Protective Effects of Biochanin A against Methotrexate-Induced Acute Liver Injury in Rats

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ABSTRACT

Background: Methotrexate (MTX) is one of the most commonly used chemotherapeutic agents in the treatment of cancer patients, however its therapeutic use is limited due to dose dependent hepatotoxicity caused by oxidative damage. Biochanin A (BCA), a naturally occurring dietary isoflavone, has strong cytoprotective, anti-inflammatory, and antioxidant properties. Although protective actions of BCA against various chemical-induced hepatotoxicity including that of carbon tetrachloride and arsenic, none of the studies was made on MTX-induced acute liver injury. Methods: Wistar rats were separated into four groups; Control, MTX, Control + BCA, MTX + BCA. BCA (50 mg/kg/day) or vehicle (dimethyl sulfoxide; 1 mL/kg/day) with the same volume was intraperitoneally injected for 5 days. At sixth day, a single dose of MTX (20 mg/kg) was injected to rats. Twenty-four h after the MTX administration, rats were sacrificed and then blood and liver samples were obtained for biochemical and histopathologic analyses. Results: MTX increased the serum AST, ALT, and LDH levels. Furthermore, malondialdehyde (an indicator of oxidative injury) and myeloperoxidase (an indicator of neutrophil infiltration) levels increased, while total glutathione levels (an indicator of antioxidant status) decreased in liver. MTX-induced hepatotoxicity was also observed histopathologically. BCA substantially improved these alterations induced by MTX administration. Conclusion: These results indicate that BCA may be useful in preventing the MTX-induced acute liver injury due to its antioxidant and anti-inflammatory properties.

Key words: Methotrexate, Hepatotoxicity, Biochanin A, Protective effects

INTRODUCTION

Methotrexate (MTX), a folic acid antagonist, has dose-dependent cytotoxic and immunosuppressive effects that has been used for many years to treat a wide range of disease including different types of inflammatory diseases and cancer.1,2 Despite current widespread use of MTX, hepatotoxicity, one of the serious complications of MTX, limits its use and leads to malabsorption and diarrhea.3,4,5 The exact underlying pathophysiological mechanism of MTX-induced hepatotoxicity is not well known. However, several studies have suggested that MTX triggers neutrophil infiltration, lipid peroxidation, and excessive release of free radicals.6,7,8 Therefore, it is believed that administration of antioxidant may inhibit the MTX-induced liver damage. Biochanin A (5,7-Dihydroxy-4′-methoxyisoflavone, BCA) is a member of a natural organic compound classified under the phytochemicals known as isoflavonoids. BCA is predominantly found in red clover, soy, peanuts, and alfalfa sprouts.9,10 Recent studies have found that BCA possesses strong anti-oxidant, anti-inflammatory, and cytoprotective effects.11,12,13,14 However, there...
are no detailed studies on potential beneficial effects of BCA against MTX-induced hepatotoxicity. In the light of above information, it was aimed to examine whether pre-administration of BCA can protect against MTX-induced liver damage.

**MATERIALS AND METHODS**

**Animals and experimental protocols**

Twenty-eight Wistar albino female rats aged 75-90 days and weighing between 250-300 g were used for this study (Experimental Research Center of Selcuk University, Konya, Turkey). They were maintained in an air-conditioned room with a 12:12 h light/dark illumination sequence at 50 ± 10% humidity and 21 °C temperature. Animals had *ad libitum* access to commercial laboratory rodent pellet diet and tap water (drinking bottle). During the experiments animals to be randomly distributed in different cages following the each procedure and all animals were housed in the same animal room. This study was conducted in accordance with the standards for the care and use of animal subjects, as mentioned in the Guide for the Care and Use of Laboratory Animals (2011, published by National Academies Press) and Animal Ethics Committee of Selcuk University approved the protocol (Approval Number: 2015/57).

Animals were randomly separated into four subgroups as follows: Control, MTX, MTX+BCA, Control+BCA. The animals in the MTX+BCA and Control+BCA groups were intraperitoneally injected with BCA (50 mg/kg/day) for 5 consecutive days. At the same time, rats in the Control and MTX groups were intraperitoneally injected with dimethyl sulfoxide (DMSO) as the solvent and vehicle of BCA at the same volume. After the BCA and vehicle administrations, MTX was given to rats in the MTX and MTX+BCA groups at a single dose of 20 mg/kg and physiological saline was intraperitoneally injected to rats in the Control and Control+BCA groups at the same volume. Variations in body weight and behavioural changes were monitored during the period of experiment. Blood and tissue samples were collected 24 h after the last injection. Rats were fasted overnight one day before the sample collection. Animals were fasted overnight and intraperitoneally injected with sodium pentobarbital (60 mg/kg) to induce general anesthesia. Approximately 1 mL blood sample was collected into the Eppendorf tubes via cardiac puncture, abdominal cavity was then opened and liver tissue was carefully removed and weighed. Following this procedure rats were immediately euthanized by cervical dislocation. The piece of liver tissue was transferred into the Eppendorf tubes and stored at -80 °C until myeloperoxidase (MPO), malondialdehyde (MDA), and total glutathione measurements. After waiting 15-20 min blood specimens were centrifuged for 5 min at 4000xg and obtained aliquots of serum were frozen and stored at -80 °C for several biochemical assays. Additionally, rest of liver tissue was harvested and fixed in the solution of 10% neutral buffered formalin for histopathological analysis.

