

Protection by L-Carnitine Against the Inhibition of Gene Expression of Heart Fatty Acid Binding Protein by Chronic Administration of Doxorubicin

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ABSTRACT

This study was designed to investigate if chronic administration of Doxorubicin (DOX) can alter the expression of the heart-type fatty acid binding protein (H-FABP) mRNA and the possibility of reversing this effect by chronic administration of L-carnitine in cardiac and skeletal muscle tissues. In this study, rats were divided into 4 groups namely: control group (injected I.P. with normal saline), DOX group (injected I.P. with DOX, 18 mg/kg, as a total cumulative dose divided in six equal doses, 3 mg/kg each, every other day over a period of two weeks), L-carnitine group (injected daily with 500 mg/kg L-carnitine) for six weeks and DOX plus L-carnitine group (injected daily with 500 mg/kg L-carnitine for six weeks starting two weeks prior and two weeks post DOX administration). Chronic administration of DOX resulted in a significant 75% decrease in H-FABP mRNA expression in both cardiac and skeletal muscle tissues, Whereas, H-FABP was not changed by L-carnitine. Daily administration of L-carnitine to DOX-treated rats completely restored the DOX-induced inhibition of H-FABP mRNA expression in cardiac and skeletal muscle tissues to normal levels. Worth mentioning is that, the inhibition of H-FABP by DOX in cardiac tissues was dose-dependent and parallel to the inhibition of palmitoyl-CoA oxidation in rat heart mitochondria. These studies suggest for the first time that: (1) chronic administration of DOX causes dose-dependent and cumulative inhibition of H-FABP gene expression and that daily administration of L-carnitine protects against this effect in cardiac tissues, (2) DOX-induced cardiotoxicity could be, at least in part, due to the inhibition of H-FABP gene expression and/or fatty acid oxidation.

Key Words: *Doxorubicin - Heart fatty acid binding protein - Cardiotoxicity - L-carnitine.*

INTRODUCTION

Doxorubicin (DOX) is an anthracycline antibiotic with a broad spectrum antitumour activi-

ty against a variety of tumours (10). Its use, unfortunately, is limited due to its specific dose-dependent cardiotoxicity [8,9,15,18]. Although several hypotheses have been postulated to explain the mechanisms of DOX-induced cardiotoxicity, the exact cause of this pathogenesis is still a subject of great debate [4,24,26,27,28].

Free fatty acids (FFA) represent the major source of energy in the normal adult working heart [21]. The inhibition of this vital pathway is usually associated with cardiomyopathy secondary to the decrease in ATP supply and the accumulation of fatty acid oxidation toxic intermediates including, long chain fatty acyl-CoA (FA-CoA) and long chain fatty acyl-carnitine (FA-carnitine) derivatives [12,17]. A growing number of studies have demonstrated that DOX induces its cardiotoxicity by the inhibition of long-chain FFA oxidation and that L-carnitine completely protects the myocardium against this effect [2,16,24]. These authors concluded that the inhibition of FFA oxidation by DOX was due to the inhibition of carnitine palmitoyl transferase I (CPT I) and long chain beta-oxidation enzymes as well as depletion of L-carnitine in cardiac tissues.

Heart-type fatty acid binding protein (H-FABP) is non-enzymatic protein located in the cytosol of cardiac myocytes and plays an important role in eliminating the toxicity of FFA and their intermediates in the cytosol by transporting these compounds to their sites of metabolic conversion [25]. Although, the effects of

DOX and/or L-carnitine on the overall pathway of FFA oxidation in the heart have been completely studied, however, their effects on the expression of H-FABP which is of major importance in this vital pathway are not studied yet. Therefore, this study has been initiated with the following specific aims: (1) to investigate whether chronic administration of DOX alters the expression of H-FABP in cardiac tissues and if so, whether these alterations are dose-dependent and may contribute to DOX-induced cardiotoxicity and (2) to investigate whether daily administration of L-carnitine has the potential ability to protect the myocardium against these alterations and if this is the case, what could be the effect of L-carnitine on the antitumour activity of DOX?

