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# Melatonin modulates inflammatory response and suppresses burn-induced apoptotic injury

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*Research Article*

# Melatonin modulates inflammatory response and suppresses burn-induced apoptotic injury

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**Abstract**

**Introduction**: Melatonin, the principal secretory product of the pineal gland, has antioxidant functions as a potent antioxidant and free radical scavenger. **Objectives** of the present study were to investigate the effect of melatonin against inflammatory response, burn-induced oxidative damage and apoptotic changes of rat liver. **Methods**: Melatonin (10 mg /kg, i.p.) was applied immediately after 30% of total body surface area (TBSA) burns on male Wistar rats. The level of malondialdehyde (MDA) as a marker of an oxidative stress was quantified by thiobarbituric method. Hepatic TNFα and IL-10 as inflammatory markers were assayed by ELISA. Using light immunochistochemistry the expression Ki67 proliferative marker was investigated. **Results**: Hepatic MDA and TNF-α levels increased significantly following burns without any change in IL-10 level. Intracellular vacuolization, hepatic cell degeneration and apoptosis occurred in rats after burns. The number of apoptotic cells was increased whereas no significant increase in Ki67 proliferative marker. Melatonin decreased the MDA and TNF-α content and increased the IL-10 level. It also limited the degenerative changes and formation of apoptotic cells in rat liver but did not increase expression of the marker of proliferation. **In conclusion**, our data show that melatonin relieves burn-induced hepatic damage associated with modulation of the proinflammatory/anti-inflammatory balance, mitigation of lipid peroxidation and hepatic apoptosis.

**Keywords**: melatonin, cytokines, lipid peroxidation, apoptosis, liver, burn

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## Introduction

Severe burn is still a serious clinical problem in emergency medicine causing damages to organs distant from the burn wound (1-3). Pathophysiology of burn-induced liver injury includes many mechanisms and is not entirely defined yet. Inflammation is proposed to be the main event in the pathogenesis of thermal trauma (4-6). The inflammatory cascade has counter balancing factors that maintain a delicate balance of pro- and anti-inflammatory mediators that regulate cellular homeostasis (7-8). Many pro-inflammatory and anti-inflammatory cytokines are involved in the pathogenesis of thermal trauma having overlapping, antagonistic and synergistic effects (9). Tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10) and C-reactive protein are thought to play an essential role in the modulation of immunological and inflammatory processes.

Hepatic apoptosis has been recognized as part of the pathological changes after burn injury (10). High apoptotic cell number after burn injury implicates hepatic damage and impairment to its function. Cytokines and oxidative stress are well known to induce hepatocytes apoptosis (11). Activation of NF-kB by hypoxia induced endothelial cell death and apoptosis in Bcl-2-dependent manner (12).

Melatonin (N-acetyl-5-methoxytryptamine) is an indoleamine endogenously produced in the pineal gland and extrapineal tissues, including liver (13). Melatonin possesses a wide variety of biologic effects. Particularly, it has sedative, anxiolytic, and anti-nociceptive effects. Melatonin and its metabolites have potent antioxidant /anti-inflammatory properties and have been highly effective in various diseases linked to inflammation and oxidative stress (14). Melatonin not only neutralizes reactive oxygen species (ROS) and reactive nitrogen species (RNS) but acts through stimulation of antioxidant systems and stabilizes cell membranes. It modulates the expression of several protective enzymes and reduces apoptosis and lipid peroxidation (15). There are no data about protection of melatonin against burn-induced apoptotic injury after burns.

The aim of the present study was to investigate the effect of melatonin on inflammatory response and burn-induced oxidative damage and apoptotic changes of rat liver.

## Materials and Methods

- *Animals*

The experimental procedure was approved by the Home Office for Care and Use of Laboratory Animals, and experiments were performed in accordance with the European Communities Council Directives 86/609/EEC. Age-matched male rats weighing between 220 and 250 g fasted for 12 h were allowed free access to water before injury. Animals were housed in 20oC and offered rat food and water ad libitum. They were kept in dark-light cycles (DL = 12:12 hours) in individual wire-bottomed cages. Thus, lights were turned off at 8:00 p.m. and turned on at 8:00 a.m. for achieving satisfactory photoperiod.

