Biochemical and Biophysical Research Communications 592 (2022) 87–92

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Meclozine ameliorates skeletal muscle pathology and increases muscle forces in *mdx* mice

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ARTICLE INFO

Article history:
Received 20 October 2021
Received in revised form
23 December 2021
Accepted 3 January 2022
Available online 7 January 2022

Keywords:
Drug repositioning
Meclozine
Duchenne muscular dystrophy

ABSTRACT

We screened pre-approved drugs for the survival of the Hu5/KD3 human myogenic progenitors. We found that meclozine, an anti-histamine drug that has long been used for motion sickness, promoted the proliferation and survival of Hu5/KD3 cells. Meclozine increased expression of MyoD, but reduced expression of myosin heavy chain and suppressed myotube formation. Withdrawal of meclozine, however, resumed the ability of Hu5/KD3 cells to differentiate into myotubes. We examined the effects of meclozine on *mdx* mouse carrying a nonsense mutation in the dystrophin gene and modeling for Duchenne muscular dystrophy. Intragastric administration of meclozine in *mdx* mouse increased the body weight, the muscle mass in the lower limbs, the cross-sectional area of the paravertebral muscle, and improved exercise performances. Previous reports show that inhibition of phosphorylation of ERK1/2 improves muscle functions in mouse models for Emery-Dreifuss muscular dystrophy and cancer cachexia, as well as in *mdx* mice. We and others previously showed that meclozine blocks the phosphorylation of ERK1/2 in cultured cells. We currently showed that meclozine decreased phosphorylation of ERK1/2 in muscles in *mdx* mice but not in wild-type mice. This was likely to be one of the underlying mechanisms of the effects of meclozine on *mdx* mice.

1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive muscle wasting disease occurring in 1 in 3500 male births [1]. DMD results from a germline mutation in *DMD* encoding the subsarcolemmal protein dystrophin. Glucocorticoid is approved for DMD [2]. In addition, antisense-oligonucleotides to induce skipping of *DMD* exons 51, 52, and 53 for resuming an open reading frame have been recently approved [3]. *Mdx* mouse carries a nonsense mutation in *Dmd* [4]. In *mdx* mouse, degeneration of muscle fibers begins by 3 weeks of age, but gradually subsides after 8.5 weeks of age, partly because of compensatory expression of a paralogous protein, utrophin [5].

Here, we applied the drug repositioning strategy to identify a clinically applicable drug for muscle regeneration, and found that meclozine, an antihistamine agent for motion sickness, had favorable effects on human myogenic cells and *mdx* mice.

2. Materials and methods

2.1. Cell culture

Hu5/KD3 human muscle progenitor cells were cultured in a dish coated with type I collagen (Corning) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2% Ultroser G (Pall Corp.), and 4.5 mg/ml glucose [6]. On day 3, the medium was changed to the differentiation medium comprised of DMEM supplemented with 2% horse serum and 1% insulin-transferrin-selenite (Gibco, ITS). Medium was changed every

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https://doi.org/10.1016/j.bbrc.2022.01.003
0006-291X/

other day. Although a biological half-life of meclozine in culture medium remained undetermined, the expression level of the meclozine-metabolizing enzyme, cytochrome P450 (CYP2D6), is low in skeletal muscle [7].

2.2. Screening of FDA-approved compounds in Hu5/KD3 cells

Each of 1,186 FDA-approved compounds (10 mM in 100% DMSO) (Prestwick Chemical) was diluted to 500 μM with DMEM. One μl of 500 μM compound was added to 49 μl culture medium to make the culture medium with 10 μM compound and 0.1% DMSO. Hu5/KD3 cells in a 96-well plate (200 cells/mm^2) were incubated for 3 days with 10 μM compound. The number of cells was evaluated by the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Aqueous One), as well as by the Cell Proliferation ELISA, BrdU (Roche), both using a plate reader (POWERSCAN4, DS Pharma). For the following confirmatory studies, meclozine was purchased from MP Biomedicals (Cat. 155341).

2.3. Evaluation of the growth of Hu5/KD3 cells

Hu5/KD3 cells seeded in a collagen I-coated 6-well plate (52 cells/mm^2) were placed in the IncuCyte ZOOM (Essen Bioscience) to evaluate the growth curve. Cell images were captured every 1 h for 3 days with a brightfield channel, and were analyzed with the IncuCyte Cell-by-Cell Analysis Software.

