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Carnitine Deficiency Aggravates Cyclophosphamide-Induced Cardiotoxicity in Rats

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Key Words
Cardiomyopathy · D-Carnitine · Carnitine deficiency · Cyclophosphamide · Mildronate · Propionyl-L-carnitine

Abstract
Background: This study examined, for the first time, the involvement of carnitine deficiency in cardiotoxicity, particularly cyclophosphamide (CP)-induced cardiomyopathy, as well as effects of carnitine supplementation with propionyl-L-carnitine (PLC) on cardiotoxicity. ***Methods:*** An animal model of carnitine deficiency was developed in rats treated with D-carnitine (DC)-mildronate (MD). Adult male Wistar albino rats were assigned to one of six treatment groups: the first three groups were injected intraperitoneally with normal saline, PLC (250 mg/kg/day), and DC (250 mg/kg/day) combined with MD (200 mg/kg/day), respectively, for 10 successive days. In groups 4–6, the same doses of normal saline, PLC and DC-MD were injected, respectively, during the 5 successive days before and after a single dose of CP (200 mg/kg). On day 6 after CP treatment, 24-hour urine was collected, then animals were sacrificed, and serum as well as hearts were isolated. ***Results:*** CP caused a significant increase in serum creatine phosphokinase isoenzyme (CK-MB), lactate dehydrogenase (LDH), urinary carnitine excretion and clearance and intramitochondrial acetyl-CoA/CoA-SH, and a significant decrease in serum free carnitine, total carnitine and adenosine triphosphate (ATP) contents in cardiac tissue. In the carnitine-depleted rats, CP induced dramatic increases in CK-MB and LDH levels, carnitine clearance and intramitochondrial acetyl-CoA/CoA-SH, as well as progressive reduction in total carnitine and ATP in cardiac tissues. Interestingly, PLC supplementation completely reversed the biochemical and histopathological changes induced by CP to the control values. ***Conclusion:*** (1) Carnitine deficiency is a risk factor which is involved in CP-related cardiomyopathy; (2) serum and urinary carnitine levels should be monitored and viewed as indices of CP-induced multiple organ toxicity, and (3) carnitine supplementation, using PLC, prevents the development of CP-induced cardiotoxicity.

Introduction

Cyclophosphamide (CP) is an oxazaphosphorine alkylating agent which is commonly used in most cancer chemotherapy and immunosuppressive protocols [1]. It

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has a broad spectrum of antineoplastic activity against different types of human tumors [2, 3]. Unfortunately, efficacy of CP is severely limited by a high incidence of multiple organ toxicity [4–6]. High therapeutic doses of CP have been associated with lethal cardiotoxicity, a combination of symptoms and signs of myopericarditis leading to congestive heart failure, arrhythmia, cardiac tamponade and myocardial depression [7]. The cardiotoxic effects of CP consist of acute, dose-dependent cardiac damage morphologically characterized by necrosis, hemorrhage and subsequently the development of fibrosis [6, 8]. In two clinical studies, the incidence rate of CP-induced cardiotoxicity was 22%, with 11% showing fatal cardiotoxicity [9, 10]. In a group of patients who never had prior anthracycline and radiation therapy, the incidence of symptomatic CP-induced cardiotoxicity was 25%, with a 12% mortality rate [11]. In addition, about 1 week after CP administration, the incidence rate of acute heart failure secondary to cardiotoxicity was 20%, with 8% mortality after bone marrow transplantation [10, 11]. The pathogenesis of CP-induced acute cardiotoxicity was attributed to the increase in free oxygen radicals and the impaired antioxidant defense mechanisms in the heart [12].

It is well documented that cachectic cancer patients are especially at risk for carnitine deficiency due to the decreased oral intake and increased renal excretion [13, 14]. In addition, numerous anticancer drugs interfere with the absorption, synthesis and excretion of carnitine in non-tumorous tissue, resulting in secondary carnitine deficiency, which is reversed by carnitine treatment without impairing anticancer therapeutic efficacy [15]. Moreover, in earlier studies, chloroacetaldehyde and thiodiglycolic acid, two major toxic metabolites of oxazaphosphorines, inhibited the oxidation of long-chain fatty acids (carnitine dependent) but not medium-chain fatty acids (carnitine independent), indicating that these compounds either sequester carnitine or inhibit long-chain fatty acid oxidation by inhibition of carnitine palmitoyltransferase I [16]. To date, the literature is devoid of studies investigating the effects of CP on serum, urine and cardiac carnitine levels in normal and carnitine-depleted rats and its relationship with CP-induced cardiotoxicity. Taken together, this prompted us to determine the role of carnitine deficiency in CP-induced cardiomyopathy and if carnitine supplementation with PLC could offer protection against this toxicity.

Materials and Methods

Animals

Adult male Wistar albino rats, weighing 230–250 g, were obtained from the Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia, and were housed in metabolic cages under controlled environmental conditions (25°C and a 12-hour light/dark cycle). Animals had free access to pulverized standard rat pellet food and tap water unless otherwise indicated. The protocol of this study has been approved by the Research Ethics Committee of the College of Pharmacy, King Saud University.

Materials

Endoxan vials (Baxter Oncology, Halle, Germany) were a kind gift of the King Khalid University Hospital Drug Store, King Saud University. Each Endoxan vial contains 500 mg CP in a dry lyophilized powder form. The content of each vial was freshly dissolved in sterile water immediately before injection. PLC, D-carnitine (DC) and mildronate (MD) were kindly supplied by Dr. Zaven Orfalian, Sigma-Tau Pharmaceuticals, Pomezia, Italy (supplied as white powder in noncommercial plastic bottles containing 100 g which was freshly dissolved in normal saline prior to injection). All other chemicals used were of the highest analytical grade.

