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Research Paper

Caffeic acid phenethyl ester (CAPE) prevents methotrexate-induced hepatorenal oxidative injury in rats

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Abstract

Objectives This study aimed to investigate the antioxidant and anti-inflammatory effects of caffeic acid phenethyl ester (CAPE) on the methotrexate (MTX)-induced hepatorenal oxidative damage in rats.

Methods Following a single dose of methotrexate (20 mg/kg), either vehicle (MTX group) or CAPE (10 µmol/kg, MTX + CAPE group) was administered for five days. In other rats, vehicle (control group) or CAPE was injected for five days, following a single dose of saline injection. After decapitation of the rats, trunk blood was obtained, and the liver and kidney tissues were removed for histological examination and for the measurement of malondialdehyde (MDA) and glutathione (GSH) levels and myeloperoxidase (MPO) and sodium potassium-adenosine triphosphatase (Na$^+$/K$^+$-ATPase) activity. TNF-α and IL-1β levels were measured in the blood.

Key findings Methotrexate administration increased the tissue MDA levels, MPO activity and decreased GSH levels and Na$^+$/K$^+$-ATPase activity, while these alterations were reversed in the CAPE-treated MTX group. Elevated TNF-α and IL-1β levels were also reduced with CAPE treatment.

Conclusions The results of this study revealed that CAPE, through its anti-inflammatory and antioxidant actions, alleviates methotrexate-induced oxidative damage, which suggests that CAPE may be of therapeutic benefit when used with methotrexate.

Keywords caffeic acid phenethyl ester; kidney; liver; methotrexate; oxidative damage

Introduction

Methotrexate is a drug commonly used in high doses in malignancies, primarily in leukaemias.[1,2] In the last 50 years, methotrexate has been used in low doses in various inflammatory diseases including psoriasis and rheumatoid arthritis.[3] However, the efficacy of this agent is often limited by severe side effects and toxic sequelae. The cytotoxic effects of methotrexate are not selective for cancer cells, but also affect normal tissues that have a high rate of proliferation, including the haematopoietic cells of the bone marrow and the actively-dividing cells of the gut mucosa.[4] Moreover methotrexate has been shown to have potential side effects on many organs, particularly on the liver and kidney.[5,6] Methotrexate produces free oxygen radicals and thus causes lipid peroxidation by affecting the lipid components of the cell membrane. Consequently, these free radicals lead to mitochondrial functional impairment.[7,8] Long-term methotrexate use, or its use in high doses, may cause hepatic steatosis, cholestasis, fibrosis and cirrhosis[9] as well as acute renal failure.[10] Accordingly, the dose of methotrexate should be lowered or the drug should be discontinued in case of hepatorenal toxicity which causes delay in the treatment of the disease. On the other hand much attention is now being paid to factors that may enhance the effectiveness of existing drugs while reducing their unwanted side effects.

Caffeic acid phenethyl ester (CAPE), a flavonoid-like compound and an active component of propolis from honeybee hives, has been used in traditional medicine for decades.[11] CAPE is a flavonoid-like compound, the anti-inflammatory, antioxidant, antiviral and anti-cancer characteristics of which have been proven in previous studies.[12–18] CAPE completely blockades the reactive oxygen species produced by neutrophils or by the xanthine/xanthine oxidase

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system. CAPE exerts antioxidant characteristics by suppressing the lipid peroxidation and inhibiting the activity of xanthine oxidase and nitric oxide synthase.[19,20] Although the antioxidative and protective effects of CAPE against methotrexate-induced renal, cerebellar and testicular toxicity have been studied,[21–23] the hepatic effects of CAPE against methotrexate toxicity are not clear yet. Furthermore the effect of CAPE on methotrexate-induced cytokines has not been studied before. Thus, we aimed to investigate the effects of CAPE treatment against methotrexate-induced oxidative injury on the main elimination organs, liver and kidney, and associated morphologic changes using biochemical methods and histological examinations.

