Food & Function

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: J. Chen, X. Sun, T. Xia, Q. Mao and L. Zhong, *Food Funct.*, 2018, DOI: 10.1039/C7FO01073G.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/food-function

View Article Online
DOI: 10.1039/C7FO01073G

Pretreatment with dihydroquercetin, a dietary flavonoid, protected against concanavalin A-induced immunological hepatic injury in mice and TNF-α/ActD-induced apoptosis in HepG2 Cells

Jiajie Chen, Xu Sun, Tingting Xia, Qiqi Mao and Liang Zhong*

Department of Gastroenterology, Huashan Hospital North, Fudan University, Shanghai China, 201907

*To whom correspondence should be addressed. Mailing address: Liang Zhong, No.108 LuXiang Road, BaoShan District, 201907, Shanghai China. Tel: +86-21-66894836, Fax: +86-21-66895845, E-mail: zl_huashan@163.com

Electronic word count: 3804

Number of figures: 5

View Article Online
DOI: 10.1039/C7FO01073G

Abstract

We have previously demonstrated the hepatoprotective effect of dihydroquercetin (DHQ) against Concanavalin A (Con A)-induced immunological hepatic injury in mice. In this study, we investigated the immunoregulatory effects of DHQ on Con A-induced liver injury in mice. DHQ administration significantly decreased the serum levels of alanine transaminase and aspartate transaminase, effectively prevented liver damage, and increased the survival rate of Con A-treated mice. Immunohistochemistry examination revealed that supplementation with DHQ obviously reduced infiltration of $CD4^+$ and $CD8^+$ T cells in the injured liver tissues. Furthermore, DHQ administration resulted in down-regulation of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-2, IL-4, IL-10), chemokine (osteopontin), apoptosis factors (Fas, FasL), transcription factors that regulate Th cell differentiation (T-bet, GATA-3), Perforin, Granzyme B, and inducible nitric oxide synthase (iNOS). *In vitro*, treatment with DHQ protected HepG2 cells against TNF-α/ActD-induced apoptosis by inhibiting the activation of caspase-3, caspase-7, and caspase-8. In addition, DHQ reduced phosphorylation of NF-kB/p65, and inhibited the expressions of pro-apoptotic factors (p53, Bax), while it up-regulated the expression of anti-apoptotic factor (Bcl-2). Our findings suggest that the immunosuppressive effects of DHQ ameliorated Con A-mediated immunological liver injury by reducing the expression of pro-inflammatory mediators and infiltration of $CD4^+$ and $CD8^+$ T cells in liver tissues, and DHQ protected HepG2 cells against TNF-α/ActD-induced apoptosis possibly via modulation of the caspase and NF-kB pathways.

View Article Online
DOI: 10.1039/C7FO01073G

***Key Words*: Dihydroquercetin, protection, Concanavalin A, Immunological hepatic injury, TNF-α, Apoptosis**

View Article Online
DOI: 10.1039/C7FO01073G

1 Introduction

Dihydroquercetin (DHQ), also called taxifolin, is a bioactive flavonoid often found in Sorghum grain [1], onions [2], milk thistle [3], artichoke [4], tamarind seeds [5], and French maritime bark [6]. An accumulating body of evidence has demonstrated the multifunctional attributes of DHQ, which include antimicrobial [4], anti-oxidative [5], anti-inflammatory [7], and anti-allergic [8] effects. More interestingly, DHQ is also found in the approved hepatoprotective drug, silymarin, used for adjuvant therapy of toxic liver injury, chronic inflammatory liver disease and liver cirrhosis [5]. We have previously demonstrated that supplementation with DHQ alone exhibited a hepatoprotective effect against Concanavalin A (Con A)-induced immunological hepatic injury in mice [9]. However the underlying mechanisms are not completely understood.

Con A-induced liver injury in mice is a classical animal model of T cell-dependent liver failure which resembles virus-induced immune injury of the liver and autoimmune liver disease. The pathogenesis of autoimmune or viral hepatitis is associated with the cytotoxicity of activated T cells and pro-inflammatory cytokines secreted by macrophages [10]. In 1992, Tiegs *et al* found that Con A-induced liver injury in mice mainly depended on the activation of $CD4^+$ T cells by macrophages in the presence of Con A [11]. The dominant cytokines which contribute to the development of hepatitis are tumor necrosis factor-alpha (TNF-α) [12], interferon gamma (IFN-γ) [13], interleukin-2 (IL-2), IL-4, IL-6, IL-8 and IL-10 [14, 15]. These factors may induce hepatic inflammation, bleeding and necrosis, due largely to reduced lysosomal stability or enrichment of nitric oxide by up-regulating inducible nitric oxide synthase (iNOS) [16]. Pretreatment of mice with antibodies against mouse CD4, TNF-α or IFN-γ was showed to protect against Con A-mediated

View Article Online
DOI: 10.1039/C7FO01073G

immunological hepatic injury [11-13], which suggests that inhibition of the over-activated immune response may have a beneficial effect in immunological liver diseases [17]. We have shown that DHQ can protect mice from Con A-induced immunological liver injury [9]. However, it is not clear whether the hepatoprotective effect of DHQ is mediated via inhibition of Con A-activated immune response.

The over-expression of pro-inflammatory cytokines induced by Con A-activated immune response may result in apoptosis of hepatocytes, in which the activation of cysteine-dependent aspartate-directed protease (Caspase) has been demonstrated to be involved [18-20]. Inhibition of Caspase can prevent TNF-α induced apoptosis in mouse hepatocytes *in vivo* and *in vitro* [21]. Recently, Nam YJ *et al* found that taxifolin (DHQ) may attenuate the proteasome inhibitor-induced apoptosis in PC12 cells by suppressing the activation of the caspase-8-dependent pathway [22]. Another *in vitro* investigation showed that taxifolin inhibited glucose induced H9c2 cells apoptosis by inhibiting caspase-3 and caspase-9 activation [23]. Therefore, we ask whether DHQ could prevent TNF-α induced apoptosis in liver cells by inhibiting the activation of caspases. In this study, we further explored the immunoregulatory effects of DHQ on Con A-induced liver injury, and investigated the mechanism of the protective effect of DHQ against TNF-α-induced apoptosis in HepG2 Cell *in vitro*.

2 Materials and Methods

2.1 Chemicals and Reagents

DHQ (03890585) and Con A (L7647) were purchased from Sigma-Aldrich (St. Louis, MO, USA). High glucose Dulbecco's modified Eagle's medium (11965118), Fetal Bovine Serum (10099141) and trypsin-EDTA (25300054) were obtained from

View Article Online
DOI: 10.1039/C7FO01073G

Thermo Scientific (Waltham, MA, USA). Primary antibodies for detecting β-actin (4967), NF-κB/p65 (6956), phospho-NF-kB/p65 (Ser536) (3033), p53 (2527), Bcl-2 (2872), Bax (2772), Caspase 3 (9662), Caspase 7 (9492), Caspase 8 (4790), and HRP-linked anti-rabbit IgG (7074P2) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies detecting for CD4 (ab25475) and CD8 (ab22378) were purchased from Abcam (Cambridge, MA, USA). MTT (ST316), RIPA lysis buffer (P0013B) and primary antibody dilution buffer (P0023A) were obtained from Beyotime Institute of Biotechnology (Shanghai, China).

2.2 Animals and experimental procedure

The animal procedures were conducted in accordance with the China National Law on animal care and use, and approved by the Animal Experiments Committee of Medical College, Fudan University (#2015 1443 A425). Male BALB/C mice aged 10-12 weeks were obtained from the Shanghai SLAC Laboratory Animal Co. Ltd., Chinese Academy of Sciences, and fed with standard laboratory mice diet and water *ad libitium*.

