

L-Carnitine protects kidney against ischemia reperfusion injury via suppression of expression of tubular kidney injury molecule (Kim-1) and Wnt proteins (Beta-Catenin)

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Abstract

L-Carnitine is a unique nutritional supplement for athletes that has been recently studied as a potential treatment for certain renal disorders. However, its efficacy in renal ischemia reperfusion injuries has not been investigated. Rats were randomly assigned to one of five groups (n = 6/group): 1, Control negative: received saline, 2, control positive I/R, rats received Sal and ischemia reperfusion at the end of fourth week and 3, L-Car +I/R group: same as Sal and ischemia reperfusion, but rats received L-Car (100 mg/kg) intraperitoneally daily for four weeks, 4, metformin +I/R group: same as Sal and ischemia reperfusion, but rats received oral metformin (300 mg/kg/day) daily for four weeks. 5, metformin +I/R group +L-Car: same as group 3, but rats received oral metformin (300 mg/kg/day) daily for four weeks. IR induced a significant increase in serum creatinine, serum K⁺ and serum KIM-1 peaked at 24 hours after I/R. KIM-1 and β -catenin expression is upregulated 168 hours after I/R. L-Car administration led to a significant decrease in serum creatinine in proportional to I/R group, serum K⁺ in proportional to I/R group, and a significant decrease in serum KIM-1 and Kim-1 expression. L-Car administration attenuated I/R-induced increase in oxidative stress marker MDA and increased antioxidant GSH activity. Moreover, the inducer of autophagy (Metformin) led to a significant decrease in serum creatinine, serum K⁺ and a significant decrease in serum Kim-1 and Kim-1 expression in renal tissue. Overall, our results suggest a potential therapeutic role of L-Car and metformin in I/R

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Introduction

Ischemia/reperfusion injury (I/R) is characterized by restriction of blood supply to an organ followed by restoration of blood flow and re-oxygenation. The inevitable injuries may occur after infarction, sepsis and organ transplantation and this phenomena exacerbate tissue damage by initiating an inflammatory cascade including reactive oxygen species (ROS), cytokines, chemokines, and leukocytes activation^{1, 2}. In the kidney, IRI contributes to pathological conditions called acute kidney injury that is a clinical syndrome with rapid kidney dysfunction and high mortality rates^{3, 4}. The pathophysiology of I/R in kidney is very complex but some pathological pathways such as activation of neutrophils, release of reactive oxygen species and other inflammatory mediators including adhesion molecules and a variety of cytokines are involved. Studies have demonstrated the beneficial effects of different agents in combat with I/R, for example, doxycycline through reducing the level of pro-inflammatory cytokines^{5, 6}, leptin by decreasing tumor necrosis factor alpha (TNF- α) level and increasing nitrite level⁷, levosimendan through antioxidant and NO-related mechanisms⁸, iloprost by suppression of lipid peroxidation⁹ and ascorbic acid via free radical scavenging and antioxidant activities^{10, 11}.

Wnt/ β -catenin signaling is an evolutionarily conserved, key developmental signaling pathway implicated in organogenesis, tissue homeostasis and disease development in multicellular organisms^{12, 13, 14, 15}. Over the past several years, substantial studies have demonstrated that Wnt/ β -catenin signaling could have a pivotal role in promoting tubular repair and regeneration after

acute kidney injuries induced by either ischemia-reperfusion injury (I/R) or nephrotoxins^{16, 17}. However, data are also mounting that aberrant activation of Wnt/ β -catenin is associated with proteinuria, renal function decline and kidney fibrosis in many forms of chronic kidney diseases, regardless of whether the injury initially occurs in renal tubule-interstitium or glomeruli^{18, 19, 20, 21, 22, 23}.

In the rat model of acute kidney injury induced by I/R, Wnt4 mRNA is reported to increase at 3 to 12 h while its protein level rises at 6 to 24 h after ischemia. This is associated with upregulation of cyclin D1 and cyclin A at 24 to 48 h, suggesting that this signaling may be instrumental for tubular cells entering the cell cycle, a key event in tubular repair and regeneration after damage. Indeed, overexpression of Wnt4 or β -catenin promotes cell cycle progression and increases the protein expression of cyclin D1 in tubular epithelial cells *in vitro*, indicating that stimulation of Wnt/ β -catenin could be reparative in ischemic acute kidney injury. In a mouse model of ischemic acute kidney injury, renal expression of multiple Wnts including Wnt1, 2, 3, 3a, 4, 7a, 8a, 8b, 10a and 16, and β -catenin are upregulated, and the magnitude of their induction is closely associated with the severity of I/R²⁴.

