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The protective effects of carnosine and melatonin in ischemia-reperfusion injury in the rat liver

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Summary

The reperfusion following liver ischemia results in hepatocyte damage and apoptosis. The aim of this study was to investigate the effects of two antioxidant agents, carnosine and melatonin, in rat liver ischemia-reperfusion injury. Five study groups were formed; I. sham, II. ischemia-reperfusion, III. ischemia-reperfusion+melatonin, IV. ischemia-reperfusion+carnosine, V. ischemia-reperfusion+melatonin+carnosine. Then 250 mg/kg carnosine and 10 mg/kg melatonin were administered intraperitoneally 30 min before ischemia and immediately after the reperfusion. Sinusoidal dilatation, congestion and neutrophil infiltration were observed in the ischemia-reperfusion group while these symptoms were less pronounced in the treatment groups. Alanine aminotransferase, aspartate aminotransferase and myeloperoxidase levels were increased in the ischemia-reperfusion group while they were lowered in the treatment groups. Glutathione level was low in the ischemia-reperfusion group while it tended to increase in the ischemia-reperfusion+carnosine administered and ischemia-reperfusion+carnosine+melatonin administered groups. There was an increase in the number of apoptotic cells in the ischemia-reperfusion group while this number was lowered in the treatment groups. Carnosine was more effective than melatonin in the reversal of structural and biochemical alterations that resulted from ischemia-reperfusion injury. The administration of melatonin and carnosine together yielded better outcomes compared to the sole administration of each agent.
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Introduction

Ischemia starts a series of biochemical reactions that cause cell dysfunction and may progress to cell death. The restoration of blood flow to the ischemic tissue is requisite to remove toxic metabolites produced during the ischemic period. However, severe metabolic disorders may appear due to the infusion of toxic metabolites and various inflammatory mediators that are formed during ischemia into the systemic circulation and reperfusion may cause further tissue damage (Carden and Granger, 2000). Ischemia-reperfusion damage (IRD) is the cellular damage that appears due to the restoration of oxygen delivery to the hypoxic organ. IRD in the liver was first observed in the liver transplant that was experimentally performed by Toledo-Pereyra et al. (1975). Congestion, progressive thrombosis and graft necrosis leading to organ failure developed in the transplanted liver (Bilzer and Gerbes, 2000). The pathophysiology of liver IRD consists of many mechanisms that cause liver damage. Various cellular and molecular interactions such as Kupffer cell activation, formation of reactive oxygen species (ROS), cytokine and chemokine secretion, vasoconstriction, impairment of nitric oxide and endothelin balance, accumulation of neutrophil leukocytes, alteration in mitochondrial permeability, unbalanced influx of calcium (Ca^{2+}) into the cell and pH paradox may be involved in the process. These complex mechanisms cause cell death, organ dysfunction and eventually organ loss (Kaplowitz, 2000; Jaeschke, 2003; Teoh and Farrell, 2003).

Carnosine (CAR) (β-alanyl-L-histidine), first described in the 1900s, was the first discovered of all neuropeptides (Gulewitsch and Amiradzibi, 1900). Muscles and nerves are diffusely distributed in tissues. Water soluble CAR functions in the cytosol where the oxidation mediators (metals and oxygen radicals) are abundantly located. Due to its biological function of scavenging active oxygen radicals, it has an antioxidant property. It acts as a scavenger of hydroxyl and superoxide radicals and, most effectively, of the singlet oxygen molecule. CAR has been observed to have a protective effect, based on its scavenging effect on hydroxyl and superoxide radicals, in brain, kidney, liver and skeletal muscle (Boldyrev et al., 1999; Fouad et al., 2007; Fujii et al., 2003, 2005; Stvolinsky et al., 1999, 2000; Zalesova and Kuleva, 1998).

Pineal gland is the main source of melatonin (MEL) (*N*-acetyl-5-metoksitriptamin) in the circulation. It is also produced in small amounts in the retina, gastrointestinal system and by leukocytes (Reiter et al., 2007). MEL is tiny (Reiter et al., 2001a, b; Reiter and Tan, 2004) and highly lipophilic, and for this reason, it is found abundantly in all parts of the cell. MEL protects the DNA, lipids and proteins against oxidative damage (Reiter et al., 2000, 2001a, b; Tan et al., 1993). The free radical scavenging and antioxidant effects of MEL have been shown in many studies (Cuzzocrea et al., 2000; Reiter and Maestroni, 1999; Cabeza et al., 2001; Pei et al., 2002).

