

ORIGINAL ARTICLES

EARLY DETECTION OF DOXORUBICIN-INDUCED CARDIOTOXICITY AND ITS PREVENTION BY α -TOCOPHEROL

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ABSTRACT

Objective: To detect doxorubicin-induced myocardial injury by quantitative estimation of cardiac specific protein, Cardiac Troponin I (cTnI) at early stage and to evaluate the cardioprotective effects of α -Tocopherol.

Study Design: Lab based randomized controlled *in-vivo* study in rabbits.

Place and Duration of Study: Department of Pharmacology in collaboration with Pathology department, Army Medical College Rawalpindi, Pakistan from Jan 2012 to Dec 2012.

Material and Methods: Eighteen healthy male adult rabbits were used. Cardiotoxicity was induced by single intravenous injection of 12 mg/kg of doxorubicin in a group of rabbits, control group was treated with normal saline only and the rabbits of third group were pretreated with α -Tocopherol 200 mg/kg of body weight for ten days before injection of doxorubicin 12 mg/kg.

Results: Doxorubicin produced severe cardiotoxicity confirmed by markedly raised serum levels of cTnI, CK-MB, LDH and grade 3 necrosis of the heart tissue in rabbits. The pre-treatment with α -Tocopherol resulted in improved serum levels of cTnI and the histological picture of heart tissue.

Conclusions: The quantitative cTnI estimation for detection of cardiotoxicity at subclinical level can lead to significant economic impact in management of cancer patients because the troponin-negative subjects can be excluded from long term cardiac monitoring programs, which require high cost imaging techniques. Furthermore, the outcome of most potent and widely used doxorubicin chemotherapy can be made successful with the concurrent use of α -Tocopherol.

Keywords: α -Tocopherol, Cardiac troponin I, Creatine kinase-MB, Doxorubicin, Lactate Dehydrogenase.

INTRODUCTION

Doxorubicin (Dox) is the most active and widely used cytotoxic agent for the adjuvant chemotherapy of frequently occurring malignancies (breast, colorectal and lung cancer and childhood malignancies) since the last four decades but its adverse effects to the myocardium prevent its use at the maximum doses for the required number of courses^{1,2}. About 20% patients receiving doxorubicin may develop adverse cardiac effects³. The quantitative estimation of sensitive biomarker Cardiac Troponin I (cTnI) leads to early recognition of cardiotoxicity and have relevant economic impact in oncological patient management⁴.

Doxorubicin is isolated from *Streptomyces peucetius*⁵. It exerts its well defined cytotoxic actions through DNA intercalation and inhibition of DNA topoisomerase II (enzyme involved in proliferation of cells)^{6,7} while cardiotoxicity is produced by virtue of its quinone group based metabolites that generate reactive oxygen species (ROS) and free radicals. Cardiomyocytes are inherently more susceptible to oxidative stress⁸. Free radicals and ROS inflict mitochondrial and nuclear DNA lesions in cardiomyocytes with disruption of mitochondrial bioenergetics and impaired expression of important cardiac proteins⁹. Doxorubicin metabolites also cause disturbances of calcium release from sarcoplasmic reticulum, lipid peroxidation¹⁰, degradation of myofilaments and cytoskeletal proteins¹¹. These processes lead to cardiomyocyte death either by necrosis or by apoptosis¹², releasing the cardiac specific contractile proteins,

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Cardiac Troponins I (cTnI) and cytosolic energy producing enzymes, Creatine Kinase MB (CK-MB) Lactate Dehydrogenase (LDH) into the circulation¹³. CK-MB and LDH are nonspecific and cTnI is becoming the most sensitive biomarker of cardiotoxicity¹⁴. Cardiac Troponin I is myocardial regulatory protein. It is 13 times more abundant in the myocardium than CK-MB. cTnI is expressed only in myocardium¹⁵. cTnI determination detects the presence of cardiotoxicity at very early stage, significantly before impairment of cardiac function can be revealed by any other diagnostic technique¹⁶. Keeping in view the absolute cardiospecificity of cTnI, we considered cTnI more favourable for the detection of myocardial injury as approved by Jaffe, Lipshultz and their associates^{16,17} especially when chemical induces cardiac necrosis¹⁸.

