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INCESSANT CYCLES OF DYSTROPHIC MYOFIBER DEGENERATION AND REGENERATION RESULT IN UBIQUITOUS CENTRALLY-NUCLEATED FIBERS IN SKELETAL MUSCLES OF X-CHROMOSOME-LINKED MUSCULAR DYSTROPHY (MDX) MOUSE

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Duchenne muscular dystrophy (DMD) is an X-linked recessive, degenerative disorder and is the most prevalent, devastating and fatal pediatric neuromuscular disorder in the world. DMD is primarily caused by loss-of-function mutations in the dystrophin gene that result in either partial and dysfunctional domains in dystrophin protein or complete absence of it, leading to contraction-induced necrosis of skeletal muscle fibers and eventual replacement with adipose and connective tissue. Ultimately, rapid and subjugating progression of myofiber degeneration and necrosis, along with gradual attenuation and failure of regenerative capacity, rather than failure of muscle development, result in the crippling phenotype of DMD. The X-chromosome-linked muscular dystrophy (*mdx*) mouse is genetically homologous to human DMD but exhibits a milder phenotype and evades severe end-stage histopathology. Histopathologic analysis revealed that skeletal muscles of *mdx* mice exhibit drastically elevated levels of centrally-nucleated myofibers, similar to humans with DMD, as a result of incessant cycles of dystrophic myofiber degeneration and regeneration.

Duchenne muscular dystrophy – dystrophin – mdx mouse – necrosis - myofiber degeneration and regeneration – centrally-nucleated fibers

Դյուշենի մկանային դիստրոֆիան (ԴՄԴ) X–քրոմոսոմի հետ կապված ռեցեսիվ, դեգեներատիվ խանգարում է և աշխարհում ամենատարածված, ամենածանրընթաց ու ամենամահացու մանկական նյարդամկանային հիվանդությունն է: Դիստրոֆին գենի մուտացիաները բերում են դիստրոֆին պրոտեինի բացակայության կամ դիսֆունկցիայի, որը հանգեցնում է կմախքային մկանների մանրաթելերի կրճատմամբ պայմանավորված նեկրոզի և նրանց՝ ճարպային և շարակցական հյուսվածքներով փոխարինմանը: Ի վերջո, ԴՄԴ-ի անդամալույծ դարձնող բարդ ֆենոտիպը պայմանավորված է ոչ թե մկանների թերզարգացմամբ, այլ մկանների արագ զարգացող դեգեներացիայով, նեկրոզով և մկանների ռեգեներացիոն ունակության աստիճանական խախտմամբ: X-քրոմոսոմով պայմանավորված մկանային դիստրոֆիայով *(mdx)* մուկը գենետիկորեն համանման է մարդկային ԴՄԴ-ին, բայց ցուցաբերում է ավելի մեղմ ֆենոտիպ և շրջանցում է վերջին փուլին բնորոշ ծանր հիստոպաթոլոգիան: Հիստոպաթոլոգիական վերլուծությունը ցույց տվեց, որ *mdx* մկան կմախկային մկանները, ԴՄԴ-ով հիվանդ երեխաների մկանների նման, պարունակում են խիստ բարձր քանակով կենտրոնական կորիզով մանրաթելեր, ինչը վկայում է դիստրոֆիկ մկաններում անընդհատ կրկնվող դեգեներատիվ և ռեգեներատիվ ցիկլերի մասին:

Դյուշենի մկանային դիստրոֆիա – դիստրոֆին – mdx մուկ – նեկրոզ – մկանաթելերի դեգեներացիա և ռեգեներացիա – կենտրոնական կորիզով մանրաթելեր

