The Effects of L-Carnitine on Cyclophosphamide-Induced Oxidative Liver and Intestinal Damage in Rats

L-Karnitinin, Siklofosfamidin Karaciğer ve İnce Bağırsak Üzerinde Oluşturduğu Oksidatif Hasara Karşı Etkileri

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ABSTRACT Objective: Cyclophosphamide (CP) is among the most commonly used anti-cancer and immunosuppressant drugs. The efficacy of this agent often is limited by severe side effects and toxic conditions. The present experimental study was undertaken to determine whether L-carnitine (LCAR), as a potent antioxidant compound, could ameliorate CP-induced oxidative liver and intestinal injury. **Material and Methods:** Thirty-two Wistar rats were divided into four (control, CP, CP + LCAR and LCAR) groups each containing 8 rats. The antioxidant agent LCAR or saline was given to rats for 5 days after administration of a single dose of CP. On the sixth day, rats were sacrificed and liver and ileum tissue samples were obtained and stored to measure reduced glutathione (GSH) and malondialdehyde (MDA) levels, myeloperoxidase (MPO) and superoxide dismutase (SOD) activities. **Results:** CP decreased GSH level and SOD activity and increased MDA levels and MPO activity in the liver and intestinal tissue homogenates. These changes except intestinal SOD activity were significantly reversed by LCAR treatment. Although intestinal SOD activity increased with LCAR treatment compared to the CP group, this increase was not significant. **Conclusion:** Our results demonstrated that, LCAR could ameliorate the depletion of GSH and SOD activities and could reduce the activities of MPO and levels of MDA in rat liver and intestinal tissues; this makes it a potential prophylactic and preventive agent against CP-induced oxidative stress. In conclusion, these data indicate that LCAR may be of therapeutic use in preventing hepatotoxicity and intestinal injury in patients receiving chemotherapeutic agents.

Key Words: L-carnitine; cyclophosphamide; oxidative stress

ÖZET Amaç: Siklofosfamid (SF), en sık kullanılan anti-kanser ve immünosüpresan ilaçlardandır. Etkili bir ilaç olmasına rağmen, sıklıkla yan etki ve toksik etkileri nedeni ile kullanımı kısıtlanmaktadır. Bu deneysel çalışmada, güçlü bir antioksidan madde olan L-karnitin (LKAR)'in SF'ye bağlı oksidatif karaciğer ve ince bağırsak hasarına karşı etkilerini araştırmayı amaçladık. **Gereç ve Yöntemler:** Otuz iki adet sıçan, her grupta sekizer sıçan olacak şekilde 4 gruba ayrıldı (kontrol, SF, SF + LKAR ve LKAR). Tek doz SF (100 mg/kg) uygulamasının ardından, serum fizyolojik ve antioksidan ajan olan LKAR (500 mg/kg), beş gün boyunca sıçanlara uygulandı. Altıncı günde sıçanlar öldürüldü ve karaciğer ve ileum dokuları çıkarılarak, indirgenmiş glutatyon (GSH), malondialdehid (MDA), miyeloperoksidaz (MPO) ve superoksit dismütaz (SOD) aktivitesini ölçmek için depolandı. **Bulgular:** SF'nin, karaciğer ve bağırsak dokusundan hazırlanmış homojenatlarda GSH düzeyi ve SOD aktivitesini düşürüp, MDA düzeyi ve MPO aktivitelerini artırdığı gözlendi. Bu değişikliklerin, intestinal SOD aktivitesi dışında LKAR tarafından geriye döndürüldüğü saptandı. Tek başına SF verilen grup ile karşılaştırıldığında, LKAR tedavisi ile intestinal SOD aktivitesi artmasına rağmen, bu artış istatistiksel olarak anlamlı değildi. **Sonuç:** Sonuçlarımız, LKAR'nin, sıçan karaciğer ve bağırsak dokularında azalmış GSH düzeyini ve SOD aktivitesini düzelttiğini, artmış MPO aktivitesi ve MDA düzeylerini ise düşürdüğünü göstermiştir. Bu da LKAR'nin, SF'nin karaciğer ve bağırsakta oluşturduğu oksidatif strese karşı potansiyel koruyucu etkilerini göstermektedir. Sonuç olarak, bu veriler, LKAR'nin, kemoterapötik ilaçlara bağlı oluşan karaciğer ve bağırsak hasarına karşı koruyucu olarak kullanılabileceğini ortaya koymaktadır.

