Schisandra chinensis extract decreases chloroacetaldehyde production in rats and attenuates cyclophosphamide toxicity in liver, kidney and brain

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Schisandra chinensis extract decreases chloroacetaldehyde production in rats and attenuates cyclophosphamide toxicity in liver, kidney and brain

Jianxiu Zhai,a, b Feng Zhangb, Shouhong Gaob, Li Chenb, c Ge Fengb, c, Jun Yin,a, *, Wansheng Chenb, c

a School of Traditional Chinese Material, Shenyang Pharmaceutical University, Shenyang, China
b Department of Pharmacy, Changzheng Hospital, Second Military Medical University, Shanghai, China
c Key Laboratory of Jiangxi Province for Research on Active Ingredients in Natural Medicines, Bioengineering Research Institute, Yichun University, Yichun, China

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carboxyphosphamide (Pubmed CID: 31515)
schizandrin A (Pubmed CID: 23915)
schizandrin B (Pubmed CID: 68781)
schisantherin A (Pubmed CID: 151529)
schisantherin B (Pubmed CID: 648572)
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Pharmacokinetics
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ABSTRACT

Ethnopharmacological relevance: Schisandra chinensis (Turcz.) Baill (S. chinensis) has been used for thousands years in China, and is usually applied in treatment of urinary tract disorders and liver injury. S. chinensis extract (SCE) has board protective effects on liver, kidney and nervous system. Schisandra lignans are generally considered as the bioactive components of SCE.

Aim of the study: To investigate the pharmacokinetic herb-drug interactions (HDIs) between SCE and cyclophosphamide (CTX). To evaluate the protective effects of SCE against CTX induced damage in rat liver, kidney and brain.

Materials and methods: The pharmacokinetic HDIs between SCE and CTX were investigated by determining plasma concentrations of CTX and three metabolites, namely 4-ketocyclophosphamide (4-Keto), 2-dechloroethylcyclophosphamide (DCCTX) and carboxyphosphamide (CPM) using a previously developed UPLC-MS/MS method. To evaluate the protective effects of SCE pretreatment, toxicity and oxidation stress assessments along with histology investigations were carried out in rat liver, kidney and brain.

Results: The equimolar produced metabolite DCCTX was chosen to reflect chloroacetaldehyde (CAA, a toxic metabolite of CTX) production in rats. Single-dose pretreatment of SCE significantly reduced Cmax and AUC0–24 h of DCCTX by 69% and 49% respectively (P < 0.05). After pretreated with SCE for 7 consecutive days, the Cmax and AUC0–24 h of DCCTX were still decreased (~25% and ~37%, P < 0.05) when compared with CTX alone group. Parallel toxicity and oxidation stress investigations showed that single-dose SCE pretreatment significantly decreased plasma BUN and Cr levels (~12% and ~46%, respectively) and reduced liver AST activity (~32%). Moreover, SCE pretreatment potentially increased the brain GSH content by 7.8-fold, and reduced MDA levels in rat liver, kidney and brain by 39%, 28% and 31%, respectively (compared with CTX alone group). The protective effects of SCE were also supported by histological observations.

Conclusion: Our experiment results suggest that S. chinensis may find use as a complementary medicine in CTX treatment.
1. Introduction

Nowadays, herbal medicines are increasingly used in cancer treatments against side-effects of anticancer drugs (Engdal et al., 2009). *Schisandra chinensis* (Turcz.) Baill. (*S. chinensis*), also known as “Wu Wei Zi”, has been used for thousands years in China for its effects of astringent, tonifying qi, promoting the production of body fluid, nourishing kidney and calming (Lu and Chen, 2009). It is usually applied in treatment of urinary tract disorders and liver injury (But et al., 1997; Fil'kin, 1952; Panossian and Wikman, 2008; Zhu et al., 1999). The extract of *S. chinensis* (SCE) is rich in schisandrine lignans, which are generally considered as the main bioactive components of *S. chinensis*. Among all schisandrine lignans, *S. chinensis* is mainly enriched in schisandrol A, schisandrol B and γ-schisandrin (Lu and Chen, 2009). Recent studies have revealed their broad protective effects on the liver, kidney and nervous system (Chiu et al., 2008; Hwang et al., 2013; Kim et al., 2006; Li et al., 2014; Lu et al., 2014) (Fig. 1). The previous findings suggest that *S. chinensis* may relieve unnecessary hepatotoxicity, nephrotoxicity and neurotoxicity in patients during cancer chemotherapy. However, the combination uses of *S. chinensis* with anticancer chemicals have been seldom investigated, and more information is needed to develop the clinical application of *S. chinensis* and to avoid unwanted herb-drug interactions (HDIs).

