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Melatonin treatment counteracts the hyperoxidative status in erythrocytes of patients suffering from Duchenne muscular dystrophy

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ABSTRACT

Objectives: To analyze whether the antioxidant melatonin could reduce the hyperoxidative status in the blood of patients with Duchenne's muscular dystrophy.

Design and methods: Ten patients aged 12.8 ± 0.9 years were treated with melatonin (60 mg at 21:00 h plus 10 mg at 09:00 h) for 9 months, and erythrocyte markers of oxidative stress were determined at 3, 6, and 9 months of treatment. Healthy age- and sex-matched subjects served as controls.

Results: Prior to treatment, the patients had higher glutathione disulfide/glutathione ratio and higher glutathione transferase and superoxide dismutase activities, and lower glutathione reductase activity than controls. After 3 months of melatonin treatment, the hyperoxidative status of these patients was counteracted, being reduced to the normal redox state between 3 and 9 months.

Conclusion: These results, together with the reduction in the inflammatory process and in muscle injury recently reported in the same patients, support the efficacy of melatonin therapy in DMD patients.

Introduction

Duchenne muscular dystrophy (DMD), a disease linked to X chromosome and affecting 1 in 3500 newborns, is a lethal disorder characterized by progressive muscle weakness [1,2]. The DMD is caused by different mutations of the gene that encodes the dystrophin, a protein of 427 kDa that usually locates in the cytoplasmic face of the sarcolemma [1,3,4]. The role of dystrophin in the muscle is unclear, and it was related to the prevention of free radical generation [5,6]. Among other considerations, dystrophic muscles show a functional ischemia during muscle contraction, with subsequent reperfusion during rest, which may imply the existence of an increased oxidative stress [7].

In this regard, increased levels of 8-hydroxy-2′-deoxy-guanosine, protein and lipid peroxidation, and lipofuscin accumulation in dystrophic muscles have been reported [8–12]. In supporting these data, an enhanced sensitivity of dystrophin-deficient muscle cells to oxidative damage was reported [13]. A hyperoxidative status was also showed in the muscle of *mdx* mice (deficient in dystrophin), including the reduction in the total glutathione content and an increase in the disulfide glutathione (GSSG)/glutathione (GSH) ratio; increased activities of the GSH cycle, glutathione peroxidase (GPx) and reductase (GRd), and reduced concentration of hydrogen isocitrate coupled with NADP (ICDH) and aconitase, two enzymes sensitive to inactivation by oxidative stress and also involved in the production of GSH [14]. Thus, the progressive loss of muscle fibers in DMD patients may involve a chronic oxidative injury that surpasses the ability of muscle regeneration [6].

Although the factors involved in the reduced ability of muscle regeneration in DMD are yet unclear, a relationship between the loss of the neuronal nitric oxide synthase (nNOS) expression secondary to dystrophin deficiency, and increased reactive oxygen species (ROS) production, seem to be involved [15]. In this regard, there is an induction of pro-inflammatory genes by ROS including cytokines through the activation of NF-kB in DMD patients [16]. Besides, nNOS, a member of the dystrophin-glycoprotein complex, produces nitric oxide ($NO^{\bullet}$), a gas involved in multiple regulatory pathways. Among others, $NO^{\bullet}$ participates in the homeostasis of the redox status because it can scavenge ROS produced by inflammatory cells [17], protecting the muscle from oxidative damage [18]. We recently reported that patients suffering from DMD showed a noteworthy pro-inflammatory status coursing with high levels of $NO^{\bullet}$ and pro-

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inflammatory cytokines including IL-1β, IL-2, IL-6, TNF-α and INF-γ [19]. The pro-inflammatory status in these patients was absolutely counteracted after 9 months of treatment with melatonin, a known antioxidant and anti-inflammatory molecule [20]. Here, we analyzed in these patients the intracellular oxidative stress status of erythrocytes before and after melatonin therapy, looking for a beneficial effect of melatonin in reducing the hyperoxidative status of these patients. Erythrocytes were used in this study because red blood cells do not contribute to oxidative damage, and we can unequivocally measure those antioxidants able of crossing the plasma membrane into the intracellular space. Therefore, the intracellular redox status obtained from erythrocytes measurement indirectly reflects the redox status of the skeletal muscle cells [21,22].