Materials and Methods

All experimental protocols were approved by the Animal Care and Use Committee of Marmara University Faculty of Medicine. Both sexes of Wistar albino rats, 200–250 g, were maintained at a constant temperature ($22 \pm 1°C$) with a 12-h light–dark cycle.

Methotrexate (Onco-Tain; Faulding Pharmaceutics Plc, Leamington Spa, UK), CAPE (Sigma, St Louis, USA) and physiological physiological saline were injected intraperitoneally. CAPE was dissolved in 0.1% dimethyl sulfoxide (DMSO). Following a single dose of methotrexate (20 mg/kg), either vehicle (MTX group) or CAPE (10 µmol/kg, MTX + CAPE group) was administered for five days. In other rats, vehicle (control group) or CAPE (CAPE group) was injected for five days, following a single dose of saline injection. At the end of the experiment rats were decapitated and blood samples were obtained for the measurement of tumour necrosis factor-alpha (TNF)-α and interleukin-1-beta (IL-1β). The levels of malondialdehyde (MDA) and glutathione (GSH), as well as myeloperoxidase (MPO) and sodium-potassium adenosine triphosphatase (Na$^+$/K$^+$-ATPase) activity were analysed in the hepatic and renal tissues. Furthermore interstitial inflammation and perihepatic and perinephritic necroses were evaluated via histological examination of the tissue sections under a light microscope.

Measurement of malondialdehyde and glutathione levels

To determine MDA and GSH levels, hepatic and renal tissue samples were homogenized in ice-cold 150 mM KCL. The MDA levels were assayed for the products of lipid peroxidation.[24] Results were expressed as nmol MDA/g tissue. The GSH levels were measured by spectrophotometric method using Ellman's reagent.[25] Results were expressed as µg GSH/g tissue. Serum MDA and GSH levels were measured using the same method.

Measurement of myeloperoxidase activity

Tissue-associated MPO activity was measured according to the procedure reported by Hillegas et al.[26] Hepatic and renal tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0) and homogenates were centrifuged at 41 400g for 10 min; pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide.

After three cycles of freezing and thawing, with sonication between the cycles, the samples were centrifuged at 41 400g for 10 min. Volumes of 0.3 ml were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H$_2$O$_2$ solution. One unit of enzyme activity was defined as the amount of MPO that caused a change in the absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Measurement of Na$^+$/K$^+$-ATPase activity

The measurement of Na$^+$/K$^+$-ATPase activity was based on the measurement of inorganic phosphate produced from 3 mM disodium adenosine triphosphate added to the incubation medium.[27] The medium (containing in mM: 100 NaCl, 5 KCL, 6 MgCl$_2$, 0.1 EDTA and 30 Tris HCL (pH 7.4)) was incubated at 37°C in water bath for 5 min. Following this pre-incubation period, Na$_2$ATP, at a final concentration of 3 mM, was added into each tube and incubated at 37°C for 30 min. After the incubation, the tubes were placed in an ice bath to stop the reaction. The mixture was then centrifuged at 3500g, and Pi in the supernatant fraction was determined by the method of Fiske and Subarrow.[28] The specific activity of the enzyme was expressed as nmol Pi mg^{-1} protein h^{-1}. The protein concentration of the supernatant was measured by the Lowry method.[29]

Biochemical analysis

Plasma TNF-α and IL-1β were analysed using enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Nivelles, Belgium) in accordance with the manufacturer's instructions and guidelines. These assay kits were particularly selected because of their high degree of sensitivity, specificity and inter-assay and intra-assay precision, and due to the fact that they require a small amount of plasma sample.

Microscopic evaluation

For the light microscope examinations, hepatic and renal tissue samples were fixed in 10% formaldehyde and processed routinely for embedding in paraffin. Tissue sections, 5 µm thick, were stained with hematoxylin and eosin (H&E) and examined under an Olympus BX51 (Tokyo, Japan) photomicroscope.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, USA). All data were expressed as mean $\pm$ standard error of the mean (SEM). Group comparisons were performed with analysis of variance followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

The serum levels of TNF-α and IL-1β were significantly higher in the MTX group than in the control groups ($P < 0.001$), whereas these levels were found to be significantly lower in the CAPE-treated MTX group than in the vehicle-treated MTX group ($P < 0.001$; Table 1).