In the present study, we aimed to compare the protective effects of CAR with MEL against liver IRD through histological (hematoxylin and eosin), immunohistochemical (apostain), electron microscopical and biochemical (ALT, alanine aminotransferase; AST, aspartate aminotransferase; GSH, glutathione; MPO, myeloperoxidase) parameters.

Material and methods

Animals and tissues

Female Wistar albino rats, weighing 200–250 g, were obtained from the Animal Research Laboratory of Dokuz Eylül University Medical School. The animals were synchronized to a 12:12 h light–dark cycle and housed at 20–22 °C before the beginning of the experiment. The animals were fed with a standard pellet diet and tap water *ad libitum*. The experiment protocol was approved by the Ethics Committee of Research on Laboratory Animals of Dokuz Eylül University Medical School.

Five study groups, each containing eight rats were formed: Sham group, ischemia-reperfusion group (IR), ischemia-reperfusion+melatonin group (IR+MEL), ischemia-reperfusion+carnosine group (IR+CAR), ischemia-reperfusion+melatonin+carnosine group (IR+MEL+CAR), respectively. The rats were fasted overnight before the experiment. The anesthesia of the animals was performed by 25 mg/kg intraperitoneal (i.p.) pentobarbital (Kojima et al., 2003) and the rats were subjected to median laparotomy under sterile conditions in the supine position. Firstly, hepatic artery, portal vein and ballast ductus were made visible in each group and then the abdomen of the animals were closed without executing any further procedures in the sham group. In the other groups, the hepatic pedicle was identified and the hepatic artery, portal vein and bile ducts were occluded with an atraumatic vascular clamp for hepatic ischemia, which lasted for 1 h. Following 1 h of ischemia, the clamp was opened and the liver was reperfused for 4 h in the IR, IR+MEL, IR+CAR, IR+MEL+CAR groups.

Thirty minutes before the clamp application and immediately before the reperfusion, 1 mL 0.9% NaCl, 10 mg/kg melatonin (Sigma-Aldrich, USA) or 250 mg/kg carnosine (Fluka-BioChemika, USA) were administered i.p. to the IR, IR+MEL and IR+CAR groups, respectively. 2 mL blood samples were taken from vena cava caudalis before scarification from each group. Three different tissue samples were taken for biochemical, electron microscopic and light microscopic evaluations. The first tissue samples taken for the biochemical tissue enzyme measurements were washed with 0.9% NaCl, packed with aluminum foil and placed into ice. The second tissue samples were placed into 2.5% glutaraldehyde in 0.1 M Sorenson's phosphate buffer (pH 7.2) for electron microscopy procedures. Later on, the rats were perfused with 10% phosphate-buffered formalin (pH 7) and tissue samples were taken for light microscopic evaluation.

Light microscopic examination

Tissue samples were fixed in 10% phosphate-buffered formalin. They were then rinsed under running tap water overnight to remove the fixative. Tissue samples were processed for embedding in paraffin wax by routine protocol. 5 μm thick sections were cut using a Leica/Reichert-Jung rotary microtome (Köln, Germany). After deparaffinization in xylene, the sections were rehydrated through a graded ethanol series, stained with hematoxylin and eosin, dehydrated through a graded ethanol series, cleared in xylene and mounted with entellan (UN 1866, Merck, Darmstadt, Germany). The slides were examined using a light microscope (Olympus BH-2, Tokyo, Japan) and the histopathological appearance of tissues in the different groups were compared.

