African Journal of Biotechnology Vol. 11(58), pp. 12222-12228, 19 July, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1577
ISSN 1684-5315 ©2012 Academic Journals

Full Length Research Paper

Melatonin inhibits endothelin-1 and induces endothelial nitric oxide synthase genes expression throughout hepatic ischemia/reperfusion in rats

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Accepted 22 June, 2012

The production of reactive oxygen species (ROS) and dysfunction of vasculature play a central role in the pathophysiology of hepatic ischemia/reperfusion (I/R) injury. The aim of this study was to evaluate the beneficial effects of melatonin on reducing liver I/R injury in rats. Four study groups were formed: (1) saline - administered, control group (Control), (2) melatonin-administered group (MEL), (3) saline - administered I/R group (I/R) and (4) melatonin-administered I/R group (MEL+ I/R). Melatonin was injected intraperitoneally (15 mg/kg) 20 min before ischemia and immediately after reperfusion. After reperfusion, blood and ischemic liver tissues were collected. The group subjected to ischemia showed a significant increase in the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, as well as an increase in hepatic malondialdehyde (MDA) concentration. These increases were significantly inhibited by melatonin. Although, I/R augmented the endothelin-1 (*ET-1*) gene expression and the level of big endothelin-1 (big ET-1) in liver tissue, melatonin attenuated these increases. Conversely, non-significant decrease in endothelial nitric oxide synthase (eNOS) mRNA expression in I/R group was significantly elevated by melatonin in MEL+ I/R group. Melatonin exerts beneficial effects on ischemia/reperfusion liver injury through its anti-oxidative function as well as regulation of hepatic microcirculation.

Key words: Melatonin, oxidative stress, ischemia/reperfusion injury, endothelin and nitric oxide synthase.

INTRODUCTION

Ischemia/reperfusion (I/R) liver injury is a complicated pathophysiologic event during hepatic surgery, trauma, hemorrhage, sepsis and liver transplantation, which may lead to hepatic dysfunction. Although the underlying mechanisms of I/R injury remain unclear, several lines of evidences suggest that production of reactive oxygen species (ROS), reactive nitrogen species (RNS) and dysfunction of endothelium play a central role in the pathophysiology of I/R liver injury (Bahde and Spiegel, 2010). The ROS generation by activated Kupffer cells or neutrophils induces a series of harmful effects including oxidative alterations in lipids and proteins, release of pro-inflammatory mediators and dysfunction of microvasculature, which together lead to cellular and tissue injury (Selzner et al., 2003).

Moreover, disturbed hepatic microcirculation after ischemia is one of the main mechanisms involved in development of I/R injury (Bahde and Spiegel, 2010). The

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Abbreviations: ROS, Reactive oxygen species; **I/R,** ischemia/reperfusion; **AST,** aspartate aminotransferase; **ALT,** alanine aminotransferase; **MDA,** malondialdehyde; **ET-1,** endothelin-1.

endothelium, a primary target of ROS, by releasing vasoconstrictors [endothelin-1 (ET-1)] and vasodilators [nitric oxide (NO)], has a prominent role in liver microcirculation (Pannen et al., 1998). More importantly, the balance between NO and ET-1 pathways affects the vascular reactivity homeostasis and therefore, disturbance of NO and ET-1 pathways associated with I/R injury (Uhlmann et al., 2001).

ET-1, one of the most potent vasoconstrictors discovered so far, is a 21-amino acid peptide; which is produced as a 212-amino acid precursor known as preproET-1. Removing signal peptide converts preproET-1 to proET-1, which is secreted into the cytoplasm. Further cleavage of proET-1 molecule by end-peptidases, generates 38-amino acid big-ET-1 molecule, which circulates in plasma with very low bioactivity and has been used as an index of ET-1 secretion. Several lines of evidence indicate that the active form of this vasoconstrictor, which is produced by the final cleavage of the C-terminal 17-amino acid residues has a crucial role in pathogenesis of I/R injury (Kirkby et al., 2008).

