International Journal of Research in Pharmacology & Pharmacotherapeutics

ISSN Print: 2278-2648 IJRPP |Vol.5 | Issue 3 | July - Sep - 2016
ISSN Online: 2278-2656 Journal Home page: www.ijrpp.com

Research article Open Access

Hepatoprotective effects of l-carnitine against cyclosporine A-induced liver injury in white albino rats; a newly proposed mechanism of action

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ABSTRACT

It is well documented that l-carnitine (L-C) has protective effects against various types of injury. Although its antioxidant action has been reported as a major mechanism, other suggested pathways may be implicated in its protective effect. This study was designed to evaluate the suggested pathways which may be implicated in the protective effect of L-C on liver injury caused by cyclosporine A (CsA). Forty-two adult male Swiss albino rats weighing 180–200 g were assigned randomly into 6 groups, 7 rats each: rats were given i.p. either sterile saline (1 ml/kg/d), L-C (50 or 200 mg/kg/d), CsA (15 mg/kg/d), or a combination of CsA and L-C for 4 weeks. The impact of L-C on the hepatic injury was assessed by estimation of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T.Bil). Superoxide dismutase (SOD), glutathione reductase (GSH-Rd), malondialdehyde (MDA) levels were measured in both serum and liver homogenates. In addition, prostaglandin E2 (PGE_2) and nitric oxide (NO) were estimated in liver homogenates. CsA treatment caused liver dysfunction, manifested by elevation in serum AST, ALT, and T.Bil levels, and associated with elevation in MDA and reduction in SOD, GSH-Rd, PGE_2, and NO levels in serum and liver homogenates. Concomitant administration of L-C induced dose-dependent improvement of liver functions, antioxidant enzymes, and MDA levels. Furthermore, the administration of L-C at a high dose ameliorated the hepatic levels of PGE_2 and NO. These findings suggest that L-C has a protective effect against CsA-induced liver injury not only by its antioxidant properties but also, by its effect on PGE_2 and NO pathways.

Keywords: L-carnitine, Cyclosporine, Liver function, Antioxidant enzymes, MDA, PGE_2, Nitric oxide.

INTRODUCTION

Despite the development of newer immunosuppressants, cyclosporine (CsA) remains a potent immunosuppressive agent that is used for treating multi-organ transplantation and autoimmune diseases [1]. It is a calcineurin inhibitor; their main mechanism of action involves inhibition of important phosphatase [2]. Currently identified side effects of CsA include hepatotoxicity [3], neurotoxicity, nephrotoxicity and hypertension [4]. In addition, it causes hyperlipidemia [5], hypertrichosis, and gingival hyperplasia [6]. It was reported that many mechanisms are involved in CsA nephrotoxicity

including inflammatory mediators, transforming growth factor-beta1 (TGF-β1), oxidative stress, and apoptotic cell death [7].

L-carnitine (L-C), the L-beta-hydroxy-gamma-N-trimethylaminobutyric acid, is a nonessential amino acid derivative which synthesized primarily in the liver and kidneys from lysine and methionine [8-9]. L-C covers an important role in lipid metabolism. It acts as an obligatory cofactor for beta-oxidation of fatty acids by facilitating the transport of long-chain fatty acids across the mitochondrial membrane as acylcarnitine esters [10]. Furthermore, since carnitine behaves as a shuttle for acetyl groups from inside to outside the mitochondrial membrane, it covers also a key role in glucose metabolism and assists in fuel-sensing [11]. The acetyl group of acetyl L-C is used to produce the antioxidant glutathione (GSH), reducing oxidative stress, and protecting cells against lipid peroxidation [12]. L-C increases hepatic expression of genes related to antioxidant enzymes following suppression of hepatic oxidative stress markers and inflammatory cytokines [13].