L-Car (L-b-hydroxy-g-N-trimethyl aminobutyric acid) is a non-protein amino acid which main sources in humans include both diet and endogenous synthesis. It is present in the free or acyl-carnitine form in the plasma¹¹. L-Car is widely found in animal foods such as meat, fish, milk and dairy products, and it can be synthesized in liver and kidney from the amino acids lysine and methionine. Oral administration of L-Car in drinking water has potential nephroprotective

effects against drug-induced nephropathy but its role in ischemia reperfusion is still defective¹¹.

We set out therefore to: (i) test the hypothesis that a unilateral renal I/R model can induce overexpression of tubular kidney injury biomarkers KIM-1 and β -catenin, if confirmed, (ii) explore the possible protective role of L-Car and inducer of autophagy metformin and their effects on oxidative stress markers, tubular kidney injury biomarker KIM-1 blood level and expression and β -catenin expression.

Materials and Methods

2.1. Experimental Animals

Thirty Sprague Dawley rats were used in the present study. The animals had free access to food and water and were housed in individual cages with a 12-hour light-dark cycle. The animals were adapted to these conditions for at least 1 week before being used in the experiments and general conditions were monitored throughout the study. All protocols and experimental procedures were approved by the Institutional Review Board (IRB) at Mansoura Faculty of Medicine on 20 April 2019 with Approval Code R.19.04.490-2019/04/20.

1.2. Study Groups

Rats were randomly assigned to one of five groups (n = 6/group): 1, Control negative: received saline (Sal group), 2, control positive I/R (Sal +I/R), rats received Sal and ischemia reperfusion at the end of fourth week and 3, L-Car +I/R group: same as Sal and ischemia reperfusion, but rats received L-carnitine hydrochloride (100 mg/ kg) intraperitoneally daily for four weeks, I/R was done at the end of fourth week¹¹, 4, metformin +I/R group: same as Sal and ischemia reperfusion, but rats received oral metformin (300 mg/kg/day) daily for four weeks, I/R was done at the end of

fourth week. 5, metformin +I/R group +L-Car: same as group 3, but rats received oral metformin (300 mg/kg/day daily for four weeks, I/R was done at the end of fourth week. Serum samples were obtained 24 hours, 72 hours, 120 hours and 168 hours after ischemia reperfusion surgery while renal tissue samples were obtained 168 hours after surgery only.

1.3. I/R surgery

After the right nephrectomy through median abdominal incision to make the solitary kidney model, the left renal artery was separated from the renal pedicle. In I/R group, the left renal artery was blocked for 45 minutes before reperfusion.

1.4. Collection of renal samples

After reperfusion, the animals were anesthetized with pentobarbital [0.6 ml/ kg] and the blood collected by heart puncture and allowed to clot for 30 minutes. Serum was separated by centrifugation at 2500 rpm for 15 minutes and used for biochemical estimations. After that rats were sacrificed by cervical dislocation and the abdomen of terminated animal was cut open quickly and the kidney was immediately removed and washed thoroughly with ice-cold 0.9 % sodium chloride solution [saline] and dried with filter paper and weighted. Then portion of the kidney was used to assess biochemical parameters.

2.5. Determination of creatinine and K^+ in serum

The assay is based on the reaction of creatinine with sodium picrate. Creatinine reacts with alkaline picrate forming a red complex. The time interval chosen for measurement avoids interference from other serum constituents. The intensity of the color formed is proportional to creatinine concentration in the plasma²⁵

Serum samples were separated by centrifugation at 3000 rpm for 15 min. The clear serum was received in dry sterile tubes and used directly for determination of potassium (K) ²⁶

2.6. Assay of lipid peroxidations marker malondialdehyde (MDA) and antioxidant reduced glutathione (GSH) Activity in renal tissues

About 50 -100 mg of kidney tissues will be homogenized in 1-2 ml cold buffer (50 mM potassium phosphate, pH 7.5, 1 mM EDTA) using mortar and pestle then centrifuged at 4,000 rpm for 15 minutes at 4°C. The supernatant will be kept at - 20 ° C until it is used for analysis. Malondialdehyde (MDA) and reduced glutathione (GSH) in the supernatant of kidney homogenates were measured using a colorimetric method according to the manufacturer's instructions (Bio-Diagnostics, Dokki, Giza, Egypt).

2.7. Measurement of tubular kidney injury molecule (Kim-1) by ELISA

Tubular Kidney injury molecule (Kim-1), as a biomarker of acute kidney injury, can sensitively reflect the renal protective effect by L-Car and metformin. It was measured using Rat Kim-1 Sandwich ELISA Kit according to the manufacturer's instructions (Rat Kim-1 ELISA Kit, Boster immunoboster. Fremont, CA, USA).

