Gandou decoction, a Chinese medicinal formula, in the treatment of hepatic injury by Wnt/β-catenin pathway regulation in models of Wilson disease

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Background: Gandou decoction (GDD) has been widely used in the treatment of Wilson disease (WD) for decades. It is optimized from the Dahuanghuanglianxiexin decoction, Yinchenhao decoction, and Huanglianjiedu decoction. It was first reported in the *Treatise on Febrile and Miscellaneous Diseases* and *A Handbook of Prescriptions for Emergencies* published in the Eastern Han Dynasty and the Eastern Jin Dynasty respectively. Hepatic injury is one of the most severe complications of WD. The current study aimed to explore the hepatic-protection effects of GDD and its exact therapeutic target, with a particular focus on the expression of oxidative stress and the Wnt/β-catenin pathway.

Methods: Hepatic injury was induced in a copper-loaded rat model using the intragastric administration of copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$). The water extract of GDD (0.4 g/kg/d) was administered twice a day for 4 weeks. Copper content and alanine aminotransferase (ALT) level, structural observation under the microscope, and immunohistochemical analysis of liver tissue were performed after the final administration. Moreover, the expression of β-catenin, GSK3β, Dishevelled-3, c-Myc, and p-GSK3β of liver tissue were detected to explore the relationship between the hepatic protection of GDD and the Wnt/β-catenin signal pathway of GDD. We also stimulated the rat hepatic cell line BRL-3A with $CuSO_4 \cdot 5H_2O$ to establish a hepatic injury cytomodel. GDD serum at a concentration of 20% was administered into the model cell for 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and flow cytometry were performed to detect cell viability, mitochondrial membrane potential (MMP), and the expression of reactive oxygen species (ROS). Meanwhile, the expression of the Wnt/β-catenin signal pathway-related proteins was evaluated.

Results: GDD reduced copper and ALT while inhibiting oxidative stress and the degeneration and necrosis of liver tissue and hepatocytes. Treatment with GDD improved cell viability and MMP while suppressing the ROS level. Furthermore, GDD rectified the expression of Wnt/β-catenin signal pathway-related proteins in both livers of the copper-loaded and copper-stimulated BRL-3A cell lines.

Conclusions: GDD had apparent therapeutic effects on the hepatic injury of copper-loaded rats and copper-stimulated BRL-3A cells. Its mechanism is related to its regulatory effect on the Wnt/β-catenin pathway rectification and oxidative stress antagonism.

Keywords: Wilson disease (WD); liver injury; Gandou decoction (GDD); Wnt/β-catenin pathway; oxidative stress

Introduction

Wilson disease (WD), also known as hepatolenticular degeneration (HLD), is an autosomal recessive inherited copper metabolism disorder, which is caused by mutations of the copper-transporting P-type ATPase (*ATP7B*) gene located on chromosome 13q14.3. It is characterized by excess copper accumulation in tissues and organs like the liver, brain, and cornea. It leads to hepatic damage, neuropsychiatric syndromes, corneal K-F ring, and other symptoms and signs. WD commonly occurs during adolescence, and its prevalence is estimated to be 1 case per 30,000 in populations of European descendants (1), and 5.87 cases per 100,000 in East Asian populations (2). Hepatic injury is one of the most common symptoms of WD patients. *ATP7B*, the pathogenic gene, is mainly expressed in the liver, and this is likely the reason why the liver is the premier and the uppermost target injury organ of WD (3). The hepatic injury occurs across the entire disease course beginning from birth, and, as the leading WD-related cause of death, has become a focal issue in the prevention and treatment of WD. Therefore, the remedy for hepatic injury is one of the most crucial aspects of WD treatment.

The metal-chelating agent, D-penicillamine (PCA), has been the preferred medication for WD therapy for decades, but its adverse reactions are too severe to be tolerated for patients. They can cause irreversible deterioration of neurology, drug allergy, and hematologic complications (4). Consequently, the development of drugs with less adverse reactions and diversified effect targets has become a critical breakthrough point for treating WD, especially with liver injury.

Traditional Chinese medicine has been widely used to treat WD, and its high therapeutic effects and low adverse reaction rate have been demonstrated (5). Gandou decoction (GDD) is a Chinese herb formula directed by "heat elimination, detoxification, diuresis, and viscus regulation", and has proven to be effective in treating WD patients, with its effective rate being 84.1% (6). Additionally, studies that showed a regulative effect on programmed cell death (PCD) after GDD administration demonstrated that GDD protected hepatocytes via varieties of pathways, including Wnt/β-catenin, extracellular signal-regulated kinase (ERK). and mitogen-activated protein kinase (MAPK). However, the exact mechanism of GDD is still unclear.

Wnt/β-catenin signaling pathway affects physiological processes such as liver growth, development, metabolism, and participates in the pathological processes of liver diseases, including liver fibrosis and liver cancer (7). Stress and cell damage such as DNA damage response (DDR) are closely related to this classic signal pathway (8). Our previous studies had found that high a concentration of copper could induce oxidative stress and inactivation of the Wnt/β-catenin signaling pathway in hepatocytes, thereby inducing hepatocyte damage. In order to further clarify the efficacy mechanism of GDD in the treatment of WD liver injury, we hypothesized that the pathophysiological process of WD liver injury is related to Wnt/β-catenin pathway and that GDD treats liver injury by activating Wnt/β-catenin signal pathway. Therefore, to confirm this hypothesis, this study aimed to investigate the effect of GDD on the WD model. We present the following article in accordance with the ARRIVE reporting checklist (available at http://dx.doi. org/10.21037/apm-20-301).

Methods

GDD preparation

Components of GDD are listed in *Table 1*. The standardized GDD extract was purchased from Tongrentang Chinese Medicine (Hefei, China) and met the standards set by the *Chinese Pharmacopoeia (2015 Edition)*. All the components were extracted with 400 mL boiling water for 2 h, and the yield was 37.5%. Standardization was conducted using high-performance liquid chromatography as in the previous study (9).

The composition of GDD of previous report of preparation and standardization performed by high performance liquid chromatography (HPLC).

To simulate the metabolic process of traditional Chinese formula for following *in vitro* experiments, we acquired the serum of GDD from mice after 15 days of GDD intragastric administration. Whole blood sera were collected 6 h after the last administration from the abdominal aorta.

