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# Melatonin treatment normalizes plasma pro-inflammatory cytokines and nitrosative/oxidative stress in patients suffering from Duchenne muscular dystrophy

Abstract: Duchenne muscular dystrophy (DMD), a lethal disorder characterized by dystrophin absence, courses with chronic inflammation, sarcolemmal damage, and skeletal muscle degeneration. Among the multiple pathogenic mechanisms proposed for DMD, oxidative stress and inflammation are directly involved in the dystrophic process. Unfortunately, there is no current treatment for DMD, and the inflammatory process is an important target for therapies. Based on the antioxidant and anti-inflammatory properties of melatonin, we investigated whether melatonin treatment may reduce the dystrophic process. Ten DMD patients aged 12.8 ± 0.98 yr, were treated with melatonin (60 mg at 21:00 hr plus 10 mg at 09:00 hr), and plasma levels of lipid peroxidation (LPO), nitrites ($NO_x$), interleukin (IL)-1$\beta$, IL-2, IL-6, tumor necrosis factor-$\alpha$, interferon-$\gamma$, and plasma markers of muscle injury, were determined at 3, 6 and 9 months of treatment. Healthy age- and sex-matched subjects were used as controls. The results show a significant increase in LPO, $NO_x$, and cytokine levels in plasma of DMD patients compared with controls. Melatonin administration reduced these values to control levels at 3 months of treatment, decreasing further 9 months later. In parallel, melatonin also reduced plasma levels of creatine kinase (CK; 50%), lactate dehydrogenase (28%), aspartate aminotransferase (28%), alanine aminotransferase (20%), and myoglobin (13%). These findings strongly support the conclusion that melatonin administration significantly reduced the hyperoxidative and inflammatory process in DMD patients, reducing the muscle degenerative process.

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## Introduction

Duchenne muscular dystrophy (DMD), the second most common genetic disease in humans, is a lethal disorder characterized by lack of dystrophin that results in chronic inflammation, sarcolemmal damage, and skeletal muscle degeneration [1]. The genetic basis of DMD is well known but the dystrophic process, i.e., necrosis, exhaustive regeneration, and secondary fibrosis, is unclear. DMD is caused by different mutations of the gene encoding dystrophin, a protein of 427 kDa usually located in the cytoplasmic face of the sarcolemma [2]. In the normal muscle, dystrophin is associated with a multimolecular complex of glycoproteins, the dystrophin glycoprotein complex (DGC), which in normal muscle forms a link between the extracellular matrix and the cytoskeleton to protect against contraction-induced membrane lesions and to regulating cell signaling [3, 4]. Moreover, DGC also plays a role of 'scaffolding' that integrates into the membrane proteins such as nitric oxide synthase (NOS) [3].

Among the multiple pathogenic mechanisms proposed in the DMD [1, 3, 5], increasing evidence suggest the involvement of oxidative stress and inflammation to explain the dystrophic process [6, 7]. In turn, the occurrence of a

functional ischemia during muscle contraction, with the subsequent reperfusion during rest, support the free radical generation responsible for the hyperoxidative status and muscle damage [8]. Moreover, recent data support the participation of free radicals in gene induction [9]. Among others, nuclear factor-kappaB (NF-kB) immunoreactivity was detected in plasma, macrophages and in the cytoplasm of all fibers in regeneration and in the 20–40% of necrotic fibers in muscle specimens of DMD patients, suggesting that NF-kB is active in the DMD [10, 11]. NF-kB is one of the transcription factors that seems to be regulated by the redox status of the cell [12, 13]. In turn, NF-kB modulates the cellular immune responses, inflammation, and proliferation in the skeletal muscle, and it plays a key role in the expression of the inducible genes, including tumor necrosis factor (TNF)-$\alpha$ and interleukin (IL)-1$\beta$ [14]. In turn, TNF-$\alpha$ can also stimulate the production of mitochondrial reactive oxygen species (ROS), thus creating a positive feedback loop whereby the increased ROS cause further activation of NF-kB as well as having their own deleterious effects on the muscle [15]. Using *mdx* mice as an animal model of DMD [16], treatment with inhibitors of the NF-kB pathway [17] or with anti-TNF-$\alpha$ antibodies [18], resulted in a reduction in muscle necrosis and inflammation. NF-kB is also activated in response to pro-inflammatory molecules such as IL-1$\beta$, IL-6, and TNF-$\alpha$. These cytokines as well as NF-kB have been found increased in DMD [7, 19]. Recent data suggest that the inhibition of lipid peroxidation (LPO), which reflects the oxidative damage to membranes, blunts NF-kB activation and reduces TNF-$\alpha$ in *mdx* mice, reducing muscle degeneration and necrosis, increasing its regeneration and reducing creatine kinase (CPK) concentration [20].