2.8. Histopathological examination of kidney tissue by Hematoxylin and Eosin

Twenty micrometer-thick sections of kidney slices were stained with hematoxylin and eosin (H&E), and the slides of kidney were stained with hematoxylin for 15 minutes and in HCl alcohol solution for 35 seconds. Then, the sections were immersed with eosin for 10 minutes and 90% ethanol for 40 seconds. After that, the section was

examined, and images of the kidney tissue were captured under light microscope.

2.9. Immunohistochemical study of a novel biomarker for the early detection of kidney tubular injury after ischemia/reperfusion injury (β - Catenin)

Serial coronal sections (40 μ m) were sliced using a freezing sledge microtome, and a 1:6 series was used for all quantitative immunohistochemistry. Peroxidase-based immunostaining was completed. In brief, following quenching of endogenous peroxidase activity where appropriate (using a solution of 3% hydrogen peroxide/10% methanol in distilled water) and blocking of nonspecific secondary antibody binding (using 3% normal serum in Tris-buffered saline (TBS) with 0.2% Triton X-100 at room temperature for 1 h), sections were incubated overnight at room temperature with the appropriate primary antibody diluted in 1% normal serum in TBS with 0.2% Triton X-100 (polyclonal anti- β -catenin rabbit antibody, Cat#YPA1340, dilution 1:200; Chongqing Biospes Co., Ltd., Chongqing, China).

2.10. RNA Extraction and First-strand cDNA synthesis

For all animals, the kidney was removed for RNA extraction under standard sterile surgical method. Total RNA was extracted from kidney tissue using Trizol Reagent [Invitrogen, USA] according to the manufacturer's description and treated with RNase-free DNase to remove any residual genomic DNA. Single stranded cDNAs were synthesized by incubating total RNA [1 μ g] with Revert Aid H Minus M-MuL V Reverse transcriptase [200 U], oligo-[dT] primer [5 μ M], Random Hexamer Primer [5 μ M], dNTPs [1 mM],

and Ribo Lock RNase-inhibitor [20 U], for 5min at 25°C followed by 60 min at 42°C in a final volume of 20 µl. The reaction was terminated by heating at 70°C for 5 min.

2.11. Real-time RT-PCR Analysis of Kim-1 Expression

Primers for the Kim-1 gene (NM 173149, forward primer: TGGCACTGTGACATCCTCAGA; reverse primer: GCAACGGACATGCCAACATA) and the house keeping GAPDH gene (NM 017008, forward primer: CCTGGAGAAACCTGCCAAGTAT; reverse primer: AGCCCAGGATGCCCTTTAGT) were designed using ABI Primer Express software (Applied Biosystems, Foster City, CA). Total RNA was reverse transcribed with ABI High-Capacity cDNA Reverse Transcription kit and then subjected to real-time PCR using ABI SYBR green PCR master mix (Applied Biosystems, Cheshire, UK). The cycle time values of Kim-1 were normalized with GAPDH, and the relative differences between control and Cd groups were calculated and expressed as percentage of controls.

2.12. Statistical Analysis

Values are presented as the mean (+/-) SD by using SPSS (SPSS, Sigma Plot Software, Inc, Chicago, IL) program statistical package for social science version 17. One-way analyses of variance (ANOVA) were used to compare each molecular variable between control negative (Saline), control positive (I/R) and experimental I/R + L-Car, I/R + metformin, I/R + L-Car + metformin groups. When a significant interaction was detected, post-

hoc t-tests were used to compare the two groups at different time points. Pearson correlation analyses were used to study the relationships between serum K⁺, serum creatinine, serum KIM-1 and KIM-1 expression in I/R group. All data were analyzed using SPSS (SPSS, Sigma Plot Software, Inc, Chicago, IL) program statistical package for social science version 17. Results were considered significant when (P <0.05).

Results

3.1. The Effects of I/R, L-Car, metformin and (L-Car + metformin) on serum creatinine level (mg %)

Table (1) showed that intervention in the left kidney disturbed renal functions. The data strongly suggest renal impairment developed 24 hours after renal reperfusion. I/R led to a significant increase in serum creatinine 24 hours after renal reperfusion in proportional to control. While it decreased, significantly, in L-Car group and metformin group in proportional to I/R group. Also, I/R led to a significant increase in serum creatinine 72 hours after renal reperfusion, in proportional to control. While it decreased, significantly, in the L-Car group and metformin group, in proportional to I/R group. Also, I/R led to a significant increase in serum creatinine 120 hours after renal reperfusion, significantly, in proportional to control. While it decreased, significantly, in L-Car group and metformin group, in proportional to I/R group. Also, I/R led to a significant increase in serum creatinine 168 hours after renal reperfusion, it increased, significantly, in proportional to control. While it decreased, significantly, in L-Car group and metformin group, in proportional to I/R group.

