1–C3)**.

(IRI) levels. Plasma glucose was determined by a glucose oxidative method[17] on blood samples obtained from the tail veins at 5, 7, and 9 weeks of age. Plasma IRI was determined by a radioimmunoassay using a polyethylene glycol method with rat plasma insulin as the standard[27] on blood samples obtained from the abdominal aorta at 9 weeks of age.

Histochemical Procedures. The muscle was placed on cork, stretched to its in vivo length, and immediately frozen in isopentane cooled in a mixture of dry ice and acetone. Serial 10-μm thick transverse sections of the muscle were cut in a cryostat set at −20°C. The sections were brought to room temperature, air-dried for 30 min, then stained for adenosine triphosphatase (ATPase) activity following acid (pH 4.3 and 4.5) and alkaline (pH 10.4) preincubation (Figs. 1, 2).[6,18,19] The muscle fibers were classified into type I (positive at preincubation pH 4.3 and 4.5, and negative at preincubation pH 10.4), type IIA (negative at preincubation pH 4.3 and 4.5, and positive at preincubation pH 10.4), and type IIC (positive at preincubation pH 4.3, 4.5, and 10.4). The fiber-type distribution of the muscle was determined from the entire transverse section of the muscle.

The sections were also stained for succinate dehydrogenase (SDH) activity, an indicator of mitochondrial capacity (Figs. 1, 2).[6,18,19] Tissue sections were digitized as gray scale images and the value of the SDH staining intensity was expressed as an optical density (OD) value on a computer-assisted image processing system (Neuroimaging System, Kyoto, Japan).[8,10] Each pixel was quantified as one of 256 gray

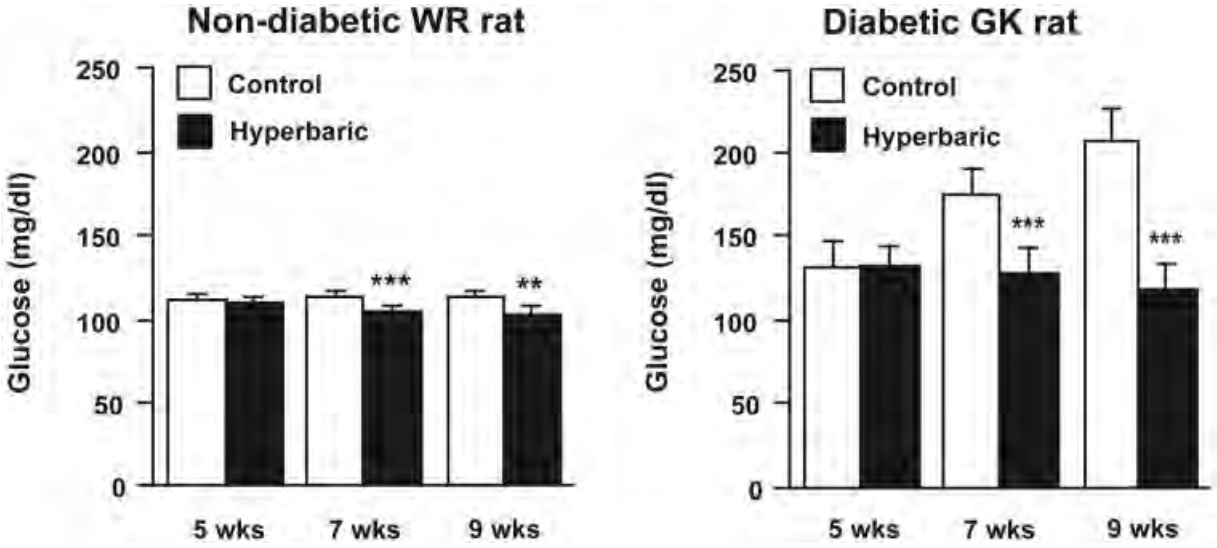

FIGURE 3. Glucose levels of nondiabetic Wistar (WR) and diabetic Goto–Kakizaki (GK) rats. Values are expressed as mean ± standard deviation ($n = 5$). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control value.

levels. A gray value of zero was equivalent to 100% transmission of light, and that of 255 was equivalent to 0% transmission of light. The OD units of all pixels within the muscle fiber were converted to a mean OD unit using a calibration photographic tablet, which has 21-step gradient density ranges of diffused density values.

Statistics. Means and standard deviations were calculated from individual values using standard procedures. Student's *t*-test was used to determine significant differences between the control and hyperbaric groups.

RESULTS

Body Weight and Food Intake. The body weights of Wistar rats at 9 weeks of age in the control and hyperbaric groups were 365.8 ± 24.6 g and 364.6 ± 19.2 g, respectively, and those of GK rats at 9 weeks of age in the control and hyperbaric groups were 217.4 ± 14.2 g and 206.8 ± 4.5 g ($n = 5$ for all groups), respectively. There was no difference in body weight of Wistar or GK rats between the control and hyperbaric groups.

The food intakes of Wistar rats at 9 weeks of age in the control and hyperbaric groups were 30.8 ± 3.8 g/day and 29.0 ± 3.3 g/day, respectively, and those of GK rats at 9 weeks of age in the control and hyperbaric groups were 9.5 ± 1.1 g/day and 9.0 ± 1.2 g/day ($n = 5$ for all groups), respectively. Wistar or GK rats in the control and hyperbaric groups had equivalent levels of food intake.

White Adipose Tissue Weight. The white adipose tissue weights of Wistar rats in the control and hyperbaric groups were 7.07 ± 2.89 g and 7.16 ± 1.28 g, respectively, and those of GK rats in the control and hyperbaric groups were 3.53 ± 0.69 g and 3.85 ± 0.57 g ($n = 5$ for all groups), respectively. There was no difference in white adipose tissue weight of Wistar or GK rats between the control and hyperbaric groups.

Fasting Plasma Glucose and Insulin Levels. The fasting plasma glucose levels of Wistar and GK rats were significantly lower in the hyperbaric groups at 7 and 9 weeks of age than in the control groups (Fig. 3).

The fasting plasma IRI levels of Wistar rats at 9 weeks of age in the control and hyperbaric groups were 1707.6 ± 526.7 pg/ml and 1514.5 ± 631.6 pg/ml, respectively, and those of GK rats at 9 weeks of age in the control and hyperbaric groups were 472.1 ± 238.0 pg/ml and 123.2 ± 41.3 pg/ml ($n = 5$ for all groups), respectively. The fasting plasma IRI levels of GK rats were significantly lower ($P < 0.05$) in the hyperbaric than control group, but there was no difference in fasting plasma IRI level of Wistar rats between the control and hyperbaric groups.

Soleus Muscle Weight. The muscle weights of Wistar rats in the control and hyperbaric groups were 0.13 ± 0.02 g and 0.13 ± 0.02 g, and those of GK rats in the control and hyperbaric groups were 0.08 ± 0.01 g and 0.09 ± 0.01 g ($n = 5$ for all groups),

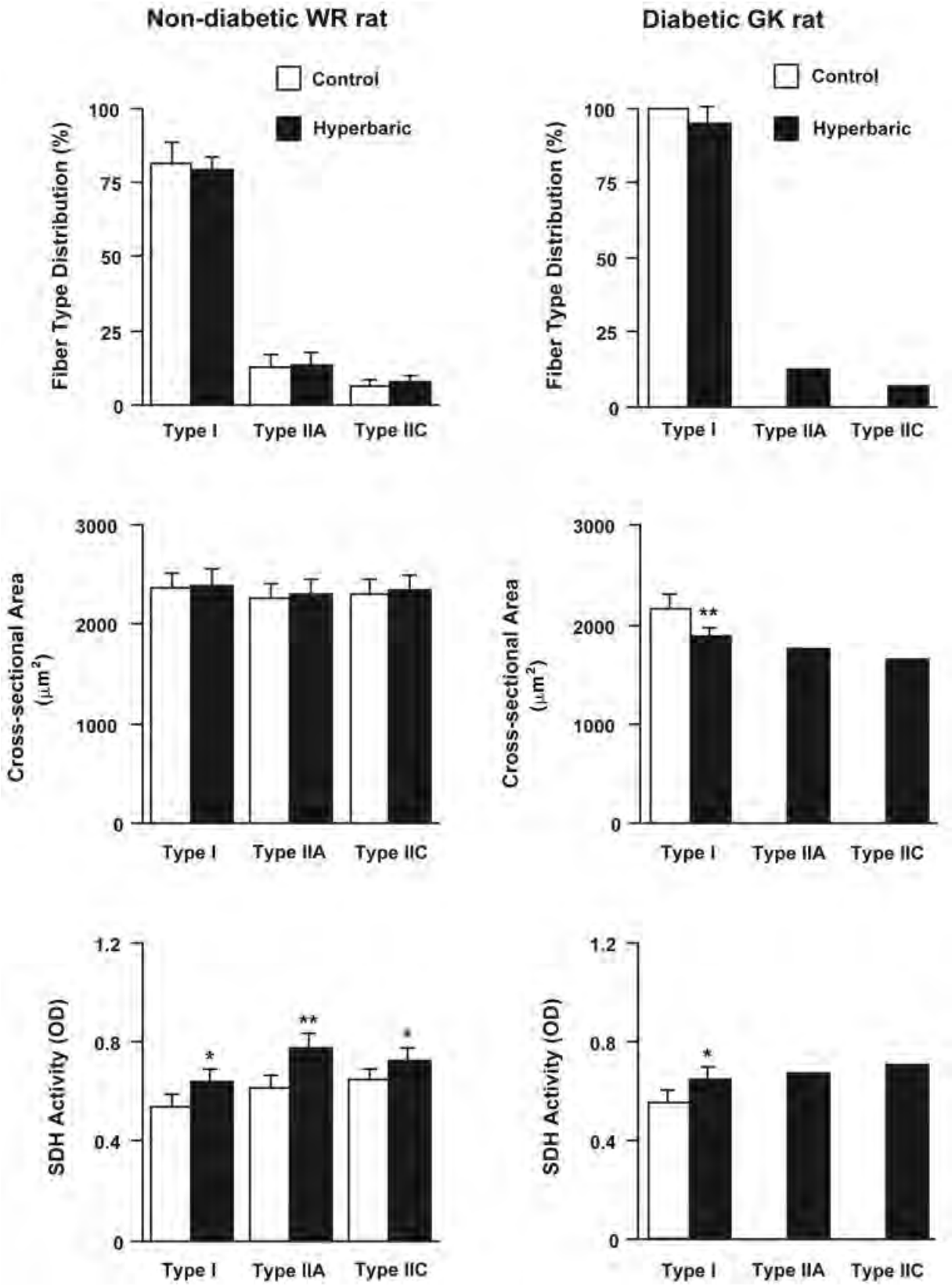

FIGURE 4. Fiber-type distributions, cross-sectional areas, and succinate dehydrogenase activities of the soleus muscle in nondiabetic Wistar (WR) and diabetic Goto–Kakizaki (GK) rats. SDH, succinate dehydrogenase; OD, optical density. Values are expressed as mean ± standard deviation [$n = 5$, except for type IIA ($n = 1$) and type IIC ($n = 2$) in diabetic GK rats of the hyperbaric group]. *$P < 0.05$, **$P < 0.01$ compared with control value.

respectively. There was no difference in muscle weight of Wistar or GK rats between the control and hyperbaric groups.

Soleus Muscle Fiber Properties. In Wistar rats there was no difference in fiber-type distribution or cross-sectional area between the control and hyperbaric groups (Fig. 4). The oxidative enzyme activities of all types of fibers were significantly higher in the hyperbaric than control group (Fig. 4).

In GK rats all fibers in the muscles of the control group were type I (Figs. 2, 4). The muscles of two rats in the hyperbaric group were composed of only type I fibers (Fig. 4). The muscles of two other rats in the hyperbaric group were composed of type I (94.0% and 92.5%) and type IIC (6.0% and 7.5%) fibers, whereas that of the other rat in this group contained type I (87.7%) and type IIA (12.3%) fibers. The cross-sectional area of type I fibers in the muscle was significantly smaller in the hyperbaric than control group, whereas the oxidative enzyme activity of type I fibers was significantly higher in the hyperbaric than control group (Fig. 4).

DISCUSSION

Skeletal muscle plays an important role in the regulation of blood glucose because it is the site with the highest level of insulin-stimulated glucose uptake and disposal.[25] It is largely accepted that type 2 diabetes mellitus is associated with impaired insulin-stimulated glucose disposal capacity, which is attributed to insulin resistance in skeletal muscle. Patients with type 2 diabetes mellitus have disrupted metabolic potentials and different patterns of fiber types in the skeletal muscles compared with nondiabetic subjects.[5,16,22] Diabetes has been associated with a high percentage of low-oxidative fibers (particularly type IIB fibers) and a low percentage of high-oxidative fibers in the fast skeletal muscles, such as the biceps femoris, vastus lateralis, and rectus abdominis muscles. We observed similar changes in the fiber-type distribution of both the fast plantaris and slow soleus muscles in diabetic rats.[29,30] Previous studies[2,3] suggested that a decreased percentage of high-oxidative fibers in the skeletal muscles combined with a reduction in glucose transporter (GLUT)-4 expression in high-oxidative fibers reduces the insulin-sensitive GLUT-4 pool in patients with type 2 diabetes mellitus and contributes to skeletal muscle insulin resistance. These results strongly indicate that changes in the fiber-type distribution of skeletal muscles in diabetic rats are due to an impairment in insulin sensitivity and glucose metabolism.

Consistent with our hypothesis that the increased availability of oxygen induced by hyperbaric exposure with high oxygen concentration has a beneficial impact on glucose and insulin levels and the metabolism of skeletal muscles, we observed that a growth-related increase in glucose level of GK rats was completely inhibited by hyperbaric exposure (Fig. 3). These findings are consistent with those in our recent study.[28] In addition, the insulin level was significantly lower in the hyperbaric than control group. Hyperbaric exposure with high oxygen concentration might therefore provide a new approach to improve glucose tolerance and insulin resistance.

In the present study, we examined the soleus muscle because it has a higher percentage of high-oxidative fibers, which are more insulin sensitive and responsive than low-oxidative fibers.[12,13,15] In addition, high-oxidative fibers are characterized by increased fatty acid oxidation, low glycolytic capacity, and high triglyceride accumulation compared with low-oxidative fibers.[14,21] A previous study[26] also observed that skeletal muscle insulin resistance in GK rats is associated with high-oxidative fiber-specific defects in the insulin-signal transduction pathway to glucose transport, suggesting that hyperglycemia affects high-oxidative fibers more severely than low-oxidative fibers, and that it selectively reduces GLUT-4 expression in high-oxidative fibers in patients and animal models with type 2 diabetes mellitus. In the present study, hyperbaric exposure with high oxygen concentration prevented diabetes-associated changes in the fiber-type distribution of the soleus muscle in GK rats. In addition, we observed that the type I fiber oxidative enzyme activity of the soleus muscle in GK rats increased after hyperbaric exposure. These results suggest that the increase in oxidative capacity of skeletal muscles is an adaptive response to hyperbaric exposure with high oxygen concentration.

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ORIGINAL ARTICLE

Effects of exposure to hyperbaric oxygen on oxidative stress in rats with type II collagen-induced arthritis

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Abstract Arthritis was induced in 9-week-old female Dark Agouti rats by injecting type II collagen. Serum levels of the derivatives of reactive oxygen metabolites (dROMs), which are oxidative stress markers, and C-reactive protein (CRP) in arthritic rats that were exposed to a pressure of 1.25 atmospheres absolute and an oxygen concentration of 36% for 3 weeks (arthritis + HBO group) were compared to those of control rats (control group) and arthritic rats that were not exposed to hyperbaric oxygen (arthritis group). The body weights of the arthritis and arthritis + HBO groups were lower than that of the control group, whereas no difference in the body weight was observed between the arthritis and arthritis + HBO groups. The serum levels of dROMs and CRP in the arthritis group were higher than those in the control and arthritis + HBO groups. No difference in the serum level of CRP was observed between the control and arthritis + HBO groups. These results indicate that the conditions of hyperbaric oxygen exposure used in this study are effective for reducing the levels of reactive oxygen species, which are overproduced during arthritis.

Keywords C-reactive protein · Derivatives of reactive oxygen metabolites · Hyperbaric oxygen exposure · Rat · Reactive oxygen species · Type II collagen-induced arthritis

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Introduction

Highly reactive and toxic transient chemical species, referred to as reactive oxygen species (ROS), are overproduced by phagocytes during the development and progression of inflammatory processes and pathogenesis in a number of diseases [1]. The excessive production of ROS can damage cellular components such as nucleic acids, lipids, proteins, membranes, and matrix components. In addition, ROS serve as important intracellular signaling and regulatory molecules that enhance the synovial inflammatory–proliferative response [2]. Oxygen metabolism plays an important role in the pathogenesis of many joint diseases. The potential sources of ROS production are numerous in the case of joint diseases. In degenerative joint diseases, proinflammatory factors such as cytokines and prostaglandins are released at sites of inflammation and destruction together with ROS [3]. The production of tumor necrosis factor (TNF)-α contributes to excessive ROS release and causes substantial reduction in the activity of antioxidant enzymes in inflammatory joints. Several other proinflammatory cytokines, including interleukin (IL)-1, IL-6, and IL-8, are produced by synovial cells; these cytokines recruit neutrophils into the synovial fluids and exert their effects by activating the intracellular signaling pathways [4]. C-reactive protein (CRP) is a

marker of inflammation and destruction of tissues such as cartilage and bone. The serum level of CRP correlates with the production of derivatives of reactive oxygen metabolites (dROMs), which are oxidative stress markers [5, 6].

An elevation in atmospheric pressure accompanied by an increase in oxygen concentration enhances the partial pressure of oxygen and increases the concentration of dissolved oxygen in the plasma. We determined that a pressure of 1.25 atmospheres absolute (ATA) and an oxygen concentration of 36% are required for obtaining effective responses with regard to oxidative metabolism [7, 8]. Previous studies [9, 10] have reported low levels of partial pressure of oxygen in the synovial fluid of arthritic joints. These joints are characterized by hypoxia, which is caused by increased oxygen demand and decreased blood flow [11–13]. Hypoxic conditions induce the production of proinflammatory cytokines and excessive ROS release in inflammatory joints. We hypothesized that increased pressure and enhanced delivery and uptake of oxygen induced by exposure to hyperbaric oxygen are effective in reducing arthritis-induced inflammation because exposure to hyperbaric oxygen may prevent and improve hypoxic conditions in inflammatory joints.

In this study, we compared the serum levels of dROMs, TNF-α, IL-6, and CRP in arthritic rats that were exposed to hyperbaric oxygen to those of control rats and arthritic rats that were not exposed to hyperbaric oxygen. We verified that the conditions of hyperbaric oxygen exposure used in this study are effective for decreasing the levels of ROS, which are overproduced during arthritis.

Materials and methods

All experimental procedures and animal care were conducted in accordance with the guidelines stated in the Guide for the Care and Use of Laboratory Animals issued by the Institutional Animal Experimentation Committee of Kyoto University.

Experimental animals

Bovine type II collagen (CII) was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. Dissolved CII was frozen at a temperature of −70°C until use. We immunized 9-week-old female Dark Agouti (DA) rats ($n = 12$), which are susceptible to induction of adjuvant arthritis, with an emulsion of 2 mg/ml of CII in incomplete Freund's adjuvant (IFA). The emulsions were prepared by homogenizing one part of dissolved CII into one part of IFA placed in an ice-water bath. The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight); thereafter, 0.2 ml of the emulsion was intradermally injected into 3–5 other sites located on the back of each rat. Following injection, half of these rats (arthritis + HBO group; $n = 6$) were exposed to a pressure of 1.25 ATA (950 mmHg) and an oxygen concentration of 36%, automatically maintained by a computer-assisted system, for 3 weeks, while the other rats (arthritis group; $n = 6$) were placed in a hyperbaric chamber under normal conditions (1 ATA (760 mmHg) and 21% oxygen concentration); therefore, this group was not exposed to hyperbaric oxygen. Nonimmunized DA rats (control group; $n = 6$) served as controls. All rats were individually housed in same-sized cages in a room maintained under controlled 12 h light–dark cycles (lights switched off from 2000 to 0800 hours) at a temperature of 22 ± 2°C with a relative humidity of 45–65%. Food and water were provided ad libitum to all groups. The body weight of each rat was measured every week. The pictures of the paws in all rats were taken when they were 12 weeks old. The arthritis severity was determined by means of visual examination, on the basis of the number of instances of knuckle swelling (0 point, no sign; 1 point, 1 or 2 digits; 2 points, 3 digits; 3 points, 4 digits; and 4 points, all digits), and the degree of arch edema (0 point, no sign; 1 point, light; 2 points, mild; 3 points, heavy; and 4 points, severe). The arthritis score of each rat was calculated by determining the average of the points assigned to individual paws. Therefore, the lowest arthritis score corresponded to 0 point, while the highest arthritis score corresponded to 8 points.

Blood sampling and biochemical measurements

Following 12 h of fasting, blood samples were collected from the abdominal aorta of the rats anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The blood samples were centrifuged (6,000*g*; 1 min) and evaluated photometrically. We used a device capable of determining the levels of free radicals and antioxidant potentials (Free Radical Analytical System 4; Health & Diagnostics; Grosseto, Italy) to measure the serum level of dROMs [14]. The serum level of dROMs was used as an index to determine the level of oxidative stress induced by measuring the amount of organic hydroperoxide (ROOH) converted into radicals that oxidize *N*,*N*-diethyl-para-phenylenediamine. The serum levels of TNF-α (R&D Systems, Inc.; MN, USA), IL-6 (R&D Systems, Inc.) and CRP (Helica Biosystems, Inc.; CA, USA) were measured by a routine laboratory method using the enzyme-linked immunosorbent assay (ELISA) kit.

Statistical analysis

Values are presented as mean and standard deviation. One-way analysis of variance (ANOVA) was used to evaluate the differences among groups. When the differences were found to be significant, further comparisons were made by performing *post hoc* tests. A probability level of 0.05 was considered to be statistically significant.

Results

Body weight

No difference in the body weight was observed among the control, arthritis, and arthritis + HBO groups at 9, 10, and 11 weeks of age (Fig. 1). In contrast, the body weights of the 12-week-old arthritis and arthritis + HBO groups were lower than that of the age-matched control group.

Arthritis severity

The rats in the control group were not arthritic (Fig. 2, A1–6). In contrast, the rats in the arthritis (Fig. 2, B1–6) and arthritis + HBO (Fig. 2, C1–6) groups presented with knuckle swelling and/or arch edema. No difference in the arthritis score was observed between the arthritis (6.0 ± 2.3, $n = 6$) and arthritis + HBO (4.8 ± 1.6, $n = 6$) groups.

Serum level of dROMs

The serum levels of dROMs in the arthritis and arthritis + HBO groups were higher than that in the control group (Fig. 3a). The serum level of dROMs in the arthritis + HBO group was lower than that in the arthritis group.

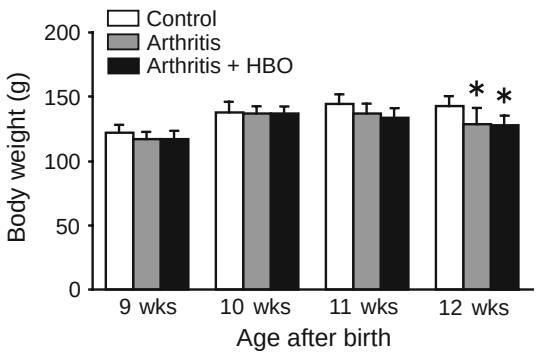

Fig. 1 The body weights of control rats and arthritic rats that were and were not exposed to hyperbaric oxygen. Data are presented as mean and standard deviation as determined from six animals. *HBO* hyperbaric oxygen exposure. $^*P < 0.05$ compared to age-matched control rats

Serum levels of TNF-α, IL-6, and CRP

Neither TNF-α nor IL-6 was detected in the rats, irrespective of whether they were arthritic and were exposed to hyperbaric oxygen. The serum level of CRP in the arthritis group was higher than those in the control and arthritis + HBO groups (Fig. 3b). No difference in the serum level of CRP was observed between the control and arthritis + HBO groups.

