PROTECTIVE EFFECTS OF TINOSPORA CORDIFOLIA AGAINST METHOTREXATE INDUCED OXIDATIVE DAMAGE IN RAT LIVER

DEEPAK, J. N.,1 RAO, S.,1 BYREGOWDA, S. M.,2 VETRIVEL, M.,1 PURUSHOTHAM, K. M.,2 SATYANARAYANA, M. L.,1 NARAYANASWAMY, H. D.1 AND RENUKAPRASAD, C.3

1Department of Veterinary Pathology, Veterinary College, Hebbal, Bangalore 560 024; 2Institute of Animal Health and Veterinary Biologicals, Hebbal, Bangalore; 3Karnataka Veterinary Animal and Fisheries Sciences University [KVAFSU], Bidar, Karnataka. E. mail: drdeepakjn@gmail.com

Received: January 30, 2015; Accepted; March 5, 2015

Abstract: The role of aqueous extract of Tinospora cordifolia [TC], in preventing methotrexate [MTX] induced oxidative damage in rat liver was studied. Sixty Wistar albino rats were divided into five groups with twelve rats in each group. Group I served as normal control group. To group II rats, MTX was administered at 5mg/kg body weight intraperitoneally for three consecutive days. Rats in group III were administered with MTX and treated with TC extract at 200mg/kg by oral gavaging for 45 days. Group IV rats were pre-treated with TC ten days prior to MTX administration and followed by TC treatment for 45 days. Rats in group V served as TC control and were administered TC at the dose of 200mg/kg body weight for 45 days. The liver samples collected at 7th, 14th, 28th, and 45th day of the study were subjected for estimation of catalase, superoxide dismutase, glutathione peroxidase and malondialdehyde [MDA]. Significant decrease [P<0.01] in the levels of antioxidant enzymes and increase in MDA levels were recorded in MTX treatment group. The TC treatment groups revealed a significant recovery [P<0.01] all enzymes and decrease in MDA levels which suggested that TC has a good antioxidant effect and could be effectively used for prevention of toxic side effects of MTX. The study also indicated that pre-treatment of TC prior to MTX administration has good prophylactic effect.

Key words: Tinospora cordifolia, Methotrexate, oxidative damage

INTRODUCTION

Methotrexate (MTX) is a chemotherapeutic drug and a folic acid antagonist widely used as a cytotoxic agent in the treatment of malignancies and various autoimmune diseases such as psoriasis and rheumatoid arthritis. However, the efficacy of MTX is limited due to its side effects, which includes hepatotoxicity [1-4]. It has also been shown that MTX toxicity has severe side-effects on the haematopoietic system [5] and liver enzymes in general [6]. The exact mechanisms of methotrexate-induced toxicity have to date not yet been elucidated [7,8]. The amelioration of MTX induced toxicity has been the prime concern during therapeutic intervention with MTX. It is thought that the detrimental effects of MTX are partly due to its direct toxic action by increasing ROS production. It has also been reported that MTX administration induces oxidative stress and significantly reduces antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in liver, intestinal mucosa and spinal cord tissues of rats [9,10]. It is however known that many of the antioxidants protect against drug induced oxidative stress by the action of certain enzymes, vitamins, and other substances [11].
Since MTX induces toxicity, a search for drugs or agents with antioxidant property has been extensively carried out \cite{13} and some of them proved partially helpful in preventing MTX toxicity, while other caused additional adverse effects. Therefore, there is a need to search for some alternative drugs for the treatment of MTX induced hepatotoxicity. Numerous medicinal plants and their formulations are being used for liver disorders in ethno-medical practices and in traditional system of medicine in India.

*Tinospora cordifolia*, a herbal creeper, is widely used in veterinary folk medicine/ayurvedic system of medicine as a general tonic for its anti-spasmodic, anti-inflammatory and anti-diabetic properties \cite{14-18}. The hepatoprotective and immunomodulatory effects of *T. cordifolia* in various drug induced toxicities have been reported in rats and mice \cite{18-21}. This study was undertaken to investigate the antioxidant enzyme status in liver of rats following methotrexate administration and to study the possible protective effect of *Tinospora cordifolia* against MTX induced oxidative damage.

**MATERIALS AND METHODS**

**Drugs and chemicals:** Methotrexate [Folitrax-15] was procured from IPCA Laboratories Ltd, Mumbai, and the aqueous stem-extract of *Tinospora cordifolia* was obtained from Kisalaya Herbals Ltd, Indore, Madhya Pradesh.

