Kafkas Üniv Vet Fak Derg
14 (2): 229-235, 2008
DOI:10.9775/kvfd.2008.59-A

Protective Effects of L-carnitine on Doxorubicine Induced Cardiomyopathy in Rabbits [1]

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[1] *This project was funded by the Scientific and Technical Research Council of Turkey, Project code: VHAG-2042*
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Yayın Kodu (Article Code): 2008/59-A

Summary

This study was aimed to investigate effect of L-carnitine (LCAR) on adverse effect of doxorubicin (DOX) on heart tissues. For this purpose, a total of 21 healthy albino New-Zealand rabbits were divided into 3 groups. Rabbits in group I (n=8) received DOX at a dose rate of 0.6 mg/kg/day body weight (BW) intra peritoneally (IP) for 6 days, group II (n=7) received DOX at a dose rate of 0.6 mg/kg/day BW, IP and LCAR at dose of 1000 mg/kg/day BW IP for 6 days and group III /n=6) received LCAR at dose of 1000 mg/kg/day BW IP for 6 days. Blood samples from auricular vein were taken from all animals at the beginning of the experiment (before drug administration) and 2 hours after drug administration daily for 6 days. Pathological examination of all animals was carried out at the end of the study. Macroscopic and microscopic alterations were noticed only in heart of the DOX-group rabbits. Cardiac troponin-I and T rapid assay tests of animals in all groups were negative throughout the study. There were no statistically significant changes in biochemical parameters on day 0 between the groups, while on subsequent days these parameters altered. Blood concentrations of troponin-I, CK-MB and LDH were significantly higher in DOX received group when compared to the groups given LCAR. The results of histopathological and biochemical analysis reveal that parenteral LCAR administration had a protective effect against adverse effect of DOX on heart and kidney.

Keywords: *Doxorubicin, Cardiomyopathie, L-carnitine, Troponine, Rabbit*

Tavşanlarda Doksorubisinle Oluşturulan Kardiyomiyopatilerde L-Karnitinin Koruyucu Etkileri [1]

Özet

Bu çalışmada Doksorubisinin (DOX) kalp dokusunda oluşturacağı yan etkiler üzerine L-karnitinin (LCAR) koruyucu etkilerinin araştırılması amaçlanmıştır. Bu amaçla, çalışmada toplam 21 adet sağlıklı albino Yeni Zelanda tavşanı üç ayrı grup oluşturularak kullanıldı. Grup I'deki tavşanlara (n=8) 0.6mg/kg canlı ağırlık (CA) dozda DOX, Grup II'deki tavşanlara (n=7) 0,6mg/kg CA dozda DOX ile 1000 mg/kg CA dozda LCAR İP, Grup III'teki tavşanlara (n=6) 1000mg/kg CA dozda LCAR İP yolla günde bir kez 6 gün süreyle uygulandı. Bütün hayvanlardan kan örneklerinin toplanması ilk ilaç kullanımından önce ve 6 gün boyunca ilaç uygulamasını takiben 2 saat sonra gerçekleştirildi. Çalışma sonunda tüm hayvanlardan patolojik değerlendirmeler için numuneler alındı. Yalnızca DOX grubundaki tavşanların kalp dokularında makroskobik ve mikroskobik değişimler belirlendi. Çalışma süresince ve tüm gruplardaki hayvanlarda kardiyak troponin-I ve T rapid assay testleri negatif olarak tespit edildi. Çalışmanın 0. gününde tüm gruplardaki hayvanlardan elde edilen biyokimyasal değerler arasında anlamlı bir farklılık belirlenmezken, çalışmanın ilerleyen günlerinde istatistiksel olarak farklılıklar tespit edildi. DOX grubundaki hayvanlardan elde edilen troponin-I düzeyleri ile CK-MB ve LDH enzim aktiviteleri LCAR kullanılan gruplarla karşılaştırıldığında istatistiksel olarak yüksek olduğu belirlendi. Histopatolojik ve biyokimyasal analiz sonuçları, parenteral LCAR uygulamalarının doksorubisinin kalp dokusu üzerindeki yan etkilerine karşı koruyucu etkilere sahip olduğunu göstermektedir.

