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EXPERIMENTAL STUDY

Protective effect of Yiguanjian decoction against DNA damage on concanavalin A-induced liver injury mice model

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Abstract

OBJECTIVE: To investigate the inhibitory effect of Yiguanjian decoction (YD) on DNA damage in Concanavalin A (Con A)-induced liver injury mice model and to explain the possible mechanism.

METHODS: Totally 120 male BALB/c mice were randomly divided into 6 groups, 20 mice each: normal group, model group, Bifendate group, YD low dose group, YD middle dose group and YD high dose group. Except normal group, liver injury model induced by Con A was established. While modeling, each mouse in YD group was given YD (0.4 mL/20 g per day) by intragastric administration (0.13 g YD for YD low dose group; 0.26 g for YD middle dose group; 0.52 g for YD high dose group). Bifendate group was given Bifendate (0.2 g $\cdot$ kg^{-1} $\cdot$ d^{-1}) by gavage. Normal group and model group were fed with same volume of physiological saline daily. After 8 weeks, the serum alanine transaminase (ALT) and aspartate transaminase (AST) were tested. The hematoxylin-eosin staining was used to evaluate the grade of liver inflammation and liver fibrosis stage. Hepatocellular DNA damage was detected by single cell gel electrophoresis technology. The protein expression of tumor necrosis factor-α (TNF-α), Bax and MutT Homolog 1 (MTH1) was detected by western blotting and enzyme linked immunosorbent assay. Bax mRNA and MTH1 mRNA were detected by Real-time Polymerase Chain Reaction (PCR).

RESULTS: YD can improve the degree of liver inflammation and fibrosis in the liver of chronic hepatitis mice, the dose effect relationship is remarkable ($P < 0.05$). YD can reduce liver cell DNA damage. The difference between YD middle dose group and model group was statistically significant ($P < 0.05$). YD middle dose group had decreased the protein expression of TNF-α in the mice liver of immunological liver injury ($P < 0.05$). YD can increase the protein expression of Bax ($P < 0.05$). Compared with normal group, the protein expression of MTH1 was decreased ($P < 0.05$), but there was no statistical significance between YD group and model group ($P > 0.05$). YD can increase the mRNA expression of Bax and MTH1 (both $P < 0.05$).

CONCLUSION: YD can effectively inhibit the DNA damage in immunological liver injury mice, the mechanism may be that it can decrease the TNF-α and increase the Bax and MTH1 expression.

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Key words: Drug-induced liver injury; Liver kidney *Yin* deficiency; DNA damage; Concanavalin A; Yiguanjian decoction

INTRODUCTION

China is a high incidence area of hepatitis B virus (HBV) infection, liver cirrhosis and hepatocellular cancer (HCC) are the leading causes of death in the crowd. About 110 000 people died from HCC each year in China, which accounted for 45% of deaths worldwide. And about 90 percent of liver cancer patients were infected with HBV.[1] Therefore, the key measure to reduce the incidence of HCC is active treatment of chronic hepatitis B (CHB), which can prevent CHB developing into cirrhosis or HCC.

The mechanism of CHB leading into HCC has not been fully elucidated, but it is closely associated with the accumulation of DNA damage. HBV can not only cause liver cell DNA damage, but also can directly or indirectly affect the repair system, then block DNA repair and eventually lead to liver cancer.[2-4] How to inhibit DNA damage and promote the repair of it is the main measure, but there is no such drugs in clinical,[5-7] we need further study.

One study found that the active ingredients of Traditional Chinese Medicine (TCM) Tanshinone has some antagonistic action on the DNA damage in peripheral blood lymphocytes of CHB patients induced by H_2O_2.[8] Another study found that grape seed proanthocyanidins (GSP) can antagonize $CdCl_2$-induced hepatic oxidative damage and may inhibit DNA damage or promote DNA repair in rats.[9] Since the pathology of chronic hepatitis B is mainly immunological liver injury, to study TCM on whether it can inhibit or repair the DNA damage in liver cell immune injury is necessary for founding effective medicine to treat HBV patients.

TCM treatment of CHB has achieved remarkable results.[6] Epidemiological findings show that *Yin* deficiency syndrome of liver and kidney is a common but important syndrome of CHB.[10,11] Yiguanjian decoction (YD) is a famous prescription which has the function of nourishing *Yin* and dispersing stagnated liver *Qi*. Clinical practice has proved YD does have good efficacy on treatment of *Yin* deficiency syndrome of liver and kidney of CHB.[12] Experimental studies have found the anti-inflammatory effect of YD is related to its inhibition of hepatic apoptosis.[13] DNA damage is the basis for conversion of hepatitis to HCC but there is no research to indicate whether YD can inhibit and repair DNA damage of liver cells. We choose immunological liver injury in mice as the experimental animal model, to observe the inhibitory effects of YD on DNA damage of Con A-induced immune liver injury, and to explore its molecular mechanism.

