Protective effects of Zhibai Dihuang Wan on renal tubular cells affected with gentamicin-induced apoptosis

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ABSTRACT

Ethnopharmacological relevance: Zhibai Dihuang Wan (ZDW) is an ancient traditional Chinese medicine composed of eight herbal ingredients and has been used to treat chronic kidney inflammation and diabetes for thousands of years. Nonetheless, the influence of ZDW on acute kidney injury is still unknown. We intended to identify the influence of ZDW on cell growth and gentamicin-induced apoptotic injury in renal tubular cells.
Materials and methods: We extracted ZDW with artificial intestinal fluid and treated rat renal tubular cells (NRK-52E) with various concentrations of the ZDW extraction. Cell proliferation and gentamicin-induced apoptosis of NRK-52E cells were evaluated using real-time proliferation monitoring and annexin V staining, respectively. Western blotting was used to evaluate the levels of Bcl-2 and caspase-3 expression. The effect of ZDW on gentamicin-induced kidney injury was also monitored in mice using the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay, and the measurement of serum creatinine and blood urea nitrogen.
Results: We found that 30 µg/ml of ZDW promoted cell proliferation of the rat renal tubular cells. ZDW also expressed a dose-dependent protective effect against gentamicin-induced apoptosis in the cells. Pretreatment with 3 µg/ml or 30 µg/ml of ZDW maximally increased Bcl-2 and decreased cleaved caspase-3 in the gentamicin-treated NRK-52E cells. Among the herbal ingredients of ZDW, only *Phellodendron amurense* Rupr., bark (Cortex Phellodendri), and *Anemarrhena asphodeloides* Bunge, rhizome inhibited both the gentamicin-induced Bcl-2 decrease and cleaved caspase-3 increase. *Phellodendron amurense* Rupr., bark and *Anemarrhena asphodeloides* Bunge, rhizome also inhibited gentamicin-induced apoptosis at particular concentrations; however, these two ingredients were less effective than ZDW. In the mouse model of gentamicin-induced nephropathy, the ZDW treatment significantly reduced apoptotic cells in the renal cortex and improved renal function.
Conclusions: Our results suggest that ZDW at adequate doses attenuates gentamicin-induced apoptotic injury in renal tubular cells and also protects kidneys from gentamicin-induced injury in mice.

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1. Introduction

Herbal medicines have been used to treat various diseases for many centuries, especially in Chinese society. Recently, the potential side effects of herbs on the kidney have been reported (Isnard Bagnis et al., 2004). In 1991, nephrologists in Belgium reported on many young women who had ingested extracts of Chinese herbs containing *Aristolochia fangchi* in diet regimens who presented with different degrees of renal failure (Vanherweghem et al., 1993). The exposure to the inadvertently added *Aristolochia* plant containing aristolochic acid (AA) was proven to be the main cause of the renal injury, urothelial-cell atypia, and carcinoma (De Broe, 2012). As traditional Chinese medicine is practiced generally in Taiwan and mainland China, AA nephropathy is prevalent in these areas and is a potential public health problem (Grollman, 2013). Most nephrologists in Taiwan recommend patients avoid traditional herbal medicine. Nonetheless, some traditional herbal medicines have shown promise in treatment of kidney disease in

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preclinical studies or clinical trials (Wojcikowski et al., 2006; Wang et al., 2012; Zhang et al., 2012). While avoiding the use of *Aristolochia* plants in herbal medicines is essential, herbal medicines are still potential candidates for treatment of certain kidney diseases.

Zhibai Dihuang Wan (ZDW), a polyherbal formula, has been used to treat chronic kidney inflammation and diabetes for thousands of years. ZDW is made from *Cornus officinalis* Siebold & Zucc (13.8%), *Rehmannia glutinosa* (Gaertn.) DC., root, baked (Radix Rehmanniae preparata, 27.6%), *Dioscorea oppositifolia* L. (13.8%), *Phellodendron amurense* Rupr., bark (Cortex Phellodendri, 6.9%), *Anemarrhena asphodeloides* Bunge, rhizome (6.9%), *Paeonia suffruticosa* Andrews, root bark (Moutan Cortex, 10.3%), *Alisma plantago-aquatica* L., rhizome (Rhizoma Alismatis, 10.3%) and *Poria cocos* (Schw.) Wolf (10.3%), and does not contain *Aristolochia*. Among these ingredients, *Cornus officinalis* Siebold & Zucc, *Rehmannia glutinosa* (Gaertn.) DC., root, baked, *Phellodendron amurense* Rupr., bark, and *Poria cocos* are reported to be beneficial for diabetic nephropathy or chronic renal injury in animal models (Yokozawa et al., 2004; Kim et al., 2008; Jiang et al., 2012; Zhao et al., 2013). ZDW without *Anemarrhena asphodeloides* Bunge and *Phellodendron amurense* Rupr., bark also had a partial protective effect on early diabetic nephropathy in rats (He et al., 2007). These findings suggest that ZDW has potentially protective effects against renal injury. However, the influence of ZDW on cell growth and apoptosis of renal tubular cells is still unclear.

Gentamicin is a widely used aminoglycoside antibiotic to treat gram-negative bacterial infections, but it induces acute kidney injury in about 30% of patients (Singh et al., 2012). In renal proximal tubular cells, inducing apoptosis is a key cytotoxic mechanism of gentamicin (Servais et al., 2006). Gentamicin induces apoptosis through a mitochondrion-mediated signaling pathway, including decreasing Bcl-x$_L$ and increasing cleaved caspase-3 and -9 (Ali, 2003; Juan et al., 2007). To date there is no ideal clinical remedy to prevent gentamicin-induced acute kidney injury. In this study, we investigated the influence of ZDW on gentamicin-induced apoptosis in renal tubular cells *in vitro* and *in vivo*. Our data revealed the protective effect of ZDW on gentamicin-treated renal tubular cells and further showed contributions of its ingredients to the protective effect of ZDW.

