Brief report

Title: Enhanced muscle regeneration in mdx mice, Duchenne muscular dystrophy animal model, proven by CD44 & MYH3 expression, on oral feeding of N-163 strain of *Aureobasidium Pullulans* produced B-Glucan.

Running title: B-glucan enhances muscle regeneration in mdx mice.

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Declarations

Ethics approval and consent to participate

Protocol approvals were obtained from SMC Laboratories, Japan's IACUC (Study Protocol no: SP_SLMA143-2208-3). This study was conducted in accordance with the Animal Research: Reporting of In Vivo Experiments Guidelines. C57BL/10SnSlc mice (3 weeks of age, male) were obtained from Japan SLC, Inc. (Japan). C57BL/10-mdx/Jcl mice (3 weeks of age, male) were obtained from CLEA Japan (Japan). All animals used in this study were cared for following guidelines: Act on Welfare and Management of Animals (Ministry of the Environment, Act No. 105 of October 1, 1973), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No.88 of the Ministry of the Environment, April 28, 2006) and Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1, 2006).

Availability of data and material

All data generated or analysed during this study are included in the article itself.

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Competing interests

Author Samuel Abraham is a shareholder in GN Corporation, Japan which holds shares of Sophy Inc., Japan., the manufacturers of novel beta glucans using different strains of Aureobasidium pullulans; a

board member in both the companies and also an applicant to several patents of relevance to these beta glucans.

Author Contribution Statement:

S.A. and N.I contributed to conception and design of the study. K.I, S.S and T.H helped with the experiments and analysis. R.S and M.R performed the literature search. S.A, and S.P. drafted the manuscript. N.Y, M.I, V.D and S.S performed critical revision of the manuscript. All the authors read, and approved the submitted version

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Abstract:

Introduction:

Duchenne muscular dystrophy (DMD) is a X-linked fatal myogenic disorder, in which lack of dystrophin leads to significant fibrosis and a progressive loss of muscle mass. Regeneration of the muscles in response to the degeneration is not adequate to contribute to restoration of muscle function. Having proven the anti-inflammatory and anti-fibrotic properties of the 1,3-1,6 B-glucan (Neu REFIX) produced by the N-163 strain of *Aureobasidium Pullulans* in clinical and pre-clinical studies, we have now examined its effects on muscle regeneration in a DMD animal model of mdx mice.

Methods:

Forty-five mice were divided into the three groups, Gr. 1 (n=15): normal mice, Gr.2 (n=15): mdx mice as vehicle, Gr.3 (n=15) mdx mice administered the N-163 β-glucan for 45 days. Expression of CD44 and Myosin heavy chain (MYH3) were evaluated in the skeletal muscles.

Results:

In the Gr.3 (N-163 group), CD44+ve staining in immunohistochemistry was more intense, with an H-score of > 2.0 as opposed to 1.0 in Gr. 2 and 0.1 in Gr. 1. Immunofluorescence staining showed 20% higher MYH3-staining positive area in Gr.3 (N-163 group) than that in the Gr.2.

Conclusion:

Successful muscle regeneration indicated by (i) increased MYH3 expression, characteristic of early myosin and muscle formation, followed by (ii) differentiation evident by an increase in CD44, upon oral feeding of N-163 B-Glucan, have been proven in this study. The earlier reported anti-fibrosis and anti-inflammatory effects in pre-clinical and human clinical studies along with the present study's findings,

unravel the potentials of this safe B-Glucan food supplement as a disease modifying adjunct in the management of DMD and other muscular dystrophies.