Discussion

Type II collagen-induced arthritis

Mice and rats with type II collagen-induced arthritis are widely used as experimental animal models of inflammatory polyarthritis with clinical and pathological features similar to those of rheumatism [15, 16]. The DA rats are susceptible to induction of adjuvant arthritis. Arthritis is observed in 74 and 100% DA rats at 2 and 3 weeks after immunization, respectively (SLC Inc.; Tokyo, Japan). The body weights of DA rats are observed to gradually decrease following immunization; the body weights are the lowest at 3 weeks after immunization and subsequently recover to the control level (SLC Inc.). In this study, we examined certain parameters of DA rats at 3 weeks after immunization because all immunized rats demonstrated high severity of arthritis, including the signs of knuckle swelling and/or arch edema (Fig. 2, B1–6). These arthritic rats may undergo further changes leading to cartilage and bone destruction when studied for a prolonged period; similar results were observed in a previous study using mice with type II collagen-induced arthritis [17].

Serum level of dROMs in arthritic rats

Oxidative stress is the condition in which the production of oxidants exceeds the capacity to neutralize them. Several factors could be involved in the generation of oxidative stress in inflammatory joints [18, 19]. ROS are produced during many metabolic processes, including mitochondrial respiration and enzyme activities. ROS concentrations are regulated by maintaining the balance between their production and elimination by antioxidants. An appropriate balance is crucial for normal cell and tissue function. In contrast, the excessive production of ROS damages cellular components, including nucleic acids, lipids, proteins, membranes, and constituents of the extracellular matrix such as proteoglycans and collagens [2]. Previous studies [20, 21] have reported that excessive production of ROS causes an accelerated damage to joint cartilage and osteoclast activation. We observed that the serum levels of

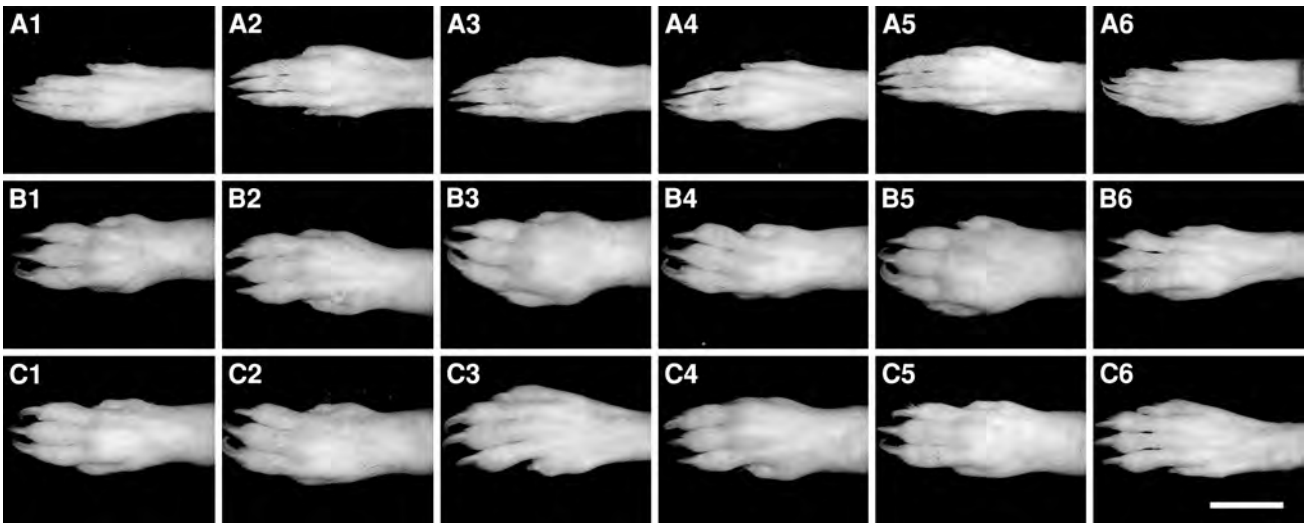

Fig. 2 The hind-paws of control (*A1–6*) and arthritic rats that were (*C1–6*) and were not (*B1–6*) exposed to hyperbaric oxygen. The *scale bar* on *C6* indicates 1 cm

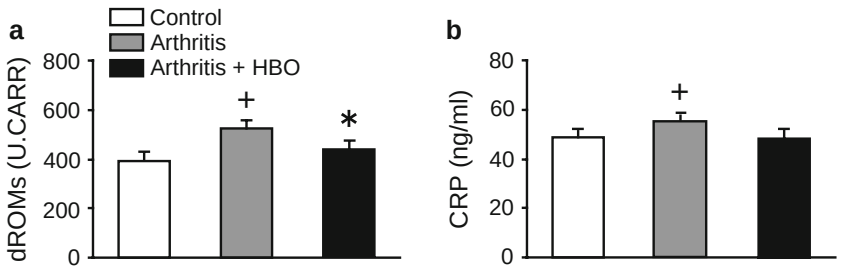

Fig. 3 The serum levels of derivatives of reactive oxygen metabolites (**a**) and C-reactive proteins (**b**) in control rats and arthritic rats that were and were not exposed to hyperbaric oxygen. Data are presented as mean and standard deviation as determined from six animals. *dROMs* derivatives of reactive oxygen metabolites; *U.CARR* unit of Carratelli, which is a conventional unit named after an Italian biologist who developed a scale based on the observations from a group of more than 5,000 nonsmoking healthy subjects with age ranging from 14 to 80 years (1 U.CARR = 0.08 mg of hydroperoxide/100 ml hydrogen peroxide); *CRP* C-reactive protein; *HBO* hyperbaric oxygen exposure. $^{*}P < 0.05$ compared to control rats; $^{+}P < 0.05$ compared to control and arthritis + HBO rats

dROMs, which serve as a marker of oxidative stress, were higher in arthritic rats than in control ones (Fig. 3A); this finding suggests that arthritis induces an excessive production of ROS.

Serum levels of TNF-α, IL-6, and CRP in arthritic rats

Several proinflammatory cytokines (TNF-α, IL-1, IL-6, and IL-8) are produced by synovial cells and recruit neutrophils to the synovial fluids in arthritic rats. However, we detected neither TNF-α nor IL-6 in the DA rats with arthritis. Previous studies [22, 23] have reported increased levels of TNF-α and IL-1β in Wistar or Lewis rats with type II collagen-induced arthritis. We were unable to elucidate the reason underlying the inability to detect cytokines in arthritic DA rats.

We observed increased serum levels of CRP in arthritic rats (Fig. 3b). Previous studies [24, 25] have reported that the serum level of CRP correlated with that of proinflammatory cytokines such as IL-6. Increased serum levels of CRP are observed in inflammatory diseases [26]. Therefore, the increased serum levels of CRP reflect the arthritis severity, including signs of knuckle swelling and/or arch edema (Fig. 2, B1–6).

Major drugs for arthritis

It is widely accepted that reduction of oxidative stress by superoxide dismutase (SOD) affects the course of inflammation. Several studies [27–30] have focused on synthetic low-molecular weight compounds that mimic the effects of SOD. Among the various families of SOD mimetics, the most promising are nitroxides (tempol) and Mn(II) penta-azamacrocyclic ligand (M40403). Tempol diminishes hydroxyl radical production and decreases the cytotoxic effects of hydrogen peroxide and peroxynitrite [27].

Furthermore, it decreases inflammation and tissue damage in rats with type II collagen-induced arthritis. M40403 decreases the release of proinflammatory cytokines such as TNF-α, probably by inhibiting the expression of the transcription factor, nuclear factor (NF)-κB [28]. The beneficial effects of M40403 have been reported in rats with collagen-induced arthritis [29, 30].

Treatment with 3,7,11,15-tetramethyl-2-hexadecene-1-ol (phytol) increases oxidative burst in vivo, decreases the autoimmune response, and ameliorates both the acute and chronic phase of arthritis in rats [31]. Furthermore, treatment with alpha-lipoic acid (LA), which is a cofactor for mitochondrial α-keto dehydrogenase complexes and which participates in S–O transfer reactions, suppresses the development of collagen-induced arthritis in mice. A previous study [32] reported that the amelioration of joint diseases by LA treatment was associated with reduction in oxidative stress, as well as with the inhibition of inflammatory cytokine activation and NF-κB DNA binding activity. Furthermore, LA-induced decreased intracellular ROS in lymphocytes obtained from the inguinal lymph nodes of arthritic mice and prevented bone destruction in vivo and osteoclastogenesis in vitro.

Effects of exposure to hyperbaric oxygen

Hyperbaric oxygen therapy leads to new vasoconstriction and hyperoxygenation, making it an effective treatment option for various clinical disorders [33–35]. This treatment involves administration of 100% oxygen concentration at a pressure greater than atmospheric pressure at sea level, usually equivalent to 2–3 ATA. However, a standard procedure of hyperbaric oxygen therapy is known to cause excessive production of ROS in several tissues and organs [33, 34], suggesting that oxidative stress, which is induced by hyperbaric oxygen therapy, accelerates the tissue damage.

We determined that a pressure of 1.25 ATA and an oxygen concentration of 36% are required for obtaining effective responses with regard to oxidative metabolism [7, 8]. In this study, we exposed the rats to moderate atmospheric pressure and oxygen concentration when compared to those exposed to classical hyperbaric oxygen therapy with 100% oxygen concentration at 2 ATA; we observed that these conditions of hyperbaric oxygen exposure inhibit the excessive production of ROS. Furthermore, these conditions of hyperbaric oxygen exposure are more cost-effective and safe; i.e. treatment at these conditions is not associated with the risk of accidents such as eardrum split. We observed that an excessive production of ROS in spontaneously hypertensive rats was inhibited by exposure to hyperbaric oxygen with 36% oxygen concentration at 1.25 ATA [36].

Intraarticular pressure is high in inflammatory joints because of decreased compliance of the joint wall, which is attributable to synovial membrane swelling and capsule fibrosis [37]. Other factors contributing to increased intraarticular pressure include movement and accumulation of synovial fluids in chronically involved joints [10]. Together with the reduced capillary density, this elevated pressure could decrease the rate of capillary blood flow and induce lowering of oxygen tension in synovial fluids and repetitive ischemia–reperfusion injury in the inflammatory joint [11–13]. This suggests that arthritic joints are characterized by hypoxia, which is caused by increased oxygen demand and decreased blood flow induced by increased intraarticular pressure. These observations show that a potential therapeutic approach for treating arthritis would be to enhance the partial pressure of oxygen and increase the levels of dissolved oxygen for the elimination of ROS. An increase in atmospheric pressure and oxygen concentration enhances the partial pressure of oxygen; this increases the oxygen tension in the affected tissues and the concentration of dissolved oxygen in the plasma, thus enhancing the activity of oxidative enzymes in the mitochondria and, consequently, the rate of oxidative metabolism in cells and tissues. Previously, we reported that the beneficial effects of hyperbaric oxygen exposure with 36% oxygen concentration at 1.25 ATA were associated with an increased oxidative enzyme activity in skeletal muscle fibers and spinal motoneurons in rats [7, 8]. In addition, the possible beneficial effects of exposure to hyperbaric oxygen have been reported; the increase in blood glucose level, which is related to growth, of type 2 diabetic rats has been reported to be inhibited by exposure to hyperbaric oxygen [38–40]. We observed that the levels of dROMs decreased in arthritic rats following exposure to hyperbaric oxygen (Fig. 3a); this indicated that the conditions of hyperbaric oxygen exposure used in this study are effective for decreasing the activity of ROS, which are overproduced in arthritic rats. However, further studies are warranted to elucidate whether these effects of exposure to hyperbaric oxygen persist over prolonged periods. In addition, clinical trials must be performed to evaluate the efficacy of exposure to hyperbaric oxygen for the treatment of arthritic patients.

The arthritis scores did not differ between the arthritic rats that were and were not exposed to hyperbaric oxygen (Fig. 2). This result indicates that the effects of exposure to hyperbaric oxygen on the dROM and CRP levels (Fig. 3) did not correspond with those on the morphological profiles of individual paws in arthritic rats. Morphological effects of exposure to hyperbaric oxygen on rats may be observed if they were treated for a prolonged period; in this study, we sacrificed the rats only 3 weeks after immunization. Further studies are required to clarify time-dependent

effects of exposure to hyperbaric oxygen on the morphological profiles of paws in arthritic rats.

Conclusion

A minor but significant decrease was induced in the serum levels of dROMs and CRP in arthritic rats after exposure to hyperbaric oxygen. We conclude that the conditions of hyperbaric oxygen exposure used in this study are effective to decrease the activity of ROS, which are overproduced at an early stage of arthritis.

Conflict of interest statement The authors declare that they have no conflict of interest related to the publication of this manuscript.

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Hyperbaric Oxygen Exposure Improves Blood Glucose Level and Muscle Oxidative Capacity in Rats with Type 2 Diabetes

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Abstract

Background: The effects of exposure to hyperbaric oxygen on blood glucose level and muscle oxidative capacity in rats with type 2 diabetes were investigated.
Methods: Five-week-old male Goto-Kakizaki rats were divided into four groups: normobaric (NN; exposed to 21% oxygen at 760 mm Hg for 8 weeks), hyperbaric to normobaric (HN; exposed to 36% oxygen at 950 mm Hg for 4 weeks, followed by 21% oxygen at 760 mm Hg for 4 weeks), normobaric to hyperbaric (NH; exposed to 21% oxygen at 760 mm Hg for 4 weeks, followed by 36% oxygen at 950 mm Hg for 4 weeks), and hyperbaric (HH; exposed to 36% oxygen at 950 mm Hg for 8 weeks).
Results: Blood glucose levels were lower in the HN, NH, and HH groups than in the NN group. Up-regulated mRNA expression levels of peroxisome proliferator-activated receptor-γ co-activator-1α were observed in the soleus muscles of the HN, NH, and HH groups and in the plantaris muscles of the HN and HH groups. The soleus muscles of the NN group contained only type I fibers, whereas those of the HN, NH, and HH groups contained type I, type IIA, and type IIC fibers. An increased percentage of type I fibers and a decreased percentage of type IIB fibers were observed in the plantaris muscles of the NH, HN, and HH groups.
Conclusions: Exposure to hyperbaric oxygen reduces high blood glucose levels and improves oxidative capacities in the skeletal muscles of rats with diabetes, and these effects are maintained under normobaric conditions even after exposure to hyperbaric oxygen.

Introduction

THE SKELETAL MUSCLES of patients with type 2 diabetes have lower oxidative capacities compared to those of healthy individuals because the skeletal muscles contain a reduced percentage of high-oxidative fibers.[1–3] Therefore, it is suggested that the lower oxidative capacity in the skeletal muscles of such patients is associated with insulin resistance and impaired glucose metabolism.

An elevation in atmospheric pressure accompanied by an increase in oxygen concentration enhances the partial pressure of oxygen and increases the concentration of dissolved oxygen in the plasma. An increase in atmospheric pressure and oxygen concentration enhances oxidative enzyme activity in mitochondria and consequently increases oxidative metabolism.[4,5] Exposure to hyperbaric oxygen reduces the growth-related increase in blood glucose level in rats with type 2 diabetes.[6] Furthermore, it inhibits both the transition of fiber types from the high-oxidative to the low-oxidative and the decrease in fiber oxidative enzyme activity in the soleus and plantaris muscles of rats with type 2 diabetes.[7,8] However, there is no evidence to clarify whether exposure to hyperbaric oxygen reduces high blood glucose levels in rats with type 2 diabetes or whether these effects are maintained under normobaric conditions even after exposure to hyperbaric oxygen.

The gene expression levels associated with oxidative metabolism in the skeletal muscles of rats with type 2 diabetes have not yet been determined. Oxidative metabolism in skeletal muscles is largely regulated by several factors such as peroxisome proliferator-activated receptor-γ (PPARγ) co-activator-1α (PGC-1α).[9] PGC-1α co-activates PPARγ, and

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therefore an increase in PGC-1α can improve insulin resistance and impaired glucose metabolism.[10]

This study investigated the effects of exposure to hyperbaric oxygen on blood glucose levels, PGC-1α mRNA expression levels, and morphological and histochemical properties of fibers in the slow soleus and fast plantaris muscles of Goto-Kakizaki (GK) rats with type 2 diabetes that exhibited high blood glucose levels. We observed that the growth-related increase in blood glucose level of GK rats was inhibited on exposure to hyperbaric oxygen.[6] Therefore, we investigated the changes in these parameters under normobaric conditions after exposure to hyperbaric oxygen.

Materials and Methods

All experimental procedures, including animal care, were conducted in accordance with the guidelines stated in the Guide for the Care and Use of Laboratory Animals issued by the Institutional Animal Experimentation Committee of Kyoto University, Kyoto, Japan.

Experimental animals

Twenty 5-week-old male GK rats were randomly assigned to four groups: normobaric (NN) group (exposed to 21% oxygen at 760 mm Hg for 8 weeks; $n=5$), hyperbaric to normobaric (HN) group (exposed to 36% oxygen at 950 mm Hg for 4 weeks, followed by 21% oxygen at 760 mm Hg for 4 weeks; $n=5$), normobaric to hyperbaric (NH) group (exposed to 21% oxygen at 760 mm Hg for 4 weeks, followed by 36% oxygen at 950 mm Hg for 4 weeks; $n=5$), and hyperbaric (HH) group (exposed to 36% oxygen at 950 mm Hg for 8 weeks; $n=5$). All rats were individually housed in same-sized cages in a room maintained under a controlled 12-h light-dark cycle (lights switched off from 20:00 to 08:00) at a temperature of $22\pm2^{\circ}$C with a relative humidity of 45–65%. Food and water were provided ad libitum to all groups.

Exposure to hyperbaric oxygen

The rats in the HN, NH, and HH groups were exposed to atmosphere at a pressure of 950 mm Hg and an oxygen concentration of 36%. To accomplish this, we designed a hyperbaric chamber for performing the animal experiments.[4] The chamber consisted of an oxygen tank containing an oxygen concentrator and air compressor. The atmospheric pressure and oxygen concentration were controlled by a computer-assisted system. We had previously determined the optimal atmospheric pressure (950 mm Hg) and oxygen concentration (36%) required for obtaining effective responses with regard to oxidative capacity in the neuromuscular system.[4,5] The hyperbaric environments were maintained for 6 h (11:00–17:00) per day for 4 (HN and NH groups) or 8 (HH group) weeks. Body weights and food intakes of individual rats were measured at 5, 9, and 13 weeks of age.

Measurements of glucose and insulin

Blood samples were obtained from the tail vein of the rats at 5, 9, and 13 weeks of age. After 12 h of fasting, blood glucose levels were determined by using the glucose oxidation method.[11] Furthermore, blood samples were obtained from the abdominal aorta of the rats at 13 weeks of age. Insulin levels were determined after 12 h of fasting by performing a radioimmunoassay with the polyethylene glycol method.[12]

Analyses of PGC-1α mRNA expression

After blood sampling, the soleus and plantaris muscles of both legs were removed. Excess fat and connective tissue were trimmed off, and the muscle wet weight was measured. Total RNA was extracted from the muscles of the left leg by using the TRIzol reagent (Invitrogen, Carlsbad, CA) and then treated with deoxyribonuclease I (Invitrogen). The first strand of cDNA was synthesized from 1.0 μg of total RNA by using the PrimeScript™ RT reagent kit (TaKaRa Bio, Otsu, Japan). To assay gene expression in the samples, we performed real-time reverse-transcription polymerase chain reaction by using the LightCycler® DX400 system (Roche Diagnostics, Mannheim, Germany) and SYBR® Premix Ex Taq II (TaKaRa Bio).[13] The following primer sets were used: PGC-1α forward, 5′-CGATG ACCCTCCTCACACCA-3′; PGC-1α reverse, 5′-TTGGCTTGA GCATGTTGCG-3′; β-actin forward, 5′-GTGACAGCATTGC TTCTGTG-3′; and β-actin reverse, 5′-AACGGTCTCACGTC AGTGTA-3′. The mRNA expression level of PGC-1α was normalized to that of β-actin.

Histochemical procedures

The soleus and plantaris muscles of the right leg were pinned onto a cork board along their in vivo length, rapidly frozen in isopentane cooled in a mixture of dry ice and acetone, and stored at −80°C until analyses. The mid portion of the muscle was mounted on a specimen chuck by using a Tissue Tek OCT compound (Sakura Finetechnical, Tokyo, Japan). Serial transverse sections, 10 μm in thickness, were cut on a cryostat at −20°C. The sections were thawed at room temperature, air-dried for 30 min, and preincubated under acidic (pH 4.3 and 4.5) and alkaline (pH 10.4) conditions for subsequent assessment of ATPase activity.[14,15] The muscle fibers in each section were classified as either type I (positive response to preincubation at pH 4.3 and 4.5, but negative response to that at pH 10.4), type IIA (negative response to preincubation at pH 4.3 and 4.5, but positive response to that at pH 10.4), type IIB (negative response to preincubation at pH 4.3, but positive response to that at pH 4.5 and 10.4), or type IIC (positive response to preincubation at pH 4.3, 4.5, and 10.4). A single common area was selected in each section and digitized as gray-level images by using a computer-assisted image-processing system (Neuroimaging System, Kyoto, Japan). The cross-sectional area of the fibers was measured by tracing the outline of each fiber in the section. Fiber type distribution and cross-sectional area were determined for approximately 500 fibers located in the central region of the muscle section.

The sections were stained to determine succinate dehydrogenase (SDH) activity, which is an indicator of mitochondrial oxidative capacity,[16,17] and to measure the number of capillaries surrounding the fibers.[18] The SDH activity and number of capillaries surrounding the approximately 500 fibers analyzed earlier were determined using a computer-assisted image-processing system (Neuroimaging System). The sectional images were digitized as gray-scale images. Each pixel was quantified as one of 256 gray levels; a gray level of 0 was equivalent to 100% light transmission, whereas that of 255 was equivalent to 0% transmission. The mean optical density of all pixels within a fiber was determined using a

calibration photographic tablet with 21 steps of gradient-density ranges and the corresponding diffused density values.

Statistical analyses

The mean and SD values were calculated from the individual values by using standard procedures. One-way analysis of variance was used to evaluate the growth-associated changes in body weight, food intake, and blood glucose level of individual groups and the differences among the age-matched groups. When the changes and differences were found to be significant, further comparisons were made using post hoc tests. A probability level of 0.05 was accepted as significant.

Results

Body weights

A growth-associated increase in body weight was observed in the NN (2.3- and 2.8-fold increase at 9 and 13 weeks compared to the weight at 5 weeks, $P < 0.05$), HN (2.0- and 2.7-fold increase at 9 and 13 weeks compared to the weight at 5 weeks, $P < 0.05$), and HH (2.1- and 2.5-fold increase at 9 and 13 weeks compared to the weight at 5 weeks, $P < 0.05$) groups (Fig. 1A). A growth-associated increase in body weight was observed in the NH group at 9 weeks (2.4-fold increase compared to the weight at 5 weeks, $P < 0.05$), whereas there was no difference in body weight of the NH group between 9 and 13 weeks.

The body weights of the HN and HH groups at 9 weeks were lower than those of the age-matched NN and NH groups (Fig. 1A). The body weights of the NH and HH groups at 13 weeks were lower than that of the age-matched NN group.

Food intakes

A growth-associated increase in food intake was observed in individual groups at 9 weeks (1.4-, 1.4-, 1.5-, and 1.3-fold increase in the NN, HN, NH, and HH groups compared to the intake at 5 weeks, respectively, $P < 0.05$) whereas there were no differences in food intake of individual groups between 9 and 13 weeks (Fig. 1B).

No differences in food intake were observed among the age-matched NN, HN, NH, and HH groups (Fig. 1B).

Fasting blood glucose and insulin levels

A growth-associated increase in glucose level was observed in the NN group (1.2- and 1.4-fold increase at 9 and 13 weeks compared to the level at 5 weeks, $P < 0.05$) (Fig. 2A). A growth-associated decrease in glucose level was observed in the HN group at 9 weeks (84% of the level at 5 weeks, $P < 0.05$), whereas there was no difference in glucose level of the HN group between 9 and 13 weeks. A growth-associated increase in glucose level was observed in the NH group at 9 weeks (1.2-fold increase compared to the level at 5 weeks, $P < 0.05$), whereas a growth-associated decrease in glucose level was observed in the NH group at 13 weeks (79% of the level at 9 weeks, $P < 0.05$). There were no differences in glucose level of the HH group at 5, 9, and 13 weeks.

The glucose levels of the HN and HH groups at 9 weeks were lower than those of the age-matched NN and NH groups (Fig. 2A). The glucose levels of the HN, NH, and HH groups at 13 weeks were lower than that of the age-matched NN group.

The insulin level of the HN group was lower than that of the NN group (Fig. 2B). The insulin level of the HH group was lower than those of the NN and NH groups.

