Melatonin improves muscle function of the dystrophic mdx<sup>5Cv</sup> mouse, a model for Duchenne muscular dystrophy

Abstract: Duchenne muscular dystrophy (DMD) is a severe X-linked muscle-wasting disease caused by the absence of the cytoskeletal protein dystrophin. In addition to abnormal calcium handling, numerous studies point to a crucial role of oxidative stress in the pathogenesis of the disease. Considering the impressing results provided by antioxidants on dystrophic muscle structure and function, we investigated whether melatonin can protect the mdx<sup>5Cv</sup> mouse, an animal model for DMD. Male mdx<sup>5Cv</sup> mouse pups were treated with melatonin by daily intraperitoneal (i.p.) injection (30 mg/kg body weight) or by subcutaneous (s.c.) implant(s) (18 or 54 mg melatonin as Melovine<sup>®</sup> implants) from 17/18 to 28/29 days of age. Isometric force of the triceps surae was recorded at the end of the treatment. The i.p. treatment increased the phasic twitch tension of mdx<sup>5Cv</sup> mice. The maximal tetanic tension was ameliorated by 18 mg s.c. and 30 mg/kg i.p. treatments. Melatonin caused the dystrophic muscle to contract and relax faster. The force–frequency relationship of melatonin-treated dystrophic mice was shifted to the right. In accordance with improved muscle function, melatonin decreased plasma creatine kinase activity, a marker for muscle injury. Melatonin treatment increased total glutathione content and lowered the oxidized/reduced glutathione ratio, indicating a better redox status of the muscle. In light of the present investigation, the therapeutic potential of melatonin should be further considered for patients with DMD.

Introduction

Duchenne muscular dystrophy (DMD) is a severe muscular dystrophy characterized by progressive muscle weakness that affects approximately one in 3500 male births. The first symptoms appear in early childhood. Typically, the patients lose ambulation by 10–12 yrs of age and die of cardiac and respiratory failure after the disease has progressed to the heart and the diaphragm. The disease is because of the absence of dystrophin, a large cytoskeletal protein ensuring the mechanical stability of the plasma membrane and proper transversal force transmission during muscle contraction [1, 2]. Mutations in the dystrophin gene cause muscle degeneration in patients with DMD and in mdx mice, the most common animal model for DMD. Mdx mice present a severe degeneration culminating around 4 wks of age in most locomotor muscles followed by an efficient regeneration that ensures almost complete structural recovery of the muscles at around 7–8 wks of age. This period is associated with a decline of muscle force production and a shift to a slower contractile phenotype [3].

At present, though the dystrophin gene and its protein products are well known, the molecular mechanisms leading to muscle degeneration are not fully understood. In addition to the loss of cytoskeletal and sarcromeral integrity, the evidence establishes a crucial role for the deregulation of calcium homeostasis, increased oxidative stress, enhanced protease activity, and impaired energy metabolism in the pathogenesis of the disease [1, 4, 5]. Many studies have suggested that excessive generation of reactive oxygen species (ROS) contribute to muscle damage and degeneration of dystrophic muscle. In particular, increased markers of oxidative stress such as protein carbonyls and lipid peroxidation by-products, DNA adducts, and lipofuscin-positive granules have been detected in muscles from both patients with DMD and mdx mice [6–8]. An increased susceptibility to oxidative stress and subsequent adaptive induction of antioxidant enzymes have been also described [9–11]. It has been shown that glutathione, a major endogenous antioxidant in cells, acting both as a ROS scavenger and as a substrate for antioxidant enzymes, is lowered in dystrophic muscle. In particular, the ratio of oxidized-to-reduced glutathione (GSSG/GSH) shifts toward a more oxidized state, indicative of oxidative stress in dystrophic muscle [12]. In support to the role of oxidative stress in the pathogenesis of DMD, a number of studies have demonstrated positive effects of antioxidants such as N-acetylcysteine [13], green tea extracts [3, 14–16], IRFI-042, a synthetic analogue of vitamin E [17], pentoxifylline [18], and SNT-MC17/idebenone [19] in muscle structure and function of dystrophic mice [20].

