

Modulation of Induced Cardiocytotoxicity and Genotoxicity of Doxorubicin in Rat by L-Carnitine.

OLA H.M. EL-HABIT*, Ph.D.; MOHAMED M. SAYED-AHMED**, Ph.D.; MOHAMED S. GABRY*, Ph.D.; MONA F.K.KHALIL*, B.Sc. and ABDEL MONEIM M. OSMAN**, Ph.D.

*Department of Zoology, Faculty of Science, Helwan University.

**Cancer Biology Department, Pharmacology Unit, National Cancer Institute, Cairo University.

ABSTRACT

Doxorubicin (DOX) is an anthracycline antibiotic with broad-spectrum antitumor activity. Its effectiveness has been limited by the occurrence of dose-related myocardial and bone marrow toxicity.

L-carnitine is tested in this study to evaluate its protective effect against DOX induced cytotoxicity and genotoxicity.

Four groups of adult female rats, each of 15 animals were used; one is used as control receiving 0.5ml of saline, the other groups received either DOX (3mg/kg), L-carnitine (100mg/kg) or a combination of the two drugs. The treatment was continued i.p. every other day for two weeks. Five animals of each group were injected with 0.2ml of colchicine 2 h before sacrifice, which took place 24 h after the last treatment.

Cardiotoxicity was assessed by measuring the serum levels of lactic acid dehydrogenase (LDH), creatine phosphokinase (CPK), glutamic oxaloacetic transaminase (GOT), Reduced glutathione (GSH), malonaldehyde (MDA) and mitochondrial palmitoyl Co-A and octanoate oxidation were also, determined in cardiac tissue homogenate. The femurs were removed and bone marrow was processed for the preparation of metaphase chromosomes and determination of mitotic activity.

DOX significantly increased LDH, CPK, GOT and MDA and significantly decreased GSH and palmitoyl Co-A. Administration of L-carnitine one hour before DOX treatment caused significant recovery for the serum enzymes LDH, CPK, GOT, and MDA, GSH and palmitoyl Co-A.

Cytogenetic analysis showed that DOX increased the incidence of chromosomal aberration 18.4% in bone marrow cells and inhibited mitosis to about 50% of its normal rate. Administration of L-carnitine one hour before treatment with DOX significantly decreased the incidence of chromosomal aberrations (14.8%) and increased mitotic activity (10.4). The results suggest that the cardiotoxicity and genotoxicity induced by DOX took place via a number of possible mechanisms. The results obtained suggest that L-carnitine could be used together with DOX as an adjuvant therapy.

Key Words: Doxorubicin - L-carnitine - Cardiocytotoxicity - Genotoxicity - Antioxidants - Anticancer.

INTRODUCTION

Doxorubicin (DOX) is an anthracycline glycoside antibiotic that possesses a potent and broad spectrum antitumor activity against a variety of human tumors [11,14]. Cardiotoxicity induced by the intake of DOX is usually mediated through lipid peroxidation and inhibition of long fatty acid oxidation in cardiac tissues [1,20,28,30]. Intercalation of DOX with DNA results in inhibition of topoisomerase-II activity, the formation of DNA single and double strand breaks, mutation and chromosomal aberrations [16,19,38,47].

DOX causes numerous changes in different metabolic reactions within cardiac cells with major adverse undesirable effects that involve cardiotoxicity and bone marrow suppression [9,14,18]. Elevation of serum enzymes such as glutamic oxaloacetic transaminase (GOT), lactate dehydrogenase (LDH), creatine phosphokinase (CKP) and cardiac isoenzymes of LDH and CPK were reported [2].

Irreversible cardiomyopathy restricts the use of DOX as an antitumor in 30-40% of the patients [24]. Combinations of DOX with radiotherapy and other regimens of chemotherapy increase the risk of inducing cardiomyopathy at even a much lower dose [42].

Several chemical compounds are known to exhibit protection against induced cytotoxicity. L-carnitine, a β -hydroxy- γ -trimethylammonium butyrate present in all mammalian tissues, oc-

curs endogenously and also can be obtained from diet. Its concentration varies between different tissues of the body with the highest concentration in the epididymis, heart and skeletal muscles [12].

