ORIGINAL ARTICLE

PROTECTIVE EFFECTS OF TRIMETAZIDINE AGAINST DOXORUBICIN-INDUCED CARDIOTOXICITY AND HEPATOTOXICITY IN MICE

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Background: Trimetazidine (TMZ) is traditionally known for cardio protection, however various experimental studies are also evaluating its protective benefits in other tissues. Doxorubicin (DOX) is an extensively used chemotherapeutic drug but is associated with a high incidence of multi-organ damage. This study was aimed at countering DOX induced cardiac and hepatic toxicity by administering TMZ in two different study designs. **Methods**: It was a laboratory based randomized controlled trial conducted on 40 BALB/c mice divided into 5 groups (n=8). In the two study designs conducted, TMZ in a dose of 10 mg/kg was given orally for five and ten consecutive days. On the third and eighth day of the respective designs, 10 mg/kg DOX was administered intraperitoneally. **Results:** DOX induced significant elevation of four biochemical markers, namely creatine kinase MB (CKMB), lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (p-value ≤0.0001). Histological changes in heart were graded to be moderate while hepatic changes were graded as mild. Trimetazidine administration for ten days attenuated the enzyme upsurge significantly with p-value ≤0.05 for ALT and ≤0.0001 for AST, LDH and CKMB. However, five-day TMZ administration caused nonsignificant restoration in ALT and CKMB level (p-value >0.05). Hepatic and cardiac histological changes were restored accordingly in both groups. **Conclusion:** Treatment with TMZ for ten days, seven of which were prior to DOX administration, was concluded to be an effective strategy to counter cardiac and hepatic toxicity of DOX.

Keywords: Alanine aminotransferase; Aspartate aminotransferase; Cardiotoxicity; Creatine Kinase MB; Hepatotoxicity; Lactate Dehydrogenase; Trimetazidine

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INTRODUCTION

Aggressive approach of chemotherapy has led to a considerable improvement in the survival of cancer patients but at the cost of a proportionate rise in life threatening adverse effects. Doxorubicin (DOX), an anthracycline antibiotic, remains one the most widely used chemotherapeutic agents against a wide range of solid and haematological malignancies.[1] A higher cumulative dose which is essential for ensuring successful chemotherapy however, leads to dose related organ damage.[2] Reactive oxygen species generation and topoisomerase II inhibition are the most common factors which kill tumour cells as well as cause normal tissue injury. Acute cardiotoxicity is the most common and most feared adverse effect seen in 40% of the patients.[3] Heart failure incidence is around 5% at a dose of 400 mg/m^2 and is calculated to be 18–48% for 700 mg/m^2.[4,5] The approved protective strategies include simultaneous administration of dexrazoxane (DEX) or the administration of liposomal-encapsulated DOX. Both are costly options associated with very difficult procurement in many countries including Pakistan.[4]

Though heart is the most affected organ but liver, the chief drug metabolizing organ, has also been stated in several animal experimental studies.[6–8] Concerning human data, the occurrence was established to be 30.4% in a study of breast cancer patients who received four cycles of DOX.[9] Hepatic insult by DOX causes toxins to accumulate in the body, and further enhances the cardio toxic potential of DOX. However, hepatotoxicity by DOX is comparatively an underexplored phenomenon and there are inconsistent conclusions about DOX use and emergent hepatotoxicity. As no agent is approved by FDA against DOX-induced hepatotoxicity, there is a tendency of using a restricted dose to avoid liver damage.[10,11]

Bearing in mind these toxicities, the clinical use of DOX is often limited to a total dose of 450–550 mg/m^2 which might affect its chemotherapeutic benefits.[12] It is therefore, crucial to introduce pharmacological modulations that would limit the

toxic potential of DOX without compromising its efficacy.

Trimetazidine (TMZ) is being used successfully for over 40 years in cardiology.[13] It is clinically an effective anti-ischemic drug used in coronary heart disease as it enhances the contractile response of chronically dysfunctional myocardium. It is approved as a long-term treatment of angina pectoris alone as well as in combination.[14,15] It diminishes fatty acid metabolism and enhances glucose metabolism instead, thus conserving cellular homeostasis. Trimetazidine is now called a "metabolic agent" due to its notable ability to reserve intracellular ATP production. It preserves membrane structures and function by restriction of intracellular acidosis and maintenance of mitochondrial function.[16,17] Owing to these antioxidant properties, it might confer remarkable protection against DOX induced damage which is caused by unwarranted production of oxygen free radicals and lipids peroxidation.[6] However, the experimental studies available on the subject provide contradictory conclusions regarding its optimal dose and schedule.[12,18,19]

