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Whole Body Periodic Acceleration Is an Effective Therapy to Ameliorate Muscular Dystrophy in mdx Mice

Francisco Altamirano¹, Claudio F. Perez², Min Liu³, Jeffrey Widrick⁴, Elisabeth R. Barton⁵, Paul D. Allen¹,2, Jose A. Adams⁶, Jose R. Lopez¹,2*

¹Department of Molecular Biosciences, School of Veterinary Medicine, University of California Davis, Davis, California, United States of America, ²Department of Anesthesiology Perioperative and Pain Medicine, Brigham & Women’s Hospital, Harvard Medical School, Boston, Massachusetts, United States of America, ³Department of Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, United States of America, ⁴Division of Genetics and Program in Genomics, Boston Children’s Hospital, Harvard Medical School, Boston, Massachusetts, United States of America, ⁵Anatomy and Cell Biology, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, United States of America, ⁶Division of Neonatology, Mount Sinai Medical Center, Miami, Florida, United States of America

Abstract
Duchenne muscular dystrophy (DMD) is a genetic disorder caused by the absence of dystrophin in both skeletal and cardiac muscles. This leads to severe muscle degeneration, and dilated cardiomyopathy that produces patient death, which in most cases occurs before the end of the second decade. Several lines of evidence have shown that modulators of nitric oxide (NO) pathway can improve skeletal muscle and cardiac function in the mdx mouse, a mouse model for DMD. Whole body periodic acceleration (pGz) is produced by applying sinusoidal motion to supine humans and in standing conscious rodents in a headward-footward direction using a motion platform. It adds small pulses as a function of movement frequency to the circulation thereby increasing pulsatile shear stress to the vascular endothelium, which in turn increases production of NO. In this study, we examined the potential therapeutic properties of pGz for the treatment of skeletal muscle pathology observed in the mdx mouse. We found that pGz (480 cpm, 8 days, 1 hr per day) decreased intracellular Ca²⁺ and Na⁺ overload, diminished serum levels of creatine kinase (CK) and reduced intracellular accumulation of Evans Blue. Furthermore, pGz increased muscle force generation and expression of both utrophin and the carboxy-terminal PDZ ligand of nNOS (CAPON). Likewise, pGz (120 cpm, 12 h) applied in vitro to skeletal muscle myotubes reduced Ca²⁺ and Na⁺ overload, diminished abnormal sarcolemmal Ca²⁺ entry and increased phosphorylation of endothelial NOS. Overall, this study provides new insights into the potential therapeutic efficacy of pGz as a non-invasive and non-pharmacological approach for the treatment of DMD patients through activation of the NO pathway.


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* Email: jrlopez@ucdavis.edu

Introduction
Duchenne muscular dystrophy (DMD) is a X-linked recessive and progressive muscle disease caused by failure to express sarcomemmal protein dystrophin [1,2]. DMD is the most common muscular dystrophy observed in children. The estimated worldwide incidence of DMD is approximately 1.35/1000 male live births [3]. Dystrophin is a key component of the dystrophin glycoprotein complex (DGC), which links the cytoskeleton of the muscle fibers to the extracellular matrix [1,2,4]. In the absence of dystrophin, DGC is functionally impaired such that mechanical stress associated with contraction leads to the degeneration of muscle fibers [5,6]. It is now well established that the lack of dystrophin expression in skeletal and cardiac mdx muscles leads to several secondary processes including inflammation, alteration of intracellular ion homeostasis, chronic degeneration and regeneration and necrosis/apoptosis of muscle fibers, metabolic alterations and interstitial fibrosis all of which exacerbate the progression of DMD [7].

Cumulative evidence suggests that in addition to its mechanical function as a molecular scaffold, dystrophin plays an important signaling role in both cardiac and skeletal muscles [8]. Thus, the absence of dystrophin is associated with intracellular Ca²⁺ and Na⁺ overload in DMD patients [9] and mdx mice [10,11], alterations in transient receptor potential channel function (TRPC) [12] and activation of several Ca²⁺-dependent intracellular signaling pathways in skeletal muscle [10,11,13].

Although the genetic defect responsible for DMD was identified decades ago [4], currently there is no effective treatment available for this devastating disease. Administration of corticosteroids and related drugs to diminish inflammation in DMD [14] have limited efficacy along with significant side effects, such as respiratory muscle weakness, hypoxemia, fatigue, and hypoventilation during sleep [15–17]. The need for new treatments have led investigators...
Overall, this study provides new insights about the potential terminal PDZ ligand of nNOS (CAPON). These effects were also generation and up-regulation of both utrophin and the carboxylation of Evans Blue. Furthermore, pGz improved muscle force diminished serum CK levels, and reduced intracellular accumulation of lactate. This suggests potential for usage in other muscular dystrophies.

