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Combined Effect of Aerobic Exercise and Normobaric Hyperoxia on Skeletal Muscle Capillary and Mitochondrial Function in Diabetic Rats

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Abstract

Background/Objectives: The combined effects of aerobic exercise and normobaric hyperoxia exposure on skeletal muscle in diabetes mellitus (DM) remain unclear. This study investigated whether their combination influences skeletal muscle capillary and mitochondrial function in diabetic rats. **Methods**: Seven-week-old male Wistar rats were randomly assigned to the following five groups: control (CON), DM, DM with aerobic exercise (DMEx), DM with aerobic exercise under 30% oxygen exposure (DMEx30), and DM with aerobic exercise under 50% oxygen exposure (DMEx50). Aerobic exercise and normobaric hyperoxia exposure were performed simultaneously in the DMEx30 and DMEx50 groups. **Results**: Combined aerobic exercise and normobaric hyperoxia significantly improved both capillary density and succinate dehydrogenase (SDH) activity in the soleus muscle, which is predominantly composed of slow-twitch fibers, in diabetic rats. In contrast, the effects were more limited in predominantly fast-twitch muscles, including the extensor digitorum longus and plantaris muscles. **Conclusions**: Combined aerobic exercise and normobaric hyperoxia may induce beneficial skeletal muscle adaptations in diabetic rats, with more pronounced effects in the predominantly slow-twitch soleus muscle than in predominantly fast-twitch muscles. These findings suggest that muscle fiber-type composition may influence responsiveness to this intervention. This combined approach may contribute to the development of novel exercise-based interventions for DM.

Keywords: diabetes mellitus; aerobic exercise; normobaric hyperoxia exposure; skeletal muscles; capillaries; mitochondrial function

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1. Introduction

Hyperglycemia associated with diabetes mellitus (DM) induces excessive accumulation of reactive oxygen species in the body [1]. Excessive reactive oxygen species accumulation damages the vascular endothelial cells and leads to microcirculatory impairment in various organs and tissues [1,2]. In skeletal muscles affected by DM, microcirculatory dysfunction is characterized by reductions in capillary density and the capillary-to-fiber ratio (C/F ratio), which are accompanied by decreased substrate utilization, impaired muscle contractility, and muscle atrophy [3,4]. Muscle atrophy in DM varies according to muscle fiber type [4,5], and skeletal muscle fibers are closely associated with the capillary network [6]. Additionally, DM has been reported to reduce the enzymatic activity of succinate dehydrogenase (SDH), which functions as a mitochondrial complex II and participates in both the tricarboxylic acid cycle and the electron transport chain [7,8]. A decline in SDH activity leads to mitochondrial dysfunction and inhibits cellular oxidative phosphorylation, resulting in skeletal muscle atrophy, impaired contractile function, and abnormalities in skeletal muscle energy metabolism [9,10]. Thus, both the capillary network and mitochondrial function in the skeletal muscle play critical roles in maintaining metabolic homeostasis and are closely associated with metabolic dysfunction in the skeletal muscle in DM.

In recent years, hyperbaric hyperoxia exposure has been shown to promote angiogenesis and improve mitochondrial function by increasing dissolved oxygen and oxygen bound to hemoglobin in the blood [11]. In DM, hyperbaric hyperoxia exposure has also been reported to improve capillary length and volume in muscle tissue and to enhance SDH activity and oxidative metabolism [12–14]. However, exposure to hyperbaric environments requires substantial changes in atmospheric pressure and has been associated with adverse events, such as inner ear and alveolar injury [15,16]. A previous study investigating hyperbaric exposure to 100% oxygen up to 3 atmospheres absolute showed a pressure-dependent increase in oxidative stress in the rat lung [17]. Conversely, our previous study examined the effects of 40 and 50% normobaric oxygen exposure on the soleus, extensor digitorum longus, and plantaris muscles in DM rats [18]. As a result, significant improvements in both the C/F ratio and SDH activity were observed only in the soleus muscle under 40% oxygen exposure. The soleus is predominantly composed of slow-twitch fibers, whereas the extensor digitorum longus and plantaris muscles consist mainly of fast-twitch fibers [19–21]. These findings suggest that the effects of normobaric hyperoxia may reflect differences in muscle fiber-type characteristics, and that mild hyperoxic exposure without changes in atmospheric pressure may provide a more favorable oxygen environment for improving skeletal muscle function in DM. Nevertheless, skeletal muscle capillary networks and mitochondrial function are regulated not only by oxygen availability but also by metabolic demand and mechanical stimuli associated with muscle contraction [22,23]. Aerobic exercise has been shown to improve skeletal muscle metabolic function through mechanisms such as angiogenesis, mitochondrial biogenesis, and signaling mediated by factors such as insulin-like growth factor-1 (IGF-1) [24,25]. However, the effects of combining normobaric hyperoxia with aerobic exercise on skeletal muscle capillaries and mitochondrial function remain unclear. Compared with hyperbaric oxygen exposure, normobaric oxygen exposure is less restricted by environmental conditions and can be more easily combined with aerobic exercise, making it potentially useful as a novel exercise strategy.

Therefore, in the present study, we aimed to investigate the effects of combined aerobic exercise and normobaric hyperoxia exposure on skeletal muscle capillaries and mitochondrial function in diabetic rats. In contrast to our previous work focusing on hyperoxia exposure alone, this study was designed to examine the combined effects of aerobic exercise and hyperoxia in the same diabetic model. In addition, we assessed circulating markers, including lipid-related blood parameters and additional markers of

oxidative stress, antioxidant capacity, and IGF-1, to evaluate the differential effects of oxygen concentration.

2. Results

The five groups were as follows: the control (CON), DM, DM with aerobic exercise (DMEx), DM with 30% oxygen exposure combined with aerobic exercise (DMEx30), and DM with 50% oxygen exposure combined with aerobic exercise (DMEx50).

2.1. Body Weight and Blood Glucose Levels

At the end of the experiment, body weight was significantly lower in all DM groups (DM, DMEx, DMEx30, and DMEx50) than in the CON group. In contrast, blood glucose levels at the end of the experiment were significantly higher in all DM groups than in the CON group (Table 1).

Table 1. Body weight (g) and blood glucose levels (mg/dL) at the beginning and end of the experiment.

