Effects of blueberry on hepatic fibrosis and transcription factor Nrf2 in rats

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Abstract

AIM: To investigate the effects of blueberry on hepatic fibrosis and NF-E2-related factor 2 (Nrf2) transcription factor in rats.

METHODS: Forty-five male Sprague-Dawley rats were randomly divided into control group (A); CCl4-induced hepatic fibrosis group (B); blueberry prevention group (C); Dan-shao-hua-xian capsule (DSHX) prevention group (D); and blueberry + DSHX prevention group (E). Liver fibrosis was induced in rats by subcutaneous injection of CCl4 and a high-lipid/low-protein diet for 8 wk (except the control group). The level of hyaluronic acid (HA) and alanine aminotransferase (ALT) in serum was examined. The activity of superoxide dismutase (SOD), glutathione-S-transferase (GST) and malondialdehyde (MDA) in liver homogenates was determined. The degree of hepatic fibrosis was evaluated by hematoxylin and eosin and Masson staining. Expression of Nrf2 and NADPH quinone oxidoreductase 1 (Nqo1) was detected by real-time reversed transcribed-polymerase chain reaction, immunohistochemical techniques, and western blotting.

RESULTS: Compared with group B, liver indices, levels of serum HA and ALT of groups C, D and E were reduced (liver indices: 0.038 ± 0.008, 0.036 ± 0.007, 0.036 ± 0.005 vs 0.054 ± 0.009, P < 0.05; HA: 502.33 ± 110.57 ng/mL, 524.25 ± 255.42 ng/mL, 499.25 ± 198.10 ng/mL vs 828.50 ± 237.83 ng/mL, P < 0.05; ALT: 149.44 ± 16.51 U/L, 136.88 ± 10.07 U/L, 127.38 ± 11.03 U/L vs 203.25 ± 31.62 U/L, P < 0.05), and SOD level was significantly higher, but MDA level was lower, in liver homogenates (SOD: 1.36 ± 0.09 U/mg, 1.42 ± 0.13 U/mg, 1.50 ± 0.15 U/mg vs 1.08 ± 0.19 U/mg, P < 0.05; MDA: 0.294 ± 0.026 nmol/mg, 0.285 ± 0.025 nmol/mg, 0.284 ± 0.028 nmol/mg vs 0.335 ± 0.056 nmol/mg, P < 0.05). Meanwhile, the stage of hepatic fibrosis was significantly weakened (P < 0.05). Compared with group A, the activity of GST liver homogenates and expression levels of Nrf2 and Nqo1 in group B were elevated (P < 0.05). The expression level of Nrf2 and Nqo1 in groups C, D, and E were increased as compared with group B, but the difference was not significant.

CONCLUSION: Blueberry has preventive and protective effects on CCl4-induced hepatic fibrosis by reducing hepatocyte injury and lipid peroxidation. However, these effects may not be related to the activation of Nrf2 during long-term of CCl4.

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Key words: Blueberry; Hepatic fibrosis; NF-E2-related factor 2; NADPH quinone oxidoreductase 1; Glutathione-S-transferase

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INTRODUCTION

Hepatic fibrosis is a common result of chronic injury to the liver[1]. Oxidative stress has recently been recognized as a fundamental factor in the pathological changes observed in various liver diseases[2-4]. Oxidative stress can cause excessive damage to hepatocytes through lipid peroxidation and protein alkylation[5,6]. NF-E2-related factor 2 (Nrf2) is an important transcription factor that regulates antioxidative stress reactions. NADPH quinone oxidoreductase 1 (Nqo1) and glutathione-S-transferase (GST) are the major phase-II detoxification enzymes controlled by Nrf2[6-7].

Blueberries are flowering plants that belong to Viscumium spp. of the family Ericaceae. The Human Nutrition Research Center (Mayer, USA) has performed a series of intensive studies of blueberries. These studies have indicated that blueberries contain anthocyanins, polyphenols and flavonoids, and appear to have the highest antioxidant capacity among the common fruits and vegetables[8-9]. Blueberries may also affect chronic disease through anti-inflammatory and antitumor mechanisms[10,11].

