Amelioration of Energy Metabolism by Melatonin in Skeletal Muscle of Rats With LPS Induced Endotoxemia

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Summary

In the literature, few studies have investigated the effects of melatonin on energy metabolism in skeletal muscle in endotoxemia. We investigated the effects of melatonin on tissue structure, energy metabolism in skeletal muscle, and antioxidant level of rats with endotoxemia. We divided rats into 4 groups, control, lipopolysaccharide (LPS) (20 mg/kg, i.p., single dose), melatonin (10 mg/kg, i.p., three times), and melatonin + LPS. Melatonin was injected i.p. 30 min before and after the 2nd and 4th hours of LPS injection. Antioxidant status was determined by glutathione (GSH) measurement in the blood. Muscle tissue was stained using modified Gomori trichrome (MGT), succinic dehydrogenase (SDH), and cytochrome oxidase (COX) and histological scored. Also the sections were then stained with hematoxylin and eosin. The stained sections were visualized and photographed. Creatine, creatine phosphate, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) levels were investigated using high performance liquid chromatography (HPLC) in muscle tissue. In the Melatonin + LPS group, blood GSH levels were increased compared with the LPS group (P<0.01). Melatonin reduced myopathic changes in the LPS group according to the histopathologic findings. In addition, ATP values were increased compared with the LPS group (P<0.05). Our findings showed melatonin treatment prevented muscle damage by increasing ATP and GSH levels in rats with LPS induced endotoxemia.

Key words

Energy • Lipopolysaccharide • Melatonin • Skeletal muscle

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Introduction

Sepsis-induced organ failure is a major cause of death in critical care units and is characterized by a massive dysregulated inflammatory response and oxidative stress. Lipopolysaccharide (LPS) triggers the production of various inflammatory mediators involved in the development of sepsis, systemic inflammatory response syndrome (SIRS), and multiple organ failure (Kamisoglu *et al.* 2015).

Skeletal tissue comprises 50-60 % of body cell mass and represents the largest organ affected by systemic inflammation. Muscle wasting is a serious complication of sepsis. Studies in patients with sepsis and experimental animals have provided evidence that myofibrillar proteins are particularly sensitive to the effects of sepsis (Wu *et al.* 2001). The ubiquitin-proteasome system has a major role in skeletal muscle proteolysis and is activated by several pathways, including proinflammatory cytokines, reactive oxygen species, and cyclooxygenases (Holecek *et al.* 2012). High-energy phosphorylated compounds and mitochondrial impairment have been reported in septic skeletal muscle (Giannesini *et al.* 2008). The effect of

sepsis on skeletal muscle mitochondria in rat models indicate decreased mitochondrial function, depletion of adenosine triphosphate (ATP) and adenosine 5'-diphosphate (ADP) stores, decreased mitochondrial protein synthesis, and damage to the mitochondrial transcription mechanism (Correa *et al.* 2015).

Melatonin is known to be an effective anti-inflammatory agent in various animal models of inflammation and sepsis. It has been demonstrated also to increase antioxidant defense and to exert protective effects on mitochondrial function (Reiter *et al.* 2008, Srinivasan *et al.* 2010).

Melatonin is mainly secreted from pineal gland is well known for being a potent free-radical scavenger, powerful antioxidant, and a regulator of mitochondrial bioenergetic function, especially ATP production *via* complexes I and IV (Paradis *et al.* 2015, Martin *et al.* 2002).

In a number of animal models of septic shock, as well as in patients with septic disease, melatonin reportedly exerted beneficial effects that reduced cellular damage and multiorgan failure (Sener *et al.* 2005, Kurcer *et al.* 2006).

It is now well documented that reactive oxygen species are involved in the pathogenesis of multiple organ failure following sepsis, which often leads to death (Heyland *et al.* 2006, Biolo *et al.* 2007).

Oxidative stress is the result of an imbalance between the production of reactive oxygen species (ROS) and their neutralization by antioxidants (Manzanares *et al.* 2009). Glutathione (GSH) is the main endogenous thiol antioxidant and plays an essential role in the protection against reactive oxygen species. GSH has a somewhat opposite effect in immunity; it is often regarded as anti-inflammatory because it inhibits the production and action of several inflammatory cytokines and chemokines (Biolo *et al.* 2007). Studies have shown that GSH is lower in sepsis (Biolo *et al.* 2007, Asci *et al.* 2015). Melatonin has also been shown to increase gene expression and enzyme activities of GSH and the mitochondrial GSH pool. In addition, melatonin has been shown to restore GSH homeostasis by stimulating mitochondrial gamma-glutamylcysteine synthase function under oxidative stress (Martin *et al.* 2000, Rodriguez *et al.* 2004).