		CON	DM	DMEx	DMEx30	DMEx50	*p*-Value [1]
BW	Before	190.8 ± 4.2 ††† (185.6–196.0)	186.8 ± 9.0 †† (175.6–198.0)	188.2 ± 7.4 ††† (179.0–197.4)	188.2 ± 2.2 †† (185.5–190.9)	190.4 ± 5.8 †† (183.2–197.6)	0.829
	After	391.4 ± 17.1 (370.2–412.6)	256.2 ± 25.3 *** (224.8–287.6)	251.8 ± 12.5 *** (236.3–267.3)	270.8 ± 30.7 *** (232.7–308.9)	262.8 ± 22.4 *** (235.0–290.6)	<0.001
GLU	Before	122.2 ± 9.0 † (111.1–133.3)	128.6 ± 5.7 ††† (121.5–135.7)	125.0 ± 8.7 ††† (114.2–135.8)	123.4 ± 7.9 ††† (113.6–133.2)	123.4 ± 5.9 ††† (116.1–130.7)	0.705
	After	142.4 ± 10.7 (129.1–155.7)	590.8 ± 20.6 *** (565.3–616.3)	589.8 ± 22.8 *** (561.5–618.1)	597.6 ± 5.4 *** (590.9–604.3)	594.6 ± 8.7 *** (583.8–605.4)	<0.001

Mean ± SD (95%CI), [1]: one-way analysis of variance, † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ for within-group comparisons between the beginning and end of the experiment (paired *t*-test), *** $p < 0.001$ for comparisons between the CON group and each DM group (Scheffé's test). BW: Body weight, GLU: blood glucose, CON: Control, DM: Diabetes mellitus, DMEx: DM + exercise, DMEx30: DM + exercise + 30% oxygen exposure, DMEx50: DM + exercise + 50% oxygen exposure.

2.2. Biochemical Parameters

2.2.1. Blood Lipid Parameters

At the end of the experiment, total cholesterol (TC) levels were significantly higher in the DM group than in all other groups, whereas high-density lipoprotein cholesterol (HDL-C) levels were significantly higher in the DM, DMEx30, and DMEx50 groups than in the CON group. In contrast, low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) levels were significantly lower in the CON, DMEx, DMEx30, and DMEx50 groups than in the DM group (Figure 1).

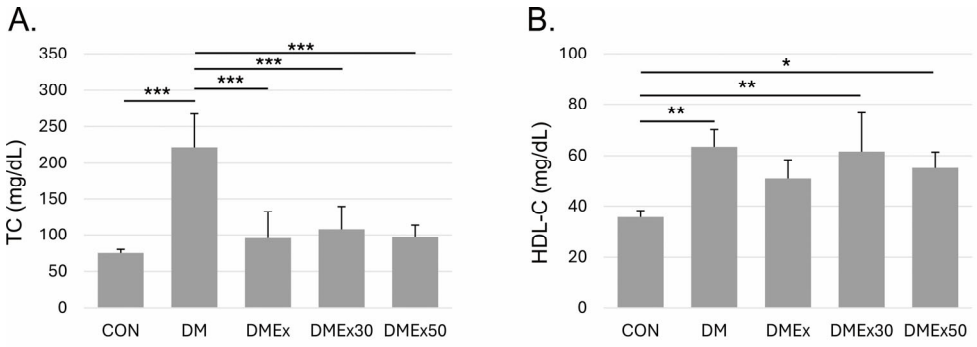

Figure 1. *Cont.*

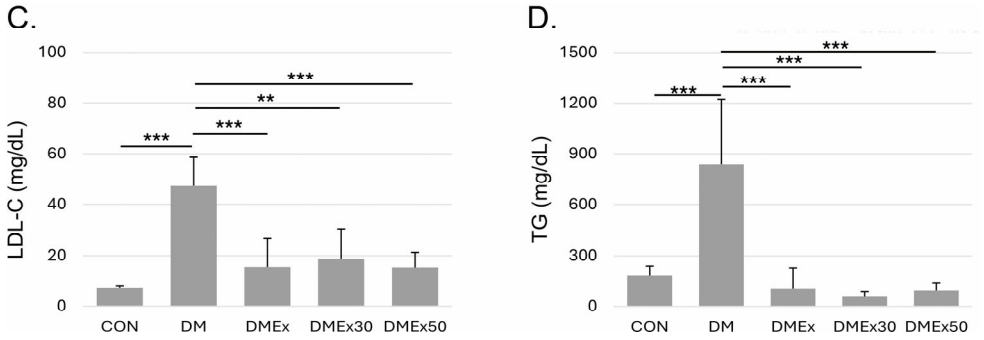

Figure 1. Blood lipid parameters at the end of the experiment. (**A**) Total cholesterol (TC), (**B**) High density lipoprotein cholesterol (HDL-C), (**C**) Low density lipoprotein cholesterol (LDL-C), (**D**) Triglyceride (TG). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for between-group comparisons (Scheffé's test). CON: Control, DM: Diabetes mellitus, DMEx: DM + exercise, DMEx30: DM + exercise + 30% oxygen exposure, DMEx50: DM + exercise + 50% oxygen exposure.

2.2.2. Blood Oxidative Stress-Related Parameters

At the end of the experiment, derivatives of reactive oxygen metabolites (d-ROMs) were significantly higher only in the DMEx50 group than in the DM group by 25.7% ($p < 0.05$). In contrast, biological antioxidant potential (BAP) was significantly higher only in the DMEx30 group than in the DM group by 250.0% ($p < 0.01$). Furthermore, the oxidative stress index (OSI), an indicator of relative oxidative stress, was significantly lower only in the DMEx30 group than in the DM group by 36.4% ($p < 0.05$) (Figure 2). These findings suggest that, in combination with aerobic exercise, higher oxygen concentrations may increase oxidative stress, whereas mild hyperoxia may enhance antioxidant capacity.

