Protective effect of Bu-zhong-yi-qi decoction, the water extract of Chinese traditional herbal medicine, on 5-fluorouracil-induced renal injury in mice

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ABSTRACT

Background: Drug-induced renal injury is a serious toxic side effect of 5-fluorouracil (5-FU) treatment. Bu-zhong-yi-qi decoction (BZYQD), a water extract of Chinese traditional herbal medicine, is widely used in Asia as an alternative treatment to reduce the side effects of chemotherapy and also improve cancer survival. However, the mechanism is unknown. This study is designed to investigate the protective effect of BZYQD on 5-FU-induced renal injury in mice.

Methods: Mice were divided into four groups: the control, 5-FU, 5-FU + low, and high BZYQD group. Mice in the three latter groups were administered 5-FU (100 mg/kg/day, intraperitoneally) for six days, and in the 5-FU + low and high BZYQD groups were given BZYQD (1 or 2 g raw herb/kg/day, intragastrically) beginning four days before 5-FU and continuing until the termination of the experiment. The right kidney fixed in formalin for histological examination and the left was homogenized to measure the levels of apoptosis-related proteins and activities of oxidative stress-related biomarkers. Blood samples were collected for measuring renal function-related biochemical indices.

Results: Renal morphology injury, increased urea nitrogen and creatinine concentration, and decreased SOD, CAT, and GSH-Px were all observed in 5-FU-administrated mice. However, BZYQD almost reversed the morphological injury as well as renal function-related indices and antioxidant enzyme activity.

Conclusion: These results suggest that BZYQD inhibits 5-FU-induced renal injury, possibly through the reduction of apoptosis and necrosis in renal tubular epithelial cells via the antioxidant mechanism. Henceforth, BZYQD may be a potential antioxidant against drug-induced oxidative stress.

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Introduction

5-Fluorouracil (5-FU), an anti-metabolite anticancer agent, is one of the most potent and widely used antineoplastic drugs. It is used for the treatment of several human malignancies, including breast, head, neck, stomach, colorectal, liver, and skin cancer. Like other chemotherapeutics, 5-FU is non-targeted in action. Its cellular toxicity targets and demolishes not only tumors but normal cells as well, leading to extensive side effects caused by proliferative inhibition, DNA damage and cell death. One of the most serious clinical side effects of 5-FU is renal toxicity. Recent studies documented that 5-FU treatment results in the production of reactive oxygen species (ROS), which causes peroxidative damage to the kidney and induces toxic effects like necrosis and apoptosis.

Xenobiotics, such as toxicants and drugs, generate ROS that play a crucial role in the initiation and progression of nephrotoxicity and renal injuries, while antioxidant agents can mitigate these deleterious effects through modification of the oxidant-antioxidant balances. Bu-zhong-yi-qi decoction (BZYQD, also called Bu-Zhong-Yi-Qi-Tang, Bojungikki-tang, and Hochu-ekki-to), is comprised of crude ingredients that are extracted from eight herbs. It is a well-known formula for traditional Chinese medicine that has been used in Asia for the enhancement of digestive capacity. Recently, a number of studies have shown that BZYQD has antioxidant properties, such as protecting the gastric mucosa from ethanol-induced acute gastric injury by reversing the decrease in antioxidant enzymes activities including the catalase (CAT), glutathione-S-transferase (GST), etc.
glutathione reductase, glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD). In addition, there has been an increasing trend for cancer patients to take BZYQD as an alternative medicine along with standard chemotherapy treatment, in which BZYQD enhances chemotherapeutics-induced cytotoxicity and protects gastrointestinal tract function. Is the protective effect of BZYQD related with its antioxidant properties? In the present study, we investigated the efficacy of BZYQD on 5-FU-induced oxidative stress and apoptosis in kidneys.

Materials and methods

Preparation and administration of BZYQD

Eight herbs (Table 1) were put into a 20-fold volume of distilled water, decocted from 80°C to 100°C, filtered and concentrated at 40°C to 80°C, and then stored in a refrigerator until use. The authenticity of BZYQD was proved by HPLC (Figure 1).