In previous studies, L-C induced endothelium-dependent relaxation in the rat aorta and the mechanism of this relaxation appeared mostly mediated by endothelial production of nitric oxide (NO) [14]. Another report has shown the involvement of endothelium on vasodilatation induced by L-C in subcutaneous human arteries, being the effect related to prostaglandin (PG) synthesis [15]. So, this study was aimed to investigate whether L-C can ameliorate the CsA-induced hepatic injury through its effect on liver function; aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin (T.Bil), antioxidant enzymes; superoxide dismutase (SOD), glutathione reductase (GSH-Rd) and malondialdehyde (MDA) levels, furthermore, to detect the mechanism of this effect through its action on liver (PGE_2) and (NO) levels.

MATERIALS & METHODS

Materials

L-C and kits for determination of PGE_2 level by enzyme-linked immunosorbent assay (ELISA) were obtained from Sigma-Aldrich (USA). CsA was purchased from Novartis Institutes for Biomedical research, Basel, Switzerland. Kits used for measuring liver functions were obtained from Egyptian Company for Biotechnology, Cairo, Egypt. Kits for determination of (SOD, GSH-Rd, MDA and NO) were obtained from Bio-diagnostic Company Pharmaceutical Industries, Egypt.

Animals

Male adult Swiss albino rats weighing 180-200 g have been used. Animals were purchased from the animal house, Faculty of Science, Sohag University Egypt. The animals were kept at standard housing place, with room temperature being maintained at 22-24ºc. They were fed on a commercial pellet diet and kept under 12 hours light / dark cycle. Animals were given a free access to food and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of faculty of medicine Sohag University.

Experimental design

Forty- two Male adult Swiss albino rats initially weighing 180 to 200 g, were randomized into 6 subgroups; seven rats each. Animals were treated daily for 4 weeks duration by intraperitoneal (i.p.) route as follow: group 1; (control group), rats received normal sterile saline (1 ml/kg/d), group 2; (L-C 50 group): rats received L-C (50 mg/kg/d) [16], group 3 (L-C 200 group): rats received L-C (200 mg/kg/d) [17], group 4; CsA group: rats received CsA (15 mg/kg/d) [18]. Group 5; (CsA + L-Car 50): rats received CsA (15 mg/kg/d) concomitant with L-C (50 mg/kg). Group 6 (CsA + L-C 200): rats received CsA (15 mg/kg/d) in association with L-C (200 mg/kg/d).

Samples collection

At the end of experimental period, the investigated animals of all groups were fasted overnight and sacrificed by decapitation, blood samples were obtained and serum was separated by centrifugation at 3000 rpm for 10 minutes at 4ºC for estimation of liver functions (AST, ALT, and T.Bil), SOD, GSH-Rd and MDA levels. Livers were quickly removed from the sacrificed rats, placed in ice cold saline solution, then divided into two parts and blotted on filter papers and weighted. The first part was homogenized using Glas-Col, LLC USA motor-driven homogenizer in 2 ml normal saline containing 0.1 M dithiothreitol per gram tissue (v/w), for determination of PGE_2 level. The second part was homogenized in 10 ml ice-cold buffer (50 mM

potassium phosphate pH 7.4) per gram tissue (v/w) for assay the hepatic SOD, GSH-Rd, MDA and NO levels. All homogenates were centrifuged at 4000 rpm for 15 min at 4ºC. The supernatant was removed and kept at -20ºC until the time of analysis.

Calculation of liver index

The liver index was calculated according to the formula: (rat liver weight / rat' weight) 100%. [19].

Biochemical analysis

Determination of liver function

AST, ALT and T.Bil levels in serum were assayed by using commercial kit according to the method of Bergmeyer et al. [20].

Determination of serum and hepatic SOD enzymes activities

Activity of SOD in serum and tissue homogenate was determined by a colorimetric method using commercially available kits. The method was described by Nishikimi et al. [21]. This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium (NBT) dye. The change in the absorbance was measured at 560 nm for control and sample at 25 ºC. SOD activity was expressed in the serum and tissues in U/ml and U/g respectively.

