ORIGINAL ARTICLE

EVALUATING THE PROTECTIVE EFFECTS OF LOVASTATIN AGAINST DOXORUBICIN INDUCED CARDIOTOXICITY IN BALB-c MICE

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Background: Doxorubicin is one of the most commonly used anti-cancer drugs that treat a large number of haematological and solid malignancies. Its usage in dose and duration is nevertheless restricted by dose related organ damage, particularly cardiotoxicity. Lovastatin is a commonly prescribed drug for hypercholesterolemia and possesses remarkable antioxidant potential. Our study was aimed at evaluating and comparing its cardioprotective effect in two pre-treatment schedules against doxorubicin-induced cardiac damage. **Methods:** In this lab-based randomized controlled experiment, 40 BALB/c mice were randomly grouped into five groups (n=8). Group 1 served as control whereas Group 2 was given doxorubicin intraperitoneally at a dose of 10mg/kg. Group 3 received 10mg/kg of oral lovastatin for five days. Groups 4 and 5 were administered lovastatin for five and ten consecutive days correspondingly and doxorubicin was given on 3rd and 8th experimental days of these groups. **Results:** Doxorubicin caused a significant rise in cardiac enzymes; Creatine kinase MB (CK-MB) and Lactate Dehydrogenase (LDH) (*p*-value ≤0.0001) whereas cardiac histological alterations were ranked as moderate. The damage was significantly attenuated by lovastatin in the ten-day study design with a *p-value* of ≤0.001 for both LDH and CK-MB whereas a slightly less efficient restoration was observed in the five-day design with a *p*-value of ≤0.001 for LDH and 0.012 for CK-MB. Histological preservation in both pre-treatment schedules was in accordance with the biological markers. **Conclusion:** In doxorubicin-based regimens, pretreatment for at least seven days with an easily available and safe statin can effectively prevent its potentially life-threatening cardiotoxicity.

Keywords: Cardiotoxicity; Creatine Kinase MB; Lactate Dehydrogenase; Doxorubicin; Lovastatin

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INTRODUCTION

The age of directed and aggressive chemotherapy has substantially improved the long-term survival of cancer patients but it has also been a source of comparable increase in life-threatening adverse effects.[1] In many instances, these dose-related adverse effects become as lethal as cancer itself. Doxorubicin (DOX) is a broad spectrum anthracycline which to date is an extensively used anti-cancer agent for several decades; it is used for treating a vast majority of solid and haematological malignancies in all age groups despite fatal complications.[2] It kills tumour cells by Topoisomerase II inhibition and subsequent DNA damage; reactive oxygen species (ROS) generation leads to apoptosis.[3] Cardiotoxicity is the most frequently occurring and potentially fatal dose limiting adverse effect of DOX which can be acute, sub-acute and chronic.[2] Acute toxicity is seen in 40% of patients; and can be detected by enzyme elevations and ECG changes. Subacute effects present as toxic myocarditis and chronic consequences are detected after months comprising of cumulative dose-related cardiomyopathy and congestive heart failure, fatal in 27–60% of cases.[4] Risk of cardiotoxicity is enhanced with greater cumulative dose; incidence rates related to dose more than 400 mg/m^2 may be up to 5% and is calculated to be 18–48% for 700 mg/m^2.[2,5] The total dose of DOX is hence limited to 450 to 550 mg/m^2.[6] The only FDA approved cardioprotective strategies include concomitant dexrazoxane (DEX) administration or that of a liposomal preparation which is both costly and have a challenging procurement.[7]

Lovastatin has been one of the most prescribed and well tolerated lipid lowering drugs. In recent years, a substantial quantity of data showing that it exerts non-lipid lowering pleiotropic effects on multiple targets has caught the attention of researchers worldwide providing possibly novel therapeutic uses for clinicians and these effects have proven to occur at low doses.[8] Lovastatin is established to be cardioprotective; this has been established by several studies with regard to cardiovascular pathologies.[9–11] The major cardioprotective effects are credited to LDL lowering, but it owes cardio protection to a multitude of other properties which are being explored. It prevents cardiotoxicity induced by DOX by a multitude of mechanisms: reduced formation of DNA double-strand breaks occurring due to inhibition of topoisomerase II; decreases neutrophil infiltration by preventing ROS generation; causes a

significant decrease in TNF-α and an increase in superoxide dismutase (SOD) activity all of which make it a cardioprotective agent with and antioxidant properties.[12] The anti-neoplastic features of statins have been receiving considerable attention in the last few decades. Pre-clinical evidence advocates that lovastatin inhibits tumour growth and induces apoptosis in specific cancer types; this and the well-established safety profile uncovers its clinical potential as a combination agent in oncology.[8,13]

