Ameliorative effect of gallic acid on methotrexate-induced hepatotoxicity and nephro toxicity in rat

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Abstract

We investigated the protective effect of gallic acid (GA) against methotrexate (MTX)-induced hepatotoxicity and nephrotoxicity. Male Wistar rats were randomized into five groups (n = 6/group): I, control; II, MTX-treated for seven days; III, pre-treated with GA for seven days, followed by MTX for seven days; IV, co-treated with MTX and GA for seven days and V, GA for seven days. MTX caused a significant increase in plasma markers of nephrotoxicity (urea, creatinine) and hepatotoxicity (Bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transferase) compared with control. Furthermore, MTX caused a significant decrease in the activities of hepatic enzymes (superoxide dismutase, catalase, glutathione S-transferase) and non-enzymic antioxidants (Vitamin C and glutathione) in comparison with control. Several authors have reported that GA possess strong antioxidant properties and a wide array of biological and pharmacological activities such as free radical scavenging, anti-apoptotic and anti-inflammatory.

The present study is therefore aimed at evaluating the protective effect of gallic acid pre-treatment and co-treatment on methotrexate-induced hepatotoxicity, nephrotoxicity and oxidative stress in rats. In this study, the role of GA in MTX induced hepatotoxicity and renal toxicity was evaluated by assessing plasma biomarkers of hepatic and renal function as well as selected oxidative stress markers (level of lipid peroxidation, enzymic and non-enzymic antioxidants).

Materials and Methods

Drug, chemicals and reagents
Methotrexate tablets is a product of West Coast Pharmaceutical Works Ltd, Gota, Ahmedabad, India; GA (3,4,5-trihydroxybenzoic acid) (Figure 1B) is a naturally occurring phenolic compound that is known for its antioxidant properties and a wide array of biological and pharmacological activities such as free radical scavenging, anti-apoptotic and anti-inflammatory.

The present study is therefore aimed at evaluating the protective effect of gallic acid pre-treatment and co-treatment on methotrexate-induced hepatotoxicity, nephrotoxicity and oxidative stress in rats. In this study, the role of GA in MTX induced hepatotoxicity and renal toxicity was evaluated by assessing plasma biomarkers of hepatic and renal function as well as selected oxidative stress markers (level of lipid peroxidation, enzymic and non-enzymic antioxidants).

Introduction

Methotrexate (MTX), (Figure 1A) is an antimitabolite and a 4-amino, 10-methyl analogue of folate that inhibit dihydrofolate reductase (DHFR), involved in the formation of tetrahydrofolate from dihydrofolate and subsequently inhibiting DNA synthesis and cell proliferation. MTX is a cytotoxic agent used in the treatment of leukaemia and various malignancies, as well as in non-neoplastic diseases such as psoriasis, rheumatoid arthritis and as anti-inflammatory and immunosuppressive agent. Earlier studies have revealed that MTX is converted mainly to metabolites connected to glutamate (MTX-glu) in cells and tissues. The complex so formed is referred to as polyglutamated forms which is responsible for most biochemical activities of MTX. MTX is known to generate reactive oxygen species (ROS) in both normal and cancer cells resulting in oxidative damage. The anti-cancer, anti-inflammatory and immunosuppressive actions of MTX has been shown to occur via ROS generation and induction of apoptosis. MTX has been reported to induce renal and hepatic toxicity via oxidative stress and its efficacy has been limited by severe organ toxicity.

In the regulation of oxidative processes, cellular systems have been equipped with several antioxidant defense mechanisms, and there are several phytochemicals of plant origin with inherent antioxidant properties that are capable of boosting cellular enzymatic and non-enzymatic antioxidant indices thus making them excellent scavengers of free radicals.

The chemoprotective properties of plant extracts have been extensively studied and are attributed to the presence of flavonoids, anthocyanins and phenolic compounds. Gallic acid (GA) is a naturally occurring phenolic compound present in green tea, gall nut, grapes, red wine, hops, oak bark etc. Several authors have reported that GA possess strong antioxidant properties and a wide array of biological and pharmacological activities such as free radical scavenging, anti-apoptotic and anti-inflammatory. Other reported biological effects include protection against doxorubicin-induced myocardial toxicity and cyclophosphamidemduced oxidative stress.

Materials and Methods

Drug, chemicals and reagents
Methotrexate tablets is a product of West Coast Pharmaceutical Works Ltd, Gota, Ahmedabad, India; GA, glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), bis-2-nitrobenzoic acid (DTNB), epinephrine, and hydrogen peroxide (H2O2) were obtained from Sigma® Chemical Company, London, UK; Assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), urea, Creatinine, total bilirubin were purchased from Randox Laboratories Ltd. (Antrim, UK). All other reagents used were of analytical grade and of highest purity.

Materials and Methods

Experimental design
Thirty rats were randomly assigned into five experimental groups (I–V) of six animals each. The animals of each group were treated as presented in Table 1. The dose for MTX (0.2 mg/kg bw) was selected based on the recommended adult dose for rheumatoid arthritis and other inflammatory diseases while the dose for GA (20 mg/kg bw) was arrived at based...
on available literature. The respective doses were based on the average weight of animals in each treatment group and administered in one mL of distilled water. The drug doses were administered once daily by oral intubation.