With regard to a reciprocal interaction between NO and ET-1, regulation of NO radical generation affects vascular function (Alonso and Radomski, 2003). NO, a potent vasodilator plays an important role in the regulation of hepatic microcirculation. It is hypothesized that endothelial nitric oxide synthase (eNOS)-derived NO production reduces I/R-induced liver injury through several mechanisms. It can regulate sinusoidal diameter, inhibit platelet aggregation and scavenge reactive oxygen species. Moreover, it can neutralize deleterious effect of ET-1 during earlier stages of reperfusion (Peralta et al., 2001; Rockey, 2003). Under normal condition, eNOS-derived NO production is under a fine regulation and hepatic perfusion is regulated by low amounts of NO. Accordingly, disturbed NO production as well as *eNOS* gene expression have a deleterious effect on liver function and eventually lead to I/R liver injury (McCuskey, 2000; Mittal et al., 1994).

Melatonin, the main secretory hormone of the pineal gland, was found to be involved in many important physiological pathways. As it is well known, melatonin, a potent antioxidant, can counteract harmful effects of free radicals and reactive oxygen intermediates. More importantly, melatonin has been reported to have a protective role in cells against oxidative stress-induced injury (Tan et al., 2002). Studies have shown that melatonin protects liver, kidney and intestine against I/R injury (Li et al., 2009; Liang et al., 2009; Pasbakhsh et al., 2008). The aim of this study was to investigate whether melatonin administration would protect against I/R injury in rat liver. Accordingly, we examined the effect of melatonin administration on vascular function through ET-1 and *eNOS* gene expression and big-ET-1 as an index for ET-1 production *in vivo* model of liver injury. Also, we investigated melatonin effect on severity of liver injury via measurements of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and malondialdehyde (MDA) levels.

MATERIALS AND METHODS

Animals

32 male Wistar rats, weighing 270 to 310 g, were provided by animal laboratory of Tehran University of Medical Sciences. The animals were housed under standard laboratory conditions (12 h light-dark cycles for seven days) with 55±5% humidity and 23±1°C temperature. All animal procedures were approved by the Institutional Animal Ethics Committee.

Hepatic ischemia and melatonin administration

After overnight fasting, rats were anesthetized with intraperitoneally administered Ketamine (Alfasan,Woerden, Holland), 7.5 mg/100 g and Xylazine (Alfasan, Woerden, Holland) 0.1 mg/100 g. Rats were divided into four groups consisting of eight rats each: (1) saline (solution of 10% ethanol in saline)-administered, control group (Control), (2) melatonin-administered group (MEL), (3) saline (solution of 10% ethanol in saline)-administered I/R group (I/R) and (4) melatonin-administered I/R group (MEL+ I/R).

Through a midline laparotomy, hepatic artery and portal vein were made visible in each rat. To create complete ischemia of the left and middle hepatic lobes, left branches of hepatic artery and portal vein were occluded by a traumatic vascular clamp. Reperfusion was permitted after 90 min by removing the clamp. The abdomen of Control and MEL groups was closed without clamp application (Baykara et al., 2009).

Twenty minutes before the clamp application and immediately after the reperfusion, melatonin (550-071-G001, Alexis) was dissolved in a solution of 10% ethanol in saline and was injected twice intraperitoneally at a dose of 15 mg/kg to MEL and MEL+ **I/R** groups. Solution of 10% ethanol in saline was administrated intraperitoneally to Control and I/R groups. After 6 h of reperfusion, serum and tissue samples were separated and stored at -70°C for biochemical investigations.

Serum aminotransferases measurements

In order to assess the severity of hepatic injury, ALT and AST levels were measured using an automated chemical analyzer (BT-3000-plus, Biotechnica, Italy) in the central laboratory of Tehran University of Medical Sciences. The values were expressed in units of activity per liter (U/L).

