

**Linda K. McLoon**

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## Focusing on fibrosis: halofuginone-induced functional improvement in the *mdx* mouse model of Duchenne muscular dystrophy

Linda K. McLoon

Departments of Ophthalmology and Neuroscience, University of Minnesota, Minneapolis, Minnesota

DUCHENNE MUSCULAR DYSTROPHY (DMD) is a devastating X-linked disease caused by mutations in the gene encoding for dystrophin and characterized by widespread muscle degeneration that leads to death (20). The major focus of research has been directed toward alleviating the primary genetic deficit, using gene therapy and myoblast transfer approaches to promote dystrophin expression in muscle fibers. Unfortunately for patients with this ultimately fatal disease, there is currently no cure. Thus the development of complementary and supportive therapies that slow the progression of the disease and allow patients to have an improved quality of life is critically important.

In the DMD patients, their skeletal muscles are subjected to ongoing cycles of skeletal muscle degeneration and regeneration. However, fibrosis and inflammation are also prominent features of the disease (30). This genetic disorder is complex, and the timetable of specific skeletal and cardiac muscle involvement varies significantly from patient to patient (27). This is due to the differential development over time of inflammatory and fibrotic changes within limb muscles, the diaphragm, and other respiratory muscles, as well as within cardiac tissue (36). The inflammatory reaction and resultant fibrosis are poorly understood but, in general, is thought to be the sequelae of inflammatory infiltrate caused by ongoing muscle necrosis in DMD patients. The fibrosis that results from this inflammatory response plays an important role in promoting the pathology that leads to patient death. Thus, despite the primary pathology of skeletal muscle degeneration, ultimately 90% of DMD patients develop fibrotic changes that result in functional cardiac anomalies and respiratory insufficiency, and 53–90% of patients die from respiratory failure (22, 37).

Increasingly, research is focusing on potential pharmacological interventions that would reduce symptoms and prolong life. However, only glucocorticoid corticosteroids are currently in clinical use (reviewed in Ref. 25). Interestingly, the use of corticosteroid to increase muscle mass in DMD patients was first attempted in 1977, where it partially normalized high serum creatine kinase and lactate dehydrogenase levels in three of five DMD boys (7). Manzur and colleagues (25) analyzed all published randomized trials of corticosteroid use for its effectiveness using a number of functional measures, including studies of all three glucocorticoid corticosteroids currently used in the clinic: prednisone; prednisolone; and, outside of the United States, deflazacourt. All were effective in increasing muscle function and strength over the course of 6 mo. However, adverse effects were very common, although not severe. These included excessive weight gain, behavioral abnormalities, and other well-known effects from this class of drug.

Other pharmacological interventions have been tested in *mdx* mice, including such disparate therapies as insulin growth factor-1, creatine, and cyclosporine A (10, 18, 34), yet contradictory results were reported with many of these drugs in different laboratories (9, 43, 45). Often a pharmacological agent will have both positive and negative effects. In the *mdx* mouse model of DMD, the administration of transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) decreased fibrosis but increased the inflammatory response (2). Collectively, these studies strongly suggest that pharmacological intervention has great potential, but the selection of the appropriate drug is critical.

The study by Huebner et al. (21) describes the positive effect of halofuginone in reducing levels of fibrosis in muscles from mature *mdx* mice, including limb, diaphragm, and heart. Even more importantly, this study demonstrates positive functional consequences of halofuginone treatment on exercise endurance and cardiac and respiratory function. These marked functional improvements suggest that halofuginone is a strong candidate for clinical testing in DMD patients since it has the potential to target and attenuate many of the symptoms that occur in DMD patients.

Halofuginone was well chosen by Huebner and colleagues (21) as a drug with the potential to modulate fibrosis in DMD patients. It was first described in 1975 for use as an anticoccidial in poultry (40). In the early 1990s, it was observed that poultry treated with the drug showed increased skin tearing (16), and even low concentrations of halofuginone inhibited collagen-1 synthesis (6, 17). The efficacy of halofuginone in reducing collagen 1 in animal models of human disease was demonstrated, including such conditions as pulmonary fibrosis (32). Halofuginone also inhibited collagen formation in vitro using fibroblasts derived from human scleroderma patients (19). In addition to inhibition of collagen-1 production, halofuginone significantly decreased tissue fibrosis by inhibiting the expression of matrix metalloproteinase-2 (MMP2) (11) and TGF- $\beta$ 1 (28). The antifibrotic effects of halofuginone prevented collagen-induced pericardial contraction using an in vitro model (26). The first published study of halofuginone in a human patient was its topical application on the neck skin of a person with cutaneous chronic graft versus host disease, a condition marked by significant skin fibrosis and contractures; after 6 mo of application, there was a marked reduction in collagen synthesis in the skin of this patient (33). A pharmacokinetic study in rats set the stage for its systemic use in human patients, showing that after intravenous administration in rats, halofuginone rapidly distributed to all tissues except the brain but most importantly, as it pertains to its potential use in DMD patients, persisted in lung and skeletal muscle longer than in plasma. No metabolites of the drug were measured in plasma or tissues, and it was only toxic at extremely high doses (42). Within two years, the first human single oral-dose *phase I* trial was performed in normal healthy volunteers with the drug being well tolerated; plasma levels were even higher than predicted (38).

