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ABSTRACT
Ingestion of a toxic dose of the analgesic drug, acetaminophen (also called paracetamol or APAP), is among the most common causes of acute liver injury in humans. We tested the hypothesis that the combined polyphenolic compounds, resveratrol (RES) and quercetin (QUR), can substantially protect against hepatocyte ultrastructural damage induced by a toxic dose of APAP in a rat model of APAP-induced acute liver injury. The model group of rats received a single dose of APAP (2 g/kg), whereas the protective group of rats was pretreated for 7 days with combined doses of RES (30 mg/kg) and QUR (50 mg/kg) before being given a single dose of APAP. All rats were then sacrificed 24 hours post APAP ingestion. Harvested liver tissues were prepared for transmission electron microscopy (TEM) staining, and liver homogenates were assayed for biomarkers of inflammation, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), and oxidative stress, such as malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx). In addition, blood samples were assayed for the liver injury enzyme alanine aminotransferase (ALT) as an indicator of liver damage. TEM images showed that APAP overdose induced acute liver injury as demonstrated by profound hepatocyte ultrastructural alterations, which were substantially protected by RES+QUR. In addition, APAP significantly (p < 0.05) modulated TNF-α, IL-6, MDA, SOD, GPx, and ALT biomarkers, which were completely protected by RES+QUR. Thus, RES+QUR effectively protects against APAP-induced acute liver injury in rats, possibly via the inhibition of inflammation and oxidative stress.

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Introduction
Acetaminophen (APAP)-induced hepatotoxicity is common in both humans and experimental animal models after an accidental or intentional ingestion of an overdose of the drug. It is the most common agent of intentional self-harm and APAP poisoning claimed the life of 284 persons aged 12 years and over between 1993 and 1996 in England and Wales, UK. In addition, about 50% of acute liver failure admitted cases in the United States of America are caused by APAP poisoning. APAP is metabolized in the liver, and hepatotoxic metabolites that represent about 10% of the whole metabolites are rapidly inactivated by glutathione (GSH) to protect the hepatocytes. But, with the drug overdose, for example, the elevated levels of liver toxic metabolites, mainly N-acetyl-p-benzoquinoneimine (NAPQI), rapidly deplete GSH and covalently modify cellular proteins, leading to the generation of high levels of reactive oxygen species (ROS) and depletion of the ATP, which results in mitochondrial damage and hepatocyte injury. However, it was postulated that depletion of 90% of GSH in hepatocytes is critically necessary for the development of cell necrosis. In addition, hepatic inflammatory cytokines are also reported to be involved in APAP-induced liver injury.

Quercetin and resveratrol are polyphenolic antioxidants found in fruits, vegetables, and grains. They have been widely known to have potent cardiovascular protective and therapeutic effects via scavenging ROS and have anti-inflammatory effects, inhibit lipid peroxidation, inhibit platelet aggregation and thrombus formation, prevent apoptosis, and promote cell survival and liver protection against hepatic sinusoidal obstruction.
and hepatic steatosis. In addition, both quercetin and resveratrol were reported to inhibit APAP-induced ROS levels in liver tissue homogenates and the blood of rats and mice. However, neither quercetin nor a combination of quercetin and resveratrol has been used before to study the protection of hepatocyte ultrastructure upon acetaminophen intoxication in an animal model. Therefore, this study was designed to investigate the degree of protection by resveratrol and quercetin given in combination against APAP intoxication to the hepatocyte ultrastructure and compare it with the level of protection provided by these agents to known liver injury biomarkers.

**Methods**

**Reagents and assay kits**

Quercetin (C15H10O7, CAS Number 117-39-5) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was prepared daily and freshly by dissolving in a normal saline solution (0.9% NaCl) to the final concentration of 50 mg/ml. Resveratrol (C14H12O3, Cat No. R5010) was also purchased from Sigma-Aldrich (St. Louis, MO, USA) and was prepared daily and freshly by dissolving in a saline solution (0.9% NaCl) containing 20% hydroxypropyl cyclodextrin (American Maize-Products Co., Hammond, IN, USA) to a final concentration of 30 mg/kg. Assay kits for the determination of malondialdehyde (MDA, Cat No. NWK-MDA01) were purchased from NWLSS (Vancouver, BC, Canada). Superoxide dismutase (SOD) assay kit was purchased from Cayman Chemical, Cat. No.706002. ELISA kits for the determination of malondialdehyde (MDA, Cat No. NWK-MDA01) were purchased from NWLSS (Vancouver, BC, Canada). Superoxide dismutase (SOD) assay kit was purchased from Cayman Chemical, Cat. No.706002. ELISA kits for the determination of interleukin (IL)-6 (Cat No. ELR-IL6-001) were purchased from RayBio, GA, USA. ELISA kits for the determination of tumor necrosis factor-alpha (TNF-α) (Cat No. ab46070) were purchased from Abcam, Cambridge, UK. Assay kits for the determination of serum levels of alanine aminotransferase (ALT) were purchased from Human Co., Germany.

