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To cite this article: Delu Che, Jiao Cao, Rui Liu, Jue Wang, Yajing Hou, Tao Zhang & Nan Wang (2018): Harpagoside-induced anaphylactic reaction in an IgE-independent manner both in vitro and in vivo, Immunopharmacology and Immunotoxicology, DOI: [10.1080/08923973.2018.1424900](https://doi.org/10.1080/08923973.2018.1424900)

To link to this article: <https://doi.org/10.1080/08923973.2018.1424900>



Published online: 22 Jan 2018.



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ORIGINAL ARTICLE



Harpagoside-induced anaphylactic reaction in an IgE-independent manner both *in vitro* and *in vivo*

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ABSTRACT

Background: Harpagoside (HAR) is an active component of *Scrophularia ningpoensis* (SN), which has anti-inflammatory and anti-immune effects. SN is used widely in China to treat various diseases. Recently, SN has been used as a traditional Chinese medicine injection and used clinically. However, allergic responses to these injections are frequently reported.

Aim: We examined whether the main component of SN, HAR, is associated with the allergic reaction to SN.

Methods: This study assessed the effects of HAR in mice and mast cell activation to characterize its anaphylactic effects and underlying mechanisms. Mice hindpaw swelling, serum allergy factor detection, enzyme-linked immunosorbent assays, and degranulation assays were performed to measure allergic mediators both *in vivo* and *in vitro*.

Results: The present study indicated that HAR induced paw swelling, interleukin-6, inositol triphosphate, tumor necrosis factor- α , and histamine increases in mice. Our *in vitro* data also showed that HAR induced β -hexosaminidase, inositol triphosphate, and interleukin-6 release, leading to mast cell degranulation. In contrast, neither C48/80 nor HAR induced local anaphylaxis in STOCK Kit^{W-sh}/HNhrJaeBsmJNju mice.

Conclusions: HAR is a potential sensitization compound in SN, and these results provide information for the safe clinical use of SN.

ARTICLE HISTORY

Received 3 August 2017

Revised 22 September 2017

Accepted 12 December 2017

KEYWORDS

Scrophularia ningpoensis;
harpagoside; mast cell;
anaphylactoid reaction; IgE-
independent

Introduction

Harpagoside (HAR), a derivative of catalpol, is an iridoid glycoside from *Scrophularia ningpoensis*¹. HAR was shown to have pharmacological activities in various diseases, such as inflammation^{2,3}, ischemia¹ and osteoarthritis, among others^{4,5}. While HAR is widely used worldwide, its allergic effects remain unclear.

Anaphylaxis is a common adverse reaction to the clinical use of drugs such as traditional Chinese medicine injection (TCMi). *Scrophularia ningpoensis* is present in various TCMi mixtures, for which adverse reactions are frequently reported. Most of these adverse reactions present as anaphylaxis, one of the most common hypoallergenic symptoms. Anaphylaxis presents diagnostic and therapeutic challenges and often manifests as a broad array of symptoms and signs that may be similar to those of other diseases.

Mast cells are classically considered to trigger allergic and anaphylactic reactions. The most widely studied mechanism by which mast cells become activated is the cross-linking of Fc ϵ RI, with mast cells responding to antigenic stimulation through the cross-linking of immunoglobulin E (IgE) bound to high-affinity receptors for IgE (Fc ϵ RI), and activation of Fc γ R after IgG binding⁶. Once activated, intracellular Ca²⁺ influx increases rapidly and triggers the release of mediators including histamine, β -hexosaminidase, proteases (mainly

tryptase), mast cell carboxypeptidase A3 and chymase, platelet-activating factor and prostaglandins⁷. These substances induce allergic responses, local swelling and asthma, which are common symptoms of allergy. A unique characteristic of mast cells is their antibody-independent responsiveness pathway^{8,9}. This pathway is activated by a variety of basic compounds collectively known as basic secretagogues, which includes peptides and amines such as substance P, mast cell degranulating peptide, neuropeptide Y, mastoparan, and compound 48/80¹⁰. In this study, we examined the anaphylactic effects of HAR both *in vitro* and *in vivo*.