Table (1): Effect of I/R, L-carnitine hydrochloride, metformin and (L-Car + metformin) on serum creatinine level (mg %) 24 hours, 72 hours, 120 hours and 168 hours after ischemia reperfusion

	Control	I/R group	L-Car +I/R group	Metformin +I/R group	L-Car + Metformin + IR
0 h.	0.59 ± 0.08	0.77 ± 0.3*	0.85 ± 0.3*	0.94 ± 0.3*	0.75 ± 0.4*
24 h.	0.52 ± 0.08	2.32 ± 0.1*	1.66 ± 0.21*#	1.72 ± 0.1*#	1.57 ± 0.1*#
72 h.	0.73 ± 0.07	1.77 ± 0.17*	0.92 ± 0.11*#	0.87 ± 0.2*#	1.43 ± 0.1*#
120 h.	0.55 ± 0.07	1.42 ± 0.19*	0.88 ± 0.1*#	0.76 ± 0.1*#	1.44 ± 0.1*
168 h.	0.61 ± 0.08	1.22 ± 0.21*	0.74 ± 0.1#	0.66 ± 0.1#	1.227 ± 0.1*

Test used: ANOVA followed by posthoc Tukey for multiple comparisons. Values are expressed as mean ± S.D. (n = 6). *(P <0.05): significant in proportional to control.# (P <0.05): significant as compared with I/R group.

3.2. The Effects of I/R, L-Car, metformin and (L-Car + metformin) on serum K⁺ level (mmol/L.)

Table (2) showed that intervention in the left kidney disturbed renal functions. The data strongly suggest renal impairment developed 24 hours after renal reperfusion. I/R led to a significant increase in serum K⁺ level, it increased, significantly, 24 hours after renal reperfusion in proportional to control. While it decreased, significantly, in the L-Car group and metformin group, in proportional to I/R group. Also, I/R led to a significant increase in serum K⁺ level 72 hours after renal reperfusion, it increased, significantly, in proportional to control.

While it decreased, significantly, in the L-Car group and metformin group, in proportional to I/R group. Also, I/R led to a significant increase in serum K⁺ level 120 hours after renal reperfusion, it increased, significantly, in proportional to control. While it decreased, significantly, in the L-Car group and metformin group, in proportional to I/R group. Also, I/R led to a non-significant increase in serum K⁺ level 168 hours after renal reperfusion, in proportional to control. While it decreased, significantly, in L-Car group and metformin group, respectively, in proportional to I/R group.

Table (2): Effect of I/R, L-carnitine hydrochloride, metformin and (L-Car + metformin) on serum K⁺ level (mmol/L.) 24 hours, 72 hours, 120 hours and 168 hours after ischemia reperfusion

	Control	I/R group	L-Car +I/R group	Metformin +I/R group	L-Car + Metformin + IR
0 h.	4.7 ± 0.2	4.9 ± 0.2	4.9 ± 0.5	4.5 ± 0.9#	4.5 ± 0.6#
24 h.	4.6 ± 0.2	5.7 ± 0.3*	5.1 ± 0.2*#	4.9 ± 0.3#	4.5 ± 1.5#
72 h.	4.77 ± 0.06	5.4 ± 0.1*	4.5 ± 0.2#	4.4 ± 0.1#	4.4 ± 1.1#
120 h.	4.38 ± 0.02	4.9 ± 0.4*	4.5 ± 0.2#	4.4 ± 0.4#	4.9 ± 1.3
168 h.	5.1 ± 0.06	5.4 ± 0.2	4.5 ± 0.2#	4.7 ± 0.2#	4.9 ± 0.7#

Test used: ANOVA followed by posthoc Tukey for multiple comparisons. Values are expressed as mean ± S.D. (n = 6). *(P <0.05): significant in proportional to control. # (P <0.05): significant as compared with I/R group.

3.3. The Effects of I/R, L-Car, metformin and (L-Car +I/R + metformin) on serum tubular kidney injury molecule (Kim-1)

Table (3) showed that intervention in the left kidney disturbed renal functions. The data strongly suggest renal impairment developed 24 hours after renal reperfusion. I/R led to a significant increase

in serum Kim-1, 24 hours after renal reperfusion in proportional to control. While it decreased, significantly, in the L-Car group and metformin group, in proportional to I/R group. Also, I/R led to a significant increase in serum Kim-1 72 hours after renal reperfusion, in proportional to control. While it decreased, significantly, in the L-Car group and metformin group, in proportional to I/R group. Also, I/R led to a significant increase in

serum Kim-1 120 hours after renal reperfusion, in proportional to control. While it decreased, significantly, in L-Car and metformin groups, in proportional to I/R group. Also, I/R led to a significant increase in serum Kim-1 168 hours after renal reperfusion, in proportional to control. While it decreased, significantly, in L-Car and metformin groups, in proportional to I/R group.