Con A was used to induce immunological liver injury in mice [16]. Animals were divided into three groups with eighteen mice in each group. Group I: negative control group (PBS+PBS) received PBS by intragastric (i.g) administration daily for 14 days and intravenous (i.v) injection of PBS in tail vien on the 4^{th} day. Group II: positive control group (PBS+Con A) received PBS (i.g) daily for 14 days and injection of Con A (i.v) at a dose of 30 mg/kg body weight on the 4^{th} day. Group III: DHQ treated group (DHQ+Con A) received DHQ (5 mg/kg body weight; i.g) daily for 14 days and injection of Con A (i.v) at a dose of 30 mg/kg body weight on the 4^{th} day. For examination of Con A hepatitis, mice were sacrificed by CO_2 asphyxiation and

View Article Online
DOI: 10.1039/C7FO01073G

histological assessment of liver inflammation was performed at predefined time points. Serum was separated by centrifugation at 2000 ×g for 15 min. Alanine transaminase (ALT) and aspartate transaminase (AST) were measured using the assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.3 RNA Isolation and Quantitative Real-time PCR Analysis

RNA extraction and cDNA synthesis were performed according to the manufacturer's instructions. Total RNA was extracted from liver tissues using TRI reagent (MBI Fermentas Inc., Burlington, Ontario, Canada) and reverse transcribed using PrimeScript RT Master Mix (TaKaRa Biotechnology Dalian Co. Ltd., Dalian, China). Primers for each gene were designed using Primer Premier 5.0 design software and were synthesized by Sangon Biotech (Shanghai, China). Real-time PCR was performed using ABI 7500 systems (ABI, California, USA) with SYBR Premix Ex Taq (TaKaRa, Dalian, China). β-actin was used as an internal control to normalize gene expression. The relative quantification was determined by ΔCt method, and normalized mRNA levels expressed as arbitrary units by transforming the numbers of cycles using $2^{\Delta Ct}$ [24].

2.4 Hematoxylin–eosin and Immunohistochemical Staining

Liver tissues were dissected and fixed in 4% paraformaldehyde, then embedded in paraffin and cut into 5-μm sections. For histopathological examination by hematoxylin–eosin staining, the deparaffinized and rehydrated sections were first stained with Harris hematoxylin for 5 min, and then counterstained with alcoholic eosin stain. The pathological changes were observed under light microscope. To characterize the distribution of $CD4^+$ and $CD8^+$ T cells, immunohistochemical

View Article Online
DOI: 10.1039/C7FO01073G

staining was performed with S-P Kit (Zymed Laboratories, San Francisco, CA, USA). Antigen retrieval was performed by microwave heating of the sections on slides in TEG buffer (10 mM Tris, 0.5 mM EGTA, pH 9.0) for 10 min at 400 W. After treatment with 1.5% blocking serum for 1 h at room temperature, the sections were incubated with primary antibodies directed against mouse CD4 (1:50) or CD8 (1:50) for 12 h at 4°C. Subsequently, these were treated with biotin-labeled secondary antibody (1:200) and streptavidin-HRP (1:200) for 30 min at 37°C, respectively, and further developed using DAB as chromogen. Counterstaining of nuclei was performed with hematoxylin. For each section, three randomly selected fields were used to screen for T cell infiltration with Image-Pro Plus software (Media Cybernetics, Inc. Rockville, MD, USA), under a light microscope.

2.5 Cell Culture and Treatment

HepG2 cell was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and maintained at 37°C in 5% CO_2. Apoptosis of HepG2 cells was induced by combining TNF-α with Actinomycin D (ActD) [25]. Cells were pretreated with 200 μM of DHQ for 6 h before TNF-α (50 ng/ml)/ActD (25n g/ml) were added to the medium. For a negative control, cells were maintained in the routine medium without any treatment. For a positive control, cells only received TNF-α/ActD without DHQ pretreatment. Twenty-four hours after addition of TNF-α/ActD, cell viability, apoptosis and protein expression were determined.

2.6 Cell Viability Assay

View Article Online
DOI: 10.1039/C7FO01073G

Cells were plated in 96-well plates with 100 μL of medium containing 5.0×10^3 cells per well, with six parallel wells for each group as described above. 24 h after addition of TNF-α/ActD, MTT assay was performed to assess cell viability as described elsewhere [20]. Accordingly, 10 μL of MTT (5 mg/mL) was added to each well, and cells were incubated for 4 h at 37°C. The medium was discarded, and 100 μL of DMSO was added to each well. After shaking thoroughly for 10 min, the OD_{490nm} of each well was read in a microplate reader (BioTek ELX800, BioTek Instrument Inc., Winooski, VT, USA).

2.7 Analysis of Cell Apoptosis by Flow Cytometry

Cells were seeded in six-well plates at a density of 5.0×10^5 cells per well, with three parallel wells for each group as described above. 24 h after addition of TNF-α/ActD, cells were collected and centrifuged at 400 ×*g* for 5 min. Cell pellets were re-suspended in 500 μL of ice-cold 70% ethanol and fixed for 24 h at -20 °C. To detect cellular DNA content, the fixed cells were centrifuged at 400 ×*g* for 5 min, and then re-suspended in PBS containing ribonuclease A and stained with propidium iodide for 30 min at 37 °C. The proportion of cells in sub-G1 phase, which represents apoptotic cells, was analyzed with BD Accuri C6 flow cytometer (BD Co., Franklin Lakes, NJ, USA).

2.8 Western Blot Analysis

Western blot was performed as previously described [26] with some modifications. Briefly, cells were lysed in RIPA lysis buffer containing PMSF and protease inhibitors, and then centrifuged for 10 min at 14,000 ×*g* at 4°C. Equal amounts of total protein were dissolved with SDS-PAGE sample loading buffer, and denatured at 95°C for 5

View Article Online
DOI: 10.1039/C7FO01073G

min. 30 μg of total protein per sample was loaded and resolved on 10% SDS-PAGE gel, and then transferred to PVDF membrane. The membranes were incubated overnight at 4°C with targeted primary antibodies (diluted in primary antibody dilution buffer according to the manufacturer's recommendation), and blotted with HRP-anti-rabbit IgG (1:2000) for 1 h at room temperature. Signals were exposed to X-ray film for 2-5 min using BeyoECL Plus chemifluorescent substrate (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. The films were developed in the developing solution and fixed.

2.9 Statistical Analysis

Data are expressed as mean ± SD. Student's *t* test was used to assess between-group differences. Values of $p < 0.05$ were considered statistically significant.

3 Results and Discussion

3.1 DHQ protected BALB/C mice against Con A-induced immunological hepatic injury

To investigate the hepatoprotective effect of DHQ against immunological injury, liver injury was induced in BALB/C mice by intravenous injection of Con A in tail vein. The histopathological features of this animal model are characterized by a rapid inflammation in the liver with obvious infiltration of neutrophils, macrophages and T cells, and a remarkable simultaneous increase in the levels of transaminases in the blood, which are similar to autoimmune hepatitis [27]. In this study, three mice from each group were sacrificed for serological testing and histopathological examination, at 24 h and 72 h after Con A challenge, respectively. Supplementation with DHQ

View Article Online
DOI: 10.1039/C7FO01073G

significantly reduced the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in mice with Con A-lesions (Fig. 1A). In accordance with this, hepatic morphological changes in DHQ pretreated mice (DHQ+Con A group) reflected much less damage as compared to that in mice who received only Con A (PBS+Con A group) (Fig. 1B). Further, we calculated the survival rate of mice, which suffered from Con A-mediated hepatic injury. After injection of 30 mg/kg body weight Con A, some mice began to die within 8 h; however, after 96 h no mortality was observed in any of the groups. Interestingly, pretreatment with DHQ effectively improved the survival rate of mice (Fig. 1C). These results are consistent with those of our previous study [9].

3.2 DHQ decreased the infiltration of $CD4^+$ and $CD8^+$ T cells in injured liver tissues

Con A-induced experimental liver injury in mice is T cell-dependent [11]. In this hepatitis model, rapid activation and infiltration of T cell is a crucial factor. Therefore, we analyzed the effect of DHQ on T-cell infiltration in the injured liver tissues. At 24 h and 72 h after Con A injection, three mice from each group were sacrificed, and the liver tissues were collected to characterize the distribution of $CD4^+$ and $CD8^+$ T cells by immunohistochemical staining with CD4 and CD8 antibodies. The infiltrating T cells were counted as the positive staining in liver tissues. Results showed that injection of Con A enhanced the infiltration of $CD4^+$ T cells (Fig. 2) and $CD8^+$ T cells (Fig. 3) in the injured liver tissues, while supplementation with DHQ remarkably attenuated these morphological changes. These findings suggest that the hepatoprotective effect of DHQ against Con A-induced liver injury in mice may partly be attributable to inhibition of infiltration of T cells induced by Con A.

