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RESEARCH ARTICLE

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Toxicity Studies on Secretio Bufonis: A Traditional Supplement in Asia

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Abstract
Objectives: This study was performed to investigate the toxicity of Secretio Bufonis (SB) on male mice and assess its no-observed-adverse-effect-level (NOAEL).
Materials and Methods: After feeding an aqueous solution of SB extracts to mice for either 1 or 8 weeks, their blood and urine were assayed and their liver and kidney morphology examined. The numerical data was analyzed by the Mann-Whitney U-test and analysis of variance test.
Results: Mice administered SB in 50 mg/kg/day for 1 week had higher heart weights and higher aspartate transaminase activities; those administered SB in 0.01 and 0.05 mg/kg/day for 8 weeks had lower creatinine concentrations; and those administered SB in 0.5 mg/kg/day for 8 weeks had higher brain weights and higher blood urea nitrogen.
Conclusions: The extracts of SB had cardiac toxicity in the short term and hepatotoxicity in the long term. The NOAEL of the extract was under 5 mg/kg/day for 1 week and under 0.25 mg/kg/day for 8 weeks.

1. Introduction

Chan Su is a traditional Chinese medicine prepared from the dried white secretion of the auricular and skin glands of the toads *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider [1]. The secretion commonly contains biogenic amines, alkaloids, peptides, proteins, and bufadienolides, one of the main components of Chan Su and a newly recognized type of natural steroids with potent bioactivities [2]. Chan Su has been used in the treatment of various diseases, including cancer, arrhythmia, and other heart diseases. Studies *in vivo* or *in vitro* have shown that HuaChanSu, an extract of a species of Chinese toad, has several functions, including the ability to kill several kinds of tumor cells, an antitumor effect, a leukopoietic effect, and increased immunity [3].

Unfortunately, HuaChanSu has obvious side effects in clinical settings because of serious toxicity to humans and, thus it had been used carefully by clinicians. Chan Su overdose can cause nausea, vomiting, diarrhea, and even general paralysis [4]. Toad toxin poisoning manifests primarily as digitalis toxicity-like cardiac effects, including bradycardia, atrioventricular conduction block, ventricular

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tachycardia, ventricular fibrillation, and sudden death [5]. The death of one Chinese woman after ingestion of a Chinese herbal tea containing Chan Su has been reported and a trial has been performed regarding the lethality to mice of Chan Su in diverse consuming types [6]. One study reported the lethal dose causing 50% death (LD_{50}) of toad skin extract in mice was ~400 mg/kg intravenously [7]. We could not locate any systemic *in vivo* trials evaluating Chan Su's toxicity and its no-observed-adverse-effect-level (NOAEL).

Aqueous extracts of SB were administered orally to male Institute for Cancer Research (ICR) mice and the pathological changes observed. The present communication presents the results of this study and evaluates the toxicity of this SB extract in male ICR mice.

2. Materials and Methods

2.1. Test material

Solid powder of whole Secretio Bufonis (SB) extracts was obtained from Dunsan Oriental Hospital (Daejeon, South Korea). Fifty gram of SB was washed with distilled water, and then boiled with 80ºC for 6 hours. Solid particles and aggregates were removed by centrifugation at 3000*g* for 30 minutes and the supernatant lyophilized. The resulting 22.5 g of lyophilized SB was stored at −20ºC until used.

2.2. Animals and administration method

Fifty male ICR mice (6 weeks old, Samtako, Korea) were used after acclimatization for 7 days. The animals were housed individually in suspended wire cages in a temperature (21–24ºC) and humidity (43–40%) controlled room with a 12:12 hour light:dark cycle and provided food (Pellet, Samyang, Korea) and water *ad libitum*. These mice were equally distributed into 10 groups (five groups for the short term toxicity study, five groups for a long term toxicity study).