MATERIALS AND METHODS

Animals

Totally 120 healthy male BALB/c mice of specific pathogen free (SPF) grade, six-month-old, weighing (20 ± 2) g, were acquired from Vital River Laboratories Animal Technology Co., Ltd. (Certificate of quality No. SCXK [Beijing] 2012-0001, Beijing, China). Mice were housed in the SPF level animal laboratory in Department of Laboratory Animal Science of Capital Medical University, maintained at constant temperature (22-24) ℃ and humidity (30%-45%) under a 12 h light/dark cycle, and all mice had free access to food and water. All experiments were approved by the Experimental Animal Ethics Committee of Capital Medical University.

Drugs

YD consists of Beishashen (*Radix Glehniae*) 9 g, Maidong (*Radix Ophiopogonis Japonici*) 9 g, Danggui (*Radix Angelicae Sinensis*) 9 g, Dihuang (*Radix Rehmanniae*) 20 g, Gouqizi (*Fructus Lycii*) 12 g, Chuanlianzi (*Fructus Toosendan*) 4.5 g. All components were purchased from the Beijing Tongrentang Drugstore(Beijing, China). The aqueous extract of YD was prepared according to the following procedures: These herbs were soaked in 6 times (v/w) distilled water for an hour and then heated to boiling and decocted for 30 min. The filtrate was then collected. The residue was decocted for 20 min with 4 times (v/w) distilled water, and then the filtrate was collected and mixed with the previously collected filtrate. YD was made at a high dose (1.3 g/mL) and a middle dose (0.65 g/mL) and a low dose (0.33 g/mL) and stored at 4 ℃ until use. Bifendate Pills made by Beijing Union Pharmaceutical Factory (Beijing, China), were powdered and dissolved in deionized water at a concentration of 10 mg/mL. Con A made by Sigma-Aldrich Co. LLC. (St. Louis, MO, USA) was dissolved in deionized water at a concentration of 2 mg/mL.

Reagents

RNAprep pure Tissue Kit and Fast Quant RT kit (With gDNase) were purchased from TIANGEN Biotech (Beijing) Co., Ltd. (Beijing, China). SYBR Premix Ex Taq (Tli RNaseH Plus) was purchased from Takara Biotechnology (Dalian) Co., Ltd. (Dalian, China). Mouse tumor necrosis factor-α (TNF-α) enzyme linked immunosorbent assay (ELISA) Kit was purchased from eBioscience, Inc. (San Diego, CA, USA). Anti-Bax and Anti-MutT Homolog 1 (MTH1) antibody were purchased from Abcam Trading Company Ltd. (Cambridge, MA, USA). Anti-TNF-α and anti-β-actin antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Dylight 680 AffiniPure Goat anti-rabbit IgG and Dylight 800 AffiniPure Goat anti-Mouse IgG were purchased from EarthOx Life Sciences (Millbrae, CA, USA). RIPA Lysis Buffer and protease inhibitors and bicinchoninic acid (BCA) protein concentration assay kit were purchased from Beijing DINOAO Biological Technology Co., Ltd. (Beijing, China).

Instruments

Instruments used included: Model 680 Microplate Reader and PowerPac™ Universal power supply and Mini-PROTEAN Tetra cell and Mini Trans-Blot cell (BIO-RAD Laboratories Ltd., Hercules, CA, USA), high speed desktop refrigerated centrifuge (Heraeus Ltd., Hanau, Germany), Applied Biosystems 7300 Real-Time PCR machine (Applied Biosystems, Inc., Carlsbad, CA, USA), and LI-COR Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE, USA).

Grouping and treatment

The animals were conveniently fed for one week and were randomly divided into six groups (n = 20 per group) by the random number table method: normal group (A), model group (B), Bifendate group (C), YD low dose group (D), YD middle dose group (E) and YD high dose group (F).

Mice in the normal group were intraperitoneally injected with physiological saline (0.1 mL/10 g body weight) once a week for eight weeks, while other groups were intraperitoneally injected with Con A (0.1 mL/10 g body weight). While modeling, each mouse in YD group was given YD (0.4 mL/20 g body weight/day) by intragastric administration (0.13 g YD for YD low dose group; 0.26 g for YD middle dose group; 0.52 g for YD high dose group). Bifendate group was given Bifendate (0.2 $g \cdot kg^{-1} \cdot d^{-1}$) by intragastric administration. The normal group and model group were fed with same volume of physiological saline daily. The administration dose of the mouse was calculated according to the equivalent body surface area method and was adjusted according to the change of weight.

Collection of samples

All mice were anaesthetized by intraperitoneal injection of 5% chloral hydrate (5 mL/kg) on the eighth weekend. Blood samples were drawn from the eye ground venous plexus, centrifuged at 3000 rpm for 15 min at 4 ℃ after an hour to separate the serum, and then the serum was kept at − 80 ℃ for liver function tests. A small piece of liver tissue was fixed in 10% formaldehyde and embedded in paraffin. The remaining liver tissue was immediately placed in liquid nitrogen and stored at − 80 ℃ before use.