Keywords: β-glucan; Duchenne Muscular Dystrophy (DMD); mdx mice; CD44; MYH3

Manuscript Text:

INTRODUCTION:

Dystrophin, a protein that gives the membrane of muscle fibres structural rigidity and integrity, when encoded by a faulty gene leads to Duchenne muscular dystrophy (DMD), an X-linked hereditary severe form of muscular dystrophy [1]. The lack of dystrophin in muscle fibres causes membrane fragility, a high risk of injury, severe inflammation, and oxidative stress, leading to muscle necrosis. Continuous damage result in fibrosis of the muscles and their dysfunction leading to loss of ambulation followed by pre-mature death in the early 20s or 30s due to cardiac and respiratory failure [1]. In muscular dystrophy, though the satellite stem cells are activated to regenerate in response to injury, the invasion of immune cells, primarily macrophages occur together with inflammation leading to improper muscle maturation and progressive replacement by fibrous-fatty tissue ensues [2].

Although a lack of dystrophin is the primary cause of the manifestations of DMD, ensuing chronic inflammation seems to be the key factor curtailing proper muscle regeneration [1]. While there is no definitive cure for the disease, approaches to control inflammation by use of corticosteroids has been a standard of care. However, due to the associated adverse effects of corticosteroids, their continuous administration in all the patients is quite difficult. While gene therapies are in clinical trials, even the approved exon skipping therapies with some benefits in basic and translational studies, haven't yet yielded a clinically satisfactory outcome, which may be attributed to insufficient amelioration of inflammation, fibrosis, apart from limited muscle regeneration and maturation [3].

In this scenario, we need a safe and easily administrable disease modifying approach. We have earlier demonstrated the anti-inflammatory, anti-fibrotic and immune-modulating effects of a biological response modifier B-glucan (BRMG) produced by the N-163 strain of *Aureobasidium pullulans* (*A.pullulans*) in pre-clinical mdx mice model [4] and human clinical studies of DMD [5]. In the mdx

animal study done earlier, this B-Glucan was able to ameliorate inflammation proven by a significant decrease in inflammation score and fibrosis, levels of plasma ALT, AST, LDH, IL-13, and haptoglobin, and an increase in levels of anti-inflammatory TGF-β [4]. In the human clinical study done in 27 patients, anti-inflammatory effects proven by a decrease in IL-6 levels, and anti-fibrotic effects by a decrease in TGF-β levels apart from improved muscle strength and increased plasma dystrophin levels [5] were demonstrated. However, with an objective to evaluate the effects of this N-163 B-Glucan food supplement on muscle regeneration and differentiation, this study was undertaken in mdx mice model of DMD.

METHODS

The guidelines for reporting in vivo animal experiments were followed when conducting this investigation. Male, three-week-old C57BL/10SnSlc mice were purchased from Japan SLC, Inc. (Japan). Male, three-week-old C57BL/10-mdx/Jcl mice were purchased from CLEA Japan (Japan). The animals were treated with care in accordance with the Ministry of the Environment's Act No. 105 of 1 October 1973's Act on Welfare and Management of Animals, its Notice No. 88 of April 28, 2006's Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain, and its Guidelines for Appropriate Conduct of Animal Experiments.

Forty-five mice were included in the study [3] and based on their body weight, the day before the start of treatment were randomized into the following three groups of 15 mice each. Randomisation was performed by body weight-stratified random sampling using Excel software.

Group 1: Normal- Fifteen C57BL/10SnSlc mice were without any treatment until sacrifice.

Group 2: Vehicle- Fifteen mdx mice were orally administered vehicle [pure water] at a volume of 10 mL/kg once daily from days 0 to 45.

Group 3: N-163 β-glucan- Fifteen mdx mice were orally administered with N-163 strain produced B-glucan (Neu-REFIX^{TM}), was provided by GN Corporation Co. Ltd at a dose of 3 mg/kg as API in a volume of 10 mL/kg once daily for 45 days.