**Animals:** Normal adult Wistar albino rats of both sex and weighing approximately 140-150 grams were procured from RRL Instruments and Animals supplier, Bangalore for the study. They were maintained under standard laboratory conditions and fed with *ad libitum* standard commercial rat feed and clean drinking water. The duration of experiment was for a period of 45 days and a prior permission was obtained from the Institutional Animal Ethics Committee [IAEC] for the conduct of the experiment.

**Experimental design:** The rats were maintained under standard laboratory conditions for a period of 15 days for acclimatization in the experimental animal house. The rats were divided, based on the body weight, into five groups with twelve rats in each group.

**Group I:** Negative control - injected with 0.5ml sterile PBS intraperitoneally on Day 1 and gavaged with PBS daily.

**Group II:** Positive control- hepatotoxicity induced with administration of methotrexate at 5mg/kg body weight intraperitoneally for three consecutive days

**Group III:** Supplemented with *Tinospora cordifolia* extract at the dose rate of 200 mg/kg body weight concurrently with administration of MTX.

**Group IV:** Supplemented with *Tinospora cordifolia* extract at the dose rate of 200 mg/kg body weight 10 days prior to induction of hepatotoxicity by MTX.

**Group V:** *Tinospora cordifolia* control-animals supplemented with aqueous extract of *Tinospora cordifolia* alone at the dose rate of 200 mg/kg body weight.

Liver tissue samples collected at 7th, 14th, 28th and 45th day of experiment and were homogenized with ice cold 0.1 M Tris-HCl buffer of pH 7.4 to make tissue homogenate [0.5 g liver crushed in 10 mL of ice cold 0.1 mol/L Tris-HCl buffer]. The homogenate was centrifuged at 3000 rpm for 10 min. The supernatant was collected and used for estimation of superoxide dismutase, and catalase, glutathione peroxidase and TBARS [MDA] levels.

**Estimation of superoxide dismutase [SOD]:**
Superoxide dismutase activity was determined by the method described by Marklund and Marklund [22]. The enzyme activity was expressed in terms of units per minute per mg of protein. One unit of SOD was defined as the amount of enzyme required to inhibit pyrogallol auto-oxidation reaction by 50 per cent.

**Estimation of catalase [CAT]:** Catalase was estimated by the method described by Caliborne [23]. Enzyme activity was expressed as imol of H$_2$O$_2$ decomposed per minute per mg of protein.

**Estimation of glutathione peroxidase [GPx]:**
Glutathione peroxidase was determined by the method described by Rotruck et al. [24]. Enzyme activity was expressed as µmol /mg protein.

**Estimation of TBARS [Malondialdehyde]:** Lipid peroxidation in the liver tissue was determined by estimation of thiobarbituric acid reactive substance
[TBARS] by the method of Yagi [25]. The unit of activity was expressed as n moles of MDA /mg of tissue.

**Histopathology:** Following sacrifice, the livers were collected and preserved in 10% neutral buffered formalin [NBF] for 48 hours and processed as per routine procedure. 4-μ thick sections were cut using a semi automatic microtome and stained with hematoxylin and eosin and observed under light microscope.

**Statistical analysis:** Statistical analysis was performed using the statistical software Graph Pad Prism, version 6.0 for Windows. Mean values and standard error were calculated and all values were expressed as Mean (± SE). The data were analyzed by two-way analysis of variance [ANOVA].

**RESULTS**

The effect of MTX administration on the antioxidant enzyme status and lipid peroxidation in liver of rats was analyzed. The results indicated that MTX caused a significant decrease [P<0.01] in the levels of SOD, CAT and GPx [Tables 1,2,3] and significant increase [P<0.001] in the level of MDA [Table 4] in Group-II when compared to other groups, throughout the duration of the experiment.

Administration of aqueous extract of *Tinospora cordifolia* ameliorated the deleterious effects of MTX which was reflected by significant recovery [P<0.01] in the levels of SOD, CAT and GPx in the animals of Group-III and Group-IV [Tables 1,2,3] and also by significant reduction [P<0.001] in the levels of MDA [Table 4].

Improvement in the levels of antioxidants [P<0.01] and MDA [P<0.001] was significant in the TC pre-treated group [Group-IV] when compared to concurrent TC treatment group [Group-III]. No significant difference in antioxidant levels and MDA was recorded in the TC control group [Group-V] when compared to the normal control group [Group-V] throughout the duration of the experiment.