Nitric oxide (NO•) can scavenge ROS produced by inflammatory cells [21]. But in *mdx* muscle, the inhibition of neuronal NO synthase (NOS) leads to a reduction in NO• levels, which may increase the ROS damage [7]. The significance of the reduced NO• levels in dystrophic muscle is unclear, although a reduction in muscle damage, inflammation and plasma CK levels after the upregulation of NO• in muscles of *mdx* mice, was reported [7].

Despite extensive research into the genetic and cellular mechanisms underlying DMD, and the promising gene therapy, there are no effective treatments for this disease to date. The existence of a hyperoxidative status and inflammation suggest that antioxidant and/or anti-inflammatory therapy may have beneficial effects in DMD. Melatonin, an endogenous antioxidant with anti-inflammatory properties, is a good candidate for this type of therapy. Melatonin, as well as its metabolites, directly scavenges reactive oxygen (ROS) and nitrogen (RNS) species, thus improving mitochondrial function and reducing the redox status of the cell [22–26]. But more importantly, melatonin induces the expression and activity of antioxidant enzymes such a as glutathione peroxidase and reductase [27, 28], thus increasing intracellular reduced glutathione (GSH) pool [29, 30]. The consequence of these actions is the LPO reduction by melatonin administration [31, 32], a finding that may be related to inhibition in NF-kB levels elsewhere described [20, 33]. Melatonin administration also inhibits the expression of some of the NF-kB-dependent genes including iNOS and COX-2 [32, 33], thus reducing the inflammatory response. These anti-inflammatory actions of melatonin are accompanied by a reduction in pro-inflammatory cytokines such as IL-1$\beta$, IL-6, TNF-$\alpha$, and interferon (INF)-$\gamma$ [34].

With these considerations in mind, we evaluated whether melatonin administration could reduce the oxidative stress and inflammation in DMD patients. Our study showed that DMD patients had increased levels of LPO and IL-1$\beta$, IL-2, IL-6, TNF-$\alpha$ and INF-$\gamma$, and melatonin treatment during 9 months inhibited these values in a time-dependent manner.

## Materials and methods

### Patients

The study was carried out on 10 pediatric patients, aged 12.8 $\pm$ 0.98 yr, who were followed in the Neuropediatric Unit of the Granada's University Hospital (Granada, Spain). These patients were diagnosed of DMD at 4–5 yr of age by genetic analyses. Only those patients suffering from DMD with no other pathology or treatment, except for corticoids, were included in the study. Informed consent was obtained from all parents and from the Hospital's Ethical Committee, according to the 1983 revised Helsinki Declaration of 1975. The final study was approved by the Andalusian's Ethical Committee of Clinical Assays (ref. 0191/06). The patients were under treatment of prednisone (0.75 mg/kg/day) before they were enrolled in the study and, due to ethical considerations, prednisone treatment was maintained along the study. Melatonin therapy consisted in the administration of two oral daily doses of the indoleamine for 9 months, one at 09:00 hr (10 mg melatonin) and the other at 21:00 hr (60 mg melatonin), respectively. The daily schedule of melatonin administration was chosen to maintain the night/day blood melatonin concentration differences. A group of 10 healthy age- and sex-matched subjects was used as a control group (C).

Blood samples were obtained from the antecubital vein in all patients at 9:00 hr before and 3, 6, and 9 months after melatonin administration. Samples were centrifugated at 3000 g for 10 min and plasma was separated and frozen at −80°C until the biochemical assays were performed.