Table (3): Effect of I/R, L-carnitine hydrochloride, metformin and (L-Car + metformin) on serum Kim-1 (pg/ml.) 0 h., 24 hours, 72 hours, 120 hours and 168 hours after ischemia reperfusion

	Control	I/R group	L-Car +I/R group	Metformin +I/R group	L-Car + Metformin + IR
0 h.	616 ± 96.8	633 ± 115.7	703± 154.6	605 ± 93	639 ± 104
24 h.	649 ± 201.8	3184± 399*	1087 ± 267*#	1059 ±110*#	1042 ±110*#
72 h.	641 ± 207.8	2289± 325*	990 ± 218*#	890 ±192*#	1039 ±90*#
120 h.	647 ± 202.8	2100± 317*	991 ± 210*#	883 ±175*#	1021 ±77*#
168 h.	655 ± 201.8	1783± 199*	874 ± 177*#	877 ±169*#	959 ±108*#

Test used: ANOVA followed by posthocTukey for multiple comparisons. Values are expressed as mean ± S.D. (n = 6). *(P <0.05): significant in proportional to control.# (P <0.05): significant as compared with I/R group.

3.4. The Molecular Effects of L-Car on malondialdehyde (MDA) and reduced glutathione (GSH)

Table (4) showed that I/R led to a significant increase in renal tissue MDA concentration, 168 hours after renal reperfusion in proportional to control. While it decreased, significantly, in L-Car group, metformin group and L-Car + metformin

group, in proportional to I/R group. Moreover, I/R led to a significant decrease in renal tissue GSH activity, 168 hours after renal reperfusion, in proportional to control. While it increased, significantly, in L-Car group, metformin group and L-Car + metformin group, in proportional to I/R group.

Table (4): Effect of I/R, L-carnitine hydrochloride, metformin and (L-Car + metformin) on malondialdehyde (MDA) (nmol/g. renal tissue) and reduced glutathione (GSH) (nmol/g. renal tissue) 168 hours after ischemia reperfusion

	Control	I/R group	L-Car +I/R group	Metformin +I/R group	L-Car + Metformin + IR
MDA (nmol/g. renal tissue)	10.55 ± 0.1	15.3 ± 1.49*	12.3 ± 1.07*#	11.3 ± 1.66*#	12.9 ± 3.11*#
GSH (nmol/g. renal tissue)	119.72± 9.32	48.76*	101.33*#	105 ± 27.4*#	94.2 ± 6.8*#

Test used: ANOVA followed by posthocTukey for multiple comparisons. Values are expressed as mean ± S.D. (n = 6). *(P <0.05): significant in proportional to control. # (P <0.05): significant as compared with I/R group.

3.5. The Effects of I/R, L-Car and metformin on morphology of tubular cells (168 hours post I/R)

Kidney sections from I/R group (Figure 1 A) showed irregular arrangement of cells with a reduction in the number of cells, and the cells

showed pyknosis (Darkly-stained nucleus and cytoplasm). The number of abnormal cells was significantly reduced in the kidney tissues obtained from (L-Car + I/R) group (Figure 1 B) and (Metformin + I/R) group (Figure 1 C) and (L-Car + Metformin + I/R) group (Figure 1 D).

