A rat model of Shuang Huang Lian injection-induced anaphylaxis

Zhonggang Li[1], Yan Gao[2], Huaishan Wang[1], and Zhaoping Liu[1]

Summary

Background: **ShuangHuangLian Injection (SHLI) has induced many serious anaphylactic diseases, becoming a threat to the public health. However, study of the mechanism of the reaction and therapeutic approaches to it have been hindered by the lack of suitable animal models.**

Objective: **We sought to develop a rat model that could mimic the characteristics of SHLI-induced anaphylaxis and the immunological changes that occur in clinical use.**

Methods: **Brown Norway (BN) rats were sensitized twice at an interval of seven days with three doses of SHLI and challenged 14 days after the last administration. Different parameters, including the symptoms, the histamine levels and the pathological changes were analyzed. Antibody and cytokine levels were determined to explore the mechanisms involved.**

Results: **Total and SHLI-specific IgE levels were significantly increased after SHLI sensitization. Systemic symptoms, local skin reactions, elevated histamine levels and decreased blood pressures were observed after challenge. Histological examination revealed that slight pathological changes occurred in lungs while no obvious alteration was found in intestine. IL-4 but not IFN-γ was significantly increased in spleen cells from SHLI-sensitized rats, indicating SHLI-induced anaphylaxis may be Th2-mediated.**

Conclusion: **This rat model may provide a useful tool to study the mechanisms in SHLI-induced anaphylaxis and suggest some novel therapeutic approaches for this anaphylaxis.** ***(Asian Pac J Allergy Immunol 2010;28:185-91)***

Key words: *Anaphylaxis; BN rats; Th2-mediated reaction; IgE*

Introduction

ShuangHuangLian Injection (SHLI) is a modern formulation that is composed of the alcohol-water extracts of three herbs: *lonicera*, *scute*, and *forsythia*. It has been widely used to treat a variety of infections for many years in China. Unfortunately, some serious anaphylactic diseases occur when the patients are treated with SHLI. One hospital reported that among 2000 cases of viral and bacterial infections that were treated with SHLI during a four-year period, two cases of anaphylactic response occurred. One developed a skin rash and the other developed serious anaphylactic shock.[1] Recently the number of cases of SHLI-induced anaphylactic disease has increased, which has become a threat to public health.[2] No reliable animal model is currently available and therefore the immunology mechanisms involved are poorly understood, restricting the diagnosis and therapy for SHLI-induced anaphylaxis.[3]

A suitable animal model should resemble the diseases closely, not only resembling the clinical and functional characteristics of the specific disease but also mimicing the basic mechanism involved. Being a high IgE response species, Brown Norway (BN) are good models for anaphylactic diseases and have been widely used to study the allergic responses induced by drugs, food protein extracts and other allergens.[4-6]

In this study we used three different doses of SHLI to sensitize BN rats twice. After sensitization, sera total IgE were quantified by

From the [1]Center for New Drugs Evaluation, Shandong University, Jinan 250012, China ;
[2] Department of Pharmaceutics, College of Pharmacy, Shandong University, Jinan 250012, China)
Corresponding author: Zhaoping Liu
Address: Center for New Drugs Evaluation Shandong University 44 Wenhua Xilu, Jinan, 250012, China
E-mail: lizhonggang1@yahoo.cn.

Enzyme-linked immunosorbent assay (ELISA) and specific IgE were determined by passive cutaneous anaphylaxis (PCA) assay. Systemic symptoms and local skin reactions were evaluated with a standardized scoring system.[7,8] The histamine levels in plasma and the blood pressures were also determined after challenge. The gastrointestinal and respiratory reactions were evaluated by analyzing intestinal and lung pathologic changes 24 hours after SHLI challenge. Moreover, the role of T cells involved in the regulation of SHLI was investigated by measuring cytokine production.

Methods

Animals and materials

Male, 11-week-old, inbred BN rats were purchased from WeiTongLiHua Co. (Beijing, China) and acclimatized for at least 5 days before the study. They were kept under SPF laboratory conditions (Centre for New Drug Evaluation of Shandong University, Jinan, China) and received the institute's grain-based open-formula diet and unfluoridated tap water. All animal procedures were approved by Shandong Provincial Animal Welfare and Care Guideline.

