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(–)-Epicatechin induces mitochondrial biogenesis and markers of muscle regeneration in adults with Becker muscular dystrophy

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Abstract

Introduction: We conducted an open-label study to examine the effects of the flavonoid (–)-epicatechin in seven ambulatory adult patients with Becker muscular dystrophy (BMD).

Methods: Seven participants received (–)-epicatechin 50 mg twice per day for 8 weeks. Pre- and postprocedures included biceps brachii biopsy to assess muscle structure and growth-relevant endpoints by western blotting, mitochondria volume measurement, and cristae abundance by electron microscopy, graded exercise testing, and muscle strength and function tests.

Results: Western blotting showed significantly increased levels of enzymes modulating cellular bioenergetics (liver kinase B1 and 5'-adenosine monophosphate-activated protein kinase). Peroxisome proliferator-activated receptor gamma coactivator-1alpha, a transcriptional coactivator of genes involved in mitochondrial biogenesis and cristae-associated mitofilin levels, increased as did cristae abundance. Muscle and plasma follistatin increased significantly while myostatin decreased. Markers of skeletal muscle regeneration myogenin, myogenic regulatory factor-5, myoblast determination protein 1, myocyte enhancer factor-2, and structure-associated proteins, including dysferlin, utrophin, and intracellular creatine kinase, also increased. Exercise testing demonstrated decreased heart rate, maximal oxygen consumption per kilogram, and plasma lactate

Abbreviations: 6MWT, 6-minute walk test; AMP, adenosine monophosphate; AMPK, 5'-adenosine monophosphate-activated protein kinase; BMD, Becker muscular dystrophy; cGMP, current good manufacturing practices; DMD, Duchenne muscular dystrophy; DAPC, dystrophin-associated protein; ECG, electrocardiogram; EM, electron microscopy; EGCG, epigallocatechin gallate; HR, heart rate; IND, investigational new drug; LKB1, liver kinase B1; MEF2a, myocyte enhancer factor-2; mtB, mitochondrial biogenesis; Myf5, myogenic regulatory factor-5; MyoD, myoblast determination protein 1; NF-κB, nuclear factor-kappaB; PGC1α, peroxisome proliferator-activated receptor gamma coactivator-1alpha; SIRT1, sirtuin 1; SkM, skeletal muscle; VO₂max, maximal oxygen consumption.

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levels at defined workloads. Tissue saturation index improved in resting and postexercise states.

Discussion: (–)-Epicatechin, an exercise mimetic, appears to have short-term positive effects on tissue biomarkers indicative of mitochondrial biogenesis and muscle regeneration, and produced improvements in graded exercise testing parameters in patients with BMD.

KEYWORDS

aerobic exercise, Becker muscular dystrophy, epicatechin, mitochondrial biogenesis, follistatin

1 | INTRODUCTION

Dystrophinopathy is characterized by a systemic mitochondrial impairment that is central to the disease pathogenesis.¹ Dystrophin deficiency leads to persistent calcium influx and toxic calcium loading of mitochondria triggering mitochondrial dysfunction that limits adenosine triphosphate (ATP) production and increases reactive oxygen species.^{1,2} Mitochondrial dysfunction further impairs Ca^{2+} buffering from myofibers and organelles, increases inflammation, decreases satellite cell activation, and worsens the clinical phenotype of dystrophinopathy.² Mitochondria also participate directly in membrane and myofiber repair localized at the site of acute myofiber injury.³ Aerobic exercise, which activates mitochondrial biogenesis,⁴ directly affects several pathways, which are targets of dystrophinopathy therapeutics and include: (a) reductions in nuclear factor-kappaB (NF- κ B) activity;⁵ (b) increased follistatin expression and inhibition of myostatin;⁶ and (c) upregulation of utrophin.⁷ Aerobic exercise itself has been shown to be beneficial functionally in both Becker muscular dystrophy (BMD) and Duchenne muscular dystrophy (DMD) patients.^{8,9}

(–)-Epicatechin, the most abundant flavanol present in cacao, appears to largely mediate the health effects ascribed to the consumption of this product. The two isoforms of epicatechin, (–) and (+), structurally resemble or mimic 11- β -hydroxypregnenolone, a naturally occurring sterol recently shown to be a potent inducer of mitochondrial biogenesis (mtB).¹⁰ Two-week oral dosing of normal 1-year-old mice with (–)-epicatechin was shown to increase skeletal muscle (SkM) and heart mtB¹¹ and exercise capacity. In a follow-up study, using a mouse model of limb-girdle muscular dystrophy, 2 weeks of (–)-epicatechin treatment led to benefits in heart and SkM, with an increase in multiple regulators of mtB, leading to improved SkM function and decreased fibrosis.¹² In a pilot study, using (–)-epicatechin-rich cocoa supplementation for 8 weeks, we reported improvements in markers of SkM structure and mtB in patients with heart failure and type 2 diabetes.^{13,14} At baseline, by electron microscopy (EM) patients had a severe loss of mitochondrial cristae, and levels of dystrophin and sarcoglycans were also significantly reduced vs normal muscle.¹⁴ With (–)-epicatechin treatment, notable recovery (vs pretreatment levels) in markers of mitochondria biogenesis and structure, muscle growth, and muscle regeneration was observed, along with increased dystrophin-associated proteins.¹³

In this study we aimed to evaluate the ability of (–)-epicatechin to improve mitochondria structure and function, and partially recover many of the key muscle fiber elements that are known to be compromised in patients with dystrophinopathy. We hypothesized that 8 weeks of (–)-epicatechin would increase muscle tissue biomarkers of mitochondrial biogenesis, improve markers of muscle structure and growth, and enhance aerobic capacity by graded exercise testing.

