Curcumin Attenuates Methotrexate-Induced Hepatic Oxidative Damage in Rats

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ABSTRACT

In the present study, we have addressed the ability of curcumin to suppress MTX-induced liver damage. Hepatotoxicity was induced by injection of a single dose of MTX (20mg/kg I.P.). MTX challenge induced liver damage that was well characterized histopathologically and biochemically. MTX increased relative liver/body weight ratio. Histologically, MTX produced fatty changes in hepatocytes and sinusoidal lining cells, mild necrosis and inflammation. Biochemically, the test battery entailed elevated activities of serum ALT and AST. Liver activities of superoxide dismutase (SOD), catalase (CAT) and level of reduced glutathione (GSH), were notably reduced, while lipid peroxidation, expressed as malondialdehyde (MDA) level was significantly increased. Administration of curcumin (100mg/kg, I.P.) once daily for 5 consecutive days after MTX challenge mitigated the injurious effects of MTX and ameliorated all the altered biochemical parameters. These results showed that administration of curcumin decreases MTX-induced liver damage probably via regulation of oxidant/anti-oxidant balance. In conclusion, the present study indicates that curcumin may be of therapeutic benefit against MTX-cytotoxicity.

Key Words: Methotrexate (MTX) – Liver damage – Oxidative stress – Curcumin (CUR).

INTRODUCTION

Methotrexate (MTX), a folic acid antagonist, is widely used as a cytotoxic chemotherapeutic agent for treatment of leukemias and other malignancies [1]. In addition, it has been used for the treatment of various inflammatory diseases such as psoriasis and rheumatoid arthritides. However, the efficacy of this agent in high doses has been associated with hepatotoxicity [1]. The underlying mechanism of hepatotoxicity caused by MTX treatment remains unclear. However, it has been reported that MTX causes oxidative stress in liver tissue [2,3]. Although 7-OH metabolite is the major pathway of MTX metabolism, MTX is metabolized and stored in hepatocytes in the polyglutamated form [4,5]. The presence of higher levels of polyglutamates causes a longer intracellular presence of the drug and this has been suggested as a mechanism for hepatotoxicity [6]. Moreover, MTX inhibits dihydrofolate reductase, it indirectly affects the synthesis of thymidilate, thereby suppressing DNA synthesis [7]. Additionally, it was demonstrated that the cytosolic nicotinamide adenine diphosphate (NADP)-dependent dehydrogenases and NADP malic enzyme are inhibited by MTX, suggesting that the drug could decrease the availability of NADPH in cells [8]. Under normal conditions, NADPH is used by glutathione reductase to maintain the reduced state of cellular glutathione, an important cytosolic antioxidant, which protects against reactive oxygen species (ROS). Thus the significant reduction in glutathione (GSH) levels induced by MTX leads to reduction in the level of the antioxidant enzyme defense system, sensitizing the cells to ROS [9].

Curcumin is the main component of the turmeric pigment of Curcuma longa. It has many beneficial effects including anti-inflammatory [10], antioxidant [11], anticancer [12], antimicrobial [13], hepatoprotective [14] and antihyperlipidemic [15] actions. These pleiotropic effects made curcumin a suitable candidate to be incorporated in this study. The main objective of this work was to address whether or not curcumin...
could have an ameliorative effect in MTX-induced liver damage of male Swiss albino rats and the mechanism(s) whereby this turmeric pigment would confer such effect.

**MATERIAL AND METHODS**

The biochemical test battery encompassed assessment of serum activities of ALT, AST, liver contents of MDA and GSH and liver activities of SOD and CAT. In addition gross examination of the liver and histopathological preparation of liver sections with routine hematoxylin and eosin double stain was done to confirm the model and unravel any possible treatment exerted by curcumin.

**Animals:**

Male Swiss albino rats weighing 150-175g were obtained from Theodor Bilharz-Institute (Giza, Egypt). The animals were housed in the animal facility of the Faculty of Pharmacy, Al-Azhar University (Assiut). The animals were fed a standard diet (El-Nasr Company, Abou-Zaabal, Cairo, Egypt) and allowed free access to water. The rats were kept under standard conditions of temperature (21±0.5º) and relative humidity (55±5) with 12-h light/12-h dark cycle.