Carnitine-Depleted Rat Model

Experimental animal models of carnitine deficiency were developed by Paulson and Shug [17], Whitmer [18] and Tsoko et al. [19]. In the current study, carnitine deficiency was induced in rats by daily intraperitoneal injection of DC (250 mg/kg/day) combined with MD (200 mg/kg/day) for 10 successive days, according to previously published studies [17–22].

Experimental Design

A total of 60 adult male Wistar albino rats were used and randomized to one of six groups (10 animals each). Rats of group 1 (control group) received normal saline (2.5 ml/kg, i.p.) injection for 10 successive days. Animals in group 2 (carnitine-depleted group) were given DC (250 mg/kg/day, i.p.) and MD (200 mg/kg/day, i.p.) for 10 successive days. Animals in group 3 (carnitine-supplemented group) were given PLC (250 mg/kg/day, i.p.) for 10 successive days. Rats of group 4 (CP group) received normal saline for 5 days before and 5 days after a single CP dose (200 mg/kg, i.p.) according to Mythili et al. [12]. Rats of group 5 (CP/carnitine-depleted rats) were given the same doses of DC-MD as group 2 for 5 days before and 5 days after a single CP dose as group 4. Rats in group 6 (CP-carnitine supplemented rats) were given the same doses of PLC as group 3 for 5 days before and 5 days after a single CP dose as group 4. Immediately after the last dose of the treatment protocol, 24-hour urine was collected for monitoring urinary carnitine excretion and carnitine clearance. Animals were then anesthetized with ether, and blood samples were drawn by heart puncture. Serum was separated to assess lactate dehydrogenase (LDH), creatine phosphokinase iso-enzyme (CK-MB), free carnitine, acylcarnitine and total carnitine. Animals were then sacrificed by decapitation after exposure to ether in a dessicator kept in a well-functioning hood, and hearts were quickly excised, washed with saline, blotted with a piece of filter paper and homogenized in normal sa-

line or 6% perchloric acid, as indicated in the procedures of measurement of each parameter, using a Branson sonifier 250 (VWR Scientific, Danbury, Conn., USA). Heart specimens from each group were removed for histopathological examination, they were fixed in 10% neutral buffered formalin, sectioned at 3 μm and stained with hematoxylin and eosin for light microscopy.

Assessment of Cardiac Enzymes

Serum activities of LDH and CK-MB were determined according to the methods of Buhl and Jackson [23] and Wu and Bowers [24], respectively.

Determination of Free and Total Carnitine in Serum, Urine and Cardiac Tissue

Total and free carnitine concentrations were determined in serum, urine and cardiac tissue according to the method reported by Prieto et al. [25]. In brief, carnitine reacts with acetyl-CoA forming acetylcarnitine in a reaction mediated by carnitine acetyltransferase enzyme. The liberated CoA-SH reacts with 5,5-dithiobis-(2-nitrobenzoic acid), forming a thiophenolate ion whose generation is proportional to the amount of carnitine and can be measured spectrophotometrically at 412 nm. Serum, urine and heart tissue were deproteinized with equal volumes of ice-cold 0.6 M perchloric acid and allowed to stand in an ice bath for 10 min. The mixture was centrifuged at 1,000 *g* at 4°C for 5 min. The supernatant was used directly for measuring free carnitine after neutralization with 1.2 M potassium carbonate. To assess total carnitine, part of the supernatant was mixed with 1 M KOH and incubated at 37°C for 20 min for the hydrolysis of acylcarnitines. Carnitine levels were computed from a calibration curve for carnitine hydrochloride.

Determination of CoA-SH and Acetyl-CoA in Isolated Rat Heart Mitochondria

Rat heart mitochondria were isolated according to the procedure of Chappell and Hansford [26]. The isolation buffer contained 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris-HCl (pH 7.4) and 1 mM EGTA. In brief, heart tissues were homogenized in mitochondrial isolation buffer and centrifuged at 1,000 *g* for 10 min at 4°C. The resulting supernatant was decanted and further centrifuged at 1,000 *g* for 10 min, and the resulting pellet (mitochondria) was resuspended in the isolation buffer. Protein concentration of the mitochondria was determined using a Bio-Rad protein assay according to the method of Bradford [27]. Free CoA-SH and acetyl-CoA were determined in isolated heart mitochondria using HPLC (Jasco, Tokyo, Japan) according to Lysiak et al. [28]. In brief, mitochondria were mixed with ice-cold 6% perchloric acid, centrifuged at 300 *g* for 5 min at 0.5°C, and the resulting supernatant fluid was neutralized to pH 6–7, then injected into HPLC. Chromatographic separation was performed using ODS-Hypersil (150 × 4.6 mm internal diameter, 5-μm column; Supelco, Gland, Switzerland). The UV detector was operated at 254 nm and set at 0.005. The mobile phase of 220 mM potassium phosphate contained 0.05% dithioglycol (A) and 98% methanol and 2% chloroform (B). The flow rate was 0.6 ml/min and the gradients were as follows: at zero time, 94% A and 6% B; at 8 min, 92% A and 8% B; at 14 min, 87% A and 13% B; at 25 min, 80% A and 20% B; at 40 min, 55% A and 45% B; at 45 min, 55% A and 45% B, and at 60 min, 94% A and 6% B.

Determination of Adenosine Triphosphate in Cardiac Tissue

Adenosine triphosphate (ATP) was determined in heart tissue using HPLC (Jasco) according to Botker et al. [29]. In brief, heart tissue was homogenized in ice-cold 6% perchloric acid and centrifuged at 1,000 rpm for 15 min at 0.5°C; the supernatant fluid was injected into HPLC after neutralization to pH 6–7. Chromatographic separation was performed at a flow rate of 1.2 ml/min using ODS-Hypersil (150 × 4.6 mm internal diameter, 5-μm column; Supelco) and 75 mM ammonium dihydrogen phosphate as mobile phase. The ATP peaks were eluted at 3.2 min and the UV detector was operated at 254 nm.