The expected, normal, human tolerance to SB is approximately <0.5–1 mg/kg/day. Based on the use of SB in animal studies and clinical applications, dosages of 0, 0.05, 0.5, 5, and 50 mg/kg/day were set for the short term toxicity study and 0, 0.01, 0.05, 0.25, and 0.5 mg/kg/day for the long term toxicity study. While the extract was orally administered to mice, distilled water was administered to the control group. The total volume administered was 0.15 mL/kg body weight. For the short term toxicity study, the groups were designated as follows: 0 mg/kg SB distilled water control group, D.W.; 0.05 mg/kg SB, SB-S1; 0.5 mg/kg SB, SB-S2; 5 mg/kg SB, SB-S3; and 50 mg/kg SB, SB-S4. For the long term toxicity study, the groups were: 0 mg/kg SB distilled water control group, D.W.; 0.01 mg/kg SB, SB-R1; 0.05 mg/kg SB, SB-R2; 0.25 mg/kg SB, SB-R3; and 0.50 mg/kg SB, SB-R4.

Representative dose preparations for each SB concentration were analyzed for homogeneity of distribution, concentration, and stability during the studies. An appropriate amount of the test substance or distilled water was administered orally to each mouse for 1 week in the short term study, and 8 weeks in the long term study. Mice were monitored for mortality, signs of gross toxicity, and behavioral changes at least once daily for the duration of the studies.

2.3. Hematology, biochemistry parameters, and urine analysis

All mice were fasted ~18 hours prior to collection a blood sample from the descending aorta under ether anesthesia at the termination of the experiment. Urine analysis, including specific gravity, pH, leukocyte, nitrite, protein, glucose, ketone, urobilinogen, bilirubin, and blood, was conducted using Bayer Diagnostics Multistix 10SG (Not. 5J06C, USA) REF 2300 (03536597) reagent strips for urine analysis and a Urine Chemistry Analyzer (Bayer Clinitek 500, USA).

2.4. Morphologic pathology evaluations

In the long term study, complete post morphologic evaluations were immediately performed on mice found dead to avoid organ autolysis and, at the termination of the study, the surviving animals were fasted overnight, weighed, and humanely killed using anesthetic ether.

Gross pathologic evaluations were performed and the weight of liver, kidney, heart, spleen, lungs, testis, and brain measured and recorded. The relative organ weights (organ to body weight ratio) were also calculated. Histopathological examination was performed on routinely prepared sections of hepatic and renal tissues; the tissues were fixed in 10% formalin immediately after removal and weighing and standard hematoxylin and eosin staining performed.

2.5. Statistics

The Mann-Whitney U-test was used to compare the homogeneity of variance in the numerical data (body weight, food and water consumption, hematology, blood chemistry, and organ weights). If there was homogeneity of variance in the data between groups, a one way ANOVA test was conducted. For these analyses, Microsoft Excel 2002 and SPSS 10.1 were

used and statistical significance was considered at $p<0.05$.

3. Results

3.1. SB-consumption group in short term toxicity study showed significantly increased heart weight

Absolute and relative weights of treated mice indicated that there were no statistically significant differences among the treated groups without considering relative heart weight. The relative heart weight of SB-S4 group mice was greater than that of the control group ($p<0.05$) (Table 1).

3.2. SB-consumption group in short term toxicity study showed higher values of AST as a biochemical parameter

The AST level in mice of the SB-S4 group was higher than in the control group ($p<0.05$) (Table 2).

3.3. SB-consumption group in long term toxicity study had a more significant absolute brain weight gain than the control group

Absolute and relative weights of treated mice indicated that there were no statistically significant differences among treated groups without considering absolute brain weight. The absolute brain weight

