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A rapid and low-cost approach to evaluate the allergenicity of herbal injection using HPLC analysis

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Abstract

Herbal medicines have ever been thought harmless, but it is obviously not true. Many adverse reports emerged with the development of their popular application in the world. Allergic reactions, especially serious immediate hypersensitivity, frequently occurred when herbal injections were used in clinic and made this ever prevailing agent nearly disappear in China. The aim of this study is to establish a rapid and economical method for the prediction of the allergenicity of herbal injections. Ovalbumin (OVA) and four other herbal injections, in which two of them were well known for their allergenicity, were selected to sensitize and stimulate the animals. Serotonin in the animal serum was detected with HPLC to reflect the anaphylactic response and compared with the other cytokines which could mediate the anaphylaxis, including histamine, IgE and β -hexosaminidase. The results suggest that serotonin can be detected quickly and has good correlation with the other allergy-related cytokines. It is a promising way for predicting the allergenicity of the herbal injections and those complicated natural products.

Keywords

Allergic reaction; herbal medicine; immediate hypersensitivity; safety evaluation; serotonin

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1. Introduction

Herbal medicines are widely used in the countries of East Asia since ancient time and have become increasingly popular in western world nowadays. Although they are considered less harmful because of their 'natural' characteristic and the long history adopted in clinic in many countries, adverse effects were reported frequently [1, 2]. The causes contribute to the phenomena are complex which might be related to the toxic ingredients, impurities, heavy metals, bacteria or other contaminants in the agents and drug interaction [3–5]. Out of them, new preparation derived from traditional pattern should be given more concerns, such as injections prepared from traditional Chinese medicine (TCM). The primary preparation of TCM is decoction (extracted with water and administered orally). Unlike western medicines, many ingredients in TCM are of unclear. If they were extracted and transformed into injections, the ratio and amounts of components in them should have been altered and all of them would enter body circulation directly so that the toxicological risk increased.

Severe adverse effects of herbal injections were reported in China over the past few decades which made their developments nearly static. There is not even one herbal injection collected in Chinese Pharmacopoeia of the latest edition (Chinese Pharmacopoeia Committee 2010). Only a few scattered contents related to general injection preparation exist in some chapter of the Pharmacopoeia, such as Formulations General Clauses in Appendix IU, the allergic reaction test method in Appendix XIII G and the guiding principles for herbal injection safety test in XVIII B. Developments of new herbal injection slow down even being inhibited. The old productions which have been used in clinic are required to re-verify their safety. One of the great harmful responses is the immediate-type hypersensitivity reaction which could lead to life-threatening health problems. Powerful approaches which could predict the allergenicity of herbal injection accurately and quickly are still deficient.

Commonly used methods (e.g. murine passive cutaneous anaphylaxis (PCA) [6, 7], measurement of the antibodies, etc. [8, 9]) always mean inevitable exhausted, expensive and time-consuming work. According to the known knowledge while anaphylactic reaction occur, depending on the antigen crosslinked IgE, mast cells and basophils would degranulate and release β -hexosaminidase [10], histamine [11], serotonin (5-HT) [12], or other cytokines [13, 14]. Those molecules play critical roles in anaphylaxis. Therefore, some specific methods, like ELISA, could be employed to detect those substances so as to reflect the allergy-related cells' status. In present study, a simple and rapid approach was discussed based on the measurement of serotonin with HPLC technique for predicting allergenic reaction of herbal injection. To our knowledge, this is first report on systematic study of the allergenicity of herbal injection with serotonin detection method. Meanwhile, the sensitized and stimulated approaches implemented on the animal model were also discussed and optimized.

2. Experimental

2.1. Reagents

Albumin chicken egg (ovalbumin, OVA) and standard serotonin were purchased from Sigma (St. Louis, MO). Guinea pig histamine and β -hexosaminidase ELISA Kits were the products of R&D systems, Inc. Tween 80 and chloral hydrate were obtained from Kemiou chemical reagent company (Tianjin, China). HPLC grade acetonitrile was from TEDIA (Fairfield, OH, USA). Other chemicals not mentioned were all of analytical grade.

2.2. Animals

Male guinea pigs weighing 250–350 g were obtained from the Laboratory Animal Center in Nanjing University of Chinese Medicine. All the animals were maintained under standard laboratory conditions and had free access to food and water. The protocols of animal experiments in this study were permitted by the University Animal Care Committee.