Table 1 The components of Gandou decoction (GDD) (9)

Composition of GDD	Part of use	Ratio in GDD
Rheum palmatum L.	Root & Rhizome	18%
Coptis chinensis Franch.	Root & Rhizome	18%
Curcuma longa L.	Rhizome	18%
Lysimachia christinae Hance	Whole herb	22%
Alisma plantago-aquatica L.	tuber	22%
Aralia quinquefolia var. notoginseng Burkill	Root & Rhizome	2%

Reagents and materials

PCA was purchased from Shangyao Xinyi Pharmaceutical Co., Ltd. (Shanghai, China). Xav-939, a Wnt/β-catenin inhibitor, was purchased from Selleck Chemicals (TX, USA). Roswell Park Memorial Institute (RPMI)-1640 and fetal bovine serum (FBS) were purchased from GE Healthcare-Hyclone (Chicago, IL, USA). Anti-beta actin antibody (ab8227), anti-β-catenin antibody (ab16051), anti-GSK3β antibody (ab32391), anti-GSK3β (phospho-Y216) antibody (ab75745), anti-DKK1 antibody (ab61275), anti-c-Myc antibody (ab39688), and anti-Dishevelled-3 antibody (ab93850) were purchased from Abcam (Cambridge, UK). Other reagents were bought from Beijing Solarbio Science & Technology Co. Ltd. (Beijing, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, lactate dehydrogenase (LDH), and enzyme-linked immunosorbent assay (ELISA) test was performed by Spectra Max-M5 Multifunctional Full-wavelength Microplate Reader (MD, USA). Flow cytometry detection was performed by Cytomics TM FC500 (Beckman, USA). Western blot was performed by a lab kit manufactured by Bio-Rad Laboratories (Philadelphia, PA, USA).

Animal experiment design and grouping

A total of 115 Sprague-Dawley (SD) rats (3 months old, 210±25 g) were grouped into the healthy control normal group and model group. Hepatic injury was induced by copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) gastric administration for 12 weeks in the model group (n=95). The concentration and dose of $CuSO_4 \cdot 5H_2O$ was 0.185% and 0.2 mL/10 g/d, while the normal control (NC) group rats (n=20) were intragastrically administered for 16 weeks with the same volume of normal saline daily. For grouping,

95 well-modeled rats were grouped into the model group (n=45), GDD group (n=25), and the PCA group (n=25), and were continuously given $CuSO_4 \cdot 5H_2O$ gastric administration for 4 weeks. During these 4 weeks, the GDD group and PCA group were treated with GDD (0.4 mL/10 g/d) and PCA (0.09 g/kg/d) respectively, while the model group rats were intragastrically administered with the same volume of normal saline daily. Liver tissue and whole blood were collected 10 h after the last administration. Each experiment repeated for 3 times.

Serum alanine aminotransferase (ALT) detection and liver tissue microscope-observation were used to assess the hepatic injury level. Liver tissue was fixed in 4% polycondensation formaldehyde solution and dehydrated for 12 h to make a wax block, which was sectioned at 3 μm and stained with hematoxylin and eosin (HE). The tissue wax was then observed under a microscope. This study was performed under a project license granted by the Ethics Committee for Animals Experiments of the Anhui University of Chinese Medicine, in compliance with national guidelines for the care and use of animals.

Immunochemistry of hepatic tissue

Liver tissues of each group were collected, incubated with primary antibody at 4 ℃ overnight after dehydration, immersed and embedded in wax, and sectioned. The secondary antibody was incubated at room temperature for 30 min. Then counterstaining, dehydrating, and mounting was performed with 3,3'-diaminobenzidine (DAB). The number of positive cells was counted by ImageJ to calculate the positive rate and the expression level of 8-hydroxyguanosine and nitrotyrosine proteins in the liver tissue of each group.

Cell culture and hepatic injury induction

The BRL-3A rat hepatic cell line was obtained from the American Type Culture Collection (ATCC) (CRL-1442), and cultured in RPMI-1640 media supplemented with 10% FBS and 1% penicillin-streptomycin mixture. Cells were cultured at 37 ℃ in an environment of 5% CO_2 and 95% air at an appropriate growth density of 3×10^4–9×10^4.

Cell experiment design and grouping

BRL-3A cell line was treated with 1640 medium as the NC group. NC group cell was stimulated by copper(II) sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) at a concentration of 325 nM (model group). Model group cell was treated with GDD serum and Wnt-inhibitor XAV-939 (respectively

defined as GDD group and XAV-939 group) to explore the curative effect and exact target mechanism of GDD. The concentration of XAV-939 was 11 nM, as suggested by the Selleck official website.

Cell proliferation and cytotoxicity assay

MTT assay was used to evaluate viability in BRL-3A. Briefly, the cells were seeded in 96-well tissue culture plates at a density of 1×10^4 cells/well, and the outermost wells were filled with phosphate-buffered saline (PBS). Then, cell lines were treated with different doses (100, 200, 300, 400, 600, 800, 1,200, and 1,600 μM) of $CuSO_4 \cdot 5H_2O$ solution, PBS, and 1640 medium for 24 h. The level of dissolved formazan was quantified by detecting absorbance measurements at 570 nm (n=6). Inhibition rates were calculated by the following formula:

$$\text{Inhibition rate} = 1 - \frac{\text{OD(copper-treated cells)} - \text{OD(PBS-treated cells)}}{\text{OD(1640 medium-treated cells)} - \text{OD(PBS-treated cells)}} \times 100\% \qquad [1]$$

Inhibitory concentration 50 (IC50) was calculated according to the inhibition rate. BRL-3A was treated with PBS, 1640 medium, $CuSO_4 \cdot 5H_2O$ solution (concentration of IC50), and different doses (5%, 10%,

15%, and 20%) of GDD serum for 24 h. Survival rates were calculated by the following formula, and the most appropriate concentration was selected based on the results:

$$\text{Survival rate} = \frac{\text{OD(GDD-treated cells)} - \text{OD(PBS-treated cells)}}{\text{OD(1640 medium-treated cells)} - \text{OD(PBS-treated cells)}} \times 100\% \qquad [2]$$

Flow cytometry analysis

After the BRL-3A was stimulated and treated for 24 h, cells were washed and collected by PBS solution and 0.25% trypsin-EDTA Solution. A fluorescent probe (DCFH-DA) was added to resuspended cells and incubated in the incubator for 20 min. Flow cytometry tests were then performed to detect the reactive oxygen species (ROS) level and mitochondrial membrane potential (MMP) of each group, and the peak plot was analyzed by WinMDI 2.9.