Determination of Reduced Glutathione and Lipid Peroxidation in Cardiac Tissues

The tissue levels of the acid soluble thiols, mainly glutathione (GSH), were assayed spectrophotometrically at 412 nm, according to the method of Ellman [30], using a Shimadzu (Tokyo, Japan) spectrophotometer. The contents of GSH were expressed as micromoles per gram wet tissue. The degree of lipid peroxidation in cardiac tissue was determined by measuring thiobarbituric acid reactive substances (TBARS) in the supernatant tissue from homogenate [31]. The homogenates were centrifuged at 3,500 rpm, and supernatant was collected and used for the estimation of TBARS. Absorbance was measured spectrophotometrically at 532 nm and the concentrations were expressed as nanomoles TBARS per gram wet tissue.

Statistical Analysis

Differences (means ± SEM, n = 10) were assessed by one-way analysis of variance followed by the Tukey-Kramer multiple comparison test; $p \leq 0.05$ was regarded as statistically significant.

Results

Effect of CP on Serum Enzymatic Indices of Cardiomyopathy

Figure 1 shows the effects of CP on indices of cardiotoxicity, serum enzyme levels of LDH (fig. 1a) and CK-MB (fig. 1b), in PLC-supplemented and carnitine-depleted rats. Administration of a single CP dose (200 mg/kg) resulted in significant 99 and 80% increases in serum LDH and CK-MB, respectively, compared to the control group. Treatment with either PLC (carnitine-supplemented rats) or the DC-MD combination (carnitine-depleted rats) for 10 successive days showed non-significant changes. Treatment with DC-MD for 5 days before and 5 days after a single CP dose resulted in significant 37 and 59% increases in LDH and CK-MB activity, respectively, compared to CP alone. Interestingly, administration of PLC for 5 days before and 5 days after CP resulted in complete reversal of the CP-induced increase in serum CK-MB and LDH levels to the control values.

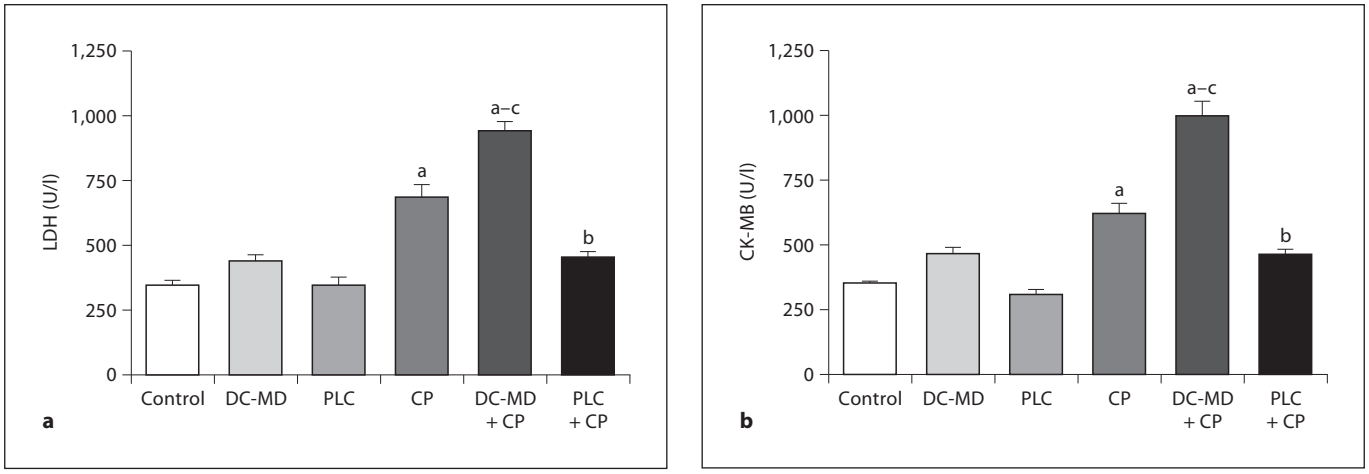

Fig. 1. Effect of CP, PLC, DC-MD and their combination on enzymatic indices of cardiomyopathy, serum LDH (**a**) and CK-MB (**b**), in rats. Data are means ± SEM (n = 10). [a–c] $p < 0.05$ vs. control (a), CP (b) and DC-MD (c), respectively (ANOVA followed by the Tukey-Kramer test).

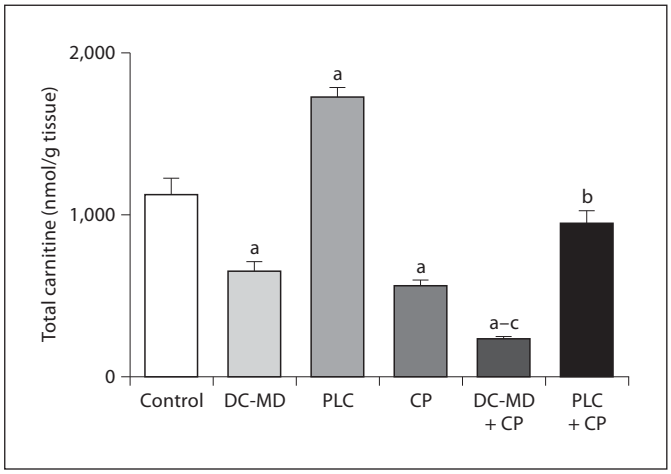

Fig. 2. Effect of CP, PLC, DC-MD and their combination on total carnitine levels in rat cardiac tissue. Data are means ± SEM (n = 10). [a–c] $p < 0.05$ vs. control (a), CP (b) and DC-MD (c), respectively (ANOVA followed by the Tukey-Kramer test).