### Lipid peroxidation assay

Plasma samples were thawed and centrifugated at 5000 g for 5 min, and 200 $\mu$L of the supernatants were used for LPO measurements. For this purpose, a commercial LPO assay kit that estimated both malondialdhehyde (MDA) and 4-hydroxyalkenals (4HDA) was used (Bioxytech LPO-568 assay kit; OxisResearch, Portland, OR, USA) [35]. LPO concentrations are expressed in $\mu$mol/L.

### Nitrite plus nitrate determination

Thawed plasma samples were deproteinized with ice-cold 6% sulfosalicylic acid, incubated at room temperature for 30 min and centrifuged at 10,000 g, 15 min; then, 50 $\mu$L supernatant were incubated with 4 $\mu$L NaOH 1.25%, 36 $\mu$L of a 14-mM phosphate dehydrogenase, 750 $\mu$M glucose-6-

phosphate, and 30 mU nitrate reductase, and 10 $\mu$L NADPH 3 $\mu$M for 60 min at room temperature. The concentration of nitrites was measured following the Griess reaction which coverts nitrite into a colored azo compound spectrophotometrically detected at 550 nm [36]. Plasma levels of $NO_x$ are expressed in $\mu$mol/L.

### Plasma cytokine assay

The Milliplex Human Cytokine immunoassay kit (Millipore Corp., Billerica, MD, USA) was used to profile expression of three inflammatory mediators (IL-1$\beta$, IL-2, IL-6, TNF-$\alpha$, and INF-$\gamma$). The assay was performed according to the manufacturer's instructions. Briefly, 50 $\mu$L of working solution containing multiple microbeads labeled with specific antibodies against each of the aforementioned cytokines were added into each well, washed twice with 200 $\mu$L of Linco-Plex wash buffer and filtered to dryness. Then 25 $\mu$L thawed plasma aliquots diluted 1:4 with the specific Linco-Plex sample diluents were added to each well and incubated for 60 min at room temperature. After a wash step (twice) with 200 $\mu$L Linco-Plex wash buffer, the beads were incubated with 25 $\mu$L of the detection antibody cocktail for 30 min at room temperature, each antibody specific to a single cytokine. After another two time wash step with 200 $\mu$L Linco-Plex wash buffer, the beads were incubated with 25 $\mu$L of the streptavidin–phycoerythrin solution for 30 min at room temperature and washed twice again. The beads were resuspended in each well with 100 $\mu$L of the Linco-Plex Sheath fluid and the concentration of each cytokine was determined using the array reader. A parallel standard curve was constructed for each cytokine. Levels are expressed in ng/L.

### Plasma melatonin determination

Melatonin was determined by HPLC with ultraviolet detection. Briefly, 500 $\mu$L plasma were extracted with 1 mL trichloromethane. The mixture was vortex for 1 min at 1400 rpm, and then centrifuged for 1 min at 5000 g. Aqueous phase was removed and the organic phase was washed thrice with 500 $\mu$L $NaHCO_3$, 50 mM, pH 10.25. Finally, 500 $\mu$L of sample was placed in a Speed Vac System for 33 min (vacuum pressure 5.1), and the dry extracts obtained were frozen at −80°C until melatonin assay. On the day of the assay, dry extracts were resuspended in 100 $\mu$L of mobile phase consisting of 100 mM sodium phosphate, 0.1 mM EDTA and acetonitrile 25%. Melatonin was then measured by HPLC (Shimazdu Europe GmbH, Duisburg, Germany) with a 150 mm × 4.5 mm Waters Sunfire C18 5 $\mu$m column (Waters Chromatography, Barcelona, Spain). After stabilizing the column with the mobile phase, samples (20 $\mu$L) were injected onto the HPLC system at a 1 mL/min flow rate, with 5-fluorotryptamine as internal standard, and the fluorescence of melatonin was measured in a fluorescence detector (Shimadzu RF-10A XL fluorescence detector) with an excitation and emission waves of 285 nm and 345 nm, respectively. Retention time was 8.9 min. A standard curve for melatonin was constructed with 4.45, 8.9, 17.9, 35.9, 71.6 and 143.2 ng/L, and the concentration of melatonin in the samples was calculated according to the peak area. Melatonin levels were expressed in ng/L.