Western blot

Liver tissue and BRL-3A cell lines were collected and homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer and centrifuged at 13,000 RPM and 4 ℃ for 10 min. Protein concentration was measured by the bicinchoninic acid (BCA) protein assay kit. After that, a 4× loading buffer was added into the protein solution

and heated for 8 min to denature the protein. Then, the protein sample was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels for electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with 5% fat-free dry milk in PBS containing 0.1% Tween-20, the membranes were incubated with primary antibodies at 4 ℃ overnight. Horseradish peroxidase-labeled secondary antibodies were used to detect the primary antibodies. Bands were visualized with enhanced chemiluminescence (ECL) kit in the automatic chemiluminescence system, and the results were analyzed with ImageJ.

Statistical analysis

Data analysis was performed using ImageJ, WinMDI (version 2.9), and SPSS 17.0. All results are presented as mean ± standard error of the mean and were compared with the group of NC and model using the Student's *t*-test
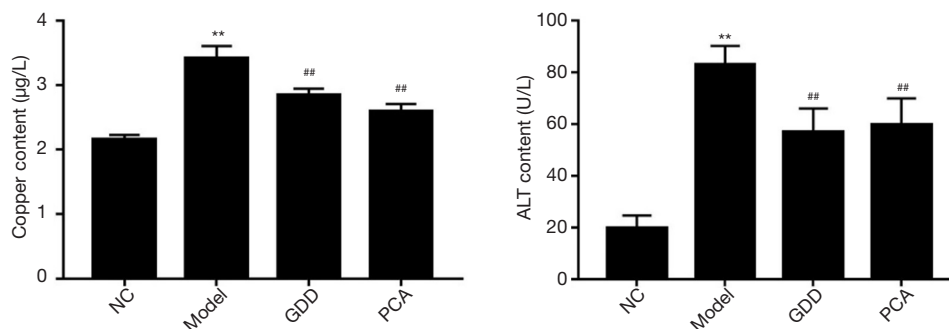
Figure 1 Effect of GDD on the biochemical indexes of copper and liver function of copper-loaded rats. The values were presented as means ± SD (n=8). **, P<0.01 compared with NC group; ##, P<0.01 compared with model group. GDD, Gandou decoction; PCA, penicillamine.

(unpaired, two-tailed). P<0.05 was considered statistically significant.

Results

Hepatic injury detection of copper-loaded rats

A total of 95 rats were hepatic injury-induced, and 95 models were successful, yielding a model success rate was 100%.

GDD reduced the level of ALT and copper

As shown in *Figure 1*, the ALT and copper results showed the following: (I) copper-loaded rats had the same impaired hepatic function as WD patients, (II) copper-loaded rats had the same copper-related biological changes as WD patients, and (III) the treatment with GDD extract strikingly ameliorated these dysfunctions. Compared with the NC rats, the ALT and copper levels of the model group rats were significantly higher (P<0.01). Compared with the model rats, these indexes in the GDD and PCA rats were significantly lower (P<0.01).

GDD ameliorated morphological and pathological changes of liver in copper-loaded rats

We collected and observed the external morphologies and pathological changes of the liver tissues from the copper-loaded rats. GDD significantly ameliorated both the morphological and pathological changes in the livers of copper-loaded rats, as shown in *Figure 2*.

HE staining in the NC rats showed that the structural arrangement of liver tissue and cells were normal and neat (*Figure 2A*). Only a few hepatocytes showed granular

degeneration, and the cytoplasm showed fine red particles. In the copper-loaded rats (*Figure 2B*), the structures of tissue and hepatocytes were disorderly and messily arranged, and most of the hepatocytes had extensive, severe vesicular degeneration and cytoplasmic vacuolation. In the GDD-treated copper-loaded rats (*Figure 2C*), hepatocytes were extensively denatured, and the cytoplasm and nucleus were lightly stained. Red spots were seen in the cytoplasm along with hepatic sinus dilatation and red plasma. In the PCA-treated copper-loaded rats (*Figure 2D*), hepatocytes were extensively denatured with granules, the cytoplasm and nucleus were lightly stained, and small red particles were seen in the cytoplasm. Compared with the copper-loaded rats, GDD- and PCA-treated copper-loaded rats had less hepatocyte degeneration, necrosis, pathological changes, and complete cell structure.

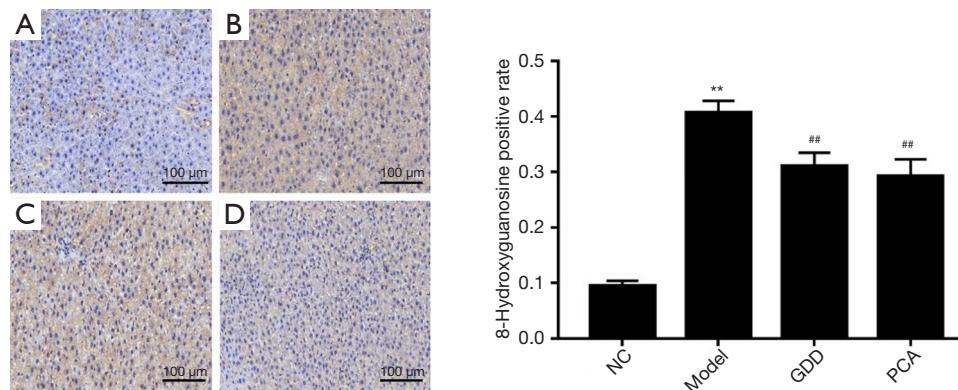### *GDD reduced the expression of 8-hydroxyguanosine and nitrotyrosine*

Immunochemistry was performed to detect the expression level of 8-hydroxyguanosine (*Figure 3*) and nitrotyrosine (*Figure 4*), 2 of the most important biomarkers of oxidative injury. GDD could significantly reduce the level of 8-hydroxyguanosine and nitrotyrosine in the liver of copper-loaded rats.

