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Comparative effects of Quercetin and SRT1720 against D-galactosamine/lipopolysaccharide-induced hepatotoxicity in rats: biochemical and molecular biological investigations

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Abstract. – OBJECTIVE: Quercetin, a plant flavonoid with potent antioxidant action, has been shown to be ameliorative against different types of liver insults, including D-Galactosamine/Lipopolysaccharide (D-GalN/LPS). The notion that its cytoprotective effects are SIRT1 mediated is still controversial. In this work, we examined whether the synthetic allosteric SIRT1 activator, SRT1720, may similarly attenuate D-GalN/LPS-induced hepatotoxicity.

MATERIALS AND METHODS: Male Wistar rats were randomly assigned into 6 groups: (1) Control, (2) Quercetin, (3) SRT1720, (4) D-GalN/LPS, (5) Quercetin + D-GalN/LPS and (6) SRT1720 + D-GalN/LPS. After twenty-four hours, the effects of these treatments were evaluated by biochemical studies, real-time PCR and Western blot.

RESULTS: D-GalN/LPS treatment downregulated SIRT1 expression and markedly increased the aminotransferase, bilirubin and conjugated diene levels. Conversely, quercetin and SRT1720 pretreatments upregulated SIRT1 expression and decreased the levels of the aforementioned markers. Quercetin had more profound effect on SIRT1 expression than SRT1720. Moreover, quercetin was more efficacious than SRT1720 in combatting the cytotoxic effects of D-GalN/LPS, as evidenced by lower markers of liver injury.

CONCLUSIONS: These results strongly suggest the involvement of SIRT1 in the cytoprotective effects of quercetin and SRT1720 against D-GalN/LPS-induced hepatotoxicity.

Key Words:
Polyphenols, Quercetin, SRT1720, SIRT1, D-Galactosamine/Lipopolysaccharide, Hepatotoxicity.

Abbreviations

AMPK = AMP-activated protein kinase; DMSO = Dimethyl sulfoxide; FoxO = Forkhead box O; HO-1 = Heme Oxygenase 1; MnSOD = Manganese superoxide dismutase; NF-κB = nuclear factor-kappaB; OTC = Over-the-counter; STACs = Sirtuin-activating compounds; SIRT1 = Sirtuin 1; TNF-α = Tumor necrosis factor alpha.

Introduction

Flavonoids are a class of polyphenolic plant compounds widely present in the human diet. Quercetin, the major representative flavanoid, is abundant in vegetables, fruits, red wine, green tea, spices and herbs. Quercetin has long been known to possess antioxidant properties. For instance, the ancient custom of preserving lard by mixing it with onion may be based on the prevention of lipid oxidation and rancidity by quercetin[1]. Furthermore, there are numerous epidemiologic studies showing an inverse relationship between chronic consumption of quercetin and oxidative-stress related diseases such as coronary heart diseases[2], cerebrovascular accidents[3], gastric cancer[4] and non-alcoholic fatty liver disease[5].

Acute liver failure (ALF) is a critical disease with numerous causes and unacceptably high mortality rates[6]. Despite great advances in modern medicine, conventional drugs used in treatment of liver disorders are often inadequate, hence the need for alternative drugs[7]. Polyphenols such as resveratrol[8], quercetin[9], curcumin[10] and silymarin[11], also found in traditional healing herbs such as *Lagerstroemia speciosa*[12] and *Prosthechea michuacana*[13], are the most promis-

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ing in treatment of liver diseases, necessitating further studies to characterize their cytoprotective mechanisms. In 2013, we found that the liver-protective effects of quercetin against D-GalN/LPS-induced hepatotoxicity were due, at least in part, to induction of HO-1[14]. Most recently, we demonstrated that the antihepatotoxic effects of resveratrol are SIRT1-dependent[15]. Furthermore, there is mounting evidence of SIRT1 involvement in the pleotropic multisystem effects of polyphenols[16,17,18].