2 | METHODS

2.1 | Trial design

This study was approved by the institutional investigational review board and all subjects signed an approved consent to participate. A single-center, open-label study of oral (–)-epicatechin 50 mg twice daily in ambulatory adults with genetically confirmed BMD was implemented with an investigator-initiated investigational new drug (IND) with the United States Food and Drug Administration. This dose was selected on the basis of attaining serum blood levels of ~100 nmol/L, as in previous studies on efficacy and metabolite levels.^{15–18}

2.2 | Sample size estimation

We utilized a convenience sample of participants because this was a single-center, open-label biomarker study and there was limited availability of genetically confirmed BMD patients. The criterion for success in this study was the presence of one or more biologic or strength and performance outcome measures with response magnitude that allows for sufficient power in a phase IIB study.

2.3 | Study participants

Seven subjects were recruited. Participants were males, 18 to 60 years of age, with genetically confirmed BMD, with average to low daily physical activity, and able to ambulate at least 150 meters without assistive devices. All were required to have discontinued nutritional, herbal, and antioxidant supplements taken with the intent of

maintaining or improving SkM strength or functional mobility for at least 2 weeks before screening. Exclusion criteria included hematology and clinical chemistry profiles outside of the normal range for BMD (including gamma-glutamyltransferase, a more specific biomarker for liver disease, compared with adenosine diphosphate and alanine aminotransferase, which can leak from skeletal muscle). No change in exercise regimen during study participation was allowed. Subjects were excluded from the study if they were enrolled in another treatment-oriented clinical trial, had a history of significant concomitant illness, had impaired renal or hepatic function, were currently using regular daily aspirin or another medication with antiplatelet effects, or had a history of symptomatic heart failure with a cardiac ejection fraction of less than 25%.

2.4 | Study drug

Participants received (–)-epicatechin orally, 50 mg twice daily (total dose 100 mg/day), for 8 weeks. Study medication was supplied as white 25-mg gelatin capsules manufactured at a current good manufacturing practices (cGMP) facility (Syngene, Karnataka, India). Subjects took two capsules in the morning at least 15 minutes before the morning meal and two in the evening at least 1 hour after the evening meal.

(–)-Epicatechin was dissolved in ethanol, treated with charcoal, and filtered to remove insoluble materials. The solvent was exchanged with purified water and dried by lyophilization in a facility certified under current cGMP. The repurified compound was tested in a cGMP-certified analysis laboratory using high-performance liquid chromatography, with identification by ¹H nuclear magnetic resonance spectroscopy-infrared; water content by Karl Fischer titration; ethanol content by gas chromatography; and general United States Pharmacopoeia (USP) tests of residue on ignition and heavy metals. Specifications required at least 90% purity and less than 5% of the enantiomer and catechin. A certificate of analysis was generated based on test results. Microbiologic tests were negative. Bulk (–)-epicatechin was formulated with inert excipients and encapsulated for the clinical study as gelatin capsules with 25 mg of (–)-epicatechin per capsule. (–)-Epicatechin powder and the capsules were found to be stable before release for the clinical study and during the duration of the study under International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) stability protocols. Purification and formulation of study drug was conducted by Epirium Bio, Inc (formerly Cardero Therapeutics, Inc, Los Altos Hills, California).

2.5 | Study procedures and evaluation

Participants had a total of seven study visits each, at baseline, at screening, at day 1, and at weeks 1, 2, 4, and 8. Evaluations of efficacy compared results at day 1 vs 8 weeks. A comprehensive safety review was conducted at each visit and a medical monitor reviewed suspected study-related adverse events throughout the investigation. Biceps brachii biopsies were obtained before and at the end of the treatment period. Muscle samples were aliquoted for electron

microscopy (EM; fixed in 2% paraformaldehyde plus 2.5% glutaraldehyde) or flash frozen in chilled isopentane for western blots.

2.6 | Study outcomes

Primary endpoints consisted of biologic indicators of treatment efficacy using SkM markers as follows:

Modulators of bioenergetics and mitochondrial biogenesis: Protein levels of liver kinase B1 (LKB1) and AMPK as well as peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC1α; a transcriptional coactivator that regulates genes involved in oxidative metabolism and mtB)^{4,19–22} by western blots.

Mitochondrial volume and cristae abundance: Four patients had muscle biopsy tissue sufficient for analysis by EM to quantitate mitochondrial volume and cristae abundance at baseline and after 8 weeks of treatment, as described in the Supporting Information online. The cristae-associated protein mitofillin was assessed in SkM specimens using western blot.

Muscle-specific regulators and contractile proteins: protein markers of muscle growth regulation (follistatin and myostatin): Markers of contractile apparatus integrity (myosin and actin α1) and markers of regeneration (myogenic regulatory factor-5 [Myf5], myoblast determination protein 1 [MyoD], myogenin, and myocyte enhancer factor-2a [MEF2a]) were measured by western blot.

Dystrophin-related proteins: Dysferlin, utrophin, and creatine kinase protein levels were assessed by western blotting.

Secondary endpoints included evaluation of function, strength, exercise metabolic testing parameters, and safety as follows:

Function: Six-minute walk test (6MWT) and times to complete motor performance tests (stand from supine, four-stair climb, and 10-meter run/walk).

