

INCREASED SERUM AND CARDIAC ACYL-CARNITINE/FREE CARNITINE RATIO DURING DEVELOPMENT OF DOXORUBICIN-INDUCED CARDIOTOXICITY

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استهدفت هذه الدراسة إلى معرفة ما إذا كان العلاج التراكمي لمادة دوكسوروبيسين يغير من مستوى مادة كارتينين في المصل والقلب وإذا كان الأمر كذلك ، فهل هذه التغيرات يجب أن ينظر إليها كآلية أو مؤشر أثناء حدوث سمية مادة دوكسوروبيسين في القلب. ولتحقيق الهدف من هذه الدراسة ، تم تقسيم 40 من ذكور الجرذان إلى أربع مجموعات. تم حقن جرذان المجموعة الأولى في الغشاء البريتوني بمحلول ملح واستخدمت كمجموعة ضابطة. أما الجرذان في المجموعة الثانية إلى الرابعة ، فقد تم حقنهم في الغشاء البريتوني يوماً بعد يوم بجرعة مقدارها 3 مجم/كجم من مادة دوكسوروبيسين للحصول على مجموعات علاجية بجرعات تراكمية مقدارها 6 مجم/كجم من مادة دوكسوروبيسين (المجموعة الثانية) ، 12 مجم/كجم (المجموعة الثالثة) و 18 مجم/كجم (المجموعة الرابعة). وبعد مرور 24 ساعة من حقن آخر جرعة من مادة دوكسوروبيسين ، تم قتل الجرذان وفصل كل من المصل والقلب. وأدى حقن مادة دوكسوروبيسين إلى زيادة ملحوظة ومعتمدة على الجرعة في نشاط إنزيم كرياتين فوسفوكيناز ولاكتات ديهيدروجيناز وزيادة نسبة إسترات مادة كارتينين/مادة كارتينين الحر ونقص ملحوظ في كارتينين الحر في المصل. وفي أنسجة القلب أدى حقن مادة دوكسوروبيسين إلى نقص ملحوظ في كارتينين الحر مقداره 46% و 63% بعد جرعات تراكمية مقدارها 12 ، 18 مجم/كجم على التوالي. علاوة على ذلك أدى حقن مادة دوكسوروبيسين إلى نقص ملحوظ ومعتمد على الجرعة في ثلاثي أدنوزين فوسفات في أنسجة القلب وفي الخلاصة فإن نتائج هذه الدراسة تقترح ما يلي :

1. نقص مادة كارتينين في القلب يجب أن ينظر له كآلية أثناء حدوث سمية بمادة دوكسوروبيسين على القلب.
2. أن الزيادة المتوازنة لنسبة إسترات كارتينين / كارتينين الحر ومؤشرات إنزيمات التسمم القلبي ممكن أن تشير إلى احتمالية إعتبار نسبة إسترات كارتينين / كارتينين الحر مؤشر أثناء حدوث سمية بمادة دوكسوروبيسين في القلب.

This study has been initiated to investigate whether cumulative doxorubicin (DOX) therapy alters serum and cardiac carnitine levels and if so, whether these alterations should be viewed as a mechanism and/or as an index during development of DOX-induced cardiotoxicity. To achieve the ultimate goal of this study, a total of 40 adult male Wistar albino rats were divided into 4 groups. In the first group, animals were injected intraperitoneally (I.P.) with normal saline (0.5 ml/200 gm body weight) and served as a normal control. Animals in the second to the fourth groups were injected every other day with DOX (3 mg/kg, I.P.), to obtain treatments with cumulative doses of 6 mg/kg (group 2), 12 mg/kg (group 3) and 18 mg/kg (group 4). At 24 hours after receiving the last dose of DOX, animals were sacrificed, serum as well as hearts were isolated and analyzed. DOX induced a significant and dose-dependent increase in serum creatine phosphokinase isoenzyme (CK-MB), lactate dehydrogenase (LDH), acyl-carnitine (AC)/free carnitine (FC) ratio and a significant decrease in serum free FC. In cardiac tissues, DOX induced a significant 46 % and 63 % decrease in FC after cumulative doses of 12 and 18 mg/kg, respectively. In contrast to FC, DOX induced a significant 70 % and 81 % increase in AC after

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cumulative doses of 12 and 18 mg/kg, respectively. Moreover, DOX treatment showed significant and dose-dependent decrease in adenosine triphosphate (ATP) level in cardiac tissues. In conclusion, data from this study suggest that: (1) Decreased myocardial carnitine level should be viewed as a mechanism during development of DOX cardiotoxicity, and (2) the parallel increase of serum AC/FC ratio and cardiotoxicity enzymatic indices, may point to the possible consideration of AC/FC ratio as a marker during development of DOX cardiotoxicity.