Among the many targets of ROS, those involved in muscle contractile function and membrane stability are of special interest with regard to muscular dystrophy. ROS
interfere with excitation–contraction coupling and muscle performance [21–24] by targeting myofibrillar proteins and proteins involved in calcium handling [21, 25]. In addition, it has been proposed that free radical injury to muscle fiber membrane may contribute to the loss of membrane integrity in muscular dystrophy [26].

The beneficial effects of melatonin (N-acetyl-5-methoxytryptamine), the main secretory product of the pineal gland, have been described in a number of experimental models. Specifically, melatonin has been shown to protect against the deleterious effects of oxidative stress in numerous tissues including skeletal muscle [27–33]. This protection is mainly attributed to the antioxidant properties of melatonin and its metabolites that range from direct scavenging of a variety of radicals and reactive oxygen/nitrogen species to the regulation of a number of processes improving the cellular antioxidant capacity [34, 35]. In particular, melatonin increases the expression and activity of γ-glutamylcysteine synthetase, the rate-limiting enzyme of glutathione synthesis [36, 37]. Besides being an antioxidant, increasing evidence supports a role for exogenous melatonin in preserving mitochondrial function [38, 39] such as interactions with the mitochondrial permeability transition pore, a major player in initiating cell death [27, 33]. Moreover, recent evidence suggests that melatonin treatment improves Ca²⁺ homeostasis during excitation–contraction coupling [31, 40, 41].

Based on this rationale, the present study investigated the therapeutic potential of melatonin given to dystrophic mdx/Cv mice during the episode of massive necrosis and decline of muscle function [3]. The primary objectives were to explore the effects of melatonin on muscle mechanical properties such as force parameters, kinetics of contraction and relaxation, and resistance to fatigue of dystrophic muscle. In addition, we evaluated the effect of melatonin treatment on creatine kinase (CK) levels, a marker for muscle injury. Finally, as glutathione can modulate muscle mechanical properties, we studied glutathione homeostasis in melatonin-treated dystrophic muscle.

**Materials and methods**

**Animals and treatments**

All the procedures involving animals were conducted in accordance with the Swiss Federal Veterinary Office’s guidelines, based on the Swiss Federal Law on Animal Welfare. The study was performed on male dystrophic mdx/Cv mice (The Jackson Laboratory, Bar Harbor, ME, USA). Mdx/Cv mice were housed in plastic cages containing wood granule bedding, maintained with 12-hr dark/12-hr light cycles and unlimited access to food and water throughout the study. In most mdx/Cv limb muscles, necrosis starts by the end of the third week and culminates around 4-5 wks postnatal [42] and is normally followed by efficient regeneration, which is completed at 7–8-wks of age [9, 43]. Mdx/Cv mice were treated from 17/18 (just before the onset of muscle necrosis) to 28/29 days old. The animals were randomly assigned to one of the following groups: daily intraperitoneal (i.p.) injection of melatonin (30 mg/kg body weight), one subcutaneous melatonin implant (18 mg), three subcutaneous melatonin implants (54 mg) or untreated. The melatonin implants were placed under ketamine (50 mg/kg)-xylazine (5 mg/kg) anesthesia. The interscapular region was shaved and a small incision was made. One or three implants were placed under the skin, which was then sutured. Implants consisted of 18 mg tablets of melatonin (Melovine®, CEVA santé animale, Libourne, France). Implants were used to provide constant, low-dose release of melatonin (250 µg per implant per day). According to the literature, this produces an elevation of melatonin plasma levels of 300–900 pmol/L for a period exceeding 70 days [44–46]. By contrast, i.p. injection allows a plasmatic peak of melatonin before rapid decrease to the baseline within 2-3 hr (half-life of approximately 20 min) [47]. For daily i.p. administration, the dosing solution was prepared freshly by dissolving melatonin powder (Sigma-Aldrich, Buchs, Switzerland) in absolute ethanol (30 mg/mL) and further diluting in 9 volumes 0.9% saline to achieve a final concentration of 3 mg/mL. Ten microlitre of this dosing solution was injected per gram body weight, yielding a final dose of 30 mg/kg body weight. This dose corresponds approximately to the daily dose received by the mice having three melatonin implants. For comparative purpose, all the mice, including the untreated ones and those receiving daily i.p. injections of melatonin, underwent interscapular surgery. A pilot experiment showed that daily i.p. injection with saline had no impact on muscle structure and function as compared to noninjected mice. Thus, the reference (placebo) group consisted of dystrophic animals that were sham-operated and received neither implant nor saline injection. Treatments were performed at 12:00 hr ± 2 hr. Data were collected from groups of 8–9 animals.

