

## Melatonin protects against burn-induced hepatic oxidative injury by inducing HO-1 via the Nrf2 pathway

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**ABSTRACT:** Melatonin exerts beneficial effects on early liver injury by modulating hepatic oxidative stress. In order to understand the protective effect of melatonin against burn-induced hepatic injury we investigated the expression of 4-hydroxynonenal (4-HNE), a main product of lipid peroxidation and mediator of oxidative injury, the inducible heme-oxygenase-1 (HO-1), an antioxidant enzyme, and the anti-oxidative stress regulator erythroid 2-related factor 2 (Nrf2) in a burn rat model. Expression and localisation of HO-1, 4-HNE and Nrf2 in liver were investigated using light immunochemistry. Thermal skin injury caused a significant elevation in hepatic 4-HNE and degenerative liver changes. Concurrently, there was increased expression of HO-1, a rate-limiting enzyme for haem degradation and an oxidative stress marker in sinusoidal endothelial cells (SECs) and hepatocytes without changes in Nrf2 expression in the liver. Melatonin (20 mg/kg b.w.) augmented the increase in HO-1 expression, upregulated Nrf2 expression and also led to decreased 4-HNE levels and reduced levels of histopathological changes in rat liver. In conclusion, our results suggest that melatonin ameliorates burn-induced liver injury through the inhibition of oxidative stress, upregulation of the antioxidant enzyme HO-1 and activation of the antioxidant Nrf2 pathway. Stimulation of cellular protective mechanisms by activating the antioxidant stress response through Nrf2 is a new mechanism for protection against liver damage in burns.

**Keywords:** melatonin; Nrf2; HO-1; 4-HNE; oxidative stress; liver

Thermal injury represents one of the most severe forms of trauma and is a serious clinical problem in emergency medicine. A severe burn is a devastating injury affecting every organ system and leading to complications with poor outcome. The liver plays an important role in metabolism, inflammation, homeostasis and host defence mechanisms (Klein et al. 2004), and is a major organ responsible for initiating multiorgan dysfunction syndrome (MODS) following burn injury. The mechanism of burn-induced injury is not completely understood; however, several mechanisms, including hypoxia, free radicals, inflammation, and apoptosis are thought to be involved (Jeschke et al. 2001). The excessive production of free radicals such as superoxide anion, hydrogen peroxide and hydroxyl radicals as well as the occurrence of lipid peroxidation products such as malondialdehyde (MDA) due to oxidative stress are associated with burn-induced liver dysfunction (Horton 2003; Bekyarova et al. 2009; Bekyarova et al. 2012). Burn reduces the antioxidant status thus

leading to a failure of the antioxidant defence against free-radical damage (Piantadosi et al. 2006; Parihar et al. 2008; Vinha et al. 2013).

In many cell types, numerous cellular responses to oxidative stress are mediated by signalling proteins that act through the antioxidant response element (ARE) and the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Dulak and Jozkowicz 2003; Lee et al. 2005). Nrf2 is a key transcription factor regulating ARE-mediated transcription of genes specialized in the detoxification of certain reactive species (HO-1), as well as phase-2 detoxifying enzymes and related stress-response proteins that play a central role in the cellular defence against oxidative injury (Osburn et al. 2006; Yang et al. 2011). HO-1 upregulation by pharmacological treatment protects against the deleterious effects of free radicals and oxidative stress, and blocks apoptosis in various cell types and animal models (Ashino et al. 2011).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a powerful antioxidant that scavenges superoxide