Key Words: Acyl-carnitine/free carnitine, doxorubicin, cardiotoxicity carnitine insufficiency.

Introduction

Doxorubicin (DOX) was among the first anthracycline antibiotics to be in clinical use in cancer chemotherapy (1). It has a broad spectrum antitumour activity against a variety of solid and hematological tumours (2). Unfortunately, the optimal clinical usefulness of DOX is usually limited secondary to the development dose-dependent and irreversible cardiotoxicity (3). Several mechanistic studies have shown that DOX induces its cardiomyopathy through several events including generation of free radicals and lipid peroxidation of cardiac membranes (4,5), cardiac calcium overload (6), formation of DOX-iron complex (7) and inhibition of beta-oxidation of long chain fatty acids with the consequent depletion of cardiac ATP (8-11). In view of irreplaceability of DOX in cancer chemotherapy, one of the research aims being pursued most intensively is the possibility of eliminating its cardiotoxicity or reducing it to an acceptable level. In this regard, various drugs have been tried including L-carnitine (12-15)

L-carnitine is a naturally occurring quaternary ammonium compound which is synthesized endogenously in kidney and liver and also obtained from dietary sources (16). L-Carnitine plays an essential role in transporting long fatty acids from the cytoplasmic compartment into mitochondria, where they are oxidized to produce energy (16). Inhibition of this vital pathway in the heart as a result of primary or secondary causes of carnitine deficiency has been shown to impair myocardial function (8,17). Moreover, decreased myocardial level of FC is thought to be part of the mechanism involved in the progression of DOX-induced heart failure and cardiomyopathy (11,12,17). Several experimental and clinical studies have demonstrated that L-carnitine has the potential ability to protect the myocardium against DOX-induced cardiotoxicity (18-22). In DOX-treated cancer patients, Yaris et al.

(23), reported that there was a trend towards decreasing serum carnitine levels with increasing the cumulative doses of DOX. In contrast, Yoon et al. (20), have reported that DOX caused significant elevations of plasma FC, AC and total carnitine (TC). Results from these studies should be carefully reviewed because the level of carnitine fractions in the myocardium during development of DOX cardiotoxicity and its relationship to plasma carnitine level are not investigated. Therefore, this work has been initiated to investigate whether cumulative doxorubicin (DOX) therapy alters serum and cardiac carnitine levels and if so, whether these alterations should be viewed as a mechanism and/or as a marker during development of DOX-induced cardiotoxicity.

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. Animals were housed in metabolic cages under controlled environmental conditions (25°C and a 12 h light/dark cycle). Animals had free access to pulverized standard rat pellet diet essentially carnitine free and tap water unless otherwise indicated. The protocol of this study has been approved by Research Ethics Committee of College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Materials

Doxorubicin was a generous gift from King Khalid University Hospital drug store. Free L-carnitine, short chain acyl-carnitine, and long chain acyl-carnitine standards were kindly supplied by Dr. Menotti Calvani, Sigma-Tau, Pomezia, Italy. All other chemicals used were of the highest analytical grade.

Experimental design and doxorubicin treatment protocol:

In this study, the DOX treatment regimen (3 mg/kg every other day) used to develop the cumulative cardiotoxicity was adopted from Beanlands *et al.* (19) with slight modification in which animals were sacrificed 24 hours after cumulative doses of 6, 12 and 18 mg/kg. To achieve the ultimate goal of this study, a total of 40 adult male Wistar albino rats were used and divided at random into 4 groups of 10 animals each. In the first group, animals were injected intraperitoneally (I.P.) with normal saline (2.5 ml/kg) and served as a normal control. Animals in the second group were injected, every other day, with DOX (3 mg/kg, I.P.) over a period of 3 days to obtain cumulative dose of 6 mg/kg. Animals in the third group were injected, every other day, with DOX (3 mg/kg, I.P.) over a period of 7 days to obtain cumulative dose of 12 mg/kg. Animals in the fourth group were injected, every other day, with DOX (3 mg/kg, I.P.) over a period of 11 days to obtain cumulative dose of 18 mg/kg. At 24 hours after receiving the last dose of DOX, animals were anesthetized with ether, and blood samples were obtained from the retro-orbital sinus of the eye. Serum was separated for measurement of lactate dehydrogenase (LDH), creatine phosphokinase iso-enzyme (CK-MB), FC, AC, TC and AC/FC ratio. Immediately after blood samples were collected, animals were then sacrificed by decapitation after exposure to ether in a desiccator kept in a well-functioning hood and hearts were quickly excised, washed with saline, blotted with a piece of filter paper and homogenized in 6 % perchloric acid using a Branson sonifier (250, VWR Scientific, Danbury, Conn., USA).