Due to challenges associated with discovering novel chemotherapeutic agents and procurement of costly and potentially hazardous dexrazoxane, strategic improvements in current chemotherapeutic regimens are crucial for ensuring safe cancer treatments in economically underprivileged countries like Pakistan. Henceforth, pharmacological modulations which either enhance the properties, reduce toxicities of antitumor agents or have intrinsic anti-cancer properties may have a high clinical impact. Considering cardioprotective effects and anti-cancer potential of lovastatin, we speculated that it might be a suitable adjuvant to counter DOX induced cardiotoxicity**.** This study explores and compares its cardioprotective potential in two different schemes of study designs which may contribute towards further studies.

MATERIAL AND METHODS

The experiment was conducted in an animal house at, the Department of Pharmacology and Therapeutics of Army Medical College (AMC). Its protocol was approved by the Ethical Committee of the Centre for Research in Experimental and Applied Medicine (CREAM), AMC. The biochemical analysis of sera and the histopathological analysis of cardiac tissue was accomplished in association with the Department of Chemical Pathology and Histopathology, AMC. Forty male and female Balb/c mice of age 2–3 months and weight 40±5 grams were acquired from the National Institute of Health (NIH), Islamabad and kept in the animal house for a week for familiarization with the new milieu. The animals were provided with a customary laboratory environment.

DOX was obtained from Bone Marrow Transplant Centre (BMTC), Rawalpindi. The pharmaceutical brand of lovastatin tablets was bought from a local pharmacy. A pilot project was carried out prior to establish the human relevant doses of the drugs in light of the literature review, and also to eliminate the fear of inducing life-threatening cardiotoxicity. DOX dose was carefully chosen to be 10 mg/kg and that of lovastatin was also concluded to be 10 mg/kg.[14–17]

It was a lab based randomized controlled experiment in which animals were grouped into 5 groups (n=8) using non-probability convenience sampling method. The study was carried out over two different time spans; the study on groups 1 to 4 was carried out for five days and the study on group 5 lasted for ten days (Table-1).

Table-1: The intervention protocol

Groups	Intervention Protocol
Group 1: Control group (n=8)	Normal saline intraperitoneal (IP) for 5 consecutive days
Group 2: DOX group (n=8)	Single IP dose of 10mg/kg on day 3.[15,18–20]
Group 3: Lovastatin only (n=8)	Per oral (PO) as 10 mg/kg/d for 5 days.[15]
Group 4: DOX + Lovastatin for 5 days (n=8)	PO Lovastatin as 10mg/kg/d for five consecutive days along with IP DOX as 10 mg/kg on 3rd day.[15,19]
Group 5: DOX + Lovastatin for 10 days (n=8)	PO Lovastatin at the dose of 10mg/kg/d for ten consecutive days along with IP DOX as 10 mg/kg on 8th day.

The DOX solution was prepared fresh in Normal Saline (NS) before administration to each group to be administered intraperitoneally. Lovastatin (strength of 20 mg) was crushed into a powder prior to its addition in NS to be given by oral gavage. At the end of the experiment, mice were sacrificed with a humane approach according to regulations of the Animal Welfare Act and Animal Welfare Regulations, 2013.[21] Ether was used for anaesthesia by the drop jar method.

Terminal samples of blood for analyses of CK-MB and LDH were collected by intra cardiac puncture on the fifth day for groups 1 to 4 and on the tenth day for group 5. It is important to note that all samples were collected 48 hours after the DOX dose.[16] All the samples were analyzed as per the principles of the International Federation of Clinical Chemistry (IFCC) on kits made by Diasys Diagnostic System, USA and evaluated on analyzer SPECTRA E.