Malondialdehyde measurements

MDA levels, an indicator of lipid peroxidation, were measured in the homogenates of liver tissues using commercially available enzyme-linked immune-sorbent assay (ELISA) kits (OxiSelect MDA Adduct; Cell Biolabs, San Diego, CA) according to the manufacture's protocol.

Big ET-1 measurement in the liver tissue

Big ET-1 peptide concentration in the liver tissue homogenates was determined by an enzyme immune-assay method using rat big ET-1 assay kit (IBL, Hamburg, Germany) according to the manufacturer's instructions.

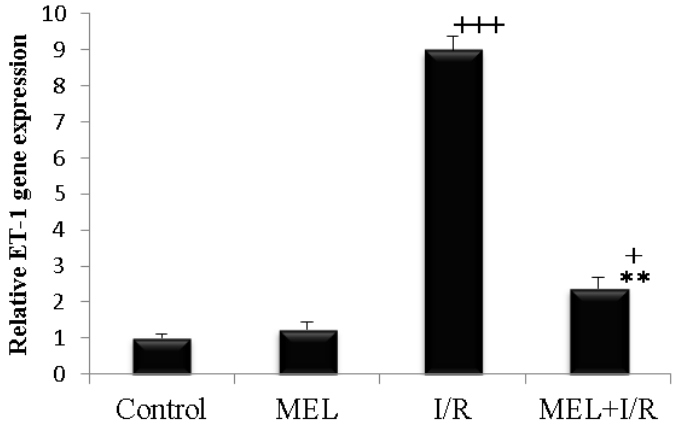

Figure 1. Elevated level of *ET-1* gene expression after hepatic I/R was diminished by melatonin. Results are expressed as a mean ± S.E.M. of 8 rats per group. $^{+,\ +++}$Significantly different from the control group (P<0.05, P<0.001). **Significantly different from the IR group (P<0.01). Control, Saline-administered control group; MEL, melatonin-administered group; I/R, saline-administered I/R group; MEL+IR, melatonin-administered I/R group.

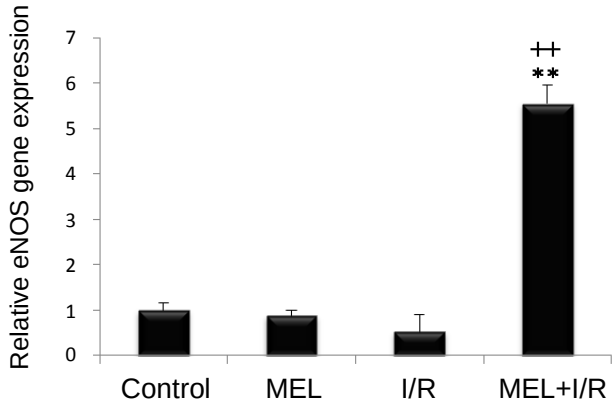

Figure 2. Elevated level of *eNOS* gene expression during I/R in MEL+ I/R group by melatonin although, a non-significant decrease occurred in I/R group. Results are expressed as a mean ± S.E.M. of 8 rats per group. $^{++}$Significantly different from the control group (P<0.01). **Significantly different from the I/R group (P<0.01). Control, Saline-administered control group; MEL, melatonin-administered group; I/R, saline-administered I/R group; MEL+ I/R, melatonin-administered I/R group.

RNA isolation and purification

Total cellular RNA isolation from liver tissues was carried out using RNeasy plus mini kit (Qiagen, USA) according to the manufacturer's protocol. The concentration and purity of the RNA were determined on a UV spectrophotometer (Thermo Scientific, USA) by measuring the absorbance at 260/280 nm absorbance ratio. Agarose gel electrophoresis (1%) with ethidium bromide staining was used to examine the integrity of the RNA.

