Avicenna Journal of Medical Biochemistry
doi:10.15171/ajmb.2017.15 2017 December;5(2):81-86 http://ajmb.umsha.ac.ir

Research Article

Antioxidant Properties of Resveratrol on Acetaminophen Induced Toxicity in Wistar Rat Liver and HepG2 Cells

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Received: 6 February 2017
Accepted: 14 March 2017
ePublished: 10 April 2017

Abstract

Background: Although acetaminophen (APAP) is considered safe at therapeutic doses, intake of high amounts of this drug can cause liver failure. In the present experiment, we examined the hepatoprotective effects of resveratrol (RES) in HepG2 cells and rat liver.

Objectives: This study aimed to evaluate the influence of RES on liver function in rat model of necrosis and HepG2 cells.

Materials and Methods: In this study, rats were randomly assigned into 4 groups (7 rats in each group) as follows; group 1: control rats (received normal saline), group 2: hepatotoxic control (control rats that received 640 mg/kg/d APAP), group 3: positive control (received 150 mg/kg N-acetylcysteine), group 4: RES (received 30 mg/kg RES). The animals were treated for 7 days. Afterwards, the levels of liver enzymes, protein carbonyl content, glutathione (GSH) level, and Tumor necrosis factor (TNF-α) level were determined.

Results: In the in vitro experiment, APAP-induced HepG2 cells were treated with RES at different concentrations and various factors such as cell viability, liver enzymes, GSH and TNF-α levels were measured.

Conclusions: Our results indicated that RES could normalize all these factors in vitro and in vivo ($P<0.05$). In fact, RES had potential hepatoprotective effect against APAP -induced hepatotoxicity in HepG2 cells and animal models mainly via dual change of oxidative stress and cytokine levels.

Keywords: Acetaminophen, Glutathione, Resveratrol, HepG2

Background

Acetaminophen (APAP) is known as the analgesic medicine in the world, and can lead to acute liver failure (ALF) following accidental or intentional overdose (1). Poisoning with APAP is attributed to nearly one-half of all cases of ALFs in Great Britain and the United States (2). It has been reported that alcohol consumption and fasting might increase APAP toxicity (3). Cytochrome p450 converts APAP into reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). In physiological conditions, glutathione quickly converts NAPQI to nontoxic metabolites. APAP at high doses increases reactive metabolite of NAPQI which might react with liver proteins, and cause liver failure (4). Before 1980s, APAP was not considered as the cause of ALF, however, one retrospective experiment from 1994 to 1996 observed a 20% incidence of APAP toxicity which caused liver failure (3). Different mechanisms have been proposed for APAP toxicity including depletion of glutathione contents, elevation of nitrogen species and reactive oxygen species (ROS) undergoing necrotic alterations, augmented oxidative stress, changes in Ca^{2+} metabolism which change signal transduction pathways, leading mitochondrial permeability transition, and ATP reduction which causes necrosis (2). Furthermore, APAP might lead to lactic acidosis often associated with coma, happening before the onset of liver toxicity or after APAP poisoning. Certainly, increased lactate levels have

been reported to be a potential death predictor (5).

APAP-induced liver toxicity has been an important issue for a few decades and various strategies have been adopted, like the administration of natural products with antioxidant properties. In fact, an increasing line of evidence established that herbal medicine (6-8) and their polyphenols have useful effects on liver injury by alleviating lipid levels, ROS and inflammatory markers. Resveratrol (RES) is a well-recognized polyphenol which is naturally found in various foods and vegetables, such as berries, grapes, peanuts, and wine(9,10).

This polyphenol has established significant hepatoprotective effects in different animal models (11-13). RES showed protection against hepatic injury induced by carbon tetrachloride (CCl_4), APAP, and ethanol (11-14). Zhou et al found that 14 days of RES administration to animal models prevented CCl4-induced oxidative stress (11). Our previous collected report established that this polyphenol has potential antioxidant, anti-inflammatory, and antidiabetic properties (15). Hence, these beneficial properties of RES might play a prominent hepatoprotective role (11).