**Biochemical analyses**

The frozen aliquots of serum were thawed and then mixed. Hepatic function markers, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as lactate dehydrogenase (LDH; a nonspecific indicator for tissue injury) were calculated with the standard auto analyzer methods on the Abbott Architect ci16000 system by using the commercial reagents in accordance with the producer’s protocols (Abbott Laboratories, Illinois, USA).

On the day of biochemical analyses in the liver tissues, tissue samples were homogenized in cold potassium phosphate-buffered solution (pH = 7.4, 50 mM, and 10 % weight/volume) on an ice cube with a homogenizer (IKA T10 basic Ultra-Turrax Homogenizer). The obtained homogenate was centrifuged at 10000 × g for 20 min and then clear supernatant was separated for biochemical measurements. MDA, an end product of lipid peroxidation, is a useful marker for oxidative stress-induced organ damage. To evaluate the antioxidant capacity of liver samples, total glutathione (reduced and oxidized forms) levels were assayed. MPO is an early biomarker of inflammation and neutrophil activation. The MDA and total glutathione levels, as well as MPO activity in liver tissues were determined with the commercial kits in accordance with the manufacturer protocol.

**Histopathological Examination**

The formaldehyde-fixed tissues were embedded into paraffin and cut in 5-μm thick sections. The obtained sections were placed on slides and stained by hematoxylin and eosin. Microscopic organ damage scoring was performed by experienced pathologist. The pathologist was unaware of the treatments and evaluated tissue sections with a conventional light microscope (Carl Zeiss Axio Lab.A1) and photographed them with the camera of Axiocam MRc5 microscope. Severity of liver damage was semiquantitatively scored by the blinded pathologist as 0 (absent), 1 (slight), 2 (moderate), and 3 (severe) for each criteria including congestion/hemorrhage, inflammation, necrosis/degeneration, and sinusoidal dilatation.
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Drugs and chemicals
MTX was purchased from Kocak Pharmaceuticals (Istanbul, Turkey). BCA (Cat. No. D2016), sodium pentobarbital (P-010), total glutathione assay kit (CS0260), lipid peroxidation (MDA) assay kit (MAK085), and MPO Colorimetric Activity Assay Kit (MAK068) were provided by Sigma Aldrich (St Louis, MO, USA). Formaldehyde, paraffin, hematoxylin, and cosin were obtained from Merck Millipore (Billerica, MA, USA).

Statistical analyses
GraphPad Prism 5 software (San Diego, California, USA) was used for analysis of data. All data were expressed as mean ± standard deviation (mean ± SD) and analyzed by using one-way analysis of variance (ANOVA) with Newman–Keuls multiple comparison post-test. A value of \( p < 0.05 \) was considered as statistically significant. \( n = 7 \) for each group. *Denotes significant difference between Controls vs. other groups. #denotes significant difference between MTX vs. MTX+BCA group (*, #, \( p<0.05; **, ##, p<0.01; ###, p<0.001 \).

RESULTS
The body weight and also weight of liver were shown in Table 1. MTX did not effect the weights of body and liver. MTX administered rats showed clinical signs such as dullness, hunched posture, anorexia, and lethargy. Besides, there was no death during the experimental period.

Effects of BCA on biochemical measurements
Increases of the serum ALT and LDH levels caused by MTX administration were completely prevented by BCA. Additionally, BCA improved the MTX-caused elevation in the serum AST levels but could not totally blocked Table 2. As shown in Table 3, liver MDA and MPO levels were elevated in the MTX group when compared with Control group (\( p < 0.001 \) and \( p < 0.01 \). Pretreatment with BCA completely blocked the MTX-caused elevations of MDA and MPO levels in liver tissues. Moreover, MTX led to a significant reduction in total glutathione levels of the liver tissues when compared with Control group (\( p < 0.01 \). BCA also improved the MTX-induced reduction of total glutathione levels in the liver but could not totally block.