**Isometric force recordings**

At the end of the treatment period, 28-29-day-old animals were anaesthetized by i.p. injection of a mixture of urethane (1.5 g/kg) and diazepam (5 mg/kg). The Achilles tendon of the right hind limb was exposed and linked to a force transducer coupled to a LabView interface for trace acquisition and analysis. The knee joint was firmly immobilized. Two thin steel electrodes were inserted into the triceps surae muscle. Muscles were electrically stimulated with 0.5-ms square pulses of controlled intensity and frequency. The stimulation–recording protocol was performed as follows. After manual settings of optimal muscle length (L₀) and optimal current intensity, a series of five phasic twitches were recorded to measure the average absolute phasic twitch force (P₀), time to peak (TTP), and time for half-relaxation from the peak (RT₁/₂). After a 3-min pause, muscles were subjected to a force–frequency test, where force was recorded using 200-ms bursts of increasing frequency (from 10 to 100 Hz by increments of 10 Hz) with one burst every 30 s. The strongest response was taken as the absolute optimal tetanic force (Pₒ). Finally, after another 3-min pause, muscles were submitted to repetitive tetanization for 4 min to assay muscle resistance to mechanical stress: Frequency was set at 60 Hz, and the decrease in tetanic muscle force was recorded while 60 stimulation sequences were delivered, each consisting of...
a 1-s tetanic burst and a 3-s rest. The responses were expressed as percentage of the maximal tension. Absolute phasic and tetanic forces were converted into specific forces (mN/mm² of muscle section) after normalization for the muscle cross-sectional area. The cross-sectional area (in square millimeter) was determined by dividing the triceps surae muscle mass (in milligram) by the product of optimal muscle length (in millimeter) and \(d\), the density of mammalian skeletal muscle \((d = 1.06 \text{ mg/mm}^3)\).

**Plasma creatine kinase level**

After the measurement of the muscle contractile properties, the mice received an intracardiac injection of heparin (30 \(\mu\)L; 3000 U/mL) and the aorta was cut. Whole blood was collected from the chest cavity in heparinized centrifuge tubes in a time window of 24 ± 4 hr after the last melatonin injection. The blood was centrifuged at 3000 g for 10 min at 4°C, and plasma was stored at 4°C. Creatine kinase determination was performed within 48 hr of plasma preparation by spectrophotometric analysis using the Catachem diagnostic kit (Investcare Vet, Middlesex, UK) according to the manufacturer’s instructions.

**Tissue sampling**

The extensor digitorum longus, soleus, gastrocnemius, tibialis, and plantaris muscles were dissected bilaterally and weighed. Diaphragm, heart, liver, kidneys, spleen, testes, peri-epididymal (white) fat, and interscapular (brown) fat were also collected and weighed. Gastrocnemius muscles were snap-frozen in liquid nitrogen and stored at −80°C until processed for further analysis.

**Glutathione analysis**

Gastrocnemius muscles were powdered in liquid nitrogen. Reduced (GSH) and oxidized (GSSG) glutathione were quantified by a 5,5′-dithio-bis(2-nitrobenzoic acid)-glutathione reductase recycling assay, essentially as described previously [15, 33]. The quantification of GSSG, exclusive of GSH, was accomplished by first derivatizing GSH with 2-vinylpyridine. The concentrations of total GSH, reduced GSH, and GSSG were determined spectrophotometrically at 405 nm with a microplate reader (Labsystems multiscan MS, Allschwil, Switzerland) from a calibration curve produced with known glutathione standards. GSH content in the samples was calculated as the difference between total GSH and GSSG levels. GSH and GSSG content were expressed per gram dry muscle.

**Data analysis**

The graphs were constructed and the data analyzed using the GraphPad Prism software (GraphPad, San Diego, CA, USA). Data are shown as mean ± S.E.M. Statistical analysis was performed using a two-tailed unpaired Student’s \(t\)-test with the placebo \(mdx^{cv}\) group used as reference for comparison to any other group. Differences with \(P\) values ≤ 0.05 were considered significant.