radicals as well as other radical oxygen species (ROS) and radical nitrogen species (RNS), protects against oxidative injury and gives rise to a cascade of metabolites (AFMK and AMK) that share its antioxidant and anti-inflammatory properties (Reiter et al. 2009; Galano et al. 2013; Mauriz et al. 2013; Zhang and Zhang 2014). Melatonin induces the expression of antioxidant enzymes such as superoxide dismutase SOD, catalase and glutathione peroxidase under conditions of elevated oxidative stress (Rodriguez et al. 2004), through activation of Nrf2 (Jung et al. 2009). Further, melatonin ameliorates oxidative stress through Nrf2 induction in experimental models of hepatic injury (Osburn et al. 2006; Aleksunes and Manautou 2007; Crespo et al. 2010; Yang et al. 2011). Activation of Nrf2 by melatonin has been described to result in an increased expression of the antioxidant enzyme haem oxygenase-1 (HO-1) and a restriction of hepatic injury (Jung et al. 2009; Crespo et al. 2010). Although experimental and clinical studies have shown that melatonin restrains oxidative liver injury (Ohta et al. 2000; Oz et al. 2006), we failed to find any reports on a possible protective effect of melatonin on burn-induced hepatic damage by activation of the Nrf2/HO-1 system. Therefore, we designed this study to investigate whether the protective effect of melatonin against burn-induced liver injury is associated with the activation of the Nrf2/HO-1 signalling pathway. Histological changes were examined and the expression of 4-hydroxynonenal (4-HNE) was determined as a marker of oxidative stress. Expression and localisation of HO-1 and Nrf2 were analysed using light immunohistochemistry.

## MATERIAL AND METHODS

**Experimental study.** The experimental procedures were approved by the Home Office for Care and Use of Laboratory Animals and were performed with careful consideration of the ethics of animal experimentation according to the International Guiding Principles for Animal Research approved in Bulgaria. Same-age male rats weighing between 220 and 250 g were fasted for 12 h, but were allowed free access to water before injury. Animals were housed in a 20 °C room and offered standard rat chow and water *ad libitum*. They were kept in dark-light cycles (DL = 12 : 12 h) in individual wire bottomed cages. The lights were turned off at

8:00 p.m. and turned on at 8:00 a.m. to achieve a satisfactory photoperiod.

After mild ether inhalation, general anaesthesia was intraperitoneally (*i.p.*) established using thiopental 30 mg/kg body weight (b.w.) In order to accomplish a 30% third-degree burn, scalding hot water (90 °C) was applied on the back of the animals for a 10 s duration. Following burn injury, 4 ml physiological saline was *i.p.* applied for immediate resuscitation. No animals died within the first 24 h post-burn period. Twenty one male rats were randomly assigned to three groups: group of vehicle treated without burns (C) ( $n = 7$ ); group of vehicle treated with burns (B) ( $n = 7$ ); and group of melatonin (10 ml/kg b.w.) treated with burns (B + M) ( $n = 7$ ). 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 *i.p.* immediately and 12 h after thermal skin injury. The groups without burns and with burns received vehicle only (5% ethanol, 5 ml/kg, *i.p.*). All animals received buprenorphine (0.3 mg/kg b.w. *i.p.*) twice daily for post-burn pain control. Twenty four hours after the burns the animals were anaesthetised with thiopental, sacrificed and livers were sampled.

**Histological examination.** Liver slices were fixed in 10% phosphate-buffered saline (PBS)-formalin and embedded in paraffin. Liver samples were subsequently sectioned (5 µm), stained with haematoxylin and eosin (H&E), and examined under a microscope (Olympus, Japan) to evaluate liver injuries.

**Immunohistochemical staining of 4-HNE, HO-1 and Nrf2.** Rat liver specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. The deparaffinised and dehydrated sections (5 µm thick) were treated with 1% hydrogen peroxide for peroxidase activity inhibition for 5 min. Then they were rinsed in 0.1M phosphate buffered saline (PBS) (pH 7.4) and treated in normal goat serum for 20 min. Subsequently, the sections were incubated with polyclonal primary antibodies for 24 h at room temperature. Rabbit anti-HO-1 antibody (Santa Cruz, USA), anti-Nrf2 antibody (Santa Cruz, USA) and 4-HNE antibody (Abcam, UK) 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-antiperoxidase complex for 20 min at

doi: 10.17221/8530-VETMED

room temperature and then rinsed in PBS. Finally, peroxidase activity was estimated using the diaminobenzidine-tetrachloride  $H_2O_2$ -method.

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

The morphometric method described by Bekyarova et al. (2013) was used to semiquantitatively assess the content of HO-1, Nrf2 and 4-HNE. The immunohistochemical staining was determined as: strong = score 3; moderate = score 2; weak = score 1; and lack of staining = score 0. The content of HO-1, Nrf2 and 4-HNE in the liver cells was defined as the number of each type of the cell content multiplied by their scoring factor and divided by the total number of cells. Morphometry was carried out on 50 cells of each sample.