Patients and methods

Patients

The study was carried out on 10 ambulant pediatric patients, aged 12.8 ± 0.98 years, who were followed in the Neuropediatric Unit of the Granada's University Hospital (Granada, Spain). These patients were diagnosed of DMD at 4–5 years 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 following data were systematically collected: age, sex, onset, and type of clinical signs; laboratory data (muscle biopsy results), skeletal muscle enzyme assessments, and genetic diagnosis. The patients were under treatment of prednisone at least 5 years ago (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 (Fagrón Ibérica, Barcelona, Spain) therapy consisted in the administration of two oral daily doses of the indoleamine for 9 months, one at 09:00 h (10 mg melatonin) and the other at 21:00 h (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-, sex-, and weight-matched group of children was used as a control group (C).

Samples

Blood samples were obtained from the antecubital vein in all patients at 9:00 am before starting with melatonin therapy, and 3, 6, and 9 months during melatonin treatment. Samples were centrifuged at 3000 *g* for 10 min and erythrocytes were separated, washed twice with cold saline, and frozen at −80 °C until the biochemical assays were performed. On the day of the experiment, washed erythrocytes were hemolized in phosphate buffer (10 mM sodium phosphate, 1 mM EDTA-Na2, pH 6.25), deproteinized with ice-cold 10% trichloroacetic acid, and centrifuged at 20000 *g* for 15 min. Supernatants were then used for the measurements.

Glutathione assay

For glutathione measurement, 10 μL supernatant was incubated with 10 μL of ethanol ophthalaldehyde solution (1 g/L) and 180 μL phosphate buffer (100 mM sodium phosphate, 5 mM EDTA-Na_2, pH 8.0) for 20 min at room temperature. Then, the fluorescence of the samples was measured at 350 nm excitation and 420 nm emissions in a plate-reader spectrofluorometer (Bio-Tek Instruments, Inc., Winooski, USA) [21]. For glutathione disulfide measurement, 200 μl aliquots of supernatants were preincubated with 80 μL N-ethylmaleimide solution (5 g/L in distilled water) for 40 min at room temperature and then alkalinized with 720 μl sodium hydroxide 0.1 N. Aliquots of 30 μL were then incubated with 10 μL ophthalaldehyde solution and 160 μl sodium hydroxide 0.1 N for 25 min at room temperature, and the fluorescence was then measured [23]. Glutathione concentrations were calculated according to standard curves previously prepared. Levels are expressed in μmoL/g Hb.

Glutathione peroxidase and reductase assays

To measure glutathione peroxidase activity, 10 μL of each supernatant were added to 240 μL of working solution containing buffer A plus 4 mM sodium azide, 60 mM glutathione, 20 mM reduced nicotinamide adenine dinucleotide phosphate and 0.5 U/L glutathione reductase. After incubation for 5 min at room temperature, the reaction was started by adding 10 μL of cumene hydroperoxide (0.3%) and the glutathione peroxidase activity was determined following the oxidation of the reduced nicotinamide adenine dinucleotide phosphate for 3 min at 340 nm in an UV spectrophotometer (Shimadzu Deutschland GmBH, Duisburg, Germany) [24]. To measure glutathione reductase activity, 35 μL of each supernatant were added to 465 μL of working solution containing buffer A plus 2.5 mM disulfide glutathione. After incubation for 5 min at room temperature, the reaction was started by adding 8.5 μL of reduced nicotinamide adenine dinucleotide phosphate 12 mM and the glutathione reductase activity was spectrophotometrically determined for 3 min at 340 nm [24]. Glutathione peroxidase and reductase activities are expressed as nmoL/min • g Hb. In both cases, non-enzymatic reduced nicotinamide adenine dinucleotide phosphate oxidation was subtracted from the overall rates.

