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Subchronic administration of mitoxantrone and the influence of enzyme inhibitors on its induced cardiotoxicity in mice: role of NRF-2/CYP2E1

P.M. EMEKA[1], H.I.M. IBRAHIM[2], I.A. ALHAIDER[1,3], M.A. MORSY[1,4], M.E. MOHAMED[1,5]

[1]Department of Pharmaceutical Sciences, College of Clinical Pharmacy, King Faisal University, Al-Ahsa, Saudi Arabia
[2]Department of Biological Sciences, College of Science, King Faisal University, Al-Ahsa, Saudi Arabia
[3]R&D, Saudi Food and Drug Authority, Riyadh, Saudi Arabia
[4]Department of Pharmacology, Faculty of Medicine, Minia University, El-Minia, Egypt
[5]Department of Pharmacognosy, College of Pharmacy, Zagazig University, Zagazig, Egypt

Abstract. – OBJECTIVE: Mitoxantrone (MTX)-induced cardiotoxicity is a clinical concern that is limiting its use. The aim of this paper, therefore, was to investigate the subchronic administration of MTX plus nonspecific/specific inhibitors of CYP450/2E1, to assess the extent of oxidative-induced injury by measuring levels of oxidative cardiac and injury biomarkers in mice and to evaluate the effects of CYP2E1 on caspase 3 activity and nuclear factor erythroid 2-related factor-2 (NRF-2).

MATERIALS AND METHODS: Mice (n = 32) were divided into four treatment groups of eight: control, MTX, MTX + 4-methlypyrazole (4MP) and MTX + disulfiram (Disf). After 6 weeks of treatments, blood and heart samples were collected.

RESULTS: Liquid chromatography-mass spectrometry (LCMS) analysis of MTX-treated plasma samples revealed several metabolites with different retention times. Cardiac antioxidant enzymes and creatine kinase (CK) levels were not significantly different among the groups. However, cardiac troponin and caspase 3 activity were significantly raised, with increased CYP2E1 expressions and reduced NRF-2 expression. Tissue damage was observed in all the treatment groups, including MTX, leading to the conclusion that MTX-induced cardiotoxicity was mediated by CYP2E1 activity, which initiated caspase 3 production, and decreased NRF-2 expression.

CONCLUSIONS: Therefore, agents that inhibit CPY2E1 expression might attenuate MTX-induced cardiotoxicity by increasing NRF-2 expression.

Key Words:
Cardiotoxicity, Mitoxantrone, Troponin, Caspase 3, NRF-2, CYP2E1.

Abbreviations

MTX = Mitoxantrone; 4-MP= 4-methylpyrazole; Disf= Disulfiram; CK = Creatine Kinase; LCMS= Liquid chromatography-mass spectrometry; NRF-2 = Nuclear factor erythroid 2-related factor 2; DNA = Deoxyribonucleic acid; RNA = Ribonucleic acid; GSH = Glutathione; PBS = Phosphate-buffered saline; ADP = Adenosine diphosphate.

Introduction

The risk of cardiotoxicity limits the use of certain drugs, particularly in chemotherapy[1], but the use of ineffective substitutes ends up prolonging treatment duration. In addition, adjusting dosages to counter adverse effects more often than not reduces steady-state drug concentrations and may even lead to drug resistance[2]. Due to negative limiting factors like cardiotoxicity[3], the decision to use effective agents has become a risk/benefit balancing act for patients who need these medications[4]. Among the culprits is mitoxantrone (MTX), an anthracenedione cytotoxic derivative used in cancer chemotherapy. This agent is used to treat solid tumours, breast cancer, acute leukaemia, prostate cancer and, recently, multiple sclerosis[5-7]. The mechanism of action of MTX includes DNA intercalation to prevent replication and RNA transcription[8]. MTX also inhibits the enzyme topoisomerase II, which can cause DNA breakage[9,10]. Despite claims that MTX is safe compared to other anthracycline analogues, studies in both human and animals show otherwise[9-12], but the mode of its toxicity is still controversial. Various reports[13] have advanced different mechanisms with result that no mitigating agent has been found. Based on three assumptions in that it is said to 1. Form complex with Fe (III), thereby initiating oxidative stress in the heart, but the use of chelators has not produced any clinically significant attenuation. 2. Causes apoptosis as well in cardiac tissues by way of activating different

Corresponding Author: Promise Madu Emeka, Ph.D; e-mail: pemeka@kfu.edu.sa

signalling pathways, which is reported as one of its antineoplastic activities. Therefore, countering this effect might compromise its anticancer activity. 3. MTX is bio-activated to toxic metabolite within the cardiac tissues which is reported to cause cardiotoxicity. MTX has also been reported to accumulate extensively in highly perfused organs, including the heart[14], which is very sensitive to oxidative stress because of its high metabolic activity; therefore, agents that induce the production of reactive oxygen species (ROS), may cause transient or permanent cardiac injury[12]. The mechanism of MTX cardiotoxicity is still evolving as some reports[3,11,15] claim that it induces apoptosis at a much lower concentration than doxorubicin does and that it induces necrosis in cumulative doses. As MTX cardiotoxicity is complex, further studies are needed to bring clarity to the conflicting reports in the literature[16]. Studies[16] have reported the ability of MTX to form reactive iron chelates that, in turn, create oxidative stress due to the generation of oxygen radicals. According to Wang et al[17] the drug has been shown to activate caspase 3, which leads to apoptosis. Accumulated evidence[15,18] shows that reactive oxygen radicals may signal apoptotic events because these two activities tend to modulate each other. MTX is activated by peroxidases that produce hydroperoxide which is linked with the alteration of mitochondrial calcium levels, causing apoptosis[19,20]. In addition, its biotransformation to an active metabolite, naphtoquinonoxaline, as described by Rossato et al[7], has been implicated in chemotherapeutic action as well as cardiotoxicity[21,22]. To elucidate the role of MTX metabolites in cardiotoxicity, some studies[23,24] employed inhibitors of CYP450 enzymes using cell-lines, isolated cardiac myocytes/tissues, while Rossato et al[7], measured MTX metabolites after a single-dose administration. Their study suggested that MTX is a substrate for CYP2E1 and can bio-activate this enzyme. Therefore, the issue of safety cannot be overemphasized as MTX now has a broad application in cancer chemotherapy[6].

Beside CYP2E1 metabolizing function, its increased expression has been linked to lipid peroxidation in liver tissues[25,26]. Evidence[26] shows that a decrease in CYP2E1 activation has been associated with a decreased drug-induced liver injury. On the other hand, reports[25] suggest that transcription factor nuclear factor erythroid 2-related factor-2 (NFR-2) plays a crucial role in protecting the cells against increased oxidative stress caused by the induction of CYP2E1 enzymes in the HepG2 cells. It is also known to regulate large numbers of cytoprotective enzyme expressions during oxidative stress[27]. They include antioxidant, anti-inflammatory as well as metabolic enzymes involved in redox homeostasis of cell tissues[27]. The present study is focused on investigating whether the subchronic administration of MTX induces apoptosis, necrosis or both, to determine a beneficial treatment model. Specifically, we (1), investigated the role of the subchronic administration of MTX and the influence of CYP450/2E1 inhibitors on induced cardiotoxicity in mice[28]; (2) examined levels of cardiac antioxidants and cytotoxin markers in different treatment groups for a link to cardiac injury mechanisms; (3) ascertained if the mode of injury were mediated via the stimulation of apoptotic pathways or due to injury. Cardiac injury and apoptosis were evaluated by measuring cardiac troponin and caspase 3 activities, while cardiac oxidative stress was assessed by determining the levels of cardiac antioxidants and myocardial injury markers along with histopathological examinations. (4) Examination of NRF-2 and CYP2E1 protein expression in relation to apoptotic biomarkers were also carried out.

Materials and Methods

Chemicals, Assay Kits, and Antibodies

Mitoxantrone (MTX) dihydrochloride was supplied by Ebewe Pharma GmbH. KG. Disulfiram and 4-methylpyrazole were from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). Assay kits for lipid peroxidation, glutathione (GSH), catalase, caspase 3 and creatine kinase (CK) were purchased from Biovision Inc. (Milpitas, CA, USA). Cardiac troponin (MyBioSource, San Diego, CA, USA). Primary antibodies against NRF-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gels for Western blotting were procured from Bio-Rad (Hercules, CA, USA).