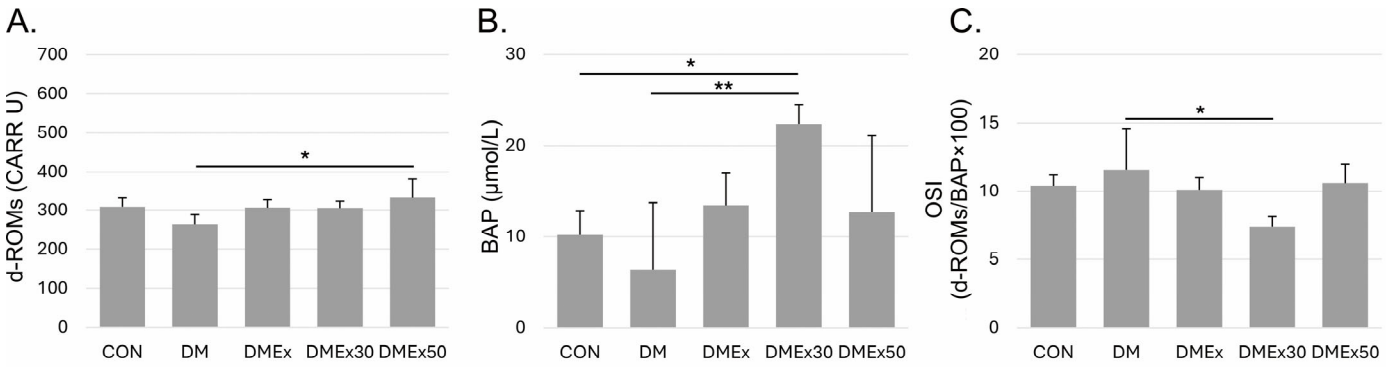

Figure 2. Blood oxidative stress-related parameters at the end of the experiment. (**A**) Derivatives of reactive oxygen metabolites (d-ROMs), (**B**) Biological antioxidant potential (BAP), (**C**) Oxidative stress index (OSI). * $p < 0.05$, ** $p < 0.01$, for between-group comparisons (Scheffé's test). CON: Control, DM: Diabetes mellitus, DMEx: DM + exercise, DMEx30: DM + exercise + 30% oxygen exposure, DMEx50: DM + exercise + 50% oxygen exposure.

2.2.3. Blood IGF-1 Levels

At the end of the experiment, IGF-1 levels were significantly lower in the DM, DMEx, DMEx30, and DMEx50 groups than in the CON group by 85.0% ($p < 0.001$), 62.4% ($p < 0.01$), 64.2% ($p < 0.001$), and 44.9% ($p < 0.05$), respectively. In contrast, compared with the DM group, IGF-1 levels were significantly higher only in the DMEx50 group by 266.5% ($p < 0.05$) (Figure 3). These results suggest that, in combination with aerobic exercise, higher oxygen concentrations may promote the increase in IGF-1.

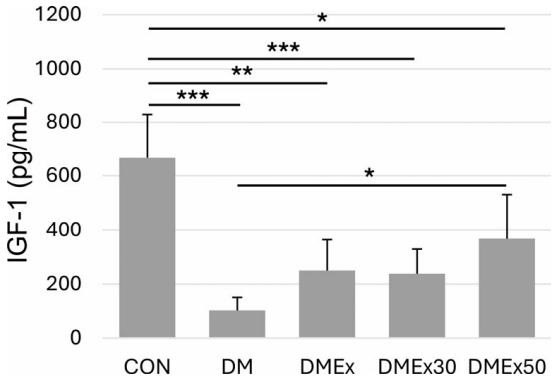

Figure 3. Blood IGF-1 levels at the end of the experiment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Scheffé's test for multiple comparisons). CON: Control, DM: Diabetes mellitus, DMEx: DM + exercise, DMEx30: DM + exercise + 30% oxygen exposure, DMEx50: DM + exercise + 50% oxygen exposure.

2.3. Histological Findings

For all histological analyses, three microscopic images were analyzed from each rat to ensure evaluation of more than 100 muscle fibers per rat (5 rats per group, 15 images in total per group). In addition, type I and type II fibers correspond to slow-twitch and fast-twitch fibers, respectively.

2.3.1. Skeletal Muscle Fiber Type Distribution and Cross-Sectional Area

In the soleus muscle, the proportion of type I fibers was significantly lower in the DM group than in the CON group by 17.3% ($p < 0.001$). In contrast, compared with the DM group, the proportion of type I fibers was significantly higher in the DMEx30 and DMEx50 groups by 18.0% ($p < 0.001$) and 11.2% ($p < 0.05$), respectively (Figure 4F). Regarding cross-sectional area, both type I and type II fibers in the DM group were significantly smaller than those in the CON group by 31.6% and 38.8%, respectively (both $p < 0.001$). In contrast, compared with the DM group, the cross-sectional area of type I and type II fibers was significantly greater in the DMEx30 group by 24.7% ($p < 0.01$) and 39.8% ($p < 0.01$), respectively, and in the DMEx50 group by 21.2% ($p < 0.05$) and 31.1% ($p < 0.05$), respectively (Figure 4G).

In the extensor digitorum longus muscle, the proportion of type I fibers was significantly higher in the DMEx group than in both the DM and DMEx50 groups by 86.7% (both $p < 0.05$) (Figure 5F). Regarding cross-sectional area, both type I and type II fibers in the DM group were significantly smaller than those in the CON group by 34.9% and 44.8%, respectively (both $p < 0.001$). Compared with the DM group, only the cross-sectional area of type I fibers was significantly greater in the DMEx group by 45.9% ($p < 0.01$) (Figure 5G).

In the plantaris muscle, the proportion of type I fibers was significantly lower in the DM group than in the CON group by 34.5% ($p < 0.05$). In contrast, compared with the DM group, the proportion of type I fibers was significantly higher in the DMEx, DMEx30, and DMEx50 groups by 58.4% ($p < 0.05$), 52.7% ($p < 0.05$), and 66.6% ($p < 0.01$), respectively (Figure 6F). Regarding cross-sectional area, in the DM group, type I and type II fibers were significantly smaller than those in the CON group by 31.2% ($p < 0.01$) and 44.2% ($p < 0.001$), respectively. In contrast, compared with the DM group, the cross-sectional area of type I and type II fibers in the DMEx group was significantly greater by 33.6% ($p < 0.05$) and 40.6% ($p < 0.01$), respectively (Figure 6G).

