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The hepatoprotective effect of carnosine against ischemia/reperfusion liver injury in rats

Amr A. Fouad [a,*], Mahmoud Abdel-Aziz El-Rehany [b], Hala K. Maghraby [c]

[a] Department of Pharmacology, Faculty of Medicine, Minia University, El-Minia, Egypt
[b] Department of Biochemistry, Faculty of Medicine, Minia University, El-Minia, Egypt
[c] Department of Pathology, Medical Research Institute, Alexandria University, Alexandria, Egypt

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Abstract

The potential protective effect of the natural antioxidant, carnosine was evaluated against ischemia/reperfusion liver injury in rats. Ischemia was induced by clamping the pedicle supplying the left hepatic lobe for 60 min followed by reperfusion for 2 h. Untreated rats exposed to ischemia/reperfusion showed significant elevation of serum aspartate aminotransferase and alanine aminotransferase levels, and malondialdehyde level and caspase-3 activity in liver homogenates associated with significant reduction in hepatic nitrite level, catalase and glutathione peroxidase activities as compared with sham-operated group. Pre-treatment with a single i.p. dose of carnosine (250 mg/kg), 30 min prior to the ischemic episode significantly attenuated the deterioration in the measured biochemical parameters observed with ischemia/reperfusion-induced liver injury. Also, light and electron microscopic examinations in ischemia/reperfusion untreated group revealed severe hepatic damage, such as cytoplasmic vacuolation, necrotic and apoptotic cell death, which was markedly ameliorated by pre-ischemic treatment with carnosine. These results strongly emphasize that carnosine can be useful as a prophylactic treatment to protect the liver against hypoxia-reoxygenation damage.

Keywords: Carnosine; Antioxidant activity; Liver; Ischemia-reperfusion; (Rats)

1. Introduction

Ischemia/reperfusion injury is a major determinant in many clinical conditions such as liver resections, hemorrhagic and other types of shock, and liver transplantation. Generation of reactive oxygen species and exhaustion of oxidative defense mechanisms are the main factors implicated in the pathogenesis of ischemia/reperfusion-induced liver damage (Bilzer and Gerbes, 2000; Galaris et al., 2006). Also, several studies have demonstrated the beneficial effect of antioxidants in protecting the liver against ischemia/reperfusion injury (Giakoustidis et al., 2006; Zhang et al., 2006; Vali et al., 2007).

Carnosine (β-alanyl-L-histidine) is an endogenous dipeptide in humans and many animal species. It is involved in various physiological functions including antioxidant, membrane-stabilizing and pH-buffering activities (Gariballa and Sinclair, 2000). The antioxidant effect of carnosine is related to its ability to inactivate reactive oxygen species, scavenge free radicals and chelate prooxidant metals (Kang et al., 2002). In previous studies, carnosine inhibited Fe^{2+}-induced oxidation of membrane lipids, oxidative modification of proteins and DNA fragmentation caused by reactive oxygen species (Gariballa and Sinclair, 2000; Stvolinsky and Dobrota, 2000). Carnosine also protected both *in vitro* and *in vivo* activity of antioxidant enzymes as ceruplasmine (Kang et al., 2002) and superoxide dismutase (Choi et al., 1999). Also, carnosine exerted neuroprotective and cardioprotective effects in PC12 cells and cardiomyoblasts exposed to hypoxia/reoxygenation, respectively (Bharadwaj et al., 2002; Tabakman et al., 2002). In addition, carnosine attenuated ischemia/reperfusion-induced renal dysfunction in rats (Fujii et al., 2003, 2005), and compensated deficit in antioxidant defense system of brain of rats and Mongolian gerbils in the post-ischemic period (Dobrota et al., 2005). However, the protective effect of carnosine in ischemia/reperfusion liver injury is not yet studied.

* Corresponding author. Tel.: +20 106872356.
E-mail address: amrfouad65@yahoo.com (A.A. Fouad).

