

RESEARCH ARTICLE

Ketogenic diet with medium-chain triglycerides restores skeletal muscle function and pathology in a rat model of Duchenne muscular dystrophy

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Abstract

Duchenne muscular dystrophy (DMD) is an intractable genetic disease associated with progressive skeletal muscle weakness and degeneration. Recently, it was reported that intraperitoneal injections of ketone bodies partially ameliorated muscular dystrophy by increasing satellite cell (SC) proliferation. Here, we evaluated whether a ketogenic diet (KD) with medium-chain triglycerides (MCT-KD) could alter genetically mutated DMD in model rats. We found that the MCT-KD significantly increased muscle strength and fiber diameter in these rats. The MCT-KD significantly suppressed the key features of DMD, namely, muscle necrosis, inflammation, and subsequent fibrosis. Immunocytochemical analysis revealed that the MCT-KD promoted the proliferation of muscle SCs, suggesting enhanced muscle regeneration. The muscle strength of DMD model rats fed with MCT-KD was significantly improved even at the age of 9 months. Our findings suggested that the MCT-KD ameliorates muscular dystrophy by inhibiting myonecrosis and promoting the proliferation of muscle SCs. As far as we can ascertain, this is the first study to apply a functional diet as therapy for DMD in experimental animals. Further studies are needed to elucidate the underlying mechanisms of the MCT-KD-induced improvement of DMD.

Abbreviations: BW, body weight; CK, creatine kinase; DGC, dystrophin-glycoprotein complex; DMD, Duchenne muscular dystrophy; eWAT, epididymal white adipose tissues; KD, ketogenic diet; KR, ketogenic ratio; LCT, long-chain triglyceride; MCT, medium-chain triglyceride; MCT-KD, ketogenic diet with medium-chain triglyceride; ND, normal diet; SC, satellite cell; TA, tibialis anterior.

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KEYWORDS

Duchenne muscular dystrophy, ketogenic diet, ketone bodies, nutrition therapy, skeletal muscle

1 | INTRODUCTION

Duchenne muscular dystrophy (DMD) causes progressive muscle weakness and atrophy due to mutations in the DMD gene encoding dystrophin, a component of the dystrophin-glycoprotein complex (DGC) that connects the cytoskeleton to the basal lamina.¹ Defects in DGC lead to fragile cell membranes, causing an influx of extracellular fluid and components, especially calcium. Abnormal calcium levels in myofibers cause muscle contraction² and increased proteolysis, resulting in persistent degeneration and muscle cell destruction. Continued muscle degeneration/necrosis leads to chronic inflammation, and the replacement of muscle with fibrous and adipose tissues causes progressive muscle weakness.³ Patients with DMD usually require a wheelchair by the ages of 8–14 years and succumb to respiratory and heart failure.⁴ The main genetic and pharmaceutical approaches to DMD are respectively aimed at increasing dystrophin expression, promoting muscle regeneration, protection, and mass, and activating dystrophin compensatory mechanisms. Genetic therapies such as exon skipping have recently been investigated. However, the delivery, effects and the safety of genetic therapies are suboptimal, indicating a need for other mono- or combination therapies.⁵

Skeletal muscle regeneration depends on the properties of satellite cells (SCs), which are adult stem cells of skeletal muscle, and their environment (stem cell niche).^{6,7} SCs reside between the basal lamina and muscle membrane and are quiescent in normal adult muscle, but damaged muscle fibers activate SC proliferation. SCs are constantly activated in DMD due to chronic muscle degeneration, leading to depletion of the stem cell pool of SCs and consequently impaired muscle regeneration.⁸ SCs are therefore an important target of DMD treatment. However, despite advances in cell transplantation therapy, this approach is still under development due to problems associated with the effects of transplanted cells, their engraftment in muscle, and the need for systemic injection.^{9,10} Therefore, therapies using regulators are needed that can control SC functions such as activation, proliferation, differentiation, and self-renewal.^{11,12}

High-fat, low-carbohydrate, moderate-protein ketogenic diets (KDs) promote the production of acetoacetate, beta-hydroxybutyrate, and acetone (ketone bodies) by shifting energy production from glucose to ketone bodies.¹³ Ketone bodies that are produced in the liver are released into the bloodstream, and serve as peripheral

energy sources for various organs, particularly the skeletal muscle and brain, under conditions of starvation and limited carbohydrate availability (including that imposed by a KD), as well as the neonatal period.¹⁴ KDs have proven effective for treating refractory epilepsy, and growing evidence supports their beneficial effects in diet-sensitive disorders such as Alzheimer disease,¹⁵ cancer,¹⁶ and diabetes.¹⁷ Ketone bodies can enhance muscle regeneration by inducing the proliferation of muscle SCs, and daily intraperitoneal injections of ketone bodies partially ameliorate symptoms in mouse models of DMD (mdx mice).¹⁸ Therefore, a KD that promotes ketone body production should improve muscle regenerative capacity. However, treating skeletal muscle diseases with a KD has the disadvantage of causing muscle atrophy.^{19,20} This might be associated with the “classic” or “standard” KD usually requiring a 3:1 to 4:1 weight ratio of fat to non-fat macronutrients, and extremely low amounts of carbohydrates and protein to increase blood ketone levels. Carbohydrates inhibit the activity of enzymes that degrade amino acids in the liver, resulting in a protein sparing effect,²¹ whereas extreme low-carbohydrate diets eliminate this effect and cause muscle catabolism.²² In addition, a decrease in protein intake beyond the appropriate range leads to a loss of muscle mass and strength.²³ A medium-chain triglyceride (MCT) KD was developed during the 1970s as an alternative to the classical diet.²⁴ Unlike long-chain triglycerides (LCTs), MCTs do not require micelle formation with bile acids, are readily absorbed into capillaries in the intestinal tract, flow directly into the portal vein without forming chylomicrons, and are rapidly converted to ketone bodies in the liver. Therefore, a KD containing MCT (MCT-KD) seems to contain sufficient protein and carbohydrates to prevent muscle catabolism, because the MCTs increase blood ketone levels more efficiently than LCTs.¹³

We speculated that an MCT-KD would improve the DMD phenotype by inducing the activation of muscle SCs. We therefore fed an MCT-KD²⁴ to DMD model rats,²⁵ which are prevalent animal models of DMD. Unlike mdx mice, DMD rats develop severe disease phenotypes such as skeletal muscle fibrosis and adipogenesis^{26,27} that are representative of late stage DMD in humans. Therefore, the effects of MCT-KD on late stage DMD pathology can be investigated in these rats.

We found that the MCT-KD led to increased blood ketone levels, improved muscle function, and improved pathology and the characteristic parameters of necrosis, inflammation, and fibrosis in a rat model of DMD. These

findings showed that the MCT-KD could be a new avenue for preventing or treating DMD.

2 | MATERIALS AND METHODS

2.1 | Animals and diets

All animal experiments conducted herein were approved by the Institutional Animal Care and Use Committees at the National Institute of Advanced Industrial Science and Technology (AIST) (Permission No: 2020-358) and the University of Tokyo (Permission No: P20-012) and complied with the guidelines for animal experiments published by AIST and the University of Tokyo.

