

The use of fluorescent dextrans as a marker of sarcolemmal injury

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Summary. We investigated the use of intravenously injected fluorescent dextran molecules (FDx) as a histological marker of sarcolemmal injury. Using fluorescent microscopy, uptake of FDx (average MW 10 kD) was assessed in sections of quadriceps muscles from three models: 1) normal (C57BL/10SnJ) mice, 2) normal mice run downhill (0, 3, and 7 days post exercise), and 3) non-exercised *mdx* (dystrophin-deficient) mice. These were compared to serial sections stained with hematoxylin and eosin (H&E). In control muscles, strong fluorescence was seen between fibers (intercellular). Intracellular FDx was observed within cells of the quadriceps from normal mice run downhill at days 0 and 3 post exercise, but not at day 7. On H&E staining, muscle pathology was not observed until day 3, with regeneration by day 7. Intracellular FDx was also observed within *mdx* muscles, particularly in fibers that appeared pre-necrotic on H&E stained sections. FDx appears to be useful as a histological marker of changes in sarcolemmal integrity associated with muscle injury from eccentric exercise or muscle disease.

Key words: Muscle injury, Histology, Fluorescein Isothiocyanate-Dextran, Eccentric, Exercise

Introduction

The detection of skeletal muscle injury as a result of exercise or disease has commonly been accomplished by observation of degeneration and necrosis using conventional histological techniques such as staining with hematoxylin and eosin (H&E). Using these methods muscle necrosis is evident 2-4 days following strenuous eccentric exercise. Regeneration is usually evident by 7 days post injury. Shortly after exercise, when CK elevation occurs, there is generally no evidence of injury detected by routine histology. The

exact mechanisms of CK release from muscle cells is unknown, but is felt to be linked to changes in sarcolemmal permeability. Recently, studies by McNeil and Khakee provided the first direct evidence that sarcolemmal integrity was substantially altered following eccentric exercise, as shown by muscle fiber uptake of albumin and fluorescent dextrans (FDx), even though conventional histological techniques did not reveal any evidence of injury (McNeil and Khakee, 1992).

The purpose of this study was to investigate the usefulness of FDx as a histological marker of sarcolemmal disruption. Fluorescent microscopy was used to assess the uptake of intravenously injected fluorescein isothiocyanate labelled dextran molecules (FDx) by quadriceps muscle fibers in three models: 1) control mice, 2) normal mice run downhill, and 3) adult *mdx* mice. The dystrophin-deficient muscles of *mdx* mice were used because it has been hypothesized that lack of dystrophin, a protein normally found on the cytoplasmic surface of the sarcolemma, results in membrane instability (Watkins et al., 1988; Campbell and Kahl, 1989; Hutter, 1992).

Materials and methods

Healthy C57BL/10SnJ and *mdx* mice were bred and maintained in the American Association for the Accreditation of Laboratory Animal Care (AAALAC) accredited, specific pathogen free facilities maintained at our university. Our institution's guidelines were followed regarding the care and use of laboratory animals. All chemicals were purchased from the Sigma Chemical Co., St. Louis, MO. All mice used were mature adults (age range 120-190 days, weighing from 27 to 32 grams).

Exercise Paradigm

Approximately 50 untrained C57 adult mice were randomly assigned to the experimental or control group.

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In our studies we used an exercise protocol similar to that used in previous studies (Armstrong et al., 1983; McNeil and Khakee, 1992). The mice were run on a variable speed treadmill, set at an decline of 20 degrees. The exercise regimen consisted of an initial 5 minute warm-up period at 18 meters per minute (m/min). This was followed by five 30 minute bouts at a speed of 21 m/min, with a 5 minute rest between bouts. Out of 8 mice that started each run, 5 or 6 usually finished and were used in the experiment. Blowing animals with a stream of air and, when necessary, electrical stimulation were used to encourage the animals to run. At the end of the final 30 minute bout, the animals panted heavily and did not willingly move around their cage. Studies were conducted immediately (day 0), and at days 3 and 7 post-exercise. Prior studies have shown that muscle necrosis peaks at day 3, with regeneration evident by day 7, with the quadriceps showing the most damage (McNeil and Khakee, 1992). The *mdx* mice were treated the same as the non-exercised C57 mice.