The focus of the present study was to examine the effect of blueberry on hepatic fibrosis and detoxification enzyme systems in rats.

MATERIALS AND METHODS

Ethical approval of the study protocol

All animal studies complied with the Animal Care and Use Guidelines of Guiyang Medical College (Guiyang, China).

Reagents and animal treatments

Forty-five male Sprague-Dawley rats (200 ± 20 g) were obtained from the Experimental Animal Center of Guiyang Medical College [Approval number SCXK (Gui-zhou) 2002-00001]. Rats were randomly divided into five groups of nine: control group (A); CCl₄-induced hepatic fibrosis group (B); blueberry prevention group (C); Dan-shao-hua-xian capsule (DSHX) prevention group (D); and blueberry + DSHX prevention group (E).

Except in the control group, liver fibrosis was induced in each group by a complex method[12]. Rats in groups B-E received subcutaneous injections of 40% CCl₄ solution (mixture of pure CCl₄ and peanut oil) and 0.3 mL/100 g twice a week for 8 wk (the first dose was 40% CCl₄ 0.4 mL/100 g). Rats were fed a high-lipid/low-protein diet (79.5% corn fatina, 20% fat, and 0.5% cholesterol) each day. Rats in group A were fed only a normal diet. At the same time, blueberry juice (1.5 g/100 g per day, po, “Rabbiteye Blueberry”; Blueberry production field of Ma-Jiang, Guizhou, China) was processed. Samples were stored at -20°C until use, and homogenized to prepare blueberry juice (1 mL blueberry juice contained about 2 g of dried blueberries). DSHX (1.0 g/kg per day, po; lot number 20081011; DSHX capsules composed of five Chinese herbal medicines, Tetrandrine, Radix Salviae Miltiorrhizae, Radix Paeoniae Rubra, Astragalus Membranaceus and Ginkgo leaf; Guiyang Pharmaceutical Company, Guizhou, China) and blueberry juice + DSHX (1.5 g/100 g + 1.0 g/kg, po, daily) were given to rats in groups C-E. After 8 wk, there were eight survivors in groups A, B, D and E, and nine in group C.

Rats were killed to collect blood and livers (rats were weighed before killing). The wet liver was weighed. The same part of each liver was removed and fixed in 10% neutral formalin; the remaining portion of liver was stored at -80°C until use. Rat serum was centrifuged (1500 r/min for 15 min at room temperature) and stored at -80°C until use.

Histopathology

After fixation in 10% formalin for 24 h, samples were embedded in paraffin. Samples were then cut into 5-μm pieces and mounted on slides. They were stained with hematoxylin and eosin (HE) for histopathological examination, and with fibrosis-specific Masson stain for evaluation of the degree of liver fibrosis[13].

Calculation of the liver index

The liver index was calculated according to the formula[14]: (liver weight/rat weight) × 100%.

Measurement of levels of hyaluronic acid and alanine aminotransferase

Concentrations of hyaluronic acid (HA) (lot number 20090501, Shanghai Institute of Naval Medicine, Shanghai, China) and alanine aminotransferase (ALT) were measured by radioimmunoassay (RIA) or by an automatic biochemical analytic instrument (Siemens Advia 1650, Bensheim, Germany).

Measurement of levels of superoxide dismutase, glutathione-S-transferase and malondialdehyde in liver homogenates

Liver homogenates were prepared. Superoxide dismutase (SOD) content was determined by the xanthine oxidase method; malondialdehyde (MDA) content was tested by the thiobarbituric acid method; and GST content examined by the dinitrobenzene combination method, according to the manufacturer’s instructions (lot numbers 20090521, 20090522, and 20090522, respectively; Jiancheng Biologic Co., Nanjing, China).

