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Original Research Article

Protective role of Bu-Zhong-Yi-Qi decoction on aristolochic acid-intoxicated zebrafish

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Abstract

Purpose: *To investigate the potential nephroprotective effects of a traditional Chinese medical prescription, Bu-Zhong-Yi-Qi Decoction (BZYQD) in an aristolochic acid (AA)-intoxicated zebrafish model.*
Methods: *A green fluorescent zebrafish line Tg (wt1b:EGFP) was used, and different exposure protocols were applied. Once a suitable protective concentration of BZYQD was found, antibody staining and real-time PCR methods were applied and the pro-inflammatory gene expressions were determined.*
Results: *The results showed that low dose (10 ppm) of BZYQD attenuates the AA-induced malformed kidney phenotype. This finding was further substantiated by an examination of the integrity of pronephric tubes in the treated embryos. Pre-treatment with BZYQD suppressed the elevated expressions of pro-inflammatory genes, including tumor necrosis factor-α and myeloperoxidase, induced by AA exposure (1.6 ~ 2.3-fold). This indicates that the nephroprotective effect of BZYQD may be mediated by suppression of pro-inflammatory gene expression. On the other hand, a high dose (500 ppm) of BZYQD caused nephrotoxic effect.*
Conclusion: *An efficient model to identify AA-protective compounds using zebrafish embryos has been successfully established. Using this strategy, it has been found that BZYQD is nephroprotective in zebrafish embryos, and might have the potential to be applied in humans.*

Keywords: *Bu-Zhong-Yi-Qi Decoction, Zebrafish; Kidney, Aristolochic acid, Nephroprotection*

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INTRODUCTION

Bu-Zhong-Yi-Qi decoction (BZYQD) is a traditional Chinese medicine composed of eight formula. Clinically, it was used to treat fatigue, poor appetite, diarrhea, as well as uterine and rectal prolapse [1]. Recently, it was used to treat myasthenia gravis, allergic rhinitis, chronic obstructive pulmonary disease, polymyositis and chronic renal failure [2-3]. Because BZYQD

possess anti-oxidant, anti-inflammatory and immune modulation properties, it was reported that BZYQD treatment attenuated xenobiotic-induced renal injury, hepatotoxicity and asthma in laboratory animals [4]. These studies indicate the importance of BZYQD in animal studies and in human use.

Aristolochic acid (AA) is a plant-produced compound which has been demonstrated that AA is toxic in fish, human and other mammals [5,6]. Furthermore, it has been reported that AA exposure is highly associated with gene mutation, kidney failure and anaemia [7]. For these reasons, it would be beneficial to find some products which are able to attenuate AA-induced toxicities. Zebrafish has been demonstrated as an efficient animal model for toxicological studies [8,9]. In this study, a green fluorescent kidney transgenic zebrafish line, Tg (*wt1b:EGFP*) [10] was used to evaluate the protective effects of BZYOD. By recording the kidney malformation rates, combined with antibody staining and RT-qPCR experiments, the possible molecular mechanism and the nephroprotective effects of BZYQD could be easily verified.

EXPERIMENTAL

Composition and preparation of Bu-Zhong-Yi-Qi Decoction (BZYQD)

BZYQD contains eight herbs: Huangqi (*Astragali radix*, 21.1 %), Renshen (*Ginseng radix*, 14 %), Baizhu (*Atractylodis rhizoma Alba*, 12.7 %), Gancao (*Glycyrrhizae radix et rhizome,* 12.7 %), Danggui (*Angelicae gigantis radix*, 14 %), Chenpi (*Citri unshius pericarpium*, 8.5 %), Shengma (*Cimicifugae rhizome*, 8.5 %), and Chaihu (*Bupleuri Radix*, 8.5 %). The method for preparation of the BZYQD was previously described [4].

In brief, BZYQD were extracted with 1000 ml of distilled water and boiling for 1 hr. When the temperature came down to room temperature, the aqueous phase of BZYQD extracts were transferred to a new flask, freeze-dried, and the dried powder extracts were stored at −20 °C. Twenty milligrams of dried powder extract was dissolved in phosphate-buffered saline (PBS) as the stock test sample solution (20 mg/mL, known as 20,000 ppm). For subsequent assays, each test solution was serially diluted with PBS.