View Article Online
DOI: 10.1039/C7FO01073G

3.3 DHQ inhibited the expression of pro-inflammatory cytokines and T cell regulating genes in Con A-damaged liver

Con A-induced hepatitis was shown to involve a lot of pro-inflammatory cytokines and chemokines [12-15]. Thus, we examined the effect of DHQ on the expression of some important pro-inflammatory cytokines and chemokines during Con A-induced liver injury in mice. As shown by the results of Real Time-PCR (Fig. 4), DHQ administration resulted in notable down-regulation of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-2, IL-4, IL-10), chemokine (osteopontin), apoptosis factors (Fas, FasL), transcription factors that regulate T-helper (Th) cell differentiation (T-bet, GATA-3), Perforin, Granzyme B, and inducible nitric oxide synthase (iNOS), which were all dramatically up-regulated on Con A challenge.

The pro-inflammatory cytokines, sharply increased after Con A injection, which predominantly contribute to the development of hepatitis. Th1 cells produce the cytokines IFN-γ, TNF-α, IL-2 and IL-12 that mediate death of hepatocytes [28, 29]. Pretreatment with anti-IFN-γ or anti-TNF-α monoclonal antibodies was shown to protect against Con A-induced hepatitis, which indicates that Th1 immune reaction is involved in mediating Con A-induced hepatitis [12, 13]. Osteopontin (a glycoprotein over-expressed by Kupffer cells, macrophages, and stellate cells) which is required for the initiation of Th1 immune response was shown to be activated in the injured liver tissues [30]. Th1 and Th2 immune reactions were deranged after Con A treatment, with Th1 immunity predominating in transgenic mice expressing osteopontin in hepatocytes [30]. In T cell-dependent hepatitis, Fas and its ligand (FasL) have also been shown to participate in liver damage, which can induce hepatocyte death [31]. Soon after Con A injection, FasL was induced in

View Article Online
DOI: 10.1039/C7FO01073G

liver and mainly expressed on the intrahepatic T cells. Administration of antibody neutralizing mouse FasL was shown to reduce the serum levels of aminotransferase [31]. T-bet, a Th1-specific T box transcription factor that controls the expression of the hallmark Th1 cytokine, IFN-γ [32]. Strikingly, all of the above factors involved in immunological liver injury were up-regulated by Con A treatment, while DHQ administration down-regulated their expression. These results indicate that DHQ attenuate Con A-induced liver damage by inhibiting the expressions of a series of pro-inflammatory mediators and T cell immune reactions.

3.4 DHQ prevented HepG2 cells from TNF-α/ActD-induced apoptosis

Apoptosis plays an important role in the development of Con A-induced liver failure [33]. TNF-α, a major pro-inflammatory cytokine involved in Con A-induced liver hepatitis, may result in apoptosis of hepatocytes [21]. TNF-α is considered to cause subsequent effects via its membrane receptor TNFR1 (which contains an intracellular death domain structure), and plays an important role in cell dissolution [34]. DHQ has been demonstrated be able to inhibit proteasome inhibitor-induced PC12 cells apoptosis and glucose induced H9c2 cells apoptosis [22, 23]. According to a previous study [25], HepG2 cells apoptosis was induced with TNF-α/ActD *in vitro* to test whether DHQ exerts anti-apoptotic effect on TNF-α/ActD-induced apoptosis. Cell viability and apoptosis assays revealed that supplementation with DHQ promoted cell survival and reduced the proportion of apoptotic cells treated by TNF-α/ActD (Fig. 5A, 5B). Cells accumulating in Sub-G1 phase were decreased from 13.54±0.48 % (TNF-α/ActD treatment) to 5.41±0.37 % (DHQ+TNF-α/ActD), $p < 0.01$.

View Article Online
DOI: 10.1039/C7FO01073G

3.5 DHQ reduced NF-kB/p65 phosphorylation and caspase activation in TNF-α/ActD treated HepG2 cells

The above study demonstrated that DHQ may prevent HepG2 cells from TNF-α/ActD-induced apoptosis. Next, we performed experiments to investigate the underlying mechanism. It was reported that DHQ may inhibit caspase-8-depentdent pathway in proteasome inhibitor-induced PC12 cells apoptosis and inhibit the activation caspase-3 and caspase-9 in glucose induced H9c2 cells apoptosis [22, 23]. Caspase-8-mediated apoptotic pathway was shown to be invovled in TNF-α/ActD-induced apoptosis [25]. Caspase-8 triggers cell apoptosis by activating caspase 3 which is responsible for PARP cleavage and DNA fracture [16]. As expected, our results of western blotting showed that DHQ treatment suppressed the cleavage of caspase-3, caspase-7, and caspase-8 (Fig. 5C). These findings suggest that DHQ treatment may inhibit the activation of those caspases.

In addition to caspases, apoptosis can also be activated by other pathways, including Fas/FasL, NF-κB p65, ROS, MAPK and AKT in Con A models [16, 31, 33, 35-37]. Further experiments in our study revealed that DHQ reduced the phosphorylation of NF-kB/p65 and the expression of pro-apoptotic factors (p53 and Bax), while it up-regulated the expression of anti-apoptotic factor Bcl-2 in TNF-α/ActD treated HepG2 cells (Fig. 5C). These findings indicate that DHQ protected HepG2 cell against TNF-α/ActD-induced apoptosis possibly via modulation of caspase and NF-kB pathways.

The above data, combined with existing evidence, suggest that DHQ may affect a series of diverse biological processes through various pathways. Teselkin YO *et al* reported that DHQ offered anti-oxidative defences in rats with tetrachloromethane (CCl_4)-induced hepatitis [38]. Pretreatment of rat with DHQ (100 mg/kg body

View Article Online
DOI: 10.1039/C7FO01073G

weight) decreased the products of lipid peroxidation and increased the blood plasma antioxidant activity. Except for liver injury, the role of DHQ was also evaluated in other animal models. Impellizzeri D *et al* found that DHQ could protect mice against bleomycin-induced lung injury [39]. The underlying mechanisms might be to prevent oxidative and nitroxidative lung injury by down-regulating cyclo-oxygenase-2 (COX-2) and iNOS expression, and to suppress apoptosis by up-regulating Bcl-2 expression. Furthermore, DHQ treatment also reduced the phosphorylation of ERK and NF-κBp65 translocation. Wang YH *et al* demonstrated that taxifolin could ameliorate cerebral ischemia-reperfusion injury through its anti-oxidative effect and modulation of NF-kB activation [40]. Taxifolin administration inhibited the infiltration of leukocyte, down-regulated the activity of NF-kB and the expression of COX-2 and iNOS, and reduced the production of reactive oxygen species (ROS) and nitric oxide (NO).

In previous study, we demonstrated that in vitro treatment of RAW264 macrophage cells with DHQ enhanced the expression of nuclear factor-erythroid 2-related factor 2 (Nrf2) and antioxidant responsive element (ARE)-regulated gene, heme oxygenase-1 (HO-1) [9]. Our current data showed that DHQ down-regulated the pro-inflammatory cytokines and chemokines. Inflammation and oxidative stress are the key events in Con A-induced hepatitis, which are regarded as essential partners that present simultaneously and interact with each other in various pathological conditions [41]. Signaling pathways such as NF-kB, Nrf2/ARE, JNK/STAT and p38 MAPK are activated by oxidative stress, which could regulate the expression of inflammatory cytokines [41, 42]. On the other hand, large amounts of ROS could be directly produced by the infiltrated immune cells and activated phagocytic cells in response to inflammatory cytokines [43, 44]. The inflammatory cytokines may also

View Article Online
DOI: 10.1039/C7FO01073G

stimulate macrophages with resulting increased iNOS expression and NO production and subsequent oxidative injury [41]. DHQ has been proved to be able to down-regulate inflammatory cytokines and the enzymes associated with oxidative properties such as COX-2 and iNOS [39, 40], but contrarily be able to up-regulate antioxidant gene such as HO-1 and NAD(P)H quinone oxidoreductase-1 (NQO1) [9, 45]. The molecular mechanisms of its anti-inflammatory and anti-oxidative activities include inhibition of NF-kB and JAK2/STAT3 pathways [39, 40, 46], and activation of the antioxidant defense pathway Nrf2/ARE [9, 45].