**Animals**

All experimental procedures were approved by the medical research ethical committee at King Khalid University and according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No.85-23, revised 1996). Sprague Dawley rats (n = 18) weighing 170–200 g were used in this study. All rats were bred and housed in the research center of King Khalid University, College of Medicine (Abha, Saudi Arabia), at a temperature of 23 ± 1°C and a 12 h light:12 h dark cycle. Rats had free access to tap water and fed standard laboratory chow during the acclimatization period.

**Experimental design**

After a one-week adaptation period, rats were randomly assigned into three groups (n= 6; each) and were distributed in their corresponding cages and classified as follows: (1) Control group: rats received normal saline daily for 7 days; (2) APAP-intoxicated group (Model group): rats received normal saline for 7 consecutive days and then given a single dose of APAP (2 g/kg, orally); and (3) RES+QUR+APAP group: rats were pretreated with RES (30 mg/kg) and 50 mg/kg QUR for 7 consecutive days and then administered with a single dose of APAP (2 g/kg, orally). APAP was administered to the desired groups one hour after the last dose of treatment on day 7, and all treatment in all groups was administered i.p. in a final volume of 1 ml. All animals were sacrificed on day 8.

**Biochemical measurements**

**Blood samples**

At the end of the experimental period, blood samples were collected by cardiac puncture under anesthesia (sodium thiopentone at 40 mg/kg body weight) after an overnight fast of 12 hours. These blood samples were collected without any anticoagulant, left for 10 min, and then centrifuged for 10 minutes at 4,000 r/min to obtain serum, which was stored at −20°C until further biochemical analysis for determination of serum liver injury enzyme ALT.

**Preparation of liver homogenates**

As we previously reported, part of the livers obtained from the rats of all groups were freshly washed with phosphate-buffered saline (PBS), pH 7.4. Then, they were homogenized with an ultrasonic homogenizer in cold phosphate buffer, pH 7.4,
containing ethylene-diamine-tetra-acetic acid (EDTA). The supernatant obtained from each rat was aliquot in separate tubes and stored at –70°C for later determination of levels of TNF-α, IL-6, MA, SOD, and glutathione peroxidase (GPx) according to manufacturer’s instructions.

**Transmission electron microscopy (TEM)**
As we previously reported\(^{26}\), small pieces of 1 mm\(^3\) of the liver were fixed at 4°C in 4% buffered glutaraldehyde (SERVA, Frankfurt, Germany) with 0.2 M cacodylate buffers (TAAB essential for microscopy, Aldermaston, Berks, UK) and processed for electron microscopic examination. Contrasted ultrathin sections with uranyl acetate and lead citrate (LobaChemie Pvt. Ltd., Colaba, Mumbai, India) were examined and photographed using the Philips EM 208S transmission electron microscope (FEI Company, Eindhoven, The Netherlands) at accelerating voltage of 80 kV.

**Determination of serum levels of ALT and liver homogenate levels of TNF-α, IL-6, MDA, SOD, and GPx**
On day 8, animals were sacrificed, and liver function was evaluated by assessing serum levels of liver injury enzyme ALT using ELISA kits according to the manufacturer’s instructions. Tissue levels of TNF-α, IL-6, MDA, SOD, and GPx were determined using ELISA kits according to the manufacturer’s instructions.

**Statistical analysis**
The data were expressed as mean ± standard deviation (SD). Data were processed and analyzed using the SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA). One-way ANOVA was done followed by Tukey’s post hoc test. Pearson correlation statistical analysis was done for the detection of a probable significance between two different parameters. Results were considered significant if \( p \leq 0.05 \).

