Toxicity of Danshen Injection in Beagle's Dogs by Repeated iv Injection

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Abstract: **Objective** To re-evaluate the potential toxicity of Danshen Injection (DI) in Beagle's dogs by repeated iv injection. **Methods** DI was iv given to the dogs at the doses of 0, 1.6, 5.4, and 16.0 g/(kg·d) (4 per sex per group) for 13 weeks. During the test period, the clinical signs, mortality, body weights, food consumption, rectal temperature, ophthalmoscopy, electrocardiography, urinalysis, hematology, serum biochemistry, organ weights, gross findings, and histopathology were examined. **Results** Dogs iv given with DI at the doses of 0, 1.6, 5.4, and 16.0 g/(kg·d) for 13 weeks had no drug-related changes in mortality, body weight, food consumption, temperature, electrocardiography, ophthalmoscopy, urinalysis parameters, and organ weights. The hematological parameter data showed a significant decrease in red blood cells and hemoglobin concentration in the high-dose group and a significant increase in activated partial thromboplastin time suggesting an effect on haemopoiesis. For biochemical parameters, a significant decrease in glucose and a significant increase in total bilirubin were observed in the high-dose group, and the latter was considered to be toxicologically insignificant as lack of histopathological correlate. However, the histopathological examinations of the injection site showed that DI could cause dose-dependent focal inflammation. **Conclusion** That the iv injection with DI into dogs at 16 g/(kg·d) for 13 weeks could cause the decreases in red blood cell parameters and glucose, as well as the lesions of the injection site. The no observed adverse effect level is 5.4 g/(kg·d), which suggests that safe clinical dosing be possible.

Key words: Beagle's dogs; Danshen Injection; iv injection; *Salvia miltiorrhiza*; repeated dose toxicity
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Introduction

Chinese materia medica (CMM) injection is made from the extraction of effective materials of natural medicine single or compound by using modern scientific techniques and methods. Since the first CMM injection, Chaihu Injection, was approved in 1954, more than 100 CMM injections have been used in clinic in China. The injections have quick action and definite therapeutic effects, and are usually used to treat acute and severe syndromes (Luo, 2010). Along with the wide application of the injections in clinic, the adverse drug reactions have been more and more reported. Furthermore, most CMM injections have not been subjected to clinical trials in large scale (Luo, 2010). In order to monitor the CMM injection market, the State Food and Drug Administration of China has started to re-evaluate the CMM injections, including the technical process, quality standard, clinical efficacy, and safety of the injections should be included.

Salvia miltiorrhiza Bunge (*Danshen*) is a Chinese herbal medicine that has been widely used for the treatment of cardiovascular disease, particularly angina pectoris, and myocardial infarction (Cheng, 2006a; 2006b; Ji, Tan, and Zhu, 2000; Yue *et al*, 2012; Zhou *et al*, 2012). According to the therapeutic theory of traditional Chinese medicine (TCM), *Danshen* has the properties of improving blood circulation, eliminating blood stasis, relieving pain, clearing heat from the blood,

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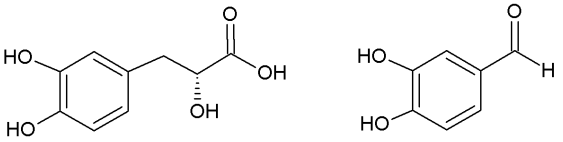
resolving swelling, and easing the mind. The previous studies (Bao *et al*, 2006; Ji *et al*, 2003; Kang *et al*, 2002; Ni *et al*, 2011; Yue *et al*, 2006; Zhang *et al*, 2010; Zou, Xu, and Tian, 1993) showed that *Danshen* extracts had antihypertension, anti-oxidative, anticoagulation, anti-thrombotic, and antifibrosis effects; *Danshen* extracts also enhanced the immune system and protect cells and mitochondria.

There are numerous pharmaceutical dosage forms of *Danshen* in China, such as tablets, capsules, granules, oral liquids, sprays, dripping pills, and injections of either *Danshen* or Compound Danshen. Danshen Injection (DI), made from the aqueous extracts of *Danshen*, is one of the most widely used CMM preparations. No major side effects have been reported on DI (Cheng, 2006a; Zhou, Zuo, and Chow, 2005). But some injected individuals suffered from gastro-intestinal reactions, such as nausea, vomiting, and abdominal pain, and in very few cases patients experienced dizziness and rashes (Wang, Yin, and Zhu, 2009). Nevertheless, in order to improve the safety, efficacy, and quality control, DI was re-evaluated in accordance with the notice of the State Food and Drug Administration of China. The present study was carried out to evaluate the toxicity from repeated iv administration of DI in Beagle's dogs.

Materials and methods

Test materials

Danshen Injection (SY100112), made from the aqueous extract of *Danshen*, was supplied by Shanghai Worldbest Anhui Jinhui Pharmaceutical Co., Ltd. (Fuyang, Anhui, China). It contained 3.2 g/mL of *Danshen* raw material, and the concentration was 50 times greater than that for human clinical use. In DI, the phenolic acids, including salvianolic acid B, danshensu, and protocatechuic aldehyde, are the major hydrophilic components and pharmacologically active ingredients (Chen *et al*, 2011; Zhang *et al*, 2005). According to the *Chinese Pharmacopoeia 2005*, danshensu and protocatechuic aldehyde were quantified for the quality control of preparation in the quality standard of DI. The structures of danshensu and protocatechuic aldehyde were shown in Fig. 1. The contents of sodium danshensu and protocatechuic aldehyde were determined by the reverse phase HPLC. The analytical test was carried out on a Kormasil C_{18} column (250 mm × 4.6 mm, 5 μm) with methanol-0.5% glacial acetic acid (16:84) as the mobile phase. The detection wavelength was 281 nm and the flow rate was 1.0 mL/min. The results showed that DI contained 22.06 mg/mL sodium danshensu and 4.89 mg/mL protocatechuic aldehyde. The injection was sealed in glass bottles and stored at room temperature. Dose formulations were prepared daily before administration by direct dilution with 5% dextrose injection.



