

The Anaphylactoid Constituents in Xue-Sai-Tong Injection

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Abstract

Xue-Sai-Tong injection, a traditional Chinese medicine with total saponins of Sanqi ginseng as active ingredients, has been used for more than 500 years to treat coronary artery disease in China. Anaphylactoid reaction induced by Xue-Sai-Tong injection was one of the main adverse drug reactions which has occurred frequently in recent years. It is of importance to elucidate its anaphylactoid constituents. The *in vivo* anaphylactoid tests indicated that the anaphylactoid mediators could be used as indexes to evaluate the anaphylactoid action. Further, the *in vitro* model based on determining the mediators release from the degranulation of mast cells and RBL-2H3 cells stimulated by Xue-Sai-Tong injection was explored. Mediators released from mast cells and RBL-2H3 cells caused by Xue-Sai-Tong injection were determined by comparison of the methods of fluorospectrophotometry, ELISA, and spectrophotometry, respectively, revealing that the histamine release induced by the Xue-Sai-Tong injection could not be assayed accurately by the method of fluorospectrophotometry because of the interference of saponins and unknown components in the injection. The rat peritoneal mast cell was also not an optimal cell model for determining histamine and β -hexosaminidase release due to the higher spontaneous release ratio during the cell collection. Thus, ELISA determination of the histamine release from RBL-2H3 cells is a suitable *in vitro* model to assay the anaphylactoid reaction

of Xue-Sai-Tong injection. Previously, abnormal hemolysis in some batches of Xue-Sai-Tong injection was observed in the course of their HD₅₀ (half hemolytic dosage) determination. This study further found that injections which exhibited an abnormal hemolysis phenomenon also caused a higher release of the anaphylactoid mediators from RBL-2H3 cells, indicating the HD₅₀ could be an auxiliary index to evaluate anaphylactoid action of the herbal injection indirectly. Research for anaphylactoid components in Xue-Sai-Tong injection indicated that proteins with over 10 KDa of molecular weight, but not ginsenosides, could be the main constituents inducing the release of anaphylactoid mediators from RBL-2H3 cells. An HPLC method for protein determination in the Xue-Sai-Tong injection was established subsequently, and the content of proteins with molecular weights of over 10 KDa in the injections showed an obviously positive correlation with the histamine release induced by the injections. In addition, taking ginsenoside-Rd coupled with BSA as an example, the hapten property of ginsenosides was studied and the ratio of ginsenoside-Rd to BSA was determined to be 8:1 by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and the ginsenoside-BSA conjugate showed a stronger action to stimulate histamine release from the RBL-2H3 cells.

Supporting information available online at <http://www.thieme-connect.de/ejournals/toc/plantamedica>

Introduction

Herbal medicine injections, such as ginseng and ginkgo leaf, are widely used in China and other countries. The complicated ingredients are the outstanding characteristic of the injections, which make their quality difficult to be con-

trolled. Thus, adverse drug reactions (ADR) of herbal injections occurred frequently [1,2]. In recent years, more and more reports indicated that the ADR of herbal injections is serious. Therefore, China's State Food and Drug Administration (SFDA) took action to stop the production of 7 kinds of herbal injections in 2006 [3].

Sanqi ginseng, the roots of *Panax notoginseng* (Burk.) F.H. Chen (Araliaceae), is an important ancient herbal medicine widely used for more than two thousands years in traditional Chinese medicine to treat atherosclerosis and cerebral infarction and ginsenosides were considered its principal ingredients responsible for the pharmacological activities [4]. Sanqi ginsenosides, with a purity of over 80%, are the active components of *Panax notoginseng* (XSTI), generally used as an anti-coronary medicine [5–8]. Hemolysis is one of saponins' properties and can lead to hemolytic ADR of injections. Clinically, XSTI was reported to possess acute anaphylactic and hemolytic ADR. Thus, prediagnostic methods for the hemolysis of injections containing ginsenosides were established in previous research [9], and HD₅₀ (half hemolytic dosage) was used as an index for the hemolytic prediagnosis of XSTI, which could indicate abnormal hemolytic reactions. Clinical exploration indicated that more than 70% of the adverse drug reaction caused by Chinese medicine injections was an anaphylactoid reaction [10]. Thus, anaphylactoid reaction is another major ADR of herbal injections.

In a continuing study, this is the first time that an optimal *in vitro* model for evaluating anaphylactoid reaction of herbal injections was established by taking XSTI as an example, and also an analytical method for determination of residual plant proteins in the injection was set up. Meanwhile the anaphylactoid constituents in XSTI were also explored.

Materials and Methods



Chemicals and reagents

Ginsenosides and oleanolic acid were isolated and identified in our laboratory [8,11,12] and their purity was determined to be over 98% by HPLC measurement.

XSTI was purchased from Harbin Zhenbao Pharmaceutical Co., Ltd. (Batch No.20090911, 20091006, 20101102, 20110303, 20111004, 20110202, 20100716, Harbin, China), Wanrong-Sanjia Pharmaceutical Co., Ltd. (Batch No.911271 and 911273, Henan, China), Kunming Pharmaceutical Co., Ltd. (Batch No.09LJ12, Kunming, China), and Kunming Xingzhong Pharmaceutical Co., Ltd. (Batch No.20110303, 20100312, 20100501). RBL-2H3 cells were from the American Type Culture Collection (ATCC). Fetal bovine serum was purchased from GIBCO Invitrogen. BSA, coomassie brilliant blue (CBB), and lysozyme were from Beijing Solarbio Science & Technology Co., Ltd. A histamine assay kit was purchased from IBL International GMBH. Histamine phosphate was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Compound 48/80 (purity, ≥98%), 4-nitrophenyl N-acetyl-β-D-glucosaminide, O-phthalaldehyde (OPT), percoll solution, trypan, toluidine, ribonuclease, and ovalbumin were purchased from Sigma-Aldrich, Co. DMEM, Triton X-100, HBSS buffer, PBS, and trypsin were purchased from Thermo Scientific Co. All other chemical reagents were purchased from Kermel Chemical Co. A multimode microplate reader was purchased from Tecan Group Ltd. A freeze-drying machine was purchased from Labconco Corporation. A centrifuge was purchased from Thermo Electron Corporation.