The animals were housed in TPX cages (CLEA Japan) and maintained in an SPF facility under controlled conditions of temperature, humidity, lighting (12-hour artificial light and dark cycles and air exchange. Viability, clinical signs (lethargy, twitching, and laboured breathing), and behaviour were monitored daily. Body weight was recorded daily before treatment. The mice were observed for significant clinical signs of toxicity, morbidity, and mortality before and after administration. The animals were sacrificed on day 45 by exsanguination through the abdominal vena cava under isoflurane anaesthesia (Pfizer Inc.). After sacrifice, the quadriceps, gastrocnemius, soleus, plantaris, tibialis anterior, extensor digitorum longus, diaphragm, and myocardium muscles were collected. Individual muscle weights were measured. Each muscle was separated, dissected, and stored for histological analysis [4].

Muscle preparation for histological analysis was done by placing the tragacanth gum on cork disk, with only enough tragacanth gum to provide foundation for the oriented muscle. The tragacanth gum was placed on one end so that the long axis of the muscle is perpendicular to the cork disc. The specimen was rapidly frozen and placed into isopentane cooled in liquid nitrogen. The frozen block was transferred on to dry ice and the isopentane was evaporated for around one hour and stored at -80 °C.

Sections were cut from paraffin blocks of the muscle (TBD) tissue using a rotary microtome (Leica Microsystems). After sectioning, each slide was coded as a number for blind evaluation. Each number was generated using the RAND function of the Excel software, sorted in ascending order, and assigned to the slides. The tissue slides were used for staining. For immunohistochemistry staining of CD44, sections were cut from the frozen blocks and fixed in acetone. Endogenous peroxidase activity was blocked using 0.03% H2O2 for 5 minutes followed by incubation with Block Ace (DaiNippon Sumitomo Pharma Co.Ltd., Japan) for 10 minutes. The sections were incubated with antibody against CD44 (Rabbit Monoclonal Anti-CD44(E7K2y) purchased from Cell Signaling Technology (Danvers, MA, USA)

For immunofluorescence staining of Myosin heavy chain (MYH3), the frozen sections were fixed in freshly prepared 4% paraformaldehyde for 15 min at room temperature and were then permeabilized with 1% Triton X-100 in PBS for 20 min at room temperature. The sections were blocked with blocking solution (0.2% gelatin, 1% BSA and 0.05% Tween 20 in PBS) for 30 min at room temperature. After blocking, the sections were immunostained with primary antibodies diluted in blocking solution at 4 ºC overnight. After washing with TBS containing 0.05% Tween 20 (TBST), the sections were incubated with secondary antibodies diluted in blocking solution for 2h at room temperature. After washing with TBST, the sections were stained with DAPI to label nuclei. The stained sections were mounted with ProLong Gold antifade reagent (Invitrogen, USA). The following antibody was used: Myh3 (sc-53091, Santa Cruz Biotechnology, 1:100). Images were taken with all-in-one fluorescence microscope BZ-X800 (Keyence, Japan). The positive areas of those biomarkers were evaluated using H-score [5] & SketchAndCalc™ software, respectively.

Statistical analyses were performed using the Prism Software 6 (GraphPad Software, USA). Statistical analyses were performed using the Bonferroni's multiple comparison test. Comparisons were made between the following groups: 1) Group 2 (Vehicle) vs. Group 1 (Normal) and Group 3 (N-163 β-glucan). Statistical significance was set at $P < 0.05$. Results are expressed as mean ± SD.

RESULTS

The mean body weight of mice in the vehicle group was significantly lower than that of mice in the normal group but there was no significant difference in the mean body weight between the vehicle group and the N-163 β-glucan.

The H-score is one of the commonly employed endpoints to gauge biomarker expression. Individual cells are first identified using the H-score algorithm and then the cells are categorised as either positive or negative depending on the relative expression of the biomarker of interest. Based on the strength of