- *Thermal injury and melatonin treatment*

After light ether inhalation, general anesthesia was performed using thiopental (30 mg/kg i.p.). In order to accomplish 30% of third degree burn hot boiling water (98$^{o}$C) was applied on the back of the animals during a period of 10 sec. For those rats which were subjected to burn injury 4 ml of physiological saline was applied i.p. for immediate resuscitation following burn injury. No animals died within the first 24 hour post-burn period. Twenty-four male rats were randomly assigned to three groups of 8 animals each: control - non-burned, non-treated (C), vehicle-treated burned group (B), and melatonin-treated burned group (B+Mel).

Either melatonin (N-acetyl-5-methoxytriptamine, Merck, Germany) in a dose of 10 ml/kg body weight (b.w.) solved in vehicle, or vehicle (ethyl alcohol 2% in physiological saline in dose 5 ml/kg) was administered,

respectively. Melatonin and vehicle were applied immediately i.p. after burns in the morning between 8:00 a.m. and 9:00 a.m. and 12 hours after burns. All animals were given buprenorphine (0,3 mg /kg i.p.b.w. twice daily) for pain control post burn. They were re-anesthetized with thiopental and sacrified 24 hours after burns as liver was sampled.

- *Biochemical analysis*

Blood was taken from the jugular vein and heparinized. Plasma was separated by centrifugation at 800 x g rpm for 10 min and aliquots were stored at -80oC until analysis. Liver was gently separated from the underlying tissue and homogenized in 1:5 w/v 50 mM phosphate buffer (pH 7,4) containing 0,1 mM EDTA, at 4000 rpm for 10 min. The homogenate was centrifuged at 800 x g rpm/15 min to discard the sediment and supernatant was frozen until analysis. All manipulations were performed at 4-8oC. Analysis was carried out immediately after thawing of the samples.

Membrane lipid peroxidation was assayed by MDA measured by its thiobarbituric acid (TBA) reactivity of hepatic homogenate using the method of Porter et al (16). Results were expressed as nmol MDA/g protein. They were determined using the extinction coefficient of MDA-TBA complex at 532 nm =1, 56 x 10-5 cm-1 M-1 solution.

- *Immunohistochemistry*

Rat liver specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. The deparaffinized and rehydrated sections (5 μm thick) were treated with 1% hydrogen peroxide for peroxidase activity inhibition for 5 min. Then they were rinsed in 0.1 M phosphate buffered saline (PBS) (pH 7.4) and treated in normal goat serum for 20 min. Subsequently, the sections were incubated with polyclonal primary antibody for 24 hours at room temperature. Monoclonal Mouse Anti-Rat Ki-67 Antigen (DAKO, USA) was used. After rinsing with PBS the sections were incubated for 20 min in goat anti-rabbit immunoglobulins at room temperature. Then they were rinsed in PBS again and treated with rabbit peroxidase-anti-peroxidase complex for 20 min at room temperature and then rinsed in PBS. Finally, peroxidase activity was estimated by the diaminobenzidine-tetrachloride $H_2O_2$-method.

Negative controls. Controls were incubated with non-immune sera instead of primary antibody.

- *Histopathological Examinations*

Tissue specimens were fixed in 10% buffered formalin (pH 7.2), dehydrated in ascending series of ethyl alcohol (70% - 100%), cleared in methyl benzoate and embedded in paraffin wax. Tissue sections of 5 μm were stained with hematoxylin and eosin (H&E) and examined using light microscope (Olympus BH-2, Tokyo, Japan). Apoptotic SECs were examined by light microscopy at a mag-nification of 400× (high power field). Approximately 500 SECs were examined in each high power field. The apoptotic index was defined as the number of stained cells per high power field.

- Statistics

Liver enzyme data were log-transformed to satisfy the assumptions required to perform parametric tests and, therefore, presented as geometric mean and 95% confidence intervals of the mean. Orthogonal contrasts in ANOVA were used to statistically analyze the difference between any two specified groups.

