Therapeutic effects of the traditional medicinal plant Ipomoea stolonifera for the treatment of liver diseases
Bai, Xueting

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Chapter 3

The protective effect of the natural compound hesperetin against fulminant hepatitis in vivo and in vitro

Xueting Bai1, 2, Peixuan Yang3, Qiaoling Zhou1, Bozhi Cai4, Manon Buist-Homan2, He Cheng1, Jiyang Jiang1, Daifei Shen1, Lijun Li1, Xiajiong Luo1, Klaas Nico Faber2, Han Moshage2*, Ganggang Shi1*

1. Dept. Pharmacology, Shantou University Medical College, Shantou, China
2. Dept. Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands
3. Health Care Center, The First Affiliated Hospital of Shantou University Medical College, Shantou, China
4. Laboratory of Molecular Cardiology, The First Affiliated Hospital of Shantou University Medical College, Shantou, China

*Last 2 authors contributed equally to this work

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Abstract

Background: Liver diseases are mostly accompanied by inflammation and hepatocyte death. Therapeutic approaches targeting both hepatocyte injury and inflammation are not available. Natural compounds are considered as potential treatment for inflammatory liver diseases. Hesperetin, a flavonoid component of citrus fruits has been reported to have anti-inflammatory properties. The aim of this study was to evaluate the cytoprotective and anti-inflammatory properties of hesperetin both in vitro and in models of fulminant hepatitis.

Methods: Apoptotic cell death and inflammation were induced in primary cultures of rat hepatocytes by bile acids and cytokine mixture, respectively. Apoptosis was quantified by caspase-3 activity and necrosis by LDH release. The concanavalin A (ConA) and D-galactosamine/LPS (D-GalN/LPS) were used as models of fulminant hepatitis. Liver injury was assessed by ALT and AST levels, liver histology and TUNEL assay and inflammation by inducible Nitric Oxide Synthase (iNOS) expression.

Results: Hesperetin blocked bile acid-induced apoptosis and cytokine-induced inflammation in rat hepatocytes. Moreover, hesperetin improved liver histology and protected against hepatocyte injury in ConA- and D-GalN / LPS-induced fulminant hepatitis, as assessed by TUNEL assay and serum AST and ALT levels. Hesperetin also reduced expression of the inflammatory marker iNOS and the expression and serum levels of TNFα and IFN-γ, the main mediators of cell toxicity in fulminant hepatitis.

Conclusion: Hesperetin has anti-inflammatory and cytoprotective actions in models of acute liver toxicity. Hesperetin therefore has therapeutic potential for the treatment of inflammatory liver diseases accompanied by extensive hepatocyte injury, such as fulminant hepatitis.
Introduction

Hesperetin is a natural compound belonging to the flavanone class of flavonoids. It is the aglycone of hesperidin (β-7-rutinoside of hesperetin), a predominant flavonoid component of citrus fruits (Fig.1). It is now well accepted that a low consumption of fatty foods and an increased intake of fruit and vegetables will reduce the risk of some life-threatening diseases and maintain a good health status [1]. World-wide, the dietary intake of citrus fruit products, and hence flavanones, is increasing every year [2]. In Western countries, the intake of hesperetin is largely dependent on dietary habits [3], whereas hesperetin is also known as a major active ingredient in the Chinese traditional medicinal herb Chenpi [4]. Because of the reported bioactivities, extensive research has been performed on hesperidin and hesperetin in various experimental models. These bioactivities include antioxidant, anti-inflammatory and anticarcinogenic effects [5, 6].

Figure 1. Structures of hesperitin and hesperidin

Most liver diseases are accompanied by inflammation and oxidative stress, regardless of the etiology of the underlying disorder. Mild and time-restricted hepatic inflammation could be considered beneficial in the restoration of tissue homeostasis, e.g. by eliminating invading pathogenic organisms and damaged or dead cells. However, excessive and uncontrolled inflammation leads to massive loss of hepatocytes as a result of apoptosis and/or necrosis [7] irreversible damage to the liver parenchyma and loss of liver function [8]. Loss of hepatocytes and loss of liver function occurs in many liver pathologies, including fulminant hepatitis, reperfusion injury, (non-)alcoholic liver diseases, cholestasis and viral hepatitis. All these conditions demonstrate high morbidity and mortality and liver transplantation is often the only life-saving treatment [9, 10]. The management of acute and chronic inflammatory liver disease is still a challenge to modern drug development, because there are currently no effective treatments that improve liver function and/or
regenerate or protect hepatic cells [11]. Therefore, there is an urgent need for novel therapeutic approaches that prevent liver injury via protection against hepatocyte cell death. In particular, the potential of herbal and dietary supplements, like hesperetin has been largely unexplored in this regard.