**Results**

**Induction of acute liver injury in rats by APAP**
We induced the disease in the model group of rats by APAP overdose (2 g/kg body weight), which was confirmed after 8 days as shown by high blood and liver tissue levels in biomarkers of liver injury and a profound damage to hepatocytes (Figure 1). APAP induced hepatotoxicity which caused about a fivefold increase in ALT (Figure 1a) and a twofold decrease in the levels of the antioxidant SOD (Figure 1b), and TEM images of liver sections (Figure 1c,d) confirmed liver injury and abnormal changes to hepatocytic ultrastructure. Representative TEM image (5,000×) of liver sections obtained from the model group (Figure 1d) shows altered hepatocyte ultrastructure and condensation of chromatin masses with no nucleolus, and cytoplasm displays many lipid droplets, swollen mitochondria, dilation of endoplasmic reticulum, and damaged intercellular space. However, a TEM image of another hepatocyte at similar magnification prepared from liver sections of the control group (Figure 1c) shows an unremarkable hepatocyte, with centrally placed nuclei and crowded cytoplasm with organelles, particularly rough endoplasmic reticulum and mitochondria.

**Resveratrol plus quercetin protects hepatocyte ultrastructural alterations induced by APAP**
To determine whether giving two antioxidants together can work to completely protect the ultrastructure of hepatocytes against damage induced by APAP, one group of rats was pretreated for 7 days with RES+QUR and then administered with a single dose of APAP (Figure 2). Representative TEM images (5,000×) of liver sections display hepatocytes with normal architecture in the control animal group (Figure 2a,b) surrounded by an intact intercellular space. It shows round centrally located nuclei with at least one nucleolus and intact nuclear membrane, and the cytoplasm displays many organelles, particularly rough endoplasmic reticulum and mitochondria. Capillary sinusoid with Kupffer cell and erythrocytes were also seen. TEM images that represent liver sections of APAP-treated rats (Figure 2c,d) display pyknotic nuclei with nuclear disaggregation of granular elements, mitochondrial swelling, lysosomes, dilation of endoplasmic reticulum, and cytoplasmic steatosis (lipid droplets). Also, damaged intercellular space and pleomorphic capillary sinusoid with Kupffer cell and erythrocytes were also seen. Treatment of APAP rats with RES+QUR
(Figure 2e,f) significantly protected the hepatocellular architecture of rats as demonstrated by normal nuclei and cytoplasmic compartments. However, very few cytoplasmic vacuoles can be seen.

**Resveratrol plus quercetin protects the modulation of liver injury enzyme and oxidative, antioxidant, and inflammatory biomarkers induced by APAP in the blood and liver tissues**

Tissue inflammation, oxidative stress, and augmentation of ALT are known to be involved in the pathology of acute liver injury in animal models and humans. To investigate the level of inhibition of modulation of these biomarkers by combined injections of resveratrol and quercetin for 7 days prior to APAP hepatic intoxication, we measured ALT, MDA, SOD, GPx, TNF-α, and IL-6 in all rat groups (Figures 3 and 4). As shown in Figure 3a–d, acute liver injury induced by APAP significantly (p < 0.05) modulated MDA, SOD, GPx, and ALT, which were completely protected by RES+QUR. In addition, APAP significantly (p < 0.05) increased liver tissue levels of TNF-α and IL-6 compared to the control group, which were also completely inhibited by RES+QUR (Figure 4a,b).

**Discussion**

In this report, we investigated the status of hepatocytic ultrastructure in APAP-induced acute liver injury in a rat model of the disease in the presence and absence of resveratrol plus quercetin. Also, our protective approach using both agents was also used to assess levels of oxidative, inflammatory, and liver injury biomarkers. Therefore, rats were pretreated for 7 days with resveratrol plus quercetin prior to the induction of acute liver injury in rats by APAP.

Blood levels of ALT (a) and liver tissue levels of SOD (b) were measured one day post oral ingestion of rats (n = 6) with APAP (2 gm/kg; model group) compared to vehicle-ingested control group (n = 6). Results represent the mean (±SD), and experiments were performed in triplicate. *p < 0.0001 versus control. TEM images (5,000×) of harvested tissues obtained from the liver of the model group (d) compared to the control group (c) rats are visualized using transmission electron microscopy. There are many fat droplets and damaged mitochondria and rough endoplasmic reticulum in the hepatocyte obtained from the liver of the model group (APAP). Arrows point to the plasma membrane surrounding the hepatocyte. Abbreviations: N, nucleus; nu, nucleolus; L, lipid droplet; m, mitochondria; RER, rough endoplasmic reticulum; ne, nuclear envelop; chr, chromatin.