Assessment of cardiac enzymes:

Serum activities of LDH and CK-MB were determined according to the methods of Buhl and Jackson (24) and Wu and Bowers (25), respectively. *Determination of carnitine levels in serum and cardiac tissue:*

Heart homogenate was prepared in ice-cold 6 % perchloric acid and centrifuged at 8000 g for 10 minutes. Similarly, 1 ml of serum was mixed with 1 ml of ice-cold 6 % perchloric acid and centrifuged at 8000 g for 10 minutes. The tissue and serum supernatants were used for the estimation of FC and

short chain AC, whereas the remaining pellets were used for determination of long chain AC after hydrolysis in 1 M KOH at 65 °C for 1 hour according to Alhomida (26). Carnitine fractions were then determined using high-pressure liquid chromatography after precolumn derivatization with L-aminoanthracene as previously described by Longo *et al.* (27). The mobile phase was prepared by mixing 700 ml of 0.1 M ammonium acetate pH 3.5 with 300 ml of acetonitrile. Chromatographic separation was performed at a flow rate of 1.3 ml/min, using a Kromasil C18, 250 x 4.6 mm I.D. 5 µm column (Saulentechnik Knayer, Berlin, Germany). The excitation and emission wavelengths of the spectrofluorimeter were 248 and 418 nm respectively.

Determination of adenosine triphosphate in cardiac tissue:

Adenosine triphosphate was determined in heart tissues using HPLC according to Botker *et al.* (28). In brief, heart tissue was homogenized in ice-cold 6 % perchloric acid, centrifuged at 3000 rpm for 15 min at 0.5 °C, and 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 X 4.6 mm I.D., 5 µm column (Supelco SA, Gland, Switzerland) and 75 mM ammonium dihydrogen phosphate as mobile phase. The peak elution was followed at 254 nm.

Statistical analysis:

Differences between obtained values (mean ± SEM, n = 10) were carried out by one way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. A P value of 0.05 or less was taken as a criterion for a statistically significant difference.

Results

The cumulative cardiotoxicity of DOX was clearly featured by the dose-dependent increase in serum cardiac enzymes CK-MB and LDH (table 1). At the highest cumulative dose, 18 mg/kg, DOX resulted in a significant 178 % and 154 % increase in CK-MB and LDH, respectively, as compared to the control group.

Table 1: Effect of doxorubicin on serum cardiomyopathy enzymatic indices, creatine phosphokinase isoenzyme (CK-MB) and lactate dehydrogenase (LDH), in rats.

Treatment groups	CK-MB (U/L)	LDH (U/L)
Control	365.2 ± 8.2	312.4 ± 12.4
DOX, 6 mg/kg	594.3 ± 24.5 *	482.1 ± 19.2*
DOX, 12 mg/kg	901.6 ± 42.1* [#]	748.2 ± 29.5* [#]
DOX, 18 mg/kg	1014.8 ± 53.4* [#]	792.6 ± 33.8* [#]

Data are presented as mean ± SEM (n =10). * and # indicate significant change from control and DOX (6 mg/kg), respectively, at p<0.05 using ANOVA followed by Tukey-Kramer as a post ANOVA test.

Table 2: Effect of doxorubicin on serum free carnitine (FC), acyl carnitine (AC), total carnitine (TC) and AC/FC ratio in rats.