Determination of serum and hepatic GSH-Rd levels

This method based on the reduction of 5,5' dithiobis (2-nitrobenzoic acid) with glutathione to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance was measured at 405nm [22]. Concentration of GSH-Rd in the serum and tissues was expressed in mg/dl and mg/g respectively.

Determination of lipid peroxidation level

MDA level is an indicator to lipid peroxidation. MDA in serum and liver tissue homogenate was determined by a colorimetric method using commercially available kits, as described by Ohkawa et al. [23]. The principle of the method is based on spectrophotometric measurement of the color formed during the reaction of a thiobarbituric acid with MDA. They react in acidic medium at a temperature of 95ºC for 30 min to form a thiobarbituric acid reactive product. The absorbance of the resultant pink product was measured at 534nm. MDA level was expressed in nmol/ml in serum and nmol/g in liver tissues.

Determination of hepatic nitric oxide (NO) level

The level of NO was determined by a colorimetric method using commercially available kits according to the method of Montgomery and Dymock [24]. This assay depends on that, in acid medium and in the presence of nitrite the formed nitrous acid diazotize sulphanilamide the product is coupled with N-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish- purple color which was measured at 540 nm. The level of NO was expressed in nmol/g tissue.

Determination of hepatic PGE_2 level

This assay employed the competitive inhibition enzyme immunoassay technique according to Fernández et al. [25]. The microtiter plate provided in this kit has been pre-coated with a goat-anti-rabbit antibody. Standards or samples were added to the appropriate microtiter plate wells with an antibody specific for PGE_2 and Horseradish Peroxidase (HRP) conjugated PGE_2 and incubated, then substrate solutions were added to each well. The reaction was terminated by the addition of a sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm.

Statistical Analysis

Data were expressed as mean values ± SE using one-way ANOVA. To assess the significant differences among treatment groups, post hoc Tukey test was carried on. The criterion for statistical significance was set at $p<0.05$ for the data. All statistical analysis were performed using SPSS statistical version 16 software package.

RESULTS

Changes of liver index in rats

The liver index showed a significant increase ($p<0.01$) in the group treated with CsA compared to the normal control group. Conversely, by addition of both low and high dosage of L-C concomitant with CsA for 4 weeks duration, the liver index decreased significantly ($p< 0.01$) compared to CsA treated group (Fig. 1).

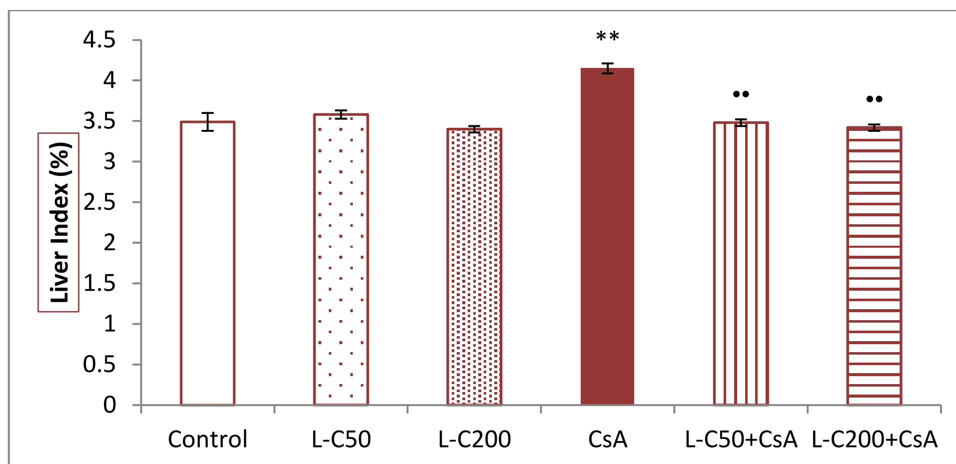

Fig. 1: Effect of l-carnitine (L-C) administration in a dose of 50 and 200 mg/kg b.w i.p on liver index in cyclosporine A (CsA)- induced liver damage in rats. Each value represents the mean ±SEM. N= 7. ** Significant at (p<0.01) vs. control group, •• Significant at (p<0.01) vs. CsA-treated group.