### Biochemical analysis

Blood samples were analyzed within 24 hr by the laboratory of biopathology of the Granada University's Hospital for plasma levels of the following markers for muscle injury: creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase ($\gamma$GT), aldolase, and myoglobin.

### Statistical analysis

Data are expressed as the mean ± S.E.M. ANOVA followed by Student's *t*-test were used to compare the means between groups (before and after the competition). A $P$ value of less than 0.05 was considered statistically significant.

## Results

The changes in plasma LPO levels are illustrated in Fig. 1. Compared with the control group (C), DMD patients had increased levels of LPO before melatonin treatment (group 0) (7.73 ± 0.25 $\mu$mol/L versus 9.84 ± 0.31 $\mu$mol/L, $P < 0.001$). Three months of melatonin treatment were enough to reduce this difference ($P < 0.01$), restoring the levels of LPO in the DMD patients to control values (7.67 ± 0.16 $\mu$mol/L). Six months later, the LPO levels decreased significantly below the controls (5.92 ± 0.21 $\mu$mol/L, $P < 0.05$).

Fig. 2 shows the changes in plasma levels of the pro-inflammatory cytokines IL-1$\beta$, IL-2, and IL-6 in DMD

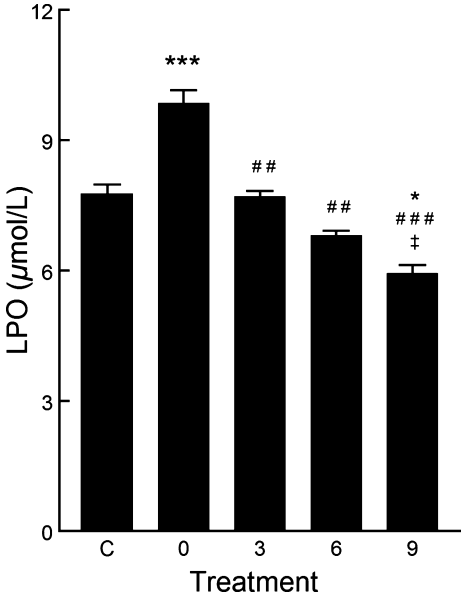


*Fig. 1.* Plasma lipid peroxidation (LPO) levels before (0) and 3, 6 and 9 months after melatonin treatment, compared with LPO levels in the control group (C). $^{*}P < 0.05$ and $^{***}P < 0.001$ versus C; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus 0.

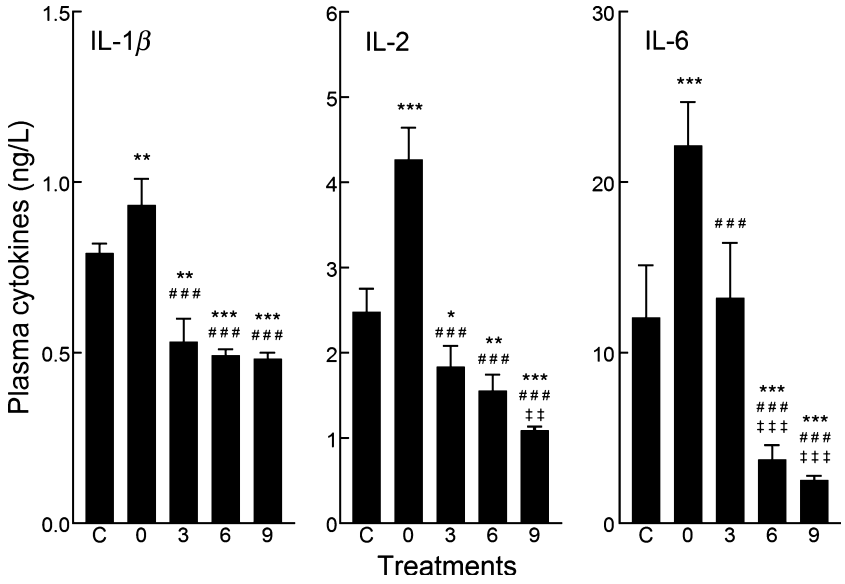


*Fig. 2.* Plasma levels of IL-1$\beta$, IL-2, and IL-6 before (0) and 3, 6, and 9 months after melatonin treatment, compared with the control group (C). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus C; $^{\#\#\#}P < 0.001$ versus 0; $^{\ddagger\ddagger}P < 0.01$ and $^{\ddagger\ddagger\ddagger}P < 0.001$ versus 3 months.