**Results**

No difference in body weight or relative weights of selected tissues was observed. The only exceptions were an increase in the spleen mass with 30 mg/kg i.p. treatment, consistent with previous reports on melatonin-treated rats [48], and a decrease in brown adipose mass with 18 mg s.c. treatment, although this effect was not seen with 54 mg s.c. and 30 mg/kg i.p. (data not shown).

The level of circulating CK is a widely used marker for the assessment of muscle injury [49]. As expected, dystrophic mice displayed high levels of CK (16030 ± 1662 U/L). Interestingly, plasma CK levels of mice treated with 54 mg s.c. and 30 mg/kg i.p. were markedly reduced compared to untreated mice (−39% and −46%, respectively). Mice treated with 18 mg s.c. displayed an intermediate reduction in CK activity (Fig. 1).

The TTP and the time for half-relaxation from the peak tension (\(RT_{1/2}\)) were determined from phasic twitch traces. The TTP of mice treated with melatonin 54 mg s.c. (15.2 ± 0.7 ms) and 30 mg/kg i.p. (14.6 ± 0.3 ms) was significantly decreased compared with the untreated dystrophic group (17.1 ± 0.5 ms), indicating that triceps surae muscle from the treated mice contracted faster (Fig. 2). Melatonin treatment tended to decrease the time required by the muscle to achieve relaxation. Significant difference was obtained for the mice receiving 30 mg/kg i.p. compared with the untreated dystrophic group, demonstrating that triceps surae can relax more rapidly upon melatonin treatment (Fig. 2).

The phasic twitch tension (\(P_t\)) was significantly higher in dystrophic mice receiving 30 mg/kg i.p. treatment.
compared with untreated ones (from 80.0 ± 2.3 to 89.0 ± 2.3 mN/mm², \( P \leq 0.05 \)) (Fig. 3A). Similarly, the maximal tetanic tension \( P_o \) was significantly higher in dystrophic mice receiving 18 mg s.c. and 30 mg/kg i.p. melatonin treatment compared with untreated ones (from 271.4 ± 8.1 to 303.1 ± 6.9 and 318.3 ± 11.4 mN/mm², respectively, \( P \leq 0.01 \)) (Fig. 3B). As a result of the effects of melatonin on \( P_t \) and \( P_o \), the phasic-to-tetanic ratio decreased from 0.305 ± 0.005 in untreated mice to 0.285 ± 0.009 and 0.276 ± 0.012 for 18 mg s.c. and 30 mg/kg i.p. treatments, respectively (Fig. 3C).

Curves connecting tension output to stimulation frequency were established. Melatonin treatments tended to shift the force–frequency curves to the right with significant differences obtained with 30 mg/kg i.p. treatment. Of note, the fraction of maximal force developed at low stimulation frequency (30–50 Hz) by dystrophic mice treated with 30 mg/kg i.p. was significantly lower than that of untreated dystrophic mice. This rightward shift is consistent with the faster contractile phenotype described above. To a lesser extent, the treatment with 54 mg s.c. also tended to cause a rightward shift \( (P \leq 0.05, \text{versus dystrophic at 70 Hz}) \) (Fig. 4).

The resistance of the triceps surae muscle to mechanical stress was assessed by repetitive tetanizations. As shown in Fig. 5A, the tension developed by untreated dystrophic mice strongly decreased during the first 30 tetanizations. Then, the loss of force was less marked and resulted in a plateau after approximately 50 tetanizations (Fig. 5A). Compared to untreated dystrophic mice, 30 mg/kg i.p. treatment increased the resistance of triceps surae to mechanical stress during the first 10 tetanizations (Fig. 5B) but showed similar residual force at the end of the assay (Fig. 5A).

The levels of total GSH and GSSG were measured in muscle from untreated and melatonin-treated dystrophic mice.

