Effects of rosmarinic acid on acetaminophen-induced hepatotoxicity in male Wistar rats

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ABSTRACT

Introduction

Acetaminophen (APAP or paracetamol or N-acetyl-p-amo-n-phenol) has been widely used as an analgesic and antipyretic drug for many years (Shah et al. 2011). The American Association of Poison Control Centers reports that APAP is one of the most common pharmaceuticals associated with both intentional and unintentional poisoning and toxicity. APAP toxicity is the most common cause of hepatic failure requiring liver transplantation (Larson et al. 2005).

The pathogenesis of APAP-induced hepatotoxicity begins by the cytochrome P450-catalysed formation of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) that directly trigger oxidative stress, mitochondrial damage and hepatocellular injury. As this injury propagates throughout the hepatic lobule, a strong host inflammatory response is activated, resulting in hepatic neutrophil infiltration and significant damage (Patel et al. 2016).

There is an emerging focus on plant products to find a highly effective and reliable drug for the prevention of APAP-induced hepatotoxicity. Rosmarinic acid (RA) (2-)[2-[(2-)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl][oxy]-3-(3,4-dihydroxyphenyl) propanoic acid] is a natural phenol carboxylic acid which is commonly found in various plants from the Lamiaceae (the mint) family, such as Rosmarinus officinalis L. (rosemary), Origanum vulgare L. (oregano), Thymus vulgaris L. (thyme), Mentha spicata L. (spearmint), Perilla frutescens (L.) Britton (perilla), Ocimum basilicum L. (sweet basil) and several other medicinal plants, herbs and spices (Shetty 2005). It has been reported that RA has diverse biological properties including antioxidant and anti-inflammatory activities (Moon et al. 2010; Chu et al. 2012). RA has also been shown to be an effective agent to decrease prooxidant status in oxidative stress-induced pathologies such as ischemia-reperfusion and liver damage (Ramalho et al. 2014). In this context, previous studies reported hepatoprotective effects of RA against liver injury induced by diabetes (Mushtaq et al. 2015), tert-butyl hydroperoxide (t-BHP) (Yang et al. 2013), carbon tetrachloride (CCl₄) (Domitrović et al. 2013) and aging (Zhang et al. 2015) in experimental models.

To our knowledge, no study has been carried out concerning the protective effects of RA on APAP-induced hepatotoxicity. Taking the above data into account, the present study was carried out to assess the protective effect of RA in APA-induced liver damage in rats.

Materials and methods

Drugs

APAP and RA were obtained from Sigma (St. Louis, MO). DTNB (2, 2'-dinitro-5, 5'-dithiobisbenzoic acid), TPTZ (2, 4, 6-tri
(2-pyridyl)-1, 3, 5-triazine), TBA (2-thiobarbituric acid), trichloroacetic acid (TCA), n-butanol, tris-hydrochloric acid, ethylenediamine tetracetic acid (EDTA), sodium acetate, glacial acetic acid, phosphoric acid, potassium chloride, tetramethoxypropene (TMP), ferric chloride (FeCl₃·6H₂O), ferrous sulphate and hydrochloric acid was obtained from Merck (Dramstadt, Germany). All other chemicals were of analytical grade and highest purity.

**Animals**

Male adult Wistar rats (8 weeks old, weighing 220–250 g) were used in the present experiments. All animals were maintained at a constant temperature (22 ± 0.5°C) with a 12 h light/dark cycle. Food and water were available ad libitum in the home cages. Rats were divided randomly into four experimental groups (n = 7). The rats were habituated to the environment for at least one week before the start of the experiment. The study protocol was approved by the Institutional Animal Ethical Committee (No. 7IB-1344) and all procedures were performed in accordance with the recommendations for the proper care and use of laboratory animals.