The GSH levels in the hepatic and renal tissues were decreased in the vehicle-treated MTX group ($P < 0.01$), while the GSH levels in both tissues were found to be restored with CAPE treatment ($P < 0.05$, Figure 1). Methotrexate

Table 1 Serum tumour necrosis factor-α and interleukin-1β levels of vehicle-treated or caffeic acid phenethyl ester (CAPE)-treated control and methotrexate groups

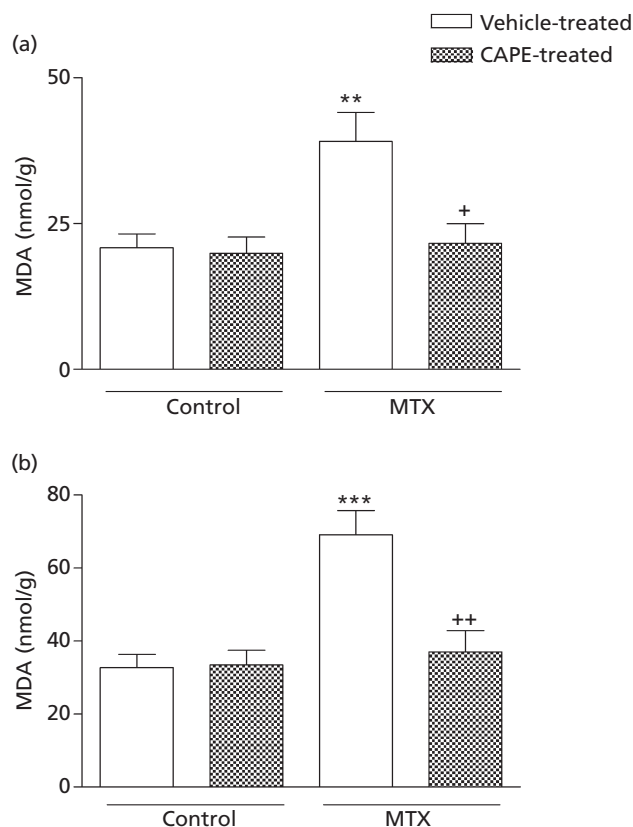|	Control		Methotrexate	
	Vehicle-treated	CAPE-treated	Vehicle-treated	CAPE-treated
TNF-α (pg/ml)	8.85 ± 1.26	9.15 ± 1.48	35.90 ± 3.83*	15.75 ± 2.87**
IL-1β (pg/ml)	10.57 ± 1.56	11.89 ± 1.72	34.57 ± 3.41*	17.82 ± 2.71**