Animals

The animals for the experiment were purchased from Dalian Medical University (Grade II). The animals were housed in an air-conditioned room (temperature, 25 °C; relative humidity,

55%) and fed *ad libitum* with standard feed and water in the course of the study. At the end of the experiment, pentobarbital sodium was used for euthanasia of the animals. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The study protocol was approved by the ethics regulations of Liaoning University of TCM (131/2010).

Cell culture

RBL-2H3 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 100 U·mL⁻¹ penicillin streptomycin at 5% CO₂ and 37 °C. Cells were subcultured using trypsin when reaching 80% confluency.

Contents analysis of ginsenosides in XSTI by HPLC

Content analysis was achieved in an Agilent 1100 series HPLC system with a pump (Agilent model G1314A VWD), and an Agilent Eclipse XDB-C₁₈ column (4.6 × 150 mm, 5 μm particle size) protected by a pre-column from the same company, eluted with water-phosphoric acid (100:0.5) (A) and acetonitrile (B) in a gradient at the flow rate of 1 mL/min. The solvent gradient for determining consisted of 19% B at the beginning, 19% at 12 min, 36% at 60 min, 50% at 75 min, 65% at 85 min, and 95% at 125 min. The UV absorption of the column effluent was determined at 203 nm. The injection volume was 10 μL for each fraction. Peak identification was performed by comparison of retention times.

Calibration: Curves were constructed using standard solutions of ginsenoside-Rg₁, -Rb₁, -Rd, and -R₁ in the concentration range 0.1–6.0 mg/mL, respectively. Each sample (10 μL) was determined in triplicate and the average detector responses were used by the software to construct the curves.

Linearity: Aliquots (10 μL) of 6 solutions of ginsenoside-Rg₁, -Rb₁, -Rd, and -R₁ were analyzed in triplicate and the average detector responses were used by the software to construct the curves.

Precision: Standard solutions of ginsenoside Rg₁ (1.02 mg/mL) were injected in sextuplicate, respectively, in order to determine the standard deviation of the method on the same day.

Anaphylactoid activity assay of XSTI *in vivo*

Twenty-four healthy male guinea pigs, weighing 250–350 g, were randomly divided into 4 groups (n = 6). XSTIs of 1.6 mL (equivalent dose of eight times injected into a human) were injected into the marginal ear vein. Compound 48/80 (3.0 mg/mL) and normal saline solutions of 1.6 mL were injected as the positive and negative control group, respectively. Their behavioral changes within 30 min were observed and recorded. Blood samples were drawn from the guinea pigs under ether anesthesia. The contents of histamine in the serum were assayed by the ELISA method. Pathological changes were observed in lung tissue sections.

Validation of the *in vitro* model for evaluating anaphylactoid mediator release

Preparation of sample solution: In order to obtain sample solutions containing a higher content of total saponins, XSTI was powdered by lyophilizing, and dissolved with saline to make solutions with different concentrations of total saponins. The sample solutions were filtered through 0.22 μm organic membranes prior to use.

Histamine and β-hexosaminidase assays of RBL-2H3 cells: 100 μL of RBL-2H3 cell suspension were plated at 5 × 10⁵ cells/mL in 96-well plates for 24 h. Then, the medium was removed and the

wells were washed twice with PBS. 200 μ L XSTI or compound 48/80 (positive drug) solutions of different concentrations (detailed concentrations are shown in **Fig. 2**) were added and incubated at 37 °C for 30 min. The contents of histamine and β -hexosaminidase in the supernatant were determined.

The histamine release assay of RBL-2H3 induced by sample solutions and compound 48/80 were assayed either using a histamine ELISA kit according to the manufacturer's directions or by using a reported fluorometric assay. To 50 μ L of supernatant from each well or histamine phosphate standard solution (in 0.1 N HCl), 50 μ L of 0.4 N NaOH and 10 μ L of 0.1 % OPT (in methanol) were added. The supernatant was then incubated at room temperature for 10 min. The reaction was stopped by the addition of 50 μ L of 0.1 N HCl. The concentration of the fluorescent product of the reaction was measured using a fluorescence microplate reader set at $\lambda_{\text{ex}} = 349$ nm and $\lambda_{\text{em}} = 448$ nm, respectively. One hundred percent release of histamine in the cell was caused by Triton X-100 (0.1 %, v/v). The release rate was determined with the formulation as follows:

$$\text{Release ratio (\%)} = (C_{\text{eg}} - C_{\text{nc}}) / (C_{100\%} - C_{\text{nc}}),$$

where C_{eg} and C_{nc} represent the content of the experimental group and the negative control group, respectively.

The β -hexosaminidase release assay of RBL-2H3 or RPMC stimulated by XSTI was obtained using the published method with minor modifications [13]. 50 μ L of supernatant was added to a 96-well plate which contained 50 μ L of substrate (4-nitrophenyl N-acetyl- β -D-glucosaminide, 1 M) dissolved with 0.1 M citrate buffer. The plate was incubated for 1.5 h at 37 °C. The reaction was stopped by adding 200 μ L Na₂CO₃-NaHCO₃ buffer (1 M) to each well and the formed *p*-nitrophenolate was measured spectrophotometrically in the microplate reader at a wavelength of 405 nm. The absorption was converted into the percentage of total cellular β -hexosaminidase content by comparison with the absorption produced by Triton X-100 according to the following equation:

$$\text{Release rate (\%)} = (OD_{\text{eg}} - OD_{\text{nc}}) / (OD_{100\%} - OD_{\text{nc}}),$$

where OD_{eg} and OD_{nc} represent the absorbance of the experimental group and the negative control group, respectively.

