Original Article

Protective effect of carvacrol against hepato-renal toxicity induced by azathioprine in rats

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Abstract

Background and aims: Azathioprine (AZA) is an immunosuppressant medication that has toxicity to kidneys and liver. This study aimed to investigate the protective activity of carvacrol (CAR) against hepatorenal toxic activity of AZA in male Wistar rats.
Methods: All study rats were divided into five groups: control (saline, ip); azathioprine-only (AZA 50 mg/kg, ip), Sily+AZA (Silymarin 50 mg/kg, gavage), CAR+AZA (CAR 10 mg/kg, gavage), and CAR+AZA (CAR 20 mg/kg, gavage) groups. Silymarin was used as the standard hepatoprotective drug. The drugs were administered once daily for 21 days in III-V groups, and a single dose of AZA was injected on the seventh day of the experiment.
Results: AZA-intoxicated rats exhibited an elevation in aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) activity in serum, as well as an increase in extent of lipid peroxidation. Activities of enzymatic antioxidants (superoxide dismutase – SOD, catalase - CAT) in the serum, liver, and kidney were decreased as for the AZA group ($P < 0.05$). Co-treatment of CAR (both doses of 10 and 20 mg/kg) lowered the serum transaminases and ALP level, the elevation of endogenous enzymes levels, and the malondialdehyde (MDA) in serum and both tissues ($P < 0.05$). This protective effect was greater in CAR 10 compared to 20 mg/kg doses, which was comparable to silymarin.
Conclusion: This study demonstrated that the renal and nephrotoxic activities of AZA could be attributed to the generated increased oxidative stress, as well as to the CAR with antioxidant effect similar to that in silymarin, which protected these tissues against AZA-induced nephrotoxicity hepatotoxicity.
Keywords: Azathioprine, Carvacrol, Silymarin, Nephrotoxicity, Hepatotoxicity

Introduction

Azathioprine (AZA) is a purine analogue, which is mainly used as an immunosuppressive agent to treat graft rejection after organ transplantation; it is also used for treating hematological malignancies, inflammatory bowel disease, autoimmune conditions, etc (1,2). AZA is classified as a cytotoxic drug whose usage is limited due to high incidence of its adverse side effects (15%–28%) including hepatotoxicity, bone marrow suppression, and gastrointestinal symptoms (2); this drug also causes severe liver injury. Although the drug has been widely used for the treatment of arthritis disorders, hepatotoxicity has been found in 2% of arthritis patients using it (3). The hepatotoxicity of AZP has been also documented in rats (4,5) so that the alanine transaminase (ALT) level increases about twice the normal level in *in vivo* rats (4). More importantly, hepatotoxicity of AZA may produce unpredictable side effects with unknown pathogenic mechanisms (6).

However, the exact biochemical mechanism of AZA-induced damage has not been understood precisely, and the oxidative stress is believed to be involved in the pathophysiology of AZA. Oxidative stress refers to an imbalance between oxidant and antioxidant capacities, and to the production of reactive oxygen species (ROS). Therefore, the use of antioxidants could be useful in reducing drug toxicity. Antioxidants are substances that inhibit oxidation and act as protective agents. Natural antioxidants such as carvacrol (CAR, 5-isopropyl-2-methylphenol) have received special attention recently due to their wide range of biological, pharmacological, and therapeutic activities against oxygen-free radicals.

CAR is a phenolic monoterpene and main component of oregano oil that has been approved by FDA as a dietary supplement and flavoring agent (e.g., used in sweets and beverages). CAR is a natural antioxidant flavonoid found in some medicinal and aromatic plants that are used in

folk medicine for therapy or preventive purposes (7). It is known to have multiple pharmacological properties including anti-inflammatory, anti-microbial, anti-viral, antitumor, anti-carcinogenic, and antidiabetic activities (8,9). Antidepressant and antinociceptive activities have also been attributed to CAR (10,11). Moreover, CAR can be applied as an agent for treatment of wound healing or as a neuroprotective promoter in neurodegenerative diseases such as ischemia, epilepsy and traumatic neuronal injury, since it attenuates acute kidney injury induced by cisplatin (12-14); CAR application may also be adopted as a new method for attenuating hyperglycemia as well as neurodegenerative diseases in experimental diabetes (15). Some studies have found that antioxidant property of CAR is the main mechanism accounting for its therapeutic activities. It can prevent oxidative damage through scavenging ROS and incrementing the activity of endogenous antioxidant enzyme (7).