Chemotherapeutic agents are infamous for causing multi-organ toxicities. Cost effective and convenient pharmacological modifications in chemotherapeutic regimens are becoming a necessity, especially in underprivileged countries with inadequate health care infrastructure. Administration of chemotherapy with full dosing protocol in the presence of a protective agent could pave way for effective as well as safer chemotherapy. Trimetazidine is one such agent with a remarkable potential to counter toxic insult conferred by DOX and related drugs. The comprehensive and comparative formulation of this study on hepatoprotective and cardioprotective potentials of TMZ in two pre-treatment schedules could contribute towards conduction of clinical trials.

MATERIAL AND METHODS

The study was carried out in the animal house of Department of Pharmacology and Therapeutics, Army Medical College (AMC), Rawalpindi. DOX was arranged from Bone Marrow and Transplant Centre (BMTC), Combined Military Hospital (CMH), while TMZ was purchased from a local pharmacy. Study protocol was approved by the Ethical Committee of Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College. The biochemical and histopathological analyses were done at the Departments of Chemical Pathology and Histopathology, AMC.

Forty Balb/c mice of age 8–12 weeks and weight 40±5 grams were procured from National Institute of Health (NIH), Islamabad and were acclimatized for a week before the start of experiment. They were kept under standard laboratory conditions of 12 hours light and dark cycle, 20±25 ˚C temperature and 70±15 percent humidity. Mice were provided with the rodent pellet diet and tap water *ad libitum* during the course of the experiment.

It was a laboratory based randomized controlled trial where animals were chosen by non-probability convenience sampling and allocated into five groups (n=8).

Experiment on groups one to four was conducted for five days and for ten days on group five. Group one served as the control group and received normal saline intraperitoneally (IP). Group two mice were injected DOX IP at a dose of 10 mg/kg on the 3rd day of experiment. Group three received TMZ orally in a dose of 10 mg/kg for five consecutive days. Group four and five received TMZ for five and ten consecutive days respectively, whereby DOX was administered on the 3rd and 8th day of the respective experiments.

Mice were euthanized according to the decorum of Animal Welfare Act and Animal Welfare Regulation; 2013.[20] Initiation of anaesthesia was attained by ether using drop jar method.

Blood samples were collected terminally by intra cardiac puncture for the analyses of CK-MB, LDH, ALT and AST. All the analyses were done by the principles laid down by International Federation of Clinical Chemistry (IFCC) using kits manufactured by Diasys Diagnostic System, USA and were analysed on the automatic analyser SPECTRA E.

After the terminal blood sampling, heart and liver were dissected out and were mixed immediately in 10% phosphate buffered formaldehyde for fixation. All the samples were later taken to laboratory and were dried in increasing alcohol concentrations, cleared with xylene and imbued in paraffin. Afterwards they were cut into four micrometre thin sections by a rotatory microtome. They were stained with Haematoxylin and Eosin (H&E) dyes and were examined thoroughly under light microscope. Histological sections of the heart were evaluated quantitatively and qualitatively. Qualitatively, DOX generated cardiac damage was identified by the presence of interstitial oedema, perinuclear vacuolization and disorganization of the myofibrils. Quantitative morphological grading was done using Bellingham's scoring method.[12] Ishak Histological Activity Index (HAI) was used to grade histopathological changes in the liver.[21]

The results of biochemical parameters were expressed as Mean±Standard Error of Mean (S.E.M).

Statistical analysis was done on SPSS version 22. One-way analysis of variance, i.e., ANOVA followed by Post Hoc Tukey test was used for multiple comparisons between the groups. The difference between the two groups was considered significant if the *p*-value was less than 0.05. Histopathological findings were analysed by the 'Chi Square test'. The difference between two observations was considered significant if the *p*-value was less than 0.05.

RESULTS

Biochemical analysis

Single injection of DOX led to a very significant (*p*-value ≤0.0001) elevation of both CK-MB and LDH (286% and 254% respectively) in group 2. Three-day administration of TMZ prior to DOX failed to prevent the enzyme upsurge of CK-MB significantly. However, longer pre-treatment in group 5 very significantly prohibited the CK-MB elevation (*p*-value ≤0.0001) (Table-1) reflecting remarkable protective potential of this study design. On the other hand, three days preconditioning did prevent the LDH upsurge by 27% (*p*-value ≤0.05) but the longer pre-treatment caused 53% attenuation of the derangement (*p* value ≤ 0.0001) (Table-1) (Figure-1).