## Results

*Changes in the Hepatic Malondialdehyde and Hepatic TNF-α and IL-10 Levels*

Hepatic TNF-α content was significantly increased by 112% ($p<0.05$) at 24 h in the burned group. TNF-α content was increased in the burned group treated with melatonin g, but the increase was significantly lower than in the burned group (Fig. 1).

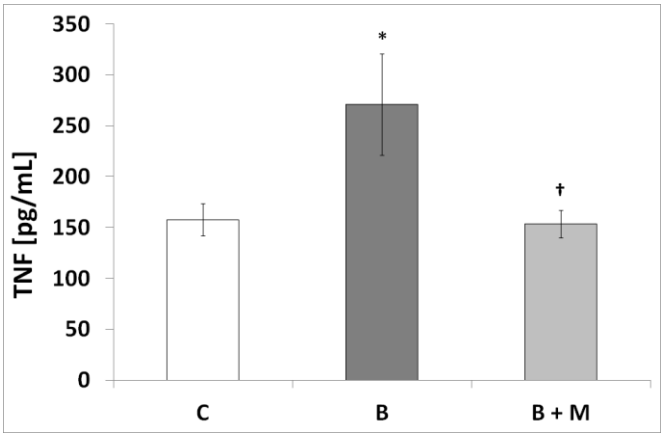


**Fig 1.** Effect of melatonin on hepatic TNF level in the early post burn period. C-control, non-burned; B- burned group , and B+Mel- melatonin-treated burned group. Results are given as mean±SEM; *p < 0.05 C vs B; **o** p < 0.05 B+mel vs B.

The hepatic IL-10 content tended to increase in both groups following burn, but was significantly higher by 44% (p<0.01) in the burned group treated with melatonin than in untreated burned group (Fig. 2). Hepatic MDA content persistently increased 166% (p < 0.01) at 24 h and was still significantly higher than the control level. However, MDA content was significantly lower in the burned group treated with melatonin (Fig.3).

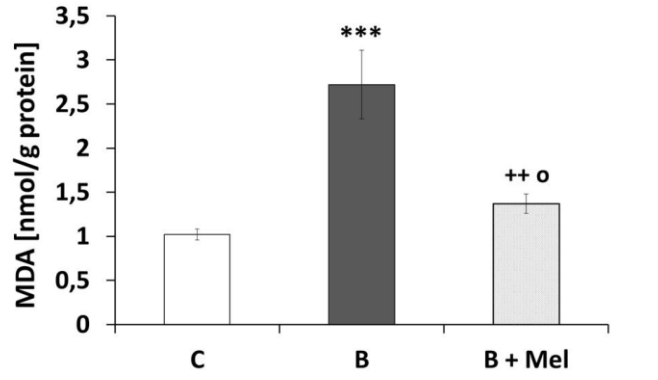


**Fig 3.** Effect of melatonin on hepatic MDA level in the early post burn period

*** P<0.01 B vsC; **o**-p<0.05 B+Mel vs C; + p<0.05 B+Mel vs B

A positive correlation was established between hepatic MDA and hepatic TNF α levels (r =0.50, p < 0.05) (Fig. 4).

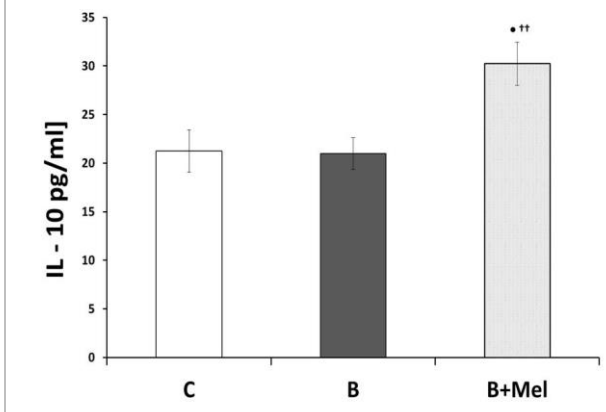


**Fig 2**. Effect of melatonin on hepatic IL-10 level in the early post burn period ++ P<0.01; B vs B+mel; **o**-p<0.05 B+Mel vs C

**Histological (H&E) changes in liver**

In the control group, hepatocytes had a normal shape and size with vesicular nuclei in (Fig. 5). In the burned group, hepatocytes showed an abnormal structure, acidophilic degeneration and vacuolization. In addition, an increased number of apoptotic cells were detected. In the burned group treated with melatonin, a reduced number of apoptotic cells was established.

