

## ORIGINAL ARTICLE

# CARVEDILOL AS A VALUABLE HEPATOPROTECTIVE AGENT AGAINST METHOTREXATE-INDUCED DAMAGE CHIEFLY DUE TO ITS ANTI-OXIDANT INHERITENCE

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**Background:** Methotrexate is a commonly used drug in several heterogenic inflammatory and malignant disorders but life-sustaining liver organ is inevitably predisposed to damage by it. Carvedilol on the other hand demonstrates anti-oxidant characteristic. The objective of this study was to explore the probable hepatic benefit it might provide when used concomitantly with methotrexate in rats. **Methods:** It was a laboratory-based randomized control trial done with collaborated effort of Pharmacology and Pathology Department, Army Medical College Rawalpindi along with National Institute of Health Islamabad, from March to July 2018. A total of 18 Sprague Dawley rats were randomly divided into 3 groups with 6 rats each. Untreated Group-I served as standard. On 7<sup>th</sup> and 14<sup>th</sup> day, 20 mg/Kg intraperitoneal methotrexate injection was given to rats in Group-II, while Group-III received carvedilol (10 mg/Kg/day) per-orally throughout with methotrexate intervention in-between. Blood was drawn for evaluation of liver function tests after giving chloroform anaesthesia on 15<sup>th</sup> day and later sacrificed for collection of liver samples for the assessment of histopathological changes and oxidative stress induction measured by glutathione and malondialdehyde levels. Results were analysed on SPSS-22 using independent *t*-test and one-way ANOVA for quantitative analysis and histological changes were interpreted via Chi-square test. **Results:** Methotrexate caused significant elevation of liver enzymes and histoarchitectural damage with concurrent glutathione depletion and malondialdehyde extension. Carvedilol brought significant fading of these detrimental changes. **Conclusion:** Carvedilol can cause attenuation of methotrexate-induced hepatotoxicity predominantly by its anti-oxidant quality thereby preserving the normal histoarchitecture as well as reducing deleterious alterations in liver enzymes, glutathione and malonaldehyde levels.

**Keywords:** Carvedilol, Methotrexate, Hepatotoxicity, Glutathione, Malondialdehyde, Anti-oxidant

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## INTRODUCTION

Methotrexate (MTX), an antimetabolite cytotoxic agent, is one of the most commonly used drug in several heterogenic inflammatory and malignant disorders.<sup>1,2</sup> However, the potential risk to cause life-threatening adverse effects including hepatic damage may put a break to its usefulness.<sup>3</sup> Multiple theories have been put forward to explain the etiopathogenesis, of which induction of oxidative stress is the most appealing. MTX polyglutamates accumulate within hepatocytes reversibly inhibiting NADPH dependant dihydrofolate reductase (DHFR) and several other oxidoreductases ultimately jamming the NADPH utilization by glutathione reductase to maintain glutathione (GSH) in its reduced state. This reduced form functions basically for trapping reactive oxygen species (ROS). An imbalance is created between the endogenous defences and ROS produced along with cessation of DNA synthesis. ROS interacting with fatty acids within biological membranes cause their dysfunction with resultant increased malondialdehyde (MDA) production, an indirect marker for accessing oxidative damage. ROS and peroxidation products also alter the

phagocytic and extracellular matrix-producing cell function thus inducing collagenous alterations within liver.<sup>4</sup> Triglyceride and cholesterol metabolism is also affected ending up in steatohepatitis.<sup>5</sup> ROS provoke opening of pores within the mitochondrial membrane thereby collapsing membrane potential and influx of protons with mitochondrial swelling affecting its ATP production.<sup>6</sup> Homocysteine accumulation due to reduced production of tetrahydrofolate and increased adenosine production also directly activates several pro-inflammatory cytokines and stellate cells.<sup>7</sup> Additionally, the 7-OH metabolite of MTX also retains cytotoxic action and being less soluble causes direct toxicity on the hepatic tissue.<sup>8</sup>

Third generation  $\beta$ -blocker Carvedilols' (CVD) utility is vast including portal hypertension, congestive cardiac failure, ischemic heart disease and arrhythmias with additional appreciable effect on glucose and lipid profile making it a preferable choice in hypertensive diabetic and dyslipidemic individuals.<sup>9–11</sup> Interestingly, preventive role in ultraviolet light induced skin malignancies has also been seen.<sup>12</sup> Hepatoprotective mechanism involves an increase in the