Although hesperidin possesses a wide range of biological activities, including hepatoprotective properties in liver injury [12-14], its aglycone-derivative hesperetin has stronger bioactivity as a result of more efficient absorption from the intestine than hesperidin [15-17], [18]. Existing studies are mainly focused on one specific in vivo or in vitro model [19, 20], but comprehensive reports on the effectiveness of different doses of orally administered hesperetin in multiple models of fulminant hepatitis are lacking. In viral and autoimmune hepatitis, activation of T-cells and macrophages is the initial event [21]. Experimental liver injury models were established that resemble fulminant human hepatitis, including TNF-α- and IFN-γ-dependent inflammatory liver injury models that allow the evaluation of hepatoprotective interventions, including medicinal plant components. In our study, immune-mediated liver injury was induced by the T-cell mitogenic plant lectin concanavalin A (Con A). Liver injury in this model is dependent on both macrophage-derived TNFα and T-cell-derived IFN-γ. In this model the expression of various cytokines is strongly induced, including IFN-γ, IL-4, and IL-2 [22]. As a second model, we used an inflammation-induced model of fulminant hepatitis. Endotoxins like LPS are known as strong stimulators of macrophages, including Kupffer cells. TNFα alone does not induce hepatocyte cell death. However, when hepatocytes are simultaneously sensitized with D-galactosamine (D-GalN), preventing hepatocyte transcription, LPS-induced TNFα becomes extremely hepatotoxic, because of massive apoptosis of hepatocytes [23].

The aim of the present study was to investigate the hepatoprotective and anti-inflammatory properties of hesperetin in acute liver injury. We demonstrate that hesperetin is anti-inflammatory and cytoprotective, in part by repression of IFN-γ expression in T-cell-mediated hepatitis and by repression of TNFα expression in the TNFα-dependent D-GalN/LPS model of liver injury.

**Methods**

**Animals**

Male Wistar rats (220-250g) were purchased from Harlan (Zeist, the Netherlands) Experiments were performed after approval by and following the guidelines of the local Committee for Care and Use of Laboratory Animals of the University of Groningen. 6 week-old male BALB/c mice (20-22g) were obtained from Hunan SJA Laboratory Animal Co. Ltd (Changsha, China NO. 43004700009427). All animals received humane care according to the legal requirements and guidelines approved by the ethics committee for the animal facility of Shantou University Medical College. All studies involving animals are reported in accordance with the ARRIVE
guidelines for reporting experiments involving animals [24, 25]. All animals were maintained under controlled conditions and had free access to standard laboratory chow and water.

**Hepatocyte isolation**

Hepatocytes were isolated from Wistar rat by a two-step collagenase perfusion procedure as described previously [26]. Cell viability was determined by trypan blue staining and was more than 85%. 112,500 cells per cm\(^2\) were plated on Vitrogen®-coated plates in William’s E medium (Life Technologies Ltd; Breda, The Netherlands) supplemented with 50 µg/mL gentamycin (Life Technologies Ltd) and penicillin-streptomycin-fungizone (Lonza, Verviers, Belgium). During the attachment period (4 h) 50 nmol L\(^{-1}\) dexamethasone (Sigma, St Louis, USA) and 5% fetal calf serum (Life Technologies Ltd) were added to the medium. Cells were cultured in a humidified incubator at 37 °C and 5% CO\(_2\).

**In vitro studies**

Experiments were started after the attachment period of 4 hours. Monolayers of cultured primary hepatocytes were treated with different concentrations of hesperetin (10, 25, 50 µmol L\(^{-1}\)) to analyze the effect on cytokine mixture (CM: 20 ng ml\(^{-1}\) mTNFα, 10 ng ml\(^{-1}\) hIL-1β and 10 ng ml\(^{-1}\) rIFN-γ)-induced inflammation for 6 h. As in vitro model of cell death we used glycochenodeoxycholic acid (GCDCA: 50 µmol L\(^{-1}\))-induced cell death for 4 h. GCDCA-induced cell death is independent of any inflammation and induces mainly apoptosis [27]. Signal transduction pathways were inhibited using 10 µmol L\(^{-1}\) of the ERK1/2 inhibitor U0126 (Promega, Madison, USA), 10 µmol L\(^{-1}\) of the p38 inhibitor SB 203580 (Calbiochem, San Diego, CA, USA), 50 µmol L\(^{-1}\) of the PI3 kinase inhibitor LY 294002 (Calbiochem, San Diego, CA, USA). All inhibitors and receptor antagonists were added to the cultured hepatocytes 30 minutes prior to the apoptotic or inflammatory stimuli. Every experimental condition was performed in triplicate wells and each experiment was repeated at least four times using hepatocytes from different rats. Cells were harvested at the indicated time points using lysis buffer for protein assay or TriZol reagent for RNA isolation.

**Liver injury experimental models**

After 7 days of adjusting, the animals were randomly divided into 10 experimental groups.

Control group (n=8): These animals were treated with the equivalent volume of PBS as used for the administration of Con A and D-GalN/LPS.

Control hesperetin group (n=8): The mice were administered hesperetin 400 mg kg\(^{-1}\) p.o in 0.5% CMC-Na solution for 10 days.
Con A group (n=15): The animals were administered the same volume of CMC-Na as used for administration of hesperetin for 10 days and were challenged with Con A (i.v.15mg kg\(^{-1}\)).

Con A + hesperetin groups: The animals received various doses of hesperetin (100, 200, 400 mg kg\(^{-1}\)) orally for 10 days before Con A injection (each group n=15).

D-GalN/LPS group (n=15): The animals were administered CMC-Na for 10 days and intraperitoneally injected with D-GalN (700 mg kg\(^{-1}\))/LPS (5 \(\mu g\) kg\(^{-1}\)).