Compared with the NC rats, 8-hydroxyguanosine- and nitrotyrosine-positive expressions of liver tissue in copper-loaded rats were significantly higher (P<0.01). Compared with copper-loaded rats, 8-hydroxyguanosine- and nitrotyrosine-positive expressions in GDD and PCA-treated copper-loaded rats were significantly lower
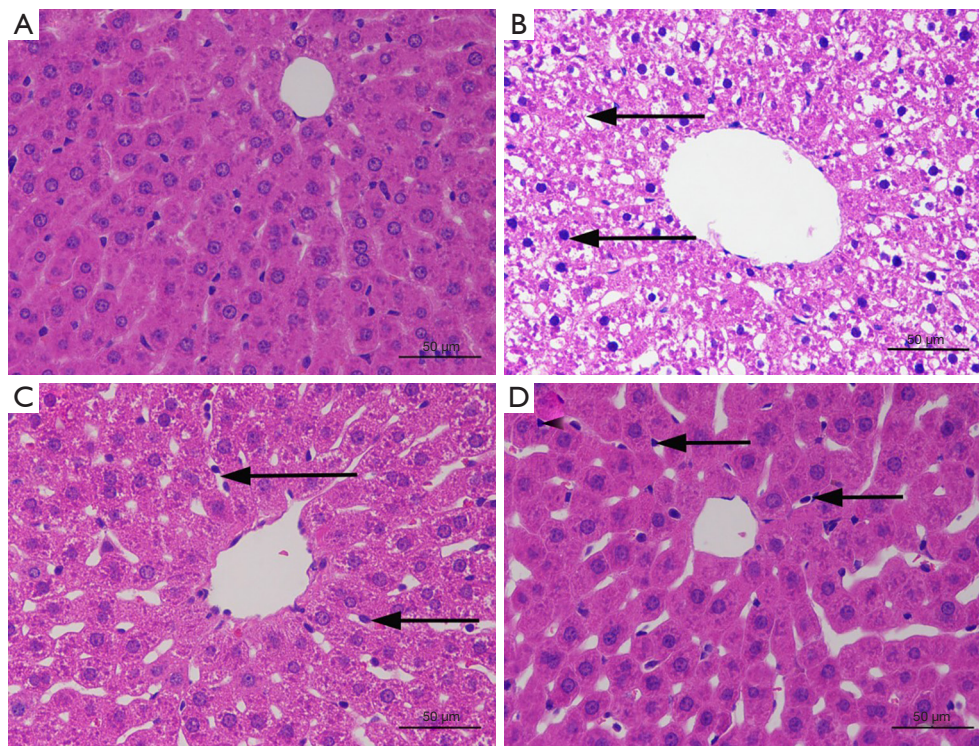
Figure 2 Effect of GDD on morphological and pathological changes of liver of copper-loaded rats. (A) Normal control group; (B) model group; (C) GDD group; (D) PCA group. H&E staining was performed to present the injury of liver (scale bar =50 μm, H&E stain, 400×, black arrows refer to tissue necrosis, vesicular degeneration and cytoplasmic vacuolation). GDD, Gandou decoction; PCA, penicillamine.

Figure 3 Effect of GDD on 8-hydroxyguanosine expression of liver of copper-loaded rats. (A) NC group; (B) model group; (C) GDD group; (D) PCA group. IHC, 200×. Wheat colored zone represented 8-hydroxyguanosine positive, and the positive rate were calculated by ImageJ. The values were presented as means ± SD (n=3). **, $P<0.01$ compared with NC group; ##, $P<0.01$ compared with model group. GDD, Gandou decoction; PCA, penicillamine.

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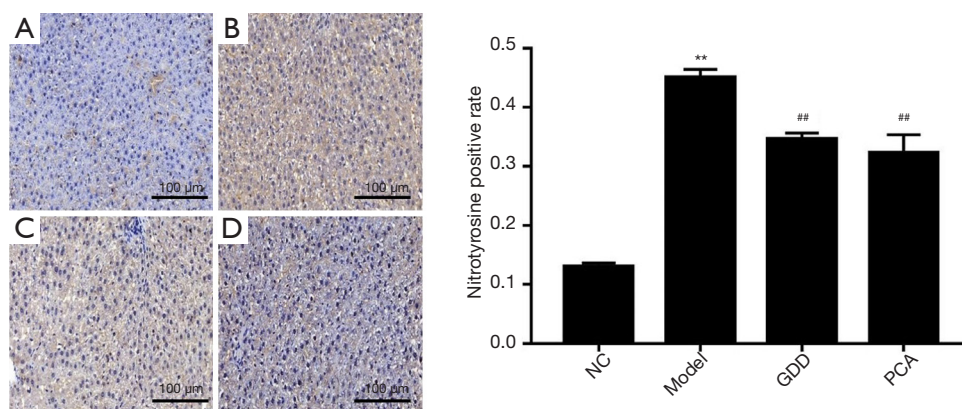
Figure 4 Effect of GDD on nitrotyrosine expression of liver of copper-loaded rats. (A) NC group; (B) model group; (C) GDD group; (D) PCA group. IHC, 200×. Wheat colored zone represented nitrotyrosine positive, and the positive rate were calculated by ImageJ. The values were presented as means ± SD (n=3). **, P<0.01 compared with NC group; ##, P<0.01 compared with model group. GDD, Gandou decoction; PCA, penicillamine.
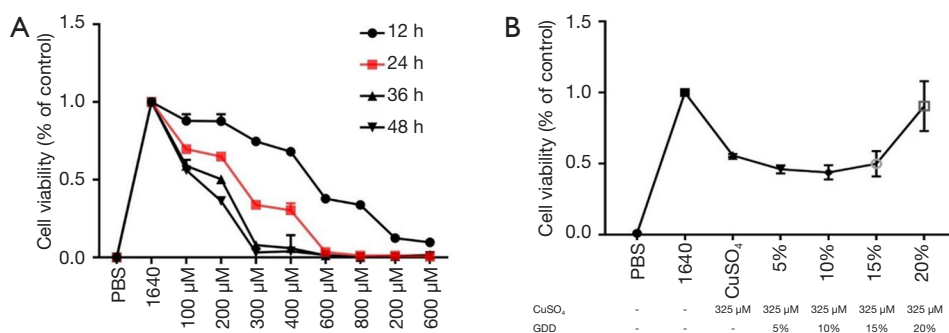
Figure 5 Protective effect of GDD on the copper-stimulated BRL-3A cell lines. (A)The damage effect of different concentration of $CuSO_4$ towards BRL-3A; (B) the protective effect of different concentration of GDD towards $CuSO_4$-stimulated BRL-3A. The values were presented as means ± SD (n=24). GDD, Gandou decoction.

(P<0.01).

GDD ameliorated the cell viability and morphology of stimulated BRL-3A

$CuSO_4 \cdot 5H_2O$ was added into the BRL-3A cell line according to an incremental concentration gradient described in section "Cell proliferation and cytotoxicity assay" above. Cell viabilities were detected by MTT 24 h after $CuSO_4 \cdot 5H_2O$ was added (*Figure 5*). Results showed that the IC50 level 24 h after $CuSO_4 \cdot 5H_2O$ (according to the formula in section "Cell proliferation and cytotoxicity assay") was 326 μM. GDD was added into $CuSO_4 \cdot 5H_2O$-stimulated BRL-3A, and the EC50 level 24 h after was close to 20% (*Figure 5*). As shown in *Figure 6*, GDD could

significantly ameliorate the viability, morphology, quantity, and density of copper-stimulated cells.