Treatment groups	Serum carnitine level (µmol/l)			
	FC	AC	TC	AC/FC
Control	48.8 ± 5.1	14.1 ± 2.1	63.0 ± 6.3	0.3 ± 0.1
DOX, 6 mg/kg	40.3 ± 4.2	26.5 ± 3.3	66.6 ± 6.4	0.66 ± 0.26
DOX, 12 mg/kg	36.5 ± 3.0	32.2 ± 4.0*	68.5 ± 6.7	0.88 ± 0.28
DOX, 18 mg/kg	8.3 ± 3.2*	46.0 ± 5.4* [#]	74.3 ± 5.6	1.62 ± 0.17 [#]

Data are presented as mean ± SEM (n =10). * and # indicate significant change from control and DOX (6 mg/kg), respectively, at p<0.05 using ANOVA followed by Tukey-Kramer as a post ANOVA test.

Table 2 shows the effect of different cumulative doses of DOX on serum FC, AC, TC, and AC/FC ratio in rats. DOX therapy resulted in dose-

dependent decrease in FC with the maximum (42 %) decrease was observed at a dose of 18 mg/kg. In contrast to FC, the level of AC showed a significant 128 % and 226 % increase after cumulative DOX doses of 12 and 18 mg/kg, respectively, as compared to the control group. Moreover, DOX treatment resulted in a significant and dose-dependent increase in AC/FC ratio with the maximum (440 %) increase at the highest cumulative dose of DOX (18 mg/kg).

Table 3 shows the effect of different cumulative doses of DOX on the levels of FC, AC, TC, and AC/FC ratio in rat heart tissues. Treatment with DOX resulted in a significant 46 % and 63 % decrease in FC at cumulative doses of 12 and 18 mg/kg, respectively, as compared to the control group. In contrast to FC, the level of AC showed a significant 70 % and 81 % increase after cumulative DOX doses of 12 and 18 mg/kg, respectively. Moreover, DOX treatment resulted in a significant and dose-dependent increase in AC/FC ratio with the maximum (400 %) increase at the highest cumulative dose of DOX (18 mg/kg).

Table 3: Effect of doxorubicin on free carnitine (FC), acyl carnitine (AC), total carnitine (TC) and AC/FC ratio in rat heart tissues.

Treatment groups	Cardiac carnitine level (nmol/ g wet tissue)			
	FC	AC	TC	AC/FC
Control	702 ± 88.3	201.2 ± 12.5	903.6 ± 68.3	0.28 ± 0.01
DOX, 6 mg/kg	533 ± 31.4	267.1 ± 9.8	800.1 ± 52.8	0.50 ± 0.05
DOX, 12 mg/kg	382 ± 16.1*	342.5 ± 22.6* [#]	724.7 ± 44.0	0.89 ± 0.13
DOX, 18 mg/kg	259 ± 12.4* [#]	364 ± 21.4* [#]	623 ± 39.4*	1.40 ± 0.38* [#]

Data are presented as mean ± SEM (n =10). * and # indicate significant change from control and DOX (6 mg/kg), respectively, at p<0.05 using ANOVA followed by Tukey-Kramer as a post ANOVA test.

Table 4: Effect of doxorubicin on adenosine triphosphate level in rat heart tissues.

Treatment groups	Adenosine triphosphate (ATP) (nmol/g wet tissue)
Control	851.3 ± 32.4
DOX, 6 mg/kg	780.2 ± 36.5
DOX, 12 mg/kg	518.6 ± 21.5* [#]
DOX, 18 mg/kg	472.3 ± 15.1* [#]

Data are presented as mean ± SEM (n =10). * and # indicate significant change from control and DOX (6 mg/kg), respectively, at p<0.05 using ANOVA followed by Tukey-Kramer as a post ANOVA test.

To investigate the effect of DOX on mitochondrial function and energy production, the level of ATP was measured in cardiac tissues (table 4). DOX resulted in a significant 39 % and 45 % decrease in ATP level after cumulative doses of 12 and 18 mg/kg, respectively, as compared to the control group.

Discussion

L-carnitine performs a crucial role in the energy production in the heart by controlling the influx of long chain fatty acids into mitochondria (16). The total body content of carnitine in adults amounts to 50-100 mmol, most of it being localized in skeletal muscles (29). Carnitine is present in both plasma and tissue as FC or bound to fatty acids as AC with relatively high concentration in cardiac tissues (14). Under normal physiological conditions, 80 % of total serum carnitine is FC and 20 % is AC with normal AC/FC ratio of 0.25 (30,31). Earlier studies reported that increased ratio of AC/FC greater than 0.4 is abnormal and creates a pathological condition known as carnitine insufficiency in which there is insufficient FC relative to increased metabolic needs (30-32). Moreover, it has been reported that AC/FC ratio is very sensitive to metabolic changes and equals to the intramitochondrial acyl-CoA/CoA-SH ratio (32).