D-GalN/LPS + hesperetin groups: Three doses of hesperetin (100, 200, 400 mg kg\(^{-1}\)) were given to mice once per day for 10 days. D-GalN (700 mg kg\(^{-1}\))/LPS (5 \(\mu g\) kg\(^{-1}\)) were administered intraperitoneally (each group n=15).

Con A from Canavalia ensiformis (Jack bean) Type IV, lyophilized powder (Sigma C2010), LPS (Escherichia coli 055:B5; Sigma L2880) and D-(+)-Galactosamine hydrochloride (Sigma G1639) were diluted in sterile endotoxin-free PBS. Mice were pretreated with hesperetin (3', 5, 7-Trihydroxy-4'-methoxyflavanone, Afar Aesar B20528) dissolved in sterile PBS containing 0.5% sodium carboxymethylcellulose CMC-Na (Aladdin) orally for 10 days once per day. Con A (15 mg kg\(^{-1}\)) was injected intravenously, and D-GalN (700 mg kg\(^{-1}\))/LPS (5 \(\mu g\) kg\(^{-1}\)) was administered intraperitoneally. Animals were sacrificed 8 h after the challenge. Blood was collected and the whole liver was harvested. The left lateral lobe was used for routine histology and the remaining lobes were frozen in aliquots in liquid nitrogen and then stored at -80°C for RNA isolation and protein assays. Serum was separated by centrifugation (15 min at 4,000 rpm) and used for serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determination and ELISA assays.

**Routine histological analysis**

Liver tissue was fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned into 4-\(\mu\)m sections. The tissue paraffin sections were deparaffinized, rehydrated following routine methods, and stained with hematoxylin-eosin (HE).

**TUNEL assay**

Apoptotic cell death was determined on paraffin-embedded sections of liver tissue by the presence of free 3'-hydroxy groups by TUNEL assay using DeadEnd™ Fluororometric TUNEL System (Promega, Beijing, China). The assays were performed as recommended by the manufacturer. For each liver tissue section, the number of TUNEL-positive cells in 3 random 20x-objective high-powered fields (containing at least one portal triad and central vein each) was counted by an investigator blinded with respect to treatment group using an Olympus IX81 microscope (Olympus, Japan).
Serum biochemical parameters

Activities of serum aminotransferases (ALT, AST) and total bilirubin (TBil) were determined by Bio-sinew kits (Chengdu, China) on Automatic Chemistry Analyzer (Acute TBA-40FR, Toshiba Medical Systems Corporation, Japan). Cytokine serum concentrations were assayed for murine TNFα, IL-4 (4A Biotech, Beijing, China), IFN-γ, (Boster, Wuhan, China), IL-6, and IL-10 (Bangyi, Shanghai, China) by enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (PCR)

RNA was isolated from mouse liver tissue using TriZol Reagent (TaKaRa, Japan). After using PrimeScript TM RT reagent Kit with gDNA Eraser (TaKaRa, Japan), quantitative real-time PCR (qRT-PCR) of liver tissue was performed using SYBR Green reagent (TaKaRa, Japan) on PCR detection system (ABI 7500, Applied Biosystems, USA). Rat mRNA from hepatocytes was isolated using Tri-reagent (Sigma-Aldrich), and assayed with TaqMan control reagents (ABI PRISM 7700, Applied Biosystems, The Netherlands). Mice primers and rat primers and probes are described in Table 1. Gene expression in vivo was normalized with respect to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and 18S for hepatocytes shown relative to control values.

Western blot analysis

Protein extracts from liver were prepared by homogenization in RIPA lysis buffer (P0013B Beyotime, Jiangsu, China). 50 microgram of protein from each sample was separated by SDS-PAGE and transferred to nitrocellulose filter membranes. Following blocking, membranes were probed with primary antibodies: mouse anti-iNOS rabbit monoclonal antibody 1:2,000 (Cell Signaling Technology, USA), mouse anti-phospho-SAPK/JNK (Thr183/Tyr185) rabbit monoclonal antibody 1:2,000 (Cell Signaling Technology, USA), anti-SAPK/JNK rabbit monoclonal antibody 1:3,000 (Cell Signaling Technology, USA). Mouse anti-GAPDH monoclonal antibody 1:3,000 (ZSGB-BIO, Beijing). All primary antibody incubations were overnight at 4°C, followed by detection using HRP-conjugated secondary mouse antibody 1:60,000 and rabbit antibody 1:80,000 (ZSGB-BIO, Beijing) at room temperature for 1 h. Total hepatocyte lysates were analyzed for Poly (ADP-ribose) polymerase (PARP) cleavage (1:1,000 Cell Signaling Technology, Beverly, Massachusetts, USA).

Analysis of hepatocyte apoptosis and necrosis

Measurement of caspase-3 activity was as described previously [26]. The arbitrary fluorescence unit was corrected for the amount of protein using BioRad protein assay kit. Cell necrosis was determined by measuring LDH release according to standard laboratory protocol. Briefly, 100 µl medium was loaded in 96-well plates followed by
addition of pyruvate and NADH. LDH activity was detected by absorbance at 340 nm for 30 min. The linear portion of the kinetic curve was calculated compared to a standard curve.

Data and statistical analysis

The data were expressed as mean ± standard error of the mean. One-way ANOVA and t-test were used to analyze the results. Results were considered statistically different when the \( P \) values were equal to or less than 0.05.