**Statistical analysis.** All experimental data were expressed as mean  $\pm$  standard error of the mean (SEM). Significant differences were determined by one-way analysis of variance (ANOVA).  $P < 0.05$  was considered as a statistically significant difference.

## RESULTS

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

Hepatocytes had a normal shape and size with vesicular nuclei. No apoptotic hepatocytes were observed in the control group. (Figure 1). In the burn group hepatocytes exhibited acidophilic degeneration and vacuolisation and there were numerous apoptotic cells. The degenerative changes were reduced and there were only a small number of apoptotic cells in the burn group treated with melatonin.

### Melatonin ameliorates burn-induced acute liver injury

4-HNE expression was found in both sinusoidal endothelial cells (SEC) and hepatocytes in the control group (Figure 2). The staining intensity of 4-HNE-positive cells varied from weak to moder-

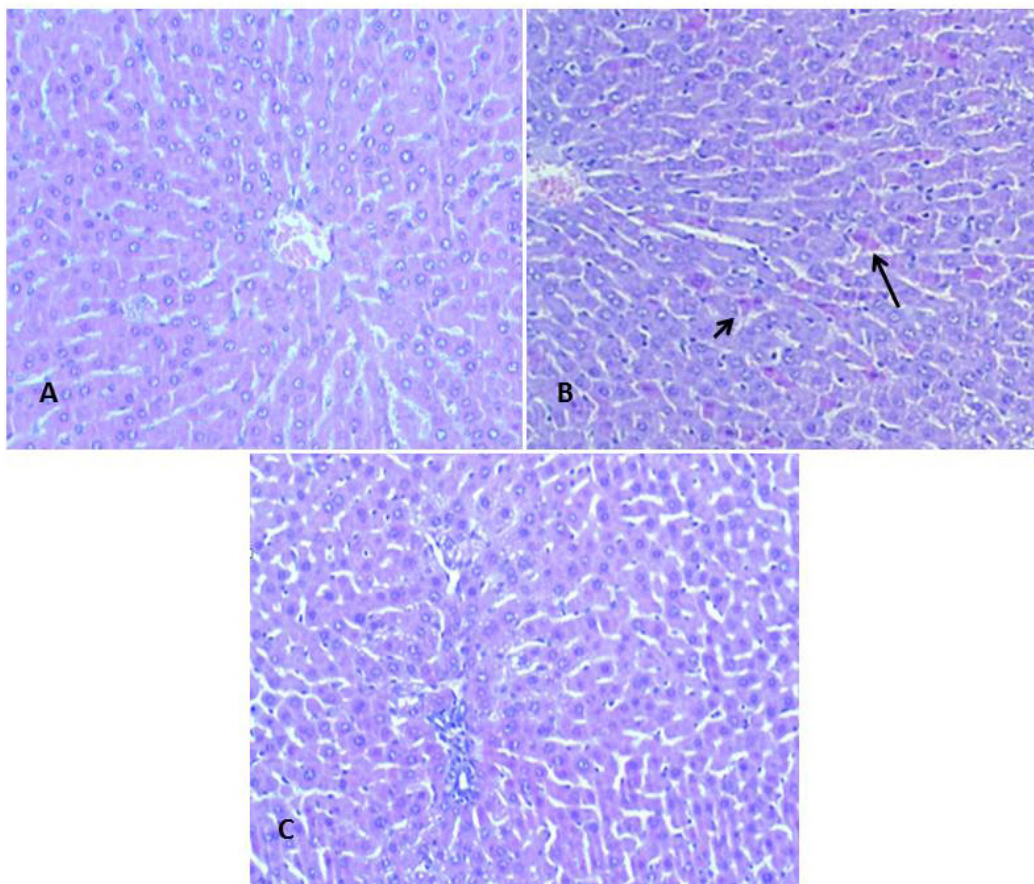


Figure 1. Effect of melatonin on burn-induced histomorphological changes in the liver of rats (A) control group, (B) burn group, (C) burn, treated with melatonin group. Original magnification  $\times 400$  H&E staining, original magnification. Arrows indicate apoptotic cells



