The Effect of Rebadioside A on Attenuation of Oxidative Stress in Kidney of Mice under Acetaminophen Toxicity

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ABSTRACT

Background: Acetaminophen (APAP) overdose causes renal and hepatic injury. It is also believed that oxidative stress has a pivotal role in APAP-induced renal injury. Therefore, protective effects of different antioxidants have been examined in APAP-induced renal and hepatic toxicity models. *Stevia rebadiana* is a plant with a high degree of natural antioxidant activity in its leaf extract. The aim of this study was to investigate the possible protective effects of rebadioside A; one of the main components of stevia extract, on APAP-induced oxidative stress in kidney of mice.

Methods: Oxidative stress was induced in kidney of BALB/c mice by the intraperitoneal (i.p.) administration of a single dose of 300 mg/kg APAP. Some of these mice also received rebadioside A (700 mg/kg) (i.p.) 30 minutes after APAP injection. Two and six hours after APAP injection, all mice were sacrificed and malondialdehyde (MDA), glutathione (GSH), free APAP, and glutathione conjugated of APAP (APAP-GSH) were determined in the kidney tissue.

Results: GSH depletion and MDA levels significantly ($P<0.05$) increased in mice treated with either APAP or APAP plus Rebadioside A, respectively in 2 and 6 hours intervals after APAP administration. Significantly ($P<0.05$) higher levels of free APAP and APAP-GSH levels detected in kidney of mice administrated with APAP plus rebadioside A compared to APAP treated ones.

Conclusion: Rebadioside A may be a potential compound in alleviation of APAP-induced oxidative stress in kidney of mice after APAP overdoses.

Keywords: Acetaminophen, Oxidative Stress, Rebadioside A.

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INTRODUCTION

Acetaminophen (APAP) is an analgesic and antipyretic drug which is safe at therapeutic doses. However, in the case of overdose, APAP is known to cause hepatic necrosis and renal failure in both humans[1,2] and animals [3, 4]. Renal damage and acute renal failure can occur even in the absence of liver injury [5]. Renal insufficiency occurs in approximately 1–2% of patients with APAP overdose [6].

At therapeutic doses, APAP is cleared from body through conjugation with glucoronic acid and sulfate in the liver and kidney. However, in the case of overdose, a portion of APAP is metabolized to N-acetyl-p-benzoquinone imine (NAPQI), a highly reactive metabolite which binds to cellular macromolecules, such as lipids and proteins, or react with glutathione (GSH) to form a conjugate (APAP-GSH) [7,8]. Lipid peroxidation [9, 10] and the depletion of intracellular glutathione (GSH) are reported to play a role in the pathogenesis of APAP-induced renal damage [11]. APAP can cause life-threatening renal damages which is consistent with acute tubular necrosis and a decrease in the glomerular filtration rate (GFR) [12, 13]; therefore, finding protective compounds acting against APAP-induced nephrotoxicity is of clinical importance.

Although N-acetyl-cysteine, as the main therapy in clinical cases acting as a GSH

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precursor, protects against APAP hepatotoxicity in humans [14], it is not protective against APAP-induced renal damage [12, 15]. Antioxidants, such as curcumin, melatonin, and vitamins E and C, have also been used to prevent APAP-induced toxicity in mice [16-18].

Stevia rebaudiana (Bertoni) is a small shrub that is native to the Amambay Mountains in Paraguay [19]. Stevia leaf extract exhibits a high degree of antioxidant activity and it has been reported to inhibit hydroperoxide formation in sardine oil with a potency greater than that of either DL -α-tocopherol or green tea extract. The antioxidant activity of Stevia leaf extract has been attributed to the scavenging of free radical electrons and superoxides [20]. In a study, the in vitro potential of ethanolic leaf extract of *S. rebaudiana* showed its significant potential for use as a natural antioxidant [21]. Rebaudioside A and stevioside are the two main Stevia extracts used by South Americans for the treatment of diabetes for long [22, 23]. Steviol glycosides found in the *S. Rebaudiana* herb and are the two predominant derivatives used in high potency sweeteners [24]. Results of a recent study showed that Stevia leaves could protect rats against streptozotocin-induced diabetes, reduce the risk of oxidative stress, and ameliorate liver and kidney damage [25].

Hence, the aim of this study was to investigate the protective effects of rebaudioside A on APAP-induced renal toxicity in mice.

