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# Halofuginone improves muscle-cell survival in muscular dystrophies

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ABSTRACT

Halofuginone has been shown to prevent fibrosis *via* the transforming growth factor-β/Smad3 pathway in muscular dystrophies. We hypothesized that halofuginone would reduce apoptosis—the presumed cause of satellite-cell depletion during muscle degradation—in the *mdx* mouse model of Duchenne muscular dystrophy. Six-week-old *mdx* mouse diaphragm exhibited fourfold higher numbers of apoptotic nuclei compared with wild-type mice as determined by a TUNEL assay. Apoptotic nuclei were found in macrophages and in Pax7-expressing cells; some were located in centrally-nucleated regenerating myofibers. Halofuginone treatment of *mdx* mice reduced the apoptotic nuclei number in the diaphragm, together with reduction in Bax and induction in Bcl2 levels in myofibers isolated from these mice. A similar effect was observed when halofuginone was added to cultured myofibers. No apparent effect of halofuginone was observed in wild-type mice. Inhibition of apoptosis or staurosporine-induced apoptosis by halofuginone in *mdx* primary myoblasts and C2 myogenic cell line, respectively, was reflected by less pyknotic/apoptotic cells and reduced Bax expression. This reduction was reversed by a phosphinositide-3-kinase and mitogen-activated protein kinase/extracellular signal-regulated protein kinase inhibitors, suggesting involvement of these pathways in mediating halofuginone's effects on apoptosis. Halofuginone increased apoptosis in α smooth muscle actin- and prolyl 4-hydroxylase β-expressing cells in *mdx* diaphragm and in myofibroblasts, the major source of extracellular matrix. The data suggest an additional mechanism by which halofuginone improves muscle pathology and function in muscular dystrophies.


## 1. Introduction

Adult skeletal myofibers are terminally differentiated. Therefore, regeneration after injury or in muscular dystrophies (MDs) depends on recruitment of resident satellite cells. Upon appropriate stimulatory signals, satellite cells are activated from the quiescent stage, undergo proliferation and myogenic differentiation, and subsequently fuse with pre-existing myofibers or with other myoblasts, forming myotubes which mature into new myofibers [reviewed in 1,2]. Therefore, the survival and proliferative potential of satellite cells determine the regenerative capacity of skeletal muscles.

Studies have shown that myogenic differentiation is accompanied by apoptosis [3–6]. However, a positive correlation between muscle wasting and apoptotic signals was observed after acute stress, such as electrical stimulation [7] and angiotensin II treatment [8], and in chronic conditions such as myostatin-null mice [9], aging [10], cachexia [11] and MDs [reviewed in 12,13]. In MDs, the muscle wasting due to loss of myofibers is accompanied by fibrosis and muscle-function failure. Necrosis is probably the major leading cause of myofiber degradation in many MDs with sarcolemmal deficiencies, but in the *mdx* mouse model of Duchenne MD (DMD), apoptosis precedes necrosis [12–15]. Apoptotic nuclei were detected in dystrophic muscles, particularly in interstitial cells such as macrophages and activated satellite cells; however, some myofibers with centrally located nuclei were also TUNEL-positive, suggesting DNA fragmentation and apoptosis [15]. Moreover, in the *mdx* mouse model of DMD and in DMD patients, upregulation of pro-apoptotic proteins such as Bax and caspases was observed in myofibers, suggesting that under pathological conditions, these myofibers undergo apoptosis [13,16,17]. Detection of apoptosis and decreased Bcl2 expression have been observed in other MDs such as Limb Girdle MD type 2C patients [18], and congenital MD type 1A [5,19,20].