**Experimental design**

Fifty-six rats were randomly divided into eight equal groups: Group I served as normal control and were orally treated with isotonic 0.9% NaCl for seven days and at day seven injected with isotonic 0.9% NaCl. Groups II, III and IV, used as RA control groups, were orally treated with RA 10, 50 and 100 mg/kg for seven days and at day seven injected with isotonic 0.9% NaCl. Group V served as hepatotoxicity control and received 0.9% NaCl for seven days and at day seven intoxicated with 500 mg/kg APAP. Groups VI, VII and VIII, used as RA treatment groups, were treated with RA 10, 50 and 100 mg/kg for seven days and at day seven injected with 500 mg/kg APAP. APAP and physiological saline were injected intraperitoneally while RA was pre-orded by gavage. After 24 h of APAP intoxication, rats were euthanized by ether and then sacrificed. About 5 mL of blood was collected after centrifugation at 4000 rpm for 10 min. The liver homogenates were centrifuged at 5000 rpm for 10 min at 4°C to prepare supernatant for enzyme assay.

**Lipid peroxidation assay**

Hepatic lipid peroxide was estimated by measuring the formed malondialdehyde (MDA). Briefly, the samples were diluted by 1.5 mL TCA (20% w/v) and centrifuged at 3000 × g for 10 min. Then, the precipitation was dissolved in sulphuric acid and 1.5 mL of the mixture was added to 1.5 mL of TBA (0.2% w/v). The mixture was then incubated for 1 h in a boiling water bath. Following incubation, 2 mL of n-butanol was added, the solution centrifuged, cooled and the absorption of the supernatant was recorded in 532 nm. The calibration curve of tetraethoxypropane standard solutions was used to determine the concentrations of TBA + MDA adducts in samples (Moore & Roberts 1998).

**Reduced glutathione assay**

Reduced glutathione (GSH) was measured spectrophotometrically based on the Ellman (1959) method. Briefly, the supernatant was centrifuged with 5% TCA to centrifuge out the proteins. To 0.1 mL of this homogenate, 2 mL of phosphate buffer (pH 8.4), 0.5 mL of DTNB and 0.4 mL of double distilled water were added. The mixture was vortexed, and the absorbance was read at 412 nm.

**Ferric reducing/antioxidant power (FRAP) assay**

The basis of FRAP assay is to reduce the colourless ferric tripyridyltriazine (TPTZ) complex to blue-coloured ferrous TPTZ by the action of electron donating antioxidants in biological samples (Benzie & Strain 1996). This ferrous TPTZ complex has an intense blue colour and can be monitored at 593 nm. The FRAP reagent consists of 300 mM acetate buffer (pH 3.6), 10 mM FeCl₃·6H₂O in the ratio of 10:1:1. Briefly, 50 μL of homogenate was added to 1.5 mL freshly prepared and prewarmed (37°C) FRAP reagent in a test tube and incubated at 37°C for 10 min. The absorbance of the blue coloured complex was read against reagent blank (1.5 mL FRAP reagent +50 μL distilled water) at 593 nm. Standard solutions of Fe II in the range of 100–1000 mM were prepared from ferrous sulphate (FeSO₄·7H₂O) in distilled water. FRAP values were expressed as nmol ferric ions reduced to ferrous form/g protein (Benzie & Strain 1996).

**Glutathione S-transferase (GST) assay**

Hepatic GST activity was determined by the method described by Habig et al. (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. Briefly, the assay mixture (3 mL) was made up of 30 μL of reduced GSH (0.1 M), 150 μL of CDNB (3.37 mg/mL), 2.79 mL phosphate buffer (0.1 M, pH 6.5) and 30 μL of liver homogenate. The reaction was allowed to run for 60 s before the absorbance was measured at 340 nm against the blank.

**Cytochrome P450 2E1 activity assay**

CYP2E1 activity was measured spectrophotometrically by the rate of oxidation of p-nitrophenol to p-nitrocatechol according to the method described by Koop et al. (1997). Briefly; 1 mL of the assay mixture (consisted of: 200 μL of rat liver microsomes,
100 μL of 1 mM NADPH, 100 μL of 10 mM ascorbic acid, 500 μL of 100 mM potassium phosphate buffer (pH 6.8) and 100 μL of 100 μM p-nitrophenol was incubated for 10 minutes at 37°C; then 0.5 mL of 0.7 N perchloric acid was added to terminate the reaction. Following centrifugation at 1300×g for 5 min, 1 mL of supernatant was mixed with 100 μL of 10 N NaOH. The absorbance of 4-nitrocatechol was recorded with a spectrophotometer at 546 nm. The CYP2E1 activity is expressed as nmol/min/mg protein.