The cardiac impairment produced by Doxorubicin requires long-term follow-up and treatment involving high medical costs¹⁹. More than half of the total cancer occurs in developing countries with low health budget²⁰. To rule out the patients for long term cardiac monitoring most of the time qualitative detection of cTnI is being done that may give false positive or negative results imposing heavy economic burden. The main aim of this study was quantitative detection of the specific and sensitive biomarker, cTnI that might prove to be helpful in cost effective management of these cases.

Several strategies for reduction of cardiac toxicity have been proposed and used²¹. However, they are expensive and protection conferred by them is not always effective¹⁹. Moreover, the efficacy of these measures depends on the early and reliable detection of cardiotoxicity as proposed and done in this study on one hand. On the other hand possible prevention by pretreatment with inexpensive α -Tocopherol, have produced noticeable decrease in cardiotoxicity and it can play a great role as adjuvant chemotherapy with doxorubicin.

Alpha-Tocopherol is a lipid-soluble, inexpensive organic compound thought to

prevent the propagation of free radical damage in biological membranes²². It inhibits lipid peroxidation²³ in the presence of endogenous Vitamin C and Selenium in cytosol²⁴.

Various studies in preclinical and clinical models showed the effectiveness of α -tocopherol in mitigating cardiac toxicity of chemotherapeutic agents like doxorubicin where oxidative stress plays a major role^{25,26}. It can prevent the pro-inflammatory cytokines and chemokines induced by doxorubicin²⁷.

MATERIAL AND METHODS

The lab based randomized controlled in-vivo study was carried out in the department of Pharmacology and Therapeutics in collaboration with Chemical Pathology after getting approval from Ethical Committee of "Centre for Research in Experimental and Applied Medicine" (CREAM), Army Medical College, Rawalpindi. Eighteen adult healthy male rabbits, weighing 1.0 to 1.5 kg were procured from the local market and kept in animal house at standard normal conditions. They were fed with fresh green fodder, cereals and tap water ad libitum. Animals were allowed to acclimatize with the environment of animal house for one week.

Pharmaceutical brand of Doxorubicin HCL (adriamycin) from Park-Davis Pak and α -Tocopherol from Merck Pak Ltd were purchased. cTnI Beckman Coulter kit was purchased from PMA while CK-MB kit and LDH commercial kit were purchased from Merck Pak Ltd.

The animals were randomly divided into three groups, group A: (n=6) Control group received 0.9% sodium chloride (NaCl) solution 2 ml daily by gavage for 11 days. Group B: (n=6) was administered doxorubicin injection 12 mg/kg body weight (BW)²⁸ with intermittent 0.9% NaCl infusion into marginal ear vein on 10th day of study. Group C: (n=6) was treated with α -tocopherol 200 mg/kg of body weight by gavage from day 1 to day 11 plus doxorubicin 12 mg/kg BW intravenously in single dose on 10th day of study.

Blood samples for the estimation of cTnI, ng/ml cTnI was recommended for diagnosis of CK-MB and LDH were taken at the necrosis³¹.

Table-1: Serum biomarkers of rabbits in group A treated with normal saline (n=6), group B treated with doxorubicin (n=6) and group C pretreated with α-tocopherol and doxorubicin (n =6).