Established rat models of DMD²⁵ were maintained by crossing female rats with a heterozygous mutation in the *Dmd* gene ($X^{Dmd}X$), with wild-type (WT) male rats. We randomly assigned newborn DMD male rats and their WT littermates to receive a normal (ND), or an MCT-KD diet (Tables 1 and 2) from 3 weeks of age for 32 weeks. The ND group was pair-fed with the MCT-KD based on daily caloric intake in a short-term experiment (individually caged) (Figures 1–4), and the others were fed with either the ND or MCT-KD ad

TABLE 1 Diet composition

Ingredients (g/kg)	AIN 93M	MCT-KD (1.95)	MCT-KD (1.43)
Casein	140	125.4	119.2
L-Cystine	1.8	1.6	1.5
Corn starch	495.7	127	120.7
Maltodextrin 10	125	0	0
Sucrose	106.7	15.6	15.1
Cellulose, BW200	50	0	0
Soybean oil	40	10	9.5
Lard	0	164.7	156.4
t-Butylhydroquinone	0.008	0.017	0.017
Mineral mix S10022C	3.5	5.6	5.3
Calcium carbonate	12.5	17.3	16.4
Potassium citrate, 1 H ₂ O	0.98	1.6	1.5
Potassium, phosphate, monobasic	8.8	14	13.3
Calcium phosphate, dibasic	0	3.5	3.3
Sodium chloride	2.6	3	2.9
Vitamin mix V10037	10	15.9	15.1
Choline bitartrate	2.5	4.0	3.8
Quest nutrition MCT oil (powder form)	0	490.6	423.1
Casein (Bulk sports)	0	0	93.1

TABLE 2 Ratios of energy sources in experimental diets

	AIN 93M	MCT-KD (1.95)	MCT-KD (1.43)
	3.88 (kcal/g)	6.16 (kcal/g)	5.85 (kcal/g)
Protein (% g)	12.36	11.10	18.06
Carbohydrate (% g)	74.70	17.47	16.59
Fat (% g)	4.00	55.58	49.44
Protein (% kcal)	13.0	7.3	12.5
Carbohydrate (% kcal)	77.5	10.4	10.4
Fat (% kcal)	9.5	82.3	77.2
Ketogenic ratio ^a	0.05	1.95	1.43

^a Defined as ratio of fat (g) to carbohydrate plus protein (g).

libitum in a long-term experiment (2–4 rats/cage) (Figure 5). The MCT-KD had a ratio of fat weight to carbohydrate plus protein weight (ketogenic ratio; KR) of 1.95 for the first 10 days followed by 1.43 for the remainder of the rearing period. The rats were maintained at 23°C under a 12-h light/dark cycle (lights on at 8:00), and water was supplied ad libitum. Food intake was measured daily, and the rats were weighed weekly. The caloric contents were 387.7, 615.6, and 584.8 kcal/100 g for ND, and MCT-KD with KRs of 1.95, and 1.43, respectively. The ratios (%) of calories derived from fat, carbohydrate, and protein were 9.5%, 77.5%, and 13.0%, respectively, for ND, 82.3%, 10.4%, and 7.3%, respectively, for MCT-KD with a KR of 1.95, and 77.15%, 10.4%, and 12.45%, respectively, for MCT-KD with a KR of 1.43. The DMD rats fed with ND or MCT-KD were fasted for 12 h from 20:00 followed by feeding for 60 min at the age of 10 weeks. The tibialis anterior (TA), soleus muscles and epididymal white adipose tissues (eWAT) dissected from sacrificed rats were weighed, and the TA was frozen in liquid nitrogen.

2.2 | Measurement of ketone bodies and glucose in plasma

Blood was sampled from rat tail veins, then blood ketone bodies (β -hydroxybutyrate and acetoacetate) and glucose concentrations were measured using Precision Xceed kits (Abbott Laboratories, Chicago, IL, USA).

2.3 | Grip test

Forelimb muscle strength was measured as grip strength²⁷ using a dynamometer (GPM-101B; Melquest, Toyama, Japan). The rats grasped a T-shaped rod connected to the dynamometer, their tails were pulled backwards, then

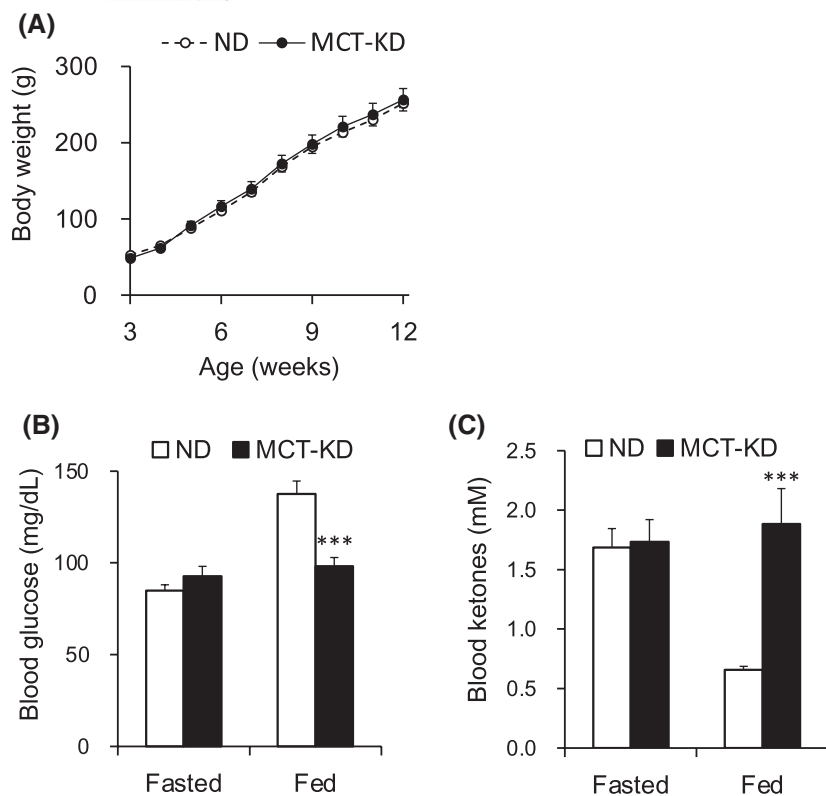


FIGURE 1 Body weight, blood glucose and ketones in DMD rats. (A) Changes in weight of DMD rats fed with ND and MCT-KD. (B, C) Fasting and postprandial (B) blood glucose and (C) blood total ketone levels after 7 weeks of feeding with ND or MCT-KD. Data are expressed as means $\pm$ SEM and were compared by two-way ANOVA followed by Tukey–Kramer multiple comparisons tests (ND: $n = 7$, MCT-KD: $n = 6$). Results of statistical comparisons between genotypes at indicated ages and conditions. * $p < .05$; ** $p < .01$; *** $p < .001$

maximum resistance (muscle strength) was averaged from 10 consecutive measurements.

2.4 | Histological and immunohistochemical analyses

Cryosections (7 μ m thick) of tibialis anterior muscle were fixed in 4% paraformaldehyde for 15 min at room temperature then stained with hematoxylin and eosin (HE) and Masson trichrome, and immunostained as described below.

