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Mild hyperbaric oxygen exposure attenuates rarefaction of capillary vessels in streptozotocin-induced diabetic soleus muscle in rats

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ABSTRACT

We examined the effects of mild hyperbaric oxygen (mHBO) exposure on capillary rarefaction in skeletal muscles of rats with diabetes. Streptozotocin (100 mg/kg) was administered to male Wistar rats via the tail vein to prepare a diabetic model. These rats were divided into 2 groups: the group with mHBO exposure (1.25 atmospheres absolute (ATA) with 36% oxygen; 3 h/day) and the group without mHBO exposure. Age-matched rats were used as the control group. Eight weeks later, the soleus of the rats was removed and then analyzed. With the onset of diabetes mel- litus, capillary number, diameter, and volume in the soleus of the rats with diabetes decreased compared with those of the rats in the control group. In addition, increased anti-angiogenic throm- bospondin-1 (TSP-1) and decreased pro-angiogenic murine double minute 2 (MDM-2) protein ex- pressions were observed in the rats with diabetes. Alternatively, mHBO exposure attenuated the decrease in capillary diameter and volume in skeletal muscles of rats with diabetes, suppressed the overexpression of TSP-1, and restored the MDM-2 expression. These results indicate the exposure of mHBO partially attenuates capillary rarefaction in diabetic soleus muscle.

INTRODUCTION

Skeletal muscle capillaries determine the muscle metabolic activity. Capillary growth increases the supply capacity of oxygen and substrates, while capillary rarefaction decreases them. Diabetes mellitus impairs the hemodynamics in skeletal muscles (Kindig *et al.* 1998; Padilla *et al.* 2006), which can affect their mobility and pathology. Capillary rarefaction in skeletal muscles has also been reported in diabetes (Mårin *et al.* 1994; Sexton *et al.* 1994; Abaci *et al.* 1999; Kivela *et al.* 2006), which results in an impaired metabolic exchange between the capillaries and muscle cells and thus leads to exercise intolerance (Olfert *et al.* 2009) and insulin resistance (Bonner *et al.* 2013).

Capillary architecture in skeletal muscles is susceptible to be changed in response to physiological and pathological conditions. Reports have shown that a decrease in capillary tortuosity, diameter, anastomosis, and volume and a decrease in the number of capillaries were observed in disused (Fujino *et al.* 2005; Kanazashi *et al.* 2013), ovariectomized (Tanaka *et al.* 2015), and diabetic (Kondo *et al.* 2015; Matsumoto *et al.* 2019) muscles. Furthermore,

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the 3-D changes in the capillary architecture, such as capillary diameter and volume, are not always accompanied by two-dimensional capillary alterations, such as capillary number. Some reports have shown that a decrease in capillary luminal diameter and volume occurred in skeletal muscles of animals with no change in capillary number (Kondo *et al.* 2015; Tanaka *et al.* 2015).

Capillarity is tuned by a balance between the pro-angiogenic and anti-angiogenic factors. Pro-angiogenic vascular endothelial growth factor (VEGF) plays an important role in angiogenesis (Olsson *et al.* 2006), while anti-angiogenic thrombospondin-1 (TSP-1) acts as an inhibitor of angiogenesis (Resovi *et al.* 2014), leading to capillary regression. These pro-angiogenic and anti-angiogenic factors are well known to be involved in a process of capillary growth and regression in various skeletomuscular conditions such as aerobic exercise (Hoier *et al.* 2012; Roudier *et al.* 2012; Delavar *et al.* 2014; Slopack *et al.* 2014), disuse (Roudier *et al.* 2010; Kaneguchi *et al.* 2014), peripheral arterial disease (Smadja *et al.* 2011), and chronic obstructive pulmonary disease (Eliason *et al.* 2010). In addition, an imbalance be- tween VEGF and TSP-1 protein expressions is ob- served along with capillary rarefaction within diabetic skeletal muscles (Kondo *et al.* 2015; Dunford *et al.* 2017; Matsumoto *et al.* 2019). Thus, the optimiza- tion of the pro-angiogenic and anti-angiogenic fac- tors is necessary to restore the capillary network in diabetic muscles.

Endurance exercise is a therapeutic countermeasure for glycemic control and effective for angiopathy in diabetic skeletal muscles (Kondo *et al.* 2015; Dunford *et al.* 2017). However, whole-body exercises, such as endurance exercises, may be difficult for someone with lower exercise tolerance or severe complications to perform continuously and effectively. Therefore, an alternative intervention that does not involve voluntary exercises is needed. Since skeletal muscles play a principal role in glucose metabolism, an intervention expected to have a systemic effect rather than a local effect is desirable.