Anahtar sözcükler: *Doksorubisin, Kardiyomiyopati, L-karnitin, Troponin, Tavşan*

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INTRODUCTION

Doxorubicin (DOX), the most effective antineoplastic drug, is highly cardiotoxic even at the therapeutic dose of 0.6 mg/kg bw. Its use is therefore limited in human [1]. Reports disclose that one third of the patient with neoplasm undergoing DOX treatment experienced irreversible cardiac disorders [2,3]. Mortensen et al.[2] reported acute or chronic cardiotoxicity in 16 of 38 cancer patients treated with DOX. Similarly, rat, undergone DOX treatment experienced irreversible lesions such as interstitial oedema, fibrotic and myocardial degenerations [3]. DOX also causes production of free radicals, oxygen radicals, direct DNA damage, inhibition of DNA damage repair [4], initiation of immune reaction in heart and kidneys and apoptosis in the gut [5]. Therefore its clinical use is prescribed as 3 days on, 3 weeks off and then 3 days on again by the physicians.

L-carnitine (LCAR) synthesized from lysine and methionin in the liver and kidney, plays an important role in the energy production in mitochondria and removal of toxic substances from the cell. L-carnitine is proved to decrease in hamsters with cardiomyopathy [6,7] this may lead to lowered fatty acid utilization and reduced production of energy through decreased ATP production [8]. L carnitine has been reported to enhance energy metabolism, to remove long chain fatty acids and to improve myocardial contraction in experimentally induced ischemic heart in dogs [9]. Histopathology results also shown that L carnitine use protected myocard against pathological changes such as necrosis, fibrosis and calcification [10].

Troponins are located on thin laments of striated muscle and play role in contraction of myofibrils. These are; troponin T (Tr-T) bound to tropo-myosine, troponin I (Tr-I) inhibiting tropomyosine and troponin C (Tr-C) binding calcium [11]. Troponins have been used as cardiac markers in addition to conventionally used parameters (LDH, CK-MB, AST) in human myocardial infarction and acute coronary diseases in recent years [12-16]. Studies have already shown the superiority of cardiac troponins over conventional parameters in human myocardial degenerations in terms of diagnosis and prognosis [12,15,17-19].

Studies involving animal subjects have also been carried out with regard to cardiac troponins and found a high proportion of amino acid homology between troponins of animal and human origin [20-22]. This has resulted in use of troponins especially cTN-I in veterinary field [23-30] but these type of studies are limited in numbers.

This led us first to evaluate human troponin kits in determining cardiomyopathy induced by DOX in rabbits and second to determine protective effect of LCAR in DOX induced cardiomyopathy where DOX was administered continuously for 6 days by evaluating histopathology, cardiac troponins and other biochemical parameters.

MATERIAL and METHODS

Animal Material

The study involved 21 healthy New Zealand albino rabbits (Laboratory Animal Unit of The University of Kafkas, Kars, Turkey) of both sexes, aged between 5-7 months old. Rabbits were fed hay and special pelleted rabbit diet (produced by Bayramoglu Yem AS, Erzurum, Turkey) and drinking water ad libitum in their individual cages during the experiment. Study animals were kept in cages (four rabbits per cage) at dimension of 70 cm in length, 50 cm in height and 70 cm in width. Animals were kept at room temperature (22-25ºC) and 12 hours daylight/12 hours night cycle. Animals were divided into three groups; DOX group (n=8), DOX+LCAR group (n=7) and LCAR group (n=6). The Laboratory Animal Care and Use Committee of Faculty of Veterinary Medicine approved the whole experimental protocol. All animals were dewormed before the experiment commenced.

Study Design

DOX group received doxorubicin (DOX) at dose of 0.6 mg/kg BW for 6 days intraperitonally (IP), DOX+LCAR group was given 0.6 mg/kg BW DOX and 1000 mg/kg BW L carnitine (LCAR), IP and LCAR group received 1000 mg/kg BW LCAR, IP. Animals were examined and sampled on day 0 and 2 hours after daily drug injections. Values determined on day 0 were considered as baseline values. Blood samples were collected from vena auricularis into plain tubes and tubes with anti-coagulant for determination of biochemical parameters and troponin levels. All rabbits were subjected to histopathology at the end of the experiment.

Biochemical Analyses

Serum and plasma were separated by centrifugation at 3000g for 10 minute and stored

at -25ºC until analyses. Cardiac Troponin-I and T (cTn-I and T) were first determined using practical assay kits (CARDI-I KIT, AboaTech®, Tromp-T Sensitive Rapid, Roche®) and serum cTn-I level was determined using a commercial ELISA kit (Combo test kit). Serum biochemistry parameters of CK-MB, LDH and AST were measured on a spectrophotometry (Tecan-spectra, Austria) using commercial kits (DDS®, Germany). Tests were carried out and the results were calculated as instructed by the manufacturers.

