Original Article

DOI: 10.5582/bst.2016.01152

Ferulic acid prevents liver injury induced by Diosbulbin B and its mechanism

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Summary **The rhizome of *Dioscorea bulbifera* Linn, traditionally used to treat thyroid disease and cancer in China, is reported to induce serious liver injury during clinical practice. Diosbulbin B (DB), a diterpene lactone, has been found to be the main toxic compound in *D. bulbifera*. The present study aims to investigate the protection of ferulic acid (FA) against DB-induced acute liver injury and its engaged mechanism. Mice were orally administered FA (20, 40, 80 mg/kg) once daily for 6 consecutive days; and then orally given DB (250 mg/kg) on the last day. Daily FA (40, 80 mg/kg) decreased DB (250 mg/kg)-induced increase in serum levels of alanine/aspartate aminotransferase (ALT/AST) and alkaline phosphatase (ALP). Histological evaluation showed that FA (80 mg/kg) ameliorated DB-induced hepatocellular degeneration and lymphocyte infiltration. Results of terminal dUTP nick-end labeling (TUNEL) staining assay showed that FA (80 mg/kg) decreased the DB-increased number of apoptotic hepatocytes. FA (40, 80 mg/kg) reduced DB-increased liver malondialdehyde (MDA) amount. FA (40, 80 mg/kg) decreased DB-increased serum levels of tumor necrosis factor alpha (TNF-α) and interferon-γ (IFN-γ), and liver myeloperoxidase (MPO) activity. FA (80 mg/kg) reversed the DB-induced decrease in expression of inhibitor of kappa B (IκB) and the increase in nuclear translocation of the p65 subunit of nuclear factor kappa B (NFκBp65). Taken together, our results demonstrate that FA prevents DB-induced acute liver injury *via* inhibiting intrahepatic inflammation and liver apoptosis.**

Keywords: Hepatotoxicity, detoxification, inflammation, apoptosis, NFκB

1. Introduction

The rhizome of *Dioscorea bulbifera* Linn. is traditionally used to treat thyroid disease and cancer in China (*1*). However, *D. bulbifera* can cause severe hepatotoxicity in clinical practice, which seriously limits the anti-tumor activity of this medicinal herb (*2*). Our previous studies demonstrated that intake of *D. bulbifera* could result in severe liver injury such as liver swelling, fatty degeneration, and even animal death (*3-5*). Diosbulbin B (DB), a diterpene lactone, was isolated from *D. bulbifera*, and showed significant antitumor activity in our previous reported study (*6*). However, our previous studies also showed that DB was the main hepatotoxic compound in *D. bulbifera*, and it caused oxidative stress-associated liver injury *(4,7)*.

Ferulic acid (FA) is found in many Chinese medicinal herbs such as Angelica sinensis (Oliv) Diels (Angelica) and *Ligusticum chuanxiong* Hort. (Ligusticum) (*8*). FA exerts multiple biological activities such as antioxidant, and anti-inflammation (*9,10*) and it is known for treatment of Alzheimer's disease (*11*), cardiovascular disease (*12*), and cancer (*13*). In addition, FA is reported to have hepato-protective activity (*14,15*). Previous study in our lab has demonstrated that *A. sinensis* root prevented liver injury induced by *D. bulbifera* rhizome (*16*). As the main active compound in *A. sinensis*, we think that FA may prevent DB-induced liver injury

Released online in J-STAGE as advance publication October 23, 2016.

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in mice. In addition, our previous study has already demonstrated that FA attenuated DB-induced liver injury in S180 tumor-bearing mice and further augmented DB-induced inhibition of tumor growth *in vivo* (*17*). The present study is designed to investigate protection by FA against acute liver injury induced by DB in mice and the potential underlying mechanism.

2. Materials and Methods

2.1. *Drugs and reagents*

DB (Figure1A) and FA (Figure 1B) were purchased from Shanghai Tauto Biotech Co., Ltd (Shanghai, China). Antibodies against inhibitor of kappa B (IκB), p65 subunit of nuclear factor kappa B (NFκBp65) and β-actin were all purchased from Cell Signaling Technology (Danvers, MA, USA). Peroxidase-conjugated goat anti-Rabbit IgG (H+L) was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Nitrocellulose membranes were purchased from Bio-Rad (Hercules, CA, USA). Enhanced chemiluminescence detection system was obtained from Millipore Corporation (Billerica, MA, USA). Nuclear/Cytosol fractionation Kit was obtained from BioVision (Palo Alto, CA, USA). Enzyme linked immunosorbent assay (ELISA) Kits for determining tumor necrosis factor alpha (TNF-α) and interferon-γ (IFN-γ) were purchased from RapidBio (West Hills, CA, USA). Kits for detecting the activity of alanine/aspartate aminotransferase (ALT/AST), alkaline phosphatase (ALP), myeloperoxidase (MPO) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. *Experimental animals*