SHLI was provided by the Second Traditional Chinese Medicine Co. (Harbin, China). Evan's Blue, heparin sodium and Compound 48/80 were purchased from Sigma (St. Louis, MO). The rat total IgE ELISA kits, histamine ELISA kits, IFN-γ and IL-4 ELISA kits were purchased from Rapid Bio Lab (Calabasas, California, USA).

Sensitization protocol and samples preparation

Groups of rats were shaved on the neck and received a subcutaneous administration of 200 mg SHLI per kilogram of body weight, 400 mg/kg and 800 mg/kg. The naïve rats were given PBS instead. The same treatment was repeated 7 days later. Rats were exsanguinated 14 days after the last administration. Pooled serum samples were prepared and stored at −80°C until analysis.

Total IgE in serum

The total IgE concentrations in the serum samples were analyzed by the rat total IgE ELISA-kit. The measurement was conducted by the manufacturer's instructions and the results were expressed as μg/ml.

Passive Cutaneous Anaphylaxis (PCA)

Sera (100 μL) from sensitized rats 14 days after the last administration were diluted two-fold in PBS and then injected intradermally to shaved sides of naive rats. 48 hours later, sera-transferred rats were challenge by injection of 1% Evan's Blue containing 400 mg/kg SHLI. The animals were sacrificed by inhalation of CO_2 after 30 minutes and the diameters of dye leakage in the underside of the skin were recorded. Blue spot with a diameter of more than 5 mm was recorded as positive reaction. After heating the sensitized serum at 56 °C for 3 h, the same operations were repeated[9].

Skin tests

Rats were tested for immediate active cutaneous hypersensitivity reactions 14 days after the last administration. Briefly, under anesthesia the skin of the rat belly was shaved the day before the test. Evan's Blue (100 μL, 5 mg/ml in PBS) was intravenously injected into the SHLI sensitized rats. Subsequently, the following test substances (50μl, filtered through 0.22-μm pore size sterile filters) were administered intradermally into the shaved abdominal skins: Compound 48/80 (20 μg/ml), PBS and SHLI (200, 800mg/kg). Rats were killed and skinned 20 minutes later. The diameter of blue lesion was measured by calipers. A diameter larger than 5 mm is regarded as positive reaction.

Systemic anaphylaxis

Symptoms of systemic anaphylaxis were observed in naïve and SHLI-sensitized rats 30 minutes after the challenge. Symptoms were evaluated by using a scoring system which was described by Li[10]: 0 = no symptoms; 1 = scratching and rubbing around the nose; 2 = puffiness around the eyes and mouth, 3 = wheezing, labored respiration, and cyanosis around the mouth and the tail; 4 = convulsion; and 5 = death.

Plasma histamine levels

Thirty minutes after challenge, blood was collected into chilled tubes containing heparin sodium solution (30 μL, 5 % in PBS). After 1000×g centrifugation for 10 minutes, plasma supernatant were collected and histamine levels were determined by histamine ELISA-kit, as described by the manufacturer.

Blood pressures

14 days after the last administration, the animals were challenged with SHLI (0.25 ml, 200 mg/kg) by intravenous administration. The blood

pressures were recorded by the multi-channel recorder 30 minutes before and after challenge.

Histology

Intestine and lung samples from sensitized rats were collected 24 hours after SHLI challenge and then fixed in neutral-buffered formaldehyde. Tissues were sliced and 5 μm sections were stained with hematoxylin and eosin (HE) for light microscopy examination.

Cytokine production

The spleen cell suspensions from SHLI-sensitized rats were cultured with and without SHLI in 96-well plates at a concentration of 2×10^6 cells/well. After 24 and 48 hours, supernatants were taken and stored at -80ºC until analyzed. The levels of IL-4 and IFN-γ were measured with rat cytokine ELISA-kits.

Statistical analyze

Data were analyzed using SPSS 11.0 (SPSS, Chicago, USA). For total IgE, histamine and cytokine levels, the differences between the groups were analyzed by one-way ANOVA and Student's t-Test. Statistical significance (P <0.05) was determined.

Results

Total IgE concentrations

Total IgE levels for 200, 400, 800 mg/kg SHLI-sensitized rats (212.0±33.5, 280.7±26.8, 324.0±17.7 ng/ml, respectively) were significantly increased (P <0.01) compared to that of the naïve rats (153.7±10.4 ng/ml). We also observed that the increase of total IgE in sera was dose-dependent (r = 0.93). These results indicate that the IgE antibody responses were determined by the SHLI sensitization dose to a certain extent.

