Pharmaceutical Sciences, 2021, 27(1), 32-45
doi:10.34172/PS.2020.60
https://ps.tbzmed.ac.ir/

Research Article

Carnosine Mitigates Biomarkers of Oxidative Stress, Improves Mitochondrial Function, and Alleviates Histopathological Alterations in the Renal Tissue of Cholestatic Rats

Omid Farshad[1], Mohammad Mehdi Ommati[2], Jale Yüzügülen[3], Akram Jamshidzadeh[1,4], Khadijeh Mousavi[4], Zahra Ahmadi[3], Negar Azarpira[5], Hasti Ghaffari[6], Asma Najibi[4], Marzieh Shafaghat[4], Hossein Niknahad[1,4*], Reza Heidari[1*]

[1] *Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz Iran.*

[2] *College of Life Sciences, Shanxi Agricultural University, Taigu, Shanxi 030801, Peoples' Republic of China.*

[3] *Eastern Mediterranean University, Faculty of Pharmacy, Famagusta, North Cyprus, Turkey.*

[4] *Department of Pharmacology and Toxicology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.*

[5] *Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.*

[6] *Department of Veterinary Sciences, Islamic Azad University, Urmia Branch, Urmia, Iran.*

Article Info

Article History:
Received: 3 June 2020
Accepted: 18 July 2020
ePublished: 2 October 2020

Keywords:
-Acute kidney injury
-Bile acids
-Cirrhosis
-Cholestasis
-Nephropathy
-Renal failure

Abstract

Background: Cholestatic liver disease primarily affects hepatic tissue. Cholestasis could also influence the function of other organs rather than the liver. Cholestasis-induced kidney injury is a severe clinical complication known as "cholemic nephropathy" (CN). Bile duct ligation (BDL) is a trustworthy experimental model for inducing CN. Although the precise mechanism of renal injury in cholestasis is not fully recognized, several studies revealed the role of oxidative stress in CN. There is no promising pharmacological intervention against CN. Carnosine (CAR) is a peptide extensively investigated for its pharmacological effects. Radical scavenging and antioxidative stress are major features of CAR. The current study aimed to evaluate the role of CAR supplementation on the CN.

Methods: CAR was administered (250 and 500 mg/kg, i.p) to BDL rats for 14 consecutive days. Urine and serum markers of renal injury, biomarkers of oxidative stress in the kidney tissue, and renal histopathological alterations were monitored.

Results: Significant elevation in oxidative stress biomarkers, including ROS formation, lipid peroxidation, oxidized glutathione (GSSG) levels, and protein carbonylation were found in the kidney of BDL rats. Moreover, renal tissue antioxidant capacity and reduced glutathione (GSH) levels were significantly decreased in the organ of cholestatic animals. Renal histopathological changes, including tubular atrophy, interstitial inflammation, tissue fibrosis, and cast formation, were detected in the kidney of BDL rats. It was found that CAR administration significantly protected the kidney of cholestatic animals.

Conclusion: The antioxidative properties of this peptide might play a fundamental role in its protective properties during cholestasis.

Introduction

Cholestasis is a critical clinical situation that could lead to the stoppage of bile flow. Cholestasis could occur by various etiologies. Alcohol and drugs, infectious liver diseases, disturbances in the metabolism of fatty acids, cancer, and gall stones cloud lead to cholestasis.[1-5] Hepatic tissue is the primary site affected by cholestasis. However, the accumulation of potentially cytotoxic molecules during cholestasis could affect the function of other organs. It has been well-known that cholestasis could significantly influence renal function.[4,6,7] Cholestasis-induced renal injury is a severe clinical complication known as "cholemic nephropathy" (CN).[6,8-12]

Although the exact mechanism included in the pathogenesis of CN is not fully recognized, several studies declared the disturbances of redox balance in the kidney of cholestatic models.[13-18] It has also been mentioned that oxidative stress is a systemic phenomenon that occurred in different tissues during cholestasis.[19-21] Protein carbonylation, depletion of kidney antioxidant capacity, and lipid peroxidation have been documented in the kidney of cholestatic animals.[13,14]

*Corresponding Author: Reza Heidari, E-mail: rheidari@sums.ac.ir and Hossein Niknahad, E-mail: niknahadh@sums.ac.ir
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Mitochondrial impairment is another complication that could play a role in the mechanism of CN-associated complications.[22] It has been found that mitochondrial function and energy metabolism is impaired in CN.[22] These events could lead to serum electrolytes imbalance because of cholestasis-associated renal injury.[22-25] Based on these data, the administration of antioxidant and mitochondria protecting molecules could have potential therapeutic value against CN.

Carnosine (β-alanyl-histidine; CAR) is a dipeptide extensively investigated for its physiological and pharmacological possessions.[26-28] A high level of CAR is present in tissues such as the brain and skeletal muscle.[29,30] Free radical scavenging activity is an essential feature of CAR.[31-33] CAR has been widely investigated for its antioxidative properties against a variety of pathological conditions.[26-28] CAR is also a well-known scavenger of lipid peroxidation end products and a carbonyl trap.[21-23,26-28] Therefore, CAR could significantly prevent the oxidation of cellular lipids and proteins.[26-28,31-33] The antioxidant properties of CAR have been repeatedly mentioned in hepatic and renal tissue.[34-38] On the other hand, there are several lines of evidence which indicate the positive effects of CAR on mitochondrial function. It has been found that CAR administration significantly enhanced ATP production and mitigated mitochondria-mediated cell death in different experimental models.[39-45] All these properties make CAR as a potential candidate for the management of CN complications.

The current study aimed to assess the effects of CAR administration against cholestasis-induced renal injury. Rats underwent bile duct ligation (BDL) operation and supplemented with CAR (250 and 500 mg/kg, i.p, 14 consecutive days). Markers of oxidative stress, various mitochondrial indices, as well as tissue histopathological alterations, were monitored.

Materials and Methods

Reagents

Sodium acetate, trichloroacetic acid (TCA), iodoacetic acid, dithiothreitol (DTT), acetonitrile HPLC grade, sucrose, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), methanol HPLC grade, thiobarbituric acid, tetrabutylammonium hydroxide, meta-phosphoric acid, potassium hydrogen phosphate monobasic (KH_2PO_4), ethylenediamine tetra-acetic acid (EDTA), and 2-amino-2-hydroxymethyl-propane-1,3-diol-hydrochloride (Tris-HCl), were obtained from Merck (Darmstadt, Germany). Carnosine, dichlorodihydrofluorescein diacetate (DFC-DA), oxidized glutathione (GSSG), reduced glutathione (GSH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Biomarkers of organ injury in serum and urine of BDL animals were measured using Pars Azmoon® kits (Tehran, Iran).