Specific pathogen free male ICR mice (18-22 g body weight) were obtained from the Shanghai laboratory animal center of Chinese Academy of Sciences (Shanghai, China). Animals were fed a standard laboratory diet and given free access to tap water, kept in a controlled room temperature (22 ± 1°C), humidity (65 ± 5 %), and a 12:12-h light/dark cycle. All animals received humane care in compliance with the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine.

2.3. *Treatment protocol*

Mice were divided into 6 groups. Mice in group 1 were used as control group. Mice in group 2 were orally administered DB (250 mg/kg, suspended in 0.5% sodium carboxyl methyl cellulose (CMC-Na)) only once on the sixth day. Mice in groups 3-5 were orally given FA suspended in 0.5% CMC-Na (20, 40 or 80 mg/kg per day) for six consecutive days, and DB was orally given two hours after the final administration of FA. Mice in group 6 were given FA only, suspended in 0.5% CMC-Na (80 mg/kg per day), for six consecutive days. Twenty-four hours later after administration of DB, blood and liver samples were collected for further research (*16-18*).

2.4. *Assay for serum ALT, AST and ALP*

The blood samples collected from all groups of mice were kept at room temperature to coagulate for 2 h. Serum was then isolated and transferred to new tubes after centrifugation at 840 × g for 15 min. Serum ALT, AST and ALP levels were detected with kits according to the manufacturer's instructions.

2.5. *Histological observation*

The liver tissues were soaked in 10 % formalin, and embedded in paraffin. Samples were cut into five micrometer sections and stained with hematoxylin-eosin for further histological assessment of tissue damage.

2.6. *TdT-mediated biotin–dUTP nick-end labelling (TUNEL) assay*

For the detection of apoptosis, paraffin-embedded sections were stained with the TUNEL detection kit according to the manufacturer's protocol. The cells showing nuclear dark-brown staining were considered to be positive staining (apoptotic cells). The apoptotic hepatocytes were counted manually in at least nine randomly selected fields from each group using a light microscope at a magnification of ×200.

2.7. *Analysis of MDA amount*

MDA amount in liver was determined using an MDA detection kit according to the manufacturer's instructions. MDA amount is expressed as nmol/mg of protein.

2.8. *ELISA analysis*

Serum contents of TNF-α and IFN-γ were measured with ELISA kits according to the manufacturer's instructions.

2.9. *Analysis of MPO activity*

Liver MPO activity was determined using a MPO detection kit according to the manufacturer's instructions. MPO activity is expressed as units/g of protein.

2.10. *Extracting cytosol and nuclear proteins*

Cytosol and nuclear proteins were extracted according to the manufacturer's instructions. Protein concentration

was determined and normalized to equal protein concentrations.

2.11. *Western blot analysis*

Liver tissue was homogenized in ice-cold lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 0.5% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 g/mL aprotinin, 10 g/mL leupeptin, 10 g/mL pepstatin A. The homogenate was centrifuged at 10,000 g for 20 min at 4°C. The supernatant was transferred to new tubes and protein concentration was assayed and normalized to equal protein concentrations. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blots were incubated with primary and horseradish peroxidase (HRP)-conjugated secondary antibodies. The protein bands were quantified by ratios of integral optic density (IOD) following normalization to β-actin, and the results were expressed as percentage of control.

2.12. *Statistical analysis*

All experimental data were expressed as means ± standard error (S.E.). Significant differences were determined by One-Way ANOVA. $p < 0.05$ was considered as statistically significant difference.

3. Results

3.1. *FA decreased DB-induced increase in serum levels of ALT, AST and ALP*

Shown in Figure 1C and 1D, DB (250 mg/kg) are increased serum levels of ALT, AST and ALP as compared with control. After treatment with FA (40, 80 mg/kg), DB-induced an increase in serum levels of ALT, AST and ALP, which were all decreased ($p < 0.05$, $p < 0.01$, $p < 0.001$). In addition, there were no significant differences in the serum levels of ALT, AST and ALP between control and FA (80 mg/kg)-treated mice ($p > 0.05$).