Cyclophosphamide (CTX, cytophosphane, Fig. 2) is an alkylating anticancer drug and a potent immunosuppressant (Binotto et al., 2003; Emadi et al., 2009). As one of the standard treatment approaches to multiple myeloma (MM) patients, high-dose CTX (HD-CTX, 2–3 g/m²) has been frequently used in autologous peripheral blood hematopoietic stem cell transplantation (ASCT) (Attal et al., 1996). Unfortunately, the application of CTX often causes serious side-effects. As a prodrug, CTX is extensively metabolized into both active and inactive metabolites (Fig. 2). The majority of CTX is transformed into the effective component phosphoramide mustard (main-chain metabolism). However, 5–10% of CTX is transformed into an inactive metabolite 2-dechloroethylcyclophosphamide (DCCTX) and a by-product chloroacetaldehyde (CAA) by CYP3A (side-chain metabolism) (Huang et al., 2000). CAA has been found to produce hepatotoxicity, neurotoxicity and nephrotoxicity (McDonald et al., 2003; Rzeski et al., 2004).

In this study, we have investigated the potential of SCE as a CTX detoxifier for patients in HD-CTX treatment against MM. On one hand, we speculated that SCE may reduce CAA toxicity by its curative effects on liver, kidney and nervous system. On the other hand, we hypothesized that SCE pretreatment may decrease CAA production, considering that *S. chinensis* has been reported to inhibit CYP3A activity in vivo (Lai et al., 2009; Wang et al., 2014).

Hence, it was our aim to investigate the pharmacokinetic HDIs between SCE and CTX, and to evaluate the protective effects of SCE against CTX induced damage in rat liver, kidney and brain. Up to now, there is no published data on the predictive value of CTX and SCE interaction.

2. Materials and methods

2.1. Chemicals and reagents

Plant Material, *S. chinensis* was collected in Liaoning province (batch no. 2010HA) in China and identified by Professor Jun Yin (Department of Pharmacognosy, Shenyang Pharmaceutical University, Shenyang, China). CTX and tinidazole (TNZ, internal standard) were obtained from Meilun Biotechnology Co., Ltd. (Dalian, China). DCCTX, 4-KetoCTX and CPM standards (purity > 98%) were obtained from Toronto Research Chemicals Inc. (Toronto, Canada). CTX (batch no. 5H071A) and Mesna injections (Endoxan, batch no. 4A177A) were obtained from Baxter International Inc. (Deerfield, America). The standards (purity > 98%) containing schisandrol A, schizandrol B, schisantherin B, deoxyschisandrin, γ-schisandrin and schisandrin C were isolated and purified from SCE, the purities were determined by HPLC with UV detection (Supplement Fig. 1). Acetonitrile and methanol of HPLC grade were obtained from Merck Company (Darmstadt, Germany). HPLC grade of formic acid was obtained from Tedia (Fairfield, OH). All other reagents were of analytical grade.

Superoxide dismutase (SOD), alanine aminotransferase (ALT), aspartate transaminase (AST), malondialdehyde (MDA), creatinine (Cr), blood urea nitrogen (BUN), glutathione peroxidase (GSH-px),...
glutathione (GSH) and oxidized glutathione (GSSG) assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Preparation and characterization of SCE

Crushed fruits of *S. chinensis* were mixed 1:10 with ethyl acetate (w/v) and then flask extracted 3 times (2 min each time) using a JHBE-50 T flask extractor. The mixture was filtered and the ethyl acetate in the filtrate was removed under reduced pressure using a RE-52A rotary evaporator at 55 °C. The residue was then collected and evaporated to a constant weight on a 40 °C water bath (yield: 20%). Determination of the main lignan contents in the extract was performed as described in a previous study with slight modification (Wei et al., 2010). Briefly, 1 g of SCE was mixed with 40 ml of ethanol aqueous solution (90%, v/v) and then ultrasonic extracted for 45 min. The extracted solution was centrifuged at 6000 rpm for 10 min 1 ml of the supernatant was filtered using a 0.45 mm membrane filter. An aliquot of 20 μl filtrate was injected for HPLC analysis.

The HPLC system included a diode array detector (190–400 nm), a quaternary solvent delivery system, a column temperature controller and an autosampler. The chromatographic data were analyzed using Agilent Chromatographic Work Station software. The mobile phase consisted of A (water–formic acid, 100:0.1, v/v) and B (acetonitrile) was programmed as follows for separation: A/B: 55/45 (0 min), 48/52 (12 min), 47/53 (15 min), 42/58 (24 min), 24/76 (40 min), 0/100 (41 min), 0/100 (55 min). The wavelength was set as 230 nm and the flow rate was 1.0 ml/min.