Animals and treatments

Forty pathogen-free Kunming mice, weighing 20–25 g were obtained from Experiment Animal Center of Liaoning University of Traditional Chinese Medicine (Shenyang, China). The animals were acclimatized to standard laboratory conditions (temperature 22–25°C, relative humidity 50–60%, and 12 h photoperiod lights on 07:00–19:00). This study was approved by the Institutional Animal Ethics Committee of Liaoning University of Traditional Chinese Medicine and the study conducted in accordance with the “Guide for the Care and Use of Laboratory Animals”. Mice were randomly divided into four groups (n = 10/group, half male and half female), including control, 5-FU, 5-FU + low BZYQD, and 5-FU + high BZYQD group. The treatments of animals in different groups were shown in Table 2.

Histopathologic evaluation of the kidney

Kidney tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (4–5 mm), and stained with hematoxylin and eosin (H&E). Kidney sections were screened to determine the type of effects present, in a random order and without knowledge of group identity.

Immunohistochemical assay for apoptosis

Cell apoptosis in kidney segments was detected by cleaved caspase 3 antibody (1:100, SAB, Fairfield, NJ) immunohistochemical assay. The results were reviewed by two independent researchers and analyzed using the MetaMorph/DP10/BX41 image analyzing system (UIC/Olympus, Chicago, IL/UIC/OLYMPUS, US/JP, Tokyo, Japan).

Western blotting analysis

Kidney tissue samples were homogenized in lysis buffer solution (13.2 mmol/L Tris-HCl, 5.5% glycerol, 0.44% SDS, and 10% β-mercaptoethanol). After the supernatants were collected, protein levels were detected by the bicinchoninic acid (BCA) assay kit (Thermo, Waltham, MA). Equal amounts of separated protein (20 μg) were fractionated by 12% or 8% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Blots were incubated overnight at 4°C with cleaved caspase 3 (1:1000, SAB) and cleaved poly adenosine diphosphate-ribose polymerase (cleaved PARP, 1:1000, SAB). Then, horseradish peroxidase-conjugated secondary antibodies (ZSGB BIO, Beijing, China) were used in conjunction with an ECL chemiluminescence detection system (Amersham, UK). Staining was quantified by scanning densitometry. The densitometer values of measured proteins were normalized to GAPDH and used as loading controls.

Renal function-related biochemical index assay

Collected blood samples were coagulated at room temperature for at least 30 min and then centrifuged at 3000 rpm. The levels of blood urea nitrogen (BUN) and creatinine in serum were estimated spectrophotometrically using commercial diagnostic kits (Jiancheng Institute of Biotechnology, Nanjing, China). The results were all expressed as mg/dL. Each experiment was performed in triplicates.

Oxidative stress-related biomarker assay

Fresh tissue supernatant of kidney samples was prepared by the method of Rehman et al. Kidneys were removed quickly, cleaned of extraneous material, and immediately perfused with ice-cold saline (0.85% NaCl). The kidneys were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%) using a homogenizer. The homogenate was altered through a cell strainer (20 mesh) and centrifuged at 800 g for 5 min at 4°C to separate the nuclear debris. The aliquot obtained was centrifuged at 12,000 rpm for 20 min at 4°C to obtain the supernatant, which was used as a source of enzymes. All biochemical estimations were completed within 24 h of animal killing.
Superoxide dismutase (SOD) activity: the determination of SOD activity was performed by using the commercial kit Superoxide Dismutase Activity Assay (CELL BIOLABS, San Diego, CA). The assay principle is based on the ability of a xanthine/xanthine oxidase (XOD) system to generate \( \text{O}_2^-/\text{CO}_2^- \) anions that are detected with a Chromagen solution. In the presence of SOD, these \( \text{O}_2^-/\text{CO}_2^- \) anion concentrations are reduced, yielding lower colorimetric signal. One unit (U) of SOD activity was defined as the amount that reduced the absorbance at 490 nm.\(^{16}\) Each experiment was performed in triplicates.

Catalase (CAT) activity: according to the previous study, CAT activity was estimated spectrophotometrically.\(^{17}\) In short, the reaction mixture consisted of 0.05 mL PMS, 1.0 mL of \( \text{H}_2\text{O}_2 \) (0.019 M), and 1.95 mL phosphate buffer (0.1 M, pH 7.4), all in a total volume of 3 mL. Changes in absorbance were recorded at 240 nm and were calculated as nmol \( \text{H}_2\text{O}_2 \) consumed/min per mg protein.