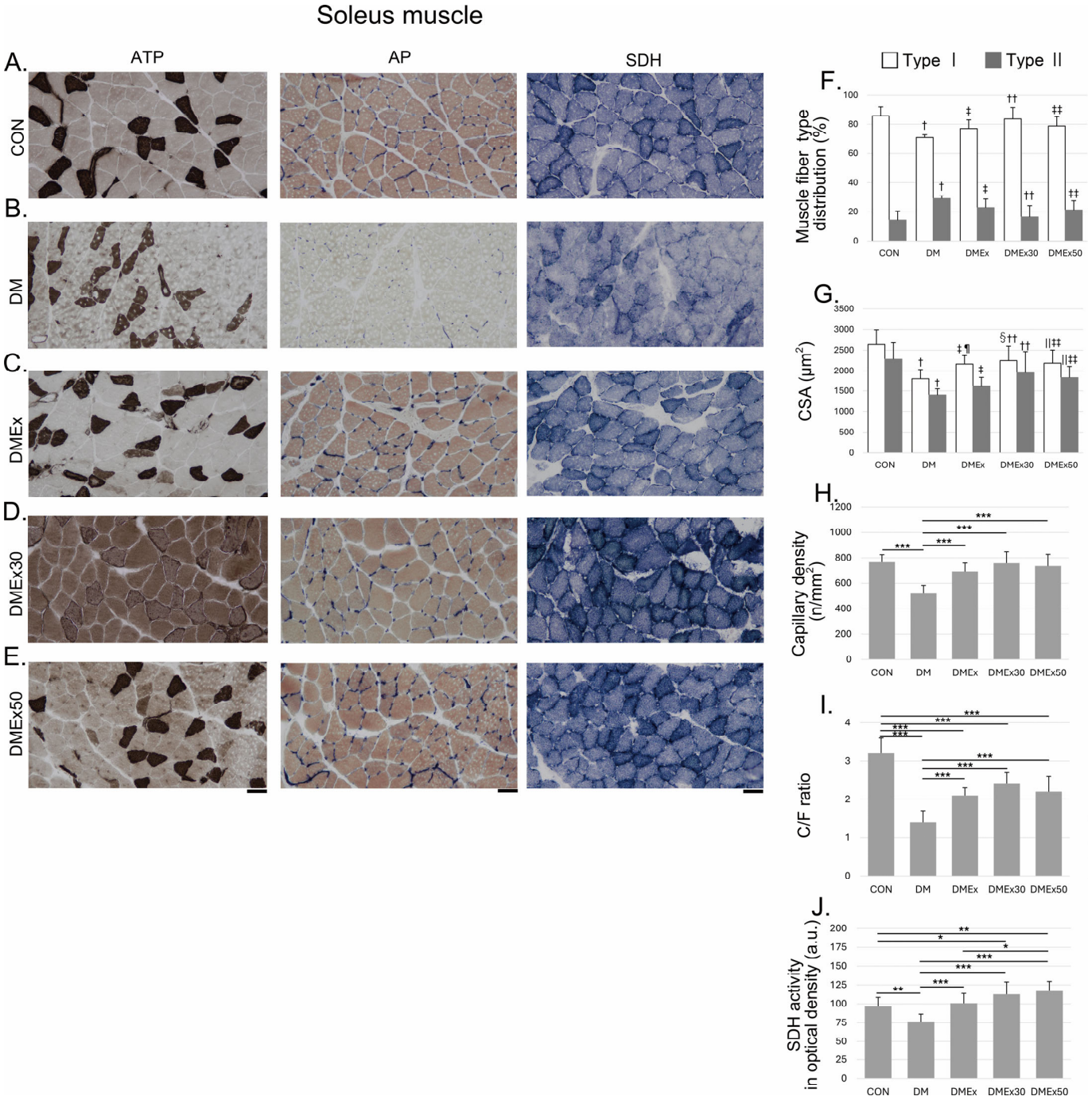

Figure 4. Histological analysis of the soleus muscle at the end of the experiment. Representative soleus muscle staining images for each group: (**A**) Control (CON), (**B**) Diabetes mellitus (DM), (**C**) DM + exercise (DMEx), (**D**) DM + exercise + 30% oxygen exposure (DMEx30), (**E**) DM + exercise + 50% oxygen exposure (DMEx50). ATP: Adenosine triphosphatase staining, AP: Alkaline phosphatase staining, SDH: Succinate dehydrogenase staining. (**F**) Muscle fiber type distribution, (**G**) Cross-sectional area, (**H**) Capillary density, (**I**) Capillary-to-fiber ratio (C/F ratio), (**J**) SDH activity in optical density. Symbols indicate significant differences between groups ($\alpha = 0.05$): †: CON vs. DM, ‡: CON vs. DMEx, §: CON vs. DMEx30, ||: CON vs. DMEx50, ¶: DM vs. DMEx, ††: DM vs. DMEx30, ‡‡: DM vs. DMEx50. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Scheffé's test for multiple comparisons). Scale bars = 100 μm.