Liver function tests

SXOUT Ⅱ semi-automatic biochemical analyzer was used to determine the content of serum alanine transaminase (ALT) and aspartate transaminase (AST).

Histopathological examination

Paraffin-embedded tissue sections were stained with hematoxylin-eosin (HE), and the grade of liver inflammation (G) and liver fibrosis stage (S) in mice were judged by the same pathologist according to the National criteria 2000 revision (Table 1).[14]

Single cell gel electrophoresis assay (SCGE) (Comet Assay) for detecting hepatocellular DNA damage

The SCGE was carried out by the method described by Speit and Hartmann.[15] 20 μL cell suspension was mixed with 70 μL 0.7% low melting point agarose and then placed on frosted slides that were pregelatinized with 1% normal melting point agarose. The slides were covered with cover glass and kept refrigerated at 4 ℃ to solidify the gel. The slides were then immersed in chilled lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl pH 10, 1% Triton X-100 and 10% DMSO) in the dark at 4 ℃ for 1.5 h. And then the slides were placed in an electrophoresis chamber, covered with electrophoresis buffer at 4 ℃ for 20 min without light. The electrophoresis ran at 300 mA and 25V for the next 20 min. The slides were then neutralized with 0.4 M Tris-HCl buffer, pH 7.5 for three times (5 min each time). After being stained with 50 μL of ethidium bromide (2 μg/mL), the sides were examined with a fluorescence microscope (excitation filter of 515-560 nm and barrier filter of 590 nm) at 400× magnification. Image analysis was performed using the Comet Assay Software Project on 100 randomly selected and non-overlapping cells. Olive tail moment [OTM, (tail mean − head mean]× [tail DNA content/ total DNA content × 100%)] was used to evaluate DNA damage of the cells.

Western blotting for detecting the protein expression of TNF-α, Bax and MTH1

Liver tissue (50-100 mg) was mixed with 0.5 mL RIPA lysis buffer and 5 μL protease inhibitors and then the total protein was extracted after homogenized and centrifuged. Protein concentrations were determined by a

Table 1 Scoring criteria of liver inflammation grade and liver fibrosis stage

Score	Liver inflammation grade		Liver fibrosis stage
	Inflammation of the portal area	Inflammation in hepatic lobule	Degree of hepatic fibrosis
0	Non-existent	Non-existent	Non-existent
1	A few inflammatory cells infiltration	Degeneration and few spotty necrosis	Periportal fibrosis and localized perisinusoidal fibrosis
2	Massive inflammatory cells infiltration	Degeneration and spotty necrosis	Fibrosis around portal area and fibrous septums
3	Moderate amounts of piecemeal necrosis	Large necrosis area	Fibrous septums and lobule structural disorder
4	Severe piecemeal necrosis	Large patchy necrosis	Early cirrhosis

BCA protein concentration assay kit. Equal amounts (50 μg) of protein in each sample were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto a NC membrane. After being blocked with 5% skim milk in Tris buffered saline tween for 1 h, the membrane was incubated overnight with the primary antibody at 4 ℃, then incubated with the secondary antibody for 1 h at room temperature. The protein bands were scanned on an Odyssey infrared fluorescent scanner (LI-COR) and analyzed with Odyssey software version 2.0. The results were compared with the densitometric signal of β-actin and the ratios were expressed as the relative protein contents.

Real-time PCR for detecting the mRNA expression of Bax and MTH1

Total RNA was extracted from liver tissue specimens by using the RNAprep pure Tissue Kit and reverse transcribed into cDNA by using the FastQuant RT kit (With gDNase) according to the manufacturer's guidelines. The expression of β-actin was used as an internal control.

The primers used for RT-PCR are as follows:
Bax forward primer: 5'-GAACAGATCATGAAGACAGGG-3'
Bax reverse primer: 5'-CAGTTCATCTCCAATTCGCC-3'
MTH1 forward primer: 5'-CCTGTTTTAGAAGGTTGTGGCA-3'
MTH1 reverse primer: 5'-GTGTGAGCAGACAGTAAGCGGT-3'
β-actin forward primer: 5'-GGCTGTATTCCCCTCCATCG-3'
β-actin reverse primer: 5'-CCAGTTGGTAACAATGCCATGT-3'

PCR reaction conditions were as follows: initial denaturation was performed at 95 ℃ for 30 s, followed by 40 cycles of denaturing at 95 ℃ for 5 s and extension at 60 ℃ for 30 s. To verify the specificity of the amplification reaction, a melting curve analysis was performed. The relative transcript amount of the target gene was normalized to that of β-actin by using the $2^{-\Delta\Delta Ct}$ method.

ELISA for detecting the protein expression of TNF-α

The frozen liver tissues were homogenized in cold PBS. The homogenate was centrifuged at 1000 rpm for 5 min and then the supernatants were collected. The amount of TNF-α in liver tissue was quantified according to the manufacturer's instructions.