Few studies have investigated the effects of melatonin on energy metabolism in skeletal tissue in sepsis models. In this study we aimed to investigate the effects of multidose melatonin treatment, which was injected before and/or after endotoxemia on both energy metabolism and histologic changes in skeletal muscle and GSH level high-dose LPS-induced endotoxemia.

Methods

Experimental procedures

The study was approved by Istanbul University Local Ethics Committee for Animal Experiments. Male Wistar Albino rats that were 8-9 weeks old and weighed an average 200 – 250 g were housed in polypropylene cages with free access to food and drink, in a light/dark cycle of 12/12 h. Rats were randomly divided into 4 groups: the control group, LPS group, melatonin group, melatonin + LPS group, each comprised 8 animals.

The control group received no treatment. The LPS group received i.p. 20 mg/kg, as a single dose; LPS from Escherichia coli O127: B8 (Sigma-Aldrich, Product No: L5668) was dissolved in 1 ml of sterile saline solution. The melatonin group received a total of 30 mg/kg in 3 i.p. doses of 10 mg/kg at 2-hour intervals; melatonin (Sigma Aldrich, Product No: M5250) was dissolved in 2.5 % ml ethyl alcohol. The fourth group was injected with a dose of melatonin (10 mg/kg, i.p.) 30 min before the LPS administration. Two further doses of melatonin were administered 2 and 4 hours after LPS administration.

All rats from all 4 groups were sacrificed at the end of the 6th hour after the first injection using sodium pentothal anesthesia (i.p. 30 mg/kg) and gastrocnemius muscles were dissected from all animals. For this purpose, the gastrocnemius muscle was cut proximally, using the same line as the skin incision. The muscle samples were rapidly frozen by liquid nitrogen.

Biochemical procedures

At the end of the experimental period, blood samples (2-3 ml) were collected from heart. Blood samples were centrifuged at 3000 rpm for 20 min. Serum concentrations of creatinine kinase, aspartate aminotransferase, alanine aminotransferase, glucose, and blood leucocyte were determined. Serum creatinine kinase, aspartate aminotransferase, alanine aminotransferase, glucose levels (Cobas autoanalyzer, DPC, Diagnostic products corporation, USA) and blood leucocytes (Beckman Coulter LH 780) were measured using the corresponding kit in an autoanalyzer.

HPLC analysis of creatine, creatine phosphate, AMP, ADP, ATP

Sample preparation

Muscle tissue samples weighing 200 mg were homogenized in 2 ml 0.42 M HClO$_4$ using a homogenizer (Ultraturrax T25) for 30 sec. A 1.0 ml supernatant was taken for adjusting pH with 1.0 M K$_2$HPO$_4$ after centrifugation at 3000 rpm for 5 min.

Measurement of creatine, creatine phosphate, and high-energy compounds

Creatine, creatine phosphate, and adenine nucleotides were evaluated with C18 column (5 µm, 250 mm x 4.6 mm, Nucleodur, USA) with isocratic elution using a KOH/KH$_2$PO$_4$ buffer (215 mM, pH 6.25), 3 mM tetrabutylammonium phosphate, and 5 % acetonitrile ion-paired reverse-phase chromatography using HPLC (Agilent 1100, USA) at 214 nm (Shellvold *et al.* 1986). Creatine, creatine phosphate, and adenine nucleotides were calculated from their external standard curves from different concentrations.

Measurement of blood reduced GSH levels

GSH levels was determined with reduced GSH measurement in blood in accordance with the method of Buetler *et al.* Blood reduced GSH levels were measured spectrophotometrically using DTNB (5,5'dithio-bis-(2-nitro-benzoic acid) reagent (Buetler *et al.* 1963). After blood samples were hemolyzed with distilled water and precipitated proteins were removed by centrifugation, phosphate buffer and DTNB were added to the supernatant. The colored complex was measured at 412 nm at 5 min using a spectrophotometer (Shimadzu, Japan). Blood GSH concentrations were calculated using a GSH calibration curve. The results were expressed as mmol/l (Buetler *et al.* 1963).