Materials and methods

Reagents

HAR and compound 48/80 (C48/80, C2313, Lot: 073M4050V) were obtained from Sigma-Aldrich (St. Louis, MO) and purified to $\geq 98\%$. Fluo-3, AM ester, and Pluronic F-127 were from Biotium (Fremont, CA). TM buffer was prepared on the day of the experiments (6.954 g/L NaCl, 0.353 g/L KCl, 0.282 g/L CaCl₂, 0.143 g/L MgSO₄, 0.162 g/L KH₂PO₄, 2.383 g/L HEPES, 0.991 g/L glucose, and 1 g/L bovine serum albumin, pH 7). Metoprolol tartrate (Lot: 100084–201102), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; CAS: 298–93–1), and Triton X-100 were obtained from Amresco (Solon, OH). Dimethyl

sulfoxide (analytical reagent, Sigma-Aldrich, MO Lot: MFCD00002089). All ELISA kits were obtained from KYM Biotech (Beijing, China). All aqueous solutions were prepared using ultrapure water produced by the MK-459 Millipore Milli-Q Plus ultra-pure water system (Billerica, MA).

Mouse model

C57 BL/6 mice were purchased from the School of Medicine Laboratory Animal Center juvenile and were aged 7–8 weeks. The experimental protocols for using mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011–0045). STOCK Kit^{W-sh}/HNIhrJaeBsmJNju mice were purchased from the Model Animal Research Center of Nanjing University. All mice were housed in the Experimental Animal Center of Xi'an Jiaotong University in individual cages in a large colony room with free access to water and were fed standard dry food twice per day. The breeding environment was 20–25 °C with a relative humidity of 40% and day-night cycle of 12/12 h. The mice were randomly divided into the following groups (5 mice in each group): control, compound 48/80 and experimental group. All experiments involving equal treatments in animals were conducted by researchers blinded to the conditions.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols for using the mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011–0045). All animals were operated on under chloral hydrate anesthesia.

Hindpaw swelling and extravasation

Adult male mice weighing 18–22 g were anesthetized by intraperitoneal injection of 0.2 ml 0.35% chloral hydrate. Fifteen minutes after the induction of anesthesia, each mouse was injected intravenously with 0.2 ml of 0.4% Evans Blue in saline. A vernier caliper was used to measure paw thickness before injection. Five minutes later, 5 µL of the test drug was administered by a microinjector in the left paw and saline was administered in the right paw as a negative control. Fifteen minutes later, paw thickness was measured again and recorded. Mice were then sacrificed by decapitation, and a photo of each paw was taken.

Paw tissue was collected, dried for 24 h at 50 °C, and weighed. Evans Blue was extracted by 24-h incubation in 500 µL acetone-saline (7:3) at 37 °C. Tissues were cut into pieces, placed in an ultrasonic machine for 10 min, centrifuged at 3000 rpm for 20 min. The supernatant was equally distributed in 200-µL aliquots into 96-well cell culture plates, and the optical density was read at 620 nm using a spectrophotometer. For studies using drug substances, mice were

injected with 5 µL of a 30 µg/mL solution of compound 48/80 as a positive control.

ELISA of allergic-related factors in serum

Adult male mice (18–22 g) were injected intravenously with different doses of HAR (0.23, 1.13, and 2.25 mg/kg), C48/80 (10.00 mg/kg), or saline, and whole blood was obtained from the orbit 30 min after injection. Blood was incubated for 30 min at room temperature and centrifuged for 15 min at 1000 × g to separate the serum. The protein levels of inositol triphosphate (IP3), histamine, interleukin (IL)-6, and tumor necrosis factor (TNF)-α in the serum were determined by enzyme-linked immunosorbent assay (ELISA) (KYM, Beijing, China) following the manufacturer's instructions.

Cell culture

RBL-2H3 cells were purchased from the China Center for Type Culture Collection and cultured at 37 °C in a 5% CO₂ atmosphere in DMEM (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA).

Cell viability assay

The effect of HAR on RBL-2H3 cell viability was evaluated using the MTT assay. Briefly, exponentially growing cells were harvested and plated in 96-well plates at a concentration of 2×10^4 cells/well. Following 24-h incubation at 37 °C, the cells were treated with various concentrations of HAR for 48 h. Subsequently, 20 µL MTT (5 mg/mL) was added to each well and the cells were incubated at 37 °C for 4 h. After discarding the supernatant, 150 µL dimethyl sulfoxide was added to each well and the optical density of the cells was determined using a microplate reader (Bio-Rad, Hercules, CA, USA) at 490 nm and expressed as absorbance values.