SOCS1-mediated pro-inflammatory suppression (suppressor of cytokine signalling) thereby decreasing the production of several pro-inflammatory chemokines and cytokines including IL-1 $\beta$  and TNF- $\alpha$  with additional reduced NF- $\kappa$ B expression. The strengthening and replenishment of IL-10 stores occurs that has several pleiotropic advantages as it inhibits phagocytic cells from generating ROS with resultant shielding of the hepatocytes of the lipid peroxidation process. It also has peculiar free radical scavenging quality. Additionally, it improved the blood flow within the hepatic channels and choked the stellate cell differentiation and Kupffer cell function thereby caused recovery of fibrotic changes in the liver tissue in previous studies.<sup>13</sup> CVD also restocks the depleted GSH within the hepatocytes but whether this benefit is derived by its  $\alpha$ -1 blocking ability still demands clarification.<sup>14</sup>

## MATERIAL AND METHODS

This randomized control trial was carried out in the Department of Pharmacology and Therapeutics along with the collaborated effort of Pathology Department, Army Medical College, and National Institute of Health after clearance from Ethical Review Committee of Centre for Research in Experimental and Applied Medicine (CREAM), AMC. Eighteen untreated, 6–8 week old, male and non-pregnant female Sprague Dawley rats weighing around 200–250 grams were procured from and retained in the animal house of NIH during the experimental duration keeping the environmental conditions optimal. They were randomly segregated into 3 groups having 6 rats each. Dosing schedule was determined keeping the weight of the animals in mind and the following dosing regimen was followed:

Group-I (Control Group): Left untreated.

Group-II (MTX Group): Received 20 mg/Kg intraperitoneally (*i.p.*) on 7<sup>th</sup> and 14<sup>th</sup> of the experiment.

Group-III (CVD+MTX Group): Received peroral 10 mg/Kg/day for 14 consecutive days with toxic drug *i.p.* intervention on 7<sup>th</sup> and 14<sup>th</sup> day.

All animals were anesthetized 24 hours after the last dose of the drugs using chloroform and 1.5–3 ml blood was drawn through cardiac puncture for the estimation of AST, ALT and ALP. Liver tissue was excised out afterwards and divided into two equal halves. Half of the portion was rinsed thoroughly using phosphate buffer solution (BPS) and homogenized on ice the same day via IKA® WERKE homogenizer. Supernatants were obtained by centrifuging the homogenized mixture in eppendorf centrifuge 5417R at 14,000 rpm for approximately 20 minutes maintaining temperature up to 4 °C. Supernatant material was analysed for GSH and MDA content using the commercially available Bioassay Technology kits

utilizing sandwich ELISA principle. The optical density (OD) of the samples were then recorded via microplate reader at 450 nm wavelength and compared with the standard solutions. GSH and MDA content was then calculated by the formula derived from the graph using Microsoft Excel software.

Half of the other tissue was immediately fixed in 10% formalin followed by series of physical and chemical reactions for the preparation of histopathological slides. Ultrastructure of the hepatic tissue was then scrutinized and graded according to Ishak Modified Histological Activity Index (HAI)<sup>15</sup>.

Statistical analysis was done using SPSS-22 where results were expressed in Mean $\pm$ SEM. Independent *t*-test and one-way ANOVA was used for multiple comparisons of quantitative variables and Chi-square test was used for qualitative variables, and  $p \leq 0.05$  was indicative of significant statistical difference.

## RESULTS

Serum AST, ALT and ALP levels were within the normal limit for the untreated Group-I. The levels rose significantly for toxic Group-II ( $p \leq 0.001$ , 0.002 and  $\leq 0.001$  respectively) demonstrating damaging effect of the MTX administration on hepatic tissue. In CVD-treated Group, the levels rose back almost closer to the normal values signifying ample protection offered by the agent (Table-1).

**Table-1: Levels of AST, ALT and ALP in Group-I, Group-II and Group-III (n=6)**

GROUPS	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
Group-I (Control)	148.5 $\pm$ 13.69	84.67 $\pm$ 4.05	105 $\pm$ 8.66
Group-II (MTX)	294 $\pm$ 21.29	160.17 $\pm$ 17.28	221.5 $\pm$ 11.53
Group-III (CVD+MTX)	187 $\pm$ 27.92	85.5 $\pm$ 9.26	174.17 $\pm$ 12.64
<i>p</i>	0.012*	0.003*	0.020*

\*Significant when Group-II and III were compared

The hepatic GSH and MDA content was within the normal limit for Group-I. In Group-II, there was a considerable fall in the GSH levels and an up rise of MDA content ( $p \leq 0.001$ ) for both respectively. Group-III exhibited elevation of hepatic GSH levels when compared with toxic Group-II and required attenuation of the MDA content depicting even lower mean levels than the control group. This indicates replenishment of the intracellular stores and blockade of lipid peroxidation process by CVD (Table-2).

