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ARTICLE

The benefit of a supplement with the antioxidant melatonin on redox status and muscle damage in resistance-trained athletes

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Abstract: Previous data showed that the administration of high doses of melatonin improved the circadian system in athletes. Here, we investigated in the same experimental paradigm whether the antioxidant properties of melatonin has also beneficial effects against exercise-induced oxidative stress and muscle damage in athletes. Twenty-four athletes were treated with 100 mg·day^{-1} of melatonin or placebo 30 min before bedtime during 4 weeks in a randomized double-blind scheme. Exercise intensity was higher during the study that before starting it. Blood samples were collected before and after treatment, and plasma was used for oxygen radical absorption capacity (ORAC), lipid peroxidation (LPO), nitrite plus nitrate (NOx), and advanced oxidation protein products (AOPP) determinations. Glutathione (GSH), glutathione disulphide (GSSG) levels, and glutathione peroxidase (GPx) and reductase (GRd) activities, were measured in erythrocytes. Melatonin intake increased ORAC, reduced LPO and NOx levels, and prevented the increase of AOPP, compared to placebo group. Melatonin was also more efficient than placebo in reducing GSSG·GSH^{-1} and GPx·GRd^{-1} ratios. Melatonin, but not placebo, reduced creatine kinase, lactate dehydrogenase, creatinine, and total cholesterol levels. Overall, the data reflect a beneficial effect of melatonin treatment in resistance-training athletes, preventing extra- and intracellular oxidative stress induced by exercise, and yielding further skeletal muscle protection against exercise-induced oxidative damage.

Key words: melatonin, oxidative stress, exercise, antioxidants, skeletal muscle, athletes.

Résumé : D'après des données antérieures, l'administration de fortes doses de mélatonine améliore le rythme circadien des athlètes. Dans la présente étude, on vérifie si, selon le même paradigme expérimental, les propriétés antioxydantes de la mélatonine sont aussi valables contre le stress oxydant de l'exercice physique et les lésions musculaires chez les athlètes. Selon un devis aléatoire à double insu, 24 athlètes prennent durant quatre semaines 100 mg·day^{-1} de mélatonine ou un placebo 30 min avant le coucher. L'intensité de l'exercice physique est plus élevée durant l'étude qu'avant celle-ci. On prélève des échantillons de sang avant et après le traitement. Le plasma est utilisé pour l'analyse de la capacité d'absorption des radicaux oxygénés (ORAC), de la peroxydation lipidique (LPO), du nitrite et du nitrate (NOx) et des produits d'oxydation avancée des protéines (AOPP); les globules rouges sont utilisés pour l'analyse du glutathion (GSH), de la concentration de disulfure de glutathion (GSSG), de l'activité de la glutathion peroxydase (GPx) et de la glutathion réductase (GRd). La mélatonine suscite une augmentation de ORAC, une diminution des taux de LPO et de NOx et s'oppose à l'augmentation de AOPP comparativement au placebo. La mélatonine est aussi plus efficace que le placebo pour diminuer les ratios GSSG/GSH et GPx/GRd. La mélatonine mais pas le placebo suscite une diminution de la créatine kinase, de la lactate déshydrogénase, de la créatinine et du taux de cholestérol total. Dans l'ensemble, les données indiquent un effet bénéfique du traitement à la mélatonine chez les athlètes entraînés contre résistance en prévenant le stress oxydant extra- et intracellulaire induit par l'exercice physique et en procurant une plus grande protection du muscle squelettique contre les lésions oxydantes induites par l'exercice physique. [Traduit par la Rédaction]

Mots-clés : mélatonine, stress oxydant, exercice physique, antioxydants, muscle squelettique, athlètes.

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Introduction

Professional athletes are exposed to a large amount of physical exercise from training programs and competitions. Intense exercise is well known to have an impact on many homeostatic systems, including the stress response. Strenuous exercise increases oxygen consumption, leading to an overproduction of reactive oxygen species (ROS). An imbalance between the generation of ROS and antioxidant defense capacity of the cell is closely associated with oxidative stress, which is involved in cell and tissue injuries and in multiple disease processes (Bloomer et al. 2010). It is well known that during intense exercise, the skeletal muscle is one of the major sources of ROS generation. Low levels of ROS regulate muscle force and adaptive responses to training by mechanisms, involving calcium release and probably influencing myofilament structure. High levels of ROS, however, reduce force production and contribute to muscular fatigue during prolonged and intense exercise (Powers et al. 2011).