Hu5/KD3 cells in an 8-well chamber slide (500 cells/mm^2, Thermo Scientific) were immunostained with an anti-myosin heavy chain antibody (MYH, 1:200, Santa Cruz, sc-20641) and a donkey anti-rabbit IgG secondary antibody (1:500, Life Technologies, A21206).

2.4. Western blotting

Hu5/KD3 cells and tibialis anterior muscle were lysed in RIPA Lysis Buffer (Santa Cruz), and were subjected to immunoblotting using antibodies against MyoD (1:1000, Santa Cruz, sc-304), MYH (1:1000, Santa Cruz, sc-20641), ERK1/2 (1:1000, Cell Signaling, 9102), phospho-ERK1/2 (1:1000, Cell Signaling, 4736), β-actin (1:1000, Santa Cruz, sc-47778), and GAPDH (1:400, SIGMA, G9545). Donkey anti-rabbit and anti-mouse IgG antibodies conjugated to horseradish peroxidase (HRP) (1:2000, GE Healthcare, NA9340V and NA931, respectively) were used as the secondary antibody.

2.5. C57BL/6J mice

All animal studies were approved by the Animal Care and Use Committee of Nagoya University. Food containing meclozine was prepared by adding 0.4 g meclozine in 1 kg food (Oriental Yeast Co.) [8], and was administered *ad libitum* to male and female C57BL/6J mice from postnatal day 16. We previously reported with this meclozine-containing food that mice took 1.05 ± 0.10 mg meclozine (mean and SD, $n = 4$) per day in 3 weeks from postnatal days 21 to 42 [8]. This gave rise to an average plasma meclozine concentration of 36.6 ± 20.1 ng/ml (mean and SD, $n = 10$ mice) [8], which was under a clinically achievable concentration of 68.4 ng/ml after a single dose of 25 mg meclozine in human [9]. When mice were sacrificed after taking meclozine for 3 weeks, transverse micro-computed tomography (μCT) images were taken with a Skyscan 1176 X-ray-computed microtomography scanner (Bruker) with a 0.5-mm aluminum filter, and were reconstructed with SkyScan DataViewer (Bruker). The cross-sectional area (CSA) was quantified using Image J (NIH).

2.6. Wild-type C57BL/10 and mdx mice

All experiments were performed with either male wild-type or male *mdx* (*mdx*/Y) mice to avoid the influence of sex. Meclozine (5 mg/kg) was intragastrically administrated with a sonde needle once a day for two weeks from postnatal week 3. Transverse μCT images were taken as above at 3 and 5 weeks of age. Grip test was performed by a force transducer (Grip Strength Meter, Columbus Instrument). Each measurement was repeated three times within 2 min, and the best score was adopted. Rota-rod (Ugo Basile) was linearly accelerated from 5 to 50 rpm over 240 s. Mice were tested four times at an interval of 15 min. Each mouse was placed in a cage implemented with a counter-equipped running wheel (Ohara Medical Corp.), and the running distance was recorded for 24 h.

2.7. Measurement of isometric tetanic force of skeletal muscle

The calf muscle was connected to a force transducer (UT-200GR; MinebeaMitsumi), and was directly stimulated by an electronic stimulator (SEN-5201; Nihon Kohden). Maximal isometric tetanic force at 100 Hz stimulation was analyzed using PicoScope oscilloscope system and software (Pico Technology).

2.8. Histology and immunohistochemistry

A 10-μm muscle section was stained with hematoxylin and eosin. The ratio of central myonuclei was blindly analyzed. Sections of soleus muscle were stained with rabbit polyclonal anti-Pax7 antibody (1:100, Invitrogen, PA1-117) and a secondary polyclonal antibody against rabbit IgG conjugated with FITC (1:200, Vector Laboratories, FI-1000), and were blindly observed.