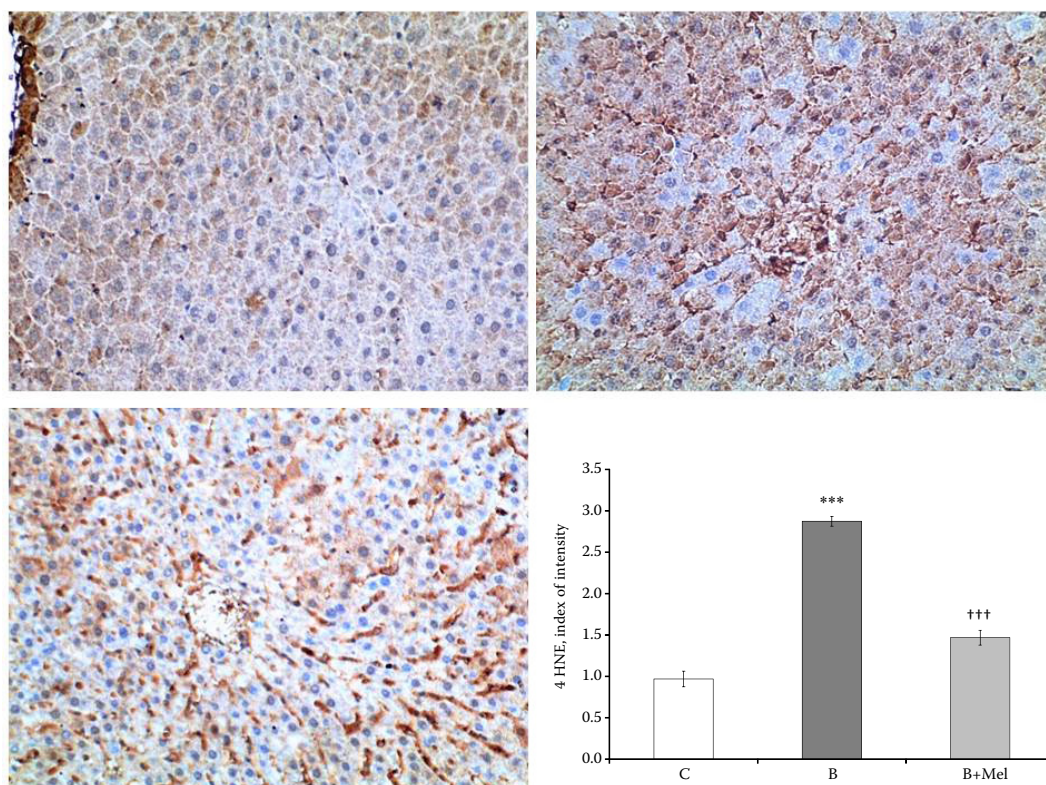


Figure 2. Immunohistochemical detection of 4-hydroxynonenal (4-HNE) in liver. (A) It is localised in both sinusoidal endothelial cells (SECs) and hepatocytes in the control group. The staining intensity of 4-HNE-positive cells was weak. (B) In the burn group, 4-HNE positivity was observed principally in the sinusoidal cells and in hepatocytes around the central vein. It was moderate to strong in the individual cells. (C) In the burn group treated with melatonin, 4-HNE positivity was observed principally in SEC and some hepatocytes around the central vein. The number of 4-HNE positive cells was lower than in the burn non-treated group; (immunohistochemistry,  $\times 400$ )

ate. The mean 4-HNE signal in the cells was  $0.95 \pm 0.095$ . In the burn group, the number of 4-HNE positive cells was elevated. The mean signal was  $2.87 \pm 0.059$ . The number of 4-HNE-positive cells was diminished in the burn group treated with melatonin. The mean content in SEC and hepatocytes was  $1.46 \pm 0.089$  which is 50% lower ( $P < 0.001$ ) than the value in the burn group.

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

Immunohistochemical analysis showed that the transcription factor Nrf2 is expressed in the cytoplasm and in the nucleus of SECs in the liver in the control group and the index of intensity was  $1.68 \pm 0.087$  (Figure 3). The immune reaction was moderate to strong in the cytoplasm of SECs in individual hepatocytes around the central vein in the burn group

and the Nrf2 signal was similar to that of the control group ( $1.72 \pm 0.103$ ). Melatonin increased Nrf2 expression in the cytoplasm and in the nucleus in both hepatocytes and SEC compared with the burn group. The mean signal was  $2.11 \pm 0.077$  ( $P < 0.05$ ).