In group-2, levels for ALT and AST were raised by 1317% and 514 % respectively reflecting the enormous toxic potential of a single DOX injection (*p*-value ≤0.0001). The prevention of ALT upsurge was not significant in group 4 (33% decrease), however in group 5, TMZ successfully prevented the elevation by 65% with a *p* value ≤0.05 (Table-2). In contrast, the short as well as the longer pre-treatment with TMZ successfully prevented the upsurge for AST with *p*-values of ≤0.05 and ≤0.0001 respectively (Table-2) (Figure-2).

Table-1: Effect of Trimetazidine (TMZ) administration for 5 and 10 days on the cardiac biochemical parameters of Doxorubicin (DOX) treated mice

	Control	TMZ	DOX	DOX/TMZ-5d	DOX/TMZ-10d
CK-MB	356.25±44.72	415.00±28.41	1374.37±149.8#	1203.75±115.2	303.12±30.58**
LDH	1878.75±196.85	2775.0±189.74	6657.5±589.02#	4833.75±405.94*	3157.37±276.33**

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

Table-2: Effect of Trimetazidine (TMZ) administration for 5 and 10 days on the hepatic biochemical parameters of Doxorubicin (DOX) treated mice

	Control	TMZ	DOX	DOX/TMZ-5d	DOX/TMZ-10d
ALT	36.5±5.11	44.25±3.19	517.37±74.59#	345.00±66.54	182.75±53.69*
AST	130.62±19.14	185.0±12.67	801.87±54.99#	520.62±67.73*	222.75±28.43**

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

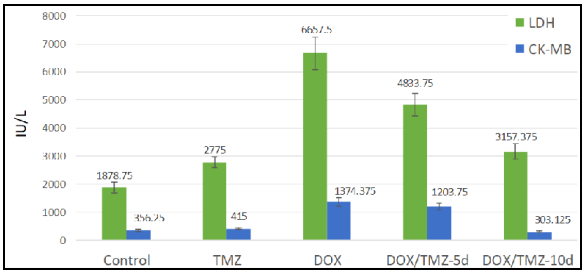

Figure-1: Graphical representation of the effects of TMZ administration for two different durations on the cardiac biomarkers of DOX treated animals.

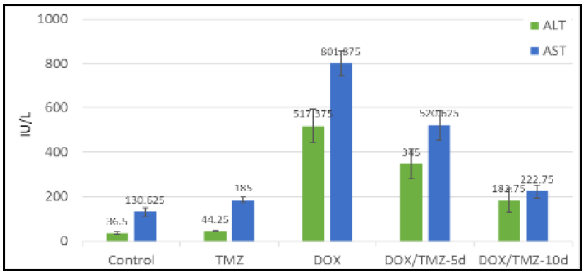

Figure-2: Graphical representation of the effects of TMZ administration for two different durations on the hepatic biomarkers of DOX treated animals.

Histopathological analysis

Heart

The histological slides were analysed under 100x and 200x. They were all then graded as per the Bellingham's classification. The slides from the control group exhibited normal architecture with no necrosis (Figure 3a). Five out of eight slides from group 2 showed moderate toxicity; 26–35% of the cells in these slides exhibited the classic DOX induced changes. They included myofibril disarrangement, perinuclear vacuolization, loss of nuclei and marked interstitial oedema (Figure 3b). In group 4, 6 out of 8 slides were categorized to have mild toxicity as they revealed minimal

disarrangement, oedema and loss of nuclei in some cells (Figure 3c). While in group 5, protection was even more profound as half the slides were graded to be normal (Figure 3d). When chi square test was applied, it demonstrated significance showing ~~the~~ prominent difference between various groups.

Liver

Single injection of DOX induced hepatic damage described by scattered areas of inflammatory cellular infiltration in portal and peri-portal region, venous congestion and dilated sinusoids (Figure 4b). The changes were assessed to be mild with a score of 4 as per the ishak HAI scale. Treatment of mice with TMZ for five days slightly prevented DOX induced pathological changes as focal inflammation and sinusoid dilation was still seen in 6 slides (Figure 4c). The ten-day administration by TMZ led to a remarkable protection as 5 out of 8 slides were graded to be normal (Figure 4d).

