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The Effect of Melatonin on Plasma Oxidant–Antioxidant Skeletal Muscle Reperfusion Injury in Rats

M Halici[1], F Narin[2], CY Turk[1], R Saraymen[2], A Baykan[3] and S Kabak[1]

[1]Orthopaedics and Traumatology Department, [2]Biochemistry Department and [3]Department of Paediatrics, Medical Faculty, Erciyes University, Kayseri, Turkey

We investigated the effects of melatonin administration on skeletal muscle ischaemia–reperfusion injury (IRI) by assessing plasma malondialdehyde (MDA), superoxide dismutase (SOD), total glutathione (GSSH), glutathione peroxidase (GPX) and myeloperoxidase (MPO) concentrations. Male Sprague-Dawley rats (*n* = 32) were randomized into four groups: group 1 served as time controls; group 2 were the test animals; group 3 received melatonin (30 mg/kg) intraperitoneally prior to the induction of ischaemia; and group 4 received melatonin (30 mg/kg) intraperitoneally prior to the reperfusion period. Administration of melatonin prior to reperfusion significantly decreased the elevated MDA concentration caused by IRI, and significantly elevated GSSH concentrations, which had been reduced by IRI. Ischaemia–reperfusion injury significantly increased activities of GPX, SOD and MPO, and melatonin administration reversed this effect. In conclusion, a pharmacological dose of melatonin showed significant protective effects against IRI by decreasing lipid peroxidation, MPO, SOD and GPX enzyme activities and regulating glutathione content.

KEY WORDS: Melatonin; Indolamine; Ischaemia–reperfusion injury; Rats; Skeletal muscle; Malondialdehyde; Glutathione; Glutathione peroxidase; Superoxide dismutase

Introduction

Skeletal muscle is highly sensitive to ischaemia–reperfusion injury (IRI), which occurs clinically during tourniquet injury, free tissue transfer, acute compartment syndrome and replantation. All of these procedures entail unavoidable periods of ischaemia. The severity of IRI is directly related to the duration of ischaemia, the tissue temperature and the specific tissue sensitivity to ischaemic injury.[1]

It is well recognized that sustained ischaemia can induce cell death and tissue necrosis, which are mainly caused by energy insufficiency. Tissue injury in many organs occurs not only during the ischaemic period, but also during reperfusion period, and reoxygenation of the ischaemic tissue may promote the generation of reactive oxygen metabolites (ROM) that have deleterious effects on various cellular functions.[2] Therapeutic interventions for reperfusion injury need to be multifactorial, therefore; it

has been shown that antioxidant therapies clearly protect against IRI to various degrees.[3–6]

Melatonin, the chief indolamine produced by the pineal gland, has been shown to be an effective antioxidant and free radical scavenger.[7,8] Melatonin, because of its small size and high lipophilicity, crosses biological membranes easily, thus reaching all compartments of a cell. There is a substantial body of evidence to show that melatonin protects DNA, lipids and proteins against oxidative damage resulting from a number of endogenous and exogenous free radical generating processes.[7,9,10] Recently, exogenously administered melatonin was shown to protect against ischaemia–reperfusion-induced tissue damage and it was suggested that this protective effect may be due to its ability to scavenge free radicals.[11–13] The protective effect of melatonin on plasma oxidant–antioxidant activity during ischaemia–reperfusion-induced skeletal muscle damage has not been studied to date, however.

We aimed to investigate the effects of pre-ischaemic and pre-reperfusion melatonin administration on skeletal muscle IRI in rats. To do this we assessed plasma concentrations of malondialdehyde (MDA), superoxide dismutase (SOD), total glutathione (GSSH), glutathione peroxidase (GPX) and myeloperoxidase (MPO).

Materials and methods

ANIMALS

This study was carried out with the prior approval of The Animal Experimental Ethics Committee of Erciyes University (Kayseri, Turkey). Male Sprague-Dawley rats (Erciyes University Medical Faculty Experimental Animals Breeding and Research Centre, Kayseri, Turkey; n = 32) weighing 298 – 367 g (mean 332 g) were used in the study. Rats were kept under standardized conditions of light (14/10 h light/dark cycle) and temperature (23 ± 2 °C), and fed standard rat chow (Aytekinler Yem Sanayi, Konya, Turkey). All procedures were performed in the Experimental Animals Breeding and Research Centre (Medical Faculty, Erciyes University).

EXPERIMENTAL GROUPS

Rats were randomized into four groups of eight rats; the total experimental duration was the same for each group of rats. Rats in group 1 were used as time controls, i.e. they remained anaesthetized for the whole duration of the study while the other experimental groups underwent surgical intervention. Group 2 comprised the ischaemia–reperfusion test animals: the microvascular clamp remained in place for 4 h continuously, after which it was removed to allow reperfusion for a further 1 h. Group 3 animals received melatonin (30 mg/kg) intraperitoneally 15 min before the induction of ischaemia, and group 4 animals received melatonin (30 mg/kg) intraperitoneally immediately before the reperfusion period.