PCA reactions

The PCA assay was conducted with or without IgE (eliminated by heating) to confirm the role of specific IgE in SHLI-induced anaphylaxis. The sera of SHLI sensitized rats elicited positive reactions, while when heated for IgE antibody inactivation negative reactions were observed (Table 1). These results show that IgE is the reactive antibody to elicit SHLI-induced anaphylaxis.

Table 1. PCA reactions after injection of immune or heated inactivate serum

SHLI sensitization doses (mg/kg)	Heat inactivation	Diamond (mm) mean±STD	Positive reaction (n= 5, %)
0	−	0.61±0.35	0
200	−	7.44±1.82$^{**\#\#}$	80
400	−	7.3±1.77$^{**\#\#}$	80
800	−	12.336±2.47$^{**\#\#}$	100
800	+	1.25±0.34	0

$^{**}P$<0.01 compared to naïve rat sera; $^{\#\#}P$<0.01 compared to heated immune sea.

Systemic reactions

Systemic reactions after challenge could mimic the anaphylaxis shock that SHLI has induced. Obvious systemic reactions in SHLI-sensitized rats were observed within 30 minutes after challenge, while naïve rats showed no reactions (Figure 1). In 800 mg/kg SHLI-sensitized rats, the most severe reactions occurred and a number of rats had convulsions (2 in 5), although no rats died.

Skin tests after challenge

The skin reactions are closely associated with IgE-induced mast cell degranulation. All rats (6 in 6) showed positive reactions to Compound 48/80 and no rats (0 in 6) demonstrated positive response to PBS. In the dose of 800 mg/kg SHLI, all rats sensitized (6 in 6) showed positive reactions, but only 66.7% (2 in 6) showed positive reactions in the dose of 200 mg/kg SHLI (Figure 2). These result indicate that the severity of skin reactions are not only associated with the type of reagent but also the dose of reagent for challenging.

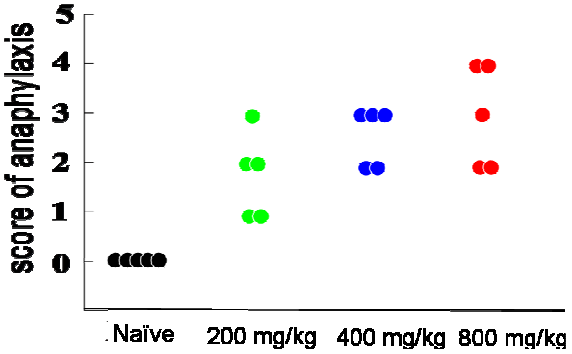

Figure 1. System anaphylaxis symptom scores in rats. SHLI-sensitized rats (200, 400, 800 mg/kg) and naïve rats (n=5) were challenged intravenously with SHLI. Thirty minutes later, the symptoms of anaphylaxis were observed and scored on a scale from 0 (no symptoms) to 5 (death), as described in section 2.6. The circles indicate individual rats.

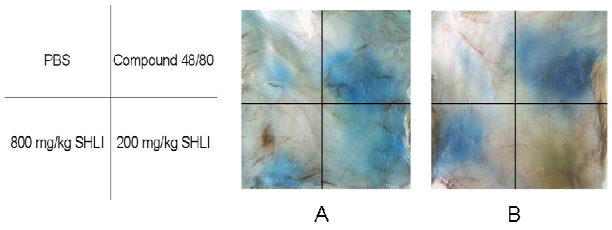

Figure 2. The skin test reactions in rats. Rats (n= 6) were sensitized to SHLI in dose of 800 mg/kg and the treatment was repeated 7 days later. 14 days after the last administration, Evan's Blue (100 μL, 5 mg/ml in PBS) was injected intravenously and then 50μl of the following test substances were administered intradermally into the shaved abdominal skin: compound 48/80 as positive control, PBS as negative control, 200 mg/kg and 800mg/kg SHLI. The diameters were measured and a diameter larger than 5 mm was regarded as positive. Both A and B showed that PBS induced negative reactions, compound 48/80 and 800 mg/kg SHLI induced positive reactions. In rat A (2 in 6), 200 mg/kg SHLI induced positive reactions, while in rat B (4 in 6) the reaction induced by 200 mg/kg SHLI was negative.