MATERIALS AND METHODS

Chemicals

Acetaminophen (APAP), rebadioside A, and trichloroacetic acid (TCA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Disodium ethylene diamine tetra acetic acid (Na2EDTA), thiobarbituric acid (TBA) for lipid proxidation assessment, hydrochloric acid, 5,5´dithio nitro benzoic acid (DTNB), glutathione (GSH), and 2-Amino-2-hydroxymethyl-propane-1,3-diol (tris base) were purchased from Merck Co. (Darmstadt, Germany). All other chemicals were of analytical grade and they were purchased either from Sigma Co. (St. Louis, MO) or Merck Co. (Darmstadt, Germany).

Animals

Male BALB/cJ mice (6-8 weeks old), weighing approximately 20–25 g, were supplied from Pasteur Institute of Iran (Tehran, Iran) and maintained in a conventional sanitary facility with the required consistent temperature and relative humidity. All animals had free access to standard diet and water *ad libitum* for at least 2 weeks prior to the experiments. The experimental protocols were approved by the Medical Ethics Committee of Tarbiat Modares University, Tehran, Iran.

Experimental Protocol

In the present study, different parameters compared among APAP, APAP+rebadioside A, and control groups. Groups of animals were treated as follows:

APAP-treated group was treated with 300 mg/ kg B.W of APAP (i.p.) (dissolved in 500 µl of normal saline) and sacrificed 2 hours after APAP administration.

APAP+rebadioside A group was treated with 300 mg/ kg B.W of APAP (i.p.) (dissolved in 500 µl of normal saline). Then it was post injected with 700 mg/kg B.W of rebadioside A (i.p.) (dissolved in 500 µl of normal saline) 30 minutes after APAP injection and sacrificed 2 hours after APAP administration.Control group was treated with 500µl of normal saline (i.p) and sacrificed 2 hours later.Time-dependent function of rebadioside A was similarly examined using other groups of animals which were sacrificed 6 hours after APAP injection.

Groups of animals were sacrificed by cervical dislocation under diethyl ether anesthesia at various times after APAP or APAP+rebadioside A injection. Kidneys of animals were removed and stored at -80 °C for further examinations.

Reduced Glutathione (GSH)

GSH concentrations were measured in the kidney homogenate applying the Ellman's reagent as described earlier [26]. In brief, 100mg of frozen tissue was homogenized at 0 °C in 4 ml of 0.2 mM disodium ethylene

diamine tetra acetic acid (Na2EDTA) and 5 ml of the homogenate was mixed with 4 ml of distilled water and 1 ml of 50 % TCA. The mixture was vortexed for 15 min and centrifuged at 3000×g for 15 minutes to precipitate the tissue protein. Then 2 ml of supernatant was diluted with 4 ml of 0.4M tris containing 0.2 mM EDTA, pH 8.9. The samples were assayed using 0.1 ml of 0.01M dithio itrobenzoic acid (DTNB) at 412 nm with Nanodrop 2000C (Thermo scientific, USA). All data were expressed in nmol/ml GSH, using a GSH standard curve prepared by applying an authentic GSH standard.

Lipid Proxidation

Lipid peroxidation levels were assayed on the basis of reaction between Thiobar bituric acid and Malone dialldehyde (MDA) as the main product formed through lipid proxidaion [27]. Briefly, kidney homogenates were prepared in phosphate buffer, and centrifuged. Then 1 ml of supernatant was diluted in sealed tubes with 2 ml of TBA indicator solution consisting of 15% (w/v) TCA, 0.375% (w/v) TBA, and 0.25N HCL. After shaking, sealed tubes were incubated in boiling water for 15 min and allowed to cool at room temperature for 10 min prior to centrifugation at 10000×g. Supernatant absorption was measured at 535nm.

HPLC Analysis

To identify free acetaminophen and glutathione conjugate of acetaminophen (APAP-GSH) in the samples, HPLC analysis was performed using the method previously described [28]. Kidney tissues were homogenized (1:5, w/v) in 10 mM sodium acetate, pH 6.5, and centrifuged at 16000×g for 20 minutes at 4 °C. Then 100µl of the supernatant was mixed with 100 µl of 20% ice-cold trichloroacetic acid for 15 minutes, and then centrifuged for 5min at 16000 ×g. Fifty µl of the supernatant was injected and assayed using reverse-phase, Agilent Technologies HPLC and ZORBAX-Eclipse-C18 (5 µm, 4.6mm, 150mm) column with Agilent DAD (1200 series) detection component at 254 nm wavelength.

Mobile phase consisted of HPLC graded methanol and water (Merck, Germany) at the ratio of 10: 90. Injection volume was 50 µl and flow rate was adjusted to 1.3 ml/min.