Experimental Animals

A total of 32 (male and female) BALB (Bagg Albino)/c mice with an average weight of 25 g, were obtained from the Department of Biological Sciences, College of Science, King Faisal University (Saudi Arabia). The mice were maintained in groups of eight per cage in controlled environmental conditions, according to the specified standards, with 12 h dark and 12 h light cycle, at 23 ± 1°C. They were allowed access to food and water

throughout the study. Animal care and experimental procedures were carried out according to the guidelines of the Research Ethics Committee at King Faisal University (KFU-REC-/2021-06-08) and that of the National Committee of Bioethics (NCBE), King Abdulaziz City for Science and Technology (KACST), Saudi Arabia.

Experimental Protocol

The mice were divided into 4 (four) treatment groups of 8 mice per group. Animals were treated once per week for 6 weeks. Group 1, the untreated control, was given 0.2 ml of phosphate buffer (PB) solution. Group II was administered with 3 mg/kg MTX[29]. Group III was treated with CYP2E1 inhibitor 25 mg/kg 4-methylpyrazole (4-MP), and 48 h later was administered with MTX. Group IV was then given CYP450/2E1 non-specific inhibitor, 300 mg/kg disulfiram (Disf) and then MTX 48 h later. All mice were dosed once a week for 6 weeks with monitoring. All administration was done *via* intraperitoneal route. Animals were monitored for signs of piloerection, hemorrhage from injection site, diarrhea, movement coordination, gasping of breath, fur colour changes and writhing as a sign of pain. At the end of the study, mice were euthanized by cervical dislocation under ether anaesthesia. Ether was administered using "open-drop" method with ether-impregnated cotton ball in a bell jar for induction followed by inhalation *via* a simple face cone. As stipulated by institutional guidelines for the use of animals, we used about five drops corresponding to 3-4 ml. The blood and heart samples were harvested at the same time. The heart samples were thoroughly rinsed in phosphate-buffered saline (PBS) to remove excess blood and then weighed before being stored at -85°C for analysis. The collected samples were used to estimate biochemical markers of cardiac antioxidants such as lipid peroxidation, GSH and catalase. Additionally, the cardiac injury markers CK, cardiac troponin and caspase 3 activity were measured. Western blotting was performed to analyze the expressions of CYP2E1 and NRF-2.

LCMS Method

A qualitative analysis of MTX and its metabolites in mice plasma, as previously reported by Brück and Brück[30], was adopted with modification. However, the present analysis was performed with LCMS/MS Agilent 6200 series TOF/6500 version Q-TOF B.06.01 (B6157). This was coupled with Agilent G6545A MS Q-TOF with a dual jet stream electrospray (ESI). LCMS was performed with voltage capacity of 3500 V and a source temperature was 350°C. A 5-10 min LC method with a flow rate of 0.3 ml/min was used for separation. The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) and performed according to Zheng et al[31].

Measurement of Myocardial Oxidative Stress Markers

Harvested heart tissues were homogenized in PBS in a ratio of 1:10 (w/v), and then, centrifuged at 13,000 g (4°C) for 30 min, after which the supernatant was collected for assay. Concentrations of MDA as a marker for lipid peroxidation was determined by measuring the levels of thiobarbituric acid-reactive substances, as described by Ohakawa et al[32]. A phosphoric acid solution (1%) and a known volume of thiobarbituric acid were added to each homogenate sample, and then, incubated at 95°C for 1 h. The concentration of the subsequent thiobarbituric acid-reactive substances was then measured spectrophotometrically at 535 nm. Catalase activity was determined spectrophotometrically according to the manufacturer's instructions. Essentially, in the assay, the catalase first reacts with H_2O_2 to produce water and oxygen; unconverted H_2O_2 reacts with an OxiRed™ probe to produce a product that is then measured at 570 nm. GSH levels were determined in accordance with the instructions of the manufacturer. Briefly, it is a colorimetric reaction of 5,5'-dithiobis (2-nitrobenzoic acid). Heart tissue levels of GSH were measured at 412 nm using a standard curve plot.

Measurement of Myocardial Enzyme Activity

CK activity was evaluated also in accordance with manufacturers' procedures. CK converts phosphoric acid into phosphocreatine and ADP, which reacts with a CK enzyme mix to form an intermediate, which reduces a colourless probe to a coloured product measured at 450 nm.

Measurement of Cardiac Roponin-I

Cardiac troponin-I (cTnI) levels in samples were assayed by Enzyme Linked-Immuno-Sorbent Assay (ELISA) kit, according to the manufacturer's instructions (Shanghai Crystal Day Biotech Co., LTD, China).

Measurement of Caspase 3 Activity

Caspase 3 activity was measured using a colorimetric assay, according to the manufacturer's

instructions. The heart tissues were homogenized in 3 ml of 10 mM phosphate buffer (pH 7.4). Detection of caspase 3 activity was predicated on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide by caspase 3. The reaction led to the consequent release of the p-nitroaniline component, which was then measured spectrophotometrically at 405 nm.

Histological Examination

The heart tissue was fixed in 10% formaldehyde, sampled and embedded in paraffin for light microscopic evaluation. Tissues were sectioned and stained with haematoxylin and eosin (H and E) and prepared slides were examined by a pathologist who was unaware of the treatment regimens. Histopathology scores were evaluated using modified grading described by Billingham et al[33]. A comparison between treatment groups and control were as follows: 0 = no pathology, 1 = mild pathology, 2 = moderate pathology, 3 = severe pathology.

Western Blotting Analysis

The protein expressions of NRF-2 and CYP2E1 in the heart tissues were determined through Western blotting using specific antibodies, according to the method described by Ahn et al[34]. The harvested tissues were homogenized with radioimmunoprecipitation assay (RIPA) lysis buffer to isolate and extract the content of the whole protein. The nuclear extracts were isolated and used for to determine NRF-2/CYP2E1 expressions. Approximately 50 µg of protein samples were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. These were later transferred on to polyvinylidene difluoride (PVDF) membranes, and 5% non-fat dried milk was used to block the non-specific sites at 20-22°C for 1 h. This was followed by washing the membranes with Tris-buffered saline and 0.1% Tween 20 (TBST). The membranes were then incubated overnight at 4°C with primary mouse monoclonal antibodies against NRF-2/CYP2E1 in a ratio of 1:1000. Thereafter, the membranes were washed with TBST, and incubated with secondary peroxidase-conjugated goat anti-mouse IgG in a ratio of 1:5000 for 1 h. The resulting bands were visualized according to the manufacturer's instructions, using an enhanced chemiluminescence system. ImageJ software (freeware; rsbweb.nih.gov/ij) was used to perform the densitometric analyses of the bands.

Statistical Analysis

The data generated are expressed as mean ± SD and were analyzed using the GraphPad Prism software version 8.2 (La Jolla, CA, USA). Two sample Student's *t*-tests between percent were used to compare the control and treated groups' percent mortality rates. Comparisons between control and treatment groups were made using one-way analysis of variance, and the differences between the groups were measured using Tukey's multiple comparisons test. Statistical significance was considered at $p < 0.05$.

Results

Mortality Rates after 6-Weeks Subchronic Administration

Table I describes the mortality rates in different treatment groups compared to control after 6 weeks of subchronic MTX, MTX + 4-MP and MTX + Disf administration. Results showed that rates were higher during the sixth week in all groups. However, mortality started being observed from the fifth week only for MTX and MTX + Disf (12.5% each) compared to control. All observed mortality was significant ($p < 0.05$).

In Table II we present the final body, absolute organ and relative organ weights. The table indicates that relative organ weights in all the treatment groups and control did not show any significant difference. However, the body weights showed significant difference compared to control.