Mild hyperbaric oxygen (mHBO) exposure, which is 1.2–1.3 atmospheres absolute (ATA) with 36% oxygen in the chamber, has been reported to facilitate the oxidative capacity of skeletal muscles and their fibers by increasing the peripheral blood flow, serum-dissolved oxygen, and energy expenditure (Ishihara *et al.* 2014; Ishihara 2019). So far, mHBO is proven to suppress a decrease in skeletomuscular oxidative capacity associated with diabetes (Fujita *et al.* 2012; Nagatomo *et al.* 2018), metabolic syndrome (Takemura and Ishihara 2017), and disuse (Takemura *et al.* 2017). Increased blood flow is a favorable factor for the formation and maintenance of capillaries in skeletal muscles (Zhou *et al.* 1998; Zhou *et al.* 2014; Mandel *et al.* 2016). Increased blood flow caused by the administration of prazosin, an α1-adrenerigic antagonist, increased the pro-angiogenic protein murine double minute 2 (MDM-2) (Dunford *et al.* 2017), which promoted capillary growth and acted as a negative regulator for TSP-1 (Prives 1998; Roudier *et al.* 2012). Therefore, these results led the authors to expect that mHBO would be effective for capillary maintenance in diabetic skeletal muscles by positively regulating pro-angiogenic and anti-angiogenic factors, such as VEGF, MDM-2, and TSP-1. However, the effects of mHBO on the capillary architecture and angiogenic factors remain unclear. Therefore, the aim was to examine the effect of mHBO on 3-D changes in the capillary structure and pro-angiogenic and anti-angiogenic factors in skeletal muscles of rats with diabetes.

MATERIALS AND METHODS

Experimental design. Fifteen male Wistar rats (age, 6 weeks) (Japan SLC, Hamamatsu, Japan) weighing between 193 g and 254 g were randomly divided into the following three groups: (1) control (CON), (2) streptozotocin-induced type 1 diabetes (STZ), and (3) streptozotocin-induced type 1 diabetes with mHBO exposure (STZ+mHBO) groups. The rats in the STZ and STZ+mHBO groups were subjected to a single injection of streptozotocin (100 mg/kg body weight) into the tail vein to induce diabetes. The successful induction of diabetes was confirmed by >250 mg/dL blood glucose levels 3 days after streptozotocin injection. All rats were provided water and food *ad libitum*. All rats were housed in a 12 : 12 h light–dark cycle (light was turned on from 07 : 00 to 19 : 00) at room temperatures of 22°C ± 2°C. This study was approved by the Institutional Animal Care and Use Committee and followed the Kobe University Animal Experimentation Regulations (Kobe, Japan). All experimental and animal care procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) of the National Institute of Health.

Exposure to mHBO. The rats in the STZ+mHBO group were exposed to mHBO following the same protocol as previously described (Takemura and Ishihara 2016, 2017). Briefly, the rats were exposed

to 1.25 ATA with 36% oxygen in a mild hyperbaric chamber for 3 h (08 : 00–11 : 00) daily for 8 weeks after the induction of diabetes. The amount of food intake of the rats with diabetes with and without mHBO exposure was equal.

Assessment of fasting blood glucose levels. Blood glucose levels of the rats in each group were measured using the Glutest Neo Super blood glucose test meter (Sanwa Kagaku Kenkyusho Co. Ltd., Nagoya, Japan). Blood samples for the test were collected from the tail vein after overnight fasting.

Sample preparation. At the end of the 8-week experimental period, the rats were fasted overnight, anaesthetized deeply by inhalation of 2% isoflurane gas *via* anesthetic masks to remove muscle samples. For use in histochemical and biochemical analysis, the left soleus muscle was dissected, weighed, and immediately frozen in dry-ice-cooled acetone. Additionally, the right soleus muscle was used to visualize the 3-D capillary architecture as described previously (Fujino *et al.* 2005; Kondo *et al.* 2011; Fujino *et al.* 2012; Tanaka *et al.* 2019). Briefly, a catheter was inserted into the right iliac artery, and 0.9% physiological saline containing heparin (1,000 IU/L) at 37°C was injected to wash out the intravascular blood and induce maximal vasodilation. Then, 8% gelatin solution containing 1% fluorescent material was injected. After refluxing 50 mL of the contrast medium, the right ankle joint was held in a maximum plantar-flexion position and the entire right hind limb was quickly immersed in ice-cooled sa- line. Fifteen minutes later, the right soleus was ex- cised, and immediately frozen in dry-ice-cooled acetone. All frozen muscle samples were stored at −80°C until analyzed.

Histological analyses. Serial transverse sections (12-μm thick) of the left soleus muscles were cut using the CM3050S cryostat microtome (Leica Microsystems, Heidelberg, Germany). The transverse sections were stained with myofibrillar ATPase following preincubation with a pH of 4.2, as described previously (Punkt *et al.* 2004), to identify type I and IIA muscle fibers and measure fiber type distribution and muscle fiber cross-sectional area (CSA). Then, the other sections were stained with succinate dehydrogenase (SDH) to determine mitochondrial oxidative potential as previously described (Kanazashi *et al.* 2019). Using the images of the serial transverse sections stained with ATPase and SDH, the SDH activity in each muscle fiber type was measured according to the quantitative histochemical methods of Nagatomo *et al.* (2018). Finally, the other sections were also stained with alkaline phosphatase (AP), as previously reported (Hansen-Smith *et al.* 1992), to visualize the capillaries. The capillary-to-fiber (C/F) ratio and capillary density were determined by counting the capillaries and myofibers on each cryosection using the microscopic images obtained by AP staining. All measurements were performed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Citrate synthase activity. Citrate synthase (CS) activity was measured from the left soleus as an indicator of mitochondrial oxidative capacity. The sample was homogenized in 10-mM trisaminomethane (Tris) (pH 7.4), 175-mM potassium chloride (KCl), and 2-mM ethylenediaminetetraacetic acid (EDTA). Homogenates were frozen and thawed thrice and then centrifuged at 3,000 ×*g* for 10 min at 4°C. Supernatants were collected and used for measuring the CS activity by Srere's method (Srere 1969).