the biomarker signal, the positive cells are further divided into high (3+), medium (2+), or low (1+) categories [6]. The proportion of the weighted sum of the number of positive cells to the total number of detected cells determines the H-score. The H-score extracts the biomarker of interest's intensity and percentage from the IHC image. The H-score was 0.1 ± 0.3 in Gr. 1 (Normal), 1 ± 0.18 in Gr. 2 (Vehicle) and 2.16 ± 0.89 in Gr. 3 (N-163), thus a significantly higher H-score in the Neu-REFIX group compared to Gr.2 and Gr.1 (p-value = 0.0002) (Figure 1). SketchAndCalc™ is an irregular area calculator app for all manner of images containing irregular shapes. When calculated using SketchAndCalc, the MYH3 positive area was 1.72 cm^2 with a total area of positively stained cells being 256 units and percentage of positive region was 0.665 in Gr.1. In Gr. 2, the MYH3 positive area was 10.78 cm^2 with a total area of positively stained cells being 272.6 units and percentage of positive region was 3.954. In Gr. 3 (Neu-REFIX) the MYH3 positive area was highest among the groups, 15.11 cm^2 with a total area of positively stained cells being 305.25 units and percentage of positive region was 4.954 (Figure 2)

DISCUSSION:

To address DMD, a disease modifying approach must have the following potentials to be an ideal agent, (i) Amelioration of inflammation and fibrosis due to lack of dystrophin and (ii) regeneration of muscle stem cells and their maturation towards functional muscle cells that should reflect in terms of improved muscle strength. Though the tissue resident satellite stem cell pool gets activated in response to muscle injury, the myofiber formation and maturation are jeopardized by a hostile environment of inflammatory nature in the tissue and therefore, addressing this inflammation becomes crucial.

Earlier studies of the N-163 B-Glucan have reported amelioration of inflammation and immune-over expression in different conditions including COVID-19 [7], Non-alcoholic steatohepatitis (NASH) [8] and multiple sclerosis [9]. The same anti-inflammatory effect was observed in human clinical study of DMD by decrease in inflammatory markers such as decrease of IL-6 and anti-fibrotic effects evident

from decrease in TGF-β levels apart from functional improvement in terms of increased plasma dystrophin levels and medical research council (MRC) grading showing muscle strength improvement [5]. The animal study that followed in mdx mice [4] for evaluation of anti-inflammatory and anti-fibrotic effects proved successful with a significant decrease in inflammation score and fibrosis, levels of plasma ALT, AST, LDH, IL-13, and haptoglobin, with an increase in anti-inflammatory TGF-β, apart from balanced regulation of the quantity of centrally nucleated fibres[4].

Having addressed the inflammation, next we sought to study the effects of the N-163 B-glucan on muscle regeneration and maturation. In the present study we have been able to prove proper muscle regeneration evident from MYH3 expression and muscle differentiation, maturation by increased CD44 expression. Regeneration of the muscle stem cells in DMD is usually considered as pathogenic, because the reservoir of residual stem cells, being they are pre-maturely forced to regenerate to compensate for the loss of muscle undergoing a pathological degeneration. Because of this pre-mature and forced regeneration, besides being considered pathogenic, the reservoir of stem cells are also depleted well before the physiological age, until when the resident stem cells should otherwise remain in situ for a non-pathological response to sporadic damage to the muscles by a regenerative response. In the present study, the outcome of muscle regeneration and maturation can be considered normal and not pathogenic [1] because of the decrease in the inflammatory markers apart from improvement in muscle function evident from increase in MRC muscle grading in the 45 days clinical trial [5] of the same N-163 B-Glucan in human patients [4]. Also, there was improvement in gut microbiome of these patients [10] which are relevant to anti-inflammatory and anti-fibrotic effects, connecting the gut-skeletal muscle axis [11] phenomena, contributing to recovery.