Soleus and plantaris muscle weights

The soleus muscle weights in the HN and HH groups were lower than those in the NN and NH groups (Fig. 3A). No differences in plantaris muscle weight were observed among the NN, HN, NH, and HH groups (Fig. 3B).

The mRNA expression levels of PGC-1α

The mRNA expression levels of PGC-1α in the soleus muscles of the HN, NH, and HH groups were higher than that of the NN group (Fig. 3C). The mRNA expression levels of PGC-1α in the plantaris muscles of the HN and HH groups were higher than those of the NN and NH groups (Fig. 3D).

Properties of soleus muscle fibers

Only type I fibers were found in the NN group (Figs. 4A–D and 5A), whereas type I, type IIA, and type IIC fibers were found in the HN (Fig. 4E–H), NH (Fig. 4I–L), and HH

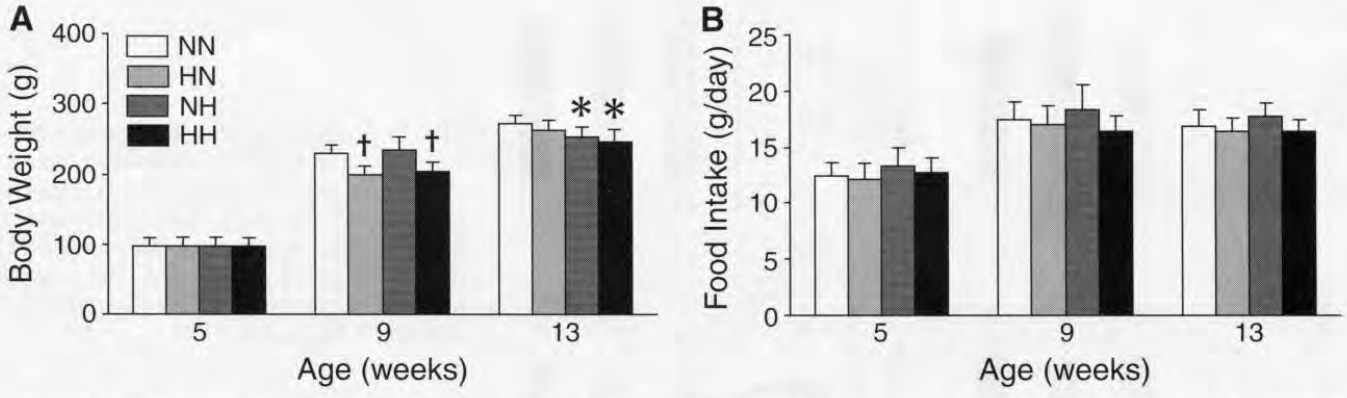

FIG. 1. (**A**) Body weights and (**B**) food intakes of the NN, HN, NH, and HH groups. Data are mean ± SD values determined for five animals. The NN group was exposed to 21% oxygen at 760 mm Hg for 8 weeks; the HN group was exposed to 36% oxygen at 950 mm Hg for 4 weeks, followed by 21% oxygen at 760 mm Hg for 4 weeks; the NH group was exposed to 21% oxygen at 760 mm Hg for 4 weeks, followed by 36% oxygen at 950 mm Hg for 4 weeks; and the HH group was exposed to 36% oxygen at 950 mm Hg for 8 weeks. $^{*}P < 0.05$ compared to the NN group; $^{\dagger}P < 0.05$ compared to the NN and NH groups.

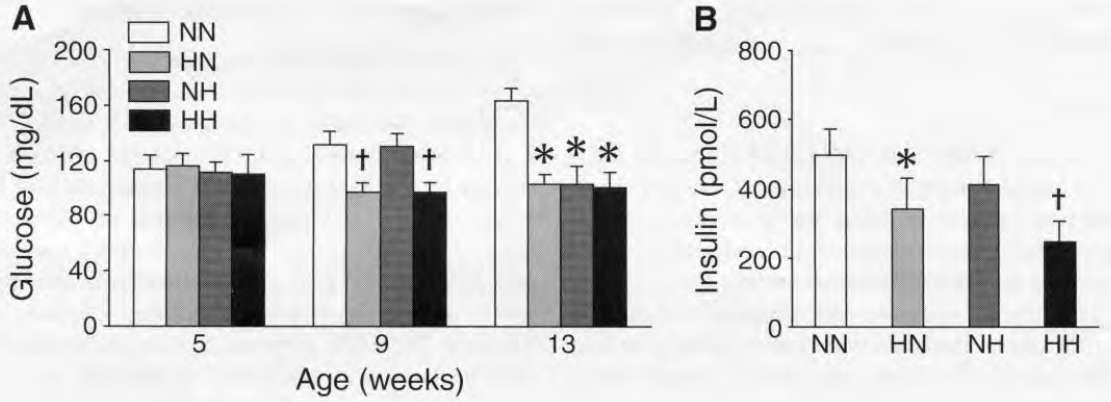

FIG. 2. (**A**) Fasting blood glucose and (**B**) insulin levels of the NN, HN, NH, and HH groups. Data are mean ± SD values determined for five animals. $^{*}P < 0.05$ compared to the NN group; $^{\dagger}P < 0.05$ compared to the NN and NH groups.

(Fig. 4M–P) groups. No differences in fiber type distribution were observed among the HN, NH, and HH groups (Fig. 5A).

No differences in fiber cross-sectional area were observed among the NN, HN, NH, and HH groups, irrespective of the fiber type (Fig. 5B).

The SDH activities in type I fibers of the HN and NH groups were higher than that of the NN group (Fig. 5C). The SDH activity in type I fibers of the HH group was higher than those of the NN and NH groups. The SDH activity in type IIA fibers of the HH group was higher than that of the NH group. The SDH activity in type IIC fibers of the HH group was higher than those of the HN and NH groups.

No differences in number of capillaries surrounding the fibers were observed among the NN, HN, NH, and HH groups, irrespective of the fiber type (Fig. 5D).

Properties of plantaris muscle fibers

The percentages of type I fibers in the HN and NH groups were higher than that in the NN group (Fig. 6A). The percentage of type I fibers in the HH group was higher than those in the NN and NH groups. The percentages of type IIA fibers in the HN and HH groups were higher than those in the NN and NH groups. The percentages of type IIB fibers in the HN, NH, and HH groups were lower than that in the NN group.

No differences in fiber cross-sectional area were observed among the NN, HN, NH, and HH groups, irrespective of the fiber type (Fig. 6B).

The SDH activity in type I fibers of the HH group was higher than that of the NN group (Fig. 6C). The SDH activities in type IIA and type IIB fibers of the HN and HH groups were higher than those of the NN and NH groups.

No differences in number of capillaries surrounding the fibers were observed among the NN, HN, NH, and HH groups, irrespective of the fiber type (Fig. 6D).

Discussion

Characteristics of muscle fibers in rats with type 2 diabetes

Mammalian skeletal muscle fibers are classified into several types based on their enzyme histochemical and immunohistochemical profiles.[19] Oxidative enzyme activity is higher in

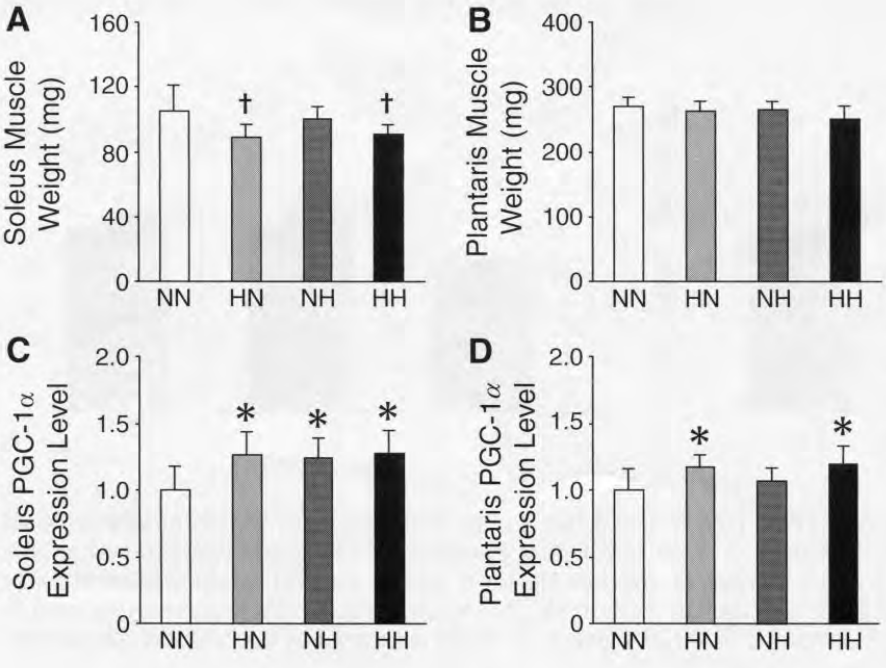

FIG. 3. (**A**) Soleus and (**B**) plantaris muscle weights and mRNA expression levels of PGC-1α in the (**C**) soleus and (**D**) plantaris muscles of the NN, HN, NH, and HH groups. Data are mean ± SD values determined for five animals. $^{*}P < 0.05$ compared to the NN group; $^{\dagger}P < 0.05$ compared to the NN and NH groups.

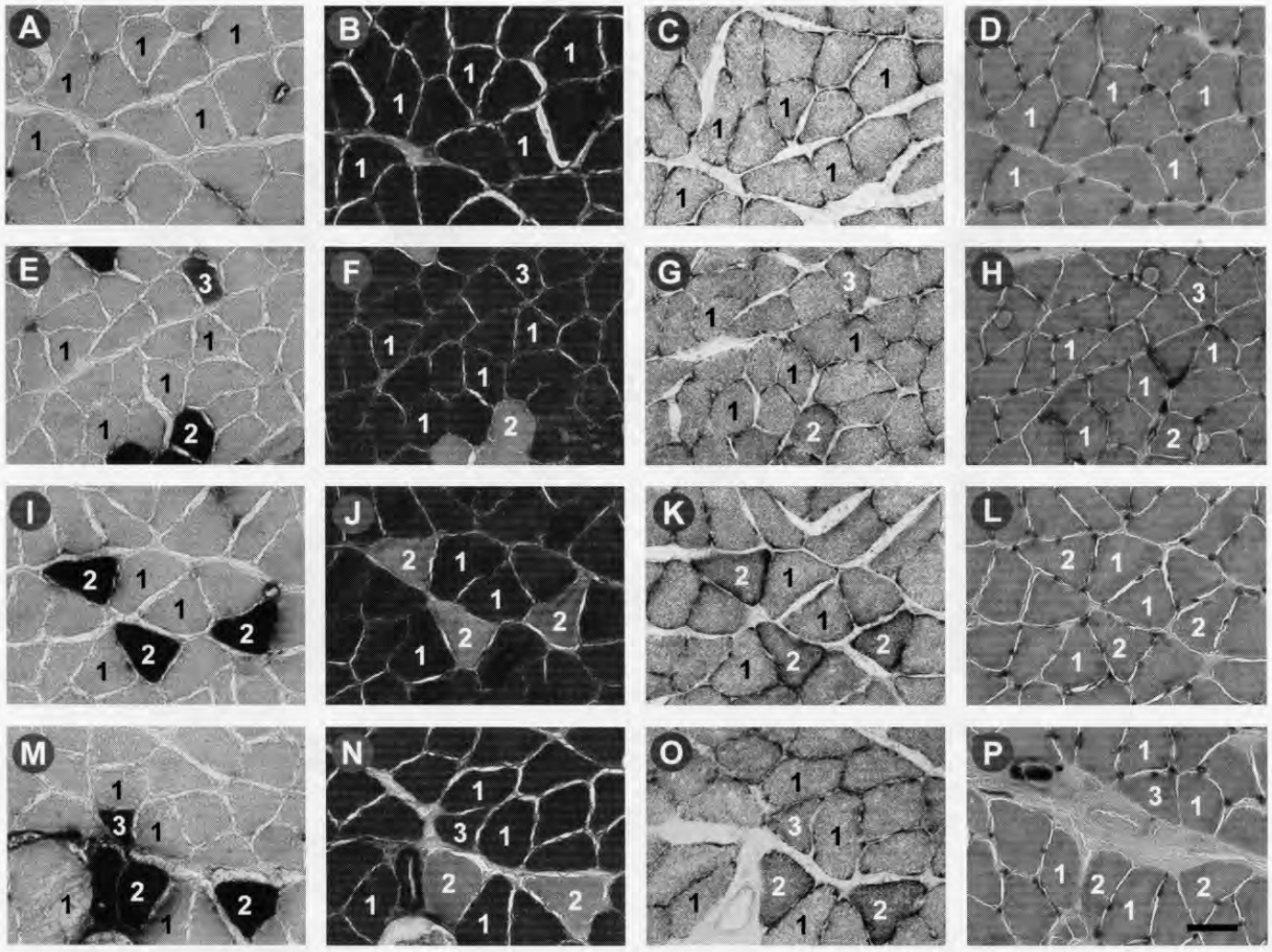

FIG. 4. Serial transverse sections of the soleus muscles in the NN, HN, NH, and HH groups. The sections of the (**A–D**) NN, (**E–H**) HN, (**I–L**) NH, and (**M–P**) HH groups were stained for ATPase activity following preincubation at (A, E, I, and M) pH 10.4 and (B, F, J, and N) pH 4.5, (C, G, K, and O) SDH activity, and (D, H, L, and P) capillarization. 1, type I fibers; 2, type IIA fibers; 3, type IIC fibers. Scale bar = 50 μm.

type I and type IIA fibers than in type IIB fibers.[15] Previous studies[1–3] have reported that patients with type 2 diabetes have a high percentage of low-oxidative type IIB fibers and a low percentage of high-oxidative type I fibers in skeletal muscles such as the vastus lateralis and rectus abdominis muscles. Furthermore, in previous studies,[20,21] we observed that obese Otsuka-Long-Evans-Tokushima Fatty (OLETF) and nonobese GK rats exhibiting type 2 diabetes have a higher percentage of low-oxidative fibers and a lower percentage of high-oxidative fibers in the soleus and plantaris muscles as compared to nondiabetic rats. This suggests that insulin resistance and impaired glucose metabolism as observed in type 2 diabetes are associated with reduced oxidative capacity in skeletal muscles. In this study, GK rats under normobaric conditions (NN group) had only type I fibers in the soleus muscle (Figs. 4A–D and 5A). This finding is consistent with our previous studies using OLETF and GK rats with type 2 diabetes.[20,21]

Effects of exposure to hyperbaric oxygen

An elevation in atmospheric pressure accompanied by an increase in oxygen concentration enhances the partial pressure of oxygen and increases the levels of dissolved oxygen in the plasma. These conditions enhance the oxidative capacity of mitochondria, thereby increasing oxidative metabolism in cells and tissues. Furthermore, an enhancement in the atmospheric pressure and oxygen concentration increases the carbon dioxide concentration, which in turn facilitates the release of oxygen from hemoglobin and causes dilation of blood vessels.

Our previous study[5] demonstrated that developing rats exposed to hyperbaric oxygen exhibited greater voluntary running activities than those maintained under normobaric conditions and that oxidative enzyme activities in soleus and plantaris muscle fibers and in spinal motoneurons innervating these skeletal muscles increased following exposure to hyperbaric oxygen. These findings suggest that the adaptation of neuromuscular units to hyperbaric oxygen enhances the oxidative capacity in muscle fibers and motoneurons, which promotes the function of the neuromuscular units. Our previous study[6] also revealed that exposure to hyperbaric oxygen inhibited the growth-related increase in blood glucose level of GK rats; this suggests that the increased availability of oxygen during exposure to hyperbaric oxygen is beneficial for oxidative metabolism in skeletal muscles of rats with type 2

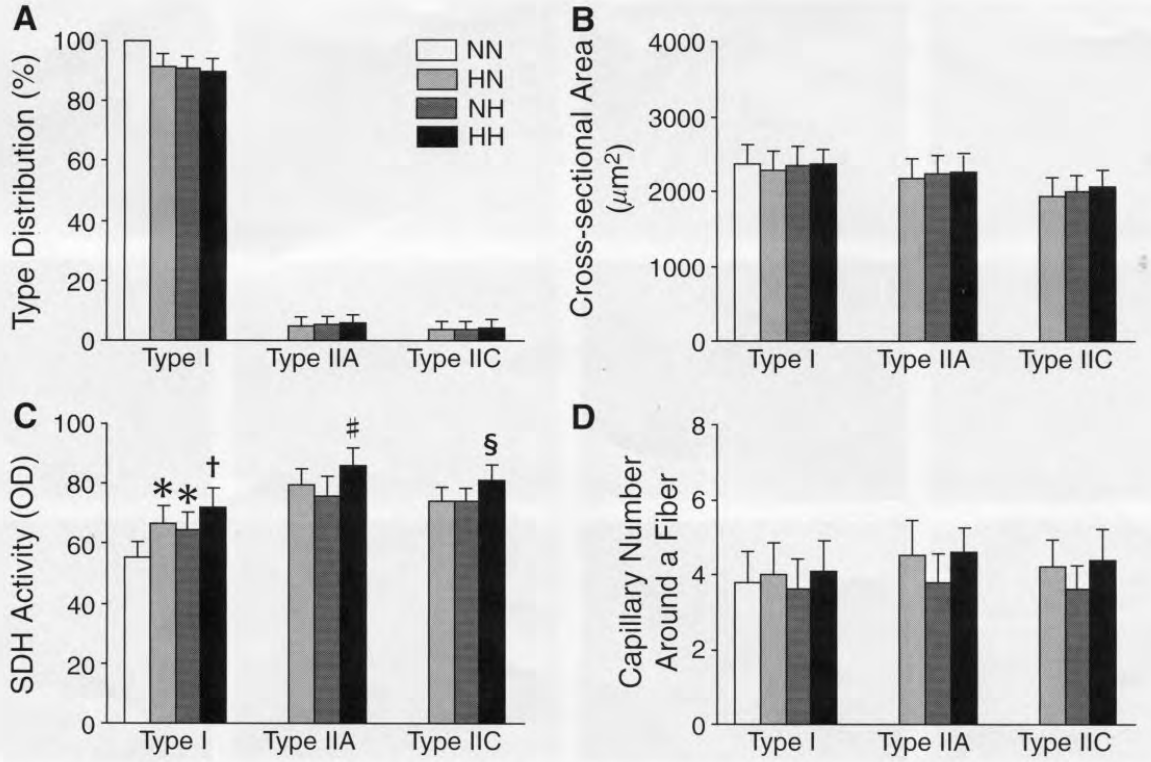

FIG. 5. (**A**) Fiber type distributions, (**B**) fiber cross-sectional areas, (**C**) SDH activities, and (**D**) numbers of capillaries surrounding the fibers in the soleus muscles of the NN, HN, NH, and HH groups. Data are mean ± SD values determined for five animals. OD, optical density. $^{*}P < 0.05$ compared to the NN group; $^{\dagger}P < 0.05$ compared to the NN and NH groups; $^{\#}P < 0.05$ compared to the NH group; $^{\S}P < 0.05$ compared to the HN and NH groups.

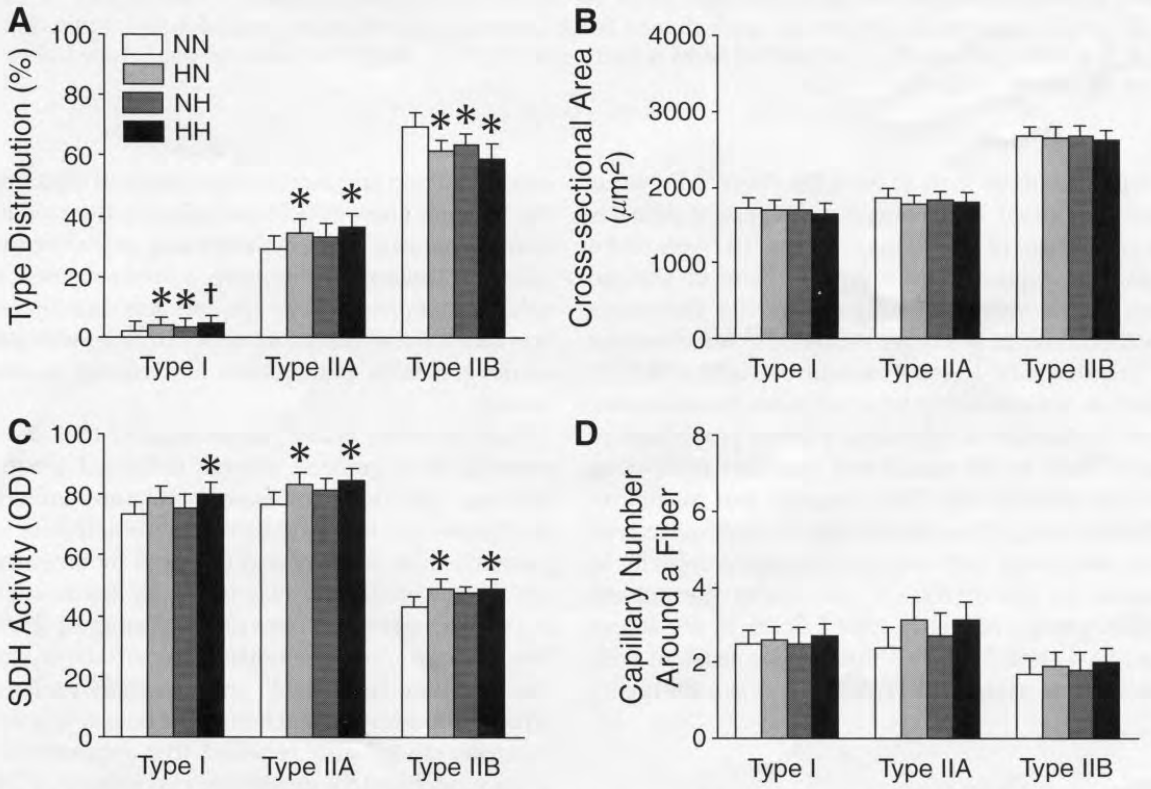

FIG. 6. (**A**) Fiber type distributions, (**B**) fiber cross-sectional areas, (**C**) SDH activities, and (**D**) numbers of capillaries surrounding the fibers in the plantaris muscles of the NN, HN, NH, and HH groups. Data are mean ± SD values determined for five animals. OD, optical density. $^{*}P < 0.05$ compared to the NN group; $^{\dagger}P < 0.05$ compared to the NN and NH groups.

diabetes. In fact, exposure to hyperbaric oxygen inhibited both the growth-related transition of fiber types from the high-oxidative to the low-oxidative and the decrease in fiber oxidative enzyme activity in the soleus and plantaris muscles of GK rats.[7,8]

In this study, we aimed to clarify whether exposure to hyperbaric oxygen reduces high blood glucose levels in rats with type 2 diabetes and whether these effects are maintained under normobaric conditions even after exposure to hyperbaric oxygen. We observed that exposure to hyperbaric oxygen reduced the high blood glucose level in rats with type 2 diabetes and that this effect was maintained after exposure to hyperbaric oxygen (Fig. 2A). The changes in blood glucose level following exposure to hyperbaric oxygen were associated with the changes in fiber type distribution and oxidative enzyme activity of the soleus (Fig. 5A and C) and plantaris (Fig.6A and C) muscles. These findings indicate that exposure to hyperbaric oxygen reverses the decreased oxidative capacity in skeletal muscles of rats with type 2 diabetes and that these effects persist for a prolonged duration under normobaric conditions. We believe that the decrease in blood glucose level after exposure to hyperbaric oxygen is associated with an improvement in diabetes because the hyperbaric (HN and HH) groups showed lower insulin levels (Fig. 2B), indicating inhibition of insulin resistance, a symptom of diabetes.

Capillary density in the vastus lateralis muscle is low in patients with type 2 diabetes.[1,22] This indicates that the presence of few capillaries is associated with a decrease in oxidative capacity of skeletal muscles, which induces insulin resistance and impairs glucose metabolism in patients with type 2 diabetes. In contrast, a large number of capillaries were observed surrounding all types of fibers, especially type IIB fibers, in the gastrocnemius muscle of patients with type 2 diabetes.[23] In this study, no differences were observed in the number of capillaries surrounding the fibers of the soleus (Fig. 5D) or plantaris (Fig. 6D) muscles. Thus, exposure to hyperbaric oxygen does not seem to induce capillary proliferation in skeletal muscles of rats with type 2 diabetes. However, further studies investigating capillary architecture and the blood flow and volume in skeletal muscles are required to analyze the effects of exposure to hyperbaric oxygen.