Animals

Thirty-two male Sprague-Dawley (SD) rats (250-300 g weight) were purchased from Shiraz University of Medical Sciences, Shiraz, Iran. Animals were housed in a standard environmental (23±1°C, 12L: 12D photoschedule, and 40% relative humidity). Animals allowed free access to a rodents' diet (RoyanFeed®, Isfahan, Iran) and tap water. All experiments were accomplished in conformity with the guidelines for care and use of experimental animals certified by Shiraz University of Medical Sciences ethics committee (#19360).

Animal model of cholestasis and experimental setup

Bile duct obstruction model was used as a reliable animal model of cholestasis. Briefly, animals were deeply anesthetized (a mixture of ketamine and xylazine; 90 mg/kg and 8 mg/kg respectively, i.p), laparotomy was made, and the common bile duct was identified and ligated. The sham operation consisted of laparotomy and bile duct manipulation.[46,47] Animals were equally allotted into four groups (n = 8 in each group; total 32 rats). Rats were treated as follows: **1**) Sham-operated (Sham; Vehicle-treated); **2**) BDL; **3**) BDL + Carnosine (250 mg/kg, i.p); **4**) BDL + Carnosine (500 mg/kg, i.p). Animals received carnosine for 14 following days. Cholestasis-associated cholemic nephropathy was assessed 14 days after BDL operation.

Serum and urine biochemistry

Commercial kits (Pars Azmoon®, Tehran, Iran) and an auto-analyzer (Mindray BS-200®, Guangzhou, China) were used to assess serum and urine biomarkers of organ injury in cholestatic rats.[48]

Renal tissue histopathology and organ weight index

Samples of the renal tissue were fixed in buffered formalin solution (0.4% w: v of NaH_2PO_4, 0.64% w:v of $Na_2H_2PO_4$, and 10% v:v of formaldehyde in deionized water). Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E). Masson's trichrome staining determined renal fibrotic changes in BDL rats.[49,50] Bile cast formation in BDL animals was assessed by Periodic Acid-Schiff (PAS) staining.[51] The organs (liver, spleen, and kidney) weight indices were measured as organ weight index = [Wet organ weight (g)/Body weight (g)] × 100.

Lipid peroxidation

Renal tissue lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay method.[46,52] The reaction mixture was consisted of thiobarbituric acid (1 mL of 0.375%, w:v), and phosphoric acid (3 mL of 1% w: v, pH = 2). Samples of tissue homogenate (500 μL of 10% w:v in KCl, 1.15% w:v), were added to the reaction mixture and mixed. Tubes were heated in an oven (100 °C, 45 min). After the incubation period, the mixture was cooled, and n-butanol (2 mL) was added. Samples were mixed (30 sec) and centrifuged (700 g, at λ = 532 nm (Ultrospec 2000® UV spectrophotometer, Pharmacia Biotech, Sweden).[53,54]

Reactive oxygen species formation

Reactive oxygen species (ROS) formation in the kidney tissue of cholestatic animals was measured using 2′, 7′-dichlorofluorescein diacetate (DCF-DA).[55,56] Briefly, kidney tissue (200 mg) was homogenized in 5 mL of ice-cooled Tris-HCl buffer (40 mM, pH = 7.4). The resulted tissue homogenate (100 µL) was mixed with Tris-HCl buffer (1 mL) and 5 µL of DCF-DA (Final concentration of 10 µM). The mixture was incubated at 37°C (15 min, in the dark). Finally, the fluorescence intensity of the samples was assessed using a fluorimeter (FLUOstar Omega® microplate reader, $\lambda_{excit.}$ = 485 nm and $\lambda_{em.}$ = 525 nm).[55,57]

Renal glutathione content

The kidney tissue reduced (GSH) and oxidized (GSSG) glutathione contents were assessed using an HPLC method. The HPLC system consisted of a 25 cm NH_2 column (Bischoff® chromatography, Leonberg, Germany) as the stationary phase.[58] The mobile phases composed of buffer A (Acetate buffer: HPLC grade water; 1:4 v: v) and buffer B (HPLC grade water: Methanol 1:4 v: v). A gradient method was used with a steady increase of buffer B to 95% in 25 minutes with a flow rate of 1 mL/min.[58] GSH and GSSG were used as standards. Tissue samples (200 mg) were homogenized in 250 mM Tris-HCl buffer (pH = 7.4; 4°C). Then 3 mL of the resultant homogenate was treated with TCA (500 µL of 50% w: v in distilled water). Samples were mixed well and centrifuged (15,000 g, 25 min, 4°C). Then, 1 mL of the supernatant was collected in 5 mL tubes, and 200 µL of the NaOH: $NaHCO_3$ (2 M: 2 M) was added. Afterward, 100 µL of iodoacetic acid (1.5% w: v in water) was added, and samples were incubated (one hour, 4°C, in the dark). After the incubation period, 500 µL of 2, 4-dinitrofluorobenzene (DNFB; 1.5% w: v in absolute ethanol) was added and incubated in the dark (25°C, 24 h). Finally, samples were centrifuged (15,000 g, 30 min) and 25 µL of the supernatant was injected into the described HPLC system.[58,59]

Renal tissue total antioxidant capacity

Ferric reducing antioxidant power (FRAP) assay measures the formation of a blue-colored ferrous-tripyridyltriazine compound from the colorless oxidized ferric (Fe^{3+}) form by the action of tissue electron-donating antioxidant systems.[46,60] In the current study, the FRAP reagent (freshly-prepared) was composed of ten volumes of acetate buffer (300 mmol/L, pH = 3.6), with one volume of TPTZ (10 mmol/L in 40 mmol/L HCl), and one volume of ferric chloride ($FeCl_3$, 20 mmol/L). Tissue samples were homogenized in an ice-cooled 250 mM Tris-HCl containing 5 mM DTT, and 200 mM sucrose (pH = 7.4). Then, 100 µL of tissue homogenate was added to 900 µL of the FRAP reagent. The mixture was incubated at 37°C (5 min, in the dark). Finally, the absorbance of developed color was measured at λ = 595 nm (EPOCH® microplate reader, BioTek® Instruments, USA).[46,61]