2.9. Statistical analysis

Statistical analyses were carried out using either unpaired Student's *t*-test, or one-way or two-way ANOVA with Dunnett's post-hoc test using SPSS ver. 21 (IBM). *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Meclozine enhances the proliferation and survival of Hu5/KD3 human myogenic progenitor cells

To search for off-label effects of pre-approved drugs on myoblasts, we first screened a library of 1,186 FDA-approved drugs using Hu5/KD3 human myogenic progenitor cells [10]. We found that meclozine, an anti-histamine agent that has long been used for motion sickness, increased the number of Hu5/KD3 cells in 3 days in the growth medium by an MTS assay (Fig. 1B). Continuous monitoring of the number of cells in the growth medium showed that statistical significance of the increased number of cells was observed when the cells reached confluency (Supplementary Fig. S1B). A BrdU assay showed that meclozine also increased the number of cells in the differentiation medium especially on day 7 (Fig. 1C), and the effect was dose-dependent (Supplementary Fig. S1A). We found that meclozine markedly attenuated the number of dead cells from differentiation days 7.0–7.5 (Supplementary Fig. S1CD). Thus, meclozine enhanced the viability of differentiating cells. We next examined the effects of meclozine on myotube differentiation of Hu5/KD3 cells (Fig. 1D). Untreated Hu5/KD3 cells were able to form myotubes in 7 days in the differentiation medium, whereas 10 μM meclozine suppressed the myotube formation and the expression of myosin heavy chain (MYH) (Fig. 1E and Supplementary Fig. 1E). In contrast, meclozine