Study period	Day 0			Day 11			p value
	Gp A (n =6)	Gp B (n =6)	Gp C (n =6)	Gp A (n =6)	Gp B (n =6)	Gp C (n =6)	
LDH (U/L) ± SEM	722 ± 25	643 ± 10	698 ± 11	760 ± 26	1421 ± 114	646 ± 55	0.000*
CKMB (U/L) ± SEC	119 ± 10	122 ± 15	119 ± 6	129 ± 4	346 ± 37	186 ± 7	0.000*
cTnI (ng/L) ± SEM	0.03 ± 0	0.03 ± 0	0.03 ± 0	0.03 ± 0	10.5 ± 0	2.85 ± 1	0.000*

Table-2: Grades versus groups cross tabulation.

			Groups			Total
			A	B	C	
Grades	Normal	Count	6	0	0	6
		% within groups	100.0%	0%	.0%	33.3%
	Mild	Count	0		1	1
		% within groups	.0%	0%	16.7%	5.6%
	Moderate	Count	0	1	4	5
		% within groups	0%	16.7%	66.7%	27.8%
	Severe	Count	0	5	1	6
		% within groups	0%	.83.3%	16.7%	33.3%
Total		Count	6	6	6	18
		% within groups	100.0%	100.0%	100.0%	100.0%

commencement of the study and on the final day after 24 hours of doxorubicin administration. The blood samples were allowed to clot at room temperature and centrifuged at 3000 rpm for 15 minutes. Serum was separated with the help of an automatic micropipette and stored in clean, dry serum storage vials at -20°C for the estimation of cTnI, CK-MB, and LDH levels to be done by specific kit methods.

The sequences of human and rabbit CK-MB are very similar and polyclonal or monoclonal antibodies specifically prepared against human cTnI have been shown to react with cTnI in the serum of rabbits²⁹.

cTnI was detected by immunoassay systems of Beckman Coulter kit (Access Accu TnI) on (Access 2) made in USA³⁰. A cut off value of 0.50

CK-MB: Estimation was based on principle of immuno-inhibition from a specific antibody of both M and B subunit of CK-MB, by optimized IFCC method of Kinetic-UV on Automated Chemistry Analyser SELECTRA E made in Netherland with kit from Merck.

Lactate Dehydrogenase was measured by UV kinetic method using commercial kit (minias Globe Diagnostics, Italy) on Automated Chemistry Analyser Selectra-E made in Netherland³².

Rabbits were sacrificed to take out the heart. Heart tissue was sectioned sagittally and sections were placed in 10% formalin for fixation then processed for histological examination. Histological sections of the myocardium of all the rabbits were assessed qualitatively and

quantitatively. Qualitatively, DOX-induced cardiac damage was recognized by the presence of marked interstitial edema, perinuclear vacuolization, disorganization and degeneration of the myocardial fibrils. Semiquantitative morphological grading was done by using Billingham scoring method³³.

Statistical analysis

The arithmetic means and SEM were calculated on the computer using SPSS 17 applying one way ANOVA, Post Hoc Tukey test and two-tailed t-test where appropriate. The results of serum analysis were expressed as means \pm SEM. The difference between two observations was considered as significant if the *p* value was found to be <0.05 . The results of histopathology were analyzed by using chi square test. The difference between the two observations was considered significant when the value was less than 0.05.

RESULTS

Cardiac Troponin I levels remained well below normal over the whole period of study i.e. within the range of $0.03 \text{ ng/L} \pm 0.00$ in Gp-A but there was marked rise of cTnI upto $10.55 \text{ ng/L} \pm 0.00$ in Gp-B which was statistically significant ($p < 0.05$) as compared to Gp-A (table-1 and fig-1).

In Gp-C cTnI levels ($2.85 \text{ ng/L} \pm 1.00$) were high as compared to Gp-A but much low as compared to Gp-B with statistically significant difference ($p < 0.05$), (table-1 and fig-1).

Serum CK-MB showed significantly high value up to $346 \text{ U/L} \pm 37$ in Gp-B as compared to control Gp-A $129 \text{ U/L} \pm 4$ ($p < 0.05$), (table-1). Again Gp-C showed less rise ($186 \text{ U/L} \pm 10.00$) of CK-MB as compared to Gp-B which was statistically significant ($p < 0.05$), (table-1).