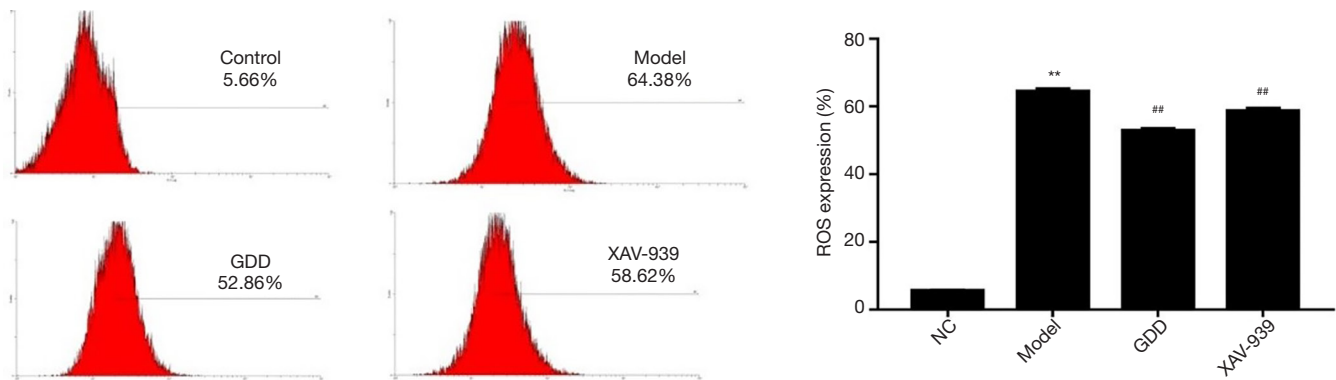### GDD reduced the expression of ROS and promoted MMP in copper-stimulated BRL-3A cells

We assessed the ROS expression and MMP after adding $CuSO_4 \cdot 5H_2O$, GDD, and XAV-939 to BRL-3A. The results were analyzed 24 h after the administration of medication. Compared with NC BRL-3A, the expression of the ROS of copper-stimulated BRL-3A cells was significantly higher (P<0.01); compared with copper-stimulated BRL-3A cells, the expression of ROS was significantly lower in GDD-treated cells. Compared with the BRL-3A of the NC group, the MMP of copper-stimulated BRL-3A cells
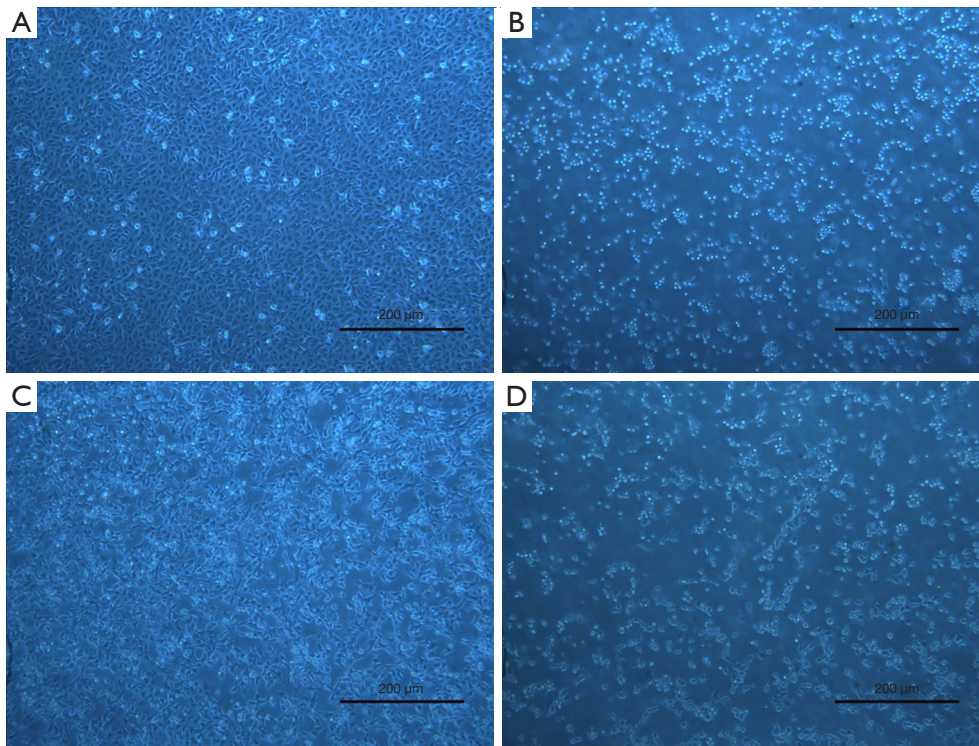
Figure 6 The cell morphology of each group. (A) Normal control group; (B) model group; (C) GDD group; (D) XAV-939 group. Observation under inverted phase contrast microscope, 100×. GDD, Gandou decoction.

