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Research Article

Early detection of doxorubicin-induced cardiotoxicity and its prevention by carvedilol

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ABSTRACT

Background: The objective was to detect doxorubicin (Dox) - induced myocardial injury at early stage by quantitative estimation of cardio specific protein, cardiac troponin I (cTnI) and to explore the cardioprotective effects of carvedilol.
Methods: The study design was lab-based randomized controlled *in-vivo* in rabbits conducted from January to August 2012. Cardiotoxicity was produced by single intravenous injection of 12 mg/kg body weight (BW) of Dox in a group of rabbits, control group was treated with normal saline only and the rabbits of third group were pre-treated with carvedilol 30 mg/kg of BW for 10 days before injecting Dox.
Results: Dox induced cardiotoxicity was depicted by markedly raised serum levels of cTnI, creatine kinase-MB, lactate dehydrogenase, and Grade 3 necrosis of the heart tissue in rabbits. The pre-treatment with carvedilol resulted in improved serum levels of these biomarkers and the histological picture of heart tissue.
Conclusions: Quantitative serum estimation of cTnI detects the presence of cardiotoxicity much before cardiac dysfunctions can be revealed by any other diagnostic technique. It can lead to significant economic impact in the management of cancer patients because the troponin-negative subjects can be excluded from long-term cardiac monitoring programs that involve high costs imaging techniques. The outcome of Dox chemotherapy can be made successful with the concurrent use of carvedilol.

Keywords: Cardiac troponin I, Doxorubicin, Carvedilol, Lactate dehydrogenase, Creatine kinase-MB, Group, Body weight

INTRODUCTION

Doxorubicin (Dox) is a potent anticancer agent for the chemotherapy of frequently occurring malignancies (breast, colorectal and lung cancer and childhood malignancies) 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 Dox may develop adverse cardiac effects.[3] The quantitative estimation of sensitive biomarker cardiac troponin I (cTnI) leads to early recognition of cardiotoxicity and have pertinent economic impact in oncologic patient management.[4]

Dox exerts its cytotoxic actions through DNA intercalation and inhibition of DNA topoisomerase II[1] 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 stress. Free radicals and ROS inflict mitochondrial and nuclear DNA lesions in cardiomyocytes with disruption of mitochondrial bioenergetics and impaired expression of cardiac proteins.[5] Dox metabolites also cause disturbances in calcium release from sarcoplasmic reticulum and lipid peroxidation, degradation of myofilaments and cytoskeletal proteins.[6] These processes lead to cardiomyocyte death either by necrosis or by apoptosis,[7] releasing the cardio-specific contractile proteins, cTnI and cytosolic energy

producing enzymes, creatine kinase MB (CK-MB) and lactate dehydrogenase (LDH) into the circulation.[8] CK-MB and LDH are non-specific while cTnI is considered most sensitive biomarker of cardiotoxicity.[9] cTnI is myocardial regulatory protein. It is 13 times more abundant than CK-MB in the myocardium. cTnI is expressed only in myocardium.[10] cTnI determination detects the presence of cardiotoxicity at very early stage, significantly before impairment of cardiac function can be revealed by any other diagnostic technique.[11] Keeping in view the absolute cardio specificity of cTnI, we considered cTnI more favorable for the detection of myocardial injury as approved by Jaffe, Lipshultz et al.[12] especially when drug causes cardiac necrosis.[13]

The cardiac impairment produced by Dox requires long-term follow-up and treatment involving high medical costs.[14] More than half of the total cancer occurs in developing countries.[15] Most of the time, qualitative detection of cTnI is being done that may give false positive or negative results imposing difficulties in cardiac monitoring. The quantitative detection of the specific and sensitive biomarker, cTnI might prove to be helpful in effective management of cancer patients.

Several strategies for reduction of cardiac toxicity have been proposed and used. However, protection conferred by them is not always effective.[16] The objective of the study was the early and reliable detection of cardiotoxicity on one hand and possible prevention with carvedilol, on the other hand, for improved outcome of adjuvant chemotherapy with Dox.

