

# Mild Hyperbaric Oxygen Improves Decreased Oxidative Capacity of Spinal Motoneurons Innervating the Soleus Muscle of Rats with Type 2 Diabetes

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**Abstract** Rats with type 2 diabetes exhibit decreased oxidative capacity, such as reduced oxidative enzyme activity, low-intensity staining for oxidative enzymes in fibers, and no high-oxidative type IIA fibers, in the skeletal muscle, especially in the soleus muscle. In contrast, there are no data available concerning the oxidative capacity of spinal motoneurons innervating skeletal muscle of rats with type 2 diabetes. This study examined the oxidative capacity of motoneurons innervating the soleus muscle of non-obese rats with type 2 diabetes. In addition, this study examined the effects of mild hyperbaric oxygen at 1.25 atmospheres absolute with 36 % oxygen for 10 weeks on the oxidative capacity of motoneurons innervating the soleus muscle because mild hyperbaric oxygen improves the decreased oxidative capacity of the soleus muscle in non-obese rats with type 2 diabetes. Spinal motoneurons innervating the soleus muscle were identified using nuclear yellow, a retrograde fluorescent neuronal tracer. Thereafter, the cell body sizes and succinate dehydrogenase activity of identified motoneurons were analyzed. Decreased succinate dehydrogenase activity of small-sized alpha motoneurons innervating the soleus muscle was observed in rats with type 2 diabetes. The decreased succinate dehydrogenase activity of these motoneurons was improved by mild hyperbaric oxygen. Therefore, we concluded that rats with type 2 diabetes have decreased oxidative capacity in motoneurons innervating the soleus muscle and this

decreased oxidative capacity is improved by mild hyperbaric oxygen.

**Keywords** Mild hyperbaric oxygen · Oxidative capacity · Soleus muscle · Spinal motoneuron · Type 2 diabetes

## Abbreviations

ATA	Atmosphere absolute
CSA	Cross-sectional area
GK	Goto-Kakizaki
HbA1c	Glycosylate hemoglobin
LETO	Long-Evans Tokushima Otsuka
OD	Optical density
OLETF	Otsuka Long-Evans Tokushima fatty
SDH	Succinate dehydrogenase
WR	Wistar

## Introduction

It is well known that non-obese and obese rats with type 2 diabetes exhibit decreased oxidative capacity, such as reduced oxidative enzyme activity, low-intensity staining for oxidative enzymes in fibers, and no high-oxidative type IIA fibers, in the skeletal muscle, especially in the soleus muscle [1, 2]. Skeletal muscle is the primary site of insulin action and glucose metabolism [3]. Reduced oxidative capacity in skeletal muscle impairs glucose metabolism and induces the development and aggravation of type 2 diabetes and its associated complications [4, 5].

The morphological and histochemical properties of fibers in skeletal muscle correspond well with those of motoneurons in the spinal cord [6, 7]. The small-sized and

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high-oxidative alpha motoneurons innervate high-oxidative fibers, whereas the large-sized and low-oxidative alpha motoneurons innervate low-oxidative fibers in skeletal muscle [7]. We suggest that spinal motoneurons of rats with type 2 diabetes exhibit reduced oxidative capacity because the skeletal muscle, and its fibers innervated by these motoneurons, show decreased oxidative capacity [1, 2]. However, there are no data available for the oxidative capacity of spinal motoneurons in rats with type 2 diabetes. This study determined the oxidative capacity of spinal motoneurons innervating the soleus muscle in non-obese rats with type 2 diabetes.

An elevation in atmospheric pressure accompanied by high oxygen concentration increases blood flow and oxygen, especially dissolved oxygen, in the plasma [8, 9]. An increase in both atmospheric pressure and oxygen concentration enhances oxidative enzyme activity in mitochondria and consequently increases oxidative metabolism in cells and tissues. We have demonstrated that mild hyperbaric oxygen at 1.25 atmospheres absolute (ATA) with 36 % oxygen increased blood flow and dissolved oxygen in the plasma, thereby improving oxidative metabolism [10]. In addition, we found that the increased blood flow and dissolved oxygen induced by mild hyperbaric oxygen were not accompanied by an excessive increase in oxidative stress. We observed that mild hyperbaric oxygen induced a growth-associated increase in neuromuscular oxidative capacity [11] and improved age-associated decrease in muscular oxidative capacity [12]. We observed that mild hyperbaric oxygen inhibited and/or improved lifestyle-related diseases, e.g., type 2 diabetes [13–16], diabetes-induced cataracts [17], and hypertension [18], in rats. In addition, mild hyperbaric oxygen inhibited development of arthritis in rats [19]. A clinical study [20] showed that mild hyperbaric oxygen reversed the increase in melanin pigmentation induced by ultraviolet B irradiation, as well as reduced senile spot size.