Anahtar Kelimeler: L-karnitin; siklofosfamid; oksidatif stres

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Cyclophosphamide (CP) is among the most commonly used anti-cancer and immunosuppressant drugs. It is used for the treatment of chronic and acute leukemias, multiple myeloma, lymphomas, and rheumatic arthritis and in preparation for bone marrow transplantation. Use of CP as an effective chemotherapeutic agent is often restricted because of its widespread side effects and toxicity that includes nausea, vomiting, alopecia, hematopoetic suppression, nephrotoxicity, immunotoxicity, mutagenicity, carcinogenicity and teratogenicity.[1,2]

CP is an alkylating agent that produces highly active carbonium ion, which reacts with the extremely electron-rich area of nucleic acids and proteins.[3] In addition; CP treatment leads to lung injury and cardiac toxicity, which are mediated by the reactive oxygen species (ROS) and lipid peroxide formation. CP treatment not only induces lipid peroxidation (LPO) but also suppresses the tissue and serum level of reduced GSH, glutathione peroxidase (GP), glutathione reductase, SOD and catalase (CAT) activities.[4] Liver disorders were also observed by the increased therapeutic dose of CP. Moreover, recent studies have shown that CP decreases anti-oxidative activity and conversely induces the oxidative system in the liver tissue experimentally. On the other hand, the toxic effects of chemotherapeutic drugs on gastrointestinal mucosa and their consequent side effects such as mucositis, diarrhea, nausea and vomiting are well known.[5]

Recent studies have shown beneficial effects of various antioxidant substances against the toxic effects of CP such as the effects of alpha-tocopherol, beta-carotene and melatonin against toxic effects of CP on bladder.[6] In addition, protective effects of another antioxidant, sgualenin, have been shown against the oxidative stress due to CP.[7] LCAR is known to be an antioxidant and prevents the accumulation of end products of lipid oxidation.[8,9] Carnitine (β-hydroxy-trimethylaminobutyric acid) plays a major role as a cofactor in the transportation of free fatty acids (FFAs) from the cytosol to the mitochondria. FFAs degrade to acyl-CoA by β-oxidation and these substances enter the tricarboxylic acid (TCA) cycle. A large amount of O_2 is consumed in this reaction and ATP is synthesized in the steps of the electron transport chain and oxidative phosphorylation. O_2 is reduced to H_2O at the end of the TCA cycle and the O_2 concentration decreases. Thus, toxic products such as ROS and products of FFA are cleared from the cell.[10]

Since LCAR has been demonstrated to have anti-oxidant properties, there is a probability that LCAR would protect against the oxidative effects of CP. Therefore, in the present study, we assessed the preventive effects of LCAR against the CP-induced oxidative liver and intestinal tissue injury.

MATERIAL AND METHODS

ANIMALS AND EXPERIMENT PROTOCOL

The structure of this study and animal experimental procedures were approved by the Ethical Committee of Kahramanmaraş Sütçü İmam University Faculty of Medicine. Thirty-two Wistar albino rats, weighing 200-230 g, were obtained from the Kahramanmaraş Sütçü İmam University Faculty of Medicine Experimental Research Center (Kahramanmaraş, Turkey). They were kept in stainless steel cages in the animal room, which was maintained on a 12-h-light / 12-h-dark cycle at 21–22°C. The rats were allowed free access to food and water *ad libitum*.

CP, LCAR and saline were injected intraperitoneally (IP). Following a single dose of CP (100 mg/kg), either saline or LCAR (500 mg/kg) was administered IP for 5 days. Wistar rats were divided into four groups.

Group 1 (Control group; n= 8): Only saline was administered.

Group 2 (CP group; n= 8): Saline was given following the administration of a single dose of CP (100 mg/kg).