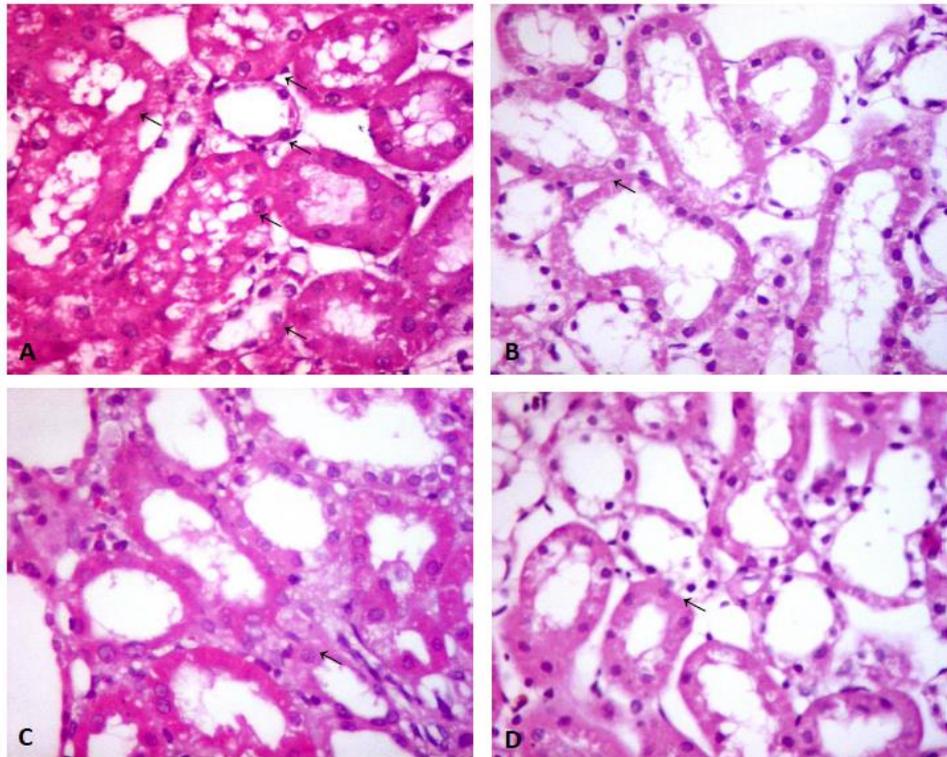


Figure (1): Histopathological examination of the renal tubules. A) H&E staining of I/R group (n=6) (H&E, 400 X): swollen proximal tubules epithelial lining with loss of brush border, even the tubules lumen is very narrow due to the swollen epithelial lining (arrowhead) B) H&E staining of L-Car + I/R group (n=6) (H&E, 400 X): L-Car administration attenuated renal injury induced by I/R. C) H&E staining of the metformin +I/R group (n=6) (H&E, 400 X): metformin administration attenuated tubular injury induced by I/R. D) H&E staining of I/R + metformin + L-Carnitine (n=6) (H&E, 400 X): attenuated renal injury induced by I/R.

3.6. The Effects of I/R, L-Car and metformin on beta-catenin expression in the renal tubules 168 hours post I/R

Kidney sections obtained from I/R group showed enhanced expressed Wnt proteins (β - Catenin) which were localized in both injured proximal and distal renal tubules (Figure 2 A). While L-Car administration led to a significant

decrease in this marker expression (Figure 2 B). Also, metformin administration led to a significant decrease in its expression (Figure 2 C). Moreover, metformin and L-Car administration led to a significant decrease in its expression (Figure 2 D).