Elevated histamine levels

Plasma histamine levels were significantly increased in SHLI-sensitized rats when compared to naïve rats (Figure 3). These results suggest that histamine is one of the major mediators to induce anaphylaxis in this rat model.

Decreased blood pressures after challenge

No changes in blood pressures were observed in naïve rats upon intravenous challenging with SHLI. However, the blood pressures of the sensitized animals decreased dramatically within 30 minutes after SHLI challenge (Figure 4). The changes of blood pressures revealed that SHLI might induce damage to the circulatory system. These results, consistent with the elevated histamine levels, indicated that the release of allergy mediators contributes to the decrease of blood pressures and then induces anaphylaxis shock in SHLI-sensitized rats.

Histological analysis

Analysis of intestinal histology showed that no inflammation associated with morphologic changes occurred in intestines of animals receiving all three doses of SHLI or in naïve rats (Figure 5). The thickened muscular layer crypt elongation and villous atrophy, commonly regarded as hallmarks of intestinal allergy, were not noticed in these histological samples. We observed that SHLI-induced immediate reactions in this model were sometimes accompanied with mild respiratory symptoms such as wheezing and labored respiration. Histological examinations revealed that lungs from SHLI-sensitized rats were slightly inflamed; there were slight eosinophil granulocyte infiltrations around the blood vessels (Figure 5).

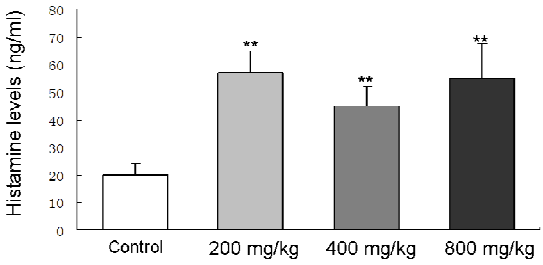

Figure 3. The histamine levels in plasma after challenge. Groups of six rats received PBS (Control), 200, 400 or 800 mg/kg SHLI, respectively. The treatment was repeated 7 days later. 14 days after the last administration rats were challenged by 200 mg/kg SHLI. The histamine levels in sera after 30 minutes were determined by ELISA-kit. Results are expressed as mean }STD. Significant differences aredonated as: $^{**}P$ <0.001 compared to thecontrol groups.

Th2 cytokine response

To determine the role that T cells and cytokines played in SHLI-induced anaphylaxis, we examined the production of cytokines by spleen cells from SHLI-sensitized and naïve rats after *in vitro* culture with SHLI. Compared with naïve rats, the IL-4 levels were significantly increased in SHLI-sensitized rats (P <0.01) and IFN-γ levels were significantly decreased (P <0.01, P <0.05) (Table 2).

Table 2. Cytokine production in spleen cell suspensions

SHLI sensitization doses (mg/kg)	Cytokine Concentrations (pg/ml)	
	IL-4	IFN-γ
0	8.41±3.03	104.69±18.57
200	26.34±10.53**	62.30±13.69**
400	25.92±9.77**	75.60±16.22*
800	31.68±11.11**	58.33±16.49**

$^{*}P$ <0.05, $^{**}P$ <0.01 compared to cytokine concentrations of naïve rats.