2.3. General antigen sensitization and stimulation protocol

OVA was dissolved in normal saline (NS) at the concentration of 5 mg/mL. Three ways for sensitizing guinea pigs were: intramuscular, subcutaneous, and intraperitoneal injection with the antigen solutions. Model animals were exposed to OVA 0.5 mL each time, qod for a total of three administrations. Then, six time point were selected for the challenge on day 6, 11, 16, 21, 26, 31 from the day 0 after sensitization. Anti-coagulant blood samples were collected before (sample A) and after (sample B) the stimulation through the anaesthetic animals' carotid artery, and the samples after the stimulation were collected at different time (15 min, 30 min). Serum were obtained through centrifugation (2000 rpm, 10 min) and then put into storage at -20 degree Celsius. Antigen solutions involved different herbal injections. Qingkailing injection was purchased from Shenwei Pharmaceutical Co. Ltd (Hebei, China) which consisted of *cape jasmine fruit*, *buffalo horn* (powder), *isatis root*, *baicalin*, *Lonicera japonica*, *cholic acid*, *pearl shell* (powder), *hyodeoxycholic acid*, and some adjuvant material. Danshen injection (*salvia miltiorrhiza* injection) was obtained from Jiangsu Shenlong Pharmaceutical Co. Ltd (Jiangsu, China) and Diammonium glycyrrhizinate injection was the products of Jiangsu Chia-Tai Tianqing Pharmacy Co. Ltd. (Jiangsu, China). Yuxingcao injection was prepared from the plant (*Houttuynia cordata* Thunb.) which was harvested in the suburb of Nanjing, China and identified by Prof. Haobin Hu (Jiangsu institute for food and drug control). A voucher specimen (No. NJUTCM-PE-20090801) was deposited at the Herbarium in the department of pharmaceutical engineering of Nanjing University of Chinese Medicine. The preparation procedure was described in brief: 2 kg fresh plant was collected for steam distillation and 200 mL initial distillation liquid was obtained for double distillation. The redistilled liquid was added in 7 g sodium chloride, 5 g Tween 80 and water for injection to yield 1000 mL solution. After filtration, sterilization, the injection was sealed for storage.

Since the immediate allergy is frequently found on their clinical application [15–18], the two injections were selected to verify the allergenicity.

2.4. HPLC analysis

HPLC analysis was carried out on Waters 510 HPLC apparatus (USA) with EC2000 ChemStation software. A heder ODS-2 column (150 mm×4.6 mm, 5 μm) was used at 25 °C while the flow rate was 0.8 mL/min. The mobile phase, which consisted of: 25 mmol/L sodium dihydrogen phosphate (with 0.5 mmol/L EDTA-Na and 3.0 mmol/L sodium heptanesulfonate, pH 4.6): acetonitrile (85: 15), was used. Equivalent methanol were added in sample serums, and then, after vortex for 30 s and the centrifugation (10000 rpm, 15 min), supernatants of the pretreated serums were collected for the detection of serotonin.

2.5. Histamine, IgE and β-hexosaminidase determination

Histamine, IgE and β-hexosaminidase in the serum sample were measured through Enzyme-Linked Immunosorbent Assay (ELISA) follow the instruction of manufacturer of the ELISA kits. The amount variation rate of released histamine, IgE and β-hexosaminidase were expressed as a percentage as below:

$$Sample(\%) = \frac{(ValueB - ValueA)}{ValueA} \times 100\%$$

2.6. Statistical analysis

The results are expressed as mean values ± standard deviation and data have been submitted to Student's *t*-test. The level of $p < 0.05$ and $p < 0.01$ was taken as statistical significance.

3. Results and discussion

3.1. Serotonin measurement

Chromatogram of serotonin is shown as Fig. 1. The chromatographic peak appeared quickly at around 8 min after the sample introduction. The linear range was from 30–3000 ng/mL (Fig. 2., Table 1) and the limits of detection (LOD) was 20 ng/mL. Serotonin is stabler than histamine. Detection of the latter need derivatization [19, 20] or more complicated analytical instruments [21, 22].

3.2. Different ways for antigen sensitization

The sensitization and stimulation protocols have a reference to Chinese Pharmacopoeia (2010). Animals were sensitized with saline, OVA and Qingkailing injection on days 1, 3 and 5 for a total of three injections. OVA were administrated through intramuscular, while Qingkailing through subcutaneous. On day 16, saline, OVA and Qingkailing injection were administrated to challenge corresponding animals through intraperitoneal. Thirty minutes later after the challenge, blood sample of the animals were collected for the analysis. As shown in Fig. 3., production of serotonin increased significantly as well as those of IgE and histamine. For the purpose to compare the impact of different sensitization way using serotonin analysis method, animals were sensitized with OVA through subcutaneous, intramuscular, and intraperitoneal, and stimulated through intravenous. As to OVA, the way through intraperitoneal was more effective. (Fig. 4.)