danshensu protocatechuic aldehyde

Fig. 1 Structures of danshensu and protocatechuic aldehyde

Animals and treatment

Male and female Beagle's dogs, approximately 6 to 8 months of age, were purchased from Nanjing Yadong Laboratory Animal Research Center (Nanjing, China). Upon arrival, the dogs were examined for health condition and quarantined for 21 d, prior to being randomly divided into experimental groups. During the experiment, they were individually housed in clean and stainless-steel cages (80 cm × 70 cm × 95 cm). Animals were housed in a room with controlled ventilation (10 air changes/h), temperature (20—26 ℃), relative humidity (40% — 70%), and light intensity (150—300 lux) during the study. Room lights were controlled automatically to provide 12 h of light and 12 h of dark. All the animals were provided with dog diet and water *ad libitum* in cages. The study was conducted at Jiangsu Center for Safety Evaluation of Drugs (Nanjing, China) according to Good Laboratory Practice (GLP) Guidelines, and was inspected by an independent quality assurance department. The protocol was designed in compliance with the *Testing Guidelines for Safety Evaluation of Drugs* (Notification [Z] GPT3-1) issued by State Food and Drug Administration of China, and approved by the Institutional Animal Care and Use Committee of Jiangsu Center for Safety Evaluation of Drugs, following the *Chinese Specifications for the Production, Care and Use of Laboratory Animals*.

A total of 32 dogs of either sex were used, with varying body weights ranging from 6.5 to 9.5 kg for the

males and 6.0 to 8.7 kg for the females. The dogs were randomly divided into four groups of eight in each group comprising of four males and four females. The dogs were iv given 0, 1.6, 5.4, and 16.0 g/(kg·d) of DI (control, low-, mid-, and high-dose groups, respectively). The doses were selected based on the results of the acute toxicity study in dogs and the dose for human use. In the previous acute study, DI iv dosed to dogs in single dose of 32 g/kg induced only transiently decreased appetite signs. So the high dose used was half of the dose used in the acute toxicity study. Both DI and the control vehicle were dosed at a volume of 10 mL/kg and the total volume given was adjusted weekly based on dog's body weight. The concentration of the dosing solutions was 0, 0.16, 0.54, and 1.60 g/mL for the control, low-, mid-, and high-dose groups, respectively. The drug was delivered in order of increasing dosage level at a rate of 3—5 mL/min by iv infusion, once daily, 6 d a week (Monday to Saturday). Each animal was scheduled to be treated for 13 weeks. The dogs in the control group were treated with 5% dextrose injection, and the others with DI. After 13 weeks of treatment, three male and three female animals in each group were sacrificed, and the remaining animals were continuously observed for another 2 weeks, whereupon they were sacrificed as well. The clinical signs, mortality, body weights, food consumption, rectal temperature, ophthalmoscopy, electrocardiography, urinalysis, haematology, serum biochemistry, macroscopic findings, organ weights, and histopathology were all examined.

Clinical observation and mortality

During the study, all the animals were individually observed twice daily for mortality and clinical signs of toxicity. The detailed clinical observations included changes in skin and fur, eyes and mucous membranes, manure, psyche states, and behavior patterns. The ophthalmoscopic examinations, electrocardiography, and clinical pathology were conducted for all animals in the study twice before the start of the treatment, after dosing during weeks 7, 13, and after the 2-week recovery period. Before the start of the treatment, there were some minor statistically significant changes between the groups but the changes were within normal ranges.

Body weights, food consumption, and temperature examinations

Individual body weight was measured weekly during the treatment period thereafter. Food consumption was calculated daily by weighing the amount of food supplied to each animal and the amount remaining the next day. A daily ration was routinely available, except during overnight fasts. The average food consumptions were calculated weekly. The rectal temperature was recorded weekly.

Ophthalmoscopy

The conjunctiva, sclera, cornea, iris, lens, and fundus of each animal were examined for abnormalities of the eyes.

Electrocardiography

Electrocardiogram (lead II) of each animal was taken for heart rate and rhythm, amplitude of the P and T wave, duration of the P wave, PR interval, QRS complex, and QT intervals by using electrocardiography (ECG-6851K, NIHON KOHDEN, Japan). QT intervals were corrected using the formulae: QTc = QT-87(60/HR-1) (van de Water *et al*, 1989).

Urinalysis

Urine samples were collected from all animals and tested for parameters including bilirubin (BIL), urobilinogen (UBG), ketone (KET), ascorbic acid (ASC), glucose (GLU), protein (PRO), occult blood (BLD), pH, nitrite (NIT), leukocyte (LEU), and specific gravity (SG). The urinalysis was performed using reagent strips that were read using a urine chemistry analyzer (Uritest-500B, Urit Medical, Guilin, China).

Hematological and serum biochemical analyses

The animals fasted overnight but were allowed access to water *ad libitum* prior to the blood sample collection. The blood samples, collected from the cephalic vein into blood collection tubes, were divided into three portions. The first part was collected into tubes containing EDTA for hematological examination, the second part was taken into trisodium citrate anticoagulant for coagulation examination, and the last was allowed to clot for biochemical analysis of serum. The tests on the following hematological parameters were conducted using an Automated Hematology Analyzer (ADVIA—120, Bayer, Germany): red blood cell (RBC) count, hemoglobin (HGB) concentration, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean

corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cell (WBC) count, percent of neutrophilic granulocyte (NEUT), percent of lymphocyte (LY), percent of monocyte (MO), percent of eosinophile granulocyte (EOS), percent of basophile granulocyte (BASO), percent of large unstained cell (LUC), platelet (PL) count, plateletocrit (PCT), mean platelet volume (MPV), platelet distribution width (PDW), reticulocyte percentage (RET), high fluorescence reticulocyte (HFR), middle fluorescence reticulocyte (MFR), and low fluorescence reticulocyte (LFR). Prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (Fbg), and thrombin time (TT) for blood coagulation were measured by Coagulometer (CA—1500, Sysmex, Germany).