LCMS Analysis of Standard and Plasma Samples of MTX, MTX + 4-MP and MTX + Disf

LCMS qualitative analysis of MTX standard compared to plasma samples of treated groups is presented in Figure 1. Photomicrograph A represents the LCMS tracing of an MTX standard sample showing a peak at retention time 6.134 min with a molecular weight of 445.2082. Photomicrograph B represents LCMS tracing of a plasma sample of mice treated with MTX alone showing different retention times of 6.6, 6.7, 6.8, 7.5, 8.6 and 9.3 min, indicating the presence of different metabolites. Photomicrograph C, is the tracing of a plasma sample of the MTX + 4-MP treated group also showing different peaks with retention times of 6.1, 6.3, 8.5, 9.2 and 9.6 min. This also suggests that other enzymes might be at play considering the number of peaks/metabolites observed in plasma sample tracings.

Photomicrograph D represents traces of MTX + Disf plasma sample with different peaks displaying retention times of 6.8, 8.6, 9.0, 9.6 and

Table I. Percentage of mortality among different treated groups (MTX, MTX + 4-MP and MTX + Disf) after 6-weeks of dosing [N (%)].

Groups/	Weeks of drug treatments					
	1st	2nd	3rd	4th	5th	6th
Control	8 (0)	8 (0)	8 (0)	8 (0)	8 (0)	8 (0)
MTX	8 (0)	8 (0)	8 (0)	8 (0)	7 (12.5)	6 (25)#
MTX +4MP	8 (0)	8 (0)	8 (0)	8 (0)	8 (0)	6 (25)#
MTX +Disf	8 (0)	8 (0)	8 (0)	8 (0)	7 (12.5)	6(25)#

Mortality at the sixth week was 25% for MTX, MTX + 4-MP, and MTX + Disf. Using two sample Student's t-tests between percent and comparing the control and treated groups % mortality, they all showed significant a difference ($^{\#}p<0.05$) when compared with control. MTX= mitoxantrone, 4-mp= 4-methylpyrazole, Disf= disulfiram.

Table II. Final weight and relative organ weights after subchronic administration of MTX, MTX + 4-MP and MTX + Disf.

Treatment groups	Body weight (gm)	Absolute organ weight (gm)	Relative organ weight (%)
Control	36.3±2.35	0.177± 0.006	0.48± 0.02
MTX	25.8± 3.38 *	0.123 ± 0.02	0.48 ± 0.06
MTX + 4-MP	26.17 ± 3.20 *	0.133 ± 0.035	0.51 ± 0.08
MTX + Disf	27.8 ± 2.39 *	0.12 ± 0.02	0.43 ± 0.052

Body weights of all the treated groups were significantly different ($p < 0.001$) compared to control. Relative organ weights were calculated as the percentage of absolute organ weight/final body weight. There were no significant differences between control and treated groups, with p-values as 0.9999, 0.6944, 0.3239, respectively, for MTX = mitoxantrone; 4-MP = 4-methylpyrazole; Disf = disulfiram.

9.8 min. It suggests the presence of metabolites, as Disf is a non-specific enzyme inhibitor. It may also suggest that the metabolism of MTX was not completely abolished.

Biochemical Cardiac Antioxidant Biomarkers Parameters (Catalase and GSH) and Lipid Peroxidation

In Figure 2, we present lipid peroxidation and cardiac antioxidant markers (A-C). A represents lipid peroxidation measured as malondialdehyde (MDA) levels in homogenized cardiac tissue. The results showed no significant changes in mice treated with MTX alone (12.05 ± 1.9 nmol/mg protein) compared to control (10.1 ± 1.2 nmol/mg protein). The same trend was observed with MTX + 4-MP (13.1 ± 2.1 nmol/mg protein). However, treatment with MTX + Disf produced a significant ($p < 0.05$) increase in lipid peroxidation (14.6 ± 1.3 2 nmol/mg protein). B represents catalase activities measured in all the treatment groups, including control. It revealed no changes in levels of catalase activities when compared to control (7.3 ± 0.6 units/mg protein). Treatment with MTX + Disf showed the highest value of 8.8 ± 2.1 units/mg protein but was not statistically significant compared to control. C shows GSH activities in cardiac tissue treated with MTX and MTX + 4-MP and MTX + Disf for 6 weeks. Treatment with MTX and MTX + 4-MP did not produce significant changes in GSH levels (26.4 ± 2.2, 29.9 ± 2.8 nmol/mg protein) compared to control (25.12 ± 1.87 nmol/mg protein). However, our results here revealed that only the treatment with MTX + Disf produced a significant ($p < 0.05$) increase in GSH levels (32.3 ± 3.62 nmol/mg protein) compared to control. Therefore, there was no significant effect on cardiac oxidative biomarkers by MTX alone.

Cardiac Injury Biomarkers (CK, Troponin and Caspase 3)

Figure 3 shows the levels of cardiac injury biomarkers measured in mice heart tissues after 6 weeks of subchronic administration of MTX and MTX + 4-MP and MTX + Disf compared to control. Figure 3A shows levels of CK in all treatment groups, indicating that MTX did not produce any significant increase in levels of CK compared to control (179.9 ± 23.3 and 160 ± 2.82 nmol/min/mg, respectively). Although, treatment groups of MTX + 4-MP and MTX + Disf did produce higher values, (217.5 ± 19.1 and 199.5 ± 29.0 nmol/min/mg) compared to MTX alone, only the MTX + 4-MP difference was significant ($p < 0.05$).