Objectives

The aim of the current experiment was to evaluate the influence of RES on liver function in rat model of necrosis and HepG2 cells.

Materials and Methods

Animals and Treatment Strategy

In this experimental study, Wistar rats were purchased from Laboratory of Animals, Research Center of Hamadan University of Medical Sciences (Hamadan, Iran). Rats were kept in animal house with free access to normal chow diet and under standard conditions at temperature 23 ± 2ºC and humidity of 60±5%, with a 12:12 dark:light cycle. After 7 days of adaptation to animal cages, the rats were randomly divided into 4 groups (7 in each group) as follows; group 1: control rats (received normal saline), group 2: hepatotoxic control (control rat that received 640 mg/kg/d APAP), group 3: N-acetylcysteine (NAC) group (received 150 mg/kg NAC), and group 4: resveratrol (RES) group (received 30 mg/kg RES) (16,17). This study was approved by Animal Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1395.383).

Sample Perpetration

After 7 days of treatment, fasted rats were anesthetized and blood samples were collected from the hearts of rats. Serum was prepared following the centrifugation of blood at 3000 ×g for 10 minutes at 4°C and then used for biochemical factors. Liver of rat was removed and washed with cold PBS and quickly frozen at liquid nitrogen and stored at -70ºC.

Liver Tests

Liver tests such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined in serum according to kit protocol using automated chemistry analyzer (Pars Azmoon, Tehran, Iran). For antioxidant test, liver of each animal was homogenized with lysis buffer containing protease inhibitors and then supernatant was used for more analysis following centrifugation at 3000 ×g for 15 minutes at 4°C.

Total Protein

The amount of total protein was determined in supernatant according to Bradford method.

Glutathione Levels

Liver and serum glutathione (GSH) were measured according to the manufacturer's instruction (ZellBio, Germany).

Protein Carbonyl Content Assay

The amount of carbonyl group formation was determined by the 2,4-dinitrophenylhydrazine (DNPH)-based method. Briefly, 300 µL of homogenate was added to tube containing 500 µL of DNPH (10 mM in 2M hydrochloric acid) and incubated at room temperature for 60 minutes. After centrifugation, 500 µL trichloroacetic acid (20%) was added to the tube and remaining pellet was washed 3 times using 1000 µL of ethanol:ethyl acetate (1:1) and re-dissolved in 1 mL 6M guanidine-HCl. The absorbance of sample was measured at the wavelength of 370 nm (18).

TNF-α Levels

The serum levels of Tumor necrosis factor (TNF-α) was measured by TNF-α kit (BioLegend, UK) according to the manufacturer's protocol.

HepG2 Cell Line

HepG2 cell line, hepatocellular carcinoma, was purchased from National Center for Cell Sciences, Pasteur Institute (Iran). Culture was grown in Dulbecco's minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C humidified incubator with 5% CO_2 (19).

Cell Viability Analysis

The effects of APAP and RES on cell viability were determined using the micro-culture tetrazolium assay (MTT) method. Briefly, HepG2 cells were seeded in a 96-well plate at a density of 5×10^5 cells/well in medium having different doses of APAP and RES (1-400 µg/mL) for 24 hours. Then 10 µL MTT solution (5 mg/mL) was

added to wells and the cells were incubated for 2.5 hours at 37°C. The supernatant was removed and formazan crystals were dissolved in 100 µL dimethyl sulfoxide (DMSO) and absorbance was measured at the wavelength of 540 nm (19).

Biochemical Assays in Culture Media

ALT, AST and lactate dehydrogenase (LDH) levels in the cell culture medium are signs of cell membrane injury. Briefly, HepG2 cells were seeded in a 24-well plate (1.5×10^5 cells/well) and incubated with 1, 10, and 100 µg/mL RES for 24 hours at 37°C. Afterwards, the levels of these enzymes were determined in the medium according to the commercial kit (Pars Azmoon, Tehran, Iran) based on the colorimetric method (20).