Protein carbonylation

The oxidative damage of proteins was assessed based on the reaction of dinitrophenylhydrazine (DNPH) carbonyl groups.[62] Briefly, 200 mg of the kidney tissue was homogenized in 5 mL of the phosphate buffer solution (pH = 7.5) containing 0.1%. v:v of triton X-100. Tissue homogenate was centrifuged (700 g, 10 min, 4°C), and 500 µL of the supernatant was treated with 300 µL of 10 mM DNPH (dissolved in HCl). Samples were then incubated at room temperature for one hour (in the dark, vortexing every 10 min).[62] Then, 100 µL trichloroacetic acid (20% w: v solution) was added, and samples were centrifuged (17,000 g, 5 min). Afterward, the supernatant was discarded, and the pellet was washed three times, with 1 mL of ethanol: ethyl acetate (1:1 v: v), and the precipitate was re-dissolved (15 min, 37°C) in 6 M guanidine chloride solution (pH = 2.3). Finally, samples were centrifuged (12,000 g, 5 min), and the absorbance of the supernatant was measured at λ = 370 nm (EPOCH plate reader, BioTek® Instruments, USA).[62]

Kidney mitochondria isolation

Isolated mitochondria were prepared based on the differential centrifugation method.[63] For this purpose, rats' kidney was washed with normal saline (NaCl 0.9% w: v, 4°C), and minced in the ice-cold isolation buffer (70 mM mannitol, 2 mM HEPES, 220 mM sucrose, 0.5 mM EGTA and 0.1 % BSA; pH = 7.4). Then, fresh isolation buffer (5 mL buffer: 1 g tissue) was added, and the minced tissue was homogenized. At the first run of centrifugation (1000 *g* for 20 min at 4 °C), unbroken cells and nuclei were pelleted. Then, the supernatant was centrifuged (10,000 *g* for 20 min at 4 °C) to pellet the mitochondria fraction. The second step was repeated three times using the fresh isolation buffer medium. Finally, the mitochondrial pellets were resuspended in a buffer (≈5 mL buffer/g tissue) containing 70 mM mannitol, 220 mM sucrose, and 2 mM HEPES (pH = 7.4). The mitochondria fractions used to assess mitochondrial swelling and mitochondrial depolarization were suspended in mitochondria swelling assay buffer (125 mM Sucrose, 10 mM HEPES, 65 mM KCl, pH = 7.2), and depolarization assay buffer (220 mM Sucrose, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 68 mM Mannitol, 10 mM KCl, 50 µM EGTA, and 10 mM HEPES, pH = 7.2).[63] Samples protein concentrations were determined using the Bradford method to standardize the obtained data.

Mitochondrial ATP levels

Mitochondrial ATP level was assessed by an HPLC method based on a previously reported protocol.[64] Briefly, isolated mitochondria (1 mg protein/mL) were treated with 100 µL of ice-cooled meta-phosphoric acid (50 % w: v, 4 °C), incubated on ice (10 min), and centrifuged (17,000 *g*, 30 min, 4 °C). Then, the supernatant (100 µL) was treated with 15 µL of ice-cooled KOH solution (1 M). Samples were filtered and injected (25 µL) into an HPLC system composed of an LC-18 column (µ-Bondapak, 25

cm). The mobile phase was consisted of 100 mM KH_2PO_4 (pH = 7 adjusted with KOH), 1 mM tetrabutylammonium hydroxide, and acetonitrile (2.5 % v: v). The flow rate was 1 mL/min, and the UV detector was set at λ = 254 nm.[64]

Mitochondrial permeabilization and swelling

Mitochondrial swelling was assessed by analyzing the changes in the absorbance of the samples at λ = 540 nm.Briefly, isolated mitochondria (0.5 mg protein/ml) were suspended in swelling buffer (125 mM Sucrose, 10 mM HEPES, and 65 mM KCl; pH = 7.2), and the absorbance was monitored (25 °C, for 30 min), using an EPOCH® plate reader (Highland Park, USA). A decrease in absorbance is connected with an increase in mitochondrial swelling. The results are reported as maximal mitochondrial swelling amplitude (ΔOD at λ = 540 nm).[65]

Mitochondrial depolarization

Mitochondrial uptake of rhodamine 123 was used for the estimation of mitochondrial depolarization.[66,67] Rhodamine 123 accumulates in the mitochondrial matrix by facilitated diffusion. When the mitochondrion is depolarized, there is no facilitated diffusion. Therefore, the amount of rhodamine 123 in the supernatant will be increased. Briefly, the mitochondrial fractions (0.5 mg protein/mL) were incubated (10 min, 37ºC, in the dark) in the depolarization assay buffer containing rhodamine 123 (10 µM final concentration). Then, samples were centrifuged (17,000 g, 2 min, 4ºC) to precipitate the mitochondria fraction, and then the supernatant fluorescence intensity was measured with a fluorometer (FLUOstar Omega®, Germany; $\lambda_{excit.}$ = 485 nm and $\lambda_{em.}$ = 525 nm).[44,66]

Mitochondrial dehydrogenase activity

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was used for the measurement of mitochondrial dehydrogenase activity.[45,68] For this purpose, mitochondria fractions (1 mg protein/mL) were incubated with 40 µL of MTT solution (5 mg/mL) and incubated at 37°C (30 min, in the dark).[69] Then, samples were centrifuged (17,000 g, 10 min), and the pellet (purple formazan crystals) was dissolved in dimethyl sulfoxide (DMSO; 1 mL). Samples were centrifuged again (17,000 g, 1 min), and the absorbance of the supernatant was determined (λ = 570 nm using an EPOCH® plate reader, USA).[45,68]

Statistical methods

Data are represented as mean±SD. The comparison of data sets was performed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparisons as the *post hoc* test. Scores of histopathological changes are given as median and quartiles. The Kruskal–Wallis test followed by the Mann Whitney U test was used for the statistical analysis of kidney tissue histopathological scores. A $P < 0.05$ was considered a statistically significant difference between groups.

Results

Symptoms of splenomegaly and hepatomegaly were evident in the BDL group (Figure 1). On the other hand, the kidney weight index was not significantly changed 14 days after BDL surgery. It was found that CAR administration (250 and 500 mg/kg) significantly decreased the liver and spleen weight indices in cholestatic animals.

Serum biomarkers of liver injury were significantly changed in cholestatic rats in comparison with the control animals (Figure 2). On the other hand, CAR administration (250 and 500 mg/kg) significantly alleviated serum biomarkers of liver injury in BDL animals (Figure 2). Kidney injury biomarkers were also changed considerably in the urine and serum of cholestatic animals (Figure 3). It was found that CAR supplementation (250 and 500 mg/kg, i.p) significantly mitigated renal injury biomarkers in both serum and urine of cholestatic rats (Figure 3).