Carvedilol is non-selective beta and alpha-adrenoceptor blocker. Experimental and clinical evidences have revealed that carvedilol exerts antioxidant, antiproliferative, and antiapoptotic[17] along with cardioprotective effects. It has recently been shown to reduce morbidity and mortality in patients with chronic heart failure.[18] By virtue of carbazole moiety and its hydroxylated metabolites it has potent antioxidant activity and has marked protection in pathological processes associated with chemotherapeutic insults.[19]

METHODS

The lab-based randomized controlled *in-vivo* study was carried out in the Departments of Pharmacology and Chemical Pathology after approval from Ethical Committee of "Centre for Research in Experimental and Applied Medicine" (CREAM), Army Medical College, Rawalpindi from January to August 2012. 18 adult healthy male rabbits, weighing 1.0-1.5 kg 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 Dox injection 12 mg/kg body weight (BW) with intermittent 0.9% NaCl infusion into marginal ear vein on 10th day of study. Group C: (n=6) was given carvedilol 30 mg/kg BW by gavage from day 1 to day 11 plus Dox 12 mg/kg BW intravenously as a single dose on 10th day of study.

Blood samples for the estimation of cTnI, CK-MB, and LDH were taken at the commencement of the study and on the final day after 24 hrs of Dox administration.

Pharmaceutical brands of Dox HCL (adriamycin) from Park-Davis Pak and Carvedilol from Ferozsons Pak Ltd. were purchased. cTnI Beckman Coulter kit from PMA while CK-MB kit and LDH commercial kit were purchased from Merck Pak Ltd.

Estimation of serum biomarkers

General principle

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 rabbits.[20]

cTnI

cTnI was detected by immunoassay systems of Beckman Coulter kit (Access Accu TnI) on (Access 2) made in USA A cut-off value of 0.50 ng/ml cTnI was recommended for diagnosis of necrosis.[21]

CK-MB

Its estimation was based on principle of immunoinhibition from a specific antibody of both M and B subunit of CK-MB, by optimized IFCC method of kinetic - ultraviolet (UV) on automated chemistry analyzer SELECTRA E made in Netherland with kit from Merck.

LDH

LDH was measured by UV kinetic method using a commercial kit (Minias GLOBE DIAGNOSTICS, Italy) on Automated Chemistry Analyzer SELECTRA E made in Netherland.

Histopathology

After approval from the institutional ethics committee rabbits were sacrificed to take out the heart. Heart tissue was sectioned sagittally, and sections were 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. Semiquatitative morphological grading was done by using Billingham scoring method.[22]

Statistical analysis

The arithmetic means and standard errors of the mean were calculated on the computer using SPSS 17 applying one-way ANOVAs, post-Tukey test and two-tailed t-test where appropriate. The results of histopathology were analyzed using the "Chi-square test." The difference between two observations was considered as significant if the p value was found <0.05.

RESULTS

Serum biomarkers

cTnI levels remained well below normal (0.03±0.00 ng/L) over the whole period of study in Group A but there was marked rise of cTnI up to 10.55±0.00 ng/L in Group B with statistically significant difference (p<0.05) as compared to Group A. In Group C cTnI levels (2.44±1.00 ng/L) were high as compared to Group A but much low as compared to Group-B with statistically significant difference (p<0.05) (Table 1).

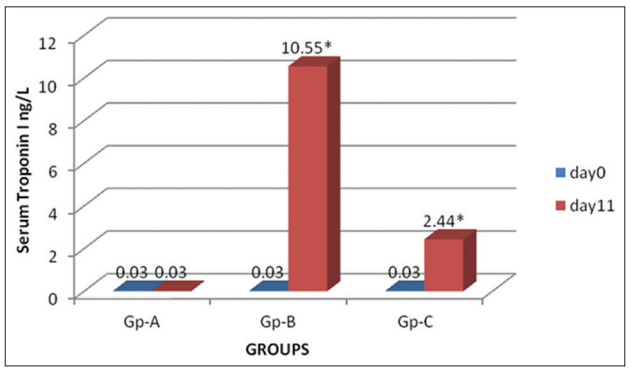

Figure 1: Comparison between serum troponin I of rabbits of Group A treated with normal saline 0.9%, Group B treated with 12 mg/kg doxorubicin (Dox), Group C treated with 12 mg/kg of Dox plus carvedilol 30 mg/kg on day 0 and day 11. *Significance <0.05, Non-significance ≠ >0.05, Group A (n=6), Group B (n=6), Group C (n=6).