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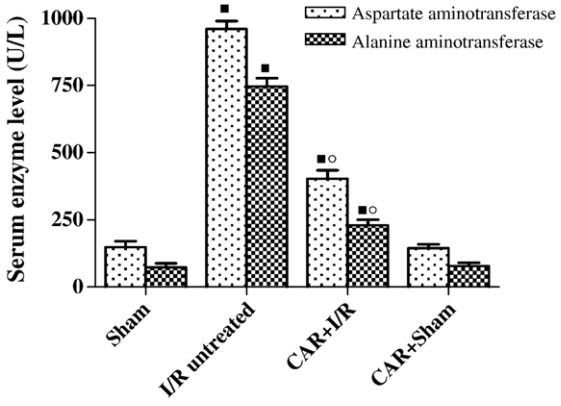

Fig. 1. Effect of ischemia/reperfusion (I/R) liver injury on serum aspartate aminotransferase and alanine aminotransferase levels in rats treated and untreated with carnosine (CAR). Data are expressed as mean±S.E.M., ■$P<0.05$ with respect to sham-operated (sham) group, °$P<0.05$ with respect to I/R untreated group.

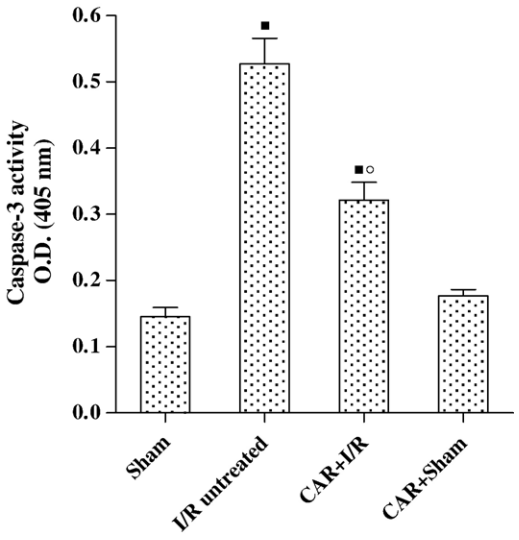

Fig. 2. Activity of caspase-3 in the liver following ischemia/reperfusion (I/R) injury in rats treated and untreated with carnosine (CAR). O.D.: optical density. Data are expressed as mean±S.E.M., ■$P<0.05$ with respect to sham-operated (sham) group, °$P<0.05$ with respect to I/R untreated group.

The present study aimed to evaluate the hepatoprotective effect of pre-ischemic treatment with a single i.p. bolus dose of carnosine in rats exposed to ischemia/reperfusion liver injury. For this purpose, serum levels of aspartate aminotransferase and alanine aminotransferase, hepatic malondialdehyde and nitrite (the stable end product of nitric oxide) levels, and catalase and glutathione peroxidase activities were measured. Also, caspase-3 protease activity was determined as a marker of hepatocellular apoptosis. In addition, light and electron microscopic examinations of liver tissue were performed.

2. Materials and methods

2.1. Animals

Thirty male Sprague–Dawley rats, weighing 180–200 g, were obtained from Othman Animal House (Abu-Rawash, Giza, Egypt). The animals were kept in a standard housing facility and were supplied with ordinary laboratory chow and water *ad libitum*. The experimental protocol was approved by the Local Animal Care Committee, and all the experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals.

2.2. Chemicals

L-carnosine and cadmium granulated (Fluka, Switzerland). Cupric sulfate, zinc sulfate, potassium dichromate and hydrogen peroxide (El-Nasr Pharmaceutical Chemicals, Egypt). Glacial acetic acid, potassium phosphate and pyridine (Prolabo, France). Sulfanilamide, *N*-naphthylethylenediamine, glutathione, glutathione reductase, nicotinamide adenine dinucleotide hydrogen phosphate (NADPH), sodium azide, sodium dodecyl sulphate, thiobarbituric acid and 1,1,3,3-tetramethoxypropane (Sigma Chemical Company, USA). Tris–hydrochloric acid buffer and urethane (BDH, England).