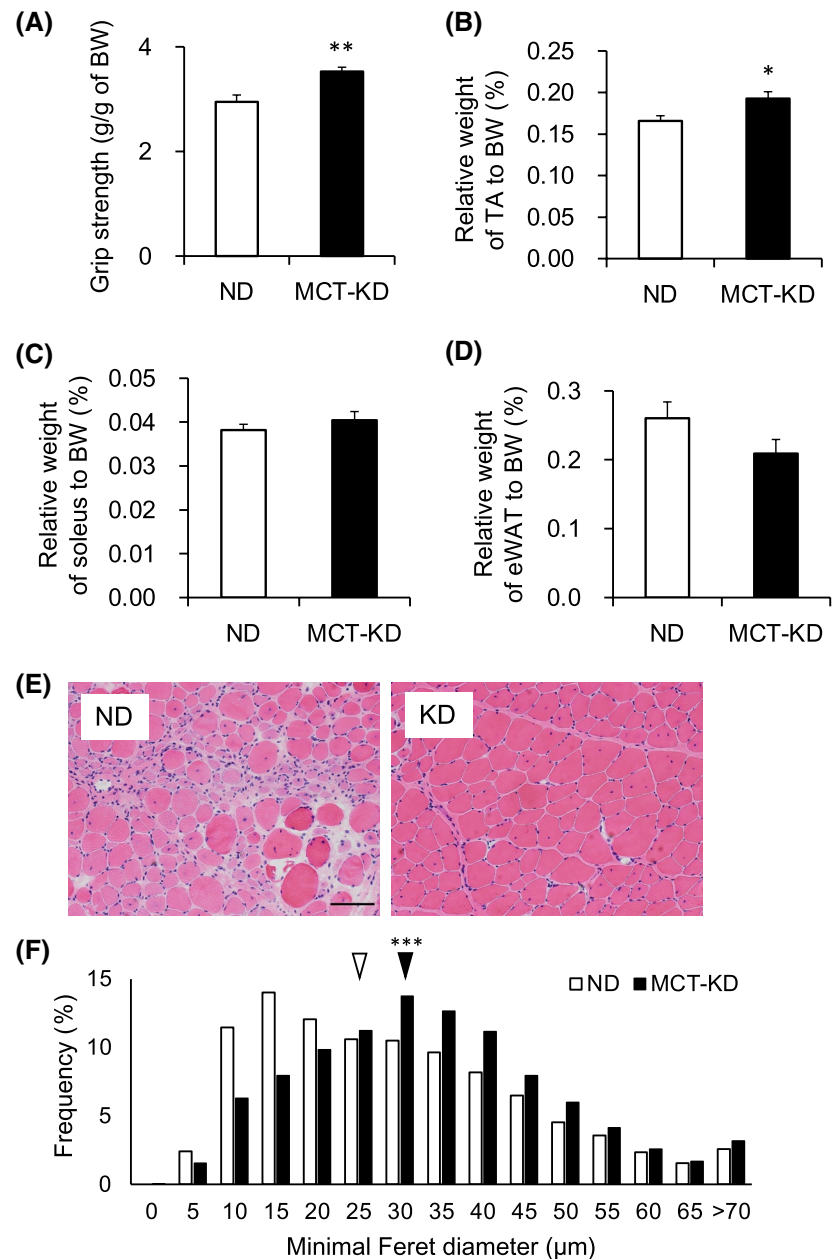
Albumin, CD11b and eMHC immunostaining: Nonspecific protein binding was blocked with 5% goat serum in phosphate-buffered saline (PBS) containing 0.1% Triton (blocking buffer) and 0.6% H_2O_2 to quench endoperoxide activity. The sections were incubated with the primary antibodies (listed below) for 2 h at room temperature, washed in PBS, and incubated with Histofine Simple stain Rat MAX-PO (Nichirei Bioscience, Tokyo, Japan) for 1 h at room temperature. After the 3,3-diaminobenzidine (DAB) reaction, nuclei were counterstained with hematoxylin. All samples were visualized using a BX51 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a DP73 digital camera (Olympus).

IgG immunostaining: Nonspecific protein binding was blocked with 5% fetal bovine serum in phosphate-buffered saline (PBS) containing 0.1% Triton (blocking buffer).

The sections were incubated with laminin primary antibody (listed below) for 2 h at room temperature, washed in PBS, the incubated with 1:100-diluted AlexaFluor-conjugated secondary goat anti-rabbit IgG (H + L) F(ab')₂ Fragment Alexa Fluor 488 (#4412; Cell signaling technology, MT, USA) and goat anti-rat IgG H&L Alexa Fluor 647 (ab150159; Abcam, Cambridge, UK) antibodies for 1 h at room temperature. Nuclei were counterstained with Hoechst 33258. All samples were visualized using a BZ-X810 all-in-one fluorescence microscope (Keyence, Tokyo, Japan). Whole tibialis anterior muscle sections were photographed, and myonecrosis was quantified as ratios (%) of IgG-positive to total fibers counted blindly in photographs.

Pax7, MyoD and Ki67 immunostaining: Nonspecific protein binding was blocked with 5% donkey serum in phosphate-buffered saline (PBS) containing 0.1% Triton (blocking buffer). The sections were incubated with primary antibodies (listed below) overnight at 4°C, washed in PBS, followed by 1:400-diluted AlexaFluor-conjugated secondary donkey anti-mouse IgG (H + L) Alexa Fluor 594 (1:400; Jackson Immuno Research Laboratories, West Grove, PA, USA) and donkey anti-rabbit IgG (H + L) Alexa Fluor 488 (1:400; Jackson Immuno Research Laboratories) antibodies for 1 h at room temperature. Nuclei were counterstained with Hoechst 33258. All samples were visualized using the BZ-X810 all-in-one fluorescence microscope. The Pax7⁺, MyoD⁺ and Ki67⁺

FIGURE 2 Grip strength, muscle weight and fiber diameter in DMD rats. (A) Muscle strength (grip test) of DMD rats after 9 weeks of ND or MCT-KD feeding. (B–D) Weight of tibialis anterior (TA) muscles (B), soleus muscles (C), and epididymal white adipose tissue (eWAT; D) relative to body weight of DMD rats after 9 weeks of feeding with ND or MCT-KD. (E) Representative HE-stained TA muscle sections from DMD rats after 9 weeks of ND or MCT-KD feeding. Scale bar = 100 μ m. (F) Distribution of minimal Feret diameter of 11474 and 8974 TA muscle fibers from DMD rats after 9 weeks of ND or MCT-KD feeding, respectively. Arrowheads, median values. (A–D) Results are expressed as means $\pm$ SEM and were compared by unpaired Student *t* tests. (ND, *n* = 7; MCT-KD, *n* = 6) (F) Data were compared by Wilcoxon rank sum tests. **p* < .05; ***p* < .01; ****p* < .001



cells were counted blindly in 5 randomly selected fields at 10 $\times$ the objective of a fluorescence microscope (Keyence, Tokyo, Japan).

The following primary antibodies were obtained from the respective suppliers: anti-albumin (1:100, mouse, clone R-Alb 214A-1; Takara Bio Inc, Otsu, Japan), anti-CD11b (1:100, mouse, clone OX-42; BioLegend, San Diego, CA, USA), anti-eMHC (1:100, mouse, clone F1.652; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-laminin (1:100, rabbit, L9393; Sigma-Aldrich Corp., St. Louis, MO, USA), anti-Pax7 (1:200, mouse, clone P3U1; Developmental Studies Hybridoma Bank), anti-MyoD (1:200, mouse, clone 5.8A; Novocastra, Newcastle upon Tyne, UK), anti-laminin (1:100, rabbit, L9393; Sigma-Aldrich Corp., St. Louis, MO, USA), and anti-Ki67 (1:200, rabbit, ab16667; Abcam, Cambridge, UK).