Histopathologic Examination

Systemic necropsy of all animals was made and macroscopic and microscopic changes were recorded.

Statistical analyses

Results were analyzed using Duncan ANOVA on SPSS for windows 10.0 and expressed in the tables as mean and standard error.

RESULTS

The concentrations of cTn-I, CK-MB, LDH and AST are given in the table 1. Range of serum cTn I, CK-MB and LDH concentrations were 0.760±0.038-1.065±0.047 ng/ml, 207.05±39.39-238.34±80.55 U/L and 22.93±1.49-25.06±4.47 in DOX, 0.773±0.010-0.882±0.044 ng/ml, 205.03±39.62-207.97±96.82 U/L and 23.53±1.06-32.53±8.16 U/L in DOX+LCAR and 0.795±0,024-0.696±0,011 ng/ml, 214.58±44.42-100.72±36.30 U/L and 23.29±1.75-11.13±2.02 in LCAR, respectively *(Table 1)*. The increase was significant within the groups when compared to the value obtained on day 0 apart from LCAR group where the values decreased ($P<0.05$). Comparison of the groups also revealed a statistically significant increase in these parameters in DOX and DOX+LCAR groups when compared to LCAR group ($P<0.05$).

AST enzyme activity did not change within the groups but a statistically significant difference was noted on day 5 and 6th of the study as these values were higher in DOX and DOX+LCAR groups when compared to LCAR group *(Table 1)*.

Practical cardiac troponin test results

All rabbits in all groups revealed negative results for cTn-I and cTn-T test (*Figure 1* and *2*).

Fig 1. Negative result of cardiac troponin-I.
Şekil 1. Kardiyak Troponin-I'ya ait negatif sonuçları

Fig 2. Negative result of cardiac troponin-T
Şekil 2. Kardiyak Troponin-T'ye ait negatif sonuçları

Necropsy findings

Gross lesions

Examination of hearts from DOX group revealed flattened cardiac apex, dilated right ventricular and thinness in right ventricular wall. Hemorrhagia was also noted in endocardium of two rabbits in DOX group. These lesions were absent in both DOX+LCAR and LCAR group apart from a slight dilatation and hypertrophy in left ventricules in two rabbits. Hearts of LCAR group were normal.

Microscopic lesions

Hyperemia was noticed in capillaries and pale areas in cytoplasm of heart muscle in DOX group. Loss of striation indicating necrosis was also noted in this group.

In DOX+LCAR group cytoplasms were completely stained but only in a few areas loss of striation was noted. In LCAR group no abnormalities were encountered.

Table 1. *Troponin-I level and CK-MB, LDH, and AST enzyme activities in rabbit of Doxorubicin (DOX) group (n=8), Doxorubucin+L-carnitine (DOX+LCAR) group (n=7) and L-carnitine (LCAR) group (n=6) (mean±standart error)*

Tablo 1. *Doxorubicin (DOX) grubu (n=8), Doxorubucin+L-carnitine (DOX+LCAR) grubu (n=7) ve L-carnitine (LCAR) grubu (n=6) tavşanlarda Troponin-I düzeyleri ile CK-MB, LDH ve AST enzim aktiviteleri (ortalama±standart hata)*