Results

Hesperetin has anti-inflammatory and cytoprotective effects on rat hepatocytes

The bile acid glycochenodeoxycholic acid (GCDCA; 50 \( \mu \text{mol L}^{-1} \)) induces apoptosis in primary rat hepatocytes with caspase-3 activity peaking after 4 h exposure [23, 28]. This model of apoptosis was used because it is not accompanied by inflammation and GCDCA does not activate NF-\( \kappa \)B and therefore, any cytoprotective effect of hesperetin is independent of an effect on inflammation. Hesperetin was added 30 min prior to GCDCA and the effect on GCDCA-induced caspase-3 activity was investigated 4 hours after GCDCA exposure. Hesperetin dose-dependently reduced GCDCA-induced caspase-3 activity in cultured primary rat hepatocytes (Fig. 2A). Maximum inhibition (-80%) was observed at 50 \( \mu \text{mol L}^{-1} \) hesperetin. Therefore, this concentration of hesperetin was used in subsequent analyses. Hesperetin alone, at 50 \( \mu \text{mol L}^{-1} \), did not modulate caspase-3 activity. In concordance, GCDCA induced cleavage of the caspase-3 substrate PARP, which was effectively inhibited by hesperetin (Fig. 2B). The anti-apoptotic effect of hesperetin was not accompanied by an increase in necrotic cell death as neither GCDCA, nor hesperetin nor the combination GCDCA and hesperetin induced LDH release from hepatocytes (Fig. 2C). To investigate the role of specific signal transduction pathways in the protective effect of hesperetin, we used several inhibitors of MAP Kinases and PI3K. Importantly, the protective effect of hesperetin against GCDCA-induced apoptosis was not abolished by inhibition of either ERK, p38 or PI3K (Fig. 2D). Hesperetin also dose-dependently reduced CM-induced \textit{Nos2} (iNOS) expression in hepatocytes (-41%, -63% and -83% at 10, 25 and 50 \( \mu \text{mol L}^{-1} \), respectively) indicating that hesperetin also has potent anti-inflammatory properties (Fig. 2E). Interestingly, hesperetin induced expression of the anti-oxidant gene \textit{Hmox1} (HO-1) about 4-fold compared to cytokine mixture alone (Fig. 2E).
Figure 2. Hesperetin has cytoprotective and anti-inflammatory effects on primary rat hepatocytes. (A) Hesperetin reduces GCDCA-induced caspase-3 activation (GCDCA: 50 µmol L-1). (B) Hesperetin prevents cleavage of PARP as assessed by Western blot. (C) GCDCA and cytokine mixture (CM) do not induce necrotic death of hepatocytes. The inhibitory effect of hesperetin on GCDCA-induced apoptosis and CM-induced inflammation is not accompanied by an increase of necrosis as assessed by LDH leakage in supernatant of cultured hepatocytes. LDH release is expressed as % of total LDH content of hepatocytes. (D) The protective effect of hesperetin is not abolished upon inhibition of the p38 and ERK MAP kinases and the PI3K pathway. (E) Hesperetin attenuates the inflammatory response of hepatocytes as assessed by iNOS mRNA determination by qPCR; All experiments were performed in duplicate wells and each experiment was repeated using hepatocytes from 5 different isolations; Values are mean ± SD, ns indicates P>0.05 not significant, *P<0.05 compared with GCDCA or CM.

Hesperetin attenuates Con A-mediated hepatitis

To translate our in vitro findings into an in vivo model, we first tested the effect of hesperetin on Con A-induced liver damage. The morphological observations are shown in Fig. 3A. Macroscopically, hesperetin reversed the dark surface color of livers with passive congestion induced by Con A (Fig. 3A). Microscopically, areas of active hepatocellular degeneration and necrosis are observed, presenting single or multiple foci of pale-staining groups of hepatocytes. In addition, congestion and inflammation (infiltration of mononuclear cells along with neutrophils) of pericentral areas is observed (Fig. 3B). Due to the limited period after Con A challenge, a normal
Figure 3. Hesperetin dose-dependently protects against Con A-mediated fulminant hepatitis. (A) Macroscopic appearance of livers indicating a beneficial effect of hesperetin to the dark surface of liver with passive congestion. (B) Hematoxylin-eosin staining of liver sections: in Con A hepatitis it shows hepatocellular degeneration (cloudy swelling), eosinophilic focus of cellular alteration with pale pink cytoplasm, condensed hypereosinophilic cytoplasm and shrunken nuclear occur spontaneously with one or two affected hepatocytes (arrows) and infiltration of inflammatory cells (mononuclear: lymphocytes and macrophages) (arrowheads), which are significantly reduced by Hst. Magnification 200X (upper panel), 400X (lower panel). (C) Quantitation of infiltrating leukocytes, represented as average number of at least 10 individual liver sections in Con A and Con A + Hesperetin groups (8
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liver sections in Control group). Magnification of measurement area is 400X. (D) Serum samples show increased total bilirubin levels in Con A-treated animals, which is (partially) reversed by hesperetin. Samples represent (from left to right): Control, Con A, Con A and increasing doses of hesperetin. (E) Serum markers of liver injury, AST, ALT and total bilirubin, are significantly induced in Con A hepatitis. Hst attenuates the rise in ALT, AST and TBil levels. (F) Serum levels of cytokines as assessed by ELISA demonstrate a significant rise of IFN-γ and TNFα in Con A hepatitis, which is reduced by Hst. Values are mean ± SD; Control group and Control Hesperetin group n=8, Con A and Con A + Hesperetin groups n=15, *P<0.05 compare with Con A.