The non-heparinized blood sample was allowed to coagulate and centrifuge at 2200 × *g* for 10 min after the sample collection. The serum was analyzed for biochemical and electrolyte parameters. The serum biochemical parameters, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), gamma glutamyl transpeptidase (γ-GT), total bilirubin (T-BIL), blood urea nitrogen (BUN), creatinine (CRE), GLU, triglyceride (TG), total cholesterol (CHO), and creatine kinase (CK) were evaluated by an Autoanalyzer (Dimension Xpand, DADE Behring, USA). Serum electrolytes, such as sodium (Na), potassium (K), and chloride (Cl) were measured by an Ion Autoanalyzer (EasyLyte, Medica, Bedford, MA, USA).

Morphological study

At scheduled termination, fasted dogs were anesthetized by sodium pentobarbital and then sacrificed by exsanguination from the carotid artery. All animals were necropsied, and descriptions of all macroscopic abnormalities were recorded. The weights of the following organs were measured: the brain, thymus, lungs, heart, liver, spleen, kidneys, adrenal glands, testes, epididymides, uterus, and ovaries. Paired organs were weighed together. The relative organ weights were calculated based on the final body weight.

The above organs from all animals and optical nerve, pituitary gland, spinal cord, salivary gland, thyroid gland, parathyroid gland, trachea, esophagus, sternum, bone marrow, pancreas, stomach, duodenum, jejunum, ileum, colon, rectum, lymph node, urinary bladder, sciatic nerve, injection site, and any abnormal tissues were fixed with 10% neutral buffered formalin solution. All organs and tissues were routinely processed, embedded in paraffin, sectioned at 3—5 μm, and then stained with hematoxylin and eosin (HE) for microscopic examination.

Statistical analysis

The quantitative data were expressed as $\bar{X} \pm s$. All results of treatment groups were compared with those of the control group. The software used for data analysis was SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). The following statistical methods were used to analyze the body weight, food consumption, hematological and biochemical parameters, and absolute and relative organ weight data: Levene's test was used for variance homogeneity. If the Levene's test indicated the significant differences between group variances for a given parameter, group comparisons of that parameter were made using the Kruskal-Wallis test; Otherwise, a Fisher's test was used. If the Kruskal-Wallis test or Fisher's test was significant, the differences between the treatment and control groups were assessed using Dunnett's test. The results of urinalysis were analyzed by the Kruskal- Wallis test followed by multiple comparisons using the Dunnett's test. *P*-values less than 0.05 were considered significant.

Results

Clinical signs and mortality

DI was iv dosed at 0, 1.6, 5.4, and 16.0 g/(kg·d) (4 per sex per group) for 13 weeks to Beagle's dogs. All animals survived the scheduled treatment and the recovery periods. Throughout the experiment, abdominal contractions and vomiting were observed in some animals in the control group and in all the treated groups during infusion or within 1 h post infusion; these signs did not exhibit a dose-response relationship. One female dog in the mid-dose group occasionally exhibited liquid/mucous feces from the day 2 until the end of the study. Otherwise, loose feces were sporadically noted in the control and treated groups, but there were no significant differences. The urine color of animals in all treated groups was brown, and was dose-dependant. After the treatment for two weeks, the color of urine in the mid- and low-dose groups became

normal. During the recovery period, the above changes were not observed.

Body weights and food consumption

During the administration period, no significant changes in the body weights were observed in all treated groups (Table 1). However, the above female dog of the mid-dose group showed a decrease in body weight gain. Meanwhile, a significant decrease in food consumption of this dog was noted from the week 10 onward. The changes were considered to be spontaneous gastroenteritis, and not related to treatment. Overall, there was no statistically significant change in food consumption in the treated groups as compared to the control group (data not shown).

Table 1 Mean body weight of Beagle's dogs treated with DI for 13 weeks and 2 weeks recovery

Time	*n*	Mean body weight / g			
		0 g·kg^{-1}·d^{-1}	1.6 g·kg^{-1}·d^{-1}	5.4 g·kg^{-1}·d^{-1}	16.0 g·kg^{-1}·d^{-1}
Week 0	8	7.41 ± 0.68	7.36 ± 0.56	7.38 ± 0.96	7.38 ± 0.99
Week 1	8	7.69 ± 0.60	7.60 ± 0.58	7.61 ± 0.97	7.61 ± 1.04
Week 2	8	7.98 ± 0.55	7.94 ± 0.59	7.90 ± 1.05	8.00 ± 0.96
Week 3	8	8.29 ± 0.46	8.28 ± 0.65	8.16 ± 1.05	8.30 ± 0.90
Week 4	8	8.60 ± 0.41	8.46 ± 0.67	8.41 ± 1.15	8.51 ± 0.95
Week 5	8	8.83 ± 0.38	8.80 ± 0.70	8.70 ± 1.13	8.85 ± 0.88
Week 6	8	9.21 ± 0.43	9.08 ± 0.70	9.05 ± 1.14	9.18 ± 0.81
Week 7	8	9.48 ± 0.41	9.31 ± 0.73	9.28 ± 1.19	9.46 ± 0.73
Week 8	8	9.70 ± 0.43	9.54 ± 0.80	9.45 ± 1.16	9.71 ± 0.66
Week 9	8	10.01 ± 0.55	9.86 ± 0.77	9.73 ± 1.37	10.15 ± 0.67
Week 10	8	10.40 ± 0.59	10.03 ± 0.79	9.90 ± 1.48	10.41 ± 0.66
Week 11	8	10.54 ± 0.71	10.40 ± 0.84	10.11 ± 1.65	10.74 ± 0.64
Week 12	8	10.84 ± 0.74	10.68 ± 0.96	10.38 ± 1.80	11.13 ± 0.54
Week 13	8	11.15 ± 0.81	10.89 ± 1.01	10.55 ± 1.94	11.45 ± 0.61
Recovery	*n*	Mean body weight / g			
		0 g·kg^{-1}·d^{-1}	1.6 g·kg^{-1}·d^{-1}	5.4 g·kg^{-1}·d^{-1}	16.0 g·kg^{-1}·d^{-1}
Week 1	2	11.20 ± 1.84	10.70 ± 1.41	10.50 ± 0.99	11.85 ± 1.20
Week 2	2	11.30 ± 1.98	10.80 ± 1.56	10.60 ± 1.13	12.20 ± 1.41

No statistical difference between control and DI groups ($P > 0.05$)

Temperature and ophthalmoscopy

Temperature decreased in the mid-dose group on the weeks 5 and 8, and increased in the low-dose group on the weeks 1 and 4 compared to that of the control group, but the temperature readings were within the normal range (data not shown). There were no ophthalmic changes in the control and treated groups, except that the above dog who suffered from gastroenteritis had an eye discharge.