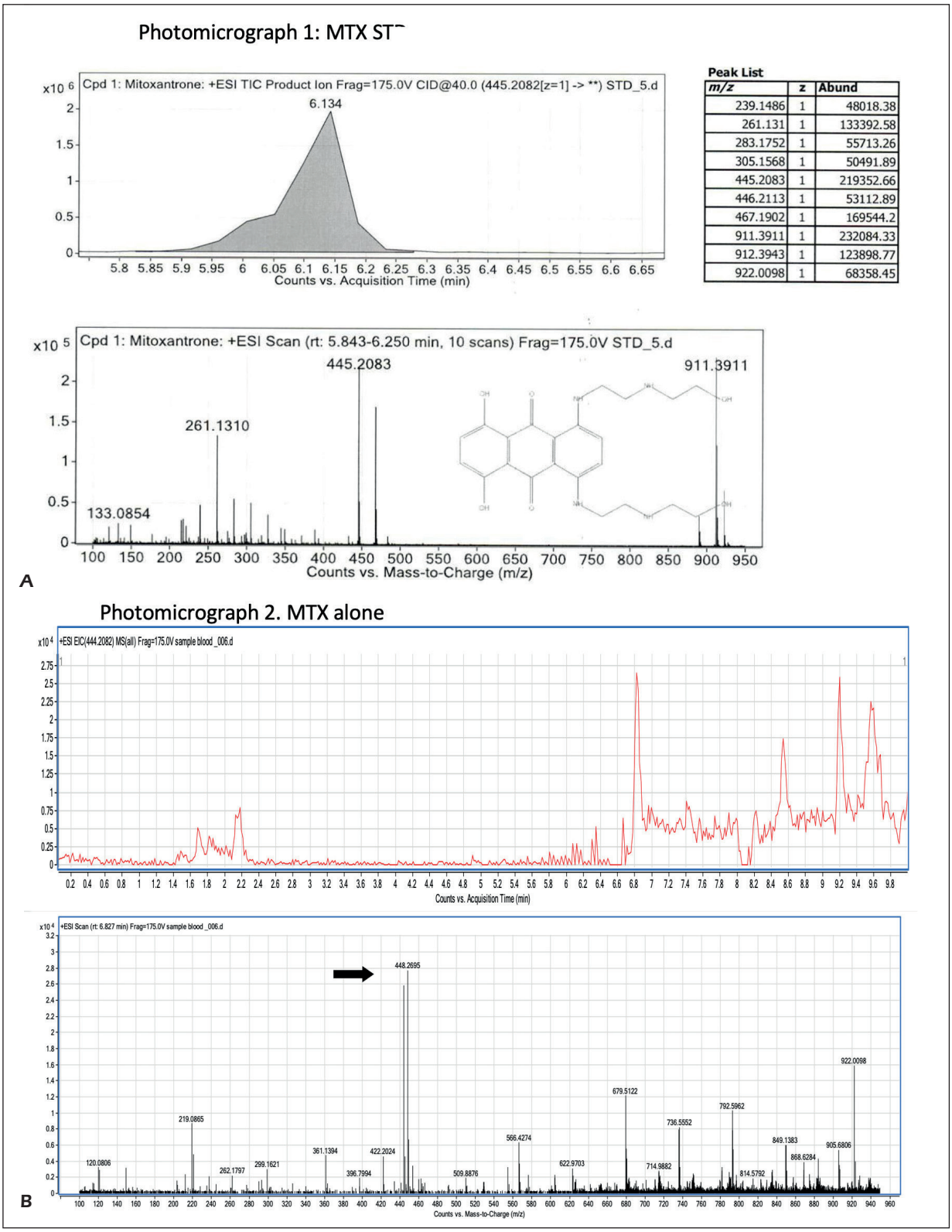

Figure 1. LCMS tracings showing MTX standard drug, MTX alone, MTX + 4-MP and MTX + Disf. Photomicrographs **(A-D)** of LCMS tracings showing MTX standard drug, MTX alone, MTX + 4-MP and MTX + Disf. Showing LCMS photomicrograph for MTX standard with MTX, MTX + 4-MP and MTX + Disf treated groups' plasma samples after 6 weeks of subchronic administration. **A**, MTX standard showing Molecular weight (MW) 445.2083, at retention time 6.134 min. **B**, MTX plasma sample with multiple metabolites as peaks, also showing different retention times.

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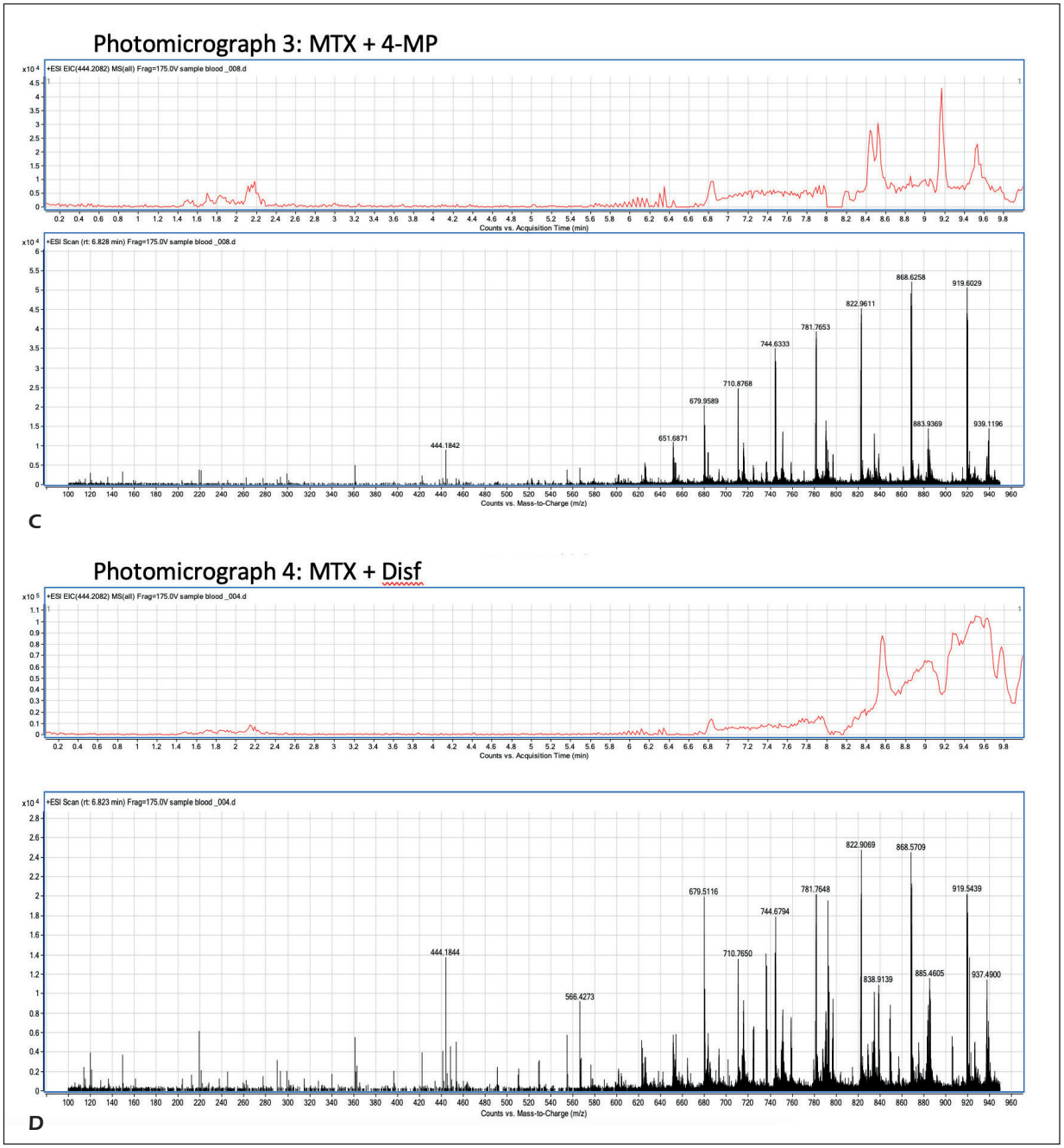

Figure 1 ***(Continued).*** **C**, MTX + 4-MP plasma samples with 6.1, 6.3, 8.5, 9.2 and 9.6 mins peaks/retention times. **D**, MTX + Disf plasma samples with different peaks with retention times of 6.8, 8.6, 9.0, 9.6 and 9.8 min. MTX=mitoxantrone; 4-MP= 4-methylpyrazole; Disf= disulfiram.

In Figure 3B, all treated groups significantly ($p < 0.001$) increased troponin levels with 13.5 ± 0.5, 12.3 ± 0.70, 13.35 ± 0.35 pg/ml respectively for MTX, MTX + 4-MP and MTX + Disf compared to the control. Figure 3C represents caspase 3 expression in all the treated groups. Our results showed that MTX-treated group significantly ($p < 0.001$) increased caspase 3 activity (3.0 ± 0.14) compared to control (1.1 ± 0.14). Comparing the MTX group with the MTX + 4-MP and MTX + Disf groups for caspase expression, we found that MTX had a better profile in increasing caspase 3 activity.