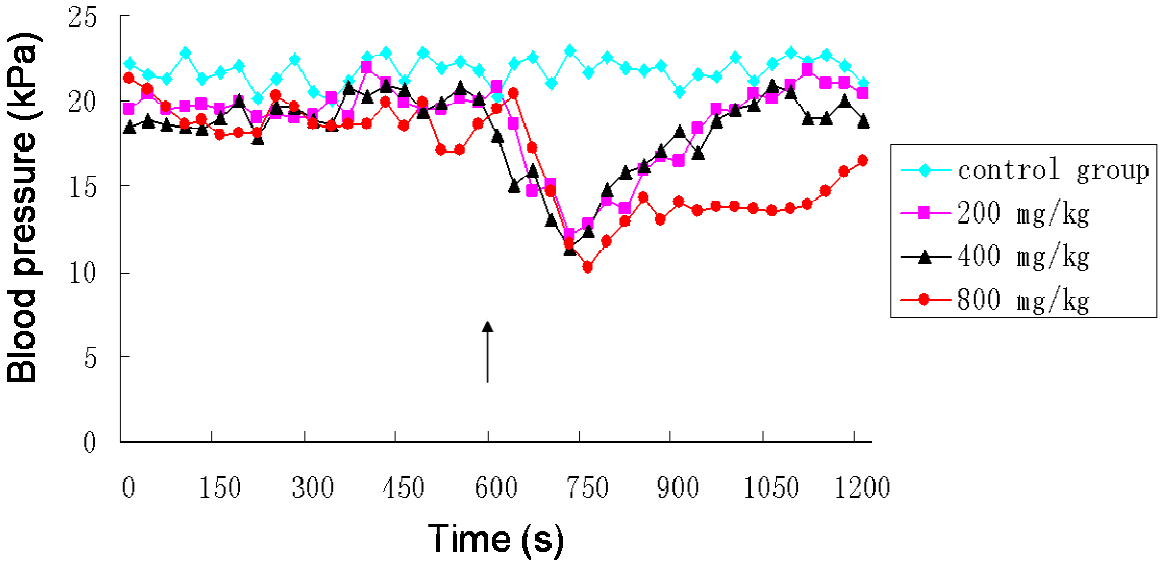

Figure 4. The blood pressure in BN rats before and after challenge. The blood pressure of naive rats and SHLI-sensitized rats (200, 400 and 800 mg/kg) were recorded for 600s. Then the rats were challenged with SHLI in 600 s and the changes of blood pressure were recorded. The blood pressure of rats was recorded every 30 s.

Discussion

In this study we have developed a rat model to mimic SHLI-induced anaphylaxis by subcutaneous sensitization and intravenous challenge. Anaphylactic symptoms were observed within 30 minutes after challenge, including systemic and local reactions. The elevation of plasma histamine, decrease of blood pressure and histology change in lungs were observed during the anaphylactic reactions. Previous studies have reported that SHLI could induce systemic reactions in BN rats. However, they failed to investigate its effect on specific organs and did not go further to investigate the mechanism involved. We not only observed the systemic and local reactions but also examined the histological injury of the digestive and respiratory system. Moreover, we determined the IgE antibody (total and specific-IgE) and the cytokine production, illustrating that Th2-mediated mechanism was involved. So this BN rat model was the first systemic and complete animal model to mimic SHLI-induced anaphylaxis, both mimicking the symptoms and exploring the mechanism involved.

Consistent with some early investigations based on BN rats[11,12], we found that BN rats were suitable for determining SHLI-induced anaphylaxis. Being a high IgE responding strain, BN rats could respond severely to SHLI, characterized by immune functions and allergic symptoms.

There was a correlation between IgE and SHLI doses. The higher doses of SHLI administrated, the more IgE was induced. Moreover, the severity of the systemic and local reactions showed the same trend with the SHLI doses. Therefore, higher doses of SHLI are prone to produce more IgE and induce more intense reactions. However, the histamine levels did not show the same pattern. That may be because most of the histamine was metabolized due to its very short half life in plasma leaving variable amounts present. This phenomenon may also be relevant to previous studies, in which lower antigen doses induced more severe reactions because of the tolerance[13]. The explanation may be that the highest doses of SHLI used in this study (800 mg/kg) was lower than the dose that is capable of inducing colonel deletion of T-cells, following which immune tolerance occurs. Therefore, compared with lower doses (200 and 400 mg/kg), higher dose (800 mg/kg) SHLI are prone to stimulate T-cells to produce more IgE antibody and then induce more severe reactions.

Most anaphylactic reactions to SHLI are regarded as immediate hypersensitivity, occurring within 24 hours after administration[14]. In immediate hypersensitivity, the role of IgE is

