Original Research

Investigation of toxicological effects of Shuanghuanglian injection in Beagle dogs by metabonomic and traditional approaches

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Abstract

In this study, clinical biochemistry, hematology, histopathology and ^{1}H nuclear magnetic resonance spectroscopy-based metabonomic approaches were applied to investigate the toxicological effects of Shuanghuanglian (SHL) injection after intravenous administration (dosed at 4, 12 and 36 mL stock/kg) in Beagle dogs for 30 d. Decreases in red blood cells, hemoglobin, mean cell volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were observed in the high-dose group. Elevated reticulocytes, total bilirubin and direct bilirubin were also observed in this group. Moreover, significant hemosiderosis and Prussian blue positivity were detected in the liver, spleen and kidney from high-dose group animals, and transmission electron microscopy examination revealed an appreciable number of acanthrocytes in the liver. These results collectively indicate that SHL injection has the potential to cause hemolytic anemia. Metabonomic analysis showed increases in serum lactate, choline and phosphocholine but a decrease in taurine in treated groups and these findings may underlie the toxicological mechanism of SHL injection. In summary, SHL injection shows hemolytic effects in Beagle dogs; moreover, serum choline and phosphocholine as well as lactate and taurine may be the biomarkers for hemolytic anemia induced by SHL injection.

Keywords: Shuanghuanglian injection, toxicity, hemolysis, metabonomic

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Introduction

Shuanghuanglian (SHL) injection, derived from three herbs, *Lonicera japonica*, Baical skullcap root and *Forsythia suspensa*, is a commonly used traditional Chinese medicine (TCM) preparation with known antimicrobial properties and officially recorded in the Chinese Pharmacopoeia (2005).[1] It has been used extensively for millions of patients with respiratory infections since 1973 due to its marked curative effects.[2] Numerous studies have shown that SHL injection possesses obvious virostatic and bacteriostatic activities against certain viruses and bacteria, such as influenza, respiratory syncytial virus, hemolytic streptococcus, and even against *Pseudomonas aerugenosa*, which can cause severe infections.[3] Recent studies indicate that the antimicrobial properties of SHL injection can be predominantly attributed to its effective ingredients, such as caffeotannic acid, saponins and baicalin.[4,5] Although SHL injection is a multi-ingredient TCM preparation, the effective ingredients, caffeotannic acid, baicalin and phillyroside, are officially recorded in the Chinese Pharmacopoeia (2005) as quality control standards.

Nevertheless, an increasing number of adverse drug reaction (ADR) cases of SHL injection have been reported,[6] and the Chinese Food and Drug Administration released an alarm over its potential toxicity in patients (http://www.sda.gov.cn/WS01/CL0078/38014_1.html). It was reported that the ADRs of SHL injection occurred in approximately 2.22–2.56% after clinical exposure and that the primary ADR observed in patients was hypersensitive response. In addition, haematuria, jaundice and hemolytic anemia, as well as gastrointestinal dysfunction, such as vomiting and diarrhea, were also observed. Although caffeotannic acid and saponins, effective ingredients of SHL injection, are thought to be involved in the pathological conditions of ADRs caused by SHL injection, the precise preclinical toxicity background remains to be fully defined.[7] Therefore, it

is necessary to conduct toxicological evaluation to obtain a comprehensive toxicological profiling of SHL injection.

Currently, the metabonomic approach has been widely applied in the toxicity research field and provided useful information with higher throughput at a lower cost than genomics, transcriptomics or proteomics.[8,9] Moreover, metabonomics is a powerful approach for the untargeted identification of potential toxicological biomarkers. Nuclear magnetic resonance (NMR) spectroscopy technique is a commonly used method to generate metabonomic datasets and uniquely suitable to detect a large range of endogenous low-molecular-weight metabolites in organisms. It is rapid, non-invasive and rich in structural and quantitative information and allows the metabolites to be analyzed simultaneously.[10,11]

In this study, we applied traditional toxicological approaches and defined biochemical and histopathological abnormalities associated with hemolytic anemia in Beagle dogs. Using a ^{1}H NMR spectroscopy-based metabonomic approach, we found that serum choline and phosphocholine, as well as lactate and taurine, may be the biomarkers of hemolytic anemia induced by SHL injection. Our findings may provide valuable information for guiding the clinical application of SHL injection and monitoring its ADRs that may arise due to accidental high-dose application.

Materials and methods

Test substance

SHL injection was provided commercially from the pharmacy. The henna transparent liquid was used as supplied without further identification. According to the manufacturer's instructions, SHL injection should be mixed with 0.9% sodium chloride solution and then injected by intravenously guttae to the patients. It is used as 1 mL stock solution/kg body weight for 5–7 d in patients with respiratory tract infection caused by viruses and bacteria.

Animals and exposure

All the animal treatments in this study were in accordance with the guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care. Twelve male and 12 female young adult healthy Beagle dogs (5.0–8.0 kg) were purchased from the Laboratory Animals Institute of Sichuan Academy of Medical Sciences and bred in the animal room of the National Chengdu Center for Safety Evaluation of Drugs. The animals were housed individually throughout the study in suspended, stainless-steel cages. Food and tap water were provided *ad labium*.

All the animals were acclimatized for at least two weeks before being randomly assigned to four groups: a control group (0.9% sodium chloride only) and three dosed groups, low, middle and high-dose groups, respectively (4, 12 and 36 mL/kg stock solution/kg, which were 4, 12 and 36-fold of clinical dosages, respectively). All the animals were intravenously administrated at the rate of 2–3 mL/min with the dose volume of 36 mL/kg for every 30 d.