Halofuginone is a novel anti-fibrotic agent which has been shown to inhibit transforming growth factor β (TGFβ)-dependent fibrosis in various animal models in which fibrosis is the hallmark of the disease [21–23, reviewed in 24]. Recently, halofuginone has been reported to

*Abbreviations:* CMD, congenital MD; DMD, Duchenne MD; ECM, extracellular matrix; ERK, extracellular signal-regulated protein kinase; DAPI, 4′,6-diamidino-2-phenylindole; MAPK, mitogen-activated protein kinase; MDs, muscular dystrophies; PI3K, phosphoinositide 3 kinase; P4Hβ, prolyl 4 hydroxylase β; αSMA, α smooth muscle actin; TGFβ, transforming growth factor β; YY1, Ying-Yang 1

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inhibit cardiac and skeletal muscle fibrosis and enhance motor coordination and balance in mouse models with early disease onset, such as *mdx* and the laminin α2-deficient $dy^{2J}/dy^{2J}$ mouse model of CMD [25,26], reviewed in [27]. Halofuginone also improved muscle histopathology and function in a dysferlin-knockout mouse model for dysferlinopathy, a late-onset MD [28]. Moreover, halofuginone treatment decreased muscle fibrosis and improved lung and cardiac muscle functions in older *mdx* mice with established fibrosis [29]. In addition to its effect on fibrosis, halofuginone has a direct effect on muscle cells and promotes myotube fusion in primary myoblasts derived from normal and dystrophic muscles [30].

The primary mechanism of halofuginone action is probably inhibition of Smad3 phosphorylation downstream of the TGFβ-signaling pathway, as shown in various cell types [24,31–34], including myoblasts derived from normal and dystrophic muscles [25–27,30]. It has been suggested that halofuginone, *via* inhibiting Smad3 phosphorylation, inhibits the differentiation of fibroblasts into myofibroblasts [24]; the latter possess migratory capabilities and are the primary source for extracellular matrix (ECM) secretion during wound healing and fibrosis [35–37].

It has been reported that halofuginone promotes the phosphorylation of Akt and mitogen-activated protein kinase (MAPK) family members, and enhances the association of Akt and MAPK/extracellular signal-regulated protein kinase (MAPK/ERK) with the non-phosphorylated form of Smad3, resulting in decreased Smad3 phosphorylation [30]. Recently, halofuginone has been reported to reduce inflammation by activating the amino acid starvation response [38].

In this study, we investigated the role of halofuginone in muscle-cell survival of normal and dystrophic mice. We report that halofuginone reduces the number of apoptotic nuclei in the *mdx* mouse diaphragm in general, and that of Pax7-expressing satellite cells and macrophages in particular, together with a reduction in Bax expression in both isolated myofibers and myoblasts, resulting in increased myoblast survival. In addition, halofuginone increases apoptosis in myofibroblasts, the major source of extracellular matrix (ECM) in MD, suggesting an additional muscle-function-enhancing mechanism.

## 2. Materials and methods

### 2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM), sera and antibiotic–antimycotic solution were purchased from Biological Industries. Collagenase and staurosporine were obtained from Sigma. Ly294002 and UO127 were purchased from Calbiochem. TGFβ was purchased from PeproTech Asia. Halofuginone bromohydrate was obtained from Halo Therapeutics, LLC.

### 2.2. Mice

C57/BL/6J (Wt) and *mdx* mice were housed in cages under constant photoperiod (12 L:12 D) with free access to food and water. The mice were injected intraperitoneally with either saline or 7.5 μg halofuginone three times a week for 2 weeks, starting at 4 weeks of age. All animal experiments were carried out according to the guidelines of the Volcani Center Institutional Committee for Care and Use of Laboratory Animals (IL-234/10).

### 2.3. Physiological parameters

Motor coordination and balance were evaluated with an accelerating single-station Rota-Rod treadmill (Med Associates, Inc.) as previously described [25]. In brief, the mice were placed one at a time on the rod, which was rotating at an initial speed of 3.5 rpm; the speed was gradually increased from 3.5 to 35 rpm over a period of 5 min, and the time that the mice stayed on the rod was recorded. The mice were subjected to three successive trials in each session, and the test was repeated on two consecutive days. The performance of each mouse was measured as the mean of its best individual performances over the three trials on the second day.