Statistical analysis

Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Differences among groups were compared by analysis of variance, comparison between samples was compared by multiple comparisons: least significant difference method. The χ^2 test was used for enumerated data. P value < 0.05 was considered statistically significant.

RESULTS

Liver function tests

Compared with normal group, the content of serum ALT and AST in model group increased significantly (both $P < 0.05$), indicating that the animal model was established successfully. The results showed that the ALT and AST levels were significantly decreased in the Bifendate group, YD low dose group, YD middle dose group and YD high dose group compared with model group (all $P < 0.05$). The results suggest that YD can improve the liver function and middle-dose YD has a better effect in protecting liver (Table 2).

Table 2 Comparison of liver function between groups ($\bar{x} \pm s$)

Group	*n*	ALT (IU/L)	AST (IU/L)
Normal	20	40.3±4.0	110.6±8.7
Model	20	144.8±68.4[a]	193.7±46.2[a]
Bifendate	20	38.0±9.1[b]	98.1±9.0[b]
Low-dose YD	20	51.5±17.3	103.9±13.8[b]
Middle-dose YD	20	32.0±1.5[b]	97.4±7.9[b]
High-dose YD	20	43.9±7.2[b]	131.2±17.3

Notes: the normal group and model group were given physiological saline (0.4 mL/20 g body weight) daily for eight weeks. Bifendate group was administered Bifendate (0.2 g/kg) by gavage. The YD groups were given the corresponding concentration of YD (0.4 mL/20 g body weight) by gavage. [a]$P < 0.05$, compared with normal group; [b]$P < 0.05$, compared with model group. ALT: alanine transaminase; AST: aspartate transaminase; YD: Yiguanjian decoction.

Changes in histopathology

The study found that in the normal group, hepatic lobule had a normal structure; cell cords were arranged radically; hepatic sinusoid and central vein were clear. The structure of liver cells in the model group could be recognized, but great necrosis foci was also observed, accompanied by a large amount of inflammatory cell infiltration and fibrous tissue proliferation. The degree of liver inflammation and fibrosis between normal group and model group existed significant differences (both $P < 0.05$). The pathohistological examination showed that liver tissues in Bifendate group, YD low, middle and high dose group were improved compared with the model group: inflammatory cell infiltration was reduced significantly; the structure of hepatic lobule was normal and cell cords were arranged radically. Inflammatory activity was decreased significantly in Bifendate group, YD low dose, middle dose and high dose group compared with the model group (all $P < 0.05$). The degree of liver fibrosis decreased significantly in Bifendate group, YD low dose, middle dose and high dose group compared with the model group (all $P < 0.05$). The results indicated that the immune liver injury mice model was established successfully. YD could decrease the degree of liver fibrosis and inflammation in mice with immune liver injury (Tables 3, 4; Figure 1).

Table 3 Comparison of liver inflammation grade between groups

Group	*n*	0	1	2	3	4	χ^2 value	*P* value
Normal	10	10	0	0	0	0	-	-
Model	10	0	0	3	4	3	20.000	0.000[a]
Bifendate	10	0	2	8	0	0	11.273	0.01[b]
Low-dose YD	10	0	0	6	4	0	10.400	0.006[b]
Middle-dose YD	10	0	8	2	0	0	15.200	0.002[b]
High-dose YD	10	0	8	0	2	0	14.667	0.002[b]

Notes: the normal group and model group were given physiological saline (0.4 mL/20 g body weight) daily for eight weeks. Bifendate group was administered Bifendate (0.2 g/kg) by gavage. The YD groups were given the corresponding concentration of YD (0.4 mL/20 g body weight) by gavage. [a]$P < 0.05$, compared with normal group; [b]$P < 0.05$, compared with model group. YD: Yiguanjian decoction.

Table 4 Comparison of liver fibrosis stage between groups

Group	*n*	0	1	2	3	4	χ^2 value	*P* value
Normal	10	10	0	0	0	0	-	-
Model	10	0	0	6	4	0	20.000	0.000[a]
Bifendate	10	0	2	8	0	0	6.286	0.043[b]
Low-dose YD	10	0	0	8	2	0	0.952	0.329[b]
Middle-dose YD	10	0	8	2	0	0	14.000	0.001[b]
High-dose YD	10	0	8	2	0	0	14.000	0.001[b]

Notes: the normal group and model group were given physiological saline (0.4 mL/20 g body weight) daily for eight weeks. Bifendate group was administered Bifendate (0.2 g/kg) by gavage. The YD groups were given the corresponding concentration of YD (0.4 mL/20 g body weight) by gavage. [a]$P < 0.05$, compared with normal group; [b]$P < 0.05$, compared with model group. YD: Yiguanjian decoction.