However, some evidence is contradictory about the effect of DHQ on the JAK/STAT pathway. Park SY *et al* reported that taxifolin could lower β-amyloid accumulation and neurotoxicity via the suppression of P-JAK2/P-STAT3/NF-κB/BACE1 signaling pathways [46]. In the study of Bito T *et al*, taxifolin inhibit IFN-γ-induced ICAM-1 expression in human keratinocytes by inhibiting the activation of JAK1/STAT1 [47]. On the contrary, Zai W *et al* found that DHQ ameliorated acetaminophen-induced hepatic cytotoxicity via activating JAK2/STAT3 pathway and modulating expression of anti-apoptotic Bcl-2 family proteins [48]. Results from another investigation showed that taxifolin prevented diabetic cardiomyopathy by inhibition of oxidative stress and cell apoptosis via inhibition of NADPH oxidase activity and activation of JAK/STAT3 [49]. Whether these conflicting observations are caused by differences in cell or animal models in different pathological conditions requires more research to confirm.

In conclusion, DHQ is a bioactive flavonoid of dietary origin that performs various functions. In our previous study, we demonstrated the hepatoprective effect of DHQ against Con A-induced immunological injury in mice, which is the classical animal model of T cell-dependent liver failure. However, the underlying mechanisms were

View Article Online
DOI: 10.1039/C7FO01073G

not clear. In this study, we investigated the immunoregulatory effects of DHQ on Con A-induced liver injury in mice. Supplementation with DHQ reduced infiltration of $CD4^+$ and $CD8^+$ T cells in the injured liver tissues, and down-regulated the pro-inflammatory cytokines and chemokines which contributed to Con A-induced hepatitis. Furthermore, we found that treatment with DHQ *in vitro* prevented HepG2 cells from TNF-α/ActD-induced apoptosis. In addition, DHQ down-regulated the activation of caspase-3/7/8 and expressions of pro-apoptotic factors, and reduced the phosphorylation of NF-kB/p65, while it up-regulated the expression of anti-apoptotic factor in TNF-α/ActD-treated HepG2 cells. Our results indicate that DHQ ameliorates Con A-mediated immunological liver injury in mice partly via its immunosuppressive effects. DHQ also prevents apoptosis of HepG2 cells induced by TNF-α/ActD, possibly via modulation of the caspase and NF-kB pathways.

Conflict of Interest

There are no conflicts of interest to declare.

Acknowledgments

This work was supported by the Science and Technology Commission Foundation of BaoShan District, Shanghai, China (No. 13-E-36); the start-up fund for young scientists in Huashan Hospital North, Fudan University (No. 2015104).

References

1. G. Wu, S. K. Johnson, J. F. Bornman, S. J. Bennett and Z. Fang, Changes in whole grain polyphenols and antioxidant activity of six sorghum genotypes under different irrigation treatments, *Food Chem.*, 2017, **214**,199-207.

View Article Online
DOI: 10.1039/C7FO01073G

2. R. Slimestad, T. Fossen and I. M. Vagen, Onions: a source of unique dietary flavonoids, *J. Agric. Food Chem*., 2007, **55**, 10067-10080.
3. S. N. Wallace, D. J. Carrier and E. C. Clausen, Batch solvent extraction of flavonolignans from milk thistle (Silybum marianum L. Gaertner), *Phytochem. Anal*., 2005, **16**, 7-16.
4. C. Pereira, L. Barros, A. M. José, C. Santos-Buelga and I. C. Ferreira, Artichoke and milk thistle pills and syrups as sources of phenolic compounds with antimicrobial activity, *Food Funct*., 2016, **7**, 3083-3090.
5. A. E. Weidmann, Dihydroquercetin: More than just an impurity? *Eur. J. Pharmacol.*, 2012, **684**:19-26.
6. P. Rohdewald, A review of the French maritime pine bark extract (pycnogenol), a herbal medication with a diverse clinical pharmacology, *Int. J. Clin. Pharmacol. Ther*., 2002, **40**, 158-168.
7. M. B. Gupta, T. N. Bhalla, G. P. Gupta, C. R. Mitra and K. P. Bhargava, Anti-inflammatory activity of taxifolin, *Jpn. J. Pharmacol*., 1971, **21**, 377-382.
8. C. Ma, L. Yang, W. Wang, F. Yang, C. Zhao and Y. Zu, Extraction of dihydroquercetin from Larix gmelinii with ultrasound-assisted and microwave-assisted alternant digestion, *Int. J. Mol. Sci*., 2012, **13**, 8789-8804.
9. M. Zhao, J. Chen, P. Zhu, M. Fujino, T. Takahara, S. Toyama, A. Tomita, L. Zhao, Z. Yang, M. Hei, L. Zhong, J. Zhuang, S. Kimura and X. K. Li, Dihydroquercetin (DHQ) ameliorated concanavalin A-induced mouse experimental fulminant hepatitis and enhanced HO-1 expression through MAPK/Nrf2 antioxidant pathway in RAW cells, *Int. Immunopharmacol.*, 2015, **28**, 938-944.
10. A. W. Lohse, M. Manns, H. P. Dienes, Z. B. K. Meyer and I. R. Cohen, Experimental autoimmune hepatitis: disease induction, time course and T-cell

View Article Online
DOI: 10.1039/C7FO01073G

reactivity, *Hepatology*, 1990, **11**, 24-30.

11. G. Tiegs, J. Hentschel and A. Wendel, A T cell-dependent experimental liver injury in mice inducible by concanavalin A, *J. Clin. Invest.*, 1992, **90**, 196-203.
12. F. Gantner, M. Leist, A. W. Lohse, P. G. Germann and G. Tiegs, Concanavalin A-induced T-cell-mediated hepatic injury in mice: the role of tumor necrosis factor, *Hepatology*, 1995, **21**, 190-198.
13. S. Küsters, F. Gantner, G. Kunstle and G. Tiegs, Interferon gamma plays a critical role in T cell-dependent liver injury in mice initiated by concanavalin A, *Gastroenterology*, 1996, **111**, 462-471.
14. T. Liang, X. Y. Chen, M. Su, H. Q. Chen, G. Z. Lu and K. Liang, Vitamin C exerts beneficial hepatoprotection against Concanavalin A-induced immunological hepatic injury in mice through inhibition of NF-kB signal pathway, *Food Funct*., 2014, **5**, 2175-2182.
15. K. Sun, X. Q. Xie, J. Xie, S. F. Jiao, X. J. Chen, X. Zhao, X. Wang and L. X. Wei, Cell-based therapy for acute and chronic liver failures: Distinct diseases, different choices, *Sci. Rep*., 2014, **4**, 6494-6502.
16. J. J. Li, K. Chen, S. N. Li, T. Liu, F. Wang, Y. J. Xia, J. Lu, Y. Q. Zhou and C. Y. Guo, Pretreatment with Fucoidan from Fucus vesiculosus Protected against ConA-Induced Acute Liver Injury by Inhibiting Both Intrinsic and Extrinsic Apoptosis, *PLoS ONE*, 2016, **11**, e0152570.
17. K. Lv, Y. Zhang, M. Zhang, M. Zhong and Q. Suo, Galectin-9 ameliorates con a-induced hepatitis by inducing cd4(+)cd25(low/int) effector t-cell apoptosis and increasing regulatory t cell number, *PLoS ONE* , 2012, **7**, e48379.
18. S. Fiorucci, L. Santucci, E. Antonelli, E. Distrutti, G. D. Sero, O. Morelli, L. Romani, B. Federici, P. D. Soldato, A. Morelli, No-aspirin protects from t

View Article Online
DOI: 10.1039/C7FO01073G

cell-mediated liver injury by inhibiting caspase-dependent processing of th1-like cytokines, *Gastroenterology*, 2000, **118**, 404-421.