Strength: Isometric knee flexor/extensor and handgrip strength by dynamometry.

Metabolism: Maximal oxygen consumption (VO₂)/kg, blood lactate levels, and heart rate by workload (watts) measured during a graded exercise cycle test.

Safety: Vital signs, medical history and adverse events, electrocardiogram (ECG), and blood clinical safety panels, including hematologic, hepatic, and renal and metabolic profiles data.

2.7 | Muscle biopsy and western blots

An open muscle biopsy was performed on the nondominant biceps brachii under local anesthesia with separate quadrants sampled at baseline and 8 weeks in six patients. The biceps brachii was chosen due to relative preservation of this muscle in BMD vs shoulder girdle, pelvic girdle, and thigh musculature and the lack of deleterious effect of biopsy on lower extremity clinical testing. The western blot methodology is detailed in the Supporting Information online. All specimens were assessed with the technician blinded to the baseline vs treatment status. Antibodies used for quantification of bioenergetic

signaling proteins, mitochondrial proteins, structural muscle proteins, indicators of regeneration, and regulators of muscle growth were from the following sources: 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK), LKB1, PGC1 α , mitofillin dysferlin, follistatin, myostatin, Myf5, MyoD, myogenin, and MEF2a (all from Abcam, Cambridge, Massachusetts, USA); dystrophin, utrophin, creatine kinase, myosin, and actin α 1 (all from Santa Cruz Biotechnology, Santa Cruz, California); and glyceraldehyde 3-phosphate dehydrogenase (all from Cell Signaling, Danvers, Massachusetts), which was used as loading control. Band intensities were digitally quantified using ImageJ software (National Institutes of Health, Bethesda, Maryland; <http://www.nih.gov>).

2.8 | EM

As described previously, muscle samples were fixed in 2% paraformaldehyde plus 2.5% glutaraldehyde (Ted Pella, Redding, California) in 0.1 mol/L sodium cacodylate (pH 7.4) on ice for 24 hours and processed for EM (see Appendix S1 in Supporting Information online). One of the six patients with pre and post muscle biopsy had a pre-treatment sample that was not adequately prepared for EM and another patient had insufficient tissue.

2.9 | 6MWT

Participants performed a 6MWT, as described elsewhere.²³ Methods were adapted for the adult population by excluding the element of constant verbal encouragement required for children.

2.10 | Graded exercise cycle testing

Participants performed a graded exercise test on an electronically braked recumbent cycle ergometer, as described elsewhere.²⁴ Modifications for BMD-related proximal weakness were made.^{8,9} The exercise test began with participants pedaling at a rate of 60 revolutions/min with no load for 1 minute (warm-up). After the warm-up period, the work rate on the cycle ergometer was started at 10 watts (W) and increased by either 10 W/min or every other minute for BMD participants until volitional exhaustion. The level of exertion was monitored by heart rate (HR) and the Borg visual analog scale.²⁵ Increments were adjusted so that the test duration was kept at between 12 and 15 minutes. Twelve-lead ECG, blood pressure, oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), and ventilation (V_{max}), using a portable metabolic cart (Model K4b²R, Cosmed, Rome, Italy) were continuously measured. After volitional exhaustion, participants were immediately placed in the supine position, where a postexercise ECG was performed within 15 to 30 seconds. Exercise tests were considered maximal if the peak HR was 85% of that predicted for age ($220 - \text{age}$) and/or the peak respiratory exchange ratio (RER; $\dot{V}CO_2/\dot{V}O_2$) was at least 1.15.

2.11 | Dynamometry

Isometric and isokinetic quantitative muscle strength of elbow and knee flexors and extensors was measured using an ergometer (Biodex Medical Systems, Shirley, New York). The highest value of three consecutive maximal strength testing efforts was recorded. Quantitative handgrip strength was assessed using a handheld dynamometer (CiTEC, Haren, The Netherlands) and recording the highest value of three consecutive tests for each hand.

2.12 | Statistical analysis

All participants who received medication for 8 weeks were included in the analysis. Values are reported as mean \pm standard error of the mean, and level of significance was set at $P < .05$. Two-sided paired Student's *t* test was used to compare changes in primary endpoint biologic indicators at baseline and after 8 weeks of treatment. To evaluate changes in metabolic testing parameters with treatment during the graded exercise testing protocol, we constructed a linear mixed-model regression using the "xtmixed" command in STATA release 12 (StataCorp, College Station, Texas) to account for differences due to use of repeated measures within participants.

3 | RESULTS

3.1 | Recruitment and baseline data

Nine participants were screened. One participant failed screening as he was not sufficiently ambulatory to perform the required functional evaluations. One participant withdrew consent during screening. The remaining seven participants began treatment. Baseline characteristics of the final study cohort are shown in TABLE 1. One participant was unable to participate in the full set of strength testing measures at baseline due to advanced level of disease. All enrolled participants completed the 8 weeks of follow-up on study medication. One participant sustained a fall at home (unrelated to study medications) that resulted in a minor injury and temporary limitation of mobility just before his 8-week evaluation and declined undergoing the 8-week biopsy or exercise test. Thus, six patients had pre- and posttreatment biopsies evaluated by western blot. Of these patients, only four had adequate samples that allowed for pre-treatment vs posttreatment EM.