PARAMETER	GROUP	DAYS							SIGNIFICANCE
		0	1	2	3	4	5	6	
Troponin-I (ng/ml)	DOX	0.760±0.038d	0.838±0.049AB,cd	0.875±0.043A,bc	0.933±0.042A,b	1.05±0.116A,a	1.038±0.026A,a	1.065±0.047A,a	P<0.001
	DOX+LCAR	0.773±0.010c	0.890±0.022A,a	0.831±0.011AB,ab	0.819±0.011B,bc	0.839±0.032B,ab	0.841±0.036B,ab	0.882±0.044B,a	P<0.01
	LCAR	0.795±0.024a	0.785±0.011C,a	0.767±0.017B,ab	0.746±0.013C,bc	0.749±0.008B,bc	0.718±0.007C,cd	0.696±0.011C,d	P<0.001
	SIGNIFICANCE		P<0.05	P<0.05	P<0.001	P<0.001	P<0.001	P<0.001	
CKMB (U/l)	DOX	207.05±39.39	241.39±84.09	217.81±77.86	229.41±72.21A	227.99±65.85A	225.62±82.07A	238.34±80.55A	
	DOX+LCAR	205.03±39.62	207.64±60.87	206.54±69.83	217.00±89.09A	205.01±54.81A	208.75±81.13A	207.97±96.82A	
	LCAR	214.58±44.42a	214.64±65.81a	152.65±62.32ab	108.12±57.25B,b	126.80±51.32B,b	115.29±35.28B,b	100.72±36.30B,b	P<0.01
	SIGNIFICANCE				P<0.05	P<0.05	P<0.05	P<0.05	
LDH (U/l)	DOX	22.93±1.49c	26.97±8.71bc	26.69±1.92A,bc	28.44±3.77A,bc	31.07±4.55A,ab	32.39±3.96A,ab	35.06±4.47A,a	P<0.01
	DOX+LCAR	23.53±1.06	27.86±4.58	27.58±4.48A	28.89±10.04A	27.06±6.99A	29.49±4.24A	31.53±8.16A	
	LCAR	23.29±1.75a	18.23±1.90b	16.82±1.87B,b	12.86±1.04B,c	11.71±2.18B,c	11.61±2.51B,c	11.13±2.02B,c	P<0.001
	SIGNIFICANCE			P<0.001	P<0.05	P<0.001	P<0.001	P<0.001	
AST (U/l)	DOX	5.62±1.97	6.53±2.60	6.25±2.23	7.88±5.16	6.59±1.97	7.25±2.26A	7.49±2.77A	
	DOX+LCAR	5.55±1.74	5.33±1.18	6.67±2.47	6.05±1.66	5.45±0.86	5.37±1.03AB	6.50±1.36A,B	
	LCAR	5.15±0.52	4.49±0.53	5.14±1.03	5.19±0.95	4.59±1.05	4.19±1.04B	4.14±1.58B	
	SIGNIFICANCE						P<0.05	P<0.05	

A,B,C: statistical significance in columns; a,b,c: statistical significance in rows P: Significance value
A,B,C: Sütün bazında gruplar arası istatistiksel önem; a,b,c:Satır bazında grup içi günlere bağlı istatistiksel önem

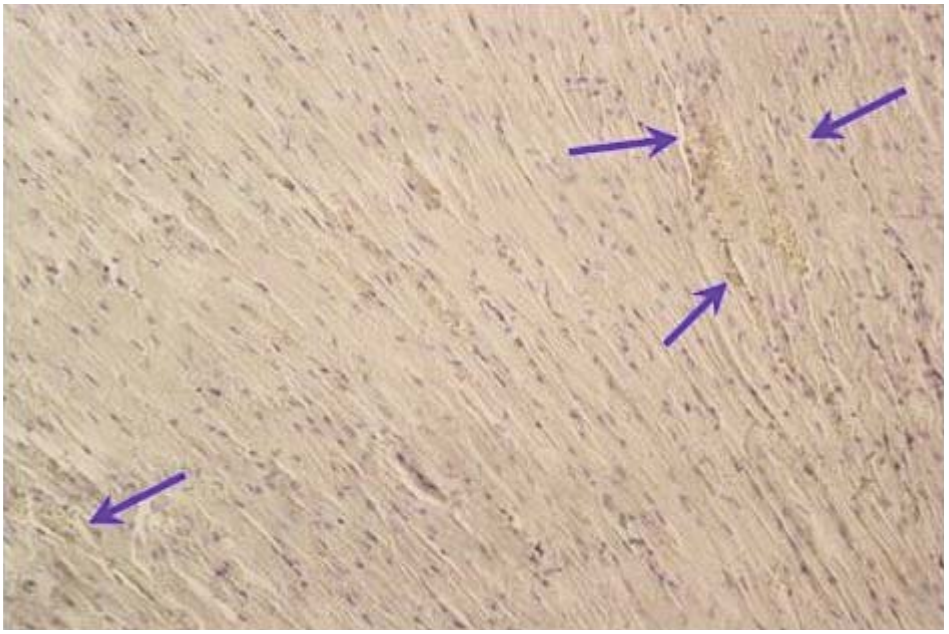

Fig 3. Microscopic view of heart of rabbits in Doxorubicine group. Severe hemorrhagic areas (blue arrow). Hemotoxylen & Eosin Staining

Şekil 3. Doxorubicin grubu tavşanların kalp kasının mikroskobik görünümü. Şiddetli kanama alanları (Mavi oklar). Hemotoksilen-Eozin Boyama

DISCUSSION

In this study it was tried to establish a model in order to evaluate the protective effect of LCAR on cardiotoxicity associated with DOX a drug well known to cause cardiac problems in cancer patients. This effect of DOX has already been reported in other species including rabbits [31-35]. In the present study conventionally used dose (0.6 mg/kg) of DOX was extended from 3 days to 6 days.