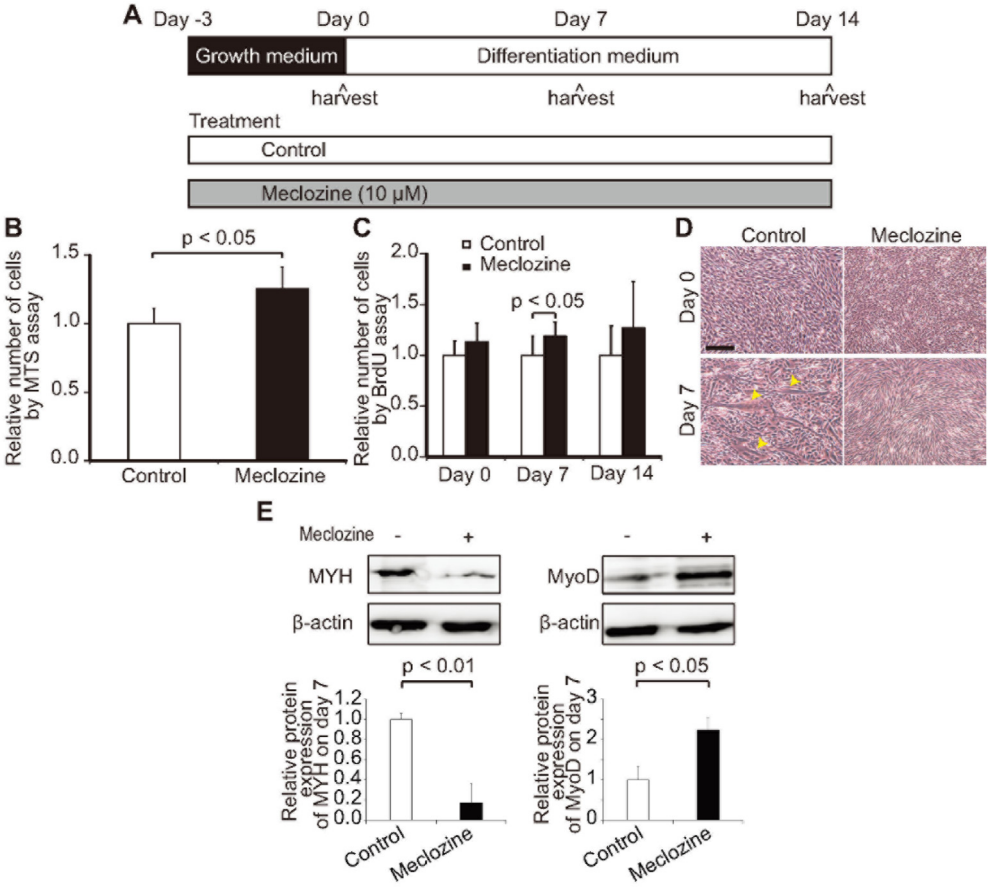

Fig. 1. Meclozine enhances the proliferation and survival of Hu5/KD3 human myogenic progenitors.
(A) An experimental scheme of Hu5/KD3 cells treated with meclozine. Hu5/KD3 cells are cultured in the growth medium for 3 days (days −3 to 0), and then in the differentiation medium for 14 more days (days 0–14) with or without 10 μM meclozine. **(B)** The number of Hu5/KD3 cells estimated by MTS assay on day 0. Absorbance at 490 nm is normalized to that without meclozine (Control). Mean and SD are indicated ($n = 6$ dishes each). Statistical significance (p) is calculated by Student's t-test. **(C)** The number of Hu5/KD3 cells estimated by BrdU assay on days 0, 7, and 14. Absorbance at 492 nm is normalized to that at 370 nm, and then to the ratio without meclozine (Control) on each day. Mean and SD are indicated ($n = 6$ dishes each). Statistical significance (p) is calculated by Student's t-test. **(D)** Representative images of myoblasts and myotubes on days 0 and 7. Arrows indicate fused myotubes with multiple nuclei. Note that 10 μM meclozine suppresses myotube formation on day 7. Scale bar = 200 μm. **(E)** Immunoblotting for MYH and MyoD in Hu5/KD3 cells treated with or without 10 μM meclozine on day 7. Ratio of MYH and MyoD to β-actin are normalized to those without meclozine (Control). Mean and SD are indicated ($n = 3$ culture dishes each). Statistical significance (p) is calculated by Student's t-test.

increased the expression of MyoD in the differentiation medium (Fig. 1E). These results suggested that meclozine delayed myotube differentiation of Hu5/KD3 cells, and also protected MyoD-positive cell against cell death.

We next asked whether the meclozine-mediated delay of myotube differentiation of Hu5/KD3 cells is reversible or not. Washing out of 10 μM meclozine on differentiation day 7 allowed Hu5/KD3 cells to differentiate into myotubes and to express MYH (Fig. 2). These results indicated that meclozine did not abrogate a potential for Hu5/KD3 cells to differentiate into myotubes.

3.2. Meclozine ameliorates amyotrophy and motor deficits in mdx muscular dystrophy mouse model

We expected that enhanced proliferation of myoblasts (Fig. 1BC) and enhanced survival of differentiating myoblasts (Supplementary Fig. S1CD) would be in favor of muscle regeneration *in vivo*. We previously reported that the half-life of meclozine was 2.66–6.43 h in an 8-week-old mouse [11]. We thus expected that, in mouse muscles, the myoblast proliferation and myoblast survival would be enhanced in the presence of meclozine, whereas myotubes would be formed in the absence of meclozine within a day.

We first examined the effect of meclozine on wild-type C57BL6/J mice. Mice were allowed to access meclozine-containing food from postnatal day 16 for 3 weeks, and were expected to take 1.05 mg meclozine per day [8]. The cross-sectional areas (CSAs) of the paraspinal, femoral, and calf muscles evaluated by reconstructed μCT images (Supplementary Fig. S2) were increased, whereas CSAs of retroperitoneal fat were decreased, in both males and females (Supplementary Fig. S3BC). This yielded high muscle-to-fat CSA ratios in treated mice (Supplementary Fig. S3D).

To test the effects of meclozine on muscle wasting diseases, *mdx* mice were intragastrically administered with 5 mg/kg/day of meclozine using a disposable sonde needle once a day for 2 weeks from postnatal week 3. We previously reported that intragastric administration of 2–6 mg/kg meclozine to an 8-week-old mouse reached the peak plasma concentrations of 60.7–352.4 ng/ml (0.13–0.73 μM), which were within the range used in clinical settings for motion sickness [11]. Administration of 5 mg/kg meclozine once a day would give rise to a reasonable plasma drug concentration, and would allow a sufficient meclozine-free period in a day for *mdx* mice. Meclozine increased the body weights of *mdx* mice more than untreated *mdx* mice (Fig. 3A). Reconstructed axial μCT images showed that meclozine tended to increase the CSAs of paraspinal muscles (Fig. 3B). Indeed, meclozine increased the masses of tibialis anterior, triceps surae, and quadriceps femoris

