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## FULL PAPER

# Nephroprotective potential of carnitine against glycerol and contrast-induced kidney injury in rats through modulation of oxidative stress, proinflammatory cytokines, and apoptosis

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**Objective:** Contrast media (CM) are a major cause of nephropathy in high-risk patients. The aim of this study was to examine the effects of carnitine (CAR) in advanced nephrotoxicity due to CM administration in rats with glycerol-induced renal functional disorder.

**Methods:** 40 rats were divided randomly into five groups ( $n=8$ ): (1) healthy group; (2) glycerol only (GLY); (3) glycerol and CM (GLY+CM); (4) glycerol, CM and 200 mg kg<sup>-1</sup> carnitine (CAR200, Carnitene®; Sigma-tau/Santa Farma, Istanbul, Turkey); and (5) glycerol, CM and 400 mg kg<sup>-1</sup> carnitine (CAR400). Kidney injury was induced with a single-dose, intramuscular injection of 10 ml kg<sup>-1</sup> body weight (b.w.) of GLY. CAR was administered intraperitoneally. CM (8 ml kg<sup>-1</sup> b.w. iohexol, Omnipaque™; Opakim Medical Products, Istanbul, Turkey) was infused *via* the tail vein to the rats in Groups 3-5.

**Results:** L-carnitine administration significantly decreased serum creatinine and blood urea nitrogen levels. Superoxide dismutase and glutathione activity increased significantly in the treatment groups compared with

the nephrotoxic groups. CAR400 significantly reduced malondialdehyde levels to healthy levels. In the treatment groups, tumour necrosis factor (TNF)- $\alpha$ , transforming growth factor  $\beta$ , interleukin  $\beta$  and caspase-3 gene expression decreased compared with the nephrotoxic groups. TNF- $\alpha$  and nuclear factor kappa-beta (NF- $\kappa$ B) protein expression increased after CM and CAR administration reduced both TNF- $\alpha$  and NF- $\kappa$ B expressions. Histopathologically, hyaline and haemorrhagic casts and necrosis in proximal tubules increased in the nephrotoxicity groups and decreased in the CAR groups.

**Conclusion:** The results reveal that L-carnitine protects the oxidant/antioxidant balance and decreases proinflammatory cytokines and apoptosis in CM-induced nephrotoxicity in rats with underlying pathology.

**Advances in knowledge:** Depending on the underlying kidney pathologies, the incidence of CM-induced nephropathy (CIN) increases. Therefore, this is the best model to represent clinically observed CIN.

## INTRODUCTION

Contrast media (CM), which are used frequently in clinical applications such as imaging techniques and angiography, are a major cause of nephropathy in high-risk patients.<sup>1</sup> CM-induced nephropathy (CIN) is a very serious complication of intravascular administration.<sup>2</sup> When a 25% increase is observed in the serum creatinine level, or when

it is  $>44 \mu\text{mol l}^{-1}$  within 24-72 h after CM administration, the condition is defined as CIN.<sup>3</sup> CM-induced acute renal injury is the third most common nosocomial cause of acute renal damage, following surgery and hypotension.<sup>4,5</sup> CIN is associated with increased mortality and morbidity (generally defined as necessitating renal replacement therapy).<sup>6</sup> The intrahospital mortality rate of patients

who have developed renal failure and require dialysis following percutaneous coronary intervention is 40%, with the 2-year mortality reaching 80%.<sup>7</sup> Preventive management,<sup>8</sup> avoidance of nephrotoxic agents and a more frequent use of low-osmolar or iso-osmolar CM have been unsuccessful in decreasing the incidence of CIN.<sup>9</sup> Although the preventive effects of many molecules against CIN have been investigated,<sup>10–12</sup> there is no standardized treatment for the disorder.

The most important reason for the failure to find a treatment for CIN is that its pathophysiology has not yet been fully elucidated. Although many hypotheses regarding the pathophysiology of CIN have been proposed, oxidative tissue injury, which has recently become the most frequently studied mechanism, is a probable pathophysiological mechanism.<sup>13</sup> Studies have shown that oxidative stress due to free radical formation and direct tubular toxicity might play an important role in the development of CIN.<sup>14</sup> Therefore, agents such as *N*-acetylcysteine (NAC) and ascorbic acid have been considered to have renal protective effects against CIN because of their antioxidant properties.<sup>12</sup> In a previous study, the effects of *L*-carnitine, an antioxidant agent, against the oxidative stress response was investigated in experimental CIN in rats, and it was shown to have a protective effect against CIN.<sup>11</sup> In that study, the effects of *L*-carnitine were investigated by administering only the CM. However, when investigated clinically, it is well known that in CIN cases, another underlying renal pathology increases the risk of toxicity because of the CM. There are significant differences between the clinical features and the pathophysiological mechanisms of CIN alone and CIN in the presence of another underlying disorder of renal function.<sup>15</sup>

We aimed to investigate the roles of free oxygen radicals and the effect of *L*-carnitine, a very powerful antioxidant, in radio-CM-induced nephrotoxicity in rats with underlying glycerol-induced renal functional disorder.

## METHODS AND MATERIALS

### Animals

40 male albino Wistar rats were used for the experiments in this study. Each rat weighed 250–300 g, and all were obtained from the Atatürk University's Experimental Animal Laboratory of Medicinal and Experimental Application and Research Center. The rats were housed in standard plastic cages on sawdust bedding in an air-conditioned room at 22 °C under controlled light conditions (14/10 h light/dark cycle). Standard rat chow and tap water were provided *ad libitum*. All animal experiments and procedures were performed in accordance with national guidelines for the use and care of laboratory animals and were approved by the Atatürk University's local animal care committee.

### Chemicals

All of the chemicals used in the laboratory experiments were purchased from the Sigma-Aldrich (Munich, Germany). *L*-carnitine (Carnitene®) was purchased from Sigma-Tau/Santa Farma (Istanbul, Turkey). Iohexol (Omnipaque™) was purchased from Opakim Medical Products (Istanbul, Turkey). Glycerol was purchased from Bikar Pharmaceutical Co. (Istanbul, Turkey).

### Experimental design

The rats were divided into five groups of eight rats in each cage: Group 1, healthy group; Group 2, glycerol only (GLY); Group 3, glycerol and CM (GLY + CM); Group 4, glycerol, CM and 200 mg kg<sup>-1</sup> carnitine (CAR200); and Group 5, glycerol, CM and 400 mg kg<sup>-1</sup> carnitine (CAR400).

### Contrast media-induced nephrotoxicity model

Underlying kidney injury was induced in Groups 2–5 *via* intramuscular administration of 25% GLY in a single 10-ml kg<sup>-1</sup> body weight (b.w.) dose, divided among the legs. In addition to kidney injury, nephrotoxicity was induced *via* intravenous administration of CM (iohexol) in a single 8-ml kg<sup>-1</sup> b.w. dose over a period of 2 min to the animals in Groups 3–5, as described previously.<sup>16</sup> All of the rats (except for the control group) received the GLY injection after 24 h water deprivation. After the GLY injection, drinking water was resumed *ad libitum*. 30 min after the GLY was injected, the first CAR dose was administered intraperitoneally to the animals in the treatment groups. 24 h after the GLY injection, the CM were infused *via* the tail vein to the animals in Groups 3–5. CAR administration continued for 4 days. All of the rats were euthanized on the fifth day after intravenous CM treatment using an overdose of a general anaesthetic (thiopental sodium, 50 mg kg<sup>-1</sup>). Whole blood samples were collected *via* the intracardiac method. The serum was immediately separated by centrifugation at 4000 rpm for 10 min at 4 °C and stored at –80 °C. The kidney tissues were dissected out immediately. Half of one kidney was kept at –80 °C for biochemical analysis, the other half of the kidney was kept in RNA stabilization reagent for molecular analysis, and the second kidney was fixed in a 10% formalin solution for histopathological analysis.

