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Hepatoprotective effect of melatonin via activation of Nrf2 and anti-apoptotic proteins in burn rats

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ABSTRACT

Objective: Burn-induced acute hepatic injury due to increased production of lipid peroxides and increased cellular apoptosis. The nuclear factor erythroid 2-related factor 2 (Nrf2) pathway is essential for cytoprotection against oxidative stress (OS). We hypothesized that the melatonin by activation of Nrf2 may shift the Bax/Bcl-2 ratio to protect rat hepatocytes against apoptosis and progressive liver injury. The aim of this experimental study was to investigate the protective effects of melatonin against burn-induced apoptotic injury and the relationship between lipid peroxides expression of transcription factor Nrf2 and apoptotic protein in burn rat model.

Methods: Melatonin was applied immediately after the burn. The expression of hepatic 4-hydroxynonenal (4-HNE), as a marker of liver peroxidative injury, Nrf2, as a marker of antioxidant defense and apoptosis-related genes Bcl-2 and Bax were evaluated using light immunohistochemistry.

Results: Burns caused an increased expression of 4-HNE, Bax, and Bax/Bcl-2 ratio, and induced apoptosis of sinusoidal endothelial cells in liver tissue. Melatonin treatment augmented the increase in Nrf2 expression, decreased both burn-induced peroxidative damage and hepatic apoptosis as evidenced by reduced expression of Bax, enhanced expression of Bcl-2.

Conclusion: Our data suggest that the activation of the transcription factor Nrf2 by melatonin is protective against OS, apoptosis, and hepatic injury in burns. The available information by melatonin's effect on the redox-sensing transcription factor Nrf2, as a regulator of antioxidant enzymes, antioxidants, and antioxidant protection of the liver is limited. Melatonin activates the Nrf2/signaling pathway and acts as a natural inducer of anti-apoptotic and antioxidant protection under the condition of burn-induced OS. This is a new cellular mechanism for protection against progressive burn-induced liver damage not only in animals but also in humans.

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Melatonin; liver; Nrf2; Bax; Bcl-2; burns

Introduction

Excessive and sustained increase in oxidative stress (OS) and apoptosis has been implicated in the pathogenesis of pathological damage in many organs, including organs in the splanchnic area. Literature data show that the 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 an increased production of proinflammatory cytokines and free radicals [2,3]. Although the mechanism underlying the progression from liver injury is still not fully elucidated, mounting evidence has suggested OS be a key driving force.

It has been reported that the oxidative injury mediated by 4-hydroxynonenal (4-HNE) may lead to changes in expression of Bcl-2 proteins known as markers of apoptosis [4]. There is an insufficient

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information on the role of 4-HNE in development of the apoptotic processes induced by burn in the cells of the liver and other organs. 4-HNE may activate intracellular kinase cascades and redox-sensitive transcriptional factor Nrf2 [5].

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is an important cytoprotective transcription factor [6]. Nrf2 is the master regulator of a network of genes, encoding antioxidant, detoxifying, and cytoprotective molecules such as heme oxygenase-1 (HO-1), glutamate cysteine ligase, thioredoxin reductase, under conditions of elevated OS [7,8], glutathione-S-transferase and NAD(P) H:quinone oxidoreductase that mediate cell survival [9]. Activators of Nrf2 have the ability to alleviate oxidative damage and apoptosis in the liver and suppress the progression of diseases [10–13].

Melatonin protects against oxidative injury and gives rise to a cascade of metabolites (N1-acetyl-N2-formyl-5-methoxykynuramine and N(1)-acetyl-5-methoxykynuramine) that share its antioxidant and anti-inflammatory properties [14–17]. Melatonin has protective roles against burn-induced oxidative damage and mitochondrial dysfunction by the upregulation of Nrf2 antioxidant signaling pathways, which may be involved in the activation of c-Jun N-terminal kinases and Extracellular Signal-regulated Kinase [18]. Although experimental and clinical studies have shown that melatonin suppresses apoptosis in liver [19,20], we failed to find any data about a possible protective effect of melatonin on burn-induced hepatic apoptotic by activation of transcription factor Nrf2.

The aim of this experimental study was to investigate the protective effects of melatonin against liver injury and the association between the OS, expression of Nrf2, and apoptotic markers in burn rat model.

Materials 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 hours were allowed free access to water before the 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 hours) in individual wire-bottomed in 40/40 cm cages throughout the experimental period. Thus, lights were turned off at 8:00 pm and turned on at 8:00 am for achieving satisfactory photoperiod.