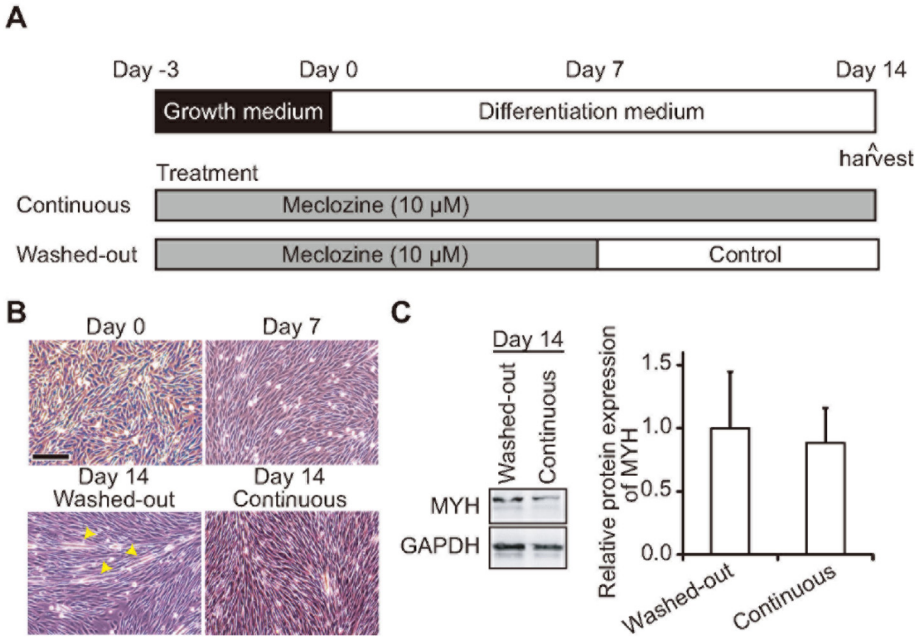

Fig. 2. Withdrawal of meclozine resumes differentiation of human Hu5/KD3 cells.
(A) An experimental scheme of Hu5/KD3 cells to examine the effect of withdrawal of meclozine on myotube differentiation. Exposure of Hu5/KD3 cells to 10 μM meclozine is continued throughout the experiment ("Continuous") or terminated on day 7 ("Washed-out"). **(B)** Representative images of Hu5/KD3 cells on days 0, 7, and 14. Note that withdrawal of meclozine enables myotube differentiation on day 14 (Day 14 Washed-out). Arrowheads point to fused multi-nucleated myotubes. Scale bar = 200 μm. **(C)** Immunoblotting for MYH of Hu5/KD3 cells on day 14. Signal intensity of MYH is normalized to that of the "Washed-out" sample. Mean and SD are indicated ($n = 3$ dishes each). No statistical difference by Student's *t*-test.

muscles (Fig. 3C) but not of paratestis and subcutaneous fats (Supplementary Fig. S4A). We next analyzed the maximal muscle strength, the exercise endurance, and the voluntary motor activity in *mdx* mice by the grip strength test, the rota-rod test, and the wheel running test, respectively. Meclozine increased the forelimb grip strength (Fig. 3D), as well as the dwell time on the rota-rod