Animals
Male 6-week old C57BL/10 and mdx male mice were obtained from the Jackson Laboratory (ME, USA). All animals were housed at 25°C with 12 h light-dark cycle and maintained with standard mouse chow and water ad libitum.

pGz in vivo and in vitro
The in vivo pGz treatment was performed on anesthetized restrained 6-week old wt and mdx mice using a reciprocal platform (Schröder, SK-180-Pro, CT, USA) at a frequency of 480 cpm, Gz±0.3 m/sec², over a one-hour daily for 8 days. After pGz treatment, animals were returned to their cages in the animal facility. All experiments were carried out 4 h after the last pGz treatment. At the conclusion of the treatment, mice were driven to the laboratory for the experiments described bellow. The in vitro pGz treatment was carried out in wt and mdx myotubes placed in an incubator (10% O₂ and 5% CO₂ at 37°C) and mounted in a reciprocal platform (described above) set at a frequency of 120 cpm for 12 h. Untreated myotubes were kept in the same incubator.

Ca²⁺ and Na⁺ selective microelectrodes
Double/barreled Ca²⁺-selective and Na⁺-selective microelectrodes were prepared as described previously [26]. The Ca²⁺ ionophore II-ETH 129 or the Na⁺ ionophore I-ETH 227 (both Fluka Sigma-Aldrich, MO, USA) were used to back-fill the Ca²⁺ and Na⁺-selective microelectrodes, respectively. Each ion-selective microelectrode was individually calibrated as described previously [26]. After making measurements of intracellular resting Ca²⁺ ([Ca²⁺]i) and resting Na⁺ concentration ([Na⁺]i) all microelectrodes were recalibrated, and if the two calibration curves did not agree within 3 mV, data from that microelectrode were discarded.

Recording of [Ca²⁺]i and [Na⁺]i in muscle fibers and cultured myotubes
Measurements of [Ca²⁺]i, and [Na⁺]i were performed both in vivo, in anesthetized (ketamine 100/xylazine 5 mg/kg) wt and mdx mice muscle fibers and in vitro on differentiated wt and mdx myotubes. For in vivo measurements, once the animal was anesthetized, a small incision was made in the skin, the muscle fascia was removed and the superficial fibers of Vastus lateralis (left leg) were exposed. A rectal temperature probe was placed, connected to a low noise heating system for maintaining animal body temperature during experimental procedures (WPI-ATC1000, FL, USA). The superficial muscle fibers were perfused with imaging solution (in mM: 140 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgSO₄, 5 glucose, and 10 Hepes/Tris, pH 7.4) and were impaled with either the Ca²⁺ or Na⁺ double-barreled microelectrode. The membrane potential signal (Vₘ) the Ca²⁺ potential (VCaE) and the Na⁺ potential (VNaE) were recorded via a high impedance amplifier (WPI Duo 773 electrometer WPI, FL, USA). The potential from the Vₘ barrel (3 M KCl) was subtracted electronically from VCaE or VNaE to produce a differential Ca²⁺-specific (VCa) or Na⁺ specific (VNa) potential that represents [Ca²⁺]i or [Na⁺]i, based on an experimental calibration curve performed with the same electrode. Vₘ VCa and VNa were filtered with a low pass filter (30–50 KHz) to improve the signal-to-noise ratio and stored in a computer for further analysis. For in vitro studies, cells were maintained in imaging solution and impaled with either a double-barreled Ca²⁺- or Na⁺-selective microelectrode and the procedures to obtain the specific potential for Ca²⁺ (VCa) or Na⁺ (VNa) were identical to that described above.

Forelimb grip strength test
Forelimb grip strength was assessed by means of a grip strength meter (Columbus Instruments, OH, USA). Wt and mdx mice were lifted over the baseplate by the tail so that its forepaws were allowed to grasp onto the steel grid. The mouse was then gently pulled back by the tail until its grip was released. Mice were tested 5 times, with one-minute interval between trials. The three highest measured values were averaged to calculate the grip strength, which was normalized by the body weight in grams.
In situ muscle function measurements