Biomarkers of oxidative stress were assessed in the

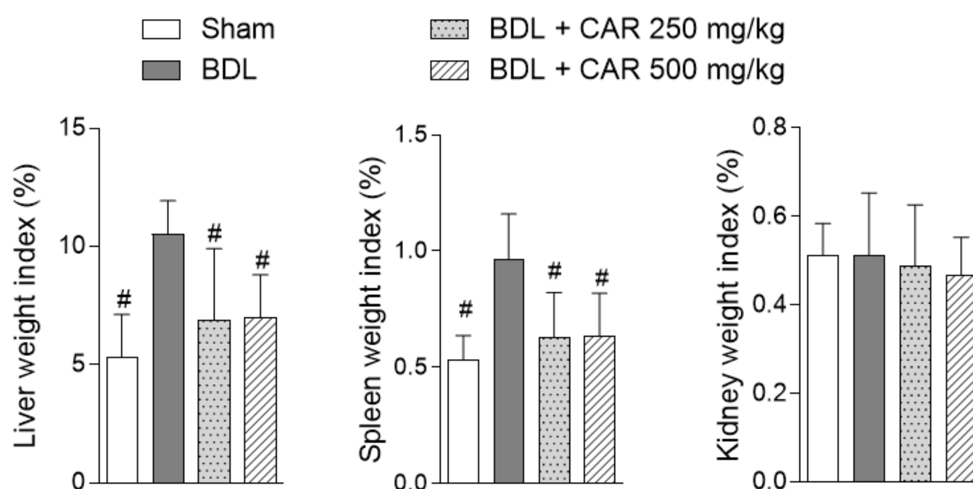

Figure 1. Organ weight indices in bile duct ligated (BDL) rats. CAR: Carnosine. Data are given as mean±SD (n = 8).#Indicates significantly different as compared with the BDL group ($P < 0.05$).

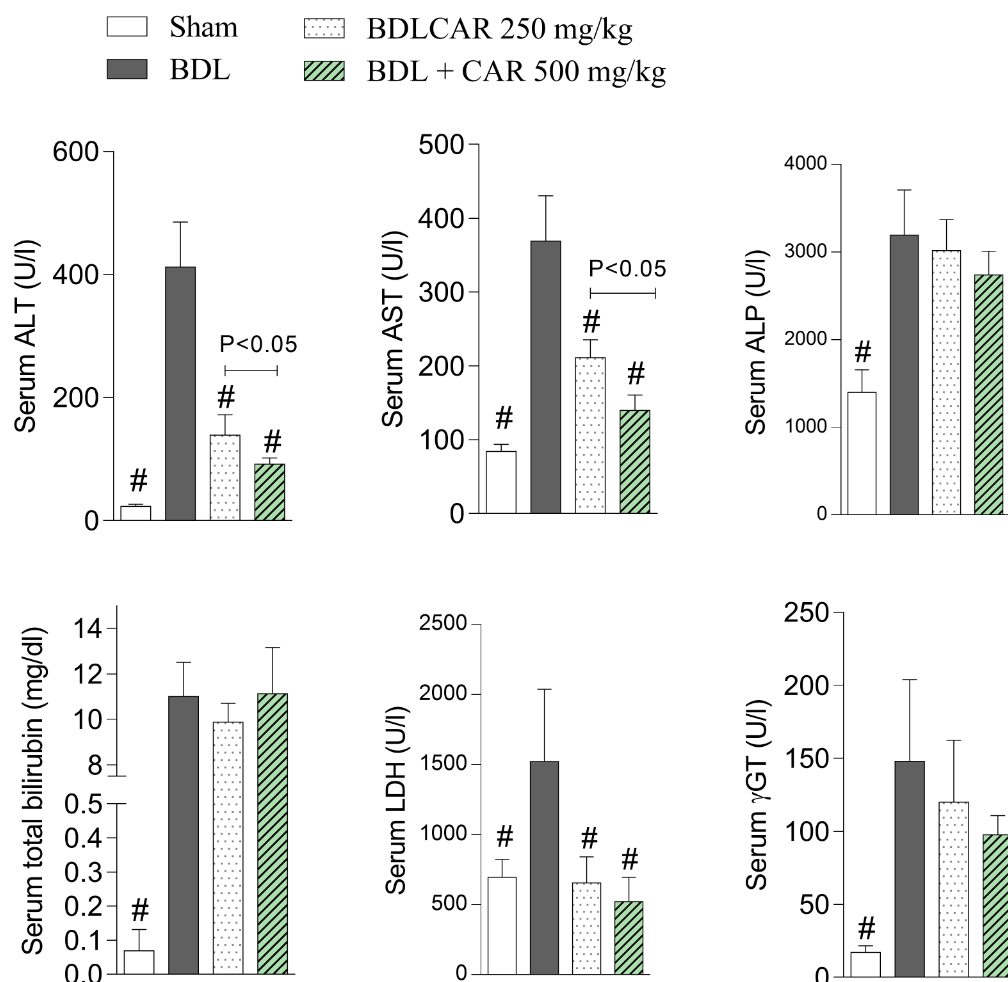

Figure 2. Serum biochemical measurements in cholestatic rats. BDL: Bile duct ligation; CAR: Carnosine.Data are given as mean ± SD (n = 8). #Indicates significantly different as compared with the BDL group (P < 0.05).

kidney of cholestatic animals (Figure 4). Significant lipid peroxidation, ROS formation, protein carbonylation, an elevated level of oxidized glutathione (GSSG) were detected in the kidney of BDL rats. Moreover, kidney GSH content was decreased, and renal antioxidant capacity was impaired in cholestatic rats. It was found that CAR (250 and 500 mg/kg, i.p, for 14 consecutive days) significantly mitigated markers of oxidative stress in the kidney of cholestatic animals. The effect of CAR on oxidative stress biomarkers was not dose-dependent in the current investigation (Figure 4).

Renal mitochondrial indices were significantly deteriorated in cholestatic rats as compared with the control animals (Figure 5). Mitochondrial depolarization, decreased mitochondrial dehydrogenase activity, depleted mitochondrial ATP levels, and mitochondrial permeabilization were detected in the kidney of the BDL group. It was found that CAR administration (250 and 500 mg/kg) significantly improved mitochondrial function in the cholestatic rats. The effects of CAR on mitochondrial indices were not dose-dependent in cholestatic animals (Figure 5).

Kidney histopathological alterations in cholestatic animals included tubular atrophy, interstitial inflammation, and hemorrhage. It was found that CAR treatment (250 and 500 mg/kg) alleviated cholestasis-induced renal histopathological changes (Figure 6), which their scores of have been listed in Table 1. Trichrome stain of renal tissue revealed significant tissue fibrosis in cholestatic rats. On the other hand, CAR administration (250 and 500 mg/kg) significantly decreased cholestasis-associated renal fibrosis (Figure 7). Significant renal cast development was also found in BDL rats. On the other hand, a significant decrease in renal cast formation was detected when BDL animals received CAR (250 and 500 mg/kg, i.p, for 14 consecutive days) (Figure 8).