2.3. Animals

Male Sprague-Dawley rats weighing 200–220 g were obtained from Shanghai Slac Laboratory Animal Co., Ltd (Shanghai, China). All the rats were acclimated for at least one week under controlled room temperature (22–24 °C) and humidity (55–60%) with a day/night cycle (12-h light and 12-h dark). The rats were fasted for 12 h before the experiments. All experiments were performed according to the guidelines of Experimental Animal Administration issued by the Ministry of Science and Technology of the People’s Republic of China (http://www.most.gov.cn) and were approved by the Laboratory Animal Ethics Committee of the Second Military Medical University.

2.4. In vivo pharmacokinetic study in rats

2.4.1. Drug administration

Rats were randomly divided into seven groups (groups 1–7, n = 6), respectively. In the first part of the experiment, six parallel groups were included for CTX pharmacokinetic investigation when rats were given a single dose of SCE at different levels. In group 1–6, SCE at doses of 0, 0.10, 0.25, 0.50, 0.75 and 1.00 g/kg was administered intragastrically (i.g.) before intravenous injection (i.v.) of CTX (300 mg/kg). In the second part of the experiment, the remaining parallel group was used to study the effects of multiple-dose SCE treatment on CTX pharmacokinetics: in group 7, 0.75 g/kg of SCE was administrated (i.g., once a day) for 7 consecutive days before CTX administration (300 mg/kg, i.v. on day 7). The dose of SCE was taken as the optimal dose level in the first part of the experiment according to the pharmacokinetic profiles. For the herb-drug combination groups, CTX was always given to the rats 30 min after the single or last dose of SCE. The 30 min time interval was determined by the preliminary pharmacokinetic study to ensure maximal exposure of lignan components from SCE when CTX was injected intravenously (Lai et al., 2009).

2.4.2. Sample collection and preparation

Blood samples were collected from the postocular vein at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after CTX injection. Immediately after the 1-h blood sample collection, rats were given an intravenous Mesna injection to avoid bladder injury. The dose was set as 420 mg/kg according to the clinical protocol. Samples were transferred to heparin tubes and centrifuged at 5000 rpm for 15 min. Plasma samples were obtained from the blood and stored at −20 °C for further analysis. Plasma samples were diluted 30-fold with plasma from untreated rats and vortexed for 2 min. Then 100 μl of each diluted sample was mixed with 400 μl acetonitrile (containing 6 ng/ml IS) and vortexed for 2 min. After centrifugation at 14,000 rpm for 10 min, 100 μl of supernatant was mixed with 400 μl aqueous solution containing 10%
acetonitrile and then vortexed for 2 min. The mixture was then centrifuged at 14,000 rpm for 10 min, and 5 μl of each supernatant was injected into the UHPLC-MS/MS system for analysis. The quality control samples were also prepared in the same manner.

2.4.3. UHPLC-MS/MS analysis

In our laboratory, we have established an UHPLC-MS/MS method for simultaneous quantification of CTX and its three metabolites in rat plasma, including two main-chain metabolites carboxyphosphamide (CPM), 4-ketocyclophosphamide (4-KetoCTX) and a side-chain metabolite DCCTX (Chen et al., 2016). Considering the difficulty of directly detecting CAA, the equally-produced metabolite DCCTX was chosen to reflect CAA production in experiment animals.

In brief, 5 μl of each sample supernatant was injected into the UHPLC-MS/MS system and separated using methanol/10 mM ammonium acetate aqueous solution as the mobile phase at a flow rate of 0.25 ml/min. The compounds were detected by tandem mass spectrometry using electrospray ionization in positive mode. The ion transitions were m/z 261.1→140.1 for CTX, m/z 275.1→142.0 for 4-KetoCTX, m/z 199.2→78.0 for DCCTX, m/z 293.1→221.1 for CPM and m/z 248.1→121.1 for IS.

2.4.4. Pharmacokinetic analysis

Pharmacokinetic profiles of CTX and its three metabolites were estimated by one-compartmental model using Data Access Service 2.0 software (Medical College of Wannan, Anhui, China). Data were expressed as mean ± SD. Comparisons between two groups were performed using one-way ANOVA followed by the LSD test in the condition of variance homogeneity or Dunnett T3 test in the case of variance heterogeneity. The differences were considered to be statistically significant at *P < 0.05 and **P < 0.01.