2.3. Experimental design

The rats were randomly assigned to four groups. The first group ($n=6$) was sham-operated and served as control, while the second group ($n=8$) received a single i.p. injection of 1 ml normal saline (vehicle of carnosine) and subjected to hepatic ischemia/reperfusion injury 30 min later. The third group ($n=8$)

Table 1
Changes in liver biochemical parameters following ischemia/reperfusion (I/R) injury in rats treated and untreated with carnosine (CAR)

Groups	Measured parameters			
	Malondialdehyde (nmol/g tissue)	Nitrite (nmol/g tissue)	Catalase (U/mg protein)	Glutathione peroxidase (U/g tissue)
Sham ($n=6$)	93.04±4.15	87.25±4.63	46.27±2.87	17.12±0.94
I/R untreated ($n=8$)	190.36±7.57[a]	58.53±3.06[a]	28.35±1.08[a]	11.29±0.46[a]
CAR+I/R ($n=8$)	127.42±3.10[a, b]	71.96±2.14[a, b]	38.77±1.66[b]	15.44±0.81[b]
CAR+Sham ($n=8$)	96.45±3.11	92.69±3.24	41.49±2.26	18.76±0.69

Data are expressed as mean±S.E.M.
[a] $P<0.05$ with respect to sham-operated (sham) group.
[b] $P<0.05$ with respect to ischemia/reperfusion untreated group.

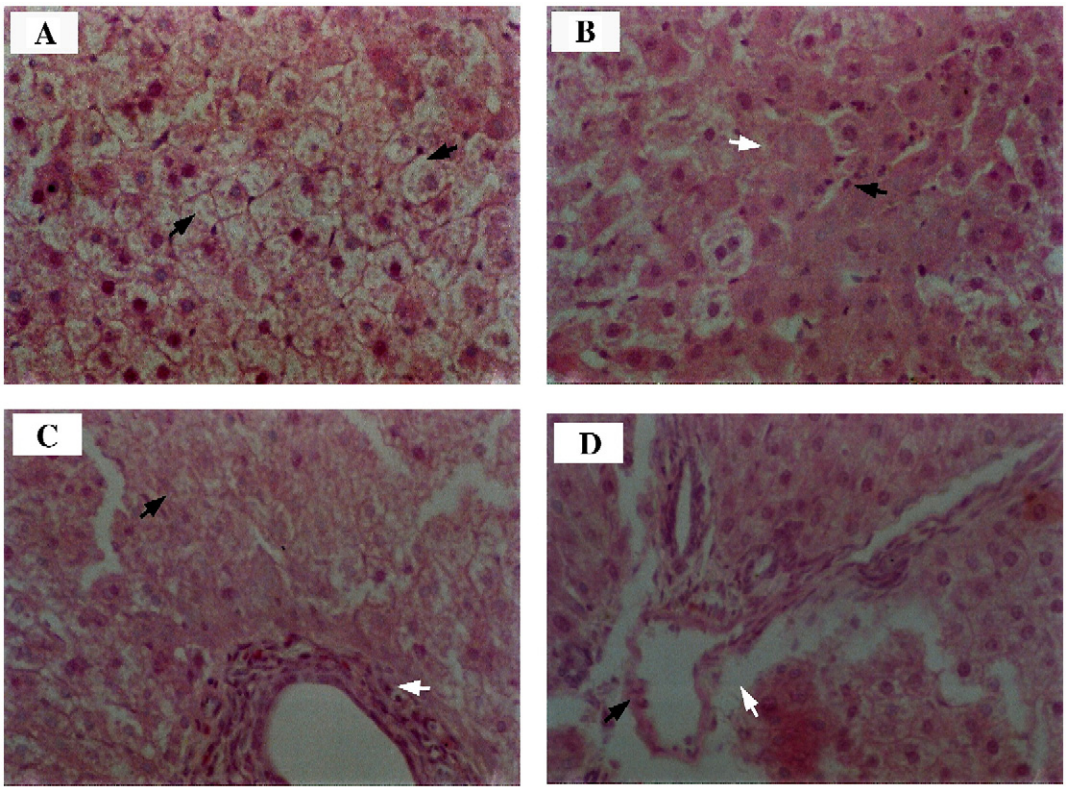

Fig. 3. Light microscopic examination (H and E, ×400) of rat liver with I/R injury showing: (A) ballooning of hepatocytes with cytoplasmic vacuolation (black arrows) and loss of definition of liver plates; (B) focal spotty necrosis of individual cells (white arrow) surrounded by relatively intact hepatocytes, and Kupffer cell hyperplasia (black arrow); (C) confluent area of coagulative necrosis with poorly stained mummified hepatocytes (black arrow) and mild portal lymphocytic infiltration (white arrow); (D) swollen and disrupted endothelium of the ectatic venule (black arrow) with delicate portal tract fibrosis (white arrow).

was treated with a single i.p. injection of carnosine, dissolved in normal saline, in a dose of 250 mg/kg (Stvolinsky and Dobrota, 2000), 30 min before ischemia/reperfusion liver injury. The fourth group ($n=8$) received a single i.p. injection of carnosine in the same dose as the third group and sham-operated after 30 min.