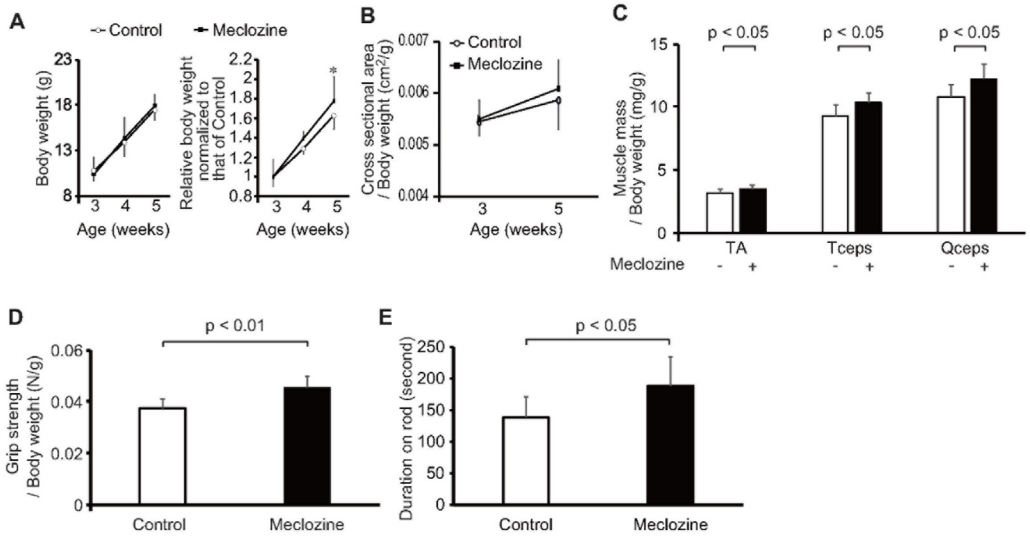

Fig. 3. Meclozine increases muscle mass and improves motor functions in *mdx* mice modeling for Duchenne muscular dystrophy.
Mdx mice are intragastrically administrated with 0.5% methylcellulose (Control) or 5 mg/kg/day meclozine in 0.5% methylcellulose for 2 weeks from 3 to 5 weeks of age.
(A) Absolute values (left) and normalized values (right) of body weights of *mdx* mice treated with ($n = 11$ mice) or without ($n = 9$ mice) meclozine. Values are normalized to an average at 3 weeks of age in the right panel. Mean and SD are indicated. *$p < 0.05$ by two-way repeated measures ANOVA followed by Dunnett's post-hoc test between the control and meclozine groups. **(B)** Quantification of cross-sectional areas (CSAs) of bilateral paraspinal muscles. The whole body μCT is taken at 3 and 5 weeks of age. CT images are reconstructed perpendicular to the spine (Supplementary Fig. S2). CSAs of bilateral paraspinal muscles in each *mdx* mouse are analyzed at the L4/5 disc level and the L5 spinous process level, and both CSAs are summed. CSAs (cm^2) are normalized to body weight (g). Mean and SD are indicated ($n = 9$ mice each). No statistical difference by two-way repeated measures ANOVA followed by Dunnett's post-hoc test between the control and meclozine groups. **(C)** Wet weights of tibialis anterior (TA), triceps surae (Tceps), and quadriceps femoris (Qceps) muscles, as well as paratestis and subcutaneous fats, in *mdx* mice treated with ($n = 11$) or without ($n = 9$) meclozine for 2 weeks. Muscle and fat masses (mg) normalized to the body weight (g) are plotted. Mean and SD are indicated. Statistical significance (p) is calculated by Student's *t*-test. **(D)** Grip strength of the forelimbs is normalized for body weight in *mdx* mice treated with ($n = 8$) or without ($n = 6$) meclozine for 2 weeks. Mean and SD are indicated. Statistical significance (p) is calculated by Student's *t*-test. **(E)** Endurance time on a rota-rod in *mdx* mice treated with ($n = 10$) or without ($n = 6$) meclozine for 2 weeks. The rotation of rota-rod is linearly accelerated from 5 to 50 rpm over 240 s. Mean and SD are indicated. Statistical significance (p) is calculated by Student's *t*-test.

(Fig. 3E). Meclozine tended to increase the voluntary exercise performance (Supplementary Fig. S4B), as well as the contractile muscle force of calf muscle (Supplementary Fig. S4C). Activities of CK and lactate dehydrogenase (LDH) in the serum, which are increased by skeletal muscle damage [12], were decreased to 40–60% of those in untreated *mdx* mice (Supplementary Figs. S4D and S4E). These results suggested that meclozine ameliorated amyotrophy and motor deficits in *mdx* mice.

3.3. Meclozine reduces central nuclei in mdx mice

Pathological features of slow-twitch (soleus) and fast-twitch (extensor digitorum longus, EDL) muscles were analyzed. Myofibers that have been injured, repaired, and regenerated exhibit centrally located myonuclei, which is a morphological hallmark for muscle degeneration in *mdx* mice [13]. The percentages of fibers displaying internalized nuclei in meclozine-treated soleus and EDL muscles (Fig. 4A) were lower than those in untreated muscles in *mdx* mice. The ratio of the Pax7-positive mononucleated cells to total myofibers of soleus muscle in meclozine-treated *mdx* mice was not changed compared to those in untreated *mdx* mice (Supplementary Fig. S4G). Thus, the percentage of regenerating myofibers represented by central myonuclei was decreased by meclozine treatment in *mdx* mouse.