**Statistical analysis**

All data are expressed as mean ± S.E.M. The analysis was performed using the SPSS statistical software package (version 21.5; SPSS, Chicago, IL). Differences among groups were tested by one-way analysis of variance (ANOVA) with the Tukey post-hoc test. Probability values less than 0.05 were considered significant.

**Results**

**Effects of RA on hepatic biomarkers**

Figure 1 demonstrates the serum activities of ALT and AST in different groups at the end of experiments. APAP treatment significantly increased ALT and AST (126.42 ± 5.2 U/L, 198.5 ± 4.6 U/L, respectively) compared to control group (80.85 ± 1.6 U/L, 163.57 ± 4.6 U/L, respectively) (p < 0.001, 0.001, respectively) (Figure 1). Although RA 10 mg/kg did not alter serum ALT and AST, RA 50 and 100 mg/kg reduced ALT (p < 0.001, 0.001, respectively) and AST (p < 0.05, 0.001, respectively) in RA-treated APAP group compared to APAP group. Furthermore, there were no significant changes in ALT and AST between RA (50 and 100 mg/kg) treated control group and control group (all p > 0.05).

As shown in Figure 2, APAP treatment decreased the concentrations of albumin (2.13 ± 0.05 g) and total proteins (4.57 ± 0.2 g) (all p < 0.001) that were prevented by RA 50 (2.13 ± 0.05 g, 2.13 ± 0.05 g, respectively) and 100 mg/kg (2.13 ± 0.05 g, 2.13 ± 0.05 g, respectively). However, there were no significant differences between RA-treated APAP groups and control group in these parameters (all p > 0.05) (Figure 2).

**Effects of RA on the liver weight**

Figure 3 represents the effects of different treatments on the liver weight of the animal groups. APAP administration induced a significant increase in the liver weight of APAP group (13.14 ± 0.28 g) compared to control group (11.25 ± 0.3 g) (p < 0.01). RA 50 and 100 mg/kg prevented the change in the liver weight of APAP treated group (11.61 ± 0.25 g, 11.15 ± 0.2 g, respectively) (p < 0.05, 0.001, respectively), however, there were no significant differences in the liver weight between group VI or APAP group treated with RA (10 mg/kg) and APAP group (p > 0.05). Furthermore, RA at any doses did not alter the liver weight of control groups at the end of the experiments (all p > 0.05).

**Effect of RA on hepatic MDA content**

The concentration of lipid peroxidative product MDA in different animal groups was shown in Table 1. MDA level was significantly enhanced in APAP group compared to the control group (p < 0.001). RA at 50 and 100 mg/kg reduced MDA in treated APAP rats compared to untreated APAP group (p < 0.001). There were no significant changes in MDA between RA (50 and 100 mg/kg) treated APAP group and control group (p > 0.05). While RA 10 and 50 mg/kg did not alter MDA levels of control groups, RA 100 mg/kg reduced MDA in control rats compared to untreated control animals (p < 0.05) (Table 1).

**Effect of RA on hepatic GST activity**

Hepatic GST activity was significantly reduced in APAP-treated rats when compared with the control (p < 0.001) (Table 1). However, RA 50 and 100 mg/kg but not RA 10 mg/kg ameliorated the APAP-induced decrease in hepatic activity of GST to the APAP group (p < 0.05, 0.01, respectively).

**Figure 1.** Effects of rosmarinic acid (RA) administration on serum ALT and AST in control (Cont), RA 10 mg/kg-treated control (Cont + RA10), RA 50 mg/kg-treated control (Cont + RA50), RA 100 mg/kg-treated control (Cont + RA100), acetaminophen (APAP), RA 10 mg/kg-treated APAP (APAP + RA10), RA 50 mg/kg-treated APAP (APAP + RA50) and RA 100 mg/kg-treated APAP (APAP + RA100) groups (n = 7) at the end of experiment. The data are represented as mean ± S.E.M. *p < 0.05 and ***p < 0.001 (as compared to control group).
Furthermore, administration of RA (50 and 100 mg/kg) increased GST activity compared to untreated control rats (all \( p < 0.05 \)).