Electrocardiogram

Some minor statistically significant changes in electrocardiographic examinations were observed in the treated groups compared to the control group, but the values did not exhibit any dose-related relationship and were within historical control ranges (data not shown).

Urinalysis, hematology, and serum chemistry

No statistically significant changes were found in urinalysis in all treated groups compared to the control group (data not shown). Changes were seen in some of the hematological parameters. A statistically significant decrease in RBCs, HGB concentration, HCT, and percent of BASO was observed in the high-dose group compared to the control group on the weeks 7 and 13 (Table 2). In addition, PCT, MPV, and PDW in the high-dose group were significantly increased at the end of the treatment. During the recovery period, there were fewer minor statistically significant changes between the treated groups and the control group but the changes were within normal ranges. The coagulation tests revealed that the APTT increased significantly in the high- and mid-dose groups on the week 7, and increased only in the mid-dose group on the week 13 (Table 3). A statistically significant increase in T-BIL appeared in the mid-dose group on the week 7 and in the high-dose group on the weeks 7 and 13 compared to the control group (Table 4). A statistically significant decrease in ALP, total PRO, and CRE was observed in the high-dose group on the week 7. Total CHO in mid-

Table 2 Hematological parameters in Beagle's dogs treated with DI for 13 weeks and 2 weeks recovery

Time	Dose / ($g \cdot kg^{-1} \cdot d^{-1}$)	RBC / ($10^{12} \cdot L^{-1}$)	HGB / ($g \cdot dL^{-1}$)	HCT / %	MCV / fL	MCH / Pg	MCHC/ ($g \cdot L^{-1}$)	RDW / %	WBC ($10^{9} \cdot L^{-1}$)	NEUT / %	LY / %	MO / %
Pretest	0	6.09	143.0	40.2	66.2	23.5	355.2	12.4	13.8	59.0	29.6	7.05
	1.6	6.05	142.9	40.4	66.7	23.6	354.4	12.5	13.3	56.2	31.7	7.12
	5.4	6.21	143.4	40.8	65.9	23.2	351.2	12.5	13.0	55.5	32.8	7.15
	16.0	6.02	140.2	39.5	65.8	23.3	354.6	12.5	12.6	50.8*	37.7**	6.64
Week 7	0	6.85	148.6	43.4	63.4	21.7	341.9	12.7	10.5	55.8	34.8	4.79
	1.6	6.56	145.4	41.6	63.4	22.1	349.2**	12.3	10.2	56.8	33.5	5.60
	5.4	6.74	147.4	42.2	62.8	21.9	348.5*	12.6	10.7	56.4	33.6	5.42
	16.0	6.05**	133.5*	38.4**	63.5	22.1	347.4*	12.6	12.2	55.9	33.2	6.9**
Week 13	0	6.96	151.8	44.6	64.3	21.9	340.8	12.8	9.8	57.0	34.9	3.89
	1.6	6.9	150.0	44.0	63.8	21.7	340.6	12.6	9.0	57.8	33.7	4.31
	5.4	6.5	141.1	41.4	63.4	21.6	341.2	13.4	10.5	58.0	32.9	5.18
	16.0	6.14**	135.1**	39.1**	63.5	22.0	345.2	12.4	9.59	56.3	33.2	7.00
Recovery	0	7.60	163.5	47.0	62.1	21.6	347.5	12.6	10.7	57.6	32.0	6.25
	1.6	7.00*	153.5	43.6	62.0	21.8	352.0	12.6	7.2	51.2	39.5*	5.00
	5.4	7.05	157.0	45.6	65.2	22.5	345.0	13.2	9.2	51.1	36.7	6.25
	16.0	6.50*	137.5	40.5	62.4	21.2	340.5	13.4	7.6	55.1	35.2	4.00

Time	Dose / ($g \cdot kg^{-1} \cdot d^{-1}$)	EOS / %	BASO / %	LUC / %	PL / ($10^{9} \cdot L^{-1}$)	PCT / %	MPV / fL	PDW / %	RET / %	HFR / %	MFR / %	LFR / %
Pretest	0	2.69	0.64	1.10	419.4	0.56	13.8	63.1	2.04	10.5	23.0	66.4
	1.6	3.36	0.78	0.86	527.4	0.66*	12.9	61.3	2.21	11.7	22.9	65.4
	5.4	2.85	0.78	0.96	541.5*	0.70	13.0	62.8	2.10	13.8	21.9	64.2
	16.0	3.28	0.70	0.96	543.2	0.70	13.6	62.8	1.72	7.35	20.2	72.5
Week 7	0	2.90	0.94	0.69	390.8	0.62	16.1	65.0	1.70	15.7	22.4	61.9
	1.6	2.82	0.76	0.55	425.1	0.68	16.3	64.8	1.39*	12.7	22.4	64.8
	5.4	3.04	0.94	0.62	445.8	0.63	17.1	64.5	1.69	16.3	22.4	61.4
	16.0	2.75	0.59*	0.61	473.6	0.73*	15.8	65.0	1.60	13.6	18.7**	67.7
Week 13	0	2.39	0.90	0.84	337.1	0.39	11.6	61.1	1.66	21.4	21.9	56.6
	1.6	2.58	0.88	0.74	348.1	0.37	10.8	59.5	1.35	15.2**	21.3	63.5*
	5.4	2.46	0.66	0.78	438.6	0.48	14.4*	62.3	1.95	18.0	21.4	60.6
	16.0	2.20	0.58*	0.82	421.4	0.63**	15.3**	66.1*	1.38	11.4**	20.0	68.6**
Recovery	0	2.40	0.60	1.00	382.0	0.62	16.5	64.0	1.50	13.2	25.8	60.9
	1.6	3.05	0.80	0.50	372.0	0.51	14.2	67.6	1.20	8.8*	24.2	66.9
	5.4	3.30	1.15	1.55	413.5	0.70	17.0	64.0	1.65	12.6	25.0	62.3
	16.0	4.05	0.65	0.95	477.0	0.68	14.8	66.1	1.60	15.2	23.6	61.2