2. Materials and methods

2.1. Reagents and preparation of ZDW

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD, USA). All other chemicals of reagent grade were obtained from Sigma Chemical Company (St. Louis, MO, USA). The powders of ZDW (batch no. 051941) and its ingredients (*Cornus officinalis* Siebold & Zucc, batch no. C810Y; *Rehmannia glutinosa* (Gaertn.) DC., root, baked, batch no. C730N; *Dioscorea oppositifolia* L., batch no. C624N; *Phellodendron amurense* Rupr., bark, batch no. C722Y; *Anemarrhena asphodeloides* Bunge, rhizome, batch no. C805N; *Paeonia suffruticosa* Andrews, root bark, batch no. C813Y; *Alisma plantago-aquatica* L., rhizome, batch no. C819N; *Poria cocos* (Schw.) Wolf, batch no. C827N) were purchased from Sun Ten Pharmaceutical Co. (Taipei, Taiwan). According to the manufacturer's instructions, 29 g of 8-herb mixture (4 g *Cornus officinalis* Siebold & Zucc, 8 g *Rehmannia glutinosa* (Gaertn.) DC., root, baked, 4 g *Dioscorea oppositifolia* L., 2 g *Phellodendron amurense* Rupr., bark, 2 g *Anemarrhena asphodeloides* Bunge, rhizome, 3 g *Paeonia suffruticosa* Andrews, root bark, 3 g *Alisma plantago-aquatica* L., rhizome and 3 g *Poria cocos* (Schw.) Wolf) was boiled in 290 ml of distilled water for 4 h at 100 °C, filtered, lyophilized to yield 7.5 g dry extracts, and then mixed with 2.2 g corn starch and 5.3 g powdered cellulose to generate 15 g ZDW.

All the herbs were verified by a herbology professor from Brion Research Institute of Taiwan, R.O.C. The voucher specimens were deposited in Herbarium of National Research Institute of Chinese Medicine, Taiwan, R.O.C. (voucher numbers: *Cornus officinalis* Siebold & Zucc - NHP-00044, *Rehmannia glutinosa* (Gaertn.) DC., root, baked - NHP-00411, *Dioscorea oppositifolia* L. - NHP-00600, *Phellodendron amurense* Rupr., bark - NHP-00369, *Anemarrhena asphodeloides* Bunge, rhizome - NHP-00196, *Paeonia suffruticosa* Andrews, root bark - NHP-00169, *Alisma plantago-aquatica* L., rhizome - NHP-00424, *Poria cocos* (Schw.) Wolf - NHP-00309). The herbal medicines (0.1 g) were digested with 1 ml of artificial intestinal fluid (1% pancreatin, 50 mM potassium dihydrogen phosphate, pH 7.5) at 37 °C for 2 h, filtered through 0.22-µm filters, and then applied in cell experiments. In addition, artificial intestinal fluid alone was also incubated at 37 °C for 2 h, filtered through 0.22-µm filters, and then applied in control groups.

2.2. Fingerprint analysis by high performance liquid chromatography (HPLC)

The quality of ZDW was measured by HPLC. In brief, 0.1 g ZDW powder was dissolved in deionic water at room temperature for 2 h, filtered through 0.22-µm filters, and then applied in HPLC analysis. One hundred µl of the solution were applied to a 250 × 4.6 mm C18-column (Grace. Columbia, USA) using a Waters HPLC system equipped with a 600 controller and a 996 photodiode array detector (Waters, St. Massachusetts, USA). The sample was then eluted with a 0–95% linear methanol gradient in deionic water, pH 7.0, over 60 min at a flow rate of 0.8 ml/min. The eluents were detected and quantified at 254 nm from 0 min to 60 min at room temperature. The analysis was carried out 10 times and the results are consistent.

2.3. Cell culture

We purchased rat renal proximal tubular cells (NRK-52E) from the Bioresource Collection and Research Center (Hsinchu, Taiwan). The cells were cultured in DMEM supplemented with antibiotic/antifungal solution and 10% fetal bovine serum. They were grown until the monolayer became confluent. The cultured cells were then cultured in the serum-free medium overnight before the experiment. ZDW extracts by artificial intestinal fluid were added to starved cells at indicated concentration, and incubated artificial intestinal fluid was applied as control treatment. After 24 h incubation, cell samples were harvested and applied in apoptosis analysis and Western blotting analysis.

2.4. Real-time monitoring of cell proliferation

To monitor cell proliferation, we used the "xCELLigence" system from Roche Applied Sciences (Indianapolis, IN, USA). NRK-52E cells were seeded in duplicate into E-plates at a density of 10000 cells/well. E-plates were then transferred to the xCELLigence RTCA DP instrument for automated real-time monitoring in a humidified incubator containing 5% CO_2 at 37 °C. After 24 h, the cells in the E-plates were starved overnight. For the proliferation assay, ZDW extracts were added to the cells for 30 min, and then 3 mmol/l of gentamicin was added to the cells. The cell index, an arbitrary unit reflecting the cell-sensor impedance, was measured every 15 min. Slope calculation was performed with the RTCA software version 1.2 (Roche Applied Sciences). Three independent experiments were performed.

2.5. Apoptosis detection

Fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) double staining was used to detect apoptosis induced by gentamicin treatment. Treated NRK-52E cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS). Specific binding of FITC-annexin V and staining with PI were performed using an apoptosis detection kit (BD Biosciences-Pharmingen, San Diego, CA, USA), according to the manufacturer's instructions. Cells were then analyzed using flow cytometry.

2.6. Western blot analysis

Twenty micrograms of NRK-52E protein lysate were applied to each lane of the blotting strip and analyzed by Western blotting. Antibodies for Bcl-2 and caspase-3 were purchased from Cell Signaling Technology (Danvers, MA, USA), and diluted 1:1000 for the assay. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected as a loading control. Relative levels of the protein bands were quantified using Quantiscan software (Biosoft, Cambridge, United Kingdom).

2.7. Animals and treatments

Male BALB/c mice 8 weeks of age and weighing 20–25 g were obtained from BioLasco Taiwan (Taipei, Taiwan). All animal experiments were approved by the Taipei Medical University Committee of Experimental Animal Care and Use. Animals were housed in a central facility, subjected to a 12-h light-dark cycle, and provided with regular rat chow and tap water. The experimental group of mice ($n=6$) for gentamicin treatment were subjected to intraperitoneal (IP) injection with gentamicin (20 mg/kg/day) for 10 days. The ZDW powder (1 or 2 g/kg) was dissolved in saline and orally administered prior to gentamicin injection each time. Mice in the control group ($n=6$) were given saline orally and intraperitoneally. All mice were anesthetized by using pentobarbital 30–50 mg/kg body weight intraperitoneally (Sigma, St. Louis, MO) 1 day after their last injection. Animals were then sacrificed while still under the effect of anesthesia, and blood samples were collected from the heart for the measurement of serum creatinine and blood urea nitrogen. The kidneys were harvested by laparotomy, and the renal cortex tissue was snap-frozen in liquid nitrogen and stored at $-80\ ^{\circ}C$.