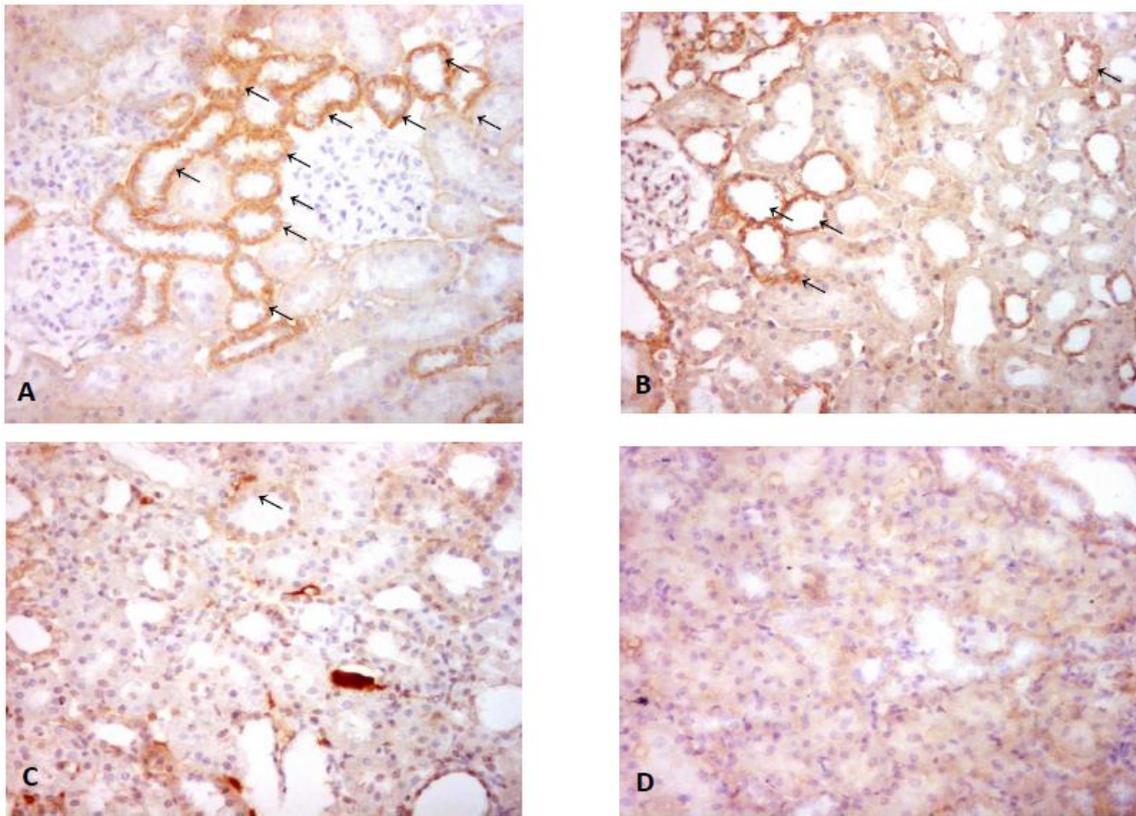


Figure (2): Enhanced expressed Wnt protein (β - Catenin) was localized in both the injured proximal and distal tubules following I/R. Histopathological examination of renal tubules. A) Immunostaining of I/R group (n=6): showed increased endogenous (β - Catenin) level, brown stain (arrowhead) (Magnification, 200X) B) L-Car administration led to a significant decrease in the biomarker of kidney injury (β - Catenin) (Magnification, 200X). C) Metformin administration led to a significant decrease in the biomarker of kidney injury (β - Catenin) (Magnification, 200X) D) Metformin and L-Car administration led to a significant decrease in the biomarker of kidney injury (β - Catenin) (Magnification, 200X).

3.7. The Effects of I/R, L-Car, metformin and (L-Car + metformin) on kidney injury molecule (Kim-1) expression in the renal tubules 168 hours post I/R

Figure (3) showed that kidney tubular injury molecule-1 (KIM-1) mRNA and protein

expressions increased in renal tissues obtained from rats subjected to I/R compared to Sal control rats. While L-Car treatment attenuated the increased expression of KIM-1 at the mRNA and protein levels. In addition, metformin + I/R, as well as, L-Car + metformin+ I/R decreased its expression.

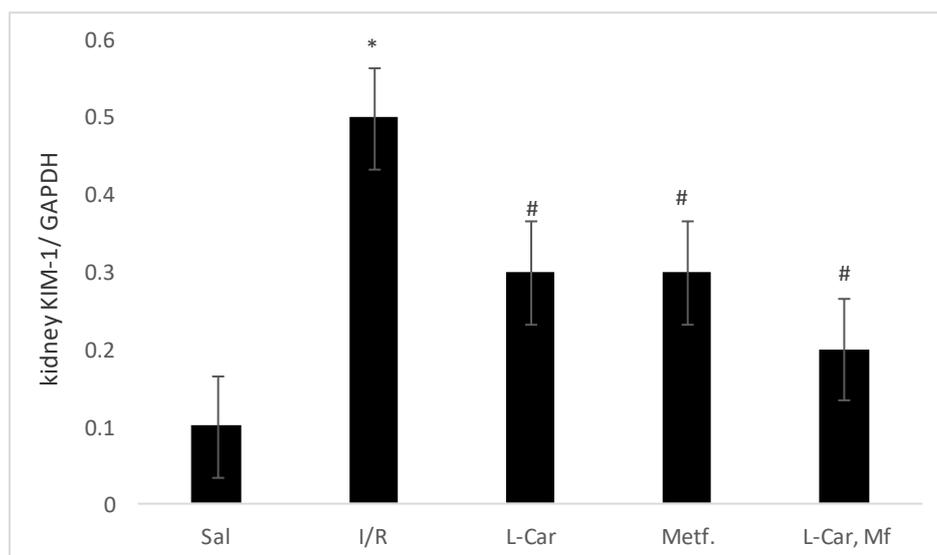


Figure (3): Products of RT-PCR for Kim-1 in Sal control group [1], I/R group [2], L-Car +I/R group [3], metformin +I/R group [4] and L-Car +metformin +I/R group [5] (168 hours post I/R). *(P <0.05): significant as compared with Sal group. # (P <0.05): significant as compared with I/R group. Sal: saline

3.8. Correlation between serum K⁺, serum creatinine, serum kidney injury molecule (Kim-1) and KIM-1 expression in renal tubules in I/R group

Table (5) showed that I/R led to a significant positive correlations between serum K⁺, serum creatinine, serum KIM-1 and KIM-1 expression.

Table (5): Correlations between serum K⁺, serum creatinine, serum KIM-1 and KIM-1 expression in I/R group

Parameters		Serum K	Serum Creatinine	Serum KIM-1	KIM-1 expression
Serum K ⁺	r		0.910	0.830	0.801
	p		0.000	0.000	0.000
Serum Creatinine	r			0.514	0.464
	p			0.010	0.022
Serum KIM-1	r				0.860
	p				0.000

Kim-1: Kidney injury molecule. r: Pearson's correlation coefficient p: Probability. P < 0.05: is considered significant. P < 0.001: is considered highly significant.