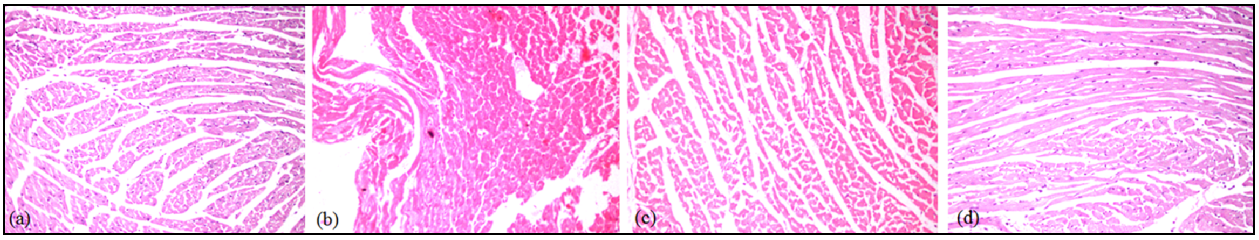

Figure-3: Photomicrograph of cardiac tissues (H&E 100x): (a) group-1 (control) with normal parenchyma (b) group 2 (DOX) with loss of nuclei, disarrayed myofibrils and interstitial oedema (c) group 4 (DOX+TMZ5d) with minimal myofibrillar disarrangement and scattered loss of nuclei (d) group-5 (DOX+TMZ10d) with restored normal parenchyma

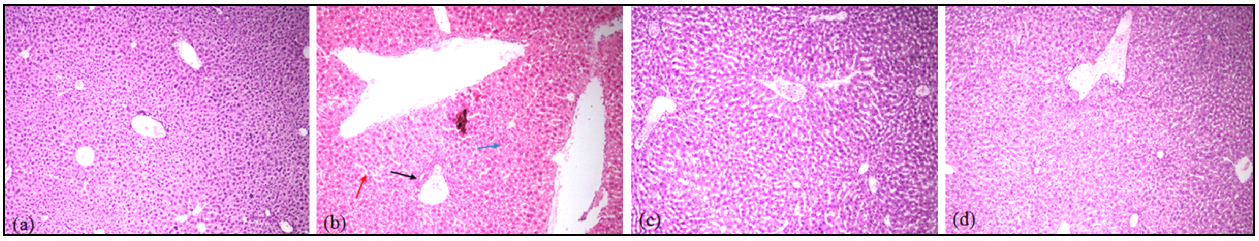

Figure-4: Photomicrograph of liver tissues (H&E 100x): (a) group-1 (control) with normal hepatic parenchyma (b) group-2 (DOX) with focal inflammation (blue arrow), portal inflammation (black arrow) and dilated sinusoids (red arrow) (c) group-4 (DOX+TMZ5d) with minimal focal inflammation and dilated sinusoids (d) group-5 (DOX+TMZ10d) with normal parenchyma

DISCUSSION

Considering the hazard of toxicities related to chemotherapeutic agents, it is very crucial to bring about pharmacological alterations in the prevalent chemotherapeutic regimens. Doxorubicin, despite its dose related toxicities, is extensively used due to excellent spectrum and outstanding efficacy. The only FDA approved drug to counter its cardiotoxicity is dexrazoxane, which is associated with difficult availability and administration.[4,22] There is no approved agent to counter other organ toxicities of this drug. The objective of this study was to test the efficacy of an agent which possesses multi organ protective potential in the face of toxic insult induced by DOX. Trimetazidine possesses potent anti-oxidant properties and also offers the desirable benefits of safety, cost effectiveness and convenience of administration. It's efficacy to counter cardiotoxicity as well as hepatotoxicity of DOX was assessed in two study designs conducted on balb/c mice, where TMZ was administered for three and eight days prior to DOX injection. Time of sampling and doses of drugs were selected after a thorough literature review and after conducting a preliminary project.

In group-2, a single dose of 10 mg/kg of DOX caused marked derangement in the cardiac and hepatic biomarkers reflecting the unusual ability of DOX to cause cellular damage. The elevation in levels of CK-MB and LDH was highly significant (*p*-value ≤0.0001) and has been observed in several other experimental studies.[23–25] The elevation in levels of ALT and AST was likewise highly significant (*p*-value ≤ .0001). The serum transaminases are sensitive markers of liver injury; ALT, especially is a highly specific diagnostic marker of liver parenchymal injury.[26,27] Such enzyme elevation has been depicted in various experimental studies.[7,27,28] Changes in the cardiac architecture were analysed to be moderate and

revealed the pathognomic lesions of disrupted myofibrillar architecture, loss of nuclei and vacuolization.[12,25,29] The changes in the architecture of liver did not correlate well with the enzyme rise and were scored 4 as per Ishak HAI depicting mild inflammation.