Glutathione transferase assay

Washed erythrocytes were hemolized in 1:20 phosphate buffer (10 mM sodium phosphate, 1 mM EDTA-Na_2, pH 6.25). Five mL of hemolisate were mixed with 5 mL Sephadex CM-50 with the same buffer (1:100); vortex for 3 min, and centrifuged at 6500 *g* for 10 min. The supernatant was maintained at 4 °C to calculate the total GST activity, following the procedure of Habig et al. [25], using 30 mM 1-chloro-2,4-dinitrobenzene as substrate. The activities were spectrophotometrically measured for 3 min at 340 nm, and expressed as μmoL/min • g Hb).

Superoxide dismutase assay

SOD activity was measured in washed and hemolized erythrocytes following the method of Misra and Fridovich [26]. This method is based on the inhibition of adrenaline autooxidation in alkaline medium by the enzyme. Briefly, an aliquot of hemolized erythrocytes was incubated in 50 mM Na_2CO_3 buffer containing 0.1 mM EDTA, pH 10.2 and 200 μL of a solution of 6 mM epinephrine in 1 mL HCl. The reaction was followed a 480 nm by 10 min in a spectrophotometer (Shimadzu Deutschland GmBH, Duisburg, Germany). SOD activity was expressed in adrenaline units (U/g Hb).

Statistical analysis

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

Results

The levels of plasma melatonin were in the normal range both in healthy controls (6.7 ± 0.5 pg/mL) and in DMD patients (9.67 ± 0.7 pg/mL). During the treatment, plasma levels of melatonin were

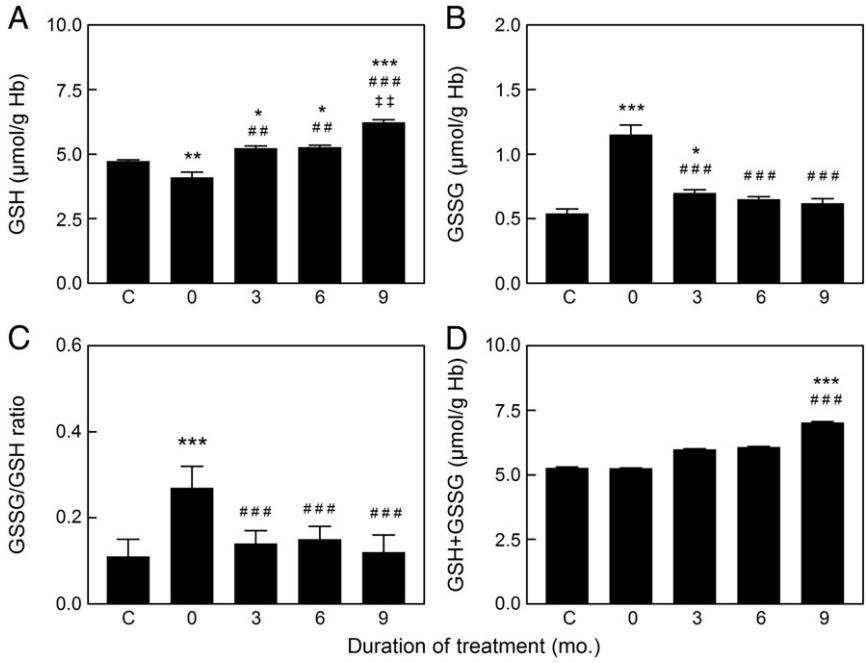

Fig. 1. Erythrocyte levels of GSH (A), GSSG (B), total glutathine (D), and GSSG/GSH ratio (C), before (0) and 3, 6 and 9 months after melatonin treatment, compared with the levels in the control group (C). *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs. C; # #$P<0.01$ and # # #$P<0.001$ vs. 0; ‡‡$P<0.05$ vs. 3 months.

stable and maintained at a pharmacological level (947 ± 57 pg/mL) (data not shown, see Ref. 19).