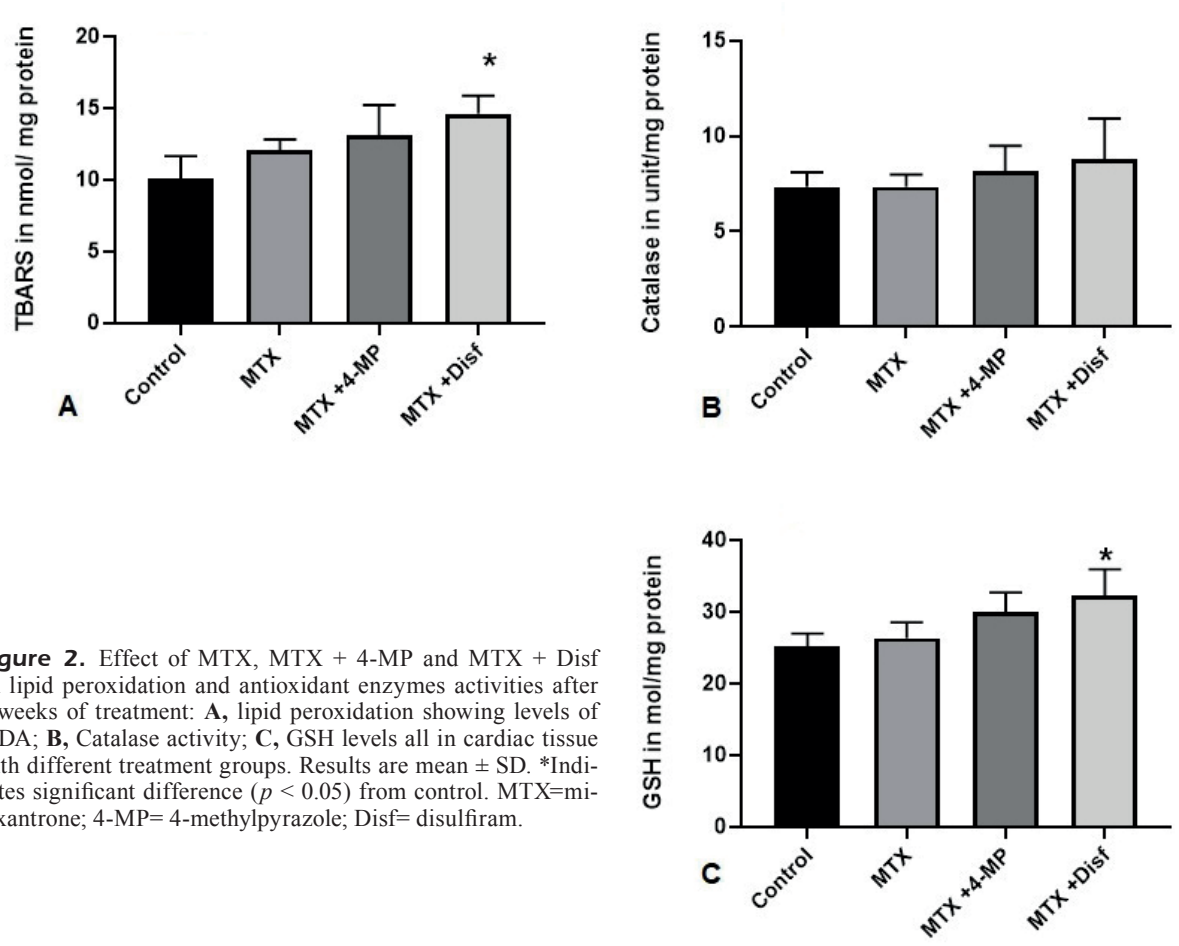

Figure 2. Effect of MTX, MTX + 4-MP and MTX + Disf on lipid peroxidation and antioxidant enzymes activities after 6 weeks of treatment: **A,** lipid peroxidation showing levels of MDA; **B,** Catalase activity; **C,** GSH levels all in cardiac tissue with different treatment groups. Results are mean ± SD. *Indicates significant difference ($p < 0.05$) from control. MTX=mitoxantrone; 4-MP= 4-methylpyrazole; Disf= disulfiram.

Western Blotting Protein Expressions of NRF-2 and CYP2E1

Figure 4A shows protein expressions of NRF-2 in different treatment groups compared to control. In the MTX treated group, NRF-2 expression was significantly ($p < 0.001$) reduced compared to the control. In addition, treatments with MTX + 4-MP and MTX + Disf also produced marked ($p < 0.001$) reduction in NRF-2 protein expression. Comparing treatment groups with MTX group, the MTX + 4-MP treatment showed a marked decrease ($p < 0.001$) in NRF-2 expression but MTX + Disf produced no significant difference. NRF-2 is known to regulate cytoprotective genes in cells, thereby reducing cellular injury. Figure 4B shows that MTX treatments significantly ($p < 0.001$) increased CYP2E1 protein expression, suggesting that the presence of MTX might have enhanced the activity of CYP2E1. It also confirms that MTX is an inducer as well as a substrate. MTX + 4-MP also comparatively increased ($p < 0.001$) the expression of CYP2E1 with respect to the control. However, the result showed no statistical difference compared to MTX alone, but MTX + Disf treatment displayed a significant ($p < 0.001$) decrease with respect to MTX treatment. This suggests an interplay between NRF-2 and CYP2E1: as one increases, the other decreases in a similar fashion.

Mice Heart Tissue Histological Changes After 6 Weeks of Subchronic Administration of MTX, MTX + 4-MP and MTX + Disf

As shown in Figure 5, A represents the control group with normal histoarchitecture of the heart with no visible pathology. B represents the MTX treatment group with red blood cell infiltration, intimal fibrosis and parenchymal infiltration of neutrophils. C is autopsied heart tissue treated with MTX showing intimal fibrosis, inflammation of cardiac tissue, red blood cell infiltration and parenchymal infiltration of neutrophils. D represents MTX + 4-MP treated heart tissue showing inflammation of cardiac tissue, red blood cells infiltration, intimal fibrosis, distorted and wavy

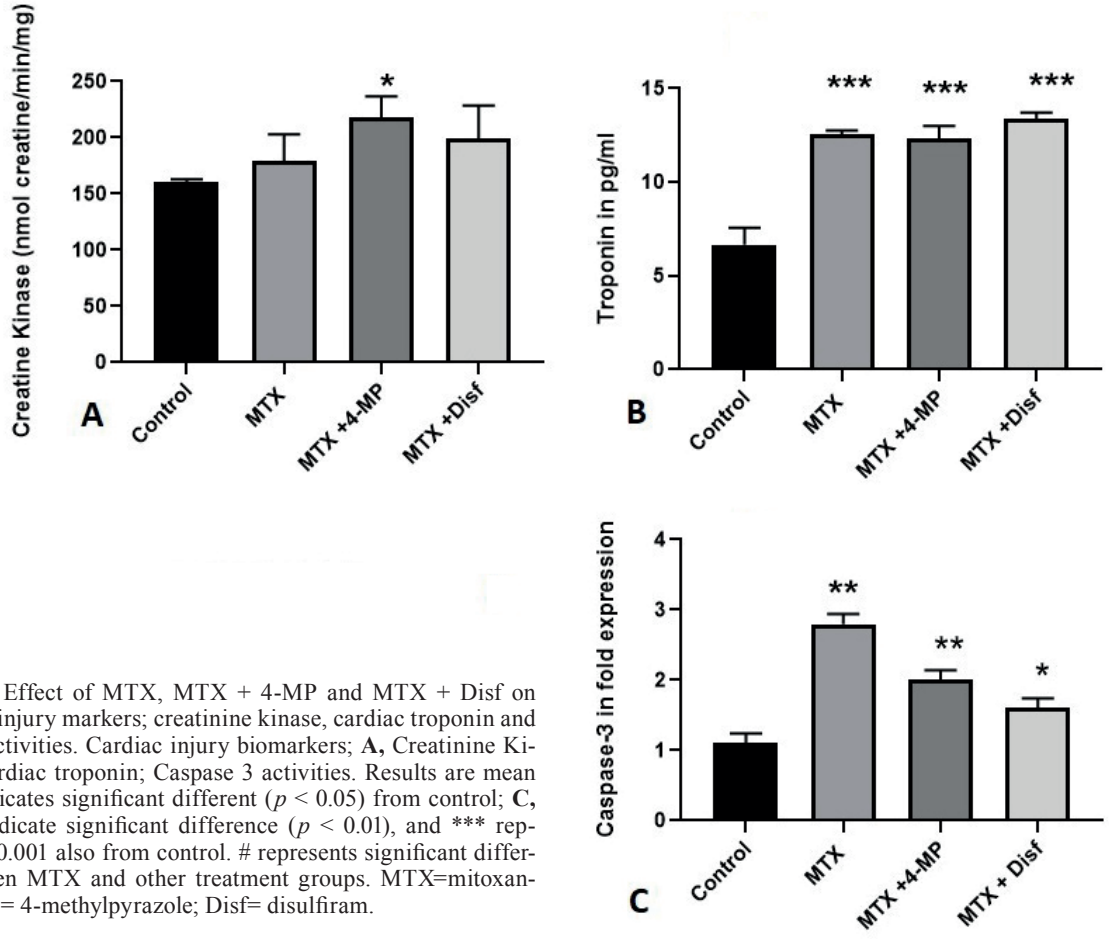

Figure 3. Effect of MTX, MTX + 4-MP and MTX + Disf on myocardial injury markers; creatinine kinase, cardiac troponin and caspase 3 activities. Cardiac injury biomarkers; **A,** Creatinine Kinase; **B,** Cardiac troponin; Caspase 3 activities. Results are mean ± SD. * Indicates significant different ($p < 0.05$) from control; **C,** while ** indicate significant difference ($p < 0.01$), and *** represents $p < 0.001$ also from control. # represents significant difference between MTX and other treatment groups. MTX=mitoxantrone; 4-MP= 4-methylpyrazole; Disf= disulfiram.

myocardial fibres with myofibril loss and parenchymal infiltration of neutrophils. E is a photomicrograph of autopsied heart tissue treated with MTX + 4-MP showing intimal fibrosis, distorted and wavy myocardial fibres and myofibrillar loss and red blood cells infiltration. F is MTX + Disf treatments showing wavy myocardial fibres, intimal fibrosis, inflammation of cardiac tissue and parenchymal infiltration of neutrophils. G shows autopsied heart tissue treated with MTX + Disf with vacuolar degeneration/mycotic apoptosis and red blood cell infiltration.