Quantitative real-time polymerase chain reaction (PCR)

To determine gene expression cDNA was generated. 1 µg of total RNA was reverse transcribed using QuantiTect reverse transcriptase kit (Qiagen, USA). The expression of candidate genes mRNA was quantified by quantitative real-time PCR (RT-PCR). Approximately 50 ng of the resulting cDNA was subjected to real time PCR using SYBR Green PCR Master Mix Reagent (Qiagen, USA), and 10 µM of each primer in a total volume 20 µl. These reactions were performed on a Step-One-PlusTM real-time (Rotor-Gene® Q). The cycling conditions were optimized as activation step of 10 min at 95°C, 45 cycles of amplification at 95°C for 15 s and 60°C for 30 s. The dissociation curve was analyzed to ensure specific product amplification and to eliminate contamination from primer dimer. All assay efficiencies were monitored using a standard curve. The resulting threshold cycles (CTs) were normalized to the β-actin housekeeper gene. All samples were analyzed independently at least twice for each gene. The housekeeping gene β-actin served as an internal control for standardization. The oligonucleotide primers for ET-1, eNOS and β- actin used in this study were purchased from Qiagen (Qiagen, USA).

Statistics

Data were analyzed using SPSS software, version 11.5. All results are presented as means ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post test. Real time PCR data was analyzed by Rest 2009 software. In all tests, the level of significance was defined as less than 0.05 (p<0.05).

RESULTS

ET-1 and *eNOS* genes expression

The quantitative analyses of *ET-1* and *eNOS* genes expression in each group are illustrated in Figures 1 and 2, respectively. While comparing Control and MEL groups, ET-1 mRNA expression in the I/R group was found to be significantly higher (P<0.001). Although there was no difference between MEL and Control groups, treatment with melatonin significantly (P<0.05) decreased ET-1 mRNA expression in MEL+ I/R group.

Although, a non-significant decreased eNOS mRNA expression was found in the I/R group in comparison to any of two other groups (MEL and Control groups) (P>0.05), melatonin administration significantly increased eNOS mRNA expression up to about five times in MEL+ I/R group (P<0.01).

Big ET-1

As shown in Figure 3, untreated I/R rats had the highest big ET-1 concentration. Melatonin administration significantly reduced (P<0.01) hepatic big ET-1 level from 4.8 ± 0.40 pg/mL in I/R group to 1.99 ± 0.16 pg/mL in MEL+IR group. However, no significant difference was found between the MEL and Control groups (P>0.05).

Lipid peroxidation (MDA)

Similarly, MDA level was significantly higher in the I/R

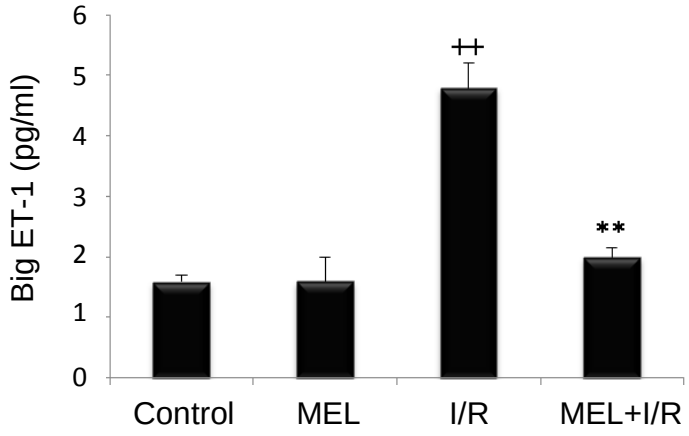

Figure 3. Decreased level of liver tissue big ET-1 after hepatic I/R by melatonin. Results are expressed as a mean ± S.E.M. of 8 rats per group. $^{++}$Significantly different from the control group (P<0.01). **Significantly different from the IR group (P<0.01). Control, Saline-administered control group; MEL, melatonin-administered group; I/R, saline-administered I/R group; MEL+ I/R, melatonin-administered I/R group.

