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Research Article

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Mechanisms of Protection With Melatonin By Hepatic Heme-Oxygenase-1 Activation In Burn

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Abstract

Background: *Melatonin, the principal secretory product of the pineal gland, functions as a potent antioxidant and free radical scavenger. Additionally, the antiapoptotic effect of melatonin has been observed. Several studies show that heme-oxygenase-1 (HO-1) possesses antiapoptotic action and prevents hepatic damages. Recent studies indicate that heme-oxygenase-1(HO-1) inhibits apoptosis and exert hepatoprotective effect.*

The aim: *of this experimental study was to investigate the protective effects of melatonin against burn-induced apoptotic injury and the association between the oxidative stress and the changed expression of hepatic HO-1 in burn rat model.*

Material and method: *Melatonin was applied immediately after the burn. The expression of hepatic 4-hydroxynonenal (4-HNE), as marker of liver peroxidative injury, hepatic HO-1, marker of antioxidant defense and apoptosis-related genes Bcl-2 and Bax was evaluated using light immunohistochemistry.*

Results: *Burns caused an increased expression of HO-1, 4-HNE, Bax and Bax/Bcl-2 ratio and induced apoptosis of sinusoidal endothelial cells (SECs) in liver tissue. Melatonin treatment augmented the increase in HO-1 expression, decreased both burn-induced peroxidative damage and hepatic apoptosis as evidenced by reduced expression of Bax, enhanced expression of Bcl-2.*

Conclusion: *Our present data suggest that melatonin suppresses burn-induced liver injury through HO-1 activation, attenuation of lipid peroxidation and modification of Bax/Bcl-2 ratio.*

Keywords: melatonin, heme-oxygenase-1, 4-hydroxy nonenal, liver apoptosis injury, burns

Introduction

Excessive and sustained increases in oxidative stress and apoptosis have been implicated in the pathogenesis of pathological damage in many organs, including organs in the splanchnic area. Literature data shows that thermal trauma induces system apoptotic response [1]. Pathophysiological mechanisms of apoptosis in hepatic injury have not been fully clarified yet. Possible mechanisms of cell death are hypoperfusion, ischemia and subsequent reperfusion, increased production of proinflammatory cytokines and free radicals [2-4]. It has been reported that oxidative injury mediated by 4-hydroxynonenal (4-HNE), a lipid peroxidation product, may lead to changes in expression of Bcl-2 proteins known as markers of apoptosis [5]. There is insufficient information on the role of oxidative stress in the development of the apoptotic process induced by thermal trauma in the cells of the liver and other organs. There is evidence that SECs are the initial targets in ischemia/reperfusion injury. Recently, it has been reported that in comparison with hepatocytes, SECs are more sensitive to burn-induced injury via an apoptotic pathway involving production of antiapoptotic proteins.

Heme-oxygenase-1 (HO-1) is an enzyme with antioxidant, antiinflammatory and antiapoptotic properties. Its expression is induced under oxidative stress conditions such as hypoxia, ischemia and exposure to proinflammatory cytokines which facilitate the generation of reactive oxygen species (ROS) [6-7] and it is involved in the protection of several cell types. Several studies suggest that HO-1 prevents hepatic damages [8-10]. HO-1 provides both antioxidant and antiapoptotic properties may be due to its products of biliverdin/bilirubin (BV/BR), iron and carbon oxide (CO). CO increases Bcl-2 expression and prevents cytochrome-C (Cyt-c) release and activation of caspases [11]. However, very little is known about the effect of HO-1 in burn-induced hepatic apoptotic injury. Inhibition of SEC apoptosis seems to be a rational therapeutic

strategy to reduce the risk of liver injury after burns.