Figure 6 is a heat-map of histopathology scores displaying levels of observed pathology by different treatment groups using Billingham's scores as previously indicated in the methodology. The MTX-treated group had lower scores compared to the other treatment groups. Autopsied heart tissues from all treatment groups had higher scores, particularly for intimal fibrosis, parenchymal infiltration of neutrophils and distorted and wavy myocardial fibers, which leads to mycotic apoptosis (Figure 6).

Discussion

Induced cardiotoxicity associated with the use of chemotherapeutic agents has become a serious concern and important adverse effect to be considered by clinicians. In this regard, the induction of cardiotoxicity by these agents is of clinical significance because it limits their use[35]. MTX was apparently developed to replace doxorubicin, which has accompanying cardiotoxicity[36]. However, cases of cardiotoxicity have been reported in about 18% of patients receiving MTX[7]. The present study revealed that prolonged use of MTX is deleterious to the heart as documented clinical evidence shows cumulative and delayed cardiotoxicity[8,37]. MTX is reportedly metabolized by the CYP450/2E1 enzyme system, which is abundant in the heart and, according to numerous reports, its metabolites produce reactive intermediates[7,21,39]. According to the report of Rossato et al[7], they suggested that metabolism of MTX contributes to its pharmacological action, and the bio-

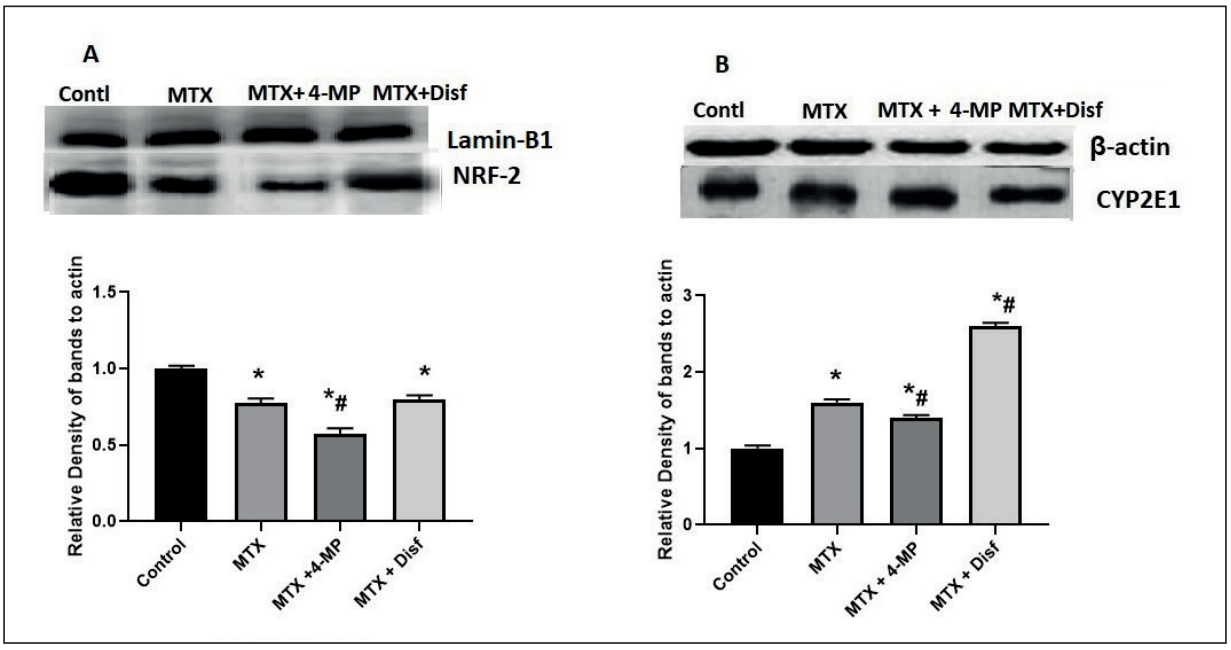

Figure 4. Effect of MTX, MTX + Disf and MTX + 4-MP on nuclear factor erythroid 2-related factor 2 (NRF-2) and CYP2E1 protein expressions. Results are represented as mean ±SD. *Represents significant difference ($p < 0.001$) between the control and treatment groups. While # represents significant difference between MTX treated and MTX +4-MP, MTX + Disf. MTX-=mitoxantrone, 4-MP= methylpyrazole, Disf = disulfiram.

transformation fate of MTX might also be involved in its cardiotoxicity. However, other reports state that it arguments the therapeutic efficacy of MTX[40]. As MTX is a very useful anticancer agent against various tumour types[7,36]. Numerous studies have, therefore, been undertaken to evaluate the mechanism of MTX-induced cardiotoxicity. In that regard, the present study examined the effects of subchronic MTX administration together with specific (4-MP) and non-specific enzyme inhibitors (Disf) in mice heart tissue *in vivo*. This is with a view to assess (1) the role of MTX alone on the heart over 6 weeks to confirm cardiotoxic effects by using the enzyme inhibitors (CYP450/2E1), the metabolism of which MTX induces[41]; (2) if MTX induces necrotic- or apoptotic-related pathways that participate in cardiac injury; and (3) pathways like NRF-2 and CYP2E1, which are known to play important protective or oxido-apoptotic roles. Although it is documented that 4-MP is a specific CYP2E1 enzyme inhibitor[28]. The present study could not confirm this, as the results showed peaks with retention times in its combination with MTX. Therefore, other enzymes might be involved in MTX metabolism. Since MTX accumulates in tissue and the elimination half-life of 4-MP is 50 h, there might be a delayed MTX effect [42]. Affect 6 weeks, a 25% mortality rate in the MTX, and other treatment groups was observed. This observation could suggest that mortality rates in groups other than MTX alone might be attributable to presence of MTX as well as its metabolites. In addition, relative organ weights did not change significantly in any treatment group compared to control, indicating that the hearts were not enlarged. Our findings, however, were contrary to the report of Armenian and Ehrhardt[43], who did observe an enlarged heart. Therefore, the present study suggests a different mechanism of MTX cardiotoxicity. From the foregoing, LCMS qualitative analysis of MTX plasma samples gave several peaks with different retention times, indicating the presence of several metabolites, which reports indicated might be responsible for MTX cardiotoxicity. MTX + 4-MP and MTX + Disf LCMS tracing also showed metabolites, and this suggested that the level of cardiotoxicity observed in this study might be due to both MTX and metabolites. Studies showed that Disf, a non-specific enzyme inhibitor, can inhibit the CYP450 isoenzyme systems, and this may have produced the MTX +Disf metabolites[44]. There is also the possibility that MTX might be metabolized by other isoenzymes non-specifically. Evidence for this came from the LCMS qualitative analysis, which showed differ-