Mice were deeply anesthetized via intraperitoneal (i.p.) injection of ketamine-xylazine mixture (80 and 10 mg/kg) and carefully monitored throughout the experiment. Additional doses were administered as needed to ensure no reflex response to toe pinch. The distal tendon of the Tibialis Anterior (TA) muscle was dissected free from surrounding tissue, individually tied with 4.0 braided surgical silk, and then cut at the most distal end. The sciatic nerve was exposed, and all its branches were cut except for the common peroneal nerve. The foot was secured to a platform, and the knee was immobilized using a stainless steel pin. The TA tendon was attached to the lever arm of a 305B dual-mode servomotor transducer (Aurora Scientific, ON, Canada). TA muscle contractions were then elicited by stimulating the distal part of the sciatic nerve via bipolar electrodes, using supramaximal square-wave pulses of 0.02 ms (701A stimulator; Aurora Scientific). Data acquisition and control of the servomotors were conducted using a LabVIEW-based DMC program (version 5.202; Aurora Scientific, ON, Canada). The muscle length was measured using digital calipers based on well-defined anatomical landmarks. The optimal muscle length (L₀) was determined by incrementally stretching the muscle using micromanipulators until the maximum isometric twitch force was achieved. Three incremental stretching the muscle using micromanipulators until the maximum isometric twitch force was achieved. Three periods were allowed between each tetanic contraction. The normalized tetanic specific force (N/g) was calculated by dividing P₀ by the muscle weight.

Serum Creatine Kinase determinations

Blood samples were obtained from anesthetized wt and mdx mice by cardiac puncture [11]. Briefly, blood was collected in sterile 1.5 mL eppendorf tube, allowed to clot in ice for 30 min and then centrifuged at 3000 rpm for 10 minutes. Creatine kinase (CK) levels were determined using the UV-kinetic method (Teco Diagnostics, CA, USA) according to the manufacturers instructions. ΔAbsorbance/min were used to calculate CK enzymatic activity and the results were expressed as International Kilo Units per liter (KUI/L).

Evans Blue uptake

To assess muscle damage, Evans Blue dye (EBD) was used as a marker of permeabilized or damaged muscle fibers. EBD was dissolved in PBS (10 mg/mL) and sterilized by filtration through 0.2 µm filters. Dystrophic mdx mice with or without 8 days of pGz treatment were i.p. injected with 0.5 mg dye per 10 g body weight (n = 2 per group). Six hours later, mice were euthanized, the skin was removed, and the animals were photographed and inspected for dye uptake into skeletal muscles, indicated by blue coloration.

Western blot protein expression analysis

Gastrocnemius muscles were dissected and minced with a pair of scissors and then homogenized with an electric homogenizer (LabGEN 7b, Cole-Parmer, IL, USA) in modified RIPA buffer (150 mM NaCl, 50 mM Tris pH = 7.4, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 5 mM EDTA, 2 mM EGTA, 1X Roche Complete Protease Inhibitor and 1X Roche PhosSTOP). Myotubes were pGz-treated, quickly washed with ice-cold PBS, and lysed with modified RIPA buffer. Total lysates were incubated in ice for 30 min and then spun down by centrifugation at 16,000 x g for 20 min. Total protein concentrations were determined using the BCA method (Thermo Scientific, IL, USA). The extracts were then heated for 5 min in Laemmli loading buffer with 50 mM DTT and 25–50 µg were separated by SDS-PAGE 4–15% gradient gels (Bio-Rad) and transferred to PVDF membranes for immunoblotting. Membranes were blocked with SEA Blocking Buffer (Thermo Scientific, IL, USA) for 1 h. The following primary antibodies were purchased from BD Transduction Labs (CA, USA): anti-utrophin (#610896, 1:1000), anti-eNOS (#610296, 1:1000), Anti-CAPON (#ab90054, 1:1000), anti-phospho-(Ser632)-eNOS (#ab76199, 1:1000) were obtained from ABCC (MA, USA), Anti-nNOS (#42368, 1:1000), anti-IkBα (#4814P, 1:1000), anti-NF-κB p65 (#8242P, 1:1000) and GAPDH (#2118S, 1:5000) were purchased from Cell Signaling (MA, USA). Primary antibodies were diluted in blocking buffer with 0.1% Tween-20, incubated overnight at 4°C, and then washed with TBS 0.1% Tween-20 (TBS-T). Primary antibodies were exposed with either anti-mouse IRDye 680 nm or anti-rabbit IRDye 800 nm antibodies (Li-COR Biosciences, NE, USA), washed, and then quantified with an Odyssey Imaging System (Li-COR Biosciences, NE, USA). Protein levels were normalized to GAPDH expression.