Group 3 (CP + LCAR group; n= 8): LCAR (500 mg/kg) was given following the administration of single dose CP (100 mg/kg).

Group 4 (LCAR group; n= 8): Only LCAR (500 mg/kg) was administered.

On the sixth day, animals were sacrificed by decapitation; liver and ileum tissues were excised and stored at -80°C for later analysis of MDA and GSH levels, and myeloperoxidase (MPO) and SOD activities.

Liver tissue and ileum samples were homogenized with 10 volumes of ice-cold 0.25 M sucrose, and were centrifuged at 14.000 rpm to measure the biochemical parameters in the resulting supernatant.

MEASUREMENT OF GSH

GSH level was determined according to Beutler.[11] The reaction mixture contained supernatant, phosphate buffer and DTNB (5.5'-dithiobis 2-nitrobenzoic acid) in a final volume of 10 mL. A blank was also prepared by using precipitating reagent and distilled water instead of supernatant. The absorbances were immediately read in a spectrophotometer at 412 nm before and after addition of DTNB. The values were determined from the standard curve. GSH level was expressed as μmol/mg protein.

MPO ACTIVITIES

MPO activity was determined by a modification of the O-dianisidine method.[12] The assay mixture, in a cuvette of 1 cm path length, contained 0.3 mL 0.1 M phosphate buffer (pH 6.0), 0.3 mL 0.01 M H_20_2, 0.5 mL 0.02 M O-dianisidine (freshly prepared) in deionized water and 10 microliters supernatant in a final volume of 3.0 mL. The supernatant was added last and the change in absorbance at 460 nm was followed for 10 min. All measurements were carried out in duplicate. One unit of MPO was defined as that giving an increase in absorbance of 0.001 per min and specific activity was given as U/mg protein.

MDA LEVEL

Lipid peroxidation level in the supernatant was expressed by MDA. It was measured according to the procedure of Ohkawa et al.[13] The reaction mixture contained 0.1 mL tissue sample, 0.2 mL of 8.1% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid. The mixture pH was adjusted to 3.5 and the final volume was adjusted to 4.0 mL with distilled water; then 5.0 mL of n-butanol and pyridine mixture (15:1,v/v) was added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the absorbance of the organic layer was measured at 532 nm. MDA level was expressed as nmol/mg protein.

SOD ACTIVITIES

SOD activity was measured according to the method described by Fridovich.[14] This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with p-iodonitrotetrazlium violet to form a red formazan dye which was measured at 505 nm. Assay medium consisted of 0.01 M phosphate buffer, CAPS (3-cyclohexilamino-1-propanesulfonicacid) buffer solution (50 mM CAPS, 0.94 mM EDTA, satd. NaOH) with pH 10.2, solution of substrate (0.05 Mm xanthine, 0.025 mM INT) and 80 UL xanthine oxidase. SOD activity was expressed as U/mg protein.

The protein concentration of tissue samples was measured with a Spectronic-UV 120 spectrophotometer by the method of Lowry et al.[15]

STATISTICAL ANALYSIS

All data were expressed as mean ± SD. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (except MPO). Since the variance in MPO averages was not equal, Kruskal-Wallis test was used for in-group evaluation and Mann-Whitney U test was utilized in dual comparisons. Values of $p< 0.05$ were considered significant.

RESULTS

CP treatment decreased the GSH levels in liver and ileum tissues significantly ($p< 0.001$) according to the controls, while LCAR treatment following CP prevented this reduction in GSH levels compared to the CP group ($p< 0.05$) (Figure 1A, B).