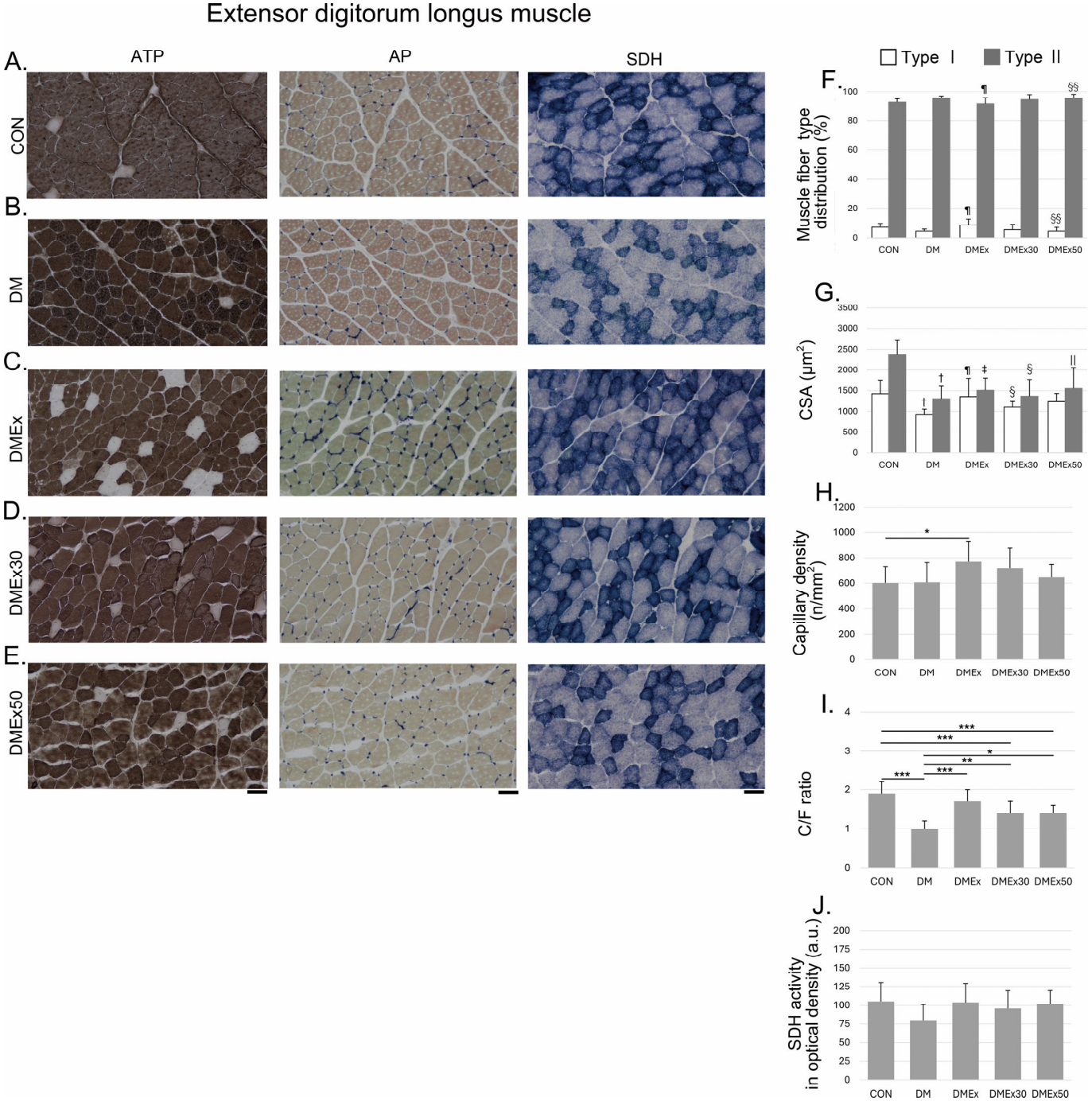

Figure 5. Histological analysis of the extensor digitorum longus muscle at the end of the experiment. Representative extensor digitorum longus muscle staining images for each group: (**A**) Control (CON), (**B**) Diabetes mellitus (DM), (**C**) DM + exercise (DMEx), (**D**) DM + exercise + 30% oxygen exposure (DMEx30), (**E**) DM + exercise + 50% oxygen exposure (DMEx50). ATP: Adenosine triphosphatase staining, AP: Alkaline phosphatase staining, SDH: Succinate dehydrogenase staining. (**F**) Muscle fiber type distribution, (**G**) Cross-sectional area, (**H**) Capillary density, (**I**) Capillary-to-fiber ratio (C/F ratio), (**J**) SDH activity in optical density. Symbols indicate significant differences between groups ($\alpha = 0.05$): †: CON vs. DM, ‡: CON vs. DMEx, §: CON vs. DMEx30, ||: CON vs. DMEx50, ¶: DM vs. DMEx, §§: DMEx vs. DMEx50. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Scheffé's test for multiple comparisons). Scale bars = 100 μm.

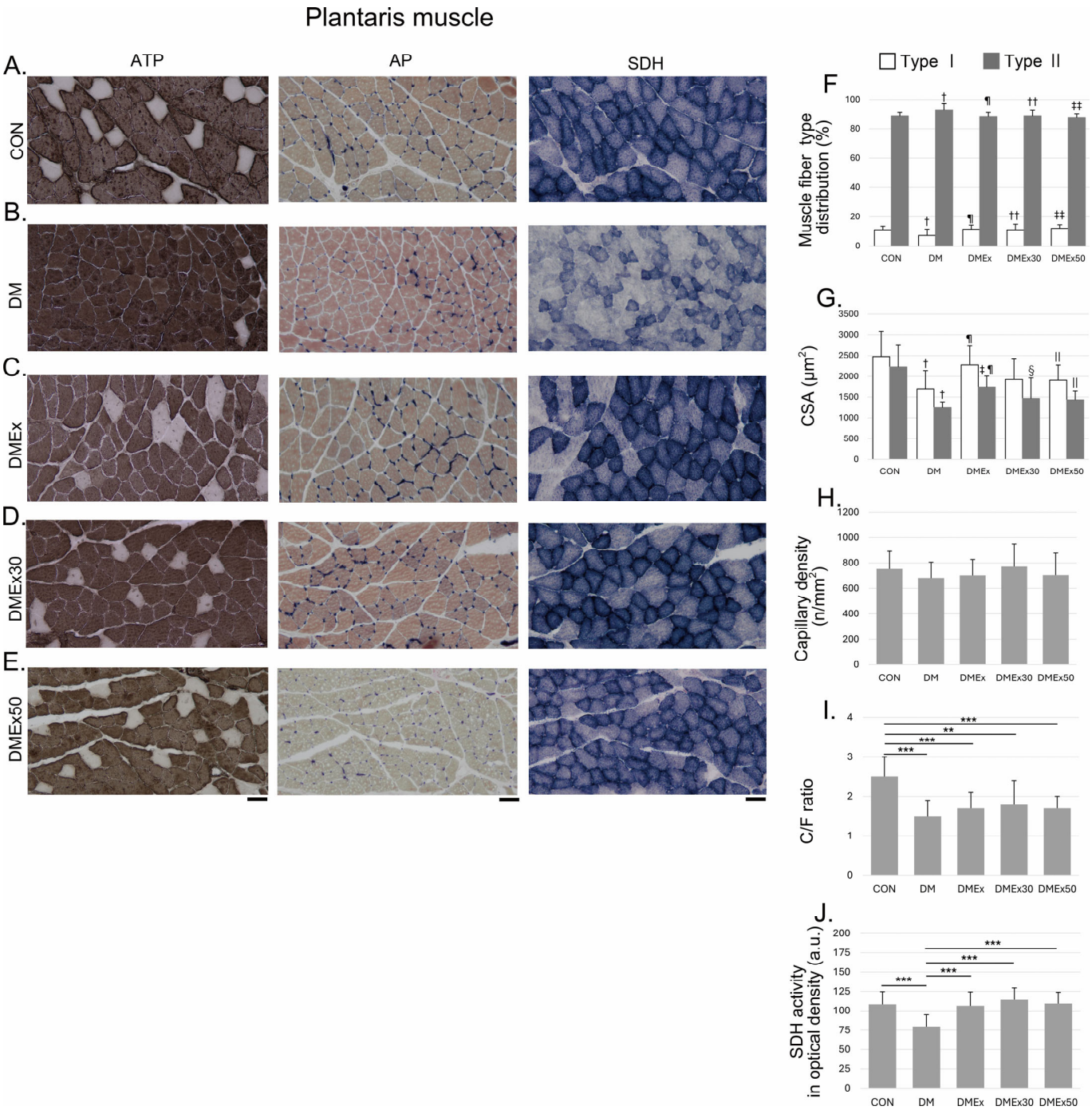

Figure 6. Histological analysis of the plantaris muscle at the end of the experiment. Representative plantaris muscle staining images for each group: (**A**) Control (CON), (**B**) Diabetes mellitus (DM), (**C**) DM + exercise (DMEx), (**D**) DM + exercise + 30% oxygen exposure (DMEx30), (**E**) DM + exercise + 50% oxygen exposure (DMEx50). ATP: Adenosine triphosphatase staining, AP: Alkaline phosphatase staining, SDH: Succinate dehydrogenase staining. (**F**) Muscle fiber type distribution, (**G**) Cross-sectional area, (**H**) Capillary density, (**I**) Capillary-to-fiber ratio (C/F ratio), (**J**) SDH activity in optical density. Symbols indicate significant differences between groups ($\alpha = 0.05$): †: CON vs. DM, ‡: CON vs. DMEx, §: CON vs. DMEx30, ‖: CON vs. DMEx50, ¶: DM vs. DMEx, ††: DM vs. DMEx30, ‡‡: DM vs. DMEx50. ** $p < 0.01$, *** $p < 0.001$ (Scheffé's test for multiple comparisons). Scale bars = 100 μm.