Fish maintenance and staging

Zebrafish, wild-type (WT; AB strain) and *Tg* (*wt1b:EGFP*) strains, were maintained using a standard protocol and kept in Tamkang University [6, 10]. Embryos were collected and staged according to the standard criteria [hours post-fertilization (hpf)] [8]. After collection, they were divided into the test groups for the subsequent BZYQD treatment experiments. All of the animal experiments in this study were approved by the Use of Laboratory Animal Committee, Tamkang University, Tamsui, New Taipei City, Taiwan (approval no. 103001).

BZYQD treatment

Three exposure protocols (methods I–III) were applied in this study. For dose titration, Tg (*wt1b:EGFP*) embryos were collected and divided into different groups (30 embryos per group), and were exposed to either water (mock-treated control: 0 ppm), or water containing the BZYQD at different concentrations (10, 100 and 500 ppm), with or without AA. All embryos were cultivated in 24-well cell culture plates, and survival rates were calculated at 48 hpf.

For the purpose of recording the defective kidney phenotypes, embryos displaying normal morphology in the regions of pronephric tube (pt) and glomerulus (gl) were classified as the embryos with normal kidneys; while embryos displaying defective morphology in either pt or gl were classified as malformed kidneys.

Histological examination and image capture

The cryosectioning protocols were performed as previously described [6]. The localization of the pt in the cryosections was visualized with immunohistochemistry using a monoclonal antibody (α6F), and assessed using an ABC staining system (Santa Cruz) and 3, 3′-diaminobenzidine (DAB) as chromogens. Sections with 10 μm in thickness obtained from the pt were stained by hematoxylin. All live or stained embryos were examined under a microscope equipped with a GFP filter and a digital camera as previously described [6,10].

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The qRT-PCR protocols were performed by following the previously described methods [6]. The primer sets were synthesized for the purpose of detecting the expression levels of *tumor necrosis factor-*α (*tnf-α)*, *myeloperoxidase* (*mpo*), *survivin* and *bax*. The housekeeping gene *β-actin* was used as an internal control for normalization in quantification (Table 1).

Table 1: Primers used in this study

Transcript	Forward primer	Reverse primer
β-actin	CAGCAAGCAGGAGTACGATGAGT	TTGAATCTCATTGCTAGGCCATT
survivin	GCGGATTTATCTCGGTTGTCTT	TGGTCTGATCATCACTTGCAAG
mpo	GAGCTCCTGCCCTTTACTAGTGTT	TCACCCGCAATGAAGCAA
tnf-α	ACGGAGGCAAAAAGCCACTT	GCAGCGCCGAGGTAAATAGT
bax	CGACAGGGATGCTGAAGTGA	ATCTCCGATCTGCTGCAAACA

Statistical analysis

All analysis in this study were carried out according to Matlab software (version 7.7 R2008b). The two-way ANOVA (analysis of variance) and the Tukey-Kramer honestly significant difference (HSD) test were further used. $P < 0.05$ was considered statistically significant.

RESULTS

Pretreatment of BZYQD attenuates aristolochic acid-induced defective kidney phenotypes

To explore the potential protective effects of BZYQD on AA-induced renal malformations, Tg (*wt1b:EGFP*) embryos were pre-treated with BZYQD (0, 1 or 10 ppm) at 12-24 hpf and then treated with AA (3 ppm) till 32 hpf, and calculated for their survival rates at 48 hpf (Figure 1 A). There were no significant differences in survival rates of each group (Figure 1 B). Next, the renal phenotypic effects in each group at 48 hpf were examined. When the zebrafish embryos were exposed to 3 ppm of AA, nearly all of the embryos displayed renal malformation phenotypes (96.30 %, Figures 1C and 1D). These malformed kidney phenotypes included a separated or a swollen gl and a curved pt (Figure 1 C). Pretreatment of BZYQD plus AA exposure led to a kidney malformation rate of 93.33 % for 1 ppm BZYQD, and 71.05 % for 10 ppm BZYQD respectively (Figure 1 C and D).

The ANOVA method was first applied to examine the effect of BZYQD dosage ('AA', '1 ppm BZYQD +AA' and '10 ppm BZYQD + AA'). The test gave a *p*-value of 0.0052, indicating a highly significant difference between BZYQD dose groups. The Tukey-Kramer HSD test was then used, and showed that the mean malformation rates (standard error, sample size) for three dose groups ('AA', '1 ppm BZYQD + AA' and '10 ppm BZYQD + AA'') are 96.30 % (6.55 %, n = 29), 93.33 % (6.21 %, n = 30), and 71.05 % (5.51 %, n = 38) respectively. Figures 1 E presents the mean malformation rates with 95% confidence intervals for these dosage groups. The above results indicated that pre-treatment of 10 ppm BZYQD has a significant protective effect against AA-induced nephrotoxicity.