Discussion

Cholestasis is a complex clinical problem that affects not only the liver but also deteriorates kidney function. Cholestasis-induced renal injury (Cholemic nephropathy; CN) is usually associated with poor prognosis and the need for organ transplantation in cholestatic/cirrhotic patients.[8,70-73] Hence, identifying the mechanisms of

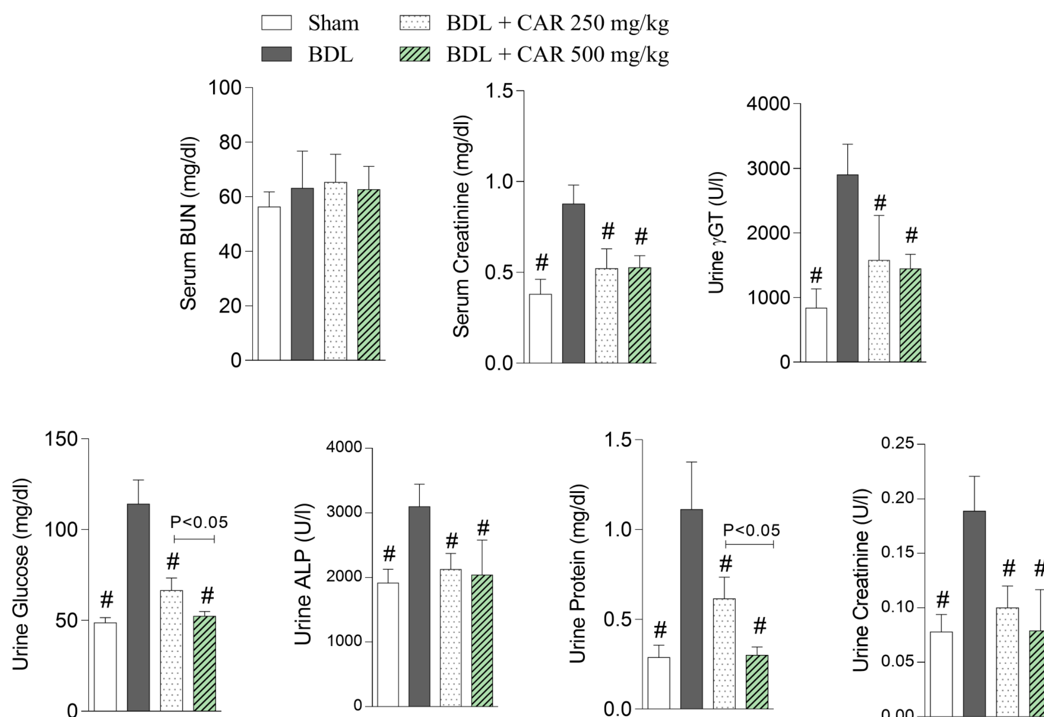

Figure 3. Urinalysis and serum biomarkers of renal injury in bile duct ligated (BDL) rats. CAR: Carnosine. Data are represented as mean ± SD (n = 8). #Indicates significantly different as compared with the BDL group (P < 0.05).

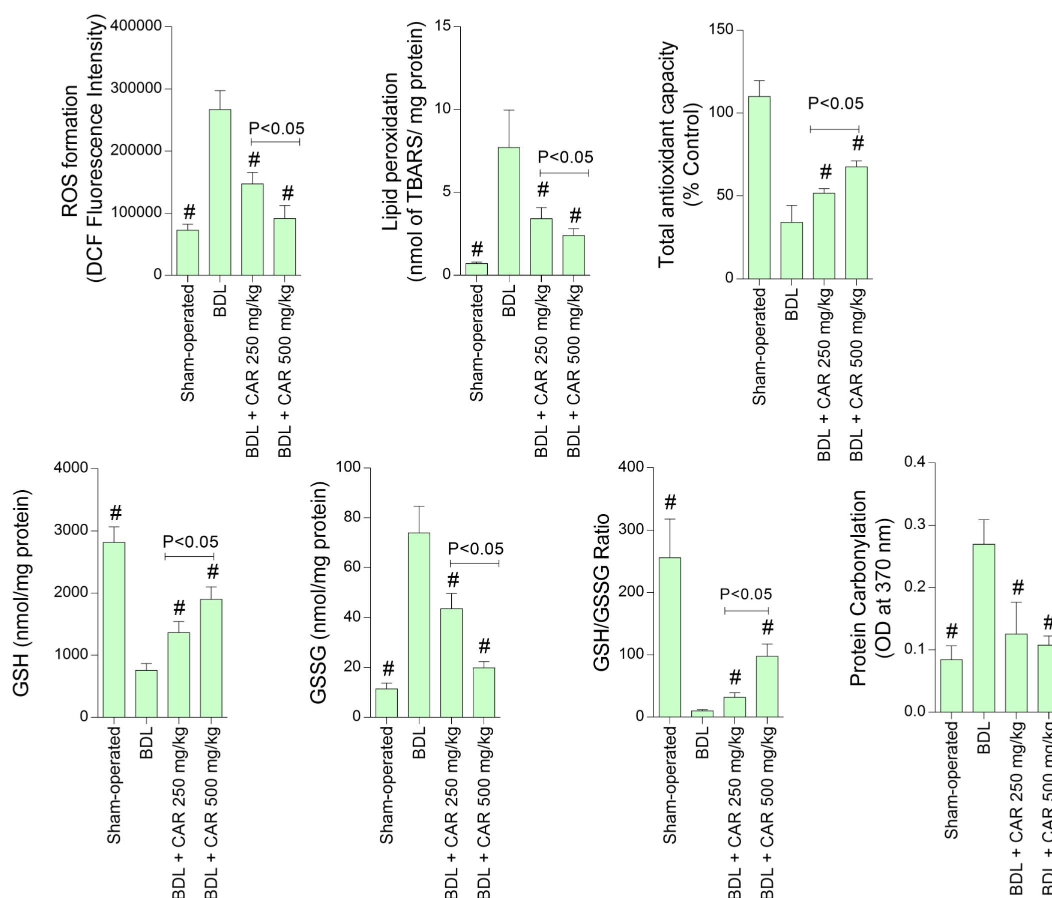

Figure 4. Markers of oxidative stress in the kidney of bile duct ligated (BDL) rats. CAR: Carnosine; ROS: Reactive oxygen species; DCF: Dichlorodihydrofluorescein; GSH: reduced glutathione; GSSG: oxidized glutathione; TBARS: Thiobarbituric acid reactive substances. Data are reported as mean ± SD (n = 8). #Indicates significantly different as compared with the BDL group (P < 0.05).