Effects of exercise

Exercise is believed to be effective in preventing and improving insulin resistance and impaired glucose metabolism.[24–26] The results of our previous study involving voluntary running exercises[27] are consistent with this view. We found that running exercises inhibited the growth-related transition of fiber types from the high-oxidative to the low-oxidative in the soleus muscles of rats with type 2 diabetes, although this inhibition was observed only in rats that ran more than 7 km/day. We conclude that both exposure to hyperbaric oxygen and exercise bring about an increase in the percentage of high-oxidative fibers and inhibit a growth-associated decrease in oxidative capacity of skeletal muscles in rats with type 2 diabetes.

Our previous study[5] found that the oxidative enzyme activity in skeletal muscle fibers and spinal motoneurons of normal rats increased after exposure to hyperbaric oxygen and that rats exposed to hyperbaric oxygen exhibited more voluntary running activity than those exposed to normobaric conditions. In this study, food intake was not different among the normobaric (NN) and age-matched hyperbaric (HN, NH, and HH) groups (Fig. 1B). On the other hand, a growth-associated increase in body weight was inhibited by exposure to hyperbaric oxygen; the body weight was lower in the hyperbaric (HN and HH) groups at 9 weeks and in the hyperbaric (NH and HH) groups at 13 weeks than in the age-matched normobaric (NN) group (Fig. 1A). These findings suggest that the oxidative metabolism in cells and tissues is enhanced on exposure to hyperbaric oxygen, thereby promoting the function of neuromuscular units. Therefore, decreased blood glucose levels of the HN, NH, and HH groups (Fig. 2A) may be associated with the higher voluntary motor activities induced by exposure to hyperbaric oxygen, although we did not monitor the spontaneous motor activities of individual rats in their cages. Further studies are warranted to elucidate whether spontaneous motor activities of individual rats in their cages are increased by exposure to hyperbaric oxygen and, if so, whether increased motor activities of individual rats in their cages are associated with a decrease in blood glucose and insulin levels.

The mRNA expression levels of PGC-1α

Oxidative metabolism in skeletal muscles is largely regulated by several factors such as PGC-1α.[9] PGC-1α co-activates PPARγ, which effectively reduces insulin resistance.[10] When PGC-1α was up-regulated in transgenic mice under the influence of a muscle creatine kinase promoter, the transition of fiber types from the low-oxidative to the high-oxidative was observed.[28] This indicates that PGC-1α regulates the proportion of high-oxidative fibers in skeletal muscles.

The mRNA expression level of PGC-1α in skeletal muscles is lower in diabetic patients than in healthy individuals.[29] Our previous study[30] found that mRNA expression levels of PGC-1α in type I fibers of the soleus muscle were lower in Zucker rats with diabetes than in controls. Patients and animals with type 2 diabetes exhibited a reduced percentage of high-oxidative fibers in skeletal muscles.[1–3,20,21] Therefore, it is suggested that stimulation of pathways involving PGC-1α increases the proportion of high-oxidative fibers, which reverse the decreased oxidative metabolism in skeletal muscles of humans and animals with type 2 diabetes. In this study, mRNA expression levels of PGC-1α in the soleus muscles of the HN, NH, and HH groups were higher than that of the NN group (Fig. 3C). Furthermore, mRNA expression levels of PGC-1α in the plantaris muscles of the HN and HH groups were higher than those of the NN and NH groups (Fig. 3D). The soleus muscles in the NN group contained only type I fibers, whereas those in the HN, NH, and HH groups contained type I fibers as well as high-oxidative type IIA and type IIC fibers (Fig. 5A). Moreover, type I fibers in the soleus muscles of the HN, NH, and HH groups exhibited higher oxidative enzyme activities than those of the NN group (Fig. 5C). The plantaris muscles of the HN and HH groups exhibited a higher percentage of type IIA fibers than that of the NN group (Fig. 6A). Moreover, type IIA and type IIB fibers in the plantaris muscles of the HN and HH groups exhibited higher oxidative enzyme activities than those of the NN groups (Fig. 6C). These results suggest that exposure to hyperbaric oxygen enhances mRNA expression levels of PGC-1α in skeletal muscles of rats with type 2 diabetes and that the

PGC-1α pathways thus bring about an increase in both the percentage of high-oxidative fibers and the fiber oxidative enzyme activity in skeletal muscles.

Differences in the effects of exposure to hyperbaric oxygen between the slow and fast muscles

In this study, we examined the slow soleus and fast plantaris muscles of rats with type 2 diabetes because we expected that exposure to hyperbaric oxygen would exert different effects on these two muscles. The normal activity patterns differ between slow and fast muscles: slow muscles exhibit activity of relatively low intensity and long duration, which is required for antigravity functions such as walking and posture maintenance; in contrast, fast muscles exhibit activity of relatively high intensity and short duration, which is required for functions demanding strength and power.[31] Insulin-induced increases in the disposal of glucose predominantly involve slow skeletal muscles that contain a high percentage of high-oxidative fibers; this indicates that the relative proportion of high-oxidative fibers in skeletal muscles is an important factor in whole-body insulin sensitivity. In comparison with fast muscles, which presumably contain low-oxidative fibers, slow muscles such as the soleus muscle presumably contain high-oxidative fibers that exhibit increased lipid storage capacity,[32] insulin binding,[33,34] insulin-stimulated glucose uptake,[35] and glucose transport protein content.[36,37]

In this study, mRNA expression levels of PGC-1α in both the soleus and plantaris muscles of the HH group were upregulated after 8 weeks of exposure to hyperbaric oxygen (Fig. 3C and D). The oxidative enzyme activity in all types of fibers in the soleus muscle of the HH group was enhanced after exposure to hyperbaric oxygen (Fig. 5C). Similarly, an increased percentage of type I and type IIA fibers, a decreased percentage of type IIB fibers, and an enhanced oxidative enzyme activity in all types of fibers were observed in the plantaris muscle of the HH group after exposure to hyperbaric oxygen (Fig. 6A and C). Therefore, we conclude that exposure to hyperbaric oxygen induces similar effects in both the slow and fast muscles.

Future scope

The body weights in the hyperbaric (HN and HH) groups were lower at 9 weeks than that in the age-matched normobaric (NN) group (Fig. 1A). Similarly, the hyperbaric (NH and HH) groups had lower body weights at 13 weeks than the age-matched normobaric (NN) group. Exposure to hyperbaric oxygen seemed to inhibit a growth-associated increase in body weight; however, the reasons for this remain unclear. Therefore, we must clarify why exposure to hyperbaric oxygen inhibits growth-associated increase in body weight. Furthermore, clinical trials must be performed to evaluate the efficacy of exposure to hyperbaric oxygen for the treatment of patients with diabetes.

Conclusions

Exposure to hyperbaric oxygen reduced high blood glucose levels in GK rats with type 2 diabetes. The lowered blood glucose levels were maintained under normobaric conditions even after exposure to hyperbaric oxygen. Exposure to hyperbaric oxygen enhanced the mRNA expression levels of PGC-1α, which induced an increase in both the percentage of high-oxidative fibers and fiber oxidative enzyme activity in skeletal muscles of GK rats. We conclude that exposure to hyperbaric oxygen, under the conditions used in this study, improves oxidative capacity of the skeletal muscles in type 2 diabetes and that these effects persist for a prolonged duration under normobaric conditions even after exposure to hyperbaric oxygen.

Author Disclosure Statement

No competing financial interests exist.

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Research Article

Hyperbaric Oxygen Exposure Reduces Age-Related Decrease in Oxidative Capacity of the Tibialis Anterior Muscle in Mice

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The effects of exposure to hyperbaric oxygen on the oxidative capacity of the skeletal muscles in mice at different ages were investigated. We exposed 5-, 34-, 55-, and 88-week-old mice to 36% oxygen at 950 mmHg for 6 hours per day for 2 weeks. The activities of succinate dehydrogenase (SDH), which is a mitochondrial marker enzyme, of the tibialis anterior muscle in hyperbaric mice were compared with those in age-matched mice under normobaric conditions (21% oxygen at 760 mmHg). Furthermore, the SDH activities of type IIA and type IIB fibers in the muscle were determined using quantitative histochemical analysis. The SDH activity of the muscle in normobaric mice decreased with age. Similar results were observed in both type IIA and type IIB fibers in the muscle. The decrease in the SDH activity of the muscle was reduced in hyperbaric mice at 57 and 90 weeks. The decreased SDH activities of type IIA and type IIB fibers were reduced in hyperbaric mice at 90 weeks and at 57 and 90 weeks, respectively. We conclude that exposure to hyperbaric oxygen used in this study reduces the age-related decrease in the oxidative capacity of skeletal muscles.

1. Introduction

A reduction in skeletal muscle mass is one of the most striking features of the aging process. Previous studies [1–3] have indicated that this reduction is due to decreases in the number and volume of individual fibers in skeletal muscles. Mammalian skeletal muscles consist of different sizes and types of fibers, for example, slow-twitch type I and fast-twitch type II fibers [4, 5]. A reduction in the number and volume of type II fibers in skeletal muscles of rats can be observed in the initial stages of the aging process [6, 7]. These changes in type II fibers are considered to be due to a transition of fiber types from type II to type I, selective loss and atrophy of type II fibers, and/or degeneration in the neuromuscular junction, which are induced by age-related disuse of type II fibers. A decrease in the number and volume of both type I and type II fibers in skeletal muscles of rats can be observed in the late stages of the aging process [6–8]. These changes in type I and type II fibers are closely related to the loss and degeneration of spinal motoneurons innervating those fibers in skeletal muscles. Furthermore, a decrease in the oxidative enzyme activity of skeletal muscles in rats was observed with increasing age [9–11].

An elevation in atmospheric pressure accompanied by an increase in oxygen concentration enhances the partial pressure of oxygen and increases the concentration of dissolved oxygen in the plasma. An increase in both atmospheric pressure and oxygen concentration enhances oxidative enzyme activity in mitochondria and consequently increases the oxidative metabolism in cells and tissues [12]; thus, it is

expected that exposure to hyperbaric oxygen facilitates the turnover of oxidative metabolism, particularly of pathways in the mitochondrial TCA cycle, thereby reducing the age-related decrease in the oxidative enzyme activity of muscle fibers. We determined that a pressure of 960 mmHg and an oxygen concentration of 36% are required for obtaining effective responses with regard to oxidative metabolism [12, 13]. This study examined the oxidative capacity of the tibialis anterior muscle in mice at different ages, which were exposed to 36% oxygen at 950 mmHg. Furthermore, the cross-sectional areas and oxidative enzyme activities of fibers, which were type-defined by ATPase activity, in the muscle of mice were determined using quantitative histochemical analysis.

2. Materials and Methods

All experimental procedures, including animal care, were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Japanese Physiological Society. This study was also approved by the Institutional Animal Care Committee at Kyoto University.

2.1. Animal Care and Treatment. We used 5-, 34-, 55-, and 88-week-old female mice in this study. The mice (the hyperbaric group; $n = 6$ in each age group) were exposed to hyperbaric conditions (950 mmHg) with a high oxygen concentration (36%), which were automatically maintained by a computer-assisted system, in a hyperbaric chamber for 6 hours (1100–1700) and were placed under normobaric conditions (21% oxygen at 760 mmHg) for 18 hours (1700–1100), while other mice (the normobaric group; $n = 6$ in each age group) were placed in a hyperbaric chamber under normobaric conditions for 24 hours. The hyperbaric chamber was 90 cm in length and 80 cm in diameter; thus, it could simultaneously house a number of rats (up to 20 cages).

All mice were individually housed in same-sized cages in a room maintained under controlled 12-hour light/dark cycles (lights switched off from 2000 to 0800) at a temperature of $22 \pm 2^{\circ}$C with a relative humidity of 45%–65%. Food and water were provided *ad libitum* to all mice.

2.2. Tissue Procedures. After 2 weeks of exposure to hyperbaric oxygen, the mice in the normobaric and hyperbaric groups were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The tibialis anterior muscles from both hind limbs were removed and cleaned of excess fat and connective tissue. Thereafter, the mice were sacrificed by an overdose of sodium pentobarbital.

The tibialis anterior muscles of the right side were quickly frozen in liquid nitrogen for measurement of succinate dehydrogenase (SDH) activity. The SDH activity was determined according to the method of Cooperstein et al. [14]. Briefly, the muscles were homogenized using a glass tissue homogenizer with 5 volumes of ice-cold 0.3 M phosphate buffer, pH 7.4. Sodium succinate was added to yield a final concentration of 17 mM. The final concentrations of the components of the reaction mixture were as follows: sodium succinate 17 μM, sodium cyanide 1 mM, aluminum chloride 0.4 mM, and calcium chloride 0.4 mM. This reaction mixture was transferred to the spectrophotometer and the reduction of cytochrome *c* was followed by observing the increase in extinction at 550 nm. The SDH activity was calculated from the ferricytochrome *c* concentration and protein content.

The tibialis anterior muscles of the left side were pinned on a cork at their *in vivo* length and quickly frozen in isopentane cooled with liquid nitrogen. The mid-portion of the muscle was mounted on a specimen chuck using a Tissue Tek OCT Compound (Sakura Finetechnical, Tokyo, Japan). Serial transverse sections (10-μm thickness) of the muscle on the chuck were cut in a cryostat maintained at -20°C. The serial sections were brought to room temperature, air-dried for 30 minutes, and incubated for ATPase activity following acid preincubation and for SDH activity [15, 16].

The ATPase activity was determined by the following procedures: (1) preincubation for 5 minutes at room temperature in 50 mM sodium acetate and 30 mM sodium barbital in distilled water, adjusted to pH 4.5 with HCl; (2) washing in 5 changes of distilled water; (3) incubation for 45 minutes at 37°C in 2.8 mM ATP, 50 mM $CaCl_2$, and 75 mM NaCl in distilled water, adjusted to pH 9.4 with NaOH; (4) washing in 5 changes of distilled water; (5) immersion for 3 minutes in 1% $CaCl_2$; (6) washing in 5 changes of distilled water; (7) immersion for 3 minutes in 2% $CoCl_2$; (8) washing in 5 changes of distilled water; (9) immersion for 1 minutes in 1% $(NH_4)_2S$; (10) washing in 5 changes of distilled water; (11) dehydration in a graded series of ethanol, passed through xylene, and then cover slipped (Figure 1). Classification into two fiber types was based on staining intensities for ATPase activity: type IIA (positive intensity) and type IIB (negative intensity) [17].

The SDH activity was determined by incubation in a medium containing 0.9 mM 1-methoxyphenazine methylsulfate, 1.5 mM nitroblue tetrazolium, 5.6 mM ethylenediaminetetraacetic acid disodium salt, and 48 mM succinate disodium salt (pH 7.6) in 100 mM phosphate buffer. The incubation time was 10 minutes; the changes in staining intensity in response to incubation reached a plateau after 10 minutes. The reaction was stopped by multiple washings with distilled water, dehydrated in a graded series of ethanol, passed through xylene, and cover slipped (Figure 1).

The cross-sectional areas and SDH activities from approximately 300 fibers, which were type-defined by ATPase activity, in the central region of the muscle section were measured by tracing the outline of a fiber and stored in a computer-assisted image processing system (Neuroimaging System, Kyoto, Japan) [18, 19]. The images were digitized as gray-level pictures. Each pixel was quantified as one of 256 gray levels that were then automatically converted to optical density (OD). A gray level of zero was equivalent to 100% transmission of light and that of 255 was equivalent of 0% transmission of light. The mean OD value of all pixels within a fiber was determined using a calibration tablet that had 21 gradient density steps and corresponding diffused density values.

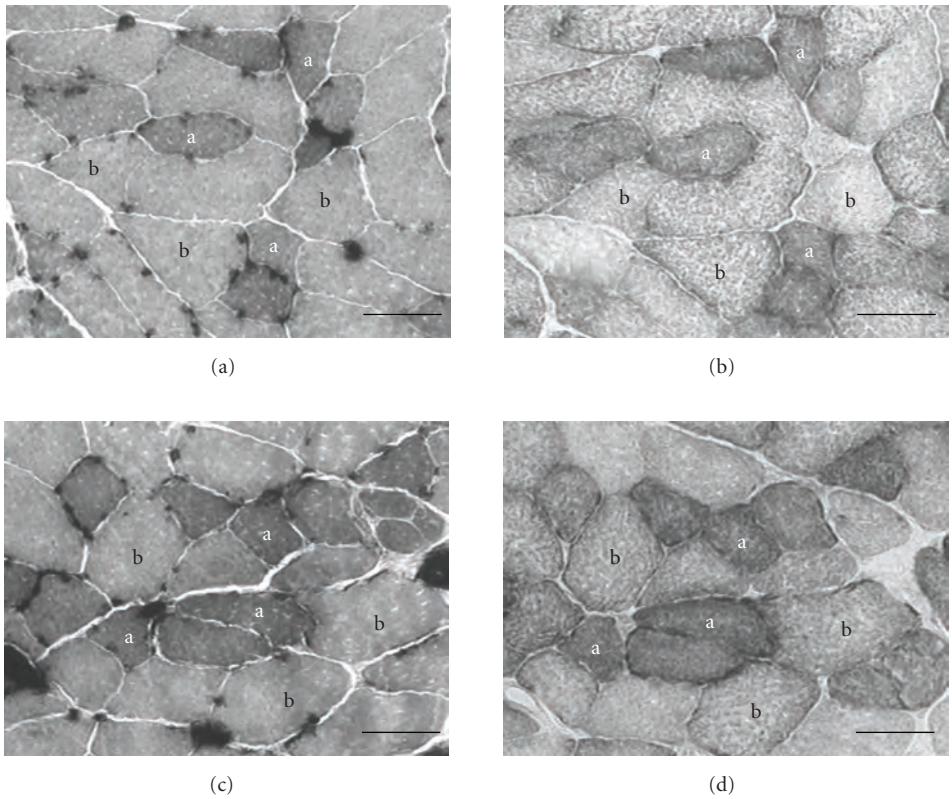

Figure 1: Serial transverse sections of the tibialis anterior muscles in the normobaric ((a) and (b)) and hyperbaric ((c) and (d)) mice at 90 weeks. (a) and (c), stained for ATPase activity following preincubation at pH 4.5; (b) and (d), stained for succinate dehydrogenase activity. a: type IIA; b: type IIB. Scale bar = 50 μm.

2.3. Statistical Analyses. The data were expressed as mean and standard deviation. One-way analysis of variance was used to evaluate the age-related changes. When the differences were found to be significant, further comparisons were made by performing *post hoc* tests. The differences between the normobaric and age-matched hyperbaric groups were determined by using the t-test. A probability level of 0.05 was considered to be statistically significant.

3. Results

3.1. Body Weight. An age-related increase in body weight was observed in the normobaric groups; the body weights at 36 and 57 weeks were greater than that at 7 weeks, and the body weight at 90 weeks was the greatest among the groups (Figure 2(a)). These results were similar in the hyperbaric groups.

There were no differences in body weight between the normobaric and age-matched hyperbaric groups, irrespective of the age.

3.2. Tibialis Anterior Muscle Weight. The muscle weights of the normobaric groups at 36 and 57 weeks were greater than that at 7 weeks (Figure 2(b)). These results were similar in the hyperbaric groups. The muscle weight of the normobaric group at 90 weeks was lower than that at 57 weeks.

There were no differences in muscle weight between the normobaric and age-matched hyperbaric groups, irrespective of the age.

3.3. SDH Activity of the Tibialis Anterior Muscle. An age-related decrease in SDH activity was observed in the normobaric groups; the SDH activities of the muscle at 57 and 90 weeks were lower than that at 36 weeks and those at 7 and 36 weeks, respectively (Figure 3). There were no differences in SDH activity of the muscle among the hyperbaric groups, irrespective of the age.

The SDH activity of the muscle in the hyperbaric group at 57 and 90 weeks was greater than that in the age-matched normobaric group.

3.4. Fiber Cross-Sectional Area in the Tibialis Anterior Muscle. There were no differences in cross-sectional area of type IIA fibers among the normobaric groups, irrespective of the age (Figure 4(a)). These results were similar in the hyperbaric groups.

There were no differences in cross-sectional area of type IIA fibers between the normobaric and age-matched hyperbaric groups, irrespective of the age.

The cross-sectional areas of type IIB fibers in the normobaric groups at 36 and 57 weeks were greater than

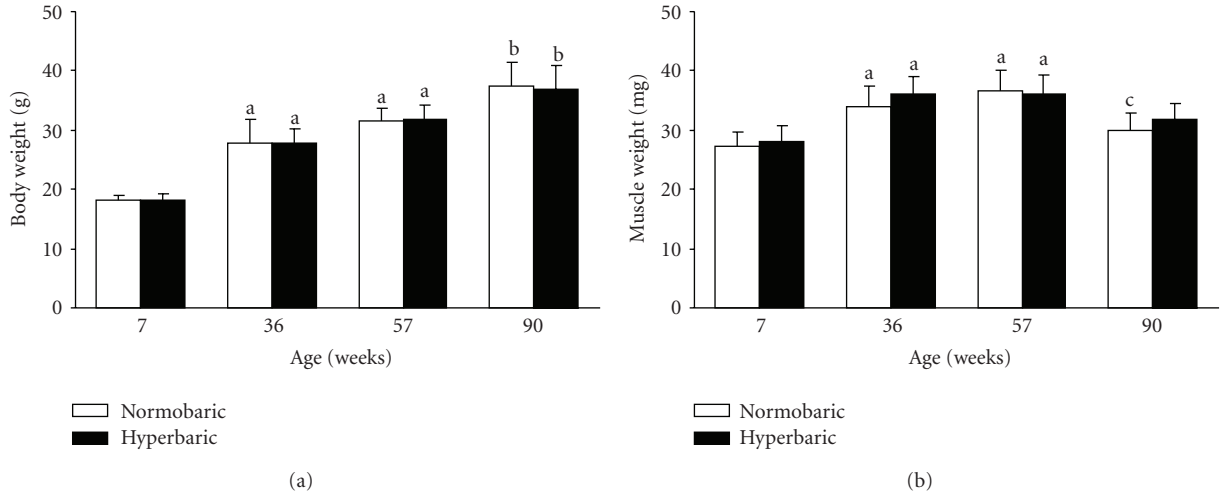

Figure 2: Body weights (a) and tibialis anterior muscle weights (b) of the normobaric and hyperbaric groups at different ages. Data are represented as the mean and standard deviation determined from six animals. The mice in the hyperbaric group were exposed to 36% oxygen at 950 mmHg for 6 hours per day for 2 weeks. $^{a}P < .05$ compared with the corresponding group at 7 weeks; $^{b}P < .05$ compared with the corresponding groups at 7, 36, and 57 weeks; $^{c}P < .05$ compared with the corresponding group at 57 weeks.

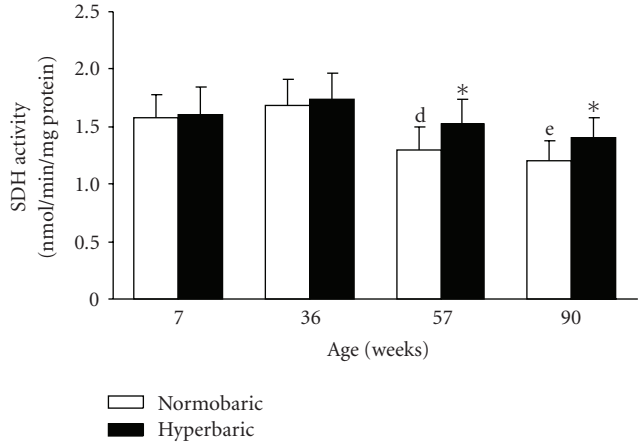

Figure 3: Succinate dehydrogenase activities of the tibialis anterior muscles of the normobaric and hyperbaric groups at different ages. Data are represented as the mean and standard deviation determined from six animals. The mice in the hyperbaric group were exposed to 36% oxygen at 950 mmHg for 6 hours per day for 2 weeks. SDH: succinate dehydrogenase. $^{d}P < .05$ compared with the corresponding group at 36 weeks; $^{e}P < .05$ compared with the corresponding groups at 7 and 36 weeks; $^{*}P < .05$ compared with the age-matched normobaric group.

those at 7 and 90 weeks (Figure 4(b)). These results were similar in the hyperbaric groups.

There were no differences in cross-sectional area of type IIB fibers between the normobaric and age-matched hyperbaric groups, irrespective of the age.