liver structure is still retained. These microscopic abnormalities were prevented or reversed by hesperetin (Fig. 3B). In fact, the histology of Con A-treated groups that received 200 and 400 mg kg⁻¹ hesperetin was similar to normal liver histology. The number of infiltrating leukocytes in liver tissues of mice with Con A induced fulminant hepatitis are significantly decreased by hesperetin, especially in portal venous areas (Fig. 3C). Serum ALT and AST were increased 75- and 6-fold, respectively after Con A treatment. Hesperetin pretreatment dose-dependently attenuated the Con A-induced increase of serum AST, ALT and TBil (Fig. 3D, E). The protective effect of hesperetin was paralleled by a significant dose-dependent decrease of serum IFN-γ levels (Fig. 3F). Con A treatment also increased TNFα and interleukin-4 (IL-4) (Fig. 3F). Co-treatment with hesperetin reduced the serum levels of these cytokines as well, albeit to a lower extent compared to IFN-γ. IL-6 and IL-10 serum levels were hardly affected by Con A treatment and hesperetin has only minor effects on the serum levels of those cytokines (Fig. 3F).

**Hesperetin attenuates Con A-induced hepatocyte apoptosis and hepatic Nos2 (iNOS) expression**

In addition to necrosis, Con A induced hepatocyte apoptosis, as detected by TUNEL staining and caspase-3 activity assay, which was markedly reduced by co-treatment with hesperetin at 200 mg kg⁻¹ (Fig. 4A, Fig. 5). Furthermore, hesperetin suppressed Con A-induced iNOS protein and mRNA (Nos2) expression (Fig. 4B, C). Con A treatment induced the hepatic mRNA levels of several inflammatory and T-cell-derived cytokines, such as TNFα, IFN-γ, IL-6, IL-10, IL-4 and IL-1β. Expression of all these cytokines was reduced by hesperetin co-treatment (Fig. 4C) and these results generally paralleled the serum levels of these cytokines (Fig. 3E).
Figure 4. Apoptotic cell death and inflammation in Con A induced fulminant hepatitis are attenuated by hesperetin. (A) Apoptosis was assessed by TUNEL assay and visualized by Alexa Fluor 488. Hesperetin significantly reduced the number of TUNEL-positive nuclei in Con A hepatitis; (B) Hst attenuated Con A-induced inflammation as assessed by iNOS Western blot analysis. GAPDH was used as a loading control. (C) Expression of cytokines in liver tissue was determined by qPCR and expressed as fold increase compared to control. Con A induced the expression of inflammatory and T-cell derived cytokines. The induction of all cytokines, except IL-1β was attenuated by hesperetin. Values are mean ± SD; Control group n=8, Con A and Con A + Hesperetin groups n=15. *P<0.05 compare with Con A.
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Figure 5. Hesperetin reduces caspase-3 activity in fulminant hepatitis. Hesperetin attenuates caspase-3 activity in fulminant hepatitis. Hesperetin (Hst) was administered to animals at 200 mg kg\(^{-1}\). D-GalN/LPS and to a lesser extent Con A, induced an increase in caspase-3 activity, which was significantly attenuated by Hst in the D-GalN/LPS model. Values are mean ± SD *P<0.05.

Hesperetin protects mice from D-GalN/LPS induced liver injury

Another well-characterized murine model of acute liver failure is LPS administration to D-GalN-sensitized mice. In this model, hesperetin showed a protective effect as demonstrated by the reversal of the darker surface of the livers as blood-filled on macroscopic appearance (Fig. 6A). In addition, hesperetin improved histology in HE-stained tissue sections. Hesperetin decreased the extent of piecemeal necrosis around central veins and the loss of normal morphology. Hesperetin co-treatment also decreased the occurrence of apoptotic bodies, hydropic degeneration, nuclear fragments, autolysis and hemorrhage (Fig. 6B). The number of leukocytes infiltrated in liver tissue of mice with D-GalN/LPS induced fulminant hepatitis are significantly decreased by hesperetin (Fig.6C). Serum markers of liver injury (AST, ALT) were reduced by hesperetin co-treatment, but hesperetin did not attenuate the increased levels of TBil (Fig. 6D, E). Remarkably, in this model hesperetin was most protective at 200 mg kg\(^{-1}\). Treatment with both 100 mg kg\(^{-1}\) as well as 400 mg kg\(^{-1}\) hesperetin resulted in less complete or no protection at all compared to 200 mg kg\(^{-1}\) hesperetin (Fig. 6A,B,C,D,E). ELISA assay on serum samples demonstrated a clear reduction of LPS/D-GalN-induced TNF\(\alpha\) level by hesperetin at 200 mg kg\(^{-1}\), but not at 100 or 400 mg kg\(^{-1}\). The effect of hesperetin on the serum levels of other cytokines (IL-6, IL-10) was less conclusive (Fig. 6F).
Figure 6. Hesperetin protect mice from D-GalN/LPS induced fulminant hepatitis. (A) Macroscopic appearance of livers. (B) Hematoxylin-eosin staining of liver sections: In D-GalN/LPS-induced fulminant hepatitis there is pale eosinophilic staining, absence of nuclear detail, nuclear fragmentation (arrows), patchy areas of blood and abundant apoptotic hepatocytes (arrowheads). Magnification 200X (upper panel), 400X (lower panel). (C) Quantitation of infiltrating leukocytes, represented as average number of at least 10 individual liver sections in D-GalN/LPS and D-GalN/LPS + Hesperetin.
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Hesperetin inhibits pro-apoptotic JNK activation and inflammation in D-GalN/LPS-challenged mice