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**Fig. 2.** Effect of melatonin treatments on the kinetics of contraction and relaxation. Electrically evoked isometric contractions of the triceps surae were recorded. (A) As an example, representative traces for dystrophic mice receiving 30 mg/kg i.p. melatonin treatment and untreated dystrophic mice are shown. The arrowheads pointing at the peaks illustrate the differences in the time to peak (TTP) and those located on the X-axis highlight the differences in the time for half-relaxation from the peak (RT_{1/2}) quantified in (B) and (C), respectively. Melatonin treatments decreased the time required for both contraction and relaxation. *\( P \leq 0.05 \); **\( P \leq 0.01 \); ***\( P \leq 0.001 \) refer to statistical significance compared to untreated dystrophic mice. Values represent mean ± S.E.M from 8 to 9 mice per group.

**Fig. 3.** Effect of melatonin treatments of isometric contraction parameters. Electrically evoked isometric contractions of the triceps surae muscles were recorded. The phasic twitch tension \((P_t)\) (A) and the optimal tetanic tension \((P_o)\) (B) were determined as described in Materials and Methods. \( P_t \) and \( P_o \) were corrected for the cross-sectional area of the muscle to express the specific forces. The phasic-to-tetanic ratio (C) was determined. Dystrophic mice receiving 30 mg/kg i.p. melatonin treatment showed a significant increase in the \( P_t \) and \( P_o \). Dystrophic mice receiving 18 mg s.c. treatment showed a significant increase in the \( P_o \). The phasic-to-tetanic ratio (C) was significantly lower in dystrophic mice receiving 30 mg/kg i.p. melatonin treatment. *\( P \leq 0.05 \); **\( P \leq 0.01 \); ***\( P \leq 0.001 \) refer to statistical significance compared to untreated dystrophic mice. Values represent mean ± S.E.M from 8 to 9 mice per group.
mice. As shown in Fig. 6A, all melatonin treatments significantly increased the levels of total GSH in dystrophic muscle (from +45% with 18 mg s.c. to +93% with 54 mg s.c.). Only 30 mg/kg i.p. melatonin treatment significantly reduced the level of GSSG (−67%) compared with untreated dystrophic muscle (Fig. 6B). As a result of the different effects on total and oxidized glutathione content, the GSSG/GSH ratio was significantly reduced by the melatonin treatments 54 mg s.c. (−75%) and 30 mg/kg i.p. treatment (−87%) (Fig. 6C).

**Discussion**

Our present study shows that melatonin given to dystrophic mdx<sup>Scv</sup> mice at the time they experience acute muscle necrosis [3, 14, 16] resulted in marked improvements of their plasma membrane stability and contractile function together with improved glutathione homeostasis. The rationale for this study was based on growing evidences pointing to a crucial role of free radical-mediated injury in the pathogenesis of dystrophic mice and patients with DMD [6, 7, 50]. In particular, it has been shown that skeletal muscle of prenecrotic mdx mice displays markers of oxidative stress [9] and an impaired glutathione homeostasis supporting the view that oxidative stress is not simply a consequence of muscle degeneration but starts before it [12]. Studies from our laboratory and from others have highlighted the benefits afforded by antioxidants such as green tea polyphenols [3, 14, 16], IRFI-42, a synthetic vitamin E analogue [17, 51], NAC [13], and others [18–20] on muscle structure and function of mdx mice.

The cellular protective effects of melatonin and its metabolites are currently attributed to its radical scavenging and antioxidant properties [34, 35]. The mechanisms by which melatonin interacts with free radicals are still only partially elucidated but it has been reported to directly scavenge the superoxide radical (O<sub>2</sub><sup>-</sup>), the strong oxidant hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the deleterious hydroxyl radical (•OH) (for reviews see [52–54]). In addition, melatonin also stimulates the expression and activities of antioxidant enzymes including glutathione peroxidase, superoxide dismutase, glutathione reductase, and γ-glutamylcysteine synthetase [29, 36, 37]. In this regard, numerous studies report on the efficacious protection provided by melatonin against muscle damage in pathological states characterized by an increase in oxidative stress such as ischemia/reperfusion injury [27–31] or sepsis [32, 38].