#### Effects of melatonin on expression of HO-1

HO-1 expression was found in SECs of the liver in the control group. However, the staining intensity of HO-1 positive cells was weak (Figure 4). The mean HO-1 signal in the cells was  $1.34 \pm 0.068$ . In the burn group, induction of HO-1 occurred principally in SEC and was moderate to strong in individual cells. The mean signal ( $2.08 \pm 0.085$ ) was significantly higher (51%,  $P < 0.001$ ) than in the control rats. The number of HO-1-positive SECs in the liver was increased in the burn group treated with melatonin, which contributed to an increase in the average sig-

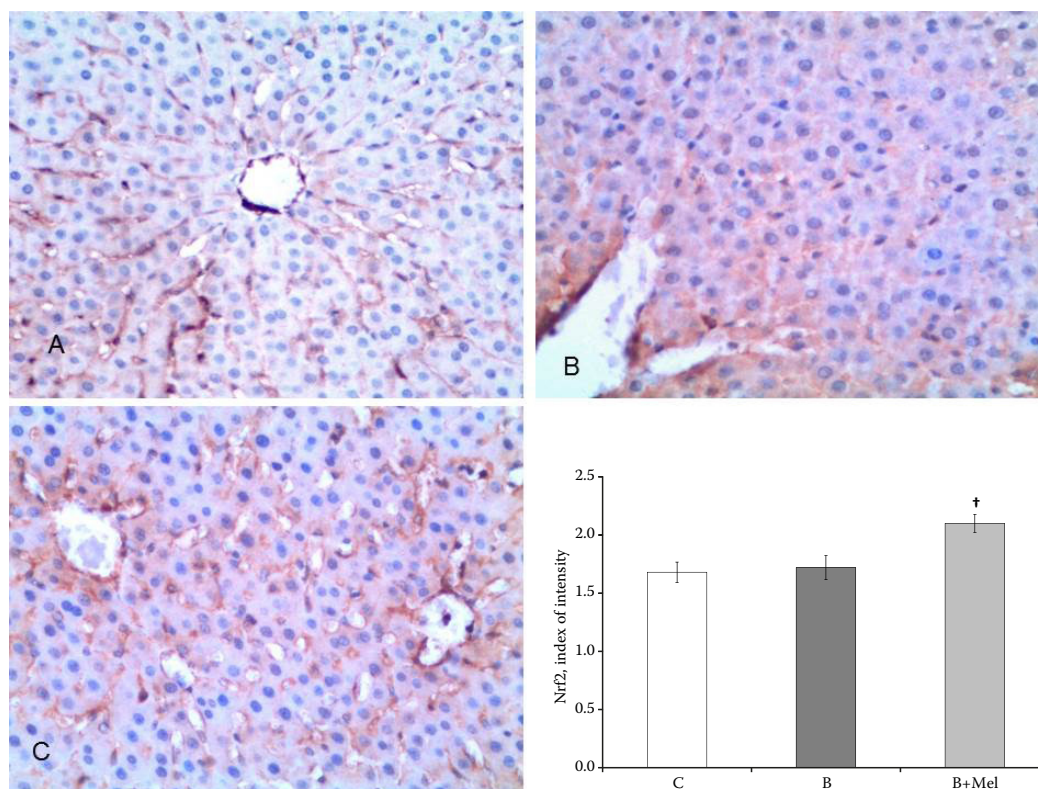


Figure 3. Effects of melatonin on expression of nuclear factor erythroid 2-related factor 2 (Nrf2). Immunohistochemical detection of nuclear factor erythroid 2-related factor 2 (Nrf2) in liver (A) It is localised in the cytoplasm and in the nucleus of SECs of the liver in the control group. The staining intensity is moderate. (B) In the burn group, Nrf2 positivity was observed in the cytoplasm of the SECs in individual hepatocytes. It was moderate to strong in the individual cells. (C) In the burn group treated with melatonin, Nrf2 positivity was observed in the cytoplasm and in the nucleus in both hepatocytes and SECs; (immunohistochemistry,  $\times 400$ )

nal of HO-1 protein in the liver to  $2.53 \pm 0.077$ , a value which was 23% ( $P < 0.001$ ) higher than in the burn group which did not receive melatonin.