TNF-α, tumour necrosis factor-α; IL-1β, interleukin-1β. *$P < 0.001$ compared with control groups; **$P < 0.001$ compared with vehicle-treated methotrexate group. Data represent mean ± SEM.
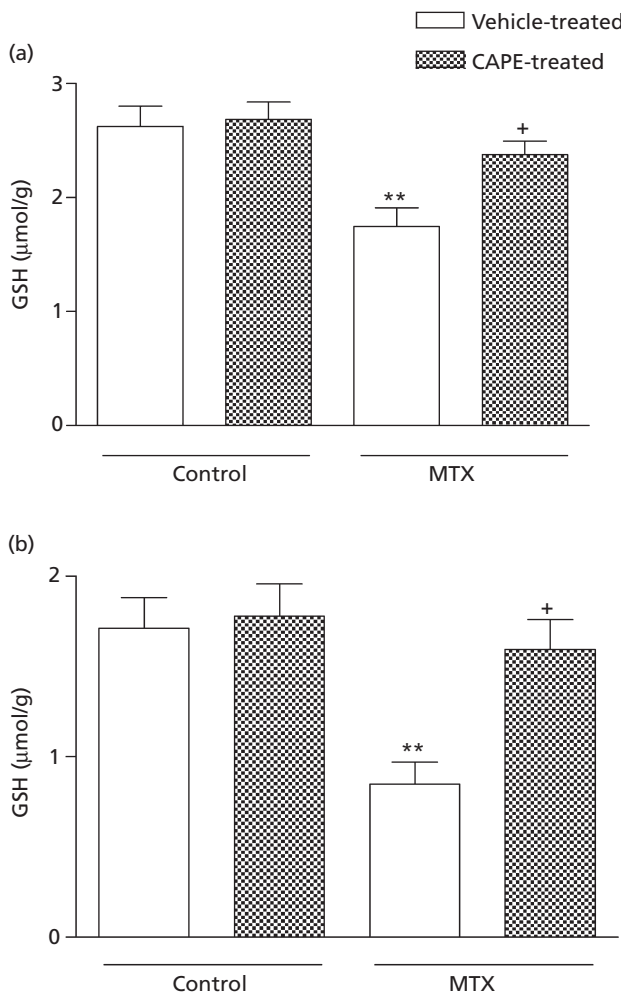
Figure 1 Glutathione (GSH) levels in the (a) hepatic and (b) renal tissues of vehicle-treated or caffeic acid phenethyl ester (CAPE)-treated control and methotrexate (MTX) groups. **$P < 0.01$ compared with control groups; +$P < 0.05$ compared with vehicle-treated MTX group.
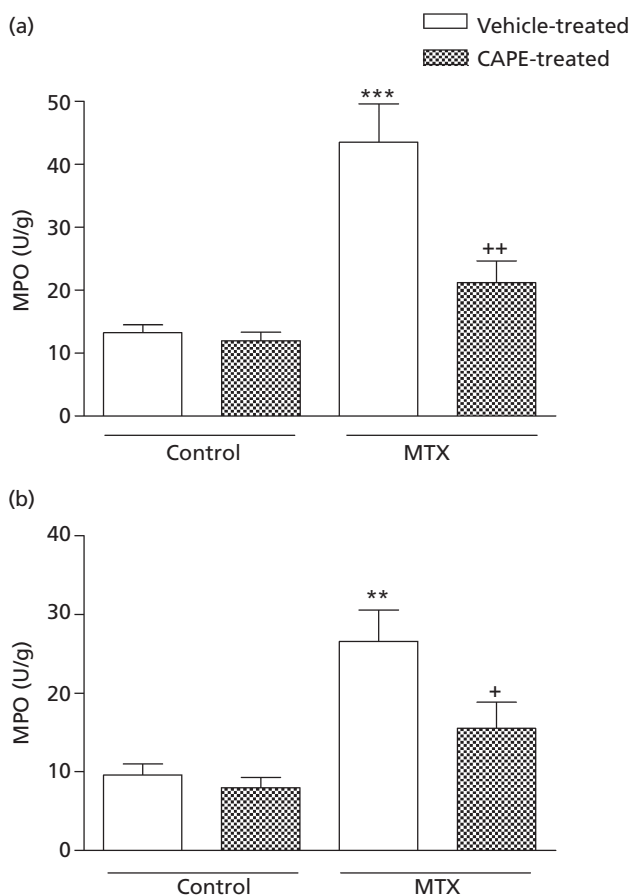

Figure 2 Malondialdehyde (MDA) levels in the (a) hepatic and (b) renal tissues of vehicle-treated or caffeic acid phenethyl ester (CAPE)-treated control and methotrexate (MTX) groups. **$P < 0.01$, ***$P < 0.001$ compared with control groups; +$P < 0.05$, ++$P < 0.01$ compared with vehicle-treated MTX group.

administration caused significant increase in the hepatic and renal tissue MDA levels ($P < 0.01$ and $P < 0.001$, respectively), which were reversed back significantly with CAPE treatment. ($P < 0.05$ and $P < 0.01$, respectively; Figure 2).