The levels of liver and ileum MDA, as products of lipid peroxidation, were increased after treatment with CP to levels that were significantly higher than the levels in the control animals ($p< 0.001$). On the

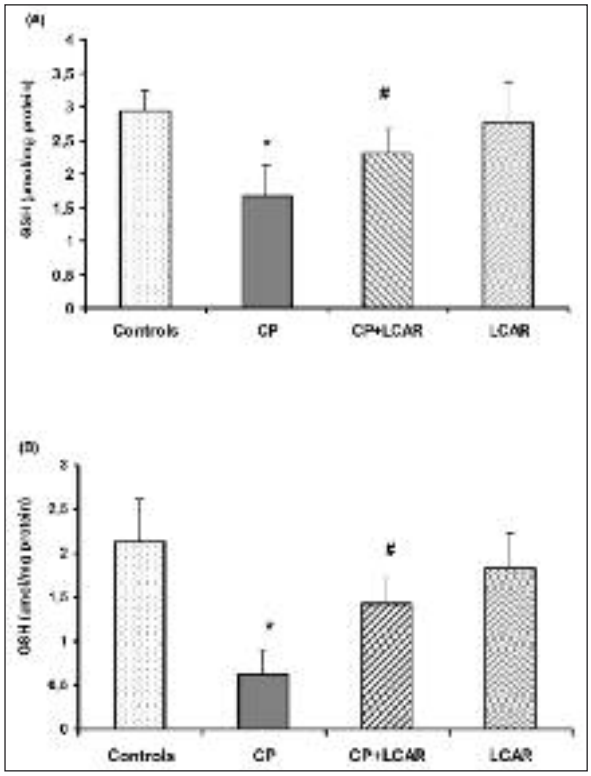

FIGURE 1: Reduced glutathione (GSH) levels in (A) liver, and (B) ileum of controls, L-carnitine (LCAR)-treated, cyclophosphamide (CP)-treated, and CP + LCAR-treated groups. *p< 0.001, compared with control; # p< 0.05, compared with the CP group. Results are mean ± SD; n= 8.

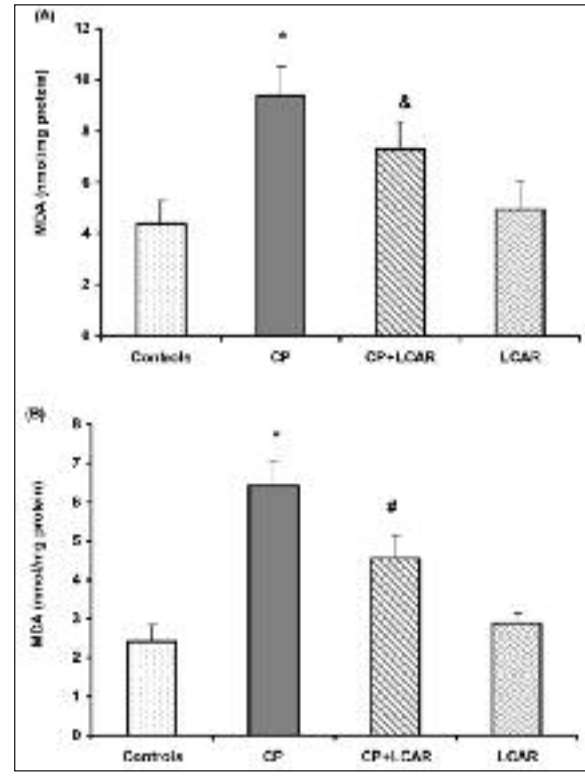

FIGURE 2: Malondialdehyde (MDA) levels in (A) liver, and (B) ileum of controls, L-carnitine (LCAR)-treated, cyclophosphamide (CP)-treated, and CP + LCAR-treated groups. *p< 0.001, compared with control; & p< 0.05, #p< 0.001, compared with the CP group. Results are mean ± SD; n= 8.

other hand, LCAR administration to the CP-treated rats significantly reduced the MDA levels compared to the CP group (p< 0.05) (Figure 2A, B).

Tissue MPO activity was measured as an indirect evidence of neutrophil infiltration. CP administration significantly elevated the liver and ileum MPO activities in comparison to controls. Supplementation of LCAR on CP-treated rats decreased the MPO activities compared to the CP group (p< 0.001) (Figure 3A, B).

Data in Table 1 demonstrate that CP administration significantly decreased SOD activities in the rat liver and ileum compared to controls (p< 0.001). The addition of LCAR to CP treatment increased the SOD activities in liver tissues compared to the activity in the CP group (p< 0.05). However, even though SOD activities were elevated compared to the CP group in ileum tissues, the difference was not significant.