L-carnitine functions as an essential cofactor in the transport of fatty acids into the mitochondria [37]. Other important roles of L-carnitine have been reported as an intramitochondrial buffer to regenerate free CoA [27], regulation of mitochondrial acyl-CoA, CoA-SH ratio [25], offers protection from ischemic injury and improves cardiac function [30,32].

In this study, the protective role of L-carnitine is evaluated against DOX-induced cardio-and genotoxicity in female albino rats. The mechanisms of its action as an antioxidant were also investigated.

MATERIAL AND METHODS

Animals:

Female albinos rats weighing 200-250g were obtained from the National Cancer Institute, Cairo University. The animals were housed in appropriate cages under standard laboratory conditions of temperature, humidity and dark-light cycle. The animals were supplied with special diet free from L-carnitine or its derivatives and water ad libitum.

Drugs:

Doxorubicin (Farmitalia-Carlo Erba, Italy) was obtained in vials containing 10mg DOX hydrochloride plus 50mg lactose in a dry lyophilized powder form. The contents of each vial were dissolved in sterile normal saline solution. L-carnitine (Sigma-Tare, Rome, Italy) was prepared in 500mg/ml solution.

Treatment:

Four groups of female rats, each of 15 animals were used, 10 for the biochemical assays and 5 for the cytogenetic study.

Group I was used as control and each animal was injected with 0.5ml saline.

Groups II, III, and IV received 0.5ml of either: DOX (3mg/Kg); L-carnitine (100 mg/Kg) or a combination of both. The dose was administered i.p. every other day over a two weeks period. Group IV, which received the combination of the drugs was given L-carnitine one

hour before DOX administration.

Animals were sacrificed 24-hours after the last treatment.

Methods:

A- Biochemical measurements:

a- Cardiac serum enzymes

Blood samples were collected from each animal and the serum was obtained by centrifugation and used for the determination of:

- 1- Lactate dehydrogenase (LDH), by the method of Buhl and Jakson [13].
- 2- Creatine phosphokinase (CPK), by the method of Szasz [39].
- 3- Glutamic oxaloacetic transaminase (GOT), by the method of Reitman and Frankel [31].

b- Cardiac tissue levels of lipid peroxidation and reduced glutathione (GSH):

The heart of each animal was quickly excised, washed with normal saline, blotted with filter paper, weighed and homogenized in ice cold-distilled water in Branson Sonifier 250 (VWR Scientific, Darbury, Conn., USA). An aliquot of 0.5ml of 20% homogenate was used for the determination of total lipid peroxidation as described by Buhl and Jackson [13]. An aliquot of 0.5ml of 20% cardiac tissue homogenate was used for the determination of GSH following the method described by Ellman [21].

c- Determination of β -oxidation of fatty acids in mitochondria:

A portion of the heart homogenate of each animal was suspended in ice cold isolation buffer and mitochondria were isolated according to the method described by Chappel and Hansford [17]. Substrate oxidation in mitochondria was measured according to the methods of Yang et al., [44]. The substrates (1-C¹⁴) palmitoyl-CoA (8.8 x 10⁴ dpm) and (1-C¹⁴) octanoate (2.2 x 10⁵ dpm) were purchased from New England Nuclear (Boston, MA, USA). The radioactivity of released ¹⁴CO₂ was measured in a liquid scintillation counter (Betamatic, Kontron, Sebai, Italy).

B- Cytogenetic assays:

1- Metaphase chromosome analysis:

Animals were injected with colchicine (1.0mg/kg) 2 hours before sacrifice. Bone marrow was aspirated from both femurs of each rat

in a test tube and a fine cell suspension was obtained. Cells were then treated with the hypotonic solution (0.075M KCl), then fixed in Carnoy's fixative. Slides were prepared and stained with 5% Giemsa solution. The slides were coded blindly to avoid bias examination. Fifty metaphases were examined microscopically using 100X-oil immersion and the aberrant cells were classified and recorded [34].

2- Mitotic index:

Bone marrow cells were examined for mitotic activity by scoring the number of cells in mitosis. Cytotoxic activity of the drug assessed by the mitotic index is calculated as percentage of dividing cells to the total number of cells counted.

Statistical analysis:

Data are expressed as means and standard errors. The Student "t" test was used to compare the significance of the observed differences between means of experimental and control groups.