Table 1 Relative organ weights of mice with oral SB for 1 week

Group–Dose (mg/kg/day)		Body weight (g)	Liver (%)	Kidney		Spleen (%)	Testis		Brain (%)	Lung (%)	Heart (%)
				Lt. (%)	Rt. (%)		Lt. (%)	Rt. (%)			
D.W.	Mean	35.8	5.7	0.7	0.7	0.4	0.3	0.3	1.4	0.6	0.4
(0)	SD	2.59	0.47	0.10	0.10	0.09	0.02	0.03	0.09	0.07	0.03
SB-S1	Mean	34.8	5.3	0.8	0.8	0.4	0.3	0.3	1.4	0.7	0.4
(0.05)	SD	1.92	0.59	0.06	0.07	0.04	0.04	0.04	0.11	0.10	0.04
SB-S2	Mean	35.6	4.3	1.9	1.9	0.9	0.8	0.8	3.7	1.7	1.2
(0.5)	SD	2.07	2.50	2.65	2.80	1.21	1.02	0.97	5.20	2.52	1.69
SB-S3	Mean	34.4	5.2	0.7	0.8	0.4	0.3	0.3	1.4	0.7	0.4
(5)	SD	2.07	0.17	0.07	0.06	0.07	0.02	0.02	0.12	0.07	0.06
SB-S4	Mean	33.6	5.4	0.7	0.8	0.4	0.3	0.3	1.5	0.7	0.5*
(50)	SD	2.07	0.70	0.08	0.06	0.06	0.02	0.03	0.08	0.06	0.04

Five mice/group; given SB in different concentrations for 1 week; organ weight and percentage to body weight; relative heart weight of SB-S4 larger than control group (0.5±0.04%, $p<0.05$). *Significantly different from values of D.W. group at $p<0.05$. Lt. = left; Rt. = right; SD = Standard Deviation; D.W. = Dextrose water; SB-S = *Secretio Bufonis* short term toxicity group.

Table 2 Blood chemical values of mice with oral SB for 1 week

Group–Dose (mg/kg/day)		Total protein (g/dL)	Albumin (g/dL)	A/G ratio (g/dL)	Creatinine (mg/dL)	BUN (mg/dL)	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	GOT (U/L)	GPT (U/L)
D.W.	Mean	3.1	1.5	1.0	0.3	22.1	99.2	76.8	73.0	45.0
(0)	SD	0.25	0.06	0.14	0.03	2.73	6.33	8.63	7.93	8.81
SB-S1	Mean	3.1	1.7	1.2	0.3	19.8	103.0	61.6	76.8	26.6
(0.05)	SD	0.05	0.06	0.09	0.03	2.41	4.83	6.94	8.42	2.05
SB-S2	Mean	3.3	1.7	1.0	0.2	20.4	104.8	64.8	72.2	25.0
(0.5)	SD	0.10	0.07	0.05	0.06	1.91	2.75	8.08	5.47	1.54
SB-S3	Mean	3.7	1.7	0.9	0.2	20.3	111.6	65.0	97.6	29.2
(5)	SD	0.32	0.11	0.16	0.04	2.12	9.09	8.51	14.88	1.14
SB-S4	Mean	3.7	1.6	0.9	0.2	20.1	112.6	68.0	98.0*	27.4
(50)	SD	0.29	0.07	0.18	0.02	1.50	11.25	7.66	7.52	3.91

GOT level in SB-S4 group higher than control group (98.0±7.52%, $p<0.05$). *Significantly different from values of D.W. group at $p<0.05$. A/G = Albumin/Globulin ratio; BUN = blood urea nitrogen; GOT = Glutamic oxaloacetic transaminase; GPT = Glutamate pyruvate transaminase; SD = Standard Deviation; D.W. = Dextrose water; SB-S = *Secretio Bufonis* short term toxicity group.

Table 3 Absolute organ weights of mice with oral SB for 8 weeks

Group–Dose (mg/kg/day)		Body weight (g)	Liver (mg)	Kidney		Spleen (mg)	Testis		Brain (mg)	Lung (mg)	Heart (mg)
				Lt. (mg)	Rt. (mg)		Lt. (mg)	Rt. (mg)			
D.W. (0)	Mean	36.8	1634.6	233.0	223.8	101.6	112.8	108.4	491.0	176.8	135.8
	SD	2.95	177.41	28.95	31.74	16.27	8.67	6.88	14.54	14.99	8.32
SB-R1 (0.01)	Mean	34.0	1491.4	240.0	225.6	96.6	107.6	100.0	461.4	176.2	136.6
	SD	2.65	280.30	35.06	11.06	18.64	7.80	6.20	40.32	14.53	12.90
SB-R2 (0.05)	Mean	36.8	1562.4	231.8	220.2	96.4	111.2	107.2	479.2	179.4	141.0
	SD	2.59	251.09	36.54	33.97	13.90	18.16	10.73	39.19	25.31	14.23
SB-R3 (0.25)	Mean	33.0	1380.6	214.8	210.8	89.0	111.0	103.2	464.4	169.8	122.6
	SD	2.55	196.74	19.52	10.35	19.20	9.35	7.43	39.51	16.27	10.43
SB-R4 (0.50)	Mean	36.4	1485.2	240.8	230.6	99.0	113.4	106.4	460.8*	173.2	128.4
	SD	3.58	248.10	30.82	27.46	16.16	7.64	6.80	13.14	19.45	13.79