## DISCUSSION

The present study demonstrates that melatonin exerts a protective effect against oxidative liver damage in a burn rat model. The expression of Nrf2 and HO-1 was significantly increased in burned rats treated with melatonin as compared with control rats and burned, non-treated rats.

Thermal skin injury is a strong inducer of oxidative stress, and lipid peroxidation is the most prominent among the many biological targets of oxidative stress (Bekyarova et al. 2009; Bekyarova et al. 2012). 4-HNE is one of the most toxic molecules of lipid peroxidation and a biomarker of oxidative injury. This lipid peroxidation by-product has high

reactivity towards the thiol and amino groups of amino acids. 4-HNE modifies essential cellular proteins, decreases glutathione levels and alters cellular redox homeostasis (Niki 2009). The accumulation of 4-HNE in the liver may increase the likelihood of tissue injury during oxidative stress and causes degenerative changes in liver. In the current study increased levels of lipid peroxidation and oxidative injury were demonstrated by increased expression of 4-HNE in the liver.

ROS are crucial for activation of the Nrf2 transcription factor (Chen and Niki 2006). It is suggested that mild oxidative stress and in particular 4-HNE might induce nuclear translocation of Nrf2. Nrf2 protects the cell against oxidative stress through ARE-mediated induction of several phase-2 detoxifying and antioxidant enzymes, particularly HO-1 (Lee et al. 2005), but also superoxide dismutase,  $\gamma$ -glutamylcysteine synthase, glutathione S-transferase and glutathione peroxi-