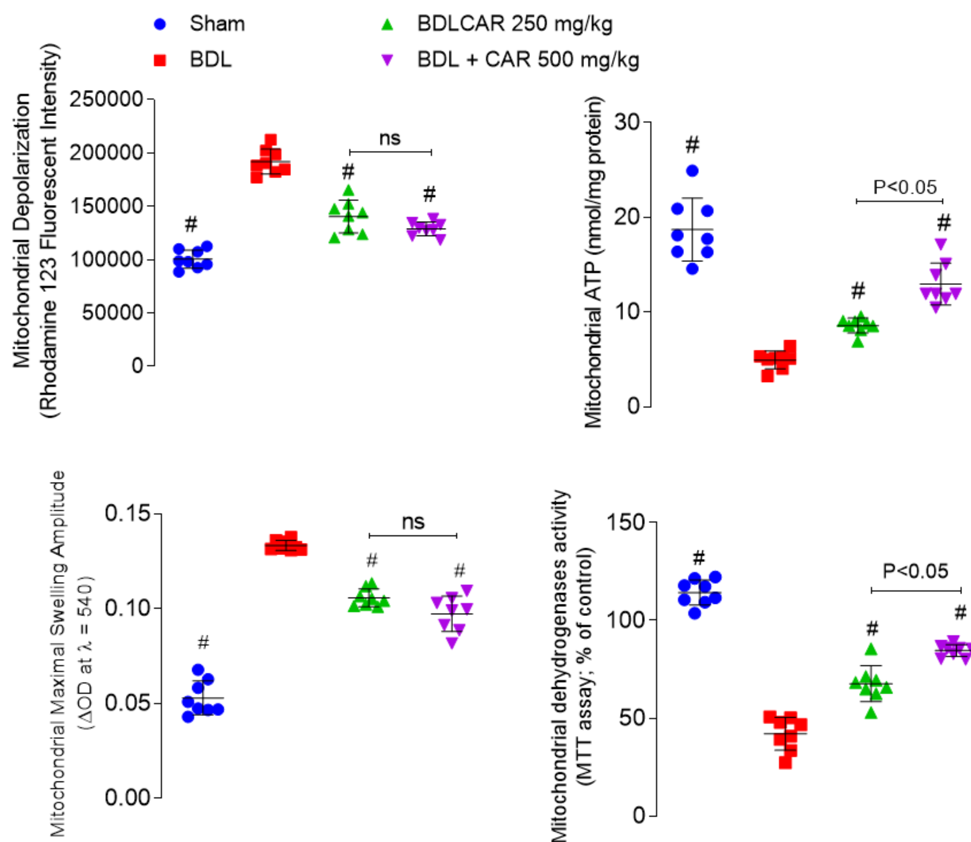

Figure 5. Effects of carnosine (CAR) administration on mitochondrial indices in the renal tissue of bile duct ligated (BDL) rats. Data are reported as mean ± SD (n = 8). # Indicates significantly different as compared with the BDL group ($P < 0.05$). ns: not significant.

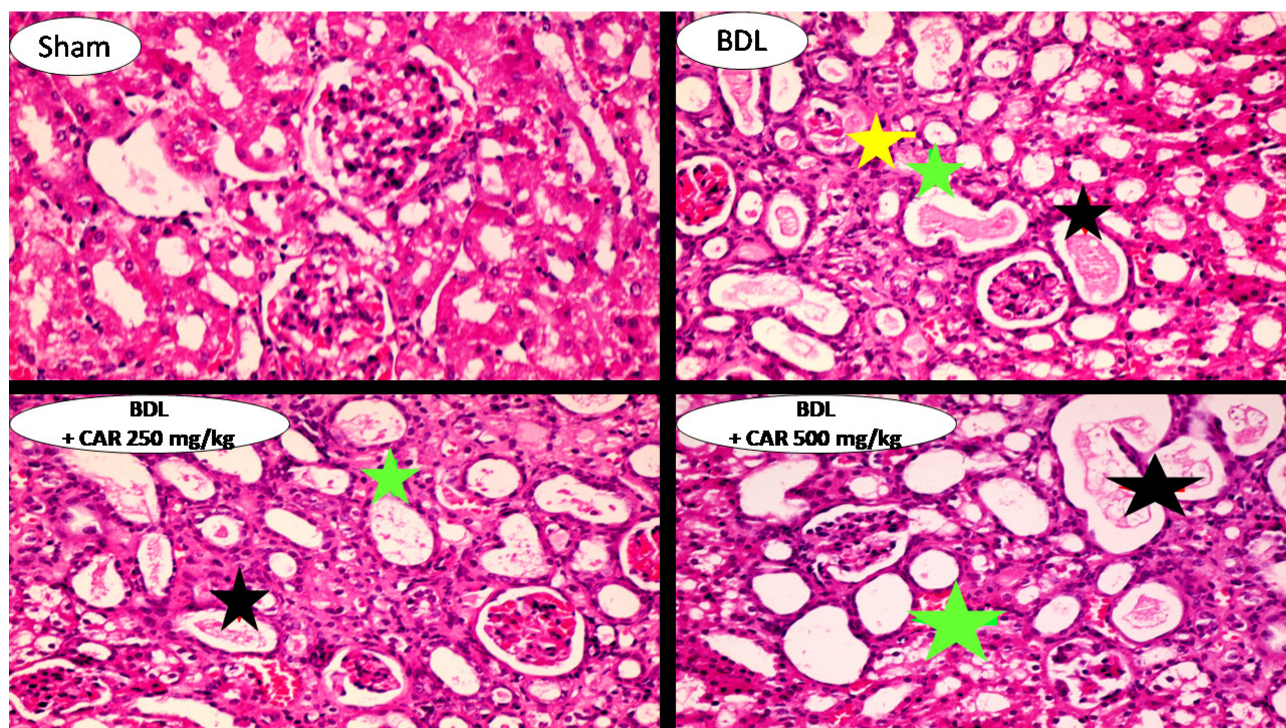

Figure 6. Effect of carnosine (CAR) treatment on cholestasis-associated kidney histopathological alterations. BDL: Bile duct ligated. Kidney tissue histopathological changes in cholestatic animals were revealed as interstitial inflammation (green star), tubular atrophy and dilation (yellow star), and hemorrhage (black stars). Carnosine administration (250 and 500 mg/kg) significantly ameliorated cholestasis-associated renal histopathological alterations (Table 1).

Table 1. Renal tissue histopathological alterations in cholestatic rats.