Following injury, skeletal muscle can heal and regenerate by the action of endogenous stem cells. Damaged fibres generate chemotactic and growth factors that trigger an immunological response, activate dormant muscle satellite cells, and cause them to proliferate and relocate to the site of injury

during this repair process [12]. Satellite cells merge with one another and with injured fibres in order to restore muscle integrity when debris is cleared. In DMD and mdx mice, the disease similarly begins with the same process; however, endogenous myogenic cells having the same genetic flaw as mature muscle, makes the newly regenerated fibres vulnerable to injury. This causes a protracted inflammatory response, recurrent cycles of degeneration and reparative regeneration, and eventually exhaustion of the stem cells in the muscle [12]. Class II or conventional myosins, or sarcomeric myosins, are found in mammalian striated muscle. Each myosin molecule consists of two heavy chains (MyHCs), two essential light chains (MLCs), and two regulatory MLCs. Several isoforms that are encoded by various genes contain MyHCs and MLCs in their various forms. Most of these genes are also expressed in growing skeletal muscle, including two MyHC isoforms called embryonic and neonatal (or perinatal) myosins, which are encoded by the genes MYH3 and MYH8, respectively [13]. These embryonic and neonatal (or perinatal) myosins are highly expressed during the early stages of muscle development, are downregulated after birth, and are re-expressed during muscle regeneration. The 20% higher MYH3 expression in the present study therefore validates the occurrence of higher muscle regeneration apart from proper stem cell activation in the N-163 B-glucan treated mice. In another recent study, CD44+ myogenic cells isolated from muscle biopsies have been reported to indicate that the cells differentiate into skeletal muscle in vitro and in vivo and restore dystrophin expression and strength [14]. Therefore, higher intensity of CD44 staining in the muscle biopsies in the present study indicates proper muscle differentiation, maturation following regeneration. All these amount to the efficacy of N-163 strain of *A.pullulans* B-Glucan in eliciting proper muscle regeneration and differentiation of the regenerated early stage muscle cells to mature muscle fibres in DMD.

This is a study in mdx mice and being an animal study, the muscle regeneration and maturation by immunohistochemical and immunofluorescence studies need to be validated in human muscle biopsies which however is invasive. Nevertheless, in the earlier human clinical study using N-163 beta-glucan,

the improvement noted in muscle strength and function by MRC scale [5] correlates with the contribution of N-163 in enhancing muscle stem cell proliferation and differentiation to functional muscle tissue. Also, in our earlier study we have proven the gut microbiome improvement in terms of inflammation [10] but not directly in terms of skeletal muscle regeneration which needs to be studied in human clinical trials. The success of the different pharmaceutical approaches is gauged by their effect on the production of dystrophin which is usually not expected out of DMT. But in our human clinical study, dystrophin levels also increased after N-163 B-Glucan food supplement [5]. Going further, understanding the molecular mechanisms connecting the reconstitution of gut microbiome, enhancement of plasma dystrophin and muscle regeneration will help in unravelling the processes that contribute to the DMT effects of this safe N-163 B-Glucan food supplement.

CONCLUSION:

Regeneration of muscles and their maturation towards muscle cells improve significantly after consumption of Neu-REFIX beta glucans, which are safe food supplements. Having earlier proven the improvement of muscle strength, anti-inflammatory biomarkers and beneficial reconstitution of gut microbiome of relevance in clinical pilot studies [5,10] along with amelioration of skeletal muscle fibrosis, inflammation in pre-clinical studies [4], this N-163 strain of *A.pullulans* produced B-1,3, 1-6 Glucans are worth considering as a disease modifying therapeutic (DMT) adjuvant in slowing down the progress of DMD after relevant validations in larger long term clinical studies.