Thermal injury and melatonin treatment

After light ether inhalation, general anesthesia was performed using thiopental (30 mg/kg ip). In order to accomplish 30% of third-degree burn, hot boiling water (90°C) was applied on the back of the animals during a period of 10 seconds. For those rats which were subjected to burn injury, 4 ml of physiological saline was applied, ip, for immediate resuscitation following burn injury. No animals died within the first 24 hours of the 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} bw) burned group (B + M). Melatonin (N-acetyl-5-methoxytryptamine; Merck, Germany) was dissolved in absolute ethanol and diluted with physiological saline. The concentration of ethanol in the final solution was 5%. Melatonin solution was administered, ip, immediately and 12 hours after thermal skin injury. The groups without burns and with burns received vehicle only (5% ethanol, 5 ml/kg, ip). All the animals were given buprenorphine (0.3 mg/kg, ip, bw) twice daily for pain control post burn. They were re-anesthetized with thiopental and sacrificed 24 hours after burns as the liver was sampled.

Immunohistochemical examination for 4-HNE, Nrf2, 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 minutes. Then, they were rinsed in 0.1 M phosphate buffered saline (PBS) (pH 7.4) and treated in normal goat serum for 20 minutes. Subsequently, the sections were incubated with polyclonal primary antibody for 24 hours at room temperature. Rabbit 4-HNE antibody (Abcam, UK), and rabbit Bcl-2, Bax, and Nrf2 antibody (Santa Cruz, USA) were used. After rinsing with PBS, the sections were incubated for 20 minutes in goat anti-rabbit immunoglobulins at room temperature. Then, they were rinsed in PBS again, treated with rabbit peroxidase-anti-peroxidase complex for 20 minutes at room temperature and then rinsed in PBS. Finally, peroxidase activity was estimated by the diaminobenzidine-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, Nrf2, 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 [21]. 4-HNE, Nrf2, Bcl-2, and Bax contents of liver were defined as the 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.

From our point of view, according to the purposes of the study, manual counting is appropriated. In the manual reading of the expression of a substance in microscopic slides, we have the possibility not only for the quantitative analysis (degree of intensity of the corresponding color) but also for the qualitative analysis of the relevant color too. Qualitative analysis provides information about the expression of the substance in the cell (including the cell structures—nucleus, cytoplasm, etc.), the type of the cell (endothelial, hepatocytes) as well as outside the cell. 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 the analysis of variance were used to statistically analyze the difference between any two specified groups.

Results

Melatonin ameliorates burn-induced liver injury

Both sinusoidal endothelial cells (SECs) and hepatocytes expressed 4-HNE in the control group (Fig. 1).