In addition to necrosis, D-GalN/LPS induced significant apoptosis as determined by TUNEL assay and caspase-3 activity assay. Hesperetin at 200 mg kg\(^{-1}\) reduced the number of TUNEL-positive hepatocyte nuclei and caspase-3 activity in liver tissue (Fig. 5 and Fig. 7A). Moreover, the D-GalN/LPS-induced activation of pro-apoptotic JNK was reduced by 49% by hesperetin treatment (200 mg kg\(^{-1}\)) (Fig. 7B). D-GalN/LPS-induced acute liver failure is characterized by a strong inflammatory response. Indeed, hepatic mRNA levels of the inflammatory cytokines TNF\(\alpha\), IL-1\(\beta\) and IL-6 were all strongly induced after D-GalN/LPS treatment and this induction was reduced by hesperetin by -61\%, -68\% and -75\%, respectively (Fig. 7C). To better characterize the molecular mechanisms underlying the protection of hesperetin, selective genes were determined by qPCR. Both FasL and TLR-4 expression were increased in the D-GalN/LPS model and this increased expression was attenuated by hesperetin at 200 mg kg\(^{-1}\). The endotoxin receptor TLR-4 and FasL are mainly expressed on macrophages in the liver, including Kupffer cells and immune cells and reflect increased inflammation. The effects of hesperetin on the expression of these markers indicate decreased inflammation (Fig. 7C).

To determine whether hesperetin is also effective when administered after the challenge (therapeutic effect), we performed a proof of concept study in which hesperetin was given 1 hour and 3.5 hours after D-Gal/LPS challenge. In this pilot experiment, using Kunming mice, hesperetin significantly reduced AST and ALT levels compared to treatment with D-GalN/LPS alone (data not shown).
Figure 7. Apoptotic cell death and inflammation in D-GalN/LPS-induced fulminant hepatitis are attenuated by hesperetin. (A) Apoptosis was assessed by TUNEL assay and visualized by Alexa Fluor 488. Hesperetin significantly reduced the number of TUNEL-positive nuclei in D-GalN/LPS induced hepatitis; (B) Hesperetin attenuated D-GalN/LPS-induced activation of JNK as assessed by Western
blot analysis for phospho-JNK. Total JNK and GAPDH were used as reference proteins. Right panel shows the quantitation of pJNK/JNK. (C) Expression of cytokines in liver tissue was determined by qRT PCR and expressed as fold increase compared to control. The inflammatory cytokines TNFα, IL-1β and IL-6 are induced in D-GalN/LPS hepatitis and this induction is attenuated by hesperetin. Furthermore, expression of FasL and TLR-4, mainly expressed on inflammatory and immune cells, is increased in D-GalN/LPS hepatitis and this increase is attenuated by hesperetin. Values are mean ± SD; Control group and Control Hesperetin group n=8, D-GalN/LPS and D-GalN/LPS + Hesperetin groups n=15, *P<0.05 compare with D-GalN/LPS.

Discussion

Use of herbal preparations can be traced back over centuries and has been described in ancient Egypt, China, India and Sumeria [29]. It is an important component of complementary and alternative medical therapies, together with dietary supplements [30] and many people consider herbal remedies as natural and free of side-effects and beneficial for health maintenance [31, 32]. Therefore, the popularity of complementary and alternative medical therapies is increasing every year. Hesperetin is a bioactive flavanone in citrus fruits and its consumption is increasing worldwide [2], although a systematic evaluation of orally administered hesperetin for the treatment of liver diseases, in particular fulminant hepatitis is still lacking. In the present study, we investigated the protective effect of hesperetin on hepatic injury using both in vivo and in vitro models. We demonstrated that hesperetin protected hepatocytes against apoptosis in an inflammation-independent model of bile acid-induced apoptosis and reduced markers of NF-κB activation like iNOS, indicative of inflammatory signaling in both macrophages as well as hepatocytes. Furthermore, hesperetin proved to be protective in two models of fulminant hepatitis, reducing both inflammation and cell injury.