3.4. Meclozine attenuates ERK phosphorylation in mdx mice

We previously showed that meclozine blocked the extracellular signal-regulated kinase (ERK) 1/2 signaling downstream of an FGF receptor, FGFR3, in an RCS rat chondrosarcoma cell line [14]. Another study similarly demonstrated that meclozine inhibited the phosphorylation of ERK in bone marrow-derived macrophages [15]. We, thus, examined the effect of meclozine on the ERK signaling in skeletal muscles of wild-type C57BL10 and *mdx* mice. Total protein was extracted from the tibialis anterior muscles, and the phosphorylation levels of ERK were determined by immunoblotting. We found that total ERK1/2, but not phosphorylated ERK1/2, was higher in *mdx* mice than wild-type mice (Fig. 4B). Meclozine had no effect on the amount of phosphorylated ERK1/2 in wild-type mice, but reduced the amount of phosphorylated ERK1/2 in *mdx* mice. Thus, meclozine was likely to suppress the ERK1/2 activity in skeletal muscles in *mdx* mouse.

4. Discussion

We found that meclozine enhanced the viability of differentiating Hu5/KD3 human myogenic progenitor cells (Fig. 1). We also observed that meclozine increased the growth of skeletal muscles in wild-type (Supplementary Figure S3) and *mdx* mice (Fig. 3AB) from postnatal weeks 3–5. How does meclozine increase the muscle mass and improve the exercise performance? We [14] and others [15] reported that meclozine blocks the phosphorylation of ERK1/2 in chondrosarcoma cell line [14] and bone marrow-derived macrophages [15]. Here, we showed that meclozine decreased phosphorylation of ERK1/2 in tibialis anterior muscles in *mdx* mice, but had no effect in wild-type C57BL10 mice (Fig. 4B). An MEK1/2 inhibitor, selumetinib, suppresses ERK1/2 activity and improves muscle function in a mouse model of Emery-Dreifuss muscular dystrophy [16] and cancer cachexia [17], in both of which phosphorylated ERK1/2 is increased in skeletal muscle. Mice bearing