3-D architecture of the capillary network. The 3-D architecture of capillary network was imaged using a confocal laser-scanning microscopy (TCS-SP; Leica Microsystems, Heidelberg, Germany) with a 488-nm argon laser according to the reports (Fujino *et al.* 2005, 2012; Kondo *et al.* 2015; Hirayama *et al.* 2017; Tanaka *et al.* 2019). Briefly, the right soleus muscles were cut longitudinally (100-μm-thick sec- tions) with the CM3050S cryostat microtome. Micro- scopic capillary images were scanned up to 50 μm in depth at 1 μm per slice thickness. The 50 images were stacked and converted into digital images to visualize the 3-D architecture of the capillary net- work. Finally, the capillary volume ($\times 10^{-2}$ mm^3/mm^3) in a 3-D image (250 μm × 250 μm × 50 μm; length × width × depth), and the capillary luminal diameter (μm) were measured using the ImageJ software program (NIH, Bethesda, MD, USA).

Western blot analysis. Portion (~20 mg) of each left soleus was homogenized in radioimmunoprecipitation assay lysis buffer containing 1-mM Na_3VO_4, 1-mM NaF, and a protease inhibitor cocktail (1 : 100, P8340; Sigma Chemicals, Perth, WA, USA) and centrifuged at 10,000 ×*g* for 10 min at 4°C, after which the supernatants were collected. The total protein concentration of the supernatants was determined according to Bradford (Bradford 1976) using the protein assay kit (Bio-Rad Laboratories, Hercu- les, CA, USA). Samples were diluted in 2× Blue

Loading Buffer and boiled at 95°C for 5 min. Proteins (20–40 μg/lane) were loaded and separated on 7.5%, 10%, or 12.5% SDS-polyacrylamide gels. The proteins were blotted on polyvinylidene difluoride (PVDF) membranes, and 1-h blocking with 5% skimmed milk in phosphate-buffered saline with Tween® 20 (PBST) was performed. The membrane was incubated using antibodies against the mitochondrial markers cytochrome oxidase IV (COX-IV) (1 : 1,000 in PBST, #4850; Cell Signaling Technology, Danvers, MA), MDM-2 (1 : 1,000 in PBST, OP115; Calbiochem, FA Jolla, CA, USA), VEGF (1 : 200 in PBST, sc-7269; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or TSP-1 (1 : 200 in PBST, sc-59887; Santa Cruz Biotechnology) over- night at 4°C, then incubated in a solution with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1 : 10,000 in PBST) or HRP-conjugated anti-mouse IgG secondary antibody (1 : 10,000 in PBST) for 1 h. The proteins were de- tected using chemiluminescent regents (Clarity™ Western ECL Substrate; Bio-Rad Laboratories, Her- cules, CA, USA) and imaged using a chemilumines- cent image analyzer (ImageQuant LAS 500; GE Healthcare, Tokyo, Japan). The chemiluminescent images were quantified using an image analysis soft- ware (ImageQuant TL; GE Healthcare) against the relative concentration of β-actin (1 : 1,000 in PBST, sc-47778; Santa Cruz Biotechnology) as an internal control.

Statistical analysis. All values are presented as mean ± standard error of the mean (SEM). The overall statistical differences were determined using one-way analysis of variance (ANOVA) followed by the Tukey *post-hoc* tests to determine specific group differences. Statistical significance was set at $P < 0.05$.

RESULTS

Body weight, soleus weight and fasting blood glucose level

Body weight, soleus weight and fasting blood glucose levels are shown in Table 1. Body weights in the STZ and STZ+mHBO groups were significantly lower than that in the CON group ($P < 0.001$ and $P < 0.001$, respectively). Absolute soleus weights in the STZ and STZ+mHBO groups were significantly lower than that in the CON group. Similarly, relative soleus weights in the STZ and STZ+mHBO groups were significantly lower than that in the CON group. Fasting blood glucose levels in the STZ and STZ+mHBO groups were significantly higher than that in the CON group, and the fasting blood glucose level in the STZ+mHBO group was significantly lower than that in the STZ group (Table 1). Thus, streptozotocin injection resulted in a reduction of body and muscle weights, and an increase of fasting blood glucose level. Meanwhile, mHBO could not ameliorate the detrimental effects of streptozotocin.

Fiber type distribution, muscle fiber CSA and SDH activity

The representative images of the soleus stained with myofibrillar ATPase and SDH in each group are shown in Fig. 1A–F. From these microscopic images of serial transverse sections, fiber type distribution, muscle fiber CSA and SDH activity were measured. The percentage of type I fibers in the STZ group was significantly lower than that in the CON group (Fig. 2A). Type I muscle fibers had no difference in CSA between the three groups (Fig. 2C). SDH activity of type I muscle fibers in the STZ group was significantly lower than that in the CON group, but maintained at near control level in STZ+mHBO group (Fig. 2E). The percentage of type IIA fibers in the STZ group was significantly higher than that in the CON group (Fig. 2B). The CSA of type IIA muscle fibers in the STZ group was significantly lower than those in the CON and STZ+mHBO groups (Fig. 2D). SDH activity of type IIA muscle fibers in the STZ group was also significantly lower than that in the CON group, but maintained at near control level in STZ+mHBO group (Fig. 2F). These data indicate that mHBO exposure had positive effects on SDH activities in type I and IIA fibers and size in type IIA fiber.