Mice were anesthetized with sodium pentobarbital. Cardiac puncture was performed to obtain blood for CK determinations. After bleeding the quadriceps muscles were removed for histology.

Creatine Kinase

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital, 65 mg per kg, and blood was removed via cardiac puncture. The blood was placed in heparinized tubes and centrifuged. After centrifugation the plasma was collected and stored at -80 °C. CK was determined on plasma diluted 1/20 in a buffer consisting of 10 mM Tris HCl, 0.25 mM sucrose, 0.2 mM EDTA, and 1.0 mM dithiothreitol at pH 7.4. CK determinations were made using a commercially-available kit (Ciba-Corning), based on the method of Szasz et al. (1976), and read at 340 nm on a Gilford automatic sampling/recording spectrophotometer.

Histology

The quadriceps were surgically excised, frozen in nitrogen-cooled isopentane, and sectioned at 10 microns in a cryostat at -20 °C. Histochemical staining procedures and qualitative analysis of the morphology and pathology of the muscle fibers were performed using a modified Dubowitz method (Dubowitz, 1985). Staining was done with H&E for general histopathology, periodic acid Schiffs (PAS) stain for glycogen, succinic dehydrogenase (SDH) for mitochondrial (oxidative) activity and acid and alkaline ATPases for fiber type classification using a modified Parry & Parslow technique (Parry and Parslow, 1981). Histopathology was shown by 1) internal nuclei, 2) inflammation/phagocytosis, and 3) necrosis.

FDx of molecular weight (MW) 10 kD was used for the fluorescence studies. The bound dextrans

are uncharged molecules which are normally impermeant to the cell membrane (McNeil and Khakee, 1992). The 10 kD weight was chosen as most suitable based on a prior study by McNeil and Khakee (1992), as well as preliminary studies done in our lab using 5, 10, 70, and 150 kD MW dextrans. We encountered problems with high background labelling using the 5 kD dextran, even in unexercised normal controls, while the heavier dextrans were not well taken up by any of the muscle models we used. Our preliminary studies also determined the FDx would not interfere with the plasma CK. A 150 mg/ml solution of FDx was prepared in phosphate buffered saline and 0.02 ml of this solution was administered to the test groups by tail vein injection. FDx was administered to the day zero group immediately after the exercise regimen and the animals terminated 4 hours later. All other animals (control C57 and *mdx*) were injected 4 hours prior to termination. The presence of FDx in muscles fibers was assessed by direct visualization using fluorescent microscopy and this was compared to the serial sections stained with H&E.

Results

Creatine Kinase

CK results are shown in Fig. 1. Downhill running significantly raise plasma CK levels at day zero, but they returned to normal levels by day 3. Plasma CK for *mdx* mice was significantly greater than that of normal control mice.

Figure 1.

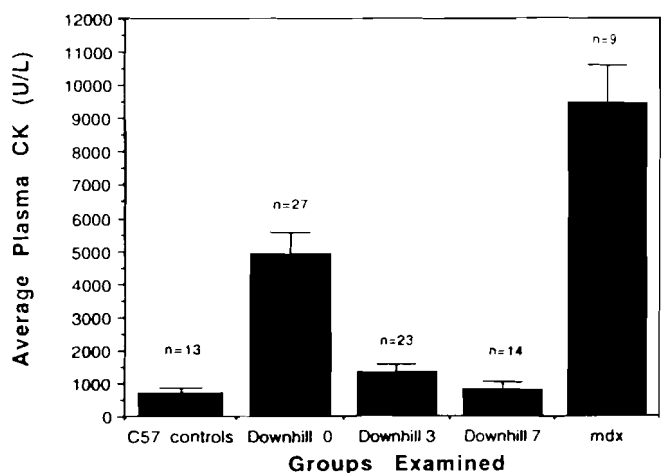


Fig. 1. Data are means \pm SEM of plasma creatine kinase levels of control mice, mice that were exercised to exhaustion on a treadmill by running downhill (downhill 0= Immediately after exercise, downhill 3= 3 days after exercise, downhill 7= 7 days after exercise), and *mdx* mice. Values for normal mice at downhill 0 and *mdx* mice are significantly elevated ($p < 0.05$) over the control value.

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Histology

Histological findings are shown in Figs. 2, 3.