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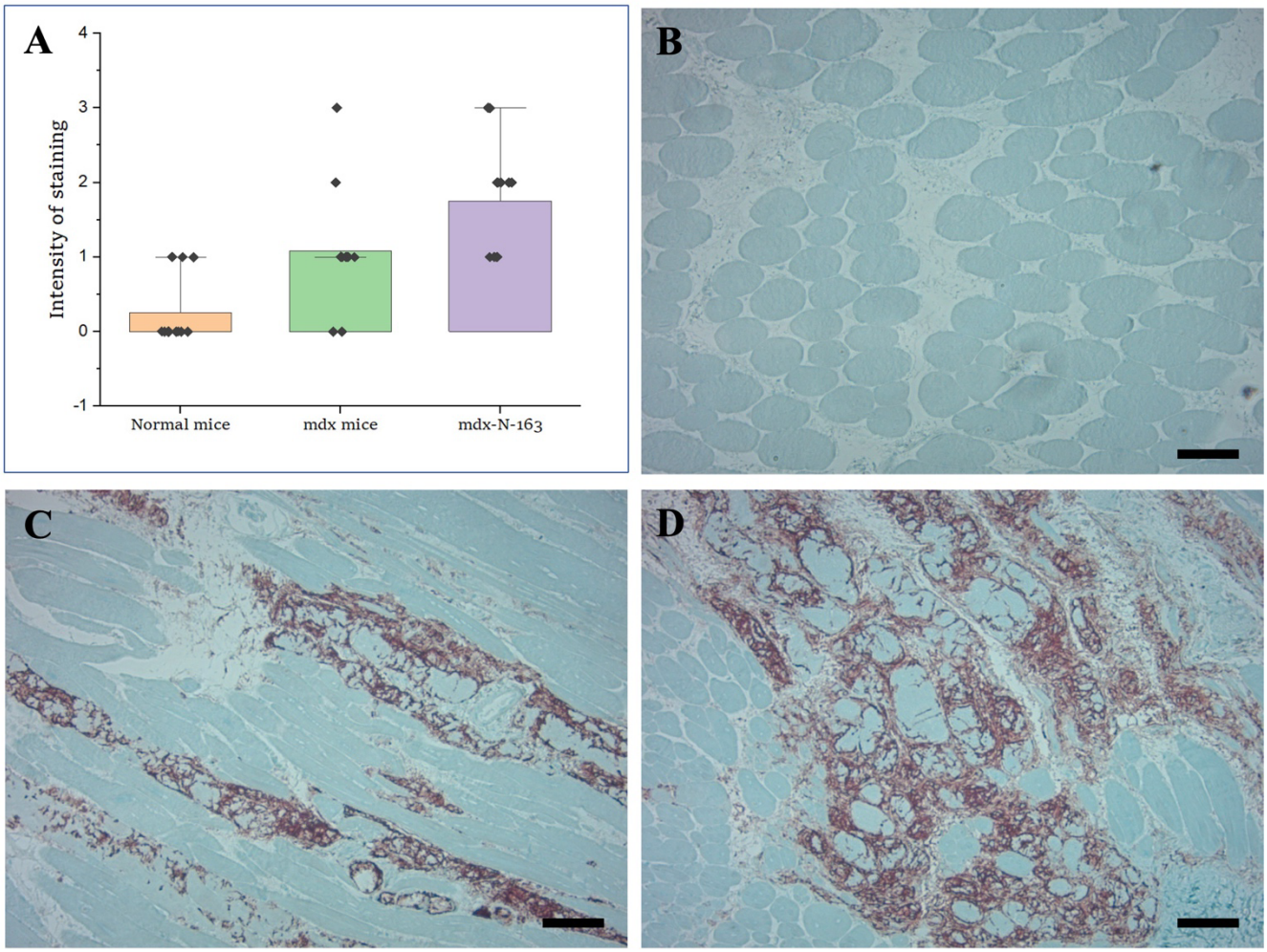

Figure 1: Immunohistochemistry staining of CD44 in muscle of mdx mice; A. Comparison of H-SCORE between the groups; B. (Gr. 1) Normal mice; C. (Gr.2) mdx mice - Vehicle group and D. (Gr.3) mdx mice – N-163 group. Scale bar: 100 µm