We hypothesized that mild hyperbaric oxygen would improve the decreased oxidative capacity of spinal motoneurons as well as skeletal muscles and their fibers [13–16]. This study investigated the effects of mild hyperbaric oxygen on the oxidative capacity of spinal motoneurons innervating the soleus muscle in non-obese rats with type 2 diabetes.

## Materials and Methods

### Ethical Statement

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 at Kyoto University, Kyoto, Japan.

### Animal Housing

Eight-week-old Wistar (WR,  $n = 16$ ) and non-obese diabetic Goto-Kakizaki (GK,  $n = 24$ ) male rats were used in this study. WR ( $n = 8$ ) and GK ( $n = 8$ ) rats were sacrificed at 8 weeks of age. The remaining GK rats were assigned randomly to either the normobaric (GK) or mild hyperbaric oxygen (GK-H) group ( $n = 8$ /group). The remaining Wistar rats were assigned as the normobaric control (WR) group ( $n = 8$ ). They were housed in individual cages and housed under normobaric conditions (1 ATA with 20.9 % oxygen). The room was maintained at  $22 \pm 2$  °C, 45–55 % relative humidity, and with a 12 h light/dark cycle (lights on from 08:00 to 20:00). All rats were given standard chow (MF, Oriental East Inc., Tokyo, Japan) and water ad libitum.

### Exposure to Mild Hyperbaric Oxygen

Rats in the GK-H group were exposed to 1.25 ATA with 36 % oxygen in a mild hyperbaric oxygen chamber (Japan Patent No. 5076067 dated September 7, 2012; Inventor, Akihiko Ishihara) for 3 h (11:00 to 14:00) daily for 10 weeks from 8 to 18 weeks of age.

### Fasting Blood Glucose and Glycosylate Hemoglobin (HbA1c) Analyses

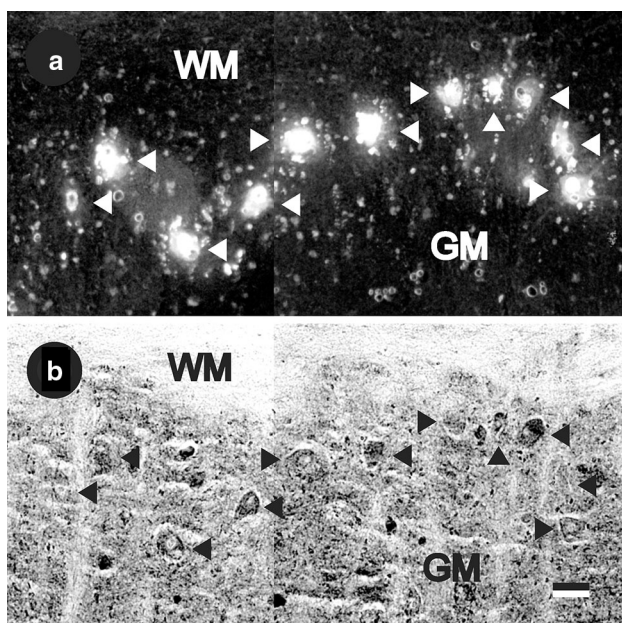
At 8 and 18 weeks of age, blood samples were obtained from the tails of conscious rats. HbA1c levels were measured using a DCA vantage analyzer (Siemens Healthcare Diagnostics, Surrey, UK). Fasting blood glucose levels were measured using a blood glucose meter GT-1650 (Arkray, Kyoto, Japan) after 12 h of fasting.