Effect of CP on Total Carnitine Levels in Rat Cardiac Tissue

The effects of CP on total carnitine levels in cardiac tissue from PLC-supplemented and carnitine-depleted rats are shown in figure 2. A single CP dose resulted in a significant 50% decrease in total carnitine in cardiac tissue, whereas daily administration of DC-MD for 10 successive days resulted in a significant 42% decrease compared to the control group. Treatment with DC-MD for 5 days before and after a single CP dose resulted in significant decreases (78, 63 and 56%) in total carnitine in cardiac tissue compared to the results of the control, DC-MD and CP groups, respectively. Interestingly, daily administration of PLC for 5 days before and 5 days after a single CP dose resulted in complete reversal of the CP-induced decrease in total carnitine in cardiac tissue to the control values.

Effect of CP on Urinary Carnitine Excretion and Carnitine Clearance in Rats

CP affected urinary carnitine excretion (fig. 3a) and carnitine clearance (fig. 3b) in PLC-supplemented and carnitine-depleted rats. Administration of a single CP dose resulted in 1.4- and 2.5-fold increases in carnitine excretion and clearance, respectively, compared to the control group. Treatment of carnitine-depleted rats with a single CP dose resulted in 5- and 1.8-fold increases in carnitine excretion and clearance, respectively, compared to CP alone. Carnitine supplementation by daily PLC administration for 5 days before and 5 days after a single CP dose resulted in 4.7- and 2.5-fold increases in carnitine excretion and clearance, respectively, compared to the control values. Treatment with PLC alone for 10 successive days resulted in significant 18- and 7.2-fold increases in carnitine excretion and clearance, respectively, whereas administration of DC-MD for 10 successive days re-

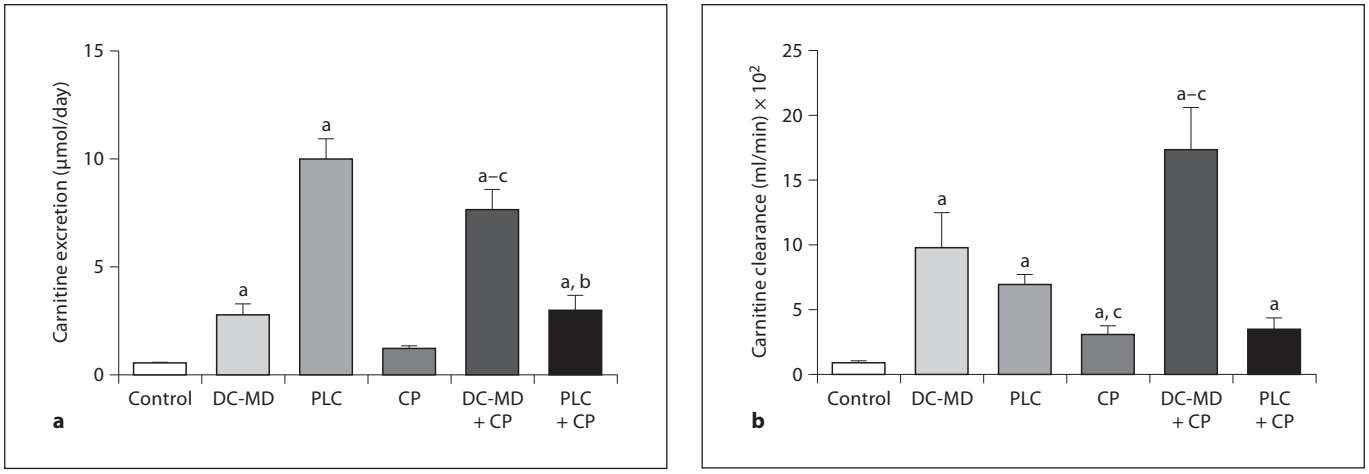

Fig. 3. Effect of CP, PLC, DC-MD and their combination on urinary carnitine excretion (**a**) and carnitine clearance (**b**) in rats. Data are means ± SEM (n = 10). [a–c] $p < 0.05$ vs. control (a), CP (b) and DC-MD (c), respectively (ANOVA followed by the Tukey-Kramer test).

Table 1. Effect of CP, PLC, DC-MD and their combination on serum levels of free carnitine (FC), acyl carnitine (AC) and total carnitine (TC) in rats

Treatment groups	Serum carnitine level, μM			
	FC	AC	TC	AC/FC
Control	33.5 ± 2.6	12.45 ± 1.7	45.97 ± 2.99	0.36 ± 0.07
DC-MD	40.4 ± 2.4	15.4 ± 1.4	55.8 ± 3.1	0.38 ± 0.02
PLC	50.4 ± 2.3[a]	53.5 ± 3.6[a]	103.9 ± 6.6[a]	0.96 ± 0.010[a]
CP	21.5 ± 1.8[a]	21.2 ± 1.2	42.7 ± 2.1	1.04 ± 0.21[a]
CP plus DC-MD	10.3 ± 2.3[a–c]	67.5 ± 4.2[a–c]	77.3 ± 5.6[a–c]	6.47 ± 0.74[a–c]
CP plus PLC	24.2 ± 2.3	40.3 ± 2.6[a, b]	64.4 ± 3.5[a, b]	1.7 ± 0.03[a, b]

Data are presented as means ± SEM (n = 10). [a–c] $p < 0.05$ vs. control (a), CP (b) and DC-MD (c), respectively (ANOVA followed by Tukey-Kramer test).

sulted in significant 4.25- and 10.5-fold increases compared to the control group.