Changes in serum AST, ALT and T.Bil

Table (1) showed that administration of CsA in a dose of 15 mg/kg b.w. i.p. for 28 successive days caused hepatotoxicity in rats as indicated by a significant increase ($p< 0.01$) in serum AST, ALT and T.Bil levels compared to the control group. Whereas, animals treated with L-C in a dose of 50 or 200 mg /kg/ day concomitant with CsA exhibited a significant decrease ($p< 0.01$) in the levels of these serum markers compared to CsA group and the L-C200 produced significant reduction ($p< 0.05$) compared to L-C50.

Table 1: Effect of l-carnitine (L-C) administration in a dose of 50 and 200mg/kg b.w i.p on cyclosporine A (CsA) -induced liver damage in rats.

Groups	L-C				L-C+CsA	
	Control	**L-C50**	**L-C200**	**CsA**	**L-C50**	**L-C200**
AST (U/L)	56.86 ± 0.83	55.43 ± 1.17	55.29 ± 0.91	128.29** ± 0.64	59.29•• ± 0.81	54.14••† ± 1.20
ALT (U/L)	91.14 ± 1.14	87.29 ± 1.36	86.00 ± 1.07	157.29** ± 1.52	97.71•• ± 2.90	90.00••† ± 0.42
T.Bil (mg/dl)	1.07 ± 0.07	0.93 ± 0.04	0.98 ± 0.07	3.71 ** ± 0.12	1.40 •• ± 0.02	1.14••† ± 0.04

Each value represents the mean ±SEM. N= 7. ** Significant at (p<0.01) vs. control group, •• Significant at (p<0.01) vs. CsA-treated group, † Significant at (p<0.05) vs. L-C50 group**.** AST: aspartate aminotransferase, ALT: alanine aminotransferase, T.Bil: total bilirubin.

Changes in serum and hepatic SOD, GSH-Rd and lipid peroxidation levels

A significant decrease ($p< 0.01$) in serum and hepatic SOD and GSH-Rd also a significant increase ($p< 0.01$) in serum and hepatic MDA were showed in animals treated with CsA alone compared to the normal control group. However, only in the serum levels of these parameters, animals treated with CsA plus L-C at low and high doses showed a significant promotion towards the control level with no significant difference between the two doses of L-C. On the other hand, in the hepatic levels of these parameters L-C at low and higher doses showed a significant amelioration ($p< 0.01$) compared to CsA-treated group, but L-C 200 had a significant effect ($p< 0.05$) compared to L-C50 group. (Table 2, 3 & Fig. 2, 3).

Table 2: Effect of l-carnitine (L-C) administration in a dose of 50 and 200mg/kg b.w i.p on cyclosporine A (CsA)- induced liver damage in rats.

Groups	L-C				L-C+CsA	
	Control	**L-C50**	**L-C200**	**CsA**	**L-C50**	**L-C200**
Serum SOD (U/ml)	9.07 ± 0.33	9.17 ± 0.31	8.87 ± 0.37	5.70** ± 0.15	7.00•• ±0.11	7.93•• ±0.11
Serum GSH-Rd (mg/dl)	125.86 ± 1.12	126.14 ± 1.50	126.43 ± 2.27	71.00** ± 2.00	122.29•• ± 1.67	129.29•• ± 1.30

Each value represents the mean ±SEM. N= 7. ** Significant at (p<0.01) vs. control group, •• Significant at (p<0.01) vs. CsA-treated group. SOD: superoxide dismutase, GSH-Rd: glutathione reductase

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Table 3: Effect of l-carnitine (L-C) administration in a dose of 50 and 200mg/kg b.w i.p on cyclosporine A (CsA)- induced liver damage in rats.