Tumor Necrosis Factor Alpha Level

Treated cells were lysed with 250 µL lysis buffer and centrifuged. Then, supernatant was used for TNF-α measurement using commercially available ELISA kits (BioLegend, UK) (21).

Glutathione Levels in Cell Lysates

The amount of GSH in cell lysates was determined in the culture supernatant according to a previous published method (22) using commercial kits with small modifications (ZellBio, Germany).

Statistical Analysis

All in vitro experiments were done in triplicate and the results were analyzed by Prism 5 software (GraphPad Software, CA). One-way analysis of variance (ANOVA) followed by Tukey post-test was used for analysis of data. Significance of difference was observed when *P* value was less than 0.05.

Results

In this experiment, MTT assay was used to determine the cytotoxicity induced by RES and APAP and their combination in HepG2 cells. The HepG2 cells were treated with different concentrations of RES and APAP for 24 hours. Our results showed that treatments of HepG2 cells with RES plus 10mM APAP significantly increased cell viability (Figure 1).

Liver Enzymes

The activity of AST and ALT enzymes, markers of liver injury, markedly elevated in the rats treated with APAP. Administration of RES significantly restored AST and ALT levels compared with hepatotoxic animals (Table 1). Under in vitro conditions, these enzymes elevated after 24-hour incubation of HepG2 with APAP compared with untreated media. However, treatment with RES significantly normalized these enzymes ($P<0.05$). The activity of LDH, the marker of cell integrity, also increased in HepG2 cells in which toxicity was induced by APAP compared with the untreated media. While, RES significantly reduced LDH levels dose-dependently (Figure 2).

Glutathione Level

APAP administration significantly decreased GSH levels compared with the control group ($P<0.001$). Treatment of rats with RES significantly augmented serum and liver GSH levels in APAP-treated liver ($P<0.01$). The levels of GSH in HepG2 cells after 24-hour exposure to APAP markedly decreased compared to APAP-induced toxic media ($P<0.05$). Treatment with RES at the dose of 1, 10, and 100 g/mL normalized GSH levels dose-dependently (Figure 3).

Tumor Necrosis Factor Alpha Levels

The serum levels of TNF-α increased in the rats in which toxicity was induced by APAP in comparison with the control rats. This cytokine level significantly reduced in RES-treated group compared to the control group. The concentration of TNF-α in HepG2 cells increased after 24-hour exposure to APAP compared to the control group ($P<0.05$). Treatment with RES at the dose of 1, 10, and 100 g/mL normalized TNF-α level (Figure 4).

Protein Carbonyl Content

Protein carbonyl content, a marker of protein oxidation, in the rat liver was markedly higher than that of

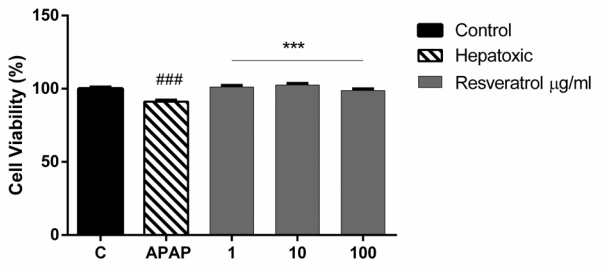

Figure 1. Changes After Exposure to Resveratrol in HepG2 Cells. Data are presented as mean ± SD. ###*P* < 0.001 compared to the control and ****P* < 0.001 compared to the hepatotoxic group. APAP; Acetaminophen.