2.8. In situ terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay

Forzen tissue sections (4 μm) of the kidney were processed with the ApopTag Fluorescein in situ apoptosis detection kit (CHEMICON International, Inc., CA, USA) according to the manufacturer's instructions. Briefly, kidney tissue slides were pretreated with proteinase K and H_2O_2, and incubated with the reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-conjugated dUTP for 1 h at 37 $^{\circ}C$, mounted with mounting solution containing PI, and finally observed with fluorescence microscopy.

2.9. Statistical analyses

Data are presented as means $\pm$ standard deviations (SDs). Statistical analyses were performed using Student t-tests, where $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Influence of ZDW on cell proliferation

For the quality control of ZDW, we employed HPLC to establish the fingerprint chromatogram as shown in Fig. 1. We first investigated the influence of ZDW extracts on proliferation of the NRK-52E cells. To mimic the gastro-intestinal environment, ZDW was digested and extracted using artificial intestinal fluid, and then applied in cell experiments. In real-time monitoring of cell proliferation, both proliferation curves and slopes showed that the cells treated with 30 μg/ml of ZDW had higher proliferation rates compared to the artificial intestinal fluid-treated control cells (Fig. 2A and B). In contrast, 300 μg/ml of ZDW significantly reduced the proliferation rates. The dosage of 30 μg/ml ZDW contains 4 μg/ml Cornus officinalis Siebold & Zucc, 8 μg/ml Rehmannia glutinosa (Gaertn.) DC., root, baked, 4 μg/ml Dioscorea oppositifolia L., 2 μg/ml Phellodendron amurense Rupr., bark, 2 μg/ml Anemarrhena asphodeloides Bunge, rhizome, 3 μg/ml Paeonia suffruticosa Andrews, root bark, 3 μg/ml Alisma plantago-aquatica L., rhizome and 4 μg/ml Poria cocos (Schw.) Wolf. However, these dosages of the ingredients reduced NRK-52E cell proliferation instead (Fig. 2C). Gentamicin significantly inhibited cell growth and even decreased the cell number index, which partially recovered with treatment by 3 or 30 μg/ml of ZDW as shown in Fig. 2A. High doses of ZDW (300 μg/ml) further increased the inhibitory effect of gentamicin on cell
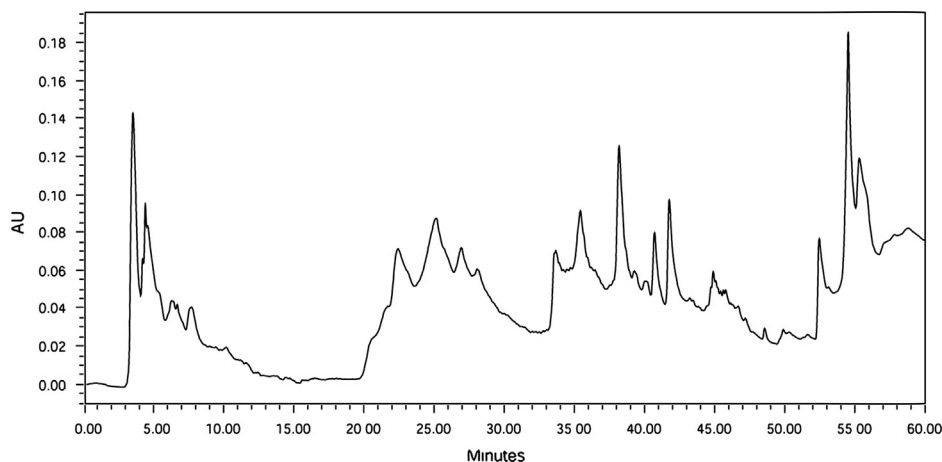

Fig. 1. HPLC chromatogram of the aqueous extract of Zhibai Dihuang Wan (ZDW).
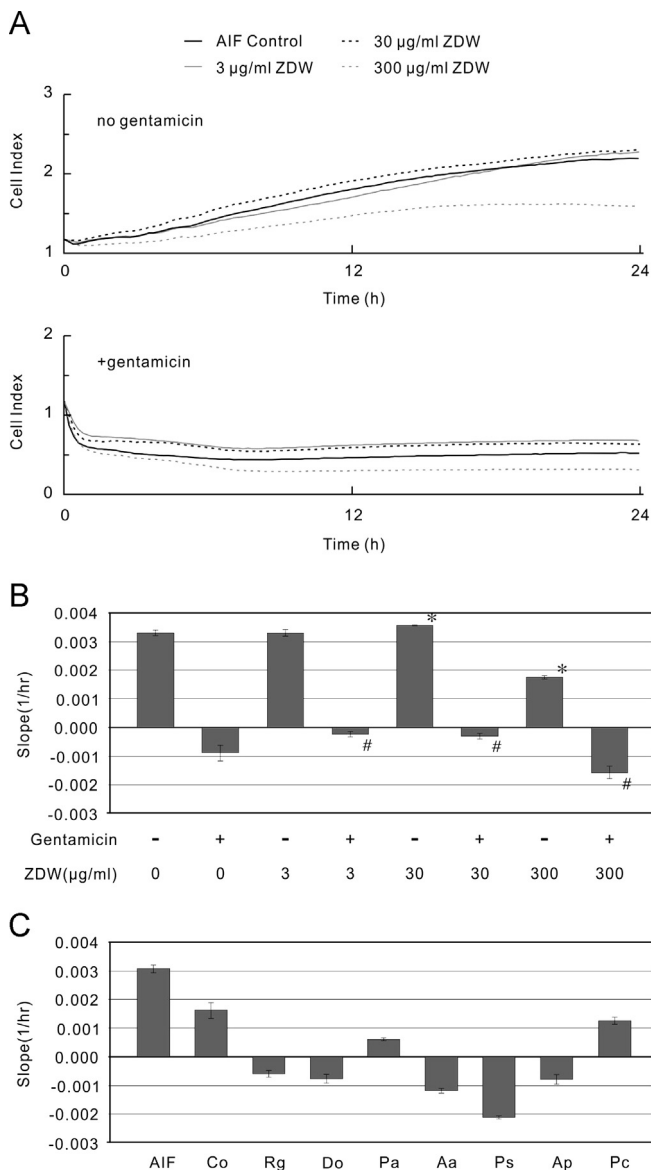
Fig. 2. Effects of Zhibai Dihuang Wan (ZDW) on the proliferative capacity of NRK-52E cells. NRK-52E cells were seeded in proliferation plates and pretreated with ZDW at the indicated concentrations or artificial intestinal fluid (30 ppm self-digested pancreatin) for 30 min and then treated with or without 3 mmol/l gentamicin and real-time cell proliferation was measured. (A) Proliferation curves monitored over 24 h. (B) Proliferation slope. Results are means ± SDs ($n=3$). *$P < 0.05$ compared with the control group. #$P < 0.05$ compared with the group treated with gentamicin alone. (C) Effects of the ingredients of Zhibai Dihuang Wan (ZDW) on the proliferative capacity. NRK-52E cells were seeded in proliferation plates and pretreated with the ingredients of ZDW or artificial intestinal fluid and real-time cell proliferation was measured. Proliferation slope is shown as means ± SDs ($n=3$). AIF, artificial intestinal fluid; Co, *Cornus officinalis* Siebold & Zucc (4 μg/ml); Rg, *Rehmannia glutinosa* (Gaertn.) DC., root, baked (8 μg/ml); Do, *Dioscorea oppositifolia* L. (4 μg/ml); Pa, *Phellodendron amurense* Rupr., bark (2 μg/ml); Aa, *Anemarrhena asphodeloides* Bunge, rhizome (2 μg/ml); Ps, *Paeonia suffruticosa* Andrews, root bark (3 μg/ml); Ap, *Alisma plantago-aquatica* L., rhizome (3 μg/ml); Pc, *Poria cocos* (Schw.) Wolf (4 μg/ml).