Data represent mean values (8 per group at pretest and weeks 7 and 13, 2 per group at 2-week recovery period)

$^{*}P < 0.05$ $^{**}P < 0.01$ *vs* control group, same as below

dose group decreased significantly on the weeks 7 and 13. Moreover, in the high-dose group, a statistically significant decrease in TG was noted on the week 7 and in GLU as well at the end of the treatment. There were fewer minor statistically significant changes in serum electrolytes between the treated groups and the control group but the values were within normal ranges (data not shown).

Organ weights

At the end of the treatment with DI, the absolute and relative weights of heart, kidneys, and adrenal glands in the low-dose group were increased in comparison to those of the control group (Table 5). The absolute brain weights in the mid-dose group were significantly increased. In addition, one female in the mid-dose group had enlarged uteri. The change was due to the individual variation of the estrous cycle. Some changes were observed in the absolute or relative organ weights in dogs at the end of the recovery period but there were not considered toxicological significance since these changes were not related with any histopathological findings.

Histopathological findings

A single female in the control group showed the congenital absence of left kidney, and right kidney enlarged significantly at the end of the treatment. The liver of a single male in the control group suffered from discoloration and enlargement at the end of the

Table 3 Coagulation parameters in Beagle's dogs treated with DI for 13 weeks and 2 weeks recovery

Treatment	Coagulation parameter	Time / s			
		0 g·kg^{-1}·d^{-1}	1.6 g·kg^{-1}·d^{-1}	5.4 g·kg^{-1}·d^{-1}	16.0 g·kg^{-1}·d^{-1}
Pretest	PT	6.14 ± 0.35	6.25 ± 0.28	6.24 ± 0.43	7.88 ± 5.23
	APTT	35.08 ± 9.89	28.65 ± 3.49	31.51 ± 2.62	31.98 ± 3.34
	Fbg	7.49 ± 1.80	8.16 ± 1.24	8.46 ± 1.26	8.96 ± 1.04
	TT	13.75 ± 1.27	12.79 ± 0.80	13.06 ± 0.44	13.11 ± 0.58
Week 7	PT	6.84 ± 0.56	6.70 ± 0.24	7.16 ± 0.51	8.28 ± 4.18
	APTT	20.58 ± 1.50	20.15 ± 1.64	23.81 ± 3.41*	31.31 ± 4.15**
	Fbg	9.29 ± 1.96	9.02 ± 1.69	9.61 ± 1.83	9.06 ± 1.45
	TT	11.89 ± 0.59	11.96 ± 0.64	12.15 ± 0.74	11.79 ± 0.35
Week 13	PT	6.84 ± 0.69	6.76 ± 0.31	7.02 ± 0.45	8.15 ± 4.01
	APTT	28.65 ± 4.41	32.76 ± 5.40	34.76 ± 6.52*	31.00 ± 7.16
	Fbg	10.05 ± 2.48	10.48 ± 1.90	10.32 ± 1.55	9.95 ± 0.90
	TT	13.71 ± 0.97	13.29 ± 0.85	13.19 ± 1.07	13.31 ± 0.90
Recovery	PT	6.30 ± 0.28	5.95 ± 0.78	6.35 ± 0.21	6.55 ± 0.35
	APTT	29.95 ± 0.35	39.50 ± 11.03	28.95 ± 6.58	32.55 ± 1.91
	Fbg	10.55 ± 0.21	11.05 ± 1.06	11.80 ± 3.11	10.25 ± 0.78
	TT	13.50 ± 0.85	13.15 ± 1.91	13.35 ± 1.20	13.20 ± 0.57

recovery period. There were no other macroscopic findings in the control group and all treated groups after 13-week treatment and 2-week recovery. After 13-week administration, focal chronic inflammation, focal perivascular hemorrhage and secondary blood vessel wall thickening of the injection site were observed in all treated groups, and they were found in three cases of the high-dose group, two cases of the mid-dose group, and two cases of the low-dose group. Focal chronic inflammation and blood vessel wall thickening of the injection site were found in one case of the high-dose group and two cases of the mid-dose group during the recovery period. In addition, vacuolization of the renal tubule, interstitial inflammation of lung and focal inflammation of liver were detected in the control and the treated groups. These findings were recorded in all groups and were within the range of normal background lesions in dogs of this strain and this age and therefore they were not considered treatment related.

Discussion

DI is a preparation of an extract from *S. miltiorrhiza*; the major pharmacological ingredients include salvianolic acid B, danshensu, and protocatechuic aldehyde. It has been clinically used in China for the prevention and treatment of coronary heart disease for several years. The repeated dose toxicity of danshensu in dogs has been reported (Li *et al*, 2009); the acute and chronic toxicity of Danshen Glucose Injection in rats and dogs has been published in Chinese (Han *et al*, 2005; Yang *et al*, 2005; 2007). But the drug standard of the injection used in this study was raised throughout every step of the production process. In order to determine the safety of the injection for human use, toxicological re-evaluation was carried out by using various methods including repeated dose toxicity in dogs. The present study was conducted following *Good Laboratory Practice Guidelines*, but similar toxicity studies on DI carried out earlier were not inspected by an independent quality assurance department.