In general, animals treated with mixed adjuvant were more easily to prompt the allergy media than those without adjuvant. This impact factor (with or without adjuvant) was test using Qingkailing injection. The results (Fig. 5.) show that adjuvant could help to prompt allergy reaction and serotonin could be used to reflect this characteristic.

3.3. Antigen stimulation

In the research of our group, the stimulation modes have less impact on the allergic response compared with the impact of sensitization modes on guinea pig. It means that the determination of sensitization modes is more important. Subcutaneous method is a sound sensitization mode through the discussion above. For the purpose to investigate the effect of stimulation, different stimulation ways were studied after the subcutaneous sensitization, which showed that the stimulation with intramuscular challenge is more sensitive, while the intraperitoneal method was more stable than that of intramuscular challenge. At the challenge time point of day 16 after the sensitization, serotonin was observed increasing to the maximum level (Fig. 6.). In order to determine the suitable time point at which serum sample was harvested after the challenge, 15 min and 30 min were investigated. The results showed that serotonin in the sample collected at 30 min increased more significantly (compared with saline group) (Fig. 7.). The reason that there were no significant differences between B (30 min) and A (15 min) in Qingkailing might be partly due to the individual differences of animals. In the guidance of Chinese Pharmacopoeia (appendix XVIII B, Chinese Pharmacopoeia Committee 2005; appendix XIII G, Chinese Pharmacopoeia Committee 2010), it is suggested to observe the anaphylactic response in 30 min. By reference to previous study, 30 min could be considered as serum harvested time point after the stimulation.

3.4. Serotonin analysis under optimal experimental condition

With the optimal condition discussed above, three injections were investigated including Danshen injection (salvia miltiorrhiza injection), Diammonium glycyrrhizinate injection and Qingkailing. The former two injections show little side effects according to the reports in clinic of China. Danshen injection was prepared from aqueous extracts of Danshen root (*Radix Salviae Miltiorrhiae*), which was deemed an extremely safe herbal plant [23]. Diammonium glycyrrhizinate injection was a single-ingredient preparation, which was actually a western medicine. The test results were shown in Fig. 8. Serotonin production provoked by DS and DG were not obvious, while that of Qingkailing still significantly higher. Danshen has been safely used for patients with coronary artery disease and other cardiovascular disease for many years. However, the injection is derived from extracts of the plant which consist of various 'mixture' substances. The main disadvantage of this type of agent is of the unclearness of what are the active components or toxic substances in them, which leads to the trouble to establish quality control for them. It might also be the main cause that most herbal medicines withdraw from European market. The chemical profile could not be consistent. This even occurs in different batches of the same product. Therefore, single-component agents should be more appropriate as 'negative' control since their pharmacological and toxicological effects are convenient to be identified. The batch number of Danshen injection in this study is 080626-3, which had been repeatedly tested and had not shown any allergic response.

3.5. Correlation of the released serotonin with IgE

Animals were sensitized with OVA and Yuxingcao injection on day 1, 3 and 5 for a total of three injections (0.5 mL/each time) through intraperitoneal way. On day 16, saline, OVA and Qingkailing injection were administrated to challenge animals through intramuscular way. 15 and 30 min later after exposure to antigens, blood sample were collected for the analysis, in which IgE was measured with ELISA and serotonin was detected with HPLC. IgE measurement with ELISA was more sensitive than that of HPLC detection of serotonin, but there are good correlations between the productions of the two cytokines, with the coefficient of correlation being 0.76. (Fig. 9.)

4. Conclusion

In this article, a feasible method for the prediction of the allergenicity of herbal medicine was reported. It has shown that serotonin has good correlation with other substances which mediated allergy, including histamine, IgE and β -hexosaminidase. Serotonin is stable and can be detected quickly with HPLC with low cost. Therefore, it is a promising way for predicting the allergenicity of those complicated multi-component agent and various natural products.