Myofiber size and Masson trichrome positive areas were quantified as described.²⁷

2.5 | FACS analysis

Immune cell infiltration in the excised quadriceps and gastrocnemius muscles of euthanized rats fed with the KD and ND diets was evaluated using a FACSVerse™ Flow Cytometer (BD Biosciences, San Jose, CA, USA). Quadriceps and gastrocnemius muscles were minced and digested for 45 min in collagenase II (0.63 mg/ml, Sigma-Aldrich Corp.), Dispase (0.32 mg/ml, Sigma-Aldrich Corp.) and DNase I (5 mg/ml, Sigma) at 37°C. Digested muscle was filtered and washed. The cells were resuspended in 40% Percoll, underlain with 80% Percoll,

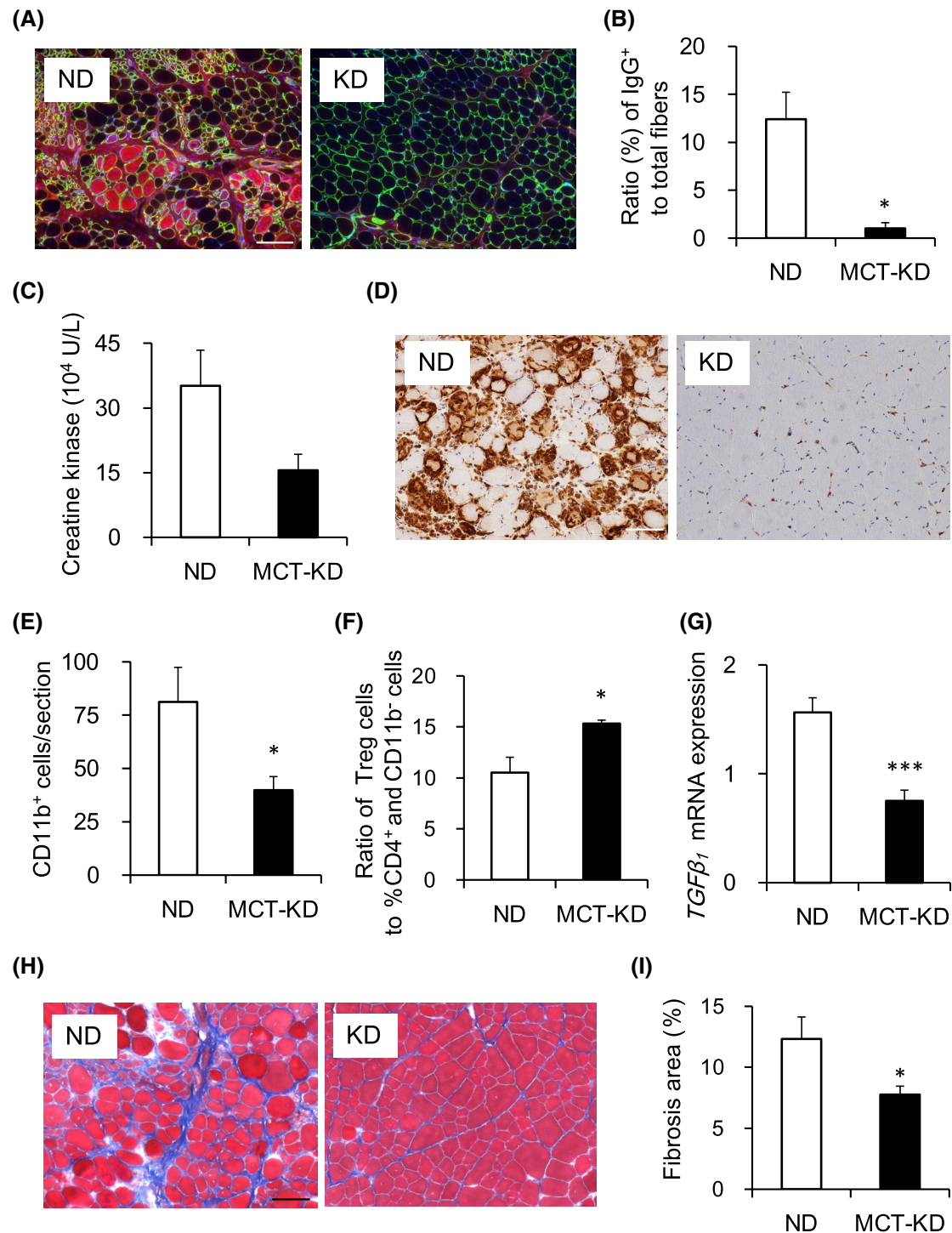


FIGURE 3 Necrosis, inflammation, and fibrosis of skeletal muscle in DMD rats. (A, B) Representative fluorescent microscopy images of TA muscle sections (A) and numbers of IgG-positive fibers per total fibers in TA muscle sections stained with IgG (red), laminin (green) and Hoechst (blue) (B). Scale bar, 200 μ m. (C) Basal serum creatine kinase levels of DMD rats fed with ND or MCT-KD for 9 weeks. (D) Immunohistochemical analysis of CD11b in TA muscle sections and (E) ratios of CD11b-positive fibers per total fibers in TA muscle sections from DMD rats fed with ND or MCT-KD for 9 weeks. Scale bar, 100 μ m. (F) Numbers of Tregs in quadriceps and gastrocnemius muscles determined by flow cytometry in DMD rats fed with ND or MCT-KD for 9 weeks. Expression of CD8 (cytotoxic T cell marker), CD11b (marker of monocytes, macrophages, NK cells, and granulocytes), CD4 (marker of helper T cells), and CD25 and Foxp3 (regulatory T cell markers). (G) Relative mRNA levels of *TGF- β_1* in DMD rats fed with ND or MCT-KD for 9 weeks (normalized by *Hprt*). (H) Masson trichrome-stained TA muscle sections and (I) positive areas per total area of TA muscle sections from DMD rats fed with ND or MCT-KD for 9 weeks. Scale bar, 100 μ m. (B, C, E–G, I) Data are expressed as means $\pm$ SEM; ND, $n = 7$; MCT-KD, $n = 6$. * $p < .05$; ** $p < .01$; *** $p < .001$ (unpaired Student t tests)