Resting Sarcolemmal Cation entry

Sarcolemmal Ca²⁺ entry rates were estimated by the rate of dye quench by Mn²⁺ entry in myotubes loaded with 5 µM Fura-2-AM as described previously [26]. Briefly, cells were exited at the isosbestic wavelength for Fura-2 (357/7 nm) and fluorescence emission at 510 nm was then captured from regions of interest within each myotube. Rate of Ca²⁺ entry were estimated from the slope of the quenched signal and expressed as fluorescence arbitrary units per second (f.u.a.u./s).

Ethics approval

All procedures for animal experimentation were done in accordance with guidelines approved by the Animal Care and Use Committee (IACUC) at University of California at Davis (Protocol number: #17298), Brigham and Women’s Hospital Harvard Medical School (Protocol number: #23456), University of Pennsylvania (Protocol number: #800950) and Mount Sinai, Medical Center (Protocol number: #14-22-A-04).

Statistical analysis

All values are expressed as mean ± SEM. Statistical analysis was performed using two-tailed unpaired t-test, or one-way analysis of variance coupled with either Tukey’s or Dunnett t-test for multiple measurements to determine significance (P<0.05).

Results

pGz reduces [Ca²⁺]i and [Na⁺], in vivo in mdx muscles

It is well documented that skeletal muscle from mdx mice and DMD patients have significantly elevated [Ca²⁺]i, [8–11,27] and [Na⁺], [13,28,29]. Since Ca²⁺ and Na⁺ overload are hallmarks of the mdx and DMD pathology, we studied the effects of pGz treatment on [Ca²⁺]i and [Na⁺], measured in vivo in the superficial fibers of Vastus lateralis muscles from both wt and mdx mice. As showed in Figure 1A, mdx muscles have a [Ca²⁺]i that is significantly higher than those of wt muscles (390±12 nM vs 121±3 nM, P<0.001). However, after pGz treatment mdx muscle fibers showed a significant reduction in the [Ca²⁺]i to 198±5 nM (P<0.001), while no significant effect was observed in wt muscles (121±3 nM, P>0.05) (Figure 1A). Likewise, [Na⁺], was significantly elevated in mdx muscles compared with wt muscles (18.1±0.2 vs 8.0±0.1 mM, P<0.001) (Figure 1B). pGz treatment significantly reduced [Na⁺], to 10.1±0.2 mM in
dystrophic muscles ($P<0.001$), without any significant effect in wt muscles (7.5±0.1 mM; $P<0.001$) (Figure 1B).

**Muscle strength is improved by pGz treatment in mdx mice**

Both DMD patients and mdx models are characterized by muscle weakness as the result of progressive muscle damage [7]. To establish if pGz treatment improves muscle force in dystrophic muscles, forelimb grip strength and tetanic specific force, developed in situ in Tibialis Anterior (TA) muscles, were measured. Comparison of normalized forelimb grip strength values (Figure 2A) revealed that mdx mice had a 46% reduction on grip strength compared to wt mice ($P<0.001$). After pGz treatment, mdx mice had normalized values of forelimb grip strength that were similar to those of untreated wt mice (Figure 2A). Similar results were observed in measurements of in situ tetanic specific force in TA muscles. In untreated mdx mice, tetanic specific force was significantly decreased in the TA muscles compared with wt mice (22.0±0.8 vs 34.9±0.7 N/g; $P<0.001$). pGz treatment for 8 days significantly improved TA muscles tetanic specific force (26.3±1.1 N/g; $P<0.05$ vs untreated mdx mice) (Figure 2B). These data show that pGz improves muscle function in mdx mice, possibly due to a reduction in muscle destruction.

**Reduction of muscle damage by pGz in dystrophic mice**

To test the hypothesis that pGz reduces muscle damage in dystrophic muscles, serum CK levels and Evans Blue dye (EBD) uptake in skeletal muscles were measured. Figure 2C shows that serum levels of CK in untreated mdx mice were significantly elevated in comparison to wt mice (14.5±1.9 vs 0.5±0.2 KUI/L; $P<0.001$). pGz treatment significantly reduced CK levels in mdx mice compared with mdx without treatment (8.8±1.2 KUI/L; $P<0.05$) (Figure 2C).

To test for increased sarcolemmal permeability pGz-treated and untreated mdx mice were i.p. injected with EBD and euthanized after six hours to obtain dye uptake. EBD is a marker of damaged and permeable muscle fibers in mouse models of muscular dystrophy and as an endpoint in therapeutic trials [30]. Figure 2D shows that mdx mice have extensive zones of intense EBD uptake (dark blue staining) in all muscle groups in the ventral, dorsal and lateral views of the hindlimbs. High levels of dye uptake in the abdominal area were observed in both untreated and pGz-treated groups, most likely the result of the i.p. injection of EBD. However, under the same experimental conditions, pGz-treated mice showed much lower levels of EBD uptake in all muscle groups. These results suggest that pGz treatment significantly decreased membrane permeability and muscle damage in mdx muscle.