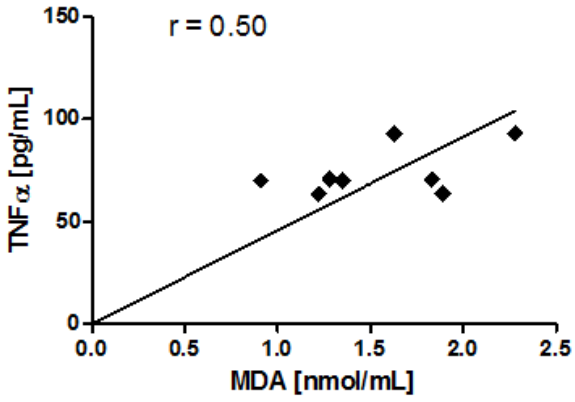


**Fig 4.** Correlation between hepatic MDA and hepatic TNF-α levels after burn

**Changes in the Expression on Ki-67-proliferative marker.** The data showed single Ki67 positive cells in hepatocytes around the central vein of the control group. K67 proliferative marker was not changed in the untreated burned group and the burned group treated with melatonin.

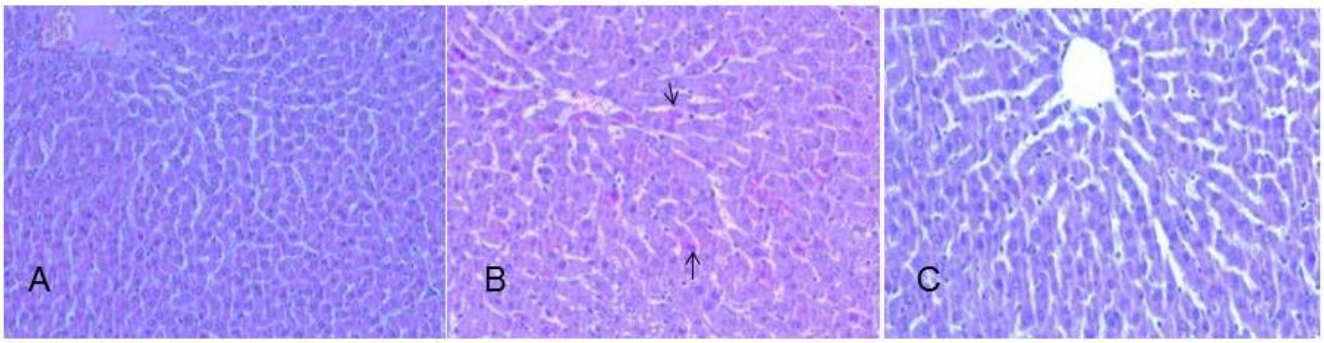


**Fig 5**. Effect of melatonin on histological changes in liver in the early post burn period. Original magnification, 400. H & E staining, original magnification. Arrows indicate apoptotic cells

## Discussion

Data from the present study show activation of inflammatory response, lipid peroxidation, degenerative damage and apoptosis in liver after thermal trauma. Melatonin inhibits inflammatory response and protects against hepatic oxidative damage and apoptosis.

Levels of TNF-α (a proinflammatory mediator) and IL-10 (an antiinflammatory mediator) in liver tissue increased significantly following severe burn (17). Our data demonstrate that TNF-α plays an important role in the occurrence and development of postburn liver damage (18). In vitro experiments demonstrated that TNF-α may directly act on rat liver cells. The application of TNF-α inhibitor preventes hepatic dysfunction in shock after burn in a guinea pig model (19).

IL-10 may inhibit inflammatory mediators such as TNF-a, upregulates humoral immune reaction and inhibits cellular immune reaction such as antigen presentation by macrophages (20). The imbalance between TNF-α and IL-10 expression may be one of the initiating factors for the development of systemic inflammatory response syndrome and multiple organ failure in the early after burn period (21).