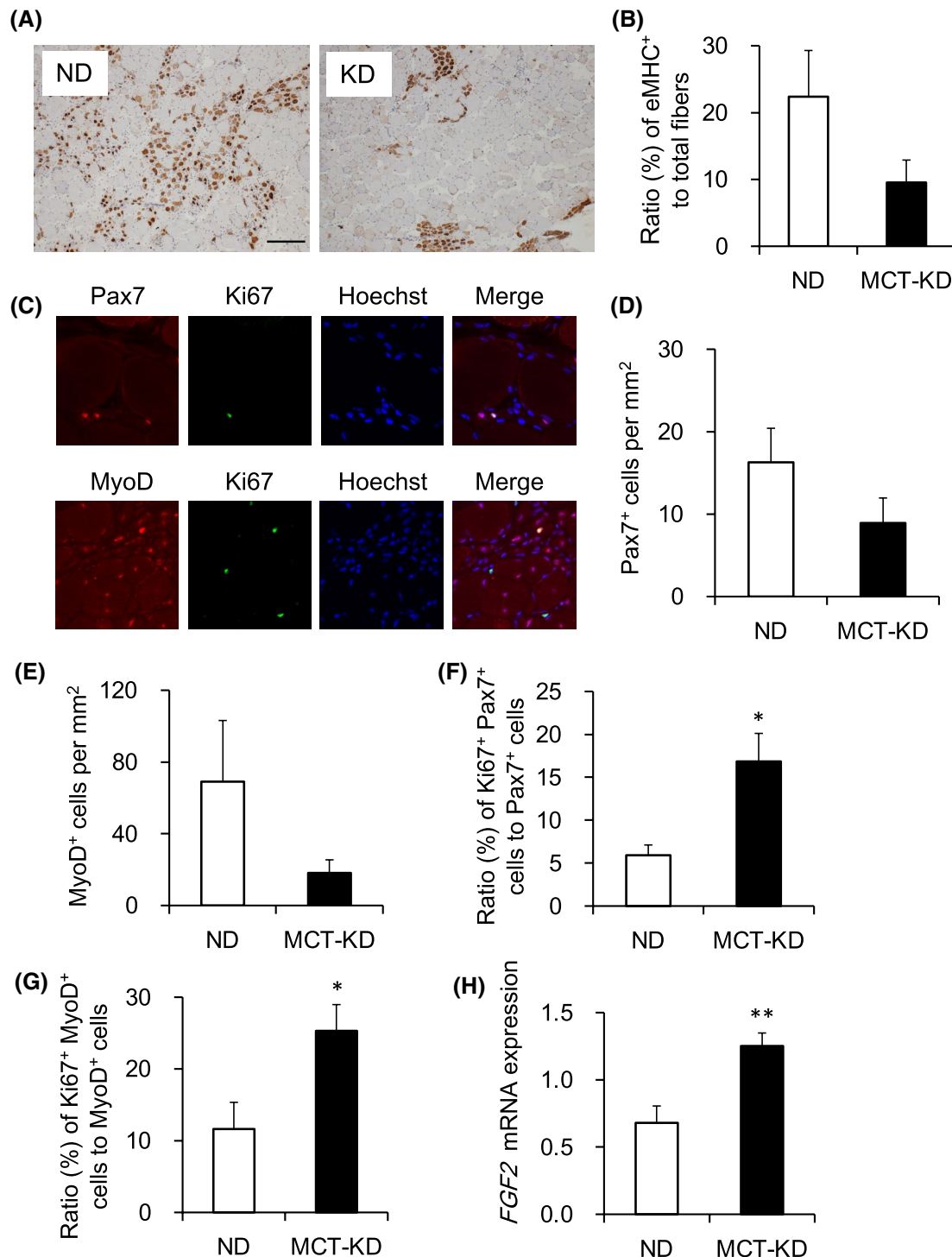


FIGURE 4 Parameters associated with regenerative capacity in DMD rats. (A) Immunohistochemical findings of eMHC in TA muscle sections and (B) eMHC positive fibers per total fibers in TA muscle sections from DMD rats fed with ND or MCT-KD for 9 weeks. Scale bar, 40 μ m. (C–G) Immunohistochemical analysis of Pax7, MyoD and Ki67 in TA muscle sections. Representative images of Pax7, MyoD and Ki67 positive cells (C). Quantitation of Pax7⁺ (D) and MyoD⁺ (E) cells in sections. Ratios (%) of Ki67⁺ cells among Pax7⁺ cells (F) and MyoD⁺ cells (G). (H) Relative mRNA levels of *FGF2* in TA muscles of DMD rats fed with ND or MCT-KD for 9 weeks (normalized by *Hprt*). (B, E, F) Data are expressed as means $\pm$ SEM (ND, $n = 7$; MCT-KD, $n = 6$) * $p < .05$; ** $p < .01$; *** $p < .001$ (unpaired Student t tests)

and spun for 20 min at 2200 rpm to separate the leukocyte fraction. The interphase containing leukocytes was collected, washed, and stained for analysis or sorting by flow cytometry. Cell suspensions were multiplex-labeled with CD8, PE; CD11b, PECy7; CD4, APC-Cy7

(1:100, BioLegend); Foxp3, APC-Cy7 (1:100, eBioscience Inc, San Diego, CA, USA) and CD25, FITC (1:100, BD Biosciences). Dead cells were stained using Invitrogen LIVE/DEADTM Fixable Red Dead Cell Stain Kit (Thermo Fisher Scientific Inc).

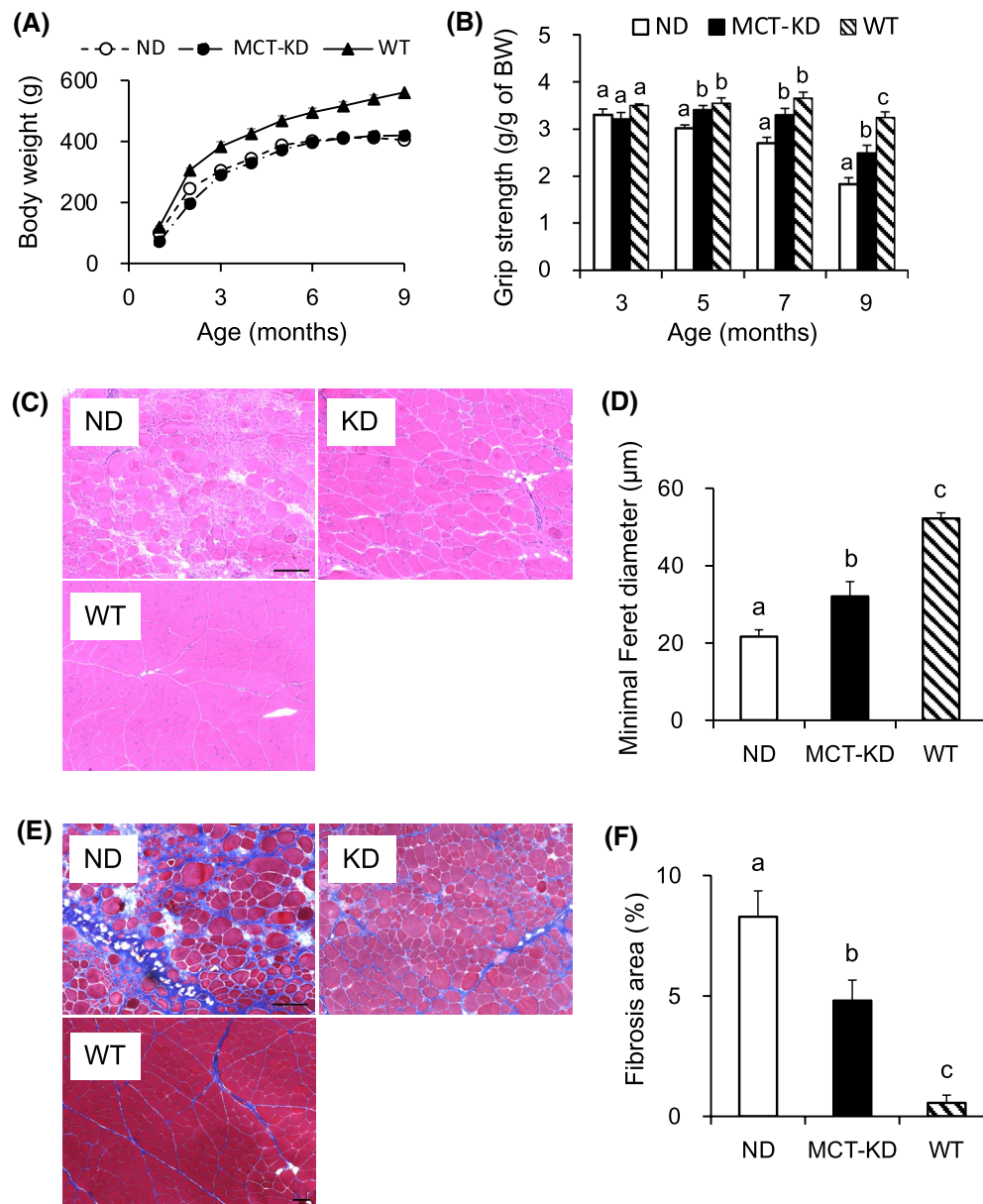


FIGURE 5 Effects of long-term MCT-KD on muscle strength, fiber diameter, and fibrosis in DMD rats at the age of 9 months. (A) Body weight changes in WT rats fed with ND and DMD rats fed with ND or MCT-KD (1 month: ND, $n = 4$, UD, $n = 8$; MCT-KD, $n = 12$; WT, $n = 5$, UD, $n = 5$; 2 months: ND, $n = 4$, UD, $n = 8$; MCT-KD, $n = 12$; WT, $n = 5$, UD, $n = 5$; 3–4 months: ND, $n = 12$; MCT-KD, $n = 12$; WT, $n = 10$; 5–9 months: ND, $n = 11$; MCT-KD, $n = 11$; WT, $n = 10$) (B) Muscle strength (grip test) of DMD and WT rats fed with ND or MCT-KD (3 months: ND, $n = 12$; MCT-KD, $n = 12$; WT, $n = 10$; 5–7 months: ND, $n = 11$; MCT-KD, $n = 11$; WT, $n = 10$). (C) Representative HE-stained TA muscle sections from WT rats fed with ND and DMD rats fed with ND or MCT-KD. Scale bar = 200 μm. (D) Median of minimal Feret diameter of TA muscle from WT rats fed with ND and DMD rats fed with ND or MCT-KD (ND, $n = 11$; MCT-KD, $n = 11$; WT, $n = 10$). (E, F) Masson trichrome-stained TA muscle sections (E) and quantification (F) of positive areas per total area of TA muscle sections from WT rats fed with ND and DMD rats fed with ND or MCT-KD (ND, $n = 11$; MCT-KD, $n = 11$; WT, $n = 10$). Scale bar, 200 μm. (A, B, D, F) Data are expressed as means $\pm$ SEM, and were compared by one-way ANOVA followed by Tukey–Kramer multiple comparisons test at each month of age. Superscript letters indicate significant difference at $p < .05$. UD, undetermined