Table 1. Effect of Different Treatments on Liver Enzymes

Groups	AST (IU/L)	ALT(IU/L)
Control	85.8 ± 4.7	44.8 ± 2
Hepatotoxic	109.2 ± 4.4 ###	63.4 ± 4.8 ###
NAC	81.2 ± 7.5**	49.4 ± 3.2 **
Resveratrol	91.2 ± 4.7 **	46.1 ± 3.1**

Data are presented as mean ± SD. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 shows significance level as compared to the hepatotoxic group (n=7). ###*P* < 0.001 shows significance as compared to the control.

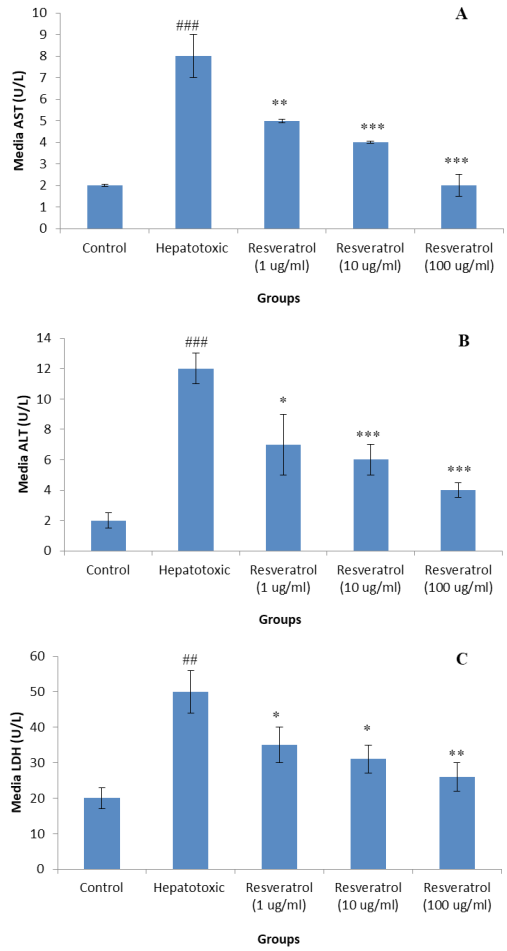

Figure 2. Effect of Resveratrol on the Levels of AST (A), ALT (B), and LDH (C) in HepG2 Cells. Data are presented as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the hepatotoxic group. ##P < 0.01 and ###P < 0.001 shows significance as compared to the control.

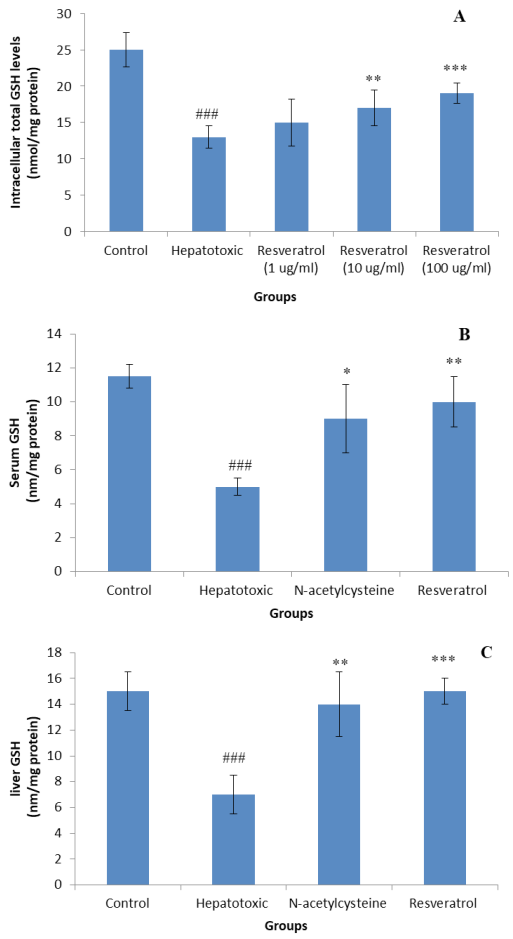

Figure 3. Effect of Resveratrol on the Levels of Reduced Glutathione (GSH) in HepG2 cells (A), in Serum (B) and Liver (C). Data are presented as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the hepatotoxic group (n=7). ###P < 0.001 shows significance as compared to the control.

control animals (P<0.001). Supplementation with RES significantly reduced carbonyl content in APAP-treated group (P<0.05) (Figure 5).