Normal controls

Fiber typing showed that the quadriceps is composed of approximately 5% type I, 35% type IIa and 65% type IIb fibers. In all control muscles, strong fluorescence was seen between fibers (intercellular), confirming adequate distribution of the FDx to the skeletal muscle (Fig. 2-C). There was no FDx observed within the cells of control muscles.

Downhill running

At day zero following downhill running none of the quadriceps muscles examined showed evidence of histopathology using H&E stain. At day zero in the mice treated with FDx, frequent intracellular fluorescence labelling was observed (Fig. 2-0). A phenomenon observed in all of the day zero muscles from mice run downhill was that focal areas of the fibers appeared to have indistinct borders. By day 3 histopathology was exhibited in 30-50% of quadriceps fibers examined. (Fig. 2-3). Necrosis was observed in areas occupied predominantly by type IIa and IIb fibers. The overall

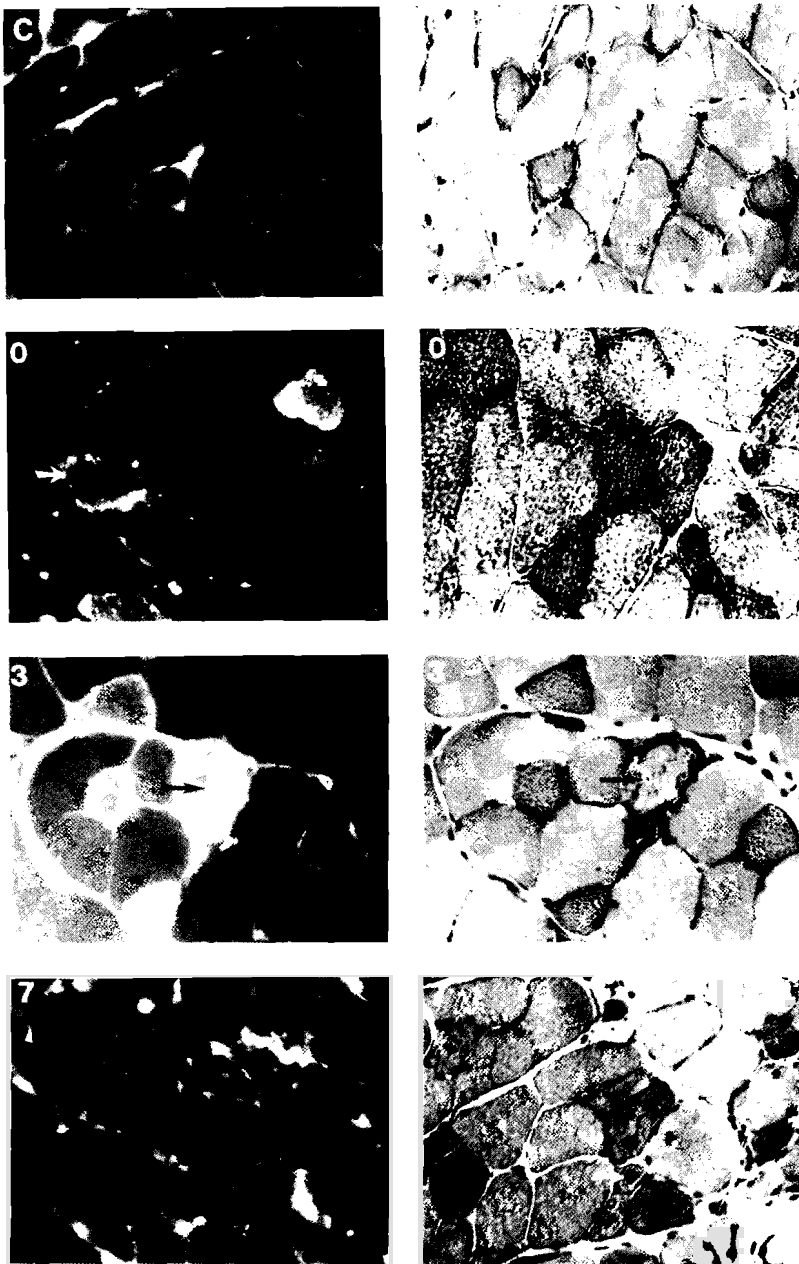


Fig. 2. Photomicrographs of quadriceps muscles from control mice, mice ran exhaustively downhill. C= control; 0= Immediately after treadmill exercise; 3= 3 days after treadmill exercise; 7= 7 days after treadmill exercise. The left side depicts sections from mice injected with 10,000 MW fluorescent dextran. The right side is sections of the same muscle stained with hematoxylin and eosin. **C.** The left FDx shows intercellular fluorescence. The right H and E does not show any abnormalities. **0.** FDx shows intracellular staining. Note cells with indistinct borders (arrow). The H&E does not show any evidence of histopathology. **3.** Intracellular fluorescence corresponding to areas of necrosis shown by H&E (arrows). **7.** Fluorescence is mainly intercellular as seen in the control. H&E staining shows areas of regeneration (arrow). x 200