2.4. Surgical procedure

The surgical technique was followed as described previously by Kooij et al. (1994). Following anesthesia with urethane (1.6 g/kg, i.p.), a mid-line abdominal incision was performed and the liver hilum was gently exposed. A fine non-traumatic vascular clip was applied at the pedicle supplying the left lobe of the liver. This allowed for selective interruption of the blood supply of the left hepatic lobe, while preserving that of the right lobe. Thus, gastrointestinal congestion and hemodynamic instability accompanying complete occlusion of the hepatic pedicle were avoided. Warm saline was injected in the abdomen which is temporarily closed. The blood flow to the left hepatic lobe was occluded for 60 min (ischemic phase), and the clip was removed after reopening the abdomen to begin the reperfusion phase which lasted for 2 h, after which the animal was sacrificed by exsanguination and sampling was done.

2.5. Sampling and biochemical analyses

The right atrium was punctured and blood was aspirated with a syringe. Blood samples were centrifuged for 10 min at 3500 g and the obtained sera were stored at −20 °C till the levels of aspartate aminotransferase and alanine aminotransferase were determined using colorimetric assay kits according to the manufacturer's instructions (Randox Laboratories Ltd., UK).

Following blood sampling, the left hepatic lobe was promptly resected, washed in cold saline and kept at −80 °C. Rat livers were subsequently homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4). The homogenates were centrifuged at 3500 *g* for 10 min at 4 °C, and the supernatant was used for determination of malondialdehyde level (Ohkawa et al., 1979), as an indicator of lipid peroxidation, and total nitrite, indirect method to measure nitric oxide level (Sastry et al., 2002). The hepatic activities of catalase (Sinha, 1972) and glutathione peroxidase (Paglia and Valentine, 1967) were also assessed. The protein content in liver homogenates was measured using colorimetric assay kit as indicated by the manufacturer (Nobel Diagnostic, Egypt).

In addition, the activity of caspase-3, which becomes activated during the cascade of events required for execution of apoptosis, was measured in the liver homogenates using colorimetric assay kit according to the manufacturer's instructions (Biosource Europe, Belgium). Briefly, the levels of chromophore *p*-nitroanilide (*p*NA)

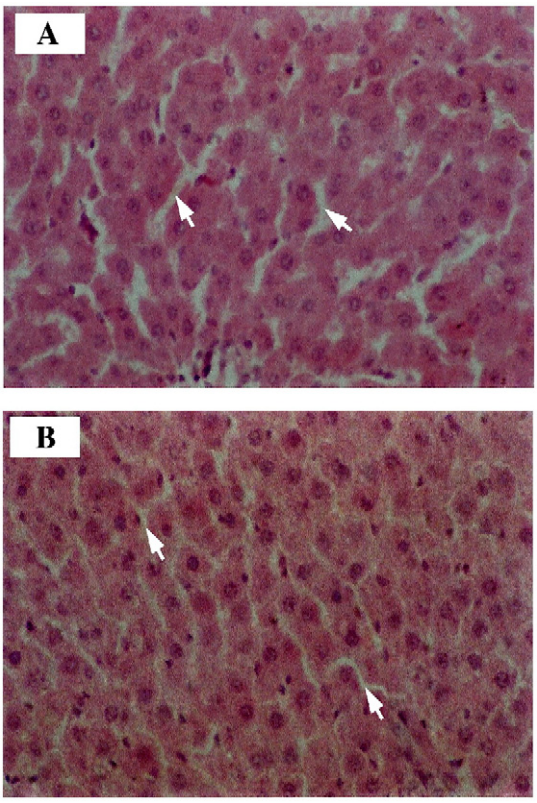

Fig. 4. Light microscopic examination (H and E, ×400) of pre-ischemic carnosine-treated (A), and sham-operated control (B) rat liver. (A) shows more or less preserved hepatic architecture without ballooning, spotty or coagulative necrosis of hepatocytes nor cytoplasmic vacuolation. Arrows denote preservation of liver plates.

released from DEVD-*p*NA by caspase-3 activity were quantified spectrophotometrically. The fold-increase in caspase-3 activity was determined by comparing the absorbance of *p*NA from samples of different groups with that of sham-operated control.