patients. Compared with their respective controls, the levels of IL-1$\beta$ (0.79 ± 0.03 ng/L versus 0.93 ± 0.08 ng/L, $P < 0.01$), IL-2 (2.47 ± 0.28 ng/L versus 4.25 ± 0.39 ng/L, $P < 0.001$), and IL-6 (12.01 ± 3.11 ng/L versus 22.10 ± 2.61 ng/L, $P < 0.001$) were significantly higher in DMD patients before melatonin treatment (group 0). Melatonin reduced IL-1$\beta$ (0.53 ± 0.07 ng/L, $P < 0.01$) and IL-2 (1.82 ± 0.26 ng/L, $P < 0.05$) below controls, and IL-6 (13.14 ± 3.30 ng/L) to control values after 3 months of treatment. IL-2 and IL-6 decreased in a time-dependent manner, reaching the lowest values at the end of treatment (IL-2, 1.11 ± 0.02 ng/L and IL-6, 2.48 ± 0.28 ng/L, $P < 0.001$). At this time, i.e., 9 months of melatonin treatment, the levels of IL-2 and IL-6 were significantly lower than those found at 3 months of treatment ($P < 0.001$).

Changes in plasma levels of TNF-$\alpha$ and INF-$\gamma$ are shown in Fig. 3. The levels of TNF-$\alpha$ (2.9 ± 0.29 ng/L versus 4.43 ± 0.61 ng/L, $P < 0.001$) and INF-$\gamma$ (17.32 ± 2.78 ng/L versus 22.10 ± 32.29 ng/L, $P < 0.01$) were significantly elevated in DMD patients compared with the control group (C) before melatonin administration. Melatonin treatment reduced these cytokines to control values at 3 months of treatment, reaching TNF-$\alpha$ (2.10 ± 0.29 ng/L, $P < 0.001$) and INF-$\gamma$ (2.48 ± 0.039 ng/L, $P < 0.001$) the lowest values after 9 months of melatonin therapy. At this time, the levels of both cytokines were significantly lower than those found at 3 months of treatment ($P < 0.001$).

Fig. 4 shows that the levels of nitrite and nitrate ($NO_x$) in DMD patients were significantly higher than controls (27.68 ± 0.41 $\mu$mol/L versus 23.46 ± 0.32 $\mu$mol/L, $P < 0.01$). Melatonin reduced $NO_x$ to control levels along the treatment.

Fig. 5 displays the plasma levels of melatonin measured along the study. Control group (C) and DMD patients had low morning levels of melatonin, compatible with normal levels of the indoleamine at this time of the day. As it was expected, at 3, 6, and 9 months of treatment, the levels of melatonin during the day remained elevated.

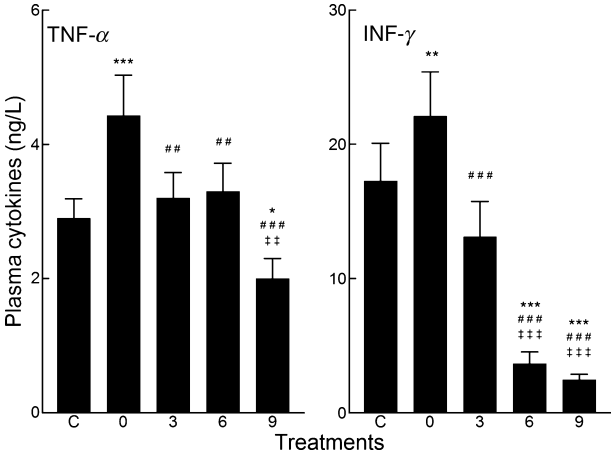


*Fig. 3.* Plasma levels of TNF-$\alpha$ and INF-$\gamma$ before (0) and 3, 6, and 9 months after melatonin treatment, compared with the control group (C). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus C; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus 0; $^{\ddagger\ddagger}P < 0.01$ and $^{\ddagger\ddagger\ddagger}P < 0.001$ versus 3 months.