SIRT1 serves as a transcriptional effector by controlling the acetyalation states of histones. It regulates transcriptional and related factors such as p53, NF-κB and FoxOs that control autophagy, inflammatory response and stress resistance[19,20]. All these attractive features have validated a prompt search of SIRT1 nutraceutical or pharmaceutical activators. Relationship between aforementioned health benefits of polyphenols and SIRT1 is still controversial. Whether polyphenols directly activate SIRT1[21], indirectly activate SIRT1 through AMPK[22] or act independent of SIRT1[23] is still elusive. Novel potent activators of SIRT1 such as SRT1720, SRT1460 and SRT2183 have recently been developed to shed more light on the therapeutic potential of SIRT1 stimulation[24].

The present report is designed to investigate whether quercetin, a natural polyphenol, and SRT1720, an established synthetic allosteric SIRT1 activator, may have any modulating effects against D-GalN/LPS-induced hepatotoxicity at biochemical and molecular biological levels.

Materials and Methods

Chemicals

Lipopolysaccharide from *Escherichia coli* K-235 (LPS), D-galactosamine hydrochloride (D-GalN), Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one with HPLC purity of > 95%), anti-mouse IgG (whole molecule)-Peroxidase antibody and mouse monoclonal anti-B-Actin antibody were purchased from Sigma-Aldrich (Prague, Czech Republic). SRT1720 was purchased from Selleckchem (Munich, Germany). SirT1 (1F3) mouse mAb antibody was from Cell Signaling Technology (Danvers, MA, USA). Power SYBR Green PCR Master Mix was purchased from Thermo Fisher Scientific (Prague, Czech Republic). ACTB RT-PCR primer set was purchased from Biomol (Hamburg, Germany) and SIRT1 RT-PCR primer set was from Qiagen (Hilden, Germany).

Animals

Male Wistar rats, 250-400 g body weight, were purchased from Velaz-Lysolaje, Czech Republic. They were given standard granulated diet and water ad libitum. They were maintained under standard conditions (12-hour light-dark cycle, 22 ± 2°C temperature, 50 ± 10% relative humidity) and received care in accordance with the ethical guidelines of the First Faculty of Medicine, Charles University in Prague.

Experimental Design

The animals were adapted for seven days before being randomly assigned into six groups and administered the following drugs intraperitoneally:

- Group 1-500 μl/kg of DMSO and 1000 μl/kg of physiologic solution;
- Group 2-50 mg/kg of quercetin dissolved in DMSO;
- Group 3-5 mg/kg of SRT1720 dissolved in DMSO;
- Group 4-400 mg/kg of D-GalN and 10 μg/kg of LPS dissolved in physiologic solution;
- Group 5-50 mg/kg of quercetin dissolved in DMSO followed by 400 mg/kg of D-GalN and 10 μg/kg of LPS dissolved in physiologic solution one hour later;
- Group 6-5 mg/kg of SRT1720 dissolved in DMSO followed by 400 mg/kg of D-GalN and 10 μg/kg of LPS dissolved in physiologic solution one hour later.

After 24 hours, the animals were euthanized by exsanguination and the blood samples were collected into heparinized tubes. The livers were excised and preserved in liquid nitrogen for RT-PCR and Western blot studies.

The above doses were selected based on our previous studies with quercetin[14] as well as studies of others dealing with SRT1720[25]. As a novel agent, pilot study was done with SRT1720 to assess the safety of the drug from 0.1-10 mg/kg and we found 5 mg/kg to be the safest effective dose.

Biochemical Investigations

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin plasma levels were detected using commercial assay

kits according to the manufacturer's instructions (Analyticon, Lichtenfels, Germany). Peroxidation of membrane lipids was assessed by formation of conjugated dienes as previously described by Farghali et al[8].

Immunoblotting

Pulverized liver samples were homogenized and lysed in NP40 lysis buffer supplemented with protease and phosphatase inhibitors. Samples were then centrifuged (12,000 rpm, 15 minutes, 4 degrees Celsius) and the supernatant was collected for further analysis. Protein concentration of each sample was determined using BIO-RAD DC protein assay kit (BIO-RAD, Hercules, CA, USA). 20 μg of proteins was fractionated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked by incubation in Tris-buffered saline containing 5% non-fat milk (for 1 hour at room temperature) and incubated with primary antibodies overnight at 4°C: SIRT1 (1:1000) and Beta actin (1:5000). The following day, the membranes were washed in TBST and incubated with anti-mouse IgG (whole molecule)-Peroxidase antibody (1:80000) at room temperature for 1 hour. Bands were detected by enhanced chemiluminescence using Super Signal West Pico Chemiluminescent Substrate (GeneTiCAs.r.o. Prague, Czech Republic) and the protein density was measured using the Quantity One software (Bio-Rad, Prague, Czech Republic).