### Injection of Fluorescent Neuronal Tracer

After blood sampling, rats were anesthetized with sodium pentobarbital (5 mg/100 g body weight, *i.p.*), and the surgical procedure was performed under aseptic conditions. A 2 % fluorescent neuronal tracer, nuclear yellow, which is retrogradely transported from the skeletal muscle to the spinal cord [21, 22], was injected using a microsyringe into multiple sites of the right soleus muscle. Care was taken to inject the neuronal tracer slowly to prevent leakage. After injecting the neuronal tracer, the skin was sutured and the rats were allowed to recover from anesthesia and surgery. The rats were sacrificed by administering an overdose of sodium pentobarbital 2 days after the injection of the neuronal tracer.

## Histochemical Analyses

The lumbosacral enlargement of the spinal cord was removed and frozen immediately in isopentane cooled in liquid nitrogen. Serial longitudinal sections (20  $\mu\text{m}$  thick) of the lumbosacral enlargement were cut in a cryostat at  $-20\text{ }^{\circ}\text{C}$ . The motoneurons innervating the soleus muscle were identified by the golden-yellow fluorescence of the nucleus imparted by nuclear yellow (Fig. 1a). The identified motoneurons in the serial sections were counted. The same sections that were used to identify the motoneurons were subsequently stained for succinate dehydrogenase (SDH) activity, a marker of mitochondrial oxidative capacity [6, 7, 23] (Fig. 1b). The cross-sectional area (CSA) and SDH activity of the identified motoneuron, in which the nucleus was visible, were measured using a computer-assisted image processing system (Neuroimaging System, Kyoto, Japan). Sectional images were digitized as gray-scale images. Each pixel was quantified as 1 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 mean optical density (OD) of all pixels, which were converted to gray-level values, within a motoneuron was determined using a calibration photographic tablet with 21 steps of gradient-density range and



**Fig. 1** Spinal motoneurons innervating the rat soleus muscle were retrogradely identified by the fluorescent neuronal tracer, nuclear yellow (a). After identification of the motoneurons, the section was stained for succinate dehydrogenase activity to determine cell body sizes and oxidative enzyme activity of identified motoneurons (b). WM white matter, GM gray matter. Scale bar 50  $\mu\text{m}$

corresponding diffused density values. The nucleus was excluded from OD measurements since it does not stain for SDH activity. The cut-off for the distinction among gamma, small-sized alpha, and large-sized alpha motoneurons was based on cell body size. Based on previous studies [24–26], there is a high probability that the very small-sized ( $<500\text{ }\mu\text{m}^2$ ) motoneurons are gamma, the small-sized ( $500\text{--}900\text{ }\mu\text{m}^2$ ) motoneurons are high-oxidative alpha, and the large-sized ( $>900\text{ }\mu\text{m}^2$ ) motoneurons are low-oxidative alpha.

## Statistics

Values are expressed as mean  $\pm$  SD. One-way ANOVA was used to determine significant differences among the five groups; WR and GK at 8 weeks and GK, WR, and GK-H at 18 weeks. When significant differences were found, individual group comparisons were performed using *Scheffé's* post hoc test. Statistical significance was set at  $p < 0.05$ .

## Results

### Body Weight

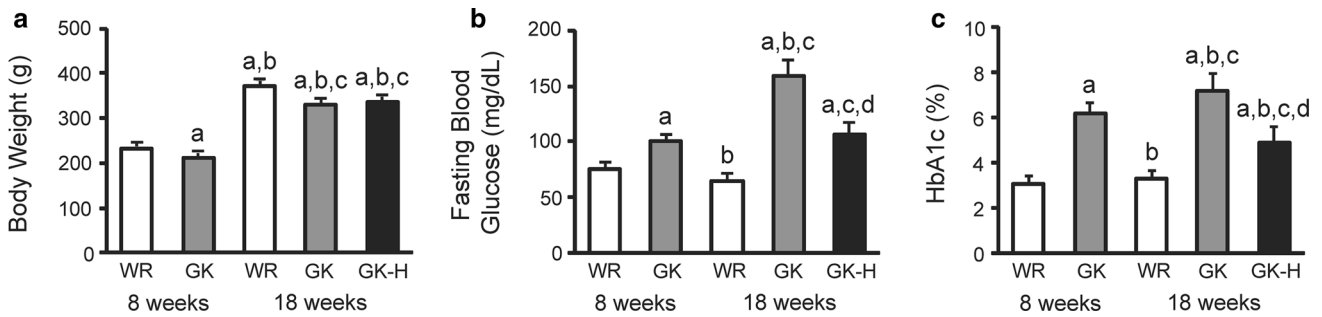
Body weight was lower in the GK group at 8 weeks of age than in the age-matched WR group (Fig. 2a). Body weight was higher in the WR, GK, and GK-H groups at 18 weeks of age than in the WR and GK groups at 8 weeks of age. Body weight was lower in the GK and GK-H groups at the 18 weeks of age than in the age-matched WR group.