2.6 | Reverse transcription-PCR

Frozen TA muscles were disrupted in RNAiso Plus (Takara Bio Inc) using a Micro Smash MS-100R (Tommy Seiko Co., Ltd., Tokyo, Japan), then total RNA was extracted.

Contaminating DNA was removed, and cDNA was synthesized using PrimeScript™ RT Reagent Kit and gDNA Eraser (Takara Bio Inc), respectively. Target amplicons were generated by reverse transcription-PCR with an annealing temperature of 57°C and 45 cycles as described¹⁹

using SYBR® Premix Ex Taq™ II (Takara Bio Inc), a LightCycler™ (Roche Diagnostics, Mannheim, Germany) and the following respective forward and reverse primers (5'→3'): *Hprt*: TGACCTTGATTTATTTGCATACC and CGAGCAAGACGTTTCAGTCCT; *TGF-β1*: CCTGGAAAGGGCTCAACAC and CAGTTCTTCTCTGTGGAGCTGA; *FGF2*: AAGAGCGACCCACACGTC and CCCTTGATGGACACAACCTCC.

The amount of target mRNA was normalized to that of *Hprt*.

2.7 | Creatine kinase activity

Serum was added to Fuji Dry-Chem slides (Fuji Film Medical, Inc, Tokyo, Japan) to measure serum creatine kinase (CK) activity using a DRI-CHEM 7000V analyzer (Fuji Film Medical, Inc).

2.8 | Statistical analysis

All data are presented as means ± SEM. Significant differences between groups were evaluated using unpaired, two-tailed Student *t*-tests. The effects of the KD on the diameter of muscle fibers were statistically evaluated using Wilcoxon rank sum tests. Multiple groups were compared using Tukey–Kramer tests. The results of statistical comparisons between genotypes at various ages are displayed. Differences were considered statistically significant at $p < .05$.

3 | RESULTS

3.1 | KD with MCT causes continuous ketosis without loss of body weight in DMD rats

We evaluated the effects of feeding DMD rats with either an MCT-KD or an ND between the ages of 3 and 12 weeks. The ketogenic ratio of fat to the carbohydrate-plus-protein weight of the MCT-KD was 1.95 for the first 10 days and 1.43 for the remainder of the study (Table 1). The ND group was pair-fed with MCT-KD based on daily caloric intake, because KD decreases appetite and food intake in mice and rats.²⁸

Figure 1A shows that BW did not significantly differ between the ND and MCT-KD DMD rats. By the age of 12 weeks, the BW of these rats was ~83% of that of DMD rats fed ad libitum (data not shown). At the age of 10 weeks, concentrations of postprandial blood glucose were significantly higher in the ND, than the MCT-KD rats, although their fasting blood glucose values were

identical (Figure 1B). Postprandial concentrations of blood ketone bodies were significantly higher in MCT-KD, than in ND rats, but identical between them under fasting conditions (Figure 1C). Blood concentrations of glucose and ketone bodies were identical between fasting and postprandial MCT-KD rats, and like those of fasting ND rats.

3.2 | Skeletal muscle dysfunction is suppressed by the MCT-KD in DMD rats

Grip strength at the age of 12 weeks was 21.4% higher in MCT-KD, compared with ND rats (Figures 2A and S1A). The relative TA muscle weight to BW was increased by 16.2% in MCT-KD, compared with ND rats, although the weight of soleus muscle did not significantly differ between the groups (Figures 2B,C and S1B,C). The relative weight of eWAT to BW was slightly, but not significantly decreased by MCT-KD (Figures 2D and S1D). We evaluated the effects of MCT-KD on skeletal muscle pathology by staining the TA muscles of DMD rats with hematoxylin and eosin (HE; Figure 2E). The MCT-KD obviously suppressed the appearance of small skeletal muscle fibers that were evident in ND rats. The distribution of the minimal Feret diameter of muscle fibers showed that MCT-KD increased the size of muscle fibers in the DMD rats (Figure 2F).

3.3 | Muscle necrosis, inflammatory cell infiltration, and fibrosis are suppressed by MCT-KD in DMD rats

Immunohistochemical analysis of IgG and albumin in sections of TA muscles from DMD rats showed that MCT-KD significantly suppressed the increase in IgG and albumin uptake, a marker of damage to muscle cell membranes^{29,30} (Figures 3A,B and S2A,B). This was in accordance with the decline in serum CK activity in MCT-KD rats (Figure 3C). The number of CD11b positive cells, a marker of monocytes, was significantly decreased, indicating that skeletal muscle damage was suppressed (Figure 3D,E). The ratio of Foxp3⁺ CD25⁺ cells, a marker of immunosuppressive regulatory T cells (Tregs), to CD4⁺ CD11b[−] cells that are associated with the suppression of muscular damage,³¹ was significantly higher in the MCT-KD, than ND rats (Figure 3F). The mRNA expression of *TGF-β1*, which regulates extracellular matrix (ECM) remodeling, was significantly decreased by the MCT-KD (Figure 3G). Masson trichrome staining showed that the MCT-KD significantly suppressed DMD-induced fibrosis (Figure 3H,I).

3.4 | Proliferation of satellite stem cells is promoted by MCT-KD

We investigated the effects of MCT-KD on muscle regeneration in the DMD rats. Immunohistochemical analysis of eMHC, a marker of regenerated myofibers, showed that the DMD rats fed with MCT-KD tended to have fewer eMHC⁺ myofibers (Figure 4A,B). Ratios of fibers with central nuclei did not significantly differ between DMD rats fed with the MCT-KD and ND (Figure S2A). We immunohistochemically assessed the SC markers, Pax7 and MyoD, in sections of TA muscle from ND and MCT-KD rats (Figure 4C–G). We also examined the proliferative potential of SCs by double staining with Ki67 and Pax7 or MyoD. The number of Pax7⁺ and MyoD⁺ cells tended to decrease in the MCT-KD rats. The ratios of Ki67⁺ Pax7⁺ cells to Pax7⁺ cells and Ki67⁺ MyoD⁺ cells to MyoD⁺ cells were significantly increased in the MCT-KD rats. The mRNA expression of *FGF2*, which promotes the proliferation of muscle SCs, was significantly increased in the TA muscles of MCT-KD rats (Figure 4H).

3.5 | MCT-KD suppressed DMD progression for 9 months

We investigated the long-term effects of MCT-KD on DMD. The DMD rats were fed with either the MCT-KD or the ND for 33 weeks from 3 weeks of age (Figure 5A,B). The reference was WT siblings of DMD rats fed with the ND. All rats were given food ad libitum and maintained in groups of 2–4 per cage. One DMD rat each fed with MCT-KD and ND died due to DMD at 4 months of age, but all other rats remained alive at 9 months of age.