General observation

During the administration periods, the body weights were recorded weekly and clinical symptoms were observed daily according to the standard operation procedures of the National Chengdu Center for Safety Evaluation of Drugs.

Clinical biochemistry and hematology

Clinical chemistry analysis of serum samples were carried out with Cobas Automatic Biochemistry Analyzer (Roche, Switzerland) using appropriate kits (Roche) and the following parameters were tested: alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), creatinine (Cr), creatine kinase (CK), alkaline phosphatase (ALP) and total bilirubin (TBIL) on days 14 and 30, and direct bilirubin (DBIL) on day 30. The urine hemosiderin was analyzed from the samples collected from the bladders on day 30. The hematology parameters were detected as follows: red blood cell (RBC), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean cell volume (MCV) and reticulocyte (Ret) by Auto Hematology Analyzer (Abbott, Princeton, NJ, USA) on days 14 and 30.

Histopathology and ultramicrostructure

All the animals were necropsied, and fresh organ weights were obtained for the kidneys, liver and spleen from all the animals. Parts of the organs above were preserved in 10% neutral buffered formalin. Organ sections were stained with hematoxylin–eosin (HE) and Prussian blue for light microscopy examination.

Liver and kidney slices (1 mm^3) from high-dose and control group animals were fixed with 1–2% OsO_4 in 0.1 mmol/L phosphate buffer (pH 7.3) for 2 h at 4°C, washed with phosphate buffer, dehydrated with ethanol (from 35% to 100%) and then infiltrated with propylene oxide and plastic mixture for 2–3 d. The embedded cubes were sectioned with ultra microtome and dyed with uranyl acetate and lead citrate prior to photomicrography under Hitachi transmission electron microscopy (TEM) operating at 80 kV.

Metabonomic studies of the serum samples

^{1}H NMR spectroscopy of serum

A volume of 2 mL blood samples were collected in the polythene tubes without any anticoagulant for NMR analysis on day 30. Prior to being centrifuged (4°C, 1800*g*, 10 min), the samples were left to clot at 4°C for one hour. The collected serums were centrifuged (4°C, 20,000*g*, 10 min) again, and then the supernatants were transferred into 0.5 mL aliquots in tubes, and stored at −80°C until analysis. The thawed serums (350 μL) were mixed with 200 μL D_2O, which was used as a locking signal, then transferred into 5 mm NMR tubes for ^{1}H NMR detection.

^{1}H NMR spectral data were acquired on a Bruker-Av II 600 MHz spectrometer (Bruker Co, Rheinstetten, Germany) at 300 K. Water signals were suppressed by presaturation. The water-suppressed Carr–Purcell–Meibom–Gill (CPMG)

spin-echo pulse sequence (RD-90°-(τ-180°-τ) n-ACQ) with a total spin-echo delay (2nτ) of 40 ms was used to attenuate broad signals from proteins and lipoproteins. Typically, 64 free induction decays (FIDs) were collected into 64k data points over a spectral width of 8992.8 Hz with a relaxation delay of five seconds and an acquisition time of 0.91 s. The FIDs were weighted by an exponential function with a 0.3-Hz line-broadening factor prior to Fourier transformation.

Data reduction and pattern recognition analysis

All the spectra were manually rephrased, baseline corrected and referenced to CH_3 resonance of creatine at δ3.05[12] and then data-reduced to 212 segments, each with a 0.04 ppm width for a spectral window ranging from 0.5 to 9.5 ppm using MestRe-c2.3 software (http://qobrue.usc.es/jsgroup/MestRe-c). The area for each segmented region was calculated. The segments of δ5.1–4.6 ppm in spectra were removed to eliminate the artifacts of the residual water resonance. All remaining spectral segments were scaled to the total integrated area of the spectrum to reduce variation in concentration. The data-sets were mean-centered prior to principle components analysis (PCA) by SIMCA-P10.0 software package (Version 10, Umetrics AB, Umea, Sweden). Two-dimensional score plots were used to visualize the separation of the samples and the corresponding loading plots were applied to identify the spectral variable contribution to the position of the spectra that were altered as a result of SHL injection.

Statistics

The data from serum clinical chemistry, hematology analysis and organ coefficient were expressed as the mean $\pm$ SD. Statistical comparisons were performed using analysis of variance followed by using Dennett's *t*-test. Fisher's exact test was applied to the findings of urine hemosiderin analysis. The criterion for statistical significance was set at $P < 0.05$.

Results

General toxicity

Symptoms of gastrointestinal dysfunction, such as anorexia and severe vomiting, were observed in high-dose group animals that were administered at 36 mL stock solution/kg dose. Additionally, the animals in the high-dose group showed weight loss during the dosing period (Figure 1). No abnormalities were observed in the other treated groups and the control group during the study period.

Clinical biochemistry and hematology

Significant changes in serum clinical biochemistry were observed in the high-dose group. TBIL increased significantly on days 14 and 30, and DBIL also showed remarkable elevation on day 30 as compared with the control (Table 1). The urine hemosiderin was also observed in the high-dose group animals. There were no changes in the levels of ALP, ALT, AST, CK, BUN and Cr from SHL injection-treated animals. Significant changes in the hematology parameters were restricted to the high-dose group animals (Table 2). Obvious decreases in RBC, HGB, MCV, MCH and MCHC were observed on both days 14 and 30 along with a compensatory increase in reticulocyte count, compared with the control group. These alterations suggested the impairment of erythrocytes and up-regulation of hematopoietic function.