Table 1 shows the time-dependent decrease in plasma CK, AST, ALT, $\gamma$GT, LDH, aldolase, and myoglobin in DMD patients treated with melatonin. Although no statistical significances were found in these parameters, a trend towards decrease in the levels of these parameters was detected with melatonin treatment. Mainly, CK decreased by almost 50%, AST by 28%; ALT by 20%; LDH by 28%; and myoglobin by 13%.

## Discussion

The data obtained in this study provide the first evidence to support the beneficial effect of melatonin in DMD patients. The results demonstrated the significant reduction in the inflammatory response and hyperoxidative status in these patients after 3 months of melatonin therapy, an effect that was maintained or even increased after 9 months of

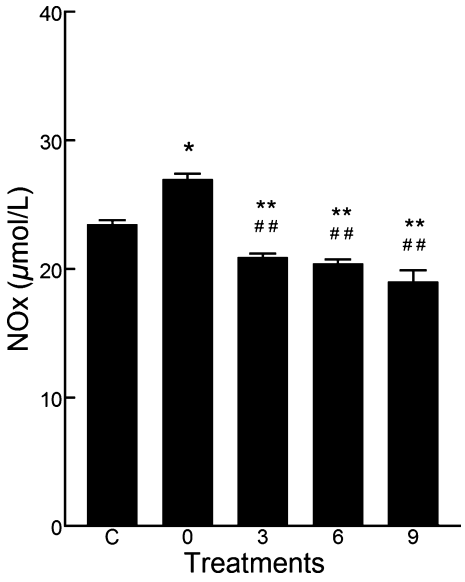


*Fig. 4.* Plasma nitrite plus nitrate ($NO_x$) levels before (0) and 3, 6 and 9 months after melatonin treatment, compared with the control group (C). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus C; $^{\#\#}P < 0.01$ versus 0.

treatment. Melatonin administration also reduced the presence of CK, AST, ALT, LDH, and myoglobin in blood of DMD patients. Together, these results support a muscle dysfunction improvement in DMD patients after melatonin therapy.

The pathogenic mechanisms of multi-systemic involvement of DMD are still unclear. Mechanical injury and membrane defects are important factors promoting dystrophic disease, but they do not fully explain the onset and progression of DMD. Increasing evidence support that

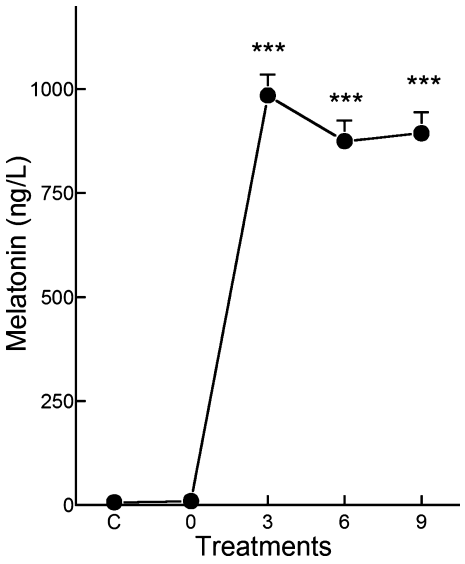


*Fig. 5.* Plasma levels of melatonin before (0) and 3, 6 and 9 months after its administration to Duchenne patients, compared with the control group (C). $^{***}P < 0.001$ versus C.

aberrant intracellular signaling cascades that regulate the inflammatory processes, contribute substantially to the degenerative process [10, 37, 38]. In this regard, intracellular signaling pathways are chronically activated in dystrophic muscle as results of lost of intact DGC signaling, calcium influx through stretch activated channels, and the generation of ROS [10, 38, 39]. Moreover, mechanical stretch as a result of DGC impairment causes the activation of NF-kB, which regulates the expression of many inflammatory genes, including cytokines, in both immune cells and muscle fibers [10, 14, 40]. Thus, inflammation and oxidative stress are important pathophysiological events causing muscle injury in DMD.