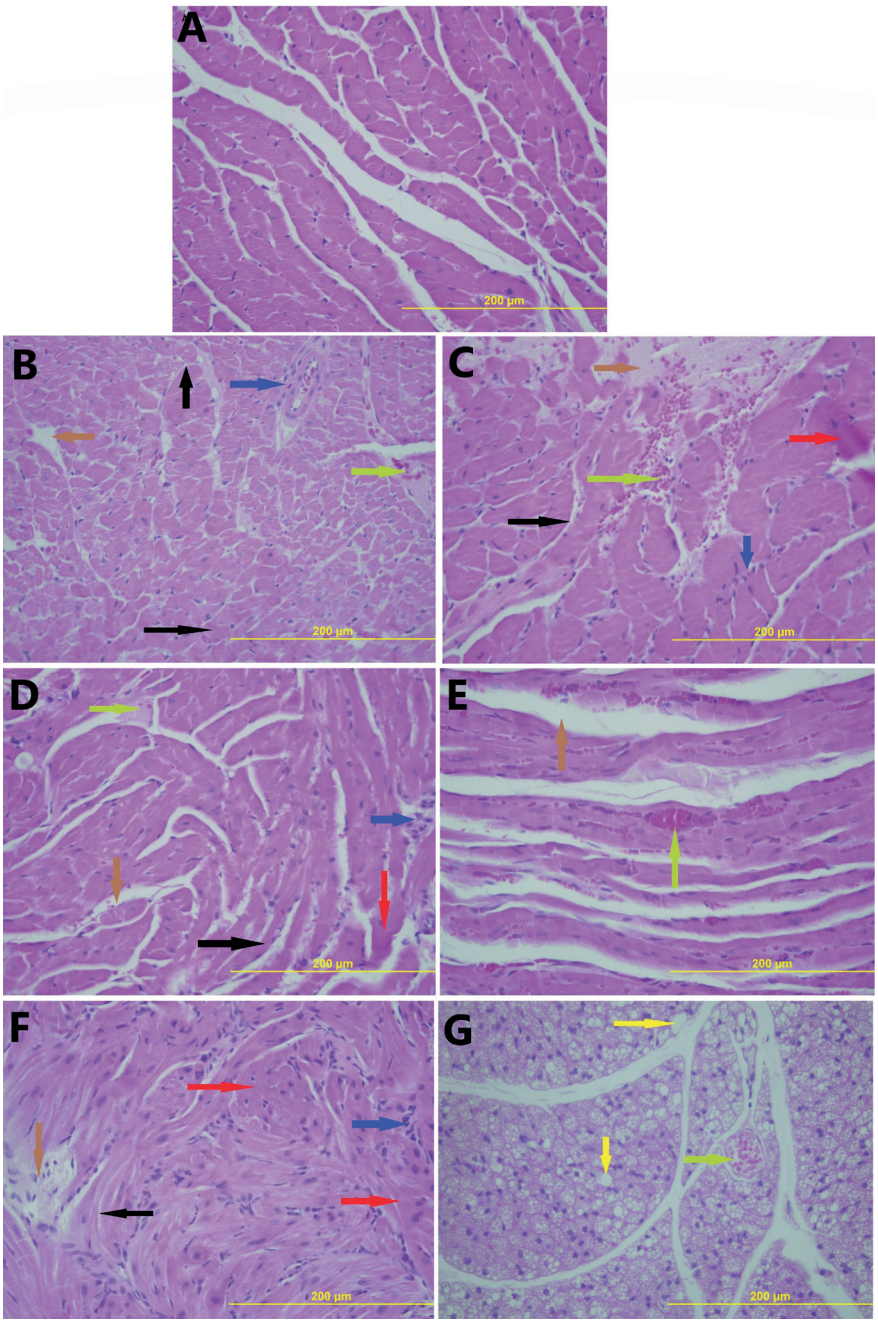

Figure 5. Histopathological analysis of heart tissues after treatments with MTX, MTX + 4-MP and MTX + Disc **(A–D**: H and E × 40). Histopathological changes in heart tissue; **(A)** control: showed normal histoarchitecture of the heart with no visible vacuolar degeneration or lesion; **(B)** MTX treated, showing red blood cells infiltration (green arrow-↑), inflammation of cardiac tissue (red arrow-↑) and blue arrow (↑) showing parenchymal infiltration of neutrophils; **(C)** Autopsied heart tissue treated with MTX showing intimal fibrosis (brown arrow-↑), inflammation of cardiac tissue (red arrow-↑), red blood cells infiltration (green arrow-↑) and parenchymal infiltration of neutrophils (blue arrow -↑); **(D)** MTX + 4-MP treated heart tissue showing inflammation of cardiac tissue (red arrow-↑), intimal fibrosis (brown arrow-↑), distorted and wavy myocardial fibres and myofibrillar loss (black arrow- ↑) and parenchymal infiltration of neutrophils (blue arrow -↑); **(E)** Autopsied heart tissue treated with MTX + 4-MP showing intimal fibrosis (brown arrow-↑), distorted and wavy myocardial fibres and myofibrillar loss (black arrow- ↑), inflammation of cardiac tissue (red arrow-↑), red blood cells infiltration (green arrow-↑) and parenchymal infiltration of neutrophils (blue arrow -↑); **(F)** MTX + Disf treatment showing wavy myocardial fibres (black arrow- ↑), intimal fibrosis (brown arrow-↑), inflammation of cardiac tissue (red arrow-↑) and parenchymal infiltration of neutrophils (blue arrow -↑); **(G)** Autopsied heart tissue treated MTX + Disf showing vacuolar degeneration/mycotic apoptosis (yellow arrow-↑) and red blood cell infiltration (green arrow-↑). MTX = mitoxantrone; 4-MP = 4-methylpyrazole; Disf= disulfiram.

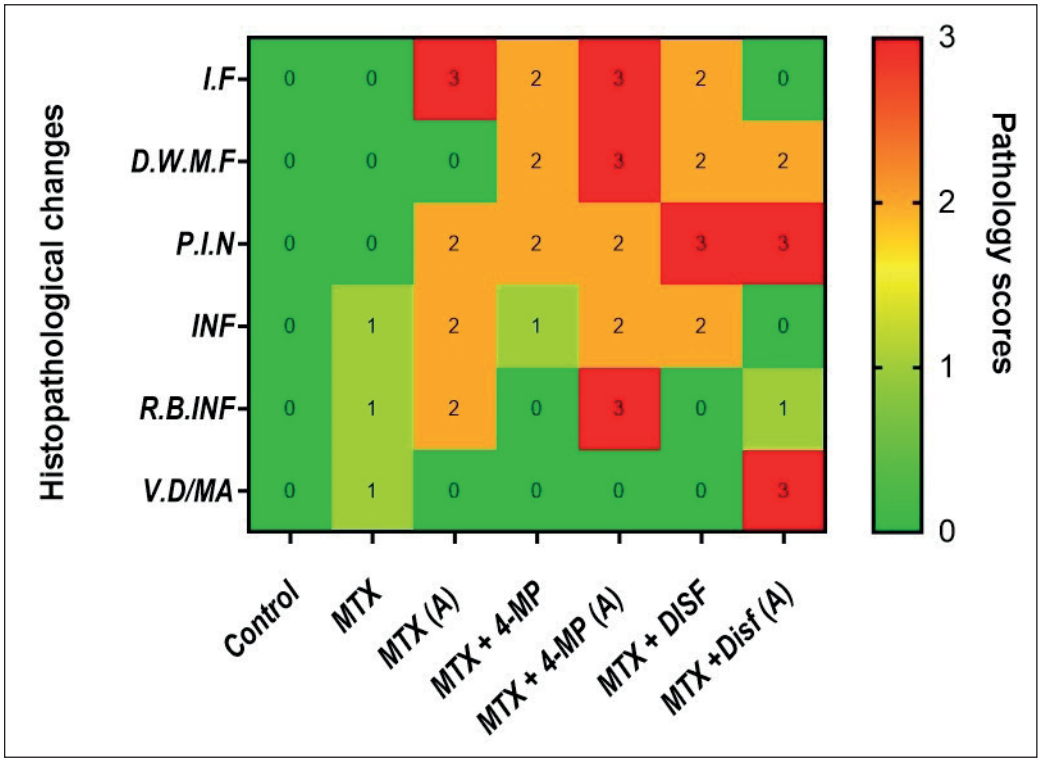

Figure 6. Heat map of cardiac histopathological changes induced by MTX, MTX + 4-MP and MTX + Disf after subchronic treatment evaluation. Histopathology score = 0 no pathology, 1 = mild pathology, 2 = moderate pathology, 3 = severe pathology. I.F= Intimal fibrosis; P.I.N=Parenchymal infiltration of neutrophils; D.W.M.F= Distorted and wavy myocardial fibres; RBC INF=Red blood cells infiltration; INFV.D/MA = Vacuolar degeneration/Mycotic apoptosis. MTX = mitoxantrone; MTX (A) = autopsied; 4-MP = 4-methylpyrazole, MTX +4-MP (A) autopsied; Disf = disulfiram, MTX + Disf (A) = autopsied.