Histopathologic procedures

The gastrocnemius muscle was taken while the animals were under anesthesia. The muscle samples were rapidly frozen in isopentane cooled by liquid nitrogen. We took 8-µm-thick serial cross-sections and stained them with modified Gomori's trichrome (MGT) method, succinic dehydrogenase (SDH), and cytochrome oxidase (COX) using standard protocols (Engel *et al.* 1963, Tanji 2008). The stained sections were visualized and photographed using a Nikon microscope (ECLIPSE 80i Nikon Corporation, JAPAN).

Both the intensity and the distribution of specific MGT, SDH and COX staining were scored. For each sample, a histological score (HSCORE) value was derived by summing the percentages of cells that stained at each intensity, multiplied by the weighted intensity of the staining [HSCORE = S Pi (i+1), where i is the intensity score (0-4) and Pi is the corresponding percentage of the cells]; staining was scored as 0= no staining, 1=weak staining, 2=middle staining, 3=dark staining, 4=very dark staining.

For hematoxylin and eosin (H&E), the muscle tissue samples were fixed in 10 % buffered formalin and embedded in parafin wax. Five-micrometer-thick sections were placed on polylysine-coated slides and stained with H&E. The slides were evaluated under a light microscopy (Olympus BX51; Olympus Corp., Tokyo, Japan) at 100 × magnification.

Statistical analysis

Data are presented as mean values ± standard deviations. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of P<0.05 were regarded as significant.

Results

Biochemical findings

Serum creatinine kinase, aspartate aminotransferase, alanine aminotransferase and blood leukocyte values were found increased in the LPS groups as compared with those of other groups (P<0.05).

In the LPS group, glucose levels were observed to be decreased compared with the control group (P<0.01). In the melatonin + LPS group; glucose levels were higher than in the LPS group (P<0.01) (Table 1).

GSH findings

Blood GSH levels were found decreased in the LPS groups as compared with the control group, but blood GSH levels were found increased in the melatonin + LPS group compared with the LPS group (P<0.01) (Fig. 1).

Histopathologic findings

COX, MGT, and SDH staining was found normal in the control group. We saw no red fibers in the control group. In the LPS group, SDH and COX had significantly increased staining (P<0.01). There were

frequent red ragged fibers in LPS group. In the melatonin group, SDH, MGT, and COX stainings were the same as those of the controls. In the melatonin + LPS groups, there were significantly less COX, MGT and SDH staining compared with the LPS groups (P<0.05). With

H&E staining, we observed weak fibre boundaries and irregular – shaped nuclei in the LPS group. The appearance of muscle fibers in the melatonin + LPS groups was the same as those of the controls (Fig. 2, Table 2).

Table 1. Values of serum creatine kinase, aspartate aminotransferase, alanine aminotransferase, glucose, leukocyte for groups.

	Control	**LPS**	**Melatonin**	**Melatonin+LPS**
Creatine kinase (U/l)	0.42±0.05	0.58±0.14[a]	0.37±0.36	0.44±0.11
Aspartate aminotransferase (U/l)	204.5±10.6	413±160.81[a]	243.4±82.6	247±52.32
Alanine aminotransferase (U/l)	78.75±28.37	98.67±56.22[a]	54.38±12.05	63.29±21.83
Glucose (mmol/l)	5.98±0.97	2.71±0.62[b]	7.27±0.79	5.36±0.65[c]
Leukocyte (10^3/µl)	1.88±0.86	4.30±1.80[a]	2.05±0.69	3.05±1.93

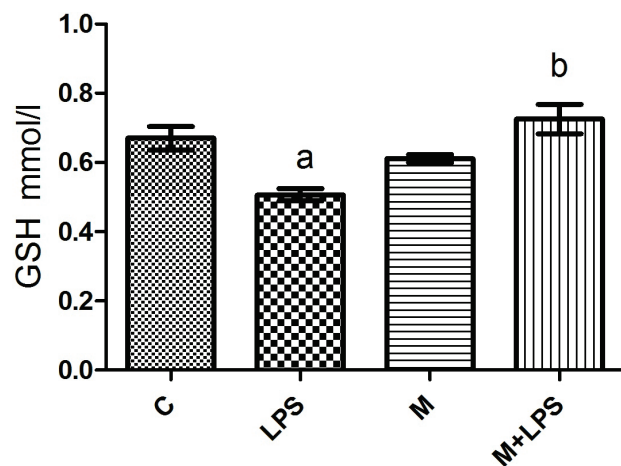[a] P<0.05, vs. control, [b] P<0.01, vs. control, [c] P<0.01, vs. LPS

Fig. 1. Levels of GSH in groups; C: Control, LPS: Lipopolysaccharide, M: Melatonin, M+LPS: Melatonin+LPS, [a] P<0.01 LPS vs. Control [b] P<0.01 M+LPS vs. LPS groups.