Real-Time PCR

Total RNA was isolated from the tissue samples using Qiagen RNeasy plus kit (Bio-Consult Laboratories, Prague, Czech Republic). Complementary DNA was synthesized from the total RNA using GeneAmp RNA PCR kit (Applied Biosystems, Prague, Czech Republic). Reverse transcription included the following phases: 10 min at 25°C for reverse transcriptase enzyme activation, 30 min at 48 °C for PCR amplification and 5 min at 95 °C for denaturation. The expressions of target mRNAs were detected by quantitative RT-PCR (ABI PRISM 7000) with SYBR Green as detection dye. Relative expression of SIRT1 mRNA was calculated using $\Delta\Delta$Ct method with ACBT mRNA as an internal control[26].

Statistical Analysis

Data are expressed as mean ± SEM (standard error of mean). Statistical evaluation of the data was performed using one-way ANOVA followed by Tukey-Kramer comparison test. $p < 0.05$ was considered to have statistical significance.

Results

Cytotoxic Effects of D-GalN/LPS in the Liver

We first sought to confirm the cytotoxic effects of D-GalN/LPS in the liver. The results show that rats treated with D-GalN/LPS developed acute hepatotoxicity within 24 hours of the insult as confirmed by a significant and dramatic increase in plasma aminotransferase levels (Figure 1). 56.2-fold increase in ALT and 3.9-fold increase in AST levels were observed, compared to the control group. Conjugated dienes in homogenate were also significantly enhanced, suggesting lipid peroxidation (Figure 2B). Furthermore, 12.6-fold increase in bilirubin, an endogenous antioxidant, suggests severe oxidative stress (Figure 2A).

Ameliorative Effects of Quercetin and Srt1720 on D-Galn/Lps-Induced Hepatotoxicity

The second objective was to find out if quercetin treatment and its synthetic counterpart, SRT1720, may have any ameliorative effects on D-GalN/LPS-induced hepatotoxicity. Alone, quercetin and SRT1720 treatments did not have any significant effects on the levels of the aforementioned markers. Pretreatment of D-GalN/LPS rats with quercetin consistently attenuated the cytotoxic effects of D-GalN/LPS. Quercetin pretreatment significantly decreased the levels of ALT (4.6 fold), AST (1.8 fold), conjugated dienes (2.7-fold) and bilirubin (4.6-fold) compared to the D-GalN/LPS group. All these data strongly suggest that the antioxidant effects of quercetin play a key role in combatting D-GalN/LPS-induced hepatotoxicity. Likewise, SRT1720 pretreatment minimized the cytotoxic effects of D-GalN/LPS. However, it was less effective than quercetin in lowering ALT (2.6-fold), AST (1.5-fold), conjugated dienes (2.3-fold) and bilirubin (2.5-fold) compared to the D-GalN/LPS group. Collectively, these results suggest that, under the present experimental conditions, quercetin was more efficacious than SRT1720 in attenuating D-GalN/LPS-induced hepatotoxicity at the given dosage schedule.