2.3.2. Skeletal Muscle Capillaries

In the soleus muscle, capillary density was significantly lower in the DM group than in the CON group by 32.2% ($p < 0.001$). In contrast, compared with the DM group, capillary density was significantly higher in the DMEx, DMEx30, and DMEx50 groups by 33.4%, 45.9%, and 41.6%, respectively (all $p < 0.001$) (Figure 4H). Regarding the C/F ratio, the DM group showed a significantly lower value than the CON group by 55.0% ($p < 0.001$). In contrast, compared with the DM group, the C/F ratio was significantly higher in the DMEx, DMEx30, and DMEx50 groups by 47.9%, 67.4%, and 54.0%, respectively (all $p < 0.001$) (Figure 4I).

In the extensor digitorum longus muscle, capillary density was significantly higher only in the DMEx group than in the CON group by 27.7% ($p < 0.05$) (Figure 5H). Regarding the C/F ratio, the DM group showed a significantly lower value than the CON group by 47.6% ($p < 0.001$). In contrast, compared with the DM group, the C/F ratio was significantly higher in the DMEx, DMEx30, and DMEx50 groups by 62.7% ($p < 0.001$), 36.6% ($p < 0.01$), and 35.9% ($p < 0.05$), respectively (Figure 5I).

In the plantaris muscle, no significant intergroup differences were observed in capillary density (Figure 6H). Regarding the C/F ratio, the DM group showed a significantly lower value than the CON group by 41.1% ($p < 0.001$). In contrast, no significant differences were observed between the DM group and the DMEx, DMEx30, or DMEx50 groups (Figure 6I).

2.3.3. Skeletal Muscle SDH Activity

In the soleus muscle, SDH activity was significantly lower in the DM group than in the CON group by 21.4% ($p < 0.001$). In contrast, compared with the DM group, SDH activity was significantly higher in the DMEx, DMEx30, and DMEx50 groups by 32.1%, 47.7%, and 53.4%, respectively (all $p < 0.001$) (Figure 4J).

In the extensor digitorum longus muscle, no significant intergroup differences were observed in SDH activity (Figure 5J).

In the plantaris muscle, SDH activity was significantly lower in the DM group than in the CON group by 26.3% ($p < 0.001$). In contrast, compared with the DM group, SDH activity was significantly higher in the DMEx, DMEx30, and DMEx50 groups by 33.3%, 43.3%, and 37.0%, respectively (all $p < 0.001$) (Figure 6J).

2.3.4. Summary of Histological Findings

In the predominantly slow-twitch soleus muscle, the proportion and cross-sectional area of type I fibers were preserved in all exercise groups compared with the DM group. In addition, both capillarization-related parameters and SDH activity were significantly improved. In contrast, these effects were limited in the predominantly fast-twitch extensor digitorum longus and plantaris muscles.

3. Discussion

This study investigated the biochemical and histological effects of combining normobaric hyperoxia exposure with aerobic exercise on skeletal muscle capillaries and mitochondrial function in diabetic rats. A novel aspect of the present study is that it extends our previous work on hyperoxia exposure alone by examining the combined effects of aerobic exercise and hyperoxia exposure in a diabetic rat model. The present findings suggest that this combined intervention may be associated not only with favorable histological adaptations in skeletal muscle, particularly in the soleus muscle, but also with improvements in lipid-related blood parameters, including LDL-C and TG. Moreover, the inclusion of circulating measurements of reactive oxygen species, antioxidant capacity, and IGF-1 may provide additional insight into the differential effects of oxygen concentration. Notably, 50% oxygen exposure was associated with a significant increase in IGF-1, although this was

accompanied by higher oxidative stress, whereas 30% oxygen exposure was associated with enhanced antioxidant capacity. Overall, the combined intervention may have improved skeletal muscle capillarization and SDH activity, and these effects appeared to reflect the characteristics of different muscle fiber types.

Exposure to normobaric hyperoxia increases both dissolved oxygen and hemoglobin-bound oxygen in the blood, thereby enhancing oxygen diffusion into the skeletal muscle [26,27]. Improvements in skeletal muscle capillarization and mitochondrial function are influenced not only by changes in oxygen availability but also by metabolic demand and increased peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) expression associated with exercise [24,28]. PGC-1α plays a role in regulating oxidative stress in skeletal muscle, and mild hyperbaric oxygen has been reported to increase Pgc-1α mRNA levels in skeletal muscle [14,29]. In the present study, the capillary density and C/F ratio improved in both the exercise-only and oxygen-combined exercise groups. These findings suggest that mechanical stimuli induced by aerobic exercise, together with altered oxygen availability under normobaric hyperoxia, may contribute to angiogenesis and mitochondrial functional adaptations in skeletal muscle.

Regarding the skeletal muscle fiber characteristics, the soleus muscle consists predominantly of oxidative muscle fibers, whereas the extensor digitorum longus and plantaris muscles are mainly composed of glycolytic fibers [30,31]. Capillary density and mitochondrial function are generally higher in oxidative fibers than in glycolytic fibers [32,33]. In the present study, compared with the DM group, all exercise groups maintained the proportion and cross-sectional area of type I fibers in the soleus muscle. In contrast, improvements in fiber cross-sectional area were limited in both type I and type II fibers in the extensor digitorum longus and plantaris muscles. Although the C/F ratio was improved in all examined skeletal muscles, capillary density showed the most marked improvement in the soleus muscle. In addition, SDH activity was also significantly improved in the soleus muscle. Taken together, these findings suggest that these interventions may exert greater effects in skeletal muscles predominantly composed of type I fibers, with responsiveness varying according to muscle fiber composition.

Analysis of lipid-related blood parameters showed significantly lower TC, LDL-C, and TG levels in all exercise groups compared with the DM group. Previous studies have reported that hyperoxia exposure can improve metabolic function and reduce TC and LDL-C levels [34]. Additionally, aerobic exercise has been shown to significantly decrease LDL-C and TG levels in diabetic rats through activation of the AMPK/Sirt1/PGC-1α signaling pathway in skeletal muscle [35,36]. The findings of the present study are consistent with those of our previous work [18]. Although oxygen exposure alone did not significantly reduce lipid-related blood parameters in the previous study, the present results suggest that the addition of aerobic exercise may have contributed to the lower levels observed in the exercise groups. These results suggest that the combination of normobaric hyperoxia and aerobic exercise may contribute to improvements in capillary density and SDH activity in skeletal muscle in DM, with possible implications for lipid metabolism.