### Determination of serum blood urea nitrogen and creatinine concentrations

Blood urea nitrogen (BUN) (lot number B0382A; BEN S.r.l, Milan, Italy) and creatinine (lot number B0914A; S.r.i) levels were detected using commercially available kits. All analyses were performed in a ChemWell® 2910 Automated EIA and Chemistry Analyzer (Awareness Technology, Inc., Palm City, FL).

### Biochemical investigation of kidney tissues

After the surgical procedures, the rat tissues were stored at –80 °C. All tissue samples from each rat were initially perfused with phosphate-buffered saline/heparin and then ground in liquid nitrogen using a TissueLyser II (Qiagen, Hilden, Germany) grinding jars set. Approximately 100 mg of the ground tissue were homogenized in 1 ml of phosphate-buffered saline homogenate buffer in an Eppendorf tube, using the TissueLyser II (Qiagen), and then centrifuged. Superoxide dismutase (SOD),<sup>17</sup> glutathione (GSH)<sup>18</sup> and malondialdehyde (MDA)<sup>19</sup> levels from the supernatant of each sample, and standards were measured at room temperature in duplicate *via* modified methods, using an enzyme-linked immunosorbent assay reader. The average absorbance of each sample and standard were calculated, a standard curve was plotted and an equation was obtained from the absorbance of the standards. Linear SOD, GSH and MDA concentrations were calculated according to this equation. The SOD, GSH and MDA levels in the tissues were expressed as U mg<sup>-1</sup>

protein,  $\text{nmol mg}^{-1}$  protein and  $\text{nmol mg}^{-1}$  protein, respectively. All data are presented as mean  $\pm$  standard deviation results based on 1 mg of protein.

#### Molecular investigations of kidney tissues

##### *Total RNA extraction and cDNA synthesis*

Total RNA extraction and cDNA synthesis were performed according to our previous data.<sup>20</sup> The tissues (20 mg) were stabilized briefly in an RNA stabilization reagent (RNAlater®; Qiagen) and then disrupted using the TissueLyser II ( $2 \times 2$  min for kidney; Qiagen). Total RNA was purified using a Qiagen RNeasy® Mini Kit according to the manufacturer's instructions in a QIAcube (Qiagen). The RNA samples were reverse transcribed into complementary DNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™; Foster City, CA). Using 10  $\mu\text{l}$ , total RNA was treated with 2  $\mu\text{l}$   $\times 10$  reverse transcription (RT) buffer, 0.8  $\mu\text{l}$   $\times 25$  deoxynucleotide (dNTPs) mix, 2  $\mu\text{l}$   $\times 10$  RT random primers, 1  $\mu\text{l}$  MultiScribe Reverse Transcriptase and 4.2  $\mu\text{l}$  diethyl pyrocarbonate-treated water (DEPC- $\text{H}_2\text{O}$ ). Reverse transcription was carried out at 25 °C for 10 min, followed by 120 min at 37 °C and finally, 5 min at 85 °C, using a Veriti® 96-Well Thermal Cycler (Applied Biosystems). cDNA concentration and quality were assessed and quantified using an Epoch™ Spectrophotometer System and Take3 Plate (BioTek®).

##### Relative quantification of gene expression

Relative tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , transforming growth factor (TGF)-1 $\beta$  and caspase-3 expression analyses were performed with the StepOnePlus™ Real-Time polymerase chain reaction (PCR) System (Applied Biosystems), using synthesized cDNA from rat kidney RNA. Quantitative PCR was run using TaqMan Probe mix, TaqMan® Probe-based technology (Applied Biosystems). Real-time PCR was performed using primers generated for rat TNF- $\alpha$  Rn00562055\_m1, rat IL-1 $\beta$  Rn00580432\_m1, rat TGF-1 $\beta$  Rn00572010\_m1, rat caspase-3 Rn00563902\_m1 and rat  $\beta$ -actin Rn00667869\_m1. The results are expressed as relative fold compared with the control animals. The expression data for  $\beta$ -actin in each tissue were used as endogenous controls. The primers and probes for  $\beta$ -actin were designed by Primerdesign (Southampton, UK). For each tissue, triplicate determinations were performed in a 96-well optical plate for both targets, using 9  $\mu\text{l}$  of cDNA (100 ng), 1  $\mu\text{l}$  of Primer Perfect Probe mix and 10  $\mu\text{l}$  of QuantiTect® Probe PCR Master Mix (Qiagen) in each 20- $\mu\text{l}$  reaction. The plates were heated for 2 min at 50 °C and for 10 min at 95 °C, and then 40 cycles of 15 s at 94 °C and cycles of 60 s at 60 °C were applied. All data are expressed as fold change in expression compared with the expression in the control group, using the  $2^{-\Delta\Delta C_t}$  method.<sup>21</sup>

##### Histological procedure

Kidneys from the rats in all groups were obtained, sectioned frontally and fixed in 10% neutral formalin for 48–72 h. Then, the tissues were processed routinely and embedded in paraffin wax, and 4- to 5- $\mu\text{m}$ -thick serial sections were cut. All tissue sections were stained with haematoxylin and eosin for histopathology and with Jones Stain (ScyTek, West Logan, UT) for basement membrane assessment and examined under a light microscope (Olympus BX51, Tokyo, Japan). Tubular necrosis, hyaline casts and haemorrhagic casts were evaluated and

scored for histopathological assessment. A minimum of five fields for each kidney slide at  $\times 100$  magnification were evaluated and assigned severity of change scores according to the following scales: Grade 0, – (negative); Grade 1, + (mild); Grade 2, ++ (moderate); Grade 3, +++ (severe); Grade 4, ++++ (most severe). The other sections were stored for immunohistochemistry.

##### Immunohistochemistry

Immunohistochemical (IHC) assessment was performed using an automated Leica BOND system, Leica BOND Dewax Solution (AR9222), Leica BOND Epitope Retrieval Solution 1 (AR9961) and Leica BOND Polymer Refine Detection (DS9800) (Leica Biosystems, Istanbul, Turkey). Epitope retrieval was performed for 20 min, followed by TNF- $\alpha$  and NF- $\kappa\text{B}$  primary antibody (Novocastra Laboratories, Newcastle-upon-Tyne, UK). IHC staining was observed under light microscopy ( $\times 100$ ).

##### Statistical analysis

SPSS® v. 13.0 software (IBM Corporation, Armonk, NY; formerly SPSS Inc., Chicago, IL) was used to conduct the statistical analysis. The results are presented as mean  $\pm$  standard deviation. Comparisons between groups were performed using one-way analysis of variance and Duncan's multiple comparison test. Significance was accepted at  $p < 0.05$ .

## RESULTS

### Biochemical results for kidney tissue

#### *Superoxide dismutase activity*

In the present study, SOD activity was significantly lower in the GLY and GLY + CM groups than in the healthy group ( $p < 0.05$ ). In addition, there were significant differences between the GLY and GLY + CM groups ( $p < 0.05$ ). In the treatment groups, SOD activity was significantly higher in both CAR dose groups, as shown in Figure 1a ( $p < 0.05$ ).