2.6. Light microscopic examination

Parts of isolated hepatic tissue were fixed in 10% formalin solution and then dehydrated in ascending grades of alcohol and embedded in paraffin. Four micron-thickness sections were taken, stained with hematoxylin and eosin solutions and examined under light microscope.

2.7. Electron microscopic examination

Small pieces of liver tissue (about 1 mm^3) were fixed as soon as possible in 3% cacodylate buffered glutaraldehyde (pH 7.4) for 1.5 h. After two rinses in the buffer overnight, the tissue was post fixed in 1% buffered osmic acid for 1.5 h at 4 °C. The specimens were then dehydrated in ascending grades of ethanol, cleared in toluene for 10 min and embedded in epon resin. Semi-thin sections were cut, stained with toluidine blue and examined under light microscope. Ultra-thin sections were obtained from the selected blocks, mounted on copper grids, stained with uranyl acetate and lead citrate and examined with transmission electron microscope (T.E.M.).

2.8. Statistical analysis

All values are expressed as mean ± S.E.M. The results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test for multiple comparisons using SPSS for Windows (version 11). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Results of biochemical analyses

The activities of serum aspartate aminotransferase and alanine aminotransferase were significantly higher in rats exposed to ischemia/reperfusion-induced liver injury as compared with sham-operated animals. Pre-treatment with a single i.p. dose of carnosine 30 min before ischemia/reperfusion caused significant reduction in the release of liver aspartate aminotransferase and alanine aminotransferase (Fig. 1).

Rats subjected to 60 min of hepatic ischemia followed by 120 min reperfusion showed significant increase in liver malondialdehyde level associated with significant decrease in nitrite level. Also, ischemia/reperfusion caused significant exhaustion of the hepatic antioxidant defense as observed by the fall in catalase and glutathione peroxidase enzyme activities (Table 1).

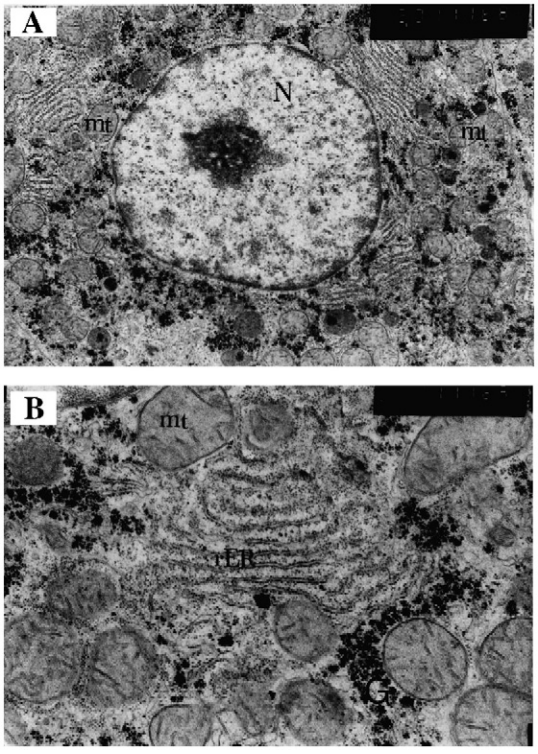

Fig. 5. Electron micrograph of control (sham-operated) rat hepatocyte showing: (A) nucleus (N) has smooth rounded contour and abundant chromatin with prominent nucleolus (T.E.M., ×7500); (B) normal mitochondria (mt) with cristae, rough endoplasmic reticulum (rER) on its external surface and glycogen (G) rosettes (T.E.M., ×14,000).