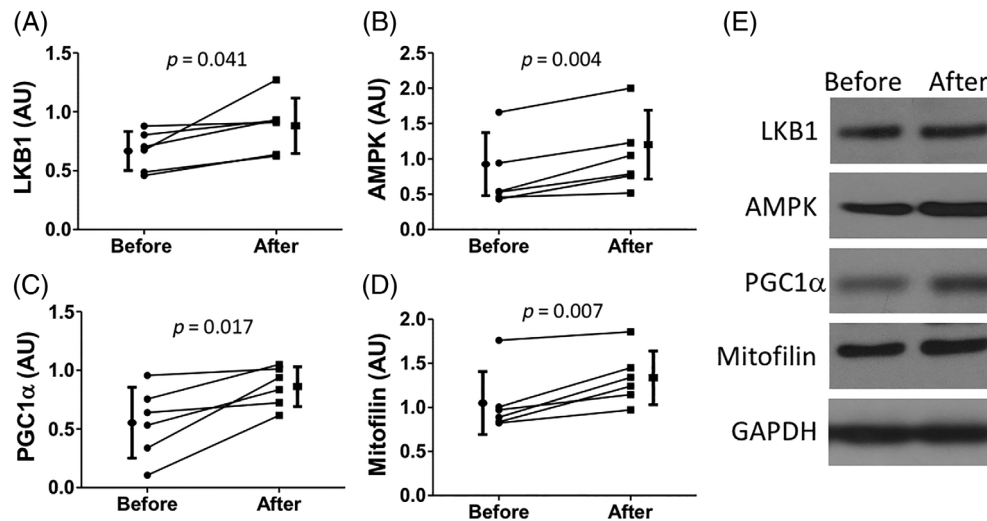
3.2 | Bioenergetic regulators and mitochondrial structural endpoints

As shown in Figure 1A-D, significant increases in the LKB1, AMPK, PGC1 α , and mitofillin were observed on western blot analysis. Based on EM imaging analysis (Figure 2A), nonsignificant increases in mitochondrial volume were observed in three of four subjects (Figure 2B).

TABLE 1 Becker muscular dystrophy patients' characteristics at baseline

Measure	Mean	SD	Minimum	Maximum
Age at screening (years)	46.6	9.5	31.5	60.0
Height (cm)	179.2	4.3	172.5	185.5
Weight (kg)	83.2	18.3	53.3	104.5
Forced vital capacity (%)	93.4	12.5	77.0	116.0
6MWD (meters)	372	87.2	245	502.0
Time to stand (seconds)	5.5	2.3	2.9	8.1
Time to climb 4 stairs (seconds)	7.4	7.4	4.4	12.3
Time to run 10 m (seconds)	7.9	7.9	5.1	12.8

Abbreviations: 6MWD, 6-min walk distance.

**FIGURE 1** Changes in bioenergetic regulators including LKB1, AMPK, PGC-1 α , and the mitochondrial cristae-associated protein mitofilin after 8 weeks of (-)-epicatechin treatment by western blot. GAPDH was used as a loading control. Data are mean \pm standard error of the mean (n = 6). Significant increases were observed on western blots for LKB1 (A), AMPK (B), PGC-1 α (C), and mitofilin (D). E, Representative western blots. AMPK, 5'-adenosine monophosphate-activated protein kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LKB1, liver kinase B1; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator-1alpha

Significant increases in mitochondria cristae were observed for all four subjects (Figure 2C).

3.3 | Muscle growth and regeneration regulators

As shown in Figure 3A-E, treatment yielded significant increases in the western blot-determined growth modulating factor follistatin, while suppressing myostatin. In addition, the structural contractile proteins myosin and actin α 1 showed significant increases. Furthermore, as shown in Figure 4A-E, there were significant increases in tissue markers known to participate in the activation of SKM regeneration, including Myf5, MyoD, myogenin, and MEF2a.

3.4 | Skeletal muscle proteins

Significant increases were seen in utrophin, dysferlin, and skeletal muscle creatine kinase by western blot analysis (Figure 5A-D).

3.5 | Graded exercise cycle testing

Overall duration of exercise and maximal attainable workload did not increase on the graded cycle exercise test with treatment. Metabolic testing evidence demonstrated improved energy efficiency at defined workloads of exercise based on reduced HR, VO_2/kg , and lactate at given levels of work consistent with and similar in magnitude to changes typically seen in response to short-duration (8-10 weeks) exercise training regimens (Figure 6A-C).

3.6 | Clinical measures of strength and function

Clinical data on timed function tests, 6MWT distance, quantitative strength, and pulmonary function are shown in TABLE S1. Our study was not adequately powered to demonstrate significant improvements in clinical measures over a short duration of treatment. With 6MWD, there were no statistically significant differences with

treatment over 8 weeks. In addition, there were no significant groupwise changes in other clinical measures, with the exception of a few non-clinically meaningful changes.