#### *Glutathione levels*

GSH levels were significantly lower in the GLY + CM and GLY groups than in the healthy group ( $p < 0.05$ ). In addition, there were significant differences between the GLY and GLY + CM groups ( $p < 0.05$ ). As seen in Figure 1b, GSH levels were significantly higher in the drug treatment groups. Administration of  $400 \text{ mg kg}^{-1}$  CAR increased GSH levels significantly to healthy levels ( $p < 0.05$ ).

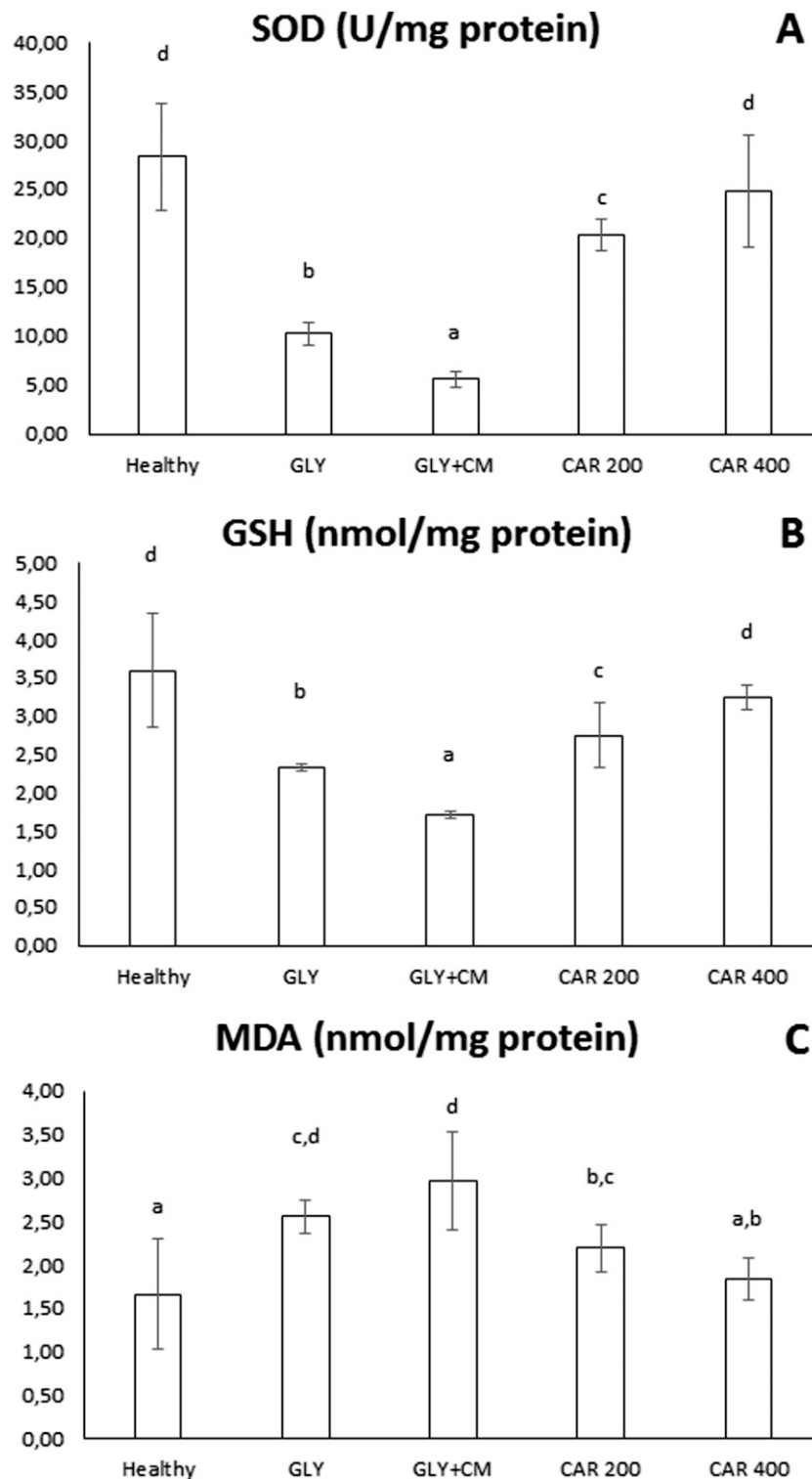
#### *Malondialdehyde levels*

As shown in Figure 1c, MDA levels were significantly higher in the GLY and GLY + CM groups than in the healthy group ( $p < 0.05$ ). In addition, there were significant differences between the GLY and GLY + CM groups ( $p < 0.05$ ). With  $400 \text{ mg kg}^{-1}$  CAR, MDA levels decreased significantly to healthy levels ( $p < 0.05$ ).

### Serum concentrations of blood urea nitrogen and creatinine

Changes in serum creatinine and BUN levels are shown in Figures 2a,b, respectively. The greatest production of BUN and creatinine serum levels was observed in the GLY + CM-induced nephrotoxicity group compared with the other groups ( $p < 0.05$ ). CAR administration significantly decreased serum

Figure 1. Effects of L-carnitine treatments on superoxide dismutase (SOD) activity, glutathione (GSH) and malondialdehyde (MDA) levels in rat kidneys. Means in the same column by the same letter are not significantly different to the test of Duncan ( $p < 0.05$ ). The values represent means  $\pm$  standard deviation. CAR, carnitine; CAR200, glycerol, contrast media and 200 mg kg<sup>-1</sup> carnitine; CAR400, glycerol, contrast media and 400 mg kg<sup>-1</sup> carnitine; CM, contrast media; GLY, glycerine.