Real-time reverse transcriptase-polymerase chain reaction analyses

Total RNA was extracted from liver tissues with Trizol reagent (lot number 13827390; Invitrogen, Carlsbad, CA, USA) and purified. This was followed by reverse transcription (lot number 00033699; Fermentas, MBI, Burling-
Table 1  Primer sequences for real-time RT-PCR analyses

| Genes   | Number | GenBank         | Reverse               |
|---------|--------|----------------|-----------------------|
| β-actin | V01217 | TCTCTCGAGCCCAAGTACTCT | GCTCAGTAAAGTGGCGCTAGAA |
| Nrf2    | NM_057152 | CCAATCTTTTCTCCACGAA | AGGCCCCATGGATTCAGTT   |
| Nqo1    | NM_017000 | CCAATCTCCACCACCTGGT | GTCCTCAGGCCATTGTTGAG   |

RT-PCR: Reverse transcriptase-polymerase chain reaction.

Table 2  Liver index, serum HA, ALT, and SOD, MDA, GST of liver homogenate in different groups (mean ± SD)

| Group | Liver index (relative liver weight) | HA (ng/mL) | ALT (U/L) | SOD (μ/μg) | MDA (nmol/mg) | GST (μ/μg) |
|-------|-----------------------------------|------------|-----------|-------------|---------------|-------------|
| A     | 0.03 ± 0.000                      | 351.75 ± 125.16 | 57.25 ± 6.88 | 1.76 ± 0.34 | 0.206 ± 0.052 | 5.95 ± 1.97 |
| B     | 0.054 ± 0.09                        | 828.50 ± 237.83 | 203.25 ± 31.62 | 1.9 ± 0.19 | 0.335 ± 0.056 | 7.30 ± 1.26 |
| C     | 0.038 ± 0.08                        | 502.33 ± 110.57 | 149.44 ± 16.51 | 1.36 ± 0.09 | 0.294 ± 0.026 | 7.37 ± 0.87 |
| D     | 0.036 ± 0.07                        | 524.25 ± 255.42 | 136.88 ± 10.07 | 1.42 ± 0.13 | 0.285 ± 0.025 | 7.35 ± 0.88 |
| E     | 0.036 ± 0.05                        | 499.25 ± 198.10 | 127.38 ± 11.03 | 1.50 ± 0.15 | 0.284 ± 0.028 | 7.81 ± 1.16 |

*P < 0.05 vs group A; °P < 0.05 vs group B. HA: Hyaluronic acid; ALT: Alanine aminotransferase; SOD: Superoxide dismutase; MDA: Malondialdehyde; GST: Glutathione-S-transferase.

After de-paraffinization, rehydration, and antigen unmasking by heat treatment, histological sections were placed in 3% H2O2 for 10 min. They were then incubated with anti-Nrf2 (lot number K2008; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-Nqo1 (lot number B1508; Santa Cruz Biotechnology) antibody (1:100) overnight at 4°C. Samples were then processed using an EnVision kit (lot number 09065A2; Millipore) and short exposure of the membrane to X-ray films. β-actin (lot number 0804104; Applied Biosystems, Foster City, CA, USA) was used for real-time reverse transcriptase (RT)-PCR analyses. The cycle time (Ct) values of the genes of interest were first normalized with β-actin from the same sample. Relative differences between groups calculated and expressed as relative increases, setting the control as 100%.

**Statistical analysis**

Data analyses were carried out using SPSS 11.5 software (Chicago, IL, USA). Quantitative data were expressed as mean ± SD and subjected to one-way analysis of variance. Ordinal data were analyzed by Ridi analysis. P < 0.05 was considered significant.