Discussion

The main findings in the present study are: (a) I/R increased serum creatinine and serum K⁺ and serum KIM-1 significantly in proportional to control, which peaked at 24 hours post ischemia/reperfusion injury (I/R) (b) β -catenin and KIM-1 expressions were upregulated in the injured renal tubules post ischemia/reperfusion injury (I/R)

(c) L-Car administration led to a significant decrease in serum creatinine, serum K⁺ and serum Kim-1, β -catenin and Kim-1 expression in proportional to I/R (d) the inducer of autophagy (Metformin) led to a significant decrease in serum creatinine, serum K⁺ and serum Kim-1, Kim-1 expression and β -catenin expression in proportional to I/R

Following I/R, the kidney often recovers its structure and function via adaptive repair and regeneration. This process can be considered as an innate wound-healing response consisting of the injury, repair and recovery phases. Both gain- and loss-of-function studies indicate that Wnt/ β -catenin plays an essential role in minimizing initial kidney damages and promoting adaptive repair and regeneration after acute kidney injuries^{27, 28, 29}. Kidney injury repair following acute kidney injuries involves several distinct biologic events including tubular damage/cell death, tubular cell proliferation, followed by resolution of renal fibroblasts and inflammation, and restoration of microvasculature. It appears that Wnt/ β -catenin signaling participates in the most majority of these reparative events, leading to accelerated regeneration and recovery. In the present study, I/R increased serum creatinine and serum K^+ level, significantly in proportional to control, which peaked at 24 hours post ischemia/reperfusion injury (I/R). Also, KIM-1 and beta-catenin expression is upregulated in the injured renal tissue 168 hours after I/R. The deteriorated kidney functions could be explained by initiation of an inflammatory cascade including reactive oxygen species (ROS), cytokines, chemokines, and leukocytes activation. During IRI, the damaged tissue produce excessive amount of ROS cause oxidative stress which changes mitochondrial oxidative phosphorylation, ATP depletion, increase intracellular calcium and activation of membrane phospholipids proteases. The blood flow during reperfusion phase of IRI can produce oxygen free radicals which leads to lipid peroxidation as main pathway of free radical tissue injuries. Formation

of free radicals develops renal tissue injury via peroxidation of membrane lipids and oxidative damage of proteins and DNA contribute to apoptosis and cell death. Also the down regulation of the antioxidant enzyme system such as catalase, superoxide dismutase, and glutathione peroxidase could be responsible for the pathophysiology of ischemia-reperfusion injury. Therefore inhibiting this pathway or prevention of free radical production is the strategy to protect the tissue during IRI.

In the present study, L-Car administration for four weeks led to a significant decrease in serum creatinine, serum K^+ , and a highly significant decrease in serum KIM-1, beta-catenin and Kim-1 expression in renal tissue. In addition, L-Car administration for four weeks attenuated I/R-induced increase in the level of oxidative stress marker malondialdehyde (MDA) and increased antioxidant GSH activity. Moreover, the inducer of autophagy (Metformin) administration for four weeks led to a significant decrease in serum creatinine, serum K^+ and a highly significant decrease in serum Kim-1, beta-catenin and Kim-1 expression in renal tissue. Overall, our results suggest a potential therapeutic role of L-Car and metformin in I/R

The role of autophagy in acute kidney injury remains controversial. Although we and others provided evidence for a renoprotective role of autophagy in acute kidney injury^{30,31,32,33}. In the present study, the inducer of autophagy (Metformin) led to a significant decrease in serum creatinine, serum K^+ and serum Kim-1. In kidneys, autophagy is essential to the homeostasis and physiological function of podocytes³⁴. Notably, autophagy induction has been demonstrated in

renal tubular cells in experimental models of acute kidney injury induced by ischemia–reperfusion and nephrotoxicants^{35, 36}. Post-ischemic acute kidney injury is the most important cause of acute kidney injury, and proximal tubular cells are the main lesion of this injury³⁷. The proximal tubule-specific autophagy-deficient mice exhibited more severe injury than control mice, with rapid accumulation of p62- and ubiquitin-positive inclusion bodies in response to I/R injury, showing that autophagy plays a protective role against I/R injury³⁷.

Conclusions

The results of the present study confirmed the renoprotective action of L-Car and metformin against I/R injury. This effect might be explained on the basis of detected L-Car and metformin actions such as antioxidant activity, anti-apoptotic effects, suppression of I/R-induced increase in the blood level and renal tissue expression of kidney injury biomarkers tubular kidney injury molecule (Kim-1) and Wnt proteins (Beta- Catenin). However, further knockout experiments will be required to study the detailed molecular mechanism of L-Car. Overall, our results suggest a potential nephroprotective effect of L-Car and metformin in I/R.

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