Larger brain weight in SB-R4 (460.8±13.14%, $p<0.05$). *Significantly different from values of D.W. group at $p<0.05$. Lt. = left; Rt. = right; SD = Standard Deviation; D.W. = Dextrose water; SB-R = *Secretio Bufonis* long term toxicity group.

Table 4 Blood chemical values of mice with oral SB for 8 weeks

Group–Dose (mg/kg/day)		Total protein (g/dL)	Albumin (g/dL)	A/G ratio (g/dL)	Creatinine (mg/dL)	BUN (mg/dL)	Total cholesterol (mg/dL)	TGs (mg/dL)	GOT (U/L)	GPT (U/L)
D.W. (0)	Mean	4.8	2.3	0.9	0.4	21.5	93.0	144.4	78.2	28.8
	SD	0.16	0.05	0.02	0.06	1.33	4.06	27.29	11.35	7.65
SB-R1 (0.01)	Mean	4.6	2.1	0.9	0.2*	23.4	85.4	107.2	137.2	36.6
	SD	0.21	0.15	0.08	0.02	2.82	7.01	27.70	33.14	15.54
SB-R2 (0.05)	Mean	4.9	2.4	1.0	0.2*	20.6	105.2	87.2	74.8	15.0
	SD	0.19	0.05	0.05	0.06	1.18	6.17	10.28	4.16	2.60
SB-R3 (0.25)	Mean	4.6	2.3	1.0	0.4	20.6	99.0	111.0	114.4	16.4
	SD	0.12	0.06	0.09	0.02	1.50	10.42	9.77	22.62	1.79
SB-R4 (0.50)	Mean	4.6	2.2	1.0	0.4	26.3*	89.0	122.8	153.6	18.2
	SD	0.30	0.06	0.07	0.04	0.32	5.70	21.76	42.08	1.85

Significantly lower Creatinine level in SB-R1 and SB-R2; higher BUN level in SB-R4 group (0.2±0.02%, 0.2±0.06%, 26.3±0.32%, respectively). *Significantly different from values of D.W. group at $p<0.05$; A/G = Albumin/Globulin ratio; BUN = blood urea nitrogen; GOT = Glutamic oxaloacetic transaminase; GPT = Glutamate pyruvate transaminase; SD = Standard Deviation; D.W. = Dextrose water; SB-R = *Secretio Bufonis* long term toxicity group; TGs = triglycerides.

of SB-R4 group was significantly greater than the control group ($p<0.05$) (Table 3).

3.4. SB-consumption group in long term toxicity study had significantly lower values for creatinine and higher values of blood urea nitrogen (BUN) than the control group

No significant hematological changes attributable to SB after the dosing period were observed without considering the creatinine and BUN values (Table 4). A significantly lower value of creatinine was observed in the SB-R1 and SB-R2 groups and higher values of BUN in the SB-R4 group.

4. Discussion

Toad venom contains two groups of toxic compounds: steroid derivatives that resemble cardiac glycosides, consisting of bufagenins (bufagins and bufadienolides) and bufotoxins, and the basic components that include epinephrine, norepinephrine, serotonin, and bufotenin [7]. These compounds have a narrow range between a treatment dose and a lethal dose.

Some people believe that microbial biotechnology can improve this supplement to produce or develop more therapeutic advantages, such as increased potency, longer half-life in the bloodstream, simpler delivery methods, and reduced side effects [8].

New compounds have been found with greater cytotoxic activities *in vitro* than cinobufagin but their cytotoxicity have not been studied, and they have not been used in the clinical setting.