### Fasting Blood Glucose Levels

Fasting blood glucose levels were higher in the GK group at 8 weeks of age than in the WR group at 8 and 18 weeks of age (Fig. 2b). Fasting blood glucose levels were higher in the GK group at 18 weeks of age than in the WR group at 8 and 18 weeks of age and the GK group at 8 weeks of age. Fasting blood glucose levels in the GK-H group at 18 weeks of age were higher than those of the WR group at 8 and 18 weeks of age and lower than those of GK group at 18 weeks of age.

### HbA1c Levels

HbA1c levels were higher in the GK group at 8 weeks of age than in the WR group at 8 and 18 weeks of age (Fig. 2c). HbA1c levels were higher in the GK group at 18 weeks of age than in the WR group at 8 and 18 weeks of age and the GK group at 8 weeks of age. HbA1c levels in the GK-H group at 18 weeks of age were higher than



**Fig. 2** Body weight (a) and fasting blood glucose (b) and HbA1c (c) levels of the WR and GK groups at 8 weeks of age and WR, GK, and GK-H groups at 18 weeks of age. WR, normal rats; GK, type 2 diabetic rats; GK-H, type 2 diabetic rats exposed to mild hyperbaric

oxygen for 3 h per day; HbA1c, glycosylated hemoglobin. Values are means  $\pm$  SD for 8 animals. <sup>a</sup> $p < 0.05$  compared with WR at 8 weeks; <sup>b</sup> $p < 0.05$  compared with GK at 8 weeks; <sup>c</sup> $p < 0.05$  compared with WR at 18 weeks; <sup>d</sup> $p < 0.05$  compared with GK at 18 weeks

those of the WR group at 8 and 18 weeks of age and lower than those of GK group at 8 and 18 weeks of age.

**Number of Motoneurons Innervating the Soleus Muscle**

There was no significant difference in the number of alpha (Fig. 3a) or gamma + alpha (Fig. 3b) motoneurons, as identified by the fluorescent neuronal tracer, innervating the soleus muscle.

**SDH Activity of Motoneurons Innervating the Soleus Muscle**

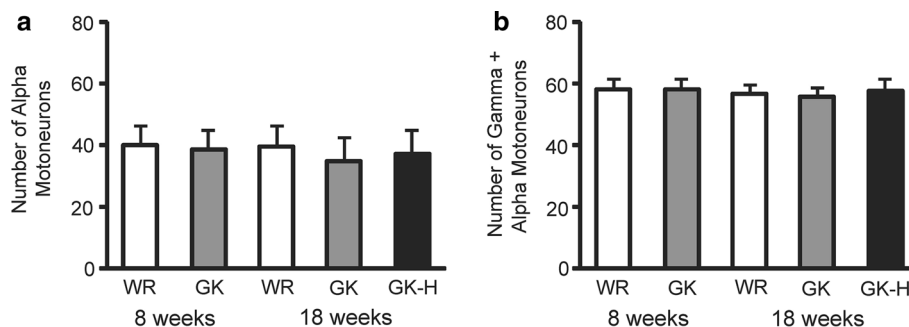
The SDH activity of small-sized alpha motoneurons innervating the soleus muscle was lower in the GK group at 8 weeks than in the WR group at 8 and 18 weeks (Fig. 4b). The SDH activity of small-sized alpha motoneurons was lower in the GK group at 18 weeks of age than in the WR group at 8 and 18 weeks. The SDH activity of small-sized alpha motoneurons was higher in the GK-H group at 18 weeks of age than in the GK group than 8 and 18 weeks.