Glutathione peroxidase (GSH-Px) activity: GSH-Px activity was assayed with a Cellular Glutathione Peroxidase assay kit (Beyotime Institute of
Figure 1. HPLC data of BZYQD. (A) and (B): BZYQT (the upper) and standard sample (the lower). The peaks indicate the existence of hesperidin (1), ammonium glycyrrhizinate (2), and astragaloside (3), which proves the authenticity of BZYQT.
According to the manufacturer’s instructions, absorbance was read at 340 nm and results were expressed as units/mg protein. Each experiment was performed in triplicates.

**Statistical analysis**

Each experiment was carried out at least three times separately. Data are expressed as means ± SD. Statistics between different groups were analyzed by ANOVA test. Value of \( p < 0.05 \) indicates statistical significance. Statistical analyses were conducted with SPSS 15.0 (Cabit Information Technology Co., Ltd, Shanghai, China).

**Results**

**BZYQD treatment inhibits renal morphology injury induced by 5-FU in mice**

In our present study, the H&E-stained sections exhibited normal histo-architecture in the control group while the 5-FU-treated group showed distorted and degenerated tubular architecture, special necrosis changes including vacuolar formation, swelling, and blebbing. However, it was found that pre- and post-treatment with BZYQD at both doses (1 and 2 g raw herb/kg/day) attenuated the 5-FU-induced histopathological changes significantly (Figure 2).

**BZYQD treatment inhibits renal tubular cell apoptosis induced by 5-FU in mice**

We have known that 5-FU can induce renal injury mainly characterized by glomerular and tubular cells apoptosis. To further examine this pathological effect, the expression of caspase-3, an apoptosis-related gene, was detected in renal sections. Cleaved caspase-3 was dramatically increased after 5-FU treatment, while BZYQD treatment significantly reduced its expression (cleaved caspase-3 positive rate: 5-FU versus 5-FU + low BZYQD, \( p < 0.05 \); 5-FU versus 5-FU + high BZYQD, \( p < 0.01 \)) (Figure 3(A)).

We further analyzed the activation of caspase-3 characteristic for induction of apoptosis as well as the inactivation of PARP, a DNA repair factor, by immunoblotting. Caspase 3 activation and PARP inactivation/cleavage were observed in 5-FU group, while these changes were markedly restored in the 5-FU + low BZYQD and 5-FU + high BZYQD groups (Figure 3(B)).

**BZYQD treatment inhibited 5-FU-induced renal injury in mice**

Based on the histopathological changes in mice induced by 5-FU, we tested reserved plasma to judge the effect of BZYQD on damaged kidneys. We found that BUN and serum creatinine, the two important markers for the kidney, were all significantly increased in the mice treated
with 5-FU when compared with the control. A marked inhibition was observed in BUN and serum creatinine in the two BZYQD administered groups (BUN: 5-FU versus 5-FU + low BZYQD, \( p < 0.05 \); 5-FU versus 5-FU + high BZYQD, \( p < 0.01 \); creatinine: 5-FU versus 5-FU + low BZYQD, \( p < 0.05 \); 5-FU versus 5-FU + high BZYQD, \( p < 0.05 \) (Figure 4).

**BZYQD treatment inhibited 5-FU-induced renal oxidative stress in mice**

Previous study suggested that 5-FU can induce renal toxicity via targeting oxidative stress and apoptotic mechanism, in which the levels of SOD, CAT, and GSH-Px are all changed according to the degree of renal injury.\(^{17}\) It was found that the three antioxidative...
enzymes were all depleted in the 5-FU treated group, but pre- and post-treatment with high dose of BZYQD significantly restored the activity of these enzymes (SOD activity: 5-FU versus 5-FU + high BZYQD, $p < 0.05$; CAT activity: 5-FU versus 5-FU + high BZYQD, $p < 0.05$; GSH-Px activity: 5-FU versus 5-FU + high BZYQD, $p < 0.05$) (Figure 5). There was no significant difference found between the control group and the low BZYQD group.