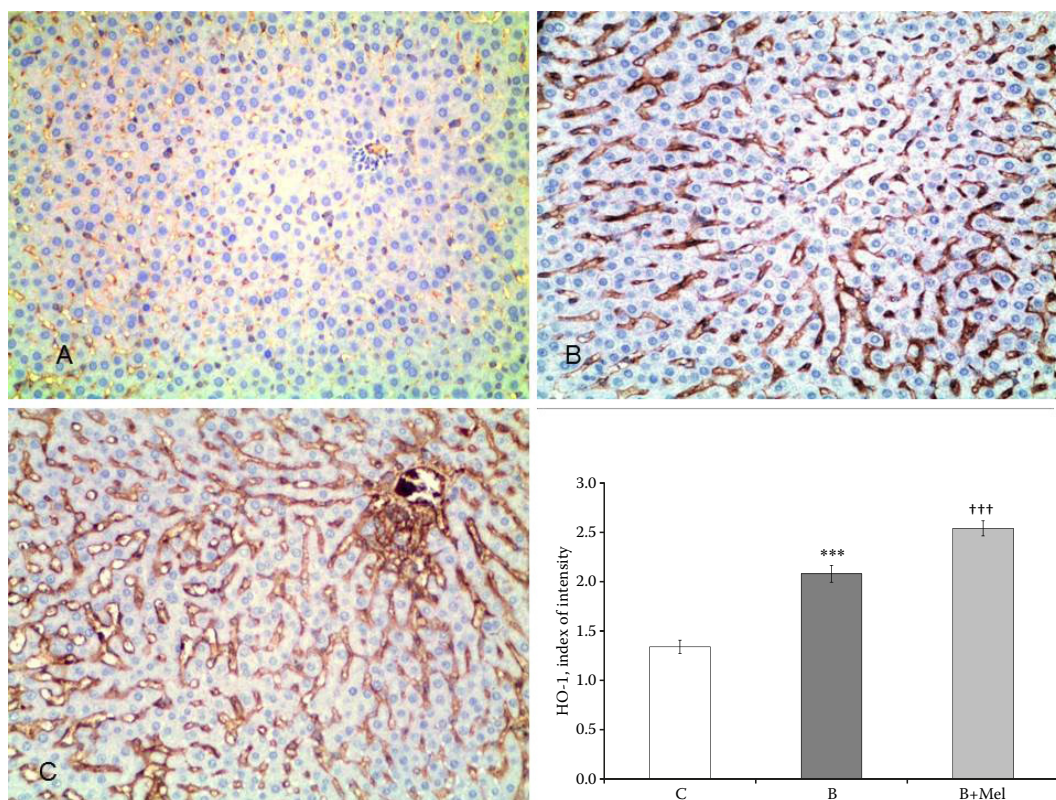


Figure 4. Effects of melatonin on expression of heme-oxygenase-1 (HO-1) in liver. Immunohistochemical detection of heme-oxygenase-1 (HO-1) in liver. (A) It is localised in SECs of the liver in the control group. The staining intensity of HO-1-positive cells was weak. (B) In the burn group, HO-1 positivity was observed in the sinusoidal cells. It was moderate to strong in the individual cells. (C) In the burn group treated with melatonin, HO-1 positivity was observed principally in the sinusoidal cells. The number of HO-1 positive cells was higher than in the burn non-treated group; (immunohistochemistry,  $\times 400$ )

dase (Aleksunes and Manautou 2007; Jung et al. 2009).

Melatonin increased Nrf2 expression, most intensively in the cytoplasm of hepatocytes, but it was also present in the nuclei of endothelial cells in the burn group. It is known that the inactive form of Nrf2 is localised 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/Keap 1 (repressor) and translocation of Nrf2 to the nucleus, where it binds to the ARE in the promoters of the genes involved in antioxidant protection such as SOD, catalase, GxP and HO-1 (Jaiswal 2004; Osburn et al. 2006). It has been reported that Nrf2 activators protect from oxidative injury (Chan et al. 2001; Farombi et al. 2008; Miller et al. 2013). We suggest that melatonin can ameliorate burn-induced liver injury through inhibition of free-radical activating lipid peroxidation, and via the elevation

of expression of the transcription factor Nrf2 and antioxidant enzymes such as HO-1.

HO-1 is a stress-responsive enzyme responsible for the breakdown of haem to biliverdin, free iron and carbon monoxide (Ryter et al. 2002). Haem is a potent oxidant, while bilirubin converted from biliverdin and carbon monoxide (CO) exhibits antioxidant activity, vasodilation and inhibition of platelet aggregation, respectively. CO has anti-inflammatory and anti-apoptotic properties (Brouard et al. 2000; Dulak and Jozkowicz 2003). Therefore, HO-1 induction might also be important in antioxidant defence and the anti-inflammatory response (Otterbein and Choi 2000; Lee et al. 2009; Ryter and Choi 2013).

Melatonin reduces the level of 4-HNE, a lipid peroxidation product, ameliorates liver histological changes and augments the increase of HO-1 activity in the liver. These findings suggest that the protective mechanism of melatonin against burn-

doi: 10.17221/8530-VETMED

induced liver injury could be closely associated with HO-1 overexpression and increased cellular antioxidant capacity. Our data are in accordance with data regarding the protective effect of melatonin on DNA damage related to the increased expression of antioxidant enzymes SOD, catalase and Gpx in *ex vivo* human skin (Fischer et al. 2013).

We and others have previously shown that melatonin, owing to its small molecular size and high lipophilic capacity, reduces liver injury in a burn rat model (Bekyarova et al. 2009; Bekyarova et al. 2013) and other models of oxidative stress (Park et al. 2007; Crespo et al. 2010; Ashino et al. 2011; Yang et al. 2011). We suggest that melatonin induces HO-1 via the Nrf2 pathway in liver in this model. Similar findings regarding upregulation of HO-1 and the Nrf2 system in other models of hepatic and oxidative stress have been reported (Osburn et al. 2006; Aleksunes and Manautou 2007).

In conclusion, our data suggest that melatonin increases HO-1 expression and thereby protects the liver against oxidative stress. This is the first report to show that melatonin activates the Nrf2/HO-1 signalling pathway and acts as a natural inducer of antioxidant protection. Stimulation of protective cellular mechanisms through activation of antioxidant transcription factors such as Nrf2 is a new mechanism for protection against burn-induced liver damage. Further research into the molecular mechanisms of Nrf2 activation should offer opportunities to develop new therapeutic approaches to victims of thermal trauma.

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Received: 2015–03–19

Accepted after corrections: 2015–09–06

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