2.5. Toxicity and oxidation stress assessments

2.5.1. Drug administration

Rats were randomly divided into three groups of nine animals each: group A-vehicle control group (saline 10 ml/kg, i.v.), group B-CTX alone group (CTX 300 mg/kg i.v. was given to rats 30 min after saline 10 ml/kg i.g.), and group C-SCE group (CTX 300 mg/kg i.v. was given to rats 30 min after single-dose of SCE 0.75 g/kg i.g.). Similarly, Mesna was intravenously given at a dose of 420 mg/kg 1 h after CTX administration to avoid bladder injury in groups B and C. Rats of all groups were administrated on day 1, and then were kept till day 7 for toxicity and oxidation stress assessments.

On day 7, after administration, rats were anesthetized with 4% chloral hydrate (10 ml/kg), blood was collected by enucleating the right eye and the rats were then euthanized. Plasma was prepared immediately after blood collection by centrifugation at 5000 rpm for 10 min. The liver, kidney and brain of the rats were removed, a portion of each tissue was fixed in formalin for histological study, the remaining tissue samples and all plasma samples were stored at −80 °C until analysis.

2.5.2. Brain injury analysis

Brain samples were homogenized in a Tissuelyser-24 homogenizer (Shanghai, China) with 9-fold volume of precooling physiological saline and then centrifuged at 3000 rpm for 15 min at 4 °C. The obtained brain supernatants were used for further GSH-px, SOD activity assessments, and for MDA, GSH, GSSG and protein analysis by using the corresponding assay kits.

2.5.3. Liver and kidney injury analysis

The plasma Cr and BUN content were determined to assess kidney injury in rats using assay kits. Liver and kidney samples were prepared similar to the brain samples. Liver and kidney supernatants were used for GSH-px, SOD activity assessments, and for MDA, GSH, and GSSG content analysis. AST and ALT activities were also assessed using liver supernatants.

2.5.4. Histological analysis

Samples of liver, kidney and brain were fixed in 10% formalin solution for 24 h and embedded in paraffin. Slices were cut (5 µm thickness) from the paraffin blocks, and sections were treated with hematoxylin and eosin (HE) and evaluated under light microscopy for histological analysis.

2.5.5. Statistical analysis

Data were analyzed using SPSS software (version 19.0) by one-way ANOVA followed by the LSD test if there was variance homogeneity, or the Dunnett T3 test in the case of variance heterogeneity to measure intergroup differences. The differences were considered to be statistically significant at *P < 0.05 and **P < 0.01.

3. Results

3.1. Quantification of lignans in SCE

The HPLC chromatogram of six lignan constituents in SCE was shown in Fig. 3. The contents of the main lignans in the prepared SCE were as follows: schisandrol A 2.50% (w/w), schisandrol B 1.03% (w/w), schisantherin B 0.36% (w/w), deoxyschisandrin 1.14% (w/w), γ-schisandrin 2.24% (w/w) and schisandrin C 0.39% (w/w).

3.2. In vivo pharmacokinetic study

The developed UPLC-MS/MS method was applied successfully to the pharmacokinetic study in rat plasma in MRM mode and the effects of SCE on the pharmacokinetics of CTX and its metabolites in vivo were studied in rats (Supplement Fig. 2). The precision and accuracy for the quality control samples met the acceptable criteria.

![Fig. 3. HPLC chromatogram of SCE with UV spectra of (1) schisandrol A, (2) schisandrol B, (3) schisantherin B, (4) deoxyschisandrin, (5) γ-schisandrin and (6) schisandrin C.](image-url)
3.2.1. Effect of different doses of SCE on the pharmacokinetics of CTX

After CTX (300 mg/kg, i.v.) administration with or without SCE pretreatment (0, 0.10, 0.25, 0.50, 0.75 and 1.00 g/kg, groups 1–6), the mean plasma concentration-time profiles of CTX, 4-KetoCTX, DCCTX, and CPM are shown in Table 1. Compared with CTX alone administration with or without SCE pretreatment at doses of 0.50, 0.75 and 1.00 g/kg (groups 4–6), the most significant decrease in DCCTX production was found in group 5 (0.75 g/kg SCE). Compared with the CTX alone group (group 1), SCE pretreatment decreased the Cmax of DCCTX by 40%, 69% and 66% respectively in groups 4–6. The most significant decrease in DCCTX production was found in group 5 (0.75 g/kg SCE). Compared with the CTX alone group (group 1), SCE pretreatment decreased the Cmax of DCCTX by 40%, 69% and 66% respectively in groups 4–6. The AUC0–24 h of DCCTX was significantly decreased by 42%, 49% and 45% respectively in groups 4–6. In group 5, the SCE pretreatment also increased the t1/2 and AUC0–24 h of CTX by 1.4- and 1.5-fold when compared with CTX alone group (P < 0.01). The co-administration did not significantly change the AUC0–24 h of 4-KetoCTX or CPM.