Here, we aimed to elucidate the oral toxicity of SB by preparing ICR mice in several groups and administering SB in a short term and a long term toxicity study. The dosages chosen were based on other available studies.

In the short term study, the mice given SB at 50 mg/kg/day showed greater increases in relative heart weight and higher AST activities than the other study groups. A heart usually grows larger as its circulatory function grows weaker and the resulting cardiomegaly can induce myocardial infarction which leads to increases in AST secretion into the bloodstream. The results here suggested that SB significantly affected the mouse heart in 1 week with consumption over 50 mg/kg/day and, thus we concluded that SB had significant toxicity in mouse heart under these conditions. On this basis, the NOAEL of SB for 1 week was considered to be under 5 mg/kg/day.

In the long term toxicity study, the groups administered SB at 0.5 mg/kg/day had larger absolute brain weight than the other groups. This increased brain weight could have some interesting implications, such as symptoms like autism or brain development. Further studies, such as observations of the behavioral changes or brain imaging studies, are needed to determine the cause of this brain hypertrophy, as the use of normal mice here obviated the chance for the present results to be expressions of congenital malformations.

We also observed that 0.01 mg/kg/day-SB administered mice and 0.05 mg/kg/day-SB administered mice had lower creatinine concentrations in their blood. Plasma creatinine, which results from catabolism of creatine phosphate in skeletal muscle, increases when renal function is poor and decreases with losses of skeletal muscle [9]. Lowering creatinine concentrations did not appear to be a useful evaluation factor of SB toxicity.

The BUN content in the mice administered SB in 0.5 mg/kg/day was higher than in other groups. BUN content usually increases when kidney filtration is failing or liver is compromised in some manner. We suggest here that SB had toxicity to mouse liver when administered at doses over 0.5 mg/kg/day for 8 weeks, indicated by the results of the long term study. Thus the NOAEL for an 8 week SB administration was considered to be less than 0.25 mg/kg/day.

Some literature has reported that SB has synergistic therapeutic efficacy with some drugs in treating cancers [10]. Combination therapy could increase efficacy and improve patients' quality of life significantly compared with chemotherapy alone [11]. Based on these results, one cancer center in the United States has been proceeding with phase I trials with a Chinese university since 2006 for evaluating the efficacy of HCS as measured by progression free survival over 4 months. In addition, randomized, placebo-controlled, blinded phase II studies of Huachansu and Gemcitabine in treating pancreatic cancer are in progress.

SB toxicity is one of the major obstacles to its usage in cancer treatment. Although some researchers seek to find means to yield SB less toxic and more effective, we need to know the toxicity of SB itself due to its current use in clinic. Otherwise, it is not possible judge the dangers of SB is and plan the appropriate limited dosages.

Here, the results of short and long term studies of aqueous SB toxicity have provided a reliable means for judging SB for clinical use. It is noteworthy that SB did not pose any dangers in normal mice at less than 5 mg/kg/day for 1 week of oral intake and 0.25 mg/kg/day for an 8 week period. But, SB water extracts may induce heart and liver toxicity when used at higher than 5 mg/kg/day for 1 week and 0.5 mg/kg/day for 8 weeks.

These present studies encountered some problems regarding the exact toxicity of SB in that we only observed changes caused and did not further examined how or why the changes occurred. We relied on information retrieved from other studies and reports that correlate similar symptoms in different circumstances. Although we looked for observable changes in liver and kidney tissues, possible changes at the molecular level that could have been present were not addressed.

Despite these shortcomings, these studies were meaningful because of SB's widespread usage and known effects, such as cardiotonic effects caused by toad skin extracts. The widespread assumption that toad skin extracts pose some dangers to the cardiovascular system was strengthened by the present results. However, SB has great possibilities as an effective antitumor drug, suggesting that there is a great need to seek means of decreasing SB's toxicity while retaining or augmenting its useful pharmacological effects.

In summary, SB was shown here to have toxicity in the mouse heart and liver and its NOAEL in this animal model was less than 5 mg/kg/day for 1 week and 0.25 mg/kg/day for 8 weeks.

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