Treatments	Tubular Degeneration	Interstitial Inflammation	Hemorrhage
Sham	0 (0, 0)*	0 (0, 0)*	0 (0, 0)*
BDL	3 (2, 3)	2 (1, 2)	2 (1, 2)
BDL + CAR 250 mg/kg	1 (1, 1)*	1 (0, 1)*	1 (0, 1)*
BDL + CAR 500 mg/kg	1 (0, 1)*	0 (0, 0)*	1 (0, 1)*

0 = absent; 1 = mild; 2 = moderate; and 3 = severe histopathological changes BDL: Bile duct ligated; CAR: Carnosine.
Data are represented as median and quartiles for eight random pictures per group.
*Indicates significantly different as compared with the BDL group (P < 0.05).

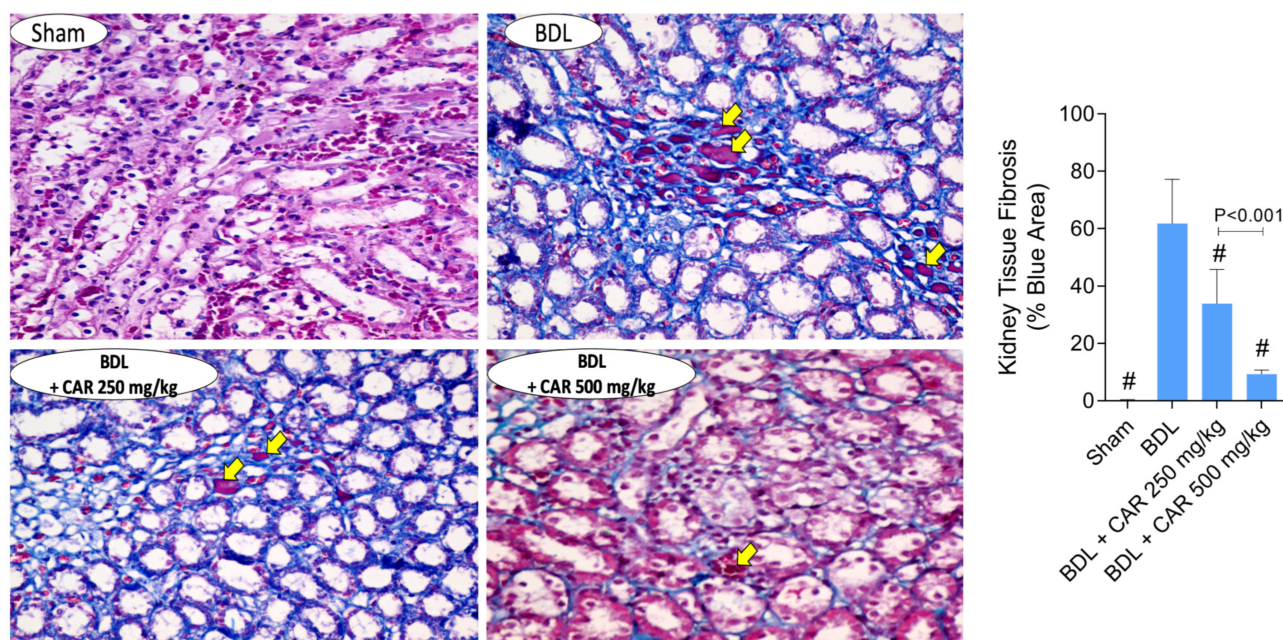

Figure 7. Trichrome staining of kidney tissue revealed significant fibrosis (blue colored area) in bile duct ligated (BDL) rats. Kidney cast formation (Figure 8) was also evident in Trichrome stain (yellow arrow heads). CAR: Carnosine. Data for tissue kidney tissue fibrosis are shown as mean±SD (n = 8). #Indicates significantly different as compared with the BDL group (P < 0.001).

CN and investigating therapeutic strategies against this complication could have tremendous clinical value. In the current study, we found that CAR treatment (250 and 500 mg/kg) could effectively mitigate renal injury in cholestatic animals. The nephroprotective effects of CAR could be mediated through the antioxidant and mitochondria protecting properties of this peptide.

CN is a severe complication developed in cholestatic patients. The prevalence of CN in cholestasis is around 18%.[74] It has been mentioned that the occurrence of CN in cholestatic patients is connected with poor prognosis and indicates the significance of organ transplantation in these patients.[75,76] Based on this data, finding new and safe therapeutic options against CN could significantly blunt its progression and decrease the renal transplantation in these patients.

Previous investigations indicate that the development of oxidative stress plays a pivotal role in the pathogenesis of CN.[77] Increased ROS levels and defected cellular antioxidant capacity have been reported in experimental models of CN.[13,21,78,79] Interestingly, signs of oxidative stress have also detected in human cases of CN.[19] Biomembrane lipids, and cellular proteins are major targets affected by ROS. In line with the previous finding, we found significant ROS formation, decreased antioxidant capacity, lipid peroxidation, and protein carbonylation in the kidney of BDL rats (Figure 4). It was found that CAR treatment significantly alleviated oxidative stress in the renal tissue (Figure 4). The influence of CAR on oxidative stress is a critical feature of this peptide which has been repeatedly mentioned in various experimental models.[27,80-82] The physiological/pharmacological properties of CAR could be mediated through several pathways.[76] First, CAR is a good carbonyl trap.[27,80-82] Therefore, this peptide efficiently scavenges reactive aldehydes produced during lipid peroxidation.[27,80-82] Moreover, the protective effects of CAR against protein carbonylation is a major feature of this peptide.[83] Second, it has been repeatedly mentioned that CAR is an excellent strategy to boost the cellular antioxidant defense system.[76] It has been found that CAR treatment increased activity enzymes such as GPx, CAT, and SOD.[76] CAR also significantly decreased mitochondrial impairment and enhanced cellular energy metabolism.[84,85] In the current study, we found that CAR