3.5. Fiber SDH Activity in the Tibialis Anterior Muscle. The SDH activity of type IIA fibers in the normobaric group at 57 weeks was lower than that at 7 weeks (Figure 5(a)). The SDH activity of type IIA fibers in the normobaric group at 90 weeks was lower than those at 7 and 36 weeks. The SDH activity of type IIA fibers in the hyperbaric group at 90 weeks was lower than that at 7 weeks.

The SDH activity of type IIA fibers in the hyperbaric group at 90 weeks was greater than that in the age-matched normobaric group.

The SDH activities of type IIB fibers in the normobaric groups at 57 and 90 weeks were lower than that at 7 weeks (Figure 5(b)). The SDH activity of type IIB fibers in the hyperbaric group at 90 weeks was lower than that at 7 weeks.

The SDH activity of type IIB fibers in the hyperbaric group at 57 and 90 weeks was greater than that of the age-matched hyperbaric group.

4. Discussion

An elevation in atmospheric pressure accompanied by high oxygen concentration enhances the partial pressure of oxygen and increases the concentration of dissolved oxygen in the plasma [20, 21]. An increase in both atmospheric pressure and oxygen concentration enhances the mitochondrial oxidative enzyme activity and consequently increases oxidative metabolism in cells and tissues. Furthermore, an increase in atmospheric pressure and oxygen concentration increases carbon dioxide concentration, which in turn facilitates the release of oxygen from hemoglobin and causes the dilation of blood vessels. We designed a hyperbaric chamber for performing the animal experiments [12]; the chamber consisted of an oxygen tank containing an oxygen concentrator and an air compressor, which automatically maintains the elevated atmospheric pressure and oxygen concentration using a computer-assisted system. We determined the optimal atmospheric pressure (950 mmHg) and oxygen concentration (36%) required for obtaining effective responses with regard to oxidative capacity in the neuromuscular system [12].

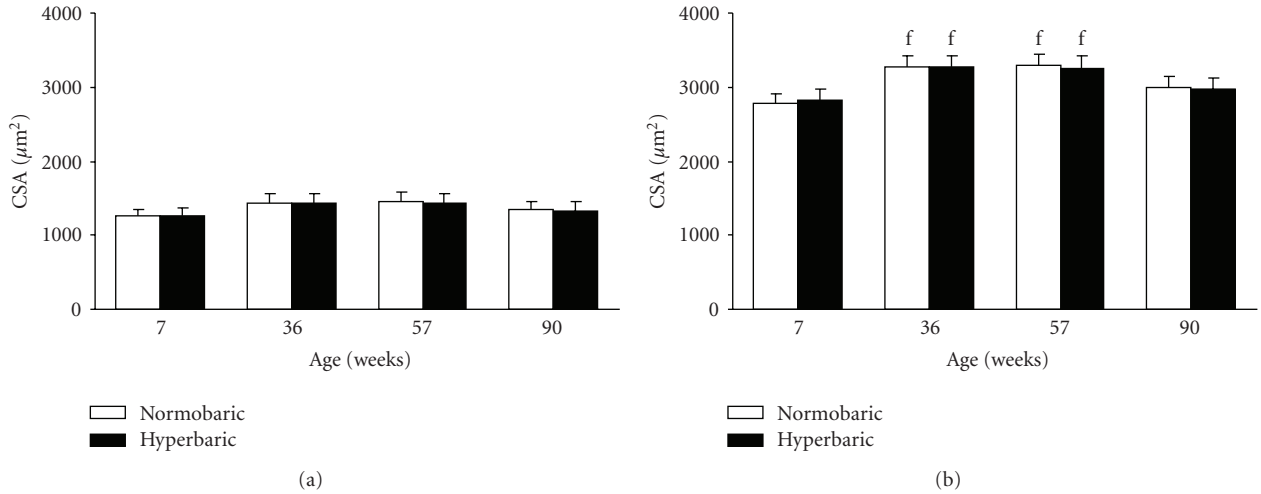

Figure 4: Cross-sectional areas of type IIA (a) and type IIB (b) fibers in the tibialis anterior muscles of the normobaric and hyperbaric groups at different ages. Data are represented as the mean and standard deviation determined from six animals. The mice in the hyperbaric group were exposed to 36% oxygen at 950 mmHg for 6 hours per day for 2 weeks. CSA: cross-sectional area. $^{f}P < .05$ compared with the corresponding groups at 7 and 90 weeks.

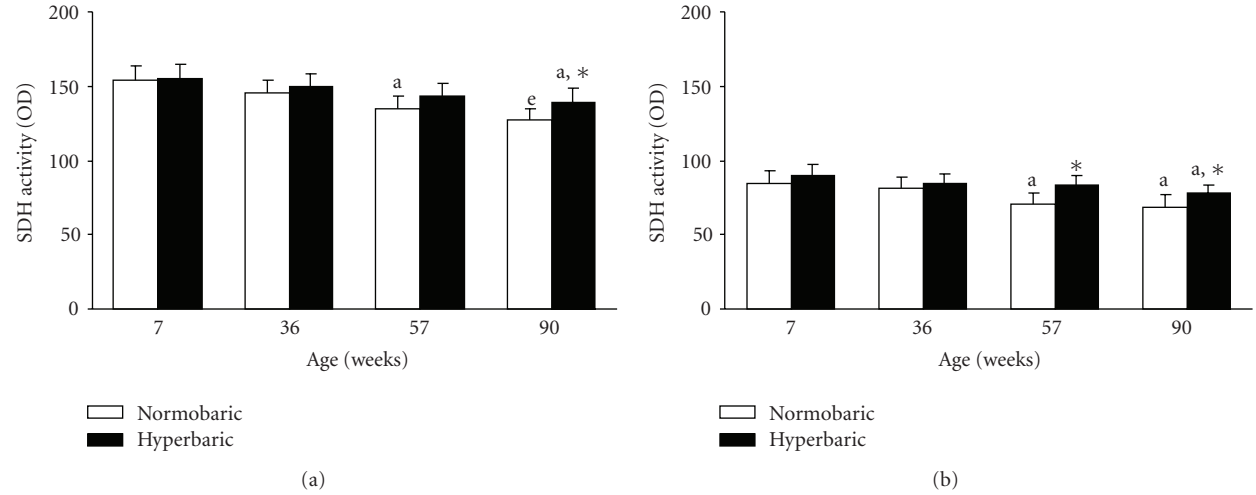

Figure 5: Succinate dehydrogenase activities of type IIA (a) and type IIB (b) fibers in the tibialis anterior muscles of the normobaric and hyperbaric groups at different ages. Data are represented as the mean and standard deviation determined from six animals. The mice in the hyperbaric group were exposed to 36% oxygen at 950 mmHg for 6 hours per day for 2 weeks. SDH: succinate dehydrogenase; OD: optical density. $^{a}P < .05$ compared with the corresponding group at 7 weeks; $^{e}P < .05$ compared with the corresponding groups at 7 and 36 weeks; $^{*}P < .05$ compared with the age-matched normobaric group.

Our previous study [13] demonstrated that young rats exposed to 36% oxygen at 950 mmHg exhibited greater voluntary running activities than those maintained under normobaric conditions. We also found that oxidative enzyme activities of fibers in the soleus and plantaris muscles and of spinal motoneurons innervating these muscles increased following exposure to hyperbaric oxygen [13]. These findings suggest that the adaptation of neuromuscular units to hyperbaric oxygen enhances the oxidative capacity in muscle fibers and motoneurons, which promotes the function of the neuromuscular units. Furthermore, our previous studies [22, 23] revealed that exposure to 36% oxygen at 950 mmHg inhibited the growth-related increase in blood glucose levels of type 2 diabetic rats and in blood pressure levels of spontaneously hypertensive rats. Exposure to hyperbaric oxygen inhibited both the growth-related transition of fiber types from high to low oxidative and the decrease in oxidative enzyme activity of fibers in the soleus and plantaris muscles of type 2 diabetic rats [24, 25]. It is suggest that exposure to hyperbaric oxygen reduces the age-related decrease in the oxidative capacity of skeletal muscles, because exposure to hyperbaric oxygen facilitates the turnover of oxidative metabolism, particularly of pathways in the mitochondrial TCA cycle.

Exercise is believed to be effective in maintaining and improving oxidative metabolism in cells and tissues. Our previous study [26] observed that exercise is effective for the prevention of a decrease in the oxidative enzyme activity of type I and type II fibers in the soleus muscles of rats, which was induced by unloading. Furthermore, our previous study [27] found that running exercises served to inhibit the growth-related transition of fiber types from high to low oxidative in the soleus muscle of rats with type 2 diabetes, although this inhibition was observed only in rats that ran more than 7 km per day.

Atrophy, loss, and decreased oxidative enzyme activity of fibers in skeletal muscles have been observed with increasing age [6, 7]. Muscle atrophy in old rats is associated with a decrease in activity levels of certain enzymes involved in oxidative metabolism [10]. These changes in skeletal muscles of rats in the initial stages of aging (60–65 weeks) are considered to be due to the age-related disuse of skeletal muscles, which results in the lowering of oxidative capacity of individual fibers. A previous study [9] observed that 96-week-old rats retained the capacity to increase the oxidative enzyme activity and mitochondrial density of skeletal muscles in response to endurance exercises. Furthermore, our previous study [28] observed that voluntary running exercises prevented atrophy of type II fibers as well as the decrease in oxidative enzyme activity of type I and type II fibers in rats in the initial stages of aging (65 weeks). Therefore, it is expected that a reduction in the decrease of oxidative metabolism in skeletal muscles, which was induced by exposure to hyperbaric oxygen as well as by aerobic exercise, should treat fiber atrophy and the decrease in oxidative capacity of skeletal muscles during the initial stages of the aging process.

We classified fibers in the tibialis anterior muscles of mice into two types on the basis of staining intensities for the ATPase activity: type IIA and type IIB. In normobaric mice, type IIA fibers were smaller than type IIB fibers, irrespective of the age (Figure 4). Type IIA fibers are more effective in supplying oxygen and nutrients for oxidative metabolism from capillaries, which are located close to the membrane, because of their small sizes. These indicate that type IIA fibers can work at a relatively low intensity and have more prolonged activities than do type IIB fibers. In this study, a reduction in cross-sectional area of type IIB fibers (Figure 4(b)), but not type IIA fibers (Figure 4(a)), was observed at 90 weeks. Low-intensity and prolonged activities, which are performed presumably using type IIA fibers, continue during increasing age, while high-intensity and short activities, which are performed presumably using type IIB fibers, decrease with increasing age. These indicate that type IIB fibers become less active with increasing age and, therefore, facilitate disuse-induced atrophy as observed in Figure 4(b). In this study, there were no differences in cross-sectional area of type IIA or type IIB fibers between the normobaric and age-related hyperbaric mice (Figure 4). Therefore, exposure to hyperbaric oxygen had no effect on fiber cross-sectional area in the muscle. This view does not match our expectations and is inconsistent with the findings observed in relation to exercise [28].

Exposure to hyperbaric oxygen reduced the age-related decrease in the oxidative enzyme activity of the tibialis anterior muscle (Figure 3). Similarly, exposure to hyperbaric oxygen reduced the oxidative enzyme activity of type IIB fibers in the muscle at 57 weeks (initial stage of aging) and those of type IIA and type IIB fibers at 90 weeks (middle to late stages of aging) (Figure 5). The changes in the oxidative enzyme activity of the tibialis anterior muscle by exposure to hyperbaric oxygen corresponded well with that of muscle fibers. We conclude that exposure to hyperbaric oxygen used in this study reduced the age-related decrease in the oxidative capacity of skeletal muscles because of the increased oxidative metabolism in cells and tissues.

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Effects of Hyperbaric Oxygenation on Blood Pressure Levels of Spontaneously Hypertensive Rats

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ABSTRACT

Five-week-old normotensive Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were subjected to hyperbaric oxygenation with an enhanced atmospheric pressure (950 mmHg) and an increased oxygen concentration (36%) for 6 h per day. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were monitored for 8 weeks of hyperbaric oxygenation period. After 8 weeks of hyperbaric oxygenation, the derivatives of reactive oxygen metabolites (dROMs) and biological antioxidant potentials (BAPs) were measured. After 5 weeks of hyperbaric oxygenation, the hyperbaric group of WKY exhibited lower SBP than the age-matched normobaric group, while there were no differences in the DBP between the normobaric and hyperbaric groups. After 3 and 7 weeks of hyperbaric oxygenation, the hyperbaric group of SHR exhibited lower SBP and DBP than the age-matched normobaric group. The hyperbaric groups of both WKY and SHR exhibited lower dROMs than the respective normobaric groups. There were no differences in BAPs between the normobaric and hyperbaric groups of WKY. In contrast, the hyperbaric group of SHR exhibited higher BAPs than the normobaric group. We conclude that the hyperbaric oxygenation conditions used in this study effectively repress hypertension.

KEYWORDS: antioxidant capacity; hyperbaric oxygenation; hypertension; oxidative stress; spontaneously hypertensive rat (SHR); sympathetic activation

INTRODUCTION

We have developed a hyperbaric chamber for animal experiments; this chamber consists of an oxygen tank with an oxygen concentrator and an air compressor that automatically maintains an elevated atmospheric pressure and an increased oxygen concentration by using a computer-assisted system (1). The elevated atmospheric pressure with high oxygen concentration increases the partial pressure of oxygen and causes more oxygen to dissolve in the plasma. Our previous studies (1,2) showed that the activity of succinate dehydrogenase (SDH), which is an indicator of mitochondrial oxidative capacity in skeletal muscle fibers and spinal motoneurons of rats, increased after hyperbaric oxygenation. Furthermore, rats subjected to hyperbaric oxygenation exhibited more voluntary running activity than those under normobaric conditions (2).

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These findings suggest that an enhanced oxidative metabolism in cells and tissues was induced by adapting neuromuscular units to hyperbaric oxygenation and therefore the function of neuromuscular units was promoted. We observed that hyperbaric oxygenation inhibited the growth-related increase in the blood glucose level of type 2 diabetic Goto-Kakizaki (GK) rats (3). Furthermore, GK rats subjected to hyperbaric oxygenation exhibited increased oxidative capacities in their skeletal muscles, i.e., an increased fiber SDH activity and a shift from the low-oxidative type to the high-oxidative type of fibers (4,5).

In rats, abnormalities of the central neural mechanisms regulating peripheral sympathetic outflow and catecholamine metabolism following neurotransmitter release from nerve endings have been associated with hypertension (6). In fact, high sympathetic activation induces development and maintenance of hypertension (7). In contrast, prolonged aerobic exercise decreases sympathetic activation in hypertensive rats, thereby reducing high blood pressure (BP) levels (8). We expected that hyperbaric oxygenation with enhanced atmospheric pressure and increased oxygen concentration would depress the sympathetic activation and therefore inhibit development of

hypertension, because the effects of hyperbaric oxygenation are similar to those of exercise. Both result in an increase in oxidative metabolism in cells and tissues. In this study, the effects of hyperbaric oxygenation on BP levels in spontaneously hypertensive rats (SHR) were examined and compared to those of normotensive Wistar-Kyoto rats (WKY). Our findings verify that hyperbaric oxygenation can be a useful modality for treating hypertension.

MATERIALS AND METHODS

All experimental procedures were conducted in accordance with *The Guidelines for the Care and Use of Laboratory Animals* published by the Institutional Animal Use Committee of the Kyoto University.

Experimental Animals and Treatments

Spontaneously hypertensive rats are animal models of spontaneous hypertension that were developed by selective repeated inbreeding of an outbred WKY colony (9). Five-week-old male WKY (n = 16) and SHR (n = 16) were randomly assigned to normobaric (n = 8) and hyperbaric (n = 8) groups. All rats were individually housed in same sized cages. All rats were maintained in a controlled environment with fixed 12:12 h light:dark cycles (lights were switched off from 20:00 to 08:00) and room temperature and relative humidity maintained at 22 ± 2°C and 45–55%, respectively. Both groups were provided food and water *ad libitum*. Prior to hyperbaric oxygenation exposure, the SBP and DBP were automatically determined in conscious rats by the indirect tail-cuff method using BP-98A (Softron, Tokyo, Japan).

Exposure to Hyperbaric Oxygenation

The rats in the hyperbaric group were subjected to an atmospheric pressure of 950 mmHg and an oxygen concentration of 36% automatically maintained by a computer-assisted system (1). The hyperbaric chamber was 90 cm in length and 80 cm in diameter; thus, it could simultaneously house a number of rats (up to 20 cages). The rats in the hyperbaric group were subjected to the hyperbaric oxygenation for 6 h (10:00–16:00) daily for 8 weeks.

Blood Sampling and Biochemical Measurements

After 8 weeks of hyperbaric oxygenation period, blood samples were obtained from the abdominal aorta following 12 h of fasting; the samples were then centrifuged (6000 rpm, 1 min), and evaluated photometrically. A free radical and antioxidant potential determination device called Free Radical Analytical System 4 (Health & Diagnostics, Grosseto, Italy) was used to automatically measure the derivatives of reactive oxygen metabolites (dROMs) and the biological antioxidant potentials (BAPs). The dROMs were used as an index to determine the level of oxidative stress by measuring the amount of organic hydroperoxide (ROOH) converted into radicals that oxidize *N,N*-diethyl-para-phenylenediamine (10,11). The BAPs were determined on the basis of the capacity of the plasma sample to reduce ferric ions to ferrous ions (11).

Statistics

The data expressed as mean and standard deviation. Significant differences in body weight, SBP and DBP between the normobaric and hyperbaric groups were analyzed with unpaired t-tests. Furthermore, one-way analysis of variance (ANOVA) was used to evaluate the significant differences in dROMs and BAPs among the groups. When the differences were found to be significant based on the ANOVA analyses, further comparisons were made using *post hoc* tests. The 0.05 level of probability was considered significant.

RESULTS

Body Weight

There were no differences in the body weight between the normobaric and age-matched hyperbaric groups of both WKY (Figure 1A) and SHR (Figure 1D) during the hyperbaric oxygenation period.

Systolic and Diastolic Blood Pressures

In WKY, SBP was lower in the hyperbaric group than in the age-matched normobaric group after 5 weeks of hyperbaric oxygenation (Figure 1B). In contrast, there were no differences in the DBP between the normobaric and age-matched hyperbaric groups during the hyperbaric oxygenation period (Figure 1C).

In SHR, SBP was lower in the hyperbaric group than in the age-matched normobaric group after 3 weeks of hyperbaric oxygenation (Figure 1E). Furthermore, DBP was lower in the hyperbaric group than in the age-matched normobaric group after 7 weeks of hyperbaric oxygenation (Figure 1F).

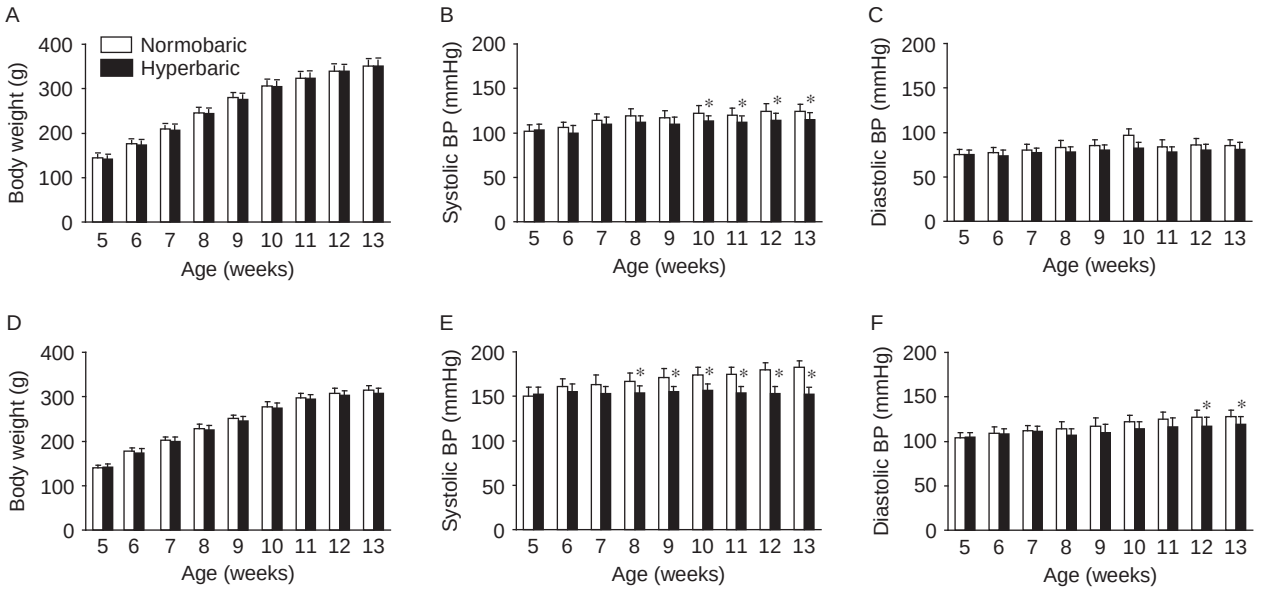

FIGURE 1 Body weights and systolic and diastolic blood pressures of normotensive Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) with and without hyperbaric oxygenation. Values are expressed as mean and standard deviation for 8 rats. (A, D), body weights of WKY and SHR; (B, E) SBP of WKY and SHR; (C, F) DBP of WKY and SHR: Abbreviation: BP-blood pressure. *$p < 0.05$ compared to the value of the age-matched normobaric group of the same strain.

The levels of dROMs and BAPs

The normobaric group of SHR exhibited the highest level of dROMs (Figure 2A). In both WKY and SHR, the levels of dROMs were lower in the hyperbaric group than in the respective normobaric group.

The level of BAPs was higher in the hyperbaric group of WKY than in the normobaric group of SHR (Figure 2B). In WKY, there were no differences in the level of BAPs between the normobaric and hyperbaric groups. In SHR, the level of BAPs was higher in the hyperbaric group than in the normobaric group.

DISCUSSION

Sympathetic Activation and ROS Production

It is widely accepted that sympathetic neuronal factors are involved in the development and maintenance of hypertension (7); sympathetic traffic to the cardiovascular system is increased in certain hypertensive animals. An enhanced sympathetic activation in hypertensive rats is mediated by overproduction of the highly reactive and toxic transient reactive oxygen species (ROS) (12). Salt-induced hypertension results

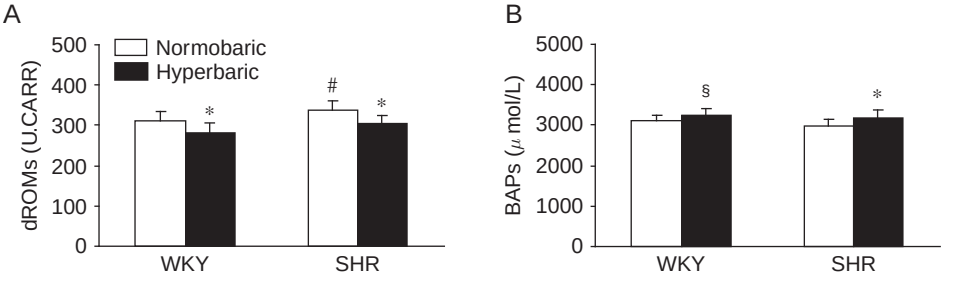

FIGURE 2 Derivatives of reactive oxygen metabolites (A) and biological antioxidant potentials (B) of normotensive Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) with and without hyperbaric oxygenation. Values are expressed as mean and standard deviation for 8 rats. Abbreviations: dROMs-derivatives of reactive oxygen metabolites; U.CARR-unit of Carratelli, which is a conventional unit named after a biologist (Italy) who elaborated a scale based on more than 5000 nonsmoking healthy subjects of ages ranging from 14 to 80 years (1 U.CARR = 0.08 mg of hydroperoxide/100 ml H_2O_2) (29); BAPs-biological antioxidant potentials. *$p < 0.05$ compared to the value of the normobaric group of the same strain. $^{\#}p < 0.05$ compared to the values of the normobaric and hyperbaric groups of WKY. $^{\S}p < 0.05$ compared to the value of the normobaric group of SHR.

from enhanced central sympathetic activation. In mice with a deficiency of adrenomedullin, which is an intrinsic antioxidant, salt-loading induced overproduction of ROS and enhanced sympathetic activation in the brain (13,14). Thus, an overproduction of ROS induces sympathoexcitation, which results in hypertension. The excessive production of ROS causes accelerated damage to cellular elements, including lipids, proteins, and nucleic acids, and can subsequently induce atherosclerosis and thrombosis (15). There are many similar results indicating that overproduction of ROS and high sympathetic activation lead not only to hypertension but also to damage of cells and tissues (16–20).