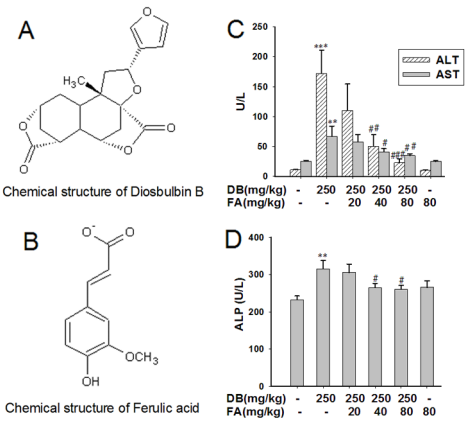

Figure 1. FA decreased the increased serum levels of ALT, AST and ALP induced by DB. Chemical structure of DB **(A)** and FA **(B)**; **(C)** ALT and AST; **(D)** ALP. Data are shown as means ± S.E. ($n = 10$). $^{**}p < 0.01$, $^{***}p < 0.001$ versus control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus DB-treated group. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DB, Diosbulbin B; FA, Ferulic acid.

3.2. *Histological evaluation of liver*

As compared to control, the liver from the mice treated with DB showed serious liver damage, indicated by hepatocellular degeneration and lymphocyte infiltration (Figure 2B). After treatment with FA (20, 40, 80 mg/kg), all these phenomena were ameliorated (Figure 2C, 2D, and 2E), and FA at 80 mg/kg was the most effective. Livers from control (Figure 2A) and FA (80 mg/kg)-treated (Figure 2F) mice showed normal histology.

3.3. *FA alleviated DB-induced cell apoptosis*

As shown in Figure 3A-a and 3A-b, there were an increased number of brown (TUNEL-positive) apoptotic hepatocytes in DB (250 mg/kg)-treated mice as compared with control. After treatment with FA (80 mg/kg), the increased number of apoptotic cells was reduced (Figure 3A-c), but FA (80 mg/kg) alone had no effect on liver apoptosis. After counting the apoptotic hepatocytes, the results showed that FA (80 mg/kg) decreased the increased apoptotic hepatocytes induced by DB ($p < 0.01$) (Figure 3B).

3.4. *FA decreased DB-induced increase of liver MDA amount*

As shown in Figure 3C, DB increased liver amount of MDA ($p < 0.01$), whereas FA (40, 80 mg/kg) reduced the increased amount of MDA induced by DB ($p < 0.01$, $p < 0.001$).

3.5. *FA reduced DB-induced increase in serum TNF-α and IFN-γ levels, and liver MPO activity*

As shown in Figure 4A and 4B, DB increased serum levels of TNF-α and IFN-γ ($p < 0.01$, $p < 0.001$), whereas FA (20, 40, 80 mg/kg) reduced the increase in serum levels of TNF-α and IFN-γ induced by DB ($p < 0.05$, $p < 0.01$, $p < 0.001$). In addition, the results of Figure 4C showed that DB-induced increase in liver MPO activity was reduced in FA (40, 80 mg/kg)-treated mice ($p < 0.05$).

3.6. *FA reversed DB-induced decrease in IκB expression and increase in NFκBp65 nuclear translocation*

As shown in Figure 5A and 5C, FA (80 mg/kg) increased

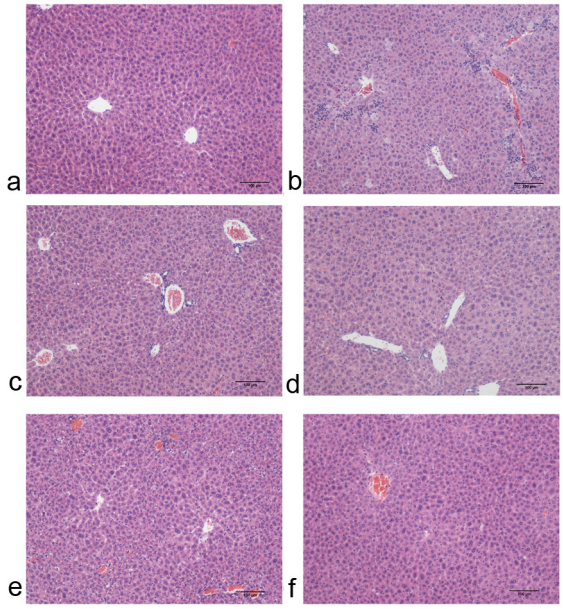

Figure 2. Histological evaluation of liver. (a) Vehicle control; **(b)** DB (250 mg/kg); **(c)** DB (250 mg/kg) + FA (20 mg/kg); (d) DB (250 mg/kg) + FA (40 mg/kg); **(e)** DB (250 mg/kg) + FA (80 mg/kg), **(f)** FA (80 mg/kg). Liver sections were stained with hematoxylin-eosin (original magnification 100 ×). DB, Diosbulbin B; FA, Ferulic acid.