Assessment of apoptotic cells

Hepatic tissue sections, prepared as described above, were mounted on slides coated with lysine, deparaffinized in xylene, dehydrated in ethanol and washed in phosphate-buffered saline (PBS, 0.1 M, pH = 7.4). Sections were incubated in 0.2 mg/mL saponin (S2149; Sigma-Aldrich, USA) for 20 min and treated with 20 μL/mL proteinase K (P6552; Sigma-Aldrich, USA) for 20 min. Sections were incubated in 50% formamide (F7503; Sigma-Aldrich, USA) in distilled H_2O at 56–60 °C for 20 min and then kept in ice-cold PBS for 5 min. They were then reacted with freshly made 3% hydrogen peroxide for 5 min, incubated with a 3% solution of non-fat milk powder for 20 min, washed with PBS and incubated in 100 μL of a 1:100 dilution of apostain monoclonal antibody F7-26 (Chemicon, USA and Canada) for 1 h at room temperature. A 1:100 dilution of peroxidase-conjugated anti-mouse IgM (Chemicon, USA and Canada) was applied for 30 min. After rinsing with PBS, sections were reacted with diaminobenzidine (DAB)/hydrogen peroxide (Zymed 00-2020, San Francisco, CA, USA), prepared according to manufacturer's instructions. Sections were counterstained with hematoxylin and mounted in entellan mounting medium (UN 1866, Merck, Darmstadt, Germany). All solutions were prepared in PBS and thorough washes between stages were performed using distilled water, unless otherwise specified. All steps were performed at room temperature unless otherwise specified.

The numbers of labeled apoptotic cells were counted in liver sections of all groups. The images were analyzed by using a computer-assisted image analyzer system consisting of a microscope (Olympus BH-2, Tokyo, Japan) equipped with a high-resolution video camera (JVC TK-890E, Japan). For quantitative analysis of apoptotic cells, approximately 1000 cells were counted in each liver section in 10 random microscopic fields viewed using a 40 × objective.

Electron microscopic examination

Samples of rat liver tissues that had been fixed by immersion in 2.5% glutaraldehyde in 0.1 M Sorenson's phosphate buffer (pH 7.2) at room temperature for 4 h were post-fixed in 1% phosphate-buffered osmium tetroxide for 2 h. The tissues were placed in a 1:1 mixture of propylene oxide: araldite and incubated at room temperature for 1 h. Following this process, they were incubated with fresh araldite at room temperature for 6–12 h, then again with fresh araldite and incubated for a further 2 h. The tissue was placed in capsules with araldite and incubated at 60 °C for 2 h. Semi-thin sections of 1 μm thick were cut using an Ultracut E ultramicrotome (Leica) and were stained with toluidine blue. Ultra-thin sections were obtained from selected blocks, mounted on copper grids, stained with uranyl acetate and lead citrate and examined using a Carl Zeiss Libra 120 transmission electron microscope.

Biochemical investigation

ALT and AST

All the blood samples were centrifuged and serum samples were separated. The ALT and AST

levels of the obtained serum samples were measured by an autoanalyzer in the Central Laboratory of Dokuz Eylül University.

Glutathione

Glutathione was measured according to the method of Schroeter et al. (1999). Briefly, after being washed with cold (+4 °C) isotonic 0.9% NaCl, the liver tissue samples were air dried. 100 mg of liver tissue was then homogenized in 1% picric acid. Absorbance was determined at 412 nm and the glutathione value was determined (nmol/mL) according to a calibration curve. Then, the concentration of glutathione (μmol) was calculated per gram tissue.

Myeloperoxidase

Myeloperoxidase was assessed according to the method of Krawisz et al. (1984). Briefly, 200–400 mg tissue samples were homogenized in 0.05 M potassium phosphate (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HETAB). The homogenates were then sonicated on ice for 10 s. Following sonication, homogenates were frozen and thawed three times and then centrifuged for 30 min at 12.500 g at 4 °C. The supernatants were used for MPO activity assay. The MPO activity was assessed by measuring the H_2O_2-dependent oxidation of *O*-dianisidine using a Cary 50 UV/vis spectrophotometer (Tokyo, Japan) at 460 nm. The results were expressed as U/g wet tissue weight.

Statistics

Results are presented as ±S.E.M. All data were analyzed by one-way analysis of variance (ANOVA) post hoc Bonferroni test. $p<0.05$ was considered statistically significant.

Results

Light microscopic examination

In the IR group, hepatocyte necrosis areas randomly disseminated in the liver parenchyma with disintegrated cell cordons, neuthrophil infiltration, expansion of sinusoids and congestion were detected. Hepatocyte damage was not observed in the liver parenchyma of the IR+CAR group except for isolated necrotic hepatocytes. Sinusoid expansion was much decreased compared to the IR group. It was observed that parenchymal integrity was maintained in the IR+MEL group. Expansion of sinusoids and cell infiltration was observed in this group compared to the IR group. Additionally, necrotic hepatocytes were rarely observed. The liver tissue of the IR+MEL+CAR group had many similarities with the sham group. Hepatocyte cordons were properly arranged, the sinusoids were of normal diameters, and congestion and neuthrophil infiltration were not observed. These observations are illustrated in Figure 1.