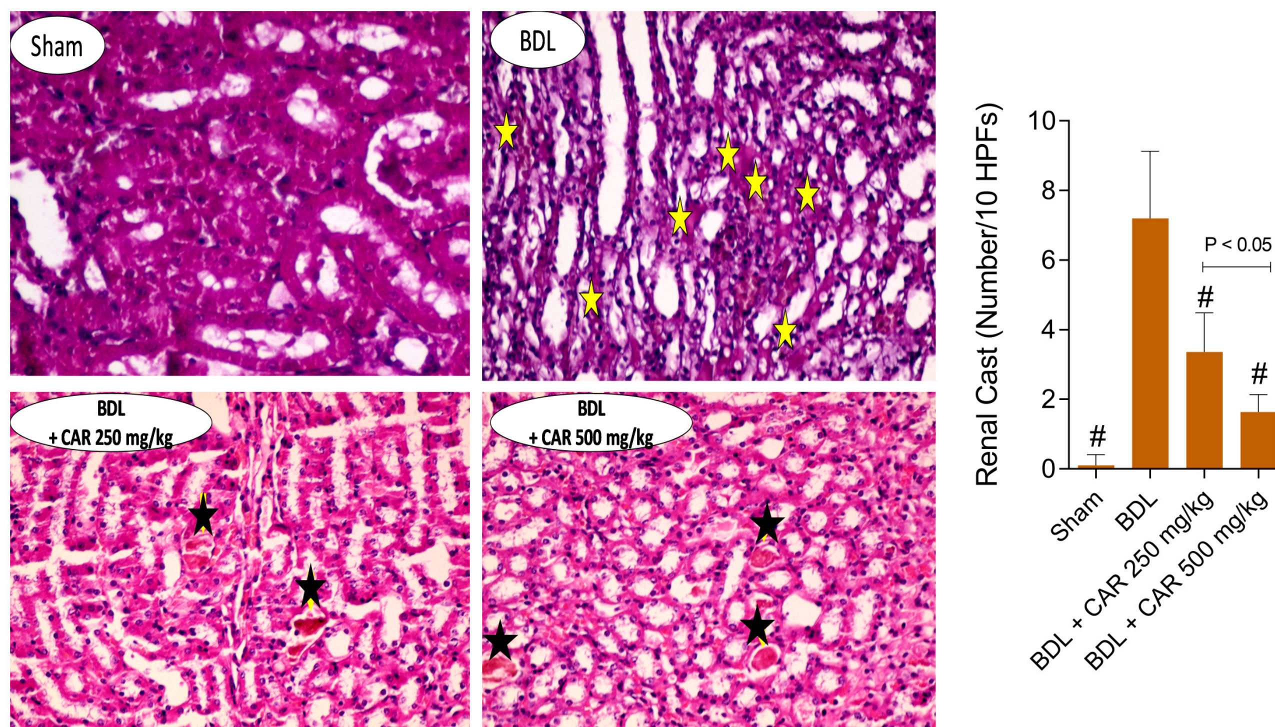

Figure 8. Effect of carnosine (CAR) supplementation on cast formation (yellow and black stars) in the kidney of cholestatic animals. BDL: Bile duct ligated; HPF: High power field (×400 magnification). Data for bile cast number is given as mean±SD (n = 8). # Indicates significantly different as compared with the BDL group ($P < 0.001$).

treatment significantly diminished oxidative stress and cellular macromolecules injury in the kidney of cholestatic animals. The nuclear factor erythroid 2–related factor 2 (Nrf2) is a fundamental signaling pathway for increasing the cellular antioxidant defense in oxidative stress.[86] Interestingly, it hs been repeatedly mentioned that CAR could significantly activate Nrf2 pathway and induce the expression of antioxidant enzymes.[87,88] The effects of CAR on such basic mechanism could provide better insight for its mechanism(s) of action and its therapeutic value in other oxidative-stress associated diseases. All these data mention the antioxidative properties of CAR as a pivotal mechanism involved in its renoprotective features in cholestatic animals.

It has also been found that kidney mitochondrial impairment could play a role in the pathogenesis of CN.[14] Mitochondria are the primary intracellular sources of ROS.[89] Hence, mitochondria-facilitated ROS formation plays a significant role in cholestasis-induced oxidative stress in the kidney.[14,89] Mitochondrial impairment in the kidney of cholestatic animals could lead to a lack of energy (ATP)-demand organ. The reabsorption of many chemicals (*e.g.,* amino acids, vitamins, glucose, *etc.*) is tightly dependent on ATP availability. Compounds such as bilirubin and hydrophobic bile acids and defect in chemical reabsorption. This situation is named Fanconi syndrome (FS).[90] FS is a prevalent complication in the cholestatic patients and animals.[91-93] Based on these data protecting renal mitochondria decrease not only mitochondria-mediated ROS formation but also improves mitochondrial function and energy metabolism. Therefore administration of mitochondria protecting agents could significantly enhance ATP level and function in Fanconi syndrome during cholestasis.[94-96] The positive effects of CAR on mitochondrial function have been repeatedly mentioned in previous investigations.[39,41-45,97] Therefore, the effects of CAR on mitochondria-facilitated ROS formation, enhancing cellular ATP levels, cellular macromolecules injury, as well as prevention of mitochondria-mediated cell death, could play a significant role in the cytoprotective properties of this peptide in CN.

The antifibrotic properties of CAR have been mentioned in various experimental models.[98-100] Different mechanisms could be involved in the renal fibrosis process. It is well known that oxidative stress and tissue fibrosis are two interconnected events.[101] The role of oxidative stress has also been highlighted in the renal tissue fibrosis process.[13-18] In the current investigation, we found that CAR possesses a significant role in decreasing renal fibrosis in CN rats. Based on these data, the antioxidative stress properties of CAR might play a significant role in renal fibrosis in cholestatic rats. The precise signaling pathways involved in the antifibrotic properties of CAR in the kidney of cholestatic animals need further studies to be revealed.

CAR is a safe agent that could be administered in high doses without significant adverse effects.[102] Moreover, this peptide has been revealed as an excellent hepatoprotective as well as a renoprotective agent in different experimental models.[103,104] Previously, we found that administration of high doses of CAR had no significant adverse effects

in critically ill rats (Cirrhotic rats), but also protects the liver tissue.[40,52,97,103] Hence, this peptide could be readily administered in human cases of CN.

Conclusion

Collectively, the data obtained from this study revealed significant protective properties of the peptide CAR against CN. The effects of CAR on oxidative stress markers, as well as the positive effects of this peptide on renal mitochondrial function, seem to play a significant role in its nephroprotective properties. Further studies are necessary to reveal the clinical implication of these data and the use of CAR as a therapeutic agent against CN.

Ethical Issues

All the experiments were performed in conformity with the guidelines for care and use of experimental animals (rats) that was approved by an ethics committee at Shiraz University of Medical Sciences, Shiraz, Iran (#19360).

Acknowledgments

This investigation was financially supported by the Vice-Chancellor of Research Affairs of Shiraz University of Medical Sciences (Grant number: 97-01-36-19360) and Shanxi Agricultural University (Grant numbers: 2018YJ33 and K271999031 for National Natural Science Foundation and Outstanding Doctors Volunteering to Work in Shanxi Province, respectively).

Conflict of Interest

The authors claim that there is no conflict of interest.

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