CS activity and COX-IV protein expression level

CS activity in the STZ group was significantly lower than those in the CON and STZ+mHBO groups (Fig. 3A). The representative images of protein blots are shown in Fig. 3B. Furthermore, we also assessed COX-IV protein content as a marker for mitochondrial content in the soleus muscle. The COX-IV protein expression level in the STZ group was significantly lower than those in the CON and STZ+ mHBO groups (Fig. 3B), indicating that mHBO exposure had a positive effect on muscle metabolism.

Capillary density and C/F ratio

Capillary density has no significant difference between the three groups (Table 2). The C/F ratios in the STZ and STZ+mHBO groups were significantly lower than that in the CON group (Table 2).

Table 1 *Body weight, soleus weight, and fasting blood glucose level*

Groups	Body weight (g)	Absolute soleus weight (mg)	Relative soleus weight (mg/100 g)	Fasting blood glucose level (mg/dL)
CON	373 ± 8	138 ± 4	37 ± 1	145 ± 9
STZ	251 ± 5*	113 ± 4*	45 ± 1*	468 ± 10*
STZ+mHBO	274 ± 9*	123 ± 4*	45 ± 1*	420 ± 11*†

CON, control; STZ, streptozotocin-induced diabetes; STZ+mHBO, streptozotocin-induced diabetes with mild hyperbaric oxygen exposure. Values are expressed in mean ± SEM. * and † represent a significant difference in the CON and STZ groups, respectively, at $P < 0.05$.

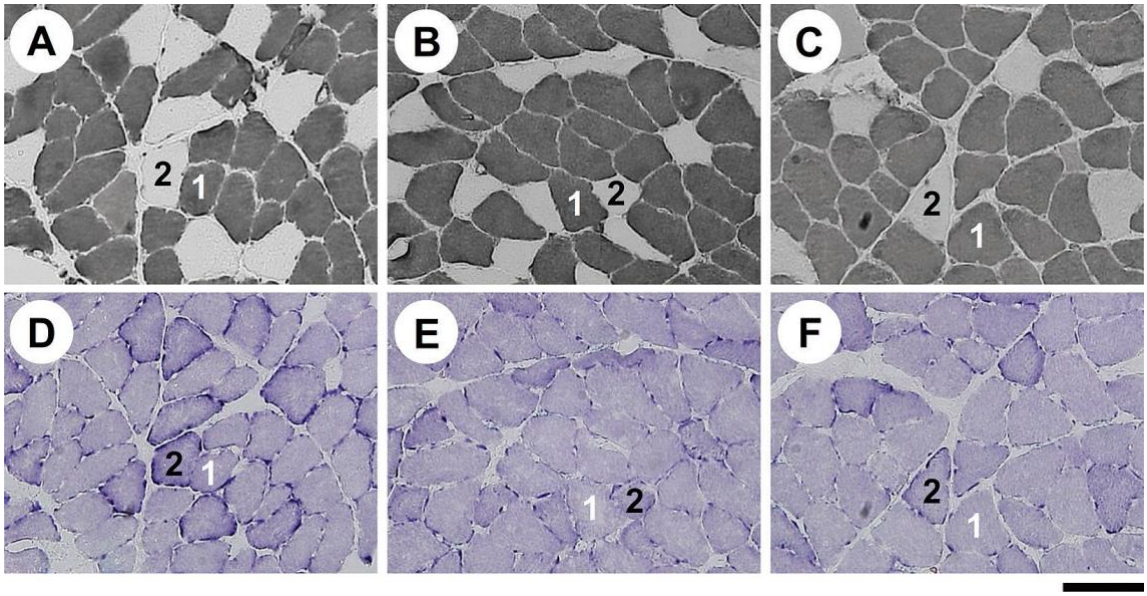

Fig. 1 Representative microscopic images of serial transverse sections of the soleus stained with ATPase (A–C) and SDH (D–F) in the CON (A and D), STZ (B and E), and STZ+mHBO (C and F) groups. CON, control; STZ, streptozotocin-induced type 1 diabetes; STZ+mHBO, streptozotocin-induced type 1 diabetes with mild hyperbaric oxygen exposure. 1, type I muscle fibers; 2, type IIA muscle fibers. Scale bar = 100 μm.

Capillary luminal diameter and volume

The representative confocal laser-scanning microscopic images of the 3-D capillary architecture in each group are shown in the Fig. 4A–C. The capillary network in the longitudinal section of the soleus was less complex in the STZ (Fig. 4B) group than in the CON (Fig. 4A) and STZ+mHBO (Fig. 4C) groups. The mean capillary diameter in the STZ group was significantly lower than those in the CON and STZ+mHBO groups (Fig. 4D). The frequency distribution of the capillary luminal diameter in the STZ group (Fig. 4G) was shifted toward smaller-diameter capillaries compared with those in the CON (Fig. 4F) and STZ+mHBO (Fig. 4H) groups. The percentage of capillaries with a diameter of less than 2.5 μm, through which erythrocytes cannot pass (Henquell *et al.* 1976), increased to a high value in the STZ group (14.9%) compared with the other groups (CON: 2.3% and STZ+mHBO: 1.9%) (Fig. 4F–H). The mean capillary volume in the STZ group was significantly lower than those in the CON and STZ+mHBO groups (Fig. 4E). These data indi- cate that mHBO exposure had positive effects on 3-D capillary architecture in diabetic skeletal muscle.