ent metabolites from different treatment groups. Therefore, their potential for toxicity needs to be further evaluated. Studies reflect differing opinions about the ability of MTX and its metabolites to induce oxidative stress as a mechanism for cardiac toxicity[12,45,46]. The present study revealed that MTX and its associated metabolites from the different treatment groups did not significantly affect cardiac antioxidative enzyme activity. This is seen with lipid peroxidation, catalase and GSH levels which were not altered compared to control. These results were consistent with the studies of Reis-Mendes et al[45], Vile and Winterbourn[47] and Dores-Sousa et al[48], However, Kadikoylu et al[12] documented increased GSH activity by MTX administration in an animal model. Overall, this study showed that MTX did not alter cardiac antioxidant profiles significantly. This could indicate that MTX-induced cardiotoxicity might not be related to induce oxidative stress. Furthermore, when evaluating the levels of cardiac enzyme activity in the presence of MTX, such as for CK, no significant changes were observed, which was also confirmed by the study of Kadikoylu et al[12]. CK, an intracellular enzyme present in large amounts in the heart among other tissues, is released when hypoxia or cellular injury disrupts the cardiomyocyte membrane[49]. Ternant et al[50] inferred that CK could be a cardiac injury biomarker since the absence of any substantial change in CK levels from MTX compared to control might indicate minimal or limited muscle damage. This finding corroborated the reports of Rossato et al[51], who said this observation might be explained as an adaptation to the effect of MTX treatments. Another cardiac enzyme evaluated was cardiac troponin, which increased considerably, an outcome that was also documented by Herman et al[11]. According to the literature, cardiac troponin is a specific and sensitive biomarker for the diagnosis of cardiotoxicity[52], and so it seems to be associated with the severity of MTX-induced cardiotoxicity[11]. In this context, a significant increase in cardiac troponin levels was observed compared to control in all treatment groups. Evidence showed

that increased troponin was indicative of inflammation and ongoing myocardial damage[53]. Furthermore, it is associated with chronic cardiac injury and poor prognosis and can occur without tissue damage[54]. Reports indicate that increased levels of cardiac troponin might be linked to apoptosis[55]. On a follow-up, the study assessed the level of caspase 3 as a means of evaluating the apoptotic pathway and found significantly elevated activity. The present report is in agreement with a recent report on MTX –induced toxicity in HL-1 cardiomyocytes, which showed an increased caspase 3 activity[56]. Caspase 3 has been described as the main executioner of apoptosis, having the chief role of causing cell death. This is evident from the fact that cells deficient in this gene, block apoptosis[15]. The ability of MTX to induce apoptosis has been associated with its antitumour mode of action, even at a much lower dose[57]. The aforementioned reports further stated that MTX activation of caspase 3 was linked to pathways that mediate intrinsic and extrinsic cell death. Indicating that it might be related to the mechanism by which MTX induces apoptosis in tumour cells[57.] Interestingly, all treatment groups in this study showed significant increases in caspase 3 activity, whereas previous studies had linked caspase 3 activity with NRF-2 gene expression[58,59]. These works documented that increased NRF-2 downstream signalling reduced caspase 3 activity in embryonic rat cardiomyoblast-derived H9C2 cells. It therefore suggests that reduced NRF-2 activity led to increased caspase 3 activity. In the present study, the expression of the NRF-2 cardiac protein in all treatment groups were examined. Hence, it was and found that they all had significantly reduced expression; however, compared to MTX alone, the MTX + 4MP group had a more marked reduction in NRF-2 expression. This finding correlated with enhanced caspase 3 activity and clearly suggested an apoptotic effect on mice myocardial tissue. Furthermore, the CYP2E1 enzyme has been reported to play a pivotal role in oxidative stress and the metabolism of drugs that act as both substrates and inducers, MTX being one of them[25,39,60,61]. Evidence also indicates that the overexpression of CYP2E1 causes cardiac dysfunction as a result of intracellular Ca^{2+} mishandling, ultrastructural damage and apoptosis[62]. According to Lu et al[63], increased activity of CYP2E1 in the heart caused by drugs, induces toxicity and activates caspase 3 activity, leading to apoptosis. These reports are consistent with the findings of our study, which found that MTX significantly increased CYP2E1 expression with a consequent increase in caspase 3 activity and decrease in NRF-2 expression. Therefore, we suggest that MTX-induced cardiotoxicity could be mediated by increased CYP2E1 expression, causing apoptosis through increased troponin and caspase 3 activity. Histological examination of cardiac tissue in all treated groups revealed observable pathological changes. Histopathological changes observed in the MTX-treated group showed red-cell infiltration and inflammation. As recently reported, inflammation may be the trigger for MTX-induced cardiotoxicity[45]. The present study revealed that the MTX + 4MP- and MTX + Disf-treated groups also produced profound pathophysiological changes compared to control. This observation is corroborated by the report of Reis-Mendes et al[40], who documented that MTX was more cardiotoxic than its metabolites, contrary to an earlier report. Additionally, autopsied cardiac tissues showed greater pathology for all the treated groups such as vacuolar degeneration and mycotic apoptosis with distorted wavy myocardial fibers. In some tissues, histoarchitecture was completely lost, which could indicate apoptotic degeneration of myocardial fibers. This study highlighted the role of MTX and its associated metabolites in inducing cardiotoxicity with reference to various reports[7,64]. Moreover, all the MTX treatment groups showed no significant alterations in antioxidant markers, including CK, a cardiac injury marker. However, cardiac troponin was increased with a consequent rise in caspase 3 activity, which was attributed to the MTX chemotherapeutic mechanism of action, apoptosis[65]. Additionally, MTX increased CYP2E1 expression, which reduced NRF-2 expression. According to numerous studies, apoptotic activity can be reduced by increased expression of NRF-2[57,66,67]. It has also been suggested that activation of Nrf2 expression can improve the prognosis of some diseases and this might have some clinical implications[68]. Therefore, based on our observations, we suggest that MTX-induced cardiotoxicity might be an apoptotic event related to increased CYP2E1 expression, which counters downstream expression of NRF-2. We suggest that apoptosis inhibitors might attenuate MTX-induced cardiotoxicity and subsequently enhance NRF-2 activity. In this regard, CYP2E1 might be a therapeutic target. In support of what this study proposed, several treatment strategies to reduce MTX cardiotoxic injury during treatment have

been tried. Some treatments to mitigate the ability of MTX to generate oxidative stress and form toxic iron (iii) radicals have not produced the desired clinical benefits [69,70]. The present study has added to and thrown more light to further help Scientist understand the mechanism of MTX adverse effect and a possible mitigating action. However, one limitation of this study is that we should have estimated the levels of MTX in the plasma at the end of the experiment. Although, it was not part of the study objective but due to MTX wide body distribution and accumulation

Conclusions

From the present study, the results obtained show that the long-term use of MTX could be deleterious to the heart due to cumulative and delayed cardiotoxicity. Additionally, we demonstrated that MTX-induced cardiotoxicity could be mediated via increased expression/induction of CYP2E1 with a consequent decrease in NRF-2 expression. Therefore, the present study as presented here contributes to the understanding of the mechanism of MTX-induced cardiotoxicity, suggesting anti-CYP2E1 as a therapeutic target.

Conflicts of Interest

The Authors declare no conflict of interest.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board Research Ethics Committee (REC) of the Deanship of Scientific Research, King Faisal University. REC REF NUMBER-KFU-REC/2021-06-08.

Authors' Contribution

All authors participated in the design, interpretation of the studies, analysis of the data and wrote the draft manuscript also reviewed the final manuscript.

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