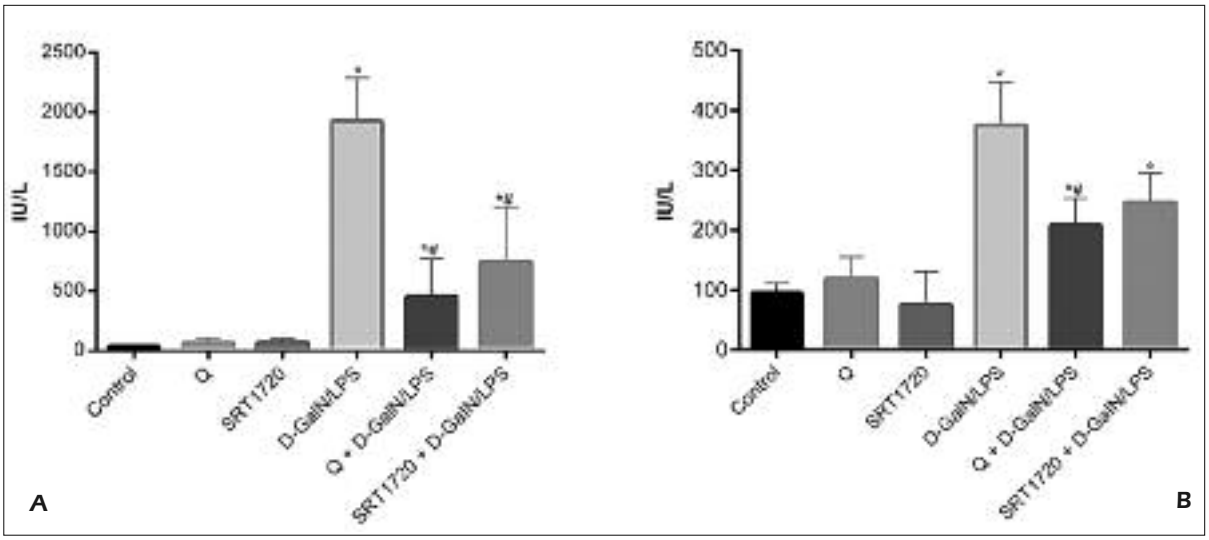

Figure 1. Effects of quercetin and SRT1720 pretreatments in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized (D-GalN/LPS) rats on plasma levels of ***(A)*** alanine aminotransferase, ALT and ***(B)*** aspartate aminotransferase, AST. Control: vehicle only; Q: quercetin 50 mg/kg; SRT1720: SRT1720 5 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + Lipopolysaccharide 10 μg/kg; Q + D-GalN/LPS: Combination of Q and D-GalN/LPS; SRT1720 + D-GalN/LPS: Combination of SRT1720 and D-GalN/LPS. *Indicates significant values ($p \leq 0.05$) compared to the control group (vehicle only); # Indicates significant values ($p \leq 0.05$) compared to the D-GalN/LPS group. The results are expressed as means ± SEM, n = 6.

Relationship Between Sirt1 Expression, D-Galn/Lps-Induced Hepatotoxicty and the Ameliorative Effects of Quercetin and Srt1720

Finally, we investigated if D-GalN/LPS, Quercetin and SRT1720 treatments may influence SIRT1 expression. For this, we did Western blot (Figure 3) and RT-PCR (Figure 4) analyses. We found that SIRT1 was ubiquitous, even expressed by animals treated with the vehicle only (control). Alone, quercetin and SRT1720 treatments significantly increased SIRT1 protein and