19. M. Al-Shamsi, A, Shahin, E. P. Mensah-Brown, A. K. Souid, Derangements of liver tissue bioenergetics in concanavalin A-induced hepatitis, *Bmc Gastroenterology*, 2013, **13**, 1-13.
20. L. Wu, C. Wang, J. Li, S. Li, J. Feng, T. Liu, S. Xu, W. Wang, X. Lu, K. Chen, Y. Xia, X. Fan, C. Guo, Hepatoprotective effect of quercetin via TRAF6/JNK pathway in acute hepatitis, *Biomed Pharmacother*, 2017, **96**, 1137-1146.
21. H. M. Ni, M. R. McGill, X. J. Chao, B. L. Woolbright, H. Jaeschke and W. X. Ding, Caspase inhibition prevents tumor necrosis factor-a-Induced apoptosis and promotes necrotic cell death in mouse hepatocytes in Vivo and in Vitro, *Am. J. Pathol*., 2016, **186**, 2623-2636.
22. Y. J. Nam, D. H. Lee, Y. K. Shin, D. S. Sohn, C. S. Lee, Flavanonol taxifolin attenuates proteasome inhibition-induced apoptosis in differentiated PC12 cells by suppressing cell death process, *Neurochem Res*., 2015, **40**, 480-491.
23. X. Sun, R. C. Chen, Z. H. Yang, G. B. Sun, M. Wang, X. J. Ma, L. J. Yang, X. B. Sun, Taxifolin prevents diabetic cardiomyopathy in vivo and in vitro by inhibition of oxidative stress and cell apoptosis, *Food Chem Toxicol*., 2014, **63**, 221-232.
24. W. H. Zhan, T. Y. Han, C. F. Zhang, C. F. Xie, M. X. Gan, K. Y. Deng, M. G. Fu and J. B. Wang, TRIM59 Promotes the Proliferation and Migration of Non-Small Cell Lung Cancer Cells by Upregulating Cell Cycle Related Proteins, *PLoS ONE*, 2015, **10**, e0142596.
25. H. S. Kim, P. A. Loughran, P. K. Kim, T. R. Billiar and B. S. Zuckerbraun, Carbon monoxide protects hepatocytes from TNF-alpha/Actinomycin D by inhibition of the caspase-8-mediated apoptotic pathway. *Biochem. Biophys. Res. Commun.*,

View Article Online
DOI: 10.1039/C7FO01073G

2006, **344**, 1172-1178.

26. N. Yao, Y. J. Li, Y. H. Lei, N. Hu, W. M. Chen, Z. Yao, M. Yu, J. S. Liu, W. C. Ye and D. M. Zhang, A piperazidine derivative of 23-hydroxy betulinic acid induces a mitochondria-derived ROS burst to trigger apoptotic cell death in hepatocellular carcinoma cells, *J. Exp. Clin. Cancer Res*., 2016, **35**, 192.
27. X. F. Tan, F. Chen, S. S. Wu, Y. Shi, D. C. Liu and Z. Chen, Proteomic analysis of differentially expressed proteins in mice with concanavalin A-induced hepatitis, *J. Zhejiang Univ. Sci. B.*, 2010, **11**, 221-226.
28. M. Inafuku, K. Nagao, A. Inafuku, T. Yanagita, N. Taira, T. Toda and H. Oku, Dietary phosphatidylinositol protects C57BL/6 mice from concanavalin A-induced liver injury by modulating immune cell functions, *Mol. Nutr. Food Res*., 2013, **57**, 1671-1679.
29. H. Y. Diao, S. Kon, K. Iwabuchi, C. Kimura, J. Morimoto, D. Ito, T. Segawa, M. Maeda, J. Hamuro, T. Nakayama, M. Taniguchi, H. Yagita, L. V. Kaer, K. Onoe, D. Denhardt, S. Rittling and T. Uede, Osteopontin as a mediator of NKT cell function in T cell-mediated liver diseases, *Immunity*, 2004, **21**, 539-550.
30. S. Mimura, S. Mochida, M. Inao, A. Matsui, S. Nagoshi, T. Yoshimoto and K. Fujiwara, Massive liver necrosis after provocation of imbalance between Th1 and Th2 immune reactions in osteopontin transgenic mice, *J. Gastroenterol.*, 2004, **39**, 867-872.
31. K. Seino, N. Kayagaki, K. Takeda, K. Fukao, K. Okumura and H. Yagita, Contribution of Fas ligand to T cell-mediated hepatic injury in mice, *Gastroenterology*, 1997, **113**, 1315-1322.
32. S. J. Szabo, S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman and L. H. Glimcher, A novel transcription factor, T-bet, directs Th1 lineage commitment, *Cell*, 2000,

View Article Online
DOI: 10.1039/C7FO01073G

100, 655-669.

33. P. Cheng, K. Chen, Y. J. Xia, W. Q. Dai, F. Wang, M. Shen, C. F. Wang, J. Yang, R. Zhu, H. W. Zhang, J. J. Li, Y. Y. Zheng, J. S. Wang, Y. Zhang, J. Lu, Y. Q. Zhou and C. Y. Guo, Hydrogen sulfide, a potential novel drug, attenuates concanavalin A-induced hepatitis, *Drug Des Devel Ther.*, 2014, **8**, 1277-1286.
34. S. J. Baker and E. P. Reddy, Transducers of life and death: TNF receptor superfamily and associated proteins, *Oncogene*, 1996, **12**, 1-9.
35. J. Li, Y. Xia, T. Liu, J. Wang, W. Dai, F. Wang, Y. Zheng, K. Chen, S. Li, H. Abudumijiti, Z. Zhou, J. Wang, W. Lu, R. Zhu, J. Yang, H. Zhang, Q. Yin, C. Wang, Y. Zhou, J. Lu, Y. Zhou and C. Guo, Protective effects of astaxanthin on ConA-induced autoimmune hepatitis by the JNK/p-JNK pathway-mediated inhibition of autophagy and apoptosis, *PLoS ONE*, 2015, **10**, e120440.
36. J. H. Lee, J. H. Won, J. M. Choi, H. H. Cha, Y. J. Jang, S. Park, H. G. Kim, H. C. Kim and D. K. Kim, Protective effect of ellagic acid on concanavalin A-induced hepatitis via toll-like receptor and mitogen-activated protein kinase/nuclear factor κB signaling pathways, *J. Agric. Food Chem.*, 2014, **62**, 10110-10117.
37. J. Xue, F. Chen, J. Wang, S. Wu, M. Zheng, H. Zhu, Y. Liu, J. He and Z. Chen, Emodin protects against concanavalin A-induced hepatitis in mice through inhibiting activation of the p38 MAPK-NF-κB signaling pathway, *Cell Physiol. Biochem.*, 2015, **35**, 1557-1570.
38. Y. O. Teselkin, I. V. Babenkova, V. K. Kolhir, A. I. Baginskaya, N. A. Tjukavkina, Y. A. Kolesnik, I. A. Selivanova, A. A. Eichholz, Dihydroquercetin as a means of antioxidative defence in rats with tetrachloromethane hepatitis, *Phytother Res.*, 2000, **14**, 160-162.
39. D. Impellizzeri, E. Talero, R. Siracusa, A. Alcaide, M. Cordaro, J. Maria Zubelia,

View Article Online
DOI: 10.1039/C7FO01073G

G. Bruschetta, R. Crupi, E. Esposito, S. Cuzzocrea, V. Motilva, Protective effect of polyphenols in an inflammatory process associated with experimental pulmonary fibrosis in mice, *Br J Nutr*., 2015, **114**, 853-865.