**RESULTS**

**Effect of blueberry on the liver index and serum concentrations of HA and ALT**

The liver index and serum concentrations of HA and ALT in group B increased greatly compared with those in group A (P < 0.05). Compared with group B, the liver index and serum concentration of HA and ALT in groups C-E were clearly reduced (P < 0.05) (Table 2).
Effect of blueberry on levels of SOD, MDA and GST in liver homogenates

The level of SOD in liver homogenates was significantly higher and that of MDA significantly lower in groups C-E compared with those in group B ($P < 0.05$) (Table 2). The level of GST was significantly higher in group B than that in group A ($P < 0.05$). The activity of GST in groups C-E was increased as compared with group B, but the differences were not significant.

Effects of blueberry on the degree of hepatic fibrosis

After HE and Masson staining, hepatocytes in group A had a radial array with the central vein, and regenerating collagen fibers were absent. The lobular structure of group-B hepatocytes was destroyed, and the hepatic plates were disordered with diffuse, fatty degeneration. Collagen fibers expanded into the hepatic parenchyma, and fibrous septa surrounding and separating the normal lobules formed. Pseudolobules were observed in a few samples, and numerous inflammatory cells infiltrated in the portal area and fibrous septa. The degree of hepatic fibrosis in group B was significantly more compared with that in group A ($P < 0.05$).

The hepatic fibrosis in groups C-E was alleviated; the fibrous septa were thinner than those in group B ($P < 0.05$). Diffuse fatty degeneration in hepatocytes was found in some samples. Bridging fibrosis, a few inflammatory cells infiltrated into the portal area, fibrous septa, and pseudolobules were also found in some samples. More detailed information about the degree of hepatic fibrosis in each group is shown in Table 3 and Figure 1.

Effect of blueberry on gene and protein expression of Nrf2 and Nqo1

Real-time RT-PCR showed that Nrf2 and Nqo1 mRNA expression was significantly higher in group B than group A ($P < 0.05$); and expression of Nrf2 and Nqo1 mRNA in groups C-E was increased as compared with group B, but the differences were not significant. Immunohistochemical and western blotting assays showed similar protein expression of Nrf2 and Nqo1 (Table 4 and Figures 2-4).

DISCUSSION

The present work demonstrated that blueberry has a therapeutic effect on CCl₄-induced hepatic fibrosis in rats. There was no difference in therapeutic effect among the blueberry, DSHX, and blueberry + DSHX groups. DSHX is a mixed preparation composed of five traditional Chinese herbal medicines. We have previously reported that DSHX is effective in preventing hepatic fibrosis [12,14,15].

Reactive oxygen species and oxidative stress have an important role in the development of hepatic fibrosis. A central role in the defense against oxidative stress has been attributed to the transcription factor Nrf2, which

![Figure 1](https://example.com/image1.png)  
**Figure 1** Liver tissue in each group of rats (HE staining, × 40 magnification). A: Light microscopy showing normal liver tissue in the control group; C-E: Pathological change in the treatment group was milder compared with that in the model group.

| Group | Degree of hepatic fibrosis | Mean rank |
|-------|---------------------------|----------|
| A     | 8  | 0  | 0  | 0  | 0  | 0 | 4.5 |
| B     | 8  | 0  | 0  | 0  | 0  | 1 | 6 | 34.25<sup>a</sup> |
| C     | 9  | 0  | 0  | 2  | 2  | 2 | 0 | 23.00<sup>a</sup> |
| D     | 8  | 0  | 0  | 1  | 1  | 1 | 1 | 22.38<sup>a</sup> |
| E     | 8  | 0  | 0  | 1  | 2  | 2 | 1 | 20.63<sup>a</sup> |

<sup>a</sup>$P < 0.05$ vs group A; <sup>b</sup>$P < 0.05$ vs group B.