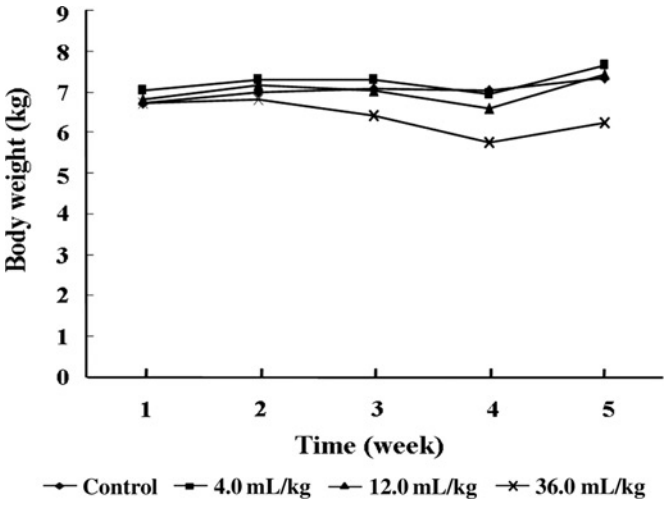

Figure 1 Body weight of Beagle dogs treated with SHL and saline injections ($N = 6$ for all groups). The animals in the high-dose group administered for three weeks showed a dramatic weight loss. SHL, Shuanghuanglian

Histopathology

The spleen and liver weights relative to body weight significantly increased in the high-dose group in comparison to the control (Table 3). This was consistent with the light microscopy findings of Kupffer's cell hyperplasia followed by hemolysis. Moreover, notable microscopic findings in the high-dose group animals were the hemosiderosis lesions in the liver, spleen and kidney, and hyperplasia and hyperpigmentation of Kupffer's cells were observed in the liver and spleen. The overt extramedullary hematopoiesis in the spleen from high-dose group animals was also identified by HE stain (Figure 2). The obvious brown pigment in the renal tubular epithelium was observed in high-dose group animals, and it was considered as haemosiderin (Figure 2f). When hemolysis occurs, plasma haemoglobin is filtered through the glomeruli of the kidneys and reabsorbed by tubular cells, where it may lead to formation of precipitates called haemosiderin. Moreover, the Prussian blue positivity in the organs including the liver, spleen and kidney were observed, which was indicated through the staining of overload iron (Figure 3). All the changes were considered as SHL injection-induced hemolysis.

Electron microscopic examination showed an appreciable number of acanthrocytes, a kind of deformed erythrocyte, in the liver from high-dose group animals. The electron dense granules and the swelling mitochondria were also detected in the endochylema of renal tubule epithelium. No morphology abnormality was observed in control animals by TEM (Figure 4).

Table 1 Effects of SHL injection on clinical chemistry parameters

Parameter	Day	Control	SHL injection (mL/kg) 4	12	36
ALP (U/L)	Day-14	114.10 ± 32.15	135.30 ± 49.17	142.25 ± 17.65	152.60 ± 32.45
	Day-30	119.20 ± 42.45	135.90 ± 44.46	151.30 ± 20.11	130.03 ± 40.07
ALT (U/L)	Day-14	26.68 ± 3.47	28.43 ± 6.91	24.63 ± 4.13	30.28 ± 18.04
	Day-30	23.24 ± 1.87	26.52 ± 6.18	22.73 ± 3.61	19.30 ± 2.65
AST (U/L)	Day-14	27.84 ± 4.50	26.77 ± 3.87	28.38 ± 5.88	36.82 ± 17.89
	Day-30	28.56 ± 1.61	26.23 ± 4.52	28.12 ± 4.93	27.33 ± 3.40
CK (U/L)	Day-14	219.0 ± 63.3	209.3 ± 59.0	234.5 ± 66.1	264.7 ± 105.9
	Day-30	230.4 ± 64.1	175.8 ± 64.5	189.5 ± 56.7	147.8 ± 16.1
BUN (mmol/L)	Day-14	2.72 ± 0.76	3.33 ± 1.67	3.26 ± 1.64	16.80 ± 20.60
	Day-30	4.04 ± 1.53	3.76 ± 1.14	3.94 ± 1.59	10.85 ± 8.51
Cr (μmol/L)	Day-14	37.88 ± 11.22	30.15 ± 9.03	33.17 ± 9.00	117.28 ± 141.93
	Day-30	44.48 ± 11.50	33.97 ± 10.38	44.05 ± 9.41	68.32 ± 31.70
TBIL (μmol/L)	Day-14	0.68 ± 0.46	1.00 ± 0.43	1.73 ± 0.45	3.00 ± 1.43*
	Day-30	1.32 ± 0.85	1.33 ± 0.33	1.87 ± 0.60	2.88 ± 1.55*
DBIL (μmol/L)	Day-30	0.006 ± 0.013	0.17 ± 0.21	0.37 ± 0.31	0.98 ± 0.64*
Urine+		0	0	0	5*
	Day-30				
Hemosiderins −		6	6	6	1

SHL, Shuanghuanglian; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; BUN, blood urea nitrogen; CR, creatinine; TBIL, total bilirubin; DBIL, direct bilirubin; ANOVA, analysis of variance
+, Represents positive result from urine hemosiderin analysis; −, represents negative result from urine hemosiderin analysis; $N = 6$ for all groups; $^*P < 0.05$ indicates significant difference between control and SHL injection-treated dogs (ANOVA followed by using Dennett's *t*-test for all the parameters except urine hemosiderin, Fisher's exact test for urine hemosiderin)