proliferative capacity. Our results suggest that low dose ZDW (3–30 μg/ml) promotes proliferation of NRK-52E cells, even in the presence of gentamicin treatment.

3.2. Influence of ZDW on apoptosis

We next evaluated the influence of ZDW extracts on the gentamicin-induced apoptosis in NRK-52E cells by annexin V/PI staining and flow cytometry. As shown in Fig. 3, ZDW extracts at

from 0.3 to 300 μg/ml did not induce significant apoptosis in NRK-52E cells. Exposure to 3 mmol/l of gentamicin markedly increased apoptotic cells. Pretreatment of the cells with ZDW significantly reduced gentamicin-induced apoptosis in a dose-dependent manner, and the inhibitory effect peaked at 30 μg/ml and decreased at 300 μg/ml of ZDW (Fig. 3). ZDW extract protected NRK-52E cells from gentamicin-induced apoptosis. To confirm this result, we also monitored the influence of ZDW on the apoptotic signaling pathway induced by gentamicin. In normal NRK-52E cells, ZDW concentrations from 0.3 to 300 μg/ml did not significantly influence the expression of Bcl-2 and the cleavage of caspase-3 (Fig. 4). Gentamicin treatment significantly reduced the expression of Bcl-2 and induced cleavage of caspase-3 in NRK-52E cells. Pretreatment of cells with ZDW improved the expression of Bcl-2 in gentamicin-treated cells, and significantly reduced gentamicin-induced caspase-3 cleavage (Fig. 4).

3.3. Influence of ZDW ingredients on apoptosis

The results revealed that the most effective concentration of ZDW was close to 30 μg/ml. In theory, 30 μg/ml ZDW contains 4 μg/
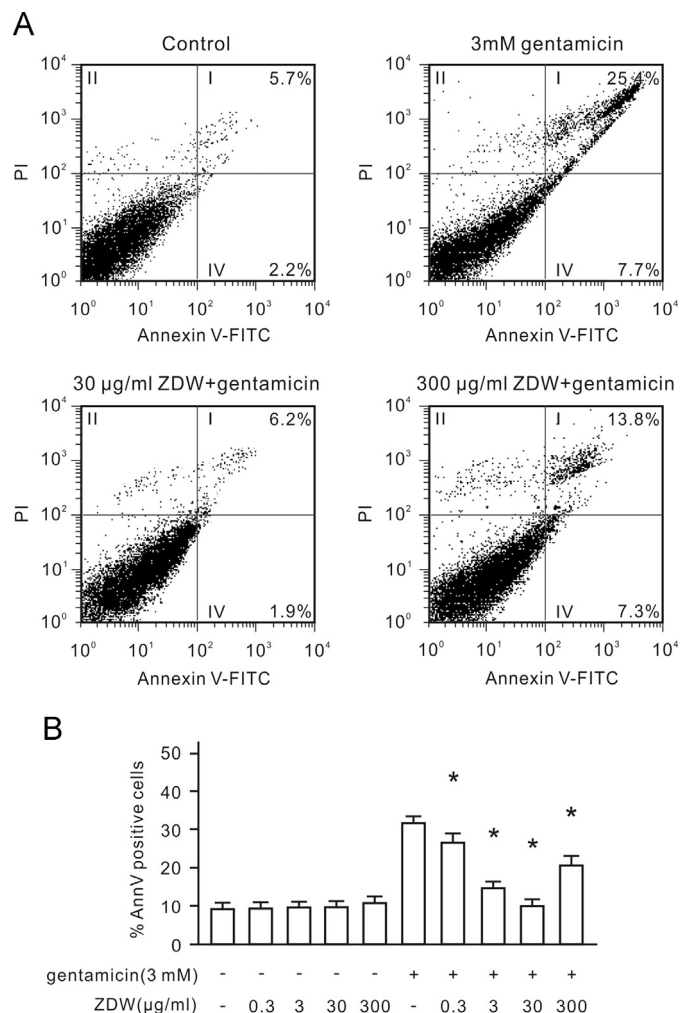