Since the liver is a central organ for the degradation of toxicological substances, and the kidneys are responsible for the exertion of approximately 2% of AZA and most of its secondary metabolites, reducing tissue damage to these organs takes on an added importance. Considering the antioxidant effect of CAR, this study aimed to investigate the potential protective effect of CAR against AZA hepatorenal toxicity, and to compare it with silymarin – a standard hepatoprotectant drug. Liver and kidneys' functions were assessed by measuring serum ALT, aspartate transaminase (AST) and ALP, creatinine, urea, endogenous antioxidants (SOD - *superoxide dismutase*, catalase - CAT) enzyme activities, and lipid peroxidation (malondialdehyde, MDA) in serum and tissue homogenate.

Materials and Methods
Animals
Male Wistar rats (200-250 g) were obtained from Pasteur Institute in Tehran. The animals were kept under controlled lighting periods (12-hour light/12-hour dark) at 25°C with enough humidity. The rats were fed with standard diets and were supplied with *ad libitum* food and water.

Chemicals
All drugs used in this study were purchased from Sigma Aldrich. AST, ALT, alkaline phosphatase (ALP), creatinine and urea kits were all purchased from Pars Azmoon Co. (Tehran, Iran).

Experimental design
In the present study, a total of 50 rats were divided into five groups, each of which included 10 rats. The groups' descriptions were as follows:
- Group I: served as control and received oral (gavage) administration of 0.5 mL of 50% ethanol daily for 21 days.
- Group II: AZA-intoxicated rats treated with single dose of AZA (50 mg/kg i.p) dissolved in ethanol

(50%) on day 7th; they received 0.5 mL of this solution orally for 21 days.
- Group III: the rats received standard reference drug silymarin (50 mg/kg/d, dissolved in 50% ethanol) via gavage for 21 days; they received a single dose of AZA (50 mg/kg) on 7th day intraperitoneally.
- Group IV and V: the rats were given CAR at a dose of 10 and 20 mg/kg/d orally for 21 days, as well as AZA 50 mg/kg i.p on day 7th; CAR was dissolved in 50% ethanol.

Biochemical methods
At the end of the study, animals were anesthetized with chloroform and blood samples were collected via cardiac puncture. After centrifugation at 3000 rpm for 15 minutes, serum was separated and stored at −20°C until biochemical analyses were performed. The liver and kidney were immediately removed, washed with ice-cold saline, and kept at -70°C in order to measure the levels of MDA, SOD, and CAT in tissue homogenates.

Biochemical parameters
Serum AST, ALT, ALP, creatinine and urea levels were determined enzymatically with commercial Kit (Pars Azmoon, Iran) and auto analyzer (BT3000, Rome, Italy).

Tissue homogenate antioxidant enzymes and malondialdehyde
The serum – renal and hepatic MDA concentration and an indicator of lipid peroxidation, was determined by the 2-thiobarbituric acid assay according to the method of Ohkawa et al (16). The CAT activity was measured by Aebi's method using hydrogen peroxide as a substrate (17). Liver and kidney SOD activity was assayed by Beauchamp and Fridivich method (18).

Measurement of serum antioxidant capacity
Serum antioxidant capacity was measured using the ferric-reducing antioxidant potential (FRAP) assay, following the technique proposed by Benzie and Strain (19).

Statistical analysis
The data were described as the mean ± SEM of studied groups using the analysis of variance test (one way ANOVA) with Duncan's post hoc tests in order for multiple comparisons. The value $P < 0.05$ was considered significant.

Results
The increase in the level of liver marker enzymes was indicative of the damage to hepatic cells. A single dose of AZA (50 mg/kg) exhibited a significant elevation in the levels of serum AST, ALT, and ALP ($P < 0.05$) compared to the control group (group I, Figure 1). The enhanced lipid peroxidation (measured as MDA) as well as the reduced activities of CAT, SOD, and the FRAP observed in AZA-only treated rats (group II) confirmed the damage to liver and kidney (Table 1 and Figure 2).
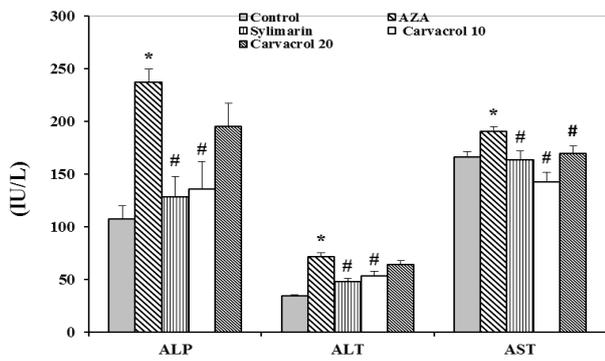
Figure 1. Effect of carvacrol on the hepatic markers in the serum of experimental groups. Values represent mean ± SEM. $P<0.05$. * as compared with control group, # as compared with AZA only group.
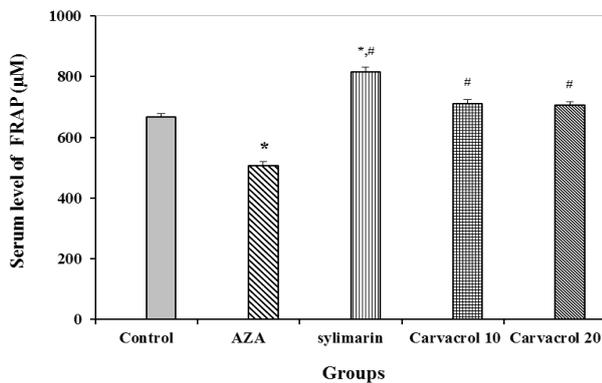