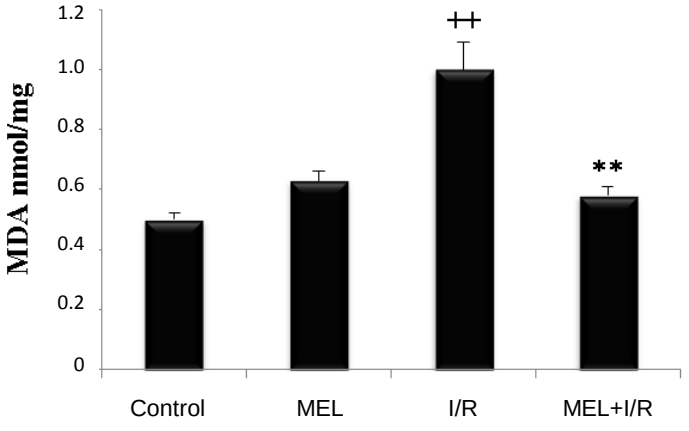

Figure 4. Attenuated MDA level in liver homogenate during liver I/R by melatonin. Results are expressed as a mean ± S.E.M. of 8 rats per group. $^{++}$Significantly different from the control group (P<0.01). **Significantly different from the I/R group (P<0.01). Control, Saline-administered control group; MEL, melatonin-administered group; I/R, saline-administered I/R group; MEL+ I/R, melatonin-administered I/R group.

rat hepatocytes compared to Control group (1.0 ± 0.09 nmol/mg protein, in I/R versus 0.5 ± 0.02 nmol/mg protein, in Control) (P<0.01). After melatonin administration, MDA level was significantly (P<0.01) decreased by nearly half in MEL+ I/R group (0.58 ± 0.03 nmol/mg protein) compared with I/R group (Figure 4).

Liver injury

As shown in Figure 5, serum ALT and AST levels in

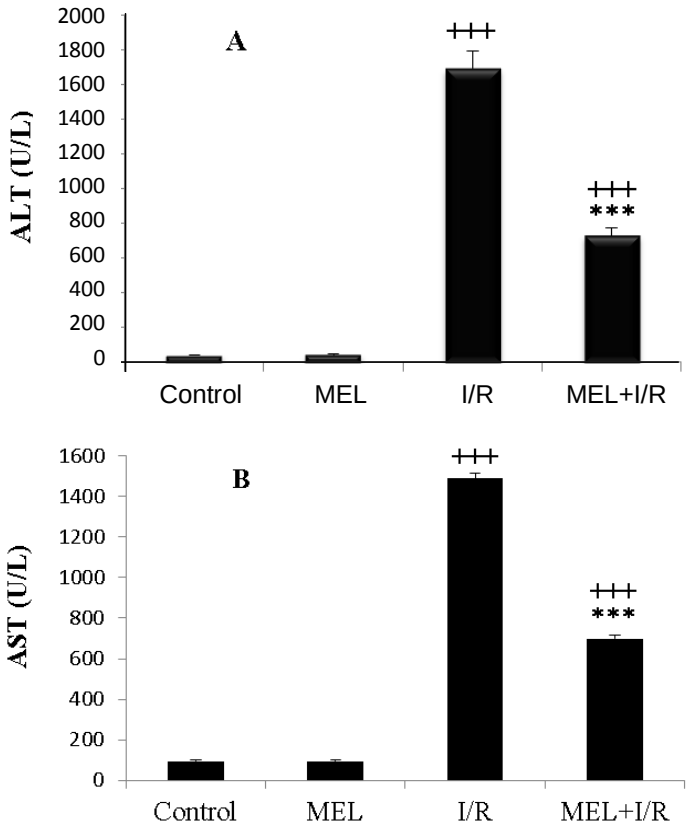

Figure 5. Effect of melatonin on the serum ALT (A) and AST (B) activity after hepatic I/R. Results are expressed as mean ± S.E.M. of 8 rats per group. $^{+++}$Significantly different from the control group (P<0.001). ***Significantly different from the I/R group (P<0.001). Control, Saline-administered control group; MEL, melatonin-administered group; I/R, saline-administered I/R group; MEL+I I/R, melatonin-administered I/R group.