### Table 1: The mean (±SE) catalase levels (μmol/min/mg protein) in liver of rats in different groups at different time intervals. Values with different superscripts in a column vary significantly (p<0.01)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days post treatment</th>
<th>7d</th>
<th>14d</th>
<th>28d</th>
<th>45d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I (NC)</td>
<td></td>
<td>47.98±2.05a</td>
<td>50.19±2.41b</td>
<td>49.12±2.93c</td>
<td>51.94±2.29a</td>
</tr>
<tr>
<td>Group-II (MTX)</td>
<td></td>
<td>19.39±0.87a</td>
<td>19.76±1.18c</td>
<td>25.19±1.01a</td>
<td>27.08±2.15b</td>
</tr>
<tr>
<td>Group-III (MTX+TC)</td>
<td></td>
<td>30.35±0.81a</td>
<td>30.24±4.73c</td>
<td>39.00±2.07c</td>
<td>47.38±1.18c</td>
</tr>
<tr>
<td>Group-IV (TC+MTX)</td>
<td></td>
<td>39.23±1.22a</td>
<td>40.10±2.35c</td>
<td>47.14±3.05c</td>
<td>48.28±1.57c</td>
</tr>
<tr>
<td>Group-V (TC)</td>
<td></td>
<td>49.78±2.25a</td>
<td>50.16±3.32c</td>
<td>49.36±1.34c</td>
<td>51.63±1.19c</td>
</tr>
</tbody>
</table>

### Table 2: The mean (±SE) glutathione peroxidase (GPx) levels (U/mg protein) in liver of rats in different groups at different time intervals. Values with different superscripts in a column vary significantly (p<0.01)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days post treatment</th>
<th>7d</th>
<th>14d</th>
<th>28d</th>
<th>45d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I (NC)</td>
<td></td>
<td>30.52±2.71a</td>
<td>32.55±2.39a</td>
<td>30.85±1.6a</td>
<td>32.24±1.96a</td>
</tr>
<tr>
<td>Group-II (MTX)</td>
<td></td>
<td>8.83±1.83a</td>
<td>10.45±0.48a</td>
<td>12.96±1.17a</td>
<td>16.49±0.5a</td>
</tr>
<tr>
<td>Group-III (MTX+TC)</td>
<td></td>
<td>15.42±0.36a</td>
<td>15.73±0.79c</td>
<td>21.79±1.83a</td>
<td>26.36±1.54a</td>
</tr>
<tr>
<td>Group-IV (TC+MTX)</td>
<td></td>
<td>22.39±0.83a</td>
<td>25.13±0.77a</td>
<td>26.77±1.56a</td>
<td>28.41±2.64a</td>
</tr>
<tr>
<td>Group-V (TC)</td>
<td></td>
<td>29.88±1.99a</td>
<td>31.04±2.35a</td>
<td>28.97±1.54a</td>
<td>30.38±0.44a</td>
</tr>
</tbody>
</table>

### Table 3: The mean (±SE) superoxide dismutase (SOD) levels (U/min/mg protein) in liver of rats in different groups at different time intervals. Values with different superscripts in a column vary significantly (p<0.01)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days post treatment</th>
<th>7d</th>
<th>14d</th>
<th>28d</th>
<th>45d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I (NC)</td>
<td></td>
<td>30.79±1.37a</td>
<td>29.65±1.45a</td>
<td>28.28±2.39a</td>
<td>27.80±1.29a</td>
</tr>
<tr>
<td>Group-II (MTX)</td>
<td></td>
<td>7.76±0.35a</td>
<td>9.23±0.37a</td>
<td>13.38±0.71a</td>
<td>14.83±1.09a</td>
</tr>
<tr>
<td>Group-III (MTX+TC)</td>
<td></td>
<td>14.28±0.65a</td>
<td>16.06±1.37a</td>
<td>21.14±2.21c</td>
<td>28.48±1.69c</td>
</tr>
<tr>
<td>Group-IV (TC+MTX)</td>
<td></td>
<td>21.65±1.06a</td>
<td>23.04±0.33a</td>
<td>29.94±2.11c</td>
<td>30.68±1.98c</td>
</tr>
<tr>
<td>Group-V (TC)</td>
<td></td>
<td>29.88±1.05a</td>
<td>31.06±1.47a</td>
<td>32.11±1.42c</td>
<td>31.73±1.74a</td>
</tr>
</tbody>
</table>