**Expression of Utrophin and CAPON is enhanced by pGz**

Several studies in the mdx mouse model have demonstrated that overexpression of utrophin (an autosomal dystrophin homologue) and/or carboxy-terminal PDZ ligand of nNOS (CAPON) improves dystrophic muscle pathology [31–33]. Furthermore, systemic administration of NO precursors has been reported to increase utrophin and CAPON levels in mdx muscles [33–35]. To determine whether the effect of the pGz treatment observed in dystrophic muscles was associated with up-regulation of utrophin and/or CAPON, the expression of both proteins in gastrocnemius muscles from non-treated and pGz-treated mdx mice was measured with Western blot analysis. Figure 3A shows that pGz treatment significantly increased utrophin protein levels by 2.1-fold in mdx mice related to untreated-mdx mice ($P<0.01$). Likewise, CAPON was significantly up-regulated by 2.2-fold after pGz treatment compared to untreated mdx mice ($P<0.05$) (Figure 3B).

**pGz increases 1xBz expression in mdx muscles**

Treatment with NO precursors, like L-arginine has been shown to reduce the inflammation and the activity of NF-kB as well as an increases in 1xBz expression in mdx muscles [35]. We studied protein expression levels of p65 (major NF-kB subunit) and 1xBz (NF-kB repressor) by Western blot in the gastrocnemius of pGz-treated and untreated wt and mdx mice. There was a 2.7-fold increase in p65 expression and 1.8-fold increase in 1xBz expression in untreated mdx muscles compared with wt muscles ($P<0.01$) (Figure 4). Whereas pGz did not have any significant effect on p65 expression in either wt or mdx muscles ($P>0.05$), pGz treatment increased 1xBz by 52% in mdx mice ($P<0.05$), without significant change in wt muscles ($P>0.05$) (Figure 4). These data suggest a reduction in the inflammatory NF-kB pathway due to a reduction in 1xBz degradation, in dystrophic muscles treated with pGz.

**Decreases in $[\text{Ca}^{2+}]$ and $[\text{Na}^+]$, by pGz in mdx myotubes**

Due to the complexity of the multi-systemic response to pGz in dystrophic mice, cultured myotubes were used as an in vitro model to study the mechanisms of mechanotransduction in skeletal muscle cells. Intracellular ion dysfunction, a hallmark of dystrophic pathology, was taken as an endpoint for the effect of pGz in skeletal muscle cells. Myotubes isolated from wt and mdx mice were exposed to pGz and then $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ were measured. Similar to the result in muscle fibers, significant differences in both $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ were observed between wt and mdx myotubes (Figure 5). Furthermore, whereas pGz treatment showed no measurable effects on either $[\text{Ca}^{2+}]_i$ (116±1 nM; $P<0.05$) or $[\text{Na}^+]_i$ (8.1±0.1 mM vs 8.2±0.1 mM; $P<0.05$) in wt myotubes, it significantly reduced both $[\text{Ca}^{2+}]_i$ (323±3 to 203±2 nM) and $[\text{Na}^+]_i$ (15.7±0.6 to 10.7±0.4 mM) in mdx cells (Figure 5). These data reveal that pGz treatment in vitro provides similar beneficial effects in myotubes to those observed in dystrophic muscles in vivo, suggesting that pGz effects are mediated by plasma membrane mechanoreceptors under these experimental conditions, and that myotubes are a
A reliable in vitro model for the study of the signaling pathways of muscle dystrophy.

Reduction of resting sarcolemmal Ca\(^{2+}\) entry by pGz in mdx myotubes

To explore if pGz reduces the abnormal sarcolemmal Ca\(^{2+}\) entry that has been observed previously in mdx skeletal muscle cells [10], we examined the rate of Fura-2 fluorescence quench by Mn\(^{2+}\) to quantify the levels of Ca\(^{2+}\) entry in dystrophic myotubes. The Mn\(^{2+}\)-quench studies indicated that untreated mdx myotubes have a significantly increased rate of resting Ca\(^{2+}\) entry compared to wt myotubes (47% increase, \(P<0.001\)) and that treatment with pGz was able to reduce resting Ca\(^{2+}\) entry to levels similar to that of wt (Figure 6). These data demonstrate that pGz treatment decreased the [Ca\(^{2+}\)]\(_i\), in part, due to a reduction in the Ca\(^{2+}\) entry from the extracellular space in dystrophic myotubes.