RESULTS

1- Effect of DOX, L-carnitine and their combination on serum LDH, CPK and GOT activity:

Table (1) shows that repeated treatment with DOX (3 mg/kg) for two weeks resulted in a highly significant two-fold increase in serum LDH, CPK and GOT levels. Treatment with L-carnitine (100 mg/kg) has no significant effect on any of the serum enzymes studied. However, administration of L-carnitine one hour prior to treatment with DOX results in a complete reversal of DOX-induced increase in serum CPK level and a significant 66% and 52% recovery for serum GOT and LDH activities, respectively.

2- Effect of DOX, L-carnitine and their combination on lipid peroxidation (MDA) and antioxidant capacity (reduced glutathione) in cardiac tissues:

Treatment with DOX results in a significant 1.5 fold increase in MDA level, but the level of reduced glutathione decreased significantly to

50% of the corresponding control value (Table 2). Treatment with L-carnitine resulted in 30% decrease of the control level of MDA but caused 30% increase in GSH (Table 2).

Administration of L-carnitine one hour before DOX treatment has reversed the effect of DOX to near normal values.

3- Effect of DOX, L-carnitine and their combination on cardiac mitochondrial β -oxidation:

Administration of DOX resulted in a significant 35% decrease in palmitoyl-CoA oxidation whereas L-carnitine caused a significant 47% increase in palmitoyl-CoA oxidation, Table (3). Administration of L-carnitine one hour before treatment with DOX resulted in complete reversal of DOX-induced decrease in palmitoyl-CoA oxidation to normal values. On the other hand, neither DOX, L-carnitine nor their combination resulted in any significant effect on octanoate oxidation.

4- Effect of DOX, L-carnitine and their combination on mitotic activity:

The effect of different treatments is given in Table (4). The mitotic index is significantly lower in DOX treatment compared with the control. L-carnitine had no effect on mitotic activity when given alone but when preceded the DOX treatment, 65% recovery of mitotic index was achieved.

5- Effect of L-carnitine on genotoxicity induced by DOX:

The data presented in Table (5) show a significant increase in the frequency of bone marrow cells with chromosomal aberrations induced by DOX treatment. L-carnitine has no effect on the incidence of chromosomal aberrations when administered singly but it has a very marked protective effect (50%) against DOX induced genotoxicity. Table (6) shows the different types of induced chromosomal aberrations in DOX treated rats. Chromatid breaks constituted more than half the types induced whereas gaps and ring chromosomes were less frequent (5.2 and 4.8%) respectively. The protective effect of L-carnitine on induced chromosomal aberrations is random suggesting no selectivity.

Table (1): Effect of DOX, L-carnitine and their combination on the levels of cardiac enzymes in albino rats.

Treatment	Serum enzyme levels (U/L)		
	LDH	CPK	GOT
Control	510 ± 24	1160 ± 64	52 ± 2
DOX	1260 ± 43	2080 ± 91*	112 ± 6*
L-carnitine	584 ± 35	1240 ± 23	45 ± 3
DOX+L-carnitine	654 ± 54*#	1140 ± 40 #	74 ± 7*#

Values represent means ± SEM (N=10).

* Significantly different from control ($p < 0.05$).

Significantly different from DOX ($p < 0.05$).

Table (2): Effect of DOX, L-carnitine and their combination on the Malonaldehyde (MDA) and reduced Glutathione (GSH) content of rats heart tissue.

Treatment	MDA	GSH
	(n mol/gm tissue)	(µ mol/gm tissues)
Control	59 ± 4	1.11 ± 0.07
DOX	90 ± 3*	0.56 ± 0.06*
L-carnitine	38 ± 2*	1.78 ± 0.08*
DOX+L-carnitine	66 ± 4 #	0.96 ± 0.01#

Values are presented as means ± SEM (N = 10).

* Significantly different from control ($p < 0.05$).

Significantly different from DOX ($p < 0.05$).

Table (3): Effect of DOX, L-carnitine and their combination on long chain fatty acid oxidation in mitochondria of cardiac tissue of rat.