Figure 7 GDD reduced the ROS expression in copper-stimulated BRL-3A cells. The expression of ROS level was presented by peak plot and the expression level were analyzed by WinMDI. The values were presented as means ± SD (n=3). **, P<0.01 compared with NC group; ##, P<0.01 compared with model group. GDD, Gandou decoction; ROS, reactive oxygen species.
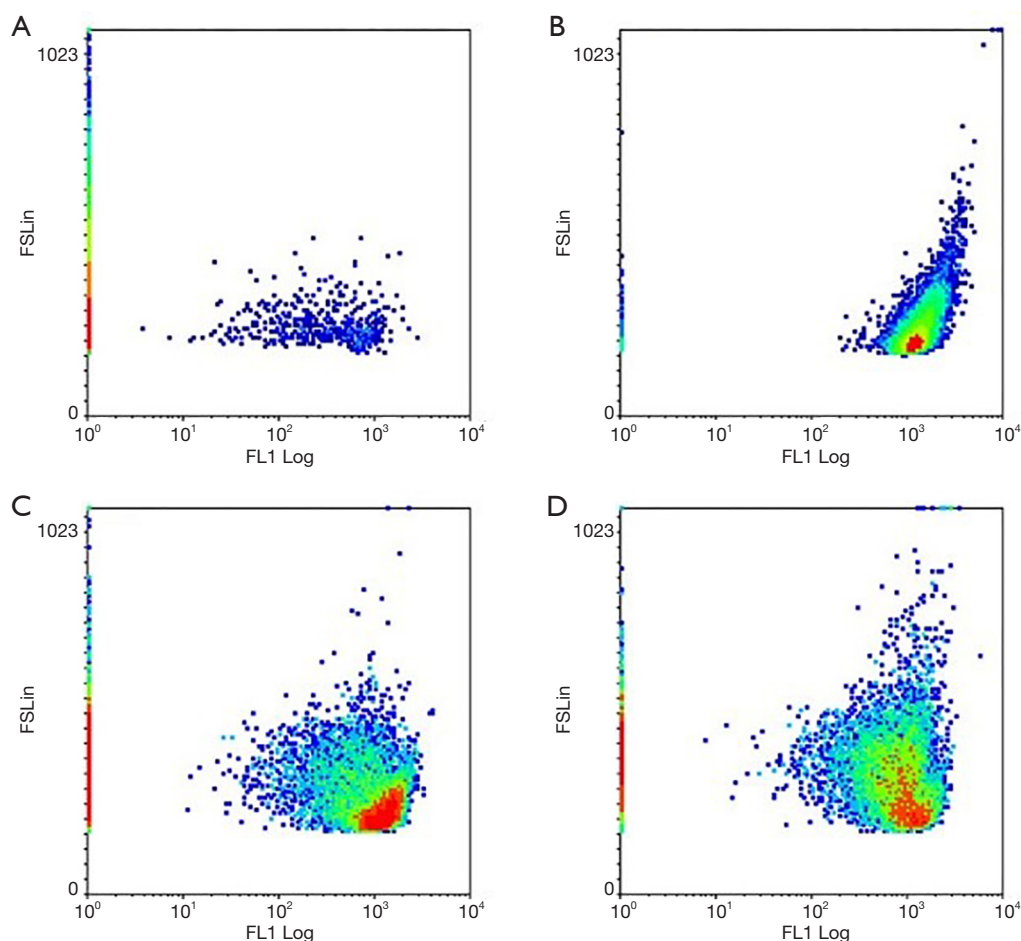
Figure 8 GDD promoted the MMP in copper-stimulated BRL-3A cells. (A) Normal control group; (B) model group; (C) GDD group; (D) XAV-939 group. Decrease of MMP were manifested as the transformation of fluorescence from red to green analyzed by WinMDI. In the figures, horizontal coordinate and vertical coordinate represent the cell density and the expression level of red/green fluorescence respectively. In NC cells, the fluorescent probe had not been loaded, thus neither the red nor the green fluorescence expressed in the cells. In copper-stimulated cells, the MMP were significantly lower and the green fluorescence were extensively distributed. GDD promoted the MMP in copper-stimulated BRL-3A and the red fluorescence rate was significantly higher. GDD, Gandou decoction; MMP, mitochondrial membrane potential.

was significantly higher (P<0.01); compared with copper-stimulated BRL-3A cells, the MMP were significantly lower in GDD-treated cells. The results thus show that GDD reduced the expression of ROS (*Figure 7*) and promoted that of MMP (*Figure 8*) induced by CuSO$_4$·5H$_2$O.

Effect of GDD on the protein expression of the Wnt/β-catenin pathway in the liver of copper-loaded rats and copper-stimulated BRL-3A