With respect to the differences in oxygen concentrations, the present study examined exposures to 30% and 50% oxygen. The results revealed that the 30% oxygen exercise group exhibited a marked increase in antioxidant capacity and a relative reduction in oxidative stress. In contrast, the 50% oxygen exercise group demonstrated increased oxidative stress and a significant elevation in IGF-1, which plays an important role in skeletal muscle growth and remodeling. Moderate oxygen exposure has been reported to enhance the antioxidant capacity in tissues and cells [37,38], whereas higher oxygen concentrations have been associated with increased IGF-1 expression [39,40]. These findings suggest that different oxygen concentrations may exert distinct physiological effects, and that moderate

oxygen levels may be more appropriate from the perspective of oxidative stress regulation. Therefore, the oxygen concentration used during normobaric hyperoxia combined with aerobic exercise may be associated with differences in physiological outcomes.

This study has some limitations. First, only two oxygen concentrations (30% and 50%) were examined, and the effects of other oxygen concentrations remain unclear. Second, although aerobic exercise was combined with hyperoxia exposure in this study, the effects of other exercise modalities, such as resistance training, were not evaluated. Third, the present findings are based mainly on histological and circulating measurements, and therefore provide only indirect evidence regarding the biological processes underlying the observed adaptations. In particular, the molecular pathways involved in angiogenesis and mitochondrial functional improvement were not directly assessed. In addition, functional outcomes of skeletal muscle, such as muscle strength or exercise capacity, were not evaluated. Future studies are therefore required to examine the effects of different oxygen concentrations and exercise modalities, to clarify the underlying molecular mechanisms using molecular biological approaches, and to determine whether these structural and circulating changes are accompanied by functional improvements in skeletal muscle.

In conclusion, this study suggests that the combination of normobaric hyperoxia exposure and aerobic exercise may improve skeletal muscle capillary density and mitochondrial function in diabetic rats and that these effects are dependent on muscle fiber characteristics. In addition, oxidative stress, antioxidant capacity, and IGF-1 responses may differ according to oxygen concentration. These findings extend our previous observations regarding the effects of normobaric hyperoxia alone and suggest that this combined approach may contribute to the development of novel exercise-based interventions for DM.

4. Materials and Methods

4.1. Experimental Animals

Seven-week-old male Wistar rats (CLEA Japan Inc., Tokyo, Japan) were used in this study. To minimize potential allocation bias, the animals were randomly assigned by drawing lots to one of five groups before baseline measurements of body weight and blood glucose were obtained: CON (n = 5), DM (n = 5), DMEx (n = 5), DMEx30 (n = 5), and DMEx50 (n = 5). Experimental diabetes was induced by intraperitoneal injection of streptozotocin (STZ; FUJIFILM Wako Pure Chemical Co., Osaka, Japan) dissolved in 0.05 mol/L citrate buffer (pH 4.5) at a dose of 100 mg/kg. Rats with blood glucose levels $\geq$ 250 mg/dL were considered to have DM [41,42]. All animals were maintained under standardized environmental conditions during the experimental period, with a temperature of 22 $\pm$ 2 °C, humidity of 55 $\pm$ 5%, and a 12 h light/dark cycle. Rats were allowed free movement within their cages and had ad libitum access to standard laboratory chow (CE-2; CLEA Japan Inc.) and water. All experimental procedures were reviewed and approved by the Animal Care and Use Committee of the Aomori University of Health and Welfare and were performed in accordance with the institutional guidelines for animal experimentation (Approval No. 23002, approved on 2 June 2023).

4.2. Experimental Protocol

Body weight was measured at the beginning and end of the experiment using a digital scale (KJ-P11/P21; TANITA Co., Tokyo, Japan).

Previous studies have suggested that oxygen concentrations between 30% and 50% under normobaric conditions may promote angiogenesis and mitochondrial biogenesis [43,44]. Additionally, moderate oxygen exposure has been reported to enhance antioxidant capacity in tissues [37]. However, oxygen concentrations below 60% under normobaric conditions did not significantly affect lung injury or survival in experimental

animals [45]. Based on these findings, oxygen concentrations of 30% and 50% were selected for this study.

For normobaric hyperoxia exposure, animals were placed in a dedicated chamber connected to medical oxygen and nitrogen gas cylinders. The chamber was equipped with gas piping and a vent system for internal pressure adjustment. Oxygen concentration within the chamber was continuously monitored using an oxygen monitor (OM-25MP11; Taiei Engineering Co., Tokyo, Japan) and controlled using a gas regulator (N-SSJ-OX-E; Yamatosangyo Co., Osaka, Japan). Oxygen concentration was maintained at the target level throughout the exposure period. Hyperoxia exposure was initiated after the onset of diabetes and was performed once daily for 60 min at the same time each day (between 9:00 and 11:00 AM) for four weeks.

For the exercise intervention, rats in the exercise groups performed aerobic exercises using a motorized treadmill (MK-680; Muromachi Kikai Co., Ltd., Tokyo, Japan). The exercise protocol was designed based on previous studies [46–48] and consisted of progressive increases in running speed as follows: 15 m/min during week 1, 18 m/min during week 2, and 22 m/min during weeks 3 and 4. To reduce stress associated with treadmill running, the rats underwent an adaptation period of three days prior to the experimental protocol, during which they ran at a speed of 12 m/min for 20–40 min. All treadmill exercise sessions were performed at a 0° inclination. During the exercise, minimal electrical stimulation was applied to the tail or hindquarters only when necessary to encourage running without causing physical injury. Normobaric hyperoxia exposure and aerobic exercise were performed simultaneously in the DMEx30 and DMEx50 groups (Figure 7).

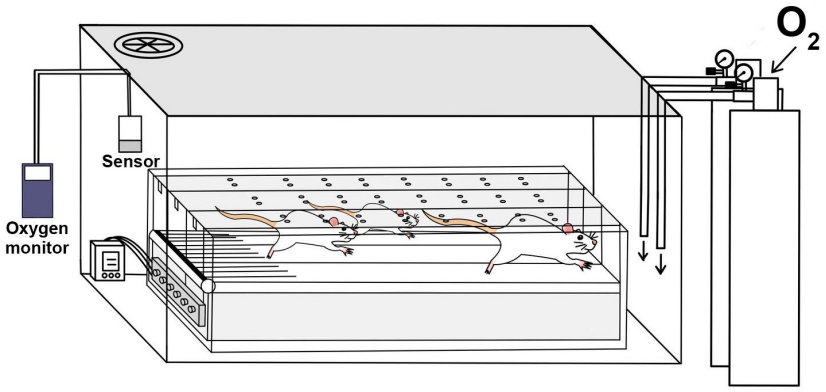

Figure 7. Combined aerobic exercise and normobaric oxygen exposure.