Compared with the control group (C), DMD patients had increased ratio of the erythrocyte GSSG/GSH before melatonin treatment (group 0) (Fig.1C, $P<0.001$), which was mainly dependent on the high levels of erythrocyte GSSG found in the patients (Fig. 1B). Melatonin reduced the erythrocyte GSSG/GSH ratio to control values after 3 months of treatment ($P<0.001$), due to a reduction in GSSG and increase in GSH levels in erythrocytes (Fig. 1A and B). The effects of melatonin were maintained up to 9 months later. Erythrocyte GSH increased in a time-dependent manner, reaching the highest values at the end of treatment (Fig. 1A, $P<0.001$). At this time, i.e., 9 months of melatonin treatment, the levels of total GSH increased significantly (Fig. 1D, $P<0.001$).

The enzymes of the GSH redox cycle in erythrocytes also changed significantly. Whereas GPx activity was similar in DMD patients and controls (Fig. 2A), GRd activity was significantly reduced in the former (Fig. 2B, $P<0.001$). Melatonin treatment reduced GPx and increased GRd activities in a time-dependent manner, with the highest effects obtained after 9 months of treatment ($P<0.001$).

DMD patients had the erythrocyte GST activity above the healthy subjects (Fig. 3A). Melatonin reduced erythrocyte GST activity along the treatment, remaining below the controls 9 months later. Erythrocyte SOD activity was higher in patients than in controls (Fig. 4, $P<0.001$), but after melatonin administration, the activity of SOD was significantly reduced during the 9 months of treatment.

Discussion

Our results show an increased oxidative stress in erythrocytes of DMD patients compared with healthy subjects. These patients also showed a significant reduction in their antioxidative defense ability. Because erythrocytes do not contribute to oxidative damage, and it was reported that biochemical abnormalities in DMD are reflected in alterations in erythrocytes, the intracellular redox status obtained from erythrocytes measurement indirectly reflects the redox status of the skeletal muscle cells [21,22]. Together, the data support the existence of an intracellular hyperoxidative status in erythrocytes of DMD patients, probably reflecting the oxidative damage present in the dystrophic skeletal muscle. Of note, the redox status in DMD patients returned to values found in healthy subjects after melatonin treatment. These results, together with those elsewhere reported [6,19], support the hypothesis of a pathogenic role for oxidative stress/inflammation in DMD, and they confirm the utility of melatonin in counteracting the oxidative stress and inflammatory status in DMD patients. Melatonin therapy also reduced the plasma levels of creatine kinase by 50% [19], supporting that blood reduction of the oxidative/nitrosative stress reflects a reduction in dystrophic muscle damage.

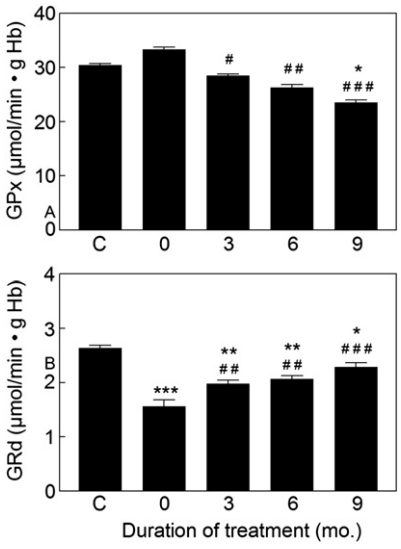

Fig. 2. Erythrocyte activities of GPx (A) and GRd (B) before (0) and 3, 6 and 9 months after melatonin treatment, compared with the levels in the control group (C). *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs. C; # #$P<0.01$ and # # #$P<0.001$ vs. 0.