3.2.2. Effect of single- and multiple-dose of SCE on the pharmacokinetics of CTX

The mean plasma concentration-time profiles of CTX, 4-KetoCTX, DCCTX and CPM are shown in Fig. 4 after CTX (300 mg/kg, i.v.) administration with or without SCE. The pharmacokinetic parameters are shown in Table 1. Compared with CTX alone group, the Cmax of DCCTX decreased markedly when CTX was administrated with multiple-dose SCE. However, after pretreated with SCE (0.75 g/kg, once daily) for 7 consecutive days, the Cmax of DCCTX (11.3 ± 2.0 μg/ml) was higher than the Cmax in single-dose group (4.6 ± 1.1 μg/ml), and was lower than the Cmax in CTX alone group (15.0 ± 2.4 μg/ml). The AUC0–24 h of DCCTX (61.7 ± 13.0 μg*h/ml) in multiple-dose group was higher than the AUC0–24 h in single-dose group (49.7 ± 9.7 μg*h/ml), and was lower than the AUC0–24 h in CTX alone group (97.8 ± 13.5 μg*h/ml). Compared with CTX alone group, the t1/2 of CTX, DCCTX and CPM was decreased by 24%, 27% and 33% respectively when CTX was administrated in combination with repetitive SCE dosing. The t1/2 of 4-KetoCTX was not different (6.4 ± 0.8 h versus 7.0 ± 1.0 h) when CTX was administrated with multiple-dose SCE for 7 consecutive days.

3.3. Toxicity and oxidation stress assessments

3.3.1. Brain injury analysis

HD-CTX administration (group B) caused a significant increase in MDA production (3.68 ± 0.68–5.62 ± 0.80 μmol/gprot) along with a decrease in protein content (3.17 ± 0.32–2.96 ± 0.24 mgprot/g) and GSH production (22.05 ± 4.76–5.25 ± 2.93 μmol/gprot) when compared with the vehicle control group (group A). In addition, GSSG production was also significantly reduced from 9.24 ± 0.85–5.41 ± 0.85 μmol/gprot (Fig. 5, C, Table 2). In SCE co-administrated group (group C), MDA production was reduced to 3.41 ± 0.48 μmol/gprot (−39%), and protein content was increased to 3.53 ± 0.35 mgprot/g (−19%), when compared with CTX alone group. In addition, a dramatical rise in GSH production (41.59 ± 4.43 μmol/gprot, +692%) along with a milder increase in GSSG production (8.17 ± 0.85 μmol/gprot, +51%) were observed in SCE co-administration group. Moreover, SCE co-administration significantly increased the ratio of GSH/GSSG to 5.24 ± 1.05, which was 5.4-fold of group A and 2.1-fold of group B (Table 2). Most of the investigated parameters in SCE pretreatment group became comparable to those in vehicle control group, suggesting the attenuating effects of SCE on the CTX-induced brain injury.

3.3.2. Liver and kidney injury analysis

HD-CTX administration (group B) led to a significant increase in the plasma BUN and Cr levels to 9.14 ± 0.47 mmol/L (+38%) and 68.26 ± 25.48 μmol/L (+140%). In addition, AST activity was markedly increased from 26.63 ± 2.74 U/gprot (group A) to 51.43 ± 10.28 U/gprot (group B) while ALT activity was slightly increased from 56.31 ± 14.36 U/gprot to 60.20 ± 16.50 U/gprot (Fig. 5, B, Table 2). Similarly, CTX injection significantly increased MDA levels to 1.60 ± 0.08 μmol/gprot (+60%) in liver and 0.88 ± 0.15 μmol/gprot (+52%) in kidney when compared with vehicle control group. Furthermore, CTX administration reduced liver GSH level to 3.89 ± 1.99 μmol/gprot (−86%), and decreased GSSG level to 8.46 ± 1.24 μmol/gprot (−79%). This led to a decrease in GSH/GSSG ratio from 0.63 ± 0.10 (group A) to 0.35 ± 0.15 (group B) (P < 0.05). In addition, the GSH-Px activity in kidney was decreased by HD-CTX administration from 373.67 ± 40.99 U/gprot to 325.69 ± 10.73 U/gprot (Table 2).