Groups		**L-C**			L-C+CsA	
	Control	**L-C50**	**L-C200**	**CsA**	**L-C50**	**L-C200**
Hepatic SOD (U/g)	31.71 ± 0.94	34.43 ±0.78	34.57 ± 0.92	12.14** ± 0.52	26.57•• ±0.72	30.00••† ± 0.62
Hepatic GSH-Rd (mg/g)	99.29 ±1.29	98.00 ±1.21	99.00 ± 0.93	63.00** ±1.15	91.29•• ± 0.75	96.14••† ± 0.83

Each value represents the mean ±SEM. N= 7. ** Significant at (p<0.01) vs. control group. •• Significant at (p<0.01) vs. CsA-treated group, † Significant at (p<0.05) vs. L-C50 group**.** SOD: superoxide dismutase, GSH-Rd: glutathione reductase.

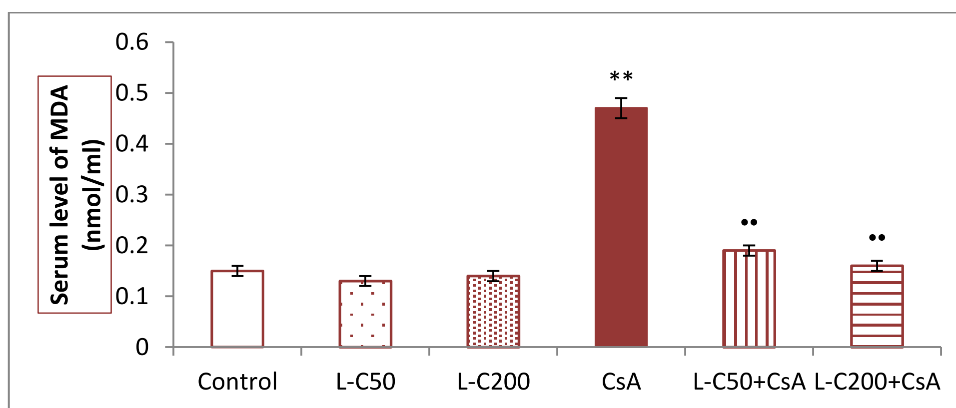

Fig. 2: Effect of l-carnitine (L-C) administration in a dose of 50 and 200mg/kg b.w i.p on serum level of malondialdehyde (MDA) in cyclosporine A (CsA)- induced liver damage in rats. Each value represents the mean ±SEM. N= 7. ** Significant at (p<0.01) vs. control group, •• Significant at (p<0.01) vs. CsA-treated group,

