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Melatonin Ameliorates Tacrolimus (FK-506)'s Induced Immunosupressive Effect in Rat Liver

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ABSTRACT

Tacrolimus (FK-506) is a powerful immunosuppressive agent that modulates neutrophil infiltration during inflammation. In this study, we sought to investigate the effects of melatonin on malondialdehyde (MDA), nitric oxide (NO), interleukin (IL)-6, and tumor necrosis factor-alpha (TNF-alpha) to oppose the negative effects of the immunosupressant FK-506. Group A was sham; group B, tacrolimus (1 mg/kg/d subcutaneously); and Group C received tacrolimus (1 mg/kg/d plus melatonin). All tissues underwent histopathologic examination. The MDA level in group B increased 53% compared with the sham group ($P < .001$); in group C, the MDA level decreased 16% compared with group B ($P > .05$). While TNF-alpha in group B increased 68.8% compared with the sham group ($P < .001$) and in group C it decreased 63.5% compared with the sham group. The IL-6 level in group B increased 81%; in group C, it decreased 13% compared with group B. NO levels in group B increased 48% compared with the sham group and increased by 15% in group B compared with group C. Thus melatonin may serve as a protective agent against the side effects of tacrolimus.

TACROLIMUS (FK-506), an immunosuppressive macrolide lactone, is produced by *Streptomyces tsukubaensis*.[1,2] In addition to being a powerful immunosuppressive agent, FK-506 modulates neutrophil infiltration during inflammation.[3,4]

Melatonin secreted by the pineal gland has many functions to regulate physiological mechanisms, such as endocrine rhythm, antigonadotropic effects, nervous system protection, immune system stimulation, protection of DNA against chemical agents, antiaging effects, antioxidant effects, and neutralization of free radicals. The antioxidant effects of melatonin arise from its ability to stimulate enzymes rather than to directly banish reactive free radicals.[5–9]

One of the most important mechanisms in cell damage related to free radicals is lipid peroxidation of membranes, resulting in structural and functional damage. Malondialdehyde (MDA) measurements have been used to indicate lipid peroxidation.[10]

Nitric oxide (NO) is an important immunoregulator of cellular immunity and organ transplantation rejection. NO plays roles to regulate vascular tonus as well as inhibit thrombocyte adhesion and aggregation. It also functions as a radical, a vasodilatator, and a neurotransmitter. It is synthesized in large quantities by activated macrophages.[11–14] The NO increase in acute also graft rejections[14–16] produces intensive infiltration of lymphocytes and mononuclear cells to grafts. The endothelium in small vessels of the graft is damaged, thromboses are formed, blood circulation ceases, and the graft becomes necrotic. While NO concentrations are about 100 to 500 nmol/L, as a result of triggering of INOS by agents such as gamma-interferon, endoxin interleukin (IL)-1 and tumor necrosis factor (TNF)-alpha, their levels increase as much as 10 times.[17]

Cytokines, small polypeptides that mediate intercellular communication, include nearly 30 members such as IL-6 and TNF-alpha, whose molecular weights range from 8 to 468 kDdal.[18,19] Neuronal expression of TNF-alpha secreted by neurons and glial cells in the central nervous system accelerates the infiltration of inflammatory cells, consequently further increasing tissue damage.[20] IL-6 is mitogenic for astrocytes and with TNF-alpha, it manifests proliferative effects.[21] By affecting hepatocytes, it increases

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the synthesis of C-reactive protein and fibrinogen and regulates acute phase reactions.[18] In this study, we sought to investigate the effects of melatonin on MDA, NO, IL-6 and TNF-alpha to mitigate the negative effects of the immunosuppressant FK-506.

MATERIALS AND METHODS

Thirty male Swiss albino rats (200–250 g) were obtained from Firat University Animal Laboratory (Elazig, Turkey). All experiments were performed in accordance with the guidelines for Animal Research from the National Institutes of Health and approved by our Committee on Animal Research. The animals were housed in stainless-steel cages under controlled temperature and humidity conditions in a quiet room with 12/12-hour light/dark cycles. Rats were maintained on a standard laboratory diet with tap water ad libitum throughout the experiment, except for an overnight fast before surgery. All surgical procedures were performed under sterile conditions. Thirty rats (200–250 g) were divided into three groups ($n = 10$): group A, sham-treated; group B tacrolimus (1 mg/kg/d subcutaneously); and group C, tacrolimus plus (melatonin). All animals underwent histopathologic examination.

We studied IL-6, TNF-alpha, NO, MDA levels tissue in homogenates prepared using an IKA Ultra-Turnax apparatus two cycles of 45 seconds at 0°C) in 0.5 molar Tris/1.5 molar NaCl/50 mmol $CaCl_2$/2 mmol sodium azide buffer (pH 7). The homogenates were centrifuged at 10,000*g* for 15 minutes at 4°C; the supernates were examined in enzyme-linked immunosorbent assays (ELISA). Rat IL-6 and rat TNF-alpha (BioSource Immunoassay kit, Camarillo, Calif, USA) levels were measured using a sandwich ELISA protocol supplied by the manufacturer of the antibodies. The resultant optical density was determined using a microplate reader at 450 nm. The results were expressed as pg/g tissue. For MDA and NO levels, liver tissue was homogenized in 1.15% KCl buffer (1:9, w/v) using a manual glass homogenizer (Tempest Virtishear, Model 278069; The Virtis Company, Gardiner, NY, USA) for approximately 5 minutes.