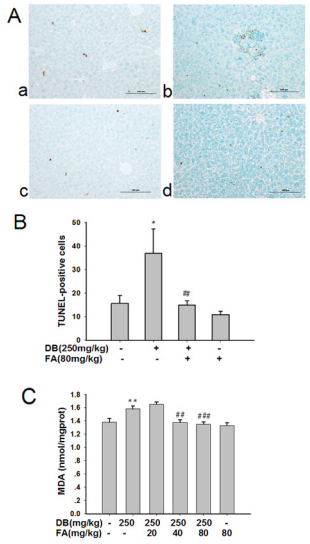

Figure 3. FA alleviated DB-induced liver apoptosis and DB-increased liver MDA amount. (A) Apoptosis was determined by TUNEL staining assay. Typical images were chosen from each group. **(a)** Vehicle control, **(b)** DB (250 mg/kg), **(c)** DB (250 mg/kg) + FA (80 mg/kg), **(d)** FA (80 mg/kg). (original magnification 200 ×). **(B)** Data are shown as means ± S.E., (n = 4). $^{*}p < 0.05$ versus control, $^{\#\#}p < 0.01$ versus DB-treated group. **(C)** FA decreased the increased liver MDA amount induced by DB. Data are shown as means ± S.E., (n = 9-10). $^{**}p < 0.01$ versus control, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus DB-treated group. DB, Diosbulbin B; FA, Ferulic acid.

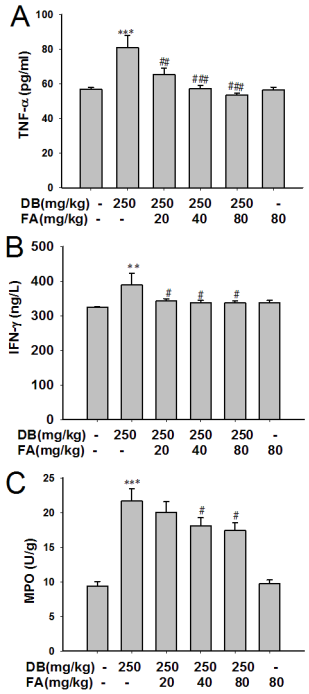

Figure 4. FA decreased DB-increased serum TNF-α and IFN-γ levels, and liver MPO activity. (A) Serum TNF-α; **(B)** Serum IFN-γ; **(C)** Liver MPO activity. Data are shown as means ± S.E., (n = 8-10). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus DB-treated group. DB, Diosbulbin B.

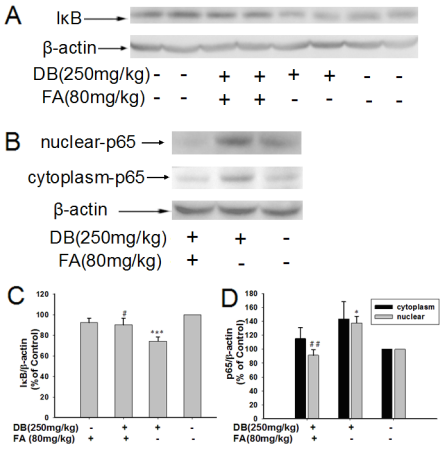

Figure 5. FA reversed DB-induced the decreased IκB expression and increased NFκBp65 nuclear translocation. (A) The expression of IκB in cytoplasm; **(B)** The expression of cytosol and nuclear NFκBp65 protein; **(C)** Quantitative densitometric analysis of IκB protein; **(D)** Quantitative densitometric analysis of NFκBp65 protein. The bands were normalized to basal β-actin expression and the vehicle control is set as 100%. Data are shown as means ± S.E., (n = 3-4). $^{**}p < 0.01$, $^{***}p < 0.001$ versus control, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ versus DB-treated group. DB, Diosbulbin B.

the decreased expression of IκB protein induced by DB ($p < 0.05$). After treatment with DB (250 mg/kg), the nuclear expression of NFκBp65 protein was increased ($p < 0.05$) (Figure 5B and 5D). In contrast, FA (80 mg/kg) inhibited this increase of NFκBp65 protein in nucleus induced by DB ($p < 0.01$) (Figures 5B and 5D).