40. Y. H. Wang, W. Y. Wang, C. C. Chang, K. T. Liou, Y. J. Sung, J. F. Liao, C. F. Chen, S. Chang, Y. C. Hou, Y. C. Chou, Y. C. Shen, Taxifolin ameliorates cerebral ischemia-reperfusion injury in rats through its anti-oxidative effect and modulation of NF-kappa B activation, *J Biomed Sci*., 2006, **13**, 127-141.
41. S. Li, M. Hong, H. Y. Tan, N. Wang, Y. Feng, Insights into the Role and Interdependence of Oxidative Stress and Inflammation in Liver Diseases, *Oxid Med Cell Longev*., 2016, **2016**, 4234061.
42. T. Hussain, B. Tan, Y. Yin, F. Blachier, M. C. Tossou, N. Rahu, Oxidative Stress and Inflammation: What Polyphenols Can Do for Us? *Oxid Med Cell Longev*., 2016, **2016**, 7432797.
43. S. K. Biswas, Does the Interdependence between Oxidative Stress and Inflammation Explain the Antioxidant Paradox? *Oxid Med Cell Longev*., 2016, **2016**, 5698931.
44. A. Ambade, P. Mandrekar, Oxidative Stress and Inflammation: Essential Partners in Alcoholic Liver Disease, *Int J Hepatol*. 2012, **2012**, 853175.
45. L. Liang, C. Gao, M. Luo, W. Wang, C. Zhao, Y. Zu, T. Efferth, Y. Fu, Dihydroquercetin (DHQ) induced HO-1 and NQO1 expression against oxidative stress through the Nrf2-dependent antioxidant pathway, *J Agric Food Chem*., 2013, **61**, 2755-2761.
46. S. Y. Park, H. Y. Kim, H. J. Park, H. K. Shin, K. W. Hong, C. D. Kim, Concurrent Treatment with Taxifolin and Cilostazol on the Lowering of β-Amyloid Accumulation and Neurotoxicity via the Suppression of

View Article Online
DOI: 10.1039/C7FO01073G

P-JAK2/P-STAT3/NF-κB/BACE1 Signaling Pathways, *PLoS One*, 2016, **11**, e0168286.

47. T. Bito, S. Roy, C. K. Sen, T. Shirakawa, A. Gotoh, M. Ueda, M. Ichihashi, L. Packer, Flavonoids differentially regulate IFN gamma-induced ICAM-1 expression in human keratinocytes: molecular mechanisms of action, *FEBS Lett.*, 2002, **520**, 145-152.
48. W. Zai, W. Chen, J. Luan, J. Fan, X. Zhang, Z. Wu, T. Ding, D. Ju, H. Liu, Dihydroquercetin ameliorated acetaminophen-induced hepatic cytotoxicity via activating JAK2/STAT3 pathway and autophagy, *Appl Microbiol Biotechnol.*, 2018, **102**, 1443-1453.
49. X. Sun, R. C. Chen, Z. H. Yang, G. B. Sun, M. Wang, X. J. Ma, L. J. Yang, X. B. Sun, Taxifolin prevents diabetic cardiomyopathy in vivo and in vitro by inhibition of oxidative stress and cell apoptosis, *Food Chem Toxicol.*, 2014, **63**, 221-232.

View Article Online
DOI: 10.1039/C7FO01073G

Figure Legends

Figure1. DHQ protected BALB/C mice against Con A-induced immunological hepatic injury. Immunological hepatic injury in BALB/C mice was induced by intravenous injection of Con A (30mg/kg body weight) in tail vein. 24 h and 72 h after administration of Con A, three mice from each group were sacrificed, the serum was separated for alanine transaminase (ALT) and aspartate transaminase (AST) test (**A**), the liver tissues were collected for histopathological examination by hematoxylin–eosin staining (**B**, arrows indicate hepatic cellular necrosis, scale bar = 50 μm). Survival rate of mice in each group was calculated (**C**).

PBS+PBS (negative control group): mice received PBS by intragastric (i.g) administration daily for 14 days and intravenous (i.v) injection of PBS in tail vein on the 4^{th} day. PBS+Con A (positive control group): mice received PBS (i.g) daily for 14 days and injection of Con A (i.v) on the 4^{th} day. DHQ+Con A (DHQ treated group): mice received DHQ (5 mg/kg body weight; i.g) daily for 14 days and injection of Con A (i.v) on the 4^{th} day. Data are presented as means ± SD (n= 3).

**p < 0.01 *vs.* PBS+PBS group; +p < 0.05 *vs.* PBS+Con A group; ++p < 0.01 *vs.* PBS+Con A group.

View Article Online
DOI: 10.1039/C7FO01073G

Figure2. DHQ decreased the infiltration of $CD4^+$ T cells in injured liver tissues. At 24 h and 72 h after injection of Con A (30mg/kg body weight) via tail vein, three mice from each group were sacrificed, and the distribution of $CD4^+$ T cells in liver tissues was examined after immunohistochemical staining with CD4 antibody (**upper panel**, scale bar = 50 μm). For each section, three randomly selected fields under light microscope were screened for infiltration of $CD4^+$ T cells with Image-Pro Plus software. Data are presented as means ± SD (n= 3) (**lower panel**).

PBS+PBS (negative control group): mice received PBS by intragastric (i.g) administration daily for 14 days and intravenous (i.v) injection of PBS in tail vein on the 4^{th} day. PBS+Con A (positive control group): mice received PBS (i.g) daily for 14 days and injection of Con A (i.v) on the 4^{th} day. DHQ+Con A (DHQ treated group): mice received DHQ (5 mg/kg body weight; i.g) daily for 14 days and injection of Con A (i.v) on the 4^{th} day.

*p < 0.05 *vs.* PBS+PBS group; **p < 0.01 *vs.* PBS+PBS group; +p < 0.05 *vs.* PBS+Con A group; ++p < 0.01 *vs.* PBS+Con A group.

View Article Online
DOI: 10.1039/C7FO01073G

Figure3. DHQ decreased the infiltration of $CD8^+$ T cells in injured liver tissues.

24 h and 72 h following Con A injection, three mice from each group were sacrificed, and the distribution of $CD8^+$ T cells in liver tissues were assessed by immunohistochemical staining with CD8 antibody (**upper panel**, scale bar = 50 μm). Three randomly selected fields under light microscope in each setion were screened for infiltraion of $CD8^+$ T cells with Image-Pro Plus software. Data are presented as means ± SD (n= 3) (**lower panel**).

PBS+PBS (negative control group): mice received PBS by intragastric (i.g) administration daily for 14 days and intravenous (i.v) injection of PBS in tail vein on the 4^{th} day. PBS+Con A (positive control group): mice received PBS (i.g) daily for 14 days and injection of Con A (i.v) on the 4^{th} day. DHQ+Con A (DHQ treated group): mice received DHQ (5 mg/kg body weight; i.g) daily for 14 days and injection of Con A (i.v) on the 4^{th} day.

*:$p < 0.05$ *vs.* PBS+PBS group.

View Article Online
DOI: 10.1039/C7FO01073G

Figure 4. DHQ inhibited the expression of pro-inflammatory cytokines and T cell regulating genes in liver. At 24 h and 72 h after injection of Con A, three mice from each group were sacrificed, and the liver tissues were collected for RNA extraction, then the cDNA was synthesized, and RT-PCR was performed in the ABI 7500 systems. β-actin was used as internal control to normalize gene expression, and the relative quantification (RQ) was determined by $2^{-\Delta\Delta Ct}$ method. The RQ of all genes of mice in PBS+PBS group were normalized to 1.00.

PBS+PBS (negative control group): mice received PBS by intragastric (i.g) administration daily for 14 days and intravenous (i.v) injection of PBS in tail vein on the 4th day. PBS+Con A (positive control group): mice received PBS (i.g) daily for 14 days and injection of Con A (i.v) on the 4th day. DHQ+Con A (DHQ treated group): mice received DHQ (5 mg/kg body weight; i.g) daily for 14 days and injection of Con A (i.v) on the 4th day.