According to previous reports, our results support the existence of a redox imbalance in the blood of DMD patients [6, 7, 41], as assessed by LPO measurement. Because LPO is a marker for oxidative damage to cell membranes, the high levels of LPO detected in DMD patients compared with controls may reflect an hyperoxidative status affecting muscular fibers in the former that may be responsible, at least in part, of the skeletal muscle dysfunction in these patients. Moreover, oxidative stress/LPO represents one of the mechanisms of activation of NF-kB, resulting in the inflammatory cascade in DMD [20]. TNF-α, another inflammatory mediator, is believed to be produced in the initial steps of DMD because even minor trauma to muscle increases its levels. TNF-α contributes to muscle necrosis, because anti-TNF-α therapy protects dystrophic skeletal muscle from necrosis [18]. Although TNF-α is mainly synthesized in macrophages, it can be also produced by muscle cells and associated with muscle regeneration [42]. Moreover, TNF-α also inhibits myogenesis by activating NF-kB [14, 43]. This cytokine is known to inhibit contractile function of skeletal muscle, and it may be related to $NO^{\cdot}$ production [44]. Additionally, TNF-α may increase mitochondrial production of ROS, that in turn regulate TNF-α/NF-kB signaling [45]. Finally, TNF-α seems to have a biphasic effect on muscle: high levels of the cytokine promote muscle catabolism, probably by a NF-kB-mediated effect, whereas low levels of TNF-α, which do not induce NF-kB, stimulate myogenesis [4]. Our results show that LPO and TNF-α, which were elevated in DMD before melatonin administration, were reduced in a time-dependent manner up to 9 months of treatment, reaching levels lower than controls. The melatonin-dependent decrease in both LPO and TNF-α probably reduced NF-kB activation, leading to a improvement of the DMD patients. Although we cannot know whether the low levels of TNF-α after melatonin treatment promoted myogenesis, the reduction in blood CK and myoglobin observed in our study probably reflects the protective effect of melatonin against muscle catabolic processes in these patients.

Together with TNF-α, our data show that IL-1$\beta$ levels are also elevated in DMD patients, supporting previous data suggesting that this cytokine contribute to inflammation in inflammatory myopathies [46, 47]. Whereas TNF-α and IL-1$\beta$ induce NF-kB and NF-kB-induced inflammatory mediators such as cytokines and chemokines, IL-1$\beta$ also inhibits insulin-like growth factor (IGF)-I stimulated

Table 1. Biochemical data obtained from DMD patients and controls

| Biochemical parameter | Control ($n$ = 10) | Treatment | | | |
|---|---|---|---|---|---|
| | | Time 0 ($n$ = 10) | 3 months ($n$ = 9) | 6 months ($n$ = 7) | 9 months ($n$ = 5) |
| CK | | 7301 ± 2219 | 4912 ± 2007 | 6149 ± 1827 | 3969 ± 1617 |
| AST | | 143 ± 37 | 110 ± 32 | 131 ± 26 | 103 ± 34 |
| ALT | | 200 ± 52 | 170 ± 55 | 192 ± 39 | 159 ± 50 |
| γGT | | 16 ± 2 | 15 ± 2 | 21 ± 2 | 17 ± 3 |
| LDH | | 1362 ± 313 | 7881 ± 313 | 1119 ± 263 | 982 ± 325 |
| Aldolase | | 27 ± 7 | 33 ± 13 | 27 ± 6 | 24 ± 8 |
| Myoglobin | | 654 ± 127 | 450 ± 98 | 505 ± 96 | – |

Data are expressed as the mean ± S.E.M. Enzyme activities are expressed in U/L, and myoglobin concentration in ng/L.

protein synthesis [48, 49]. So, IGF-I regulation by IL-1$\beta$ may be important in dystrophic muscle impairment. Cytokine networks are complex, and when TNF-$\alpha$ is removed from an in vivo system, as in mdx/TNF$^{-/-}$ mice, other proinflammatory cytokines such as IL-12 and IFN-$\gamma$ may be up-regulated to overcome the TNF-$\alpha$ deficiency [18, 50]. Besides INF-$\gamma$ and IL-2, IL-6 levels were also elevated in our study prior to melatonin treatment, which was related to the dystrophy progress in DMD patients [19, 51]. In our hands, melatonin treatment also inhibited these cytokines in a time-dependent manner, collaborating with the down-regulation of NF-kB inflammatory cascade. Thus, melatonin could interfere with inflammatory cascades activated during the dystrophic process.