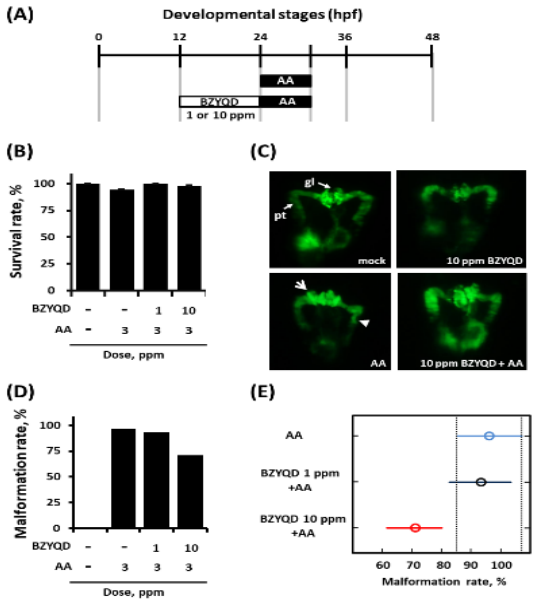

Figure 1: Kidney phenotypes of zebrafish embryos after prevention of BZYQD. A. Exposure schemes of AA treatment with or without low doses (1-10 ppm) of BZYQD prevention. B. Survival rates of zebrafish embryos after the indicated exposure. C. Morphological changes of Tg (*wt1b:EGFP*) embryos after exposure to AA with or without BZYQD prevention. *Representative* figures are *shown here.* All photos were taken at 48 hpf. An arrow and triangle indicate the defective sites in gl and pt. D. Survival rates of Tg (*wt1b:EGFP*) embryos for each treatment group at 48 hpf. E. The Tukey-Kramer HSD test. Two group means are significantly different if their intervals are disjointed

Histopathological features of AA-induced malformed kidney pretreated with BZYQD

Next, the renal abnormal phenotypes were examined from the histological level by α6F antibody. The pt in the control embryo showed a compact structure composing of a single layer of epithelial cells (Figure 2 A and B). The AA-exposed embryos displayed disorganized and broken epithelial cells around the pt, indicating the impaired integrity in the regions (compare Figure 2 B, B1-2 vs 2 C, C1-2). However, the

α6F positive cells of pt from 10 ppm BZYQD-pretreated embryos exhibited more regular and compact structures resembling those in mock control group (compare Figure 2 B, B1-2 vs 2 D, D1-2). The findings provided further support for the view that BZYQD pretreatment ameliorates AA-induced defective renal phenotypes.

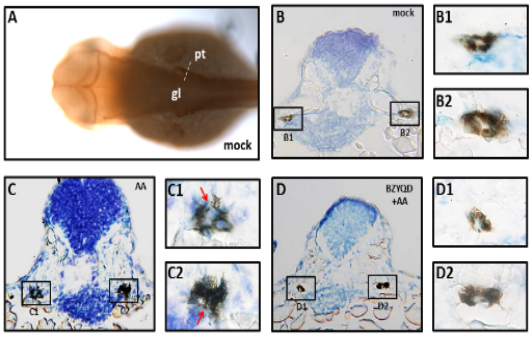

Figure 2: BZYQD pretreatment attenuate AA-Induced defective pronephric tubes. Immunohistochemical staining of the basolateral marker, α6F, was performed on transverse sections of pt from 48 hpf zebrafish embryos after exposure to 0 ppm (mock control, A, B) or 3 ppm of AA without (C) or with 10 ppm of BZYQD pretreatment. D. Sections were stained with α6F and counterstained with hematoxylin. Each group contains six embryos with similar result, and only one representative image for each group is shown here. B1-2, C1-2 and D1-2 were closer views of the boxed regions in B, C and D, respectively. Red arrow indicates the defective sites in pt

Pretreatment with BZYQD suppressed AA-induced pro-inflammatory gene expression

It has been reported that the expressions of pro-inflammatory genes, *tumor necrosis factor-α* (*tnf-α*) and *myeloperoxidase* (*mpo*) were up-regulated by AA treatment [6]. As shown in Figure 3, the expressions of *tnf-α* and *mpo* in the 10 ppm BZYQD+AA groups were decreased by 2.3- and 1.6-fold as compared with the AA group, respectively (Figures 3 A and B). However, pretreatment of BZYQD had minimal effect on the expression of pro-apoptotic gene *bax,* and anti-apoptotic *survivin* gene (Figure 3 B). These results suggest that the attenuation of AA-induced renal malformation by pretreatment of BZYQD may be mediated by the suppression of pro-inflammatory gene expressions.