The protective effects of melatonin against the deleterious effects caused by oxidative stress are well documented (Acuña-Castroviejo et al. 2011). Melatonin is a singularly diverse free radical scavenger that directly interacts with a variety of oxygen- and nitrogen-based radicals and related reactants. In addition, melatonin protects against molecular damage in all regions of the cell, i.e., in both the lipid and aqueous compartments. Furthermore, it protects against lipid peroxidation, protein oxidation, and against both mitochondrial and nuclear DNA damage by free radicals (Tan et al. 2007). These direct radical scavenging actions of melatonin are receptor-independent processes (Tan et al. 2007). One of the most attractive properties of melatonin, which distinguishes it from most antioxidants, is that its metabolites also have the ability to scavenge ROS and reactive nitrogen species. The continuous protection exerted by melatonin and its metabolites, referred to as the antioxidant cascade, makes melatonin highly effective in protecting organisms from oxidative stress (Tan et al. 2007). The antioxidant efficacy of melatonin, which synergizes with other antioxidants, is based not only on its direct free radical scavenging actions but also in its ability to enhance the expression and activities of a variety of antioxidant enzymes and to induce the synthesis of glutathione, the main intracellular antioxidant (Galano et al. 2013).

Animal and human studies have shown that the scavenging of ROS with the use of antioxidants delays muscle fatigue, and that melatonin, owing to its antioxidant activity, may reduce free radical-mediated muscle damage resulting from exercise (Hara et al. 1996, 1997; Powers et al. 2011). Taking this into account, it is highly probable that melatonin treatment can improve redox status, reducing exercise-induced oxidative muscle damage of athletes (Escames et al. 2012). There is not much information concerning high doses of melatonin treatment and antioxidant response of athletes to exercise. However, high doses of the indoleamine were able to resynchronize the circadian system affected by exercise (Leonardo-Mendonça et al. 2015). Thus, the aim of the present study was to investigate whether melatonin treatment at the same doses, i.e., 100 mg·day^{-1} for 30 days, prevents against exercise-induced oxidative stress and muscle damage in athletes during training.

Materials and methods

Participants

Twenty-four resistance-trained volunteer students (all males) from the Faculty of Sport Sciences of University of Granada were enrolled in the study. Informed consent was obtained from all participants. The Ethics Committee of the University of Granada approved the study, and it was performed in accordance with the 2008 revised Helsinki Declaration of 1975. Moreover, the policies established by the U.S. Department of Health, Education, and Welfare and the American Physiological Society were followed. All volunteers were healthy subjects without a significant previous medical history, non-smokers, and they were not taking any medication or supplements. Subjects' standing height and weight were measured, and their body mass index was calculated.

Research design

The study was conducted as a randomized double-blind design during October–November, when the period of intensive training for these students was undertaken. Subjects were randomized to experimental (melatonin; $n = 12$) or control (placebo; $n = 12$) group. The experimental group was supplemented with 100 mg·day^{-1} p.o. of melatonin during 4 weeks, administered 30–60 min before bedtime; the control group was equally treated with placebo. Placebo and melatonin jars contained gelatinous capsules of the same size and colour and with the same excipients (lactose and colloidal silica), except for melatonin in the former. The jars were the same in size and colour in both treatments, and they were marked with an unknown code for the participants to identify their content once the study ended. Melatonin treatment started on day 0 and it was maintained until the end of the study (day 28). Blood samples were collected prior to (S1) and after (S2) melatonin treatment.

Physical training during the study consisted of 8 sessions per week lasting approximately 1–1.5 h each (10 h/week), comprising resistance training (5 sessions), weight training (2 sessions), and aerobic running (1 session). All subjects were instructed to avoid training during the 24 h prior to the days of blood sample (S1 and S2). The study prioritized resistance over strength. Weekly routine of resistance consisted in 3 sessions between 60%–75% of maximal strength, with higher volume than intensity, and 2 sessions between 80%–90% of maximal strength, with a volume constant and increasing the intensity. A combination of distances and profiles using both race and bike were used for these purposes. Regarding the strength, after calculating the one-repetition maximum for each subject, the usual training in the 2 sessions was focused on the strength pyramid, i.e., performing 3 sessions of 8–12 repetitions at 75% of maximal weight, and with rest periods to stretch.

Dietary intake

To estimate the average energy and nutritional intake, participants recorded their dietary intake during 3 consecutive days (one being a weekend day), prior to and during the last week of treatment. Nutritional intake was compared, taking into account the dietary reference intake of Food and Nutrition Board's, Institute of Medicine 2000 (Ervin et al. 2004).

Blood samples

Blood samples (10 mL) were collected from the antecubital vein between 0700 h and 0800 h after 8–10 h of fasting, using vacutainer CPT tubes (Ref. No. 362782, Beckton Dickinson, Madrid, Spain). Blood was centrifuged at 3000*g* for 10 min at 4 °C, plasma was collected, the buffy coat was discarded and the erythrocytes were washed with cold saline thrice. Aliquots of plasma and erythrocytes were either maintained at 4 °C or frozen at –80 °C until the assays were performed. A total of 2 blood samples pre- and post-treatment (S1 and S2) were taken during the study period as mentioned above.