MATERIAL AND METHODS

Animals: Male Sprague-Dawley rats, weighing 200-250 g and female Swiss Albino mice, weighing 20-22 g, were obtained from the animal house of the National Cancer Institute (NCI), Cairo, Egypt. Animals were allowed free access to standard diet essentially free from L-carnitine derivatives and water ad. libitum. A line of Ehrlich ascites carcinoma (EAC) cells was supplied through the courtesy of Dr. C. Benckuijsen, Amsterdam, the Netherlands and maintained in female mice by weekly I.P. transplantation since 1982.

Material: [$1-^{14}\text{C}$] palmitoyl-CoA, was purchased from New England Nuclear (Boston, MA, USA). Sigma was the source of bovine serum albumin (BSA, essentially fatty acid free) and palmitoyl-CoA. DOX was a generous gift from NCI drug store. L-carnitine (a product of Sigma-Tau pharmaceuticals) was kindly supplied by Dr. Menotti Calvani, Sigma Tau, Pomezia, Italy. All other chemicals used were of the highest analytical grade.

Doxorubicin and l-carnitine administration protocol:

A total of 120 rats were used and divided into four groups, 30 rats each: 1) normal control rats injected intraperitoneally (I.P.) with 0.9% sodium chloride solution (no mortality was observed in this group), 2) rats injected I.P. with a total cumulative dose (18 mg/kg) of DOX, divided into six equal doses (3 mg/kg each) given every other day over a period of two weeks, ac-

ording to Beanlands et al. [5]. At the end of this period, signs of congestive heart failure including ascites and liver rigidity were evident. The mortality was approximately 50% in this group. 3) Rats injected I.P. daily with L-carnitine (500 mg/kg) over a period of six weeks (no mortality was observed in this group) and 4) rats injected with L-carnitine plus DOX. L-carnitine was injected I.P. (daily dose of 500 mg/kg of body weight) for six weeks, starting two weeks prior and ending two weeks post DOX administration (The mortality was 15% in this group). At 24 hrs after the last dose, H-FABP mRNA was determined in the heart and soleus muscle and palmitoyl-CoA oxidation was determined in heart mitochondria isolated from animals in each of these four groups. Moreover, the expression of H-FABP during development of DOX cardiotoxicity was determined in cardiac tissues 24 hrs after each dose of DOX.

Isolation and determination of H-FABP mRNA (Northern Blotting):

Rat hearts and soleus muscles were quickly removed from animals, washed with saline and frozen immediately in liquid nitrogen until use. Frozen tissues were grounded in a mortar using dry ice and liquid nitrogen to become powdered. The total RNA was extracted from powdered tissues using Trizol reagent (monophasic solution of phenol and guanidine isothiocyanate) according to the single step RNA isolation method developed by Chomezynski and Sacchi [11]. The purity and concentration of isolated RNA were measured using Shimadzu 160U spectrophotometer at wave lengths 260 and 280 nm. The 260/280 ratio for isolated RNA by this technique was 1.6-1.8. RNA was then loaded on RNA agarose gel-electrophoresis. RNA bands were then transferred to a nylon membrane and H-FABP mRNA was located by hybridization with radiolabeled probe followed by autoradiography. The density of the bands was measured using LKB 2202 ultrascan laser densitometer then scanned.

Isolation of rat heart mitochondria:

Rat heart mitochondria were isolated 24 hrs after the end of the chronic treatment protocol according to Chappel and Hansford [10]. The isolation buffer contained 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris-HCl (pH 7.4) and 1 mM EDTA.

Palmitoyl-CoA oxidation in rat heart mitochondria:

Palmitoyl-CoA oxidation in mitochondria was measured using the method of Yang et al. [29]. The reaction mixture contained in a final volume of 0.9 ml, 50 mM Tris-HCl (pH 7.4), 120 mM KCl, 0.5 mM L-carnitine, 0.5 mM EDTA-K2 (pH 7.4), 2 mM KPi and 0.1 mg/ml BSA were placed in a 25-ml Erlenmeyer flask. To this suspension, 0.1 ml of 400 μ M [1-¹⁴C] palmitoyl-CoA was added. Substrate oxidation was initiated by the addition of rat heart mitochondria (0.5-1 mg). The incubation was continued under shaking at 37°C for 30 min. An injection of 0.3 ml of 1 M hyamine hydroxide was administered through the septum into the center well to absorb the released ¹⁴CO₂ and the reaction was terminated by injecting 1 ml of 7% perchloric acid through the septum into the incubation medium. The flasks were shaken continuously for additional 2 hr at 37°C. Then, the plastic center well was removed, placed into a scintillation vial containing 10 ml of Scinti Verse BD and counted in a liquid Scintillation Counter (Betamatic Kontron, Sebai, Italy).