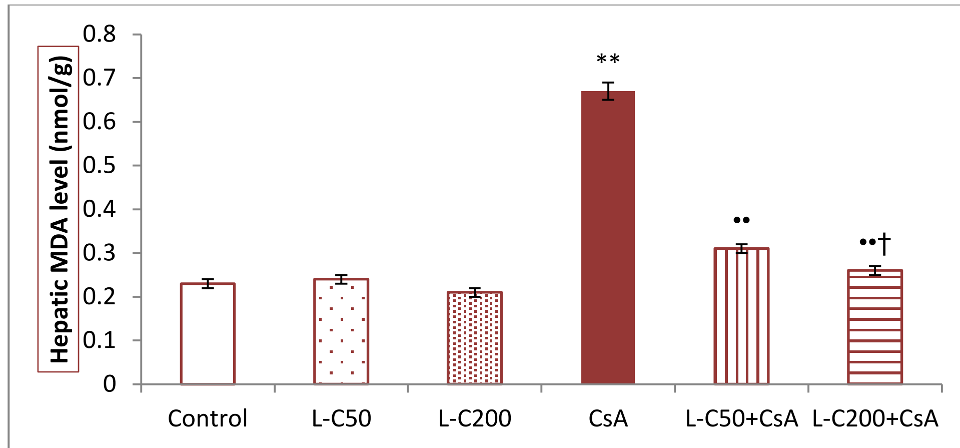

Fig. 3: Effect of l-carnitine (L-C) administration in a dose of 50 and 200mg/kg b.w i.p on hepatic level of malondialdehyde (MDA) in cyclosporine A (CsA) -induced liver damage in rats. Each value represents the mean ±SEM. N= 7. ** Significant at ($p<0.01$) vs. control group, •• Significant at ($p<0.01$) vs. CsA-treated group, † Significant at ($p<0.05$) vs. L-C50 group**.**

Changes in the hepatic nitric oxide (NO) levels

Administration of CsA in a dose of 15 mg/kg/day for 4 weeks duration produced significant reduction ($p<0.01$) in hepatic NO level compared to control group. However, L-C supplementation in low and high doses concomitant with CsA exhibited significant elevation ($p<0.01$) in the hepatic NO levels compared to CsA group and the (L-C200) produced more significant effect ($p<0.05$) compared to (L-C50) group and the L-C200 led to elevation in hepatic NO to reach near to the control level ($p=0.303$) (Fig. 4).

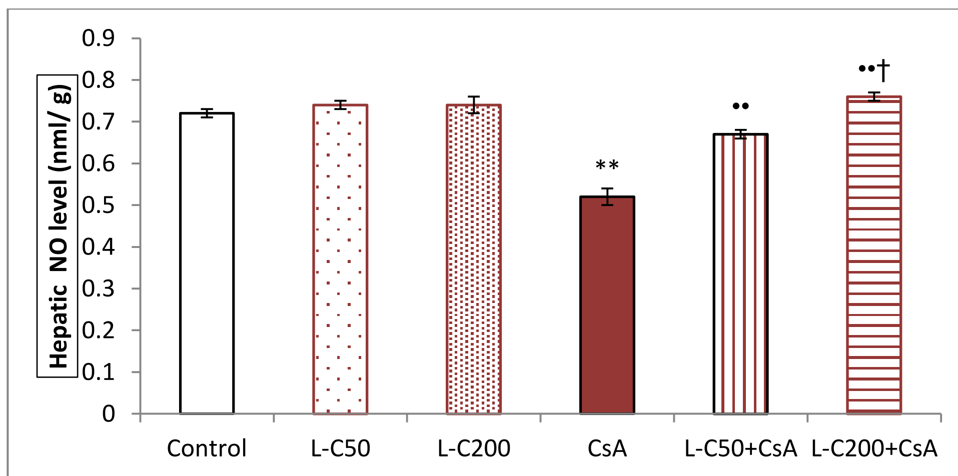

Fig. 4: Effect of l-carnitine (L-C) administration in a dose of 50 and 200mg/kg b.w i.p on hepatic level of Nitric oxide (NO) in cyclosporine A (CsA) -induced liver damage in rats. Each value represents the mean ±SEM. N= 7. ** Significant at ($p<0.01$) vs. control group, •• Significant at ($p<0.01$) vs. CsA-treated group, † Significant at ($p<0.05$) vs. L-C50 group**.**

Changes in hepatic prostaglandin E_2 (PGE_2) levels

As shown in Fig. 5, PGE_2 contents in the liver homogenate were significantly decreased (p<0.01) in the CsA-treated group compared to the normal control group. On the other hand, administration of 50 mg/kg bw L-C to CsA-intoxicated rats resulted in a significant elevation (p<0.05) in PCE_2 however, the 200 mg/kg b.w. tested dose produced highly significant elevation (p<0.01) in PGE_2 compared to the CsA-treated group.