Data were reported in µg/ml using a calibration curve set for the marker (acetaminophen) where peak areas were correlated to the corresponding concentrations. According to the retention time, the marker was identified from the extract again and the relative concentrations were further estimated according to their corresponding peak areas. The occurrence of APAP-GSH conjugate as a metabolite of APAP was confirmed by a mass spectrometer. APAP-GSH was quantified by the APAP standard curve, as the molar extinction coefficient of APAP and its conjugated metabolites at 254nm are essentially the same [29]. The results were expressed as mean standard deviation obtained from two independent experiments.

Statistical Analysis

Differences between groups were evaluated using Kuriskal-Walis test followed by Man-Whitney test, which are applied for analysis of data which are not normally distributed, using SPSS software 16. A P-value of 0.05 was considered significant for all analyses.

RESULTS

Effects of rebadiosiede A on APAP-induced GSH depletion

As it is shown in Figure 1, the administration of 300 mg/kg B.W of APAP (i.p.), with or without post treatment with rebadioside A (700 mg/ kg B.W) (i.p.) with a 30-minute interval, led to a significant ($P<0.05$) depletion of GSH 2 hours after APAP injection (i.p.). In addition, there was a replenish in renal GSH level of mice under APAP (300mg/kg B.W) administration in the 6-hour period after APAP injection compared to the 2-hour period after APAP injection; however, as the results show rebadioside A failed to play a role in GSH increase, as no significant differences in GSH levels were observed between these two groups 6 hours after APAP injection.

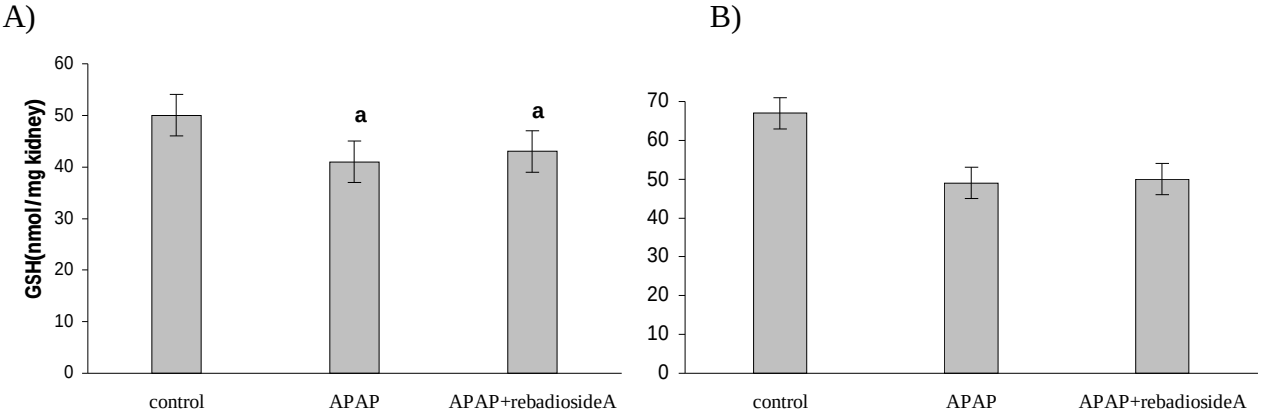

Figure 1. Time dependent GSH level after APAP dosing with or without rebadioside A post treatment.

GSH levels in kidneys were determined A) 2 and B) 6 hours after APAP dosing, Control groups received normal saline, APAP treated groups were injected with 300 mg/kg B.W acetaminophen (i.p.) and APAP+rebadioside A groups received 700 mg/ kg B.W rebadioside A 30 minutes after 300 mg/kg B.W APAP (i.p.). Each value is presented as mean±SE. a- indicates a statistically significant ($P<0.05$) difference for GSH level in APAP or APAP+rebadioside A treated mice compared to the normal control.

Effects of rebadioside A on MDA formation in kidneys of mice under APAP challenge

Figure 2 depicts that 2 hours after APAP (300 mg/kg B.W) administration, MDA level which is indicative of lipid peroxidation increased significantly in kidneys of mice treated with neither APAP nor APAP+rebadioside A in comparison to the vehicle. Although MDA level in APAP and APAP+rebadioside A groups was significantly ($P<0.05$) higher than the vehicle, it did not significantly differ between APAP and APAP+rebadioside A groups 2 hours after APAP (300 mg/kg B.W) administration. In addition, the results showed that the administration of 300 mg/kg B.W of APAP significantly increased levels of MDA formation in both APAP and APAP+rebadioside A groups compared to the vehicle in the 6-hour time interval after APAP injection, whereas post-treatment with rebadioside A (700 mg/kg B.W) significantly reduced MDA in APAP+rebadioside A group in comparison to the group treated with APAP alone.