Histopathological changes in liver by BCA
The structures of liver samples were normal in the Control Figure-1A and Control+BCA Figure 1D groups. However, MTX caused substantial histopathological changes in the liver samples including mild to moderate sinusoidal and portal hemorrhage, mild inflammatory mononuclear cell infiltration in periportal area and parenchyma, dilatation of sinusoids, centrilobular and periportal degeneration and necrosis Figure 1B. When the rats were treated with BCA the liver Figure 1C were totally prevented from the histopathological damage caused by MTX. Furthermore, beneficial effects of BCA on MTX-caused organ injury were also shown in Table 4 with histopathological damage scoring.

DISCUSSION
MTX is now used to treat a wide variety of disorders. Approaches for reducing the MTX treatment-induced hepatotoxicity complications are valuable for success of the treatment and improving the life quality of the

| Table 1: General outcome parameters of the rats. |
|-------------------------------|----------------------------|------------------|
|                               | Body weight (g)           | Liver weight (g) | Liver-Body weight Ratio (%) |
|                               | Initial                  | Final            |                                |
| Control                       | 272 ± 13                 | 276 ± 16         | 22.71 ± 2.14                   | 8.25 ± 0.93 |
| MTX                           | 267 ± 15                 | 276 ± 14         | 22.02 ± 2.16                   | 7.99 ± 1.01 |
| MTX+BCA                       | 264 ± 15                 | 278 ± 18         | 23.86 ± 1.95                   | 8.64 ± 1.11 |
| Control+BCA                   | 277 ± 8                  | 297 ± 15         | 23.14 ± 1.35                   | 7.82 ± 0.78 |

| Table 2: Serum biochemical markers. |
|-----------------------------------|-----------------|-----------------|-------------------|
|                                  | AST (IU/L) | ALT (IU/L) | LDH (IU/L)        |
| Control                          | 76 ± 27    | 34 ± 12    | 232 ± 68          |
| MTX                              | 244 ± 142  | 99 ± 67    | 463 ± 208         |
| MTX+BCA                         | 165 ± 71*   | 57 ± 28*   | 291 ± 193*           |
| Control+BCA                     | 69 ± 23    | 30 ± 5     | 222 ± 69          |

| Table 3: Tissue oxidative stress parameters. |
|-------------------------------------------|-----------------|-----------------|-------------------|
|                                  | Liver MDA (nmol/mg) | Liver glutathione (nmol/mg) | Liver MPO (mU/mL) |
| Control                          | 6.83 ± 0.47     | 189 ± 40        | 57 ± 12           |
| MTX                             | 9.98 ± 2.29**   | 74 ± 42**       | 92 ± 37**         |
| MTX+BCA                         | 6.73 ± 0.85**   | 137 ± 31**      | 40 ± 17**         |
| Control+BCA                     | 6.66 ± 0.89     | 185 ± 52        | 38 ± 13           |
Table 4: Histopathological organ damage scores.

<table>
<thead>
<tr>
<th></th>
<th>congestion/hemorrhage</th>
<th>Inflammation</th>
<th>necrosis/degeneration</th>
<th>sinusoidal dilatation</th>
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<tr>
<td>Control</td>
<td>0.14 ± 0.38</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>MTX</td>
<td>1.14 ± 0.38***</td>
<td>0.86 ± 0.69*</td>
<td>0.71 ± 0.49</td>
<td>0.57 ± 0.53**</td>
</tr>
<tr>
<td>MTX+BCA</td>
<td>0.29 ± 0.49***</td>
<td>0.14 ± 0.38**</td>
<td>0.14 ± 0.38</td>
<td>0.14 ± 0.38</td>
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<tr>
<td>Control+BCA</td>
<td>0.14 ± 0.38</td>
<td>0.00 ± 0.00</td>
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Figure 1: Effects of BCA on MTX-induced histopathological changes in the liver samples. The photographs show the representative histopathological liver sections that were obtained from the rats in Control (1A), MTX (1B), MTX+BCA (1C), and Control+BCA (1D) groups. The liver tissues show normal histopathological structures in the Control group. There are also normal liver in the Control+BCA group. Figure-1B shows the liver tissues in the MTX group with mild to moderate sinusoidal portal hemorrhage (4-point star), mild inflammatory mononuclear cell infiltration in the parenchyma and portal area (arrow), dilatation of sinusoids (6-point star), centrilobular and periportal degeneration and necrosis (arrowhead). Figure-1C: normal liver architecture in MTX+BCA group.