**Discussion**

**Fasting Blood Glucose and HbA1c Levels**

At 8 and 18 weeks of age, GK rats with type 2 diabetes showed higher blood glucose and HbA1c levels compared with age-matched normal WR rats (Fig. 2b, c). The increased blood glucose and HbA1c levels of GK rats in this study correspond well with those observed in GK rats at 7 [14], 9 [2, 14], 15 [27, 28], and 20 [2] weeks of age. In this study, the GK rats had developed diabetes by 8 weeks of age.

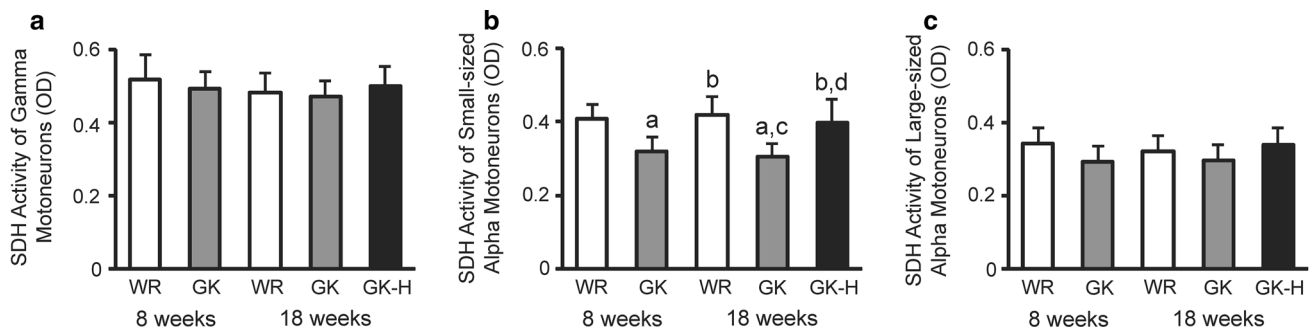
The blood glucose and HbA1c levels were lower in GK rats exposed to mild hyperbaric oxygen than in age-matched GK rats not exposed to mild hyperbaric oxygen (Fig. 2b, c), indicating that mild hyperbaric oxygen improved the blood glucose and HbA1c levels of GK rats. This improvement corresponds well with previous findings [13–16] although the blood glucose and HbA1c levels of GK rats exposed to mild hyperbaric oxygen did not attain the normal level of these parameters as observed in WR rats (Fig. 2b, c). We concluded that mild hyperbaric



**Fig. 3** Number of alpha (a) and gamma + alpha (b) motoneurons innervating the soleus muscle of the WR and GK groups at 8 weeks of age and WR, GK, and GK-H groups at 18 weeks of age. WR,

normal rats; GK, type 2 diabetic rats; GK-H, type 2 diabetic rats exposed to mild hyperbaric oxygen for 3 h per day. Values are means  $\pm$  SD for 8 animals





**Fig. 4** SDH activity of spinal motoneurons innervating the soleus muscle of the WR and GK groups at 8 weeks of age and WR, GK, and GK-H groups at 18 weeks of age. SDH, succinate dehydrogenase; OD, optical density. Spinal motoneurons were divided into three types according to their cell body sizes; gamma ( $<500 \mu\text{m}^2$ , **a**), small-sized

alpha ( $500\text{--}900 \mu\text{m}^2$ , **b**), and large-sized alpha ( $>900 \mu\text{m}^2$ , **c**) motoneurons. Values are means  $\pm$  SD for 8 animals. <sup>a</sup> $p < 0.05$  compared with WR at 8 weeks; <sup>b</sup> $p < 0.05$  compared with GK at 8 weeks; <sup>c</sup> $p < 0.05$  compared with WR at 18 weeks; <sup>d</sup> $p < 0.05$  compared with GK at 18 weeks

oxygen is effective in improving the blood glucose and HbA1c levels of rats with type 2 diabetes.

### Skeletal Muscle Properties of Humans and Animal Models with Type 2 Diabetes

Skeletal muscles in humans and animal models of type 2 diabetes have decreased oxidative capacity. In clinical studies [29–32], a low percentage of high-oxidative type I fibers and a high percentage of low-oxidative type II fibers, especially type IIB fibers, are observed in the vastus lateralis muscle of patients with type 2 diabetes. The altered fiber-type composition is correlated closely with insulin concentrations, which regulate myosin synthesis in skeletal muscle.