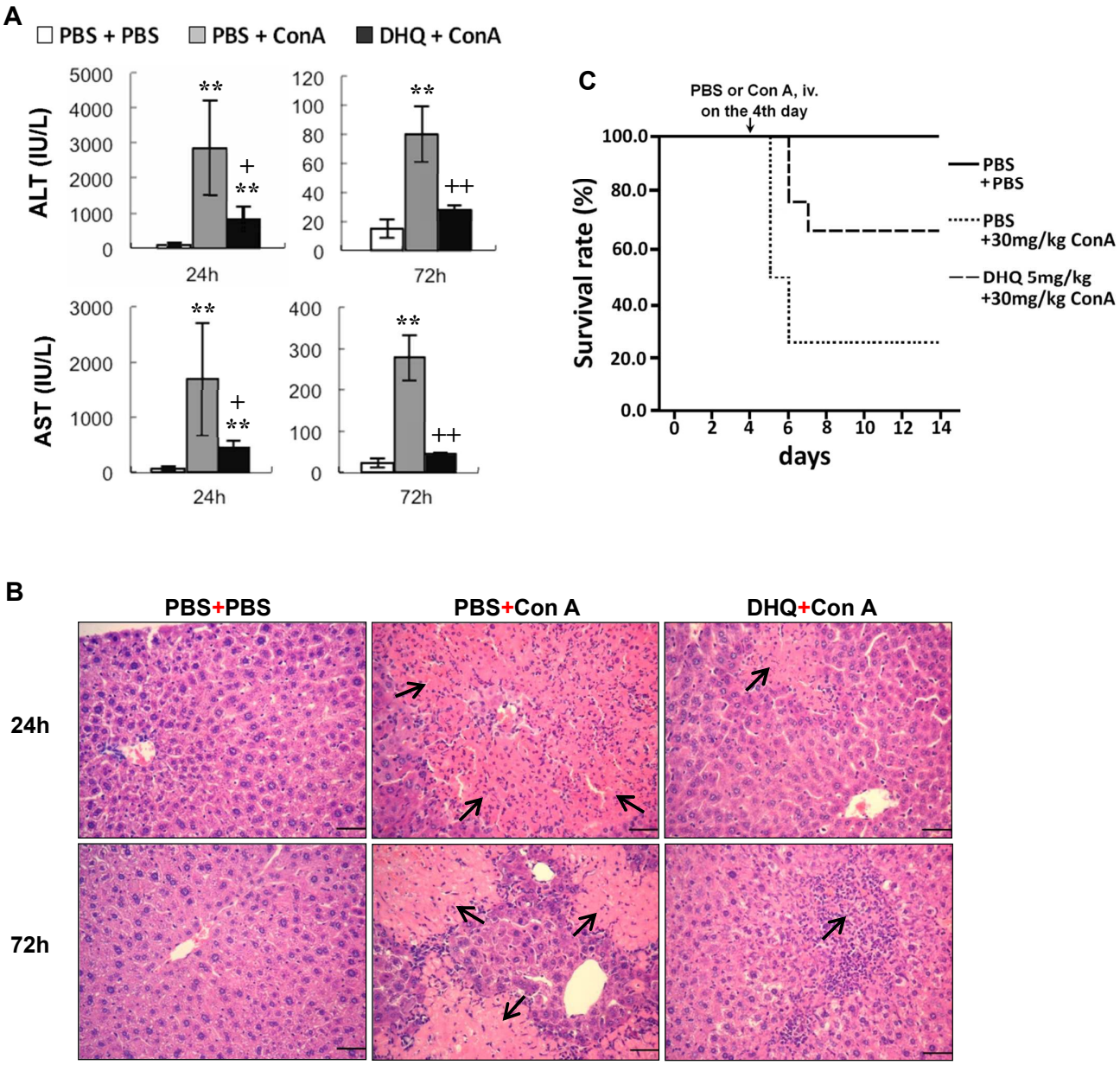
View Article Online
DOI: 10.1039/C7FO01073G

Figure 5. DHQ prevented HepG2 cells from TNF-α/ActD-induced apoptosis. TNF-α/ActD was used to induce apoptosis in HepG2 cells. Cells were pretreated with 200 μM of DHQ for 6 h before TNF-α (50 ng/mL)/ActD (25 ng/mL) were added to the medium. For negative control, cells were maintained in the routine medium without any treatment. For positive control, cells only received TNF-α/ActD without DHQ pretreatment. 24 h after addition of TNF-α/ActD, the cell viability (**A**), apoptosis (**B** arrows indicate the sub-G1 peak) and protein expression (**C**, arrows indicate the cleaved caspase) were detected. Data are presented as means ± SD (n= 3).

* $p < 0.05$ *vs.* control group; ** $p < 0.01$ *vs.* control group; ++ $p < 0.01$ *vs.* TNF-α/ActD group.

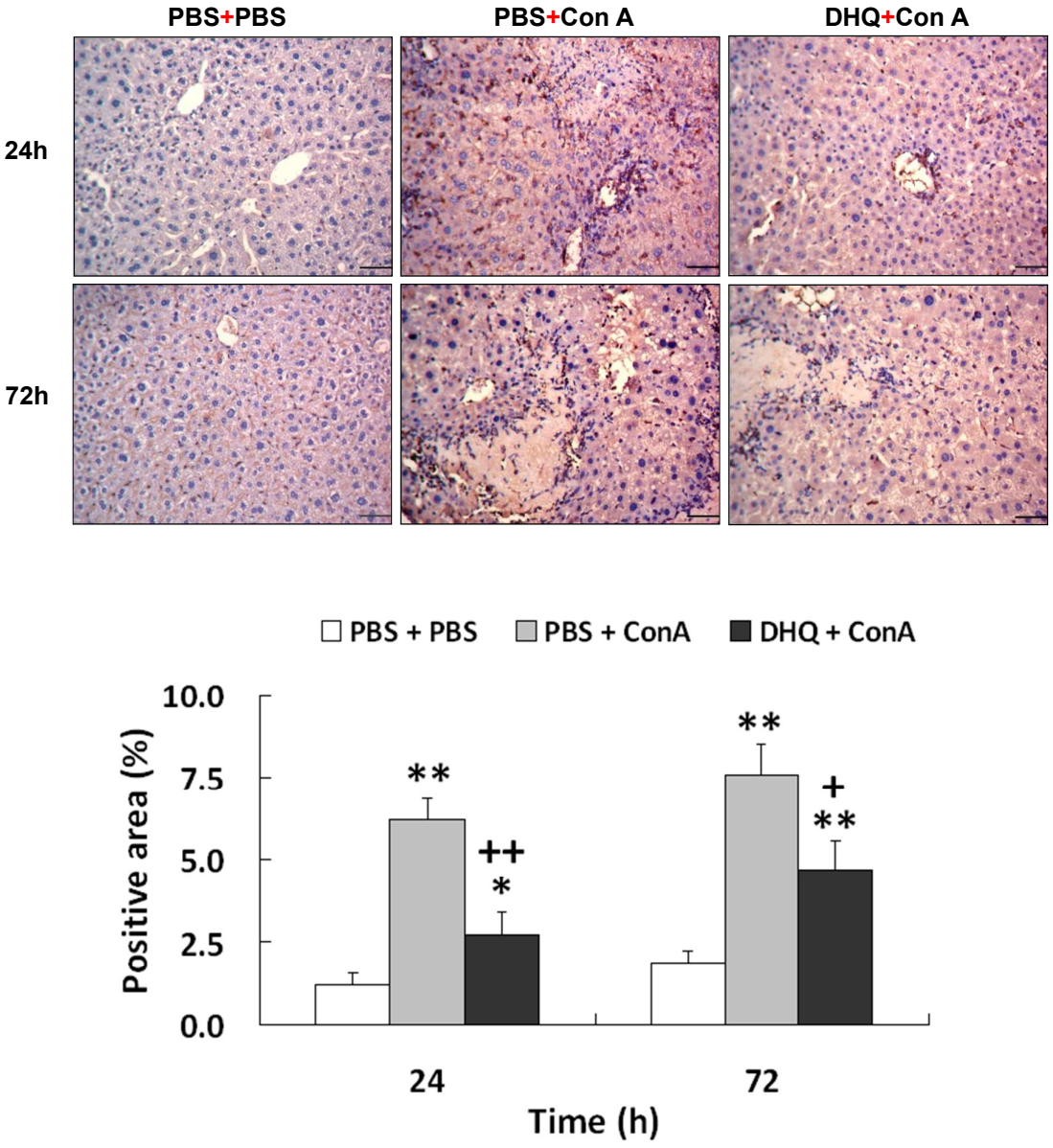
View Article Online
DOI: 10.1039/C7FO01073G