pGz increases eNOS phosphorylation in dystrophic muscle cells

Beneficial effects of pGz in skeletal muscle have been associated with eNOS activation [36]. Therefore, here we studied the effect of pGz treatment in the time course of eNOS activation in

Figure 2. pGz increases muscle strength and reduces muscle damage in mdx mice. A. Averaged forelimb grip strength normalized to body weight evaluated after 8 days of pGz treatment. B. In situ tetanic specific force measured in Tibialis Anterior after 8 days of pGz treatment. C. Serum Creatine Kinase (CK) measurements and D. Evans blue uptake in skeletal muscles from both untreated and pGz-treated mdx mice. Data are expressed as mean ± S.E.M. from N mice, *\(P<0.05\) and ***\(P<0.001\), unpaired two-tailed t-test.
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dystrophic myotubes, measured by phosphorylation at Ser632. Cultured mdx myotubes were exposed to pGz for increasing amounts of time and then quickly lysed and subjected to Western blot analysis. As showed in Figure 7A, pGz causes a transient increase in eNOS phosphorylation with significant activation after the first 30 min of treatment, reaching a maximum value (1.7 \( \pm \) 0.2 fold relative to basal levels) after 1 h of treatment, which is followed by a decline to untreated levels by 3 h of treatment.

To further assess the contribution of eNOS on pGz-mediated reduction of \([\text{Ca}^{2+}]_i\), dystrophic myotubes were pre-incubated with L-NAME (1 mM) for 30 min and then treated with pGz. L-NAME was kept in the media for the entire experiment for both the untreated and pGz-treated group. pGz treatment significantly reduced the \([\text{Ca}^{2+}]_i\) from 311 \( \pm \) 65 to 153 \( \pm \) 65 \text{nM} in mdx myotubes (51% reduction, \( P \), 0.001), however L-NAME treatment almost completely blunted its ability to lower \([\text{Ca}^{2+}]_i\) (Figure 7B). Since, L-NAME blocks all cNOS, we can not discard the potential participation of nNOS in the pGz-induced \([\text{Ca}^{2+}]_i\) reduction observed in dystrophic myotubes even though its expression in down regulated due to lack of dystrophin.

Expression of eNOS and nNOS in pGz-treated mdx muscles

pGz treatment is associated with an increase in total nNOS and eNOS protein expression in endothelial cells and cardiac muscle [37]. To further study the role of nNOS and eNOS pathways in the effects of pGz on skeletal muscles we studied the expression levels of both proteins in \( wt \) and mdx mice subjected to pGz treatment. Figure 8A shows representative Western blot analysis of eNOS and nNOS expression in \( wt \) and mdx gastrocnemius muscles with and without pGz treatment. Average normalized data indicate that that pGz treatment increased eNOS expression by 24% compared to untreated-mdx muscles, but did not reach statistical significance (\( P > 0.05 \), Fig. 8B) without any detectable effect on the expression of nNOS (\( P > 0.05 \), Figure 8C).

Discussion

Despite of the identification of the molecular defect responsible for DMD several decades ago, there are still no effective cures for the disease. There remains a profound need for alternative therapeutic strategies that can improve muscle strength, ameliorate the dystrophic pathology, and enhance patient quality of life. Here we have examined the benefit of pGz, an enhancer of NO signaling, to treat the\( mdx \) mouse, a DMD experimental model [38–40]. pGz is a non-invasive, drug free approach, which is produced by applying sinusoidal motion to a supine body in a...
Periodic Acceleration Improves Muscle Pathology in *mdx* Mice

headward-footward direction using a motion platform [39], causing additional pulses to the circulation, increasing pulsatile shear stress to the vascular endothelium and the release of NO [41]. Previous studies have shown that NO therapy has beneficial effects in dystrophic mouse models [22,34,35]. Thus, diverse NO-based therapies have been developed to restore intracellular NO homeostasis in *mdx* muscle, like treatment with L-arginine a substrate of nitric oxide synthase for NO synthesis [42], NO donors [43,44] and more recently to amplify the NO-cGMP signaling pathways with phosphodiesterase (PDE5A) inhibitors. Thus, pGz represent a new and safe therapeutic option for the treatment of DMD based on activation of NO signaling pathway.