In this study, the level of dROMs, which are indices of oxidative stress, was higher in normobaric SHR than in normobaric WKY (Figure 2A), suggesting that production of ROS was greater in SHR than in WKY. These results correspond with our recent study using SHR (21). Therefore, it is conceivable that inhibition of ROS overproduction leads to a decrease in high BP and prevents further complications.

Effect of Exercise on Hypertension

In general, prolonged aerobic exercise reduces BP and prevents hypertension in animals (22,23) and humans (24,25). Exercise reduces sympathetic activation in hypertensive rats, thus reducing high BP and preventing the development of hypertension (8,26). A previous study (26) observed that not only sympathetic tonus but also parasympathetic tonus were attenuated after exercise in hypertensive rats. The effects of exercise on BP levels in hypertensive rats were investigated by assuming a mechanism which involves calcium-dependent dopamine synthesis in the brain (8). An exercise-induced enhancement in dopamine levels inhibited sympathetic nerve activity via dopamine D_2 receptors present in the brain, which ultimately reduced high BP.

Effect of Hyperbaric Oxygenation on Hypertension

An increase in atmospheric pressure accompanied by a high concentration of oxygen enhances the partial pressure of oxygen and causes more oxygen to dissolve in the plasma; this leads to increased oxidative enzyme activity in the mitochondria and consequently, an enhanced rate of oxidative metabolism in cells and tissues. In fact, the findings of our previous studies (1,2) suggest that the beneficial effects of hyperbaric oxygenation were associated with increased oxidative enzyme activities in skeletal muscle fibers and spinal motoneurons in rats. In addition, possible beneficial effects of hyperbaric oxygenation have been reported in previous studies (3–5); the growth-related increase in the blood glucose level in type 2 diabetic rats, which had lower oxidative capacities in skeletal muscles (27,28), has been reported to be inhibited by hyperbaric oxygenation. These findings indicate that hyperbaric oxygenation inhibits the decrease in the oxidative metabolism of skeletal muscles in type 2 diabetic rats.

We hypothesized that overproduction of ROS induces sympathoexcitation and thus, hypertension, while hyperbaric oxygenation, which induces an increased oxidative metabolism, eliminates ROS and leads in the maintenance of normal BP. In this study, SBP and DBP of SHR decreased following hyperbaric oxygenation (Figures 1E and 1F). Furthermore, the level of dROMs, which are indices of oxidative stress, decreased (Figure 2A) and the level of BAPs, which are indices of antioxidant capacity, increased (Figure 2B) following hyperbaric oxygenation. This indicates that hyperbaric oxygenation induced a minor but significant decrease in production of ROS in hypertensive rats.

An enhancement of the oxidative metabolism in cells and tissues increases the carbon dioxide concentration in the surrounding region and this, in turn, facilitates oxygen release from hemoglobin and causes dilation of blood vessels. Therefore, it is suggested that vascular enlargement by hyperbaric oxygenation is one of the factors that effectively prevents high BP.

In this study, the effects of hyperbaric oxygenation on BP levels differed between WKY and SHR; SBP and DBP decreased in SHR (Figures 1E and 1F), while SBP (Figure 1B), but not DBP (Figure 1C) decreased in WKY. Furthermore, in SHR, the SBP decreased after 3 weeks of hyperbaric oxygenation, while in WKY, it decreased after 5 weeks of hyperbaric oxygenation. This phenomenon, in which the depressor effect of hyperbaric oxygenation on BP was prominent in SHR, can be explained by a higher prevailing sympathetic activation in SHR than in WKY. Therefore, hyperbaric oxygenation effectively stabilized BP in SHR; however, the beneficial effect of hyperbaric oxygenation was not apparent in WKY because normal BP had been previously maintained.

CONCLUSION

Hyperbaric exposure with high oxygen concentration used in this study decreased SBP and DBP in hypertensive rats. Therefore, the conditions of hyperbaric oxygenation are effective for preventing and improving hypertension. However, clinical trials are needed to evaluate the efficacy of hyperbaric oxygenation for treatment of patients suffering from hypertension.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Skin Research and Technology

Hyperbaric oxygen improves ultraviolet B irradiation-induced melanin pigmentation and diminishes senile spot size

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Background: The effects of exposure to hyperbaric oxygen on ultraviolet B (UVB) irradiation-induced melanin pigmentations of skins and on senile spot sizes of faces were investigated.
Methods: In the first experiment, male subjects were irradiated with UVB on their upper arms for inducing erythema and the subsequent melanin pigmentation. They were exposed to a hyperbaric environment at 1.25 atmospheres absolute (ATA) with 32% oxygen for 1 h/day, three times per week. In the second experiment, female subjects were exposed to a hyperbaric environment at 1.25 ATA with 32% oxygen for 1 h/day, two times per week.
Results: In the first experiment, melanin pigmentations lightened after 4 weeks of exposure to hyperbaric oxygen. In the second experiment, senile spot sizes became small after 12 weeks of exposure to hyperbaric oxygen.
Conclusion: We concluded that exposure to hyperbaric oxygen used in this study accelerates both the fading in melanin pigmentation and the decrease in senile spot size.

Key words: epidermis – erythema – hyperbaric oxygen – melanin pigmentation – senile spot size – ultraviolet B irradiation

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Cells and tissues need oxygen to produce energy and maintain their metabolism and function. The epidermis has direct access to oxygen and obtains considerable oxygen from the atmosphere; therefore, the epidermis can maintain a higher oxygen level than many internal tissues (1). When UV is irradiated on the skin, melanocytes are assumed to exhibit a series of oxidative stresses (2), suggesting that considerable oxygen is needed to eliminate oxidative stress and to damaged epidermis.

Cells and tissues receive oxygen in two ways: one is from oxygen binding to hemoglobin in red blood cells, which flow in blood vessels, and another is from oxygen dissolved in the tissue fluids including the plasma. Previous studies (3, 4) observed that exposure to hyperbaric oxygen induces an increase in the amount of dissolved oxygen in the plasma. Hyperbaric exposure at 2–3 atmospheres absolute (ATA) with 100% oxygen is generally used for therapy of temporary hypoxia (5), tissue repair after burn (6), intractable ulcer (7, 8), and open fractures and crush injuries (9).

Hyperbaric exposure with moderate atmospheric pressure and oxygen concentration has been investigated (10); hyperbaric exposure at 1.25 ATA with 36% oxygen enhances the oxidative enzyme activity of cells and tissues (10, 11), therefore inducing inhibition in the growth-associated increase in the blood glucose level of type-2 diabetic Goto-Kakizaki rats (12–14) and in the blood pressure level of spontaneously hypertensive rats (15). These studies (12–15) concluded that hyperbaric exposure with moderate atmospheric pressure and oxygen concentration has a beneficial effect on oxidative metabolism in cells and tissues, although the amount of dissolved oxygen is greater by hyperbaric exposure at 2–3 ATA with 100% oxygen than that at 1.25 ATA with 36% oxygen.

It is suggested that keratinocyte proliferation and epidermal cell regeneration are activated by an enhanced oxidative metabolism, which is

induced by exposure to moderate hyperbaric oxygen. In this study, we examined whether hyperbaric exposure at 1.25 ATA with 32% oxygen has an effect on ultraviolet B (UVB) irradiation-induced melanin pigmentations of upper arm skins and on senile spot sizes of faces.

Materials and Methods

This study was approved by the Experiment Committee of Kao Corporation, Tokyo, Japan, and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and the Guiding Principles published by the Physiological Society of Japan. Signed informed consent was obtained from all subjects before experiment.

The first experiment

Erythema and the subsequent melanin pigmentation of 14 healthy male subjects (31–44 years old) were induced by a UVB irradiation device (FL20SE UV light; Toshiba, Japan) (16). None of these subjects had skin diseases or a history of photosensitivity. In order to determine the minimal exposure dose (MED) for recognizing erythema of individual subjects, UVB with a peak radiation wavelength of 350 nm was irradiated from 190.04 to 427.59 mJ/cm^2 with an increment of 25% on 1.5 cm^2 diameter at six different sites of the ventral side of their right upper arms. The MED was assessed 24 h after UVB irradiation. Thereafter, the UVB, which corresponded to the double dose of individual MEDs, was irradiated on 1.5 cm^2 diameter of the ventral side of their left upper arms.

In this study, erythema was observed after 4 weeks of UVB irradiation. Therefore, we assigned subjects into the control (CO; $n = 7$) and hyperbaric oxygen (HO; $n = 7$) groups as these two groups had a similar degree of erythema. Subjects in the HO group were exposed to 1.25 ATA with 32% oxygen for 1 h/day, three times per week for 4 weeks. The hyperbaric chamber was designed to automatically maintain an increased level of oxygen concentration and atmospheric pressure. Subjects in the CO group were maintained under a normobaric environment at 1 ATA with 21% oxygen.

The luminance value (L^*), which is used as an index for the level of melanin pigmentation, was determined by an image-processing method using a videomicroscope interfaced with a computer 1, 2, 3, and 4 weeks after exposure to hyperbaric oxygen. The L^* is one of the three indices of chromatically designated $L^*a^*b^*$, which matches the Commission International de l'Eclairage standard observer response (17). The change in L^* of spot (ΔL^*pig.), which was irradiated by UVB, was measured every week by a colormeter (CM2600D; Minolta, Tokyo, Japan). In order to cancel the change in skin color during the test period, we measured the change in skin color on the control spot (ΔL^*skin) next to the spot, which was irradiated by UVB. The $\Delta\Delta L^*$ value ($\Delta\Delta L^* = \Delta L^*\text{pig.} - \Delta L^*\text{skin}$) was determined to assess the change in melanin pigmentation on the spot.

The second experiment

Seven healthy female subjects (30–41 years old), who had senile spots on their faces, were exposed to 1.25 ATA with 32% oxygen for 1 h/day, two times per week for 12 weeks. None of these subjects had skin diseases or a history of photosensitivity.

A total of 13 senile spots (one or two spots per subject) were selected. The images of senile spots were taken using a digital microscope (Hi-Scope Advanced KH-3000; HiROX, Tokyo, Japan) before exposure to hyperbaric oxygen as baseline and after 3, 7, and 12 weeks of hyperbaric oxygen (Fig. 1). The images were separated into melanin and hemoglobin chromophores (18). The images of melanin chromophores were converted to binary pictures for making clear contours of spots, and thereafter, their sizes were measured by an image-processing soft (Photoshop CS4; Adobe Systems, Tokyo, Japan).

Statistics

The data were presented as mean and SD. One-way analysis of variance (ANOVA) was used to evaluate the time-course changes in melatonin pigmentation and senile spot size. When there were overall significant differences based on the ANOVA analyses, individual group comparisons were made using Scheffé's *post hoc* tests. A probability level of 0.05 was considered to be statistically significant.

Results

Effect of exposure to hyperbaric oxygen on UVB irradiation-induced pigmentation

Figure 2 shows photographs of melanin pigmentations in the CO and HO groups before

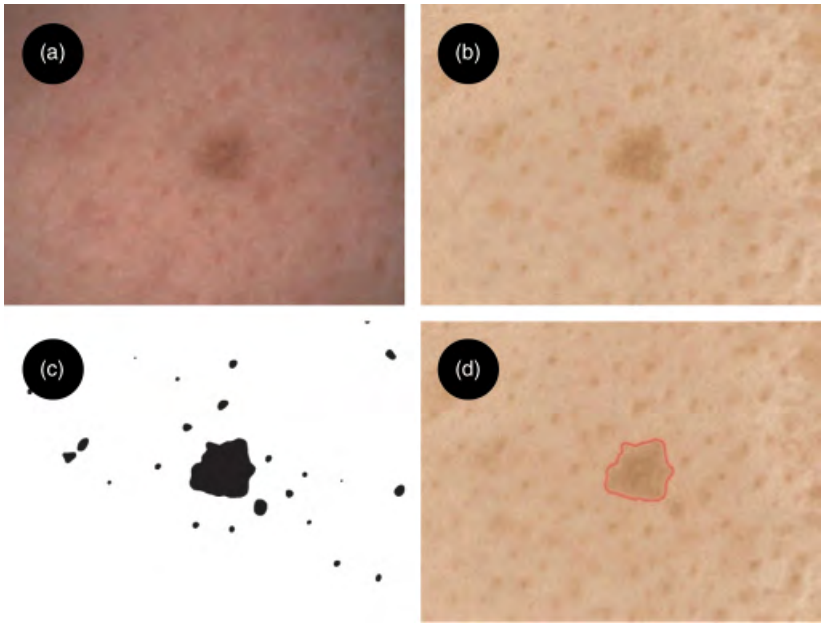

Fig. 1. Image processing for the senile spot size measurement. The original image (a) was modified to the one including the noise-reduced component (b). The image was converted to the binary picture (c) for making a clear contour of the spot, and thereafter, its size was measured by an image-processing soft (d).

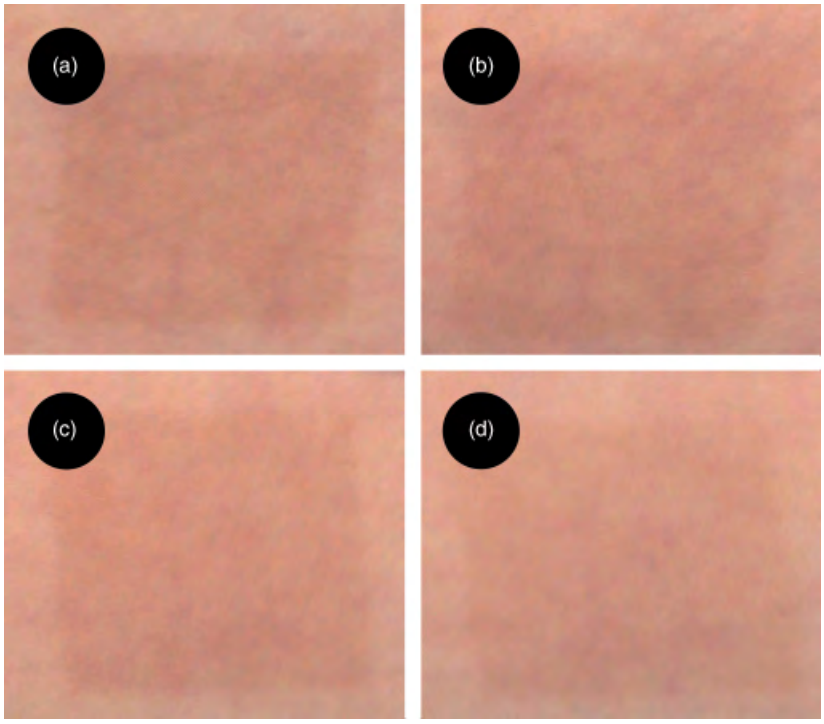

Fig. 2. Photographs of ultraviolet B-induced pigmentations in the control (a, b) and hypebaric oxygen (c, d) groups. (a, c) control and hyperbaric groups before exposure to hyperbaric oxygen, respectively; (b, d) control and hyperbaric groups after 4 weeks of exposure to hyperbaric oxygen, respectively.

and 4 weeks after exposure to hyperbaric oxygen. The melanin pigmentations turned light following exposure to hyperbaric oxygen (Fig. 3); the $\Delta\Delta L^*$ of the HO group was significantly higher than that of the CO group after 4 weeks of exposure to hyperbaric oxygen, although there was no difference in the $\Delta\Delta L^*$ between the CO- and age-matched HO groups during 1–3 weeks of exposure to hyperbaric oxygen.

Effect of exposure to hyperbaric oxygen on senile spot size

Figure 4 shows the photographs and images of senile spots before and 12 weeks after exposure to hyperbaric oxygen. Senile spot sizes gradually decreased following exposure to hyperbaric oxygen. The spot size after 12 weeks of exposure to hyperbaric oxygen was significantly smaller than that before exposure to hyperbaric oxygen; the average senile spot size after 12 weeks of exposure to hyperbaric oxygen was 82% compared with that before exposure to hyperbaric oxygen (Fig. 5).

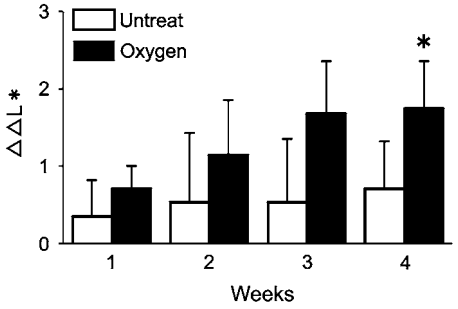

Fig. 3. Changes in pigmentation (ΔΔL) following exposure to hyperbaric oxygen. The data indicate the means and SD obtained from seven subjects. Subjects in the HO group were subjected to exposure to hyperbaric oxygen after 4 weeks of recovery from UVB irradiation because erythema was observed at 4 weeks after UVB irradiation. *P<0.05 compared with the age-matched CO group.*

Discussion

Exposure to hyperbaric oxygen

Hyperbaric exposure at a pressure >1 ATA, usually at 2–3 ATA, with 100% oxygen has been used successfully as an adjunctive therapy for many clinical disorders related both to ischemia and/or hypoxia (5–9). In contrast, a previous study (19) reported that cataracts, particularly nuclear cataracts, which are major cause of loss

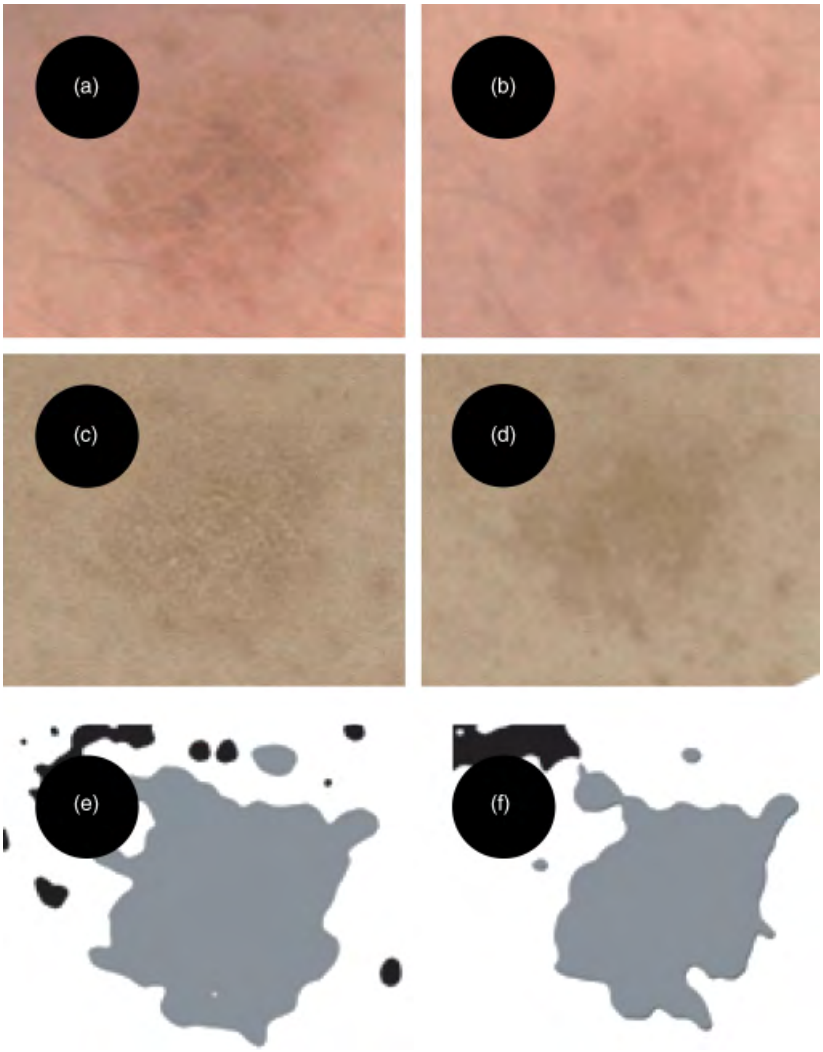

Fig. 4. Images of the senile spots before (a, c, and e) and 12 weeks after exposure to hyperbaric oxygen (b, d, and e). (a, b) cross-polarized images; (c, d) images including components of melanin pigmentation; (e, f) binary pictures.

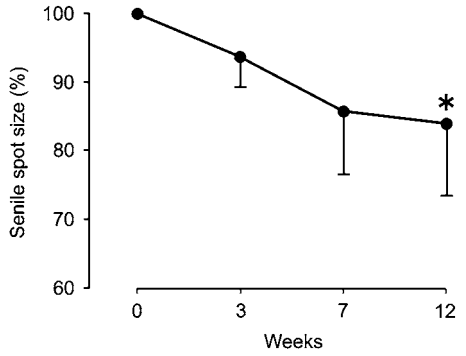

*Fig. 5. Changes in senile spot size (%) following exposure to hyperbaric oxygen. The data indicate the means and SD obtained from 13 spots of 11 subjects. *P < 0.05 compared with the value before exposure to hyperbaric oxygen.*

of lens transparency, in 17- to 18-month-old guinea-pigs were induced by hyperbaric exposure at 2.5 ATA with 100% oxygen for 2–2.5 h, three times per week for up to 100 times. Similarly, myopia and cataract developed in human lenses by prolonged hyperbaric exposure at 2–2.5 ATA with 100% oxygen for 1.5 h, one time per day from 150 to 850 times (20), but have been observed rarely after only 48 times (21).

Exposure to hyperbaric oxygen is considered to cause excessive production of reactive oxygen species (ROS) in several tissues and organs (22–25) and ROS have the ability to act as important signaling molecules (26). A standard procedure of hyperbaric exposure at 2–3 ATA with 100% oxygen has the potential to induce and accelerate myopia and cataract. Furthermore, hyperbaric oxygen-induced oxidative stress levels depend not only on the pressure but also on the exposure duration; the pressure from 2.5 to 3 ATA and the duration from 90 to 120 min resulted in a pronounced change in oxidative stress level (24, 25).

The formation of ROS (27) and lipid peroxides (28) in rat brain increases after exposure to hyperbaric oxygen at a more increased pressure level. Furthermore, exposure to hyperbaric oxygen causes elevation of blood pressure level and lowering of both the heart rate and the blood glucose level, which were augmented in the presence of hypertension, diabetes, and both hypertension and diabetes (29). Especially, diabetic patients cause a greater elevation in blood pressure level after exposure to hyperbaric oxygen compared with hypertensive patients and healthy individuals.

In this study, we used hyperbaric exposure at 1.25 with 32% oxygen because these moderate pressures and oxygen concentrations enhance the oxidative enzymatic activity of cells and tissues (10, 11). Previous studies observed that hyperbaric oxygen at 1.25 ATA with 36% oxygen induced an inhibition in the growth-associated increase in the blood glucose level of type-2 diabetic Goto-Kakizaki rats (12–14) and in the blood pressure level of spontaneously hypertensive rats (15). Furthermore, overproduction of ROS in spontaneously hypertensive rats was inhibited after exposure to moderate hyperbaric oxygen (15). These studies (12–15) conclude that hyperbaric exposure with moderate atmospheric pressure and oxygen concentration has a beneficial effect on enhancement and improvement in the oxidative metabolism of cells and tissues.

Effect of exposure to hyperbaric oxygen on melanin pigmentation

Many agents, which are mostly ointments, have been investigated for improvement in damaged skin by erythema, peeling, and stinging (30–32). Erythema and the subsequent pigmentation were suppressed and in a parallel manner by corticosteroids and indomethacin that were applied immediately after UVB irradiation (16). Chemical mediators, especially prostaglandins, released in the inflammatory process have an important role in the induction of erythema (33, 34). Histamine is also involved in the erythema formation in skins of animals and humans (35), probability by enhancing prostaglandins synthesis in UVB-irradiated skin in the earlier phase of inflammation (36). Furthermore, these mediators stimulate melanocytes of skins in animals (37, 38) and humans (39). It is suggested that the suppression of UVB irradiation-induced pigmentation is due at least to the reduction in prostaglandin synthesis through the inhibition of cyclooxygenase by indomethacin and the induction of annexin of lipocortin by corticosteroids (40).