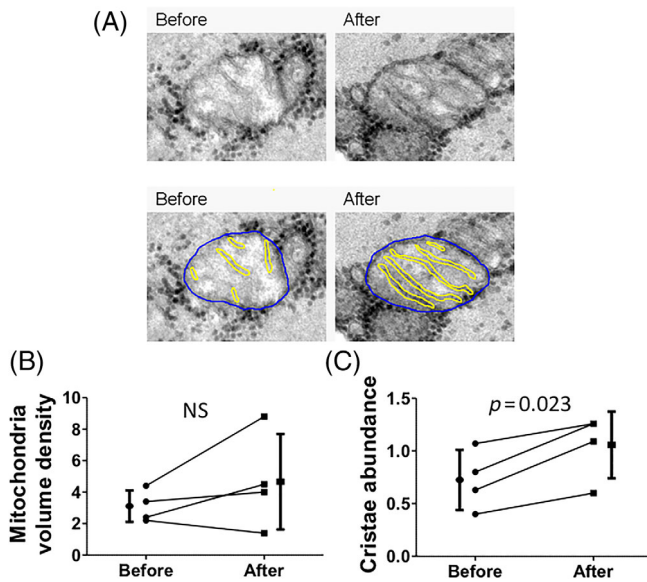


FIGURE 2 Changes in mitochondrial structure-related endpoints with 8 weeks of (-)-epicatechin treatment. A, Cristae density quantified using imaging software. B and C, Quantitative results derived using electron microscopy. Data are expressed as mean \pm standard error of the mean ($n = 4$). Based on EM imaging analysis (A), nonsignificant increases in mitochondrial volume were observed in three of four subjects (B). C, Significant increases in mitochondria cristae were observed for all four subjects

3.7 | Safety outcomes

Reported adverse events in participants were mild to moderate and consistent with a benign side-effect profile of the study drug, which was well tolerated. There were 15 adverse events in six participants reported and tracked during the 8-week treatment phase of the study. Seven of the 15 events were related or possibly related to the study biopsy procedure. Events included biopsy site infection ($n = 1$, moderate), bruising at the biopsy site ($n = 6$, mild), non-biopsy-related flulike symptoms ($n = 2$, mild), rash ($n = 1$, mild), suspected varicella (shingles) ($n = 1$, mild), muscle soreness ($n = 2$, mild), and intermittent headache ($n = 1$, mild). The headache case was considered possibly related to and is a known effect of (-)-epicatechin. All adverse events were followed until resolution.

4 | DISCUSSION

Results from this 8-week pilot study demonstrate the capacity of (-)-epicatechin to increase bioenergetic modulators including PGC1 α (a transcriptional coactivator of mitochondrial biogenesis), regulate muscle growth (increase follistatin and decrease myostatin levels systemically), enhance skeletal muscle regeneration, and to increase proteins potentially linked to maintaining sarcolemmal membrane integrity. The increase in tissue levels of creatine kinase is consistent with less leak from the intracellular space. Clinically, the treatment led to improved aerobic efficiency, similar to the expected response seen with short-term aerobic exercise training (8-10 weeks), namely decreased heart rate, VO_2/kg , and blood lactate levels at defined workloads. We

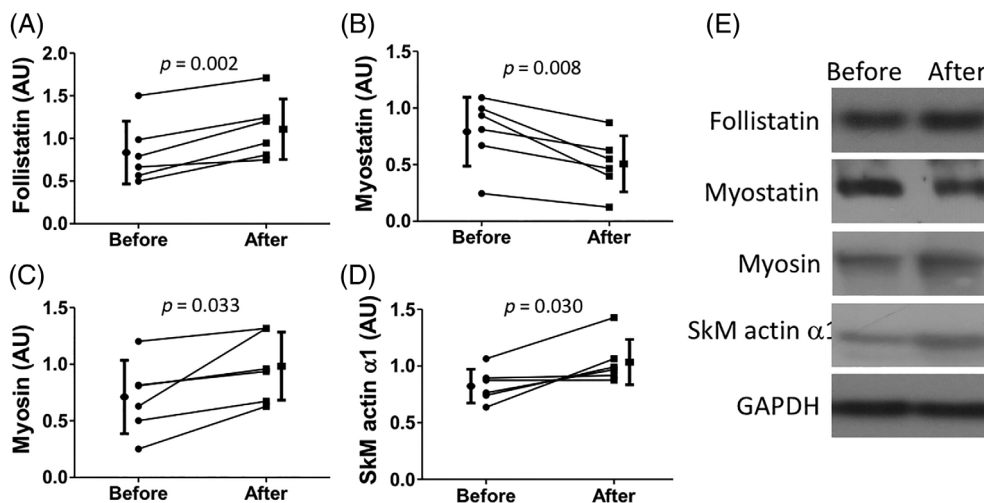


FIGURE 3 Changes in regulators of skeletal muscle growth regulators and muscle fiber contractile apparatus with 8 weeks of (-)-epicatechin treatment by western blot. Glyceraldehyde 3-phosphate dehydrogenase was used as loading control. Data are mean \pm standard error of the mean ($n = 6$). There were significant increases in the western blot-determined growth-modulating factors follistatin (A), while suppressing myostatin (B). In addition, the structural contractile proteins showed significant increases on western blot, including myosin (C) and actin $\alpha 1$ (D). Representative western blots are shown in E. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SkM, skeletal muscle