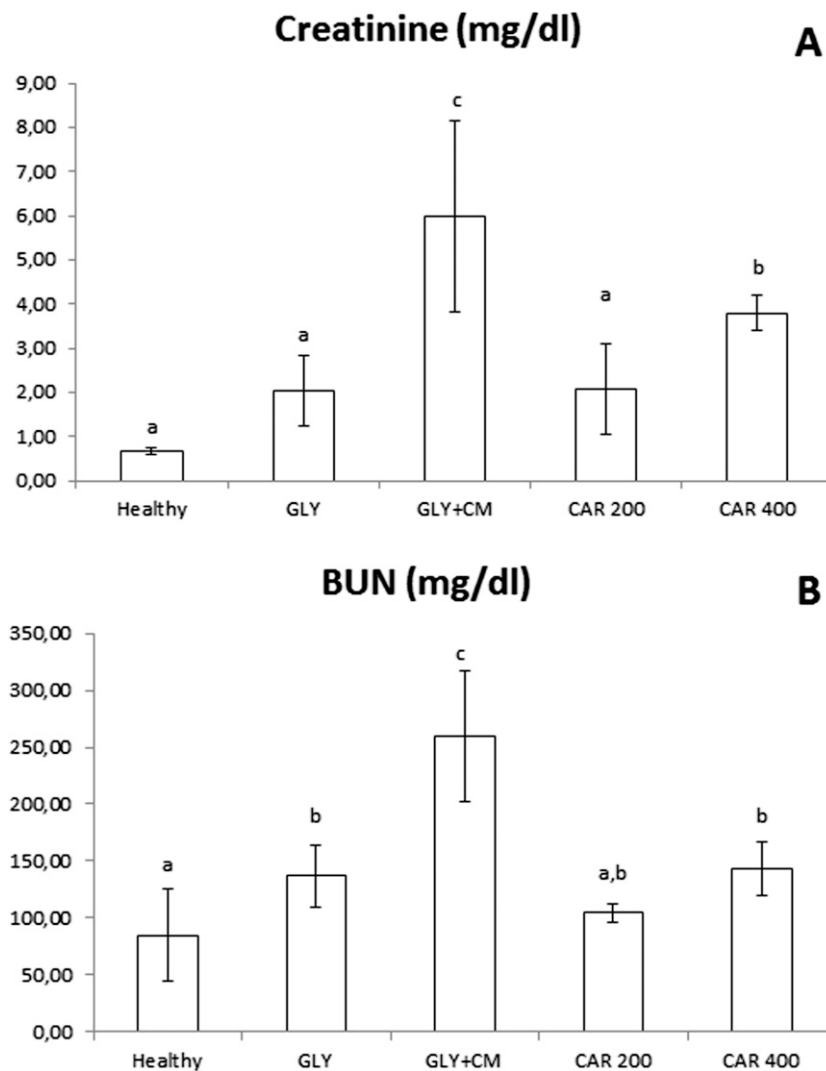


enzyme activity compared with the GLY + CM group ( $p < 0.05$ ). In addition, 200 mg kg<sup>-1</sup> CAR decreased BUN and creatinine levels significantly to healthy levels ( $p < 0.05$ ).

#### Molecular results

As shown in Figure 3a, TNF- $\alpha$  gene expression increased in the GLY and GLY + CM groups (10.92-fold and 27.9-fold,

Figure 2. Effects of L-carnitine treatments on blood urea nitrogen (BUN) and creatinine levels in rats' serum. Means in the same column by the same letter are not significantly different to the test of Duncan ( $p < 0.05$ ). The values represent means  $\pm$  standard deviation. CAR, carnitine; CAR200, glycerol, contrast media and 200 mg kg<sup>-1</sup> carnitine; CAR400, glycerol, contrast media and 400 mg kg<sup>-1</sup> carnitine; CM, contrast media; GLY, glycerine.



respectively) compared with the healthy group ( $p < 0.05$ ). The administration of 400 mg kg<sup>-1</sup> CAR resulted in a significant downregulatory effect on TNF- $\alpha$  mRNA expression, by 8.34-fold ( $p < 0.05$ ).

As shown in Figure 3b, TGF-1 $\beta$  mRNA level was significantly higher in the GLY and GLY + CM groups than the control group, by 7.73-fold and 13.78-fold, respectively ( $p < 0.05$ ). TGF-1 $\beta$  expression decreased in the kidney tissues of the 200- and 400 mg kg<sup>-1</sup> CAR groups. TGF-1 $\beta$  expression decreased by 2.7-fold in the 400 mg kg<sup>-1</sup> CAR group, and TGF-1 $\beta$  mRNA level decreased by 5.69-fold in the 200 mg kg<sup>-1</sup> CAR group compared with the control group ( $p < 0.05$ ).

We investigated IL-1 $\beta$  mRNA expression in the kidney tissues of rats using real-time PCR (Figure 3c). Compared with the control group, the IL-1 $\beta$  mRNA levels of the GLY and GLY + CM groups were significantly higher, by 19.21-fold and 29.9-fold,

respectively ( $p < 0.05$ ). The administration of 200 and 400 mg kg<sup>-1</sup> CAR resulted in a significant downregulatory effect on IL-1 $\beta$  mRNA expression, by 16.1-fold and 10.28-fold, respectively ( $p < 0.05$ ).

As shown in Figure 3d, caspase-3 gene expression increased in the GLY- and GLY + CM-induced nephrotoxicity groups (4.55-fold and 8.54-fold, respectively) compared with the healthy group ( $p < 0.05$ ). The administration of 400 and 200 mg kg<sup>-1</sup> CAR resulted in a significant downregulatory effect on caspase-3 mRNA expression, by 3.25-fold and 2.8-fold, respectively ( $p < 0.05$ ).

#### Histopathological results

Histopathological changes in the kidneys of all groups were examined and scored, and the results are provided in Table 1. The control group did not exhibit any histopathological lesions (Figure 4a). By contrast, severe lesions were seen in the cortex

Figure 3. Effects of L-carnitine treatments on relative mRNA expression levels of tumour necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$ , transforming growth factor (TGF)- $\beta$  and caspase-3 rats' kidneys. Expressions of mRNAs were detected by quantitative real-time polymerase chain reaction analysis.  $\beta$ -actin was used as the reference gene. Results are expressed as relative fold compared with control animals. Gene-specific probes were used as outlined under the Methods and materials section. The relative expression levels were calculated by the  $2^{-\Delta\Delta CT}$  method. Means in the same column by the same letter are not significantly different to the test of Duncan ( $p < 0.05$ ). The values represent means  $\pm$  standard deviation. CAR, carnitine; CAR200, glycerol, contrast media and 200 mg kg $^{-1}$  carnitine; CAR400, glycerol, contrast media and 400 mg kg $^{-1}$  carnitine; CM, contrast media; GLY, glycerine.

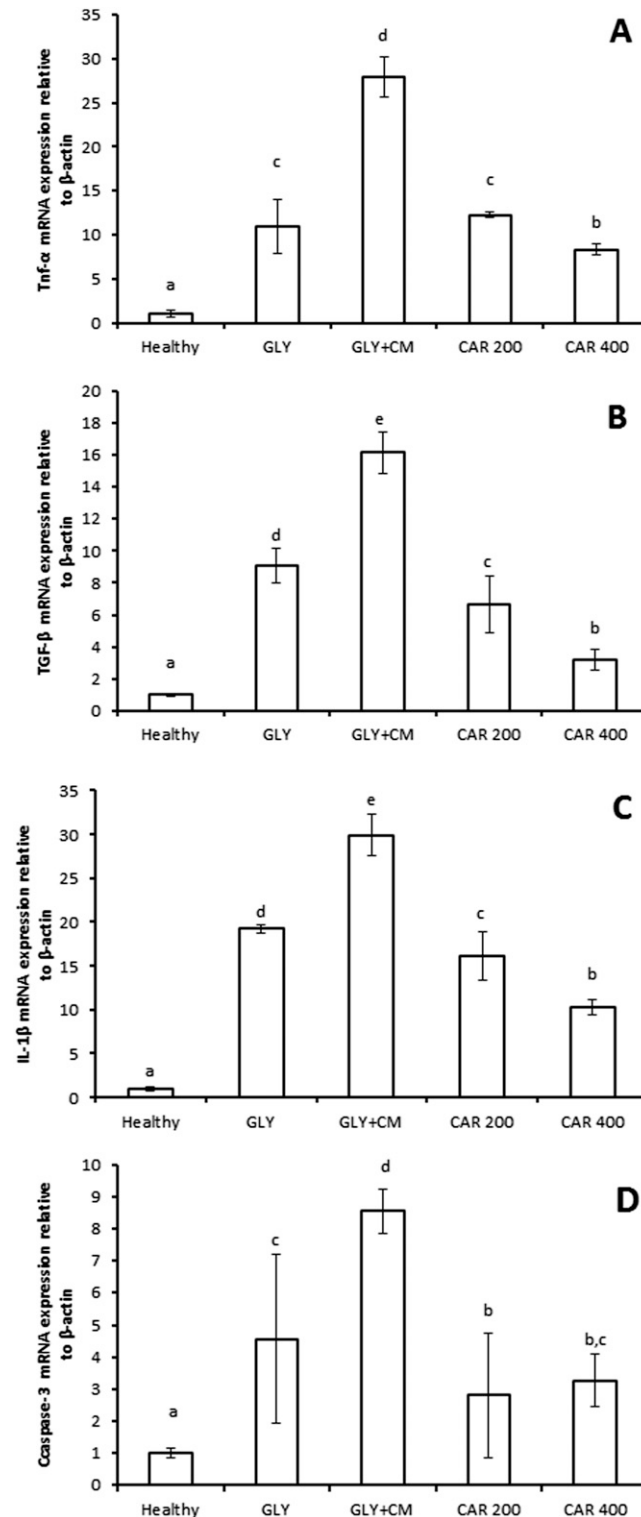


Table 1. Histopathological scores in groups

Groups	Healthy	GLY	GLY + CM	CAR200	CAR400
Tubular necrosis	–	+	+++	+++	+
Hyaline casts	–	++	++++	+++	+
Haemorrhagic casts	–	++	++	+	+