Methotrexate administration increased the neutrophil infiltration and, accordingly, the MPO values in the hepatic and renal tissues as compared with the control groups ($P < 0.001$ and $P < 0.01$, respectively), whereas MPO activity in these tissues significantly decreased in the CAPE-treated MTX group ($P < 0.01$ and $P < 0.05$, respectively; Figure 3).

The Na$^+$/K$^+$-ATPase activity in the hepatic and renal tissues were lower in the vehicle-treated MTX group as compared to the control groups ($P < 0.05$). On the other hand, the Na$^+$/K$^+$-ATPase activity in the hepatic and renal tissues was significantly increased in the CAPE-treated MTX group ($P < 0.05$, Figure 4).

As shown in Figure 5, degenerated hepatocytes, dilatation and vascular congestion in sinusoids and inflammatory cell infiltration around the portal area and central vein, seen in the liver parenchyma following methotrexate treatment (Figure 5c), were replaced with the liver parenchyma showing
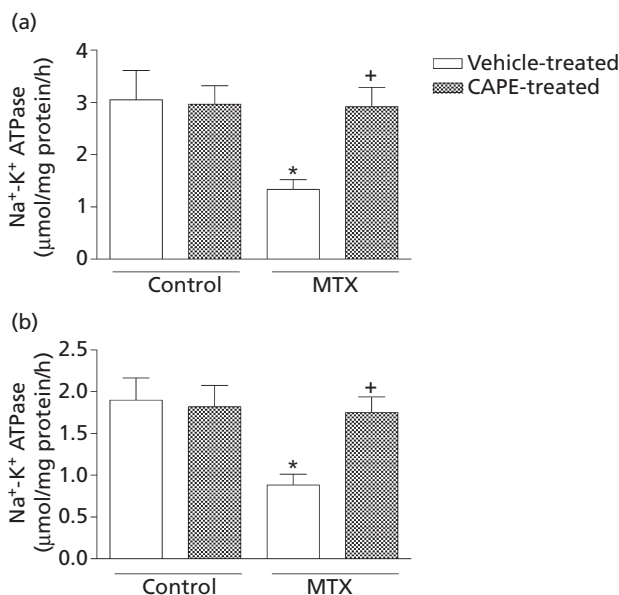
(a)

(b)

Figure 3 Myeloperoxidase (MPO) activity in the (a) hepatic and (b) renal tissues of vehicle-treated or caffeic acid phenethyl ester (CAPE)-treated control and methotrexate (MTX) groups. **$P < 0.01$, ***$P < 0.001$ compared with control groups; ++$P < 0.01$, +$P < 0.05$ compared with vehicle-treated MTX group.

(a)

(b)

Figure 4 Na^+/K^+-ATPase activity in the (a) hepatic and (b) renal tissues of vehicle-treated or caffeic acid phenethyl ester (CAPE)-treated control and methotrexate (MTX) groups. *$P < 0.05$ compared with control groups; +$P < 0.05$ compared with vehicle-treated MTX group.

normal hepatocytes, decreased number of activated Kupffer cells and mild sinusoidal congestion in most regions of the CAPE-treated group (Figure 5d).

Furthermore methotrexate treatment caused glomerular congestion and degeneration, dilatation in Bowman's space and tubular degeneration in the kidney (Figure 5g). On the other hand, in the CAPE-treated MTX group the kidney showed mild glomerular and tubular degeneration (Figure 5h). In control (Figure 5a and 5e) and CAPE-treated control (Figure 5b and 5f) groups, liver and kidney parenchyma showed regular morphology.

Discussion

Our findings revealed that CAPE, with its free radical scavenging properties, prevented methotrexate-induced lipid peroxidation and neutrophil infiltration of the hepatic and renal tissues in rats, while the depleted antioxidant GSH level and the inhibited Na^+/K^+-ATPase activity were increased back to control levels. Furthermore, CAPE treatment decreased the plasma cytokines and improved the tissue morphological changes caused by methotrexate.