Heart has great propensity of damage by DOX due to a multitude of mechanisms; the altered redox status, formation of a cascade of reactive oxygen species (ROS), impaired homeostasis of intracellular iron, mitochondrial membrane swelling and adenosine triphosphate (ATP) depletion.[1,3,5,12] The ROS have the tendency to react with macromolecules causing damage to the membranes. Hence these underlying mechanisms caused the cardio specific cytosolic enzymes to be released into the circulation of the animals under study and be detected as the markers of DOX induced toxicity. As in case of heart, structural breakdown of the defence system by oxidative stress led to hepatocyte cell death and hence the leakage of liver cytosolic components which was reflected in the form of raised enzymes.[8,30]

In group 3, TMZ was administered alone to check if the drug itself had a potential of harm to the organs under study. In group 4, TMZ was administered for five consecutive days whereby DOX was administered on day three. In this group, the enzymatic changes were partially prevented as there was no significant attenuation of two of the four biochemical parameters, i.e., CK-MB and ALT (p-value>0.05). The levels for LDH and AST were, however, significantly reduced with p-values $\leq$0.05. This deficient reversion of damage was also reflected in the microscopic examination of both heart and liver. Many slides of heart from this group exhibited loss of nuclei and minimal myofibrillar oedema and disarrangement. Regarding the architecture of liver, slides still displayed focal inflammation and sinusoidal dilation. The results of this short design were contradictory to those obtained after three-day administration of TMZ by Salouege *et al.*[12] We therefore decided to extend the pre-treatment for at least seven days while keeping our study design analogous to the design of small study. Longer pre-treatment in group 5 reduced the enzyme upsurge for CK-MB, LDH and AST significantly with p-value of $\leq$0.0001 while the p value was calculated to be $\leq$0.05 for AST. In group 5, TMZ successfully prevented the hepatic and cardiac histopathological changes as most slides in this group revealed nearly normal parenchyma for both organs.

Trimetazidine is a widely prescribed antianginal agent with prominent anti-oxidant properties and cardioprotective benefits. Trimetazidine with its free radical scavenging ability decreases the production of ROS and minimizes their toxicity on cellular macromolecules such as membrane lipids preventing cell lysis.[17] It is reported that TMZ also forms some metabolites with antioxidant property when metabolized by the liver.[19] Currently, a multitude of experimental studies are evaluating its protective benefits for other organs in the face of genotoxic insult. Several routes, times, and doses of administration have been proposed but there prevails a degree of contradiction in the findings. For instance, it has been reported to improve cisplatin induced oxidative liver damage in experimental rats with ten days of administration.[16] Salouege *et al* evaluated its protective benefits after three days of administration against DOX induced damage.[12] In another experimental animal study of hepatotoxicity, two protective doses, i.e., 5 and 10 mg/kg of TMZ were analysed and definitive improvement in terms of biochemical levels and histopathological scores was seen at 10 mg/kg.[19] Boussaid *et al* stated that TMZ in a dose of 10 mg/kg per day for at least seven consecutive days was efficient in providing cellular and mitochondrial defense.[18]

The results of this study revealed that it is significantly beneficial to increase the number of pre-treatment days with TMZ to attain optimal hepatoprotection and cardio-protection against DOX induced insult.

CONCLUSION

Pre-treatment with trimetazidine is concluded to be an effective strategy to counter the cardiac and hepatic toxicity of the widely used anti-cancer drug doxorubicin. Trimetazidine is easily available, cost effective and is well tolerated orally. It is established that prior administration of trimetazidine for at least seven days conferred optimal cardio and hepatoprotection. Its inculcation in the widely practiced chemotherapeutic regimens of doxorubicin is an effective substitute to the costly dexrezozane and liposomal encapsulated forms.

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AUTHORS' CONTRIBUTION

AS: Literature search, conceptualization of study design, data collection, data analysis and interpretation, write-up. KF: Conceptualization of study design and data interpretation. ML: Data collection and analysis. AK: Conceptualization of study design. AA, KA: Proof reading

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