Figure 2. Effect of carvacrol and silymarin on the serum level of FRAP in AZA intoxicated rat. Values represent mean ± SEM. $P<0.05$. * as compared with control group, # as compared with AZA only group.

Oral administration of silymarin and CAR 10 and 20 mg/kg for 21 days reversed these parameters towards control levels (Group III-V). Administration of CAR 10 mg/kg blocked the AZA-induced elevations of serum ALT (sALT), serum AST (sAST) and serum ALP (sALP) compared to AZA intoxicated rats (group III, $P<0.05$), while CAR 20 mg/kg (group IV) significantly lowered only sAST activity compared to AZA alone. All these parameters were found recovered to near normal levels in silymarin treated rats (Group II). The results of biochemical analyses of rat serum are shown in Figure 1.

Table 1 and Figure 2 show the activities of SOD, CAT, and MDA contents in liver, kidney, and FRAP level,

respectively. Rats treated with CAR at the doses of 10 and 20 mg/kg significantly decreased the elevated lipid peroxidation levels ($P<0.05$), and restored the altered SOD and catalase levels towards the almost normal levels in liver and kidney tissues. The results were comparable to those obtained in silymarin treated group. Silymarin used as standard reference also exhibited significant hepatorenal protective activity ($P<0.05$). In addition, CAR significantly prevented the decrease of FRAP in the serum of AZA intoxicated rat ($P<0.05$) (Figure 2). AZA-induced nephrotoxicity was reflected in significant increase in levels of kidney markers and lipid peroxides. Table 2 show that pretreatment with CAR at the doses used in this study significantly decreased ($P<0.05$) serum urea, creatinine and uric acid levels compared to AZA-administered rats. In silymarin-treated group a noticeable reduction ($P<0.05$) was observed in the levels of kidney markers.

Discussion

Our study results confirmed the involvement of oxidative stress in the pathogenesis of AZA-induced toxicity, and revealed that CAR co-administration decreased serum amino transaminases and marked inhibition of the lipid peroxidation (MDA production); however, remarkable increases of endogenous antioxidant enzymes (SOD and CAT) and FRAP were also found in a rat model of AZA-induced organ toxicity. These findings supported the notions that CAR protected liver and renal from AZA-induced damage, and the protection of CAR may have been associated with its anti-oxidative properties.

AZA is an immunosuppressant drug that is widely used in clinical settings for preventing graft rejection and treating various autoimmune and dermatological diseases; however, its side effects limit its use (2). Liver injury is one of its side effects and is estimated to occur in 2% of AZA-treated patients (3). AZA is metabolized in the liver to mercaptopurine (6-MP) through the reduction of glutathione and, subsequently, the conversion to inactive 6-thiouric acid by xanthine oxidase. AZA metabolism leads to intracellular GSH depletion, mitochondrial injury, depletion of cellular ATP, and cell death of hepatocytes (20).