Administration of DI by iv infusion to dogs for a period of 13 weeks was well tolerated. During the administration, the changes of urine color were apparently related to the treatment. Coupled with the parameters of urine, renal function of the blood serum, and renal histopathology, there was no significant nephrotoxicity. Changes may be due to metabolites of *Danshen* in the urine, rather than the treatment-related toxicity.

Liquid/mucous feces occurred in one female dog of the mid-dose group during the administration, but no obvious lesion was detected in gastrointestinal histo-pathology. The changes were considered to be spontaneous

Table 4 Serum biochemical indexes in Beagle's dogs treated with DI for 13 weeks and 2 weeks recovery

Time	Dose / ($g \cdot kg^{-1} \cdot d^{-1}$)	AST / ($U \cdot L^{-1}$)	ALT / ($U \cdot L^{-1}$)	ALP / ($U \cdot L^{-1}$)	TP / ($g \cdot L^{-1}$)	ALB / ($g \cdot L^{-1}$)	γ-GT / ($U \cdot L^{-1}$)	T-BIL / ($\mu mol \cdot L^{-1}$)
Pretest	0	34.1	34.6	164.5	57.8	32.7	6.15	2.49
	1.6	36.6	37.4	160.2	56.9	33.8	6.11	2.79
	5.4	35.4	33.8	163.4	56.9	32.9	5.59	2.49
	16.0	35.1	38.5	154.6	56.6	34.2	5.73	2.89
Week 7	0	35.6	43.6	147.6	61.0	34.2	6.11	3.10
	1.6	43.0*	48.9	143.5	60.1	33.9	5.46	3.14
	5.4	31.5	40.9	151.1	59.2	34.1	5.64	3.44*
	16.0	30.9	38.2	120.6*	56.7*	33.3	5.41	4.16*
Week 13	0	40.5	49.1	128.0	60.7	32.2	5.54	3.08
	1.6	43.1	50.4	131.1	61.4	32.5	4.93	3.32
	5.4	37.9	42.4	159.0	61.7	32.5	5.16	3.35
	16.0	39.0	44.9	112.2	58.4	32.5	4.83	4.04**
Recovery	0	49.0	47.5	138.0	60.9	29.2	6.15	3.10
	1.6	43.0	52.5	102.5	61.2	32.4	5.35	2.95
	5.4	39.0	42.0	113.5	60.7	33.0	5.55	2.90
	16.0	37.5	50.0	99.0	59.5	31.9	3.85	3.20

Time	Dose / ($g \cdot kg^{-1} \cdot d^{-1}$)	BUN / ($mmol \cdot L^{-1}$)	CRE / ($\mu mol \cdot L^{-1}$)	GLU / ($mmol \cdot L^{-1}$)	TG / ($mmol \cdot L^{-1}$)	CHO / ($mmol \cdot L^{-1}$)	CK / ($U \cdot L^{-1}$)
Pretest	0	3.59	40.4	5.26	0.49	4.88	115.0
	1.6	3.05	37.0	5.81*	0.45	4.32	128.6
	5.4	2.85	39.1	5.25	0.53	4.28	121.6
	16.0	3.51	37.2	5.60	0.42	4.28	138.5
Week 7	0	3.49	53.6	5.53	0.53	6.09	122.9
	1.6	3.01	49.5	5.63	0.48	5.41	170.9**
	5.4	3.06	49.9	5.31	0.46	4.99*	107.9
	16.0	3.02	44.4*	5.33	0.35*	5.10	122.2
Week 13	0	3.76	54.8	5.25	0.41	6.09	141.8
	1.6	3.75	56.1	5.53	0.41	5.84	143.0
	5.4	3.48	48.4	5.09	0.43	5.10*	147.8
	16.0	3.75	49.1	4.80**	0.40	5.56	161.9
Recovery	0	3.40	46.6	5.05	0.81	6.15	189.5
	1.6	3.10	47.8	5.50	0.44	4.95	167.0
	5.4	2.50	46.2	5.00	0.48	4.40	180.5
	16.0	3.05	41.4	5.20	0.39	4.90	221.5

gastroenteritis, and not related to the treatment because it was only in the mid dose. There were no changes in body weight gain and food intake with the treated animals except above female dog in the mid-dose group. The dog who had a slow weight gain and bad appetite may be related to its own spontaneous gastroenteritis.

There were no toxicologically significant changes in body temperature, ophthalmic examinations, or electrocardiographic examination because all findings were within the range of normal dogs. A significant treatment related decrease in RBC count, HGB concentration, and HCT in the high-dose group was observed. The results suggested that the iv administration of DI at a dose of 16.0 g/(kg·d) for 13 weeks affects haemopoiesis in Beagle's dogs. The changes were consistent with the findings reported in the sub-chronic toxicity study of danshensu in dogs (Li *et al*, 2009). A similar adverse reaction of DI on RBC was not reported in clinical use. Nevertheless, clinicians should monitor for potential hematology changes in patients administered with *Danshen*. A statistically significant increase in APTT was observed in the high- and mid-dose groups on the week 7 and this parameter was significantly increased in the mid-dose group at the end of the administration. Although there was no statistically significant change, this parameter in the high-dose group was higher than that in the control group. The results suggested that DI influenced the coagulation function in Beagle's dogs. This may be due to the effect of "activating blood and resolving stasis"

Table 5 Absolute and relative organ weights in Beagle's dogs treated with DI for 13 weeks and 2 weeks recovery