Fig. 3. Protective effect of Zhibai Dihuang Wan (ZDW) against gentamicin-induced apoptosis in NRK-52E cells. NRK-52E cells were pretreated with ZDW at the indicated concentrations for 30 min or artificial intestinal fluid (30 ppm self-digested pancreatin), and then treated with 3 mmol/l of gentamicin for 24 h. Treated cells were then stained with annexin V/propidium iodide and analyzed using flow cytometry. (A) Representative flow cytometric data. The sum of cell percentages in quadrants I and IV represents the percentage of apoptosis. (B) The apoptotic percentage in cells treated with ZDW. Results are means ± SDs ($n=3$). *$P < 0.05$ compared with the gentamicin alone group.
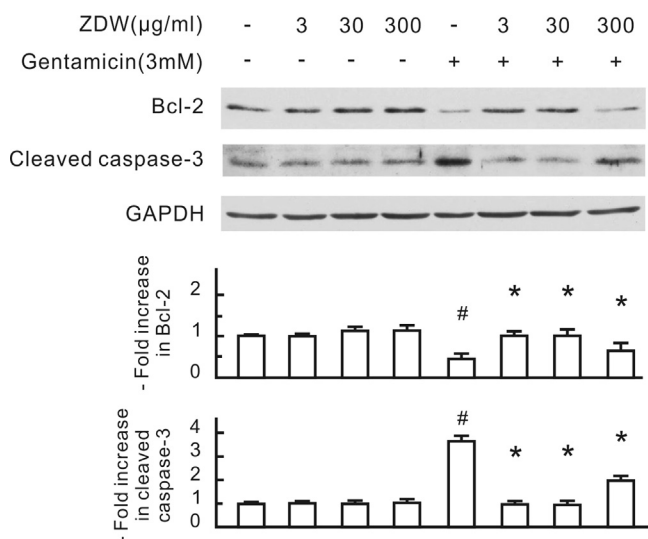
Fig. 4. Influence of Zhibai Dihuang Wan (ZDW) on gentamicin-induced apoptotic signals. NRK-52E cells were pretreated with ZDW at indicated concentrations for 30 min or artificial intestinal fluid (30 ppm self-digested pancreatin), and then treated with 3 mmol/l of gentamicin for 24 h. Bcl-2 and cleaved caspase-3 were detected using Western blotting with appropriate antibodies. GAPDH was used as the loading control. Relative increases in the protein bands are also presented in bar chart form. Results are expressed as mean ± SDs ($n=3$). *$P < 0.05$ compared with the gentamicin alone group. #$P < 0.05$ compared with the control group without any treatment.

Fig. 5. Effect of individual Zhibai Dihuang Wan (ZDW) ingredients on gentamicin-induced apoptotic signals. NRK-52E cells were pretreated with the each of the ingredients of ZDW at indicated concentrations or artificial intestinal fluid (30 ppm self-digested pancreatin) for 30 min and then treated with 3 mmol/l of gentamicin for 24 h. Bcl-2 and cleaved caspase-3 were detected using Western blotting with appropriate antibodies. GAPDH was used as the loading control. The representative blots were chosen from 3 experiments performed in duplicate. * Denotes obvious increase compared with the gentamicin alone group. # Denotes obvious decrease compared with the gentamicin alone group.

ml *Cornus officinalis* Siebold & Zucc, 8 μg/ml *Rehmannia glutinosa* (Gaertn.) DC., root, baked, 4 μg/ml *Dioscorea oppositifolia* L., 2 μg/ml *Phellodendron amurense* Rupr., bark, 2 μg/ml *Anemarrhena asphodeloides* Bunge, rhizoma, 3 μg/ml *Paeonia suffruticosa* Andrews, root bark, 3 μg/ml *Alisma plantago-aquatica* L., rhizome and 3 μg/ml *Poria cocos*. However, the extracts of these ingredients at the indicated concentrations did not influence the proliferation of NRK-52E cells as revealed by real-time monitoring of cell proliferation (data not shown). We further monitored the ingredients of ZDW separately for their effects on the expression of Bcl-2 and caspase-3 cleavage of gentamicin-treated NRK-52E cells. Fig. 5 shows that *Cornus officinalis* Sieb. et Zucc, *Rehmannia glutinosa* (Gaertn.) DC., root, baked, *Dioscorea opposita*, *Paeonia suffruticosa* Andrews, root bark, *Alisma plantago-aquatica* L., rhizome and *Poria cocos* did not influence the expression of Bcl-2 and caspase-3 cleavage in gentamicin-treated cells. Only 0.2 μg/ml *Phellodendron amurense* Rupr., bark and *Anemarrhena asphodeloides* Bunge, rhizoma at 4 and 60 μg/ml inhibited both gentamicin-induced Bcl-2 decrease and caspase-3 cleavage increase at the same time (Fig. 5). Similar to the Western blotting results, annexin V/PI staining revealed that *Phellodendron amurense* Rupr., bark at 0.2 μg/ml and *Anemarrhena asphodeloides* Bunge, rhizoma at 4 and 60 μg/ml significantly inhibited gentamicin-induced apoptosis (Fig. 6). The other herbal ingredients didn't express antiapoptotic effect on gentamicin-treated NRK-52E cells as revealed by flow cytometry (data not shown). ZDW contains 6.9% *Anemarrhena asphodeloides* Bunge, rhizoma. The dosage of 60 μg/ml of *Anemarrhena asphodeloides* Bunge, rhizoma was well over the quantity contained in 300 μg/ml of ZDW, which was the highest dosage used in this study. The *Phellodendron amurense* Rupr., bark dosage of 0.2 μg/ml was similar to the quantity of *Phellodendron amurense* Rupr., bark contained in 3 μg/ml of ZDW. *Anemarrhena asphodeloides* Bunge, rhizoma dosage of 4 μg/ml was close to the quantity of *Anemarrhena asphodeloides* Bunge, rhizoma contained in 30 μg/ml of ZDW. The ZDW concentrations of both 3 and 30 μg/ml had significant anti-apoptotic effects (Fig. 2), which may partially result from
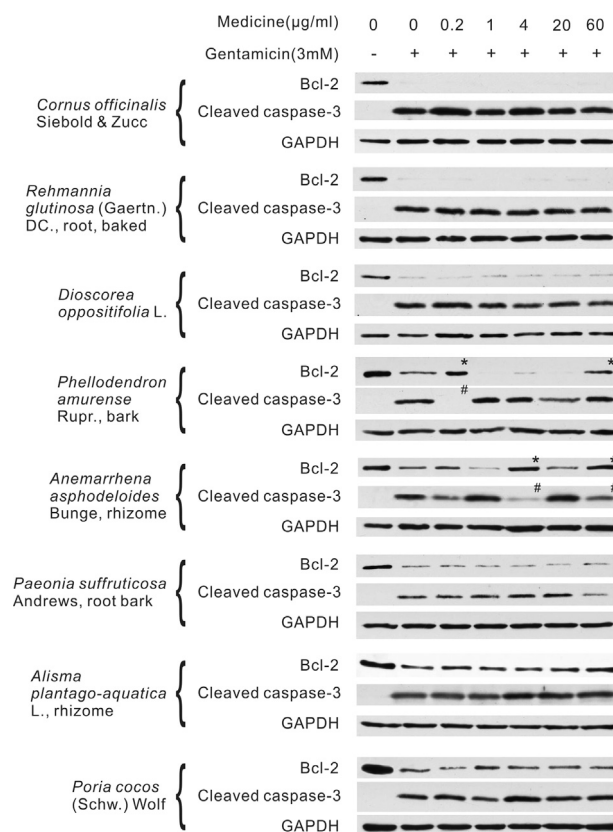
Phellodendron amurense Rupr., bark and *Anemarrhena asphodeloides* Bunge, rhizoma.