Histamine and β -hexosaminidase assays of rat peritoneal mast cells: Rat peritoneal mast cells (RPMC) were obtained by peritoneal lavage with buffered salt solution (BSS) according to the reported method [13] with minor modifications. Male Wistar rats (200–300 g) were killed in an atmosphere saturated with CO₂. 15 mL BSS was injected i.p. and the abdomen was gently massaged for 2 min. The crude cell suspension was aspirated following a midline incision and centrifuged for 5 min (4 °C, 1000 rpm). Cells were washed three times with BSS. The crude cells were purified using a percoll/BSS density gradient. After centrifugation for 15 min (4 °C, 2500 rpm), RPMC whose viability and purity were more than 95 % were separated and resuspended in BSS (5×10^5 cells/mL).

100 μ L of RPMC were added to tubes containing 200 μ L of sample solution with different concentrations. The test tubes were centrifuged for 10 min (4 °C, 1000 rpm) after incubating at 37 °C for 30 min. Then, 50 μ L of the supernatant was used to assay the release rate of histamine and β -hexosaminidase by the ELISA method and spectrophotometry, respectively.

Anaphylactoid activity assay of XSTI *in vitro*

100 μ L of RBL-2H3 cell suspension were plated at 5×10^5 cells/mL in 96-well plates for 24 h. Then, the medium was removed and the wells were washed twice with PBS. 200 μ L Xue-Sai-Tong sample solutions (37.5 mg/mL) of different batches were incubated at 37 °C for 30 min with the cells in wells, then histamine content in the supernatant was determined by the ELISA method, and the release rate was calculated according to the above method.

Anaphylactoid activities assay of ginsenosides in RBL-2H3 cells

100 μ L of RBL-2H3 cell suspension were plated at 5×10^5 cells/mL in 96-well plates for 24 h. Then, the medium was removed and the wells were washed twice with PBS. 200 μ L of ginsenosides and oleanolic acid standard solutions of different concentrations (10, 100, 1000 μ M) were incubated at 37 °C for 30 min with the cells in wells, then the contents of histamine and β -hexosaminidase in the supernatant were determined by the ELISA method and spectrophotometry, respectively, and the release rate of histamine and β -hexosaminidase were assayed according to the above method. All the compounds were dissolved in DMSO, which had no allergic activity on the RBL-2H3 cell line at a concentration of less than 2 %.

Anaphylactoid mediators released from RBL-2H3 cells by fractions with different molecular weights in XSTI

Distilled water was added to dry ultrafiltration devices (Millipore) with different sizes (3 KDa, 10 KDa, and 30 KDa) for pre-cooling at 4 °C. After removing the cold water, XSTIs (2 mL) were added to the ultrafiltration devices above, and then the injections were centrifuged for 1 h (8000 rpm, 4 °C). The remaining protein solution in the device was made up to 2 mL in a volumetric flask with normal saline solution.

100 μ L of RBL-2H3 cell suspension were plated at 5×10^5 cells/mL in 96-well plates for 24 h. Then, the medium was removed and the wells were washed twice with PBS. 200 μ L of protein solutions with different molecular weights as above were incubated at 37 °C for 30 min with the cells in wells, then the contents of histamine in the supernatant were determined by the ELISA method, and the release rate of histamine was calculated according to the above method.

Determination of proteins in XSTI by Bradford method

Coomassie brilliant blue G-250 (25 mg) was added to a mixture solution with 95 % ethanol (12.5 mL) and 85 % phosphoric acid (25 mL), and then the solution was diluted to 250 mL with distilled water to make a standard solution.

XSTI (2 mL) was added to the ultrafiltration device (10 KDa) and then centrifuged at 8000 rpm for 1 h at 4 °C. The remaining solution in the ultrafiltration device was removed precisely and diluted to 2 mL with distilled water. The sample solution (200 μ L) was added to the tubes and diluted to 1 mL with distilled water. The content of proteins was assayed in accordance with the standard curve.

Determination of proteins in XSTI by HPLC

Distilled water was added to the dry ultrafiltration devices (10 KDa) for pre-cooling at 4 °C. After removing the cold water, XSTI (2 mL) was added to the ultrafiltration devices, and then the injection was centrifuged at 8000 rpm for 1 h (4 °C). In order to get rid of the interference of micromolecules in the injection, the filtrate was poured out and 2 mL of distilled water was added to the

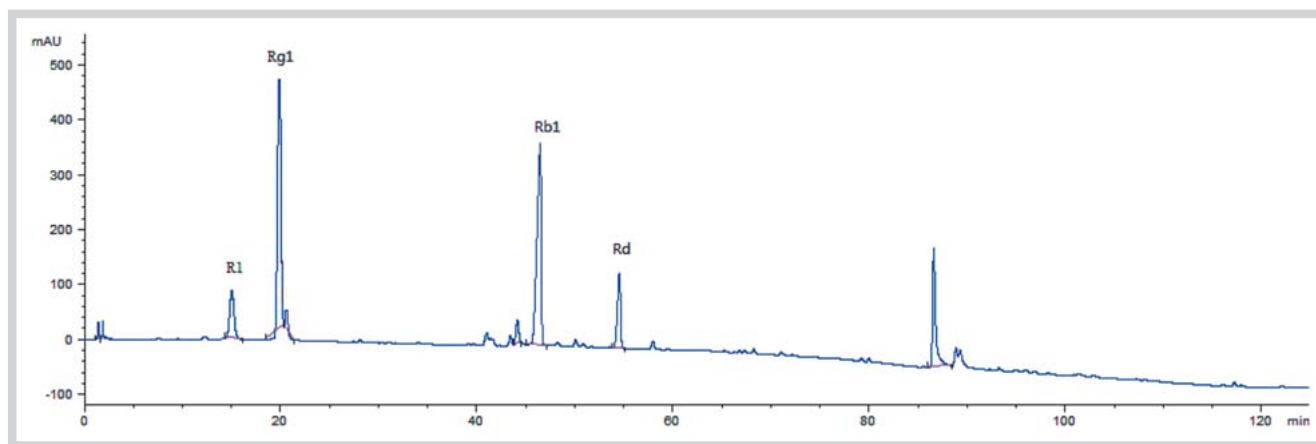


Fig. 1 HPLC chromatogram of XSTI. (Color figure available online only.)

ultrafiltration devices, and then the solution was centrifuged at 8000 rpm for 30 min. The operation was repeated for 5 times. The remaining sample solution in the ultrafiltration device was removed precisely and diluted to 2 mL with distilled water. All the sample solutions were filtered through 0.45 μ m organic membranes prior to HPLC analysis.