High dose of BZYQD caused defective renal phenotypes

Based on the stages of kidney development, three exposure protocols were designed in this study (method I, 12 - 36 h, specification and epithelialization), method II (24 - 36 h, nephron patterning), method III (24 - 48 h, nephron patterning and angiogenesis)]. For titration of BZYQD dose, zebrafish embryos were collected and exposed to the selected concentrations using different exposure protocols, and determining the survival rates at 48 hpf. The results showed that more than 90% of the embryos were alive at 48 hpf after exposure to 10 to 500 ppm of BZYQD, using method I and II. Most of the embryos also survived after exposure to 10 and 100 ppm with method III exposure protocol. However, the survival rates decreased to 73.3 ± 10.7% (n = 30, N = 3) when the exposure dose was increased to 500 ppm. Thus, we proposed that exposure to 10 - 100 ppm of BZYQD is unable to affect the embryos' survival, and that these dosages may be well suitable for the subsequent toxicological evaluation.

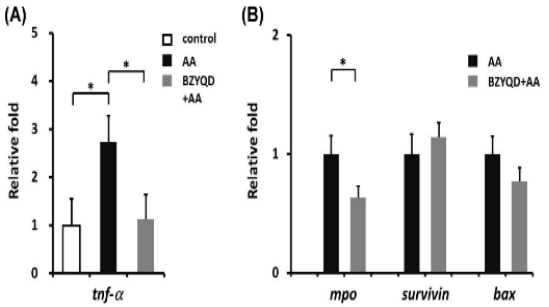

Figure 3: Quantitative gene expression was analyzed by RT-qPCR. Total RNA was extracted at the 48 hpf, and the relative expression levels of indicated genes were determined by RT-qPCR. A. Gene expression results of the indicated genes between mock control and 500 ppm of BZYQD-treated embryos. B. Gene expression results between mock control and AA-treated embryos with or without 10 ppm of BZYQD prevention. Data in triplicate were normalized for zebrafish *β-actin* expression. Error bars represent the SD. **P* < .05, ***p* < .01; Student's t-test

We next assessed the potential teratogenic effect of BZYQD on kidney development using the transgenic line Tg (*wt1b:EGFP*) by the above-mentioned exposure protocols (protocol I, II and III). While the kidneys of embryos treated with 10 ppm BZYQD appeared normal, higher doses (100 and 500 ppm) of BZYQD caused higher percentage of malformed kidney phenotypes (Figure 4 A and B).

The following statistical methods were used to further dissect the association of methods, doses and malformations. First, we applied the two-way ANOVA to the whole data (Methods I, II, III; Dose: 10, 100, 500 ppm) for testing the effects of method and dose on the malformation rate. The results showed that the p-values for method and dose effects are 0.001135 and 0.001138 respectively, indicating that there is at least one group that has significant difference. The Tukey-

Kramer HSD test was then used to pairwise-compare the marginal mean malformation rates for three treatment methods, adjusted by dose effects. It reported the adjusted mean malformation rates for method I, method II, method III are 13.3, 58.2, and 64.2 % respectively, with common standard error being 3.67 %, and also identified the malformation rates of method II and method III group as significantly higher than the malformation rate of method I at family wise error rate 0.05 (Figure 4 C). On the other hand, the marginal mean malformation rates for 10, 100 and 500 ppm groups are 23.8, 35.2, and 76.7 % respectively, with common standard error being 3.67 % and the identified malformation rates for two low dose groups do not differ from each other, but differ significantly from the high dose group (500 ppm) at familywise error rate 0.05 (Figure 4 D).