Serum CK-MB showed significantly high value up to 346±37 U/L in Group B as compared to control Group A 129±4 U/L (p<0.05) (Table 1 and Figure 2). Again Group C showed less increase (197±10.00 U/L) of CK-MB as compared to Group B and was statistically significant (p<0.05) (Table 1).

Serum LDH raised markedly i.e. up to 1421±114.00 U/L in Group B as compared to Group A with value of p<0.005 (Table 1). Group C depicted no rise in value of LDH (463±55.00 U/L). On the comparison with Group B, value of p was significant (0.000) (Table 1).

Histological examination

Histological examination of section of rabbit hearts from the Group A showed normal morphology (1 grade

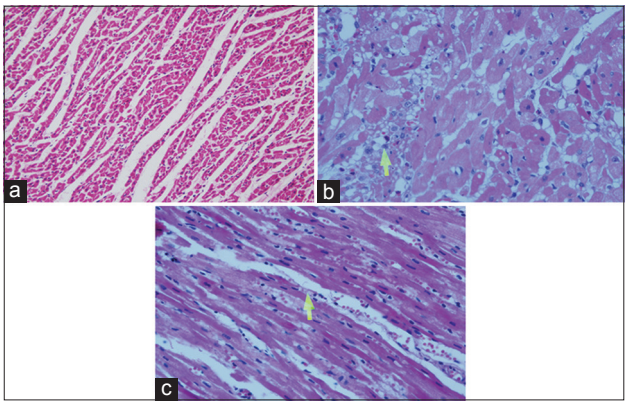

Figure 2: (a) Microscopic picture of (H and E) - stained biopsy specimens of rabbit cardiomyocytes from normal (Group A) showing normal architecture (×300), (b) micrograph of rabbit cardiomyocytes treated with 12 mg/kg of Doxorubicin (Dox) showing marked degree of vacuolization and disrupted myofibril arrangement and swollen nuclei and infiltration with inflammatory cells (×300), (c) micrograph of rabbit cardiomyocytes after treatment with Dox 12 mg/kg plus carvedilol 30 mg/kg (×300) showing less degree of damage as compared to Group B.

Table 1: Serum biomarkers of rabbits in Group A (n=6) treated with normal saline, Group B (n=6) treated with Dox and Group C (n=6) pre-treated with carvedilol and doxorubicin.

Serum tests	Study period						
	Day 0			Day 11			*P* value
	Group A	Group B	Group C	Group A	Group B	Group C	
LDH (U/L)	722	643	799	760	1421	630	
SEM±	25	10	36	26	114	25	0.000*
CK-MB (U/L)	119	122	133	129	346	197	
SEM±	10	15	10	4	37	10	0.000*
cTnI (ng/L)	0.03	0.03	0.03	0.03	10.5	2.44	
SEM±	0	0	0	0	0	1	0.000*

Dox: Doxorubicin, SEM: Standard error mean, LDH: Lactate dehydrogenase, cTnI: Cardiac troponin I, CK-MB: Creatine kinase MB, *: ???

Table 2: Grades*groups cross tabulation of histopathological analysis using the "Chi-square test".

Grades	Groups			Total
	A	B	C	
Normal				
Count	6	0	0	6
% within groups	100.0	0	16.7	33.3
Mild				
Count	0		1	1
% within groups	0	0	66.7	5.6
Moderate				
Count	0	1	4	5
% within groups	0	16.7	16.7	27.8
Severe				
Count	0	5	1	6
% within groups	0	83.3	0.00	33.3
Total				
Count	6	6	6	18
% within groups	100.0	100.0	100.0	100.0

necrosis) Figure 2a. Microscopic examination of Group 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 showed marked interstitial edema, infiltration with inflammatory cells, vacuolization and nuclear material clumping. In some of the sections neutrophils infiltration of muscle fibers with loss of myofibril arrangement was seen Figure 2b. In Group C, heart sections showed less damage. Statistically, 66.7% of the heart section revealed mild necrosis of Grade 1 only and complete prevention was exhibited by 16.7% and a similar number showed moderate toxicity Figure 2c.