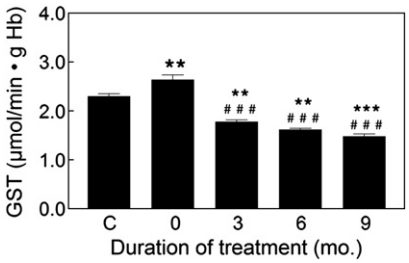

Fig. 3. Erythrocyte activities of GST (A) and termostable GST (GSTt, B) before (0) and 3, 6 and 9 months after melatonin treatment, compared with the levels in the control group (C). $^{**}P<0.01$, and $^{***}P<0.001$ vs. C; # # #$P<0.001$ vs. 0.

Because DMD seems to involve oxidative stress-dependent muscle injury [6], the progress of this disease could be clarified with the determination of biomarkers of free radicals in blood. Although GSH is one of the most abundant and physiologically important antioxidants in the skeletal muscle [14], no information regarding the key components of the GSH system in dystrophin-deficient muscle and their response to oxidative stress in DMD patients is yet available. Here, we analyzed in detail the GSH cycle and the metabolic pathways involved in the regeneration of GSH. According with previous studies in *mdx* mice, our data further support the existence of an imbalance in the redox status in the blood of patients with DMD [9,13,19,27,28]. Our study is consistent with the observation that the levels of GSSG and the GSSG/GSH ratio are higher in dystrophic patients than in healthy subjects, coinciding with a decline of GSH [14]. Because the GSSG/GSH ratio is the best index of oxidative stress status into de cell [29], our data are consistent with a high intracellular hyperoxidative status in DMD patients.

Changes in GSSG and GSH levels in the blood may also reflect changes in the enzymes of the GSH redox cycle [30], GPx and GRd. GPx plays a key role for the elimination of peroxides, oxidizing GSH to GSSG, whereas GRd recycles GSSG back to GSH [30,31]. Since GSH can also act as a direct scavenger of free radicals through electron donation [32], keeping a suitable activity of GRd is critical to yield a sufficient GSH pool for the antioxidant defense of the cell. The slight increase in GPx activity in DMD patients here found was reported elsewhere [33], and it could reflect a compensatory mechanism of the organism against oxidative damage. Nevertheless, the important inhibition of the GRd activity found in erythrocytes of DMD patients explains the high GSSG levels and high GSSG/GSH ratio in these patients. Even more, the disruption of the GSH cycle supports the inability of dystrophic subjects to defend themselves against free radical damage, which accounts for the significant membrane damage elsewhere reported in DMD patients [19].

Glutathione S-transferase (GST) is a subgroup of GST family essential in the detoxification of products derived from oxidative stress [34,35]. Under normal conditions GST, along with other antioxidant

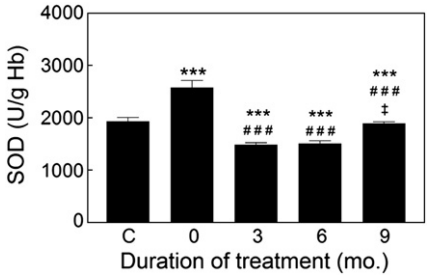

Fig. 4. Erythrocyte activities of SOD before (0) and 3, 6 and 9 months after melatonin treatment, compared with the levels in the control group (C). $^{***}P<0.001$ vs. C; # # #$P<0.001$ vs. 0; ‡$P<0.05$ vs. 3 months.

enzymes, such as SOD and GPx, provide the cell with protection against a range of harmful electrophiles produced during oxidative damage [36]. The increased activities of both GST and SOD in DMD erythrocytes suggest the existence of an elevated redox status [37,38] that, in the case of SOD, reflects the high production of O_2^{-}• by the patients, which is dismutated to H_2O_2. The slight increase in GPx activity found in DMD patients confirms this pathway.