After the collection of blood samples, the heart was dissected and transferred to phosphate buffered formaldehyde for fixation. Cardiac tissue sections were stained with Haematoxylin and Eosin (H&E) dyes and examined with a light microscope under 100x and 200x. They were assessed quantitatively and qualitatively; photomicrographs of individual slides were taken. Qualitatively, DOX generated cardiac damage was recognized by the presence of interstitial oedema, perinuclear vacuolization and disorganized myofibrils.[22] Bellingham's scoring method was used to categorize quantitative morphological categorizing.[16]

Findings of biochemical parameters were described as Mean±Standard Error of Mean (S.E.M). Statistical analysis was carried out on Special Package for the Social Sciences (SPSS) version 22. Multiple comparisons between the groups were done using one-way analysis of variance, i.e., ANOVA and then Post Hoc Tukey test. The difference between the two groups was affirmed significant if the *p-value* was ≤ 0.05. Histopathological observations were evaluated by the 'Chi Square test'

and difference between two observations was significant if the *p* value was ≤0.05.

RESULTS

Single DOX injection caused very significant (*p* value ≤0.0001) rise of both CK-MB and LDH (286% & 254% correspondingly) in group 2. In contrast, the group that received lovastatin did not exhibit any significant change in the values of CK-MB and LDH (Table-2). In group 4, which received lovastatin orally at a dose of 10 mg/kg for five days, the decline in the upsurge of CK-MB values was significant with a *p*-value 0.012 (Table-2 & 3). The ten day administration, however, produced a more significant reduction in the *CK-MB* levels with a *p* value of ≤0.0001 and it appeared significant to increase the duration of lovastatin administration from 5 to 10 days for cardio protection with reference to the CK-MB levels (*p* value 0.010) (Table-3). The administration of 10 mg/kg/day of lovastatin for both 5 and 10 days brought down the value of LDH significantly (Table-2) with a p-value of ≤0.0001 in both the groups and with reference to the LDH levels, it was also evaluated that there is benefit of prolonged administration of ten days cardio protection as is evident by the *p* value 0.022 (Table-3). Grading of slides in the control group remained unremarkable as they all exhibited normal architecture with no necrosis (Figure 1a). The slides in the DOX group showed significant alteration. Around six out of eight were graded to have moderate toxicity changes as 26–35% of the cells showed the classical DOX associated changes namely myofibril disarrangement, perinuclear vacuolization, loss of nuclei and prominent interstitial oedema (Figure-o1b).

Five out of eight slides were graded to be mild in the groups that received lovastatin for five days. The ten day administration offered remarkable protection as five slides were ranked as normal (Figure-1c & 1d). Chi square test demonstrated significance indicating the prominent difference between groups.

Table-2: **Effect of 5- and 10-day lovastatin administration on the cardiac enzymes in Doxorubicin (DOX) treated mice**

	Control	Lova	DOX	DOX/Lova-5 d	DOX/Lova-10 d
CK-MB	356.25±44.72	259.62±14.18	1374.37±149.8$^{\#}$	931.87±75.85*	477.62±35.31**
LDH	1878.75±196.85	1581.5±101.23	6657.5±589.02$^{\#}$	4089.12±265.53**	2513.37±144.94**

#Significant compared to control (*p* value ≤0.0001). *Significant compared to DOX (*p* value ≤0.05). **Highly significant compared to DOX (*p* value ≤0.0001)

Table-3: Comparison between Group 2, 4 & 5 by Post Hoc Tukey (following One Way ANOVA)

	Groups		Significance
CK-MB	2	4	0.012*
	2	5	≤0.001*
	4	5	0.010*
LDH	2	4	≤0.001*
	2	5	≤0.001*
	4	5	0.022*