MDM-2, VEGF, and TSP-1 protein expression levels

The representative images of protein blots are shown in Fig. 5A. The protein expression level of MDM-2 in the STZ group was significantly lower than that in the CON group and tended to a lower value compared with that in the STZ+mHBO group (Fig. 5B). The VEGF protein expression levels between all groups were not different (Fig. 5C). Meanwhile, the TSP-1 protein expression level in the STZ group was significantly higher than those in the CON and STZ+mHBO groups (Fig. 5D). Thus, streptozotocin

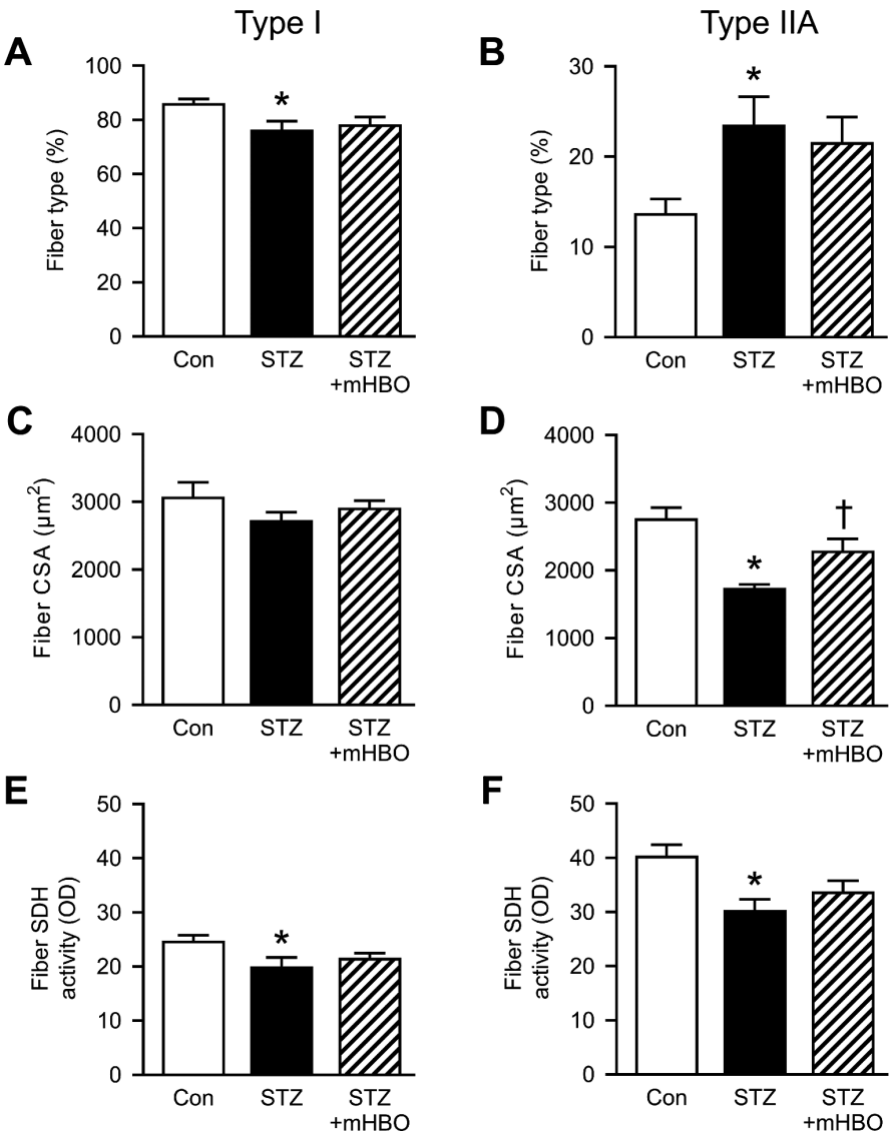

Fig. 2 Fiber type distribution (A and B), muscle fiber CSA (C and D) and SDH activity (E and F) of type I muscle fibers (A, C and E) and type IIA muscle fibers (B, D and F) of the soleus in each group. The following abbreviations are used in the figure: CON, control; STZ, streptozotocin-induced type 1 diabetes; STZ+mHBO, streptozotocin-induced type 1 diabetes with mild hyperbaric oxygen exposure. Values are expressed in mean ± SEM. * and † represent a significant difference in the CON and STZ groups, respectively, at $P < 0.05$.

injection resulted in a reduction of MDM-2 protein expression level and an increase of TSP-1, one of anti-angiogenic factors, protein expression level. Meanwhile, mHBO exposure ameliorated the detrimental effects of streptozotocin.

DISCUSSION

The novel findings of this study are that mHBO exposure attenuated 3-D capillary rarefaction and restored alterations in anti-angiogenic TSP-1 protein expression and pro-angiogenic MDM-2 protein expression within the soleus of rats with streptozotocin-induced diabetes. These results suggest that mHBO exposure is an effective therapeutic countermeasure to capillary rarefaction in skeletal muscles of rats with diabetes.