Data presented here demonstrate that DOX increased serum cardiotoxicity enzymatic indices

(LDH and CK-MB). This effect could be a secondary event following DOX-induced lipid peroxidation of cardiac membranes with the consequent increase in the leakage of LDH and CK-MB. Increased release of cardiac enzymes by DOX had been previously reported (5,11,33) In cardiac tissues, the observed decrease in both FC and TC after DOX treatment was dose-dependent with the maximum decrease at the highest cumulative dose (18 mg/kg). This decrease in FC and TC levels in cardiac tissue was parallel to the increase in cardiotoxicity enzymatic indices, LDH and CK-MB, which may point to the possible role of decreased myocardial carnitine during development of DOX-induced cardiotoxicity. Decreased cardiac carnitine content has been previously reported in many forms of DOX-dependent and independent cardio-myopathies (11,12,17,30,34) In contrast to FC and TC, DOX treatment caused dose-dependent accumulation of AC and AC/FC ratio in cardiac tissues (table 3). This effect could be a secondary event following the decrease in FC with the consequent inhibition of long chain fatty acid oxidation and accumulation of toxic fatty acid intermediates including long chain acyl-CoA and long chain AC. It is well documented that these intermediates are amphiphilic and have detergent-like effects, altering membrane integrity, inhibiting several membrane-bound enzymes, provoking free radical injury and decreasing myocardial contractility (35). Increased AC in the heart by DOX could be explained on the basis that impaired mitochondrial function leads to esterification of FC with acyl groups. This results in progressive reduction of the FC pool in the heart which could lead to a metabolically deleterious carnitine insufficiency (30).

It is well known that liver and kidney are the main organs responsible for the endogenous synthesis of FC which is then transported into cardiac and skeletal muscles (16). Therefore, in the current study, the observed decrease in myocardial FC could be a secondary event following DOX-induced inhibition of the endogenous synthesis of FC. This hypothesis is supported by reports from several recent studies which have demonstrated that DOX therapy is associated with severe hepatotoxicity (36,37) and nephrotoxicity (38-40) and that L-carnitine supplementation have the potential ability to protect against DOX-induced renal and hepatic dysfunction (38,41). Moreover, in vitro and in vivo studies have reported that DOX inactivates

cardiac carnitine palmitoyltransferase1 by competing with L-carnitine for its binding site on the enzyme, and increases the leakage of L-carnitine from cardiac myocytes (9,20). Contrary to our results, McFalls *et al.* (42) reported that cardiac carnitine levels were not significantly changed by DOX (2 mg/kg/week, for 6 weeks). This discrepancy between our results and the aforementioned study could be due to the difference in experimental design regarding the dosing and schedule of DOX therapy and the time of carnitine assessment.

In this study, the observed increase in serum AC and AC/FC ratio were parallel to their increase in the heart. This effect could be a consequence of carnitine insufficiency when esterified carnitine is exported out of the mitochondria. Previous study reported that increased serum AC could indicate impaired transport of long chain fatty acids into mitochondria and abnormal beta-oxidation pathway (30). Moreover, the observed increase in serum AC/FC ratio by DOX was dose-dependent and parallel to the increase in cardiotoxicity enzymatic indices, LDH and CK-MB, which may point to the possible consideration of serum AC/FC ratio as an index of DOX-induced cumulative cardiotoxicity. 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 (43). Depletion of the heart from carnitine by DOX therapy would impair the beta-oxidation of long chain fatty acids with the consequent decrease in ATP production and heart contractile function. This was supported by the marked decrease of ATP levels in heart tissues observed after the higher cumulative dose of DOX (table 4). This hypothesis is supported by the data presented in previous studies which have reported that carnitine supplementation completely reversed the development of DOX-induced cardiotoxicity by replenishing the myocardium with adequate carnitine for its energy production (9,13,17). In conclusion, data from this study suggest that: (1) Decreased myocardial carnitine level should be viewed as a mechanism during development of DOX cardiotoxicity, (2) serum AC/FC ratio could be used as a marker during development of DOX cardiotoxicity. This will open new perspectives for monitoring serum levels of FC, AC, and AC/FC ratio in cancer patient receiving DOX-containing combination chemotherapy protocols.

Acknowledgements

The present work was supported by operating grants from Research Center, College of Pharmacy, King Saud University (CPRC 171).

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