HPLC findings

There were no changes in values of creatine for the experimental groups (P>0.05). In the LPS group, creatine phosphate levels were decreased compared with the control group (P<0.05). AMP values were found decreased in the LPS and melatonin + LPS groups compared with the control group (P<0.05). ADP values were found increased in the LPS group as compared with the control and melatonin + LPS groups (P<0.01). ATP values were found higher in the melatonin group than in the control group (P<0.01), and also increased in the melatonin + LPS group compared with the LPS group (P<0.05). In the LPS group, we observed significantly decreased ATP values compared with controls (P<0.05) (Fig. 3).

Discussion

Sepsis is an exaggerated whole-body inflammatory response to infection that may lead to multiple organ failure, in particular of the liver, kidney, and muscle. Skeletal muscle, the most abundant tissue in the human body, is essential for the maintenance of body composition and physical activity (Hasselgren *et al.* 2005).

Sepsis should be defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer *et al.* 2016). Sepsis associates with leukocytosis or leukopenia, major hemodynamic changes, and higher values of aspartate aminotransferase, and alanine aminotransferase. Our results in the LPS group were in agreement with the literature. The activation and accumulation of leucocytes in acute sepsis models have been shown to cause tissue damage in the liver due to endotoxin infusion with Gram-negative bacteria (Windsor *et al.