3.4. Protective effect of ZDW on the kidneys

The protective effect of ZDW on gentamicin-induced apoptosis was further proven in a mouse animal model. Mice were treated with gentamicin (20 mg/kg/day) or saline as controls; the experimental groups were treated with ZDW (1 or 2 g/kg/day) in addition. At the end of the treatment period (9 days), the kidneys were harvested by laparotomy and sectioned for *in situ* TUNEL assays. As shown in Fig. 7, the nuclei in kidney sections were revealed as bright spots stained with PI. The scattered and bright nuclei stained by TUNEL could be detected over the entire renal cortex in gentamicin-treated animals, yet they were rarely detected in the control specimens. ZDW treatment reduced gentamicin-induced apoptotic nuclei in a dose-dependent manner. Most of the TUNEL-labeled nuclei were seen in the proximal tubule epithelium. ZDW inhibited the *in vivo* gentamicin-induced cell apoptosis in rat renal tubular cells. The renal function of the experimental mice was also monitored by measuring the concentration of blood urea nitrogen and serum creatinine. Fig. 8 shows that the concentrations of blood urea nitrogen and serum creatinine were elevated in the gentamicin-treated groups, and ZDW treatment significantly inhibited these gentamicin-induced increases. These results suggest that ZDW expresses a renal protective effect *in vivo*.
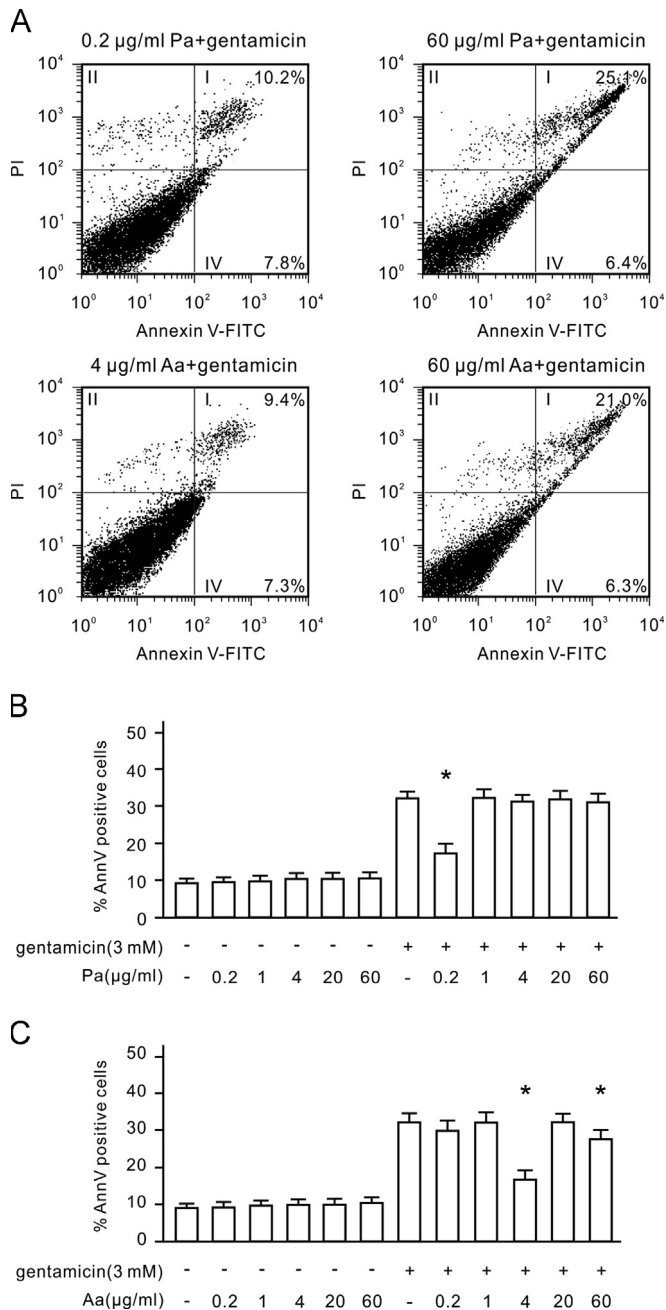
Fig. 6. Protective effects of *Phellodendron amurense* Rupr., bark and *Anemarrhena asphodeloides* Bunge, rhizoma against gentamicin-induced apoptosis in NRK-52E cells. NRK-52E cells were pretreated with *Phellodendron amurense* Rupr., bark or *Anemarrhena asphodeloides* Bunge, rhizoma at indicated concentrations for 30 min or artificial intestinal fluid (30 ppm self-digested pancreatin), and then treated with 3 mmol/l of gentamicin for 24 h. Treated cells were stained with annexin V/ propidium iodide and analyzed using flow cytometry. (A) Representative flow cytometric data. The sum of cell percentages in quadrants I and IV represents the percentage of apoptosis. (B) The apoptotic percentage in cells treated with *Phellodendron amurense* Rupr., bark. (C) The apoptotic percentage in cells treated with *Anemarrhena asphodeloides* Bunge, rhizoma. Results are the means ± SDs ($n=3$). *$P < 0.05$ compared with the gentamicin alone group. Pa, *Phellodendron amurense* Rupr., bark. Aa, *Anemarrhena asphodeloides* Bunge, rhizoma.