–, Grade 0 (negative); +, Grade 1 (mild); ++, Grade 2 (moderate); +++, Grade 3 (severe); +++++, Grade 4 (more severe); CAR200, glycerol, contrast media and 200 mg kg<sup>-1</sup> carnitine; CAR400, glycerol, contrast media and 400 mg kg<sup>-1</sup> carnitine; GLY, glycerol only; GLY + CM, glycerol and contrast media.

and medulla of the GLY-administrated rats. The renal sections showed moderate to severe hyaline casts and haemorrhagic casts and mild to severe tubular necrosis (Figures 4b–e). Necrosis was seen in the cortical segments of the proximal tubules, and less extensive changes were observed in the medulla. There were no

differences in Jones Stain for basement membrane assessment among the groups (Figures 5a–e).

Immunohistochemistry results are provided in Table 2. TNF- $\alpha$  (Figure 6) and NF- $\kappa$ B (Figure 7) immunopositivity were

Figure 4. Haematoxylin and eosin results in rats' kidney tissues; magnification  $\times 200$ . Healthy group (a); glycerol only group (b); glycerol and contrast media group (c); glycerol, contrast media and 200 mg kg<sup>-1</sup> carnitine group (d); and glycerol, contrast media and 400 mg kg<sup>-1</sup> carnitine group (e). (a) Normal architecture of the kidney in the healthy group. (b) Hyaline casts (arrowheads) and haemorrhagic casts (arrows), and tubular degeneration is seen mildly. In addition, increased glomerular cellularity (asterisk) is seen. (c) Haemorrhagic casts (arrows) are seen widely. In addition, hyaline casts (arrowheads) are present. Some glomeruli are seen enlarged and cellularity (asterisk) is increased. (d) Mild hyaline casts and haemorrhagic casts (arrows) are seen. Tubular degeneration and basophilic casts (arrowheads) are also present. (e) Glomeruli are seen hyperaemic (arrows). Glomerular cellularity (asterisk) is seen. Kidney histology is seen similar to control group animals except focal hyperaemic parenchyma.

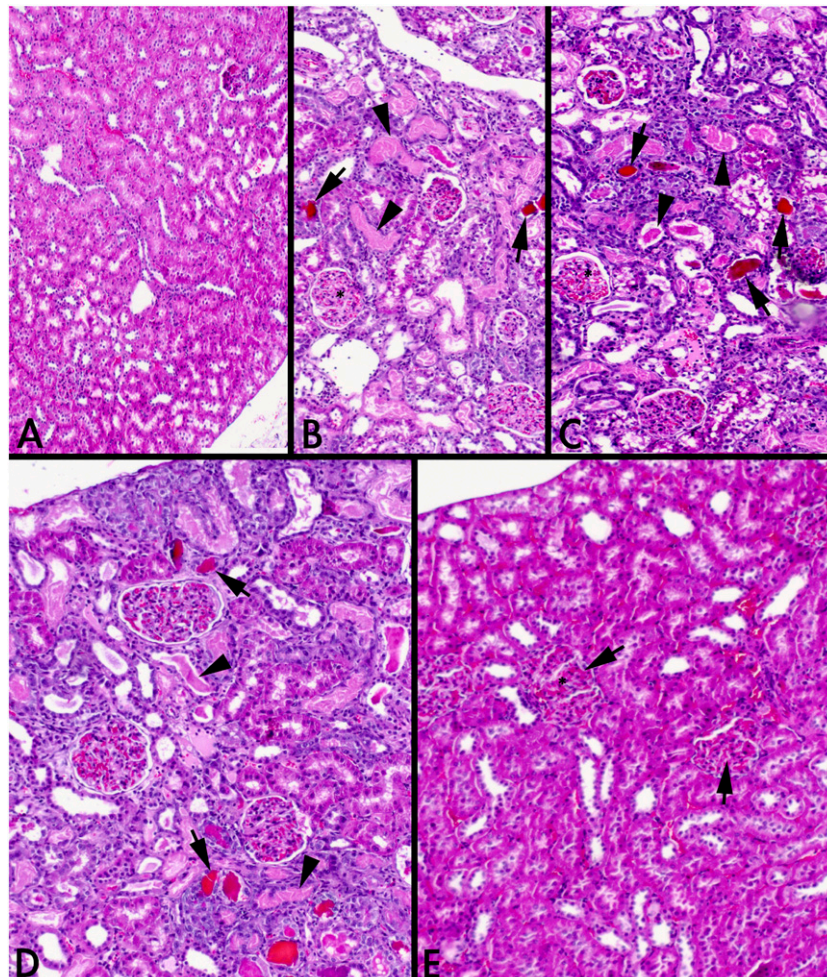
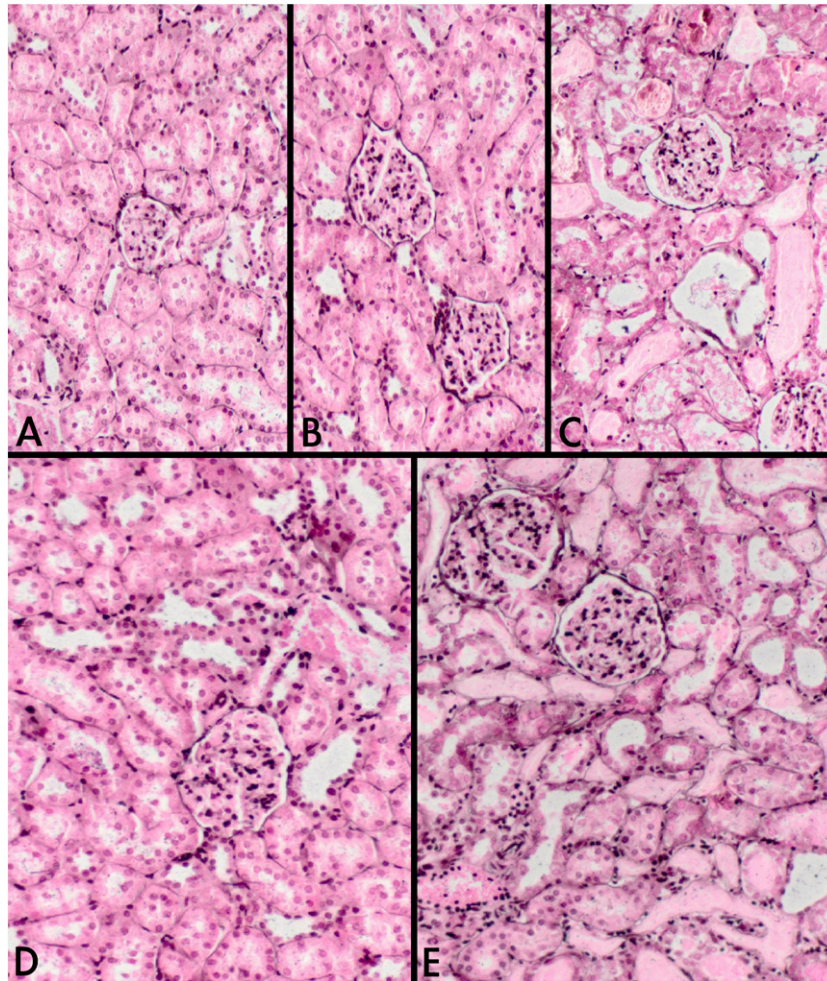