The grip strength findings showed stronger muscles in the MCT-KD, than the ND DMD rats at the ages of 5, 7, and 9 months (Figures 5B and S3A), and the MCT-KD rats at the age of 7 months were as strong as they were at the age of 4 months, whereas the ND-treated DMD rats lost muscle strength with age. The muscle strength of the MCT-KD rats at the age of 7 months did not significantly differ from that of WT rats fed with the ND. Unlike at 3 months of age, the TA, soleus muscle, and adipose tissue weight did not significantly differ between ND and MCT-KD (Figure S3B–F). We evaluated the effects of MCT-KD on skeletal muscle pathology by staining TA muscles with HE (Figure 5C). The MCT-KD suppressed the appearance of small skeletal muscle fibers that was evident in ND rats. The distribution of the minimal Feret diameter of muscle fibers showed that MCT-KD increased the size of muscle fibers in the DMD rats (Figure 5D), and Masson trichrome staining showed that MCT-KD significantly suppressed DMD-induced fibrosis (Figure 5E,F).

Serum CK activity did not significantly differ between the ND and MCT-KD groups (Figure S3H).

4 | DISCUSSION

We fed genetically mutated DMD model rats with a KD containing medium-chain triglycerides (MCT-KD) to evaluate whether it could function as nutritional therapy. The MCT-KD significantly increased muscle weight, fiber diameter, and muscle strength, and significantly suppressed the muscle necrosis, inflammation, and subsequent fibrosis that are characteristic manifestations of DMD in these rats. The MCT-KD promoted the proliferation of muscle SCs, suggesting enhanced muscle regeneration. Furthermore, the DMD model rats fed with MCT-KD retained the significantly improved muscle strength, increased muscle fiber diameter, and inhibited muscle fibrosis even by the age of 9 months. The MCT-KD applied herein maintained a state of nutritional ketosis that mimicked starvation, similar to the conventional KD. However, in contrast to previous findings,^{19,20} the MCT-KD did not induce muscle atrophy. These results suggested that the MCT-KD is an ideal KD that contains sufficient protein and carbohydrates to suppress muscle atrophy (protein-sparing effect), while inducing nutritional ketosis.

The MCT-KD was effective over 9 months, and side effects were not noticeable. The suppression of myofiber necrosis and inflammation seemed responsible for the MCT-KD-induced amelioration of a series of pathological processes of muscular dystrophy that are the main causes of subsequent fibrosis and muscle weakness.³ In addition, MCT-KD promoted the proliferation of muscle SCs. These findings suggested that the MCT-KD ameliorated the pathogenesis of DMD by suppressing muscle necrosis and inflammation while promoting muscle regeneration.

A possible mechanism for the suppression of myonecrosis by MCT-KD might be the suppression of cell membrane fragility. Cell membranes become disrupted and fragile in patients with a dystrophin deficiency, and such membranes release extracellular components, especially calcium, which causes myonecrosis.² The MCT-KD apparently reduced the fragility of muscular cell membrane, since the numbers of albumin-positive fibers and serum CK activities decreased. Dietary supplementation with NAD⁺ increases the expression of structural proteins such as α -dystrobrevin and δ -sarcoglycan and decreases necrotic areas in mdx mice.³² Furthermore, SIRT1, which is activated by an increased ratio of NAD⁺:NADH, is involved in the fusion of intracellular vesicles and the attachment of vesicles to damaged membranes.³³ A KD can decrease glycolytic flux and increase the NAD⁺:NADH

ratio in the brain.³⁴ Therefore, MCT-KD might have suppressed myonecrosis by conferring robustness to the myocyte membrane and promoting repair after injury through increasing the NAD⁺:NADH ratio followed by the subsequent activation of SIRT1.

Levels of Tregs are elevated in muscles of patients with muscular dystrophy and in mdx mice, and their depletion enhances immune cell infiltration, muscle damage, and increases inflammation.³¹ Transforming growth factor beta and interleukin (IL)-10 that are associated with Treg function, cause macrophages to change into the anti-inflammatory M2 (M2) type,³⁵ which inhibits macrophage proliferation.³⁶ Therefore, the decreased infiltration of macrophages into muscle might have resulted from an increased ratio of Tregs to CD4⁺ cells in MCT-KD rats. The ratio of Tregs to CD4⁺ cells in the spleen of rats fed with the MCT-KD did not change, suggesting that the MCT-KD promoted the recruitment, proliferation, differentiation, and retention of Tregs in muscle. The ratio of Tregs to CD4⁺ cells is lower in the damaged muscles of aging, than young mice due to the reduced proliferative capacity of Tregs, which can be restored by injecting IL-33.³⁷ A KD can increase Treg ratios (%) by suppressing mTOR and HIF-1 α .³⁸ Thus, MCT-KD might suppress inflammation by increasing Tregs through promoting Treg proliferation and differentiation in muscle *via* IL33, mTOR, and HIF-1 α .

The numbers of eMHC⁺ myofibers tended to decrease in DMD rats fed with the MCT-KD, which would reflect less muscle damage, since regenerated myofibers appear in response to damage. The MCT-KD apparently helped to promote SC proliferation. Acetoacetic acid is a type of ketone body that promotes the activation and proliferation of skeletal muscle SCs through the MEK-ERK1/2-cyclin D1 signaling pathway.¹⁸ Consistent with previous findings, we found that the MCT-KD tended to promote the proliferation of Pax7 and MyoD positive SCs and up-regulate *FGF2* expression. Basic fibroblast growth factor 2 activates the ERK1/2 pathway, which is important for the G1 to S phase transition of muscle SCs,³⁹ suggesting that FGF2 mediates the MCT-KD-induced promotion of SC proliferation.

We showed that the MCT-KD suppressed myonecrosis, promoted the proliferation of muscle SCs, and inhibited the progression of DMD, suggesting its potential application as a dietary approach to treating DMD in the absence of an established alternative. Steroids are the standard therapy, but the effects are inadequate and side effects are serious. Advanced exon skipping and viral vector-based strategies have recently been applied to treat DMD, but their effects are also inadequate, and the side effects were harmful. Furthermore, exon skipping depends on the mutation profile of DMD and can only be applied to specific patients. The present findings

showed that MCT-KD could be a novel approach to treating DMD, as it inhibited DMD progression in rats even at the terminal stage. In addition, MCT-KD seems independent of genetic background, which might overcome the disadvantages of steroids and exon skipping. Because an MCT-KD causes the liver to systemically supply ketone bodies to the bloodstream, even after and between meals, systemic circulation or dosing intervals need not be considered. In summary, a KD can probably treat DMD with less harmful side effects and more patient benefits than genetic approaches. As far as we can ascertain, this is the first report to describe the nutritional value of DMD in experimental animals. Further studies are needed to elucidate the mechanisms underlying the improvement of DMD elicited by MCT-KD.

In conclusion, MCT-KD ameliorated the pathological manifestations characteristic of DMD, such as muscle weakness, myonecrosis, inflammation, and fibrosis, by inhibiting muscle destruction and promoting muscle regeneration in rat models of DMD. These findings have important clinical implications and provide a potential rationale for using MCT-KD to treat muscular dystrophy and related diseases in humans. However, the effects of MCT-KD on the myocardium and its side effects on other organs are not fully understood, thus justifying further investigation into the potential application of MCT-KD to treat DMD.

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DISCLOSURES

The authors declare no conflicts of interest associated with this manuscript.

AUTHOR CONTRIBUTIONS

Yuri Fujikura participated in the study design, conducted the experiments, analyzed the data, and wrote the manuscript. Hidetoshi Sugihara participated in the study design, experiments, manuscript preparation. Masaki Hatakeyama participated in the study design and provided helpful suggestions about the composition of MCT-KD. Keitaro Yamanouchi and Katsutaka Oishi participated in the study design, manuscript preparation, and supervised the study.