Electron microscopic examination

The sham rat hepatocytes had a normal chromatin structure with round nuclei. The quantity and the structure of the mitochondria in the cytoplasm were normal. Ribosomal endoplasmic reticulum (RER) cisternae had a normal appearance. Spaces of Disse and bile canaliculi appeared normal. The IR

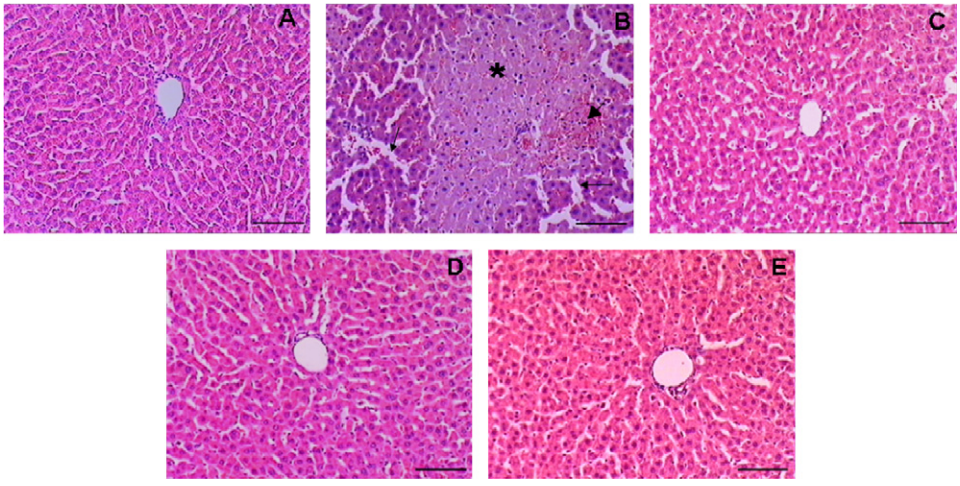


Figure 1. The effect of carnosine and melatonin treatment in the liver of rats (hematoxylin and eosin stain). Representative photomicrographs of hepatocyte necrosis (*), congestion (◀), expansion of sinusoid (→): (A) sham, (B) IR untreated, (C) IR+MEL-treated, (D) IR+CAR treated, (E) IR+MEL+CAR treated groups (scale bar = 100 μm).

group showed mitochondrial swelling, vacuolization and dilated ductuli biliferi. Some hepatocytes had heterochromatic and apoptotic nuclei. The IR+CAR group hepatocytes showed normal architecture but a few cells had lipid droplets. IR+MEL group mitochondria and RER had normal structure and appeared in normal quantity. The nucleus had a normal appearance. In the IR+MEL+CAR group, all the cellular structures in hepatocytes were similar to those seen in the sham group. The numbers and the structure of the mitochondria were normal and RER cisternae had a normal appearance. Bile canaliculi and space of Disse appeared normal. The nucleus and chromatin had normal structures. These observations are illustrated in Figure 2.

Assessment of apoptotic cells

A significant difference in numbers of apoptotic cells was determined between the IR and sham group ($p = 0.01$). Although there was no difference between MEL and CAR groups, in the CAR+MEL group the apoptotic cell count was decreased statistically compared to the MEL group. These results are illustrated in Figures 3 and 4.

Biochemical investigation

ALT and AST

When comparing sham and IR groups, the AST level in the IR group was found to be statistically higher ($p = 0.011$). It was determined that the AST levels were significantly lowered in all the treatment groups. However, a significant difference was not found between these groups. When comparing sham and IR groups, the ALT level in the IR group was higher ($p = 0.011$). ALT levels were significantly lowered in all the treatment groups. CAR was more effective compared to MEL ($p = 0.008$). MEL+CAR administration was the most effective intervention ($p = 0.002$). These finding are illustrated in Figure 5 and summarized in Table 1.