Serum LDH raised markedly i.e. up to $1421 \text{ U/L} \pm 114.00$ in Gp-B as compared to Gp-A and value of *p* was significant ($p < 0.005$) (table-1). Gp-C depicted no rise in value of LDH ($646 \text{ U/L} \pm 55.00$). On comparison with Gp-B, value of *p* was significant (0.000) (table-1).

Histological examination of section of rabbit hearts from the Gp-A showed normal morphology without any signs of insult to the cardiac tissue (grade 1 necrosis) fig-2(A).

Microscopic examination of Gp-B revealed signs of massive necrosis. None of them were normal. Among total rabbit hearts 83.3% showed grade 3 necrosis and 16.7% of grade 2 necrosis (table-2). The sections of free wall of ventricles showed marked interstitial oedema, infiltration with inflammatory cells, vacuolization and nuclear material clumping. In some of the sections neutrophils infiltration of muscle fibres with loss of myofibril arrangement was seen. Fig-2(B).

In Gp-C, heart sections showed less damage. Statistically only 16.7% revealed severe necrosis, 66.7% of sections were with moderate grade 3 necrosis and 16.7% mild changes in morphology exhibiting grade 2 necrosis fig-2(C).

DISCUSSION

The present study was aimed to detect doxorubicin induced cardiotoxicity by quantitative detection of the specific biomarker, cardiac troponin I and to determine the cardioprotective potentials of α -tocopherol, a known cellular antioxidant.

In our study, group of animals (Gp-B) that received doxorubicin in single toxic dose of 12 mg/kg body weight showed highly deranged serum biomarkers of cardiac injury, with significant difference in serum LDH ($p < 0.001$) and increase of 59.32%, serum CK-MB ($p < 0.000$) with increase of 146.49% and serum cTnI ($p < 0.000$) values with increase of 33210.53% as compared to control group of animals (Gp-A). The microscopic examination of heart sections of Gp-B revealed grade 4 necrosis in most of the tissue while the histological picture of Gp-A animal was completely normal ($p < 0.000$).

Comparable changes were observed by many other researchers following the use of doxorubicin in single or in cumulative doses in rabbits. Markedly raised levels of LDH, CK-MB

and cTnI were observed by Che and his colleagues³⁴ and Simunek¹¹. Alteration in cardiac

doxorubicin. Preclinical studies both in vitro and in vivo done by Sawyer and his colleagues¹² and