In order to construct the standard curve of proteins with different molecular weights, the retention time of four standard proteins (BSA, ovalbumin, ribonuclease, and lysozyme) were determined at the concentration of 200 μ g/mL with an injection of 20 μ L of their standard solutions. The calibration curve was constructed by plotting the analyte to retention time (y) against the logarithm of molecular weights (x). All the calibration curves were fitted using a least squares linear regression model $y = ax + b$.

Content analysis was achieved in an Hitachi LC-7100 series HPLC system with a TSK-Gel G2000SW_{XL} (7.8 $\times$ 300 mm, 5 μ m) eluting with a mobile phase (0.1 M PBS/0.1 M Na₂SO₄, aq., pH = 6.7) at a flow rate of 0.8 mL/min. UV absorption of the column effluent was determined at 280 nm. The injection volume was 50 μ L for each fraction. All the standard solutions were filtered through 0.45 μ m organic membranes prior to HPLC analysis. Peak identification was performed by comparison of retention times. This method was validated in terms of linearity, repeatability, limit of detection (LOD), and limit of quantitation (LOQ).

Anaphylactoid mediators released from RBL-2H3 cells by ginsenoside-Rd-BSA conjugate

Ginsenoside-Rd-BSA conjugate was synthesized using a previously reported method with minor modifications [14]. 0.7 mL of methanol solution (80%, v/v) containing ginsenoside-Rd (10 mg) was added to a water solution (0.5 mL) containing NaIO₄ (4 mg). The solution was stirred at room temperature for 2 h. Carbonate buffer solution (50 mM, pH 9.6, 1.0 mL) containing BSA (10 mg) was added to the above reaction solution and the mixture was stirred at room temperature for another 5 h. Then, the mixture solution was centrifuged with an ultrafiltration device (3 kDa) at 8000 r/min for 1 h, and then lyophilized to produce ginsenoside-Rd-BSA conjugate (13 mg).

100 μ L of RBL-2H3 cell suspension were plated at 5×10^5 cells/mL in 96-well plates for 24 h. Then, the medium was removed and the wells were washed twice with PBS. 200 μ L of sample solutions with different concentrations (0.04, 0.2, 1.0 mg/mL) were added and incubated at 37 $^{\circ}$ C for 30 min with the cells, and then

the contents of histamine and β -hexosaminidase in the supernatant were determined according to the above method.

Determination of ginsenoside-Rd-BSA conjugate by matrix-assisted laser desorption tof mass spectrometry

The mixture was placed inside a MALDI TOF/TOF 4800 plus mass spectrum (ABI Sciex) and irradiated with an N₂ laser (337 nm, 3 ns pulse). The ions formed by each pulse were accelerated by a 20-kV. Matrix solutions containing trifluoroacetic acid (0.1%, v/v) were prepared with acetonitrile and absolute methanol at the ratio of 7 : 3. 0.6 μ L of the equivalent volume mixture of sample solutions with different concentrations (16 pmol/ μ L, 8 pmol/ μ L, 4 pmol/ μ L, 2 pmol/ μ L) and the matrix solution was dropped on the sample target, dried at room temperature, and measured with a linear model. The data was conducted by 4000 Series Explorer Software.

Supporting information

Details regarding the anaphylactoid activity assay of Xue-Sai-Tong injection and the determination of ginsenoside-Rd-BSA conjugate by MALDI-TOF as well as the MALDI mass spectrometry of G-Rd-BSA conjugate are available as Supporting Information.

Results and Discussion



Content analysis of the main ginsenosides in XSTI was performed firstly by HPLC (● Fig. 1). The contents of ginsenoside-R₁, -R_{g1}, -R_b, and -R_d in the injection were determined to be 16.77%, 22.48%, 37.96%, and 8.2% respectively.

The *in vivo* test of anaphylactoid reaction of XSTI was assayed using the method mentioned in China's Pharmacopoeia, indicating that the XSTI which showed normal hemolysis and abnormal hemolysis in a previous experiment [9] could not make the behavior change in guinea pigs. All the injections were qualified according to China's Pharmacopoeia. Histamine and β -hexosaminidase are the main mediators in mast cells and granulocytes released by stimulation of the drug at certain concentrations directly [15]. Our results indicated that the histamine content in the serum of the experimental groups (● Table 1) was significantly higher than that of the negative control group ($p < 0.01$). Meanwhile, structure variation of pulmonary alveoli and inflammatory cell

Table 1 Histamine content in the serum of guinea pigs.

Group	Dosage	Histamine content ($\mu\text{g} \cdot \text{L}^{-1}$)
Saline	1.0 mL	7.06 ± 0.16
Injection (20091108)**	1.6 mL	$39.66 \pm 4.21^*$
Injection (20091006)**	1.6 mL	$41.08 \pm 5.71^*$
Compound 48/80	2 mg	$49.32 \pm 7.12^*$

Note: * $p < 0.01$; ** 20091108 and 20091006 were the XSTI with normal hemolysis and abnormal hemolysis *in vitro* in previous research

Table 2 Anaphylactoid mediators released from RPMC stimulated by Xue-Sai-Tong injection.