Glutathione reductase and myeloperoxidase

The GSH level was found to be statistically lower in the IR group compared to the sham group ($p = 0.006$). The GSH levels in the CAR and CAR+MEL groups were statistically higher ($p = 0.002$, $p = 0.002$, respectively). However, there was no statistical difference between the MEL group and IR group ($p > 0.05$). There was a statistically significant increase in the MPO levels of the IR group compared to the sham group ($p = 0.08$) and a statistically significant decrease in MPO levels in the CAR and CAR+MEL groups ($p = 0.024$, $p = 0.015$, respectively). A statistically significant decrease was not observed in the MEL group compared to the IR group ($p > 0.05$). These results are summarized in Table 2.

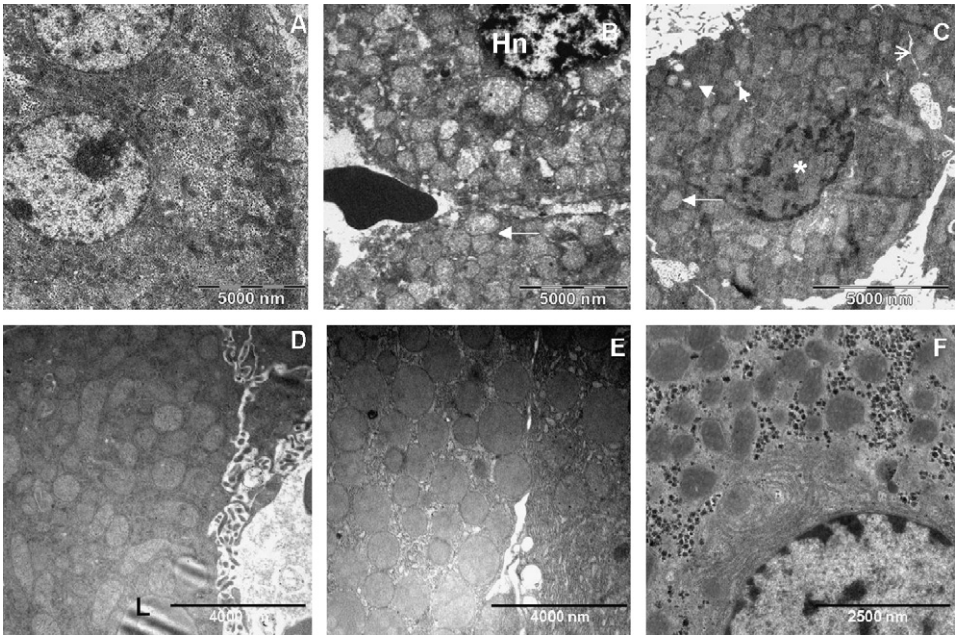


Figure 2. Electron micrographs of hepatocytes of rats in all groups. Apoptotic nuclei (*), heterochromatic nuclei (Hn), mitochondrial swelling (⟵), mitochondrial vacuolization (⇠), dilated ductuli biliferi (⇾), lipid droplet (L): (A) sham, (B–C) IR untreated, (D) IR+CAR treated, (E) IR+MEL treated, (F) IR+MEL+CAR treated groups.

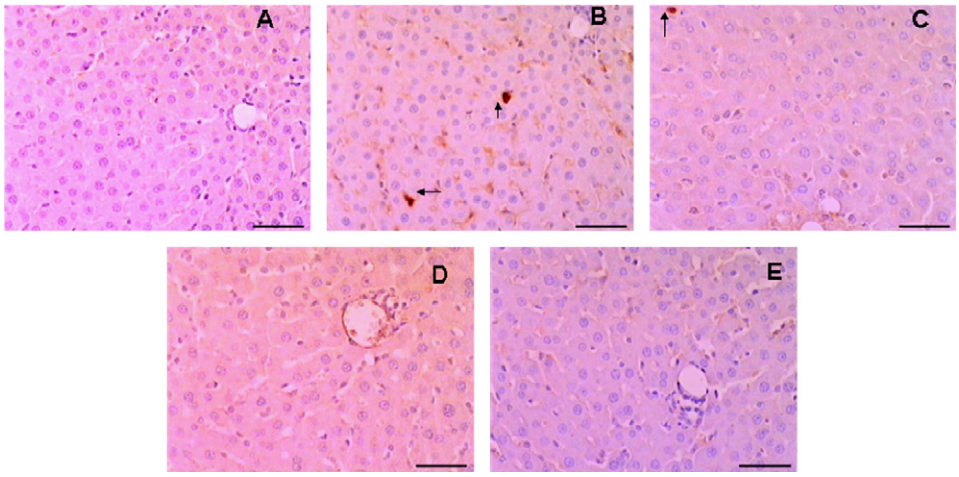


Figure 3. The effect of carnosine and melatonin treatment on apoptosis in the liver of rats. Representative photomicrographs of apostain-positive cells (arrows): (A) sham, (B) IR, (C) IR+MEL treated, (D) IR+CAR treated, (E) IR+MEL+CAR treated groups (scale bar = 50 μm).