Figure 1. Induction of acute liver injury in rats by APAP.

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injury by APAP overdose. Here, we report the ability of resveratrol plus quercetin to markedly inhibit hepatocyte ultrastructural alterations and completely inhibit the induction of biomarkers of inflammation, oxidative stress, and liver injury in APAP-induced acute hepatotoxicity in rats.

Our conclusions are supported by the data indicating that APAP caused a profound damage to the hepatocyte ultrastructure (Figures 1d and 2c,d) and induced biomarkers of liver injury, ALT (Figure 1a); oxidative stress, MDA (Figure 3a); and inflammation, TNF-α and IL-6 (Figure 4a,b), which were substantially protected by RES+QUR (Figures 2–4). Also, our data demonstrate that APAP caused an inhibition of the antioxidants, SOD and GPx, which were significantly augmented by RES+QUR to control levels (Figure 3b,c).

Our results were thus consistent with our working hypothesis that the combined polyphenolic compounds, resveratrol (RES) and quercetin (QUR),...
Figure 3. Resveratrol and quercetin protect against APAP-induced modulation of MDA, SOD, GPx, and ALT biomarkers in rats.
Liver homogenate levels of MDA (a), SOD (b), and GPx (c) and blood levels of ALT (d) were measured at the end of the experiment, one day post APAP ingestion in different groups of rats used in this study; Control group, APAP group, and RES+QUR+APAP group. Results represent the mean (±SD); n = 6 for each group. Experiments were performed in triplicate. *p < 0.05 versus control, **p < 0.05 versus APAP.

Figure 4. Resveratrol and quercetin inhibit APAP-induced TNF-α and IL-6 biomarkers in rats.
Liver homogenate levels of TNF-α (a) and IL-6 (b) were measured at the end of the experiment, one day post APAP ingestion in different groups of rats used in this study; Control group, APAP group, and RES+QUR+APAP group. Results represent the mean (±SD); n = 6 for each group. Experiments were performed in triplicate. *p < 0.05 versus control, **p < 0.05 versus APAP.
can substantially protect against hepatocyte ultrastructural damage caused by a toxic dose of APAP in a rat model of APAP-induced acute liver injury.

Promising therapeutic effects of quercetin and resveratrol on hepatotoxicity-induced acute and chronic liver injuries via the inhibition of oxidative and nitrosative stress, inflammation, apoptosis, and NF-kB pathways were reported\textsuperscript{28-30}, which are in agreement with our findings shown in Figures 3 and 4 and our recent report.\textsuperscript{31} However, conflicting data on the protective/treatment effects of resveratrol alone against APAP-induced acute liver injury in rats have been reported.\textsuperscript{32,33} A recently published work\textsuperscript{32} reported significant inhibition of liver iNOS immunostaining and hepatocyte ultrastructure damage by RES (10 mg/kg) given 20 minutes post APAP (1 g/kg) injection in rats. However, administering triple-dose RES (30 mg/kg) at the same time with APAP (1 g/kg) to rats caused a weak inhibition of blood ALT and no effect on the ALT levels in cultured hepatocytes prepared from these animals.\textsuperscript{33} In addition, the same group\textsuperscript{33} found no significant pathological changes in the liver tissue of the model group (APAP) stained with H&E. However, our H&E images demonstrated substantial liver damage induced by APAP (data not shown). Our treatment protocol (2 g/kg APAP) versus their treatment protocol (1 g/kg APAP) could be the reason for such differences.

Finally, our biochemical data that showed RES +QUR completely inhibited APAP-induced biomarkers of liver injury, inflammation, and oxidative stress are almost in agreement with our TEM images that showed a substantial protection of hepatocyte ultrastructure, which points to the importance of electron microscopy as a tool besides the biochemical investigations to monitor the progress of acute liver injury. Therefore, we conclude that pretreatment with resveratrol plus quercetin can effectively protect against hepatocyte ultrastructural alterations and inhibits inflammatory and oxidative stress biomarkers in a rat model of APAP overdose-induced hepatotoxicity.

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Disclosures

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