**Drugs and chemicals:**

Curcumin was purchased from Fluka Chemical CO. (GmbH, Steinhem, Germany) and methotrexate was purchased from David Bull Laboratories, Mulgrave – Victorica, Australia. All other chemicals used were of the finest analytical grade.

**Experimental design:**

Animals were divided into 5 groups, each of 8 rats. Group I: The rats were injected I.P with (0.2ml/100 g bw) saline once daily for 5 days. Group II: The rats were injected DMSO (the vehicle of curcumin solution) (50%v/v) (0.2ml /100g bw, I.P) once daily for 5 consecutive days. Group III: The rats were injected I.P with curcumin in a dose of 100mg/kg once daily for 5 consecutive days. Group IV: The rats were injected I.P with curcumin in a dose of 100mg/kg bw once daily for 5 consecutive days. Group V: The rats were injected I.P with MTX in a single dose of 20mg/kg [1,16]. Group V: The rats were injected I.P with a single dose of MTX (20mg/kg) followed by I.P injection of curcumin (100mg/kg) for 5 consecutive days. On the sixth day, rats were anaesthetized with ether and retroorbital blood samples were withdrawn. Serum was separated following centrifugation at 4000 rpm for 10min. at 4ºC and stored at −20ºC till biochemical analyses. Then, the animals were euthanized by cervical dislocation. Livers were dissected out, blotted dry and weighed. Livers were cut into small pieces and homogenized in ice-cold 0.15 M KCl (w/v) using Potter Elvejheim homogenizer (Berlin, Germany) to give a final concentration of 10% homogenate. Two livers from each group were kept in 10% formol saline prior to histological examination.

**Methods:**

1- **Biochemical analyses:**

1.1- **Determination of serum ALT and AST activities:** Liver function was assessed by measuring serum activities of ALT and AST [17].

1.2- **Determination of thiobarbituric acid reactive substance content in liver homogenate (TBARS):** TBARS in tissue homogenate was estimated as malondialdehyde (MDA) by using the method of Mihara and Uchiyama [18]. In brief, an aliquot of 0.5ml of 10% liver homogenate was pipetted into a 10ml centrifuge tube followed by 3ml of 1% orthophosphoric acid and 1ml of 0.6% thiobarbituric acid. The tubes were incubated in a water bath at 95ºC for 45min. The mixture was cooled and 4ml of n-butanol were added, mixed and separated by centrifugation at 2000 rpm for 10min. Absorbance was then measured at 535 and 520nm against blank, using a Shimadzu spectrophotometer UV, 1201 (Japan).

1.3- **Determination of glutathione level in liver homogenate (GSH):** GSH was estimated spectrophotometrically by the method of Ellman [19]. Protein in liver homogenate was precipitated with 10% trichloroacetic acid and the contents were centrifuged at 2000 rpm for 5min. An aliquot of the clear supernatant (0.1ml) was taken and mixed with 1.7ml of 0.1mM potassium phosphate buffer (pH.8) and 0.1ml of Ellman’s reagent. The optical density was measured at 412nm against a blank.
1.4- Determination of superoxide dismutase (SOD) activity in liver homogenate: The enzymatic activity of SOD was assessed according to the method of Marklund [20]. An aliquot of 10% liver homogenate (100 µl) was added to 25 µl pyrogallol (24 mmol/L prepared in 10 mmol HCl) and the final volume was adjusted to 3 ml using Tris HCL buffer (0.1 M, pH 7.8). The change in absorbance was recorded spectrophotometrically at 420 nm. SOD activity was expressed as U/mg protein.

1.5- Determination of total protein in liver homogenate: Total protein was determined in the liver homogenate by the method of Lowry et al. [21].

1.6- Determination of catalase (CAT) activity in liver homogenate: CAT activity was assayed by the method of Clairborne [22]. 100 µl of liver homogenate (10%) were added to 2.9 ml of 19 mmol/L H₂O₂ solution prepared in potassium phosphate buffer (0.1 M, PH 7.4). The decrease in absorbance was measured at 240 nm. Enzyme activity was expressed as U/mg protein.

2- Histopathological examination:
Livers were kept in 10% formalin solution for 24 hours using Hartz Technique [23]. Tissues were then embedded in paraffin blocks and 5 micron-thick sections were obtained from the blocks and stained by hematoxylin and eosin. The tissue sections were then examined microscopically. Examination was blindly carried out by the pathologist.