Determination of the oxygen radical absorption capacity in plasma

Oxygen radical absorption capacity (ORAC) values in plasma were calculated following a previously published procedure (Prior et al. 2003). Briefly, 100 μL of plasma was mixed with 200 μL of ethanol and 100 μL of distilled water. Then, 400 μL of hexane were added, mixed, allowed to stand for 2 min, and centrifuged at 7700*g* for 10 min at 4 °C. The hexane layer was collected and another 400 μL of hexane were added to the original tube. The 2 hexane extracts were combined and dried under nitrogen flow. Then, the dry extract was dissolved in 250 μL of acetone and diluted with 750 μL of an acetone–water mixture (50:50, *v*/*v*). The aqueous solution was deproteinized with 400 μL of 0.5 N perchloric acid and centrifuged at 7700*g* for 10 min at 4 °C. Two-hundred μL

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of the supernatant were mixed with 800 μL of 75 mmol·L^{-1} phosphate buffer, pH 7.4.

To quantify the ORAC in the samples, 75 μL of each hydrophilic and lipophilic extract were added to a microplate. Then, 75 μL of 70 nmol·L^{-1} fluorescein solution was added, followed by 37.5 μL of a 300 mmol·L^{-1} AAPH (2,2′-azobis-2-methyl-propanimidamide, dihydrochloride) solution. The fluorescence of the samples was determined in a thermostatized (37 °C) plate reader spectrofluorimeter (Polarstar Optima, BMG Lab technologies), with an excitation and emission wavelengths of 490 and 545 nm, respectively. A Trolox standard curve of 10 points (10–100 mmol·L^{-1}) was constructed, and the ORAC concentration was expressed in μmol of Trolox equivalents.

Determination of glutathione and glutathione disulphide

Both glutathione (GSH) and glutathione disulphide (GSSG) were measured using *o*-phthalaldehyde as a fluorescent reagent in washed red blood cells and expressed as μmoL·g Hb^{-1} (Hissin and Hilf 1976). Washed red blood cells were haemolysed in phosphate buffer (10 mmol·L^{-1} sodium phosphate, 1 mmol·L^{-1} EDTA-Na2, pH 6.25), deproteinized with ice-cold 10% trichloroacetic acid, and centrifuged (Avanti 30, Beckman Instruments, Inc., Palo Alto, Calif., USA) at 20 000*g* for 15 min at 4 °C. Supernatants were used for the determinations. For GSH measurement, 10 μL of the supernatant were incubated with 10 μL of the ophthalaldehyde–ethanol solution (1 g·L^{-1}, *w*/*v*) and 180 μL of phosphate buffer (100 mmol·L^{-1} sodium phosphate, 5 mmol·L^{-1} ethylenediaminetetraacetic acid (EDTA)-Na2, pH 8.0) for 20 min at room temperature. For GSSG measurement, 200 μL of the supernatants were pre-incubated with 80 μL *N*-ethylmaleimide–water solution (5 g·L^{-1} *w*/*v*) for 40 min at room temperature, and then alkalinized with 720 μL 0.1 N NaOH. Aliquots of 30 μL were incubated with 10 μL of the ophthalaldehyde solution and 160 μL 0.1 N NaOH for 25 min at room temperature. Then, the fluorescence of the GSH and GSSG samples was measured at 350 nm excitation and 420 nm emissions in a plate-reader spectrofluorimeter (Bio-Tek Instruments, Inc., Winooski, Vt., USA). Standard curves for GSH and GSSG were constructed, and the GSH and GSSG concentrations were calculated accordingly and expressed as μmol·g^{-1} Hb.

Measurement of glutathione peroxidase and reductase activities

Washed red blood cells were haemolysed in phosphate buffer (10 mmol·L^{-1} sodium phosphate, 1 mmol·L^{-1} EDTA disodium salt, pH 6.25), and centrifuged at 20 000*g* for 15 min. Supernatants were then used for glutathione peroxidase (GPx) and reductase (GRd) measurements in the presence of nicotinamide adenine dinucleotide phosphate. GPx and GRd activities are expressed as μmol·min^{-1}·g^{-1} Hb. In both cases, nonenzymatic reduced nicotinamide adenine dinucleotide phosphate oxidation was subtracted from the overall rates (Jaskot et al. 1983).

Nitrite plus nitrate determination

Due to the high instability of the nitric oxide molecule, measurements must be made indirectly by the determination of nitrites (NOx), which are the compounds formed after the reaction of nitric oxide with water. Moreover, NOx are rapidly oxidized to nitrates, which should be reduced again to NOx with nitrate reductase to obtain a reliable value of the amount of nitric oxide produced during inflammation. The concentration of NOx was measured following the Griess reaction, which converts nitrite into a coloured azo compound, spectrophotometrically detected at 550 nm. Plasma levels of NOx plus nitrates are expressed in μmol·L^{-1} (Granger et al. 1996).

Determination of lipid peroxidation in plasma

Plasma samples were thawed and centrifuged (Avanti 30, Beckman Instruments, Inc., Palo Alto, Calif., USA) at 5000*g* for 5 min at 5 °C, and 200 μL of the supernatants were used for lipid peroxidation (LPO) measurement. For this purpose, a commercial LPO assay kit that estimated both malonaldhehyde and 4-hydroxyalkenals was used (Bioxytech LPO-586 assay kit, Oxis Research, Portland, Ore., USA). Absorbance was read at 586 nm, and lipid peroxidation concentrations are expressed in μmol·L^{-1} (Esterbauer and Cheeseman 1990).

