Paracetamol Poisoning Induces Acute Liver Injury in Rats: Inhibition of miR-155/CD45 Axis-Mediated Antioxidant Depletion and Hepatotoxicity Using Quercetin and Resveratrol

La Intoxicación por Paracetamol Induce Daño Hepático Agudo en Ratas: Inhibición de la Depleción de Antioxidantes Mediado por el Eje miR-155/CD45 y Hepatotoxicidad Usando Quercetina y Resveratrol

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SUMMARY: Ingestion of an overdose of paracetamol (also called acetaminophen, or APAP) induces hepatotoxicity that can lead to liver failure. The link between the pro-inflammatory microRNA-155 (miR-155) and leukocyte infiltration (CD45) in APAPantioxidant depletion and liver toxicity with and without the natural polyphenolic compounds, quercetin (QUR) plus resveratrol (RES) has not been previously studied. Therefore, acute hepatic injury was induced in rats by 2 g/kg APAP (single dose, orally) and another group started QUR (50 mg/kg) plus RES (30 mg/kg) treatment one week prior to APAP ingestion. Animals were culled 24 hours post the paracetamol treatment. APAP overdose induced hepatic and blood levels of miR-155 expression, CD45 (leukocyte common antigen) immunostaining, degenerated hepatocytes, and hepatic injury enzymes; alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which were markedly decreased by QUR+RES. Whereas, APAP intoxication ameliorated liver tissue levels of the antioxidants, glutathione peroxidase and superoxide dismutase that were augmented by QUR+RES. Moreover, a significant (p<0.05) correlation between miR-155/CD45 axis and liver tissue injury was observed. These findings show that paracetamol intoxication augments miR-155/CD45 axis-mediated modulation of antioxidants and liver injury in rats, and is protected by QUR+RES.

KEY WORDS: Acute liver injury; Paracetamol poisoning; miR-155; CD45; Antioxidant; Quercetin; Resveratrol.

INTRODUCTION

Paracetamol (APAP) is the most common drug used for suicide and nonfatal poisoning that induces acute liver injury in humans (McGill *et al.*, 2012). APAP toxicity is the leading cause of death in the USA from acute liver failure (Lazerow *et al.*, 2005), and it is estimated that about half of the admitted cases of acute liver failure in the USA are due to paracetamol poisoning (Larson *et al.*, 2005). In England and Wales, UK, paracetamol overdose caused the death of 127 persons aged 12 and over years between 1999-2001 (Hawton *et al.*, 2004). The liver is the site of paracetamol metabolism and hepatotoxic metabolites are rapidly inactivated by the endogenous hepatic antioxidant glutathione (GSH) to protect liver cells from the deleterious effects of these metabolites (James *et al.*, 2003). However, it was suggested that the development of cell necrosis occur when 90 % of glutathione in hepatocytes is depleted

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(Henderson *et al.*, 2000). Ingestion of toxic dose(s) of APAP lead to the elevation of liver harmful metabolites that deplete the endogenous GSH and increase the production of the reactive oxygen species (Hinson *et al.*, 2004). This caused mitochondrial damage and hepatic injury (Hinson *et al.*, 2004). In addition, hepatic leukocyte infiltration and miR-155 induced liver injury and liver fibrosis is well documented (Jaeschke & Hasegawa, 2006; Bala *et al.*, 2016).

Quercetin and resveratrol are natural polyphenolic compounds found in fruits such as apple and grapes, grains and vegetables (Cudmore et al., 2012). They demonstrate cardioprotective properties by scavenging free radicals (Hung et al., 2000), inhibit LDL oxidation (Frankel et al., 1993), inhibit inflammation (Al-Ani, 2013), and hepatoprotection (Faghihzadeh et al., 2015; Zhang et al., 2017). Additionally, both compounds were reported to partially protect against paracetamol poisoning by reducing oxidative stress levels in the blood and liver tissue homogenates of mice and rats (Singh et al., 2011). However, the protection of the hepatic tissue by a combination of quercetin and resveratrol via interfering with miR-155, leukocyte infiltration, and antioxidant levels upon paracetamol intoxication in an animal model has not been investigated before. As a result, our aim herein was to investigate the miR-155/CD45 axis-mediated liver tissue injury induced by APAP, and the level of protection provided by quercetin plus resveratrol.

MATERIAL AND METHOD

Animals. The protocol used for this work was approved by the ethical committee at King Khalid University. Rats (Sprague Dawley) with a weight between 170-200 gm were maintained in a clean facility at temperature of 22 °C with a 12h light/dark cycle and had free access to water and food.