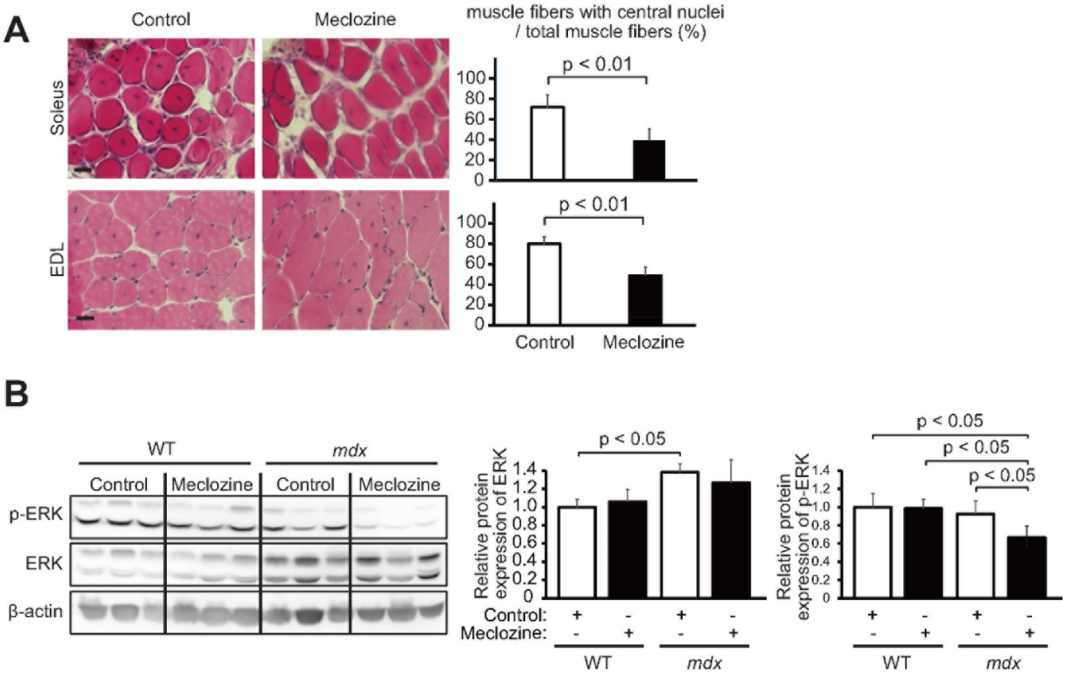

Fig. 4. Meclozine ameliorates muscle pathology and attenuates the ERK activity in skeletal muscle in *mdx* mice.
Mdx mice are intragastrically administrated with 0.5% methylcellulose (Control) or 5 mg/kg/day meclozine in 0.5% methylcellulose for 2 weeks from 3 to 5 weeks of age.
(A) Representative hematoxylin and eosin staining of the soleus and EDL muscles of *mdx* mice treated with or without meclozine for 2 weeks ($n = 3$ each group). The ratio (%) of the number of myofibers with central nuclei to the number of total myofibers in *mdx* mice with ($n = 67$ and 73 myofibers for soleus and EDL, respectively) or without meclozine ($n = 90$ and 113 myofibers for soleus and EDL, respectively). Scale bar = 20 μm. Mean and SD are indicated. Statistical significance (p) is calculated by Student's t-test. **(B)** (Left panel) Immunoblotting for total ERK and phosphorylated ERK (p-ERK) of tibialis anterior muscles harvested from wild-type C57BL/10 and *mdx* mice treated with (meclozine, $n = 3$ mice) or without (control, $n = 3$ mice) meclozine. (Right panel) The expression levels of total ERK and p-ERK are normalized for β-actin, and also for the ratio in wild-type C57BL/10 mice without meclozine. Mean and SD are indicated ($n = 3$ mice each). Statistical significance (p) is calculated by one-way ANOVA followed by Dunnett's post-hoc test between all possible pairs.

C26 undifferentiated carcinoma cells display a marked loss of body weight and muscle mass, as well as decrement of hindlimb grip strength, which are associated with increased phosphorylated ERK in gastrocnemius muscle. Daily injection of an ERK inhibitor, PD98059, to the C26-bearing mice ameliorates the muscle depletion and the weakness [18]. Moreover, an angiotensin receptor blocker decreases phosphorylation of ERK1/2 and suppresses the fibrosis of skeletal muscles in *mdx* mice [19]. All the findings including ours point to the notion that the increased skeletal muscle mass and the improved exercise performance (Fig. 3DE and Supplementary Fig. S4BC) by meclozine are likely to be accounted for by suppression of phosphorylation of ERK1/2 in skeletal muscles.

We and others reported that meclozine is a potential therapeutic agent for colon cancer [20], achondroplasia [8], and Parkinson's disease [21]. Meclozine has long been used for motion sickness as an anti-histamine agent in adults and children. The current study showed that 5 mg/kg/day of meclozine exhibited no major adverse effects in wild-type and *mdx* mice from 3 to 10 weeks of age. Sleepiness, headache, vomiting, dry mouth, tired feeling, and drowsiness are adverse-effects of meclozine, which stimulated the development of other classes of anti-histamine agents with a better pharmacological profile [22]. We expect that meclozine and other derivatives originating from meclozine will become a novel therapeutic option for treating patients with DMD and other muscle-wasting diseases in the future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Dr. Naohiro Hashimoto at National Center for Geriatrics and Gerontology, Japan for providing Hu5/KD3 human myogenic cell line, as well as Ryusaku Esaki, Harumi Kodama, and Keiko Itano at Nagoya University Graduate School of Medicine for their technical assistance. We would also like to thank Central Institute for Experimental Animals, Japan for providing C57BL/10 and *mdx* mice. The studies were supported by Grants-in-Aid from the Japan Society for the Promotion of Science (JP21K06392, JP21H02476, JP20K06925, 18K19291, and JP20H03561); the Ministry of Health, Labour and Welfare of Japan (20FC1036); the Japan Agency for Medical Research and Development (21gm1010002, 21ek0109488, and 21bm0804005), the National Center of Neurology and Psychiatry (2-5), and the Hori Sciences and Arts Foundation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.01.003.

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