Unlike many internal tissues, the epidermis has direct access to atmospheric oxygen and obtains considerable oxygen from the atmosphere; therefore, the epidermis can maintain higher oxygen levels than internal tissues (1). However, chronic wounds of the skin in regions of poor blood supply, e.g., chronic ulcers, have been proposed to be refractory to healing owing to insufficient blood supply and oxygen availability. Therefore, patients utilize dissolved oxygen increased by hyperbaric oxygen or the wound is treated topically with elevated oxygen

concentrations. Furthermore, melanocytes are assumed to exhibit a series of oxidative stresses during UVB irradiation-induced melanogenesis (2), suggesting that considerable oxygen is needed for the repair and maintenance of epidermis after erythema formation. These views indicate that the epidermis is exposed to local tissue hypoxia, which was induced by limited supply of oxygen under conditions of chronic wound and melanogenesis.

We postulated that increased oxygen induced by exposure to hyperbaric oxygen is effective for repair from damage in the epidermis, which was induced by UVB irradiation. In this study, melanin pigmentation lightened after 4 weeks of exposure to hyperbaric oxygen (Fig. 3). It is suggested that the increase in blood volume and dissolved oxygen into damaged epidermis was induced by exposure to hyperbaric oxygen. Although inhibition in the overproduction of ROS under epidermis formation would be expected following exposure to hyperbaric oxygen, we did not measure changes in the ROS level in this study. Further studies including animal experiments are needed to elucidate whether exposure to hyperbaric oxygen has an effect on inhibition in the overproduction of ROS, which may be linked to epidermis formation.

Effect of exposure to hyperbaric oxygen on senile spot size

A previous study (18) observed seasonal changes in senile spot sizes of faces in 105 female subjects (20–60 years old). A total of 115 senile spot sizes were determined quantitatively in four different seasons: March, June, September, and December. The senile spot sizes became largest in June; however, the change in senile spot size narrow: within the 5% range during a year. Therefore, it is concluded that there were no seasonal changes in the senile spot size. In this study, the senile spot size after 12 weeks of exposure to hyperbaric oxygen was 82% compared with that before exposure to hyperbaric oxygen (Fig. 5). We suggest that decreased spot sizes after exposure to hyperbaric oxygen are due to an increased oxidative metabolism, which were considered in our first experiment using UVB irradiation-induced melatonin pigmentation and its inhibition by exposure to hyperbaric oxygen.

Conclusion

Hyperbaric exposure at 1.25 ATA with 32% oxygen used in this study accelerated both the fading in melanin pigmentation and the decrease in senile spot size because of increased oxidative metabolism.

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ORIGINAL ARTICLE

Effect of exposure to hyperbaric oxygen on diabetes-induced cataracts in mice

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Abstract

Background: The growth-associated increase in the blood glucose level of animals with Type 2 diabetes is inhibited by moderate hyperbaric exposure at 1.25 atmospheres absolute (ata) with 36% oxygen, presumably due to an increase in oxidative metabolism. However, there are no data available regarding the effect of moderate hyperbaric oxygen (HBO) on diabetes-induced cataracts.

Methods: Four-week-old mice with Type 2 diabetes and cataracts were exposed to 1.25 ata with 36% oxygen, 6 h daily, for 12 weeks, followed by normal conditions at 1 ata with 21% oxygen for 16 weeks (cataract + hyperbaric group). Levels of blood glucose and derivatives of reactive oxygen metabolites (dROMs), used as an index of oxidative stress, and the turbidities of the lenses from these mice at 4, 8, 12, 16, and 32 weeks of age were compared with those of control and diabetic (cataract group) mice not exposed to HBO.

Results: Non-fasting and fasting blood glucose levels were lower in the cataract + hyperbaric group at 12, 16, and 32 weeks of age than in the age-matched cataract group. The levels of dROMs were lower in the cataract + hyperbaric group at 16 and 32 weeks of age than in the age-matched cataract group. The turbidities of the peripheral and central regions of the lenses were lower in the cataract + hyperbaric group at 12, 16, and 32 weeks of age than in the age-matched cataract group.

Conclusions: Hyperbaric exposure at 1.25 ata with 36% oxygen delays cataract development and progression in mice with Type 2 diabetes.

Keywords: cataract, hyperbaric oxygen, lens turbidity, mouse, Type 2 diabetes.

Significant findings of the study: We found that hyperbaric exposure at 1.25 ata with 36% oxygen inhibits and delays cataract formation in mice with Type 2 diabetes.
What this study adds: The findings provide significant insight into developing clinical trials for the prevention of cataract formation in patients with Type 2 diabetes.

Introduction

An elevation in atmospheric pressure accompanied by high oxygen concentration enhances the partial pressure of oxygen and increases blood flow and the concentration of dissolved oxygen in the plasma.[1,2] We have reported previously that moderate hyperbaric exposure with a pressure of 1.25 atmospheres absolute (ata) and an oxygen concentration of 36% is required for effective responses with regard to oxidative

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metabolism in the neuromuscular system (i.e. spinal motoneurons and the skeletal muscle fibers they innervate).[3,4] In previous studies,[5–9] we observed that the development and progression of lifestyle-related diseases were delayed and improved by moderate hyperbaric exposure at 1.25 ata with 36% oxygen. The growth-associated increase in the blood glucose level of rats with Type 2 diabetes was attenuated by exposure to moderate hyperbaric oxygen (HBO).[5–7] The enhanced blood glucose level and decreased oxidative enzyme activity observed in the skeletal muscles of rats with Type 2 diabetes[10,11] were restored to normal values after exposure to moderate HBO.[8] In addition, the growth-associated increase in the blood pressure of spontaneously hypertensive rats was attenuated by exposure to moderate HBO.[9]

It is widely known that the polyol pathway is the main contributor to diabetes-induced cataract formation (i.e. excess blood glucose is reduced to sorbitol, which is responsible for cataract formation).[12] The increased availability of oxygen following exposure to moderate HBO inhibits the growth-associated increase in the blood glucose level of rats with Type 2 diabetes and, in turn, may delay the accumulation of sorbitol in the lens, suggesting that exposure to moderate HBO inhibits diabetes-induced cataract formation as well as hyperglycemia.

In the present study, 4-week-old mice with Type 2 diabetes and cataracts were subjected to moderate hyperbaric exposure at a pressure of 1.25 ata with 36% oxygen, 6 h daily, for 12 weeks. Thereafter, the mice were subjected to normal conditions at 1 ata with 21% oxygen for 16 weeks. The levels of blood glucose and derivatives of reactive oxygen metabolites (dROMs), used as an index of oxidative stress, and the turbidities of the peripheral and central regions of the lenses were measured after 4, 8, 12, and 16 weeks of exposure to moderate HBO and after 16 weeks of resuming normal conditions. Data were compared at each time point to age-matched control and Type 2 diabetic mice that were not exposed to HBO.

Methods

Experimental animals

All experimental and animal care procedures were conducted in accordance with the Guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals. This study was approved by the Institutional Animal Experimentation Committee of Kyoto University (Kyoto, Japan).

An animal model for Type 2 diabetes and cataracts was developed by repeated inbreeding of normal ICR mice with glucose tolerance at the upper limit of the normal range at the Research Center of Animal Models for Human Diseases, Fujita Health University (Toyoake, Japan).

Four-week-old mice were randomly assigned to one of three groups ($n = 5$ per group): (i) a control group, ICR mice without Type 2 diabetes, cataracts, or exposure to HBO; (ii) a cataract group, mice with Type 2 diabetes and cataracts, but no exposure to HBO; and (iii) a cataract + HBO group, mice with Type 2 diabetes and cataracts and exposed to HBO. The mice in the control and cataract groups were exposed to 1 ata (760 mmHg) with 21% oxygen throughout the study. The mice in the cataract + HBO group were exposed to 1.25 ata (950 mmHg) with 36% oxygen, automatically maintained by a computer-assisted system in a hyperbaric chamber for 6 h (1000–1600 hours) daily for 12 weeks. Thereafter, the mice in the cataract + HBO group were subjected to normal conditions at 1 ata (760 mmHg) with 21% oxygen for 16 weeks.

All mice were housed individually from 4 to 32 weeks of age in a room maintained under a controlled light–dark cycle (lights on from 0700 to 1900 hours) at a temperature of 22 ± 2°C and relative humidity of 45–55%. Food and water were provided *ad libitum* to all groups. Body weights were measured when the mice were 4, 8, 12, 16, and 32 weeks of age.

Blood sampling and biochemical measurements

Blood samples were obtained from the tails of fully conscious mice and analyzed using a blood glucose meter (GT-1650; Arkray, Kyoto, Japan). Non-fasting blood glucose (NFBG) levels were measured at 4, 8, 12, 16, and 32 weeks of age. Fasting blood glucose (FBG) levels also were measured after 12 h of fasting at the same points. Blood samples were centrifuged at 6000 r.p.m. for 1 min and evaluated photometrically. A free radical and antioxidant potential determination device (Free Radical Analytical System 4; Health & Diagnostics, Grosseto, Italy) was used to automatically measure the level of dROMs. The dROMs were used as an index of oxidative stress in the present study because they measure the amount of organic hydroperoxide (ROOH) converted into radicals that oxidize *N*,*N*-diethyl-*para*-phenylenediamine.[13–15]

Analyses of lens turbidity

After blood sampling at 4, 8, 12, 16, and 32 weeks of age, photographs of the lenses were acquired in anesthetized mice (sodium pentobarbital, 5 mg/100 g, *i.p.*). Images were stored using a computer-assisted

image-processing system (Neuroimaging System, Kyoto, Japan). Images of the peripheral and central regions of the lenses were digitized and converted to gray scale density images. Each pixel in the images was quantified as one of 256 gray levels; a gray level of 0 was equivalent to 100% light transmission, whereas a gray level of 255 was equivalent to 0% light transmission. The optical density (OD), which was converted using gray level values, was determined using a calibration photographic tablet with 21 steps of gradient-density ranges and the corresponding diffused density values. Lower density values indicated cataracts; therefore, lower OD values indicated higher levels of turbidity.[16,17]

Statistical analyses

All data are presented as the mean ± SD. Two-way analysis of variance (ANOVA) was used to assess the main effects of growth (4, 8, 12, 16, and 32 weeks of age) and treatment (control, cataract, and cataract + HBO groups) or the interactions between growth and treatment on body weight, levels of NFBG, FBG, and dROMs, and the digitized gray scale densities of the peripheral and central regions of the lenses. If overall significant differences were found for the main or interaction effects, Scheffé's *post hoc* test was used to determine the specific source of the difference. Statistical significance was set at $P < 0.05$. All analyses were performed using SPSS Statistics 19 (IBM, Armonk, NY, USA).

Results

Body weight

There were significant main effects for growth ($F = 193.05$; $P < 0.05$) and HBO ($F = 278.31$; $P < 0.05$) and their interaction ($F = 14.07$; $P < 0.05$) on body weight. Body weight was greater in the control, cataract, and cataract + HBO groups at 8, 12, 16, and 32 weeks than in the same groups at 4 weeks (Fig. 1). However, there was no growth-associated change in the body weight of these three groups from 8 to 32 weeks.

There was no difference in body weight among the control, cataract, and cataract + HBO groups at 4 weeks (Fig. 1). In contrast, body weight was lower in the cataract and cataract + HBO groups at 8, 12, 16, and 32 weeks than in the age-matched control group.

Blood glucose level

There were significant main effects for growth ($F = 73.07$; $P < 0.05$) and HBO ($F = 583.76$; $P < 0.05$) and their interaction ($F = 29.83$; $P < 0.05$) on NFBG levels. There was no growth-associated change in the NFBG level of the control group from 4 to 32 weeks (Fig. 2). In contrast, the NFBG level was higher in the cataract group at 12 weeks than in the same group at 4 weeks. Furthermore, the NFBG level was higher in the cataract group at 16 and 32 weeks than in the same group at 4, 8, and 12 weeks. The NFBG level was higher in the cataract + HBO group at 16 and 32 weeks than in the same group at 4 and 8 weeks.

The NFBG level was higher in the cataract and cataract + HBO groups at 4, 8, 12, 16, and 32 weeks than in the age-matched control group (Fig. 2). The NFBG level was lower in the cataract + HBO group at 12, 16, and 32 weeks than in the age-matched cataract group.

There were significant main effects for growth ($F = 58.51$; $P < 0.05$) and HBO ($F = 236.33$;

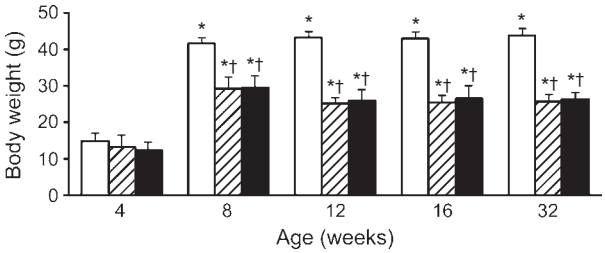

Figure 1 Body weights of the control (□),diabetic cataract (▨), and diabetic cataract + hyperbaric oxygen (HBO)-treated (■) groups of mice at 4, 8, 12, 16, and 32 weeks of age. Values are the mean ± SD ($n = 5$ per group). *$P < 0.05$ compared with the same group at 4 weeks of age; †$P < 0.05$ compared with the age-matched control group.

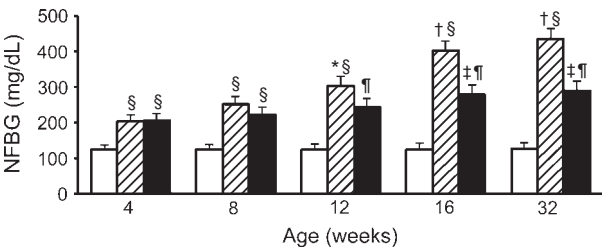

Figure 2 Non-fasting blood glucose (NFBG) levels in the control (□), diabetic cataract (▨), and diabetic cataract + hyperbaric oxygen (HBO)-treated (■) groups of mice at 4, 8, 12, 16, and 32 weeks of age. Values are the mean ± SD ($n = 5$ per group). *$P < 0.05$ compared with the cataract group at 4 weeks of age; †$P < 0.05$ compared with the cataract group at 4, 8, and 12 weeks of age; ‡$P < 0.05$ compared with the cataract + HBO group at 4 and 8 weeks of age; §$P < 0.05$ compared with the age-matched control group; ¶$P < 0.05$ compared with the age-matched control and cataract groups.

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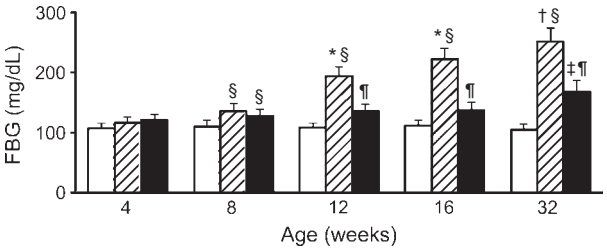

Figure 3 Fasting blood glucose (FBG) levels in the control (□), diabetic cataract (▨), and diabetic cataract + hyperbaric oxygen (HBO)-treated (■) groups of mice at 4, 8, 12, 16, and 32 weeks of age. Values are the mean ± SD (n = 5 per group). *P < 0.05 compared with the cataract group at 4 and 8 weeks of age; †P < 0.05 compared with the cataract group at 4, 8, and 12 weeks of age; ‡P < 0.05 compared with the cataract + HBO group at 4, 8, 12, and 16 weeks of age; §P < 0.05 compared with the age-matched control group; ¶P < 0.05 compared with the age-matched control and cataract groups.

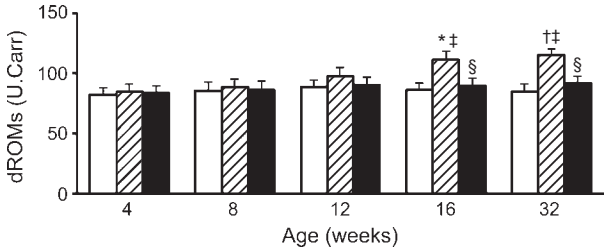

Figure 4 Levels of derivatives of reactive oxygen metabolites (dROMs) in the control (□), diabetic cataract (▨), and diabetic cataract + hyperbaric oxygen (HBO)-treated (■) groups of mice at 4, 8, 12, 16, and 32 weeks of age. Values are the mean ± SD (n = 5 per group). 1 U.Carr = 0.08 mg hydroperoxide/100 mL H_2O_2. *P < 0.05 compared with the cataract group at 4 and 8 weeks of age; †P < 0.05 compared with the cataract group at 4, 8, and 12 weeks of age; ‡P < 0.05 compared with the age-matched control group; §P < 0.05 compared with the age-matched cataract group.

$P < 0.05$) and their interaction ($F = 28.98$; $P < 0.05$) on FBG levels. There was no growth-associated change in the FBG level of the control group from 4 to 32 weeks (Fig. 3). In contrast, the FBG level was higher in the cataract group at 12 and 16 weeks than in the same group at 4 and 8 weeks. Furthermore, the FBG level was higher in the cataract group at 32 weeks than in the same group at 4, 8, and 12 weeks. The FBG level was higher in the cataract + HBO group at 32 weeks than in the same group at 4, 8, 12, and 16 weeks.

The FBG level was higher in the cataract and cataract + HBO groups at 8, 12, 16, and 32 weeks than in the age-matched control group (Fig. 3). The FBG level was lower in the cataract + HBO group at 12, 16, and 32 weeks than in the age-matched cataract group.

Level of dROMs

There were significant main effects for growth ($F = 12.81$; $P < 0.05$) and HBO ($F = 33.76$; $P < 0.05$) and their interaction ($F = 5.80$, $P < 0.05$) on the level of dROMs. There was no growth-associated change in the level of dROMs in the control and cataract + HBO groups from 4 to 32 weeks (Fig. 4). There was no growth-associated change in the level of dROMs in the cataract group from 4 to 12 weeks. However, the level of dROMs was higher in the cataract group at 16 weeks than in the same group at 4 and 8 weeks. Furthermore, the level of dROMs was higher in the cataract group at 32 weeks than in the same group at 4, 8, and 12 weeks.

The level of dROMs was higher in the cataract group at 16 and 32 weeks than in the age-matched control group (Fig. 4). The level of dROMs was lower in the cataract + HBO group at 16 and 32 weeks than in the age-matched cataract group.

Lens turbidity

There were significant main effects for growth ($F = 43.49$; $P < 0.05$) and HBO ($F = 488.82$; $P < 0.05$) and their interaction ($F = 31.33$; $P < 0.05$) on the turbidity of the peripheral region of the lens. There was no growth-associated change in the turbidity of the peripheral region of the lens in the control group from 4 to 32 weeks (Figs 5,6). A growth-associated increase in the turbidity was observed in the peripheral region of the lens in the cataract group from 8 to 32 weeks. The turbidity in the peripheral region of the lens was higher in the cataract group at 12 weeks than in the same group at 4 and 8 weeks. The turbidity in the peripheral region of the lens was higher in the cataract group at 16 weeks than in the same group at 4, 8, and 12 weeks. Furthermore, the turbidity in the peripheral region of the lens was higher in the cataract group at 32 weeks than in the same group at 4, 8, 12, and 16 weeks. The turbidity in the peripheral region of the lens was higher in the cataract + HBO group at 32 weeks than in the same group at 4 weeks.

The turbidity in the peripheral region of the lens was higher in the cataract and cataract + HBO groups at 4, 8, 12, 16, and 32 weeks than in the age-matched control group (Fig. 6). The turbidity in the peripheral region of the lens was lower in the cataract + HBO group at 12, 16, and 32 weeks than in the age-matched cataract group.

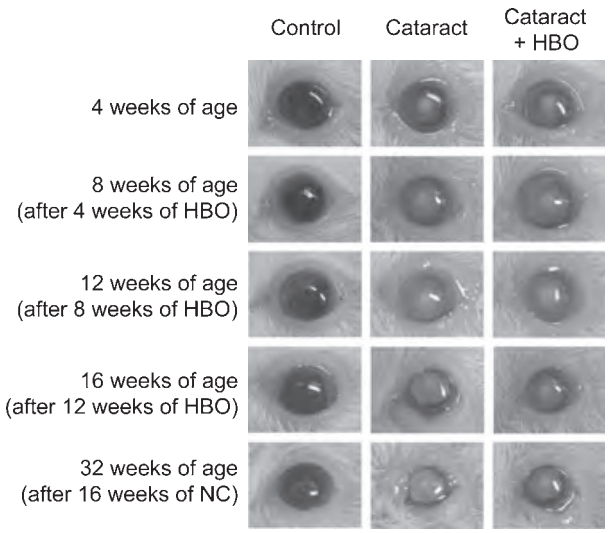

Figure 5 Photographs of lenses of mice in the control, cataract, and cataract + hyperbaric oxygen (HBO)-treated groups. Mice in the control and cataract groups were maintained under normal conditions throughout the study, whereas mice in the cataract + HBO group were exposed to HBO for 12 weeks from 4 to 16 weeks of age and thereafter maintained under normal conditions for 16 weeks from 16 to 32 weeks of age. NC, normal condition.

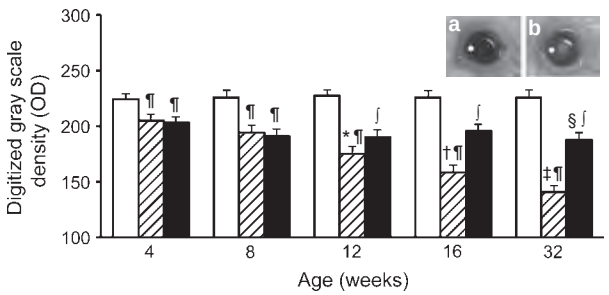

Figure 6 Digitized gray scale densities of the peripheral regions of the lenses in the control (□), diabetic cataract (▨), and diabetic cataract + hyperbaric oxygen (HBO)-treated (■) groups of mice at 4, 8, 12, 16, and 32 weeks of age. Insets, the white circles show the areas of measurement in the peripheral regions of the lenses in mice without (a) and with (b) cataracts. The digitized gray scale density value shows the average optical density (OD) level within the area; lower OD values indicate higher levels of turbidity. Values are the mean ± SD ($n = 5$ per group). $^{*}P < 0.05$ compared with the cataract group at 4 and 8 weeks of age; $^{\dagger}P < 0.05$ compared with the cataract group at 4, 8, and 12 weeks of age; $^{\ddagger}P < 0.05$ compared with the cataract group at 4, 8, 12, and 16 weeks of age; $^{\S}P < 0.05$ compared with the cataract + HBO group at 4 weeks of age; $^{\P}P < 0.05$ compared with the age-matched control group; $^{\int}P < 0.05$ compared with the age-matched control and cataract groups.

There were significant main effects for growth ($F = 4.17$; $P < 0.05$) and HBO ($F = 1888.10$; $P < 0.05$) and their interaction ($F = 8.84$; $P < 0.05$)

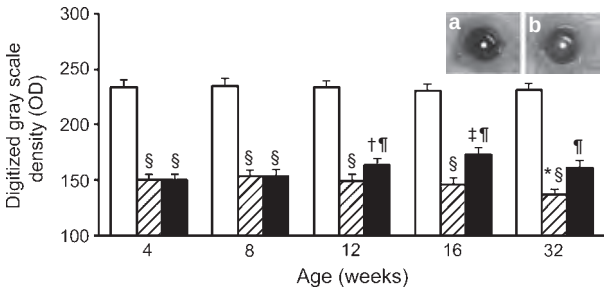

Figure 7 Digitized gray scale densities of the central regions of the lenses in the control (□), diabetic cataract (▨), and diabetic cataract + hyperbaric oxygen (HBO)-treated (■) groups of mice at 4, 8, 12, 16, and 32 weeks of age. Insets, the white circles show the areas of measurement in the central regions of the lenses in mice without (a) and with (b) cataracts. The digitized gray scale density value shows the average optical density (OD) within the area; lower OD values indicate higher levels of turbidity. Values are the mean ± SD ($n = 5$ per group). $^{*}P < 0.05$ compared with the cataract group at 4, 8, and 12 weeks of age; $^{\dagger}P < 0.05$ compared with the cataract + HBO group at 4 weeks of age; $^{\ddagger}P < 0.05$ compared with the cataract group at 4 and 8 weeks of age; $^{\S}P < 0.05$ compared with the age-matched control group; $^{\P}P < 0.05$ compared with the age-matched control and cataract groups.

on the turbidity of the central region of the lens. There was no growth-associated change in the turbidity of the central region of the lens in the control group from 4 to 32 weeks (Figs 5,7). The turbidity in the central region of the lens was higher in the cataract group at 32 weeks than in the same group at 4, 8, and 12 weeks. The turbidity in the central region of the lens was lower in the cataract + HBO group at 12 weeks than in the same group at 4 weeks. Furthermore, the turbidity in the central region of the lens was lower in the cataract + HBO group at 16 weeks than in the same group at 4 and 8 weeks.