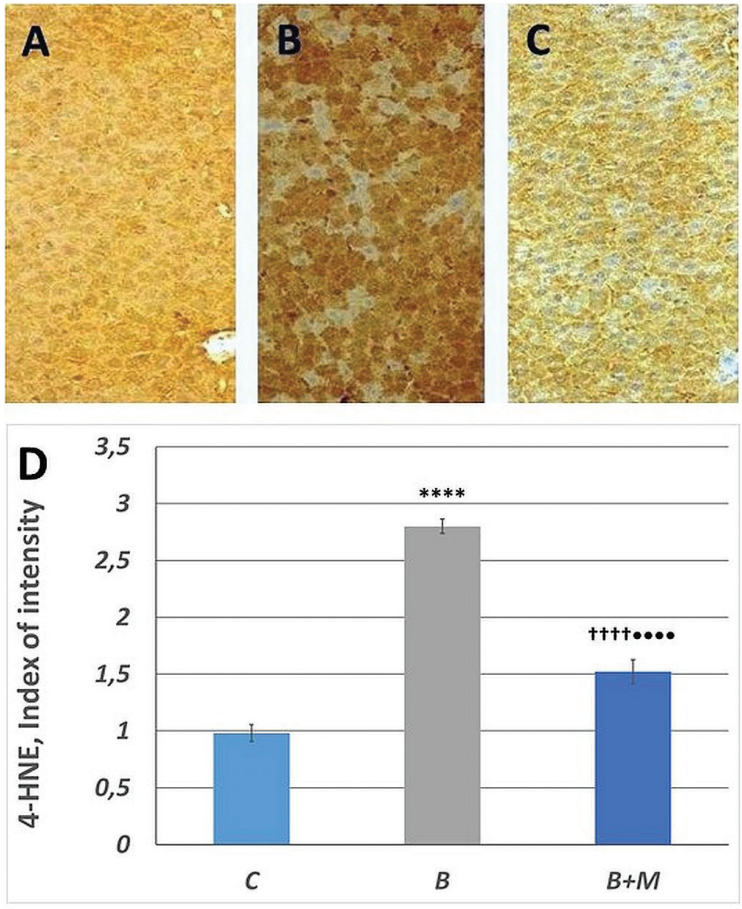

Figure 1. Effect of melatonin on the expression of 4-HNE. Immunohistochemical detection of 4-HNE in the liver. (A) Controls; (B) burned rats; and (C) burned rats, treated with melatonin. The antigen site appears as a brown color. Representative images. Original magnification, 400×; (D) score index of 4-HNE positive immunostained cells. Results are given as means ± SEM. ***$p < 0.001$ *vs.* control group; $^{\dagger\dagger\dagger}p < 0.001$ *vs.* burned, non-treated group. Controls (C); burned rats (B); and burned rats, treated with melatonin (B + M).

The staining intensity of 4-HNE positive cells was predominantly weak. The mean 4-HNE content in the cells was 0.98 ± 0.07. In the burned group, 4-HNE intensity was increased in SECs and in hepatocytes mainly around the central vein. The mean content was 2.8 ± 0.06 ($p < 0.001$). The burned group treated with melatonin 4-HNE positive cells was localized mainly in SEC and in some hepatocytes around the central vein. The mean content in both SECs and hepatocytes was 1.52 ± 0.10. It was lower (50%, $p < 0.001$) than in burned non-treated group.

Effects of melatonin on expression of apoptosis-related Bcl-2

The expression of Bcl-2 was detected in SECs in the control group (Fig. 2). The mean Bcl-2 content in the cells was 1.40 ± 0.10. In the burned group, Bcl-2 expression remained low (1.66 ± 0.11) and did not show significant changes compared with controls. The expression Bcl-2 was increased in SECs of the burned group treated with melatonin (Fig. 2). It was moderate to strong in SECs and their mean content (2.3 ± 0.12) was significantly higher 63% ($p < 0.001$) than this of the controls.

Effects of melatonin on expression of apoptosis-related Bax

Bax expression was detected mainly in the SECs in the control group (Fig. 3). Cell intensity ranged from mild to moderate (1.42 ± 0.29). In the burned group, Bax expression in SECs was moderate to strong (2.34 ± 0.11). Bax content was significantly higher by 60% ($p < 0.01$) as compared with the control group. Bax expression was reduced by 62% ($p < 0.01$) in the burned group treated with melatonin. Bax content was similar to the control values.