Effect of CP on Serum Levels of Free, Acyl- and Total Carnitine in Rats

The effects of CP on the serum levels of free, acyl- and total carnitine of PLC-supplemented and carnitine-depleted rats are shown in table 1. Administration of PLC for 10 successive days resulted in significant (50, 329, 126 and 267%) increases in serum free, acyl- and total carnitine, and the ratio of acylcarnitine/free carnitine, respectively. A single CP dose resulted in a significant 35% decrease in free carnitine and a significant 247% increase in the acylcarnitine/free carnitine ratio compared to the control group. Treatment with DC-MD for 5 days before and 5 days after a single CP dose resulted in significant (69, 74 and 52%) decreases in serum free carnitine and significant (68, 39 and 81%) increases in serum total carnitine levels compared to the control, DC-MD and CP groups, respectively. Interestingly, daily administration of PLC for 5 days before and after a single CP dose resulted in a significant 72% reversal of the CP-induced decrease in serum free carnitine compared to the control values.

Effect of CP on Acetyl-CoA and CoA-SH in Isolated Rat Heart Mitochondria

Table 2 shows the effects of CP on the level of CoA-SH, acetyl-CoA and CoA-SH/acetyl-CoA in heart mitochondria isolated from PLC-supplemented and carnitine-

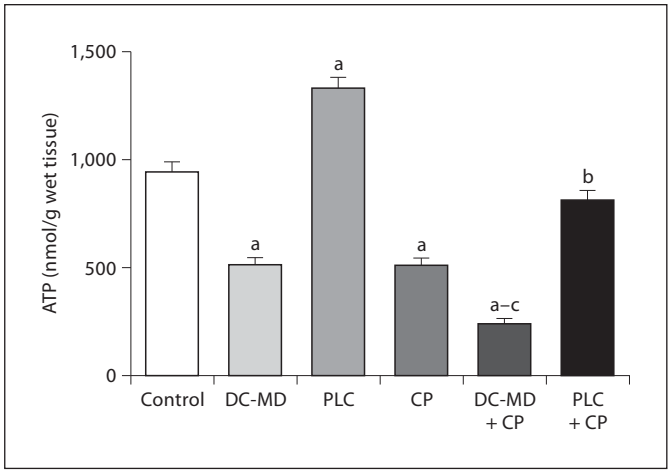

Fig. 4. Effect of CP, PLC, DC-MD and their combination on ATP in rat cardiac tissue. Data are means ± SEM (n = 10). [a–c] $p < 0.05$ vs. control (a), CP (b) and DC-MD (c), respectively (ANOVA followed by the Tukey-Kramer test).

Table 2. Effect of CP, PLC, DC-MD and their combination on acetyl-CoA, CoA-SH and the acetyl-CoA/CoA-SH ratio in isolated rat heart mitochondria

Treatment groups	Acetyl-CoA nmol/mg protein	CoA-SH nmol/mg protein	Acetyl-CoA/ CoA-SH ratio
Control	14.4 ± 0.72	2.7 ± 0.13	5.4 ± 0.28
DC-MD	16.98 ± 0.9	1.99 ± 0.08[a]	8.48 ± 0.21[a]
PLC	3.16 ± 0.156[a]	7.17 ± 0.22[a]	0.44 ± 0.029[a]
CP	13.4 ± 0.4	1.57 ± 0.14[a]	8.76 ± 0.56[a]
CP + DC-MD	15.6 ± 0.96	1.14 ± 0.067[a, c]	13.8 ± 0.87[a–c]
CP + PLC	9.05 ± 0.19[a, b]	2.48 ± 0.12[b]	3.68 ± 0.13[b]

Means ± SEM (n = 10). [a–c] $p < 0.05$ vs. control (a), CP (b) and DC-MD (c), respectively (ANOVA followed by the Tukey-Kramer test).

depleted rats. Treatment with PLC alone resulted in a significant 166% increase in the CoA-SH level and significant (78 and 92%) decreases in acetyl-CoA and acetyl-CoA/CoA-SH, respectively, compared to the control group. A single CP dose resulted in a significant 42% decrease in CoA-SH and a significant 62% increase in the acetyl-CoA/CoA-SH ratio. Carnitine deficiency following DC-MD treatment for 10 successive days resulted in a significant 26% decrease in CoA-SH and a significant 57% increase in the acetyl-CoA/CoA-SH ratio compared to the results of the control group. Administration of DC-MD for 5 days before and 5 days after a single CP dose resulted in significant (58 and 27%) decreases in CoA-SH and (156 and 49%) increases in acetyl-CoA in isolated rat heart mitochondria compared to the results of the control and CP groups, respectively. Interestingly, daily administration of PLC for 5 days before and 5 days after a single CP dose resulted in complete reversal of CP-induced changes in CoA-SH and acetyl-CoA/CoA-SH levels in mitochondria to the control values.

Effect of CP on ATP Levels in Rat Cardiac Tissue

Figure 4 shows the effects of CP on ATP levels in cardiac tissue from PLC-supplemented and carnitine-depleted rats. Treatment with PLC for 10 successive days resulted in a significant 42% increase in ATP in cardiac tissue, whereas DC-MD resulted in a significant 45% decrease compared to the control group. Administration of a single CP dose resulted in a significant 54% decrease in ATP in cardiac tissue compared to the control group. In carnitine-depleted rats, CP resulted in significant (74, 53 and 53%) decreases in ATP in cardiac tissue compared to the results of the control, DC-MD and CP groups, respectively. Interestingly, carnitine supplementation by daily administration of PLC for 5 days before and 5 days after a single CP dose resulted in complete reversal of the CP-induced decrease in ATP in cardiac tissue to the control values.

Effect of CP on Oxidative Stress Biomarkers in Rat Cardiac Tissues

Figure 5 shows the effects of CP on TBARS (fig. 5a) and GSH levels (fig. 5b) in cardiac tissue from PLC-supplemented and carnitine-depleted rats. CP resulted in a significant 98% increase in TBARS and a significant 64% decrease in GSH in cardiac tissue compared to the control group. Treatment with DC-MD for 5 days before and after a single CP dose resulted in a significant 41% decrease in TBARS compared to the CP group and significant (29 and 262%) increases in GSH compared to the results of the control and CP groups, respectively. Daily administration of PLC for 5 days before and 5 days after a single CP dose resulted in complete reversal of the CP-induced increase in TBARS and the decrease in GSH in cardiac tissue to the control values.