^{1}H NMR spectroscopic and pattern recognition analysis of serum

The CPMG ^{1}H NMR spectra reflected the lower-molecular weight metabolites presented in the serum. ^{1}H NMR chemical shifts and assignments of endogenous metabolites in serum were conducted according to previous literatures.[12–16] We utilized a pattern recognition (PR) method like PCA to uncover the latent biochemical information and characterize novel biomarkers from ^{1}H NMR spectra. The prominent changes of original ^{1}H NMR spectra were the elevation of lactate (Figure 5), which was an anaerobic metabolism product. Applying PCA to the data-sets of the ^{1}H NMR spectra, the score plot showed that the high-dose and middle-dose groups were clearly separated from the control along PC1 with PC2 (Figure 6a). The corresponding loading plot showed that the elevated lactate (δ1.34), choline (δ3.22) and phosphocholine (δ3.62 and 4.22), along with 3-D-hydroxybutyrate (δ1.22 and 2.42) and acetate (δ1.94) signals were obvious in serum spectra obtained from the high-dose group and responsible for this shift (Figure 6b). The decreases in amino acids, as an

Table 3 Effects of SHL injection on ratio of organ weights/body weights

Organ	Control (g/100 g body weight)	SHL injection (g/100 g body weight) 4 mL/kg	12 mL/kg	36 mL/kg
Liver	3.10 ± 0.31	3.51 ± 0.17	3.62 ± 0.30	3.97 ± 0.29*
Spleen	0.30 ± 0.06	0.29 ± 0.09	0.27 ± 0.06	0.44 ± 0.11*
Kidney	0.49 ± 0.08	0.50 ± 0.06	0.50 ± 0.03	0.90 ± 0.23

SHL, Shuanghuanglian; ANOVA, analysis of variance
$N = 6$ for all groups; $^*P < 0.05$ indicates significant difference between control and SHL injection-treated dogs (ANOVA followed by using Dennett's *t*-test)

Table 2 Effects of SHL injection on hematology parameters

Parameter	Day	Control	SHL injection (mL/kg) 4	12	36
RBC (10^{12}/L)	Day-14	6.01 ± 0.34	6.08 ± 0.30	5.77 ± 0.64	4.66 ± 0.16*
	Day-30	6.19 ± 0.57	5.97 ± 0.20	5.84 ± 0.46	4.65 ± 0.44*
HGB (g/L)	Day-14	134.2 ± 9.0	133.8 ± 4.2	128.5 ± 12.1	98.0 ± 2.8*
	Day-30	136.4 ± 12.4	130.2 ± 5.2	129.5 ± 7.9	92.2 ± 8.6*
MCV (fl)	Day-14	66.44 ± 0.72	65.48 ± 1.86	66.98 ± 1.38	66.00 ± 1.79
	Day-30	66.52 ± 1.18	65.22 ± 1.83	66.92 ± 1.47	62.55 ± 1.35*
MCH (pg)	Day-14	22.34 ± 0.33	22.02 ± 0.73	22.27 ± 0.61	21.07 ± 0.76*
	Day-30	22.02 ± 0.40	21.78 ± 0.53	22.20 ± 0.70	19.83 ± 0.63*
MCHC (g/L)	Day-14	336.6 ± 2.1	336.2 ± 2.7	333.0 ± 4.2	319.2 ± 9.6*
	Day-30	331.4 ± 1.8	333.8 ± 5.0	331.8 ± 4.7	317.3 ± 1.4*
RET (%)	Day-14	0.84 ± 0.36	0.60 ± 0.17	0.81 ± 0.20	5.95 ± 1.85*
	Day-30	0.61 ± 0.33	0.46 ± 0.12	0.80 ± 0.35	3.39 ± 1.88*

RBC, red blood cell; HGB, hemoglobin; MCV, mean cell volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RET, reticulocyte; SHL, Shuanghuanglian; ANOVA, analysis of variance
$N = 6$ for all groups; $^*P < 0.05$ indicates significant difference between control and SHL injection-treated dogs (ANOVA followed by using Dennett's *t*-test)