Discussion

Results from this experiment showed that RES had potential antioxidant properties in the in vitro (HepG2 cells) and in vivo conditions (animal models). HepG2 are commonly used as in vitro alternative to primary human hepatocytes. This type of cell line is used extensively as a preclinical model for liver-toxicity experiments, drug metabolism, and drug discovery (23).

Certainly, the liver disease remains one of the main causes of morbidity and mortality in the world. Consequently, there is a critical need to find potential hepatoprotective components (2). Various agents with plant origin have been examined for their normalizing influence on liver damage, in both in vitro and in vivo experiments. For instance, polyphenols and other natural products have been documented to have many useful effects (11). Among them, RES is a useful polyphenol found in grapes, red wine, and peanuts, which has been shown to have numerous pharmacological properties such as potential anti-inflammatory, antioxidant, and anticancer effects (9,11). This agent protects liver against CCl4 and ethanol-induced damage (13). Moreover, the results of Masubuchi et al showed that RES protects liver against APAP-induced injury through controlling the Th1/Th2 and TNF-α levels (24).

In this experiment, RES potentially protected liver from APAP-induced liver toxicity. It has been reported that APAP at short time can lead to liver injury. For instance, Forouzandeh et al showed that single dose of APAP (500 mg/kg) significantly led to liver damage in animal model, whereas administration of *Teucrium polium* for 5 consecutive days significantly normalized liver enzymes and histological changes (25). Janbaz e al reported that administration of APAP at the dose of 640 mg/kg led to liver injury in rats and treatment with rutin normalized liver damage (25). Recent experiments have

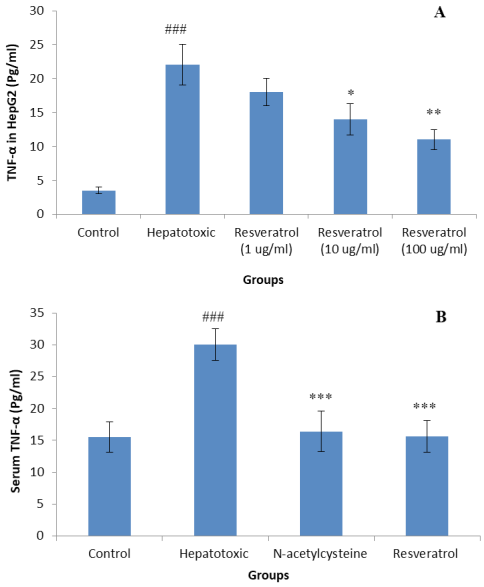

Figure 4. Effect of Resveratrol on TNF-α Levels in HepG2 cells (A) and Rat Serum (B). Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to the hepatotoxic group (n=7).

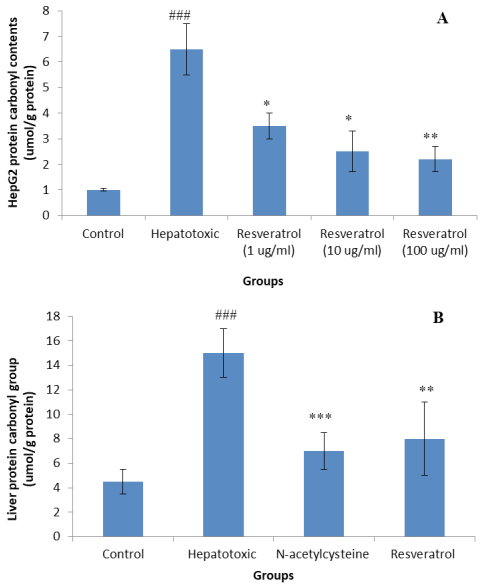

Figure 5. Effect of Resveratrol on Protein Carbonyl Content in HepG2 cells (A) and Rat Liver (B). Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to the hepatotoxic group (n=7). ###$P < 0.001$ shows significance as compared to the control.