Determination of advanced oxidation protein products

Advanced oxidation protein products (AOPP) were measured by spectrophotometry on a microplate reader and were calibrated with chloramine-T solution that in the presence of potassium iodide absorbs at 340 nm (Witko-Sarsat et al. 1996). In test wells, 200 μL of plasma diluted 1/5 in PBS was placed on a 96-well microliter late, and 20 μL of acetic acid was added. In standard wells, 10 μL of 1.16 mol·L^{-1} potassium iodide was added to 200 μL of chloramine-T solution (0–100 mmol·L^{-1}) followed by 20 μL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm on the microplate reader against a blank containing 200 mL of PBS, 10 μL of potassium iodide, and 20 μL of acetic acid. The AOPP concentrations were expressed as μmol·L^{-1} of chloramine-T equivalents.

Quantification of biochemical and hematologic parameters

Biochemical parameters in all samples were analysed in the clinical analysis service of Virgen de las Nieves's University Hospital of Granada by routine methods. Metabolic impairment was assessed by measuring the changes in serum concentrations of cholesterol and triglycerides as markers for lipid metabolism alteration, and glucose as marker for carbohydrate metabolism alteration. Serum concentration of creatine kinase (CK) and lactate dehydrogenase (LDH) were used as markers for muscle status. We assessed liver status by measuring the serum concentration of aspartate aminotransferase (AST), a non-specific marker for hepatic parenchymal injury, and alanine aminotransferase (ALT), a specific marker for hepatic parenchymal injury. Renal status was assessed by measuring the serum concentration of creatinine as indicator of glomerular filtration rate, and urea and uric acid as indicators of impaired excretory function of the kidney and (or) increased catabolism. Haematocrit, haemoglobin, erythrocyte, and leukocyte concentrations were also measured.

Statistical analysis

Statistical analyses were performed with the software SPSS 16.0 (SPSS Inc. Released 2007. SPSS for Windows, Version 16.0. Chicago, SPSS Inc.). Group data were expressed as means ± SD. All data were assessed for normal distribution with the Shapiro–Wilk test. All the data were analysed using mixed model repeated-measures general linear model, with treatment (melatonin vs. placebo) as the between-subject factor and time (pre-treatment vs. post-treatment) as the within-subject factor. Effects of each factor and combined factors (interaction) on each parameter were analysed. A $p < 0.05$ was considered as statistically significant.

Results

Anthropometry and nutritional status

Anthropometric data, energy, and nutritional intake of the subjects have been previously published (Leonardo-Mendonça et al. 2015). All athletes were of similar age and anthropometric parameters. The estimated energy and nutritional intake of participants had not changed during the study period, and no significant differences were found among groups.

Redox status

Figure 1 shows the total hydrophilic and lipophilic antioxidant capacity of plasma measured by ORAC. After the 4-week training period, melatonin enhanced the hydrophilic ($p < 0.001$) and lipophilic ORAC ($p < 0.001$) values, whereas placebo treatment only increased hydrophilic ORAC ($p < 0.05$). Thus, melatonin was more

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Fig. 1. Total, hydrophilic, and lipophilic antioxidant capacity of plasma measured by oxygen radical absorbance capacity (ORAC) during pre- and post-treatment with placebo or melatonin. * $p < 0.05$, and ** $p < 0.01$ vs. placebo; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. pre-treatment.

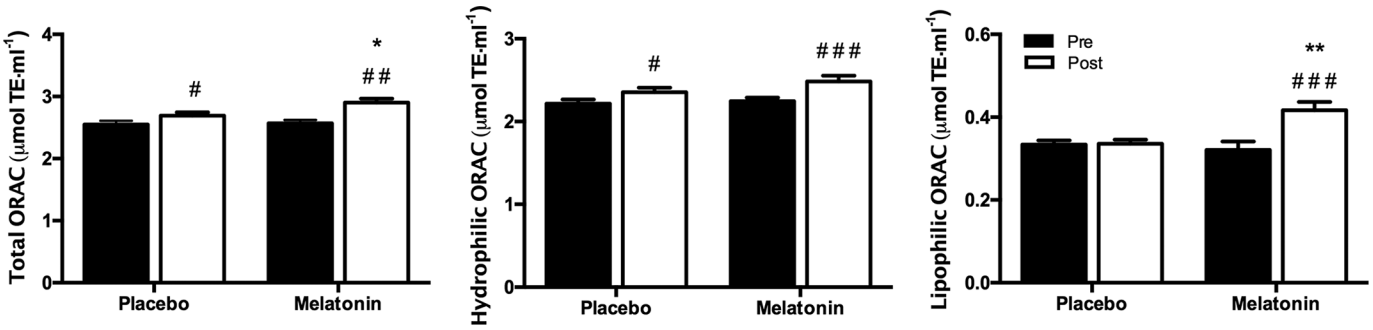


Fig. 2. Lipid peroxidation (LPO), advanced oxidation protein products (AOPP), and nitrite (NOx) levels in plasma during during pre- and post-treatment with placebo or melatonin. ** $p < 0.01$, and *** $p < 0.001$ vs. placebo; # $p < 0.05$, and ### $p < 0.001$ vs. pre-treatment.