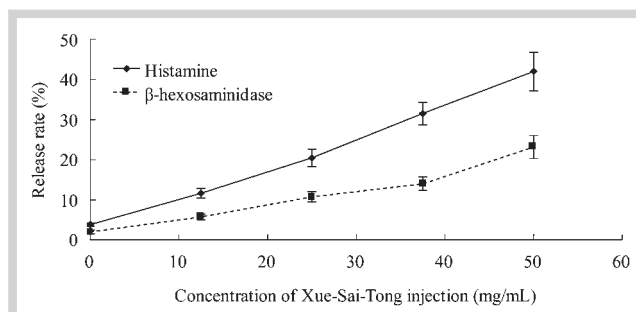
Concentration of injection (mg/mL)	β -hexosaminidase release rate (%)	Histamine release rate (%)
0	42.35 ± 5.13	62.34 ± 4.02
11.1	49.51 ± 4.87	59.51 ± 5.79
22.2	42.11 ± 4.05	53.11 ± 3.16
33.3	46.58 ± 5.21	66.58 ± 6.63
Compound 48/80 (1000 $\mu\text{g}/\text{mL}$)	82.17 ± 6.09	96.17 ± 4.38

infiltration could be observed in the experimental groups of lung tissue sections. The result showed that XSTI has potential anaphylactoid activity *in vivo* and histamine could be an index to evaluate the anaphylactoid ability of the herbal medicine injection.

The fluorescence excitation and emission wavelengths of histamine were determined to be 349 nm and 448 nm, respectively, by a fluorescence microplate reader. The standard curve of histamine was constructed, and the equation was calculated as follows: $y = 0.3812x + 7.4503$ ($R^2 = 0.9999$), in which x is the fluorescence intensity and y is the concentration of histamine.

Histamine released from RBL-2H3 cells stimulated by sample solutions with different concentrations was determined using the above fluorospectrophotometry method, indicating that fluorescence intensity of histamine in the experimental group increased as the sample solutions' concentrations increased, but the intensity was almost the same with that of the sample solution itself (blank group). One reason for this phenomenon could be the interference of unknown components in the injection and another probable reason is the hydroxyls in the saponin molecules, which could induce aldol condensation with OPT. So, fluorospectrophotometry is not a suitable method to assay the histamine release of cells stimulated by XSTI.

The results of degranulation of RPMC indicated that the RPMC could be directly stimulated by compound 48/80 (1000 $\mu\text{g}/\text{mL}$) to release anaphylactoid mediators (histamine and β -hexosaminidase). The release ratios of histamine and β -hexosaminidase were $96.2 \pm 4.4\%$ and $82.3 \pm 6.9\%$, respectively. But, there was no difference in release ratios of allergic mediators between the negative control group and the experimental group (Table 2). Other research also indicated that mast cells play a crucial role in the development of anaphylaxis, but the degranulation research of mast cells is often hampered by difficulties originating from the isolation and primary culture of the cells [16–18]. So, RPMC was also not an optimal cell model for evaluating the sensitization of

**Fig. 2** Mediators released from RBL-2H3 stimulated by XSTI.

the herbal medicine injection or other drugs with degranulation slightly due to its obvious spontaneous release rate of histamine and β -hexosaminidase.

Recently, the continuous cell line of rat basophilic leukemia, RBL-2H3, was considered a useful tool to take the place of mast cells for *in vitro* work [13]. So the RBL-2H3 cell line was applied to appreciate the anaphylactoid reaction of XSTI. On the aforementioned research, ELISA and spectrophotometric methods were selected for determining the histamine and β -hexosaminidase release from the cells, respectively. Mediators released from RBL-2H3 cells stimulated by XSTI were evaluated. It showed a similar tendency that the ratio of histamine release was higher than the β -hexosaminidase release stimulated by the injection at the same concentration (Fig. 2). It was deemed that histamine was more sensitive than β -hexosaminidase in evaluating the degranulation of RBL-2H3 cells. And ELISA was the suitable method with little interference to assay the histamine release from RBL-2H3 cells.

Anaphylactoid mediators released from RBL-2H3 cells induced by 11 batches of XSTI with different concentrations were assayed, and the results (Fig. 3) indicated that some batches of XSTIs (20090911, 20091006, 20101102, 20110303, 911271, 911273) which showed abnormal hemolysis in previous research, obviously enhanced the release of anaphylactoid mediators from the cells. So, HD_{50} could be an auxiliary indicator to evaluate the anaphylactoid ability of the herbal injection indirectly.

Anaphylactoid assaying of individual ginsenosides was also performed and the results are shown in Table 3. All the ginsenosides tested could not stimulate the RBL-2H3 cells to release anaphylactoid mediators (histamine and β -hexosaminidase) at the concentrations of 10 and 100 μM , but ginsenoside-Ro, -Rd, -Rb₃, and -Rc could induce these mediators released from RBL-2H3 cells at the concentration of 1000 μM . Ginsenoside-Rd, one of the main components in XSTI, had no anaphylactoid ability at the same concentration as its content in the injection. So, ginsenosides in the injection were not the main factors inducing mediators released from RBL-2H3 cells.

The result of histamine released from RBL-2H3 cells by fractions of XSTI with different molecular weights showed that the components whose molecular weights were above 10 kDa in XSTI were the major constituents inducing anaphylactoid mediators released from RBL-2H3 cells (Fig. 4).

Protein is a major anaphylactoid constituent, but only LOD determination, not the quantitative determination method, was stipulated in China's Pharmacopeia. Firstly, the protein determination with the Bradford method was explored with 2.96%, 2.68%, and 2.89% for precision, repeatability, and stability, respectively. The content of proteins in the injections with different batches was

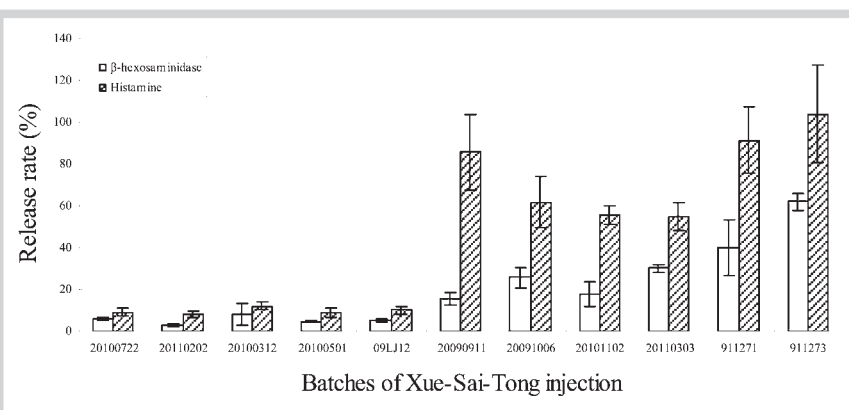


Fig. 3 Anaphylactoid mediators released from RBL-2H3 cells induced by XSTI (37.5 mg/mL) of different batches.