DISCUSSION

Our study demonstrated that CP administration caused oxidative tissue damage, as assessed by increased lipid peroxidation and decreased GSH levels and SOD activities in the liver and ileum while LCAR treatment prevented the oxidative damage. In addition, CP-induced elevation in tissue MPO activity was restored by LCAR treatment.

Recent advances showed that oxygen radicals and hydrogen peroxides were linked with the development of several pathological processes associated with anti-tumor drugs. Cellular mechanism of toxicity is mediated by an increase in free radicals through intracellular phosphoramide mustard and acrolein, the principle alkylating metabolites. The major factor for therapeutic and toxic effects of CP is the requirement of metabolic activation by hepatic microsomal cytochrome P450 mixed functio-

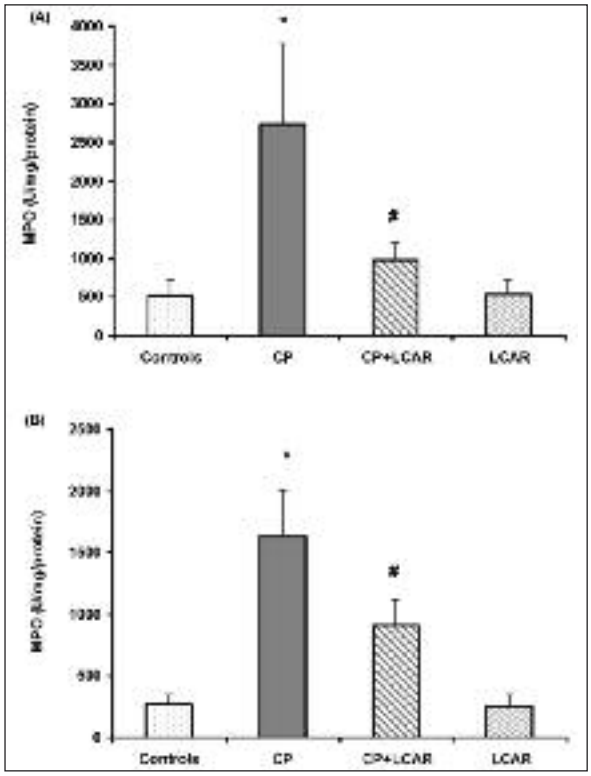

FIGURE 3: Myeloperoxidase (MPO) levels in (A) liver, and (B) ileum of controls, L-carnitine (LCAR)-treated, cyclophosphamide (CP)-treated, and CP + LCAR-treated groups. *p< 0.001, compared with control; #p< 0.001, compared with the CP group. Results are mean ± SD; n= 8.

TABLE 1: Effect of LCAR and CP on SOD levels in the liver and ileum tissues of rat.

Groups	Superoxide dismutase (U/mg protein)	
	Liver	Ileum
Controls (n= 8)	8.71 ± 1.72	2.43 ± 0.55
CP (n= 8)	3.72 ± 1.57*	1.05 ± 0.30*
CP+LCAR (n= 8)	5.87 ± 1.34**	1.59 ± 0.39
LCAR (n= 8)	6.75 ± 1.35	2.26 ± 0.49

Data are presented as mean ± SD. Superoxide dismutase(SOD) levels in liver and ileum of controls, L-carnitine (LCAR)-treated, cyclophosphamide (CP)-treated, and CP + LCAR-treated groups. *p< 0.001, compared with control; **p< 0.05, compared with the CP group.

nal oxidase system.[16] Hepatic dysfunction was the most common regimen-related toxicity reported in patients treated with CP.[17] Recent experimental studies have shown that CP induces the oxidative damage in liver tissue and various antioxidants have beneficial effects against that damage.[4,18] On the other hand, few studies have shown beneficial effects of some antioxidants such as andrographis paniculata extract and vitamine E against the oxidative damage of CP on intestinal tissue.[19,20] However, the antioxidant effects of LCAR have recently been disclosed in many studies. Furthermore, LCAR has been shown to have protective effects on oxidative injury in the intestine and liver.[21,22]

In addition, LCAR has been demonstrated to have an alleviating effect on the oxidative injury of liver caused by various chemotherapeutic agents in many experimental studies.[23,24] Moreover, beneficial effects of L-CAR have been disclosed in an acute liver deficiency model.[25] Although the effects of antioxidant substances such as N-acetylcysteine against the toxic effects of CP were demonstrated in different studies, the effects of L-CAR against CP toxicity have not been investigated sufficiently. Hence, this is the first study to examine the effects of LCAR on CP-induced oxidative liver and intestinal injury in rats.