Control group were 41.8 ± 1.7 and 94.7 ± 7.1 U/L. These amino transferases levels increased dramatically (P<0.001) in I/R group to 1693.2 ± 82.6 and 1489.3 ± 26.7 U/L, respectively. ALT and AST levels significantly decreased after melatonin administration in MEL+ I/R group (P<0.001).

DISCUSSION

It is now clearly established that oxidative stress, defined as excess production of ROS or imbalance between ROS and antioxidants, could be an early event in the pathogenic mechanism of I/R injury. The production of ROS by ischemia-stimulated Kupffer cells is an important mechanism for liver reperfusion injury (Arai et al., 2007; Jaeschke, 2003). It has been shown that ROS-induced lipid peroxidation perturbs bio-membrane. They seem to disturb fine organizations, modification of integrity and permeability of cell membranes (West and Marnett, 2006). Moreover, lipid peroxidation is associated with carcinogenic and mutagenic effects as well as expansion

of liver I/R damage (Atalla et al., 1985; Jaeschke, 2003; West and Marnett, 2006).

This study shows that ischemia followed by reperfusion significantly enhanced MDA level as a major lipid peroxidation product. As anticipated, aminotransferases levels similarly increase in IR rat hepatocytes. This appears to be due to reactivity of MDA with the free amino groups of proteins and phospholipids (Dalle-Donne et al., 2006; Esterbauer et al., 1991). These structural modifications can lead to hepatocyte damage and increment of ALT and AST levels during hepatic I/R injury and lead to liver dysfunctions.

Furthermore, in line with previous reports (Li et al., 2008; Sener et al., 2003), data collected demonstrates that melatonin, significantly decreases ALT, AST and MDA levels after reperfusion. One possible explanation for this observation is that melatonin serves not only as an inducer of antioxidative enzymatic systems but also as a direct scavenger of ROS, in turn leading to decreased oxidative stress-induced hepatic I/R injury (Tan et al., 2002).

Previous reports have shown that melatonin had a protective effect on reducing I/R injury. Kurcer et al. (2007) determined that melatonin reduced the MDA level in rats with methanol-induced liver injury. Kaçmaz et al. (2005) showed that melatonin administration reduced the MDA, ALT and AST in rats with I/R-induced oxidative organ damage. Also, ROS can trigger endothelium injury by disturbing interplay between vasodilators and vasoconstrictors (Pannen et al., 1998). For that reason, any abnormalities in microvasculature have been shown to contribute significantly to I/R injury. On other hand, disturbed fine balance between endothelin and NO production was found to be associated with micro-circulatory impairment during reperfusion and in turn lead to liver injury induced by hepatic ischemia-reperfusion (Kawada et al., 1993; Vollmer et al., 1994). In present study, as expected, rat liver injury was associated with enhancement of *ET-1* gene expression as well as hepatic big ET-1 level. Moreover, our results indicate that melatonin significantly lowers hepatic *ET-1* gene expression and big ET-1 level in the MEL+ I/R group.

Goto et al. (1994) demonstrated that the ET-1 concentration increased during reperfusion period in rats with I/R injury and diminution of hepatic microcirculatory disturbance as well as serum ALT levels was observed after administration of ET-1 antiserum. It has been hypothesized that ET-1 can trigger sinusoidal constriction, through contraction of hepatic stellate cells. Also, diminution in ET-1 can reduce hepatic I/R injury (Zhang et al., 1994).

Scommotau et al showed that treatment of *in vivo* liver injury model with endothelin receptor antagonist (ERA) and L-arginine as NO donor restored the delicate ET/NO balance. Also, blocking of ET receptor as well as providing a NO donor improved microcirculation, and delivery of blood and oxygen to the liver and in turn lead to reduce hepatic I/R injury (Scommotau et al., 1999).