Methotrexate is an antimetabolite that impairs the DNA synthesis by competitively inhibiting folic acid metabolism. In the liver, methotrexate is metabolized to 7-hydroxy-methotrexate (7-OH-MTX), which is a major extracellular metabolite, via an enzymatic system.[30] Methotrexate is stored in a polyglutamate form in the cell. The amount of intracellular polyglutamate increases and the level of folic acid decreases with methotrexate use, leading to hepatocyte necrosis.[31] An increase in its polyglutamate form increases the intracellular level of methotrexate. This mechanism is considered the reason for the hepatotoxic effect of methotrexate. The hepatotoxic and nephrotoxic effects of methotrexate have been reported in various clinical and experimental studies.[1,21,32] Administration of methotrexate in high doses may result in acute renal failure possibly due to the precipitation of methotrexate or 7-OH-MTX in the renal tubules. This nephrotoxicity leads to delayed methotrexate elimination,[33] which further increases the toxicity.

It is well known that oxidative damage targets cell lipids, especially those within membrane bilayers, containing a large quantity of unsaturated fatty acids, nucleic acids and proteins, despite the tissue's intrinsic oxidative defence system.[34] Lipid peroxidation, mediated by oxygen free radicals, is believed to be an important cause of destruction and damage to cell membranes and has been suggested to be a contributing factor to the development of methotrexate-mediated tissue damage.[21–23,30] Furthermore membrane-bound proteins are highly vulnerable to both direct oxidative modification and changes in their lipid environment. Na^+/K^+-ATPase is one cellular oxidative stress target.[34] It has been demonstrated that during free radical attack lipid peroxidation products increase and due to disorder of the lipid bilayer Na^+/K^+-ATPase activity is suppressed.[35] In this study, a single dose of methotrexate caused significant tissue injury since MDA, an end product of lipid
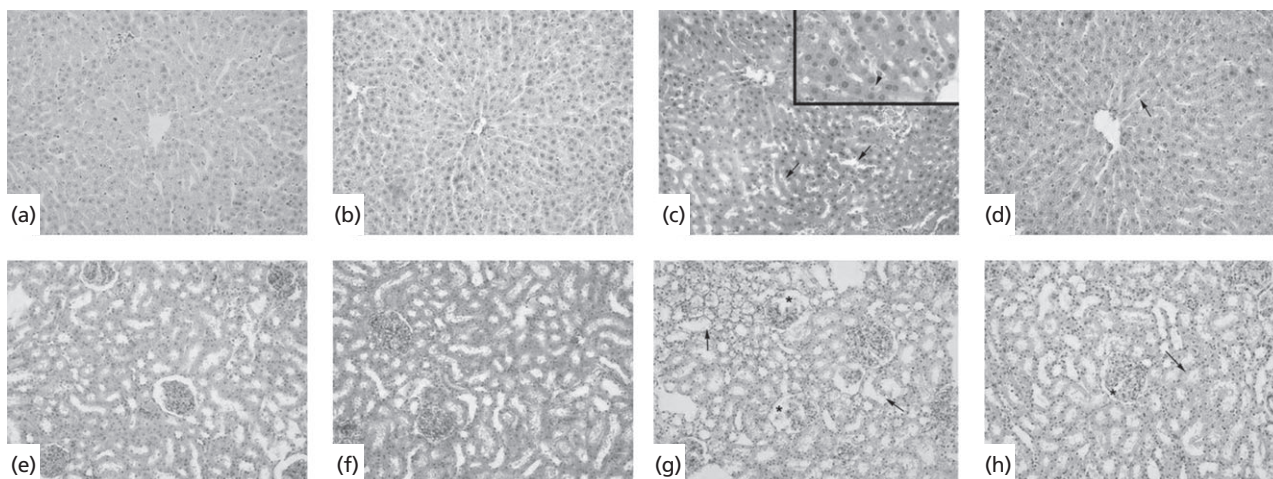
Figure 5 Photomicrographs of rat liver and kidney tissues. Vehicle and CAPE-treated control groups: normal histological appearance of liver (a, b), and kidney (e, f). Vehicle-treated MTX group: degenerated hepatocytes (▶) and sinusoidal dilatation and congestion (→) in liver (c); severe glomerular (*) and tubular cell degeneration (→) in kidney (g). CAPE-treated MTX group: normal appearance of sinusoids and hepatocytes (→) in most regions of the liver (d); normal glomerular (*) and tubular (→) structures in most regions of the kidney (h). H&E staining, original magnifications: ×200; inset: ×400.