Effect of L-carnitine on the antitumour activity of DOX:

In this experiment, 2 X 10⁶ EAC-cells were transplanted subcutaneously in the right thigh of the lower limb of each mouse. Mice with a palpable solid tumour mass (100 mm³) that developed within 7 days after implantation were divided into 4 groups and treated as the following: In the first group, animals were treated I.P. with single 18 mg/kg DOX, whereas, the second group animals were treated I.P. with single 500 mg/kg L-carnitine. Animals in third group were injected with a combination of DOX and L-carnitine exactly as the first and second regimens, whereas, the last group served as control and injected I.P. with normal saline. The change in tumour volume was measured every other day using a vernier caliper and calculated by the following formula according to Osman et al. [22].

$$\text{Tumour volume (mm}^3\text{)} = 4/3 \pi (A/2)^2 \times B/2$$

Where A is the minor tumour axis and B is the major axis.

Tumour growth delay (TGD) was then calculated as the additional days for each individu-

al treated tumour to reach 500 mm³ beyond that of the control group.

Determination of protein:

Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Richmond, VA, USA) according to the published method of Bradford [6].

RESULTS

The effects of DOX, L-carnitine and their combination on the H-FABP mRNA expression in cardiac and soleus muscle tissues are shown in Table (1) and Fig. (1). Chronic administration of DOX resulted in a significant 75% decrease in H-FABP mRNA expression in both cardiac (Fig. 1A) and soleus muscle (Fig. 1B) tissues compared to normal controls. Interestingly, daily administration of L-carnitine to the DOX-treated rats completely restored the DOX-induced inhibition of H-FABP mRNA expression in cardiac and soleus muscle tissues to normal levels.

Table (2) shows the effects of single (3 mg/kg) and different cumulative dose levels (6,9,12,15 and 18 mg/kg) of DOX on H-FABP mRNA expression during development of DOX cardiotoxicity. DOX resulted in a significant cumulative and dose-dependent decrease in the expression of H-FABP mRNA in cardiac tissues.

The effects of DOX, L-carnitine and DOX plus L-carnitine on ¹⁴CO₂ released from the oxidation of [1-¹⁴C] palmitoyl-CoA (an index of Carnitine palmitoyl transferase I) in isolated rat heart mitochondria are shown in Fig. (2). DOX induced a significant 48% decrease in palmitoyl-CoA oxidation compared to normal controls (4.7 \pm 1.2 for DOX vs 7.2 \pm 1.9 for control), whereas L-carnitine resulted in a significant 66% increase. On the other hand, daily administration of L-carnitine to DOX-treated rats resulted in complete reversal of DOX-induced decrease in the oxidation of palmitoyl-CoA to the normal values.

Table (3) shows the effects of single dose of DOX (18 mg/kg), L-carnitine (500 mg/kg) and their combinations on the growth of SEC. Combined treatment by DOX and L-carnitine showed the same TGD (6.9 \pm 0.8 days) as for DOX alone (6.6 \pm 0.5 days).

Table (1): Effect of chronic administration of DOX, L-carnitine and DOX plus L-carnitine on H-FABP mRNA expression in rat cardiac and soleus muscle tissues.

Groups	H-FABP mRNA expression	
	Cardiac tissues	Soleus muscle
Control	3.69±0.36	1.20±0.07
DOX	0.90±0.21*	0.30±0.07*
L-carnitine	3.56±0.18	1.46±0.05
DOX + L-carnitine	3.50±0.17#	2.45±0.15*,#

Data are presented as the mean ± SEM of four separate experiments. Data were normalized to the 18 S contents. * Indicate significant change from control ($p < 0.05$), whereas # Indicate significant change from DOX ($p < 0.05$).

Table (2): Dose-response relationship for the effect of different cumulative dose levels of DOX on H-FABP mRNA expression in rat cardiac tissue.