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amount of intracellular fluorescence had increased by day 3 and correlated with pre-necrotic fibers visible on H&E stains. By day 7, 30-40% of the quadriceps fibers showed evidence of regeneration as indicated by the presence of small, centrally nucleated fibers. (Fig. 2-7). Evidence of inflammation (mononuclear cells) was still present at day 7 in 1-15% of fibers. By day 7 post exercise the pattern of fluorescence was similar to that of the controls.

mdx

H&E sections from the quadriceps of *mdx* mice showed widely scattered pre-necrotic cells and evidence of regeneration (as indicated by internal nuclei) (Fig. 3-a). The corresponding FDx section (Fig. 3-b) of the *mdx* quadriceps showed fluorescence within the muscle cells, especially those that are pre-necrotic.

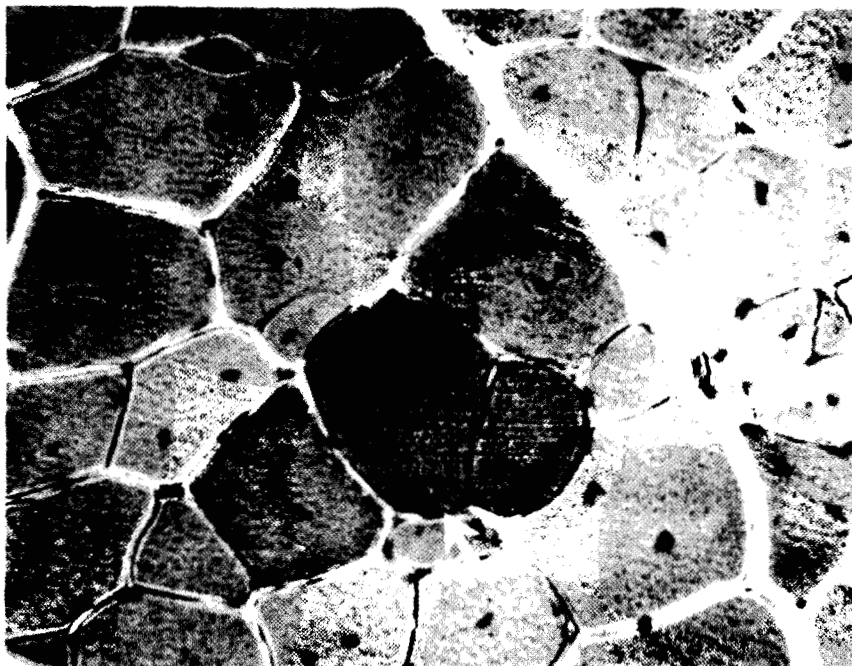
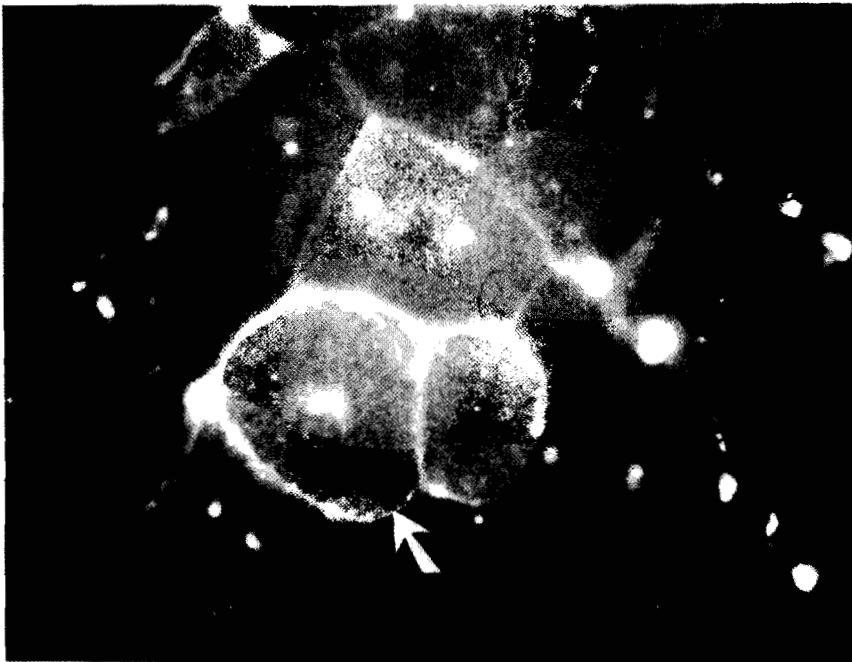