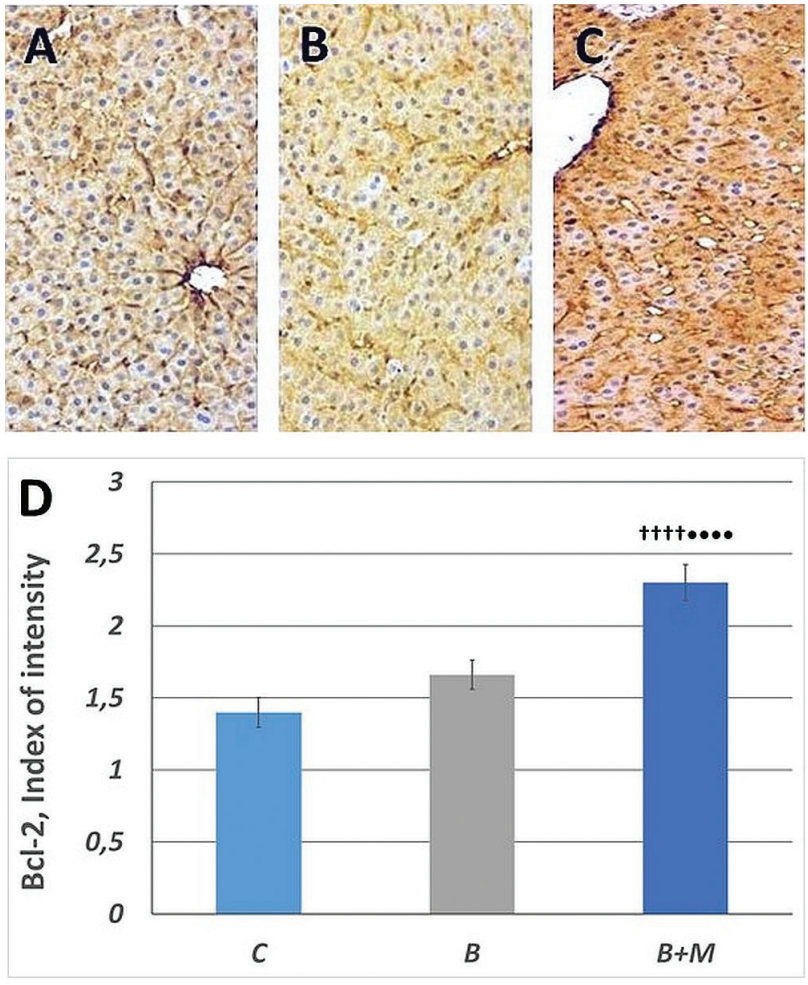

Figure 2. Effect of melatonin on the expression of Bcl-2. Immunohistochemical detection of Bcl-2 in the liver. (A) Controls; (B) burned rats; and (C) burned rats, treated with melatonin. The antigen site appears as a brown color. Representative images. Original magnification, 400×; (D) score index of Bcl-2 positive immunostained cells. Results are given as means ± SEM. *$p < 0.05$ *vs.* control group; $^{\dagger\dagger\dagger}p < 0.001$ *vs.* burned, non-treated group. Controls (C); burned rats (B); and burned rats, treated with melatonin (B + M).

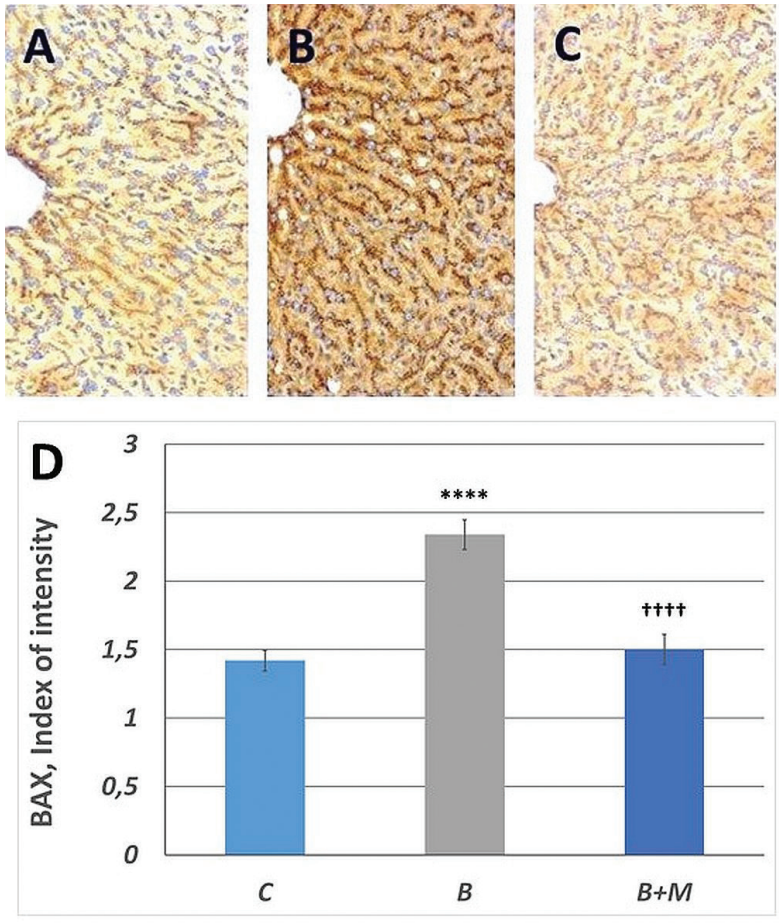

Figure 3. Effect of melatonin on the expression of Bax protein. Immunohistochemical detection of Bax in the liver. (A) Controls; (B) burned rats; and (C) burned rats, treated with melatonin. The antigen site appears as a brown color. Representative images. Original magnification, 400×; (D) score index of Bax positive immunostained cells. Results are given as means ± SEM. $^{**}p < 0.01$ *vs.* control group; $^{††}p < 0.01$ *vs.* burned, non-treated group. Controls (C); burned rats (B); and burned rats, treated with melatonin (B + M).