* 1994). Hypoglycemia occurs in the majority of domestic animals during septicemia (Lang *et al.* 1992). Melatonin treatment reversed glucose values in septic rats. Studies indicated that MT1 and MT2 melatonin receptors in the liver may be involved in regulating blood glucose (Mühlbauer *et al.* 2009). In addition, creatine kinase level was increased in our study. Elevation of creatine kinase is observed in 31 – 80 % of patients in settings of systemic inflammatory response syndrome, sepsis, or septic shock. Skeletal muscle ischemia due to sepsis-related hypotension explains the elevation of creatine kinase (Ammann *et al.* 2001).
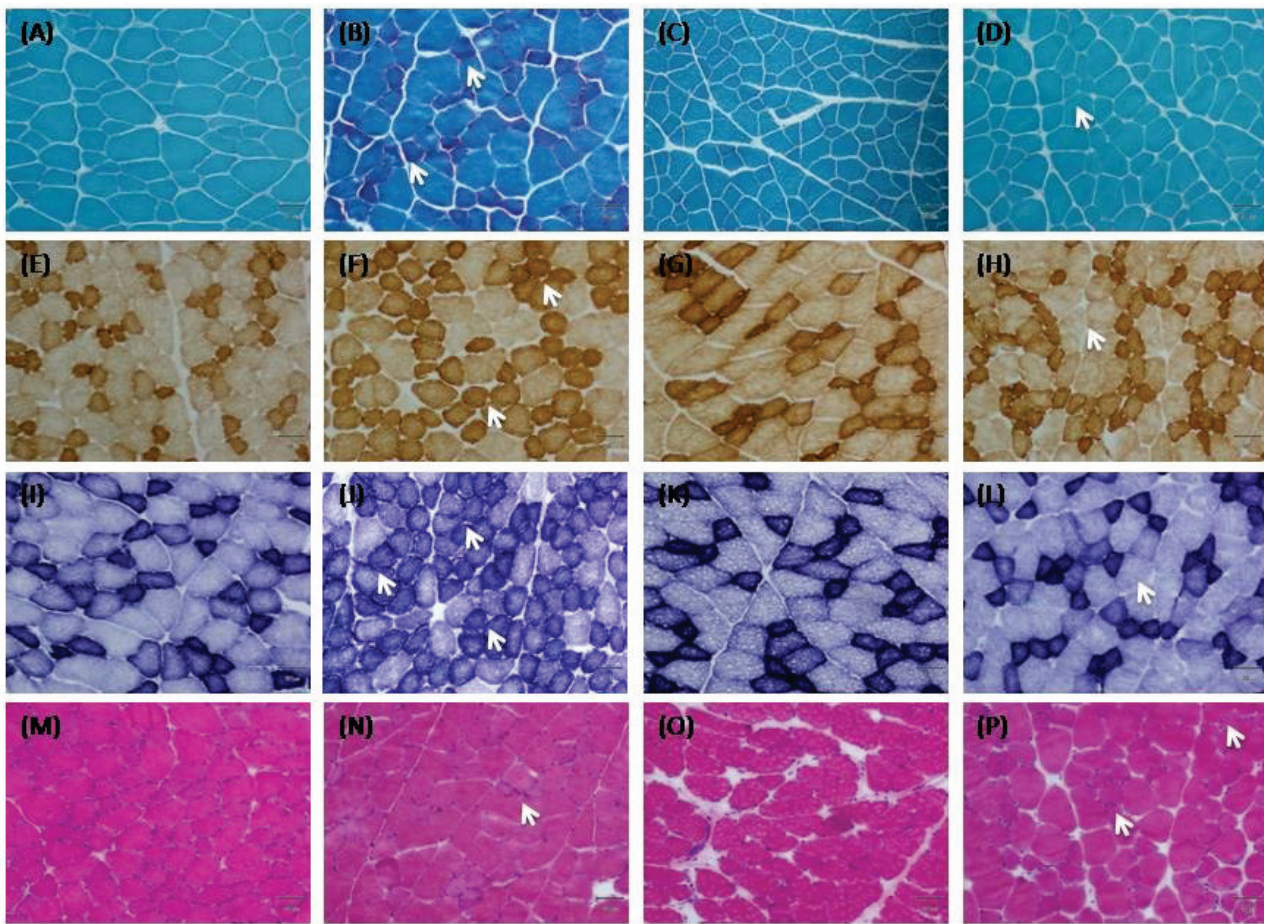
Fig. 2. Muscle tissue sections were stained control group with Modified Gomori Trichrome (**A**), cytochrome oxidase (**E**), succinate dehydrogenase (**I**). Tissue sections were stained LPS group with Modified Gomori Trichrome (**B**) cytochrome oxidase (F), succinate dehydrogenase (**J**). Tissue sections were stained Melatonin group with Modified Gomori Trichrome (**C**), cytochrome oxidase (**G**), succinate dehydrogenase (**K**). Tissue sections were stained Melatonin + LPS group with Modified Gomori Trichrome (**D**), cytochrome oxidase (**H**), succinate dehydrogenase (**L**). Sections of muscle tissue from control (**M**), LPS (**N**), Melatonin (**O**), Melatonin+LPS (**P**) stained with H&E, × 100 magnification. In image N; white arrows indicate weak fibre boundaries and irregular – shaped nuclei in the LPS group.