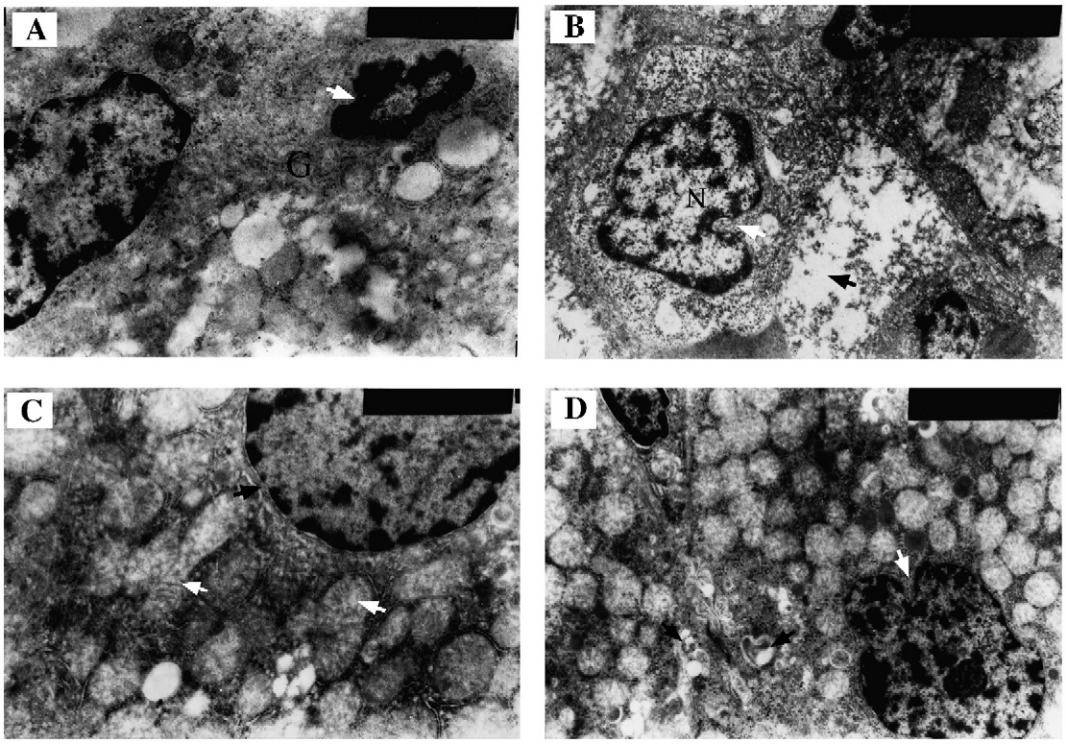

Fig. 6. Electron micrograph (T.E.M., ×12,000) of hepatocytes of rat with I/R injury showing: (A) an apoptotic nucleus having dense clumped marginal chromatin with fragmentation (white arrow) and dispersed glycogen (G) granules; (B) irregular nucleus having nuclear pouch (white arrow) and cytoplasmic vacuolation (black arrow); (C) dilated ruptured mitochondria with distorted cristae (white arrows) and nuclear pores (black arrow); (D) irregular nucleus with fragmented chromatin (white arrow) and apoptotic bodies (black arrows).

Hepatic lipid peroxidation induced by ischemia/reperfusion was significantly suppressed as a result of pre-treatment with carnosine. This was accompanied by a significant restoration of hepatic antioxidant defense (nitrite level, catalase and glutathione peroxidase activities) (Table 1).

Also, caspase-3 activity in liver homogenates was increased by three folds in rats exposed to ischemia/reperfusion injury, while carnosine-treated animals showed only two fold-increase in comparison to sham-operated group (Fig. 2).

Pre-treatment with carnosine in sham-operated rats produced no significant change in all the measured parameters.

3.2. Results of light microscopic examination

In general, ischemia/reperfusion caused marked damage of rat hepatocytes in the form of ballooning degeneration, cytoplasmic vacuolation, focal and confluent hepatocellular necrosis, and portal tract fibrosis with endothelial swelling and disruption (Fig. 3). Pre-ischemic treatment with carnosine markedly attenuated the ischemia/reperfusion-induced histopathological changes in rat liver (Fig. 4).