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REFERENCES

- Lapidos KA, Kakkar R, McNally EM. The dystrophin glycoprotein complex. *Circ Res*. 2004;94:1023-1031.
- Yasuda S, Townsend D, Michele DE, Favre EG, Day SM, Metzger JM. Dystrophic heart failure blocked by membrane sealant poloxamer. *Nature*. 2005;436:1025-1029.
- Verhaart IEC, Aartsma-Rus A. Therapeutic developments for Duchenne muscular dystrophy. *Nat Rev Neurol*. 2019;15:373-386.
- Mercuri E, Muntoni F. Muscular dystrophies. *Lancet*. 2013;381:845-860.
- Fairclough RJ, Wood MJ, Davies KE. Therapy for Duchenne muscular dystrophy: renewed optimism from genetic approaches. *Nat Rev Genet*. 2013;14:373-378.
- Wang YX, Rudnicki MA. Satellite cells, the engines of muscle repair. *Nat Rev Mol Cell Biol*. 2012;13:127-133.
- Kuang S, Gillespie MA, Rudnicki MA. Niche regulation of muscle satellite cell self-renewal and differentiation. *Cell Stem Cell*. 2008;2:22-31.
- Ribeiro AF, Souza LS, Almeida CF, et al. Muscle satellite cells and impaired late stage regeneration in different murine models for muscular dystrophies. *Sci Rep*. 2019;9:11842.
- Ūsas A, Mačiulaitis J, Mačiulaitis R, Jakubonienė N, Milašius A, Huard J. Skeletal muscle-derived stem cells: implications for cell-mediated therapies. *Medicina*. 2011;47:469.
- Motohashi N, Shimizu-Motohashi Y, Roberts TC, Aoki Y. Potential therapies using myogenic stem cells combined with bio-engineering approaches for treatment of muscular dystrophies. *Cells*. 2019;8:1066.
- Zeng L, Akasaki Y, Sato K, Ouchi N, Izumiya Y, Walsh K. Insulin-like 6 is induced by muscle injury and functions as a regenerative factor. *J Biol Chem*. 2010;285:36060-36069.
- Riuzzi F, Sorci G, Sagheddu R, Donato R. HMGB1-RAGE regulates muscle satellite cell homeostasis through p38-MAPK- and myogenin-dependent repression of Pax7 transcription. *J Cell Sci*. 2012;125:1440-1454.
- D C Harvey CJ, Schofield GM, Williden M, McQuillan JA. The effect of medium chain triglycerides on time to nutritional ketosis and symptoms of keto-induction in healthy adults: a randomised controlled clinical trial. *J Nutr Metab*. 2018;2018:2630565.
- Robinson AM, Williamson DH. Physiological roles of ketone bodies as substrates and signals in mammalian tissues. *Physiol Rev*. 1980;60:143-187.
- Augustin K, Khabbush A, Williams S, et al. Mechanisms of action for the medium-chain triglyceride ketogenic diet in neurological and metabolic disorders. *Lancet Neurol*. 2018;17:84-93.
- Klement RJ, Champ CE, Otto C, Kämmerer U. Anti-tumor effects of ketogenic diets in mice: a meta-analysis. *PLoS ONE*. 2016;11:e0155050.
- Geng S, Zhu W, Xie C, et al. Medium-chain triglyceride ameliorates insulin resistance and inflammation in high fat diet-induced obese mice. *Eur J Nutr*. 2016;55:931-940.
- Zou X, Meng J, Li L, et al. Acetoacetate accelerates muscle regeneration and ameliorates muscular dystrophy in mice. *J Biol Chem*. 2016;291:2181-2195.
- Nakao R, Abe T, Yamamoto S, Oishi K. Ketogenic diet induces skeletal muscle atrophy via reducing muscle protein synthesis and possibly activating proteolysis in mice. *Sci Rep*. 2019;9:19652.
- Tinsley GM, Willoughby DS. Fat-free mass changes during ketogenic diets and the potential role of resistance training. *Int J Sport Nutr Exerc Metab*. 2016;26:78-92.
- Nakano K, Katsuzaki M, Mizutani M, Ashida K. Further studies on the effect of dietary carbohydrate and fat on protein metabolism in rats. *J Nutr*. 1972;102:283-290.
- Noakes M, Foster P, Keogh J, James A, Mamo J, Clifton P. Comparison of isocaloric very low carbohydrate/high saturated fat and high carbohydrate/low saturated fat diets on body composition and cardiovascular risk. *Nutr Metab*. 2006;3:7.
- Campbell WW, Trappe TA, Jozsi AC, Kruskall LJ, Wolfe RR, Evans WJ. Dietary protein adequacy and lower body versus whole body resistive training in older humans. *J Physiol*. 2002;542:631-642.
- Huttenlocher PR, Wilbourn AJ, Signore JM. Medium-chain triglycerides as a therapy for intractable childhood epilepsy. *Neurology*. 1971;21:1097-1103.
- Nakamura K, Fujii W, Tsuboi M, et al. Generation of muscular dystrophy model rats with a CRISPR/Cas system. *Sci Rep*. 2014;4:5635.
- Grady RM, Teng H, Nichol MC, Cunningham JC, Wilkinson RS, Sanes JR. Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. *Cell*. 1997;90:729-738.
- Sugihara H, Teramoto N, Nakamura K, et al. Cellular senescence-mediated exacerbation of Duchenne muscular dystrophy. *Sci Rep*. 2020;10:16385.
- Deemer SE, Plaisance EP, Martins C. Impact of ketosis on appetite regulation—a review. *Nutr Res*. 2020;77:1-11.
- Grounds MD, Terrill JR, Al-Mshhdani BA, Duong MN, Radley-Crabb HG, Arthur PG. Biomarkers for Duchenne muscular dystrophy: myonecrosis, inflammation and oxidative stress. *Dis Model Mech*. 2020;13:dmm043638.
- Bencze M, Periou B, Baba-Amer Y, Authier FJ. Immunolabelling myofiber degeneration in muscle biopsies. *J Vis Exp*. 2019;5:154.
- Villalta SA, Rosenthal W, Martinez L, et al. Regulatory T cells suppress muscle inflammation and injury in muscular dystrophy. *Sci Transl Med*. 2014;6:258ra142.
- Zhang H, Ryu D, Wu Y, et al. NAD⁺ repletion improves mitochondrial and stem cell function and enhances life span in mice. *Science*. 2016;352:1436-1443.
- Fujiwara D, Iwahara N, Sebori R, et al. SIRT1 deficiency interferes with membrane resealing after cell membrane injury. *PLoS ONE*. 2019;14:e0218329.
- Xin L, Ipek Ö, Beaumont M, et al. Nutritional ketosis increases NAD⁺/NADH ratio in healthy human brain: an in vivo study by 31P-MRS. *Front Nutr*. 2018;5:62.
- Okeke EB, Uzonna JE. The pivotal role of regulatory T cells in the regulation of innate immune cells. *Front Immunol*. 2019;10:680.
- O'Farrell A-M. IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways. *EMBO J*. 1998;17:1006-1018.
- Kuswanto W, Burzyn D, Panduro M, et al. Poor repair of skeletal muscle in aging mice reflects a defect in local, interleukin-33-dependent accumulation of regulatory T cells. *Immunity*. 2016;44:355-367.

38. Ni FF, Li CR, Liao JX, et al. The effects of ketogenic diet on the Th17/Treg cells imbalance in patients with intractable childhood epilepsy. *Seizure*. 2016;38:17-22.
39. Jones NC, Fedorov YV, Rosenthal RS, Olwin BB. ERK1/2 is required for myoblast proliferation but is dispensable for muscle gene expression and cell fusion. *J Cell Physiol*. 2001;186:104-115.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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