From among the multiple mechanisms hypothesized for AZA-induced tissue injury, lipid peroxidation and oxidative stress have been frequently proposed as the

Table 1. The activities of SOD, CAT and MDA concentrations in liver and kidney homogenates of rats of control and experimental groups

Variable	MDA level (µM) in			SOD activity(U/L) in		CAT activity(U/L)	
	Liver	Kidney	Serum	Liver	Kidney	Liver	Kidney
I (Control)	0.81±0.03	18.5±0.4	0.73±0.08	158±2.2	181.7±2.6	5.66±0.09	1.52±0.04
II (AZA)	2.06±0.06*	39.3±1.3*	1.76±0.07*	90.1±2.2*	86.1±2.5*	3.7±0.06*	0.88±0.1*
III (Sily+AZA)	1.03±0.03#	22.4±0.4#	0.55±0.07#	133.3±1.4#	174.5±3.2#	5.03±0.09#	1.27±0.03#
IV (CAR10+AZA)	1.21±0.02#	23.5±0.9#	1.27±0.02	127.2±1.8#	131.9±2.9 #~	4.5±0.05#	1.2±0.02#
V (CAR20+AZA)	1.41±0.05#	25.4±0.9#	1.22±0.05	130.3±1.6#	155.7±3#	4.28±0.06#	1.18±0.03#

Values represent mean ±S EM. *$P<0.05$ vs. Control, ~ $P<0.05$ vs. Silymarin (Sily), # $P<0.05$ vs. AZA-only group.

Table 2. Effect of CAR on AZA-induced kidney function test in serum

	Creatinine (mg/dL)	Uric Acid (mg/dL)	Urea (mg/dL)
I (Control)	0.66±0.02	1.3±0.1	37±1.2
II (AZA)	0.79±0.03*	1.7±0.9*	44±1.2*
III (sily+AZA)	0.76±0.02	1.2±0.1#	38±1.3#
IV (CAR10+AZA)	0.7±0.03#	1.1±0.1#	33±2.4#
V (CAR20+AZA)	0.74±0.02	1.3±0.03#	36±1.6#

Values represent mean ± SEM. *$P<0.05$ vs. Control, # $P<0.05$ vs. AZA-only.

major mechanisms; also, therapy with dietary antioxidants has been known to reduce AZA toxicity (20,21). However, the pathophysiology of AZA-associated toxicity has not been understood adequately. In this study, a single dose of AZP induced hepatotoxicity as revealed by significant increases in the activities of serum parameters of liver injury (AST, ALT, and ALP) compared to the control animals; this result is in line with the findings from previous studies (4,5). These intracellular enzymes are released from the liver cells into the circulation in case of structural integrity damage, and the levels of these marker enzymes are proportional to the extent of injury. Similarly, AZA significantly increased MDA levels in the blood, liver, and kidneys.

Measurement of MDA as oxidative stress biomarker can explain leakage of cellular ALT, AST, and ALP to circulation due to AZA-induced tissue injury. In the present study, AZA toxicity decreased the levels of the endogenous liver antioxidants CAT and SOD enzymes. Lowered activities of these antiperoxidative enzymes result in the accumulation of highly reactive free radicals, leading to loss of cell membrane integrity and membrane function. Compared to normal control, moreover, the intoxicated rats showed significant decrease in antioxidant potential based on what was implied from the reduced FRAP values. The FRAP test quantified the ferric reducing ability of serum or plasma , an estimate of the non-enzymatic antioxidant power (19).

This study result was in agreement with the findings from previous studies which had demonstrated the involvement of oxidative stress and lipid peroxidation in AZA induced toxicity (2,22,23). In addition, glutathione (GSH) plays a key role in the detoxification of ROS, conjugation, and elimination of toxic compounds (24). Although GSH levels were not measured in our study, a significant decrease in GSH levels in animals exposed to AZA had been reported in previous studies.

Moreover, AZA caused marked impairment in renal function (significant higher levels of urea, creatinine and uric acid) alongside with significant oxidative stress in the kidney. In a study by El-Ashmawy and Bayad, AZA-induced renal damage and its amelioration by treatment with folic acid and grape seed extract were reported for experimental animal model (21). Also, significant higher serum creatinine, lower glomerular filtration rate, and creatinine clearance have been previously found in human subjects and rats treated with AZA (21,25,26). Our study

finding revealed that the nephrotoxicity of AZA could have been due to generation of ROS with consequent adverse effects on the functions of the kidney, which was consistence with the results from previous studies reporting the involvement of oxidative stress and lipid peroxidation in AZA-induced liver toxicity. Moreover, our finding indicated that pretreatment with CAR may have been useful in preventing AZA-induced hepatorenal toxicity.

On the other hand, natural compounds that are rich in antioxidants can reinforce the activity of the endogenous antioxidant system against adverse effects resulting from the toxicity of many chemicals. In this regard, some studies have demonstrated the efficiency of CAR in scavenging-free radicals (i.e., nitric oxide, superoxide radicals, peroxyl radicals, and hydrogen peroxide) (27). The presence of the hydroxyl group (OH), which is associated with the aromatic ring, is the reason for highly antioxidant activity of CAR (28). The antioxidant action of CAR has been reported to be equal to ascorbic acid, butyl hydroxyl toluene, and vitamin E (29). According to the biochemical results from both serum and tissue examinations, CAR has shown important protective effect against AZA-induced liver and kidney toxicity through attenuating the oxidative stress and increased antioxidant enzyme activity.