Melatonin (N-acetyl-5-methoxytryptamine) is an indole amine endogenously produced in the pineal gland and and extrapineal tissues, liver including [12]. Melatonin exerts a wide variety of biologic effects such as sedative, anxiolytic, and antinociceptive. It is a powerful antioxidant and scavenger and possesses antiinflammatory and antiapoptotic properties [13]. Melatonin exerts a protective effect on liver in other experimental models including ischemia/reperfusion, hemorrhagic shock and various hepatotoxic agents such as endotoxin and this protection is mediated through HO-1 induction [14]. Melatonin is an important antiapoptotic agent in various tissues by modulating expression of antioxidants and decreasing proapoptotic proteins such as Bax [15]. According to a previous study, melatonin protects mice from lipopolysaccharide/D-galactosamine-induced apoptotic liver damage [16]. In addition, pretreatment with melatonin prevents from ischemia/reperfusion-induced hepatic necrosis and apoptosis [15]. Although experimental and clinical studies have shown that melatonin suppresses apoptosis in liver [17-18] we failed to find any data about a possible protective effect of melatonin on burn-induced hepatic damage by activation of antioxidant enzyme HO-1.

The aim of this experimental study was to investigate the protective effects of melatonin against liver injury and the association between the oxidative stress and the changed expression of HO-1 in burn rat model.

Material and Methods

Animals

The experimental procedure was approved by the Home Office for Care and Use of Laboratory Animals and performed with a strong consideration for ethics of animal experimentation. 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 a 20°C and offered rat chow and water ad libitum. They were kept in dark : light cycles (DL = 12:12 h) 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 (90oC) 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 h of post-burn period. Twenty-four male Wistar rats were divided into three equal (n=8) groups: 1. control - non-burned, non-treated group (C); 2. burned, non treated (B). 3. treated with melatonin (10 ml/kg(-1) b.w.) burned group (B + M). All the 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 sacrificed 24 h after burns as liver was sampled.

Immunohistochemical Examination for 4-HNE, HO-1, Bcl-2 and Bax

Rat liver specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. The deparaffinized and dehydrated 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 h at room temperature. Rabbit 4-HNE antibody (Abcam, UK), rabbit anti-HO-1 antibody (Santa Cruz, USA) and rabbit Bcl-2 and Bax antibody (Santa Cruz, USA) were 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, 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 diaminobenzydine-tetrachloride H_2O_2-method.

Negative controls were incubated with non-immune sera instead of primary antibody. Morphometric method was used to assess quantitatively contents of 4-HNE, HO-1, Bcl-2 and Bax. Enzyme content was determined as: strong, score 3; moderate, score 2; weak, score1, and lacking, score 0 on the basis of the occurrence of immunodeposits [19]. 4-HNE, HO-1, Bcl-2 and Bax contents of liver were defined as the enzyme content of each cell was multiplied by their scoring factors and divided by total number of cells. Morphometric investigation was performed on 50 cells from each sample.

Additionally a computer program Image J (Wayne Rasband) as a control method was used. From our point of view according to the purposes of the study the manual counting is appropriated. In the manual reading of the expression of a substance in microscopic slides we have the possibility not only for quantitative analysis (degree of intensity of the corresponding color), but qualitative analysis of the relevant color too. Qualitative analysis provides information about the expression of the substance in the cell ((including the cell structures - nuclueus, cytoplasm, etc.), the type of the cell (endothelial, hepatocytes) as well as outside the cell - extracellular space and concomitant changes in the microscopic structure, lacerations, apoptosis and others. This analysis avoids any errors reporting artifacts staining close to the test substance.