Total dose of DOX	H-FABP mRNA expression in cardiac tissues
Control	3.16±0.60*
DOX, 3 mg/kg	3.24±0.33*
DOX, 6 mg/kg	2.07±0.48*,#
DOX, 9 mg/kg	2.14±0.55*,#
DOX, 12 mg/kg	1.91±0.58*,#
DOX, 15 mg/kg	1.50±0.41*,#
DOX, 18 mg/kg	0.51±0.31#

Data are presented as the mean ± SEM of four separate experiments. Data were normalized to the 18 S contents. Doses with different symbols indicate the presence of significant ($p < 0.05$).

Table (3): Tumour growth delay of Solid Ehrlich carcinoma after administration of a single dose of DOX and/or L-carnitine.

Groups	Tumour growth delay (days)
DOX (18 mg/kg)	6.6±0.5
L-carnitine (500 mg/kg)	1.1±0.3
DOX + L-carnitine	6.9±0.8

Data are presented as mean ± SEM (n=10). The doses of DOX and L-carnitine were 18 mg/kg and 500 mg/kg, respectively.

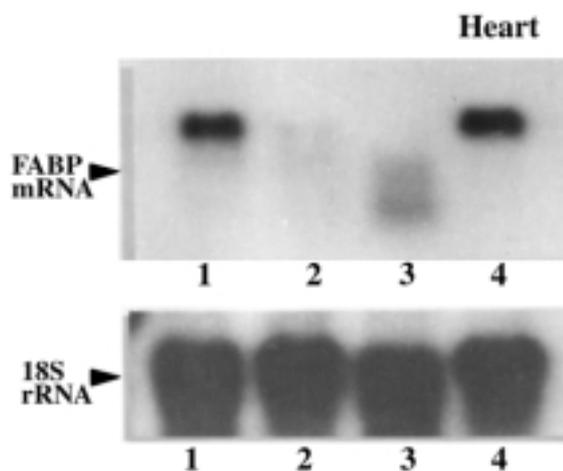


Fig. (1,A)

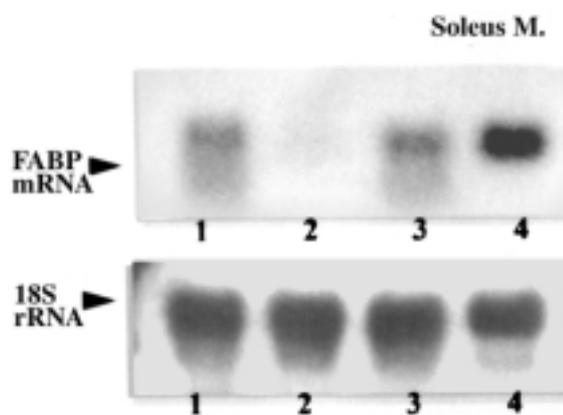


Fig. (1,B)

Fig. (1): Northern blot analysis of RNA isolated from heart (Fig. 1A) and soleus muscle (Fig. 1B) tissues demonstrating the effect of DOX, L-carnitine and their combination on H-FABP mRNA expression in normal rats (upper). It is normalized with 18S (lower). The different lanes represents, 1-control, 2-DOX, 3-L-carnitine, 4-DOX+L-carnitine.

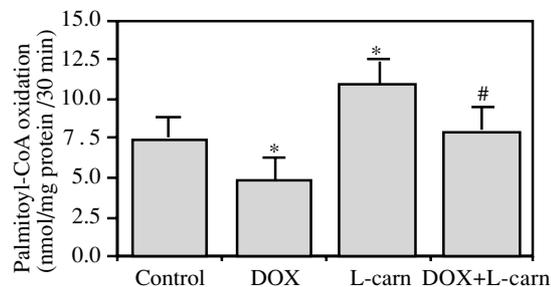


Fig. (2): Effect of chronic administration of DOX, L-carnitine and DOX plus L-carnitine on palmitoyl-CoA oxidation in rat heart mitochondria. Data are presented as the mean ± SEM of four separate experiments. * indicates significant change of DOX and L-carnitine vs control ($p < 0.05$), whereas # indicates significant change of DOX + L-carnitine vs DOX ($p < 0.05$).