Skeletal muscle capillaries are susceptible to structural changes in response to muscle conditions (Kano *et al.* 2000; Fujino *et al.* 2012; Kondo *et al.* 2015; Tanaka *et al.* 2015, 2019). Studies assessing capillarization in diabetic skeletal muscles demon-

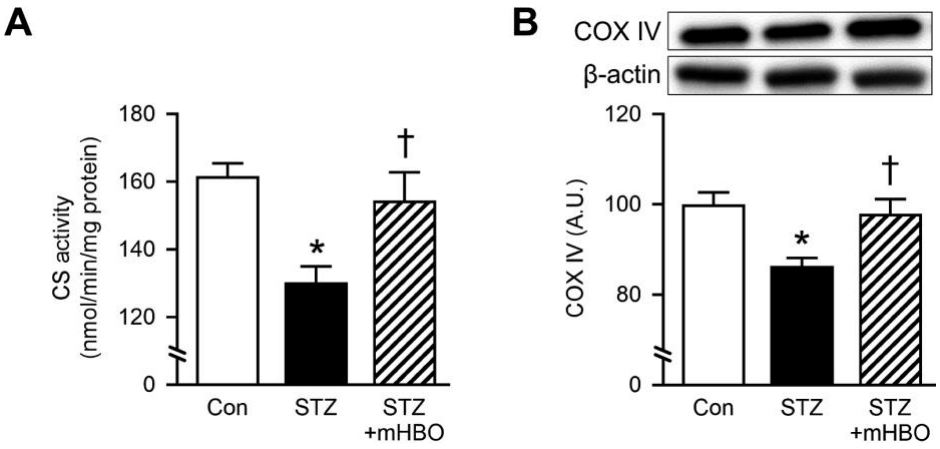

Fig. 3 CS activity (A) and protein expression level of COX-IV (B) of the soleus in each group. Values are expressed in mean ± SEM. * and † represent a significant difference in the CON and STZ groups, respectively, at $P < 0.05$.

Table 2 *Capillary density and capillary-to-fiber ratio*

Groups	Capillary density (capillaries/mm^2)	C/F ratio
CON	621 ± 17	2.47 ± 0.07
STZ	711 ± 32	2.25 ± 0.07*
STZ+mHBO	661 ± 41	2.23 ± 0.03*

CON, control; C/F, capillary-to-fiber; STZ, streptozotocin-induced type 1 diabetes; STZ+mHBO, streptozotocin-induced type 1 diabetes with mild hyperbaric oxygen exposure. Values are expressed in mean ± SEM. * and † represent a significant difference from the CON and STZ groups, respectively, at $P < 0.05$.

strated that diabetes induced capillary rarefaction confirmed by a decrease in capillary number, capillary luminal diameter, or volume (Kondo *et al.* 2015; Matsumoto *et al.* 2019). Consistent with these reports, the STZ group in this study had decreased capillary number, luminal diameter, and volume in the soleus. Moreover, capillaries with diameters of less than 2.5 μm, through which erythrocytes cannot pass (Henquell *et al.* 1976), were more frequently observed in the STZ group than those in the CON and STZ+mHBO groups. These results suggest that 3-D capillary rarefaction occurred in the soleus of streptozotocin-treated rats in this study. Muscle mitochondrial oxidative activity also limits muscle metabolic function. To assess the effects on muscle oxidative enzymatic capacity, SDH activity of the muscle fibers, CS activity and COX-IV protein content in the soleus were measured. As observed in this study, SDH activity, CS activity, and COX-IV protein expression decreased in the diabetic muscles of the rats in the STZ group, which mean lower oxidative capacity. Along with these results, the soleus of the rats in the STZ group fell into a state of decreased metabolic function due to capillary rarefaction and decreased mitochondrial oxidative capacity.

The capillary structure is tightly tuned by a dynamic balance between pro-angiogenic and anti-angiogenic factors (Olfert and Birot 2011; Olfert 2016; Olfert *et al.* 2016). The pro-angiogenic protein VEGF stimulates vascular formation by recruitment and proliferation of endothelial cells (Olsson *et al.* 2006). Despite its significant involvement in capillary growth, VEGF has little participation in capillary rarefaction in several conditions (Roudier *et al.* 2010; Kanazashi *et al.* 2014; Tanaka *et al.* 2015; Mandel *et al.* 2016; Matsumoto *et al.* 2019). Alternatively, TSP-1 acts as an inhibitor of angiogenesis by its antiproliferative and proapoptotic effects. A decrease in capillary number, diameter, and volume is accompanied by an increased TSP-1 expression in the soleus of the hind limbs of unloaded (Tanaka *et al.* 2019), ovariectomized (Tanaka *et al.* 2015), and diabetic (Kondo *et al.* 2011) rats in previous studies. Consistent with these reports, the capillary rarefaction observed in the STZ group in this study was due to an alteration in the protein expression of TSP-1, but not VEGF. The authors also measured MDM-2 protein expression to assess the angiogenic response to streptozotocin-induced diabetes. MDM-2 may be involved in capillarization in skeletal muscles partially by regulating VEGF and TSP-1 expressions (Roudier *et al.* 2012; Aiken *et al.* 2016). Additionally, lower capillarization and decreased MDM-2 expression were observed in models of streptozotocin-induced diabetes (Matsumoto *et al.* 2019) and type 2 diabetes (Roudier *et al.* 2012). These results suggest that decreased MDM-2 ex-