Compound	Concentration (μ M)	β -hexosaminidase release rate (%)	Histamine release rate (%)
Re	10	1.70 $\pm$ 0.34	1.02 $\pm$ 0.51
	100	0.58 $\pm$ 0.27	2.32 $\pm$ 0.66
	1000	1.41 $\pm$ 0.21	1.30 $\pm$ 1.33
Rg ₁	10	-0.15 $\pm$ 0.48	2.27 $\pm$ 0.13
	100	1.31 $\pm$ 0.62	1.65 $\pm$ 0.17
	1000	0.19 $\pm$ 0.82	2.18 $\pm$ 0.13
Rb ₁	10	3.10 $\pm$ 2.33	1.28 $\pm$ 0.30
	100	0.34 $\pm$ 0.34	0.87 $\pm$ 0.08
	1000	0.39 $\pm$ 0.14	0.99 $\pm$ 0.05
R ₁	10	6.93 $\pm$ 0.32	2.57 $\pm$ 0.25
	100	7.95 $\pm$ 0.79	2.83 $\pm$ 0.29
	1000	5.81 $\pm$ 0.36	4.20 $\pm$ 3.55
Rd	10	-0.40 $\pm$ 0.47	7.16 $\pm$ 1.24
	100	1.40 $\pm$ 1.13	7.40 $\pm$ 1.66
	1000	5.58 $\pm$ 1.03	42.77 $\pm$ 1.23
Oleanolic acid	10	0.71 $\pm$ 1.01	6.46 $\pm$ 0.27
	100	1.21 $\pm$ 0.50	5.12 $\pm$ 1.15
	1000*	13.74 $\pm$ 14.19	3.59 $\pm$ 0.40
Ro	10	1.00 $\pm$ 0.60	9.52 $\pm$ 2.54
	100	4.20 $\pm$ 1.71	19.98 $\pm$ 0.70
	1000	7.19 $\pm$ 3.12	77.65 $\pm$ 0.00
S-Rg ₂	10	1.49 $\pm$ 1.11	6.80 $\pm$ 0.43
	100	1.35 $\pm$ 1.11	10.52 $\pm$ 6.18
	1000	0.85 $\pm$ 0.20	10.86 $\pm$ 3.89
Rh ₁	10	-0.33 $\pm$ 0.19	6.36 $\pm$ 0.75
	100	-0.66 $\pm$ 0.47	5.78 $\pm$ 0.83
	1000	-0.66 $\pm$ 0.47	4.27 $\pm$ 0.45
Rg ₃	10	-0.47 $\pm$ 0.19	5.22 $\pm$ 2.65
	100	0.60 $\pm$ 0.19	6.50 $\pm$ 1.06
	1000*	3.21 $\pm$ 1.13	7.90 $\pm$ 0.08
Rb ₂	10	0.12 $\pm$ 1.00	16.45 $\pm$ 2.47
	100	0.00 $\pm$ 0.50	14.86 $\pm$ 2.76
	1000	1.30 $\pm$ 0.33	14.21 $\pm$ 3.93
Rb ₃	10	0.47 $\pm$ 0.84	9.25 $\pm$ 1.96
	100	1.65 $\pm$ 0.17	14.21 $\pm$ 0.73
	1000	5.08 $\pm$ 3.34	46.29 $\pm$ 13.85
Rc	10	1.65 $\pm$ 1.17	13.89 $\pm$ 3.30
	100	2.13 $\pm$ 0.17	10.61 $\pm$ 4.01
	1000	18.79 $\pm$ 2.01	124.49 $\pm$ 4.37
F1	10	6.96 $\pm$ 0.34	2.01 $\pm$ 0.52
	100	7.34 $\pm$ 0.50	1.69 $\pm$ 1.14
	1000	7.03 $\pm$ 0.43	5.38 $\pm$ 1.44

Note: * insoluble particles of compounds were produced at this concentration

Table 3 Anaphylactoid activity of ginsenosides *in vitro*.

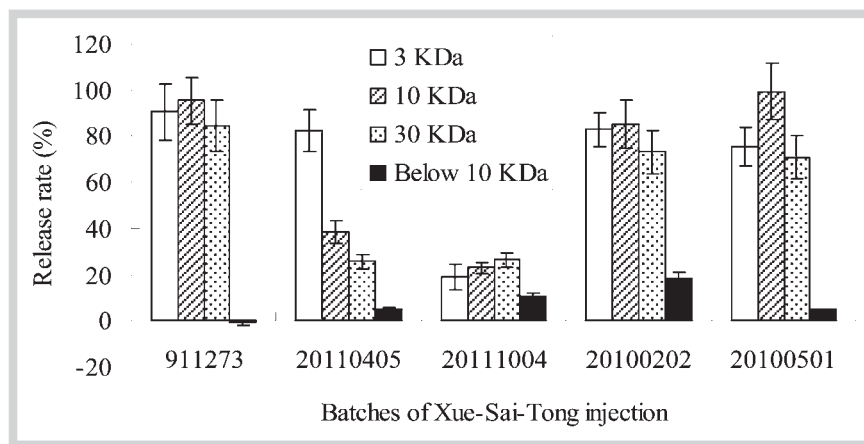


Fig. 4 Histamine released from RBL-2H3 cells induced by protein with different molecular weights.