peroxidation, is increased while membrane-bound enzyme Na^+/K^+-ATPase activity is depressed due to membrane damage. Furthermore, tissue injury was also observed at microscopic level. On the other hand, CAPE treatment following methotrexate administration significantly reduced the MDA levels and increased the enzyme activity, while normal histological appearance was observed in both liver and kidney in the CAPE-treated MTX group.

Glutathione is required for many critical cell processes and plays a particularly important role in the maintenance and regulation of the thiol-redox status of the cell.[36] Thus depletion of tissue GSH is one of the primary factors that permit tissue injury. In our study, in parallel to increase in MDA levels, GSH was depleted indicating that methotrexate-caused tissue injury is associated with oxidative stress. Since there is evidence for the role of reactive oxygen metabolites in mediating renal and hepatic toxicity of some xenobiotics and in the pathogenesis of organ failure,[21–23,30,36] free radicals are expected to play a role in methotrexate-induced hepatic, renal and gut toxicity.[37,38] CAPE was reported to protect the nuclear DNA, membrane lipids and, presumably, cytosolic proteins from oxidative damage, neutralizing the toxicity of the hydroxyl radical, singlet oxygen and, possibly, the peroxyl radical and the superoxide anion.[39] Furthermore Ilhan *et al.* demonstrated that CAPE interferes with enzymes involved in GSH synthesis, since γ-glutamyl cysteinyl synthetase and other GSH-linked detoxifying enzymes were also increased.[40] Similarly Motawi *et al.* also showed that CAPE increased GSH levels through its antioxidant effects.[41] In agreement with the previous studies,[32,37,40,41] in this study methotrexate administration caused GSH depletion in both tissues while CAPE restored the GSH, a key antioxidant for protection against oxidative damage.

Besides its antioxidant effects CAPE has a strong anti-inflammatory effect by means of preventing thrombocyte aggregation, inhibiting the synthesis of eicosanoids, such as prostaglandins and leukotrienes, and preventing the release of the mediators that play a role in inflammation.[42,43] Biochemical mediators released during inflammation intensify and propagate the inflammatory response. There is evidence that free radicals trigger the accumulation of leukocytes in the tissues and activate enzymes secreted by neutrophils (including MPO, elastase and proteases) and release more free radicals which further increase the damage. Therefore, MPO plays a fundamental role in the oxidant production by neutrophils.[44,45] In this study methotrexate caused significant increase in the plasma TNF-α and IL-1β levels, indicating systemic inflammatory response, while tissue MPO levels, an index of polimorphonuclear leukocyte infiltration, is also found to be increased. Thus, elevated MPO levels in the hepatic and renal tissues indicate that neutrophil accumulation contributes to methotrexate-induced oxidative organ injury. On the other hand methotrexate-induced elevation in MPO activity and plasma cytokines was decreased with CAPE treatment.

Conclusions

The findings of this study illustrate that CAPE is capable of reducing methotrexate-induced hepatorenal oxidative injury through its anti-inflammatory and antioxidant effects, which were evaluated both biochemically and histologically. Thus, our data suggest that CAPE may be of therapeutic use in the prevention of hepatic and renal toxicity in patients receiving toxic chemotherapeutic agents. Significant improvement of the side effects of anti-cancer drugs on the elimination organs can lead to better tolerance of these drugs and a more efficient therapy for oncology or rheumatology patients can be reached.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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