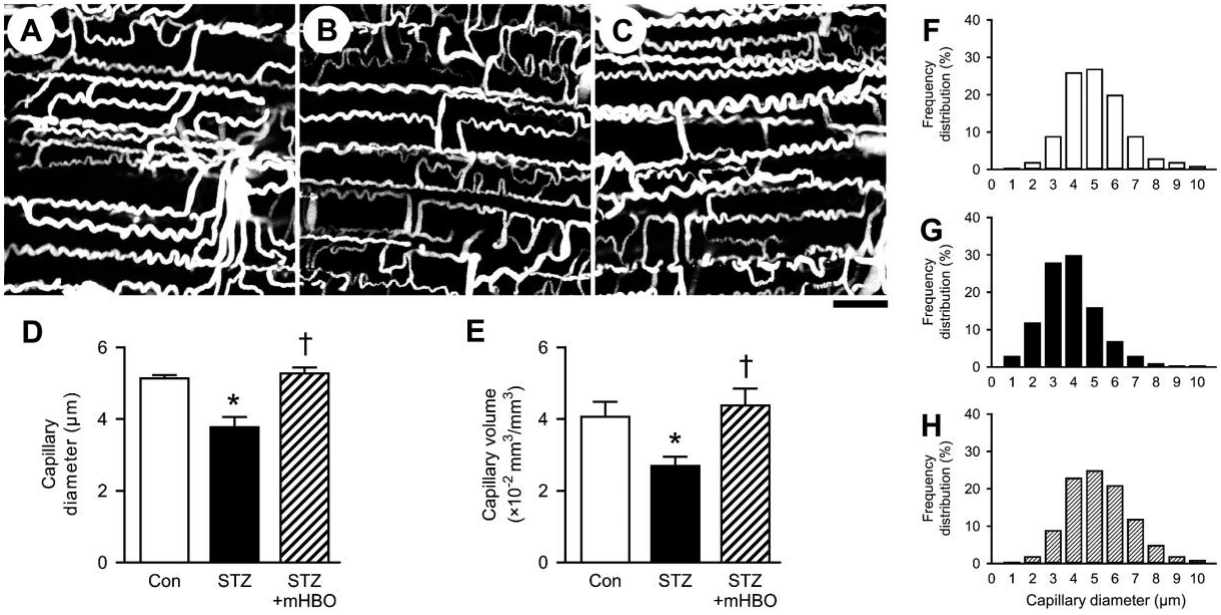

Fig. 4 Representative confocal laser-scanning microscopic images of the 3-D capillary architecture in the soleus of a rat in the CON (A), STZ (B), and STZ+mHBO (C) groups. Scale bar = 100 μm. Mean capillary luminal diameter (D) and capillary volume (E) in the soleus of each group. Frequency distribution of capillary luminal diameter in the CON (F), STZ (G), and STZ+mHBO (H) groups. Values are expressed in mean ± SEM. * and † represent a significant difference in the CON and STZ groups, respectively, at $P < 0.05$.

pression can explain the low capillarization in the STZ group observed in this study.

The authors found for the first time that mHBO exposure has positive effects on capillarization and imbalance between pro-angiogenic and anti-angiogenic factors. In this study, increased TSP-1 and decreased MDM-2 protein expressions were reversed in the soleus of rats with streptozotocin-induced diabetes exposed to mHBO. MDM-2 expression has a possible relationship with increased blood flow (Dunford *et al.* 2017). An increase in blood flow can induce angiogenesis independently of oxidative demand (Milkiewicz *et al.* 2001; Baum *et al.* 2004; Akerstrom *et al.* 2014). Furthermore, increased blood flow in diabetic muscle attenuates capillary rarefaction by reversing decreased MDM-2 and/or increased TSP-1 protein expressions (Dunford *et al.* 2017; Matsumoto *et al.* 2019). mHBO exposure has been reported to increase peripheral tissue blood flow (Ishihara *et al.* 2014). Therefore, it is speculated that the increase in blood flow by mHBO exposure promotes MDM-2 induction and results in TSP-1 downregulation, leading to the attenuation of capillary rarefaction in the soleus of rats with diabetes. These results suggest that mHBO exposure is useful in maintaining the 3-D structure of skeletal muscle capillaries under angiostatic stimulation due to diabetes.