4. Discussion

In this study, we focused on the effect of ZDW on gentamicin-induced apoptosis in rat proximal renal tubular cells. The principal findings showed that ZDW (3 and 30 µg/ml) attenuated gentamicin-induced apoptotic injury in NRK-52E cells (Fig. 3). Pretreatment of ZDW also inhibited gentamicin-induced apoptotic signals, including
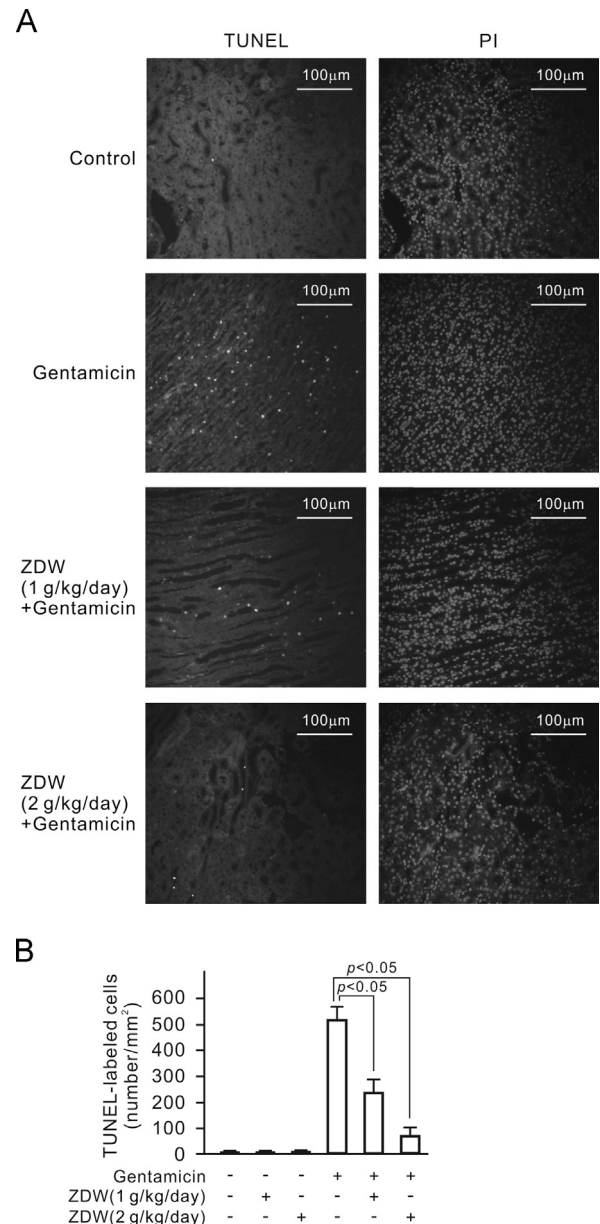

Fig. 7. Protective effects of Zhibai Dihuang Wan (ZDW) against gentamicin-induced renal tubular cell apoptosis *in vivo*. Mice were or were not fed ZDW and then were injected with saline or gentamicin. Apoptotic cells in the kidneys of experimental animals were detected using terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL). In panel A, the nuclei stained with TUNEL were revealed as bright spots in the cortex sections from untreated and treated mice. The identical fields stained for TUNEL were also stained using PI to reveal the positions of the cell nuclei. The numbers of TUNEL-labeled cells per mm^2 of cortex area in each sample were compiled and demonstrated in panel B. Data are the means ± SDs ($n=6$).

Bcl-2 decrease and caspase-3 cleavage increase (Fig. 4). In a mouse model of gentamicin toxicity, the ZDW treatment significantly reduced apoptotic cells in the renal cortex and improved renal function (Figs. 7 and 8). Our results suggest that ZDW protects renal tubular cells from gentamicin-induced apoptosis. Additionally, we also found that 30 µg/ml ZDW promoted the proliferation of NRK-52E cells (Fig. 2), which could contribute to the recovery of renal tubules from acute kidney disease. However, the high-dose ZDW (300 µg/ml) reduced the cell proliferation and its antiapoptotic effect also decreased (Figs. 2 and 3). These results show that ZDW at high doses is cytotoxic to renal tubular cells. Therefore, we suggest the
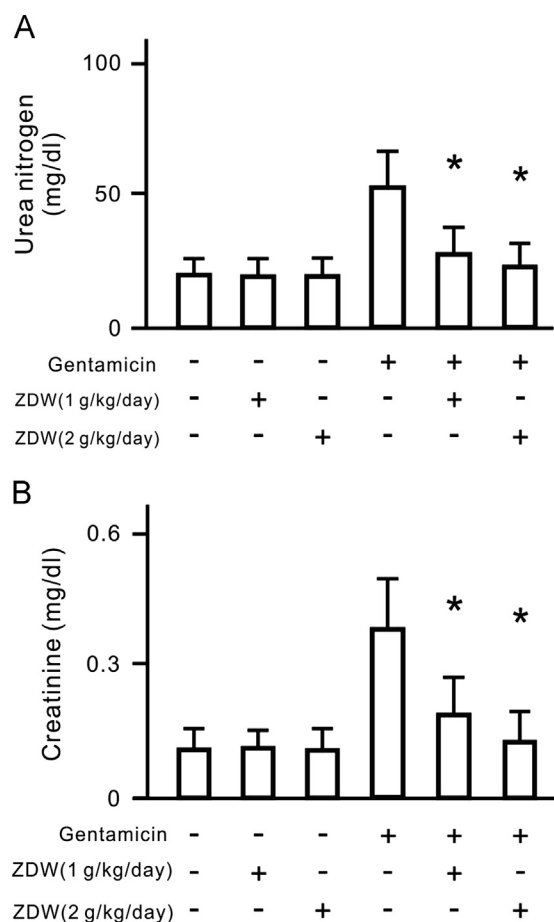
A

B

Fig. 8. Protective effects of Zhibai Dihuang Wan (ZDW) on renal function. (A) Concentrations of blood urea nitrogen in treated mice. Results are means $\pm$ SD ($n=6$). $^*P < 0.05$ compared with the gentamicin treatment alone. (D) Concentrations of serum creatinine in treated mice. Results are the mean $\pm$ SD ($n=6$). $^*P < 0.05$ compared with the gentamicin treatment alone.

administration of ZDW at adequate doses would be beneficial for patients with gentamicin-induced kidney injury.