Effect of CP on Histopathological Changes in Rat Cardiac Tissue

CP induced histopathological changes in cardiac tissue in PLC-supplemented and carnitine-depleted rats (fig. 6). Cardiac tissue of control rats showed bundles of

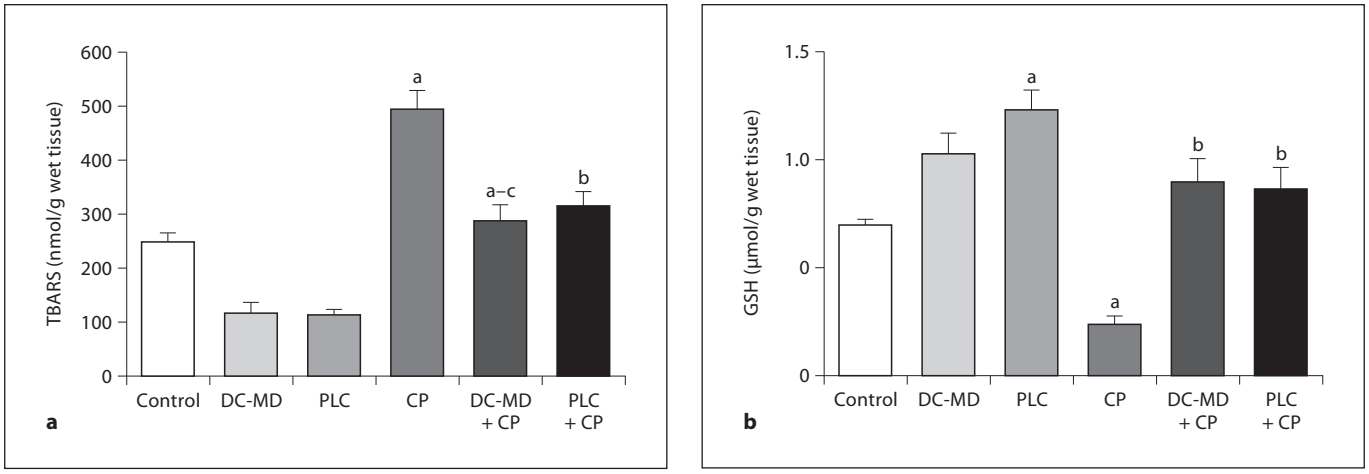

Fig. 5. Effect of CP, PLC, DC-MD and their combination on TBARS (**a**) and reduced GSH levels (**b**) in rat cardiac tissue. Data are means ± SEM (n = 10). $^{a-c}$ $p < 0.05$ vs. control (a), CP (b) and DC-MD (c), respectively (ANOVA followed by the Tukey-Kramer test).

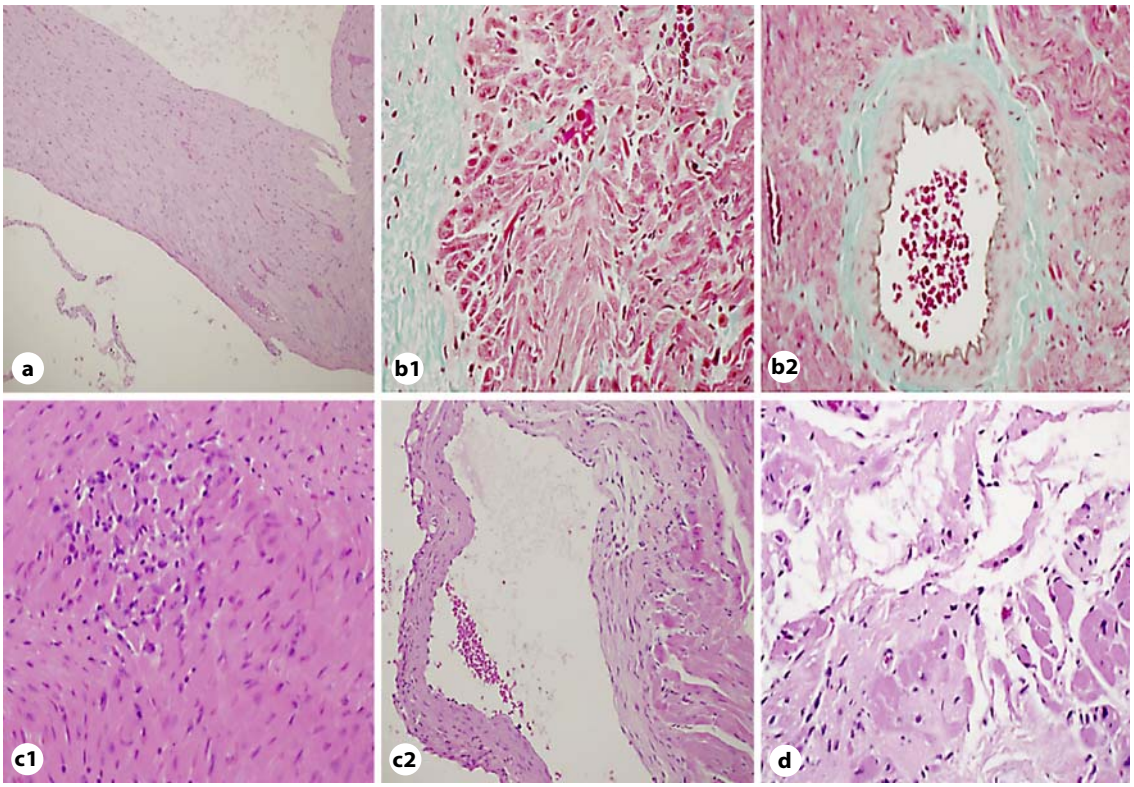

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Fig. 6. Effect of CP, PLC, DC-MD and their combination on histopathological changes in rat cardiac tissue. **a** Heart of a control rat showing unremarkable endomyocardium and vessels (×4). **b** Heart of a rat treated with CP alone showing focal subendocardial and interstitial fibrosis, rare myocyte dropout (b1, ×40) and mild perivascular fibrosis (b2, ×40). **c** Heart of a rat treated with CP plus DC-MD showing progressive myocarditis (c1, ×20), and subendocardial fibrosis and focal hypereosinophilia of myocytes (c2, ×40). **d** Heart of a rat treated with CP plus PLC showing minimal vascular myocyte degeneration and minimal focal ventricular fibrosis (×40).