We have found that treatment of *mdx* mice with pGz for even a short period of time (8 days) significantly reduced the intracellular Ca\(^{2+}\) and Na\(^{+}\) overload previously reported by us and others [8,10,11,13,27] both *in vivo* in muscle fibers from *mdx* mice and in cultured *mdx* myotubes. Ca\(^{2+}\) and Na\(^{+}\) overload have been shown to be deleterious to skeletal muscle fibers, and are associated with either necrotic or apoptotic cell death [29,45]. These results suggest that the benefits of pGz would not only arise from paracrine effects of the surrounding endothelium but also from direct effects on the muscle cells, possible through activation of mechanoreceptors. Examination of other cell types has also

**Figure 6. Resting rate of Mn\(^{2+}\) quench of Fura-2 fluorescence in pGz-treated *mdx* myotubes.** A. Representative traces and B. average resting rates of Mn\(^{2+}\) quench. Data are expressed as mean ± S.E.M. **P<0.01, ***P<0.001, ANOVA-Tukey’s. doi:10.1371/journal.pone.0106590.g006

**Figure 7. pGz activates eNOS in dystrophic myotubes.** A. Myotubes were treated with pGz (120 cpm, 12 h) for the indicated times, quickly lysed in modified RIPA buffer and eNOS phosphorylation (Ser632) was assessed by western blot. B. L-NAME treatment blocks the pGz-induced reduction of [Ca\(^{2+}\)] in *mdx* myotubes. Data are expressed as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001, ANOVA-Dunnett’s (A) and Tukey’s. (B). doi:10.1371/journal.pone.0106590.g007

**Figure 8. nNOS and eNOS protein levels in muscles from pGz-treated mice.** Gastrocnemius were dissected, homogenized and protein expression was assessed by western blot. A. Representative western blot. B. Quantification of eNOS and C. nNOS. Each lane in the western blot represents a muscle sample obtained from a different mouse. Data were normalized with GAPDH. Data are expressed as mean ± S.E.M. from N mice, *P<0.05, **P<0.01, ***P<0.001, ANOVA-Tukey’s. doi:10.1371/journal.pone.0106590.g008
shown that mechanical activity may also be a positive regulator of NO activity and/or expression and therefore NO production. For example, shear stress applied to endothelial cells in vitro induces an increase in eNOS mRNA and protein levels [46,47]. Similarly, compressive loads applied to bones or cyclic strains applied to osteoblasts or osteocytes stimulate NO activity [48]. Thus, it is possible that outside vessel luminal walls the pGz signaling cascade may be activated through cellular mechanotransduction, mechanism by which cells convert mechanical signals into biochemical responses, increasing physiological NO production by modulating cNOS activity.

In addition, our study indicates that pGz induced a significant reduction in resting sarcolemmal Ca²⁺ entry in cultured mdx myotubes. Dystrophic muscle cells are characterized by increased activity of transient receptor potential channels (TRPC), up-regulation of the reverse mode of Na⁺/Ca²⁺ exchanger (NCX1) [13] and hyper-nitrosylation of RyR1 [49] all processes known to either directly or indirectly modulate sarcolemmal Ca²⁺ fluxes. Thus, a reduction in cationic influx induced by pGz tends to restore the intracellular Ca²⁺ and Na⁺ homeostasis in mdx muscle cells.

Although, we do not precisely know the mechanisms by which pGz improves Na⁺ and Ca²⁺ homeostasis in mdx cells it is possible that this could be linked to the increased expression of utrophin and CAPON. Our experimental findings demonstrated that pGz treatment increased utrophin expression in mdx muscles by 2.1-fold which provide membrane stabilization, and CAPON by 2.2-fold which might restore nNOS localization to the sarcolemma, a key criterion for normal NO synthesis in skeletal muscle [33]. Utrophin is slightly smaller than dystrophin (395 kDa) and its primary structure is very similar to that of dystrophin, particularly in the N- and C-terminal ends that bind other proteins. Transgenic overexpression of utrophin in mdx mice has been shown to improve muscle function [50,51]. On the other hand, CAPON expression has been associated to an increased in NOS activity and nNOS expression, previously identified as treatments that reduce disease severity in mdx mice [52,53]. In muscle cells, the localization of nNOS is impaired in the absence of dystrophin, which impacts muscle function, satellite cell activation and NO production [5,54]. In neurons, the adaptor protein CAPON regulates nNOS localization and activity [55,56]. CAPON expression has been previously demonstrated in skeletal muscles, and its expression is increased in regenerating skeletal muscle cells [33]. Furthermore, in cardiomyocytes CAPON overexpression induced up-regulation of nNOS and enhanced intracellular NO production, which could both be blocked by treatment with L-NAME [57]. It is very likely that utrophin and CAPON overexpression may have some positive impact on mdx muscle cells including a decrease of sarcolemmal Ca²⁺ and restitution of nNOS localization to the sarcolemma. Further experiments need to be carried out to clarify this issue in pGz-treated skeletal muscle cells.