The herbal medicines used in the cell experiments were digested and extracted using artificial intestinal fluid containing pancreatin. Pancreatin is composed of amylase, lipase, and protease. In principle, the bioaccessible molecules in herbal medicines can be obtained using a laboratory-based extraction that mimics the gastrointestinal environment in such details as chemicals and enzymes present, temperature, exposure times, and pH. In our system, renal tubular cells had a chance to interact with the bioaccessible molecules in herbal medicines, but not the raw materials. The effective bioaccessible molecules of ZDW in our system are still unknown. Detecting absorption of ZDW in renal tubular cells is difficult so far because there is no suitable target to monitor. However, the artificial intestinal extracts of ZDW exerted a dose-dependent effect in cellular experiments. These experiments suggest that the artificial intestinal extracts of ZDW contain effective bioaccessible molecules acting on renal tubular cells. On the other hand, oral ZDW also dose-dependently expressed renal protective effect in gentamicin-treated mice (Figs. 7 and 8). These data imply that the effective molecules of the artificial intestinal extracts of ZDW may also exist in the kidneys of ZDW-treated mice. Our results show pharmacological consistency between the cellular studies with artificial intestinal extracts of herbal medicines and the animal studies with oral herbal medicines. The application of artificial intestinal fluids in cellular studies is available recently. In Caco-2 cells, artificial intestinal fluids were used to investigate the

cellular uptake characteristics of L-valyl-ara-C (Cheon et al., 2006). The oral bioavailability of soil contaminants was measured *in vitro* using artificial intestinal fluids (Ellickson et al., 2001). Bioaccessibility of arsenic in various types of rice was also studied using an *in vitro* gastrointestinal fluid system (He et al., 2012). Artificial gastrointestinal extraction yielded results in reasonable agreement with clinical measurements of arsenic bioavailability measured by urinalysis in adult humans following ingestion of arsenic-containing traditional Chinese medicine (Koch et al., 2007). Additionally, pancreatin in artificial intestinal fluid will be self-digested and lose its enzymatic activity during 37 °C incubation. The concentration of pancreatin in cell experiments was also very low (0.3~30 ppm). The influence of pancreatin on cell proliferation and apoptosis is very limited. We suggest that artificial intestinal fluid extraction is one of the ideal extraction methods for traditional herbal medicines in cell culture experiments.

Among the herbal ingredients of ZDW, only *Phellodendron amurense* Rupr., bark and *Anemarrhena asphodeloides* Bunge, rhizome inhibited gentamicin-induced apoptosis at particular concentrations (Figs. 5 and 6). *Phellodendron amurense* Rupr., bark and *Anemarrhena asphodeloides* Bunge, rhizome should play a role in the protective effect of ZDW in NRK-52E cells. *Phellodendron amurense* Rupr., bark contains a number of alkaloids such as berberine, palmatine, and jatrorrhizine. *Phellodendron amurense* Rupr., bark is a known anti-inflammatory agent (Mori et al., 1995; Uchiyama et al., 1989) and reduces oxidative stress in the kidneys of diabetic rats (Kim et al., 2008). *Anemarrhena asphodeloides* Bunge is widely used in Chinese traditional medicines. The rhizomes of *Anemarrhena asphodeloides* Bunge have antidiabetic, antiplatelet aggregation, and diuretic activities (Takahashi et al., 1985). Timosaponins isolated from the rhizomes of *Anemarrhena asphodeloides* Bunge suppresses platelet aggregation and AA-induced superoxide generation in human neutrophils (Zhang et al., 1999a, 1999b). *Anemarrhena asphodeloides* Bunge also plays a crucial protective role in ischemia-induced brain injury (Oh et al., 2007). In contrast to ZDW, *Phellodendron amurense* Rupr., bark and *Anemarrhena asphodeloides* Bunge, rhizome expressed anti-apoptotic effects over a limited concentration range (Figs. 3 and 6). Additionally, 30 μg/ml ZDW promoted cell proliferation, but all the ingredients in 30 μg/ml ZDW reduced cell proliferation instead (Fig. 2C). This result suggests that the proliferation-promoting effect of ZDW may not result from its ingredients individually. The protective effect of ZDW could result, at least in part, from synergistic effects caused by the ingredients or from the generation of modified molecules formed in the pharmaceutical compounding processes of ZDW. Traditionally many Chinese medicine practitioners believe that a polyherbal formula is usually more effective than a single herbal medicine, which is supported by our results.

In this study, the gentamicin concentration applied in the cell culture experiments was similar to previously published studies (Juan et al., 2007). Gentamicin concentrations of 1–3 mmol/l effectively induced apoptosis in renal tubular cells, and this concentration range is close to the *in vivo* kidney levels found during gentamicin antimicrobial treatments in mice and rats (El Mouedden et al., 2000a, 2000b; Sandoval and Molitoris, 2004; Servais et al., 2005). In other words, the gentamicin concentrations employed in this *in vitro* study were similar to the concentrations found in mouse kidneys *in vivo* during gentamicin treatment. In our mouse experiment, gentamicin was administered at 20 mg/kg intraperitoneally once daily, and gentamicin-induced apoptosis was observed in the mouse kidneys (Fig. 7). This dosage is suggested as equal maintenance doses for an adult patient with an estimated creatinine clearance of 90 ml/min (El Mouedden et al., 2000a). Additionally, the protective dosage of ZDW was 1 to 2 g/kg/day in this study. A mouse is 7 to 8 times less metabolically

efficient than a human is. Considering the difference between the metabolic rates of mice and humans, the ZDW dosage in animal studies is close to the traditional application of ZDW in humans. These findings provide the basis for future investigations in humans.

Caspase-dependent apoptotic signaling plays a key role in gentamicin-induced apoptosis. In the mitochondrial pathway, caspase-3 is an executioner caspase that can be cleaved to active by caspase-9 that is activated from procaspase-9 by cytosolic cytochrome c (Padanilam, 2003; Jiang and Wang, 2004). The mitochondrial release of cytochrome c is regulated by the Bcl-2 family proteins that bind to the mitochondrial outer membrane and block cytochrome c efflux (Yang et al., 1997). In our study, gentamicin increased caspase-3 cleavage and reduced the Bcl-2 expression in NRK-52E cells. These mitochondria-mediated apoptotic phenomena were reversed by ZDW treatment. Based on the findings of this study, we suggested that ZDW protects renal tubular cells from gentamicin-induced apoptotic injury through inhibition of the mitochondrial pathway.

5. Conclusions

In summary, ZDW at adequate doses promoted cell proliferation and inhibited gentamicin-induced apoptosis in rat renal tubular cells. ZDW also reduce gentamicin-induced renal toxicity *in vivo*. ZDW could have a therapeutic potential for patients with gentamicin-induced kidney injury.

Acknowledgements

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