Table 2. H-scores values for COX, MGT and SDH stainings of experimental groups.

Experimental Groups	COX	MGT	SDH
Control	48.75±8.53	14.25±4.34	27.5±9.57
LPS	162.5±35[a]	100±16.32[a]	110±31.6[a]
Melatonin	52.5±9.57	18.75±4.78	32.5±9.57
Melatonin + LPS	55±12.9[b]	20±9.12[b]	33.75±11.08[b]

[a] P<0.01 LPS vs. control, [b] P<0.05 Melatonin+LPS vs. LPS

It was shown that melatonin administration counteracted the alterations of mitochondrial GSH pool by restoring Glutathione Reductase (GRd) during sepsis (Escames *et al.* 2006). In our study, we found low GSH levels in LPS-treated rats. The decline in GSH/GSSG after LPS challenge has been demonstrated in different models of septic shock (Victor *et al.* 2004). In our study, melatonin treatment increased GSH levels in septic rats. Melatonin has been found to significantly reduce LPS-induced free radical damage in a variety of organs (Sewerynek *et al.* 1995, Escames *et al.* 2003). Sewerynek *et al.* (1995) first demonstrated protection against LPS-

induced damage after melatonin administration. Melatonin's direct antioxidant activity and its stimulatory effect on antioxidant enzyme activities may have clinical implications for the treatment of different pathologic conditions. It is suggested that melatonin has a potent glutathione peroxidase stimulating effect in response to the free-radical damage in severe sepsis. The evidence that melatonin increases gene expression for glutathione peroxidase suggests a potential mechanism of action at the DNA level in different tissues (Tan *et al.* 1993, Antolin *et al.* 1996). A stimulatory effect of melatonin on brain glutathione peroxidase activity was also reported by Barlow-Walden *et al.* (1995).
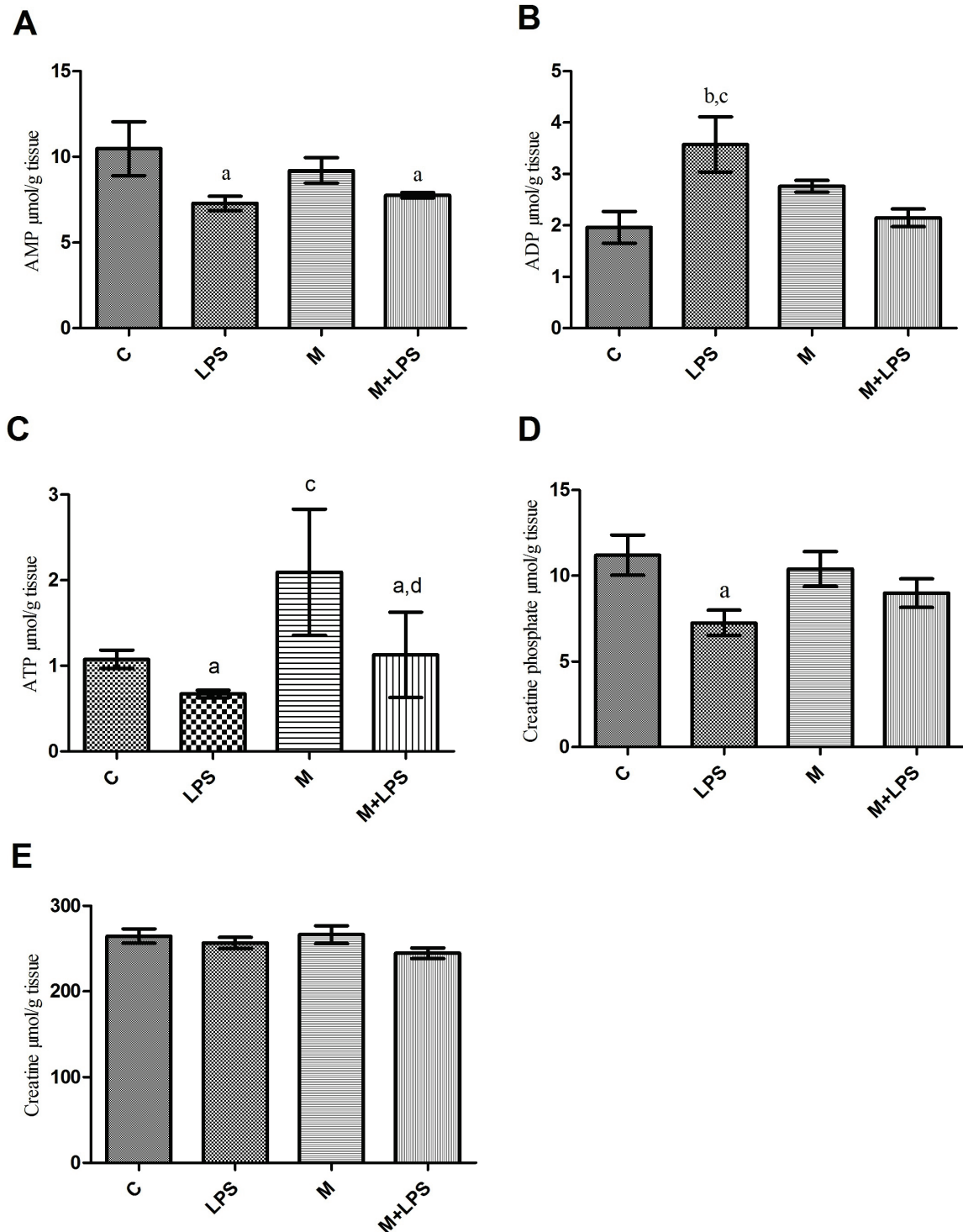

Fig. 3. Levels of AMP (**A**), ADP (**B**), ATP (**C**), Creatine phosphate (**D**), and Creatine (**E**) in groups. C: Control, LPS: Lipopolysaccharide, M: Melatonin, M+LPS: Melatonin+LPS; [a] P<0.05 vs. Control, [b] P<0.01 vs. Control, [c] P<0.01 vs. LPS, [d] P<0.05 vs. LPS.