Virtually all liver diseases are accompanied by inflammation. Mild and time-restricted hepatic inflammation contributes to the restoration of tissue homeostasis. In contrast, continuous and uncontrolled inflammation leads to massive loss of hepatocytes and loss of liver function [8] as a result of apoptosis or necrosis [33]. Inflammation and hepatocyte death results in a vicious cycle: inflammation drives hepatocyte injury and death via increased generation of apoptotic cytokines and reactive oxygen species by inflammatory cells, whereas debris of injured and dead hepatocytes drive inflammation. Because of this vicious cycle, any treatment for liver injury should ideally combine anti-inflammatory actions on inflammatory cells and cytoprotective actions on hepatocytes. Natural products are increasingly considered in the treatment of inflammatory diseases, such as rheumatoid arthritis. Recently, we described the anti-inflammatory activity of the n-butanol extract from Ipomoea stolonifera in acute models of inflammation [34]. The n-butanol extract of Ipomoea stolonifera contains five major components, including hesperetin. In preliminary in vitro experiments, hesperetin demonstrated to have potent anti-inflammatory activity. Therefore, hesperetin was chosen to be evaluated in this study for the treatment of inflammatory liver diseases.
Hesperetin protected hepatocytes against bile acid (GCDCA)-induced apoptosis. GCDCA-induced apoptosis is dependent on the mitochondrial pathway and is accompanied by the activation of mitochondria-specific caspase-9. Importantly, GCDCA does not induce an inflammatory response in hepatocytes, demonstrating that the cytoprotective effect of hesperetin is independent of any anti-inflammatory effect of hesperetin. Furthermore, the protective effect of hesperetin was independent of ERK MAP kinase, p38 MAP kinase and PI3K signaling. Previously, we reported on the protective effect of the therapeutic bile acid tauroursodeoxycholic acid (TUDCA) on GCDCA-induced apoptosis, which is dependent on intact p38/ERK and PI3K signaling [35, 36]. Therefore, hesperetin and TUDCA act via different mechanisms and the combination of hesperetin and TUDCA may be even more protective than either compound alone. In our study, we determined apoptosis by measuring caspase-3 activity and cleavage of the caspase-3 substrate PARP. So far, in all our studies we always observed a strict correlation between caspase-3 activity and end-points of apoptosis, such as nuclear condensation. Importantly, the reduction of apoptotic cell death by hesperetin was not accompanied by an increase in alternative modes of cell death, such as necrosis as determined by LDH release.

To further dissect the anti-inflammatory and cytoprotective effects of hesperetin, we also evaluated the effect of hesperetin on the cytokine-induced inflammatory response in hepatocytes. We used NF-κB-dependent induction of Nos2 (iNOS) as a marker of hepatocyte inflammation. Hesperetin significantly inhibited the induction of Nos2 by cytokines in hepatocytes. Of note, the cytokine mixture alone or in combination with hesperetin did not induce any apoptosis in hepatocytes, again underscoring the independency of the anti-inflammatory and cytoprotective effects of hesperetin. Interestingly, hesperetin induced HO-1 expression, suggesting an opposite effect of hesperetin on iNOS and HO-1 regulation. A reciprocal regulation of iNOS and HO-1 was previously described in intestinal epithelial cells [37]. The induction of HO-1 by hesperetin could, in fact, contribute to the protective effect of hesperetin, since HO-1 is known as a protective and anti-oxidant gene [38, 39].

The in vitro effects of hesperetin were confirmed in two mouse models, the T-cell/IFN-γ-mediated model of Con A-induced fulminant hepatitis and the TNFα-mediated model of D-GalN/LPS-induced fulminant hepatitis [21, 40]. In these models, animals were sacrificed 8 h after the challenge, permitting the monitoring of changes in hepatic mRNA expression and apoptosis [41, 42].

Apoptotic signaling within the cell is transduced mainly via the “death receptor” subgroup within the TNF protein superfamily, including TNFα, CD95L (also known as FasL) and the TNF-related apoptosis-inducing ligand (TRAIL) [43]. LPS leads to rapid activation of macrophages and high expression of TNFα in macrophages [44]. Resistance to TNFα cytotoxicity is particularly important in hepatic injury and this resistance is mainly dependent on intact NF-κB-signaling [27]. Therefore, in models of acute TNFα toxicity, NF-κB signaling needs to be suppressed and this is usually accomplished by D-GalN or actinomycin D. In the D-GalN/LPS model of fulminant
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hepatitis, we observed a very strong inflammatory response, accompanied by extensive liver damage and hepatocyte cell death. Both the inflammatory response and cell injury were strongly inhibited by hesperetin. At least part of the protective effect of hesperetin is due to suppression of inflammation as indicated by reduced leukocyte infiltration and reduced expression of apoptotic cytokines like TNFα and FasL and reduced expression of the endotoxin receptor TLR-4 and the apoptotic ligand FasL. It has previously been shown that Fas/FasL interactions contribute to inflammation in chronic cholestatic liver injury [45]. The reduction in hepatic TLR-4 and FasL expression could be the result of diminished expression of these receptors on inflammatory cells, and/or a reduced infiltration of inflammatory cells due to less cellular injury in the liver. On the other hand, we cannot exclude a direct cytoprotective effect of hesperetin on hepatocytes, as observed in GCDCA-induced hepatocyte apoptosis.

Interestingly, in preliminary experiments using Kunming mice, we also demonstrated that hesperetin protects against D-GalN/LPS-induced fulminant hepatitis when given twice at 200 mg kg⁻¹ (1 h and 3.5 h) after challenge, indicating that hesperetin is also therapeutically effective (data not shown).