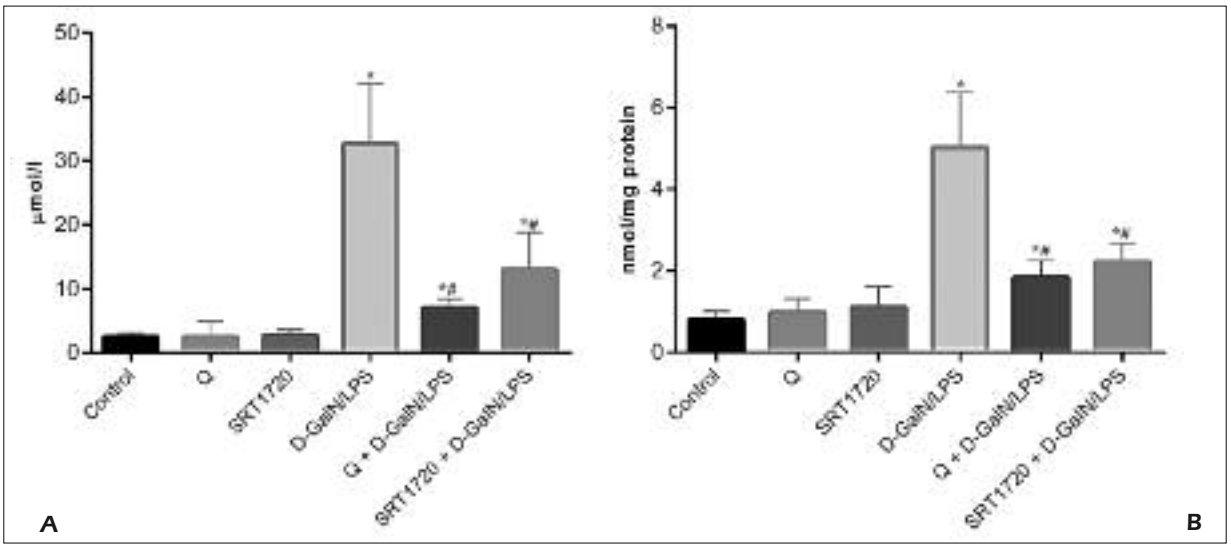

Figure 2. Effects of quercetin and SRT1720 pretreatments in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized (D-GalN/LPS) rats on the levels of ***(A)*** total bilirubin in plasma and ***(B)*** conjugated dienes in liver homogenate. Control: vehicle only; Q: quercetin 50 mg/kg; SRT1720: SRT1720 5 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + Lipopolysaccharide 10 μg/kg; Q + D-GalN/LPS: Combination of Q and D-GalN/LPS; SRT1720 + D-GalN/LPS: Combination of SRT1720 and D-GalN/LPS. *Indicates significant values ($p \leq 0.05$) compared to the control group (vehicle only); #Indicates significant values ($p \leq 0.05$) compared to the D-GalN/LPS group. The results are expressed as means ± SEM, n = 6.

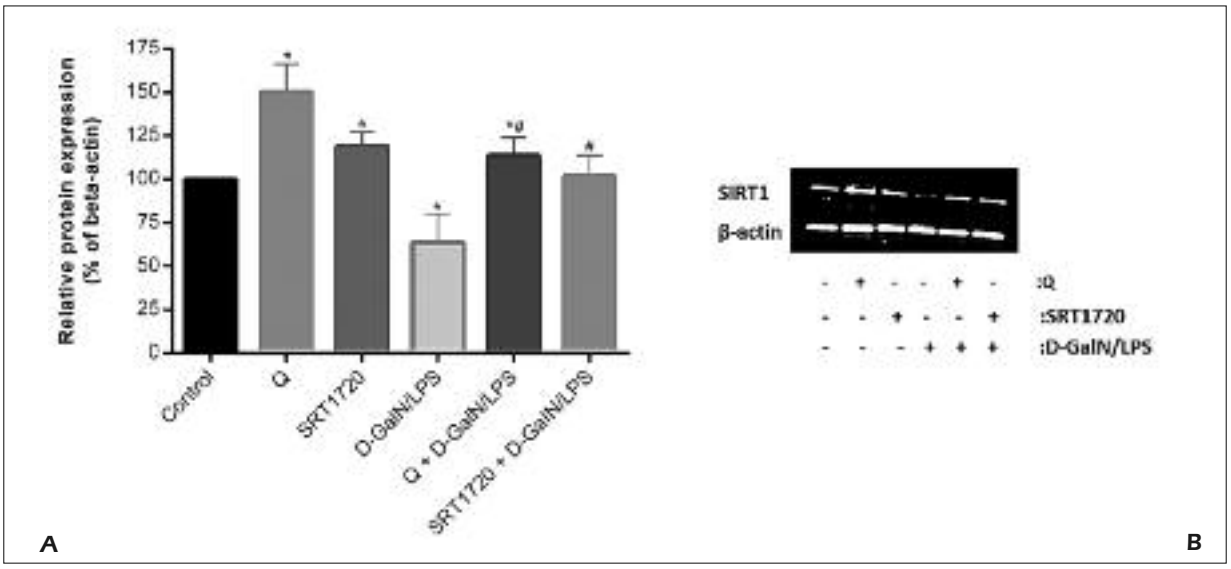

Figure 3. Effects of quercetin and SRT1720 pretreatments on SIRT1 protein expression in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized (D-GalN/LPS) rats. ***A,*** Quantification of SIRT1 protein expression levels by densitometry. Bands were detected using VersaDoc™ MP 5000 System and analyzed by Quantity One 1-D Analysis Software. In each panel, the intensity of a given SIRT1 band was normalized to the intensity of the corresponding β-actin band. Control: vehicle only; Q: quercetin 50 mg/kg; SRT1720: SRT1720 5 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + Lipopolysaccharide 10 μg/kg; Q + D-GalN/LPS: Combination of Q and D-GalN/LPS; SRT1720 + D-GalN/LPS: Combination of SRT1720 and D-GalN/LPS. *Indicates significant values ($p \leq 0.05$) compared to the control group (vehicle only); # Indicates significant values ($p \leq 0.05$) compared to the D-GalN/LPS group. The results are expressed as means ± SEM, n = 4. ***B,*** Representative Western blot image is shown below.