Effects of melatonin on expression of nuclear factor erythroid 2-related factor 2 (Nrf2)

Transcription factor Nrf2 was expressed in the cytoplasm of the SECs and hepatocytes in the control group. The Nrf2 intensity was 1.64 ± 0.11 (Fig. 4). The Nrf2 expression was moderate to strong in the cytoplasm of the SEC and in some hepatocytes mainly around the central vein. After thermal trauma, Nrf2 expression was insignificantly increased (1.72 ± 0.11) in the cytoplasm of the SECs and hepatocytes compared with controls. Melatonin increased Nrf2 expression in both cytoplasm and nucleus in hepatocytes and SEC compared with the burned group. The Nrf2 content was moderate to strong. The mean content was 2.11 ± 0.11 ($p < 0.05$).

Discussion

Beneficial effects of melatonin on cellular OS in liver and gastric mucosa, induced by burns have been established in our previous study [22–25]. The results of the current study show that melatonin activates the expression of transcriptional factor Nrf2 expression as well as modulates apoptotic proteins (Bax and Bcl-2), and thereby protects the liver against OS, apoptosis, and progressive injury in burns.

Burn-induced excessive free radical production might eventually overwhelm antioxidant defenses and generate highly toxic 4-HNE lipid peroxides. Mitochondria are very sensitive to the damaging action of lipid peroxides (HNEs), which oxidize SH-groups (cysteine and methionine) of glutathione, change the redox status, and are an early and powerful activator of apoptotic signals [7]. The translocation of the Bax protein from the cytosol and its transfer to the external mitochondrial membrane, resulting in leakage of cytochrome C and other pro-apoptotic proteins and activation of the programmed cell death [7, 26,27].

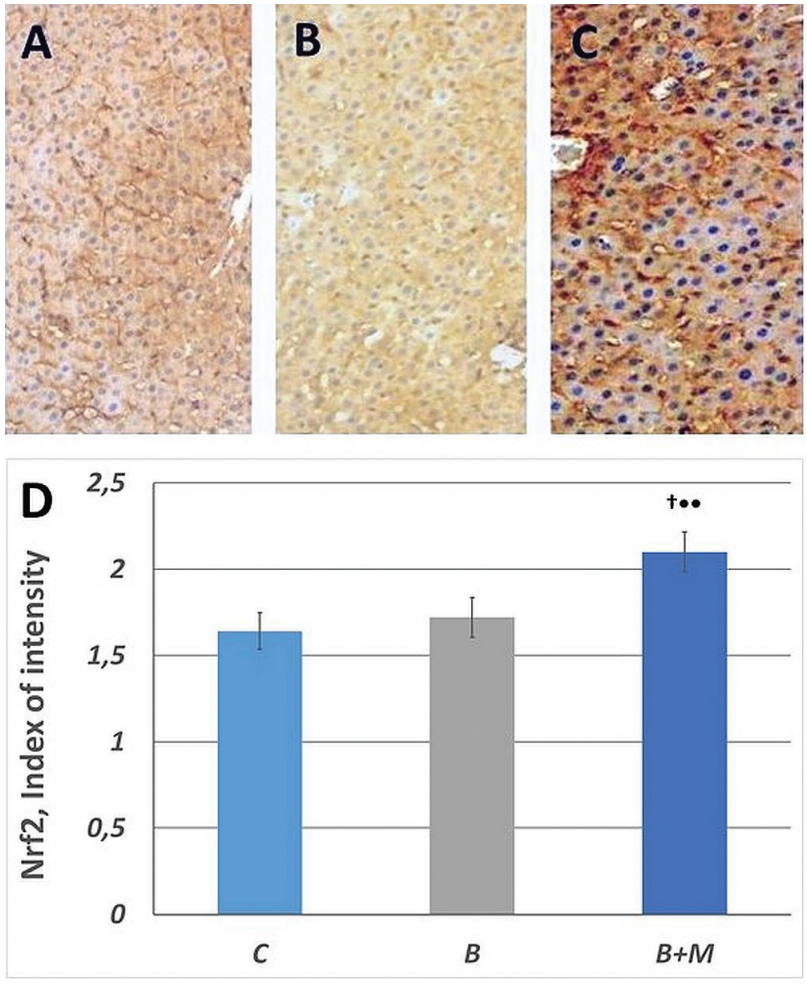

Figure 4. Effect of melatonin on the expression of Nrf2 protein. Immunohistochemical detection of Nrf2 in the liver. (A) Controls; (B) burned rats; and (C) burned rats, treated with melatonin. The antigen site appears as a brown color. Representative images. Original magnification, 400×; (D) score index of Bax positive immunostained cells. Results are given as means ± SEM. $^{**}p < 0.01$ *vs.* control group; $^{††}p < 0.01$ *vs.* burned, non-treated group. Controls (C); burned rats (B); and burned rats, treated with melatonin (B + M).