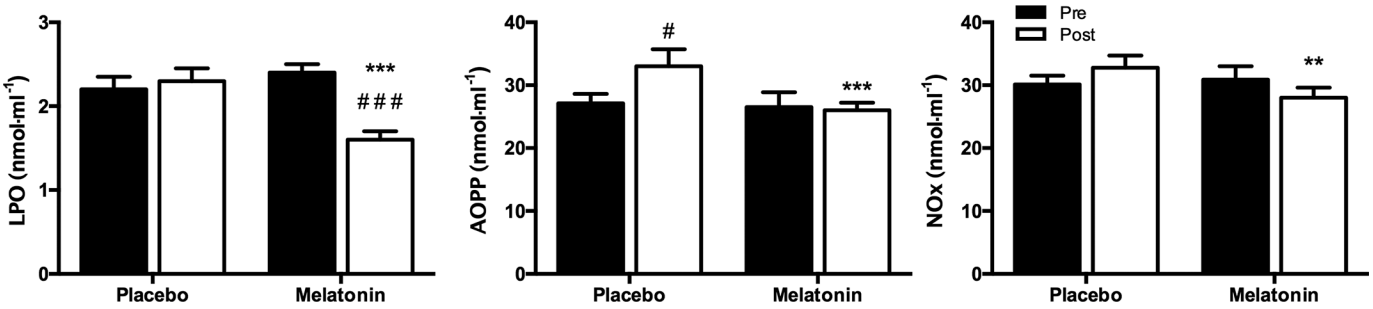
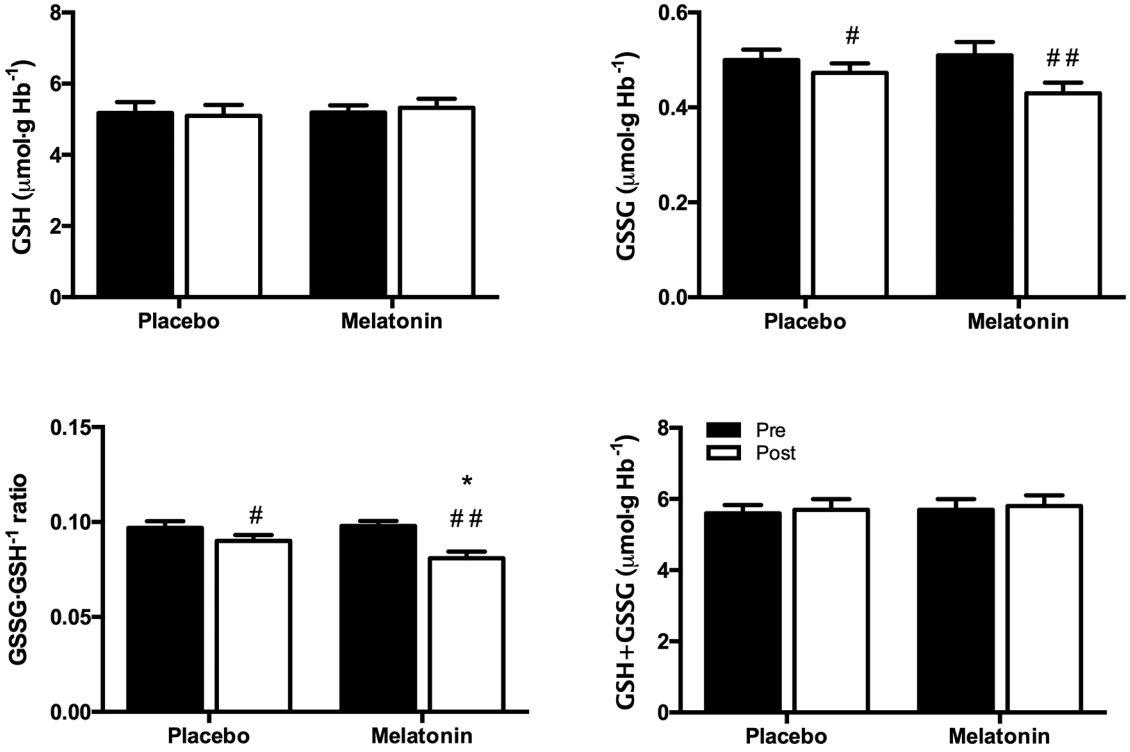


Fig. 3. Changes in the erythrocyte levels of glutathione (GSH), glutathione disulphide (GSSG), GSSG·GSH^{-1} ratio, and GSH+GSSG during pre- and post-treatment with placebo or melatonin. * $p < 0.05$ vs. placebo; # $p < 0.05$, and ## $p < 0.01$ vs. pre-treatment.