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The deleterious effects of oxidative damage caused by MTX could be attributed to lipid peroxidation. MDA levels, an end product of lipid peroxidation chain and a useful marker for lipid peroxidation, are known to be increased in response to oxidative stress status. The result of increased liver MDA levels in the present study suggests the presence of increased lipid peroxidation induced by MTX. MPO, a heme-containing enzyme and mainly located in neutrophils, is released in high amounts in the process of inflammation. MPO generates hypochlorous acid utilizing the hydrogen peroxide and chloride. Hypochlorous acid is a strong oxidizing agent that produces the free radicals during the oxidation of hydrazines. Glutathione, a small tripeptide and the major free thiol in most mammalian cells, plays a role in many important biological processes including removal of hydroperoxides, detoxification of xenobiotic, scavenging free radicals, DNA synthesis, and maintenance of the oxidation state of protein sulfhydryls. AST and ALT are present in high concentration in the hepatocytes, however loss of hepatocyte membrane integrity due to lipid peroxidation and free radicals leads to release of AST, ALT, as well as LDH into the bloodstream.

In our study we showed that administration of MTX caused oxidative tissue injury as determined by increased lipid peroxidation, excessive free radical production via activated neutrophils, whereas depletion of antioxidant defense system in the liver in accordance with the literature. Furthermore, we also observed histopathological deleterious effects of MTX administration in the liver. Thus, agents like BCA that have strong antioxidative and anti-inflammatory properties may inhibit the MTX-induced liver injury.

BCA has shown potent antioxidant, anti-inflammatory, and cytoprotective effects on various experimental studies such as lipopolysaccharide/D-galactosamine-induced acute liver injury, ethanol-induced gastric mucosal ulceration, and cerebral ischemia/reperfusion injury. Arsenic and carbon tetrachloride are well-known hepatotoxins that lead to hepatocyte degeneration, necrosis, and inflammation. Jalaludeen et al. showed that BCA ameliorated the arsenic-induced hepatotoxicity in rats. Furthermore, it was shown that BCA has protective effects against hepatotoxicity induced by carbon tetrachloride. However, to the best of our knowledge, there is no study examining the possible protective effects of BCA on MTX-induced acute liver injury.

In our study, it was found that BCA prevented the hepatotoxicity induced by MTX administration in rats. This could be due to suppression of the lipid peroxidation, free radical production, and neutrophil activity, whereas boost of the antioxidant capacity and hepatocyte membrane integrity and function.

patients. Liver is the main organ for the metabolism of MTX, therefore elevated free radical generation in the hepatic tissues due to use of MTX is associated with the hepatotoxic effects. Free radicals mainly target the cell membrane lipids that can lead to cellular damage via peroxidation of lipids. The liver is highly vulnerable to oxidative stress due to its high lipid ingredient. The deleterious effects of oxidative damage caused by MTX could be attributed to lipid peroxidation. MDA levels, an end product of lipid peroxidation chain and a useful marker for lipid peroxidation, are known to be increased in response to oxidative stress status. The result of increased liver MDA levels in the present study suggests the presence of increased lipid peroxidation induced by MTX. MPO, a heme-containing enzyme and mainly located in neutrophils, is released in high amounts in the process of inflammation. MPO generates hypochlorous acid utilizing the hydrogen peroxide and chloride. Hypochlorous acid is a strong oxidizing agent that produces the free radicals during the oxidation of hydrazines. Glutathione, a small tripeptide and the major free thiol in most mammalian cells, plays a role in many important biological processes including removal of hydroperoxides, detoxification of xenobiotic, scavenging free radicals, DNA synthesis, and maintenance of the oxidation state of protein sulfhydryls. AST and ALT are present in high concentration in the hepatocytes, however loss of hepatocyte membrane integrity due to lipid peroxidation and free radicals leads to release of AST, ALT, as well as LDH into the bloodstream.

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CONCLUSION

Our study reports that BCA has hepatoprotective effects against acute liver injury caused by MTX by reducing oxidative stress and inflammatory process. According to these results, pretreatment with BCA may provide a novel therapeutic approach for prevention of MTX-induced hepatotoxicity. Additional investigations are also required to show the clinical implication of BCA in patients receiving MTX.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS USED

MTX: Methotrexate; BCA: Biochanin A; DMSO: dimethyl sulfoxide; MPO: Myeloperoxidase; MDA: malondialdehyde; AST: Aspartat aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; ANOVA: One-way analysis of variance.

REFERENCES

In this study, we planned to investigate the possible protective effects of BCA on MTX-induced liver injury in rats. Rats were injected with a single dose of MTX (20 mg/kg) to induce acute liver injury. Rats were pretreated with BCA for 5 days (50 mg/kg) to prevent the MTX-induced organ damage in the liver. BCA administration to rats prevented histopathological and biochemical alterations induced by MTX.

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