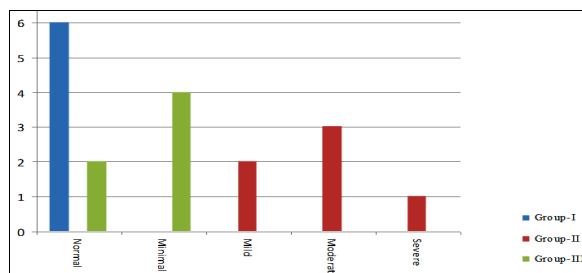
**Table-2: GSH and MDA for all groups (n=6)**

GROUPS	GSH (nmol/l)	MDA (nmol/ml)
Group-I (Control)	24.05 $\pm$ 0.93	1.19 $\pm$ 0.03
Group-II (MTX)	14.23 $\pm$ 0.20	2.31 $\pm$ 0.17
Group-III (CVD+MTX)	17.87 $\pm$ 0.33	1.07 $\pm$ 0.14
<i>p</i>	$\leq 0.001$ *	$\leq 0.001$ *

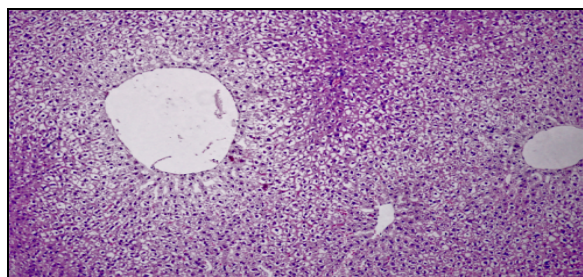
\*Significant when Group-II and III were compared

Individual slides were examined under  $\times 100$  power lens and graded according to HAI (Figure-1). Control group showed normal histological findings (Figure-2).

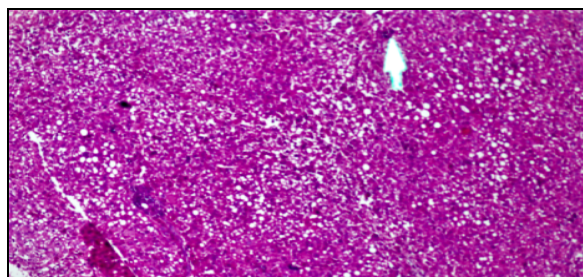
Identifiable alterations with MTX use included central vein congestion, hydropic and steatotic degenerative changes with increased granulations and ballooning of hepatocytes, inflammation in peri-portal areas, bridging and focal lytic necrosis (Figure-3). Group-III demonstrated almost normal cellular structure with mild inflammatory infiltrates around portal tracts (Figure-4).



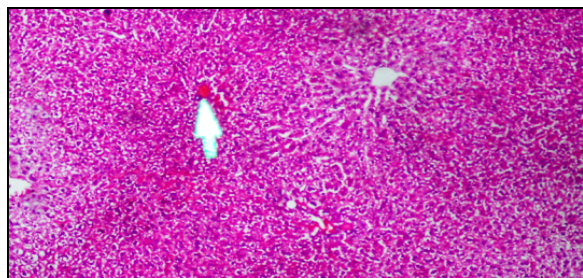
**Figure-1: Histopathological grading in groups according to HAI**



**Figure-2: Hepatic tissue photomicrograph in Group-I displaying normal architecture (H&E ×100)**



**Figure-3: Hepatic tissue photomicrograph in Group-II displaying steatotic transfiguration with pointer at focal lytic necrosis (H&E ×100)**



**Figure-4: Hepatic tissue photomicrograph in Group-III displaying roughly normal parenchyma with no central vein congestion but mild peri-portal infiltrates (H&E ×100)**