Another explanation for mHBO-induced capillary maintenance may have been the relation of mHBO exposure to improved muscle oxidative capacity. In a study, mHBO exposure increased mRNA expression for mitochondrial biogenic factors such as peroxisome proliferators-activated receptor γ coactivator-1α (PGC-1α) in several conditions (Takemura *et al.* 2017; Nagatomo *et al.* 2018). PGC-1α possesses an angiogenic effect through VEGF expression and is mediated by a molecular mechanism independent of increased blood flow-induced shear stress or hypoxia-induced hypoxia-inducible factor-1α (HIF-1α) expression (Arany *et al.* 2008). Indeed, as in previous studies (Takemura *et al.* 2017; Nagatomo *et al.* 2018), the authors found the protective effects of mHBO exposure on SDH activity, CS activity, and COX-IV protein content in the soleus of rats with diabetes in this study, which was associated with restoring mitochondrial oxidative capacity. Since decreased mitochondrial oxidative capacity in the soleus was suppressed by mHBO exposure, there might be an increase in mitochondrial biogenic factors such as PGC-1α in the soleus of the rats in the STZ+mHBO group. Thus, it is possible that there may be some positive effects of mHBO exposure on capillarization in diabetic skele-

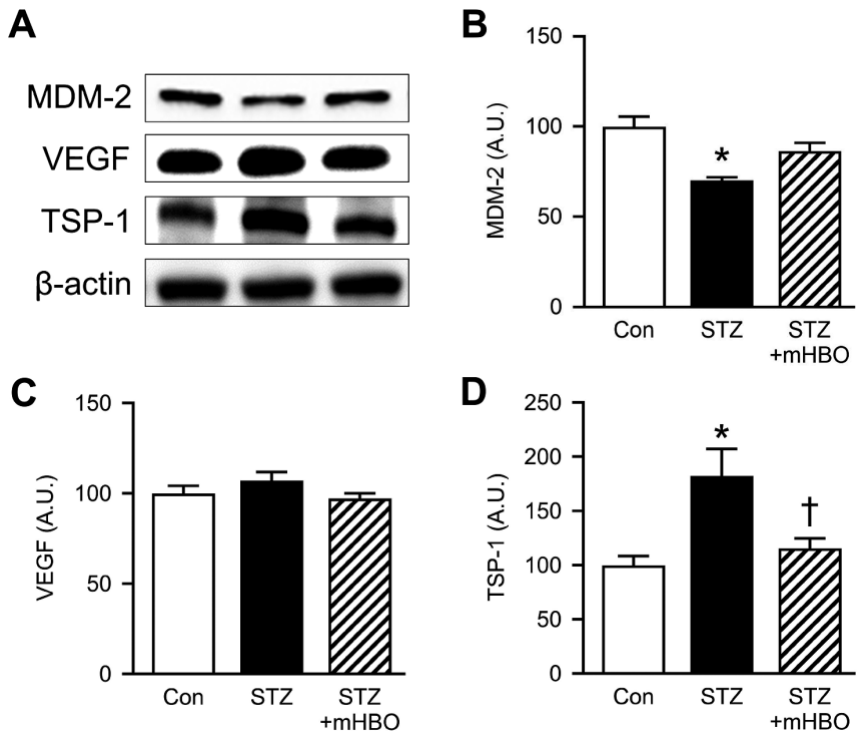

Fig. 5 (A) Representative Western blots for MDM-2, VEGF, TSP-1, and β-actin in each group. Mean protein expression levels of MDM-2 (B), VEGF (C), and TSP-1 (D) in the soleus of each group. Values are expressed in mean ± SEM. * and † represent a significant difference in the CON and STZ groups, respectively, at $P < 0.05$.

tal muscles by activating PGC-1α/VEGF-mediated capillary growth, which may have helped offset capillary rarefaction in the early phase of diabetes induction.

In this study, blood glucose level in the STZ+ mHBO group was lower than that in the STZ group. Improvement of mitochondrial oxidative capacity (Nagatomo *et al.* 2018) and capillarization (Akerstrom *et al.* 2014) in skeletal muscles has a close relationship to glucose consumption. Thus, this helps in lowering hyperglycemia. Consistent with these reports, blood glucose improvement was observed in the STZ+mHBO group in which oxidative capacity and capillary network of the soleus were improved by mHBO exposure. Because it has been reported to increase tissue blood flow, blood-dissolved oxygen, and energy expenditure (Ishihara *et al.* 2014; Ishihara 2019), mHBO exposure can be expected to possess a systemic effect, including the skeletal muscles of the whole body. Indeed, it has been reported that mHBO exposure improves oxidative capacity not only in the slow-twitch soleus muscle (Nagatomo *et al.* 2018) but also in fast-twitch extensor digitorum longus (Fujita *et al.* 2012) or plantaris (Matsumoto *et al.* 2007). In addition, it has been reported that angiogenesis in fast-twitch muscles is easier to occur than slow-twitch or cardiac muscles (Egginton 2009). From these reports and the results of this study, there was a possibility that mHBO exposure to diabetic rats has positive systemic effects, includ- ing microvasculature maintenance, in all muscles, that is, both slow-twitch and fast-twitch muscles, contributed by the improvement of blood glucose level.

In conclusion, the authors found that mHBO exposure was an effective treatment to attenuate capillary rarefaction in the soleus associated with diabetes. The results of this study suggest that mHBO exposure can be applied as a therapeutic countermeasure for skeletal muscle angiopathy due to diabetic and hyperglycemic states. However, this study has some limitations. Since we induced diabetes in 6-week-old rats with streptozotocin administration, it should be considered as a possible involvement of capillary and mitochondrial growth impairment within the skeletal muscle. Therefore, further investigation using mature animals should be conducted to exclude the skeletal muscle vascular and mitochondrial growth impairment. Another limitation is that we did not determine acute effects of mHBO exposure

on pro- and anti-angiogenic factors within the diabetic muscle. Future study should be performed to examine the effects of a single exposure of mHBO on these angiogenic factors within skeletal muscle.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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