Recent investigations have shown that muscle necrosis in mdx mice is associated with alterations in glutathione status. In prenecrotic mdx muscle, although total glutathione content was similar in both dystrophic and normal muscle, the fraction of oxidized glutathione (GSSG/GSH) was elevated twofold in dystrophic muscle. By contrast, in the postnecrotic period, dystrophic muscle had significantly less total glutathione than normal muscle, and the GSSG/GSH ratio was similar to that observed in the prenecrotic period [12]. Here, at the time of acute muscle necrosis, we
found that total glutathione was significantly reduced in dystrophic muscle. Moreover, a twofold increase in the GSSG/GSH ratio was observed in dystrophic muscle compared to normal one (Hibaoui Y et al., unpublished observations). Interestingly, in our study, both routes of melatonin administration increased total GSH content and decreased GSSG/GSH ratio, indicating that melatonin treatment contributes to the establishment of a highly reduced intracellular environment in dystrophic muscle. These results are consistent with our previous study performed in skeletal muscle cells where 24-hr incubation with 100 μM melatonin resulted in more than 50% increase in the total GSH level. Using such cells and isolated mitochondria, we demonstrated that melatonin prevented the disruption of glutathione homeostasis caused by an exogenously applied oxidant [33]. Melatonin-induced increase in glutathione levels may involve stimulation of γ-glutamylcysteine synthetase expression and activity [36].

Creatine kinase is a protein that normally resides in the cytoplasm of muscle cells and that leaks out of the myofibers through tears in the membrane [2, 4]. Free radical injury may contribute to loss of membrane integrity in muscular dystrophy, most likely by membrane protein oxidation and lipid peroxidation. The treatments with 54 mg s.c. and 30 mg/kg i.p. melatonin strongly decreased CK levels. The decrease in circulating CK levels with melatonin treatment suggests improvement of plasma membrane integrity of dystrophic muscle. In agreement with this result, melatonin has been shown to reduce lipid peroxidation and thus to prevent loss of integrity of the cell membranes [55, 56]. Along the same line, we showed previously that melatonin counteracts the deleterious effects of a short-chain cell-permeant lipid hydroperoxide analogue in skeletal muscle cells [33]. In addition, recent evidence shows that in mdx muscle, antioxidants counteract the deficit of sarcocellular expression of β-dystroglycan, a protein normally associated with dystrophin which is essential for the structure of the muscle cytoskeleton [13, 57]. Therefore, it is possible that melatonin-mediated stabilization of the plasma membrane and reduction in CK is secondary to prevention of membrane protein alterations.

Phasic twitch tension, tetanic tension, and the force–frequency relationship were all improved in dystrophic mice treated by 30 mg/kg i.p. of melatonin (and to a lesser extent by 18 mg s.c. melatonin). These results are of special interest as we have previously shown that at this age, dystrophic mice displayed lower phasic twitch and tetanic tensions and a shift to the left of the force–frequency curve compared to normal mice [3]. The improvement of muscle function is likely due to melatonin-dependent prevention of oxidative alterations of the contractile machinery and regulators. The rate of contraction and the tension developed during contraction partly depend on the nature and regulation of myosin chains expressed in the muscle [58]. In particular, redox-sensitive thiols on myosin light chains modulate the ATPase activity of the myosin heavy chains and their force generation. In addition, ROS have been shown to alter the structure and function of other myofilament proteins such as troponin C, tropomyosin, and actin, likely by oxidation of critical thiol groups [21, 23, 25]. In this context, the results obtained with 54 mg s.c. treatment are intriguing: this dosing yielded no improvement of dystrophic muscle force production although it decreased CK levels and improved GSH homeostasis. In line with this, Reid et al. proposed a dual effect of ROS and antioxidants on isometric force production in muscle. In fact, low levels of ROS are essential for muscle force production and a moderate increase in ROS induces an increase in force whereas high levels of ROS depress it. Accordingly, under conditions where force is depressed by high levels of ROS, low levels of antioxidants can ameliorate the redox balance and increase muscle force production. However, high levels of antioxidants can result in an excessive shift toward the reduced state and eventually yield to less functional benefits than lower doses of antioxidants [21, 59, 60]. We hypothesize that the 54 mg s.c. treatment, constantly providing the mouse with high levels of melatonin, would finally exceed the optimal antioxidant dosing.