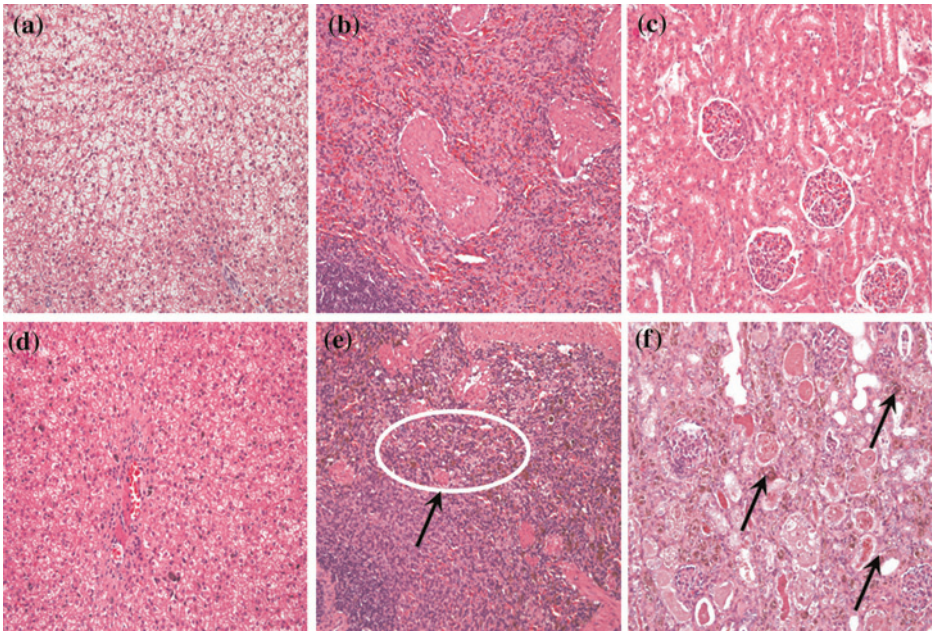

Figure 2 Photomicrographs of the liver, spleen and kidney (HE stain). The pictures (a), (b) and (c) represent the liver, spleen and kidney, respectively, of the control group (×200), and exhibit no abnormal morphology. (d) The liver of the high-dose group animals showing the hyperplasia and hyper pigmentation of Kupffer's cells (×200). (e) The hyperplasia and hyper pigmentation of Kupffer's cells and extramedullary hematopoiesis were observed in the spleen of high-dose group animals, as shown by the arrow (×200). (f) The brown pigment in renal tubule epithelium was detected in the kidney of the high-dose group animals, as shown by the arrow (×200). HE, hematoxylin–eosin (A color version of this figure is available in the online journal)

energy resource, such as valine (δ0.98 and 1.06), leucine (δ0.94), glycine (δ3.54), alanine (δ1.46 and 3.78) and glutamine (δ2.10) together with taurine (δ3.42) and trimethylamine-N-oxide (TMAO, δ3.26) signals were also identified (Table 4).

Discussion

Although ADRs of SHL injections in patients have been identified, there is still little information concerning its pre-clinical toxicity background and metabolic profiles following SHL administration. In the present study, the

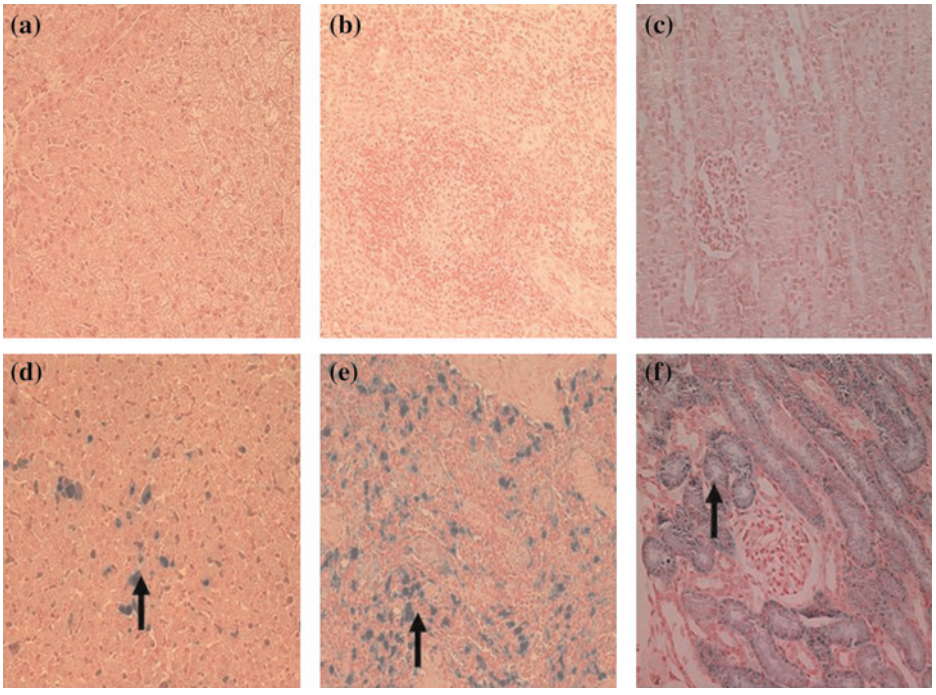

Figure 3 Liver, spleen and kidney sections stained with Prussian blue for iron detection. No abnormal morphology was observed in the control group by Prussian blue stain. (a), (b) and (c) represent liver, spleen and kidney, respectively, from the control animals (×200). (d), (e) and (f) represent liver, spleen and kidney, respectively, from the high-dose group animals and show iron overloaded. Arrows exhibit Prussian blue positivity (A color version of this figure is available in the online journal)