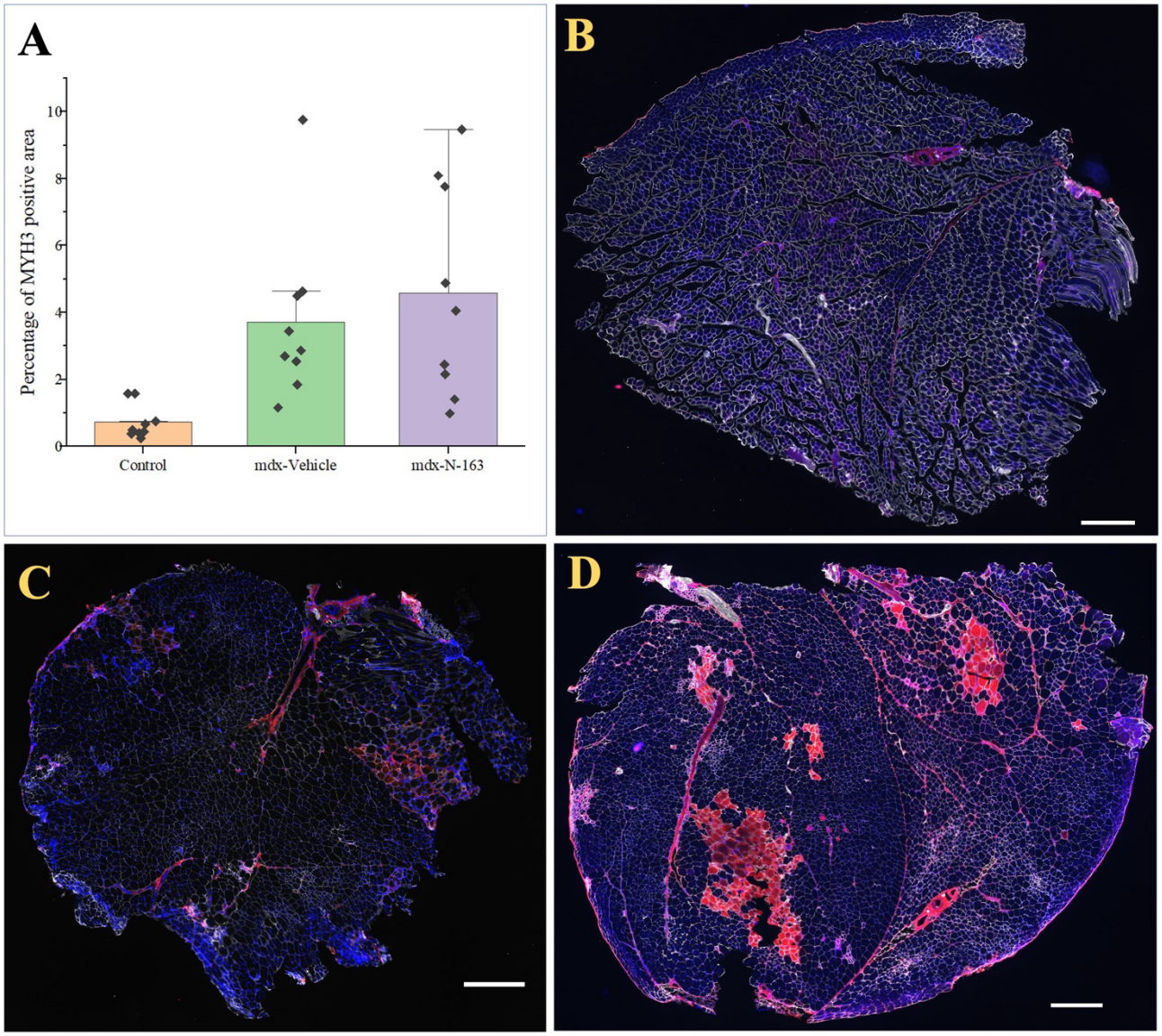

Figure 2: A. Percentage of MYH3 positive fibres in the three groups; B. (Gr. 1) Normal mice; C. (Gr.2) mdx mice - Vehicle group and D. (Gr.3) mdx mice – N-163 group; Scale bar: 100 µm