efficient than placebo in enhancing total ORAC levels ($p < 0.05$). Regarding the plasma LPO, AOPP, and NOx levels, melatonin reduced more efficiently LPO ($p < 0.001$), AOPP ($p < 0.001$), and NOx ($p < 0.01$) levels compared with the placebo (Fig. 2). Melatonin also reduced the erythrocyte GSSG·GSH^{-1} ratio more significantly than the placebo ($p < 0.05$, Fig. 3). The erythrocyte activities of GPx and GRd, and the GPx·GRd^{-1} ratio, are depicted in Fig. 4. The training realized during the 4 weeks of study increased GPx activity in all subjects, although the melatonin group shows a lower increase than placebo group ($p < 0.01$). Melatonin also prevented the GRd

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Fig. 4. Profile in erythrocyte glutathione peroxidase (GPx), glutathione reductase (GRd), and GPx·GRd^{-1} ratio during pre- and post-treatment with placebo or melatonin. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. placebo; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. pre-treatment.

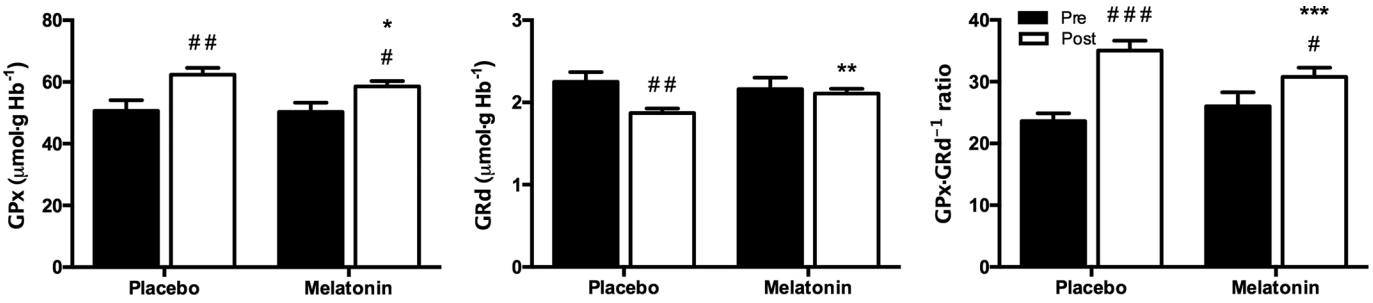


Table 1. Biochemical and hematologic data of subjects before (pre) and after (post) treatment.

	Placebo (n = 12; Mean ± SD)		Melatonin (n = 12; Mean ± SD)	
Variable	Pre	Post	Pre	Post
Biochemical parameters				
Glucose (mg/L)	76.9±1.8	81.33±1.9	77.5±2.2	80.3±2.1
Total cholesterol (mg/L)	175.1±9.5	176.3±6.9	173.5±7.8	163.9±5.3$^{\#,*}$
HDL cholesterol (mg/L)	61.4±2.8	58.5±2.8	52.3±2.7	48.8±2.9
LDL cholesterol (mg/L)	100.4±8.7	99.3±16.0	104.8±6.2	95.4±5.0
Triglycerides (mg/L)	66.6±5.9	91.7±16.0	82.2±9.5	96.6±10.4
Urea (mg/L)	35±2.1	34.9±3.1	36.3±2.9	37.2±1.6
Creatinine (mg/L)	0.96±0.0	0.99±0.0	0.94±0.0	0.91±0.0*
Uric acid (mg/L)	5.7±0.3	5.5±0.2	5.3±0.4	5.3±0.3
AST (UI/L)	23.5±2.0	23.5±1.8	25.4±3.0	26.8±5.1
ALT (UI/L)	22.6±2.7	24.9±3.4	24.0±3.0	25.6±3.2
CK (UI/L)	250.1±23.3	263.1±14.6	263.7±22.0	192.8±19.0$^{\#,*}$
LDH (U/L)	186.5±10.1	185.5±12.2	199.3±7.6	174±4.6$^{\#,*}$
Hematologic parameters				
Haemoglobin (g/dL)	15.5±0.4	15.4±0.4	16.0±0.2	16.0±0.2
Haematocrit (%)	45.1±0.9	46.5±0.9	46.3±0.5	47.4±0.7
Leucocytes (×10^3/μL)	5.4±0.3	6.3±0.5	5.7±0.3	6.1±0.4
Erythrocytes (×10^3/μL)	5.2±0.1	5.3±0.1	5.3±0.1	5.4±0.1

Note: Values are means ± SD. HDL, high-density lipoproteins; LDL, low-density lipoproteins; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CK, creatine kinase; LDH, lactate dehydrogenase.

$^{*}p < 0.05$ vs. placebo.

$^{\#}p < 0.05$ vs. pretreatment.

reduction in the athletes, which was apparent in the placebo group ($p < 0.01$). Therefore, exercise increased the GPx·GRd^{-1} ratio in both groups, but melatonin treatment reduced this increment significantly compared with the placebo ($p < 0.001$).