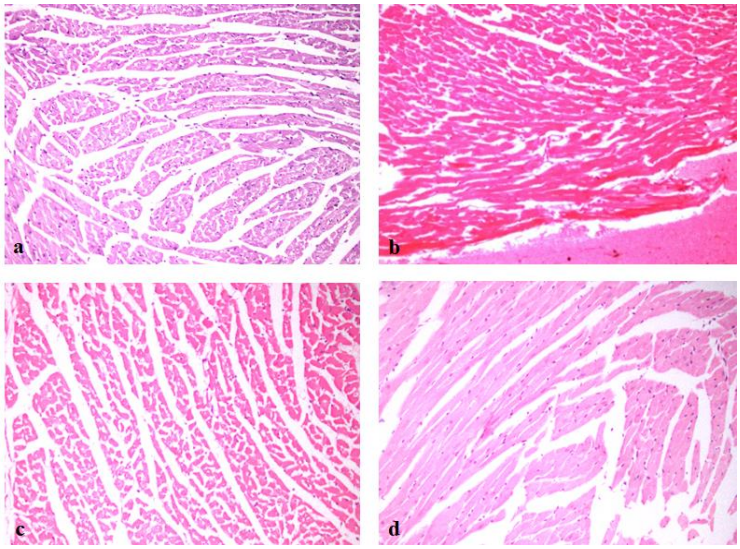

Figure-1: Photomicrographs of cardiac tissues (H&E 100x): (a) group 1 (control) with normal architecture (b) group 2 (DOX) with lack of nuclei, myofibrils disarrangement and marked interstitial oedema (c) group 4 (DOX/ Lova5d) with nominal myofibrillar disarray and scattered nuclei loss (d) group 5 (DOX/ Lova10d) with re-established normal histological architecture

DISCUSSION

Keeping in view the potential of their disproportionate adverse effects, only time tested anti-cancer agents would be preferred for a long time. Doxorubicin (DOX), although causes dose related cardiac damage, is still in extensive use for decades due to its broad spectrum and excellent efficacy. The single FDA approved drug to prevent its cardiotoxicity is dexrazoxane (DEX), the administration and availability of which is laden with difficulties[2,23] and is being discouraged lately due to concerns about its safety[7,9,24] There is now a critical need for alternate protective measures which are practical and safe, and this study contributes towards the continuous spectrum of research on discovering a safer and easily available protective agent. Lovastatin possesses effective anti-oxidant properties along with the advantages of safety, cost-effectiveness and suitability of use. This experiment was specially designed for comparative analysis of lovastatin's cardioprotective potential in two pre-treatment schedules in the setting of DOX induced toxicity. Moreover, in light of the literature review, it was ensured that the dosage and administration of both drugs simulated the pattern and dose of human administration.[15,18]

In our study, the control group received normal saline intraperitoneally in a comparable volume of 0.2 ml for a period of five consecutive days. Mice were sacrificed on day five; biochemical markers and histopathological findings were all unremarkable as per the expectation. In group 2, toxicity was introduced by a single intraperitoneal injection of DOX in a dose of 10 mg/kg, the relevant human therapeutic dose.[15,16] Experiment was conducted for five days on this group whereby DOX was administered on day 3 and intraperitoneal saline on the remaining days. Sampling was done 48 hours after the DOX dose; serum creatine kinase (CK-MB) and lactate dehydrogenase (LDH) were found to be significantly elevated compared to the control group. The raise was in accordance with many studies.[16,20,22,25,26] The histopathological changes were analyzed to be moderate showing classic lesions of interrupted myofibrillar architecture and vacuolization. These findings too were significant in comparison with the control and were in accordance with many studies.[16,18,25,27,28]

Acute Cardiotoxicity is a well-established phenomenon in DOX treated cancer patients and can present acutely within 24 hours[2,23] and presents as raised biochemical markers and electrocardiographic (ECG) changes[29,30]. In our study, acute toxicity was generated by a single dose of DOX and CK-MB and LDH were selected as the biomarkers.[16,20,22,25,26] The gold standard of confirming acute cardiotoxicity by DOX, however, remains endomyocardial biopsy of the right ventricle due to high sensitivity and specificity[18,27,30,31] and this has also been inculcated in our study.