When compared with CTX alone group, SCE co-administration...
reduced the plasma BUN and Cr levels to 8.01 ± 0.32 mmol/L (−12%, P < 0.05) and 36.82 ± 13.05 μmol/L (−46%) respectively (Fig. 5.D, Table 2). In addition, AST activity was significantly decreased to 34.98 ± 5.95 U/gprot (−32%) after SCE pretreatment. The ALT activity showed a decrease trend to 53.00 ± 5.61 U/gprot without statistical difference. SCE co-administration also decreased liver and kidney MDA

<table>
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<tr>
<th>Groups</th>
<th>CTX (μg/ml)</th>
<th>t1/2 (h)</th>
<th>AUC0–24 h (μg*h/ml)</th>
<th>4-Keto CTX (μg/ml)</th>
<th>t1/2 (h)</th>
<th>AUC0–24 h (μg*h/ml)</th>
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<tbody>
<tr>
<td>1</td>
<td>207.9 ± 15.6</td>
<td>1.4 ± 0.2</td>
<td>455.9 ± 477</td>
<td>6.1 ± 2.8</td>
<td>7.0 ± 1.0</td>
<td>26.8 ± 5.1</td>
</tr>
<tr>
<td>5</td>
<td>212.3 ± 15.5</td>
<td>2.0 ± 0.3</td>
<td>673.0 ± 105.8</td>
<td>3.0 ± 1.0</td>
<td>14.3 ± 1.0</td>
<td>26.5 ± 4.2</td>
</tr>
<tr>
<td>7</td>
<td>156.0 ± 15.9</td>
<td>1.0 ± 0.2</td>
<td>247.5 ± 26.8</td>
<td>6.7 ± 1.9</td>
<td>6.4 ± 0.8</td>
<td>37.4 ± 4.3</td>
</tr>
</tbody>
</table>

Data are the mean ± S.D. (n = 6). One-way analysis of variance with post hoc test was conducted.

* P < 0.05 compared with group.
** P < 0.01 compared with group 1.

Fig. 5. Effects of CTX injection with and without SCE (0.75 g/kg) co-administration, in group A, B and C: (A) liver, kidney and brain MDA contents; (B) liver ALT and AST activities; (C) GSH content, GSSG content and GSH/GSSG ratios in liver and brain; (D) plasma BUN and Cr levels in rats. Group A: vehicle control group, group B: CTX alone group, group C: SCE co-administration group. Values are expressed as mean ± SD, n = 9 in each group; One-way analysis of variance with post hoc test was conducted; # P < 0.05, ## P < 0.01 compared with group A; *P < 0.05, **P < 0.01 compared with group B.
levels to 1.05 ± 0.07 μmol/gprot (−28%) and 0.63 ± 0.11 μmol/gprot (−28%), respectively (Fig. 5. A, Table 2). Moreover, SCE co-administration significantly increased the liver GSH/GSSG ratio from 0.44 ± 0.18–2.31 ± 0.30 and increased the kidney GSH-Px activity to 373.32 ± 15.61 U/gprot (Table 2). However, there was no significant difference among groups A–C for kidney GSH and GSSG levels and liver GSH-Px activity (data not shown).

3.3.3. Histological analysis

The histological evaluation revealed significant morphological changes in liver, kidney and hippocampus tissue after HD-CTX administration (group B). SCE administration attenuated the CTX-induced central vein endothelium injury and hepatocyte necrosis in liver tissue (Fig. 6. C). In parallel, HD-CTX administration resulted significant glomerular atrophy and division in kidney tissue (Fig. 6. E). Prominent tubule cell degeneration and abscession of the tubular epithelium, as clear signs of nephrotoxicity (Borch and Pleasants, 1979), were found after HD-CTX dosing. The much milder damage was observed in the SCE co-administration group (group C) (Fig. 6. F), suggesting the renal protective effect of SCE. In brain tissue, vacuolar degeneration and disorganization of nerve cells were observed after HD-CTX administration (Fig. 6. H), but the severity was lessened with SCE pretreatment (Fig. 6. I).

4. Discussion

SchisandrA d, schisandrol B, deoxyxyschisandrin and y-schisandrin, the ingredients which are richly contained in S. chinensis, have been reported with various degrees of CYP3A-inhibitory effects since 2004 (Iwata et al., 2004; Li et al., 2013, 2012; Wan et al., 2010). Thus, we hypothesized that SCE pretreatment may block the CYP3A-mediated metabolism of CTX and reduce CAA production. However, long-term SCE administration has been demonstrated to induce CYCP3A expression by activating orphan nuclear receptor pregnane X receptor (Lai et al., 2009; Mu et al., 2006). Therefore, the repetitive SCE dosing might increase CAA production in CTX-treated rats. In this study, both single- and multiple-dose SCE pretreatment were conducted to investigate pharmacokinetic interactions between SCE and CTX. The dose of CTX (300 mg/kg for rats) was designed on the basis of its common usage in MM patients for stem cell mobilization (2 g/m²).