Statistical analysis:
Data analysis was achieved using the GraphPad Instat software (Version 2.0 Philadelphia, 1993). Data were expressed as mean ± SD. Comparisons were done using one-way ANOVA followed by Tukey-Kramer as post ANOVA test. Criterion for significance was chosen to be at \( p \leq 0.05 \).

RESULTS
Methotrexate treatment increased the serum activities of ALT and AST (51% and 45%) and (36% and 32%) compared with the control saline and DMSO-treated groups, respectively. Curcumin treatment for 5 consecutive days following MTX significantly decreased the serum activities of ALT and AST by ~21% and ~14%, respectively, compared to MTX treated rats (Table 1). The content of liver MDA as a product of lipid peroxidation was significantly increased after administration of MTX by 99% compared to control saline and vehicle treated animals. However, administration of curcumin after MTX significantly decreased MDA level by ~24% compared to MTX treated group (Fig. 1). GSH liver content decreased significantly after MTX treatment by ~73% compared to control saline group. Administration of curcumin after MTX restored the GSH level by about 87% compared to rats that received MTX alone (Fig. 2). The liver SOD and CAT activities were decreased after MTX treatment by about ~50% and ~52%, respectively, compared to the control saline group. Administration of curcumin following MTX significantly increased the activities of the SOD and CAT by about 57% and 90%, respectively, compared to the MTX group (Figs. 3, 4). Treatment of rats with MTX increased the relative liver weight by 62% compared to the control saline group. However, treatment of rats with curcumin reduced the relative liver weight by ~24% compared with the MTX treated rats (Fig. 5).

Table (1): Effect of curcumin on the serum levels of ALT and AST of MTX-treated rats.

<table>
<thead>
<tr>
<th></th>
<th>ALT (mg/dl)</th>
<th>AST (mg/dl)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>29.65±1.29</td>
<td>60.50±2.97</td>
</tr>
<tr>
<td>DMSO</td>
<td>30.95±1.33</td>
<td>62.50±3.17</td>
</tr>
<tr>
<td>CUR</td>
<td>28.08±1.63</td>
<td>58.10±2.14</td>
</tr>
<tr>
<td>MTX</td>
<td>44.73±2.74</td>
<td>82.32±5.39</td>
</tr>
<tr>
<td>MTX+CUR</td>
<td>35.50±3.20</td>
<td>70.78±6.07</td>
</tr>
</tbody>
</table>

DMSO: Dimethyl sulfoxide.
CUR: Curcumin.
MTX: Methotrexate.

Data are expressed as mean ± SD (n=6). Curcumin (100 mg/kg, IP) was given for 5 consecutive days. MTX (20 mg/kg, IP) was given as a single dose. Multiple comparisons were achieved using one-way ANOVA followed by Tukey-Kramer as post ANOVA test. (a) Significantly different from control saline at \( p < 0.05 \). (b) Significantly different from MTX at \( p < 0.05 \).
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Data are expressed as mean ± SD (n=6). Curcumin (100mg/kg, IP) was given for 5 consecutive days. MTX (20mg/kg, IP) was given as a single dose. Multiple comparisons were achieved using one-way ANOVA followed by Tukey-Kramer as post ANOVA test. (a) Significantly different from control groups at p<0.05. (b) Significantly different from MTX at p<0.05.

Histopathological examination of the normal liver tissue sections showed normal structure of hepatocytes recorded in (Photo A). However, after MTX treatment, marked pleomorphisms and apoptotic bodies were seen as acidophil bodies. Additionally, cholestasis, steatosis, fatty changes in hepatocytes and sinusoidal lining cells, mild necrosis and inflammation, lobular disarray as well as mild dilatation of central
lobular sinusoid were also observed after MTX treatment (Photo B-1, B-2, B-3). Treatment with curcumin after MTX mitigated pleomorphism, apoptosis, necrosis and inflammation, as well as fatty changes, improved with some cholestasis and steatosis (Photo C).

Histopathological studies of liver in control and experimental groups of rats A: Section of liver tissue from control rats showing normal structure, H&E 40. B-1: Section of liver tissue from MTX-treated rats showing acidophilic bodies, vacuolation and lymphocytic infiltration (arrow) H&E 40. B-2: Section of liver tissue from MTX-treated rats showing haemorrhage, marked pleomorphism and vacuolation (arrow) H&E 40. B-3: Section of liver tissue from MTX-treated rats showing sinusoidal dilatation and pleomorphism (arrow) H&E 40. C: Section of liver tissue from MTX group treated with curcumin showing less vacuolated with restoration of lobular architecture (arrow) H&E 40.
DISCUSSION

Methotrexate (MTX), a folic acid antagonist, is widely used as a cytotoxic chemotherapeutic agent for several malignancies and various inflammatory diseases.