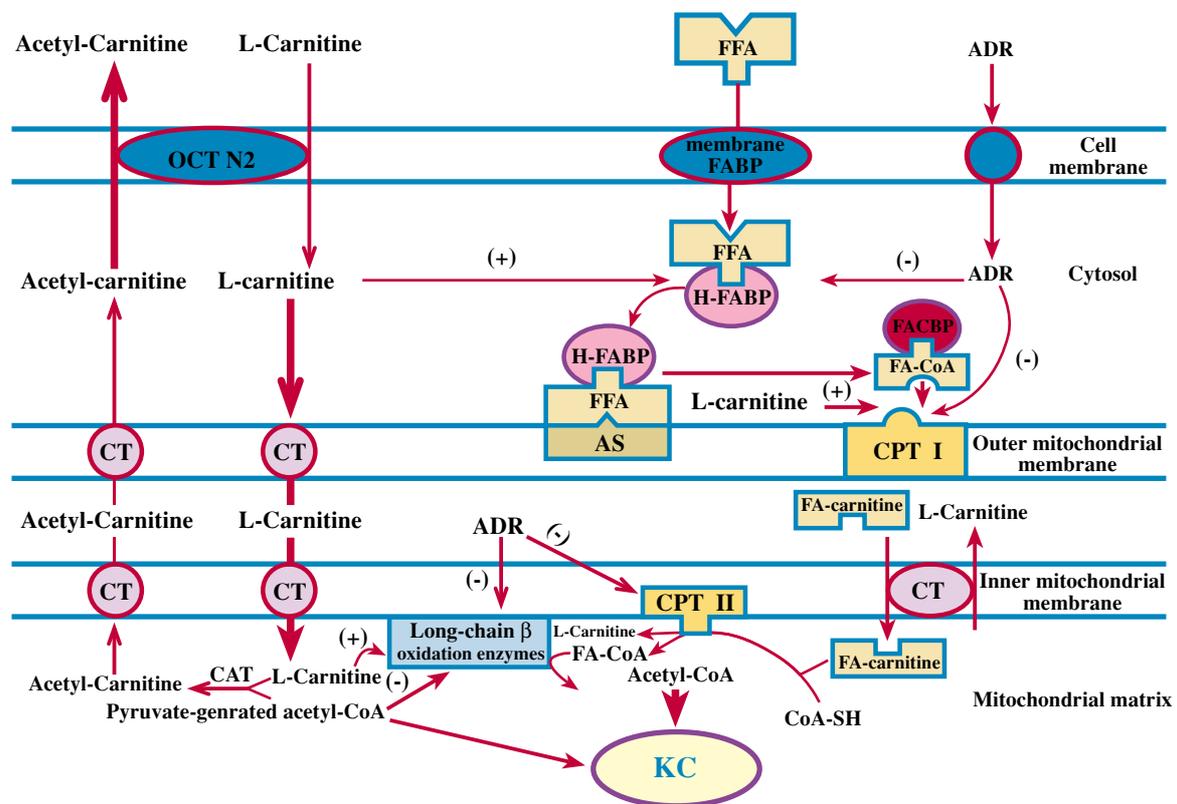


Fig. (3): Inhibition of H-FABP mRNA expression in the heart as a novel mechanism for DOX-induced cardiotoxicity and for L-carnitine mediated protection against this effect (modified Sayed-Ahmad et al. [24]).

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|--------------|---|--------|---|
| FFA | : Free fatty acid, ADR = DOX | CT | : Carnitine/acylcarnitine translocase |
| FA-CoA | : Fatty acyl-CoA | CAT | : Carnitine acetyl transferase |
| FA-carnitine | : Fatty acyl-carnitine | CPT I | : Outer carnitine palmitoyltransferase |
| H-FABP | : Heart-type fatty acid binding protein | CPT II | : Inner carnitine palmitoyltransferase |
| FACBP | : Fatty acyl-CoA binding protein | OCTN2 | : Organic cation/carnitine transporter number two (-) and (+) indicate inhibition and stimulation respectively. |

DISCUSSION

Data from this study have clearly demonstrated for the first time the severe inhibition of H-FABP gene expression by chronic administration of DOX in rat heart and soleus muscle. This effect could be explained on the bases of the inhibition of DNA, RNA and protein synthesis by DOX as part of its cytotoxic mechanism. Our results are consistent with previous studies in which the inhibition of long-chain FFA oxidation by chronic administration of DOX was reported [2,5]. Interestingly, administration of L-carnitine to DOX-treated rats, completely reversed the effect of DOX and restored the DOX-induced inhibition of H-FABP mRNA expression to its normal values. These protective effects of L-carnitine against DOX-induced inhibition of both protein synthesis, including cell-protecting heat shock protein [27]