Fig. 3. Photomicrographs of quadriceps muscles from adult *mdx* mice. The left side depicts sections from mice injected with 10,000 MW FDx. The right side is sections of the same muscle stained with hematoxylin and eosin. **a.** FDx shows intracellular staining. **b.** H&E shows areas of regeneration as indicated by central nuclei and pre-necrotic cells (arrow). x 200

Discussion

Our study demonstrates that FDx has potential use as an early marker of sarcolemmal injury. In the mice run downhill, significant elevation of plasma CK immediately after exercise confirmed that muscle injury had occurred. Although at day zero no evidence of histopathology was detected using H&E sections, there were areas of FDx positive cells throughout the quadriceps muscles, as well as cells present with indistinct borders, indicating widespread sarcolemmal disruption. These observations support the work of McNeil and Khakee (1992), who made similar findings in rat skeletal muscle at 2 and 24 hours after exercise. Following exhaustive eccentric exercise they detected the presence of intracellular albumin and 10 kD FDx in rat skeletal muscle, indicating changes in sarcolemmal permeability, and suggested that these plasma membrane disruptions are an early form of structural damage to eccentrically exercised muscles. The fact that muscles from control mice did not take up the FDx (as muscles from the downhill run and *mdx* mice did) indicates that the membrane disruptions are distinct from endocytotic and exocytotic routes of entry into the cell.

At day 3 we observed wide areas of necrosis, pre-necrosis and FDx positive cells throughout the quadriceps muscle. The indistinct borders were no longer evident and it appeared on serial sections that the necrotic and pre-necrotic cells correlated with the FDx positive cells. Thus areas of the quadriceps muscle were apparently unable to adapt to the injury observed at day zero and the initial disruption progressed to necrosis and cell death. The increased permeability of the necrotic cells to the FDx is not surprising since these cells were undergoing active destruction by macrophages and lysosomal enzymes. Thus, there appears to be an association between sarcolemmal disruption as indicated by intracellular FDx labelling and progression to degeneration following exercised-induced injury.

At day 7 in the quadriceps there were indications of regeneration (small, centrally nucleated fibers) and the FDx pattern had returned to that of controls. This indicates that the sarcolemma had returned to normal and had become impermeant to FDx.

The *mdx* mouse was used as a model of muscle with sarcolemmal instability as a result of a genetic defect. The *mdx* mouse muscles undergo early bout of degeneration, infiltration by macrophages and necrosis, followed by regeneration. The muscles of mature *mdx* mice are made up of mostly regenerated fibers. Nonetheless, plasma CK remains significantly elevated

even after regeneration is complete (Glesby et al., 1988). The uptake of the FDx into the *mdx* quadriceps provides evidence that these regenerated muscles have a sarcolemmal defect that allows uptake of FDx and have different membrane properties than the normal regenerated muscle of the C57 mice studied.

Further experiments are needed using different molecular weight dextrans, as well as charged molecules, to better ascertain the nature of permeability changes in the sarcolemma in both injury and disease. Alternative fluorescent colors could be used for different size dextrans, allowing for concomitant administration of different size markers. Different timing sequences of administering the labelled compounds should be investigated, particularly in the early phases of the post-injury model. Nonetheless, FDx appears to be useful for early detection of sarcolemmal injury not shown by standard histological techniques.

Acknowledgements. Supported by Research and Training Center Grant H133B80016 from the National Institute on Disability and Rehabilitation Research, United States Department of Education.

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Accepted January 25, 1994