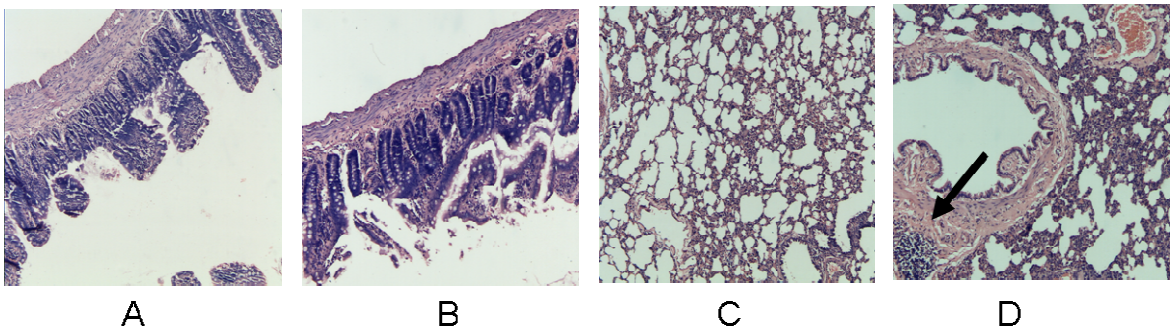

Figure 5. Histological changes in intestines and lungs of BN rats after SHLI challenge. Intestines from naïve rats (A) and SHLI-sensitized rats (B) showed neither thickened muscular layer crypt elongation nor villous atrophy. Lungs from naïve rats (C) showed neither inflammatory cells nor mucus cells, while SHLI-sensitized rats (D) showed slight inflammatory cells accumulation but no mucus cells were found (arrow). A-D: HE ×50.

pivotal. Although some studies have found that there was no increase in total IgE during some allergy diseases[15], the total IgE levels have been widely used as a diagnosis parameter both in clinical diagnosis[16,17]. In this study the relationship between the total IgE levels and the anaphylaxis was confirmed, indicating that the total IgE may be used as a potential indicator for predicting the SHLI-induced anaphylaxis. In this study we detected that the specific-IgE increased significantly in sera of SHLI-sensitization rats. The PCA reactions were abrogated by heat inactivation of immune sera, thereby strongly implicating the active role of IgE in this rat model[18]. These results suggested that SHLI-induced anaphylaxis might be mediated by IgE.

Systemic and local anaphylactic symptoms were observed in this model, mimicking the anaphylactic diseases in clinical practice. We scored the systemic anaphylaxis reactions and consequently found SHLI could induce different levels of reactions in a dose-related manner. In order to reproduce the local skin reactions, such as the skin rash, we performed skin test with different doses of SHLI and other reagents[19]. The results showed that high doses of SHLI are capable to elicit positive reactions the same as Compound 48/80, a histamine release reagent[20]. However, not all doses of SHLI could induce positive reactions, indicating the severity of skin reactions was associated with the dose of reagent used for challenge[21].

The physical effects of SHLI mainly focused on circulatory systems, where the histamine levels elevated significantly and blood pressures decreased dramatically. It is probable that the decrease of blood pressures, due to the release of histamine into the plasma, contributes to the anaphylaxis shock in the clinical situation. Histological analysis of the intestines of SHLI-sensitized rats showed that no obvious pathological damage occurred after challenge. This suggests that SHLI, as an injection, seldom induces gastrointestinal allergy, which coincides with the findings in allergy cases reported in clinical practice. There were eosinophil granulocyte infiltrations in the lung samples, leading to inflammation in the respiratory system. This indicates that the respiratory system might be another target organ when SHLI-induced anaphylaxis occurs. However, these damages were minor compared to the respiratory injuries cause by protein allergens, where obvious respiratory symptoms have been recorded and marked pathology alternations have been observed[22,23]. This might be explain by the fact that components of SHLI are small molecular compounds. Therefore, the skin, rather than the respiratory and gastrointestinal systems, is prone to becoming the major target organ for anaphylactic reactions[24,25]. More attention should be paid to the skin rash induced by SHLI in clinical diagnosis and therapy.

Cytokine production is regarded as a general feature of anaphylaxis[26,27]. In this study we determined IL-4 (Th2-prone) and IFN-γ (Th1-prone) in the spleen cells of SHLI-sensitized rats to explore the related molecular events[28,29]. SHLI could stimulate remarkable type 2 cytokine production with relatively high levels of IL-4 but little levels of IFN-γ. These data were consistent with the IgE increase in the immune sera,

suggesting that the anaphylaxis induced by SHLI may be mediated by Th2 cells.

In summary, this rat model manifests the same immunological parameters (IgE, cytokines) and symptoms (systemic reactions, local reactions) as the SHLI-induced anaphylaxis in clinical practice. Besides, this model mimics the changes of certain physiological indicators (histamine levels, blood pressure) and some pathological changes (respiratory systems). Therefore, this rat model, based on BN rats, provides a useful means for studying the diagnosis and therapy of SHLI-induced anaphylaxis.

Acknowledgements

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