normal muscle fibers, unremarkable endomyocardium and unremarkable vessels (fig. 6a). On the other hand, animals treated with CP alone showed degenerative changes manifested as focal subendocardial and interstitial fibrosis, rare myocyte dropout and mild perivascular fibrosis (fig. 6b). Cardiac tissue injury was aggravated in cardiac sections of rats treated with CP plus DC-MD, revealing progressive myocarditis, subendocardial fibrosis and focal hypereosinophilia of myocytes (fig. 6c). Interestingly, heart specimens from rats treated with CP plus PLC showed minimal vascular degeneration of myocytes and minimal focal fibrosis in the ventricles (fig. 6d).

Discussion

Several clinical and experimental studies reported that administration of high doses of CP can cause cardiotoxicity within 10 days of its administration [12, 14, 15, 32, 33]. Although cardiotoxicity is related to dosage, previous anthracycline treatment, previous thoracic irradiation, age >50 years and the presence of left-ventricular dysfunction, no definitive risk factors have been identified yet. This prompted us to investigate, for the first time, whether carnitine deficiency and its metabolic consequences play a role in cardiotoxicity, particularly in CP-induced cardiomyopathy, and if confirmed, whether carnitine supplementation using PLC could prevent this toxicity.

Data presented here demonstrate that CP increased indices of cardiotoxicity, serum enzymes LDH and CK-MB, and caused severe histopathological lesions in cardiac tissue, in agreement with previous reports [12–16]. This increase in serum cardiac enzymes could be due to CP-induced generation of reactive oxygen species (ROS) and lipid peroxidation of cardiac membranes with the consequent leakage of LDH and CK-MB from damaged cardiac myocytes. It is well known that theses enzymes travel from the heart to the blood stream during myocardial necrosis [34]. This hypothesis is confirmed by our data demonstrating CP-induced increases in TBARS, an index of lipid peroxidaton, and decreases in GSH, an index of antioxidant activity, in cardiac tissue. Earlier and recent studies applying similar experimental conditions reported that CP-induced acute cardiotoxicity was attributed to the increase in ROS and impaired antioxidant defense mechanisms in the heart, and that antioxidant compounds including lipoic acid [12, 15] and squalene [35] attenuated CP-induced cardiotoxicity. It is well documented that ROS and oxidative stress play an important role in multiple organ toxicity induced by anticancer drugs [36, 37]. Fascinatingly, administration of PLC for 5 days before and 5 days after a single CP dose completely prevented the increase in LDH and CK-MB induced by CP, suggesting that PLC may have potential protective effects against CP-induced cardiac damage. This effect could be due to cardiac membrane stabilization by the L-carnitine portion of PLC with the consequent decrease in the release of cardiac enzymes. Indeed, the interaction of L-carnitine with sarcolemmal phospholipids and mitochondrial membranes has been reported previously [38]. Moreover, via its antioxidant defense mechanisms against ROS generation in cardiac and other tissue, PLC could protect cardiac membranes against CP-induced lipid peroxidation [39]. The antioxidant effect of PLC has been confirmed by our results indicating that PLC induced complete reversal of the CP-induced increase in TBARS and decrease in GSH in cardiac tissue to the control values. Recently, Todorova et al. [33] stated that oral glutamine attenuated CP-induced cardiotoxicity through the increase in cardiac GSH. In contrast, administration of CP to carnitine-depleted rats produced a progressive increase in LDH and CK-MB activity as well as massive degenerative changes in cardiac tissue (fig. 6).

L-Carnitine plays a crucial role in energy production in the heart by controlling the influx of long-chain fatty acids into mitochondria [40]. The total body content of carnitine in adults amounts to 50–100 mmol, most of it being localized in skeletal muscles [41]. Carnitine is present in both plasma and tissue as free carnitine or bound to fatty acids as acylcarnitine, with relatively high concentrations in cardiac tissue [42]. Under normal physiological conditions, 80% of total serum carnitine is free carnitine and 20% is acylcarnitine, with a normal ratio of acylcarnitine to free carnitine of 0.25 [43, 44]. Earlier studies reported that acylcarnitine/free carnitine ratios >0.4 are abnormal and create a pathological condition known as carnitine insufficiency, in which there is insufficient free carnitine relative to increased metabolic needs [43–45]. Moreover, it has been reported that the acylcarnitine/free carnitine ratio is very sensitive to metabolic changes and reflects the intramitochondrial acyl-CoA/CoA-SH ratio [46].