Blood analysis

Biochemical and hematologic parameters were analysed in the subjects of the study (Table 1). The placebo group did not show any significant change in biochemical parameters, with only a tendency to increase creatinine and CK. Melatonin treatment decreased significantly total cholesterol, CK, and LDH, and yielded lower creatinine levels than placebo. Glucose, triglycerides, urea, uric acid, AST and ALT levels remained unchanged in all subjects. Regarding hematologic parameters, no significant differences were found post-treatment or among groups.

Discussion

To our knowledge, this study represents the first evaluation of the antioxidant efficacy of 100 mg·day^{-1} melatonin treatment in healthy athletes under habitual resistance-training conditions. The results show that melatonin administration improved antioxidant defense and the oxidative and nitrosative status, reducing skeletal muscle damage. Importantly, melatonin did not negatively affect lipid and carbohydrate metabolism and did not alter liver and renal functions. Haematological parameters including haematocrit, haemoglobin, and erythrocytes and leukocytes levels, were also unmodified by melatonin. These results, together with the previously reported ability of melatonin to improve the circadian system in the same athletes under the same experimental paradigm (Leonardo-Mendonça et al. 2015), support the efficacy of this indoleamine to promote physiological health during exercise.

A first point to discuss is the dose of melatonin used here. It is thought that the oral melatonin dose required for its antioxidant action should be considerably higher than that given for modulation of the circadian cycle. However, the actual dose required in men is unclear, particularly because there are not many studies using large oral doses of melatonin in humans. In a recent study, oral doses of melatonin ranging from 20 to 100 mg·day^{-1} were well tolerated in healthy volunteers, with no safety concerns and no clinically relevant changes in any physiological or biochemical measures (Galley et al. 2014). In a previous study, we showed that 100 mg·day^{-1} during 30 days, administered at bedtime to athletes, significantly improved their altered circadian rhythms, including the sleep/wake rhythm (Leonardo-Mendonça et al. 2015) without side effects. Pharmacokinetic and subcellular distribution of melatonin in rats allowed calculating the human equivalent dose, which ranged between 50 to 500 mg·day^{-1} (Venegas et al. 2012). In line with these studies, we expected here that administration of 100 mg·day^{-1} of melatonin would result in beneficial effects

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against exercise-induced on oxidative stress and muscle damage in athletes, without any side effects of interest.

To determine the antioxidant capacity of melatonin, we first analysed ORAC, which reflects the relative strength of the antioxidant defense of the subjects. It is known that exercise enhances ORAC in well-trained athletes (Tanskanen et al. 2010), and melatonin augments ORAC values greater than Trolox, GSH, and vitamins C and E (Sofic et al. 2005). In our study, we found that melatonin increased the lipophilic ORAC component, yielding a higher increase in total plasma antioxidant capacity than placebo, which only enhanced the ORAC hydrophilic component. Then we analysed the components of the endogenous antioxidant system that could underlie such antioxidant action of melatonin. Exercise per se decreased GSSG levels and the GSSG·GSH^{-1} ratio, the intracellular redox index (Lu 2013), suggesting an adaptation of the antioxidant defences in trained subjects (Concepcion-Huertas et al. 2013; Leonardo-Mendonca et al. 2014). A further reduction in GSSG levels and in GSSG·GSH^{-1} ratio was observed after melatonin treatment, reflecting that the athletes maintained an antioxidant reserve that can be used if it is adequately stimulated, as in the case of melatonin. The GSH·GSSG^{-1} cycle is under the control of two enzymes, GPx and GRd. GPx eliminates peroxides, oxidizing GSH to GSSG, whereas GRd recycles GSSG back to GSH (Hara et al. 1996). The significant rise of GPx activity found here could reflect a response against exercise-induced peroxides such as LPO (Concepcion-Huertas et al. 2013; Serrano et al. 2010). Melatonin reduced LPO levels, and thus further GPx activity is not required when melatonin is present. In turn, GRd is highly sensitive to inactivation by oxidative damage (Martin et al. 2000), and its reduction after exercise may reveal the higher oxidative status in this group of athletes, compared with the melatonin group. The prevention of GRd activity reduction is consistent with the lower LPO and AOPP levels in melatonin-treated athletes.

The markers for oxidative damage used here include plasma LPO and AOPP. LPO is a sensitive marker for oxidative damage to cell membranes, reflecting plasma hyperoxidative status caused by exercise (Fatouros et al. 2010). Melatonin, which is concentrated by cellular membranes (Venegas et al. 2012), significantly reduced LPO levels, providing further antioxidant protection at this level. The increased GPx activity in the placebo group is compatible with enhanced peroxides elimination, thus preventing LPO during exercise. AOPP reflects oxidation of amino acid residues, such as tyrosine, leading to the formation of dityrosine, protein aggregation, cross-linking, and fragmentation. Whereas LPO represents a rapid process of membrane lipid peroxidation induced by ROS, AOPP levels are also related to inflammatory responses (Witko-Sarsat et al. 1998); these findings may explain the different degree and timing of damage to lipids and proteins, respectively. Additionally, our data show that AOPP accumulation coexists with unchanged LPO levels in the placebo group, and these changes were accompanied with an increased GPx activity. This probably reflects that AOPP may be a more accurate marker of oxidative stress than LPO, a hypothesis proposed elsewhere (Witko-Sarsat et al. 1996).