DOX induced cardiotoxicity in rabbits as indicated by hemorrhagic areas in myocard, capillary hyperemia, loss of striation and cellular infiltration. This histo-pathological findings well corresponds to previous studies [33-36]. The toxic effects of DOX were inhibited by injection of LCAR as was evident from biochemical and pathological result of the group received DOX plus LCAR. LCAR is well known to play role in beta oxidation, removal of toxic substances and more importantly in blocking of apoptosis in cardiac myocytes through inhibition of sphingo-myelin-ceramid pathway [37-41] as in vitro studies already reported that LCAR inhibited sfingo-myelinase (SMase) through cell signaling leading to inhibition of ceramide production [42].

Experiments in laboratory animals shown that free carnitine concentration decreased while acyl carnitine increased during cardiac ischemia resulting in accumulation of long chain fatty acids in cell membrane and in insufficient synthesis of ATP and cellular energy production [8]. Injection of exogenous carnitine has been proven to reduce long chain fatty acid metabolites, to improve myocardial energy metabolism and to enhance cardiac output of myocard. This improvement had also been evidenced by histopathology as adverse changes in myocard (necrosis, fibrosis, and calcification) reported to be protected [10]. Similar results were also obtained in this study as LCAR application along with DOX resulted in such protection when compared to DOX group.

Cardiac markers are routinely used for the determination and evaluation of myocardial damage in both man and animals. Cardiac troponin-I and T have recently been used for this purpose. cTn-I is the most specific for myocardial damage and is raised after even minor cardiac injuries [43]. cTn-I is as sensitive as or even more sensitive than CK-MB and serum cTn-I concentration increases rapidly (within 5 to 7 hours) soon after myocardial injury and peaks within 12 hours of onset and remains higher than CK-MB in blood. cTn-I

concentration is proportional to the extend and severity of myocardial injury [14]. Similarly, cTn-I was more sensitive than CK-MB and LDH in this study as cTn-I concentrations began to significantly increase at 1st day of DOX administration and remained high throughout the study while CK-MB rised at 3rd day and LDH increased at 2nd day of the DOX administration.

Practical kits of cTn-I and T were negative in this study while serum cTn-I was 0.696±0.011-1.065±0.047 ng/ml. Serum cTn-I level found in this study may explain why practical kits were negative as these kits are designed for human use and can only be positive when and if troponin levels are above 0.3 µg/L.

The group received LCAR had lower cTn-I concentrations and intracellular enzyme activity in the present study as previously reported [44]. This may be attributed to the fact that LCAR enhanced cell membrane stability through activating energy synthesis from fatty acids, avoiding membrane lipid peroxidation, playing role in cellular receptors and effecting transporting proteins [45,46].

Serum cardiac markers (cTn-I, CK-MB, LDH and AST) concentration were lower in DOX plus LCAR group. This is attributed inhibition of DOX related cellular damage by LCAR as DOX is well known to cause inhibition of long chain fatty acid oxidation [47] and to enhance lipid peroxidation [48] so that oxygen consumption is increased and ATP and protein synthesis are decreased [41]. A previous study involving human cancer cases undergone DOX treatment already revealed that use of LCAR decreased irreversible cardiac injury and CK-MB concentrations [1] which was also the case in our study.

The findings obtained in this study might be of help in shedding light on toxicity due to antineoplastic drugs. Use of LCAR may be of use in extending continuous use of DOX beyond suggested 3 days to 6 days as cardiac markers especially cTn-I were lower and myocardial injury was of minimal extent. As previous studies already stated secondary carnitine deficiency during antineoplastic drug treatments, LCAR may be adviced to be used along with DOX treatment.

The results indicated that cTn-I may also be of valuable marker in determination of myocardial injuries in animals and LCAR avoided cardiotoxicity to some extent.

Acknowledgement

Authors are thanking full to Dr. Adnan AVCI the director of PROGREMED Inc. for providing troponin kits and TUBITAK for financial support.

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