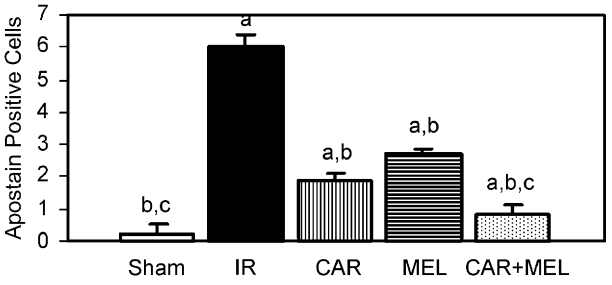


Figure 4. Quantitative analysis of apostain-positive cells in the rat livers. Data are mean ± S.E.M. $^{a}p<0.05$ compared with the sham group; $^{b}p<0.05$ compared with the IR untreated group; $^{c}p<0.05$ compared with the IR+MEL.

Discussion

Liver pathophysiology consists of many mechanisms that have an impact on liver damage at different levels. These mechanisms include cellular and molecular interactions, Kupffer cell activation, ROS formation, cytokine and chemokine secretion, vasoconstriction, nitric oxide, imbalance between endothelin and nitric oxide, accumulation of neutrophil leukocytes, alteration in the mitochondrial permeability, balanced calcium influx into the cell and pH paradox. These complex mechanisms result in cell death, organ dysfunction and eventually organ loss (Kaplowitz, 2000).

In the study reported here, light microscopic investigations revealed congestion, sinusoidal and lymphatic expansion, regional hepatocellular vacuolization and hepatocyte swelling. Ultrastructural investigations revealed impairment of mitochondrial structure, swelling, staining differences and neutrophil accumulation. The biochemical investigations on liver homogenates showed that myeloperoxidase (MPO) activity (an indirect indicator of neutrophil infiltration) was increased and glutathione (GSH) level was decreased (Sener et al., 2003; Yuan et al., 2005). Crockett et al. (2006) observed sinusoidal congestion, cytoplasmic vacuolization, hepatocellular necrosis and neuthrophil infiltration in IR-applied liver tissues and determined high ALT levels. Tuncer et al. (2007) reported polymorphonuclear cell infiltration, hepatocyte necrosis and sinusoidal expansion in an IR-applied group along with an increase in ALT and AST levels. Diffuse hepatocyte necrosis, hemorrhagia, neuthrophil infiltration, an increase in the number of apoptotic cells and ALT and AST levels in IR-applied liver tissue were reported by Peralta et al. (2001). Similarly, in our study, inflammation, sinusoidal expansion, congestion and diffuse hepatocyte necrosis areas were seen. Space of Disse and RER cisternae were significantly expanded. Mitochondrial swelling and increased mitochondrial number was observed on ultrastructural investigation. Serum AST and ALT and liver tissue MPO levels were increased, while the GSH level was lowered.

Due to its water solubility, CAR functions in the cytosol where oxidation mediators that dissolve in the water (metals and oxygen radicals) are high. It has an antioxidant property in connection with its function of scavenging active oxygen radicals. It is the scavenger of hydroxyl and superoxide radicals and, predominantly, the singlet oxygen molecule (Stvolinsky et al., 1999; Guiotto et al., 2005; Mozdzan et al., 2005). Its antioxidant effect has been tested in many experimental models. Fujii et al. (2003) determined in their kidney IR model that the administration of CAR reduces IRD in the