Address for reprint requests and other correspondence: L. K. McLoon, Dept. of Ophthalmology, Univ. of Minnesota, Rm. 374 LRB, 2001 6th St. SE, Minneapolis, MN 55455 (e-mail: mclooo01@umn.edu).

The potential of halofuginone to serve a modulating role in fibrosis formation in DMD patients is based in its well-characterized anti-fibrotic properties. However, the specific effects seen by Huebner and colleagues (21) in decreasing fibrosis, collagen I and III content, and total collagen protein in *mdx* dystrophic muscle are novel, although not surprising, and particularly exciting because these changes were seen in older *mdx* mice, not in young *mdx* mice most often used in testing drug effects (21). Its ability to decrease long-standing fibrosis makes halofuginone attractive as a potential pharmacological intervention in DMD patients. Whereas the *mdx* mouse model does not completely recapitulate the pathology of human DMD patients, there is increasing evidence that the aging *mdx* mouse shows progressive weakness and muscle deterioration with age (35). The *mdx* mice have a reduced life span compared with normal mice (5). Similar to human DMD patients, the diaphragm in the older *mdx* mouse shows significant increases in muscle fiber loss, fibrosis, and inflammatory cell invasion; even limb muscles such as gastrocnemius show increased extracellular space and inflammatory cell infiltrate in old *mdx* mice (31). The older *mdx* mice also develop cardiomyopathy, characterized by increasing fibrosis in the ventricular wall and heart conduction system, resulting in pacing abnormalities (39). The reduction in collagen expression in myonuclei by halofuginone correlates with a recent study showing that development of fibrosis within *mdx* muscles is in part due to specific upregulation of collagen 1 and 3 synthesis in myotubes and satellite cells (1). The muscles from both *mdx* mice and DMD patients express increased levels of MMP2 and TGF- $\beta$ 1 (15, 44, 46), and the antifibrotic action of halofuginone specifically inhibits these pathways (11, 13).

The functional improvements in exercise endurance as well as cardiac and respiratory function are an exciting result because these changes were seen in older *mdx* mice. Increased exercise endurance in the halofuginone-treated *mdx* mice shows that the decrease in collagen content in muscles with long-standing fibrosis can be sufficiently reduced to result in improved muscle performance with exercise. Preventing deterioration in ambulatory ability is important for patients' quality of life, and the functional improvement in exercise endurance in the *mdx* mice after halofuginone treatment suggests that it may be efficacious in human patients also. This is an exciting finding, since exercise often exacerbates the progression of symptoms (9, 41).

Whereas limb skeletal muscle pathology often abates due to the formation of revertant fibers in the *mdx* mouse, the diaphragm muscles show increased fibrotic changes and muscle loss with age (31). The halofuginone-induced increases in baseline tidal volume as well as their smaller response to a methacholine challenge when compared with the untreated *mdx* mice are an important improvement in respiratory function of these older *mdx* mice (21). A previous study in *mdx* mice showed that genetic deletion of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) resulted in increased respiratory function (14). Part of the mechanism of action for halofuginone is a significant decrease in TNF- $\alpha$  secretion; this may account for the ability of halofuginone to improve respiratory function in the *mdx* mice (24). Since 53–90% of DMD patients die from respiratory failure (23), if patient trials demonstrate the same improvement in respiratory function as seen in the *mdx* mice treated with

halofuginone, this drug would be an important addition to the pharmacological management of these patients.

Halofuginone also improved cardiac function in these older *mdx* mice (21). In the advent of improved ventilation intervention in DMD patients, cardiomyopathy is increasingly the cause of death (3). Huebner and colleagues (21) showed that halofuginone specifically improved ventricular wall motion and appeared to prevent the progression of cardiomyopathy normally seen in this time period in the *mdx* mouse. Advanced-stage DMD patients most often develop dilated cardiomyopathy (29) or significant myocardial fibrosis of the left ventricle or heart conduction system, the latter causing left ventricular wall motion abnormalities (12). In the halofuginone-treated *mdx* mice, improvement in ventricular wall motion (21) supports further testing of this drug as a potential pharmacological intervention for DMD patients. The widespread reduction of fibrosis caused by halofuginone in all these tissues is striking and unusual. Myostatin-null/*mdx* mice, for example, showed improved skeletal muscle function (4), yet there was no reduction in cardiac hypertrophy or fibrosis in these double knock-outs (8).

Halofuginone administration to older *mdx* mice proved to be a potent antifibrotic treatment. In addition to reduction in collagen synthesis, significant functional improvements were measured, including increased exercise endurance, increased pulmonary functional capacity, and improved cardiac function (21). These changes were seen in older dystrophic animals, suggesting that even long-standing fibrotic changes could be altered by systemic delivery of this drug.

There are certainly many questions left to answer. Many drugs that showed promise in the *mdx* mouse model for DMD did not prove efficacious in trials with human DMD patients. However, for the DMD patients and their families, there is great potential for a rapid translation of this drug into a human patient trial should further studies confirm these findings, since halofuginone is currently given to human patients who suffer from a variety of conditions characterized by excessive fibrosis. The possibilities suggested by the present study are intriguing and promising.

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