### 2.4. Cell preparation

Primary myoblasts and fibroblasts from the diaphragm of *mdx* mice were prepared as described previously [30,39]. Primary cultures and C2 myogenic cell line [40] were grown in DMEM supplemented with 20% (v/v) fetal calf serum. Cells were plated sparsely at $4 \times 10^3$ or $5 \times 10^4$ cell/cm$^2$ for C2 muscle cells and primary myoblasts and fibroblasts, respectively, for 1 day, after which the medium was replaced with fresh medium, with or without 10 nM halofuginone. In some cases cells were immunostained for fibroblast-specific protein1 using a monoclonal antibody (Abnova, 1:100).

### 2.5. Single-myofiber preparation and immunostaining

Single muscle myofibers were isolated from the gastrocnemius muscle of 6-week-old *mdx* and Wt mice, as described [41,42]. Briefly, mice were sacrificed and the gastrocnemius muscles were carefully removed. The outer connective tissue was removed and the muscles were immersed in a 2.5-ml solution of 0.28% (w/v) collagenase type I in DMEM for 60 min for Wt mice or for 90 min for *mdx* mice. The collagenase-treated muscle was then transferred into horse serum (HS)-coated Petri dishes containing 10 ml of DMEM with 10% (v/v) HS (growing medium) for full coverage of the digested fibers, and triturated with a wide-mouth pipette. Myofibers were then washed three times with 10 ml growing medium and placed in 90-mm coated plates. For immunostaining, the myofibers were transferred to 35-mm plates, fixed with 4% paraformaldehyde followed by incubation with Triton X-100 (0.5% v/v in PBS) and blocking with 20% (v/v) goat serum in PBS. The myofibers were incubated with polyclonal anti-Bax (1:150, Santa Cruz Biotechnology) and polyclonal anti-Bcl2 antibodies (1:150, Calbiochem) overnight at 4 °C, followed by incubation with Alexa 594 goat anti-rabbit (1:300, Jackson) secondary antibody for 1 h at room temperature, and nuclei were then stained with 4′,6-diamidino-2-phenylindole (DAPI). The myofibers were visualized under a fluorescence microscope (Olympus) with a DP-11 digital camera (Olympus).

### 2.6. TUNEL staining

TUNEL staining was performed with the MEBSTAIN Apoptosis Kit Direct (Medical & Biological Laboratories Co.) according to the manufacturer's protocol. This method detects nucleosome-sized DNA fragments by tailing their 3′-OH ends with digoxigenin nucleotides using terminal deoxynucleotidyl transferase (TdT). Paraffin-embedded muscle sections were deparaffinized and rehydrated, then pretreated with 20 μg/ml proteinase K for 20 min at 37 °C. The slides were then incubated with the TdT buffer containing the enzyme and FITC-labeled nucleotides for 1 h to allow the tailing reaction to occur. After washes, the slides were stained with DAPI. In case of double-immunostaining, slides were immunostained with primary monoclonal antibody to β-dystroglycan (1:50; Abcam) or to polyclonal antibodies to either Pax7 (1:100; Abcam), Bax (1:50), α smooth muscle actin (SMA, 1:100; Cell Marque), prolyl-4 hydroxylase ($P_4H\beta$, 1:100; Proteintech Group, Inc.), macrophages (1:200; Acris Antibodies) followed by secondary Alexa 488 donkey anti-mouse or Alexa 594 goat anti-rabbit antibody (1:300; Jackson), prior to the TUNEL staining. Sequential images of the entire diaphragm from five slides from each mouse ($n = 5$) were taken under a fluorescence microscope using DP-11 digital camera. The numbers of all green-fluorescent TUNEL-positive nuclei, DAPI nuclei or double-stained cells were counted using CELL B software (Olympus).