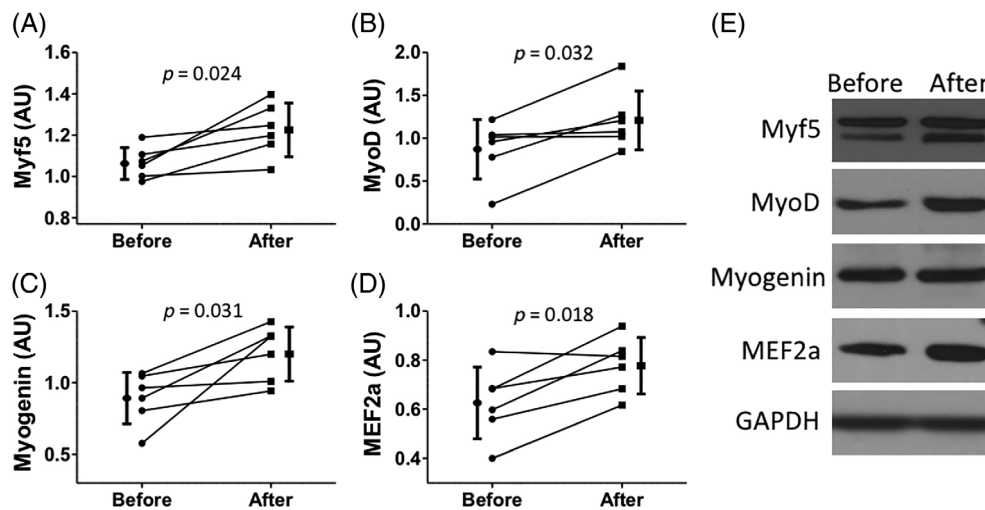


FIGURE 4 Changes in skeletal muscle regeneration-related proteins by western blots with (-)-epicatechin treatment. Glyceraldehyde 3-phosphate dehydrogenase was used as loading control. Data are expressed as mean \pm standard error of the mean ($n = 6$). There were significant increases in tissue markers known to participate in the activation of skeletal muscle regeneration including Myf5 (A), MyoD (B), myogenin (C), and MEF2a (D). Representative western blots are shown in E. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MEF2a, myocyte enhancer factor-2; Myf5, myogenic regulatory factor-5; MyoD, myoblast determination protein 1

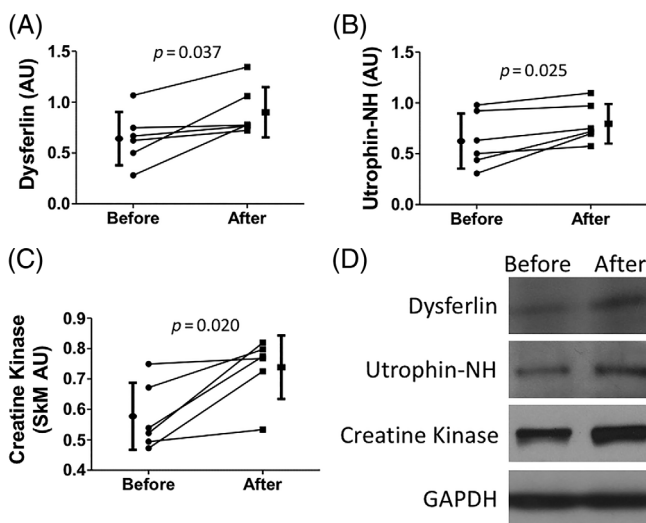


FIGURE 5 Changes on western blots in skeletal muscle proteins, including dysferlin, utrophin-NH, and intracellular skeletal muscle creatine kinase, before treatment and after 8 weeks of (-)-epicatechin. Glyceraldehyde 3-phosphate dehydrogenase was used as loading control. Data are expressed as mean \pm standard error of the mean ($n = 6$). There were significant increases in dysferlin (A), Utrophin-NH (B), and intracellular creatine kinase (C). Representative Western blots are shown in D. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SkM, skeletal muscle

did not observe an improvement in any of the clinical measures including 6MWT, time to complete motor performance tests, and isometric strength by dynamometry, although in select subjects positive responses were recorded. This lack of response in clinical measures may be due to the short duration of the study and the small sample size.

All these findings provide preliminary data suggesting that longer term administration of (-)-epicatechin could positively impact the pathogenesis of dystrophinopathy, specifically through mitochondrial biogenesis, improved cellular energetics, and improved regeneration of myocytes, leading to the possibility that (-)-epicatechin will be part of a combinatorial therapeutic approach to BMD and DMD. Although (-)-epicatechin is derived from natural plant-based sources, the (+)-enantiomer of epicatechin is only available through synthetic processes. The (+)-epicatechin molecule appears to be more potent than (-)-epicatechin,¹⁰ but the two molecules have similar pharmacokinetic, metabolism, and safety profiles. The favorable shift noted in all these tissue biomarkers (many of which are recognized targets for dystrophinopathy treatment) and the apparent lack of adverse effects observed in this study with (-)-epicatechin, support the need for further properly powered and longer term clinical trials in BMD and DMD using either (-)-epicatechin or the (+)-epicatechin enantiomer.

4.1 | LKB1/AMPK/SIRT1/PGC-1 α signaling

AMPK is a key player in maintaining cellular energy homeostasis and is a sensor of AMP/ATP or adenosine diphosphate/ATP ratios.²⁶ Exercise, which is perhaps the most extreme metabolic stress experienced in normal humans, leads to activation of AMPK in muscle.^{27,28} LKB1 has been identified as an important upstream AMPK activator in cells.²⁹ AMPK also appears to stimulate mitochondrial biogenesis by activating PGC1 α , a protein that is a transcriptional coactivator that regulates the genes involved in mitochondrial biogenesis and energy metabolism.^{4,20-22} Studies have also indicated that SIRT1 expression is increased after a single prolonged bout of exercise³⁰ and after

physical training.³¹ Although we did not measure SIRT1 in this study, it is noteworthy that other investigators have shown acute increases in SIRT1 with (–)-epicatechin treatment.¹³ PGC1 α overexpression also increases type 1 muscle fiber count, improves resistance to eccentric contraction–induced injury and fatigue, and reduces evidence of muscle tissue necrosis in *mdx* mice.³²