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References

1. Singh, Divya; Gupta, Rajiv; Saraf, Shubhini A. Herbs-are they safe enough? an overview. *Crit Rev Food Sci Nutr*. 2012; 52(10):876–98. [PubMed: 22747079]
2. Ernst, Edzard. Harmless Herbs? A Review of the Recent Literature. *The American Journal of Medicine*. 1998; 104(2):170–178. [PubMed: 9528737]
3. Ernst, Edzard. Adverse effects of herbal drugs in dermatology. *Br J Dermatol*. 2000; 143(5):923–929. [PubMed: 11069498]
4. Woodward KN. The potential impact of the use of homeopathic and herbal remedies on monitoring the safety of prescription products. *Hum Exp Toxicol*. 2005; 24(5):219–33. [PubMed: 16004184]
5. Iten F, Reichling J, Saller R. Adverse effects and interactions of phytotherapeutic drugs. *Ther Umsch*. 2002; 59(6):283–291. [PubMed: 12125177]
6. Nunomura, Satoshi; Yoshimaru, Tetsuro; Ra, Chisei. Na-Tosyl-Phe chloromethyl ketone prevents granule movement and mast cell synergistic degranulation elicited by costimulation of antigen and adenosine. *Life Sciences*. 2008; 83:242–249. [PubMed: 18634805]
7. Wershil, Barry K.; Mekori, Yoseph A.; Murakami, Tatsuo; Galli, Stephen J. 125 I-fibrin deposition in IgE-dependent immediate hypersensitivity reactions in mouse skin. Demonstration of the role of mast cells using genetically mast cell-deficient mice locally reconstituted with cultured mast cells. *J Immunol*. 1987; 139(8):2605–2614. [PubMed: 3655368]
8. Nakano, Takanari; Takahashi, Hidenori; Miyazaki, Shuichi; Kawai, Shin-ichiro; Shinozaki, Rina; Komoda, Tsugikazu; Nagata, Atsuo. Antigen-specific free immunoglobulin light-chain antibodies: Could it be a new diagnostic marker for patients with allergy? *Clinical Biochemistry*. 2006; 39(9): 955–959. [PubMed: 16814273]
9. Ahlstedt, Staffan; Murray, Clare S. In vitro diagnosis of allergy: how to interpret IgE antibody results in clinical practice. *Primary Care Respiratory Journal*. 2006; 15(4):228–236.

10. Huang, Feng; Tong, Xiaoyun; Deng, Huaming; Fu, Liangqing; Zhang, Ronghua. Inhibition of the Antigen-Induced Activation of RBL-2H3 Cells by Gab2 siRNA. *Cellular & Molecular Immunology*. 2008; 5(6):433–438. [PubMed: 19118509]
11. Jutel, Marek; Watanabe, Takeshi; Klunker, Sven; Akdis, Mübeccel; Thomet, Olivier AR.; Malolepszy, Jozef; Zak-Nejmark, Teresa; Koga, Ritsuko; Kobayashi, Takashi; Blaser, Kurt; Akdis, Cezmi A. Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. *Nature*. 2001; 413:420–425. [PubMed: 11574888]
12. Aketani, Saeko; Teshima, Reiko; Umezawa, Yoshio; Sawada, Jun-ichi. Correlation between cytosolic calcium concentration and degranulation in RBL-2H3 cells in the presence of various concentrations of antigen-specific IgEs. *Immunology Letters*. 2001; 75:185–189. [PubMed: 11166374]
13. Benhamou, Marc; Blank, Ulrich. Stimulus-secretion coupling by high-affinity IgE receptor: New developments. *FEBS Letters*. 2010; 584:4941–4948. [PubMed: 20851120]
14. Lim, Kye-Taek. Inhibitory effect of glycoprotein isolated from *Opuntia ficus-indica* var. *saboten* MAKINO on activities of allergy-mediators in compound 48/80-stimulated mast cells. *Cellular Immunology*. 2010; 264(1):78–85.
15. Zhao, Yubin; Xiao, Ying; Xie, Yanming. Correlation between change of immunotoxicology indexes of cerum and the occurrence of anaphylaxis in patients induced by qingkailing injection: a clinical study. *Zhongguo Zhong Yao Za Zhi*. 2011; 36(18):2579–83. (Article in Chinese). [PubMed: 22256770]
16. Wu, Jiarui; Zhang, Bing. Epidemiological characteristics of anaphylactic shock caused by Qingkailing injection. *Traditional Chinese Drug Research & Clinical Pharmacology*. 2007; 18(1): 74–77. (Article in Chinese).
17. Zeng, Congyan; Mei, Quanxi. Allergic Shock Induced by Houltuynia Cordata Injection: An Analysis of 62 Cases. *Evaluation and Analysis of Drug-Use in Hospitals of China*. 2008; 8(11): 865–867. (Article in Chinese).
18. Yang, Xiaoqing; Yao, Hai; Huang, Yimin; Liu, Fei. Literature analysis of allergic reactions associated with Yuxingcao injection in 120 cases. *Adverse Drug Reactions Journal*. 2005; (6):421–423. (Article in Chinese).
19. Hiroyuki, Kikuchi; Tomoaki, Tsutsumi; Rieko, Matsuda. Performance evaluation of a fluorecamine-HPLC method for determination of histamine in fish and fish products. *Shokuhin Eiseigaku Zasshi*. 2012; 53(2):121–127. (Article in Japanese). [PubMed: 22688027]
20. Jin, Gaowa; Cai, Youqiong; Yu, Huijuan; Qian, Beilei. Determination of histamine in canned fish by high performance liquid chromatography with pre-column derivatization. *Se Pu*. 2010; 28(11): 1099–1102. (Article in Chinese). [PubMed: 21381430]
21. Koyama, Junko; Taga, Shiori; Shimizu, Kae; Shimizu, Maki; Morita, Izumi; Takeuchi, Atsuko. Simultaneous determination of histamine and prostaglandin D2 using an LC-ESI-MS/MS method with positive/negative ion-switching ionization modes: application to the study of anti-allergic flavonoids on the degranulation of KU812 cells. *Anal Bioanal Chem*. 2011; 401(4):1385–1392. [PubMed: 21725829]
22. Yoshida, Tatsuo; Hamada, Hirotoishi; Murakawa, Hiroshi; Yoshimoto, Hidekazu; Tobino, Toshiaki; Toda, Kei. Determination of histamine in seafood by hydrophilic interaction chromatography/tandem mass spectrometry. *Anal Sci*. 2012; 28(2):179–182. [PubMed: 22322812]
23. Cheng, Tsung O. Cardiovascular effects of Danshen. *International Journal of Cardiology*. 2007; 121(1):9–22. [PubMed: 17363091]