Time	Dose / ($g \cdot kg^{-1} \cdot d^{-1}$)	Brain		Thymus		Lungs		Heart		Liver	
		Abs / g	Rel / %	Abs / g	Rel / %	Abs / g	Rel / %	Abs / g	Rel / %	Abs / g	Rel / %
Week 13	0	77.2 ± 5.2	0.69 ± 0.06	13.2 ± 7.0	0.12 ± 0.06	86.3 ± 6.4	0.78 ± 0.07	74.0 ± 6.3	0.66 ± 0.04	233.3 ± 30.0	2.11 ± 0.39
	1.6	82.3 ± 5.3	0.76 ± 0.10	14.3 ± 4.0	0.13 ± 0.04	84.2 ± 4.8	0.79 ± 0.09	83.3 ± 3.7*	0.77 ± 0.09*	234.0 ± 26.1	2.15 ± 0.12
	5.4	87.7 ± 10.1*	0.87 ± 0.24	12.7 ± 3.9	0.12 ± 0.02	78.7 ± 14.2	0.76 ± 0.12	70.5 ± 14.9	0.67 ± 0.08	246.5 ± 31.9	2.45 ± 0.78
	16.0	81.2 ± 6.7	0.71 ± 0.07	15.7 ± 3.3	0.14 ± 0.03	89.2 ± 10.6	0.78 ± 0.10	74.8 ± 5.1	0.66 ± 0.06	250.7 ± 32.9	2.19 ± 0.29
Recovery	0	82.0 ± 8.5	0.73 ± 0.05	12.9 ± 1.1	0.12 ± 0.03	75.0 ± 11.3	0.67 ± 0.02	69.0 ± 12.7	0.61 ± 0.00	247.5 ± 47.4	2.26 ± 0.82
	1.6	85.5 ± 12.0	0.79 ± 0.00	15.4 ± 4.8	0.15 ± 0.06	84.5 ± 19.1	0.78 ± 0.06	82.0 ± 0.00	0.77 ± 0.11	229.0 ± 15.6	2.13 ± 0.16
	5.4	82.0 ± 9.9	0.77 ± 0.01	10.6 ± 0.8	0.10 ± 0.02	80.0 ± 7.1	0.76 ± 0.01*	77.5 ± 5.0	0.73 ± 0.03*	208.5 ± 9.2	1.97 ± 0.12
	16.0	88.5 ± 7.8	0.73 ± 0.02	10.8 ± 3.2	0.09 ± 0.04	89.0 ± 9.9	0.73 ± 0.03*	83.5 ± 5.0	0.69 ± 0.04	269.5 ± 17.7	2.23 ± 0.40

Time	Dose / ($g \cdot kg^{-1} \cdot d^{-1}$)	Spleen		Kidney		Adrenal gland		Testes and epididymis		Uterus and ovaries	
		Abs / g	Rel / %	Abs / g	Rel / %	Abs / g	Rel / %	Abs / g	Rel / %	Abs / g	Rel / %
Week 13	0	27.3 ± 7.8	0.24 ± 0.06	39.9 ± 3.3	0.36 ± 0.04	0.90 ± 0.16	8.10 ± 1.22	14.8 ± 2.6	0.13 ± 0.01	3.35 ± 0.60	0.030 ± 0.007
	1.6	26.8 ± 6.5	0.24 ± 0.05	44.9 ± 3.0*	0.41 ± 0.02*	1.15 ± 0.15**	10.63 ± 1.42**	17.4 ± 2.6	0.16 ± 0.03	2.61 ± 0.71	0.024 ± 0.005
	5.4	26.4 ± 10.9	0.25 ± 0.09	43.8 ± 8.4	0.42 ± 0.08	1.00 ± 0.21	9.56 ± 1.64	16.8 ± 1.9	0.14 ± 0.02	8.26 ± 8.86	0.079 ± 0.079
	16.0	26.4 ± 5.5	0.23 ± 0.05	46.6 ± 7.0	0.41 ± 0.06	0.94 ± 0.13	8.22 ± 1.07	11.4 ± 2.0	0.10 ± 0.02	3.24 ± 1.13	0.028 ± 0.098
Recovery	0	33.2 ± 6.8	0.29 ± 0.01	45.2 ± 10.1	0.40 ± 0.02	1.07 ± 0.25	9.40 ± 0.56	15.7	0.16	2.99	0.030
	1.6	28.0 ± 12.9	0.25 ± 0.08	43.6 ± 9.4	0.40 ± 0.03	0.84 ± 0.08	7.78 ± 0.39	19.1	0.16	2.29	0.019
	5.4	22.1 ± 3.5	0.21 ± 0.01*	45.8 ± 8.6	0.43 ± 0.03	0.97 ± 0.21	9.07 ± 1.00	25.7	0.22	2.93	0.026
	16.0	23.6 ± 2.4	0.19 ± 0.03**	47.6 ± 8.0	0.39 ± 0.02	1.03 ± 0.01	8.46 ± 0.87	15.9	0.12	4.21	0.032

Values are means and standard deviation (3/sex/group at 13-week administration, 1/sex/group at 2-week recovery period)

(*Huoxue Huayu*) of *Danshen*. The other statistically significant changes in hematological parameters were not considered to be treatment related because they did not show a dose-dependent relationship, and some were recorded before the administration.

There were statistically significant changes in some biochemical parameters in the treatment groups compared with the control group. Among these parameters, an increase in total BIL level was considered to be treatment related. Coupled with a normal organ weight and histopathology of the liver, it suggested that there was no significant hepatotoxicity. At the end of the administration, a statistically significant decrease in GLU appeared in the high-dose group indicating that DI had hypoglycemic activity in Beagle's dogs. In our sub-chronic toxicity study on DI in rats, a significant decrease in the TG level was observed (Li *et al*, 2009; Wang *et al*, 2012); However, a statistically significant decrease in TG was observed only on week 7 in the current study.

The macro- and histopathology findings recorded in all groups, except for focal chronic inflammation of the injection site, were within the range of spontaneous background lesions, which may be observed in the dogs of this strain and this age. At the end of the administration, the lesions of the injection site were

identified in all treatment groups, and their severity increased while the injection dose increased. The inflammation in the low-dose group was not detected at the end of a 2-week recovery period. These changes were partially due to mechanical stimulation, and mainly caused by the stimulation of high concentration injection. In this study, the concentration of DI in the high-, mid-, and low-dose groups was 25, 8, and 2.5 times higher, respectively, than those for human clinical use. The same adverse reaction induced by iv administration was not reported during the clinical application of DI. Therefore, the clinical risk of local irritation is very low.