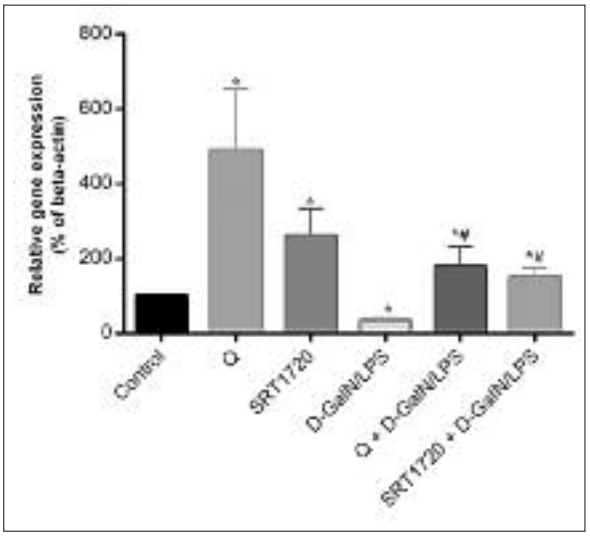

Figure 4. Effects of quercetin and SRT1720 pretreatments on SIRT1 gene expression in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized (D-GalN/LPS) rats. Q: quercetin 50 mg/kg; SRT1720: SRT1720 5 mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + Lipopolysaccharide 10 μg/kg; Q + D-GalN/LPS: Combination of Q and D-GalN/LPS; SRT1720 + D-GalN/LPS: Combination of SRT1720 and D-GalN/LPS. *Indicates significant values ($p \leq 0.05$) compared to the control group (vehicle only); #Indicates significant values ($p \leq 0.05$) compared to the D-GalN/LPS group. The results are expressed as means ± SEM, n = 6.

gene expression. In either case, the upregulation was more pronounced with Quercetin (1.5-fold protein expression, 4.9-fold mRNA expression) than SRT1720 (1.2-fold protein expression, 2.6-fold mRNA expression). Treatment of animals with D-GalN/LPS markedly decreased SIRT1 expression levels. Pretreatment of D-GalN/LPS rats with quercetin or SRT1720 raised SIRT1 expression towards normal. Compared to the D-GalN/LPS group, quercetin pretreatment increased SIRT1 protein level by 1.8-fold and SRT1720 by 1.6-fold. Similar findings were obtained with RT-PCR. Pretreatment of D-GalN/LPS rats with quercetin increased SIRT1 gene expression by 4.6-fold and SRT1720 by 2.7-fold. These findings clearly show that, under the set experimental conditions, quercetin had more profound effect on SIRT1 expression than SRT1720. Higher SIRT1 levels correlated to minimal liver damage.

Discussion

More than half of the portal blood comes directly from the gastrointestinal tract, making the liver the most susceptible organ in the hu-

man body to intoxication by xenobiotics. There are numerous hepatotoxins encountered on a daily basis, ranging from microbial toxins[27] to common OTC drugs like acetaminophen[28]. One of the most feared complication of hepatotoxicity is acute liver failure (ALF), a rapid deterioration in liver function leading to coagulopathy, encephalopathy and multiple organ failure within days[29]. ALF is a formidable clinical problem with very poor prognosis. The current available medications are of doubtful efficacy and safety, hence the dire need for alternative drugs.