Figure 5. Jones staining results in rats' kidney tissues; magnification  $\times 400$ . Healthy group (a); glycerol only group (b); glycerol and contrast media group (c); glycerol, contrast media and  $200\text{ mg kg}^{-1}$  carnitine group (d) and glycerol, contrast media and  $400\text{ mg kg}^{-1}$  carnitine group (e). (a–e) All groups' Jones staining results are similar to those of the healthy group animal kidney.



observed in the glomeruli and tubular epithelium. Different grades of severity were seen between groups. Some vascular endothelia were also positive for TNF- $\alpha$  and NF- $\kappa$ B, in different severity levels.

The kidneys of the rats in the control group exhibited a mild or weak positive result for both TNF- $\alpha$  (Figure 6a) and NF- $\kappa$ B (Figure 7a). TNF- $\alpha$  was mildly expressed in the control and all other groups, except for Group 5 (Figures 6a–e). The maximum expression levels of TNF- $\alpha$  and NF- $\kappa$ B were found to be in Group 3 (Figures 6c and 7c). The kidneys of the rats in Group 2

(Figure 7b) and Group 5 (Figure 7e) demonstrated moderate NF- $\kappa$ B expression, whereas Group 3 demonstrated severe NF- $\kappa$ B expression (Figure 7c).

## DISCUSSION

Although it has been investigated in many studies, there is still a high incidence rate and a high mortality rate associated with CIN. Thus, many experimental and clinical studies are being conducted with the aim of preventing CIN. Yao *et al*<sup>22</sup> have suggested that this injury might be composed of inflammation, oxidative stress injury and apoptosis. In the present study, we

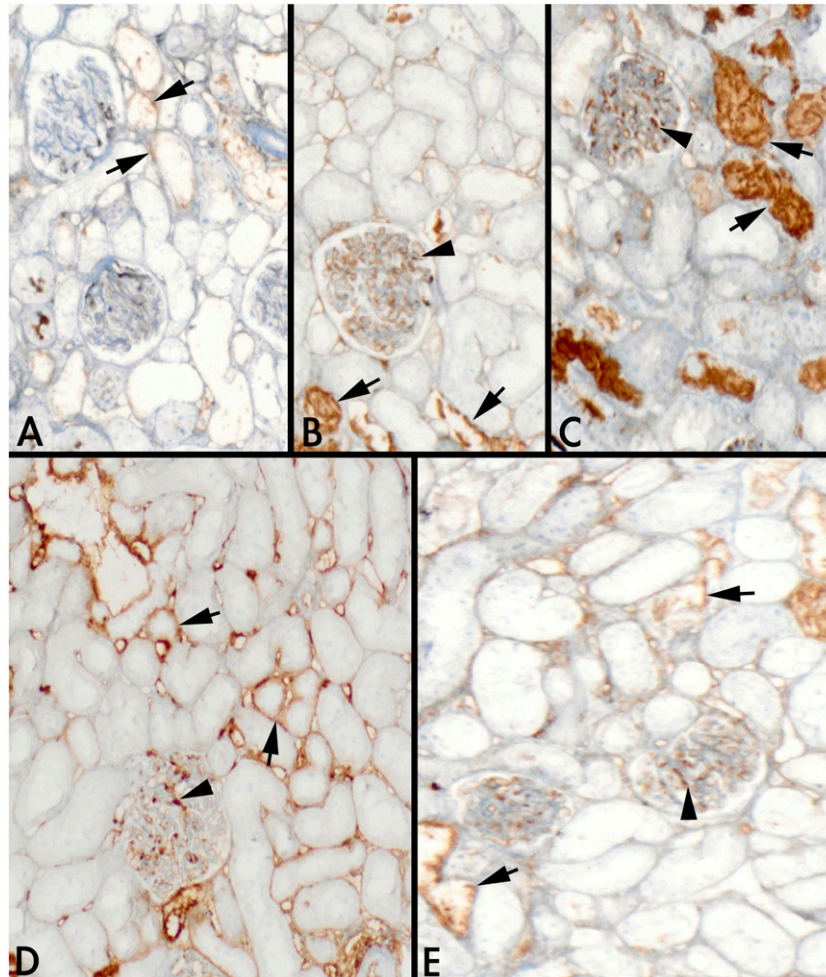
Table 2. Tumour necrosis factor (TNF)- $\alpha$  and nuclear factor kappa-beta (NF- $\kappa$ B) expression scores in groups

Groups	Healthy	GLY	GLY + CM	CAR200	CAR400
TNF- $\alpha$	+	+	+	+	+
NF- $\kappa$ B	+	++	+++	++	++

–, Grade 0 (negative); +, Grade 1 (mild); ++, Grade 2 (moderate); +++, Grade 3 (severe); +++++, Grade 4 (more severe); CAR, carnitine; CAR200, glycerol, contrast media and  $200\text{ mg kg}^{-1}$  carnitine; CAR400, glycerol, contrast media and  $400\text{ mg kg}^{-1}$  carnitine; CM, contrast media; GLY, glycerol only; GLY + CM, glycerol and contrast media.



Figure 6. Illustration of immunohistochemical tumour necrosis factor- $\alpha$  immunopositivity in rats' kidney tissues; magnification  $\times 200$ . Healthy group (a); glycerol only group (b); glycerol and contrast media group (c); glycerol, contrast media and 200 mg kg $^{-1}$  carnitine group (d); and glycerol, contrast media and 400 mg kg $^{-1}$  carnitine group (e). (a) Weak immunolabelling is seen in tubulus epithelium (arrows) and interstitium. 3,3'-diaminobenzidine (DAB) chromogen and haematoxylin counterstain. (b) Slight immunolabelling is seen in glomeruli (arrowhead) and tubulus epithelium (arrows). DAB chromogen and haematoxylin counterstain. (c) Severe immunopositivity is seen in glomeruli (arrowhead) and tubulus epithelium (arrows). DAB chromogen and haematoxylin counterstain. (d) Mild immunopositivity is seen in glomeruli (arrowhead) and tubulus epithelium (arrows). DAB chromogen and haematoxylin counterstain. (e) Slight immunopositivity is seen in glomeruli (arrowhead) and tubulus epithelium (arrows). DAB chromogen and haematoxylin counterstain.