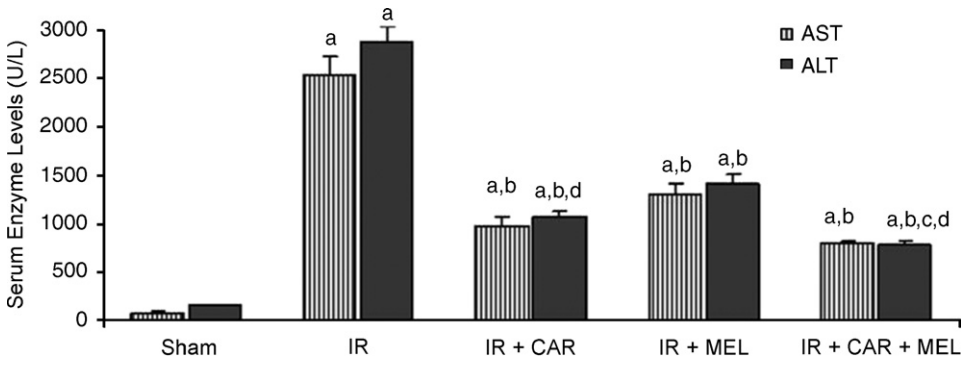


Figure 5. Change in serum enzyme levels following ischemia-reperfusion (IR) injury in rats untreated and treated with CAR and MEL. Data are mean ± S.E.M. [a]$p<0.05$ compared with the sham group; [b]$p<0.05$ compared with the IR untreated group; [c]$p<0.05$ compared with the IR+CAR; [d]$p<0.05$ compared with the IR+MEL.

Table 1. Changes in serum alanin amimotransaminase (ALT) and aspartate amimotransaminase (AST) levels following ischemia-reperfusion (IR) in rats treated with IR+CAR, IR+MEL and IR+CAR+MEL

Groups	Measured parameters	
	ALT (U/L)	AST (U/L)
Sham ($n=8$)	148.25 ± 6.35	81.5 ± 9.56
IR ($n=8$)	2887 ± 260.73*	2542.66 ± 183.12*
IR+CAR ($n=8$)	1067.57 ± 60.36*,†,§	967.42 ± 111.16*,†
IR+MEL ($n=8$)	1413.16 ± 88.64*,†	1290.16 ± 116.91*,†
IR+CAR+MEL ($n=8$)	788.85 ± 31.04*,†,‡,§	802.28 ± 26.14*,†

Data are mean ± S.E.M.
*$p<0.05$ compared with the sham group.
†$p<0.05$ compared with the IR untreated group.
‡$p<0.05$ compared with the IR+CAR.
§$p<0.05$ compared with the IR+MEL.

Table 2. Changes in liver biochemical parameters following ischemia-reperfusion (IR) in rats treated with IR+ CAR, IR+MEL and IR+CAR+MEL

Groups	Measured parameters	
	Myeloperoksidase (U/g)	Glutathione (μmol/g)
Sham ($n=8$)	0.99 ± 0.7	5.65 ± 0.48
IR ($n=8$)	1.8 ± 0.21*	3.98 ± 0.81*
IR+CAR ($n=8$)	1.16 ± 0.13†	4.99 ± 0.3†,‡
IR+MEL ($n=8$)	1.34 ± 0.2	3.23 ± 0.16
IR+CAR+MEL ($n=8$)	1.08 ± 0.16†	5.28 ± 0.17†,‡

Data are expressed as mean ± S.E.M.
*$p<0.05$ compared with the sham group.
†$p<0.05$ compared with the IR untreated group.
‡$p<0.05$ compared with the IR+MEL.

kidney. Stvolinsky et al. (1999) showed that CAR has a neuroprotective effect in brain ischemia induced animals, and in another study the same authors showed that CAR prolongs the life duration in global ischemia applied animals (Stvolinsky et al., 2000). Boldyrev et al. (1999) reported that CAR protected cerebellar granular cells against free radical damage. The same researchers also showed that CAR protected the neuronal cells against ROS (Boldyrev et al., 2004). Hipkiss (1998) reported that CAR protected rat brain endothelium cells against MDA toxicity. Lee et al. (1999) showed that CAR has a protective effect against isolated rat heart reperfusion damage. Kang et al. (2002) showed that CAR prevented the DNA fragmentation that 12-*O*-tetradecanoylphorbol-13-acetate and hydrogen peroxide cause in epithelial cells of the liver in a culture environment. They reported that this prevention is associated with the inhibition of lipid peroxidation by CAR and its superoxide dismutase and/or free radical scavenger feature. Fouad et al. (2007) reported that, in a partial hepatic ischemia-reperfusion model, pre-ischemic administration of CAR alleviated hepatic damage and ALT, AST, MDA levels were decreased while glutathione peroxidase level was increased in IR. Light microscopically, degeneration, cytoplasmic vacuolization of the hepatocytes, focal necrosis and fibrosis around the portal space were observed, while these changes resolved in the CAR-administered group. Ultrastructural examination revealed chromatin condensation, margination and fragmentation, dilation of the mitochondrial cristae and crystalysis in the IR group while in the CAR-administered group normal structure was retained. Similarly, in the study reported here, we observed that the pathological changes found in the IR group were significantly reduced with carnosine. With the exception of isolated necrotic hepatocytes, we did not observe hepatocyte damage in the liver