**Effect of blueberry on levels of SOD, MDA and GST in liver homogenates**

The level of SOD in liver homogenates was significantly higher and that of MDA significantly lower in groups C-E compared with those in group B ($P < 0.05$) (Table 2). The level of GST was significantly higher in group B than that in group A ($P < 0.05$). The activity of GST in groups C-E was increased as compared with group B, but the differences were not significant.

| Group | Degree of hepatic fibrosis | Mean rank |
|-------|---------------------------|----------|
| A     | 8  | 0  | 0  | 0  | 0  | 0  | 0  | 4.5 |
| B     | 8  | 0  | 0  | 0  | 0  | 1  | 6  | 34.25<sup>a</sup> |
| C     | 9  | 0  | 0  | 2  | 2  | 2  | 0  | 23.00<sup>a</sup> |
| D     | 8  | 0  | 0  | 1  | 1  | 1  | 1  | 22.38<sup>a</sup> |
| E     | 8  | 0  | 0  | 1  | 2  | 2  | 1  | 20.63<sup>a</sup> |

<sup>a</sup>$P < 0.05$ vs group A; <sup>b</sup>$P < 0.05$ vs group B.

**Effect of blueberry on gene and protein expression of Nrf2 and Nqo1**

Real-time RT-PCR showed that Nrf2 and Nqo1 mRNA expression was significantly higher in group B than group A ($P < 0.05$); and expression of Nrf2 and Nqo1 mRNA in groups C-E was increased as compared with group B, but the differences were not significant. Immunohistochemical and western blotting assays showed similar protein expression of Nrf2 and Nqo1 (Table 4 and Figures 2-4).

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Reactive oxygen species and oxidative stress have an important role in the development of hepatic fibrosis. A central role in the defense against oxidative stress has been attributed to the transcription factor Nrf2, which
Table 4  Expression of Nrf2 and Nqo1 in each group (mean ± SD)

| Group | n  | Relative amount of mRNA | Protein expression (%) | Protein level |
|-------|----|-------------------------|------------------------|--------------|
|       |    | Nrf2                   | Nqo1                   | Nrf2         | Nqo1         |
| A     | 8  | 46.86 ± 6.49            | 13.38 ± 4.37           | 57.50 ± 26.14| 15.25 ± 3.62 |
| B     | 8  | 254.88 ± 55.96          | 68.38 ± 12.61          | 78.75 ± 15.29| 42.50 ± 16.26|
| C     | 9  | 333.44 ± 90.53          | 72.78 ± 15.94          | 85.11 ± 13.65| 54.44 ± 15.09|
| D     | 8  | 277.00 ± 67.38          | 70.63 ± 20.17          | 80.00 ± 21.38| 50.63 ± 18.98|
| E     | 8  | 345.00 ± 72.60          | 77.88 ± 27.30          | 87.25 ± 14.16| 58.13 ± 15.80|

*a*P* < 0.05 vs group A.

Figure 2  Expression of Nrf2 protein in rat livers (immunohistochemical, × 400 magnification, arrows indicate cytolymph/nucleus-positive cells). A: Cytolymph-positive cells in the control group; B-E: More cytolymph/nucleus-positive cells in the model group and each treatment group compared with the control group.

Figure 3  Expression of Nqo1 protein in rat livers (immunohistochemical, × 400 magnification, arrows indicate cytolymph-positive cells). A: Few cytolymph-positive cells in the control group; B-E: More cytolymph-positive cells in the model group and each treatment group compared with the control group.
Blueberries can also increase expression of protective effects against acute hepatic injury in rats. Several studies have reported significant increases in the activity of phase-II detoxifying enzymes such as Nqo1, GST and heme oxygenase-1 (HO-1)\(^{[17,18]}\). Activation of Nrf2 may be a novel strategy to prevent or ameliorate toxin-induced liver injury and fibrosis\(^{[17]}\). Curcumin has been shown to attenuate dimethylnitrosamine-induced liver injury in rats through Nrf2-mediated induction of HO-1\(^{[19]}\). Aleksunes \textit{et al}\(^{[20]}\) have proposed that Nrf2 is crucial for cytoprotection by coordinately activating detoxification genes and preventing the pathogenesis of liver disease.