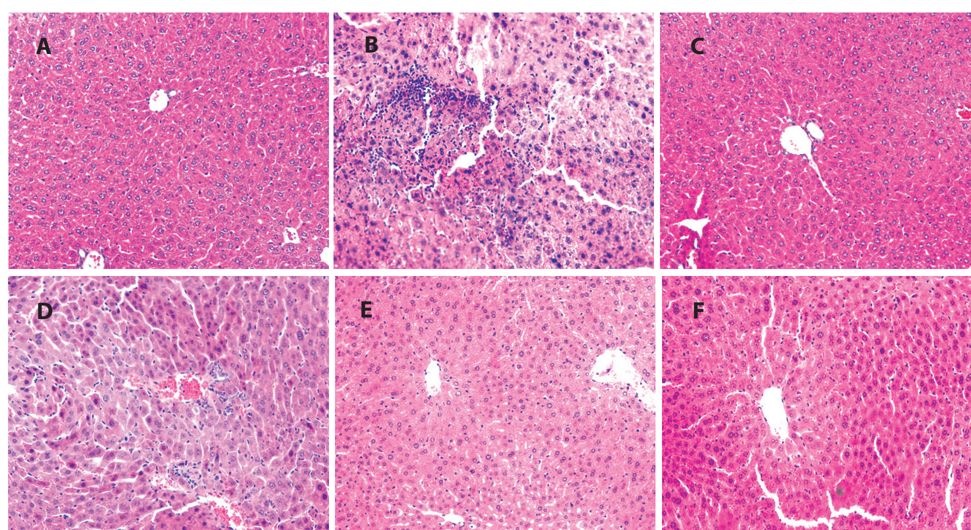

Figure 1 Pathological changes in the groups (HE staining, × 200)
A: normal group; B: model group; C: bifendate group; D: YD low dose group; E: YD middle dose group; F: YD high dose group. The normal group and model group were given physiological saline (0.4 mL/20 g body weight) daily for eight weeks. Bifendate group was administered Bifendate (0.2 g/kg) by gavage. The YD groups were given the corresponding concentration of YD (0.4 mL/20 g body weight) by gavage. YD: Yiguanjian decoction; HE: hematoxylin-eosin.

Concentration-response relationship analysis

With the increase of drug concentration, the degree of inflammation of the liver tissue was on the decline, the rank correlation coefficient is − 0.708, *P* value is 0.000, there was statistical difference between groups.

With the increase of drug concentration, the degree of liver fibrosis was on the decline, the rank correlation coefficient is − 0.648, *P* value is 0.000, there was statistical difference between groups. The study suggests that YD can improve the degree of liver inflammation and fibrosis in the liver of chronic hepatitis mice, the dose effect relationship is remarkable.

Hepatocellular DNA damage detected by SCGE assay

Compared with the normal group and YD middle dose group, the OTM in model group increased significantly ($P < 0.05$) (Figure 2, Table 5).

Protein expressions of TNF-α, Bax and MTH1

Western blotting showed that the expression of TNF-α was markedly reduced by YD and Bifendate treatments

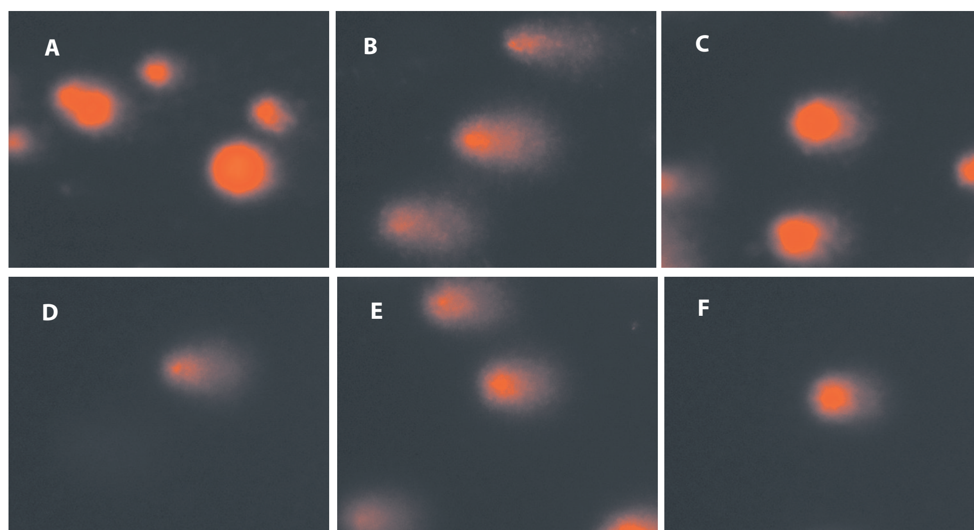

Figure 2 Liver cells DNA comet image in the groups (Ethidium bromide staining, × 400)
A: normal group; B: model group; C: bifendate group; D: YD low dose group; E: YD middle dose group; F: YD high dose group. The normal group and model group were given physiological saline (0.4 mL/20 g body weight) daily for eight weeks. Bifendate group was administered Bifendate (0.2 g/kg) by gavage. The YD groups were given the corresponding concentration of YD (0.4 mL/20 g body weight) by gavage. YD: Yiguanjian decoction.