High TNFα level activates cell-death pathways and promotes the production of reactive oxygen species and lipid peroxidation and thus hepatic injury (22). Furthermore, hepatocytes are much more sensitive to TNF-α and free radicals damaging effect when antioxidant capacity is low.

Liver tissue is subjected to severe peroxidation damage due to ischaemia/ hypoxia following severe burn (23). In the present study, levels of MDA in liver tissue were significantly increased, demonstrating severe lipid peroxidation in liver tissue following severe burn. MDA content was significantly decreased in the burned group treated with melatonin. However, this suggests that melatonin attenuates free radical activated burn-induced lipid peroxidative injury of hepatic tissue.

It appears that oxidative stress plays an important role in the primary cell and tissue destruction, and thus the secondary inflammatory reaction. ROS/RNS may activate the transcription factor NF- kB and increase the level of TNFα, which has a cytotoxic activity and may cause hepatic injury. On the other hand, increased production of TNFα and free radicals increases the expression of transcription factor NF-kB, likely forming a vicious cycle between oxidative stress and inflammation by the activation of NF-kB. In support of this idea is the correlation between levels of TNFα and MDA in the liver after thermal skin injury ($r = 0.50$, $p < 0.05$) (Fig.4). Such a positive relationship between markers of lipid peroxidation and inflammation in liver was found in other experimental models of thermal trauma (24-26).

In the present study melatonin decreased the hepatic tissue TNF-α and MDA levels. In addition, it increased liver tissue IL-10. It may be assumed that the protective effect of melatonin against burn-induced oxidative injury

is associated with modulation of the pro-inflammatory/ anti-inflammatory balance.

Melatonin significantly decreases the TNF-α and increases the IL-10 content in liver tissue in other pathological conditions (27-28) and this effect may be due to its anti-inflammatory properties. Melatonin has the capability of scavenging both oxygen and nitrogen-based reactants including ONOO- and blocking transcriptional NF-kB factor which induces pro-inflammatory cytokines (29). Thus melatonin affects the pro- and anti-inflammatory balance and restricts hepatic damage from uncontrollable inflammation, and this may be the main mechanism for the protective effect of melatonin against burn-induced oxidative damage.

Our data demonstrate that thermal injury induces degenerative changes in liver and increases number of apoptotic cells in this organ. Oxidative damage of mitochondria causes an elevation of pro-apoptotic Bax protein and of anti-apoptotic Bcl-2 protein in various diseases related with oxidative stress (30). Hence high temperature causes higher Bax/Bcl-2 ratio, which was similar to previous findings, and also elevated BAX/BCL-2 ratio suggesting the susceptibility of these cells to apoptosis (31). Proinflammatory cytokine TNFα triggers extrinsing apoptotic pathway through the binding of TNFα with cell-death receptors. The increased Bax proapoptotic protein expression may associate with translocation of proapoptotic Bid protein to mitochondria by the Fas/TNF-R1 pathway (29)

The present study shows that melatonin restricts burn-induced degenerative changes modulation of the pro-inflammatory/anti-inflammatory balance and decreased the number of apoptotic cells in liver. Melatonin attenuates the oxidative stress, activates antiapoptotic-regulating proteins and protects against apoptosis (15). Treatment with melatonin increases the glutathione synthesis and antioxidant defense and modulates important steps in both mitochondrial and the death receptor apoptotic pathway.

The current study shows that the proliferative marker K67 was not changed in the thermal injured rats compared to the controls. Previous studies showed that the proliferation of hepatocytes increased on the second day after experimental thermal trauma (32). Probably, differences in experimental models of thermal injury can explain the difference in the results. Our data show that melatonin inhibits apoptosis of hepatocytes, but it does not stimulate their proliferation in the acute period after burns.

## Conclusions

In conclusion, our data show that thermal trauma activates proinflammatory processes, causes oxidative damage and apoptosis in liver. The protection afforded by melatonin against burn-induced hepatic apoptotic injury probably occurs through modulation of the balance pro/anti-inflammatory cytokines and mitigation of lipid peroxidation.

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