## DISCUSSION

Drug induced liver injury is very underreported yet is one of the common cause for drug withdrawal worldwide.<sup>16</sup> Moreover, it might end up in organ failure requiring liver transplant whose facility is not yet available in our country. Hence, regular monitoring of the patient on such toxic drug is the safest bet to secure one's life.<sup>17</sup> Hepatic issues caused by methotrexate (MTX) and solutions to protect the organ off it have been studied widely over many years by the researchers and becomes specially important when the person is on multiple drug therapy for varied co-morbid conditions.<sup>3</sup> It has been seen that high dose MTX or low dose chronic use often ends up with liver function test (LFTs) derangements and histoarchitectural damage via oxidative stress induction as one of the probable mechanisms involved.<sup>5</sup> In a survey, derangements in LFTs were seen in 31% of the cases after three years low dose usage which led to temporary or permanent discontinuation of the therapy in 7% and 23% respectively. Chronic cumulative doses over four years survey caused fibrosis (mild=15.3% and severe=1.3%) and cirrhosis in 0.5% of the cases.<sup>18</sup> In addition to the above statistics, a one year survey at Armed Forces Institute of Pathology Pakistan showed derangements in LFTs in 8.6% cases, with overall 27% cases of reported MTX toxicity.<sup>19</sup> Despite this, it is still being widely prescribed because it is cheap, easily available and demonstrates brilliant effectiveness. A 2 year survey also shows that only rheumatoid arthritis (RA) patients using MTX had cardiovascular issues in 35.38% of the cases, of which 13.79 and 6.6% had hypertension and ischemic heart disease (IHD) respectively. Reciprocally, 85.05 and 88.09% of the hypertensive and IHD patients had a positive RA factor.<sup>20</sup> Alarming, 64% of the elderly hospitalized Pakistani population is prescribed wrong medication with deteriorative health consequences.<sup>21</sup>

Therefore, this project was specially drafted to evaluate hepatoprotective benefit of Carvedilol (CVD), a very useful cardioactive agent prescribed in our population, and quantify the protection offered by it against MTX. If proven rewarding, this will help reduce the medication burden put on the patient and also improve the drug prescription quality, the need of which is essential in our medical practice. To our knowledge, the hepatoprotective benefit of our target drug has never been tested against MTX which makes our study exclusive.

After multiple trials during our experiment, an adequate hepatotoxic model was driven using MTX in 20 mg/Kg *i.p.* dose on the 7<sup>th</sup> and 14<sup>th</sup> day of the 15 day experiment. Using Group-I as the standard, LFTs for this set of group rose to almost double the amount which is significant from statistical point of view. When

graded through standard scoring chart, slides demonstrated mild to severe alterations from normal histology. These alterations were statistically significant when studied side by side with Group-I and were in accordance with the preceding studies in which the dosing pattern of using MTX was the major differentiating point, but they all caused significant and important deterioration of the liver tissue.<sup>22-24</sup> Additionally, an important decline in the levels of GSH and an obvious and statistically significant elevation of MDA content was seen in rat liver tissue. These changes point towards the hindrance of body's defensive capability thus making the cells vulnerable to damage by reactive oxygen species (ROS) generated by the use of the toxic drug causing disruption of the cells double layered membranous structure as gauged by MDA content indirectly. The studies done by Ali and Savran with their coworkers demonstrated the same variations in oxidative stress markers as in our study.<sup>25,26</sup> This hence clearly notifies the ill health effects on the hepatic tissue of rats by MTX use in human relevant doses.

Group-III received CVD (10 mg/Kg/day) per orally. This dosing schedule was found to be affective to rectify acetaminophen induced liver damage in a previous study<sup>27</sup> but the same dose ameliorated the derangements caused by MTX in our study as well. In detail, a significant reduction in the levels of AST, ALT and ALP was evident which reveals that CVD treated rat livers were protected adequately off the harmful effects of MTX. This was linked with the remarkable improvement of alterations in the histoarchitecture caused by the toxic drug showing almost normal histological framework. These deductions were comparable with multiple researches in the past in which CVD demonstrated its anti-oxidant, anti-inflammatory and immunomodulatory potential conferring adequate shielding of liver against alcohol and acetaminophen induced damages respectively.<sup>13,14,27</sup> The critical contraction of GSH content due to MTX in the liver was also halted by the added use of CVD, thus preserving this crucial endogenous defensive entity. CVD also paralyzed the escalation of MDA marker by MTX in the hepatic tissue. This beneficial effect was consistent with former groundwork in which ischemia as well as chromium and paraquat were used to create heart, kidney and lung toxic prototypes with noteworthy oxidative stress induction.<sup>28-30</sup> Important to mention here is that CVD also has beneficial out-turn against rheumatoid arthritis (RA) and is comparable with the standard dexamethasone and MTX therapy in a latest study. Microscopic joint structure improvement along with GSH strengthening and amelioration of MDA content as well as improvement of immunological, inflammatory and rheumatoid factor markers was seen in that study.<sup>31</sup> Conclusively, it can be hypothesized that CVD might replace or provide additional improvement

in RA when used with MTX, and also on the other hand protect the hepatic tissue from the damaging effects of the drug. This reduces the load of medication on the patient and therefore saves one of the unnecessary use of dietary hepatoprotective supplements or conventional protective agents.

## CONCLUSION

Collateral use of carvedilol with methotrexate is of therapeutic benefit shielding the liver effectively if used together for several ailments keeping the liver function test derangements, oxidative burden and histological alterations under check.

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