4. Discussion

The increased levels of serum ALT, AST and ALP are commonly used to indicate liver injury (*19*). Our results of ALT, AST and ALP analysis demonstrate that FA can prevent DB-induced liver injury. In addition, such protection is further evidenced by histological evaluation of liver. Previous reports demonstrate that FA can attenuate ischemia/reperfusion or carbon tetrachloride-induced liver injury (*14,15*). Meanwhile, in our previous study, FA can not only ameliorate DB-induced liver injury in tumor-bearing mice, but also increase the anticancer effect of DB (*17*). The present study further evidenced the detoxification of FA against DB-induced acute liver injury, and the present and previous studies indicate the potential value for development of the combined application of DB with FA for cancer treatment.

Apoptosis is the process of programmed cell death, which may occur in various organisms including liver (*20*). In situ detection of apoptosis by using TUNEL assay is a commonly used method to analyze the existence of apoptosis (*21*). Our present results demonstrate that DB can induce hepatocyte apoptosis, whereas FA can prevent DB-induced apoptosis in liver. Our previous studies have already demonstrated that DB and *D. bulbifera* induced oxidative liver injury (*3,4,7*), and the present study is the first report concerning DB-induced liver apoptosis.

Oxidative stress plays an important role in drug-induced liver injury (*22*). Reactive oxygen species (ROS) are very active and can react with lipid, and MDA is one of the main end products, thus MDA is often used as an indicator to assess oxidative injury (*23*). Our previous studies have already demonstrated that DB and *D. bulbifera* can induce oxidative liver injury (*3,4,7*). The present results demonstrate that FA can alleviate DB-induced liver oxidative injury *in vivo*.

TNF-α is a pro-inflammatory cytokine produced principally by activated macrophages, and previous reports demonstrated that TNF-α was involved in alcoholic hepatitis (*24*), and ischemia/reperfusion-induced liver injury (*25*). IFN-γ is a cytokine that is critical for regulating innate and adaptive immunity, and it is also reported to be involved in various toxins-induced liver injuries such as acetaminophen and carbon tetrachloride (*26,27*). MPO exists in neutrophils and its activity is generally used to assess the infiltration of neutrophils (*28*). The increased MPO activity is found in carbon tetrachloride, α-naphthylisothiocyanate, and trauma-hemorrhagic shock-induced liver injury (*29-31*). The present study demonstrates that DB increases serum levels of TNF-α and IFN-γ, and elevates liver MPO activity, which indicates the occurrence of hepatic inflammation. Furthermore, FA can decrease those increased TNF-α, IFN-γ, and MPO activities, which suggests that FA can ameliorate DB-induced immunological liver injury.

It is well known that transcription factor NFκB plays a critical role in regulating host immune and inflammatory responses (*32,33*). In un-stimulated cells, NFκB exists in cytoplasm associated with the inhibitory protein IκB (*34*). The predominant form of NFκB is a heterodimer composed of p50 and p65 (Rel A) subunits, and NFκB is activated in response to primary (viruses, bacteria, UV) or secondary (inflammatory cytokines) pathogenic stimuli (*32,35*). Stimulation induces the release of NFκB from IκB and translocation to the nucleus, where it binds to the DNA at specific κB sites, and thus initiates the expression of target genes such as TNF-α (*36,37*). Our results demonstrate that FA reverses DB-induced translocation of p65 into the nucleus and decreased expression of cytosol IκB. Those results suggest that FA attenuates DB-induced immunological liver injury *via* inhibiting NFκB activation.

In conclusion, the present study demonstrates that FA, the major compound in *A. sinensis*, can prevent DB-induced liver injury, which may contribute to the detoxification of *A. sinensis* against liver injury induced by *D. bulbifera*, and provides strong experimental evidence of potential combined application of *A. sinensis* and *D. bulbifera* in the clinic. In addition, the amelioration of FA against DB-induced immunological liver injury *via* inhibiting NFκB activation may be the main mechanism involved in the protection of FA against DB-induced acute liver injury.

Acknowledgements

This work was supported by a grant from State major science and technology special projects during the 12th five year plan (2015ZX09501004-002-002).

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(Received August 3, 2016; Revised September 16, 2016; Accepted September 29, 2016)