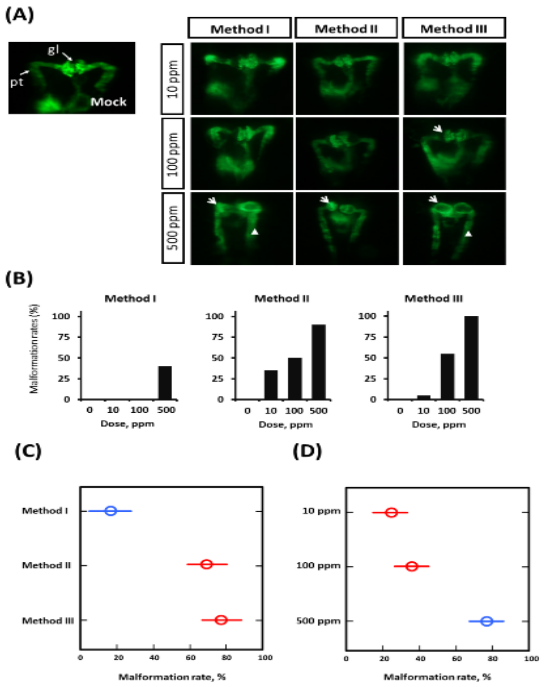

Figure 4: BZYQD-induced kidney defective phenotypes are dose-dependent. A. Morphological changes of Tg (*wt1b:EGFP*) embryos after exposure to 10, 100, and 500 ppm of BZYQD. Representative figures are shown here. All photos were taken at 48 hpf. An arrow and triangle indicate the defective sites in gl and pt, respectively. B. Kidney malformation rates of zebrafish embryos after BZYQD treatments using exposure methods I, II, and III. C, D. The Tukey-Kramer HSD test reported the marginal mean malformation rates and corresponding 95 % confidence intervals for the Dose groups, which were adjusted for Method effects

On the basis of these observations, we suggested that low dose (10 ppm) BQYZD exposure is non-toxic to kidneys, but high dose (500 ppm) is nephrotoxic. This finding was substantiated through evaluation of the integrity of pronephric ducts by α6F monoclonal antibody staining. High dose of BQZYD-treated (500 ppm) embryos displayed disorganized and broken epithelial cells around the pt regions (Figure 5).

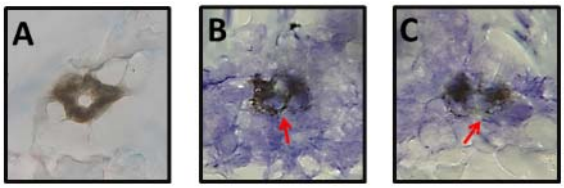

Figure 5: High dose of BZYQD impaired the integrity of pt. Immunohistochemical staining of α6F was performed on transverse sections of the pt from a 48 hpf zebrafish larvae without (A) or with 500 ppm of BZYQD. (B-C) Sections were stained with α6F and counterstained with hematoxylin. Red arrow indicates the defective sites in pt

DISCUSSION

It was reported that AA-induced organ toxicities are mainly due to inflammation [6]. AA exposure resulted in significant increase in the expression levels of pro-inflammatory genes, including *tnf-a* and *mpo* [6]. Therefore, those chemicals or natural products with anti-inflammatory effect would have the potential for AA-protective activity. In the present study, we showed that pre-treatment with BZYQD suppressed the elevated expression of pro-inflammatory genes induced by AA exposure. In this regard, which components are responsible for anti-inflammatory activity in BZYQD is an important issue that should be discussed.

Previous research thought the anti-inflammatory effect of BZYQD comes from the antioxidant properties of four components: glycyrrhizin, hesperidin, nodakenin and liquiritin [3,4]. For example, glycyrrhizin inhibits LPS-induced inflammatory mediator production in endometrial epithelial cells, and to have chemopreventive activities against UVB-radiation-induced carcinogenesis in SKH-1 mouse [11,12]; Hesperidin has the protective effect against acute alcoholic injury [13]; nodakenin is effective for treating bacterial infections, pain, diarrhea, and mast cell-mediated allergic disease [14]; liquiritin can suppress UVB-induced skin injury and attenuate rheumatoid arthritis through prevention of inflammation [15,16].

Dose is an important issue for toxicological research. In this study, we prepared 20000 ppm (20 mg/mL) of BZYOD solution as stock material from double the amount of the daily

recommended human dose. In other words, daily recommended human dose is 10000 ppm (10 mg/mL). The body weight of an adult human is around 60 kg, whereas the body weight of an adult zebrafish is around 1 g. After calculation, a recommended zebrafish dose of BZYOD is around 0.167 ppm. The results showed that 1-10 ppm (6 - 60 folds) is nearly non-toxic, but 100-500 ppm is toxic (600 - 3000 folds). On the basis of these observations, we proposed that 60-fold of BZYOD exposure might be the safety bottom line in the zebrafish model.

CONCLUSION

An efficient model to identify AA-protective compounds using zebrafish embryos has been successfully established. Using this strategy, it has been found that BZYQD is nephroprotective in zebrafish embryos, and might have the potential to be applied in humans. On the basis of these observations, 1-10 ppm of BZYQD is nephroprotective in a zebrafish model.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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