Fig. 1.
Chromatogram of serotonin

Fig. 2.
Linear regression diagram of serotonin

Fig. 3. IgE, histamine and serotonin responses following the exposure to antigen for 30 min after different sensitization. A) IgE production, 30 min after challenging with OVA and Qingkailing injection, B) histamine production, 30 min after challenging with OVA and Qingkailing injection, C) Serotonin production, 30 min after challenging with OVA and Qingkailing injection. * $P < 0.05$; ** $P < 0.01$, vs. NS group ($n=10$)

Fig. 4. Serotonin production through 3 different ways sensitization with OVA. OVA 1, subcutaneous; OVA 2, intramuscular; OVA 3, intraperitoneal. * $P < 0.05$, vs. saline group (n=10)

Fig. 5.

Serotonin production using Qingkailing injection through subcutaneous way with different adjuvants. Q, Qingkailing injection without adjuvant; Q1, Qingkailing injection with Aluminum hydroxide (0.5 %, m/V; 1:1 mixed with the injection); Q2, Qingkailing injection with Aluminum trichloride (0.5 %, m/V; 1:1 mixed with the injection). * $P < 0.05$, vs. saline group (n=10)

Fig. 6. Serotonin production at different stimulation time point after the sensitization. A, 6 day; B, 11 day; C, 16 day; D, 21 day; E, 26 day; F, 31 day. ** $P < 0.01$, vs. saline group (n=10)

Fig. 7. Serotonin production after the exposure to antigen for A) 15 min and B) 30 min. * $P < 0.05$; ** $P < 0.01$, vs. saline group (n=10)

Fig. 8. Serotonin production after treating with three clinical used injections under the optimal condition. QKL, Qingkailing injection; DS, Danshen injection; DG, diammonium glycyrrhizinate injection. * $P < 0.05$, vs. saline group ($n=9$)

Fig. 9.

IgE and serotonin production after sensitization and stimulation with OVA and Yuxingcao injection. OVA1, IgE or serotonin production after the exposure to OVA for 15 min; OVA2, IgE or serotonin production after the exposure to OVA for 30 min; Y1, IgE or serotonin production after the exposure to Yuxingcao injection for 15 min; Y2, IgE or serotonin production after the exposure to Yuxingcao injection for 30 min. * $P < 0.05$; ** $P < 0.01$, vs. saline group ($n=9$)

Table 1

Linear regression data of serotonin

Concentration (ng/mL)	Peak area
30	15.82
60	30.95
120	58.11
150	69.7
300	150.16
600	304.8
750	362.13
1500	743.72
3000	1515.31