Melatonin (Sigma, St. Louis, MO, USA) was dissolved in absolute ethanol and further dilutions were made in saline. The final concentration of ethanol was 1%. Group 2 rats were given 1% alcohol in saline (5 ml/kg) 15 min before ischaemia was induced. Group 1 rats received the same treatment at a similar time-point.

SAMPLE PREPARATION

Ketamine (20 mg/kg Ketalar®, Eczacibasi, Istanbul, Turkey) and xylazine (5 mg/kg Rompum®, Bayer, Leverkusen, Germany) was given intraperitoneally. The ischaemia cycle was caused by microvascular clamping of the left femoral vessels. Blood samples for

biochemical testing were taken from the left femoral vein using a 22G intravenous cannula (Mediflon®, Eastern Medikit Ltd, Delhi, India) before the rats were killed.

Heparinized blood samples were centrifuged at 430 ***g*** for 15 min at 4 °C. After separating the plasma, samples were stored at –20 °C until they were analysed.

All procedures were carried out in full compliance with Turkish Law 6343/2, Veterinary Medicine Deontology Regulation 6.7.26, and with the Helsinki Declaration of Animal Rights.

BIOCHEMICAL ANALYSIS

Plasma MDA concentrations were measured using the double heating method.[14] The concentration of thiobarbituric acid reactive substances (TBARS) was calculated by the absorbance coefficient of malondialdehyde-thiobarbituric acid complex and expressed in nmol/ml. Enzymatic activity of SOD was measured using the method described by Sun *et al.*[15] and the activity was expressed as U/ml. The MPO activity, used as an index of neutrophil accumulation, was determined by the method described by Klebanoff[16] and expressed as U/l. The GPX activity was determined according to Paglia and Valentine[17] using hydrogen peroxide as the substrate and expressed as U/ml. The GSSH was measured using the glutathione reductase-5-5'-dithiobis-(2-nitrobenzoic acid) recycling assay of Tietze.[18] Total glutathione data were expressed as µmol/ml.

STATISTICAL ANALYSIS

Kruskal-Wallis non-parametric variance analyses were used to test for statistical significance of *P*-values obtained between treatment groups. All groups were compared with each other using the Mann–Whitney *U*-test. A P-value < 0.05 was considered significant.

Results

Table 1 shows the mean (± SD) plasma antioxidant enzyme concentrations in rats from the four treatment groups following the interventions.

Discussion

Aerobic organisms possess antioxidant defence systems that deal with ROM produced as a consequence of aerobic respiration. The biological effects of free radicals are controlled enzymatically *in vivo*, by a wide range of antioxidant enzymes such as SOD, GPX and catalase. Small changes in their physiological concentrations may have a dramatic effect on the resistance of cells to oxidative damage to lipids, proteins and DNA.[2,19,20]

During IRI, the release of ROM (H_2O_2) by hypoxic endothelium appears to be a primary event in the initiation of local and systemic inflammation.[21] Neutrophil adhesion during IRI is determined, to a large extent, by selectin concentrations in the damaged endothelium.[22] Adherent neutrophils subsequently migrate across the endothelium into the underlying ischaemic tissue and release ROM via MPO. Myeloperoxidase plays a fundamental role in oxidant production by neutrophils, which are a potential source of ROM and considered to be the major effector cells in cellular injury long after tissue ischaemia has been reversed.[8,20,23,24] In this study, melatonin administration inhibited MPO activity, which was increased by ischaemia–reperfusion.

Lipid peroxidation, mediated by ROM, is believed to be an important cause of the damage to (and destruction of) cell membranes, since polyunsaturated fatty acids of the cellular membranes are degraded by this process with the consequent disruption of membrane integrity. Membrane peroxid-

TABLE 1:
Plasma antioxidant enzyme activities (mean ± SD) in 32 male rats (eight rats per group) following surgically induced ischaemia–reperfusion of skeletal muscle under four different treatment conditions

Group	Treatment	Malondialdehyde (nmol/ml)	Total glutathione (µmol/ml)	Glutathione peroxidase (U/ml)	Superoxide dismutase (U/ml)	Myeloperoxidase (U/l)
1	Time control	0.89 ± 0.19	174.74 ± 28.15	0.97 ± 0.07	1.08 ± 0.18	173.50 ± 142.35
2	Ischaemia–reperfusion	1.29 ± 0.19[a]	122.70 ± 15.15[a]	1.33 ± 0.19[a]	1.35 ± 0.10[c]	309.19 ± 88.15[c]
3	Melatonin (30 mg/kg) prior to ischaemia	0.98 ± 0.34	148.68 ± 19.63[a,b]	1.13 ± 0.01[b]	1.22 ± 0.07[c]	218.69 ± 63.95
4	Melatonin (30 mg/kg) prior to reperfusion	0.95 ± 0.15[b]	149.91 ± 10.98[b]	1.11 ± 0.03[a,b]	1.21 ± 0.09[a,b]	210.21 ± 38.77[c]

[a] $P < 0.001$ compared with group 1.
[b] $P < 0.005$ compared with group 2.
[c] $P < 0.05$ compared with group 1.

ation can lead to cell lysis.[2,20] In our study, the concentration of MDA in rats was significantly increased by ischaemia–reperfusion. Our results demonstrated that melatonin treatment reduced the IRI-induced elevation of MDA concentrations back to control values.