Experimental design. 24 rats were allocated into four groups (6/group) after one week acclimatization. Firstly, the control group (Control), rats received saline solution daily for one week. Secondly, QUR+RES group, rats were given 50 mg/kg QUR and 50 mg/kg RES for one week. Thirdly, the model group (APAP), rats received saline solution for one week and then given 2 g/kg APAP (single dose, orally). Fourthly, the protective group of rats (QUR+RES+APAP), rats were pre-treated with QUR+RES for one week and then given 2 g/kg APAP (single dose, orally). Blood samples were collected after experiment completion on day 8 under anesthesia with sodium thiopentone utilizing cardiac puncture. Following that, animals were culled and liver tissue samples were collected.

Histological examination. Liver specimens from various groups were fixed in 10 % formal saline for 24 hours prior to alcohol dehydration. Paraffin blocks were prepared using standard methods. Sections of 5 mm in thickness were deparaffinized, rehydrated, and were subjected to hematoxylin and eosin (H&E) stain to study the morphological changes. The H&E stained liver sections were inspected for the morphological features to assess the degree of apoptotic/ necrotic hepatocyte injury and were scored (semi-quantitative of 0-5; 0: normal; 1: minimal-mild; 2: mild-moderate; 3: moderate-severe; 4: severe; 5: extensive cell loss).

Immunohistochemistry of CD45. As previously reported (Mirdad *et al.*, 2022), deparaffinized 5 mm thick sections were used for immunohistochemical staining. Following antigen retrieval in the presence of a citrate buffer, sections were incubated overnight at 40C with anti-cluster of differentiation (CD) 45 (Abcam, Cambridge, UK). Liver sections were then incubated for half an hour at room temperature with the secondary antibody, followed by counterstained with Meyer's hematoxylin.

Liver miR-155 real-time PCR (qRT-PCR). miR-155 was amplified as recently described (Dawood *et al.*, 2022) using specific primers for miR-155 (sense, 5'-CGCAGTTAATGCTAATTGTGATAG-3'; antisense, 5'-TCCAGTTTTTTTTTTTTTTTTTCAAGGT-3'), and the endogenous control suitable for miR-155, snU6RNA (sense, 5'- ATACAGAGAAGATTAGCATGGCC-3'; antisense, 5'- GTCCAGTTTTTTTTTTTTTTTTTTTTCGAC-3'). The relative expression was estimated using the manufacturer's software.

Determination of blood levels of ALT and AST, and tissue levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx). Liver injury enzymes ALT and AST were evaluated in the blood using ELISA kits from Human Co., Germany, guided by manufacturer's instructions. Liver tissue levels of SOD (Cat. No. 706002) and GPx, (Cat NO. 703102) activities were measured (Cayman Chemical, Ann Arbor, MI, USA).

Statistical analysis and morphometry. Data were processed and analyzed using the SPSS version 10.0 (SPSS, Inc., Chicago, Ill., USA). One-way ANOVA was performed followed by Tukey's post hoc test. Pearson correlation statistical analysis was performed for detection of a probable significance between two different parameters. Results were considered significant if $p \le 0.05$. Morphometry of the areas % of CD45 immunostaining were performed using "Leica Qwin 500 C" image analyzer (Cambridge, UK) as recently reported (Dawood *et al.*, 2022).

RESULTS

Quercetin plus resveratrol inhibit miR-155 and leukocyte infiltration induced by APAP in liver tissue. Infiltration of leukocytes such as neutrophil granulocytes, monocytederived macrophages, and T lymphocyte upon liver injuries are well documented, and all leukocytes express the leukocyte common antigen, CD45 (Hu et al., 2018). In addition, miR-155 knockout decreased leukocytes infiltration (Eisenhardt et al., 2015). To determine the magnitude of miR-155 expression and leukocytes infiltration into liver tissue caused by APAP poisoning and the degree of inhibition by QUR+RES in rats, we evaluated miR-155 expression and CD45 protein levels in rats' groups (Fig. 1). APAP intoxication augmented liver tissue levels of miR-155 (Fig. 1A) and CD45+ leukocytes (Figs. 1C and 1D), which were substantially decreased by quercetin plus resveratrol in the QUR+RES+APAP group (Figs. 1A, 1E, and 1F). Nevertheless, QUR+RES effects were found to remain significant (p<0.05) in comparison with the control rats.

Quercetin plus resveratrol inhibit APAP-induced modulation of antioxidants and liver injury. Degree of modulation of the antioxidants (SOD and GPx) and liver injury enzymes (ALT and AST) by APAP in the presence and absence of QUR+RES was assessed. APAP caused a significant (p<0.05) decrease in hepatic tissue levels of the antioxidants SOD (Fig. 2A) and GPx (Fig. 2B), which were increased by QUR+RES, reaching levels compared to control group. Whereas, APAP caused a sharp rise in ALT (Fig. 2C) and AST (Fig. 2D), which were ameliorated by QUR+RES to levels compared to control group.