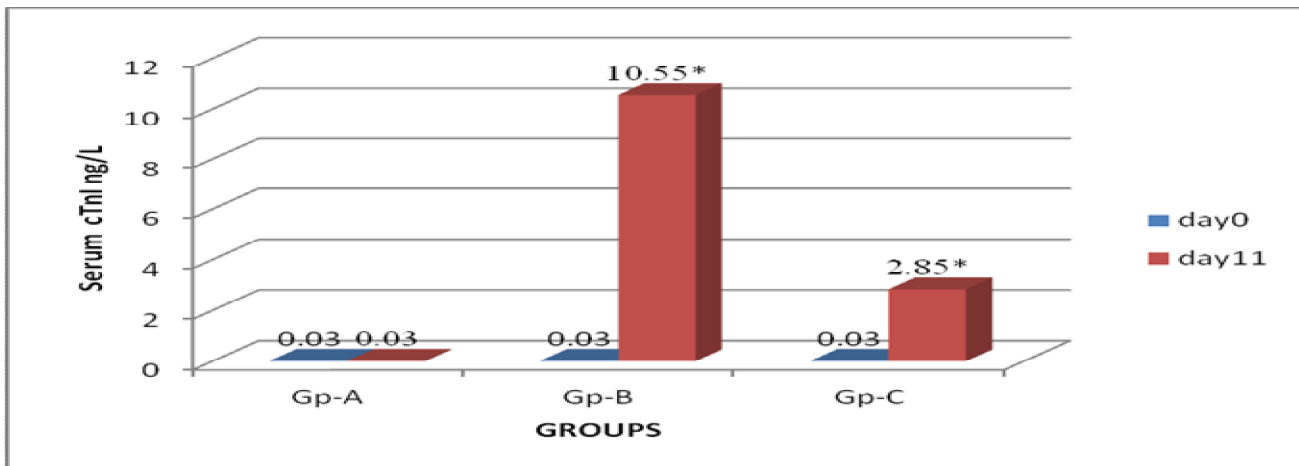


Figure-1: Comparison between Serum Troponin I of rabbits of Gp-A treated with normal saline 0.9%, Gp-B treated with 12 mg/kg Doxorubicin, Gp-C treated with 12 mg/kg of Doxorubicin plus α -Tocopherol 200 mg/kg on day 0 and day 11.

Significance * < 0.05 , Gp-A (n=6), Gp-B (n=6), Gp-C (n=6)