Table 5 Comparison of liver cells OTM among groups ($\bar{x} \pm s$)

Group	n	OTM
Normal	10	7.0±1.3
Model	10	57.6±7.4[a]
Bifendate	10	19.0±4.0[b]
Low-dose YD	10	39.2±3.5
Middle-dose YD	10	30.6±5.9[b]
High-dose YD	10	26.7±3.5[b]

Notes: the normal group and model group were given normal saline daily for eight weeks. Bifendate group was administered Bifendate by gavage. The YD groups were given the corresponding concentration of YD by gavage. [a]$P < 0.05$, compared with normal group; [b]$P < 0.05$, compared with model group. OTM: olive tail moment; YD: Yiguanjian decoction.

($P < 0.05$). Expressions of Bax and MTH1 in model group were apparently decreased compared with normal group (both $P < 0.05$). The protein level of Bax in YD middle dose group increased significantly versus the model group ($P < 0.05$). Expression of MTH1 in YD middle dose group was increased against the model group but there was no significant difference ($P > 0.05$) (Figure 3, Table 6).

Comparison of mRNA for Bax and MTH1 by RT-PCR

The mRNA expressions of Bax and MTH1 in model group were significantly reduced compared with normal group (both $P < 0.05$). The mRNA expressions of Bax and MTH1 in YD middle dose group had a significant increase compared with model group (both $P < 0.05$) (Table 7).

Comparison of TNF-α by ELISA

The protein expressions of TNF-α in liver tissue existed significant difference between normal group and model group ($P < 0.05$). Compared with model group,

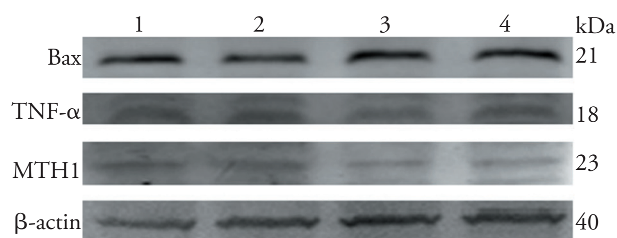

Figure 3 Protein expression of Bax, TNF-α, MTH1 and β-actin in groups
1: Normal group; 2: Model group; 3: Bifendate group; 4: YD middle dose group. The normal group and model group were given normal saline daily for eight weeks. Bifendate group was administered Bifendate by gavage. The YD groups were given the corresponding concentration of YD by gavage. TNF-α: tumor necrosis factor-α; MTH1: MutT Homolog 1; YD: Yiguanjian decoction.

Table 6 Comparison of protein expressions among groups ($\bar{x} \pm s$)

Group	Bax	TNF-α	MTH1
Normal	1.00±0.14	0.37±0.08	1.00±0.17
Model	0.73±0.12[a]	1.05±0.26[a]	0.90±0.52[a]
Bifendate	0.84±0.11[b]	0.38±0.15[b]	0.95±0.25
Middle-dose YD	1.22±0.05[b]	0.53±0.06[b]	1.02±0.28

Notes: the normal group and model group were given normal saline daily for eight weeks. Bifendate group was administered Bifendate by gavage. The YD groups were given the corresponding concentration of YD by gavage. [a]$P < 0.05$, compared with normal group; [b]$P < 0.05$, compared with model group. TNF-α: tumor necrosis factor-α; MTH1: MutT Homolog 1; YD: Yiguanjian decoction.

TNF-α in YD middle dose group decreased significantly ($P < 0.05$) (Table 8).

DISCUSSION

Con A-induced immunological liver injury mice mod-

Table 7 Comparison of mRNA expressions among groups ($\bar{x} \pm s$)

Group	*n*	Bax	MTH1
Normal	6	1.40±0.53	0.66±0.22
Model	6	0.89±0.31[a]	0.54±0.34[a]
Bifendate	6	1.36±0.33[b]	0.72±0.44[b]
Middle-dose YD	6	1.16±0.56[b]	0.71±0.68[b]

Notes: the normal group and model group were given normal saline. Bifendate group was given Bifendate. The YD groups were given the corresponding concentration of YD. [a]P < 0.05, compared with normal group; [b]P < 0.05, compared with model group. MTH1: MutT Homolog 1; YD: Yiguanjian decoction.

Table 8 Comparison of TNF-α expression among groups ($\bar{x} \pm s$)

Group	*n*	TNF-α (pg/mL)
Normal	6	375±50
Model	6	564±25[a]
Bifendate	6	468±62[b]
Middle-dose YD	6	472±87[b]

Notes: normal group and model group were given normal saline. Bifendate group was given Bifendate. The YD groups were given the corresponding concentration of YD. [a]P < 0.05, compared with normal group; [b]P < 0.05, compared with model group. TNF-α: tumor necrosis factor-α; YD: Yiguanjian decoction.

el was prepared in this study, and the results of serum ALT and AST and liver pathology suggested that the model was established successfully. Experiment results of SCGE showed that there was evident DNA damage on the liver cells of model mice. The results suggest that YD, a Chinese medicine for nourishing liver and kidney, can improve the liver function and the degree of liver fibrosis and inflammation in mice with immune liver injury. Within a certain range, the effect of the improvement of liver tissue inflammation and fibrosis degree will be enhanced with increasing dose. The study suggests that the dose effect relationship is remarkable.