Quercetin plus resveratrol protect against the induction of liver tissue damage by APAP. At day 8, liver tissue sections were stained with H&E and examined using light microscopy following H&E staining. Compared with a normal liver parenchyma in control group (Fig. 3A), APAP substantially damaged the hepatocytes, and induced numerous inflammatory cell infiltration, pericentral necrotic areas, cytoplasmic vacuolation, dilated and congested blood vessels, and haemorrhage (Fig. 3B, and data not shown). Pre-treatment with quercetin plus resveratrol protected the architecture of liver. However, few infiltrated cells in few fields were still seen (Fig. 3C). The degree of acute liver injury was significantly (p<0.05) lower in the treated animals (Fig. 3D). Nevertheless, QUR+RES effects were found to remain significant in comparison with the control rats.

Correlation between hepatocyte injury Score and miR-155, CD45, antioxidants, and biomarkers of liver injury. We further estimated the correlation between hepatocyte injury score and liver tissue and blood levels of miR-155, CD45, SOD, GPx, ALT, and AST. This correlation is important in drawing an association between these



Fig. 1. Quercetin (QUR) plus resveratrol (RES) ameliorate APAP-induced miR-155 and CD45 expression. miR-155 levels were assessed using qRT-PCR (A). CD45 immunohistochemistry of liver sections (D, x100; B, C, and E x200) from the control (B), APAP (C and D), and QUR+RES+APAP (E) rats' groups are illustrated. (F) Degree of CD45 immunostaining in liver sections from groups above is exemplified in histograms. Presented p values are significant; *p<0.05 versus control, **p<0.05 versus APAP. miR-155: microRNA-155; APAP: paracetamol; CV: central vein; PT: portal tract; CD45: cluster of differentiation 45 (leukocyte common antigen).

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Fig. 2. Quercetin (QUR) plus resveratrol (RES) augment antioxidants and inhibit liver injury enzymes caused by APAP. Tissue and blood levels of SOD (A), GPx (B), ALT (C), and AST (D) were assessed 8 days after the start of the pretreatment protocol and one day post liver intoxication by APAP in rats' groups are shown. Presented p values are significant; for A and B: *p<0.05 versus control, **p<0.05 versus QUR+RES, ***p<0.05 versus APAP. For C and D: *p<0.05 versus control, **p<0.05 versus APAP. SOD: superoxide dismutase; GPx: glutathione peroxidase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; APAP: paracetamol.

Fig. 3. Quercetin (QUR) plus resveratrol (RES) ameliorate APAP-induced liver tissue damage. H&E stained images (x200) of harvested tissues obtained one day post liver intoxication by APAP from the liver of the control (A), APAP (B), and QUR+RES+APAP (C) rats' groups. Note the presence of hemorrhage (B, arrow), and infiltration of inflammatory cells (curved arrows). (D) Degree of liver injury in rats from groups above is exemplified in histograms. Presented p values are significant; *p<0.05 versus control, **p<0.05 versus APAP. APAP: paracetamol.

parameters involved in acute liver pathology. Hepatocyte injury score displayed a significant (p<0.05) correlation with miR-155 (r = 0.924) (Fig. 4A), CD45 (r = 0.899) (Fig. 4B),

SOD (r = - 0.687) (Fig. 4C), GPx (r = - 0.819) (Fig. 4D), ALT (r = 0.895) (Fig. 4E), and AST (r = 0.908) (Fig. 4F).



Fig. 4. Correlation between hepatocyte injury Score and miR-155, leukocyte infiltration, antioxidants, and liver injury markers. Degree of hepatocyte damage was evaluated in all rat groups at day 8 inorder to draw a link between hepatocyte injury and tissue and blood levels of miR-155 (A), CD45 (B), SOD (C), GPx (D), ALT (E), and AST (F). miR-155: microRNA-155; CD45: CD45: cluster of differentiation 45 (leukocyte common antigen); SOD: superoxide dismutase; GPx: glutathione peroxidase; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

DISCUSSION

This report investigated hepatic microRNA-155/ leukocyte infiltration (CD45) mediated antioxidants inhibition (SOD and GPx) and liver injury in a rat model of paracetamol (APAP)-induced acute liver toxicity in the presence and absence of the polyphenolic compounds QUR+RES. We also assessed levels of liver injury enzymes. Here, we report (i) the induction of hepatic miR-155/CD45 microRNA and protein expression by APAP in rats associated with the amelioration of biomarkers of antioxidants, and liver injury; (ii) effective inhibition of these parameters by QUR+RES; and (iii) a significant link between hepatic tissue damage and miR-155, leukocyte infiltration, antioxidants, and liver injury enzymes (Fig. 5). This further corroborates our recent report on the protective effect of QUR+RES against hepatocyte ultrastructural alterations induced by APAP overdose (Al Humayed et al., 2019).