**Effect of RA on hepatic contents of GSH**

Hepatic contents of GSH in different animal groups are shown in Table 1. APAP administration caused a significant decrease in GSH compared with control group \( (p < 0.001) \). RA administration (50 and 100 mg/kg) to APAP group caused a significant increase in GSH compared to untreated APAP group \( (p < 0.05, p < 0.01, \text{respectively}) \). However, only RA at 100 mg/kg increased GSH of control rats compared to untreated control animals \( (p < 0.05) \). Furthermore, there were no significant differences in GSH between RA-treated APAP groups and untreated control group (Table 1).
**Effect of RA on hepatic CYP2E1 activity**

APAP administration induced a significant increase in P2E1 activity compared with control group \((p < 0.001)\) (Table 1). RA 10 mg/kg did not change the enzyme activity in neither control nor APAP group \((p > 0.05)\). However, RA 50 and 100 mg/kg reduced CYP2E1 activity in APAP groups compared to untreated APAP group \((p < 0.05)\). Furthermore, there were no significant differences between RA 50 and 100 mg/kg-treated APAP group and untreated control group at the end of experiments \((p > 0.05)\) (Table 1).

**Effect of RA on hepatic levels of FRAP**

As indicated in Table 1, there was a significant reduction in total antioxidant power (FRAP value) of liver homogenate in APAP group compared to control group \((p < 0.001)\). RA at 100 mg/kg (but not at 10 and 50 mg/kg) increased FRAP of control-treated animals compared to untreated control group \((p < 0.01)\). Furthermore, RA 50 and 100 mg/kg increased FRAP values in APAP groups compared to the untreated APAP group \((p < 0.05)\) (Table 1). There were no significant differences in FRAP value of APAP-treated rats and untreated control group at the end of study \((p > 0.05)\).

**Effects of RA on histology changes in liver**

Figure 4 shows the results of histopathological evaluation in normal control and RA control groups. The liver tissues of these animal groups showed normal histological structures (Figures 4A–D). APAP induced severe hepatic necrosis, inflammatory cell infiltrations and haemorrhage in liver sections of lead group in Figure 5. While RA 10 mg/kg-treated APAP group still shows destructive changes in liver histology (Figure 6), RA administration at 50 mg/kg induced some regions of recovery with only apoptotic cells in the liver sections (Figure 7). RA 100 mg/kg restored the normal architecture of the hepatic lobules and the hepatocytes (Figure 8).

**Discussion**

It has been well established that functional foods containing physiologically active compounds from plants have potential to improve health and longevity. Among them, some compounds such as RA are known to have physiological properties such as antioxidant, anti-inflammatory and anti-cancer activities (Yang et al. 2013). The present results clearly demonstrated, for the first time, that oral administration of RA dose dependently elicited significant hepatoprotective effects in a rat model of liver injury which was achieved through inhibition of hepatic CYP2E1 activity and lipid peroxidation. Furthermore, RA protected hepatic GSH and GST reserves and total tissue antioxidant capacity.

APAP is a safe and effective analgesic when used in therapeutic doses. However, in overdose APAP is metabolized to NAPQI predominantly via CYP2E1, the classical cytochrome P450 isozyme that plays the most important role in APAP bioactivation. The catalytic activity of CYP2E1 is associated with increased production of reactive oxygen species (ROS) and
deterioration of antioxidant defence (Zanger & Schwab 2013; Dadkhah et al. 2015). In turn, CYP2E1 inhibitors were shown to reduce free radical production and ameliorate lipid peroxidation (Domitrović et al. 2014). Thus, the effect of RA on the activity of CYP2E1 was further measured. The observed increase in hepatic CYP2E1 activity of APAP-treated group is in agreement with several previous reports (Tan et al. 2008; Yao et al. 2015; Xie et al. 2016). Our results showed that RA 50 and 100 mg/kg caused marked inhibition on the CYP2E1 enzymatic activity in APAP group. Notably, the inhibition of hepatic CYP2E1 activity by RA agrees with CYP2E1 inhibiting properties of RA in experimental model of cisplatin-induced nephrotoxicity (Domitrović et al. 2014).