Regarding the resistance to mechanical stress, fast-contracting, fatigue-sensitive type IIb fibers are the most susceptible fibers in dystrophic models. It has been shown that they die first, sparing the fatigue-resistant,
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fast-contracting type II A and slow-contracting type I fibers until regeneration is completed [10]. The 30 mg/kg i.p. treatment increased the resistance to fatigue during the first phase of the assay, suggesting a partial protection of the fatigable type II A fibers with this treatment. In age-matched dystrophic mice, green tea extracts or pentoxifylline were not able to improve the resistance to mechanical stress of triceps surae [3].

The faster contraction and relaxation of muscles from melatonin-treated mice correlates with glutathione homeostasis and reflects an improvement of the excitation-contraction coupling in dystrophic muscle. Intracellular Ca\(^{2+}\) concentration, the initiator of skeletal muscle contraction, is predominantly controlled by the ryanodine receptor (RyR) and the sarcoplasmic reticulum (SR)-Ca\(^{2+}\) ATPase (SERCA), with the former releasing the Ca\(^{2+}\) stored in the SR to initiate contraction and the latter sequestering Ca\(^{2+}\) back to the SR to initiate relaxation [24, 25, 61]. Mitochondria also take up cytoplasmic Ca\(^{2+}\) released during twitch and tetanic responses in skeletal muscle fibers [62, 63]. All these events, namely SR and mitochondrial Ca\(^{2+}\) re-uptake, Ca\(^{2+}\) release from the stores and Ca\(^{2+}\) influx, are impaired in dystrophic skeletal muscle cells [61, 64, 65] and modulated by ROS [66]. In line with this, the increase in muscle GSH content provided by melatonin treatment is likely to attenuate Ca\(^{2+}\) impairment in mdx muscle by preventing the oxidation of the RyR [67, 68]. Furthermore, the increased kinetics in muscle relaxation of dystrophic mice treated by 30 mg/kg i.p. of melatonin might result from a facilitated Sr Ca\(^{2+}\) re-uptake. Consistent with this, recent studies have revealed that melatonin restores SR Ca\(^{2+}\) handling disrupted by oxidative stress by enhancing the expression and activity of the SERCA [31, 40, 41]. Also, studies from our laboratory and others have shown that melatonin increased the capacity of mitochondria to sequester Ca\(^{2+}\) [27, 33]. Specifically, we showed that 1 \(\mu\)m melatonin increased by more than 40% the mitochondrial Ca\(^{2+}\) retention capacity [33], likely accounting for improved Ca\(^{2+}\) buffering capacity in vivo.

Recently, Chahbouni et al. [69] provided novel evidence that melatonin, given to patients with DMD, efficiently reduces CK levels, nitrosative/oxidative stress markers, and pro-inflammatory cytokines in the plasma. It is likely that similar anti-inflammatory and anti-oxidant effects occurred in our melatonin-treated dystrophic mice, contributing to muscle fiber stabilization and muscle function improvement. Melatonin, through reduction in ROS levels, could inhibit different steps of the nuclear factor-kappa B (NF-\(\kappa\)B) signaling pathway that plays a crucial role in inflammatory and muscle-wasting processes in DMD [17, 50, 51]. In this respect, melatonin has been shown to inhibit NF-\(\kappa\)B activation and the expression of nitric oxide synthase in skeletal muscle [70].

Finally, oxidative stress is considered as an important target for drug-based therapies and strategies to prevent damage in dystrophic muscle. To our knowledge, no investigations have been conducted with melatonin on dystrophic muscle function although it has been shown to be highly effective in muscle pathologies involving oxidative stress [27–32]. Here, we found that treatment of dystrophic mice with melatonin ameliorates muscle function during the onset of muscle necrosis. Muscle from melatonin-treated animals clearly displayed phenotypical improvements of their contractile function. Compared to the untreated ones, theycontracted and relaxed faster, developed higher specific tensions, and had more stable membranes. Our results support redox modulation as a likely mechanism in the amelioration of the dystrophic contractile function by melatonin treatment. Further studies are needed to address the precise mechanisms and targets involved in these improvements, in particular in relation with Ca\(^{2+}\) homeostasis. Based on the present findings on dystrophic muscle function and the recent results showing reduction in oxidative stress and inflammation by melatonin in plasma from patients with DMD [69], the therapeutic potential of melatonin should be further considered for patients with DMD.

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