and long chain FFA oxidation in cardiac tissues were reported [2,24]. The question now arises, how does the inhibition of H-FABP play a role in DOX cardiotoxicity and how does L-carnitine relief this effect. Since DOX cardiotoxicity is characterized by being cumulative and dose-dependent, it is possible that the observed dose-dependent and cumulative inhibition of H-FABP mRNA expression by DOX in the myocardium (Table 2), may contribute in the pathogenesis of DOX-related cardiotoxicity. Moreover, the inhibition of H-FABP expression could inhibit the oxidation pathway of FFA (Fig. 3), which provide up to 70% of the overall energy required for cardiac functioning [21,24], with the subsequent accumulation of FFA and their toxic intermediates, including long chain FA-CoA and long chain FA-carnitine. As shown in Fig. (3), FFA are accumulated secondary to the inhibition of their delivery by H-

FABP from cell membrane to the outer mitochondrial membrane where they are activated into long chain FA-CoA by acyl-CoA synthetase (AS). Long-chain FA-CoA are accumulated following inhibition of their delivery by fatty acyl-CoA binding protein (FACBP) to the CPT I, which is responsible for their translocation into the mitochondria where long-chain beta-oxidation enzymes are located on the inner aspect of the inner mitochondrial membrane (Fig. 3). These accumulated compounds are amphiphilic and have detergent-like effects, altering membrane integrity, inhibiting several membrane-bound enzymes, provoking free radical injury and decreasing myocardial contractility [3,17,20,25].

In this study, the inhibition of H-FABP and its contribution of DOX-induced cardiotoxicity is confirmed by the data presented which have demonstrated the inhibition of palmitoyl-CoA oxidation by DOX in rat heart mitochondria (Fig. 2). Previous studies have clearly demonstrated the inhibition of CPT I and depletion of L-carnitine in DOX-dependent [2,7,16] and DOX-independent [23] cardiomyopathies. Recently, inhibition of CPT I and depletion of L-carnitine by DOX have been described to induce apoptosis of cardiac myocytes [4]. The protective effects of L-carnitine against DOX-induced apoptosis of cardiac myocytes [4], inhibition of cell-protecting heat shock protein synthesis [27] and lipid peroxidation in cardiac tissues have been reported [19]. As shown in Fig. (3), L-carnitine is transported into cardiac myocytes through organic cation/carnitine transporter number two (OCTN2) [13] and within the myocytes into mitochondria through carnitine/acylcarnitine translocase (CT). In cytosole, L-carnitine preserves the expression of H-FABP which protects the cardiac membrane integrity by buffering the toxicity of FFA and their toxic intermediates. Whereas, in mitochondria, L-carnitine increases the oxidation of FFA by increasing its mitochondrial transport through CPT I and/or the increase in mitochondrial CoA-SH/acetyl-CoA ratio. Previously, Abdel-Aleem et al. [1] reported that L-carnitine increased the mitochondrial efflux of pyruvate-generated acetyl-CoA in the form of acetyl carnitine in a reaction mediated by carnitine acetyl transferase (CAT).

Our results also suggest the existence of H-FABP in skeletal muscle but to a much lesser

extent than cardiac tissue. This is consistent with the previous study by Heuckeroth et al. [14] that found H-FABP to exist in skeletal muscle. However, the observed increase in H-FABP by L-carnitine in soleus muscle and not in the heart (Table 1) could be explained on the basis that the heart already has quite abundant amount of H-FABP and so no need to increase its expression to meet the increase in beta-oxidation. Worth mentioning is that L-carnitine has no effect on the antitumour activity of DOX since additional administration of L-carnitine to DOX-treated animals, showed a similar TGD pattern as for DOX treated animals (Table 3). Despite the amount of data in the literature, the present observations suggest for the first time a novel mechanism for DOX-induced cardiotoxicity and for L-carnitine mediated protection against this toxicity. In conclusion, this study demonstrated the inhibition of H-FABP mRNA expression by chronic administration of DOX and the relief of this effect by chronic administration of L-carnitine. DOX-induced cardiotoxicity could be, at least in part, due to inhibition of H-FABP gene expression. The addition of L-carnitine to chemotherapeutic regimens containing DOX is useful in preventing its cardiotoxicity without interfering with its antitumour activity.

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