To investigate the protective effect of GDD on the copper-

accumulating hepatic injury, we examined the expression of the protein, including β-catenin, GSK3β, p-GSK3β, Dishevelled-3, and c-Myc, in the livers of copper-loaded rats and copper-stimulated BRL-3A cells. The trends in the expression of the proteins for both the liver of copper-loaded rats and copper-stimulated BRL-3A cells were as follows: compared with NC, the expression of β-catenin, p-GSK3β, and c-Myc in models were significantly lower (P<0.01), while the expression of GSK3β and Dishevelled-3 were significantly higher (P<0.01). Compared with the model, the expression of β-catenin, p-GSK3β, and c-Myc in
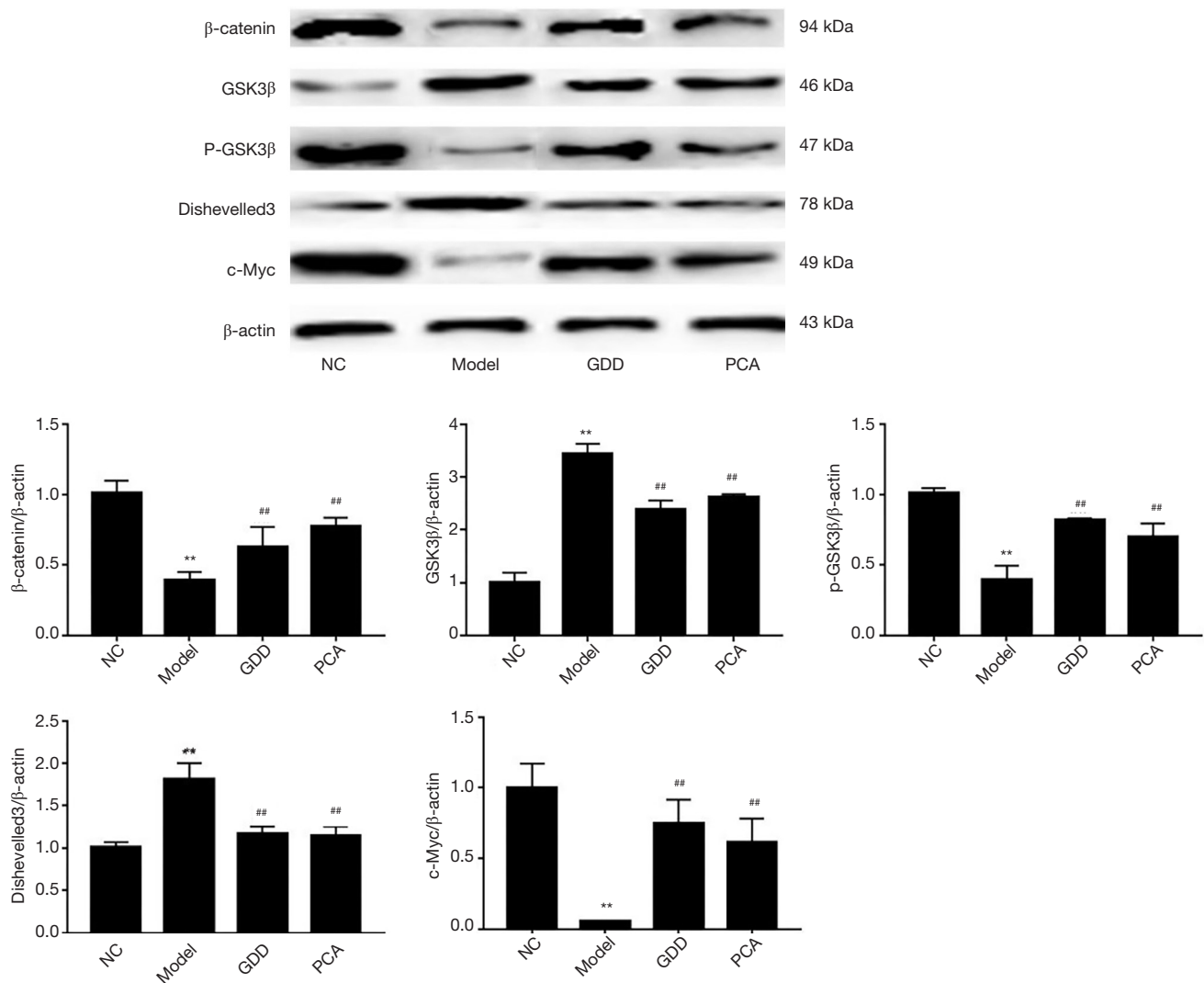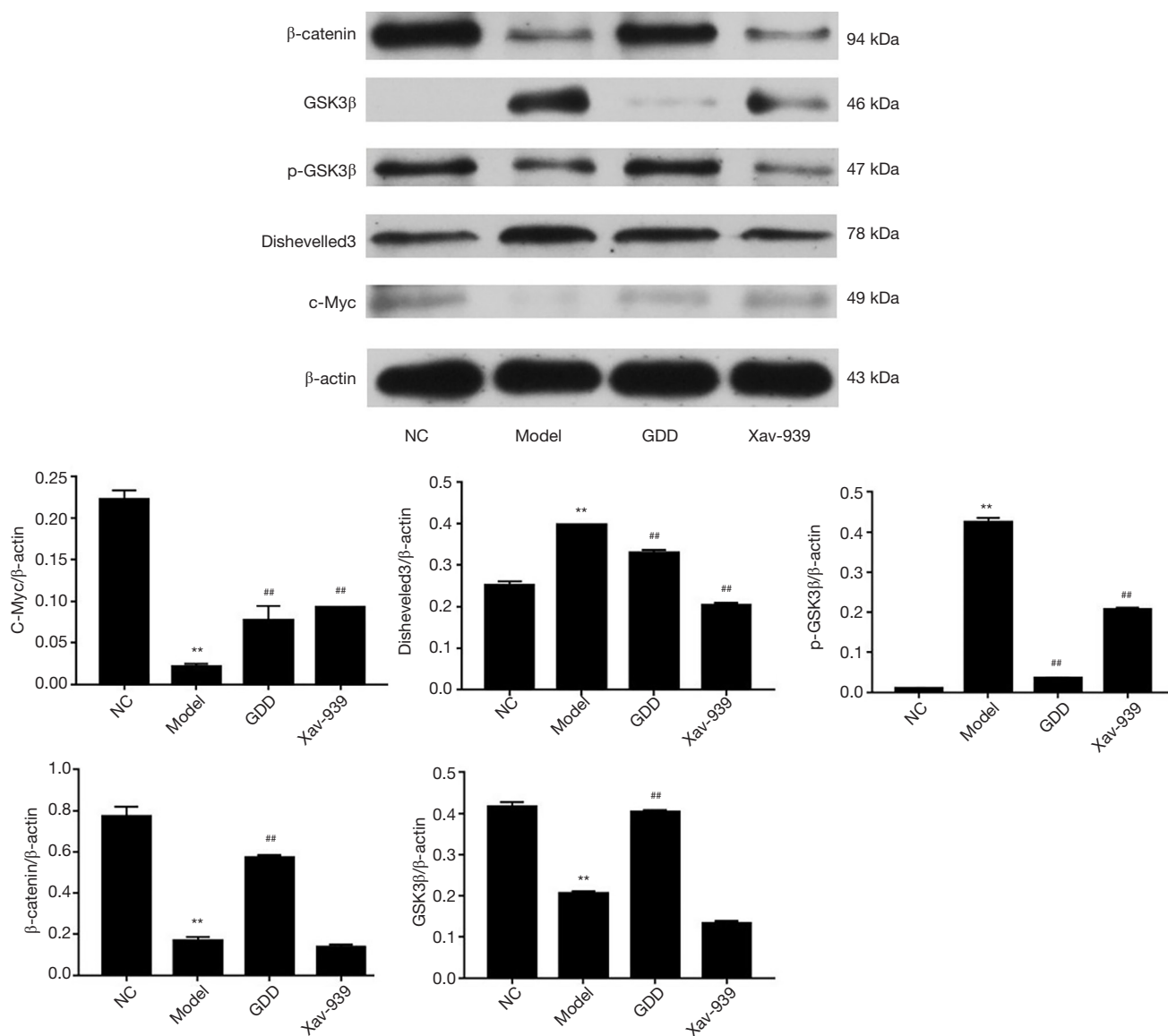
Figure 9 Effect of the GDD on the Wnt/β-catenin pathway in the liver of copper-loaded rats. Western blotting bands for liver β-catenin, GSK3β, p-GSK3β, disheveled3 and c-Myc proteins, and relative quantitative data are shown. Data are presented as mean ± SD (n=3). **, P<0.01 compared with the NC group; ##, P<0.01 compared with the model group. GDD, Gandou decoction; PCA, penicillamine.

the GDD-treated model was significantly higher (P<0.01). Furthermore, the expression of GSK3β and Dishevelled-3 was significantly lower (P<0.01). The results and statistical data are shown in *Figures 9,10*.