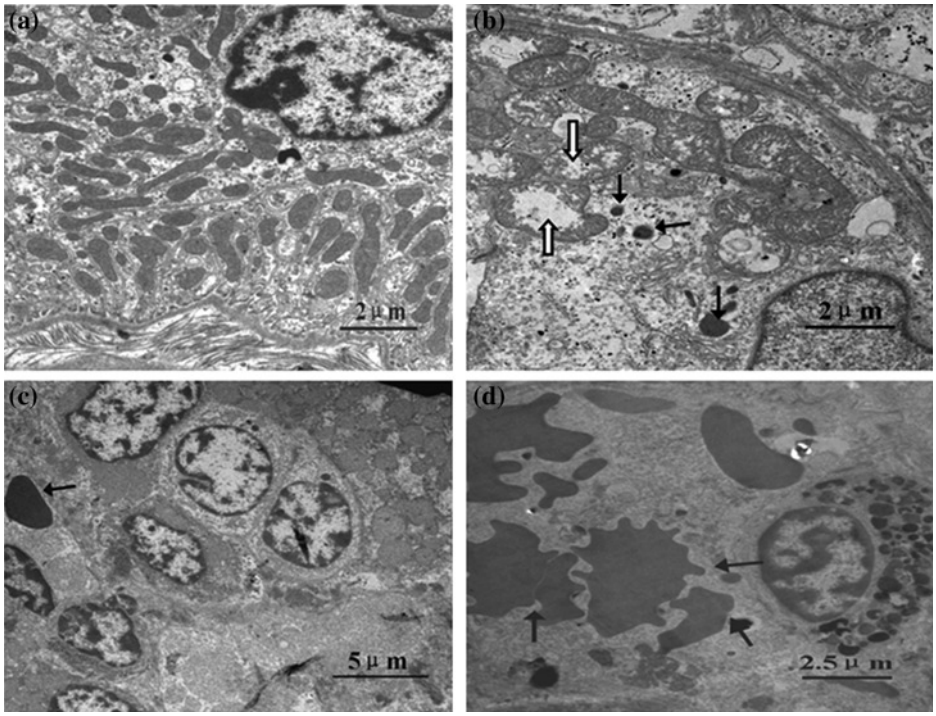

Figure 4 Photomicrographs of the liver and kidney (TEM). (a) No morphology abnormality of the kidney was observed in control by TEM (×12,000). Calibration bar: 2 μm. (b) The electron dense granules, as the black arrows show, and the swelling mitochondria, as the white arrows show, were observed in the endochylema of the renal tubule epithelium from the kidney of high-dose group animals (×12,000); the bar indicates 2 μm. (c) The erythrocytes in the liver of the control showing the smooth cytomembrane, as shown by arrow (×5600). Calibration bar: 5 μm. (d) Acanthrocytes were detected in the liver of high-dose group animals, as shown by the arrows (×8000); bar indicates 2.5 μm. TEM, transmission electron microscopy

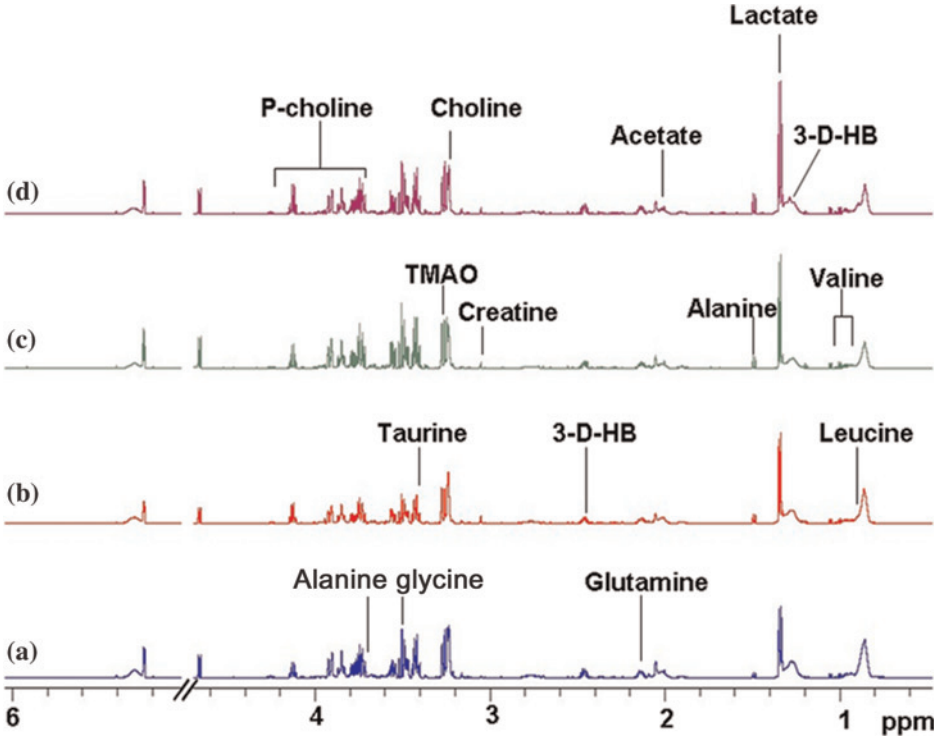

Figure 5 600 MHz CPMG ^{1}H NMR spectra of the serum samples from Beagle dogs treated with SHL injection for 30 d: (a) control; (b) 4 mL/kg; (c) 12 mL/kg; (d) 36 mL/kg. SHL, Shuanghuanglian; NMR, nuclear magnetic resonance (A color version of this figure is available in the online journal)