Treatment	Rate of substrate oxidation (n mol / mg protein / 30 min)	
	Palmitoyl-Co A	Octanoate
Control	7.2 ± 1.9	15.7 ± 2.3
DOX	4.7 ± 1.2*	13.9 ± 2.9
L-carnitine	10.6 ± 2.2*	16.3 ± 4.1
DOX+L-carnitine	8.2 ± 2.3#	14.3 ± 3.7

Values represent means ± S.D (N=10).

* Significantly different from control ($p < 0.05$).

Significantly different from DOX ($p < 0.05$).

Table (4): Effect of DOX, L-carnitine and their combination on mitotic index of bone marrow cells of rats.

Treatment †	Total No. of cells	No. of cells in mitosis	Mitotic index ± S.E
	Control	5000	617
DOX	5000	316	6.32 ± 4.22*
L-carnitine	5000	648	12.96 ± 5.47
DOX+L-carnitine	5000	520	10.4 ± 1.87*#

† Five animals per group.

* Significant from control group ($p < 0.05$).

Significant from DOX group ($p < 0.05$).

Table (5): Effect of DOX, L-carnitine and their combination on the frequency of aberrant cells of bone marrow in rats.

Treatment †	No. of Cells	Total aberrant cells.		Cells with one aberration.		Cells with more than aberration.	
		No	%	No	%	No	%
		Control	250	5	2	5	2
DOX	250	46	18.4	3	1.2	23	9.2*#
L-carnitine	250	34	13.6	2	0.8	15	6*#
DOX+L-carnitine	250	12	4.8*	1	0.4	8	3.2*

† Five animals per group.

* Indicate significance from control group ($p < 0.05$).

Indicate significance from DOX group ($p < 0.05$).

Table (6): Effect of DOX, L-carnitine and their combination on the frequency of chromosomal aberrations of bone marrow cells in rat.

Treatment †	No. cells scored	Types and frequency of chromosomal aberration									
		Total		Chromatid break		Chromatid gap		Ring chromosome		Centric fusion	
		No	%	No	%	No	%	No	%	No	%
Control	250	5	2	1	0.4	2	0.8	1	0.4	1	0.4
Dox	250	60	24*	31	12.4*	13	5.2*	12	4.8*	4	1.6
L-carnitine	250	4	1.6	1	0.4	1	0.4	1	0.4	1	0.4
DOX+L-carnitine	250	37	14.8*#	17	6.8*#	9	3.6*	7	2.8*	4	1.6

Five animals per group.

* Indicate significance from control group ($p < 0.05$).

Indicate significance from DOX group ($p < 0.05$).

DISCUSSION

Chronic administration of DOX to rats significantly increased cardiotoxicity manifested by elevation of the serum LDH, CPK and GOT levels. Our results are in good agreement with those previously reported [40,41]. However, the treatment with L-carnitine one hour before DOX administration resulted in either complete reversal or considerable recovery of the three serum enzyme activities. L-carnitine is well known for its ability to stabilize cardiac cell membranes and preserve their integrity, which consequently results in regulating cardiac enzymes release [8].

Generation of free radicals with the subsequent lipid peroxidation in cardiac tissues, is an important mechanism by which DOX induces its cardiotoxicity [29,41]. Several powerful antioxidants such as curcumin, probucol, natural flavenoid antioxidants and captopril have been examined as potential protective agents against DOX related cardiotoxicity [3,41]. In this study L-carnitine has been investigated as a potential protector against DOX induced oxidative damage in the myocardium of rats. The results obtained revealed that repeated treatment with DOX increased MDA production in cardiac tissues. The increase in MDA is likely to follow DOX induced increase in free radical generation and or decrease in lipid peroxidation enzymes. These results correlate positively with other studies where oxidative stress, lipid peroxidation and mitochondrial dysfunction have been associated with DOX induced cardiomyopathy [28].

Reduced glutathione is an important endogenous antioxidant which control the enzymatic and non-enzymatic detoxification of reactive oxygen species generated by many compounds [41]. In the present study, DOX caused a significant decrease in GSH level in cardiac tissues. The decreased concentration of cardiac GSH induced by DOX was observed in other studies [41]. Moreover, it has been reported that exogenous administration of GSH to mice provide significant protection against DOX cardiomyopathy [47].