Although hesperetin protects against apoptotic cell death, the dominant mode of cell death in our models of fulminant hepatitis appears to be necrotic cell death. Although apoptotic cell death was convincingly demonstrated in both models by counting TUNEL-positive cells and measuring caspase-3 activity, the extent of apoptotic cell death is probably limited compared to necrotic cell death in these models. Nevertheless, both indicators of apoptotic cell death were reduced by hesperetin, indicating that hesperetin also prevents apoptotic cell death in vivo. Therefore, in our models of fulminant hepatitis, the main protective effect of hesperetin is probably via its action on inflammatory cells resulting in the attenuation of the inflammatory response, less production of inflammatory and apoptotic cytokines and less generation of reactive oxygen species. It should be noted that the contribution of necrotic and apoptotic cell death varies between different liver diseases and that apoptosis is especially relevant in mild to moderate hepatitis like viral hepatitis, (non-)alcoholic steatohepatitis, whereas necrosis is dominant in fulminant hepatitis and acetaminophen intoxication.

Several studies have convincingly demonstrated signaling through the c-Jun N-terminal kinase (JNK) as a critical mechanism of TNFα-induced apoptosis [46], but also in oxidative stress-induced hepatocyte apoptosis [26, 47]. Wang et al. particularly demonstrated that hepatic injury was markedly decreased in mice lacking JNK2 [48] although JNK1 has also been implicated in TNFα-induced hepatitis [44]. Additionally, JNK is essential for development of hepatitis and is required for TNFα expression in hematopoietic cells including resident inflammatory cells in the liver (e.g. Kupffer cells and NKT cells) [49]. Administration of hesperetin attenuated hepatic activation of JNK as determined by Western blot analysis for phosphorylated JNK1/2 (Fig. 6B). It has been demonstrated recently that JNK is also involved in
necrotic cell death and that inhibition of JNK attenuates necrotic liver injury [50, 51]. In our study we observe activation (phosphorylation) of JNK and a reduction of JNK activation by hesperetin. Therefore, we conclude that hesperetin can directly inhibit necrotic cell death via inhibition of JNK activation. JNK can be activated by numerous agents, including TNFα, Fas/FasL, reactive oxygen species and bile acids. Therefore, in our models of fulminant hepatitis, with increased expression of TNF and other inflammatory cytokines, as well as generation of reactive oxygen species, it is very likely that JNK is activated via one or more of these ligands.

In contrast to the D-GalN/LPS treatment, the major contributors to liver injury in the Con A model are non-soluble membrane-bound TNFα expressed on macrophages (Kupffer cells) [52] and IFN-γ, expressed in T cells like natural killer T (NKT) cells, which are particularly abundant in the liver. Indeed, high expression of several T-cell cytokines, including IFN-γ, IL-4 and IL-2 has been implicated in Con A-induced hepatitis. Tagawa et al. showed that Con A hepatitis is suppressed in IFN-γ−/− mice [53]. In hepatitis B virus-induced hepatitis, NKT cells are recruited to the hepatic parenchyma and contribute to inflammation by releasing cytokines like IL-4 and IFN-γ [54]. In addition to having a direct toxic effect on hepatocytes, IFN-γ may also sensitize liver cells to TNFα-mediated toxicity. Of note, our data demonstrate that hesperetin significantly and dose-dependently reduce serum levels and mRNA expression of IFN-γ and serum level of IL-4. These data support the conclusion that therapeutic administration of hesperetin can also be considered for immune-mediated liver injury and that hesperetin also affects T-cells.

The anti-inflammatory action of hesperetin in Con A-induced fulminant hepatitis is underscored by the reduction of iNOS, a marker for inflammation [55]. This finding paralleled the \textit{in vitro} observation, although in the Con A model we did not distinguish between hepatocyte and Kupffer cell expression of iNOS. It is likely that expression of iNOS in both cell populations is reduced. Preliminary experiments indicate that hesperetin also reduces iNOS expression in the murine macrophage cell line RAW264.7 (data not shown).
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**Figure 8.** Potential mechanism of hesperetin in fulminant hepatitis. In acute and chronic liver injury, activation and infiltration of inflammatory cells (Kupffer cells, neutrophils) and death of functional hepatocytes occurs. Hesperetin protects hepatocytes from apoptotic cell death induced by TNFα/FasL, activating ‘death receptors’ and IFN-γ, produced by T cells like Natural Killer T (NKT) cells. Hesperetin has both anti-inflammatory effects on inflammatory cells and protective effects on hepatocytes and these effects are independent. The anti-apoptotic effects of hesperetin are in part due to reduced activation of the pro-apoptotic MAP kinase JNK.

In Fig.8, we propose a potential mechanism of the protective action of hesperetin in fulminant hepatitis. It is important to stress that we propose effects of hesperetin on both the inflammatory and T-cell populations as well as on the hepatocytes. In summary, we demonstrate the therapeutic potential of hesperetin in fulminant hepatitis based on both anti-inflammatory actions of hesperetin on inflammatory cells as well as direct cytoprotective effects of hesperetin on hepatocytes. Therefore, hesperetin has the potential to stop the vicious cycle of inflammation causing cell death and cell death leading to more inflammation as observed in many inflammatory liver diseases. We propose that hesperetin is a promising candidate to be evaluated in clinical studies for the treatment of inflammatory liver diseases, including viral hepatitis, (non-)alcoholic steatohepatitis and fulminant hepatitis.
References


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