Batches	Histamine release rate (%)	Proteins* (μg/mL)	Proteins** (μg/mL)
0911273	103.86 ± 23.40	1199.6 ± 13.9	237.43
20110303	54.62 ± 0.68	889.2 ± 10.1	98.48
20100312	9.87 ± 0.52	939.2 ± 6.1	12.57
20100501	20.29 ± 2.17	991.3 ± 4.4	49.46
20111004	25.06 ± 3.72	827.34 ± 7.26	42.23
20091006	61.84 ± 12.01	904.7 ± 10.0	120.05
20100716	12.77 ± 0.94	810.0 ± 8.0	12.29
20110202	19.78 ± 1.62	893.3 ± 5.4	45.97

Table 4 Histamine release from RBL-2H3 cells induced by XSTIs and the content of protein (MW ≥ 10 KDa) of the injection.

Note: * results determined by the Bradford method; ** results determined by HPLC

determined by the Bradford method using the equation: $y = 0.0064x + 0.0244$ ($R^2 = 0.9916$), in which x is the concentration of BSA and y is the absorbance. Bradford analysis indicated that the protein content of these injections was higher than the LOD as mentioned in China's Pharmacopoeia, but comfortable to its LOD test according to the method issued by China's Pharmacopoeia. The result indicated that proteins in XSTI could not be determined accurately by the Bradford method owing to the interference of components in the injections.

Further, an HPLC method for the protein determination in the injection was established. The equation of retention time to molecular weights is: $y = -4.1806x + 29.135$ ($R^2 = 0.9983$), in which x is the logarithm of molecular weights and y is the retention time. So, the retention time of proteins whose molecular weights were over 10 KDa should be less than 12.5 min. The protein content of XSTI was further assayed by HPLC (Table 4). The linearity of the standard calibration curve was constructed to give the following equation: $y = 6.7251x - 0.4378$ ($r = 0.9995$), in which x is the weight of proteins and y is the peak area. The LOD and LOQ for the method were 30 ng and 100 ng, respectively. The results of the correlation analysis indicated that the content of proteins whose molecular weights were over 10 KDa in the injections showed a significantly positive correlation with the histamine release induced by the injections, and the correlation coefficient was 0.989.

In addition, the hapten property of ginsenosides was studied by taking -Rd-BSA as an example. The release of anaphylactoid mediators from RBL-2H3 cells induced by the ginsenoside-Rd-BSA conjugate could be clearly observed at the concentration of 1.0 mg/mL (Table 5).

The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrum of the ginsenoside-BSA conjugate

Table 5 Anaphylactoid mediators released from RBL-2H3 induced by G-Rd-BSA conjugate.

Concentration of G-Rd-BSA conjugate (mg/mL)	Release rate (%)	
	β-hexosaminidase	histamine
0.04	0.29 ± 0.06	2.12 ± 0.09
0.2	1.61 ± 0.07	4.9 ± 0.1
1.0	52.7 ± 4.2	67.0 ± 6.3

showed that the conjugate appeared at m/z 74181.7832 (average for quadruplicates, c.v.: 0.37%) which disclosed that the coupled ratio of ginsenoside-Rd-BSA was 8:1 on the basis of the molecular weight of 66267 Da for BSA, according to previous research [18], and 947 for ginsenoside-Rd.

In this study, anaphylactoid ADR of XSTI was assayed *in vivo* and *in vitro*. An optimal *in vitro* model for evaluating anaphylactoid mediators (histamine and β-hexosaminidase) release induced by XSTI was established for the first time. This model could also be utilized in evaluating the degranulation of other herbal medicine injections. Proteins whose molecular weights were above 10 KDa were the main anaphylactoid constituents in XSTI. HD₅₀, an index for hemolytic prediagnosis of an herbal medicine injection, could also be an indicator to evaluate the anaphylactoid ability of the herbal injection.

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Conflict of Interest



No conflict of interest exists regarding the submission of this manuscript, which was approved by all authors for publication.

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Supporting Information

The Anaphylactoid Constituents in Xue-Sai-Tong Injection

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Anaphylactoid activity assay of Xue-Sai-Tong injection *in vivo*

Twenty-four healthy male guinea pigs, weighing 250-350 g were randomly divided into 4 groups (n = 6). Xue-Sai-Tong injections of 1.6 mL (equivalent dose of eight times injected to human) were injected into the marginal ear vein. Compound 48/80 (3.0 mg/mL) and normal saline solutions of 1.6 mL were injected as the positive and negative control group, respectively. Pathological changes were observed in lung tissue sections. Structure variation of pulmonary alveoli and inflammatory cell infiltration could be observed in the experimental groups of lung tissue sections. The result showed that Xue-Sai-Tong injection has potential anaphylactoid activity *in vivo* and histamine could be an index to evaluate the anaphylactoid ability of an herbal medicine injection.