Discussion

WD is a hereditary disease caused by copper metabolism dysfunction. The pathogenic gene *ATP7B* is mainly expressed in the liver, and its functional defects cause ceruloplasmin and copper to be unable to be excreted from

the body so that a large amount of copper accumulates in the liver first. Thus, the liver is the prime organ of the defect and the main target organ when considering treatment. Copper-loaded rats were shown to have copper metabolism disorder and liver damage performance similar to WD patients. Therefore, the copper-loaded rats are regarded as an appropriate animal model for the therapeutic research, pathogenesis, and efficacy mechanism of WD. BRL-3A cell lines separated from the liver tissue of normal rats have been found to stimulate the pathological and therapeutic process. We added $CuSO_4 \cdot 5H_2O$ solution to

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Figure 10 Effect of the GDD on the Wnt/β-catenin pathway in copper-stimulated BRL-3A cells. Western blotting bands for liver β-catenin, GSK3β, p-GSK3β, disheveled3 and c-Myc proteins, and relative quantitative data are shown. Data are presented as mean ± SD (n=3). **, P<0.01 compared with the NC group; ##, P<0.01 compared with the model group. GDD, Gandou decoction.

BRL-3A to simulate the pathological process of copper accumulation in hepatocytes. GDD is a Chinese medicine formula that has been widely used in WD treatment for decades. It is directed by "heat elimination, detoxification, diuresis, and viscus regulation", and has been proven to be efficient in treating WD patients, with an effective rate of 84.1% (6). As described above, the exact mechanism of GDD's cytoprotective effect has not been well understood. Thus, we attempted to explore the pathogenic mechanism

of WD and the therapeutic theory of GDD further by combining the animal and cell experiments.

Copper is a crucial trace element in the human body, but it is also a major factor in liver injury in WD patients. Results showed that the copper content in the liver of GDD-treated copper-loaded rats was lower than that in copper-loaded rats. Results of the ALT test showed that ALT was lower after GDD treatment. Pathological observation of the liver slices indicated that there was a

milder liver injury of the GDD treated copper-loaded rats. The evaluation of BRL-3A also gave evidence for the hepatic protection of GDD, which ameliorated the cell viability, morphology, quantity, and density of copper-stimulated cells. In summary, GDD showed a hepatic-protection effect in both copper-loaded rats and copper-stimulated BRL-3A cells.

Oxidative stress is regarded as a mainly pathological factor of liver or neurological dysfunction in WD (10). 8-hydroxyguanosine is a hydroxyl radical generated during oxidative stress or oxygen-containing metabolism (11). An abnormal increase in free radicals is associated with various aging and neurodegenerative diseases such as atherosclerosis, along with brain and heart ischemia-reperfusion injury (12). Nitrotyrosine is formed by peroxynitrite ($ONOO^-$) and nitric oxide (NO) in the body, and its content can reflect the degree of oxidative stress in tissue and cells (13). The abnormal content of metal elements such as copper and iron in cells and free radicals can cause oxidative stress (14). Results showed that the levels of 8-hydroxyguanosine and nitrotyrosine the liver of copper-loaded rats were significantly lower after GDD treatment. The biomarkers of oxidative stress are ROS and MMP, with MMP being a landmark event in the early stage of apoptosis. Rising ROS level and declining MMP levels represent the deterioration of redox imbalance and the level of oxidative stress. Results showed that in copper-stimulated BRL-3A cells, ROS levels were significantly lower, and MMP levels were significantly higher after GDD treatment. Our results showed that the therapeutic effects of GDD might be related to its ability to inhibit oxidative stress.

Recent research has shown that the Wnt/β-catenin pathway has a close relationship with liver fibrosis, hepatic carcinoma, rehabilitation of cerebrovascular disease, etc. (15). This classic signal pathway was first reported by Fagotto (16) in 1997, and it is believed to be closely related to physiological processes such as growth, development, and metabolism. β-catenin and p-β-catenin proteins are important downstream links in Wnt/β-catenin signaling pathway, and p-β-catenin is a phosphorylated form of β-catenin (17). When Wnt has not been activated, β-catenin and GSK3β bind to a degradation state (18). Inhibition of GSK3β can change this binding state with β-catenin protein. p-GSK3β, as a phosphorylated form of GSK3β, can improve the decrease of GSK3β and can dephosphorylate the target protein while increasing the expression level when the Wnt signal is activated (19). c-Myc protein is an important DNA-binding protein

in the nucleus (20), and its overexpression can cause apoptosis (21). Dishevelled-3 can react with receptor proteins, phosphatases, and kinases (22). When it is activated, it can inhibit GSK3β, increase β-catenin protein expression, and activate the Wnt/β-catenin signaling pathway (23). The experimental results showed that the expression of β-catenin, p-GSK3β, and c-Myc proteins in the liver of copper-loaded rats, and copper-stimulated cells were significantly lower than of the NC rats and normal BRL-3A cells, and the expression levels of GSK3β and Dishevelled-3 were significantly increased. Thus, the inactivation of the Wnt/β-catenin signaling pathway might be one of the pathological changes of liver injury in WD. Copper accumulation in liver and hepatocytes induce toxicity injury while the inactivation of the Wnt/β-catenin signaling pathway prevents physiological self-healing, which intensifies copper-induced hepatic injury. After GDD medication, the expression of β-catenin, p-GSK3β, and c-Myc proteins in the liver of copper-loaded rats and copper-stimulated BRL-3A cells was significantly upregulated, while the expression of GSK3β and Dishevelled-3 was significantly downregulated. These results clearly show that GDD can rectify the expression of proteins in the Wnt/β-catenin signaling pathway.

Past research has confirmed that Wnt/β-catenin signaling pathway is closely related to oxidative stress (24-26). This study found that a high concentration of copper inhibited Wnt/β-catenin signaling pathway and induced oxidative stress injury in rat liver and hepatocyte cell lines. Meanwhile, GDD reduced the copper content and ALT level in the liver of copper-loaded rats, and the activation of the Wnt/β-catenin signaling pathway improved the self-repair of the liver and hepatocytes, thereby reducing the hepatic oxidative stress-related injury. Although the copper-chelating effect of PCA is better than GDD, the regulation of the GSK3β and DKK1 proteins was not as significant as GDD. Xav-939 is a widely accepted inhibitor of the Wnt/β-catenin signaling pathway; however, its application could not undo the therapeutic effect of GDD in this study. A lack of consistency in the trends observed suggests that the hepatic-protection effect of GDD does not only arise from its copper-expelling ability, as the inactivation of Wnt/β-catenin signaling pathway was triggered by a high concentration of copper.

Furthermore, when Wnt/β-catenin signaling pathway was completely inhibited, the hepatic-protection effect was still effective. This result implies that the therapeutic effect of GDD is independent of Wnt/β-catenin signaling

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pathway. Multiple-therapeutic-targeting is a unique characteristic of Chinese medicinal formulae, and GDD is no exception. It is hoped that this insight can further propel research into the therapeutic mechanism of GDD, which warrants further study.

Footnote

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