The soleus muscles have high oxidative capacity and are required to function against gravity, e.g., maintaining posture and walking [33], indicating that the soleus muscles function most effectively at relatively low intensity for long durations. The rat soleus muscle contains type I, type IIA, and type IIC fibers; type IIA and type IIC fibers have high oxidative enzyme activity, whereas type I fibers have low oxidative enzyme activity [34, 35]. In previous studies using animal models of type 2 diabetes, Yasuda et al. [1, 2] found that both obese Otsuka Long-Evans Tokushima fatty (OLETF) and non-obese GK rats exhibited a decreased percentage of high-oxidative type IIA and type IIC fibers and an increased percentage of low-oxidative type I fibers in the soleus muscle during postnatal development. In addition, there are no high-oxidative type IIA and type IIC fibers in the soleus muscle of adult OLETF and GK rats.

Skeletal muscle plays a major role in insulin-stimulated glucose uptake. Type 2 diabetes is associated with impaired insulin-stimulated glucose uptake and disposal capacity, which is attributed to insulin resistance in skeletal muscle. The altered pattern of fiber types in skeletal muscles of humans and animal models with type

2 diabetes is tightly linked to impaired glucose tolerance and insulin sensitivity.

### Effect of Mild Hyperbaric Oxygen on Skeletal Muscle Properties

A decrease in the percentage of low-oxidative type I fibers and an appearance in high-oxidative type IIA and type IIC fibers, which are not detected in the soleus muscle of type 2 diabetic rats [1, 2], are observed following exposure to mild hyperbaric oxygen [14]. A high percentage of low-oxidative type I fibers with no high-oxidative type IIA fibers is observed in the soleus muscle of obese OLETF rats compared with that in normal Long-Evans Tokushima Otsuka (LETO) rats [1]. In addition, a growth-associated increase in the percentage of low-oxidative type IIB fibers and a decrease in the percentage of high-oxidative type I and type IIA fibers are observed in the plantaris muscle of OLETF rats compared with that in LETO rats [15]. In contrast, there were no differences in the fiber-type composition of the soleus and plantaris muscles between LETO and OLETF rats after OLETF rats were exposed to mild hyperbaric oxygen. The increased blood flow and availability of oxygen induced by mild hyperbaric oxygen has a beneficial impact on the type shift of fibers in skeletal muscle of diabetic rats to a normal pattern.

Endurance exercise restores decreased oxidative capacity in skeletal muscle of rats with type 2 diabetes [36]. The percentage of type I fibers in the soleus muscle is related to running distance in exercised OLETF rats. Endurance exercise inhibits a diabetes-induced type shift of fibers from high-oxidative type II to low-oxidative type I in the soleus muscle of OLETF rats that run distances of more than 7000 m/day. In contrast, changes in the fiber-type composition in the plantaris muscle of OLETF rats are inhibited by endurance exercise, irrespective of the distance they run. Thus, endurance exercise can inhibit the diabetes-induced type shift of fibers from high-oxidative to

low-oxidative in skeletal muscle of rats with type 2 diabetes because of increased muscle oxidative capacity. However, the increase in the blood flow following an endurance exercise is induced mostly in active skeletal muscles, but not in internal organs. In addition, because of the increased atmospheric pressure and oxygen concentration, mild hyperbaric oxygen has an advantage in that it can increase the amount of dissolved oxygen in the plasma, which does not occur with endurance exercise.

### Motoneuron Property of Animal Models

The neuromuscular unit is comprised of an alpha motoneuron in the dorsolateral region of the ventral horn in the spinal cord and the fibers in the skeletal muscle that the motoneuron innervates. The metabolic properties of alpha motoneurons are tightly related to those of their target muscle fibers, indicating that there is matching between the cell size and metabolic properties of the alpha motoneurons and the muscle fibers that they innervate. There is an inverse relationship between motoneuron cell body size and oxidative capacity [6, 7]. In general, alpha motoneurons are classified into low-oxidative and high-oxidative types. The high-oxidative alpha motoneurons have small cell body sizes, whereas the low-oxidative alpha motoneurons have large cell body sizes. There were no data available concerning effects of mild hyperbaric oxygen on motoneuron properties of rats with type 2 diabetes. Therefore, this study examined the effects of mild hyperbaric oxygen on motoneuron properties of rats with type 2 diabetes.