NO is rapidly oxygenated to NO_2 and further to NO_3. Thus direct assessment of NO is almost impossible (in vivo). So the combined production of NO_2 and NO_3 was used as an estimate of NO synthesis in vitro and in vivo. Nitrate was assayed by a modification of the cadmium-reduction method.[10] The produced nitrite was determined by diazotization of sulfanilamide with coupling to naphthylethylene diamine (NNDA). After samples were deproteiniazed with the Somogil reagent, the nitrate was reduced by Cu-coated Cd in glycine buffer (pH 9.7). The reduction followed pseudo-first-order reaction kinetics over a 90-minute interval. After mixing, absorbances were read against a blank at 545 nm at 20 to 60 minutes. The results are expressed as μmol/mg tissue.

Tissue MDA was determined by the method of Uchiyama and Mihara.[11] A 3-mL aliquot of 1% phosphoric acid and 1 mL of 0.6% thiobarbituric acid solution were added to 0.5 mL of 10% tissue homogenate pipetted into a tube. The mixture was heated in boiling water for 45 minutes. After cooling, the color was extracted into 4 mL of n-butanol. The absorbance was measured in a spectrophotometer (Ultraspec Plus, Pharmacia LKB Biochrom, UK) at 532 nm. The amounts of lipid peroxides were calculated as thiobarbituric acid-reactive substances of lipid peroxidation, which are shown as nmol/mg tissue.

Statistical Analysis

The statistical package for social sciences (SPSS) version 13.00 was used for the statistical analysis. Individual group parameters were assessed using the Mann-Whitney *U* test. The results are shown in the text as mean values ± standard deviations for all comparisons with significance defined as $P < .05$.

RESULTS

MDA levels in group B increased 53% compared with the sham group ($P < .001$). In group C, MDA levels decreased by 16% compared with group B ($P > .05$). While TNF-alpha in group B increased 68.8% compared with the sham group ($P < .001$), it decreased in group C has 63.5% compared with the sham group. IL-6 levels in group B increased by 81%, and in group C, decreased by 13% compared with group B. While NO levels in group B increased 48% compared with the to sham group, they increase a 15% in group B compared with group C. The results are shown in Table 1.

DISCUSSION

FK-506 has been reported to produce damage to the liver, intestine, kidney, and myocardium.[22–25] FK-506 modulates neutrophil infiltration and inhibits the production of TNF, which increases neutrophil aggregation and cellular adherence.[26]

Melatonin can regulate electron transfer, detoxify reactive intermediate products, and control peroxidative reactions. Recent investigations have reported that melatonin is a powerful endogenous antioxidant.[27,28] It is not easily oxidized, does not undergo auto-oxidation, and does not participate in the redox cycle or in reactions producing hydroxyl radical. Besides melatonin, none of the metabolites of melatonin have pro-oxidative activity. It has been reported that the hydroxyl radical-neutralizing property of melatonin is 5 times greater than that of glutathione, 15 times greater than mannitol and 2 times greater than vitamin E.

Table 1. MDA, TNF-alpha, IL-6, NO Levels as Mean ± SD in All Groups

Groups*	MDA	TNF-α	IL-6	NO
Group A	47.94 ± 1.59[b,c]	4.853 ± 0.445[b]	1.6 ± 0.03[b,c]	74.57 ± 1.80[c]
Group B	89.90 ± 8.80[a]	7.421 ± 0.166[a]	2.9 ± 0.10[a]	110.29 ± 2.85[a]
Group C	74.30 ± 3.64[a]	4.723 ± 0.147[b]	2.5 ± 0.03[b]	93.57 ± 3.48[b]

Group A: sham; group B: FK-506; group C: melatonin + FK-506. MDA, malondialdehyde; TNF-alpha, tumor necrosis factor-alpha; IL-6, interleukin-6; NO, nitric oxide; SD, standard deviation; ANOVA, analysis of variance.

ANOVA was applied. In each column, [a]compared to group A: $P < .05$; [b]compared to group B: $P < .05$; [c]compared to group C: $P < .05$.

Active macrophages and monocytes are primary sources of TNF under pathological conditions. Proinflammatory agents found in plasma, such as complement factors, cytokines (IL-6, IL-8, TNF), platelet-activating factor, and leukotrienes, also cause endothelial damage. As a T-cell blocker, FK-506 exhibits immunosuppressive effects to inhibits cytokine secretion by T cells, thereby modulating neutrophil infiltration.[29–32]

As a result, intraperitoneal administration of melatonin might protect the livers of FK-506- treated rats by reducing the severity of oxidative stress and increasing the levels of antioxidant enzymes. We believe that melatonin might be used to protect but further clinical and experimental studies are needed to verify its antioxidative and hepatoprotective effects.

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