Skeletal muscle energy metabolism is rapidly altered during sepsis. A previous investigation has shown that changes in high-energy phosphate metabolism occur early in the course of infection (Wallimann *et al.* 1992). Van Lambalgen *et al.* (1994) demonstrated that creatine phosphate and ATP levels were significantly decreased in muscle tissue of endotoxemic rats. In our study we observed significantly decreased ATP values during endotoxemia. Brealey *et al.* (2002) reported depressed ATP levels in muscle biopsies taken from patients with sepsis.

There are relationships between the severity of sepsis, mitochondrial damage, and bioenergetic dysfunction in human and experimental animal models of sepsis (Zang *et al.* 2007). In the present study, ATP values were found higher in the melatonin group. Melatonin treatment improved low energy levels in rats with sepsis.

Melatonin is selectively taken up by mitochondrial membranes. Two main considerations suggest a role for melatonin in mitochondrial homeostasis. First, mitochondria produce high amounts of ROS and reactive nitrogen species. Second, mitochondria depend on GSH uptake from the cytoplasm; therefore, the antioxidant effect of melatonin and its ability to increase GSH levels may be of great importance for mitochondrial physiology (Urata *et al.* 1999).

Mitochondrial dysfunction is thought to play an important role in the pathogenesis of many different disease states. It has been proposed that an acquired defect in oxidative phosphorylation prevents cells from using molecular oxygen for ATP production and potentially causes sepsis-induced organ dysfunction (Fink *et al.* 2002). Inhibition of electron flow through the transport chain and uncoupling of electron transport from ATP synthesis have been described in sepsis (Callahan *et al.* 2001).

ADP values were found to be increased in rats with sepsis as compared with other groups. Lara *et al.* demonstrated that free intracellular ADP concentrations were greatly increased in the gastrocnemius muscle of rats with sepsis fed with a creatine analog. Lara *et al.* (1998) proposed that high levels of ADP reflected increased ATP consumption induced by systemic infection during sepsis. We found that creatine phosphate levels were decreased in the LPS group compared with controls. Previous studies have shown that creatine phosphate breakdown was accerelated during early sepsis to maintain ATP levels within 24 hours of cecal ligation and puncture in rodents. A 20 % decrease in phosphocreatine stores has been observed in resting gastrocnemius muscle to compensate for this, alongside increased ATP consumption induced by systemic infection (Jacobs *et al.* 1991).

Lara *et al.* showed that there was altered muscle bioenergetics in septic animals depleted of creatine phosphate compounds. It has been proposed that creatine phosphate stores are used to buffer ATP levels during increased metabolic demand and that creatine phosphate plays an important role in the control of myocellular energy status during sepsis (Lara *et al.* 1998).

Creatine phosphate levels maintain myocellular ADP levels; regulate mitochondrial respiration, glycogenolysis, and glycolysis (Radda *et al.* 1986).

We showed that an injection of melatonin increased creatine phosphate levels in endotoxemic rats, but this increment was not significant.

In our study, myopathic changes were seen in the muscle sections of the majority of animals in the LPS group. The presence of red ragged fibers was often increased. Red ragged fibers are an important marker for mitochondrial disease and are often present in large numbers in the skeletal muscle of patients with mitochondrial myopathy and encephalomyopathy (Di Maura *et al.* 1993). We observed that treatment with melatonin reduced the amount of red ragged fibers and preserved the structure of myofibers in rats with endotoxemia. Red ragged fibers are considered to represent a compensatory proliferation of mitochondria in a state of insufficient energy production from oxidative phosphorylation (Iwaki *et al.* 1993). It has been shown that melatonin improved low energy levels and was a potent antioxidant. Accordingly, it was suggested that melatonin could prevent muscle damage (Sánchez *et al.* 2015).

In conclusion, many studies have shown that melatonin has antiinflammatory and antioxidant effects in human and animal models of sepsis, but few studies have shown that melatonin ameliorates skeletal energy metabolism during endotoxemia. We demonstrated that treatment with multidoses of melatonin injected before and after LPS may prevent muscle damage during endotoxemia by increasing ATP and GSH levels. We suggest that melatonin treatment restored energy levels during the septic state.

Conflict of Interest

There is no conflict of interest.

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