### Effect of Mild Hyperbaric Oxygen on Motoneuron Oxidative Capacity

Heavy ion radiation [37] and exposure to microgravity [38–42] induce decreased oxidative capacity of spinal motoneurons in rats. The oxidative enzyme activity of all motoneurons was decreased following administration of heavy ion radiation to the spinal cord at doses of 40, 50, and 70 Gy compared with that of non-irradiated controls [37]. The decreased oxidative capacity in the irradiated motoneurons returned to the oxidative capacity of non-irradiated controls after a 6-month recovery period.

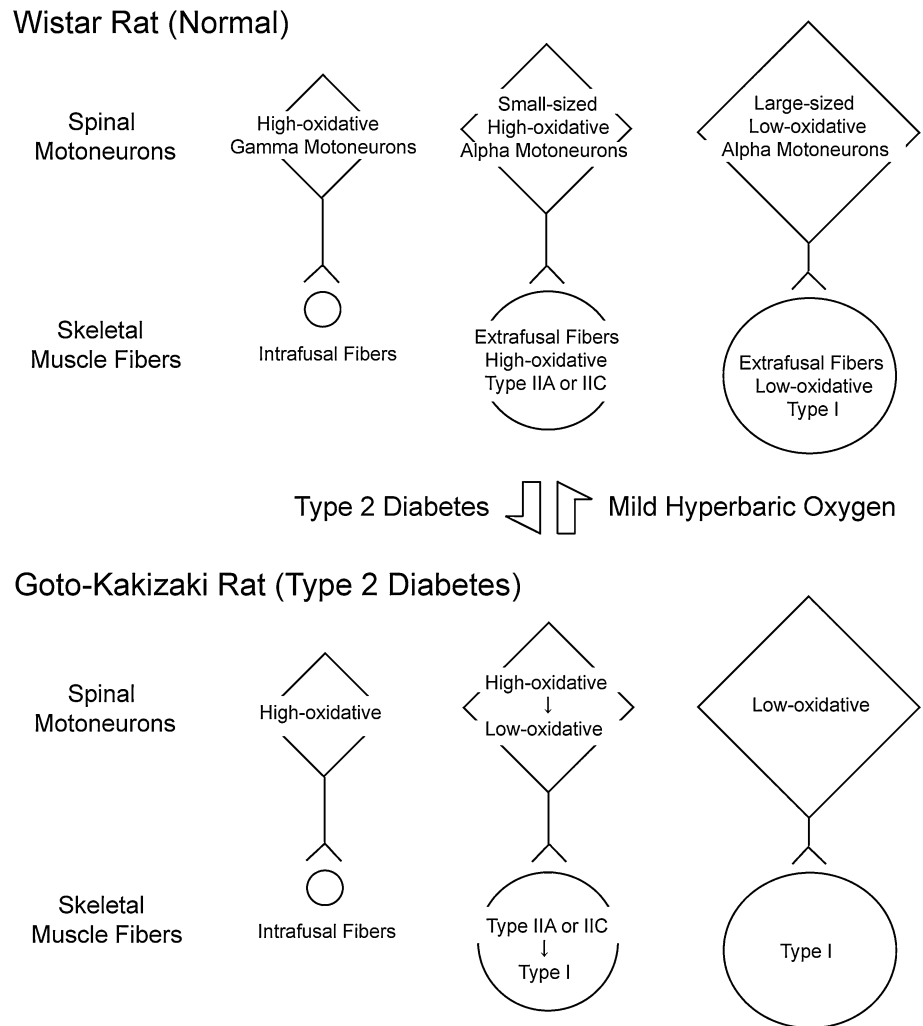
Previous studies [43, 44] observed atrophy of fibers, a type shift of fibers from high-oxidative to low-oxidative, decreased oxidative enzyme activity, and down-regulation of the mRNA expression of heat shock proteins in skeletal muscles, especially in the slow muscle, of rats following exposure to microgravity. In addition, previous studies [38–40] observed that the oxidative capacity of small-sized and high-oxidative alpha motoneurons, but not gamma or large-sized and low-oxidative alpha motoneurons, in the lumbar segment of the rat spinal cord decreased after exposure to