Blueberry is a small round berry and has high cellular antioxidant activity. Increasing fruit consumption is a logical strategy to increase antioxidant intake and reduce oxidative stress\(^{[21]}\). Studies have suggested that fractions from blueberries may alter phase-II detoxification enzymes in cell culture\(^{[22]}\). Boateng \textit{et al}\(^{[23]}\) and Reen \textit{et al}\(^{[24]}\) have reported significant increases in the activity of phase-II enzymes GST-\(\alpha\) and NADPH quinine reductase in rats after supplementation with freeze-dried berries. Blueberry and probiotics exert protective effects on acute liver injury induced by d-galactosamine and lipopolysaccharide. They reduce the injury to hepatocytes (including inflammation and secretion of pro-inflammatory cytokines), improve barrier functions, and have antioxidant activity\(^{[25]}\). Proanthocyanidin isolated from blueberry leaves may be used against hepatitis C virus by inhibiting viral replication\(^{[26]}\).

Our previous work has shown that blueberries can increase the level of mRNA expression of Nrf2, Nqo1, and HO-1 in rat liver if given orally for 21 d, and have good protective effects against acute hepatic injury in rats\(^{[27,28]}\). Blueberries can also increase expression of Nrf2 and HO-1 in primary hepatic stellate cells (data not shown).

### Conclusion

The present study tested the hypothesis that blueberries may have preventive protective effects on CCI\(_4\)-induced hepatic fibrosis, and that these effects may be related to the activation of phase-II enzymes in rat livers. We found that, in the prevention groups, liver weight and serum levels of HA and ALT were significantly reduced, and liver fibrosis was alleviated. The level of SOD in liver homogenates increased and MDA level decreased. These results indicated that blueberry had certain therapeutic effects on CCI\(_4\)-induced hepatic fibrosis in rats through inhibition of liver inflammation and lipid peroxidation. There was no statistically significant difference in therapeutic effect among the blueberry, DSHX and blueberry +DSHX groups, which indicates that blueberry and DSHX did not act in synergy.

Compared with the normal group, GST activity and expression of Nrf2 and Nqo1 were increased in the model group (\(P < 0.05\)). GST activity and expression of Nrf2 and Nqo1 were also increased in the blueberry, DSHX and blueberry + DSHX groups compared with the model group (particularly in the blueberry and blueberry + DSHX group), but these increases were not significant at the concentrations of blueberry used (\(P > 0.05\)).

In summary, in the present study, blueberries possessed a therapeutic effect on CCI\(_4\)-induced hepatic fibrosis in rats through inhibiting liver inflammation and lipid peroxidation, and may not be related to the induction of phase-II enzymes through the activation of Nrf2 in rat liver during long-term of CCI\(_4\). More detailed study is needed.

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### COMMENTS

**Background**

Conventional drugs used in the treatment of liver diseases inevitably have side effects. An increasing number of natural substances have been studied to explore if they have protective effects on the liver. Blueberries have unique effects on human retinal, brain and tumor cells, but reports about the effects of blueberries on liver diseases are lacking.

**Research frontiers**

Reactive oxygen species and oxidative stress have an important role in the development of hepatic fibrosis. Blueberries have high cellular antioxidant activity. Recent reports have suggested that proanthocyanidin isolated from blueberry leaves can be used against hepatitis C virus by inhibiting viral replication.

**Innovations and breakthroughs**

The present study showed that blueberries have therapeutic effects on CCI\(_4\)-induced hepatic fibrosis in rats, through inhibition of liver inflammation and lipid peroxidation. This protective effect may not be related to the activation of NF-E2-related factor 2 in rat livers.

**Applications**

Increasing consumption of blueberries is a reasonable strategy to increase antioxidant intake, and may lead to a reduced risk of hepatic disease.

**Peer review**

The authors present some data from their research on the effectiveness of blueberries on liver fibrosis induced in laboratory animals. Readers of the journal may find it interesting to read the data.
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