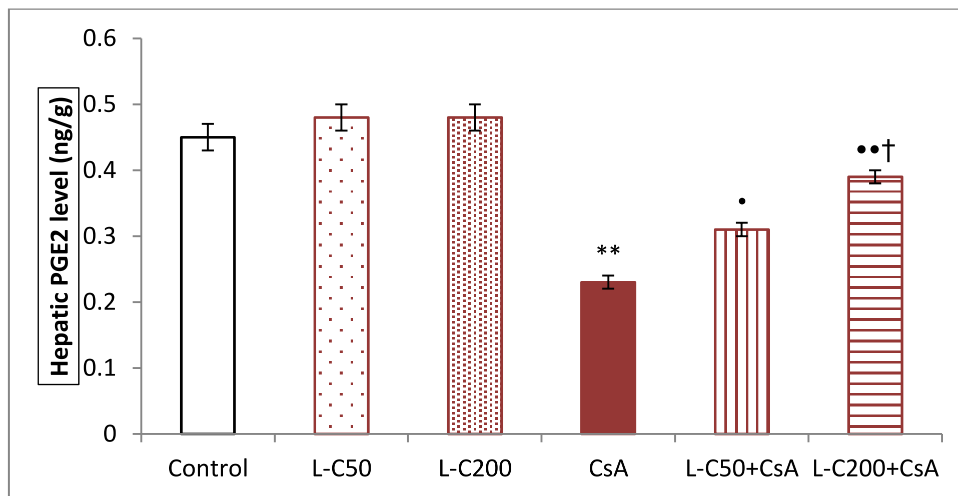

Fig.5: Effect of l-carnitine (L-C) administration in a dose of 50 and 200mg/kg b.w i.p on hepatic level of prostaglandin E_2 (PGE_2) in cyclosporine A (CsA)- induced liver damage in rats. Each value represents the mean ±SEM. N= 7. ** Significant at (p<0.01) vs. control group, • Significant at (p<0.05) and •• Significant at (p<0.01) vs. CsA-treated group, † Significant at (p<0.05) vs. L-C50 group**.**

DISCUSSION

The essential adverse effect of CsA is nephrotoxicity, yet there are also reports describing damage to other organs, such as the liver, heart, central nervous system, and testicles [26-29]. The present study was done to evaluate the protective role of L-C against the changes produced by CsA-induced hepatotoxicity.

Our findings revealed that administration of CsA induced hepatic damage which was evidenced by increased levels of AST, ALT, and T.Bil, also, increase in the liver index. These findings are in agreement with the results of experimental studies of other authors, which showed that elevated levels of these parameters due to their release into the circulation after the cellular damage has occurred as evidence of liver toxicity and elevation in liver index indicated that CsA induced hypertrophy of liver tissue and oedema [3,30-31]. However, co-supplementation of L-C in low and high doses was found to alleviate the changes in AST, ALT, T.Bil and liver index which induced by CsA, where, the higher dose had the more prominent effect than the low dose. Stabilization of the cell membrane may be a reason for this effect [32]. The elevation in liver index induced by CsA was reduced by L-C due to L-C ameliorated liver inflammation and pro-inflammatory markers through regulating carnitine-dependent peroxisome proliferator-activated receptor (PPAR) signalling [33].

SOD and GSH-Rd are considered as the first-line cellular antioxidant enzymes against oxidative damage [34]. SOD prevents the inhibition of glutathione by scavenging superoxide radicals and glutathione in turn prevent the inhibition of SOD by scavenging H_2O_2 [35]. Our results showed a significant decrease in SOD and GSH-Rd activity in serum and liver homogenates following CsA administration. These findings are in agreement with many studies [3, 30, 36-38].

MDA is the most important oxidation by-product of lipid breakdown which can show the extent of lipid peroxidation in many organs [39-40]. In the present study, MDA levels in serum and liver

samples of rats administrated CsA were significantly elevated and this finding was closely similar to those observed by other studies [3,30,36-38]. The ability of CsA to produce oxidative stress is by an increase of intramitochondrial Ca^{++}, and inhibition of mitochondrial Krebs cycle, antioxidant enzymes and ATP production [27].