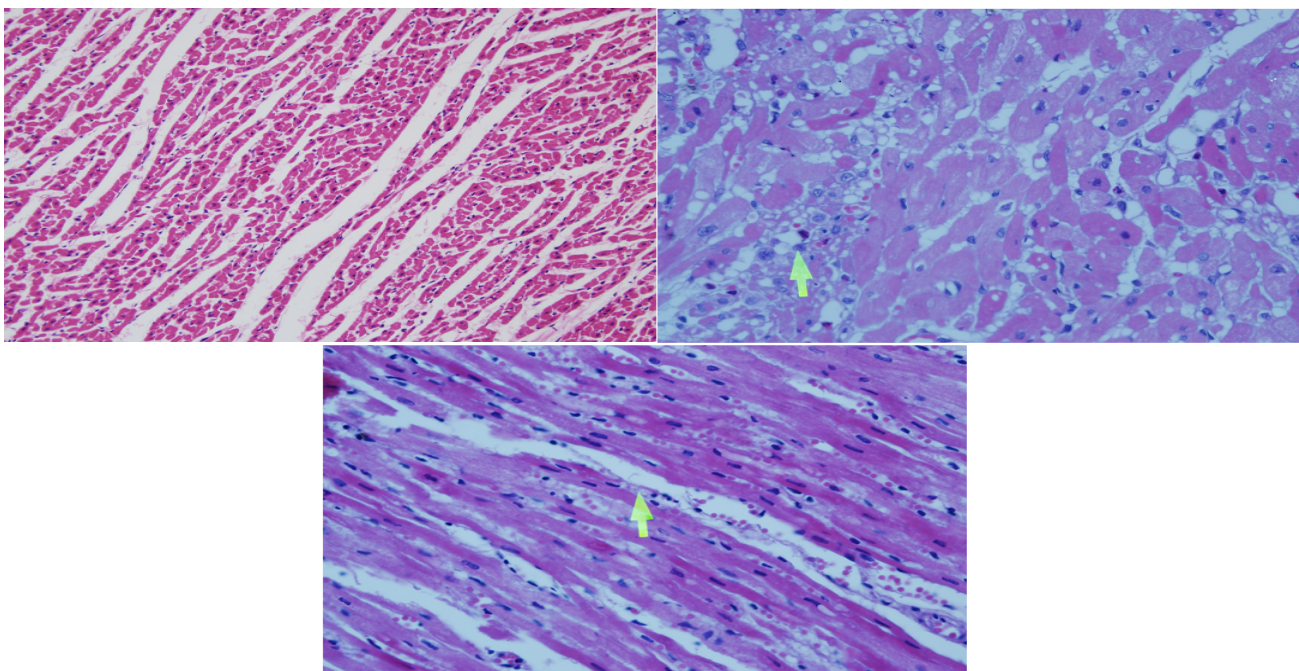


Figure-2: Clockwise from top left: A. Microscopic picture of (H & E)-stained biopsy specimens of rabbit cardiomyocytes from normal (Gp-A) showing normal architecture (300x); B. Micrograph of rabbit cardiomyocytes treated with 12 mg/kg of Doxorubicin showing marked degree of vacuolization and disrupted myofibril arrangement and swollen nuclei and infiltration with inflammatory cells (300x); C. Micrograph of Rabbit cardiomyocytes after treatment with Doxorubicin 12 mg /kg plus α -Tocophrol 200 mg/kg (300x) showing less degree of damage as compared to Gp-B.

tissue morphology is in agreement with previous reports showing cardiac injury²⁶ following the administration of cardiotoxic dose of

clinical study by Cardinale and colleagues¹⁴, in breast cancer patients established that doxorubicin caused a significant ($p < 0.001$) and

dose-dependent cardiomyocyte apoptosis and myocytes death at 24–48 h after the injection as seen in our histopathological reports. Furthermore, many other researchers had documented the elevated concentrations of troponin I, CK-MB and LDH associated with doxorubicin-induced cardiomyocytes death and our results were found consistent with them.

Doxorubicin is front-line therapy to treat a large number of different malignancies including breast cancer and a significant fraction (20%) of patients experience drug-induced cardiotoxicity leading to long-term morbidity or mortality³⁵.

So in order to design the cardioprotective treatment and interventional strategies, there is requirement for a highly sensitive marker of the damage to be measured. Cardiac Troponin I is myocardial regulatory protein. It is 13 times more abundant in the myocardium than CK-MB and is released into the circulation when damage to the myocyte has occurred becoming a marker with high specificity for cardiac injury and is replacing CK-MB. cTnI is expressed only in myocardium. No examples of cTnI expression in healthy or injured skeletal muscle or in other tissue types are known. cTnT is probably less cardiac specific¹⁵.

Keeping in view the absolute cardiospecificity of cTnI, we considered cTnI more favourable for the detection of myocardial injury as approved by Jaffe, Lipshultz and their associates^{16,17} especially where chemical is said to induce cardiac necrosis³³.

In clinical study, Jaffe et al¹⁶ while comparing the imaging techniques and estimation of serum CK-MB and cTnI, ascertained that cTnI determination detects the presence of cardiotoxicity very early, significantly before impairment of cardiac function can be revealed by any other diagnostic techniques. Cardiac troponins, have been incorporated into the National Cancer Institute (NCI) for classification of cardiotoxicity of anticancer therapy³⁶.

The second part of our study was carried out to determine the cardioprotection provided by α -

tocopherol on Dox-induced cardiotoxicity. Gp-C animals received α -tocopherol 200 mg/kg 10 days before the exposure of doxorubicin. The results of LDH showing significant difference ($p < 0.000$) with 27.60% less increase, CK-MB ($p < 0.000$) with 46.31% less increase and cTnI ($p < 0.000$) with 73.01% less increase when compared to Gp-B. Histological changes were also comparable ($p < 0.000$). Similar findings were observed by Hadi et al²⁶ *in vivo* research study in which rats were exposed to intraperitoneal doxorubicin after pretreatment with α -tocopherol. Few other studies in rabbit performed in the past showed the effectiveness of α -tocopherol in mitigating cardiac toxicity of acute high doxorubicin doses. In another rabbit model with chronic Dox-induced toxicity, α -tocopherol exerted protective effect when was administrated in high dosage as a pretreatment or given in combination with vitamin A, reducing myocardial damage^{37,38}.

CONCLUSION

This *in vivo* study provides the evidence that quantitative estimation of cTnI may be helpful for early detection of Dox-induced cardiotoxicity for appropriate intervention to prevent its progression.

Conflict of Interest: This work was partly supported by grant from National University of Sciences and Technology (NUST), Islamabad, Pakistan.

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