Thus, we can consider that the increased activities of GST and SOD and, in a lesser extent, GPx, found in DMD patients constitute one of the first responses of the organism against free radical attack. It is important to note that the activity of the GST and GPx enzymes requires GSH and, thus, their effectiveness depends on the GSH pool in the cell [36]. In turn, the GSH pool in the cell depends on the activity of GRd, which reduces GSSG to GSH. The DMD patients showed a reduced activity of GRd, which explains the diminished GSH levels in their erythrocytes. Thus, the detoxifying efficiency of GST and GPx is overlapped by the decreased GSH availability and thus, reactive oxygen species remain active [36]. This explains also the high levels of lipoperoxidation elsewhere reported [12,19,37,38], and the high GSSG/GSH ratio here showed.

From these data we suggest that the main cause in reducing the efficacy of the endogenous antioxidative system in DMD patients depends on their inability to maintain the GSH homeostasis. Consequently, an antioxidant therapy should include a compound able to restore the GSH pool in the cell. A molecule that fulfills this feature is melatonin, an endogenous antioxidant. Although originally identified as a product of the pineal gland with chronobiotic properties, it is now known that most of the organs and tissues of the human body also produce melatonin [39]. There are now substantial evidences supporting the antioxidative and anti-inflammatory properties of the indoleamine. Melatonin scavenges both oxygen and nitrogen species [40–42], yielding a series of metabolites that are also free radical scavengers [43,44], constituting the so-called the antioxidant melatonin's cascade [40]. Moreover, melatonin and its metabolites do not only scavenge ROS and RNS directly, but also induces the expression of the antioxidant enzymes including GPx, SOD, GRd [41,45].

In vitro and in vivo studies have reported a protective role of melatonin against muscle oxidative/inflammatory damage in different models of disease including aging and sepsis [46–48]. Additionally, melatonin also reduces inflammatory injury in cardiac muscle due to acute exercise [49]. The main locus of melatonin action is the mitochondria, increasing their bioenergenetic activity and reducing the oxygen toxicity [50,51], thus reducing mitochondrial permeability transition and muscle cell death [52]. Altogether, these studies provide enough scientific bases for the clinical use of melatonin in DMD [53].

Our results show that 3 months of melatonin administration were enough to increase GSH levels even upon the control levels. Several mechanisms involve the high efficiency of melatonin on GSH homeostasis. Firstly, melatonin scavenges ROS directly, reducing the consumption of GSH by GST and GPx [54]. Secondly, melatonin increases GRd activity, supporting an increase in the available GSH. Thirdly, melatonin increases the activity of the gamma-glutamylcysteine-synthase, an enzyme required for GSH synthesis [55]. All these features of melatonin allow it to maintain the GSH pool of the cell, reducing the oxidative stress [56]. Consequently, the GSSG/GSH ratio, the main intracellular redox index [29], returned to normal values after melatonin therapy, suggesting that the intracellular oxidative stress is now under control. Although 3 months of treatment were enough to reduce the redox status to control values in DMD patients, melatonin further increased the GSH pool up to 9 months of treatment, probably reflecting the increase in GSH synthesis [55]. That melatonin therapy maintains the ROS production under control was further supported because the activities of the detoxifying enzymes, GPx, GST and SOD, decreased with the treatment. Normalization of the redox status in DMD patients by melatonin was followed by a sustained reduction in the markers of muscle necrosis, including

50% reduction in creatine kinase, 28% reduction in lactate dehydrogenase, and 13% reduction in myoglobin [19], suggesting a reduction in muscle necrosis.

From a therapeutic point of view, established interventions in DMD include pharmacological use of glucocorticoids, psychosocial management, physical therapy, skeletal muscle, respiratory and cardiac managements, and exercise and nutritional considerations [57,58]. Emergent therapies to restore the dystrophin expression, such as antisense oligonucleotide therapies [59,60] and stem cell therapy [61], are being evaluated. The results of this study, and those reported elsewhere in the same patients [19], and in other children's oxidative stress status [62], suggest that melatonin therapy may significantly improve the course of the disease in DMD patients. Whereas there is no current treatment for DMD, melatonin should be implemented as routine therapy of DMD.

Acknowledgments

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