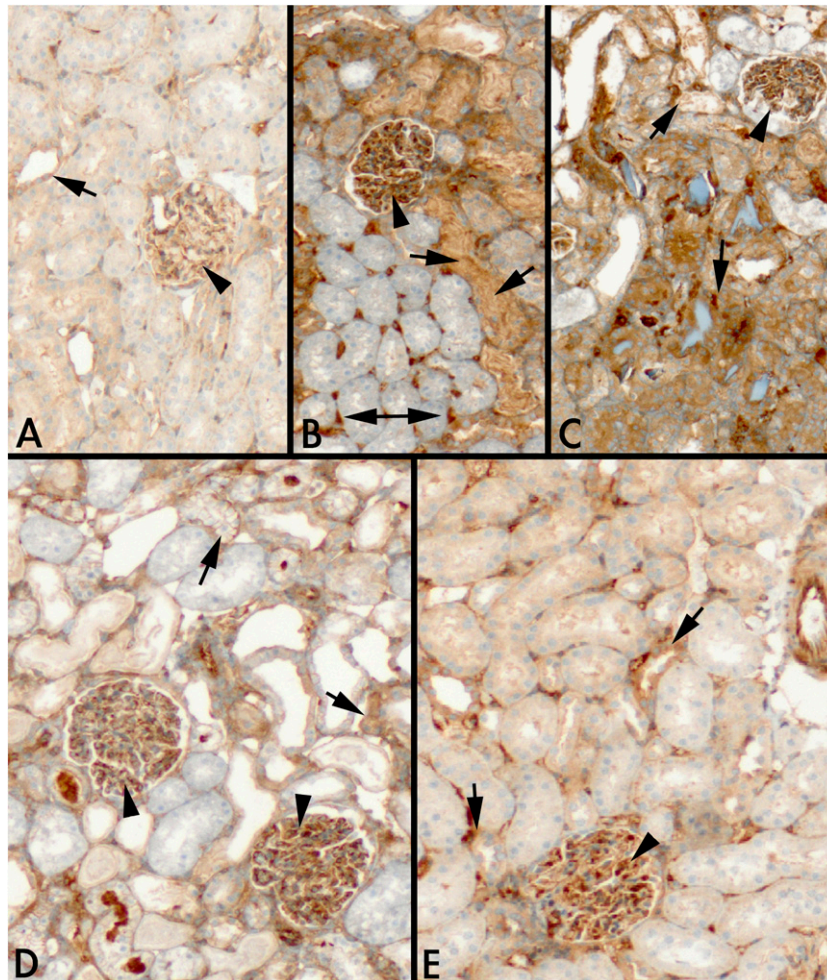


used molecular, histopathological and biochemical analyses to investigate the effects of two dosage levels (200 and 400 mg kg $^{-1}$ ) of L-carnitine, a powerful antioxidant, on injury due to CIN. Our results showed that administering L-carnitine significantly increased antioxidant levels, decreased oxidative parameters and reduced elevated renal injury biomarkers such as serum creatinine and BUN. We also determined that L-carnitine positively regulated elevated proinflammatory cytokine levels. In addition, we used various histopathological staining methods to show CM-induced renal injury and determined that tissue damage was more severe in the rats that received GLY treatment.

In nephrotoxicity, increases in the most significant markers of renal function tests, such as serum creatinine and BUN, should be monitored. Decreases in the levels of these biomarkers should

be helpful in showing the degree of improvement in renal function after drug administration.<sup>23</sup> In this context, previous experimental studies have reported increased creatinine and BUN levels in rats administered with GLY and CM.<sup>24</sup> Yenicieroglu et al<sup>25</sup> reported that serum creatinine levels increased significantly in their radiocontrast group compared with the control group. In a previous study, ebselen, an agent that has an antioxidant effect, was used in radio-CM-induced nephrotoxicity, and it decreased BUN and creatinine levels.<sup>26</sup> In light of this information, we used L-carnitine in glycerol and CM-induced nephrotoxicity, and our results supported those of previous studies. Although we observed significant increases in the BUN and creatinine levels of the GLY + CM group, the BUN and creatinine levels of the two treatment groups (200 and 400 mg kg $^{-1}$  CAR) significantly approached healthy values.

Figure 7. Illustration of immunohistochemical nuclear factor kappa-beta immunopositivity in rats' kidney tissues; magnification  $\times 200$ . Healthy group (a); glycerol only group (b); glycerol and contrast media group (c); glycerol, contrast media and  $200 \text{ mg kg}^{-1}$  carnitine group (d); and glycerol, contrast media and  $400 \text{ mg kg}^{-1}$  carnitine group (e). (a) Slight immunopositivity is seen in glomeruli (arrowhead) and light-positivity intubular basal layer and vascular endothelium (arrow). 3,3'-diaminobenzidine (DAB) chromogen and haematoxylin counterstain. (b) Moderate immunopositivity in glomeruli (arrowhead) and tubular epithelium (arrows). Some tubules have no positivity (two-headed arrow). DAB chromogen and haematoxylin counterstain. (c) Severe immunopositivity in glomeruli (arrowhead) and especially in tubular epithelium (arrows). DAB chromogen and haematoxylin counterstain. (d) Moderate immunopositivity in glomeruli (arrowheads) and especially in tubular epithelium (arrows). DAB chromogen and haematoxylin counterstain. (e) Severe immunopositivity in glomeruli (arrowhead) and particularly in tubular epithelium (arrows). DAB chromogen and haematoxylin counterstain.



In addition to renal injury biomarkers such as serum creatinine and BUN, many studies have shown that oxidative stress plays an important role in contrast and glycerol-induced renal injury and nephrotoxicity.<sup>27,28</sup> To explain the mechanisms related to CM-induced oxidative injury, we should start with renal medullary hypoxia.<sup>29</sup> The external region of the medulla is very sensitive to hypoxia, and the effect of CM on the medulla affects the delicate flow of oxygen and decreases blood pressure in the area.<sup>30</sup> Decreasing blood pressure results in increasing vasoconstrictor agents in that area.<sup>31</sup> Oxygen deprivation also impairs tubular reabsorption, thus leading to renal tubular and epithelial cell damage.<sup>32</sup> These mechanisms contribute to the cytotoxic effect of the CM. The damaged renal tubular cells, released vasoconstrictor agents and decrease in vasodilator effect lead to increasing levels of reactive oxygen species (ROS).<sup>33,34</sup>

Meanwhile, the decrease in nitric oxide production in the descending vasa recta damages the endothelial cell structure and increases oxidative stress.<sup>31,34</sup> The decreasing nitric oxide production in the descending vasa recta also increases the production of angiotensin-II, leading to greater vasoconstrictor effects and increased levels of free oxygen radicals.<sup>34</sup>

Another mechanism for increasing ROS is neutrophil infiltration, one of the most important factors in the production of free oxygen radicals.<sup>35</sup> Relevant to our subject, we can explain why CAR, which is a powerful antioxidant, exhibited an effect in our study. In a report presented by Jabbari et al,<sup>36</sup> L-carnitine administration prevented the development of CIN in a diatrizoate meglumine group. The most important difference between these studies and our study is that the other studies did

not use GLY, which makes kidneys more sensitive to the CM. This is the best model to represent clinically observed CIN, because the incidence of CIN is very low in normal kidneys, and depending on the underlying kidney pathologies, the incidence of CIN increases significantly. Therefore, our study is unique.