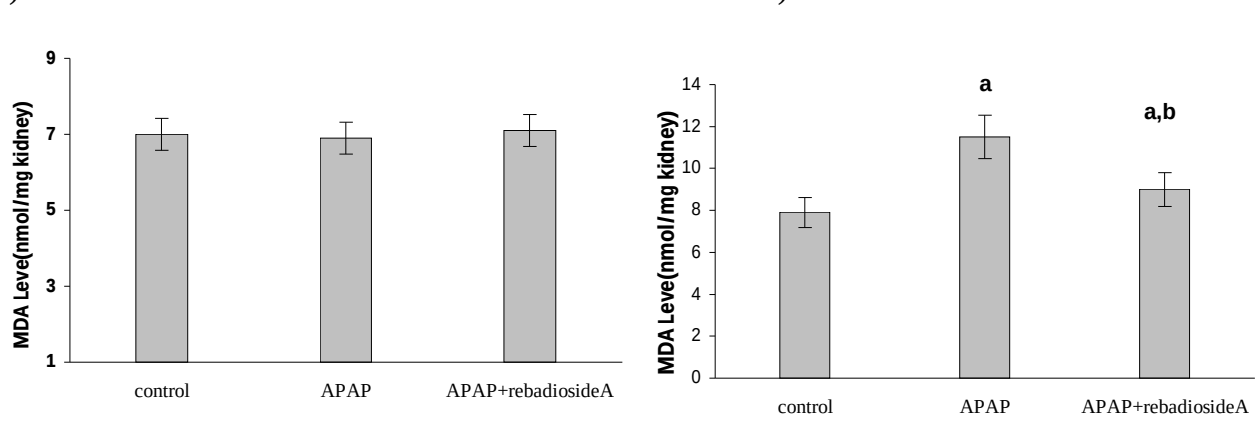

Figure 2.Time-dependent effect of rebadioside A post-treatment on APAP-induced MDA formation in kidneys of mice.

MDA levels in kidneys were determined: A) 2 and B) 6 hours after APAP dosing. Control groups received normal saline, whereas APAP treated groups were injected with 300 mg/kg B.W acetaminophen (i.p.) and APAP+rebadioside A groups received 700 mg/ kg B.W rebadioside A 30 minutes after 300 mg/kg B.W APAP (i.p.). Each value is presented as mean±SE. **a** indicates a statistically significant (P<0.05) difference for GSH level in APAP or APAP+rebadioside A treated mice compared to the normal control. **b** indicates a statistically significant (P<0.05) difference for GSH level between APAP+rebadioside A treated and APAP treated groups.

Effects of rebadiosiede A on APAP-GSH conjugate formation in kidneys of mice under APAP treatment

Table. 1 indicates that the administration of 300 mg/kg B.W of APAP was followed by formation of APAP-GSH conjugate detected with approximate retention time of 5.3 minutes (Figure 3) and the conjugate level significantly decreased due to post treatment of mice under APAP with 700 mg/ kg B.W of rebadioside A. In addition, results from the assessment of renal un-metabolized (free) APAP with approximate retention time of 5.8 (Figure 3) showed that post treatment of rebadioside A left significantly higher levels of free APAP in kidneys of mice under APAP administration 2 hours after APAP injection in comparison to mice treated with APAP alone. Furthermore, no detectable free APAP was observed in kidneys of mice with APAP or APAP+rebadioside A treatment 6 hours after APAP injection.

Table 1. HPLC analysis of mice kidneys.

A)		
Treatment groups	**Sacrifice time(h)**	
	1	6
APAP	172.9779±12.86	22.78265±0.66
APAP+rebadioside A	75.75369±13.90	13.3104±2.48*
B)		
Treatment groups	**Sacrifice time(h)**	
	1	6
APAP	117.41 ± 11.06	N.D
APAP+rebadioside A	274.76 ± 8*	N.D

Values (µg/gr kidney) are expressed as mean ± SD for five mice in each group.

A) Glutathione conjugate of acetaminophen (APAP-GSH) and **B)** free acetaminophen (APAP) were detected in kidneys of mice after APAP dosing with or without rebadioside A post treatment. APAP treated groups were administrated 300 mg/kg B.W APAP (i.p.), and APAP + rebadioside A groups received 700 mg/ kg B.W rebadioside A (i.p.) 30 minutes after 300 mg/kg B.W acetaminophen administration (i.p.).

*indicates a statistically significant difference for APAP-GSH levels in APAP + rebadioside A treated mice compared to the APAP treated mice in two groups with the same sacrifice time (ND = non-detected).