Although, the precise pathways underlying the augmentation of endothelin production following I/R are not fully understood, it has been proposed that pro-inflammatory mediators as well as elevated level of ROS can provoke endothelin release from activated Kupffer and stellate cells (Ferrari, 1994; Lopez-Ongil et al., 1998). In endothelial cells, ET-1 production can be reduced by NO, thus its generation can be augmented by inhibitors of NOS (Boulanger and Lüscher, 1991; Mitsutomi et al., 1999). A decrease in ET-1 concentration and an elevation of NO can lessen the intensity of I/R injury (Scommotau et al., 1999).

Insufficient NO production is a main cause for vaso-constriction during reperfusion after hepatic ischemia. Furthermore, eNOS inhibition diminishes liver microcir-culation and enhances I/R injury (Harbrecht et al., 1995; Nishida et al., 1994; Wang et al., 1995).

Administration of L-arginine attenuates reperfusion injury, via inducing NO production (Shiraishi et al., 1997). In endothelial cells expression of eNOS is inhibited by increased ROS levels (Hansson, 2005). In sinusoidal endothelial cells, eNOS-derived NO can oppose stellate cell activation and vasoconstriction and can reduce failure of liver during I/R (Clemens, 1999). Investigation using genetically eNOS deficient mice demonstrated the critical role of eNOS in decreasing I/R injury (Kawachi et al., 2000). Melatonin can increase the eNOS mRNA and protein during inflammation, therefore it can protect human umbilical vein endothelial cells against lipopolysaccharide stimulated damage (Dayoub et al., 2011). Koh (2008) examined the effect of melatonin on the levels of NOS isoforms in an ischemic brain injury model. They found that melatonin prevented decrease of eNOS following ischemic brain injury. Thus melatonin has neuroprotective effects. Genetic overexpression of eNOS reduces hepatic I/R injury in comparison to control mice (Duranski et al., 2006).

Although, Park et al. (2007) showed a significant increase of eNOS mRNA during I/R, we find a non-signi-ficant decrease in the gene expression probably owing to use a different genre of rats. Based on our results, melatonin can counter the non-significant decrease and significantly increased eNOS mRNA expression in MEL+ I/R group. Although, the possible mechanisms for this elevation are complicated, we believe that the reduction of ROS and tumor necrosis factor (TNF)-α by melatonin is the main contributors. Yoshizumi et al demonstrated that TNF-α extremely reduced the half-life of eNOS mRNA (Yoshizumi et al., 1993). In addition, melatonin protects endothelial cells, the only eNOS producing cells, against direct destroying effect of ROS (Tan et al., 2002). eNOS expression in MEL group showed no difference compared to Control group. One possible explanation is that in physiological situations in which ET-1 and NO is at a fine balance and microcirculation is normal, there are not stimulant mechanisms responsible for melatonin

inducing action on eNOS expression. It seems reasonable to assume that melatonin causes little vaso-constriction (Savaskan et al., 2001), thereby regulating whole body oxygen consumption in physiological condition (melatonin might hinder augmentation of oxygen consumption resulting from vasodilation).

Zhang et al. (2006) showed that melatonin ameliorates the impaired expression of ET-1 and *eNOS* gene expression during hepatic I/R. Thus I/R cause ROS overproduction, induces ET-1 and inhibits eNOS mRNA expression. All of these changes trigger microvascular dysfunction and subsequent I/R injury.

Conclusion

I/R stimulates production of ROS, which attacks and damages directly the endothelial and hepatic cells and also they provoke an imbalance between ET-1 and NO, which disturbs microcirculation in hepatic sinusoids. Melatonin has hepatocellular protective effects against warm I/R injury probably through mechanisms including direct scavenging of ROS and reducing lipid peroxidation. In addition, melatonin improves hepatic microcirculation through inhibition of the ET-1 and induction of the *eNOS* gene expression.

ACKNOWLEDGEMENT

The authors would like to thank Tehran University of Medical Sciences and Health Services for financial support of this study.

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