GSH depletion has been reported in renal injuries such as ischaemia/reperfusion. When injected intramuscularly, GLY causes renal GSH depletion.<sup>37</sup> Sinha et al<sup>38</sup> reported that an important mediator of arsenic-induced nephropathy was free oxygen radicals. In another CIN study, reduced SOD levels in nephrotoxicity groups increased with ebselen treatment.<sup>26</sup> In cisplatin-induced nephrotoxicity, significantly increased MDA levels were observed in groups administered with cisplatin.<sup>39</sup> In accordance with these studies, we showed that oxidative stress injury due to nephrotoxicity increased in our study. SOD and GSH levels were significantly lower in the renal tissues of the groups administered with the low CAR dose (200 mg kg<sup>-1</sup>) than in those of the GLY and GLY + CM groups. However, the SOD and GSH levels in the renal tissues of the rats administered with the high CAR dose (400 mg kg<sup>-1</sup>) were significantly closer to the healthy group. In this study, MDA levels increased significantly in the GLY and GLY + CM groups and decreased significantly in the CAR400 group. In previous studies that support our results, *N*-acetylcysteine, a GSH precursor, and ascorbic acid, a vitamin that has an antioxidant effect, were used in CIN and caused a decrease in free oxygen radicals and lipid peroxidation.<sup>40</sup>

As oxidative stress, another factor that plays a role in the pathogenesis of nephrotoxicity is the inflammatory process. In this process, macrophages released because of inflammation increase the production of proinflammatory cytokines in addition to the oxidant release.<sup>41–43</sup> In all cases that have developed an inflammatory response, proinflammatory cytokines, mainly TNF- $\alpha$  and IL-1 $\beta$ , are released.<sup>44</sup> Although increasing TNF- $\alpha$  is also observed during oxidative stress, an increase in ROS is also a messenger for increasing TNF- $\alpha$ .<sup>45,46</sup> In a study conducted on nephrotoxic rats that were administered with cisplatin, increased TNF- $\alpha$  levels were observed.<sup>39</sup> Similarly, in a GLY-induced nephrotoxicity study, TNF- $\alpha$  concentration increased significantly in rats that had nephrotoxicity.<sup>47</sup> Zhang et al<sup>48</sup> reported that the increases and decreases in IL-1 $\beta$  and TNF- $\alpha$  were parallel among renal injury-induced groups. In carboplatin-induced nephrotoxicity, TNF- $\alpha$  and oxidant levels increased, and *L*-carnitine administration significantly reduced levels of free oxygen radicals and TNF- $\alpha$ .<sup>49</sup> Similarly, in our study, TNF- $\alpha$  level decreased in the rats administered with CAR. The results of previous studies support our findings. In our real-time PCR results, the TNF- $\alpha$  mRNA expression level of the GLY + CM group increased significantly, and there was no significant difference between the CAR200 and GLY groups. However, there was a significant decrease in the TNF- $\alpha$  level of the CAR400 group compared with the GLY and GLY + CM groups.

In the cyclosporin-induced nephrotoxicity experiment, the development of interstitial fibrosis induced by cyclosporin was associated with TGF-1 $\beta$  released from macrophages, and the TGF-1 $\beta$  level was elevated in relation to cyclosporin.<sup>50</sup> In

another study related to this topic, IL-1 $\beta$  and TGF-1 $\beta$  levels increased in mice with induced nephrotoxicity.<sup>51</sup> The most important reason for increased TGF-1 $\beta$  levels following administration of a nephrotoxic agent is the fibrotic factor, as it is the main cytokine responsible for the progression of nephrotoxicity.<sup>52</sup> TGF-1 $\beta$  upregulation causes a breakdown of the renal extracellular matrix, leading to sclerosis and progressive renal failure. In our study, in agreement with the previous studies, TGF-1 $\beta$  level increased, but it decreased following the administration of drug therapy. Our drug therapy protected the renal mesenchymal cells and decelerated the renal injury process by decreasing TGF-1 $\beta$ .

Another important pathway that affects nephrotoxicity is apoptosis.<sup>22</sup> Caspase-3, an important marker of apoptosis, initiates apoptosis in renal tubules in the presence of nephrotoxicity.<sup>53</sup> In *in vitro* cell culture studies related to CM, severe cellular damage and apoptosis were demonstrated in endothelial and renal tubular epithelial cells.<sup>31</sup> In the present study, caspase-3 levels in the kidneys of rats administered with radio-CM increased significantly; however, those levels decreased significantly after CAR was administered. Caspase-3 level increased significantly in cisplatin-induced nephrotoxicity; after CAR was administered, the level decreased to near-normal values.<sup>54</sup> In our study, caspase-3 level, which was elevated due to nephrotoxicity, decreased after CAR was administered. A mechanism that explains the increasing apoptosis is the declining GSH levels, which enhances apoptotic cellular stimulus.<sup>55</sup> Other previous studies have also shown that apoptosis is associated with the increases observed in TNF- $\alpha$  and TGF-1 $\beta$  levels.<sup>56–61</sup>

Finally, when we analyzed the histopathological data, the results supported the molecular and biochemical results. Histopathologically, the renal histology of the rats impaired after GLY and GLY + CM administration started to improve following the administration of CAR. The improved BUN and creatinine levels in CIN kidneys in which CAR had been administered support our histopathological results. In addition, our IHC study of TNF- $\alpha$  supported our gene expression study results analyzed with real-time PCR. NF- $\kappa$ B levels increased in the nephrotoxicity groups, and the IHC staining method showed that CAR administration decreased those levels. Similar studies in the literature concluded that the NF- $\kappa$ B level identified by IHC plays an important role in nephrotoxicity injury.<sup>62</sup> Xu et al<sup>63</sup> showed that radio-CM administration increased NF- $\kappa$ B levels. We can explain that increase by several mechanisms. The first mechanism is that increases in IL-1 $\beta$  and TNF- $\alpha$  lead to an increase in NF- $\kappa$ B.<sup>64,65</sup> An increase in free oxygen radicals increases NF- $\kappa$ B as well.<sup>66</sup> However, there is an important relationship between TGF-1 $\beta$  and NF- $\kappa$ B.<sup>67</sup> In our study, NF- $\kappa$ B increased in parallel to radio-CM administration, correlated with the increasing levels of TNF- $\alpha$ , IL-1 $\beta$ , TGF-1 $\beta$  and free oxygen radicals. CAR administration reduced NF- $\kappa$ B in parallel to the other parameters. In another study, however, it was reported that CAR administration increased NF- $\kappa$ B.<sup>68</sup>

Although the preventive effects of many molecules against CIN have been investigated,<sup>10–12</sup> there is no standardized treatment for this disorder. Studies have shown that oxidative

stress due to free radical formation and direct tubular toxicity may play important roles in the development of CIN.<sup>14</sup> Therefore, agents such as NAC and ascorbic acid have been considered to have renal protective effects against CIN because of their antioxidant properties.<sup>12</sup> In addition, it is well known that when CIN cases are clinically investigated, another underlying renal pathology increases the risk of toxicity due to the CM.<sup>15</sup> Owing to all of these factors, we investigated the role of free oxygen radicals and the effect of L-carnitine. In accordance with our results and taking into account its many effects, we can suggest the use of L-carnitine as a prophylactic agent in patients with renal disorder who will be receiving radio-CM. Our results indicate that L-carnitine can be a drug of choice for both radiologists and nephrologists in daily practice.

## CONCLUSION

In the pathogenesis of developing complications in CIN cases, increased cytokine levels and apoptosis may play roles, along with oxidant/antioxidant balance. The results of this study reveal that L-carnitine protects the oxidant/antioxidant balance and decreases proinflammatory cytokines and apoptosis. Histopathologically, a significant improvement in renal tissues was observed using various staining techniques. With its many effects, L-carnitine treatment may be an effective option for patients with renal injuries if they have additional injury due to CIN. Future clinical and experimental studies would be beneficial.

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