The NAPQI-induced depletion of cytosolic and mitochondrial GSH leads to liver injury (Bhadauria 2010) which was confirmed in our study by concurrent decrease in liver GSH. Depletion of hepatic GSH increases the susceptibility of liver cells to oxidative stress (Tan et al. 2008; Omidi et al. 2014).

As an intracellular GSH-related enzyme, GST is considered to be an important component of the detoxification system (El Morsy & Kamel 2015). This study showed that APAP significantly reduced hepatic GSH and GST in rats whereas RA (50 and 100 mg/kg) inhibited these decreases. These findings suggest that GSH and GST participate in the protective actions of RA against hepatic oxidative stress injury induced by APAP.

Furthermore, APAP administration also resulted in an increase in lipid peroxidation, as indicated by the significant increase in MDA. Increased MDA level in APAP-treated rats indicates the main role of lipid peroxidation in initiation of oxidative stress. Excessive ROS generation triggers the process of lipid peroxidation in cell membranes and causes the destruction of cell components and cell death. Other studies have also shown that hepatic lipid peroxidation increases during APAP toxicity (Dadkhah et al. 2006, 2015). Our work indicated that RA 50 and 100 mg/kg reduced the formation of MDA in the liver and protected the total tissue antioxidant capacity and hepatic GSH reserves in both control and APAP-treated rats which are in
agreement with previous reports (Iuvone et al. 2006; Ramalho et al. 2014).

In the current study, APAP-induced liver toxicity was also confirmed by biochemical measurements and histopathological observations. Increased levels of serum AST and ALT indicated deterioration in the hepatic functions due to toxic effects of APAP. Damage to hepatocyte membranes will cause release of inflammatory cell infiltrations and haemorrhage in liver sections (El-Agamy et al. 2014). Furthermore, the decreased concentration of serum albumin observed here could be attributed to the interference of APAP with protein synthesis. Generally, these results may indicate degenerative changes and hypofunction of the liver (Ramadan et al. 2013). These results confirmed prior reports that APAP has a harmful and stressful influence on hepatic tissue (Omidi et al. 2014; El Morsy & Kamel 2015). In the present study, RA (50 and 100 mg/kg) treatment protected against liver damage induced by APAP as indicated by the improvement in the biochemical parameters and the liver weight.

Histological examination of the liver tissue of the animals treated with APAP also supported damage to hepatic structural integrity by revealing severe destruction of hepatic architecture, necrosis, and infiltration of inflammatory cells. Similar observations have been previously reported (Boholooi et al. 2013; Dadkhah et al. 2015). The protective effect of RA was further confirmed in the liver by histopathological examination. Administration of RA 50 and 100 mg/kg significantly attenuated hepatocyte necrosis and inflammatory cell infiltration into APAP-injured liver.

In agreement with our findings, Domitrovic et al. (2013) reported the attenuation of acute liver injury following RA administration in CCl₄-intoxicated mice. In addition, RA decreased t-BHP-induced liver damage (Yang et al. 2013) and reduced hepatic injury in a rat diabetic model (Mstagh et al. 2015).

Furthermore, there is evidence that apoptotic cell death and inflammatory responses play a significant role in pathogenesis of APAP-induced hepatotoxicity (Jaeschke et al. 2014). It has been indicated that RA has anti-apoptotic and anti-inflammatory actions in previous reports (Domitrovic et al. 2013, 2014), therefore these two mechanisms may be also involved in the effects of RA in the present study.

Conclusions
Taken together, our findings demonstrated that RA, as well as food rich in this compound, could be beneficial in the amelioration of some aspects of acute liver toxicity induced by APAP by inhibition of hepatic CYP2E1 activity and lipid peroxidation. RA protected hepatic GSH and GST reserves and total tissue antioxidant capacity. Furthermore, RA treatment attenuated necrosis, inflammatory cell infiltrations and haemorrhage in liver sections of lead exposed rats. Further clinical studies are required to confirm its therapeutic efficacy in humans.

Disclosure statement
The authors state no conflict of interest.

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