DISCUSSION

The study was conducted to detect Dox induced cardiotoxicity by quantitative estimation of the specific biomarker, cTnI and to observe the cardioprotective potentials of carvedilol *in vivo* in rabbits.

Dox produced highly deranged serum biomarkers of cardiac injury and heart tissue sections revealed Grade 4 necrosis in Group-B animals with a significant difference in values as compared to Group A animals. Serum cTnI was increased to 33210.53% ($p<0.000$), serum LDH to 59.32% ($p<0.001$), and serum CK-MB to 146.49% ($p<0.000$). Comparable changes were observed by many other researchers following the use of Dox in single or in cumulative doses in rabbits.[6,23] Preclinical studies both *in vitro* and *in vivo* done by Sawyer and his colleagues[7] and clinical study by Cardinale and Sandri,[9] in breast cancer patients established that Dox caused a significant ($p<0.001$) and dose-dependent cardiomyocyte apoptosis and myocytes death at 24-48 hrs after the injection as seen in our histopathological reports.

In order to design the cardioprotective treatment and interventional strategies, there is a requirement for a highly sensitive marker of the damage to be measured. cTnI a myocardial regulatory protein, is many times more abundant than CK-MB in the myocardium. It is released into the circulation when damage to the myocyte has occurred becoming a marker with high specificity for cardiac injury as cTnI is expressed only in myocardium. In clinical study Jaffe et al.[11] 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 cardiac dysfunction can be revealed by any other diagnostic techniques. cTn, have been incorporated into the National Cancer Institute for classification of cardiotoxicity of anticancer therapy.[24]

The second part of our study was carried out to evaluate the cardioprotection provided by carvedilol. Group C animals were pre-treated with 30 mg/kg carvedilol for 10 days before the exposure of Dox. The pre-treatment was done to maintain the steady state concentration of carvedilol for the preconditioning of cardiomyocytes. The levels of cTnI showing significant difference ($p<0.000$) with 76.87% less increase, CK-MB ($p<0.000$) with 42.93% less increase and LDH ($p<0.000$) with 57.42% less increase in Group C as compared to Group B. Histological changes were also comparable with significant difference ($p<0.000$). The histological picture also changed from Grade 3 necrosis to only of Grade 1 while one-fourth sections showed quite normal architecture of myofibrils. Our findings are in consistent with *in vivo* research study carried out by Hadi et al. 2012 and Arozal et al. 2010[25,26] who studied the cardioprotective effects of carvedilol after cumulative dose of Dox in rats. In *ex-vivo* study on atrial myocytes, Molenaar et al. in 2006[27] established that accumulation of carvedilol in the myocytes accounts for its beneficial effects on heart. Spallarossa et al. (2004) carried out *in vitro* study on cultured cardiac muscle cells and established that pre-treatment with carvedilol decreased free radical release and apoptosis in cardiomyocytes.[28]

Increased oxidative stress, nuclear DNA lesions, mitochondrial dysfunctions, interference in signaling pathway and apoptosis have been implicated in the cardiotoxicity of Dox. Pereira et al. (2012)[29] in his review article explained the cardioprotective effects of carvedilol studied by many researchers.[29] Carvedilol is a non-selective β-adrenergic blocking agent with potent intrinsic antioxidant activity. Clinical trial carried out by Kalay et al. 2006.[30] showed preserved left ventricular diastolic and systolic function with cardiac function enhancement. However, further clinical trials need to be performed with larger study groups.

CONCLUSION

This *in vivo* study provides the evidence that quantitative estimation of cTnI may be helpful for early detection of Dox-induced cardiotoxicity and for appropriate intervention to prevent its progression. Dox is frontline therapy in breast cancer. About 90,000 in one million women in Pakistan suffer from breast cancer, and 40,000 die each year.[31] Dox chemotherapy can be made successful by concurrent use of carvedilol and detection of toxicity at a subclinical level can lead to relevant impact on cancer patient management.

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Conflict of Interest: None declared
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