Our results showed that melatonin treatment prevented significantly the increased plasma AOPP levels, further confirming previous reports (Eskiocak et al. 2007). These results do not only reflect the antioxidant role of melatonin but may also suggest an anti-inflammatory action.

To further demonstrate this hypothesis, we measured plasma levels of NOx, which are an indirect measurement of NO. Exercise is related to skeletal muscle damage and subsequent inflammatory response, leading to the activation of inducible NOS (iNOS) that increases NO production (Concepcion-Huertas et al. 2013). This pathway may explain the trend to enhance NOx levels found here in the placebo group. Melatonin, which potently inhibits iNOS expression and activity (Crespo et al. 1999; Escames et al. 2003; Garcia et al. 2015), significantly reduced NOx levels when compared with the placebo group.

To assess whether melatonin may be safely used in athletes, we next examined metabolic, liver, renal, and muscle status as well as hematologic parameters. The placebo group did not show significant changes in biochemical and haematological parameters during the study, and melatonin did not modify any haematological parameters. Serum glucose and triglycerides were not affected by the melatonin, which was reported to preserve glycogen and increase glucose uptake into skeletal muscle (Ha et al. 2006). The ability to store and maintain muscle glycogen has long been considered to be the most important limiting factor in the successful performance of submaximal endurance events (Kaya et al. 2006). Moreover, total cholesterol levels decreased in the melatonin group, without affecting significantly the proportion of HDL and LDL, supporting similar results in melatonin-treated runners (Ochoa et al. 2011) and in patients with dyslipidaemia (Hussain 2007). The lack of changes in AST, ALT, urea, and uric acid suggest the absence of hepatic and renal injury after melatonin treatment. The melatonin group, however, had lower creatinine, CK, and LDH levels, strongly supporting the protective effect of melatonin against skeletal muscle damage during exercise (Chahbouni et al. 2010, 2011).

Finally, we assessed the energy profile of the dietary intake of the subjects enrolled in this study. This profile was close to that recommended by the dietary reference intake (Otten et al. 2006) for males aged 19–30 years, and similar to that reported elsewhere in resistance-trained subjects (Concepcion-Huertas et al. 2013), in cyclists (Serrano et al. 2010) and in Spanish university students who play sports regularly (Leonardo Mendonça et al. 2012). Following the recommendation of the American College of Sports and Medicine (2009), athletes do not need a diet substantially different from that recommended in the dietary guidelines for the general population. The lipid profile was similar to previously reported in athletes (Teixeira et al. 2009) and in university students (Leonardo Mendonça et al. 2012). No differences in the nutritional intake and anthropometric characteristics were found between the melatonin and placebo groups, ensuring that we had a homogenous sample.

Our findings confirm the beneficial effects of melatonin treatment on athletes, without any undesirable side effects that could affect athletes' performance. These findings positively support the use of melatonin during athletes' training, because of its global efficacy in exercise physiology. There is not much information related to melatonin effects on muscle physiology and remodelling after exercise, but it seems that treatment with melatonin may protect muscle from exercise-induced oxidative stress damage (Escames et al. 2012). Animal and human studies showed that the use of melatonin to reduce ROS production delays muscle fatigue during exercise (Hara et al. 1996, 1997). Furthermore, studies in dystrophinopathies demonstrated the beneficial effects of melatonin administration in these patients (Chahbouni et al. 2010, 2011). Overall, other mechanisms involved in muscle improvement at the time of exercise, including a direct effect on muscle mitochondrial function and myogenesis, which have been suggested elsewhere (Martin et al. 2000; Powers et al. 2011), deserve additional research. Moreover, since no direct exercise performances were measured in the present study, we cannot conclude that exercise performance was better in the athletes taking melatonin than in those taking placebo, and additional studies to address this question should be done.

We conclude that melatonin supplementation at doses of 100 mg·day^{-1} during 4 weeks, 30–60 min before bedtime, enhances the efficiency of the endogenous antioxidant defense system leading to redox equilibrium, and yields skeletal muscle protection against exercise-induced oxidative damage without adverse effects (Zhang and Zhang 2014). These data agree with the efficacy of the same dose of melatonin to improve the circadian system dur-

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ing exercise as reported elsewhere (Leonardo-Mendonça et al. 2015). Thus, chronodysruption and oxidative damage, both events that could reduce exercise efficiency, can be simultaneously prevented by melatonin, a unique molecule involving chronobiotic and antioxidant properties. Because melatonin has no side effects even after its chronic administration at high doses (Galley et al. 2014), it can be safely used in athletes.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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