Conclusion

Above all, the present study shows that the administration of DI by daily in dogs at 16 g/(kg·d) for 13 weeks could cause the decreases in RBC parameters and GLU, as well as the lesions of the injection site. The no observed adverse effect level is 5.4 g/(kg·d) DI in this 13 week dog toxicology study by iv administration.

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References

Bao YX, Wong CK, Leung SF, Chan AT, Li PW, Wong EL, Leung PC, Fung KP, Yin YB, Lam CW, 2006. Clinical studies of immunomodulatory activities of Yunzhi-Danshen in patients with nasopharyngeal carcinoma. *J Altern Complement Med* 12: 771-776.

Chen YC, Cao WW, Cao Y, Zhang L, Chang BB, Yang WL, Liu XQ, 2011. Using neural networks to determine the contribution of danshensu to its multiple cardiovascular activities in acute myocardial infarction rats. *J Ethnopharmacol* 138: 126-134.

Cheng TO, 2006a. Danshen: a versatile Chinese herbal drug for the treatment of coronary heart disease. *Int J Cardiol* 113: 437-438.

Cheng TO, 2006b. Danshen: what every cardiologist should know about this Chinese herbal drug. *Int J Cardiol* 110: 411-412.

Han J, Liu XJ, Xu Y, Wang WS, Ma ZQ, Xiong Y, 2005. Long-term toxicity study of salvia miltiorrhiza glucose injection in rats. *J Wannan Med Univ* 24: 248-251.

Ji X, Tan BK, Zhu YC, Linz W, Zhu YZ, 2003. Comparison of cardioprotective effects using ramipril and DanShen for the treatment of acute myocardial infarction in rats. *Life Sci* 73: 1413-1426.

Ji XY, Tan BK, Zhu YZ, 2000. *Salvia miltiorrhiza* and ischemic diseases. *Acta Pharmacol Sin* 21: 1089-1094.

Kang DG, Yun YG, Ryoo JH, Lee HS, 2002. Anti-hypertensive effect of water extract of danshen on renovascular hypertension through inhibition of the renin angiotensin system. *Am J Chin Med* 30: 87-93.

Li G, Gao Y, Li S, Li C, Zhu X, Li M, Liu Z, 2009. Study on toxicity of danshensu in beagle dogs after 3-month continuous intravenous infusion. *Toxicol Mech Methods* 19: 441-446.

Luo JB, 2010. Thinking about the healthy development of traditional Chinese medicine injection. *Mode Tradit Chin Med Mater Med* 12: 497-501.

Ni Q, Wang J, Li EQ, Zhao AB, Yu B, Wang M, Huang CR, 2011. Study on the protective effect of the Mixture of Shengmai Powder and Danshen Decoction on the myocardium of diabetic cardiomyopathy in the rat model. *Chin J Integr Med* 17: 116-125.

van de Water A, Verheyen J, Xhonneux R, Reneman RS, 1989. An improved method to correct the QT interval of the electrocardiogram for changes in heart rate. *J Pharmacol Methods* 22: 207-217.

Wang L, Yin T, Zhu MW, 2009. Retrospective study of the adverse drug reaction/event induced by Danshen injection. *Chin J Pharmacovigilance* 6: 227-231.

Wang M, Liu J, Zhou B, Xu R, Tao L, Ji ML, Zhu LP, Jiang J, Shen J, Gui XL, Gu LL, Bai WX, Sun W, Cheng JM, 2012. Acute and sub-chronic toxicity studies of Danshen injection in Sprague-Dawley rats. *J Ethnopharmacol* 141: 96-103.

Yang JR, Ding BP, Chen GX, Gui CQ, Fu WS, Xu CY, Liu XP, 2005. Study on a long-term toxicity test of Danshen glucose injection in Beagle dog. *J Wannan Med Univ* 24: 14-19.

Yang JR, Gui CQ, Xiong Y, Chen GX, Ding BP, Song JG, 2007. Study on acute and topical toxicity of Danshen glucose injection. *J Wannan Med Univ* 26: 11-14.

Yue KK, Lee KW, Chan KK, Leung KS, Leung AW, Cheng CH, 2006. Danshen prevents the occurrence of oxidative stress in the eye and aorta of diabetic rats without affecting the hyperglycemic state. *J Ethnopharmacol* 106: 136-141.

Yue QX, Xie FB, Song XY, Wu WY, Jiang BH, Guan SH, Yang M, Liu X, Guo DA, 2012. Proteomic studies on protective effects of salvianolic acids, notoginsengnosides and combination of salvianolic acids and notoginsengnosides against cardiac ischemic-reperfusion injury. *J Ethnopharmacol* 141: 659-667.

Zhang JL, Cui M, He Y, Yu HL, Guo DA, 2005. Chemical fingerprint and metabolic fingerprint analysis of Danshen injection by HPLC-UV and HPLC-MS methods. *J Pharm Biomed Anal* 36: 1029-1035.

Zhang XP, Jiang J, Yu YP, Cheng QH, Chen B, 2010. Effect of Danshen on apoptosis and NF-kappaB protein expression of the intestinal mucosa of rats with severe acute pancreatitis or obstructive jaundice. *Hepatobiliary Pancreat Dis Int* 9: 537-546.

Zhou L, Zuo Z, Chow MS, 2005. Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J Clin Pharmacol* 45: 1345-1359.

Zhou R, He LF, Li YJ, Shen Y, Chao RB, Du JR, 2012. Cardioprotective effect of water and ethanol extract of *Salvia miltiorrhiza* in an experimental model of myocardial infarction. *J Ethnopharmacol* 139: 440-446.

Zou ZW, Xu LN, Tian JY, 1993. Antithrombotic and antiplatelet effects of rosmarinic acid, a water-soluble component isolated from radix *Salviae miltiorrhizae* (danshen). *Acta Pharm Sin* 28: 241-245.