Мышечная дистрофия Дюшенна (МДД) является рецессивным дегенеративным расстройством, сцепленным с Х-хромосомой, и самым распространенным, тяжелотекущим и смертельным педиатрическим нервно-мышечным заболеванием в мире. При МДД мутации гена дистрофина приводят к частичной или полной дисфункции протеина дистрофина или к его отсутствию вообще, в результате чего развивается некроз волокон скелетных мышц, обусловленный их сокращением, и перерождение их в жировую и соединительную ткань. В итоге инвалидизирующий фенотип МДД обусловлен не недоразвитием мышц, а их быстропрогрессирующей дегенерацией и некрозом и постепенной потерей регенерационной способности мышц. Модель мыши с мышечной дистрофией, сцепленной с Х-хромосомой (*mdx*), генетически гомологична МДД человека, но проявляется менее выраженным фенотипом и обходит тяжелую гистопатологию, характерную для конечной стадии болезни. Гистопатологический анализ показал, что скелетные мышцы *mdx* мыши имеют резко повышенный уровень центральноядерных мышечных волокон, как и у больных МДД детей, что является результатом непрерывно повторяющихся циклов дегенерации и регенерации дистрофических мышечных волокон.

Мышечная дистрофия Дюшенна – дистрофин – mdx мышь – некроз – дегенерация и регенерация мышечных волокон – центральноядерные мышечные волокна

Duchene muscular dystrophy (DMD), an X-chromosome-linked recessive disorder, is the world's most common, severe, and lethal pediatric neuromuscular disorder [9, 10, 15, 17]. It is caused by loss-of-function mutations, mainly large-scale deletions and duplications, in the dystrophin gene that result in either complete absence or loss of critical functional domains of dystrophin protein [10, 15-17]. This initiates a complex cascade of prominent muscular dystrophy events leading to contraction-induced necrosis of skeletal muscle fibers and their eventual replacement with adipose and connective tissue [9, 10, 15-17].

Various animal models of muscular dystrophy have been identified, generated and investigated with the X-chromosome-linked muscular dystrophy (*mdx*) mouse currently being the most highly utilized animal model of DMD [1, 4, 12]. The *mdx* mouse has loss of-function mutation in the highly conserved dystrophin gene (premature stop codon in exon 23) resulting in deficiency of the highly conserved dystrophin protein in muscle thus it is genetically homologous to human DMD [1, 13, 18]. Both *mdx* mice and boys with DMD have striking elevations in serum creatine kinase (SCK) levels and total calcium content in muscle cells [5, 11, 14]. However, *mdx* mice exhibit a more benign clinical phenotype compared to the human disease as they remain reproductively viable, appear behaviorally normal, have near normal life expectancy, exhibit cage activity indistinguishable from wild type mice and do not develop crippling muscle weakness [2, 4, 15].

In the early stages of the disease the human DMD and *mdx* mouse skeletal muscles share common histopathologic characteristics such as incessant cycles of widespread degeneration and necrosis of individual dystrophic muscle fibers with infiltration of macrophages, accompanied by extensive myofiber regeneration, resulting in ubiquitous fiber size variation and large numbers of centrally-nucleated fibers in humans [5, 20]. However, gradual impairment of the dystrophic muscle fiber regenerative capability in humans with DMD results in failure to replace necrotic myofibers, causing fibrosis and progressive decrease in muscle fiber numbers, and eventually resulting in an insufficient number of muscle cells for mobility and respiration [3, 7, 10]. On the contrary, in the later stages the disease process in *mdx*, the mice is considerably milder and less progressive as the characteristic gradual failure of myofiber regenerative capability, extensive connective tissue proliferation and fatty infiltration observed in human DMD muscles are less evident in the *mdx* muscles [2, 5, 6, 8, 19, 20]. Thus, despite undergoing extensive

skeletal muscle degeneration at an early age, the *mdx* mouse somehow mitigates the severe end-stage muscle histopathology seen in humans with DMD [2, 5, 6, 8, 19, 20]. This study presents an in depth analysis of the pathology of gastrocnemius muscles of adult *mdx* mice compared to age-matched wild-type mice.