Bcl-2 and Bax protein, major mediators of apoptosis, are mainly activated via the internal (mitochondrial) signaling pathway. The expression of proapoptotic Bax protein can be also increased under the action of external death signals [28,29]. The increased Bax proapoptotic protein expression may be associated with translocation of pro-apoptotic Bid protein to mitochondria by the Fas/TNF-R1 pathway [30]. There is a strong evidence that Fas/FasL representing one possible mechanism for burn-induced hepatic apoptosis and dysfunction [31].

In the current study, we observed that an increase in Bax protein levels and a significant decrease in Bcl-2 protein levels in the liver of the burned group when compared with the control group. These results correspond to the changes reported by other authors, who detect elevated Bax protein levels and decreased Bcl-2 protein expression after burns [32,33].

Cell fate, apoptosis, or survival depend on the balance between Bax and Bcl-2 proteins [9]. The imbalance between pro- and anti-apoptotic proteins, mediating apoptosis has been found in several models of liver injury, ethanol, stress, and other factors [34,35]. Our results showed that an elevated Bax/Bcl-2 ratio, predominantly for Bax elevation in burn group than in the control one.

Additionally, 4-HNE inhibits mitochondrial respiration, DNA and protein synthesis and has been shown to induce apoptosis in a variety of cell [36,37]. Glutathione (GSH) protection of cellular macromolecules like DNA, proteins, and lipids against the toxic effect of 4-HNE, underscores its central antiapoptotic function. Increased HNE levels accompanied by a loss of GSH may promote

mitochondrial permeability transition pore opening which is a critical factor for cell death [38].

Glutathione S-transferase (GST) can modulate cell survival and death signaling by regulating the intracellular concentration of 4-HNE [39]. This enzyme catalyzes detoxification of lipid aldehyde including 4-HNE with GSH. Detoxification of 4-HNE by GST can be diminished due to depletion of GSH [40] which may result in higher 4-HNE levels [41].

Elevation of 4-HNE and depletion of intracellular GSH levels may lead to post-translational modifications of many proteins, including glutathione S-transferase [42]. The accumulation of 4-HNE-modified proteins (carrying specific 4-HNE adducts) in mitochondria are associated with cell death in many liver diseases [43]. The decrease in glutathione, a very potent anti-oxidant, renders liver vulnerable to oxidative injury [22,23] and cell death [44].

Thermal injury is a strong inducer of OS. Redox-sensitive transcription factor Nrf2 plays an important role in the antioxidant response pathways against excessive production of free radicals in burns. High HNE levels have a damaging effect and cause free radical-induced oxidative injury. HNE in low levels acts as a potential activator of Nrf2 which enhances cellular antioxidant capacity and protects against oxidative injury. Depending on its level, 4-HNE exerts harmful or protective effects associated with the activation of antioxidant defense mechanisms [26].

Our results showed an elevation of 4-HNE levels and a slightly increased expression of Nrf2, mainly in hepatocytes cytoplasm in animals with burns versus controls.

Nrf2 activates genes that encode phase II detoxifying enzymes and antioxidant enzymes such as glutathione S-transferases and heme oxygenase and mediates the regulation of redox balance and antioxidant defense in cells. Additionally, the anti-apoptotic Nrf2 effect may be associated with increasing expression of glutathione-S-transferase, alcohol dehydrogenases, and aldehyde dehydrogenase [45]. Significant amounts of these enzymes are found in mitochondria. They detoxificate 4-HNE to less reactive chemical species and decrease its pro-apoptotic action [46].

Niture and Jaiswal report that Nrf2 releases Bcl-xL in mitochondria, increases in Bcl-xL heterodimerization with Bax in mitochondria, and reduces cellular apoptosis. It mediates elevation of the anti-apoptotic protein Bcl-2 [9], upregulates Bcl-2/Bax expression, and decreases expression and activity of caspases 3 and 9 [47].