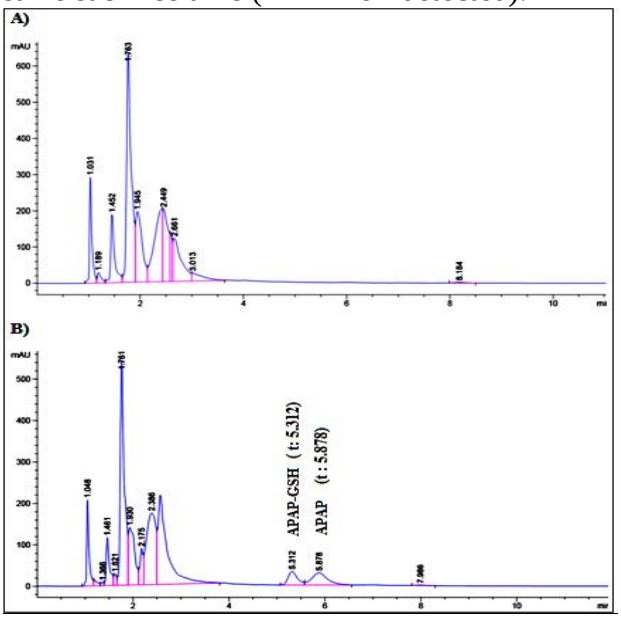

Figure 3. Representative high performance liquid chromatogram.

A) Control; resulted from kidney of a mouse one hour after 500 µl PBS i.p. injection,
B) Acetaminophen conjugate of glutathione (APAP-GSH) and free Acetaminophen (APAP) with approximate retention times of 5.3 and 5.8 minutes, respectively. Data were obtained from the kidney of a mouse 1hour after treatment with 300 mg/ kg B.W APAP i.p. To produce the chromatograms, detection was used at 254 nm.

DISCUSSION

The present study was designed to determine the mode of action of rebadioside A as a possible protective agent against

APAP- induced oxidative stress in mice. Several investigations have shown the association between APAP-induced nephrotoxicity and lipid peroxidation [30, 16]. Administration of nephro-toxic doses of APAP to rats has also been shown to result in development of oxidative stress-induced damage in renal tissues. This effect was indicated by the increased degree of lipid peroxidation, inhibition of enzymatic antioxidants, and depletion of non-enzymatic antioxidants (e.g., intracellular GSH) in kidney [31].

The present study showed that the administration of 300 mg/kg B.W of APAP did not increase MDA level as the main indicator of lipid peroxidation in a 2-hour interval. There was not a significant difference between control and treated groups. However, APAP injection led to significantly higher levels of MDA level in 6 hours. In other group, post treatment with rebadioisde A after APAP injection significantly diminished lipid peroxidation in kidney of mice 6 hours post APAP injection. Therefore, it seems that treatment with rebadioside A affects the attenuation of APAP-induced oxidative stress and consequential lipid peroxidation.

The present study also demonstrated that GSH depletion, which is predominantly considered as a consequential event following APAP-induced oxidative stress, occurred both in APAP and APAP + rebadioside A treated groups after APAP injection. However, GSH was replenished in both APAP and APAP+rebadioside A groups 6 hours after APAP administration with no significant differences between these groups.

Previous studies have demonstrated that APAP-induced nephrotoxicity might be due to metabolic activation of APAP to the reactive metabolite, NAPQI [16, 32]. Therefore, when large doses of APAP are ingested, there is more severe GSH depletion as well as massive production of metabolites, particularly APAP-GSH. In this regard, some studies have shown an association between the protective effects of some agents and reduction in APAP-GSH accumulation in sera of animals under APAP toxicity [33, 34].

In the present study, assessment of APAP-GSH conjugate and un-metabolized APAP in kidney of animals showed that post treatment with rebadioside A following APAP injection in mice results in detection of lower levels of APAP-GSH and also higher level of free APAP in comparison to the group treated with APAP alone. This suggests that rebadioside A may have a protective effect against APAP-induced oxidative stress through decreasing APAP metabolism to APAP-GSH which leads to the excretion of more free APAP through urine. In this regard, results of previous studies also suggest a contributory role for APAP-GSH in the biochemical toxicology of APAP-induced kidney injury in male CD-1 mice [35]. It has also been demonstrated that GSH-derived metabolites of APAP, such as APAP- Cys, have the capacity to contribute to APAP nephrotoxicity in mice [15].

CONCLUSION

The findings of this study suggest that although rebadioside A failed to prevent initiation of APAP-induced oxidative stress, as evidenced by GSH depletion and lipid peroxidation in kidneys of mice, it attenuated lipid peroxidation later via reducing APAP conversion to its activated metabolite, namely NAPQI which produced APAP-GSH conjugate in kidneys of mice.

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