Our result revealed that L-C increased the levels of serum and hepatic SOD and GSH-Rd associated with a reduction in MDA levels with no significant difference between low and high doses in serum measures. Based on our results, we can assume that CsA intoxication, which is mainly attributed to the induction of oxidative stress, is reversed following treatment with L-C, possibly via scavenging of reactive oxygen species (ROS). These results were in good agreement with other results obtained by Sepand et al.[41] who revealed that L-C attenuated the changes in liver function and ameliorated the hepatic, heart, and brain damage induced by arsenate toxicity. L-C significantly decreased the elevated levels of MDA in different organs which are in agreement with Barhwal et al.[42]. Moreover, L-C maintained the GSH content of various organs at near normal values in rats exposed to arsenate and it possibly acts as a free radical scavenger [43]. Carnitine moiety of L-C plays a significant role in the oxidation of fatty acids, and its acetyl moiety is involved in the maintenance of acetyl-CoA levels. These parts promote the production of glutathione [44].

PGE_2 and NO are two of the major intrarenal vasodilators, which protect the kidney from ischemia. However, the relation of hepatic PGE_2 and NO with L-C protection against CsA-induced hepatic toxicity is a new pathway and it isn't fully studied.

PG is able to protect the liver from ischemic insult. The mechanism by which PG exert this beneficial effect may be related to the reduction of excessive release of TNF-alpha and IL-1beta from Kupffer cells [45].

In the present investigation, a significant decrease in both hepatic PGE_2 and NO levels in CsA exposed rats. This result is inconsistent with other studies which revealed that CsA decreased intra-renal PGE_2 production mainly by decreasing COX-2 expression [46]. Other studies proved that CsA induced reduction in serum and liver [3], renal [47], retinal [48], and gingival endothelial [49] NO levels.

Concomitant supplementation of L-C with CsA led to an elevation in hepatic PGE_2 and NO levels in both low and high doses compared to CsA group, and the higher dose produced a more significant effect than the lower dose. These results are in agreement with many studies which revealed that L- C improves the bioavailability of NO in rat aorta [50] and in the fetal lamb pulmonary vasculature [51]. In addition, L-C increased plasma level of NO in fructose-fed hypertensive rats [52]. Bueno et al.[53] suggest that this increased production could be related to the increase in expression or activity of endothelial NO synthase.

L-C is known to enhance the formation of arachidonic acid from linoleic acid in isolated hepatocytes [54] also it attenuated cardiac fibrosis by the same pathway [55]. Chao et al. [56] proved that L-C protect against doxorubicin-induced apoptosis by induction of PGI2 synthesis in cardiomyocytes, this, in turn, leads to inhibition of ROS generation and activation of SOD and catalase. In addition, cellular PGI2 augmentation can activate PPARα [57] which leads to decrease the expression of NADPH oxidase and ROS generation [58]. Chao et al.[56] suggested that PG and activating PPARα are necessary for antioxidant effect of L-C.

The increase of PG synthesis elicited by L-C has been previously described in both rat gastric mucosa [59], macrophages and lymphocytes probably by transferring arachidonic acid to the biosynthetic pathway of PGs [60].

In conclusion, we can conclude that CsA produced free radicals which resulted in an elevation of lipid peroxidation and biochemical perturbations in the blood and liver. In addition, L-C in the higher dose had a protective effect against CsA-induced hepatic damage in rats more prominent than the lower dose. Contributing to the alleviation of CsA triggered typical hepatotoxic effect, the hepatoprotective effect of L-C is likely due to its ability to scavenge free radicals, and due to up regulation of NO and PGE_2 pathways.

Acknowledgement

I am thankful to pharmacology department Faculty of medicine Sohag University for providing the facilities for carrying out this work.

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