4.3. Biochemical Analysis

The blood samples were collected after a period of fasting. Blood glucose levels were measured at baseline (before the induction of diabetes) and at the end of the experiment. Approximately 50 µL of blood was collected from the tail vein and analyzed using a glucometer (OneTouch VerioVue; LifeScan IP Holdings LLC, Tokyo, Japan). Additional blood samples were collected from the tail veins at the end of the experiment. Whole blood was centrifuged at 3000 rpm for 15 min to obtain serum. TC, HDL-C, LDL-C, and TG levels were measured using an automated biochemical analyzer (BioMajesty JCA-BM8060; JEOL Ltd., Tokyo, Japan).

Oxidative stress markers were assessed using the REDOXLIBRA system (Wismerll Co., Tokyo, Japan), which measured d-ROMs and BAP. d-ROM values are expressed in Carratelli units (CARR U), where 1 CARR U corresponds to 0.08 mg hydrogen peroxide/dL [49]. BAP values were expressed in µmol/L [49]. The oxidative stress index was calculated as (d-ROM/BAP × 100).

Serum IGF-1 concentrations were measured using a commercial ELISA kit (IGF-1 ELISA Kit, KE10032, 96 assays; Cosmo Bio Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions.

4.4. Histological Analysis

At the end of the experiment, deep anesthesia was induced in all animals using an intraperitoneally injected anesthetic mixture composed of medetomidine hydrochloride (1.875 mL), midazolam (2.0 mL), butorphanol tartrate (2.5 mL), and physiological saline (18.63 mL). The solution was administered at 0.5 mL per 100 g of body weight. After confirmation of deep anesthesia, a thoracotomy was performed, and perfusion fixation with physiological saline was performed via insertion of a needle into the left ventricle to preserve tissue morphology. The skeletal muscles were then rapidly excised. The soleus muscle (predominantly composed of slow-twitch fibers), extensor digitorum longus, and plantaris muscles (predominantly composed of fast-twitch fibers) were analyzed. Transverse muscle sections perpendicular to the longitudinal axis of muscle fibers were prepared using a cryostat (cryo-star NX70; PHC Corporation, Tokyo, Japan) at −20 °C. Tissue samples were collected from three regions of each specimen. The section thickness was 10 μm for ATPase staining and 12 μm for alkaline phosphatase (AP) and SDH staining.

4.4.1. ATPase Staining

ATPase staining was performed to evaluate skeletal muscle fiber type distribution and cross-sectional area [50,51]. The sections were pretreated in a solution containing 0.1 M hydrochloric acid and barbiturate acetate for 10 min. They were then incubated for 30 s in a solution containing 0.1 M sodium barbiturate and 0.18 M calcium chloride. Subsequently, the sections were incubated for 45 min in a solution containing adenosine 5′-triphosphate dissolved in 0.1 M sodium barbiturate and 0.18 M calcium chloride. Following incubation, the sections were washed three times in 1% calcium chloride, treated with 2% cobalt chloride for 3 min, rinsed eight times with 0.01 M sodium barbiturate solution, and subsequently exposed to 1% ammonium solution for 1 min. After staining, the sections were dehydrated in alcohol and xylene and mounted using a xylene-based medium. The cross-sectional area of muscle fibers was measured with image analysis software (WinROOF 2021; Mitani Corporation, Tokyo, Japan). More than 100 muscle fibers were analyzed from three microscopic images per specimen. The proportions of type I and type II fibers were determined by macroscopic observation.

4.4.2. AP Staining

AP staining was performed to evaluate skeletal muscle capillaries [24,52]. The sections were prefixed in a formalin–acetone solution and subsequently incubated for 60 min at 37 °C in a reaction solution consisting of 0.02% 5-bromo-4-chloro-3-indolyl phosphate toluidine salt and 0.1% nitro blue tetrazolium in 0.2 M borate buffer. After incubation, the sections were post-fixed with 4% paraformaldehyde, dehydrated with alcohol and xylene, and mounted. The stained sections were photographed using a light microscope (BX53; Olympus, Tokyo, Japan) equipped with a digital camera (DP-28; Olympus). The illumination conditions were kept constant during image acquisition. Because the capillary distribution may vary according to the muscle fiber type and location within the muscle, image analysis was standardized for the muscle belly and deep regions. Capillary and muscle fiber numbers were counted from microscopic images (100× magnification) using the binarization function of WinROOF2021 software. The C/F ratio was calculated as follows: (number of capillary-to-number muscle fibers) × 100. Capillary density was defined as the number of capillaries per field area (1.0739976 mm^2).

4.4.3. SDH Staining

SDH staining was performed to evaluate the mitochondrial oxidative enzyme activity [53,54]. The sections were incubated for 4 h at 37 °C in 0.2 M phosphate buffer containing nitro blue tetrazolium and 0.2 M sodium succinate. Following staining, the sections were imaged using a light microscope (BX53; Olympus, Tokyo, Japan) equipped with a digital camera (DP-28; Olympus) under constant illumination settings. Image analysis was performed on the same regions used for AP staining (muscle belly and deep regions). SDH activity was quantified as the optical density using image analysis software (WinROOF2021; Mitani Corporation, Japan). The images were converted to grayscale and evaluated on a scale of 0–255. Because higher grayscale values represent greater brightness, grayscale inversion was performed to accurately reflect SDH staining intensity, and the mean inverted grayscale value was used as the SDH activity index.

4.5. Statistical Analysis

Statistical analyses were conducted using R version 4.3.0. Body weight and blood glucose levels at the beginning and end of the experiment were compared using paired *t*-tests. One-way analysis of variance, followed by Scheffé's post hoc test, was used to compare all measured variables among the groups. Statistical significance was defined as $p < 0.05$.

Author Contributions: I.S., Y.K. and S.L. conceived the experiment. I.S., Y.K., Y.U., H.I., S.O. and S.L. performed the experiments. I.S., T.S., I.K., T.N. and S.L. analysed the data. I.S., Y.K. and A.I. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this article.

Conflicts of Interest: The authors reported no potential conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AP	Alkaline phosphatase
ATPase	Adenosine triphosphatase
BAP	Biological antioxidant potential
C/F ratio	Capillary-to-fiber ratio
d-ROMs	Derivatives of reactive oxygen metabolites
HDL-C	High-density lipoprotein cholesterol
IGF-1	Insulin-like growth factor-1
LDL-C	Low-density lipoprotein cholesterol
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
SDH	Succinate dehydrogenase
TC	Total cholesterol
TG	Triglyceride

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