Nrf2 has also been shown to protect against OS-induced cell death by upregulation of antioxidant enzymes, involved in GSH biosynthesis. It has been reported that natural activators of Nrf2 may protect against oxidative injury [48,49]. Nrf2 has a crucial role in the maintenance of cellular redox homeostasis by regulating the biosynthesis, utilization, and regeneration of glutathione, and by controlling the production of reactive oxygen species by mitochondria [15]. Activation of Nrf2 down-regulated Bax and decreased apoptotic cell death in response to antioxidants [9].

In our model, the melatonin-treated group, compared with the burned group significantly reduced 4-HNE levels and significantly increases Bcl-2 protein expression. There was a similar increase in the pro-apoptotic Bax protein. Moreover, melatonin significantly decreased the Bax/Bcl-2 index through the elevation of the Bcl-2 level. It has been reported that natural activators of Nrf2 may protect against oxidative injury [50–52].

The present study demonstrates that the melatonin increases Nrf2 expression and it is even more intensive in the cytoplasm of hepatocytes, but it is also present in the nuclei of endothelial cells after burns (Fig. 4). Nrf2 is localized in the cytoplasm bound to Keap1, a cytoskeleton-associated protein. A possible mechanism by which melatonin increases Nrf2 expression is related to the dissociation of Nrf2/Keap1 (repressor) and translocation of Nrf2 to the nucleus, where it binds to ARE in the promoter of the genes involved in the antioxidant protection [53].

Nrf2 down-regulated Bax and decreased apoptotic cell death in response to antioxidants [9]. Our results demonstrate that the antioxidant melatonin may activate the Nrf2/ARE signaling pathway, increases expression of anti-apoptotic Bcl-2, and protects against apoptosis in the liver in burn rats. A recent study shows that melatonin reverses the expression of apoptotic proteins through inhibition of free-radical activating lipid peroxidation (malondialdehyde) and elevation of expression of heme-oxygenase [54]. Additionally, the expression of proapoptotic Bax protein by melatonin can also be increased under the action of external death signals [49,50]. Melatonin reduces the levels of cytotoxic TNF-α as well as another cytokines and diminishes their deleterious effects on mitochondria through NFkB activation [55,56]. By lowering the levels of toxic 4-HNE and increasing of glutathione

levels, melatonin activates transcription factor Nrf2, decreases expression of pro-apoptotic Bax protein, and increases of proapoptotic Bcl-2 protein expression.

Melatonin restricts the formation of 4-HNE and increases GSH levels and might protect the liver against oxidative injury and apoptosis. Besides, melatonin may restrict modification of glutathione S-transferase which have a vital role in the detoxification of 4-HNE [57]. Melatonin is a powerful direct antioxidant and plays a direct role in mitochondrial homeostasis by improving mitochondrial respiration, increasing the level of ATP synthesis, and reducing the harmful decrease in the mitochondrial membrane potential that can trigger the apoptotic cascade [58,59]. Additionally, melatonin preserves the disturbed mitochondrial integrity through maintenance of mitochondrial GSH in liver injury caused by alcoholism and high fructose diet [60].

Our data demonstrate the key role of the activated Nrf2/ARE signaling pathway in the prevention of burn-induced apoptotic injury through the mechanism that involves inhibition of accumulation of 4-HNE generation, activation of cytoprotective proteins including antioxidants, phase II detoxifying, and anti-apoptotic proteins. The beneficial effect of melatonin is associated with both decreased Bax protein expression and/or increased expression of the Bcl-2 protein and down-regulated Bax/Bcl-2 ratio along with increasing Nrf2 expression. We suggest that the melatonin suppresses damaging effect of 4-HNE on mitochondria, modulates expression of pro- and anti-apoptotic protein, and shift Bax/Bcl-2 ratio to protect rat hepatocytes against apoptosis.

Conclusion

Our data suggest that the activation of the transcription factor Nrf2 by melatonin is protective against OS, apoptosis, and hepatic injury in burns. The available information by melatonin's effect on the redox-sensing transcription factor Nrf2, as a regulator of antioxidant enzymes, antioxidants, and antioxidant protection of the liver is limited. Melatonin activates the Nrf2/ARE signaling pathway and acts as a natural inducer of anti-apoptotic and antioxidant protection under the condition of burn-induced OS. This is a new cellular mechanism for protection against progressive burn-induced liver damage not only in animals but also in humans. Therefore, the melatonin merits consideration as a therapeutic agent in the treatment of burn injuries.

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