3.3. Results of electron microscopic examination

The control (sham-operated) rat hepatocyte shows normal architecture and cell organelles (Fig. 5). Ischemia/reperfusion-induced hepatocellular apoptosis as evidenced by nuclear chromatin condensation, margination and fragmentation, nuclear pouches, nuclear pores and swollen ruptured mitochondria with distorted cristae (Fig. 6). Again, pre-treatment with carnosine almost restored the normal picture that was disrupted by ischemia/reperfusion (Fig. 7).

4. Discussion

Ischemia-reperfusion liver injury is a frequent clinical complication with high morbidity and mortality. Reperfusion of previously ischemic hepatic tissue initiates complex cellular events that eventually results in necrosis and apoptosis of liver cells (Kim et al., 2004; Nagai et al., 2007). Several factors are contributed to the pathogenesis of ischemia/reperfusion liver injury including ATP depletion, phospholipase and protease activation, increased endothelin-1 formation and neutrophil infiltration. However, generation of reactive oxygen species in the reperfusion phase plays the fundamental role (Seo and Lee, 2002; Totsuka et al., 2005).

Carnosine is a natural water-soluble antioxidant which suppresses reactive oxygen species generation and scavenges lipid peroxidation products during free radical reactions both *in vitro* and *in vivo* (Holliday and McFarland, 2000; Prokopieva et al., 2000). The prophylactic and therapeutic effects of carnosine against ischemia/reperfusion-induced tissue damage were evidenced in previous studies. Pre-ischemic treatment with carnosine decreased mortality and severity of neurological symptoms, and kept most of the learning parameters in rats exposed to brain ischemia (Gallant et al., 2000). Carnosine

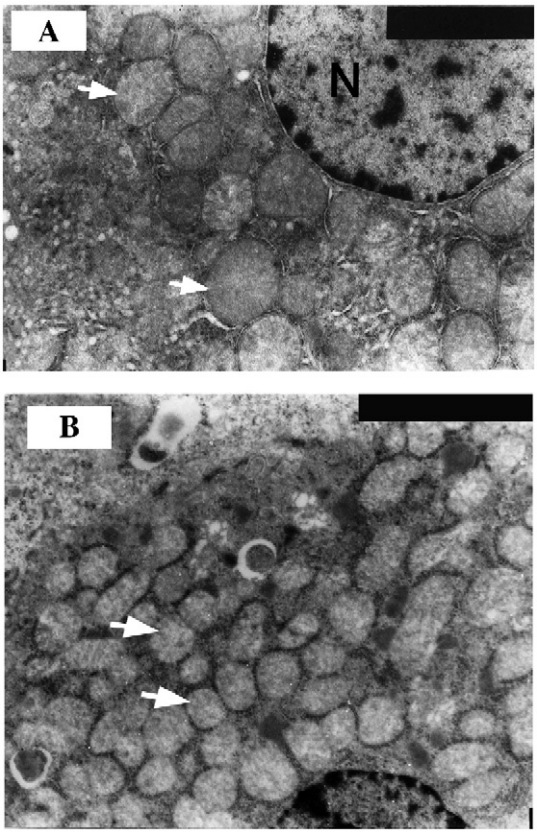

Fig. 7. Electron micrograph (T.E.M., ×12,000) of carnosine-treated rat hepatocyte showing more or less normal nucleus (A), and normal mitochondria with cristae (white arrows in A and B).

treatment for 7–14 days in rats and Mongolian gerbils after brain ischemic episode had pronounced neuroprotective effect and significantly reduced the elevated malondialdehyde level (Dobrota et al., 2005). During cardiac ischemia, carnosine preserved coronary blood flow, decreased loss of lactate dehydrogenase and myoglobin release from cardiomyocytes, and suppressed the development of ischemic contracture (Stvolinsky and Dobrota, 2000). Prior treatment with carnosine significantly attenuated ischemia/reperfusion-induced renal dysfunction and improved renal damage observed by histopathological examination in rats (Fujii et al., 2003, 2005).