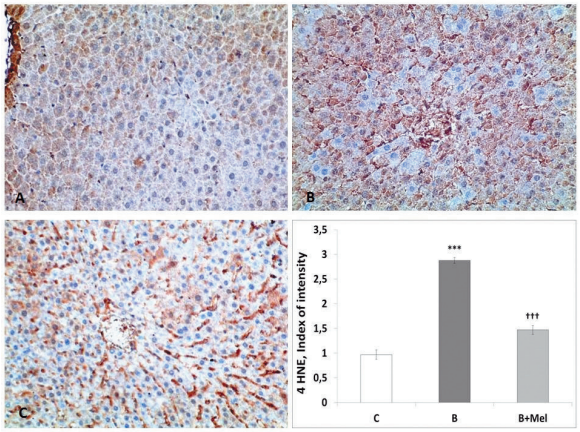
Statistics

Our 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

Melatonin ameliorates bur-induced liver injury

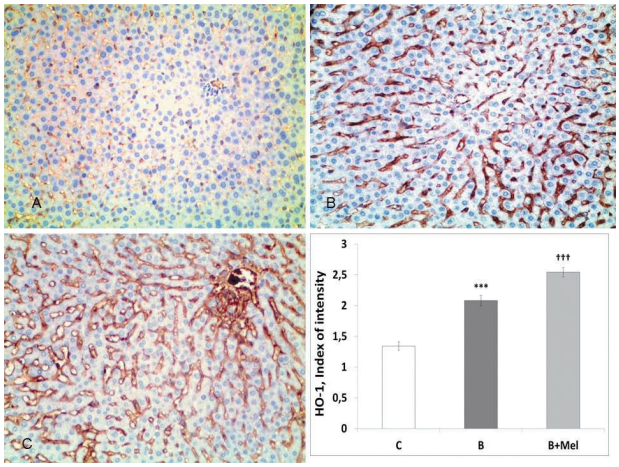
4-HNE expression was found in both sinusoidal endothelial cells (SEC) and hepatocytes in the control group **(Fig.1)**.



The staining intensity of 4-HNE positive cells varied from weak to moderate. The mean 4-HNE content in the cells was 0,95 ± 0.095. In the burned group the number of 4-HNE positive cells was increased in SECs and mainly in hepatocytes around the central vein. The mean content was 2.87± 0.059. In the burned group reated with melatonin, induction of 4-HNE positive cells was principally in SEC and some hepatocytes around the central vein. The mean content in SECs and hepatocytes was 1, 46 ±0.089, which is lower (50%, p<0.001) than this of the burned non treated group.

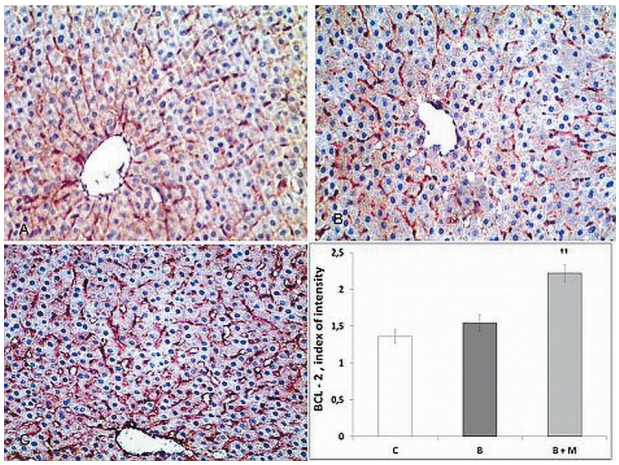
Effects of melatonin on expression of hepatic HO-1

HO-1 expression was found in SECs of the liver in the control group. The staining intensity of HO-1 positive cells was weak. The mean HO-1 content in the cells was 1.35±0.068 **(Figure 2)**. In the burned non treated group, induction of HO-1 was principally in the sinusoidal endotelial cells (SECs). It was moderate to strong in the individually cells. Their mean content (2.83 ±0. 0.085) was significantly higher (51%, p<0.001) than this of the control rats. The number of HO-1 positive SEC in the liver is increased in burned treated with melatonin group, which contributes to an increase in the average content of HO-1 protein in the liver (2.53 ± 0.077), which is 23% (p <0.001) higher than in the burned non trerated group (Fig.2).