The research suggested that YD can reduce the liver cell DNA damage. The possible mechanisms may be that YD can decrease the protein expression of TNF-α and increase the mRNA expression of Bax and MTH1.

Con A-induced liver injury mice model, one of T-cells mediated model, is a good simulation of the pathological process of human viral hepatitis. Research suggested the presence of DNA damage in Con A-induced acute liver injury mice,[16] and our test results confirmed that Con A-induced chronic liver injury also existed DNA damage. The reason may be related to increased secretion of inflammatory cytokines such as TNF-α. TNF-α caused DNA damage by the formation of 8-hydroxy-2 deoxyguanosine which was believed to be one of the most abundant DNA lesions resulting from oxidative stress.[17] The study found that YD can significantly inhibit the secretion of TNF-α then reduce the inflammatory environment of liver cells, thereby inhibiting the hepatocyte DNA damage.

Acute inflammation can remove damaged tissue and restore the balance between organizational structures, but chronic inflammation may cause malignant transformation of cells. Many inflammatory mediators such as TNF-α, interleukin (IL)-6 and IL-10 are confirmed to participate in the initiation and progression of cancer, especially TNF-α plays an important role in the early stage of tumor. High levels of TNF-α have efficacy in the treatment of cancer, but sustained low levels of TNF-α leads to the development of cancer by producing reactive oxygen species (ROS) and reactive nitrogen species (RNS). OH· and O^{2-} in ROS can cause DNA strand breaks then cause base deletion, oncogene activation and inactivation of tumor suppressor genes and ultimately may lead to tumorigenesis.[18] Activation of oncogenes and inactivation of tumor suppressor genes are most frequently gene changes in the process of tumor, abnormal expression of these genes affect the biological behavior and clinical manifestations of tumor.[19] The biological function of oncogene Bcl-2 is to increase the cells resistance to various apoptotic stimuli. Bcl-2 helps DNA damaged cells to survive and enables aggregation of mutant product. Studies have found that transgenic mice with overexpression of Bcl-2 prone to cancer.[20] Tumor suppressor gene Bax can inhibit Bcl-2 and promote cell apoptosis by forming heterodimers with Bcl-2.[21] Our research has found that YD may increase the mRNA and protein expression of Bax, thereby promote cell apoptosis so to prevent tumor cells.

As the main genetic material of our body, the importance of DNA stability is self-evident. Oxidative damage occurs when free radicals attack DNA, the body can repair the damaged DNA through self repair mechanisms, so as to maintain the stability of DNA. There are many ways to repair DNA oxidative damage in the body like base-excision repair (BER), nucleotide excision repair, mismatch repair. BER is the major repair mechanism that removes oxidized and alkylated. There are about 100 species of enzymes participate in DNA oxidative damage repair, 8-oxoguanine DNA glycosylase (OGG1) and MutT homolog 1 have been studied the most.[22] MTH1 protease can hydrolyze 8-oxoG in the nucleotide pool, reduce the free 8-oxoG binding to the DNA chain and reduce mutation caused by base A and C counterpoint during DNA replication. The abnormal expression or defect of MTH1 gene is associated with cancers like colon cancer.[23] The study found that the mRNA expression of MTH1 in model group decreased and increased in YD groups, indicated that YD can promote the expression of MTH1. Whether YD play a role in intervening OGG1 expression needs further research.

Although the exact mechanism of HCC is unclear, the

risk increased with the degree of hepatitis.[24] Tissue microenvironment plays an important role in the process of hepatitis transforming into HCC. The inflammatory microenvironment of chronic hepatitis can not only increase the hepatocellular gene mutation, but also promote proliferation of genetic mutation cells.[25] How to restrain the DNA damage caused by inflammation of liver cells, becomes the key to the prevention of hepatitis to liver cancer. In this study, SCGE assay was used to detect the DNA damage in immune injury liver cells, so as to observe the effect of YD inhibiting DNA damage. The results suggested that YD can effectively inhibit the DNA damage in immunological liver injury mice. It has instructive significance for the clinical application of the drug to prevent hepatitis transforming into HCC.

REFERENCES

1 **Ren YQ**, Li X, Zhao Y. Hepatitis B vaccine and hepatitis B and liver cancer. Qi Lu Yi Xue Jian Yan 2005; 16(3): 29-30.

2 **Zhu DQ**, Huang ZY. DNA damage and liver cancer. Shi Jie Hua Ren Xiao Hua Za Zhi 2007; 15(16): 1775-1780.