In the current study, CP significantly decreased free carnitine in serum and total carnitine in cardiac tissue, and increased serum acylcarnitine, the acylcarnitine/free carnitine ratio and urinary carnitine clearance. Our results are unique due to the lack of available experimental

or clinical data about the role of endogenous carnitine during the development of CP-induced multiple organ toxicity. The observed decrease in the cardiac carnitine content could be a secondary event following CP-induced inhibition of endogenous synthesis and/or inhibition of tubular reabsorption of carnitine. If this hypothesis proves correct, then administration of CP should be associated with acute kidney dysfunction. Under similar experimental conditions, a previous study reported that a single CP dose (200 mg/kg) increased acute nephrotoxicity indices, BUN and serum creatinine, in rats [46]. Since the kidney is the major site for endogenous carnitine biosynthesis and 95% of filtered carnitine is reabsorbed by the proximal tubules of the nephron, therefore kidney damage induced by CP may lead to inhibition of endogenous carnitine biosynthesis and increases its urinary excretion with the consequent secondary deficiency in the molecule. This is in line with our data which showed that urinary carnitine excretion and carnitine clearance were increased by CP administration. The increased urinary excretion of carnitine reported in our study is in good agreement with the only study performed on 5 patients in whom ifosfamide, an alkylating oxazaphosphorine agent, increased urinary carnitine excretion [47]. In earlier and recent studies, increased urinary excretion of carnitine was an early marker in cisplatin- and carboplatin-induced nephrotoxicity [48–50]. It is worth mentioning that the increase in urinary carnitine excretion by PLC administration is not an index of toxicity but rather due to the pharmacokinetic profile of PLC, as reported by Pace et al. [51]. The increased serum levels of acylcarnitine and the acylcarnitine/free carnitine ratio and consequently in the tubular fluid after PLC administration could have contributed to the saturation of their respective tubular reabsorption processes. Also, competition may occur between PLC, L-carnitine and acetylcarnitine for the tubular reabsorption transport systems [51]. Moreover, renal metabolism of PLC to L-carnitine and acetylcarnitine, followed by migration of the locally formed metabolites directly to the urine, could also be involved [52]. In reality, all three mechanisms (saturation, competition and renal metabolism) are likely to be contributing to the observed increase in renal excretion of carnitine after PLC administration [51–53].

Our data demonstrate that CP administration significantly decreased intramitochondrial free CoA-SH, an indispensable activator in most of the mitochondrial energy-providing systems (tricarboxylic acid cycle, fatty acid β-oxidation and pyruvate oxidation). This effect could be explained on the basis of the high reactivity of CP metabolites, including chloroacetaldehyde, thiodiglycolic acid, phosphoramide mustard and acrolein, with SH-containing molecules. Earlier studies have documented that the metabolic pathway of oxazaphosphorines leads to the formation of chloroacetyl-CoA with the consequent depletion of free CoA-SH [54]. L-Carnitine is known to detoxify acyl-CoA moieties with the formation of acylcarnitine and subsequent release of free CoA-SH, thus preserving substrate utilization and ATP production in mitochondria. This detoxification mechanism leads to an increased secretion of carnitine derivatives in urine with subsequent secondary deficiency in the molecule. Indeed, after treatment with ifosfamide, chloroacetylcarnitine has been detected in urine.

This progressive decrease (78%) in carnitine levels in cardiac tissue induced by CP in carnitine-depleted rats was paralleled by the marked increase in LDH and CK-MB and the massive degenerative changes in cardiac tissue, which may point to the possible involvement of carnitine deficiency in CP-induced cardiotoxicity. Most probably, DC-MD (via carnitine depletion) and CP (partly due to oxidative stress and partly due to carnitine depletion) produced such myocardial damage. This aggravated cardiomyopathy could be explained on the basis of myocardial carnitine deficiency with subsequent impairment in fatty acid oxidation and ATP production. It is well known that L-carnitine is an essential cofactor for mitochondrial transport and oxidation of long-chain fatty acids, which are the preferred substrates for ATP production in normal, well-oxygenated adult myocardium [55]. In the heart, depletion of carnitine and CoA-SH by either CP, DC-MD or both would impair β-oxidation of long-chain fatty acids and pyruvate oxidation, with the consequent decrease in ATP production and heart contractile function. This was supported by the marked decrease in ATP and free CoA-SH levels in heart tissue of carnitine-depleted rats, which renders cardiac cells vulnerable to damage by CP. Our hypothesis is consistent with earlier studies which have documented that chloroacetaldehyde and thiodiglycolic acid, two major toxic metabolites of oxazaphosphorines, inhibited the oxidation of long-chain fatty acids (carnitine dependent) but not medium-chain fatty acids (carnitine independent), indicating that oxazaphosphorines either sequester carnitine or inhibit long-chain fatty acid oxidation by inhibiting carnitine palmitoyltransferase I [56, 57].

In the current study, carnitine supplementation with PLC prevented the CP-induced decrease in cardiac carnitine content, intramitochondrial CoA-SH and ATP production. PLC produced cardioprotective effects by

providing the myocardium adequate carnitine for energy production. Possibly, PLC has a higher affinity for CoA-SH:carnitine acetyltransferase in mitochondria and may be metabolized into free L-carnitine and propionyl-CoA [58, 59]. The L-carnitine portion of PLC will increase fatty acid oxidation by increasing its mitochondrial transport through carnitine palmitoyltransferase I and/or decreasing the intramitochondrial acetyl-CoA/CoA-SH ratio. The propionyl-CoA formed by PLC metabolism in mitochondria will stimulate substrate oxidation since it can be converted into succinyl-CoA in a reaction mediated by propionyl-CoA carboxylase, thus increasing the flux of acetyl-CoA via the tricarboxylic acid cycle. Indeed, our results showed that PLC significantly increased free CoA-SH and decreased acetyl-CoA and the acetyl-CoA/CoA-SH ratio. Of note, carnitine supplementation had no effect on the antitumor activity of anticancer drugs [60, 61]. In conclusion, data from this study suggest, for the first time, that (1) carnitine deficiency plays an important role in cardiotoxicity, particularly in CP-related cardiomyopathy; (2) monitoring of serum and urinary carnitine levels may help to indicate CP-related toxicity, and (3) carnitine supplementation with PLC prevents the development of CP-induced cardiotoxicity. Further studies on the effects of carnitine supplementation in oxazaphosphorines-treated cancer patients may help to elucidate cancer-related fatigue and chemotherapy-induced multiple organ toxicity.

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