Figure 1



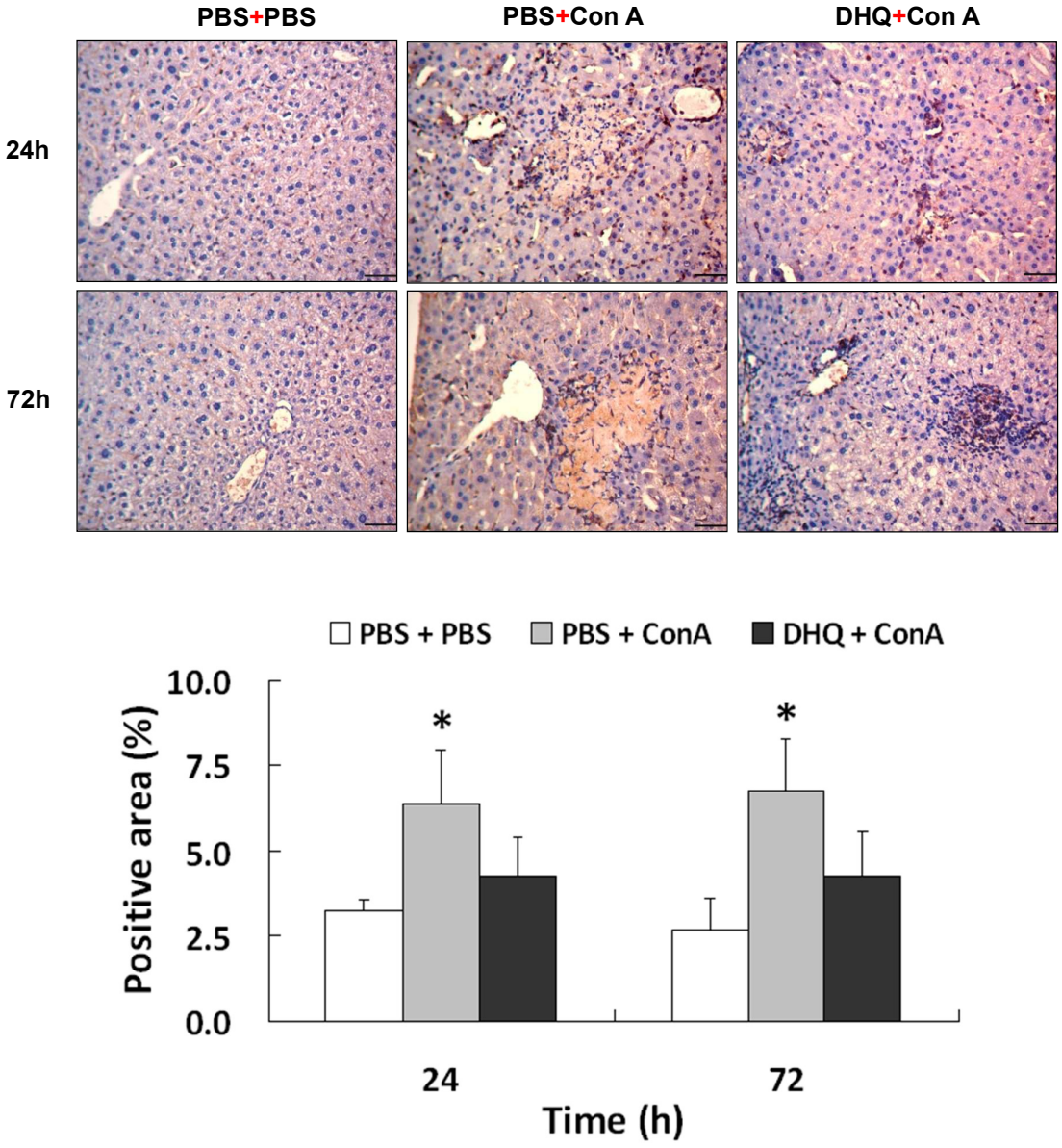
View Article Online
DOI: 10.1039/C7FO01073G

Figure 2



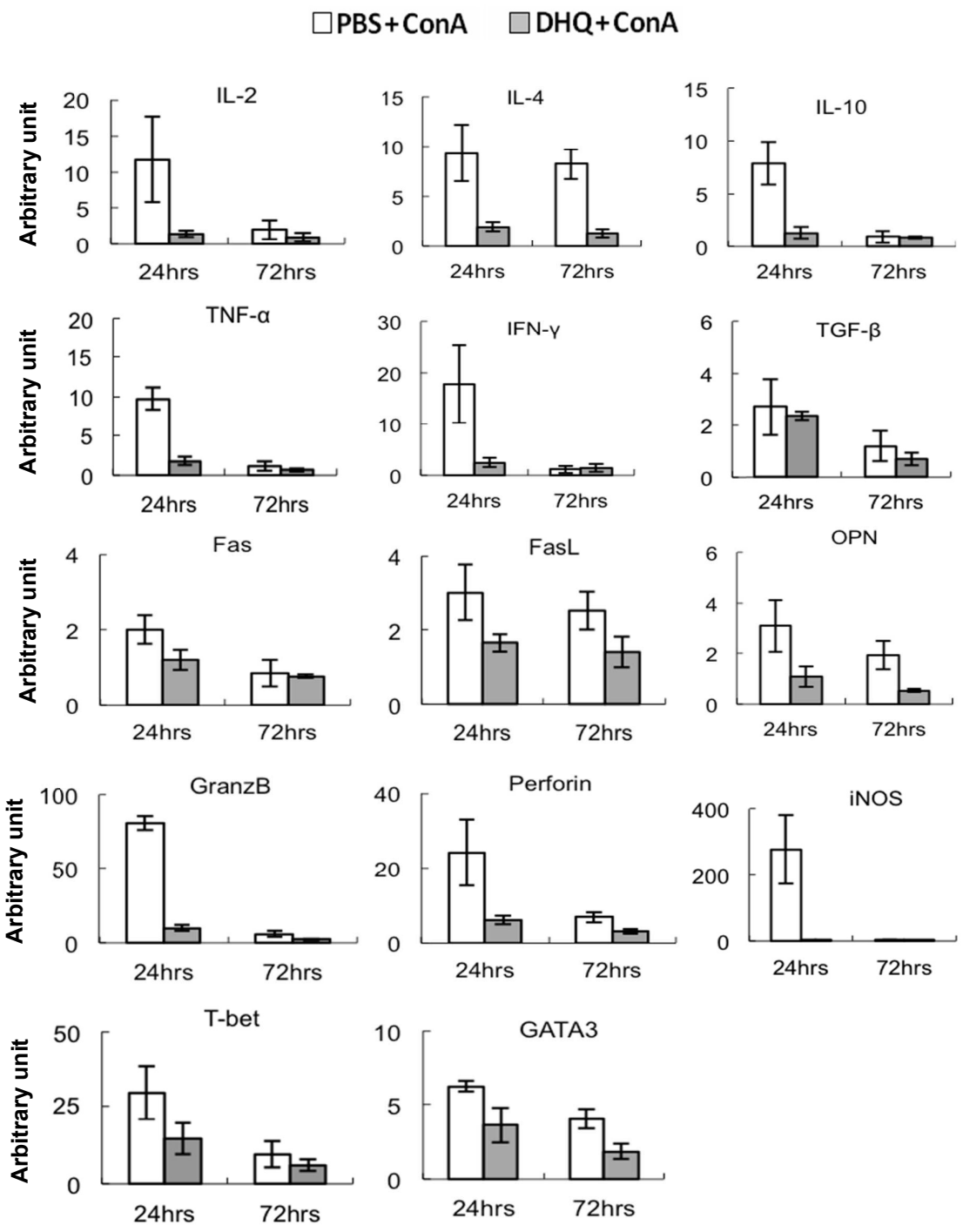
View Article Online
DOI: 10.1039/C7FO01073G

Figure 3



View Article Online
DOI: 10.1039/C7FO01073G

Figure 4



View Article Online
DOI: 10.1039/C7FO01073G

Figure 5