**Plasma and tissue preparation**

Blood samples were collected from each animal, via retro orbitals plexus in heparinized sample tubes (Li heparin). Animals were sacrificed and liver was collected from each animal for preparation of the post-mitochondrial fraction (PMF).

Centrifugation of blood samples were done at 4000 rpm for 5 minutes in a bench centrifuge (Analytica, Athens, Greece). The plasma obtained were stored at −4°C for subsequent plasma assays. Liver samples were rinsed in ice-cold 1.15% KCl and homogenized in 4 volumes of 0.01 M potassium phosphate buffer (pH 7.4). The homogenates obtained were subjected to centrifugation at 12,500×g for 15 min at −4°C in a refrigerated centrifuge (Eppendorf UK Ltd., Stevenage, UK) and supernatants (PMF) were collected in sample tubes and used for subsequent biochemical assays.

**Biochemical analysis**

**Total protein**

The protein concentration in the liver PMF was determined according to the biuret method of Gornall et al.

**Biomarkers of renal function**

Plasma level of creatinine and urea were determined with RANDOX® diagnostic kits following the manufacturer’s procedure. The method for creatinine assays was based on Jaffé and the method of Tietz was employed for plasma urea determination.

**Biomarkers of hepatic function**

Plasma total bilirubin (TBILI) level, and activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate transaminase (AST), and gamma glutamyl transferase (γ-GT) were assayed using RANDOX® diagnostic kits based on the manufacturer’s procedure. Assay of TBILI level was based on the method of Tietz et al. Activity of ALP was determined according to the method of Tietz et al. Plasma activities of ALT and AST were measured according to the method of Reltman and Frankel. The plasma activity of γ-GT was determined following the method described by Szasz.

**Biomarkers of oxidative stress**

The level of hepatic reduced glutathione (GSH) was determined following the method of Jollow et al. Ellman’s reagent reacts with reduced glutathione, and the chromophoric product resulting from the reaction has a molar absorption at 412 nm. The level of vitamin C in the liver PMF was determined according to Jagota and Dani. Vitamin C in samples reacts with Folin-phenol reagent resulting in blue chromophore which has maximum absorption at 760 nm. Hepatic GST activity was measured following the method of Habig et al. The procedure described by Misra and Fridovich was used for the determination of hepatic superoxide dismutase activity. Hepatic catalase activity was determined by the method described by Singha. Hepatic level of lipid peroxidation (LPO) was determined by the method described by Vashney and Kale. This assay involved the reaction between thiobarbituric acid and malondialdehyde (MDA) a product of lipid peroxidation to yield a stable pink chromophore which absorbs maximally at 532 nm.

**Statistical analysis**

Data are presented as the mean ± standard deviation (SD) of six replicates. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Duncan’s multiple comparison between control and treated rats in all groups using Sigma plot statistical package (Systat Software Inc., San Jose, CA, USA). P-values less than 0.05 (P<0.05) were considered statistically significant.

**Results**

**Protective effects of gallic acid on MTX-induced changes in markers of hepatic and renal toxicity**

As presented in Table 2, administration of MTX caused a significant (P<0.05) increase in plasma creatinine and urea levels (124% and 68.5% respectively) compared to control. However, pre-treatment and co-treatment of GA with MTX attenuated the observed elevated plasma urea and creatinine levels when compared with the MTX-treated group.

The plasma level of total bilirubin (TBILI) and alkaline phosphatase (ALP) activity (biomarkers of hepatobiliary damage) increased

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**Table 1. Experimental design.**

<table>
<thead>
<tr>
<th>Treatment-Groups</th>
<th>Treatments-Duration</th>
<th>Day 1-7</th>
<th>Day 8-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (CTRL)</td>
<td>-</td>
<td>Control; distilled water</td>
<td></td>
</tr>
<tr>
<td>II (MTX)</td>
<td>-</td>
<td>0.2 mg/kg bw MTX</td>
<td></td>
</tr>
<tr>
<td>III (MTX + GA) (pre-treated)</td>
<td>20 mg/kg bw GA</td>
<td>0.2 mg/kg bw MTX</td>
<td></td>
</tr>
<tr>
<td>IV (MTX + GA) (co-treated)</td>
<td>-</td>
<td>0.2 mg/kg bw MTX + 20 mg/kg bw GA</td>
<td></td>
</tr>
<tr>
<td>V (GA)</td>
<td>-</td>
<td>20 mg/kg bw GA</td>
<td></td>
</tr>
</tbody>
</table>

CTRL: control; MTX: methotrexate; GA: gallic acid.