Materials and methods. All animal experimental procedures were conducted in strict accordance with the guidelines of the United States National Institutes of Health (NIH) Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Subcommittee on Research Animal Care (SRAC), which serves as the Institutional Animal Care and Use Committee (IACUC) for Massachusetts General Hospital (MGH) and Harvard Medical School (HMS). Adult wild-type (WT) (C57BL/10ScSnJ) and *mdx* (C57BL/10ScSn-DMD^{mdx}/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in standard mouse cages in a pathogen-free facility, under constant room temperature and humidity, and 12/12 hour light/dark cycle. Both water and Prolab IsoPro RHM 3000 food (LabDiet, St. Louis, MO, USA) were available *ad libitum*.

Adult male WT (n = 5) and mdx (n=5) mice were euthanized at 28 weeks of age via carbon dioxide inhalation and cervical dislocation. Skeletal muscles were excised and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA) and frozen in liquid nitrogencooled isopentane (Sigma-Aldrich, St. Louis, MO, USA) for cryosectioning. Ten micron-thick (10μm) gastrocnemius muscle transverse mid-belly cross-sections, obtained at -22^{o}C with a Microm HM505E cryostat (Microm International GmbH, Walldorf, Germany), were collected onto Tissue Path Superfrost Plus Gold slides (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -80^{o}C in a Revco Ultima II freezer (Thermo Fisher Scientific, Waltham, MA, USA) until analysis.

Hematoxylin and Eosin (H&E) staining was carried out on gastrocnemius muscle cryosections from all mice. Formaldehyde-fixed cryosections were re-hydrated in graded alcohols (ice-cold 100% ethanol for 60 s, 95% ethanol for 60 s, 70% ethanol for 60 s, and 50% ethanol for 60 s), and washed in distilled water (3 times, 60 s each). All slices were then sequentially immersed in Mayer's Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) for 20 s followed by distilled water, Bluing Reagent (Lerner Labs, Pittsburgh, PA, USA), 70% ethanol, and Eosin Y (Acros Organics, Geel, Belgium) for 30 s each. Afterwards, all slices were dehydrated in graded alcohols (50% ethanol, 70% ethanol, 95% ethanol (2 separate rounds), and 100% ethanol (2 separate round) for 30 s each) and lastly cleared in Xylenes (Fisher Scientific) for 5 min. The H&E-stained slices were then cover-slipped with Permount mounting media (Thermo Fisher Scientific, Waltham, MA, USA) and imaged, keeping imaging parameters identical across all images. All H&E-stained slice images were analyzed for centrally-located nuclei with the entire field of each 10X-magnification image used during analyses.

All images were captured on a Nikon Eclipse 80i fluorescent microscope (Nikon Instruments, Tokyo, Japan) equipped with a Retiga 4000R Q-Imaging CCD Camera (North Central Instruments, Plymouth, MN, USA). NIS Elements Basic Research 3.0 (Nikon Instruments, Tokyo, Japan), Image J (National Institutes of Health, Bethesda, MD, USA), Adobe Photoshop CS4 Extended (Adobe Systems, San Jose, CA, USA), GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) and SigmaPlot 13 (Systat Software, San Jose, CA, USA) scientific data analysis and graphing software were used for image analyses and figure preparation.

Results and Discussion. H&E-stained skeletal muscles of adult WT mice exhibited a dense arrangement of characteristically uniform muscle fibers with peripherally-located nuclei (fig. 1). By contrast, pathology of age-matched mdx skeletal muscles revealed myocytes with predominantly centrally-located nuclei as well as substantial variability in myofiber cross-sectional area. These findings are indicative of various stages of widespread and persistent degeneration and regeneration of muscle fibers. Additionally, skeletal muscles of adult *mdx* mice exhibit more prominent regions connective tissue compared to age-matched WT mice (fig. 1) of fiber degeneration with infiltration of inflammatory cells and increased interstitial connective tissue compared to age-matched WT mice (fig. 1).