Muscle from dystrophic mice demonstrated muscle weakness compared with muscles from wt mice of the same age. Decrements in force generation in dystrophic muscle could have resulted from muscle plasma membrane fragility, impairments in the steps of excitation-contraction coupling, and/or altered contractile protein function in mdx muscle [58]. Furthermore, dystrophic skeletal muscle cells have a diminished SR Ca²⁺ loading due to RyR1 and IP₃R leak at resting conditions [10] and a reduced Ca²⁺ transient elicited by membrane depolarization [59,60]. All of these factors may compromise muscle force generation in mdx mice. Mdx muscles subjected to pGz treatment displayed increased forelimb grip strength and a significant improvement of TA specific force.

The study presented here is the first to directly test the ability of pGz to improve skeletal muscle strength in dystrophic mice by performing in vivo force measurements in the TA. These results are consistent with the hypothesis that a normalization of [Ca²⁺], by pGz treatment might improved excitation-contraction coupling, decreased muscle damage and/or increased muscle regeneration and thereby force generation in dystrophic muscles. Further research is required to elucidate the mechanisms responsible by which pGz enhances muscle force in mdx muscle fibers.

In addition, it has been reported that pGz improves muscle recovery after eccentric exercise in human subjects [61]. Eccentric arm exercises were carried out as 10 sets of 10 lengthening contractions on a seated preacher curl bench using a dumbbell (first day only), followed by 30 min of passive recovery, and 45 min pGz (140 cpm, 45 min per day). pGz treatment significantly increased muscle strength recovery after eccentric exercise, and attenuated CK response even though the reduction was not statistically significant [61].

It is very well established that lack of dystrophin results in fragility of skeletal muscle fibers, due to loss of muscle fiber membrane integrity, leading to dramatic muscle deterioration [7]. Our studies with EBD in pGz-treated mdx mice show a marked reduction of dye incorporation in the majority of the hind limb muscles. Moreover, pGz treatment significantly reduced serum CK activity in mdx mice, another way to test plasma membrane integrity, suggesting that it was responsible for a significant reduction in membrane damage and permeability of the dystrophic muscles.

Moreover, pGz increases expression of IkBα (1.5-fold), a member of a family of cellular proteins that function to inhibit the pro-inflammatory NF-kB transcription factor in mdx muscle fibers. Similar results have been observed with L-arginine treatment showing a decreased IkBα degradation in mdx diaphragm [35]. It has been reported that restoring intracellular Ca²⁺ is indirectly implicated in NO production through the modulation of the NF-kB pathway in mdx mice [10]. A decrease in [Ca²⁺], and a restoration of the NF-kB pathway, most likely via regulation of NO intracellular homeostasis would ameliorate muscle destruction in mdx muscle fibers.

Although the molecular mechanism by which pGz mitigates muscle damage in mdx mice is still unknown, it is likely to involve NO pathways. NO is gaseous messenger that conveys information based on rates of production, localization and concentration [62,63] and whose protective effect in DMD results from multiple site of actions. Increases in NO signaling has been shown to reduce muscle infiltration [35,64], decrease intracellular Ca²⁺ release [65], reduce chronic elevation of [Ca²⁺], in mdx cardiomyocytes [66], increase the expression of cytoskeletal proteins in the integrin complex [67] and utrophin and CAPON [33,42,65,68]. In the present study we found that pGz increases eNOS phosphorylation in mdx myotubes that peaked after 1 h, returning to a non-significant level after 12 h. These results suggest that salutary effect of pGz appears to be mediated through the NO signaling pathway.

In summary, in this study we have demonstrated that pGz meets several criteria for promising an effective therapy for DMD. These conclusions are supported by the fact that pGz was efficient in correcting the intracellular ion dyshomeostasis and the enhanced Ca²⁺ entry in mdx muscle cells. Most importantly, pGz treatment increased muscle strength measured by forelimb grip strength test.
and in situ force measurements in TA muscles. Furthermore, pGz treatment significantly decreased muscle injury evidenced by a reduction of Evans Blue incorporation in the limb muscles and a treatment significantly decreased muscle injury evidenced by a and in situ force measurements in TA muscles. Furthermore, pGz

References


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Author Contributions

Conceived and designed the experiments: JRL FA JAA. Performed the experiments: FA CPF MI, JW JRL. Analyzed the data: FA CPF MI, JW ERB JRL. Contributed reagents/materials/analysis tools: CPF ERB PDA JAA JRL. Contributed to the writing of the manuscript: FA CPF JW ERB PDA JAA JRL.