### Table 4: The mean (±SE) malondialdehyde (MDA) levels (n moles/mg of tissue) in liver of rats in different groups at different time intervals. Values with different superscripts in a column vary significantly (p<0.001)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days post treatment</th>
<th>7d</th>
<th>14d</th>
<th>28d</th>
<th>45d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-I (NC)</td>
<td></td>
<td>0.90±0.10a</td>
<td>1.01±0.02a</td>
<td>1.01±0.03a</td>
<td>1.03±0.05a</td>
</tr>
<tr>
<td>Group-II (MTX)</td>
<td></td>
<td>5.74±0.47b</td>
<td>8.82±0.27b</td>
<td>7.32±1.14b</td>
<td>5.34±1.06b</td>
</tr>
<tr>
<td>Group-III (MTX+TC)</td>
<td></td>
<td>5.56±0.20c</td>
<td>5.09±0.07c</td>
<td>2.93±0.12c</td>
<td>2.56±0.07c</td>
</tr>
<tr>
<td>Group-IV (TC+MTX)</td>
<td></td>
<td>3.13±0.16d</td>
<td>1.58±0.05d</td>
<td>1.30±0.08d</td>
<td>1.10±0.06d</td>
</tr>
<tr>
<td>Group-V (TC)</td>
<td></td>
<td>0.69±0.30a</td>
<td>1.03±0.02a</td>
<td>0.98±0.07a</td>
<td>1.05±0.04a</td>
</tr>
</tbody>
</table>
Histopathological changes in the liver of MTX control group included congestion, centrilobular vacuolar degeneration, multifocal necrosis, periporal mononuclear cell infiltration and presence of pre-apoptotic and apoptotic cells throughout the parenchyma [Figs. 1,2]. The severity of the lesions in the TC treated animals were reduced compared to MTX control animals and showed mild degeneration, congestion and presence of only a few apoptotic and pre-apoptotic cells [Figs. 3,4].

**DISCUSSION**

In the present study, a significant increase in the malondialdehyde [MDA] and decrease in GPx, catalase and SOD levels in MTX control group was recorded. Such observations were also made by several earlier workers [7, 26-29].

The antioxidants play an important role in protection against damage caused by reactive oxygen species [ROS]. The antioxidants breakup the chains formed during the propagation process by providing a hydrogen atom or an electron to free radical and receive the excess energy possessed by the activated molecule [30]. The function of intracellular GPx is degradation of H$_2$O$_2$ and hydro peroxides of free fatty acids and GPx catalyses degradation of H$_2$O$_2$ and hydro peroxides of phospholipids and also inhibits peroxidation process. SOD converts superoxide radicals to hydrogen peroxide and subsequently to water [31,32].

The endogenous sources of ROS include mitochondrial electron transport chain, cytochrome P 450s, NADPH oxidase and xanthane dehydrogenase/oxidase. The observation of the present study indicated that MTX administration caused oxidative stress with increased lipid peroxidation and a decrease in antioxidant enzymes. Lipid peroxidation mediated by oxygen free radicals was evaluated by estimation of malondialdehyde [MDA] which is a 3-carbon aldehyde, a end product of lipid peroxidation and a marker of oxidative stress. Under normal conditions, protection against ROS is by utilization of NADPH by glutathione reductase to maintain the reduced state of cellular glutathione which is an important cytosolic antioxidant [33]. However, in MTX induced toxicity, cytosolic NADPH and NADP enzymes are inhibited by MTX which decreases the availability of NADPH in cells, thus subjecting the cells for ROS injury.

GSH is one of the most important molecules in the cellular defense against chemically reactive toxic compounds. The reduced form of GSH is necessary for detoxification of xenobiotics. The reduction in GSH levels induced by MTX causes suppression of antioxidant enzyme defense system sensitizing the cells to ROS [7,34,35]. Increased levels of plasma MDA in MTX hepatotoxicity could be attributed to overproduction of ROS and a deficiency of antioxidant defense [13,36]. MDA released after peroxidation of lipids is reported to cause irreversible damage to cells and organelle contents accounting for tissue damage [13].

Study shows an improvement in the levels of MDA, SOD, catalase and GPx was observed in MTX control group by 45th day which could be attributed to the partial reversion in the MTX induced toxicity that naturally occurs with discontinuation of the drug. The improvement observed in the histo-morphology of liver also substantially supports the present observation.

**REFERENCES**


**Explanation of figures:**

**Fig. 1:** Section of liver from MTX control animal at 7th day of experiment showing centrilobular vacuolar degeneration. H&E X 100.

**Fig 2:** Section of liver from MTX control animal at 7th day of experiment showing congestion, vacuolar degeneration and presence of pre-apoptotic and apoptotic hepatocytes. H&E X 200

**Fig. 3:** Section of liver from *Tinospora cordifolia* treated (Group-III) animal at 14th day of experiment showing improved architecture with a few pre-apoptotic and apoptotic hepatocytes. H&E X 100

**Fig. 4:** Section of liver from *Tinospora cordifolia* pre-treated (Group-IV) animal at 14th day of experiment showing almost normal architecture of liver. H&E X 100