Treatment of rats with MTX induced an experimental model of liver damage that was well characterized histopathologically and biochemically. It induced marked pleomorphism and apoptotic bodies which were seen as acidophil bodies. Additionally, fatty changes in hepatocytes and sinusoidal lining cells, mild necrosis and inflammation, lobular disarray as well as mild dilatation of central lobular sinusoid were observed.

The histological changes induced by MTX treatment were confirmed biochemically. MTX provoked notable elevation in serum activities of ALT and AST, reflecting impaired liver function. The increase in ALT and AST is in harmony with previous reports [24,25]. ALT is a cytosolic enzyme of the hepatocyte and an increase in its activity in serum reflects a leakage in plasma membrane permeability, which in turn, is associated with cell death. ALT is considered to be one of the best indicators of liver necrosis [26]. ALT and AST are the major critical enzymes in the biological processes. They are involved in the breakdown of amino acids into \( \alpha \)-keto acid, which is routed for complete metabolism through the Krebs cycle and electron transport chain [27].

In the present study, MTX significantly altered the oxidant/antioxidant balance. MTX increased MDA level accompanied with decreased GSH content and SOD & CAT activities. Similar results were previously reported by other investigators [1,28,16]. Oxidative stress or oxidative cellular damage with its dual of free radical generation and profound lipid peroxidation are hallmarks of MTX toxicity [29]. Actually, the nadir in liver GSH content promoted by MTX represents an alteration in the cellular redox state, suggesting that the cells could be more sensitive to reactive oxygen metabolites [30] and leads to a reduction in the effectiveness of the antioxidant enzyme defense system [9]. The experimental data indicate that exaggerated inhibition of glucose-6-phosphate dehydrogenase by MTX contributes to a decrease of the availability of NADPH, an inhibition of glutathione reductase activity and finally an inhibition of GSH cycle [31]. Similarly, Rouse et al. [32] documented that intravenous administration of glutamine protects liver cells from MTX-induced oxidant injury by increasing intracellular GSH level. Additionally, the reduction in the activities of SOD and CAT may be due to the increase in MDA level or their inactivation by \( \text{H}_2\text{O}_2 \) or other ROS [16,33].

Curcumin has shown pleiotropic beneficial effects in many iatrogenic organ maladies [15]. In this study, it apparently ameliorated the gross and histological alterations in hepatocytes induced by MTX. It decreased the relative liver/body weight ratio. It also mitigated pleomorphism, apoptotic bodies, necrosis and inflammation as well as dilatation of central lobular sinusoid. It improved fatty changes with minimal microvesicular steatosis and hepatocytes became more granular.

Recently, the protective effect of curcumin on rat liver injury induced by CCl4 was demonstrated by Fu et al. [25]. They showed that curcumin administration prevented ALT and AST increases and improved liver function in CCl4-induced liver damage.

Our results showed that curcumin exhibited anti-oxidant effects not only on the non-enzymatic defense system (GSH), but also on the enzymatic one such as SOD and CAT. Treatment with curcumin following MTX apparently increased liver GSH content and increased SOD & CAT activities compared to MTX-treated animals. Similar effects of curcumin were also reported in many inflammatory conditions including gentamicin nephropathy [34], liver damage [35], type 2 diabetes [36] and renal ischemia/reperfusion injury [37]. Additionally, lipid peroxidation was markedly decreased in this study following the administration of curcumin after MTX. Similar antioxidant effects of curcumin were reported by other investigators using paracetamol [24] and aflatoxin [33] induced liver damage models.

Data so far obtained from this study would suggest that administration of curcumin after MTX challenge may have beneficial effects that could possibly be ascribed, in part, to its regulation of the oxidant/anti-oxidant balance.
Acknowledgement:
The authors express their gratitude to Dr. Ihab Shafik Atta, lecturer of pathology, Faculty of Medicine, Al-Azhar University (Assuit), for his help in histopathological examination.

REFERENCES
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