Recent evidence suggests that reactive oxygen metabolites play a basic role in the hepatotoxicity of various xenobiotics and medications.[26-28] On the other hand, it is increasingly apparent that lipid hydroperoxide may play an important role in mediating cellular and molecular events in degenerative pathophysiological processes that lead to intestinal disorders.[28] Various studies have shown that lipid peroxidation products and consequently increased oxidative stress have an important role in the toxic effect of CP on the liver and the intestine.[19,20,29] In addition to their direct damaging effects on tissues, free radicals seem to trigger the accumulation of leukocytes in the tissues involved and thus aggravate tissue injury indirectly through activated neutrophils. Activated neutrophils located in inflammatory foci secreting MPO into the extracellular space were shown to be able to convert hydroperoxides into free radicals, initiating lipid peroxidation.[30] MPO is an enzyme found predominantly in neutrophils and has been used as an effective quantitative index of inflammation.[31] In our study, elevated MPO activity and MDA levels in liver and ileum tissues may indicate that neutrophil accumulation and lipid peroxidation contribute to

CP-induced oxidative liver and intestinal injury. Hence, the results also suggest that LCAR has a preventive effect through the suppression of neutrophil infiltration and lipid peroxidation.

GSH with its sulfhydryl group functions in the maintenance of sulfhydryl groups of other molecules as a catalyst for disulfide exchange reactions and in the detoxification of foreign compounds, hydrogen peroxide and free radicals. The protective role of sulfhydryl compounds was demonstrated in the intestine and liver.[32] GSH plays an important role in the detoxification of xenobiotics and various chemicals. The depletion of GSH by intracellularly released acrolein was suggested as a major mechanism of cytotoxicity of CP.[33,34] In addition, GSH has been reported to be an effective protector against cyclophosphamide-induced urotoxicity in experimental models.[35] On the other hand, reports have shown that glutathione monoethyl ester can selectively protect the liver from high-dose CP.[36] Andrographis paniculata extract, which is an atioxidant, was reported to decrease the toxic effects of CP on liver and intestine via GSH.[19] The protective roles of glutathione and GSH-dependent enzymes against noxious compounds in the gastrointestinal tract have been documented. In our study, CP administration caused significant decreases in GSH levels in liver and intestinal tissue, which was restored by the antioxidant LCAR.

On the other hand, SOD bears a vital significance for prevention against oxidative stress. Decrease and restoration of SOD activities with antioxidants in liver damage caused by cysplatine has been documented by recent studies.[37,38] In our recent studies we observed that methotrexate had oxidative effects on liver and intestine, SOD activities decreased significantly and effects were restorated by a strong antioxidant, N-acetylcysteine.[39,40] Besides, Hague et al showed significant decrease in SOD levels due to CP and restoration of that decrease by Aqueous extract of walnut.[4] The present study indicated a significant decrease in SOD activities by CP in the liver and intestinal tissues and an increase by LCAR treatment in both tissues; however, this increase was significant only in the liver tissue. Nonetheless, significant increase in SOD activities in the liver tissue was manifested by LCAR treatment compared with the CP group. The insufficient increase in SOD activities in intestinal tissue is thought to stem from its consumption while detoxifying the excess amount of superoxide anions.

CONCLUSION

The results of the present study demonstrate that, LCAR ameliorates the depletion of GSH and SOD activities and reduces the activities of MPO and levels of MDA in rat liver and intestinal tissues. These effects make it a potential prophylactic and preventive agent against CP-induced oxidative stress. In conclusion, these data indicate that LCAR may be of therapeutic use in preventing hepatotoxicity and intestinal injury in patients receiving chemotherapeutic agents.

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