4.2 | Skeletal muscle regeneration and growth

There are well-recognized transcriptional factors that participate in modulating SkM regeneration, including MyoD, myogenin, MEF2a, and Myf5.^{33,34} Their upregulation and activation has been documented extensively in vitro and in vivo as indicators of regeneration-like responses in SkM, leading to the formation of new myofibers. Two families of transcription factors, MyoD and MEF2, are known to control myogenesis in the embryo, in cultured muscle cells, and in muscle regeneration. Knockout studies in mice have shown that members of the MyoD family, such as MyoD and Myf5, function as myogenic determination factors, whereas myogenin acts as a differentiation factor.^{35,36} Earlier studies in *mdx* mice demonstrated that the expression of both MyoD and myogenin genes is necessary in the regenerative process for the proliferation of satellite cells (myoblasts) and for the development of early regenerating fibers (myotubes) in dystrophic muscles.³⁷ In this study, treatment with (–)-epicatechin positively impacted MyoD, myogenin, MEF2a, and Myf5, supporting activation of regenerative pathways and possible synthesis of new myofibers from satellite cells—findings that need to be confirmed in future studies.

As the bioenergetics status of the diseased muscle improves, it allows for the recovery and/or enhancement of key structural components of SkM. Dysferlin is a protein that has been proposed to aid in the “patching” of disrupted cell membranes. In this study, (–)-epicatechin treatment led to a significant increase in dysferlin levels. Coupled with this result were increases in utrophin levels. (–)-Epicatechin has also helped restore other dystrophin-related proteins, including sarcoglycans and dystroglycans, in animal models.¹²

Transgenic mice expressing high levels of follistatin showed a dramatic increase in muscle mass ranging from 194% to 327% relative to controls, which resulted from a net effect of an increase in fiber count as well as fiber diameter.³⁸ The effect of follistatin overexpression is

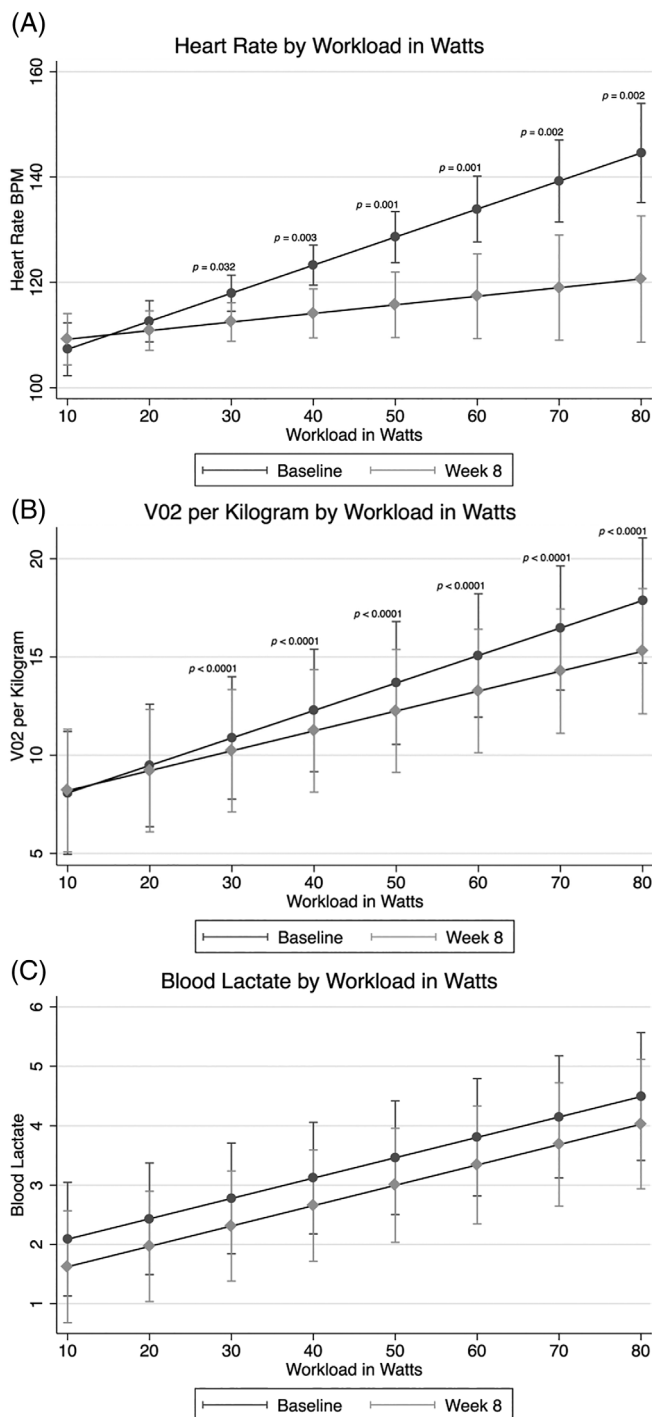


FIGURE 6 Results from graded exercise testing with recumbent cycle ergometer at defined work outputs (measured in watts, X axis) after 8 weeks of (–)-epicatechin treatment indicating reduced energy cost of exercise and improved muscle energy utilization and respiratory efficiency. A, Heart rate response during graded exercise testing at increasing workloads at baseline and after 8 weeks of treatment. There were statistically significant reductions in exercise-induced heart rate elevation at workloads of at least 30 W in the 6 subjects studied pre- and posttreatment. B, VO₂ per kilogram during graded exercise testing at increasing workloads at baseline and after 8 weeks of treatment. There were statistically significant reductions in VO₂ per kilogram at workloads of at least 30 W. C, Blood lactate levels during graded exercise testing at increasing workloads at baseline and after 8 weeks of treatment. Posttreatment blood lactate levels after treatment were reduced at baseline and significantly throughout the graded exercise test. After treatment, participants demonstrated blood lactate levels that were, on average, 0.47 mmol/L lower than baseline (95% CI, –0.81 to –0.12, $P = .009$) when controlling for level of work. All data are expressed as mean \pm standard error of the mean. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SkM, skeletal muscle