3 **Yang SF**, Chang CW, Wei RJ, Shiue YL, Wang SN, Yeh YT. Involvement of DNA damage response pathways in hepatocellular carcinoma. Biomed Res Int 2014; 2014: 153867.

4 **Buitrago-Molina LE**, Marhenke S, Longerich T, et al. The degree of liver injury determines the role of p21 in liver regeneration and hepatocarcinogenesis in mice. Hepatology 2013; 58(3): 1143-1152.

5 **Zhou XB**. Advances in chronic hepatitis B patients antiviral therapy compliance. Zhong Guo Yi Yao Zhi Nan 2012; 10(10): 453-455.

6 **Wang BH**. Progress in research of hepatitis B treatment. Re Dai Yi Xue Za Zhi 2013; 13(8): 1054-1056.

7 **Deng X**, Liang J, Wu FS, Li YB, Tang YF. Effects of the Ganning formula on liver fibrosis in patients with chronic hepatitis B. J Tradit Chin Med 2011; 31(4): 282-287.

8 **Liang XJ**, Tang YH, He W, Gong XB, Huang YX. Analysis of Tanshinone on the effect of DNA damage in peripheral blood lymphocytes of chronic hepatitis B patients induced by H2O2. Zhong Xi Yi Jie He Gan Bing Za Zhi 2005; 15(2): 74-76.

9 **Zou ZH**, Yang CC, Li BL. Study on protective effect of grape seed proanthocyanidins on liver damage induced by cadmium. Zhi Ye Yu Jian Kang 2013; 16(12): 1418-1420.

10 **Liu WL**, Li XH, Yu M, Hu JH, Duan YP, Zhang Y. Study on interrogated symptom of chronic hepatitis B. Zhong Hua Shi Yong Zhong Xi Yi Za Zhi 2004; 4(17): 3228-3230.

11 **Ye YA**, Jiang F, Zhao ZM, et al. Chinese medical pattern distribution of chronic type hepatitis B. Zhong Yi Za Zhi 2007; 48(3): 256-258.

12 **Li XH**. Clinical observation of modified Yiguanjian on the treatment of liver and kidney Yin deficiency, blood stasis type of chronic hepatitis B. Beijing Zhong Yi 1996; 15 (4): 22-24.

13 **Liu WL**, You HJ, Gao LY, et al. Study on effect of Yiguanjian on TNF-α signal transduction in hepatitis mouse. Zhong Hua Zhong Yi Yao Za Zhi 2010; 25(4): 597-599.

14 The Chinese medical association of infectious diseases and parasites epidemiology branch, liver disease branch jointly revised. Viral hepatitis prevention plan. Zhong Hua Chuan Ran Bing Za Zhi 2001; 19(1): 56-62.

15 **Speit G**, Hartmann A. The comet assay (single cell gel test), a sensitive genotoxicity test for the detection of DNA damage and repair. Methods Mol Biol 1999; 113: 203-212.

16 **Zhao DM**, Liu GT. Protective effect of bicyclol on concanavalin A-induced liver nuclear DNA injury in mice. Zhong Hua Yi Xue Za Zhi 2001; 81(14): 844-848.

17 **Wheelhouse NM**, Chan YS, Gillies SE, et al. TNF-alpha induced DNA damage in primary murine hepatocytes. Int J Mol Med 2003; 12(6): 889-894.

18 **Kim JI**, Park YJ, Kim KH, et al. hOGG1 Ser326Cys polymorphism modifies the significance of the environmental risk factor for colon cancer. World J Gastroenterol 2003; 9 (5): 956-960.

19 **Lan B**, Liu BY, Chen XH, et al. Inhibition of polo like kinase gene expression induces apoptosis in gastric cancer cells. Zhong Hua Wei Chang Wai Ke Za Zhi 2006; 9(1): 62-65.

20 **Gobé G**, Rubin M, Williams G, Sawczuk I, Buttyan R. Apoptosis and expression of Bcl-2, Bcl-XL, and Bax in renal cell carcinomas. Cancer Invest 2002; 20(3): 324-332.

21 **Köhler T**, Schill C, Deininger MW, et al. High Bad and Bax mRNA expression correlate with negative outcome in acute myeloid leukemia (AML). Leukemia 2002; 16(1): 22-29.

22 **Nie JH**, Chen ZH, Liu X, et al. Oxidative damage in various tissues of rats exposed to radon. J Toxicol Environ Health A 2012; 75(12): 694-699.

23 **Koketsu S**, Watanabe T, Nagawa H. Expression of DNA repair protein: MYH, NTH1, and MTH1 in colorectal cancer. Hepatogastroenterology 2004; 51(57): 638-642.

24 **Nikolaou K**, Sarris M, Talianidis I. Molecular pathways: the complex roles of inflammation pathways in the development and treatment of liver cancer. Clin Cancer Res 2013; 19(11): 2810-2816.

25 **Grivennikov SI**, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell 2010; 140(6): 883-899.