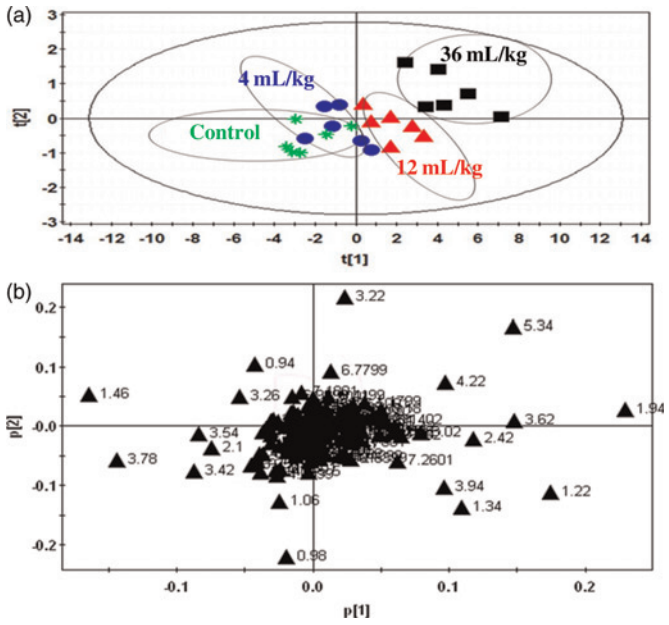

Figure 6 PCA scores and loading plots based on CPMG ^{1}H NMR of serum samples from the control and SHL injection-treated animals on day 30: (a) scores plots; (b) loadings plot derived from CPMG ^{1}H NMR of serum samples corresponding to (a). * Control group; • 4 mL/kg; ▲ 12 mL/kg; ■ 36 mL/kg. PCA, principle components analysis; NMR, nuclear magnetic resonance (A color version of this figure is available in the online journal)

Table 4 Summary of the variations from serum metabolites induced by SHL injection

Metabolite	Chemical shift (ppm)	Variation following SHL injection
Valine	0.98 and 1.06	↓
Alanine	1.46 and 3.78	↓
3-D-hydroxybutyrate	1.22 and 2.42	↑
Leucine	0.94	↓
Acetate	1.94	↑
TMAO	3.26	↓
Glutamine	2.10	↓
Choline	3.22	↑
Phosphocholine	3.62 and 4.22	↑
Lactate	1.34	↑
Taurine	3.42	↓
Glycine	3.54	↓
Creatine	3.05	–

SHL, Shuanghuanglian; TMAO, trimethylamine-N-oxide
Variations compared with control samples: ↑, relative increase in signal; ↓, relative decrease in signal; –, no change

NMR-based metabonomic approaches coupling with clinical chemistry and histopathology methods were used to investigate the toxicological profiling of high-dose SHL injection.

No abnormality was observed in the low-dose group treated with 4 mL stock solution/kg SHL injection. Therefore, we conclude that this dosage is safe for the Beagle dogs, and the no observed adverse effect level of SHL injection for the Beagle dogs can be established as 4 mL stock solution/kg.

An obvious decrease in erythrocytes and HGB, MCV, MCH and MCHC, and an increase in Ret were observed in the high-dose group. These results implied that the animals predominantly suffered from anemia syndrome. Moreover, elevated TBIL and DBIL in serum were found, suggesting that the anemia syndrome could be attributed to erythrocyte impairment. Compensatory proliferation of erythrocytes in the spleen and a significant increase in Ret in peripheral blood were observed, indicating that extramedullary hematopoiesis occurred to compensate the loss of the RBC. These findings were confirmed by the histopathological and microstructural examination, which revealed an appreciable number of acanthrocytes in livers in high-dose group animals. The electron dense granules and the swelling mitochondria were also detected in the endochylema of renal tubule epithelium. These findings indicated that SHL injection could damage erythrocytes and cause hemolytic anemia, which was consistent with previous ADR reports induced by SHL injection in patients.

To elucidate metabolic profiles after SHL injection administration and to identify potential biomarkers of the toxicity, we utilized metabonomic analysis for serum samples to identify the potential targets of toxicity. Interestingly, we found marked changes in some metabolites, which were closely associated with cell membrane and energy metabolism.

Choline and phosphocholine are breakdown products of phosphatidylcholine, and play an important role in the integrity of cell membranes and lipid metabolism.[12] They are often released under pathological conditions from their stores in cell membranes, and considered as biomarkers of membrane injury (Figure 7b). Therefore, the increased choline and phosphocholine caused by SHL injection were considered to be attributed to RBC membrane disruption.[17] Interestingly, we noticed a decrease in serum taurine, which could stabilize cell membrane and maintain intracellular Ca^{2+} concentration.[18–20] The decrease in serum taurine could be explained by the increased utilization together with the decreased source.[21] On the one hand, SHL injection-treated animals consumed more serum taurine to repair the impaired erythrocytes; on the other hand, serum amino acids, the raw materials to produce taurine (Figure 7b), were decreased since the change in energy resource. Therefore, the reduction in serum taurine not only reveals the impairment of SHL injection on erythrocytes but also supports the changes in energy metabolism caused by gastrointestinal dysfunction, such as anorexia and vomit. Actually, administration of taurine showed a preventive and therapeutic effect on erythrocytes against the exogenous toxicant showing impairment effects on erythrocytes.[22,23] Therefore, we speculate that patients suffering from hemolyisis caused by SHL injection would benefit from taurine.

SHL injection also resulted in increased serum lactate. Interestingly, previous studies reported an elevated serum lactate in patients with hemolytic anemia.[24] Because lactate is a consequence of anaerobic metabolism, we deduce that two main aspects may contribute to the elevation of serum lactate (Figure 7a). First, the lactate released from endochylema after the injury of erythrocytes. Additionally, the hemolytic anemia induced by SHL injection aggravated the hypoxia condition, resulting in accelerated anaerobic metabolism.[25]

Based on aforementioned results we presume that serum choline, phosphocholine, lactate as well as taurine may be the putative toxicological biomarkers for hemolytic anemia induced by SHL injection. Because hemolysis can cause