**Discussion**

5-FU, the pyrimidine antimetabolite, was used in the treatment of various types of cancers.19 Its metabolic products not only lead to cellular toxicity toward tumor cells directly, but also target normal cells and lead to extensive side effects like nephrotoxicity.20,21 5-FU-induced renal injury is characterized by pathological changes such as glomerular and tubular cells apoptosis or necrosis.17 In our present study, we observed typical pathogenic changes of 5-FU-induced renal toxicity. These changes include morphological damages such as distorted and degenerated tubular architecture, and necrosis or apoptosis changes in renal tubular epithelial cells, as shown by increase in renal function-related biochemical indices (evidence of BUN and serum creatinine).

Although the mechanisms underlying 5-FU-induced renal toxicity is not fully clear, the 5-FU causes severe oxidative stress in normal tissue which is manifested as lipid peroxidation as increased via reducing antioxidative enzymes content or activities.17 SOD and CAT are two important antioxidative enzymes which protect cells, tissues, and organs from damage caused by oxidative stress. The former can catalyze the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen, while the latter can induce decomposition of hydrogen peroxide into water and oxygen.1,17 GSH is a unanimous antioxidant that defends against exogenous toxic injury by augmenting the defense against ROS via scavenging of free radicals. It does so by directly donating a hydrogen atom and neutralizing free radicals (hydroxyl radicals). Depletion of GSH in tissue damages cellular defense against oxidative stress.17 Our results showed 5-FU administration depleted antioxidative enzymes like SOD, CAT, and GSH reservoirs—which is in concurrence with an earlier finding that 5-FU treatment leads to oxidative stress and apoptotic damage in kidneys of mice.

Evidences indicate that many traditional herbal medicines have good antioxidant effects, among which BZYQD can protect the gastric mucosa from ethanol-induced acute gastric injury and alleviate chemotherapy-related fatigue. These protective effects might be induced by increasing antioxidant status.8,22 Previous study has reported that there are mainly four components in BZYQD: liquiritin, nodakenin, hesperidin, and glycyrrhizin.8 All of them have different degrees of antioxidant activity. Liquiritin attenuates advanced glycation end products-induced endothelial dysfunction by reducing ROS generation.23 Nodakenin plays a role in antioxidant activities via *in vitro* activities against lipopolysaccharide-induced nitric oxide production.24 Hesperidin ameliorates heavy metal-induced brain toxicity and trichloroethylene-induced nephrotoxicity by reducing antioxidant enzymes like SOD, CAT, and GSH-Px.25,26 Glycyrrhizin acid protects renal tubular epithelial cells from high glucose-induced injury by reducing antioxidant enzymes.27 Pretreatment with BZYQD before administering 5-FU in the mice, which substantially attenuated the 5-FU-induced renal histopathological changes such as distorted and degenerated tubular

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**Figure 5.** Effects of BZYQD on the antioxidant enzymes SOD, CAT, and GSH-Px on 5-FU-induced renal redox imbalance. (A) SOD levels. (B) CAT levels. (C) GSH-Px levels. SOD: superoxide dismutase; CAT: catalase; GSH-Px: glutathione peroxidase (ANOVA followed by Bonferroni’s test). Each bar represents the mean ± SD ($n = 10$/group).
architecture, and necrosis or apoptosis changes in renal tubular epithelial cells. In addition, BZYQD-treated mice showed lower renal injury indices (less BUN and serum creatinine) and increased anti-oxidant activities (higher antioxidant enzymes like SOD, CAT, and GSH-Px) compared with the 5-FU treatment group. Our findings are in part consistent with those previous studies that BZYQD can protect the gastric mucosa from ethanol-induced acute gastric injury via antioxidant mechanism.4,5 We reason that the increasing antioxidant status of BZYQD might come from the antioxidant effects of its four components: liquiritin, nodakenin, hesperidin, and glycyrrhizin.

In addition to the neuroprotection mechanism by inhibiting intracellular accumulation of ROS in scopolamine-induced mice and the gastroprotective mechanism by increasing antioxidant status in alcohol-induced rats,6,8 our data also showed that BZYQD can protect renal tubular epithelial cells from 5-FU-induced apoptosis or necrosis. This suggests a multifactorial mechanism, possibly involving its renoprotective effects and antioxidant properties. In conclusion, BZYQD is a good alternative treatment in reducing chemotherapy side effects and may be a potential antioxidant against drugs and toxicant-induced oxidative stress.

Disclosure statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service, and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “Protective effect of Bu-zhong-yi-qi decoction, the water extract of Chinese traditional herbal medicine, on 5-fluorouracil-induced renal injury in mice”.

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