This was encouraging to conduct the present study in which pre-treatment with carnosine, in a single i.p. dose of 250 mg/kg efficiently suppressed hepatic tissue injury induced by ischemia/reperfusion in rats. This was evidenced by significant improvement in the disturbed biochemical parameters (elevated serum aspartate aminotransferase and alanine aminotransferase levels, and hepatic malondialdehyde and caspase-3 activity with reduced liver catalase and glutathione peroxidase activities), and amelioration of hepatocellular necrosis and apoptosis observed in light and electron microscopic examinations. These results are in agreement with several previous studies which demonstrated the effectiveness of various antioxidants in attenuating ischemia/reperfusion-induced liver damage (Liu et al., 2003; Lee and Lee, 2005; Zhang et al., 2006).

The protective effect of carnosine against ischemia/reperfusion-induced tissue damage is primarily due its prominent antioxidant activity. Carnosine suppresses the production of reactive oxygen species which significantly contributes to the development of ischemia/reperfusion tissue injury (Gariballa and Sinclair, 2000). It also prevents generation of peroxynitrite radical, a highly reactive nitrogen oxide species, implicated in cell apoptosis (Pietraforte et al., 2007).

Carnosine also has a metal-chelating effect. It efficiently binds divalent ions of transition metals including iron (Decker et al., 1992). This is of special importance considering the role of iron in amplification of reactive oxygen species generation and development of peroxidation during ischemia (Omar et al., 1989). By interrupting such an amplification loop, carnosine can substantially reduce hepatic tissue damage following ischemia/reperfusion. In addition, carnosine has a buffering activity allowing it to bind excess protons and decrease lactate accumulation which are among the most important pathogenic factors in ischemic injury. Reduced lactate accumulation may be also due to improved microcirculation in the ischemic tissue by carnosine (Stvolinsky and Dobrota, 2000).

It is well known that endogenous nitric oxide reacts with superoxide anion, which is rapidly produced during reperfusion phase, drastically reducing the available nitric oxide (Kawashima et al., 2000). Nitric oxide protects against liver cell necrosis and apoptosis during hypoxia and reoxygenation (Kim et al., 2004). This is probably due to conservation of cellular bioenergetics for maintenance of tissue homeostasis during hypoxia (Taniai et al., 2004), attenuation of sinusoidal neutrophil adherence (Jaeschke et al., 1990), and inhibition of platelet aggregation thus maintaining microvascular perfusion (Harbrecht et al., 1992). Also, nitric oxide provides an antiapoptotic action by inhibiting caspase-3 activity via S-nitrosylation of the enzyme (Maejima et al., 2005), and inducing heat shock protein 70 expression (Kim et al., 1997). This is in accordance with the present results which demonstrated that pre-ischemic carnosine treatment significantly increased the reduced nitric oxide level and decreased the elevated caspase-3 activity in rat liver exposed to ischemia/reperfusion.

Moreover, carnosine was found to protect against excitotoxic cell death by antinecrotic and antiapoptotic mechanisms independently of effects on reactive oxygen species (Boldyrev et al., 1999). This can be attributed to the anti-glycation effect of carnosine with reduced generation of advanced glycation end products responsible for tissue damage and cell apoptosis (Yan and Harding, 2005).

Histidine, cleaved from carnosine by serum carnosinase enzyme, or its metabolite histamine may be involved in the action of carnosine. The suppressive effect of carnosine on hyperglycemia induced by intracranial injection of 2-deoxy-D-glucose was attenuated by thioperamide, the histamine H_3-receptor antagonist (Yamano et al., 2001). Also, the protective effect of carnosine on amygdaloid-kindled seizures in rats was completely antagonized by H_1-receptor antagonists pyrilamine and diphenhydramine, but not by the H_2-antagonist zolantidine (Jin et al., 2005). Therefore, it can be speculated that the protective effect of carnosine in ischemia/reperfusion liver injury is mediated through the histaminic receptors. However, this needs to be clarified by further experiments.

In conclusion, the present results demonstrated that pre-treatment with carnosine significantly compensated deficits in the hepatic antioxidant defense system and ameliorated liver damage induced by ischemia/reperfusion. Thus, carnosine can be considered a potential candidate as an anti-ischemic medication to minimize ischemia/reperfusion injury which is a major clinical challenge, particularly in liver transplantation.

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