D-GalN/LPS is a reputable model of hepatotoxicity that closely mimics ALF seen clinically. In this model, LPS stimulates Kuppfer cells to release NF-κB[30] and a wide variety of proinflammatory cytokines[31], including TNF-α[32]. LPS also increases production of reactive oxygen species (ROS) through NADPH oxidase[33]. D-GalN, on the other hand, depletes uridine nucleotide pool in hepatocytes, inhibits RNA and protein synthesis, leading to dramatic sensitization to the cytotoxic effects of LPS in the liver[34]. Hence, the combined effect of these two drugs is massive inflammation and apoptosis that resembles severe clinical hepatitis. In the current study, the fact the D-GalN/LPS drastically increased aminotransferase plasma levels, vividly demonstrate a failing liver. Elevation of conjugated dienes, a lipid peroxidation marker, indicate that oxidative stress plays a pathological role in D-GalN/LPS-induced hepatotoxicity[35]. D-GalN/LPS also increased total bilirubin levels. There are two possible explanations for this finding. Firstly, it might reflect an adaptive response to the oxidative challenge. For instance, oxidative stress can induce HO-1, which can cleave heme into iron, carbon monoxide and biliverdin, metabolic precursor of bilirubin[36]. All these heme degradation products have antioxidative, antiinflammatory, and/or antiapoptotic actions[37]. Secondly, bilirubin is cytotoxic at high concentrations, as seen in pathological conditions such as cholestasis[38]. A significant number of studies have shown that high bilirubin levels can increase production of ROS, disrupt integrity of membranes and trigger apoptosis of hepatocytes[39,40,41]. Nonetheless, bilirubin was much more sensitive to D-GalN/LPS treatment than AST, hence it is justifiable to use the former, alongside ALT, in clinical screening of liver intoxication. Consistent with our previous experimental study[15], D-GalN/LPS downregulated SIRT1 through an unknown mechanism. There is an accumulating evidence that oxidative stress may directly or indirectly control SIRT1 expression. For instance, oxidative stress can induce expression of microRNAs such as miR-34a, which could bind to the 3'UTR of SIRT1 mRNA and inhibit SIRT1 expression[42]. Moreover, ROS can covalently modify SIRT1 and mark it for proteasomal degradation[43]. It is therefore logical to speculate that hepatic SIRT1 downregulation contributes to the cytotoxic effects of D-GalN/LPS in the liver.

Quercetin pretreatment upregulated SIRT1 and attenuated the cytotoxic effects of D-GalN/LPS. The relationship between polyphenols and SIRT1 is still open for debate. Initially, it was generally accepted that polyphenols were allosteric SIRT1 activators[21]. However, the legitimacy of polyphenols as direct SIRT1 activators has been questioned[44]. Recent evidence suggests that the cytoprotective effects of polyphenols could be mediated by other mechanisms such as AMPK activation[45], which could occur independent of SIRT1[46]. To address this ambiguity, we treated some of the animals with SRT1720, a highly potent allosteric SIRT1 activator[24]. Likewise, SRT1720 increased SIRT1 expression and minimized the liver damaging effects of D-GalN/LPS. In spite of not ruling out direct SIRT1 activation in our study, these findings strongly suggest the involvement of SIRT1 upregulation in the cytoprotective effects of both quercetin and SRT1720. The mechanism by which STACs upregulate SIRT1 was not documented in this study. However, there are some scanty reports available that polyphenols and related compounds activate FoxO1[47], which is a positive SIRT1 transcriptional regulator[48]. Strikingly, quercetin was more efficacious than SRT1720 in ameliorating liver intoxication. Regardless of its high potency, in this study, SRT1720 lagged behind quercetin in all parameters. This may simply be due to higher upregulation of SIRT1 expression with quercetin. SIRT1 is associated with many health benefits. Its antiinflammatory effects are ascribed to downregulation of NF-κB[49] and associated pro-inflammatory cytokines such as COX-2[50] and iNOS[51]. Furthermore, SIRT1 can induce antioxidant enzymes, MnSOD[52] and catalase[53], through deactylation and activation of FoxO3[54]. Notably, both quercetin and SRT1720 decreased bilirubin plasma levels, despite of SIRT1 upregulation. The

most probably explanation for this finding is that SIRT1 fine-tuned bilirubin levels to optimal antioxidant ranges to negate its cytotoxic effects[55]. Bilirubin is only cytoprotective at physiological ranges, as demonstrated in a number of *in vivo* and *in vitro* studies[56,57].

Conclusions

D-GalN/LPS downregulates SIRT1 and triggers oxidative damage to the liver. Quercetin and SRT1720 upregulate SIRT1 expression and combat the cytotoxic effects of D-GalN/LPS. Quercetin induces SIRT1 expression more than SRT1720 and is more efficacious in attenuating D-GalN/LPS-induced hepatotoxicity.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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