Effects of melatonin on expression of Apoptosis-Related Bcl-2

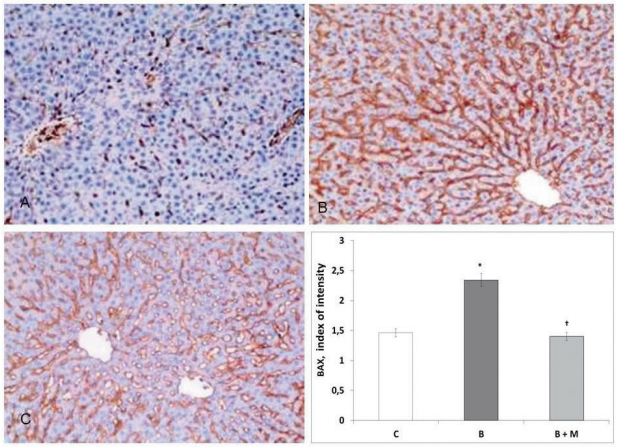
The expression of Bcl-2 was detected in sinusoidal endothelial cells (SECs) in the control group (Figure 3). The mean Bcl-2 content in the cells was 1.36 ± 0.48 (Figure 3). In the burned group, Bcl-2 expression remained low (1.54 ± 0.69) and did not show significant changes compared to controls. The expression Bcl-2 was increased in SECs of the burned group treated with melatonin **(Figure 3)**.



It was moderate to strong in sinusoidal endothelial cells and their mean content (2.22 ± 0.74) was significantly higher 63% (p < 0.001) than this of the controls.

Effects of melatonin on expression of Apoptosis-Related Bax

The data showed that Bax expression was detected mainly in the SECs in the control group **(Figure 4)**. The intensity of the immune reaction ranged from mild to moderate (1.46 ± 0.29) Bax expression in SECs was moderate to strong (2.34 ± 0.59) in the burned group and the number of positive cells was significantly higher by 60% (p < 0.01) compared to controls. The expression Bax was reduced by 62% (p < 0.01) in the burned group treated with melatonin maintaining the level close to the control values.



Discussion

Severe burn is associated with high oxidative stress which will trigger inflammatory response and apoptosis [1]. Excessive ROS production might eventually overwhelm antioxidant defenses and generate highly toxic lipid peroxides. Our results show that levels 4-HNE, a major aldehydic product of lipid peroxidation, significantly increase in the liver at 24 h after burns. 4-HNE causes modification of important biomolecules including proteins, DNA, and phospholipids containing amino group [20]. Accumulation of 4-HNE decreases glutathione (GSH) pools and antioxidant defense and may cause oxidative liver injury [21]. 4-HNE alters mitochondrial energy metabolism, protein mitochondrial function and apoptosis [22].

HO-1 is stress-responsible enzyme and plays an important role against oxidative stress [6-7]. Several studies have reported that HO-1 induction exerts protective effect against oxidative injury under pathological conditions such as ischemia/reperfusion, endotoxin, and hepatotoxins [14, 23-25] and is one of the most sensitive and reliable indicators of cellular oxidative stress [26]. Recent experimental evidence shows that HO-1-deficient cells and mice are susceptible to the accumulation of free radicals and to oxidative injury after endotoxin administration [27]. Our results show an increased expression of HO-1 protein in SECs and some hepatocytes in our experimental model.

Melatonin treatment enhances the increase in the HO-1 activity in liver, reduces the levels of lipid peroxidation products, and attenuates liver histological changes. HO-1 is responsible for breakdown of heme to bilirubin, free iron and CO [7]. Bilirubin has antioxidant properties [6]. Free iron sequestration by ferritin lowers the prooxidant state of the cell [28]. These findings suggest that the protective mechanism of melatonin against burn-induced

liver injury might be closely associated with HO-1 overexpression and its increased cellular levels may improve the redox balance in the liver cells. It is quite possible that by this mechanism, melatonin modulates and stimulates redox-sensitive signaling pathways thus contributing to the increased antioxidant protection.