parenchyma. Widenening of the sinusoids was significantly less than the IR group and ultrastructural examination was normal, other than identification of a few cells with lipid droplets. Biochemical tests showed that AST, ALT and MPO levels were decreased, while GSH level was elevated.

MEL, directly infused into the systemic circulation reaches the target cells via membrane receptors, in spite of its lipophilic nature, subsequent to its synthesis. Thus, MEL is able to protect the cell membrane, organelles and core against free radical damage (Reiter et al., 2000, 2001a, b). Reiter and Maestroni (1999) determined that MEL has an immunomodulatory and antioxidant effect in the organism. Sewerynek et al. (1996) showed that MEL reduced polymorphonuclear leukocytes and lipid peroxidation products against the oxidative damage in the liver IR and caused an increase in glutathione levels. Kaçmaz et al. (2005) reported that the gluthiathone level that was decreased due to liver IR was increased by melatonin and MEL also decreased the MPO level and caused a significant decrease in ALT, AST and MDA levels. However, in the study conducted by Okatani et al. (2003), liver gluthathione peroxidase activity remained unchanged during ischemia, while it decreased significantly during reperfusion. These authors showed that the administration of MEL did not have an effect on this decrease. As they investigated the mitochondria damage in the liver IR intrastructurally, they observed disintegration and swelling in cristae. However, they showed a decline of symptoms in the MEL-administered animals. Similarly, the administration of MEL did not cause an increase in the GSH level in the study reported here. However, the administration of MEL together with CAR caused a statistically significant increase in this level. Compared to IR, the MPO level was found to be decreased; but this decrease was not statistically significant. On the other hand, ALT and AST levels caused a statistically significant decrease in the MEL-administered group. In their studies on total hepatic ischemia applied animals, Sener et al. (2003) reported that the hepatocytes were markedly swelled and showed vacuolization, while congestion was present in the enlarged sinusoids. They observed well-protected liver parenchyma and hepatocytes and congestion was not present. Okatani et al. (2003) examined the mitochondrial damage in liver during IR ultrastructurally and showed disintegration and swelling of the cristae. They found that administration of MEL reduced these changes. In the present study, similar histological findings were noted in the MEL-administered group such that RER and the mitochondria were normal and IR-induced changes were reduced.

In the previous experimental models of liver IRD, apoptosis determination by immunohistochemical labeling was not performed. In the literature, the studies rather focused on the determination of apoptotic cells via terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) Both Saxton Nina et al. (2002) and Sun et al. (2004) showed that the cells stained with TUNEL were increased in the IR group. However, the reliability of studies conducted using TUNEL are limited. TUNEL stains all the cells with broken DNA sequence regardless of their necrotic or apoptotic nature (Malhi et al., 2006). For this reason, we preferred to use a histochemical labeling method, as it gives a more reliable measure of apoptotic index (Prochazkova et al., 2003). In our study, the number of apoptotic cells was increased significantly in the IR group compared to that of the control group. The administration of sole CAR and MEL individually decreased the number of apoptotic cells. CAR+MEL administration decreased apoptosis significantly.

The findings of the study reported here indicate that CAR had a protective effect in IR damage of the liver and this effect is higher than MEL. Further, a combination of carnosine and melatonin administration yield significantly better results than carnosine alone. This suggests that carnosine and/or melatonin may be acting synergistically with other antioxidants. In this study, carnosine and melatonin were used in combination for the first time in IR damage of the liver and histopathological findings were confirmed ultrastructurally. Moreover, in this study, histochemical labeling for apoptosis allowed a more accurate determination of apoptotic index.

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