**Table 2. Ameliorative effect of gallic acid pre-treatment and co-treatment on methotrexate-induced changes in plasma biomarkers of renal and hepatic function in rat.**

<table>
<thead>
<tr>
<th>Group Parameter</th>
<th>Control</th>
<th>MTX</th>
<th>MTX + GA (pre-treated)</th>
<th>MTX + GA (co-treated)</th>
<th>GA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.98±0.13</td>
<td>2.2±0.1*</td>
<td>1.4±0.1*</td>
<td>1.5±0.1*</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>35.0±1.2</td>
<td>50.0±2.6*</td>
<td>43.2±2.3*</td>
<td>45.5±3.2a</td>
<td>38.9±2.9</td>
</tr>
<tr>
<td>TBILI (mg/dL)</td>
<td>0.36±0.02</td>
<td>0.86±0.03*</td>
<td>0.47±0.02*</td>
<td>0.49±0.02a</td>
<td>0.43±0.02</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>157.4±6.4</td>
<td>406.8±7.3*</td>
<td>268.8±1.9*</td>
<td>327.4±1.4*</td>
<td>287.0±7.5</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>21.5±1.6</td>
<td>43.6±2.4*</td>
<td>26.9±1.6*</td>
<td>33.0±2.2a</td>
<td>26.8±2.4</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>147.2±5.3</td>
<td>281.1±6.3*</td>
<td>195.3±4.8*</td>
<td>240.5±7*</td>
<td>193.4±3.8</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>1.5±0.2</td>
<td>3.9±0.2*</td>
<td>2.1±0.1a</td>
<td>2.3±0.1*</td>
<td>1.7±0.2</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± S.D for six rats in each group. *Significantly different from the control (P<0.05); a significantly different from the methotrexate group (P<0.05); values in parenthesis represent percentage (%) increase.
significantly (P<0.05) in rats by 138.8% and 158% respectively following MTX treatment (Table 2). However, the levels of TBILI and ALP activity were significantly ameliorated in the plasma of animals pre-treated or co-treated with GA when compared with the MTX group.

MTX treatment also caused a significant increase in the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transferase (γ-GT) (biomarkers of hepatocellular toxicity) in the plasma of rats by 102.8%, 93% and 106.7% respectively compared to values in control (Table 2). Pre-treatment and co-treatment of GA with MTX significantly ameliorated the elevated activities of plasma ALT, AST, and γ-GT when compared to MTX-treated group.

Protective effects of gallic acid on MTX-induced changes in markers of oxidative stress. Hepatic SOD and catalase activity (Table 3) were significantly reduced in the MTX-treated group by 34.0% and 47.8% when compared with control (P<0.05). Also, hepatic GST activity (Figure 2) was also significantly reduced by 38.3% in the MTX-treated rats when compared with the control. However, GA pre-treatment and co-treatment significantly ameliorated the MTX-induced decrease in hepatic activities of SOD, CAT, and GST relative to the MTX-treated group (P<0.05).

Furthermore, hepatic ascorbic acid (AA) and GSH level (Figures 3 and 4) were significantly (P<0.05) decreased following treatment with MTX by 37.9% and 38.9% when compared with the control. Conversely, pre-treatment and co-treatment with GA significantly (P<0.05) protected against the MTX-induced decrease in hepatic AA and GSH levels when compared with the MTX group. In addition, the hepatic MDA level rose significantly (P<0.05) in the MTX-treated rats by 58.8% when compared with the control (Figure 5). However, GA pre-treatment and co-treatment attenuated the increase in hepatic MDA relative to the MTX-treatment group.
Discussion and Conclusions

Methotrexate (MTX) is a chemotherapeutic agent indicated in conditions such as autoimmune diseases, inflammatory myopathies, leukemia etc. However it is known to be associated with side effects including hepatotoxicity and nephrotoxicity; often mediated by reactive oxygen species (ROS). Recent studies have demonstrated the protective roles of phytochemicals in drug-induced organ toxicity. Gallic acid employed in the present study is a potent natural antioxidant with numerous biological activities including hepatoprotective activity.

Biomarkers of renal function: urea and creatinine were considered in this study. Urea and creatinine are metabolic products removed by the kidney from circulation. Increase in their plasma level is an indication of loss in renal function. The increase in plasma urea and creatinine observed in this study is in agreement with previous report on MTX. The attenuation of the level of plasma urea and creatinine by GA is an indication of the involvement of free radicals in MTX-mediated toxicity. The amelioration of hepatic LPO by GA in the study may be related to the free radical scavenging properties of gallic acid.

In summary, current findings suggest that gallic acid has the potential to protect against Methotrexate-induced hepatotoxicity and nephrotoxicity. In addition, the mechanism of protection by gallic acid may involve free radical scavenging. Therefore, gallic acid may be employed as a co-therapy in MTX chemotherapy as a protection against chemotherapy-associated oxidative damage to tissues.

Research highlights
- Chemotherapy-associated oxidative stress is a relevant side effect of most anticancer agents.
- Methotrexate (MTX) acts as an antimetabolite as its anticancer mechanism.
- Gallic acid is a plant derived antioxidant.
- Hepatotoxicity, nephrotoxicity and oxidative stress was observed following exposure to methotrexate.
- Administration of gallic acid as a pre-treatment or co-administered with MTX ameliorated MTX induced toxicity in rats.

References