Another interesting aspect of this study is the role of NO• on the dystrophic process. NO• is generated by two types of NOS enzymes, i.e., constitutive and inducible. Constitutive NOS such as nNOS is a $Ca^{2+}$-calmodulin-dependent enzyme. It is now accepted that in dystrophic fibers the high cytosolic levels of $Ca^{2+}$ may increases nNOS activity and NO• production [4]. Some studies, however, suggest that nNOS is not involved in dystrophin deficiency [4, 46, 47], although other data showed that the loss of normal levels of NO• production by dystrophic muscle exacerbates inflammation and membrane injury in muscular dystrophy [4, 48]. Cytokines play an important role in the regulation of iNOS in dystrophic muscle [4] and so, whereas TNF-$\alpha$ and IFN-$\gamma$ stimulate the expression of iNOS, IL-4 and IL-10 decrease iNOS it [14, 50, 51]. However, although dystrophin-deficient muscles show a reduction in the expression of nNOS, the inflammatory process in dystrophic muscle due to NF-kB activation lead to an increased expression of iNOS, the inducible, $Ca^{2+}$-independent NOS enzyme, that in turn produces high amounts of NO•. NO• can function as an anti-inflammatory/antioxidant and even cytoprotective molecule at low concentrations, corresponding to the NO• produced by nNOS. But, after induction of iNOS, the high amounts of NO• are responsible for cytotoxic effects, mainly due to the inhibition of mitochondria respiration and the nitrosation/nitrosylation effects on proteins [52, 53]. So, the beneficial effects of the nNOS-dependent NO• production in dystrophic muscle are surpassed when cytotoxic levels of NO• are reached after iNOS induction. This explains why L-arginine given to mdx mice may have beneficial effects: L-arginine acts as a substrate for nNOS yielding cytoprotective NO• levels, but impedes iNOS expression blocking NF-kB [54]. Other studies, however, showed that L-arginine supplementation may promote iNOS-mediated muscle damage [50]. Anyway, during chronic dystrophic process there is a significant inflammatory reaction involving NF-kB-dependent iNOS expression and NO• production. The elevated levels of NO• reported in our study may reflect the chronic activation of iNOS, an effect that was counteracted after melatonin administration. The inhibitory effect of melatonin on iNOS expression and activity has been extensively reported [55–57], and specifically reported in skeletal, diaphragmatic, and cardiac muscles [58, 59].

Because NF-kB plays a central role in DMD and free radicals influence its activation, antioxidants have been tested in experimental models of DMD such as the mdx mice [4]. Among them, epigallocatechin gallate, a component of green tea extract [60], and curcumin, a dietary component of the spice turmeric [61], haven shown protective effects reducing NF-kB activation and iNOS expression, decreasing TNF-$\alpha$ and IL-1$\beta$ levels. Other nutritional interventions, including CoQ administration, did not show significant effects on NF-kB levels and/or improvement in DMD patients [62]. Together, these studies suggest than inhibitors of NF-kB may be useful for DMD therapy. Herein, our study shows the inhibitory effects of melatonin on the NF-kB inflammatory cascade. Because NF-kB in macrophages and myofibers promotes muscle degeneration in DMD [14], the inhibition of NF-kB in the early stage of dystrophy can reduce the inflammatory burden, which might in turn slow initial exhaustion of the regenerative capacity in dystrophic muscles [7]. Thus, sustained inhibition of the NF-kB pathway by melatonin at the time of DMD diagnosis could allow dystrophic muscles to recuperate and reinitiate muscle repair in the late phase of the disease [7].

Overall, our results indicate that compared with normal muscles, the following changes are present in the dystrophic muscles: (i) a hyperoxidative status reflecting a membrane muscular damage as assessed by LPO levels, (ii) a nitrosative status depending on the iNOS induction in degenerated muscles, and (iii) an inflammatory status reflecting the muscle degenerative process. These degenerative events in DMD patients seem to be controlled, at least in part, by melatonin administration, suggesting the utility of melatonin therapy in DMD patients.

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