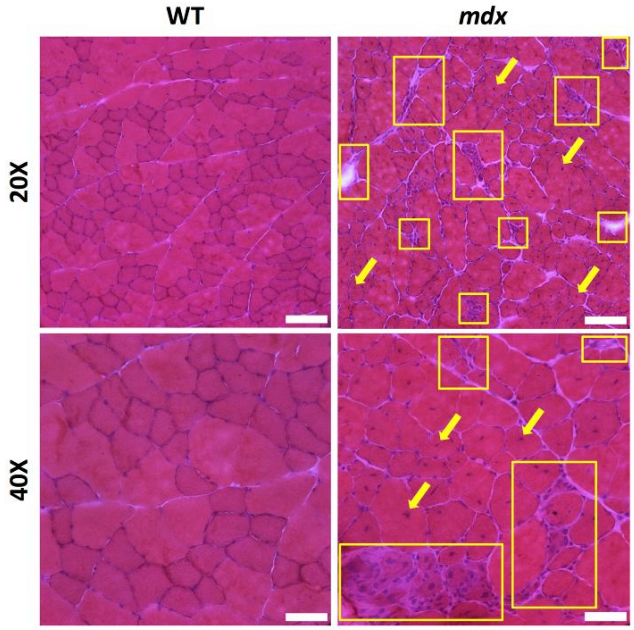

Fig. 1. Histopathology of hematoxylin and eosin-stained gastrocnemius muscles of adult agematched WT and *mdx* mice. Arrows point to examples of centrally-located nuclei of individual myofibers. Rectangles accentuate areas of necrosis of dystrophic muscle fibers with connective tissue proliferation and fat-ty infiltration. Scale bar equals 100µm for 20X-magnification images and 50µm for 40X-magnifi-cation images.

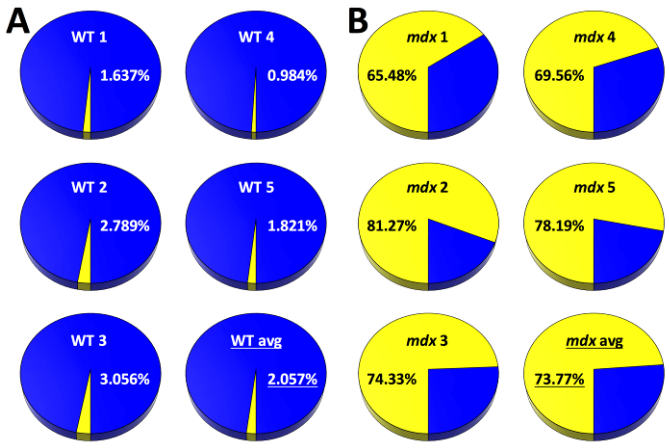

Fig. 2. Drastically elevated number of centrally-nucleated myofibers was identified in gastrocnemius muscles of adult *mdx* mice (B) compared to age-matched WT mice (A).

Quantitation of centrally-nucleated myocytes in H&E-stained cryosections revealed that adult WT mice contained predominantly peripherally-nucleated myofibers in skeletal muscles with centrally-nucleated myofibers representing merely 2% of all fibers (fig. 2A). In contrast, skeletal muscles of age-matched mdx mice contained predominantly centrally-nucleated myofibers, representing approximately 74% of all fibers. Thus gastrocnemius muscles of adult mdx mice contained 3486% more centrally-nucleated fibers than age-matched WT mice ($p<0.005$) (fig. 2B).

In summary, histopathologic analysis of centrally-nucleated myocyte counts from H&E-stained skeletal muscles (fig. 1, 2) revealed that adult *mdx* mice exhibit drastically elevated levels of non-peripherally-nucleated muscle fibers compared to age-matched WT mice. This finding, in conjunction with variations in individual fiber size, is indicative of pervasive cycles of dystrophic muscle fiber degeneration and regeneration in skeletal muscles of *mdx* mice, similar to humans with DMD.

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