Table 2
Effects of SCE pretreatment on Bun, Cr, Protein contents, MDA, GSH, GSSG levels, GSH-Px, ALT and AST activities and GSH/GSSG ratio in rat brain, kidney and liver.

<table>
<thead>
<tr>
<th></th>
<th>A-Brain</th>
<th>GSH (μmol/gprot)</th>
<th>GSSG (μmol/gprot)</th>
<th>GSH/GSSG ratio</th>
<th>Protein content (mg/gprot)</th>
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<td>Group A</td>
<td>3.7 ± 0.7</td>
<td>22.1 ± 4.8</td>
<td>9.2 ± 0.9</td>
<td>2.5 ± 1.0</td>
<td>3.2 ± 0.3</td>
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<td>Group B</td>
<td>5.6 ± 0.8*</td>
<td>5.3 ± 2.9**</td>
<td>5.4 ± 0.9**</td>
<td>1.0 ± 0.3**</td>
<td>3.0 ± 0.2</td>
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<tr>
<td>Group C</td>
<td>3.4 ± 0.5*</td>
<td>41.6 ± 4.4*</td>
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<td>3.5 ± 0.4</td>
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</table>

<table>
<thead>
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<th></th>
<th>B-Kidney</th>
<th>GSH-Px (U/gprot)</th>
<th>BUN (mmol/L)</th>
<th>Cr (μmol/L)</th>
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</thead>
<tbody>
<tr>
<td>Group A</td>
<td>0.58 ± 0.1</td>
<td>373.7 ± 41.0</td>
<td>6.6 ± 0.5</td>
<td>28.44 ± 14.1</td>
</tr>
<tr>
<td>Group B</td>
<td>0.88 ± 0.2**</td>
<td>325.7 ± 10.7**</td>
<td>9.1 ± 0.5**</td>
<td>68.26 ± 25.5**</td>
</tr>
<tr>
<td>Group C</td>
<td>0.63 ± 0.1*</td>
<td>373.3 ± 15.6</td>
<td>8.0 ± 0.3</td>
<td>36.82 ± 13.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>C-Liver</th>
<th>GSH (μmol/gprot)</th>
<th>GSSG (μmol/gprot)</th>
<th>GSH/GSSG ratio</th>
<th>ALT (U/gprot)</th>
<th>AST (U/gprot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>1.0 ± 0.2</td>
<td>28.3 ± 6.8</td>
<td>41.1 ± 7.3</td>
<td>0.6 ± 0.1</td>
<td>56.3 ± 14.4</td>
<td>26.6 ± 2.7</td>
</tr>
<tr>
<td>Group B</td>
<td>1.6 ± 0.1**</td>
<td>3.9 ± 2.0**</td>
<td>8.5 ± 12.7**</td>
<td>0.4 ± 0.2</td>
<td>60.2 ± 16.5</td>
<td>51.4 ± 10.3**</td>
</tr>
<tr>
<td>Group C</td>
<td>1.1 ± 0.1*</td>
<td>5.7 ± 1.3*</td>
<td>2.1 ± 1.3</td>
<td>2.3 ± 0.3</td>
<td>53.0 ± 5.6</td>
<td>35.0 ± 6.0*</td>
</tr>
</tbody>
</table>

** P < 0.01 compared with group B.
* P < 0.05 compared with group A.
# P < 0.05 compared with group B.
## P < 0.01 compared with group B.

Though a higher dose of CTX (4 g/m²) could give a better CD34+ yield compared with 2 g/m², it is not generally recommended to avoid more severe toxicity (Quach et al., 2015). Determinations of phosphoramidate mustard and CAA have been found hardly achieved by UPLC-MS methods. Instead, plasma concentrations of two main-chain metabolites, 4-KetoCTX and CPM were monitored to measure the status of main-chain metabolic pathway of CTX (De Jonge et al., 2005). The plasma concentration of the equimolar produced metabolite DCCTX was determined to reflect CAA production (De Jonge et al., 2005).