microgravity. In contrast, there were no changes in the oxidative capacity of alpha motoneurons in the ventromedial region of the ventral horn in the lumbar segment [41] or in the dorsolateral region of the ventral horn in the cervical segment [42] of the rat spinal cord after exposure to microgravity. These responses in the oxidative capacity of alpha motoneurons correspond well with those of fibers in the skeletal muscles that the motoneurons innervate. The findings collectively indicate that the oxidative capacity of the small-sized alpha motoneurons innervating high-oxidative fibers in skeletal muscle with gravity-dependent functions is decreased by exposure to microgravity with atrophy and decreased oxidative capacity in the slow muscle and its fibers that were innervated by the small-sized alpha motoneurons.

It is expected that mild hyperbaric oxygen would improve decreased oxidative capacity in spinal motoneurons of rats with type 2 diabetes, because the metabolic properties of alpha motoneurons are tightly related to those of their target muscle and its fibers [7] and mild hyperbaric oxygen has been shown to improve the decreased oxidative capacity of muscles in rats with type 2 diabetes [14–16]. The present study found that mild hyperbaric oxygen improved the decreased oxidative capacity of small-sized and high-oxidative alpha motoneurons innervating the slow muscle of rats with type 2 diabetes (Fig. 4b). The improved oxidative capacity in motoneurons corresponds well with those observed in fibers in the soleus muscle that the motoneurons innervate [11, 45], indicating that the properties and responses of motoneurons and their innervating fibers are related closely under diabetic conditions, as well as under normal conditions (Fig. 5). However, which changes in the oxidative capacity of motoneurons and their innervating fibers occur earlier and/or are triggered by mild hyperbaric oxygen was not clear in this study.

It is possible that the response to mild hyperbaric conditions involved changes in physiological properties as well as metabolic properties, i.e., oxidative capacity, of motoneurons innervating the soleus muscle of rats with type 2 diabetes, although we did not measure physiological properties including conduction velocity, input resistance, and duration of the after hyperpolarization period of motoneurons. Previous studies [46, 47] observed that there is a relationship between the physiological and morphological (i.e., cell body size) properties of motoneurons innervating the skeletal muscle. In addition, there is a relationship between the morphological and metabolic properties, e.g., smaller-sized motoneurons have higher oxidative enzyme activity in the extensor digitorum longus [6], tibialis anterior [7], and soleus [21, 23, 25, 26] neuron pools in rats. In this study, with our previous findings observed in the soleus muscle of rats with type 2 diabetes [1, 2] and exposed to mild hyperbaric oxygen [14, 16], it is suggested that physiological properties of motoneuron shift

**Fig. 5** Three types of soleus neuromuscular units in normal WR rats. Type 2 diabetes induces decreased oxidative capacity in small-sized and high-oxidative alpha motoneurons innervating the soleus muscle, which shifts fiber types from type IIA and type IIC to type I [1, 2]. In contrast, mild hyperbaric oxygen restores these changes to normal [14, 16]



from slow to fast type with type 2 diabetes, whereas mild hyperbaric oxygen inhibit such changes in the motoneuron's physiological properties.

## Conclusions

This study determined the oxidative capacity of motoneurons innervating the soleus muscle of rats with type 2 diabetes. In addition, this study examined the effects of exposure to mild hyperbaric oxygen at 1.25 ATA with 36 % oxygen on the oxidative capacity of motoneurons innervating the soleus muscle of rats with type 2 diabetes. Spinal motoneurons innervating the soleus muscle were retrogradely identified using a fluorescent neuronal tracer. Thereafter, cell body sizes and SDH activity of identified motoneurons were analyzed. We concluded that rats with type 2 diabetes have decreased oxidative capacity in the motoneurons innervating the soleus muscle and that the decreased oxidative capacity of the motoneurons induced

by type 2 diabetes was improved by mild hyperbaric oxygen.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical Approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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