documented the beneficial effects of RES against liver injury and alcohol-induced fatty liver in animal models (12). This polyphenol is nontoxic and well-tolerated in laboratory animals from 20 to 750 mg/kg/d in a 1 or 3-month experiments (26). Our results showed that the levels of ALT and AST significantly augmented in APAP -treated animals compared with the untreated control animals. The concentration of these enzymes increased after hepatocyte injury with the secretion of transaminase enzymes into the plasma. The levels of blood ALT and AST decreased in the RES-treated animals compared to the APAP-treated group. In this experiment, RES administration to HepG2 cell line at the dose of 1, 10, and 100 µg/mL over a period of 24 hours markedly reduced ALT, AST and LDH activities. Liver necrosis was determined by quantification of LDH secretion into the culture medium. Total LDH secretion is often related to complete HepG2 death (27).

Our study also showed supplementation with RES in APAP-induced hepatotoxicity illustrated strong anti-inflammatory effect via reducing TNF-α level. It has been reported that APAP-induced toxicity increases hepatocyte necrosis, which leads to infiltration of monocyte and neutrophil. In this condition, Kupffer cells motivate pro-inflammatory markers like TNF-α (2). We showed that APAP markedly increased TNF-α levels, which was restored by RES and NAC treatment. Administration of RES up to 24 hours to HepG2 cells significantly reduced TNF-α compared with the untreated control cells. This polyphenol normalized liver action likely due to the suppression of secretion of inflammatory markers such as TNF-α (9). Numerous experiments have documented that RES reduces inflammation in both in vivo and in vitro conditions by suppressing the release of pro-inflammatory cytokines (11). Blazka et al (28) reported that APAP significantly augmented TNF-α level in mice. Furthermore, they showed anti-TNF-α moderately prevented hepatotoxicity induced by APAP (2).

Since liver injury induced by APAP is mediated by its free radical metabolites, suppression of free radical production or antioxidant capacity plays a main role in the protection against APAP-induced liver toxicity (25). Our findings showed that treatment with RES restored oxidative stress in liver, determined by glutathione levels and carbonyl groups. The APAP–induced hepatotoxicity results in the formation of N-acetyl-p-benzoquinone imine (NAPQI), highly toxic reactive agent, leading to glutathione reduction and oxidative stress. Therefore, intentional and/or accidental use of high amounts of APAP often leads to severe injury and necrosis of hepatocytes (2,3). In this experiment, treatment with RES and NAC significantly increased glutathione in APAP-treated liver compared with the untreated controls. In vivo experiment also showed that supplementation with RES up to 24 hours significantly increased glutathione content.

Conclusions

Since APAP can lead to liver toxicity through several steps, RES might have protective effect by changing these processes. Firstly, RES showed potential antioxidant activity. ROS production is proposed as a main factor

in APAP-induced hepatotoxicity. Another possible mechanism was the anti-inflammatory effects of RES, which could prevent the liver injury. RES also reduced lipid accumulation in the liver. In this experiment, RES showed hepatoprotective effects in vitro and in vivo.

Authors' Contributions

EAO designed the experiment and wrote the draft of the manuscript. MM, HR and RM managed the biochemical analysis. MP wrote the in vitro protocol. FM approved the experiment and reviewed the manuscript. All authors read and approved the final version.

Conflict of Interest Disclosures

The authors declare no potential conflicts of interest relevant to this article.

Acknowledgements

This study was supported by a grant from Hamadan University of Medical Sciences (9509025023).

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