significantly greater than the increase in muscle size in myostatin-null animals.³⁹ This suggests that the mechanism by which follistatin enhances muscle growth is likely through regulating the action of several members of the transforming growth factor-beta family and not exclusively through its ability to block the myostatin pathway. Al-Zaidy and colleagues,⁴⁰ provided rationale for potential advantages of follistatin gene therapy over myostatin inhibition with binding proteins in the treatment of muscular dystrophy, and demonstrated the clinical benefits of follistatin gene therapy in adults with BMD. Myostatin levels are downregulated (vs healthy individuals) in patients with DMD to a greater extent than those with BMD.^{41,42} Both exercise and (–)-epicatechin acutely increase follistatin levels in the bloodstream,^{6,15} and follistatin is associated with regulation of muscle growth³⁸ and inflammation.⁴³

4.3 | Previous human studies focused on skeletal muscle effects of (–)-epicatechin

Humans with other diseases have also showed (–)-epicatechin benefits. Taub and colleagues¹³ showed that patients with type 2 diabetes mellitus and heart failure had significant perturbations in their muscle membrane structure at baseline with abnormal dystrophin and dystrophin-associated proteins (DAPC). Five patients with type 2 diabetes and stage II/III heart failure consumed dark chocolate and a beverage containing approximately 100 mg of (–)-epicatechin per day for 3 months. Posttreatment patients showed improved levels of oxidative phosphorylation proteins, and increased mitofilin, SIRT1 and PGC1 α . In addition, there was increased expression of Tfam (a gene involved in mitochondrial biogenesis and energy metabolism). Subsequently these patients demonstrated recovery/enhancement of DAPC protein levels and sarcomeric microstructure, and, in a coordinated fashion, they showed alterations in markers of SkM growth/differentiation consistent with myofiber regeneration. In addition, their VO₂max increased by 24%.¹⁴ Oxidative stress was also reduced.²⁴

In a study of normal, older sedentary adults, also conducted by Taub and colleagues,¹⁸ 20 sedentary subjects (~50 years old) were randomized to consume 20 g of placebo or dark chocolate containing (–)-epicatechin for 3 months. Seventeen subjects completed the trial. In the dark chocolate group, VO₂max increased, with no changes seen with placebo. Muscle biopsy showed significant increases in protein levels for LKB1, AMPK, and PGC1 α and

4.4 | Impact of (–)-epicatechin on aging and survival in an animal model

In a recent study,⁴⁴ epicatechin was directly compared with its analog epigallocatechin gallate (EGCG) and also in their active forms (phosphorylated AMPK and LKB1) as a 0.25% concentration in drinking water administered to 20-month-old male C57BL mice fed standard chow. The results show that supplementation with epicatechin for 37 weeks strikingly increased the survival rate from 39% to 69%,

whereas EGCG had no significant effect on survival. Consistently, (–)-epicatechin improved physical activity and delayed age-related degeneration of skeletal muscle (quadriceps).

4.5 | Limitations

Limitations of this study include the small number of subjects, the open-label design, and the use of within-subjects controls with BMD rather than a placebo-treated group in a randomized, double-blind, placebo-controlled trial design. Only one dose was studied and future studies should include a dose-ranging component. Histologic changes should be explored in the future in dystrophinopathy patients treated with (–)-epicatechin. In addition, the impact of myosin heavy chain-determined muscle fiber type on pre- and posttreatment mitochondrial content should be explored in future work. Despite limitations in statistical power and the short duration of treatment duration with (–)-epicatechin, the consistency and magnitude of the biomarker results and exercise effects on the graded exercise testing in all patients suggest a unique potential of this therapeutic approach to improve muscle energetics and function and possibly ameliorate disease progression. A larger, placebo-controlled, multicenter trial with a longer treatment duration is warranted to determine whether (–)-epicatechin has an effect on slowing or stabilizing disease progression and functional decline.

5 | CONCLUSIONS

In conclusion, this study has provided limited, but intriguing evidence as to the potential of (–)-epicatechin for use as a treatment for dystrophinopathies to positively impact regulatory mechanisms of mitochondrial biogenesis and follistatin production. This could lead to improved muscle bioenergetics, reduced oxidative stress, and better muscle regeneration, and could positively impact muscle structure without risking an additional breakdown of sarcolemmal membranes from contraction-induced injury. Short-term clinical responses of this exercise mimetic were consistent with an aerobic exercise training effect. The apparent safety profile of the compound raises hope that this or an alternative chemistry with this mechanism of action could be useful in the long-term treatment of BMD and other muscular dystrophies. Finally, improved bioenergetics through mitochondrial biogenesis may represent a complementary therapeutic approach to anti-inflammatory treatment and dystrophin restoration strategies for dystrophinopathy.

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6 | ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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