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Depletion of the antioxidant hepatic glutathione and augmentation of oxidative stress by APAP causes liver injury associated with the infiltration of leukocytes, inflammation, apoptosis, and necrosis (Williams et al., 2010). Cytotoxic insult to liver tissue increases hepatic infiltration of leukocytes such as neutrophil granulocytes, monocytederived macrophages, and T lymphocyte to combat the insult (Foureau et al., 2015). In acute liver injury, recruiting leukocytes from blood circulation to the liver is an early response to cellular stress and inflammation (Jaeschke & Hasegawa, 2006). Moreover, in CD18-deficient mice, APAPinduced acute hepatotoxicity is characterized by increased accumulation of neutrophils in liver tissue, inflammation, and liver injury (Williams et al., 2010). These reports are consistent with our data which also correlate similar parameters brought about by APAP overdose augmented the MOMENAH, M. A.; EBRAHIM, H. A.; ALZAMIL, N. M.; ALFAIFI, M.; ALSHAHRANI, M. Y.; KAMAR, S. S.; HAIDARA, M. A.; AL-ANI, B. & DAWOOD, A. F. Paracetamol poisoning induces acute liver injury in rats; inhibition of mir-155/cd45 axis-mediated antioxidant depletion and hepatotoxicity using guercetin and resveratrol. Int. J. Morphol., 40(5):1174-1180, 2022.



hepatic proinflammatory miR-155, leukocyte infiltration, and liver injury, as well as the inhibition of hepatic antioxidant levels in rats (Figs. 1 to 3). In addition, our data suggests that effective inhibition of APAP-induced hepatic intoxication by QUR+RES mirrors previous study that also demonstrated effective inhibition of APAP-induced acute kidney damage as demonstrated by APAP caused a profound kidney ultrastructural alterations associated with inflammation and oxidative stress, which were treated by resveratrol and quercetin (Dallak et al., 2022).

Furthermore, (i) overexpression of miR-155 induced infiltration of leukocytes and inflammation were reported in a mouse model of cute hepatic injury induced by LPS (Xin et al., 2020); (ii) increased heart tissue miR-155 expression associated with increased levels of CD45 protein expression was reported in a mouse model of experimental autoimmune myocarditis (Yan et al., 2016); and (iii) in ischemia-reperfusion injury model, augmentation of miR-155 increased the recruitment of inflammatory cells (Eisenhardt et al., 2015). These studies are in agreement with our data on miR-155 (Fig. 1). However, another study(Yuan et al., 2016) though demonstrated an upregulation of liver and blood miR-155 upon APAP intoxication similar to our finding, but they proposed this microRNA as a therapy since miR-155 inhibition aggravated liver injury, which is in disagreement with our findings shown in Fig. 5. In summary, we have demonstrated that in a rat model of paracetamol intoxication-induced liver damage, the activation of miR-155/CD45 axis-mediated antioxidant reduction and hepatic injury, which is protected by quercetin and resveratrol. This suggests that the manipulation of these factors would provide a potential therapeutic target in preventing acute liver failure induced by paracetamol poisoning.

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RESUMEN: La ingestión de una sobredosis de paracetamol (también llamado acetaminofeno o APAP) induce hepatotoxicidad que puede provocar insuficiencia hepática. El vínculo entre el microARN-155 proinflamatorio (miR-155) y la infiltración de leucocitos (CD45) en el agotamiento de APAPantioxidante y la toxicidad hepática con y sin los compuestos polifenólicos naturales, quercetina (QUR) más resveratrol (RES) no ha sido previamente investigado. En este estudio, se indujo daño hepático agudo en ratas con 2 g/kg de APAP (dosis única, por vía oral) y otro grupo comenzó el tratamiento con QUR (50 mg/ kg) más RES (30 mg/kg) una semana antes de la ingestión de APAP. Los animales se sacrificaron 24 horas después del tratamiento con paracetamol. La sobredosis de APAP indujo niveles hepáticos y sanguíneos de expresión de miR-155, inmunotinción de CD45 (antígeno leucocitario común), degeneración de los hepatocitos y daño hepático enzimático; alanina aminotransferasa (ALT) y aspartato aminotransferasa (AST), disminuyeron notablemente con QUR+RES. Mientras que la intoxicación con APAP mejoró los niveles de antioxidantes, glutatión peroxidasa y superóxido dismutasa en el tejido hepático los que aumentaron con QUR+RES. Además, se observó una correlación significativa (p<0,05) entre el eje miR-155/CD45 y la lesión del tejido hepático. Estos hallazgos muestran que la intoxicación por paracetamol aumenta la modulación mediada por el eje miR-155/CD45 de los antioxidantes y la lesión hepática en ratas, y está protegida por QUR+RES.

PALABRAS CLAVE: Daño hepático agudo; Intoxicación por paracetamol; miR-155; CD45; Antioxidante; Quercetina; Resveratrol.

RES:

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