Glutathione is an important component of the intracellular mechanism that protects against various noxious stimuli, including oxidative stress. Reduced glutathione, as the main component of the endogenous non-protein sulphydryl pool, is known to be a major low molecular weight scavenger of free radicals in the cytoplasm, however.[25] As a consequence of their exposed sulphydryl groups, non-protein sulphydryls bind a variety of electrophilic radicals and metabolites that may be damaging to cells.[18,26] It was reported that glutathione levels and the activities of GPX and glutathione reductase, which are critical components of the glutathione–redox cycle, were significantly reduced due to oxidative stress. One hypothesis is that an impairment of the antioxidant defence mechanism could permit enhanced free radical-induced tissue damage.[7] Our study showed that depletion of plasma GSSH was restored by melatonin treatment. The activity of GPX (which detoxifies H_2O_2 while oxidizing GSSH to reduced GSSH) was depressed during the H_2O_2 ischaemia–reperfusion; this probably contributed further to the resulting oxidative damage by making H_2O_2 available for conversion to OH.[27 – 30] Melatonin inhibited GPX activity, which had been increased by ischaemia–reperfusion, however.

Superoxide dismutase is an antioxidative metalloenzyme that catalyses the dismutation of O_2^- into O_2 and H_2O_2, which affords protection against free radical damage. It can, therefore, be associated with increased oxidative stress as the enhancement of SOD activity will increase H_2O_2 production. If H_2O_2 is not efficiently removed by GPX and/or catalase, the effect of increased SOD may be to cause oxidative damage.[27] In our study, melatonin treatment reduced SOD activity, which was increased by ischaemia–reperfusion in a similar way to GPX and MPO activity.

The pineal hormone melatonin was recently shown to have free radical scavenging ability that reduced lipid peroxidation, which can be devastating to the functional integrity of cell membranes. Melatonin can also scavenge both the hydroxyl radical, a very potent inducer of lipid peroxidation, and the peroxyl radical, which propagates the process of lipid peroxidation. Peroxidation of membrane lipids can disrupt membrane fluidity and cell compartmentation, which can result in cell lysis. Thus, oxygen radical-induced lipid peroxidation may contribute to the impaired cellular function and necrosis associated with reperfusion of ischaemic tissues.[7,8,10 – 13,16] Besides directly neutralizing a number of free radicals and nitrogen species, melatonin stimulates several antioxidative enzymes, such as SOD, GPX and glutathione reductase, which increases its efficiency as an antioxidant. Melatonin may therefore be important in determining cellular glutathione homeostasis and may also determine the total amount of glutathione within the cell. Neutrophil accumulation initiated by reperfusion has, in addition, been shown to be significantly attenuated by melatonin.[7, 8,10 – 12]

Melatonin is normally found in the human circulation, but the antioxidant effect of melatonin in humans probably occurs at pharmacological concentrations. No serious side-effects or risks have been associated with the use of melatonin. The physiological dose-dependent effects of the

hormone, however, have not yet been fully evaluated in those receiving large doses for prolonged time periods.[7–9] Lieberman *et al.*[31] suggested that melatonin, administered in pharmacological quantities (240 mg/day), has significant but short-acting sedative-like properties. In addition, in a study of 40 patients with advanced malignant melanoma treated with high doses of melatonin (up to 700 mg/day), six had transient decreases in the size of some tumour masses.[32]

Exogenously administered melatonin at pharmacological doses has been found to be effective in the prevention of ischaemic injury and tissue damage.[11–13] De La Lastra *et al.*[33] suggested that when melatonin was administered at doses of 5 mg/kg, 10 mg/kg and 20 mg/kg, the highest antioxidant effect was determined after the 20 mg/kg dose regimen. Jaworek *et al.*[34] showed that melatonin attenuated pancreatic damage induced by ischaemia–reperfusion when used at 25 mg/kg or 50 mg/kg. In recent studies, melatonin at pharmacological doses (10 mg/kg or 100 mg/kg) exerted a therapeutic effect on cholestatic liver injury in rats with bile duct ligation.[35]

We conclude that exogenously administering a pharmacological dose of melatonin has significant protective effects against IRI in the rat skeletal muscle model. This is achieved by decreasing lipid peroxidation, MPO, SOD and GPX enzyme activities and regulating glutathione content.

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Address for correspondence
Dr M Halici
Orthopaedics and Traumatology Department, Medical Faculty, Erciyes University,
38039 Kayseri, Turkey.
E-mail: mehmethalici@hotmail.com