Oxidative stress is recognized as an important mechanism of apoptosis. ROS involvement is suggested in apoptosis of hepatocytes [29, 30] and endothelial cells [31].

The results of the present study demonstrate an increased expression of 4-HNE and a greater number of apoptotic hepatic cells. Several hypotheses have been published about the pro-apoptotic activity of 4-HNE as a marker of lipid peroxidation and oxidative stress: i) direct damage and mitDNA activation of the mitochondrial apoptotic pathway; ii) inhibition of mitochondrial proteins (channels) as adenine nucleotide translocase (ANT) due to SH-group oxidation, which increases the permeability of mitochondrial membrane Cyt-c release; iii) inhibition of antiapoptotic Bcl-2 proteins [5].

ROS overproduction might induce apoptosis by inducing Fas ligand to interact with Fas and forms a death-inducing signal complex (DISC) [32], activates caspases such as caspase-8 and caspase-3 to recruit hepatocytes to apoptosis [33].

In the present study, we observe significantly increased Bax protein levels in SECs at the periphery of the sinusoidal space in liver. Our data show that the increased levels of 4-HNE, a marker of lipid peroxidation and oxidative stress, is accompanied by elevation of proapoptotic Bax protein, however, there are no changes of antiapoptotic Bcl-2 protein of burn-treated animals when compared with the control group. The large number of Bax positively stained SECs and some hepatocytes and the increased Bax/Bcl-2 ratio might increase the susceptibility of these cells to apoptosis.

HO-1 regulates mitochondrial function by activating Bcl-2 and Bcl-xL, preventing Cyt-c release and activation of caspases [34]. Increased HO-1 expression augments Bad, inhibits Cyt-c release and enhances cell survival [35]. Collectively, the protective effect of HO-1 on oxidative damage-induced apoptosis may be mediated via both the extrinsic pathway and the intrinsic apoptosis signaling pathways. Both antioxidant and anti-apoptotic properties of HO-1 could be due to its products of BV/BR, CO and iron. BV/BR protects against reactive oxygen species [36]. Iron induces ferritin, which in turn prevents lipid peroxidation [37]. HO-1 protects the cell against oxidant-induced cell death by regulating the intracellular redox active iron [28]. CO exhibits also antiinflammatory and antiapoptotic properties, which are mediated by activation of p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway [6]. The antiapoptotic effect of CO is involved in the inhibition of Fas/FasL expression. CO increases Bcl-2 expression, prevents Cyt-c release and apoptosis activation [11]. Clearly, further studies are still necessary to clarify how HO-1 affects these two apoptosis signaling pathways.

Our data show that melatonin significantly decreases Bax expression and increases Bcl-2 expression. We suggest that melatonin might modulate both Bax and Bcl-2 expression as well as Bax/Bcl-2 ratio to protect rat hepatocytes from burn-induced apoptosis. The antiapoptotic effect of melatonin is associated with its antioxidant role because melatonin protects liver from oxidative stress by scavenging the hydroxyl radicals and inhibiting generation of the lipid peroxidation product 4-HNE.

Melatonin is a known powerful antioxidant and antiinflammatory agent. It exerts beneficial effects against mitochondrial dysfunction, the major source of ROS and reactive nitrogen species (RNS) [4, 38, 39]. Melatonin prevents mitochondrial Bax translocation and the collapse of mitochondrial membrane potential [40]. It inhibits free radical-mediated mitochondrial-dependent hepatocyte apoptosis and liver damage induced during malarial infection [41] suggests that HO-1 induction by melatonin treatment attenuates apoptosis injury, which could be regarded as alleviation of lipid peroxidation and modulation of Bcl-2/Bax ratio in liver in burned rats.

In conclusion, our present data suggest that melatonin suppresses burn-induced apoptosis injury through HO-1activation, attenuation of lipid peroxidation and modification of Bax/Bcl-2 ratio.

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