The turbidity in the central region of the lens was higher in the cataract and cataract + HBO groups at 4, 8, 12, 16, and 32 weeks than in the age-matched control group (Fig. 7). The turbidity in the central region of the lens was lower in the cataract + HBO group at 12, 16, and 32 weeks than in the age-matched cataract group.

Discussion

Cataracts develop for a variety of reasons, including aging, long-term exposure to ultraviolet light, exposure to radiation, and secondary effects of metabolic diseases, such as diabetes; they are usually a result of denaturation of lens protein.[18] The present study was conducted to determine the effects of exposure to

moderate HBO on the development and progression of cataracts in mice with Type 2 diabetes. The diabetic mice with cataracts used in the present study were originally developed in Fujita Health University and had a lower body weight than the control mice at 8–32 weeks (Fig. 1), indicating that these mice had diabetes, but were not obese at adult stages.

The polyol pathway and oxidative stress

The polyol pathway in the lens is mediated by two enzymes, namely aldose reductase, which catalyses the reduction of glucose to sorbitol using NADPH as a cofactor, and sorbitol dehydrogenase, which catalyses the conversion of sorbitol to fructose using NAD^+ as a cofactor.[12] Transgenic mice that overexpress aldose reductase do not show any morphological abnormalities in the lens under normal conditions, thereby indicating that overexpression of aldose reductase alone does not induce changes in the lens.[19] However, when diabetes is induced by streptozotocin injection, the transgenic mice exhibiting overexpression of aldose reductase develop cataracts, and the rate of development of the cataracts corresponds to the level of aldose reductase expression in the lens. These results indicate that both aldose reductase and blood glucose levels are key factors in the pathogenesis of diabetes-induced cataracts.

Cataracts are characterized by an accumulation of sorbitol which, in turn, is mediated by aldose reductase activity.[20] The polyol pathway is the major contributor to diabetes-induced cataracts because an increased flux of glucose through this pathway leads to diabetic lesions in the lens and large quantities of glucose are reduced to sorbitol. This process is directly responsible for cataract formation.

Diabetes causes increased oxidative stress in the polyol pathway through hyperglycemia,[21,22] which is considered to play an important role in the pathogenesis of various diabetic complications, including cataracts.[12,23] Decreased antioxidant capacity also is associated with diabetes-induced cataract formation. A previous study reported that the lenses of mice lacking the antioxidant enzyme copper–zinc superoxide dismutase exhibited elevated levels of superoxide radicals and were more prone to develop hyperglycemia-induced cataracts *in vitro* than the lenses of wild-type mice.[24] It is expected that the increased availability of oxygen with exposure to moderate HBO inhibits the growth-associated increase in the blood glucose level of mice with Type 2 diabetes, which, in turn, may inhibit the accumulation of sorbitol and oxidative stress in the lens and thus delay cataract formation.

Hyperbaric oxygen therapy and exposure to moderate HBO

Hyperbaric exposure at 2–3 ata with 100% oxygen has been used as a therapy for many clinical disorders related to ischemia, hypoxia, tissue repair after burn, intractable ulcer, open fractures, and crush injuries.[25–29] Animals and humans are subjected to hyperbaric exposure at 2–3 ata with 100% oxygen. A previous study reported that cataracts in 17–18-month-old guinea pigs were induced by hyperbaric exposure at 2.5 ata with 100% oxygen for 2–2.5 h, three times per week, up to 100 times.[30] Similarly, myopia and cataracts developed in human lenses after prolonged hyperbaric exposure at 2–2.5 ata with 100% oxygen for 1.5 h, once daily, from 150 to 850 times,[31] but were observed rarely after only 48 times.[32] Thus, hyperbaric exposure at 2–3 ata with 100% oxygen has the potential to induce and accelerate myopia and cataracts. Furthermore, hyperbaric exposure at 2–3 ata with 100% oxygen is considered to cause excessive production of reactive oxygen species in several tissues and organs,[33,34] suggesting that overproduction of oxidative stress induced by exposure to HBO accelerates cataract formation. Oxidative stress levels depend not only on the pressure, but also on the duration of exposure; pressures from 2.5 to 3 ata and durations from 90 to 120 min have been reported to result in a pronounced increase in oxidative stress.[35,36] Furthermore, hyperbaric exposure at 2–3 ata with 100% oxygen increases blood pressure levels and lowers both heart rate and blood glucose levels, which were augmented in the presence of hypertension, diabetes, and both hypertension and diabetes.[37]

We have reported that moderate hyperbaric exposure at 1.25 ata with 36% oxygen is required for effective responses with regard to oxidative metabolism in the neuromuscular system (i.e. motoneurons in the spinal cord and their innervating fibers in the skeletal muscle).[3,4,38,39] The growth-associated increase in the blood glucose level of rats with Type 2 diabetes was inhibited by moderate hyperbaric exposure at 1.25 ata with 36% oxygen.[5–7] Furthermore, the increased blood glucose levels and the decreased oxidative enzyme activities of the skeletal muscles in rats with Type 2 diabetes were restored following exposure to moderate HBO.[8]

Effects of exposure to moderate HBO on diabetes-induced cataracts

In the present study, the increase in the blood glucose level of mice with Type 2 diabetes was inhibited by moderate hyperbaric exposure at 1.25 ata with 36% oxygen (Figs 2,3). These results are consistent with the

findings of our previous studies,[5–8] in which the growth-associated increase in the blood glucose level of rats with Type 2 diabetes was inhibited by exposure to moderate HBO. In addition, the increase in the oxidative stress level of mice with Type 2 diabetes was inhibited by exposure to moderate HBO (Fig. 4). In our previous study,[9] the increase in the blood pressure and overproduction of oxidative stress in rats with hypertension were inhibited by exposure to moderate HBO. In the present study, the cataract + HBO group at 12–32 weeks of age exhibited lower turbidity in both the peripheral (Fig. 6) and central (Fig. 7) regions of the lenses compared with the age-matched cataract group. These findings indicate that the HBO regimen used in the present study delays diabetes-induced cataract formation.

Conclusion

Hyperbaric exposure at 1.25 ata with 36% oxygen inhibits the increase in blood glucose levels and delays cataract development and progression in mice with Type 2 diabetes. This effect most likely is related to attenuation of the expected increase in the aldose reductase activity and overproduction of sorbitol and oxidative stress.

Acknowledgments

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Disclosure

The contents of this manuscript have not been published elsewhere. The authors declare that they have no competing interests.

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Research Article

Oxygen Concentration-Dependent Oxidative Stress Levels in Rats

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Introduction. We determined derivatives of reactive oxygen metabolites (dROMs) as an index of oxidative stress level (oxidant capacity) and biochemical antioxidant potential (BAP) as an index of antioxidant capacity in rats exposed to different oxygen concentrations. *Methods*. Male Wistar rats were exposed to 14.4%, 20.9%, 35.5%, 39.8%, 62.5%, and 82.2% oxygen at 1 atmosphere absolute for 24 h. Serum levels of dROMs and BAP were examined by using a free radical and antioxidant potential determination device. The morphological characteristics of red blood cells were examined by phase contrast microscopy. *Results*. There were no differences in the levels of dROMs in rats exposed to 14.4%, 20.9%, and 35.5% oxygen. However, the levels of dROMs increased in the rats exposed to 39.8% and 62.5% oxygen. The levels of dROMs were the highest in the rats exposed to 82.2% oxygen. There were no differences in the levels of BAP with respect to the oxygen concentration. Morphological changes in the red blood cells induced by oxidative attack from reactive oxygen species were observed in the rats exposed to 39.8%, 62.5%, and 82.2% oxygen. *Conclusion*. Our results suggest that exposure to oxygen concentrations higher than 40% for 24 h induces excessive levels of oxidative stress in rats.

1. Introduction

Supplemental oxygen is used in treatment and as a countermeasure for acute and chronic diseases. When pilots of unpressurized aircrafts fly to areas at high altitudes, when climbers ascend high-altitude peaks and outpace their ability to acclimatize, or when divers inhaling compressed air return to the surface, the external pressure on the body decreases and the dissolved inert gases come out of solution in the form of bubbles in the body on depressurization [1, 2]. The resulting decompression sicknesses and air embolisms are initially treated by inhalation of oxygen-enriched air or exposure to mild hyperbaric oxygen at 1.25 atmospheres absolute (ATA) until hyperbaric oxygen therapy (100% oxygen delivered at 2-3 ATA) is administered [3–6]. Hypoxic or breathless patients with chronically obstructive pulmonary disease (COPD), who have low levels of oxygen in their blood, require oxygen at concentrations greater than that in room air to achieve arterial oxygen saturations between 88% and 92% [7].

Oxygen therapy with or without pressure is associated with the risk of oxygen toxicity and excessive oxidative stress. Oxidative stress plays as key role in the pathogenesis of many diseases and their complications; the generation of free radicals and increased levels of oxidative stress are associated with atherosclerosis, cataract, retinopathy, myocardial infarction, hypertension, diabetes, renal failure, and uremia [8–10]. However, there are no data available on changes in the oxidative stress level and antioxidant capacity after exposure to different concentrations of oxygen. The analytical measurement of oxidative stress markers has been difficult because of the short half-life and high reactivity of the majority of reactive oxygen species and the applicability of measurement methods [11]. Blood samples are the appropriate biological materials for assessing the status of oxidants and antioxidants. A unique system for the

evaluation of oxidative stress levels and antioxidant capacity in the blood has been developed [12]. This evaluation approach is based on the free radical analytical system that mainly analyzes lipid hydroperoxides, which are relatively stable in the blood. This system has been used for both animal and human sera, which confirms its applicability [13–15].

In this study, we examined the derivatives of reactive oxygen metabolites (dROMs) as an index of oxidative stress levels (oxidant capacity) and the biochemical antioxidant potential (BAP) as an index of antioxidant capacity in rats exposed to different concentrations of oxygenat 1 ATA for 24 h.

2. Materials and Methods

2.1. Experimental Animals. All experimental and animal care procedures were performed in accordance with the guidelines stated in the Guide for the Care and Use of Laboratory Animals issued by the Institutional Animal Experiment Committee of Kyoto University (Kyoto, Japan).

2.2. Exposure to Different Concentrations of Oxygen. Ten-week-old male Wistar rats weighing between 200 g and 226 g were divided into 6 groups ($n = 5$ for each group). The individual groups were exposed to air containing low or high concentration of oxygen in a chamber ($75 \times 130 \times 85$ cm) at 1 ATA for 24 h by using a low-oxygen inhaler (Terucom Corp., Yokohama, Japan) or an oxygen concentrator (Ikiken Corp., Sayama, Japan), respectively. When the air chamber contained less or more than 20.9% oxygen, air with 2 different concentrations of oxygen from 2 tubes was transported to the aspirator: one tube contained normal air (20.9% oxygen) and the other tube had air containing 13% or 84% oxygen, which was procured from the low-oxygen inhaler or the oxygen concentrator, respectively. Then, the aspirator pumped mixed air into the chamber at the rate of 1 L/min. The low or high oxygen concentration in the chamber was adjusted by separately regulating the air flow from these 2 tubes. Normal air was transported by only 1 tube, while the air containing 20.9% oxygen was retained in the chamber. The oxygen concentration in the chamber was determined by using an oxygen monitor (Max O_2+AE; Maxtec Inc., UT, USA) attached to the chamber. The rats were maintained in individual, uniformly sized standard cages ($30 \times 40 \times 20$ cm) in the chamber. The room was maintained at $22 \pm 2^{\circ}$C with 45%–55% relative humidity. Food and water were provided *ad libitum*.

2.3. Measurements of dROMs and BAP. The levels of dROMs and BAP were determined after the rats were exposed to different concentrations of oxygen. Blood samples were obtained from the tail of fully conscious rats and evaluated photometrically. A free radical and antioxidant potential determination device (Free Radical Analytical System 4; Health & Diagnostics, Grosseto, Italy) was used to automatically measure the levels of dROMs and BAP.

The dROMs are used as an index to determine the level of oxidative stress (oxidant capacity) by measuring the amount of organic hydroperoxide (ROOH) converted into radicals that oxidize *N,N*-diethyl-*p*-phenylenediamine [12, 16]. The levels of dROMs were expressed in Carr units (1 U·Carr corresponds to 0.08 mg hydroperoxide/100 mL H_2O_2). The BAP is used as an index to determine the biological antioxidant capacity and is measured on the basis of the capacity of the plasma sample to reduce ferric ions to ferrous ions. After blood samples were obtained, the rats were killed by intraperitoneal overdose of sodium pentobarbital.

2.4. Red Blood Cell Morphology. Using blood samples, the morphological profiles of red blood cells were observed by phase contrast microscopy (Nikon 80iF-PH-15; Tokyo, Japan).

2.5. Statistics. Means and standard deviations were calculated from the individual values by using standard procedures. One-way analysis of variance (ANOVA) was used to evaluate the mean differences among the 6 groups. When ANOVA analyses revealed significant differences in mean values, the groups were further compared using Scheffé's *post hoc* tests. A probability level of 0.05 was considered significant.

3. Results

The levels of dROMs in rats exposed to 39.8% and 62.5% oxygen were higher than those in the rats exposed to 14.4%, 20.9%, and 35.5% oxygen (Figure 1(a)). The level of dROMs in the rats exposed to 82.2% oxygen was the highest among the 6 groups. There were no differences in the levels of BAP among the 6 groups (Figure 1(b)). Morphological changes in red blood cells were observed in the rats exposed to 39.8%, 62.5%, and 82.2% oxygen (Figure 2).

4. Discussion

4.1. Exposure to Low Concentration of Oxygen. Acclimatization at high altitude results in changes in the respiratory, cardiovascular, and hematologic systems, which enhance oxygen delivery to the cells and tissues [17]. Decompression sickness occurs between initial hypoxic conditions and the onset of acclimatization, and the incidence and severity of the sickness depend on the rate of ascent, the altitude attained, and physiological susceptibility of individuals [5].

There is little data available regarding the oxidative stress level under low concentrations of oxygen. In this study, we examined the oxidative stress levels in rats exposed to low concentrations of oxygen. In rats exposed to 14.4% oxygen for 24 h, which is equivalent to the oxygen concentration at 3500 m/11500 feet altitude, no change was observed in the oxidative stress level (Figure 1(a)). Therefore, we conclude that low concentrations of oxygen do not induce excessive oxidative stress.

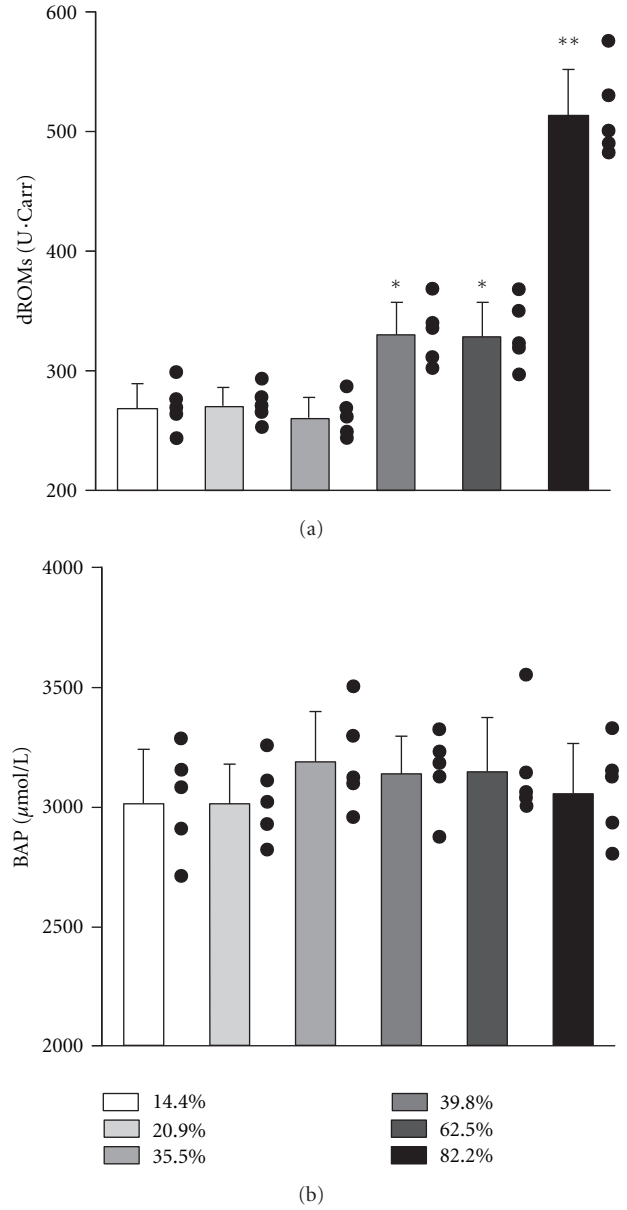

Figure 1: Levels of derivatives of reactive oxygen metabolites (a) and biochemical antioxidant potential (b) of rats after exposure to different concentrations of oxygen for 24 h. Values are expressed as the mean and standard deviation ($n = 5$ for each group). Five dots on the right side of the bar are the individual values of rats in each group. dROMs: derivatives of reactive oxygen metabolites; BAP: biochemical antioxidant potential. $^{*}P < 0.05$ compared with values of 14.4%, 20.9%, and 35.5% oxygen; $^{**}P < 0.05$ compared with values of 14.4%, 20.9%, 35.5%, 39.8%, and 62.5% oxygen.

4.2. Exposure to High Concentration of Oxygen. Hyperbaric oxygen at 2-3 ATA with 100% oxygen induces acute changes, such as increased blood pressure and further reduction in heart rate [18], and causes chronic diseases like cataract formation [19–22]; hyperbaric oxygen is generally safe when pressures do not exceed 3 ATA and the length of treatment is less than 120 min [23, 24]. However, hyperbaric oxygen has been reported to increase the levels of reactive oxygen species [25–27].

Patients with COPD inhale high concentrations of oxygen, generally up to 50% oxygen (fraction of inspired oxygen, FIO_2) when the partial pressure of oxygen in the arterial blood (PaO_2) is below 55 mmHg; these patients occasionally exhibit voluntary respiration failure, consciousness disturbance, and atelectasis when inhaling high concentrations of oxygen.

Oxygen therapy with or without pressure might induce excessive levels of oxidative stress. Excessive levels of oxidative stress are associated with many diseases, including atherosclerosis, cataract, retinopathy, myocardial infarction, hypertension, renal failure, and uremia [8–10]. In this study, we examined the oxidative stress levels in rats exposed to high concentrations of oxygen.

No change in the oxidative stress level was observed at 35.5% oxygen for 24 h (Figure 1(a)). We previously examined the effects of mild hyperbaric oxygen at 1.25 ATA with 36% oxygen on the neuromuscular system, including spinal motoneurons and their innervating muscle fibers [28, 29], type 2 diabetes [30–33], hypertension [34], type II collagen-induced arthritis [35], age-related decline in muscle oxidative capacity [36], and diabetes-induced cataracts [37] in mice and rats. Therefore, the data observed in this study suggest that mild hyperbaric oxygen at 1.25 ATA with 36% oxygen is effective for the inhibition and improvement of many metabolic diseases [28–37], without producing excessive levels of oxidative stress.

In contrast, 39.8% and 62.5% oxygen for 24 h induced excessive levels of oxidative stress, and the level of dROMs was the highest at 82.2% oxygen (Figure 1(a)). Oxidative stress level increases when the production of reactive oxygen species is markedly greater than the intrinsic antioxidant defenses. Patients with COPD inhale high concentrations of oxygen, and the possibility of accumulating excessive levels of oxidative stress will be greater when the inhaled oxygen concentration (FIO_2) is high. Therefore, we conclude that exposure to 40% oxygen for 24 h is a threshold for inducing an excessive level of oxidative stress.

In our previous study [15], we observed that obese rats with metabolic syndrome accompanied by insulin resistance, impaired glucose metabolism, and dyslipidemia had lower levels of BAP than normal Wistar rats. In addition, we observed that mild hyperbaric exposure at 1.25 ATA with 36% oxygen improves the levels of BAP in rats with hypertension [34]. These studies [15, 34] suggest that changes in the levels of BAP are reflected as an index of antioxidant capacity. In this study, we expected that exposure to higher concentrations of oxygen would decrease the levels of BAP compared with exposure to 20.9% oxygen because the levels of dROMs were increased by exposure to 39.8%, 62.5%, and 82.2% oxygen (Figure 1(a)). However, there was no difference in the levels of BAP among different concentrations of oxygen (Figure 1(b)). These results suggest that the antioxidant capacity is not affected by both low and high concentrations of oxygen, and thus, the antioxidant capacity did not change, at least after 24 h. However, we did not examine antioxidant enzyme levels of rats exposed to different concentrations

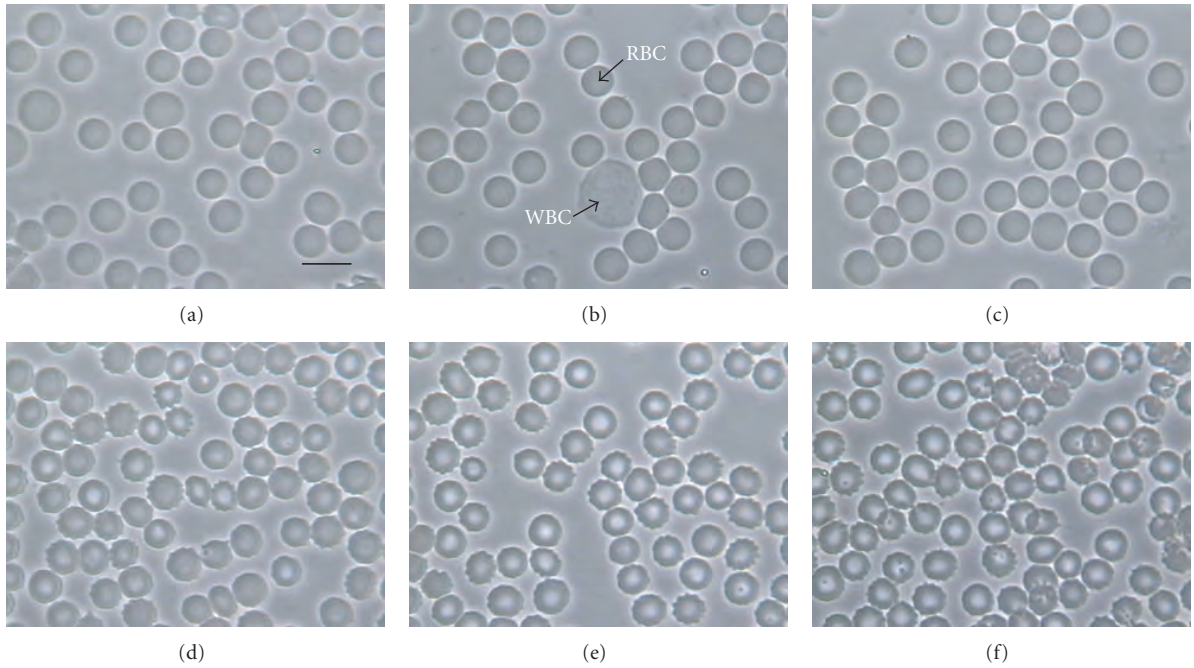

Figure 2: Morphological features of red blood cells after 24 h of exposure to different concentrations of oxygen: (a) 14.4%, (b) 20.9%, (c) 35.5%, (d) 39.8%, (e) 62.5%, and (f) 82.2%. RBC: red blood cell; WBC: white blood cell. Scale bar is 10 μm.

of oxygen. In our subsequent study, we plan to examine the levels of BAP and antioxidant enzymes, for example, superoxide dismutase, catalase, and glutathione peroxidase, in rats exposed to different concentrations of oxygen for more than 24 h.

Transformed red blood cells, which were induced by oxidative attack from reactive oxygen species, were observed by exposure to 39.8%, 62.5%, and 82.2% oxygen (Figure 2); these findings are consistent with the increased levels of dROMs at these oxygen concentrations (Figure 1(a)). Therefore, we conclude that morphological changes in the red blood cells are linked to increased levels of oxidative stress.

5. Conclusion

Exposure to oxygen concentrations higher than 40% for 24 h induces excessive levels of oxidative stress.

Conflict of Interests

The authors declare that they have no conflict of interests.

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