The concomitant use of single-dose SCE markedly decreased the Cmax and AUC0–24h of DCCTX in HD-CTX treated rats. When rats were pretreated with repetitive SCE administration, the reduction of DCCTX blood concentration was still significant when compared with CTX alone group, but to a lesser extent when compared with single-dose group. The above results suggest that single-dose SCE pretreatment could significantly reduce CAA production in HD-CTX treated rats. However, the detoxification might be less effective when SCE is repetitively administered prior to CTX treatment. Meanwhile, the AUC0–24h of two main-chain metabolites, 4-KetoCTX and CPM, showed no significant change after single-dose SCE pretreatment (Supplement Fig. 2), suggesting that the main-chain metabolic pathway was less likely to be effected by SCE administration. Thus, the combination of single-dose SCE pretreatment followed with CTX administration was recommended as the optimal combination regimen. Interestingly, the decreased DCCTX production (compared with control group) was found along with lower CTX blood exposure in rats receiving multiple-dose SCE administration (Fig. 4). According to a recent study from Wang et al., repetitive Schisandra chinensis administration could increase the expression of CYP2C, which plays an important role in the main metabolic pathway of CTX (De Jonge et al., 2005; Wang et al., 2014). Considering that CTX was mainly activated by the CYP2B/2C metabolic pathway in rats, it was possible that CTX was eliminated more rapidly in multiple-dose group than control group because of main-chain metabolism induction, even with blocked side-chain metabolic pathway.

In this study, lower CTX-induced toxicity in liver and kidney were observed in rats treated with the optimized combination regimen. Using AST and ALT as markers of liver injury (Banas et al., 2008), the hepatoprotective effect of SCE was evidenced by the significantly decreased hepatic AST activity. Moreover, SCE pretreatment potently
decreased plasma BUN and Cr levels. Along with the histological findings, the above results suggest the SCE pretreatment significantly attenuated CTX-induced liver and kidney injury. In addition, SCE pretreatment significantly increased GSH level and inhibited lipid peroxidation in rat brain. As the toxic metabolite of CTX, CAA could cause GSH depletion and ultimately result in lipid peroxidation (MacAllister et al., 2013; Singh et al., 2010). Lipid peroxidation has been demonstrated to cause neurotoxicity by causing synaptic loss and nerve terminal dysfunction (Singh et al., 2010). Studies have shown that GSH plays an important role in detoxification of CAA (Sood and O’Brien, 1993; Speen et al., 2015). It was found that SCE pretreatment could increase the brain GSH content (7.8-fold of CTX alone group and 1.9-fold of vehicle control group), indicating that the SCE pretreatment could increase the resistance against CAA toxicity. Also, the MDA content in rat brain was potently decreased after SCE pretreatment, suggesting that the lipid peroxidation was reduced. In the histological study, SCE pretreatment was found significantly attenuated vacuolar degeneration in rat brain.

According to previous reports, schisandrin A, γ-schisandrin and schisandrol B are the main components contributed to the detoxification with their antioxidative or other beneficial efficacies. Schisandrin A, γ-schisandrin and schisandrol B have been found to potently attenuate liver injury induced by D-galactosamine, acetaminophen, and CCl₄ (Li et al., 2014; Lu et al., 2014). Also, γ-schisandrin and schisandrol B have been reported with renal protective effects against CCl₄ and gentamicin (Chiu et al., 2008; Hwang et al., 2013). Despite the antioxidative (or other beneficial) properties of SCE, it was possible that the attenuation of CTX toxicity partly owed to CYP3A inhibition, which led to reduction of CAA blood exposure. By in vitro CYP3A inhibition assays, SCE was confirmed with obvious inhibitory effect on CYP3A activity (Supplement Fig. 8). In general, the experiment results suggested that the attenuation of CTX-induced toxicity was a comprehensive result of the reduction of the toxic metabolite CAA and direct protective effects of SCE.

The Cmax of DCCTX (equivalent to CAA) was 80 μM in experiment rats. According to previous studies, CAA did not cause kidney damage below the concentration of 500 μM in vitro (Dubourg et al., 2001; MacAllister et al., 2013). However, data here demonstrate significant nephrotoxicity in the biochemical assay and histological investigations (CTX alone group). This inconsistency could be explained by the complicated CAA metabolism in vivo. For example, the in vivo concentrations of CAA depended not only on their formation, but also their elimination.

Other than a famous herbal medicine, *S. chinensis* is widely used as a food additive with its unique flavor and health benefits in China (Lee et al., 2013; Ma et al., 2011). In 2002, *S. chinensis* was approved to be used as a general food ingredient by the Ministry of Health, People’s Republic of China (Chau and Wu, 2006). According to Chen et al., the maximal tolerance dose of *S. chinensis* extract was found to be 58.88 g/kg for mice (which is 40.76 g/kg for rats, and is 54.3-fold of the dosage used in this study) (Chen et al., 2006). Along with the non-toxic characteristic, our experimental results indicate that SCE has the potential as a CTX detoxifier.

However, there are some limitations in this study. In this study, the rats in control/single-dose group were given saline/SCE only once, while rats in multiple-dose group were i.g. with SCE for 7 consecutive days. An improved investigation is needed for optimizing SCE-CTX combination regimen by removing the interference of repetitive i.g.