The Doxorubicin induced cardiotoxicity is attributed to several mechanisms including the modified redox status which generates a cascade of reactive oxygen species (ROS), mitochondrial membrane damage due to increased permeability, mitochondrial deoxyribonucleic acid (DNA) damage, mitochondrial swelling and adenosine triphosphate (ATP) depletion. The ROS tend to react with lipids, proteins and other macromolecules causing damage to the membranes. The disrupted mitochondrial bioenergetics cause the release of pro apoptotic proteins which induce apoptosis of myocytes**.** This causes the release of cardio specific cytosolic enzymes into the circulation to be identified as the diagnostic markers of DOX induced cardiotoxicity.[16,18,22]

Lovastatin alone was administered in group 3, to verify if this drug could produce cardiac damage or not; we did not observe any biochemical and histopathological alterations in this group. In groups 4 and 5, we administered lovastatin in the human relevant dose of 10 mg/kg/d orally for five and ten days correspondingly. DOX was given on 3rd and 8th days respectively in groups 4 and 5. The sampling time in both groups was 48 hours after the dose of DOX. In group 4, the three-day pre-treatment brought down levels of CK-MB less markedly (*p* values $\leq$0.012) and markedly for LDH (*p* values $\leq$0.001); this was also reflected in the microscopic examination as several slides from this group still revealed nuclei loss and negligible myofibrillar oedema and disarrangement. We then concluded to prolong the pre-treatment for seven days while keeping both pre-treatment experimental designs similar. The ten day lovastatin administration in group 5 was more efficient in decreasing the biochemical markers; longer pre-treatment prevented enzyme surge for both CK-MB and LDH highly significantly with *p*-value of $\leq$0.0001. Histopathological observations were also in absolute accord; alterations in the cardiac architecture were more efficiently restored. Altogether, it was revealed that lovastatin protected mice from DOX induced cardiac damage in both study schemes but more efficaciously if mice were pre-treated with lovastatin for a week (Table-3).

Lovastatin's pre-treatment for a week prior to DOX administration has never been studied before. Its protective potential has been tested on anthracycline-induced late cardiotoxicity analyzed

three months after administration of low doses of DOX however, it has not been studied for managing equally hazardous DOX induced acute cardiotoxicity.[32,33] Henninger *et al*. investigated the hepatoprotective potential of lovastatin following the administration of DOX in mice[15] while in another experimental study on mice, the protective role of atorvastatin was ascertained against DOX-induced cardiotoxicity in two treatment schedules[28].

The mechanism in preventing such damage is mainly lipid- independent inhibition of isoprenoids and their associated molecular events and reduction of acute pro-inflammatory and pro-fibrotic stress response caused by anthracycline treatment.[10,12] Lovastatin can be a promising agent to preclude unfavourable cardiac complications in DOX therapies. The pleiotropic biological activities of statins including antioxidant, anti-inflammatory, anti-apoptotic and cytoprotective abilities support that they are potent cardioprotective agents to guard cardiac tissue against DOX-induced damage and potentiate DOX-based chemotherapies.[8,10,24,34] A wide range of clinical and epidemiological studies have also ascertained that statins may augment efficacy and prevent limitations related to conventional chemotherapy, signifying that statins should be considered in combined cancer therapies.[8,13] Besides an innumerable number of animal studies, this view is also supported by previous retrospective clinical studies which revealed reduced risk of heart failure in DOX -based therapy of breast cancer who were co-administered with statins for cardiovascular reasons.[33,35]

These data and our findings, therefore, support that adding lovastatin, which is already widely used as an anti-lipid drug, into current DOX-based anticancer regimens is highly advantageous and might be a favourable cardioprotective substitute to DEX. These findings however need further investigation to achieve a more precise pre-treatment schedule for human administration in DOX based regimens.

CONCLUSION

Lovastatin's pre-treatment is proposed to be a successful approach to mitigate the cardiotoxicity of the commonly used chemotherapeutic agent doxorubicin. It has been concluded that the preceding administration of lovastatin for at least a week provides optimum cardio protection and its introduction in the extensively practised doxorubicin-based chemotherapy may be used as an alternative to costly dexrazoxane and liposomal preparations.

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AUTHORS' CONTRIBUTION

AS: Literature search, the conceptualization of the study, data collection and analysis, data interpretation, write-up. KA: Data interpretation. AA: Data interpretation, proof reading. SR, AZ: Proof reading. TT: Analysis of data.

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