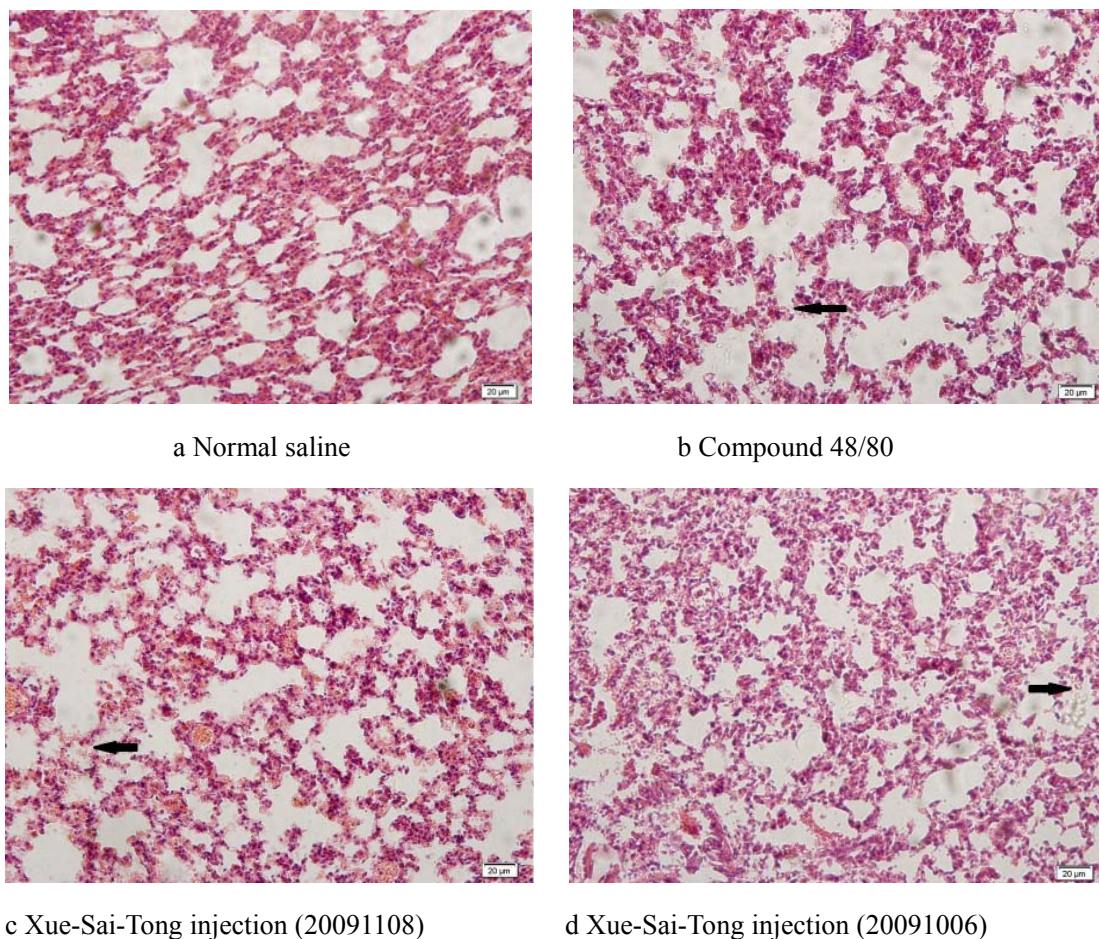


Fig. 1S Pathological changes of lung tissue caused by Xue-Sai-Tong injection (10 × 20)

a: control; b: compound 48/80; c: 20091108, normal hemolysis; d: 20091006,

abnormal hemolysis.

“→” : Pathological changes of pulmonary alveoli.

Determination of ginsenoside-Rd-BSA conjugate by matrix-assisted laser desorption tof mass spectrometry

Ginsenoside-Rd-BSA conjugate was synthesized as follows: 0.7 mL of methanol solution (80%, v/v) containing ginsenoside-Rd (10 mg) was added to a water solution (0.5 mL) containing NaIO₄ (4 mg). The solution was stirred at room temperature for 2 h. Carbonate buffer solution (50 mM, pH 9.6, 1.0 mL) containing BSA (10 mg) was added to the above reaction solution and the mixture was stirred at room temperature for another 5 h. Then, the mixture solution was centrifuged with an ultrafiltration device (3 KDa) at 8000 r/min for 1 h, and then lyophilized to produce ginsenoside-Rd-BSA conjugate (13 mg).

The mixture was placed inside a MALDI TOF/TOF 4800 plus mass spectrum (ABI Sciex) and irradiated with an N₂ laser (337 nm, 3 ns pulse). The ions formed by each pulse were accelerated by a 20-kV. Matrix solutions containing trifluoroacetic acid (0.1%, v/v) were prepared with acetonitrile and absolute methanol at the ratio of 7:3. 0.6 µL of the equivalent volume mixture of sample solutions with different concentrations (16 pmol/µL, 8 pmol/µL, 4 pmol/µL, 2 pmol/µL) and matrix solution were dropped on the sample target, dried at the room temperature, and measured at the linear model. The data was conducted by 4000 Series Explorer Software.

The MALDI tof mass spectrum of ginsenoside-BSA conjugate is shown in **Fig. 2S**. It showed the mass spectra of the conjugate of ginsenoside-Rd and BSA. A sharp peak coinciding with the conjugate appeared at m/z 74181.7832 (average for quadruplicates, c.v.: 0.37%), using a molecular weight of 66267 Da for BSA. According to previous research. the calculated value of the ginsenoside-Rd (947) residue is 7254 resulting in 8 molecules of ginsenoside-Rd conjugated with BSA.

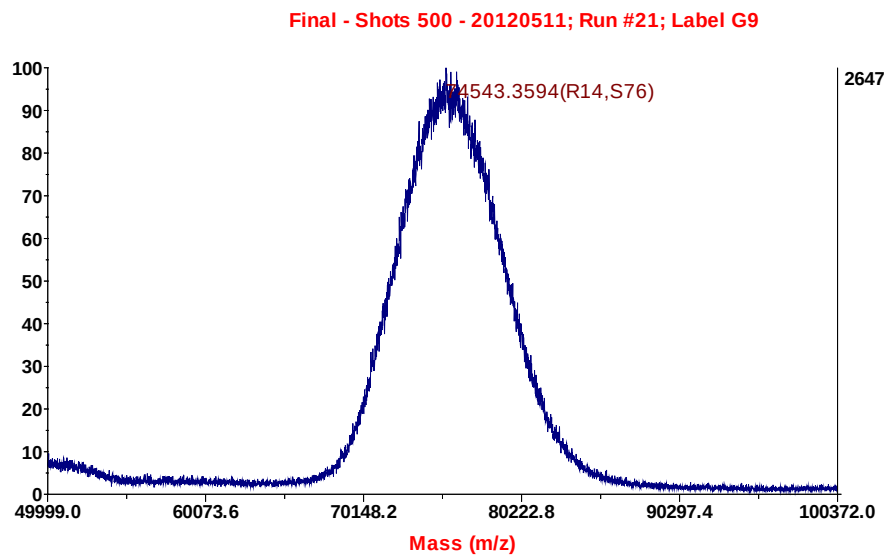


Fig. 2S. MALDI mass spectrometry of the G-Rd-BSA conjugate