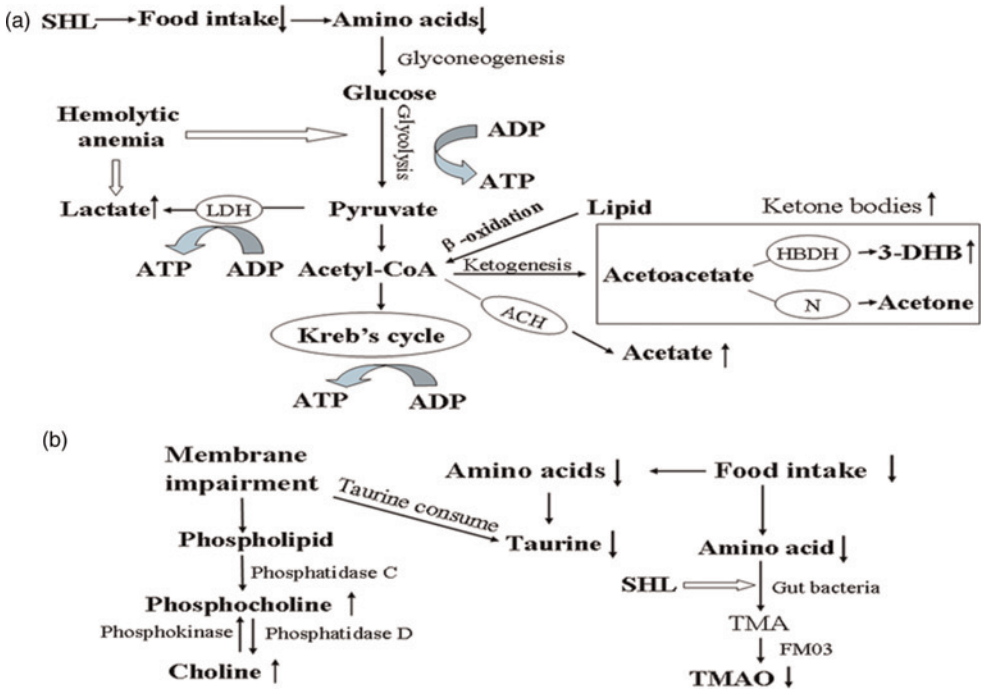

Figure 7 (a) Schematic representation of the energy metabolic network (glycolysis, glyconeogenesis, lipid metabolism, etc.). (b) Schematic representation of the degradation pathway phospholipid of RBC membrane from SHL injection-treated animals on day 30 and the effects of SHL injection on TMAO. Elevation (↑) or reduction (↓) in metabolites as observed in SHL injection-treated animals. SHL, Shuanghuanglian; RBC, red blood cell; TMAO, trimethylamine-N-oxide (A color version of this figure is available in the online journal)

both anemia and acute renal failure, therefore, it may be necessary to monitor the potential hemolytic effects of an accidental overdose of SHL injection and the biomarkers above would be helpful for monitoring.

Ketones, such as plasma 3-D-hydroxybutyrate (3-DHB), are indicative of enhanced β-oxidation and can be utilized as an alternative energy source when glucose is limited. Acetate is an end product of fatty acid oxidation. The increased serum 3-DHB and acetate may denote a shift in energy metabolism toward fatty acid β-oxidation and ketogenesis. This notion was supported by the cage-side observations such as marked anorexia and vomit in the dogs (Figure 7a).[14] The decreased serum amino acids in dosed animals, such as valine, leucine, glycine, alanine and glutamine, may represent an accelerating glyconeogenesis from amino acid to glucose to maintain the necessary blood glucose. Because TMAO is produced by gut bacteria and SHL injections possess antimicrobial effects, the declined serum TMAO may be attributed to the disruption of intestinal bacteria (Figure 7b).

The score plot derived from the ^{1}H NMR serum spectra showed that the high-dose and middle-dose groups were clearly separated from the control along PC1 with PC2, indicating that the low-molecular-weight serum metabolites in these two groups are different from the control. However, the clinical chemistry and hematology examinations could not identify the difference between the middle-dose group and control. Therefore, these findings highlight that metabonomic investigation may be more sensitive in toxicological investigation as compared with traditional approaches.

Several saponins have been identified or tentatively characterized from SHL injection.[26–29] It was reported that when erythrocytes were incubated with saponins in isotonic buffer, the membrane becomes permeable to hemoglobin, and then the ghosts of saponinlyzed erythrocytes formed.[30,31] Taken above, we speculate that the hemolytic effect may be attributed to saponins in SHL injection. The saponins in SHL injection may interact with lipid membranes of cells and form insoluble complexes with cholesterol leading to formation of pores, permeabilization of cells and subsequent loss of hemoglobin.[30,32–34] As a result, damaged erythrocytes and the hemoglobin released from the cytoplasm were defined as intravascular hemolysis. Furthermore, saponins possess detergent-like properties and can increase the permeability of cell membranes without destroying erythrocytes, but the deformed RBCs emerged. Because the deformed RBCs lost the ability to pass through epithelial gaps, they were phagocytized by the macrophage in the liver and spleen, and considered as extravascular hemolysis.[35]

In summary, the high-dose SHL injection showed the membrane impairment and hemolytic effects in Beagle dogs, which was supported by clinical chemistry, hematology and TEM analysis. NMR-based metabonomic investigations revealed increases in serum choline, phosphocholine, ketone body and lactate, but decreases in trimethylamine N-oxide, taurine, leucine, valine, glycine and glutamine. These findings highlight the toxicological profiling of high-dose SHL injection, and were in line with the clinical ADR observations.

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and CH conducted the experiments. GY and XC wrote the manuscript. All individuals who made contributions to this study are included as authors in this paper.

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