Furthermore, the tendency of marker enzymes AST, ALT, and ALP at a near normal level in the CAR+ AZA group is a indicative of the anti-hepatotoxicity effect of CAR. According to these data, we believed that CAR protected hepatocytes in a similar fashion to that of standard hepatoprotective drug silymarin. In our study, silymarin (50 mg/kg) produced a significant reduction in elevated serum levels of hepatic enzymes. Silymarin has hepatorenal protective properties because of its antioxidant activities and the capacity to scavenge free radicals (30).

An antioxidant activity of CAR was confirmed by FRAP capacity method. Increased antioxidant capacity may have explained the reduced MDA levels in the treated groups and the ensuing decrease in serum ALP, AST and ALT. The ability of CAR to enhance the levels of antioxidants along with its anti-lipid peroxidative activity suggested that this compound could have been potentially useful in counteracting AZA-induced-free and radical-mediated tissue damage. The protective effect of CAR against oxidative stress has been already reported. Selimoğlu Şen et al, for example, have demonstrated that CRV is beneficial in providing protection against MTX-induced lung toxicity by scavenging free radicals and boosting the endogenous antioxidant system to help restore normal lung histology (31). It has been shown that CAR exerts protective effects against diethylnitrosamine-induced oxidative stress and liver damage by augmenting host antioxidant defense mechanisms (32). Also, Suo et al have demonstrated that CAR provides protection against I/R injury in both rat liver and BRL cells, and the PI3K-Akt signaling pathway is involved in the protective mechanisms of CAR on I/R injury (33).

In addition, CAR significantly decreased the elevated levels of serum urea, creatinine and uric acid, which indicated renal protection of CAR against oxidative damages induced by AZA and maintenance of renal function. CAR has the ability to suppress oxidative damage caused by MTX-induced oxidative stress in rat serum and kidney tissues, which was in line with our study result (34). It was shown that CAR had a protective effect on the renal function of gentamicin-induced-nephrotoxicity in rats (35), as well as a preventive potential in favor of the kidney against the damage caused by cyclophosphamide-induced oxidative (36). Furthermore, it was demonstrated that CAR treatment could have reduced renal injury induced by bilateral renal I/R via anti-inflammatory, antioxidant, and cytoprotective effects (37). Moreover, CAR had significant protective and antioxidant effects against cyclophosphamide-induced nephrotoxicity in rats (36). CAR also had protective effect against cisplatin-induced nephrotoxicity via antioxidant, anti-inflammatory, and anti-apoptotic activities (38). In another study, Samarghandian et al showed protectective effect of CAR against chronic stress induced oxidative damage in brain, liver, and kidneys(10).

Several studies have measured the effective dose of CAR against drug-induced toxicity. Even though previous studies had reported 25 mg/kg (39) and 15 mg/kg (36) body weight of CAR as the maximum protective dose, our study found that a dose of 10 mg/kg body weight of CAR had maximum efficacy. Similarly, a study by Gunes et al revealed that adding 10 mg/kg of CAR provided more marked protection than that produced by 5 mg/kg of CAR in cyclophosphamide-induced kidney (36). In the research study by Aristatile et al, on the other hand, it was indicated that 20-mg dose of CAR had stronger effect than CAR with higher doses (40 and 80 mg) (40).

Conclusion

According to our study results, the involvement of oxidative stress in AZA-induced injury was confirmed by the decrease of serum and tissues MDA levels, the increase of SOD and CAT activities in liver and kidney, and the suppression of the increased serum ALT, AST and ALP levels by the antioxidant CAR in AZA-treated rat. Moreover, the ability of CAR to raise the levels of antioxidants together with its anti-lipid peroxidative activity was indicative of the fact that this compound may have been potentially useful for counteracting free radical-mediated tissue damage resulting from AZA toxicity. However, it is recommended that further investigations be carried out in order for illustrating the exact protective mechanism(s) of CAR, as well as determining the safety, efficacy, and therapeutic importance of these findings in clinical practices.

Conflict of Interests
The authors declare that they have no conflict of interests.

Ethical Approval
All procedures were conducted in accordance with ethical norms approved by the Ethics Committee of Shahrekord University of Medical Sciences, Shahrekord, Iran (Ethic number IR. SKUMS. REC.1395.151).

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