In this study, administration of L-carnitine increased significantly the level of GSH and decreased significantly the level of MDA in rat cardiac tissues. The administration of L-carnitine before the treatment with DOX re-

versed the depletion of GSH and the increase in MDA induced by DOX. Similar results were reported [35,46]; where L-carnitine was found to possess similar actions and produce complete protection against cisplatin induced nephrotoxicity and bleomycin induced pulmonary toxicity by increasing the antioxidant defense mechanism [5].

The present work also demonstrates that repeated administration of DOX caused a progressive inhibition of palmitoyl-CoA oxidation but has no effect on octanoate oxidation in isolated mitochondria. Our results are consistent with the data reported by Sayed Ahmed [36] who concluded that the inhibition of palmitoyl-L-carnitine oxidation by DOX is secondary to the depletion of ATP and the accumulation of toxic fatty acids intermediates to cell death.

Administration of L-carnitine one hour before treatment with DOX provided excessive exogenous source of L-carnitine which compensate for any depletion of endogenous L-carnitine and hence maintained the normal metabolic processes and protected against DOX induced cardiotoxicity. Supplementation with L-carnitine has also been shown to protect cardiac structure and improve cardiac function in DOX induced cardiomyopathy [8,15,35].

The increase of LDH level in serum and extracellular fluid suggests an increased leakage of this enzyme from mitochondria as a result of toxicity induced by treatment with DOX. This index has been recently used in other studies to test for cardiotoxicity [43,45]. The generation of free radicals by DOX in the form of DOX semiquinone free radical has been suggested to play a major role in its cardiotoxic effects [7]. Moreover, the free radical may induce destructive acute myocardial injury leading to lysis of a number of myocytes, as well as loss of cytoplasmic membrane integrity. Administration of L-carnitine one hour prior to DOX treatment has resulted in 52% decrease in LDH. A likely mechanism for L-carnitine to exert its protective effect is through its ability to stabilize plasma membranes which may result in the consequent decrease in cytoplasmic enzyme release [8]. L-carnitine acts as an intramitochondrial buffer to remove excess acyl meioties allowing regeneration of free CoA to be used to maintain metabolic functions of mitochondria [10,12].

The mitotic index is one of the standard in-

dices commonly used for measuring cytotoxicity. A significant decrease in mitotic activity (48%) was observed in groups of rats treated with DOX. This significant inhibition of mitotic activity of bone marrow cells observed in this study may be correlated with DNA damage or programmed cell death (apoptosis) induced by DOX toxicity [32,33]. It has also been suggested that DOX can form DOX-DNA complex that significantly modifies the ability of a specific class of nuclear enzymes "helicases" to dissociate duplex DNA into DNA single strands. The entire process of strand separation is thus hindered, limiting replication and cell propagation [7]. L-carnitine did not cause any significant change in mitotic activity of bone marrow cells when administered singly but it offered significant protection to mitosis and a recovery of 38% was achieved when given one hour earlier before DOX.

The specific mechanisms by which L-carnitine exerts these antitoxin effects are unknown. However, L-carnitine via its antioxidant property reduces free radical formation by DOX and also increases endogenous antioxidants such as GSH which protect DNA from the damaging effect of free radicals.

The effect of L-carnitine on DOX induced genotoxicity was also investigated in this study. Repeated treatment with DOX showed a significant increase in the incidence of structural chromosomal aberrations in bone marrow cells of rats. The results are in good agreement with *in vivo* and *in vitro* studies reported by Au et al. [6] and Larramendy et al. [23]. The induction of genetic damage by DOX has been attributed to the trapping of topoisomerase II-DNA complexes, or its ability to intercalate into DNA and to the generation of free radicals in cell [4]. The formation of iron-DOX complex, which generates free radicals and induce lipid peroxidation have also been considered a participating mechanism [26].

Administration of L-carnitine one hour prior to DOX treatment decreases the frequency of DOX induced chromosomal aberrations by 38%. These results demonstrate the efficacy of L-carnitine as a protective agent against DOX induced genotoxicity by acting as an efficient antioxidant. Other antioxidants such as metallothionein and desferrioxamine have been used efficiently against DOX induced genotoxicity [22].

The results presented in this report demonstrate the efficient role of L-carnitine as a protective agent against DOX induced cardiotoxicity and genotoxicity in rat and suggests its use as an adjuvant therapy with the antitumour doxorubicin to reduce its undesirable side effects .

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