Intracellular Ca²⁺ mobilization assay

All drug substances were diluted to the required concentrations in calcium imaging buffer (CIB; NaCl 125 mM, KCl₃ Mm, CaCl₂ 2.5 mM, MgCl₂ 0.6 mM, HEPES 10 mM, glucose 20, mM, NaHCO₃ 1.2 mM, sucrose 20 mM, brought to pH 7.4 with NaOH). The incubation buffer consisted of 1 µL Fluo-3, 4 µL Pluronic F-127, and 995 µL CIB. RBL cells were plated at 5×10^3 cells per well in 96-well plates and washed twice with CIB. Cells were incubated for 30 min, washed twice, and imaged immediately. For calcium imaging, the cells were magnified by 200X and one photo per second was taken under blue light. Cells were identified as responding if [Ca²⁺]_i increased by at least 50% after injecting the substances.

β-hexosaminidase degranulation assay

Cells in the exponential growth phase were harvested and seeded to 96-well microtiter plates (2×10^4 cells/well). After incubation at 37 °C overnight, the cells were treated with different concentrations of HAR or C48/80 (30 µg/mL). Reactions

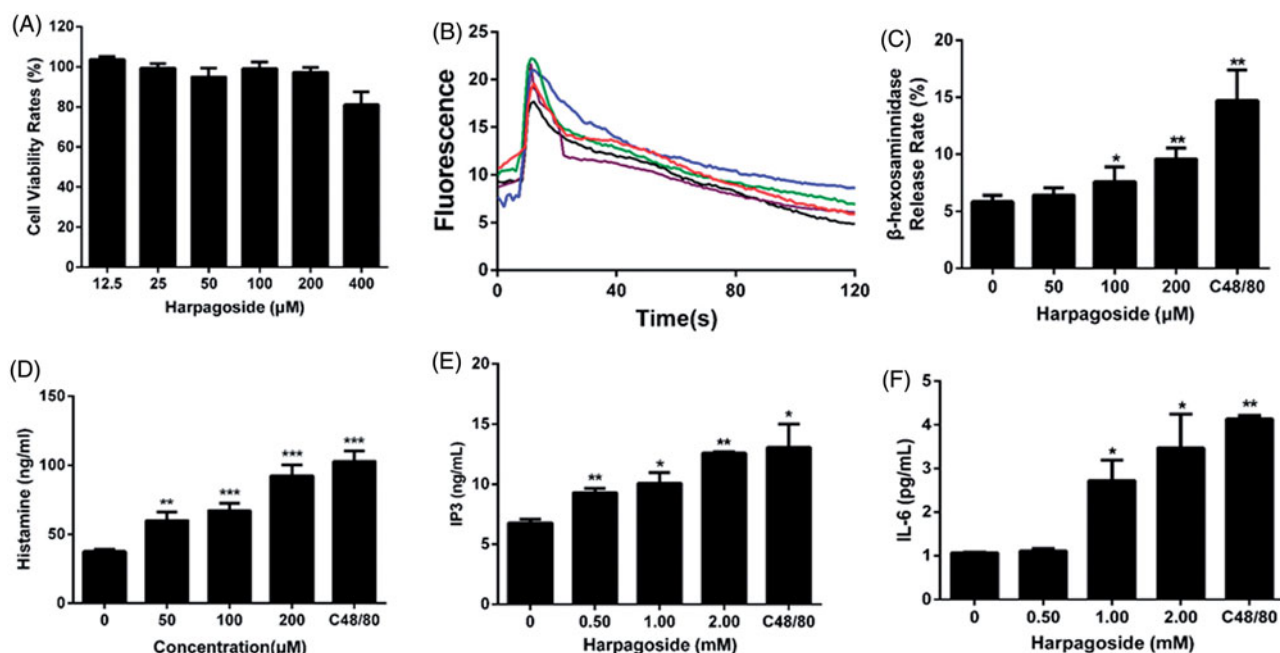


Figure 1. A: Cells were treated with several doses of HAR for 24 h and cell viability was detected by MTT assay. B: HAR increased intracellular calcium ion (Ca^{2+}) concentration in RBL-2H3 cells. C: Cells were stimulated with the indicated concentrations of HAR for 30 min. The enzymatic activity of released β -hexosaminidase was measured. Release of β -hexosaminidase is shown as %. D: Different concentrations of HAR caused the release of histamine, shown in ng/mL. E–F: Cells were stimulated with the indicated concentrations of HAR for 30 min. IP3 and IL-6 were detected by ELISA. * $p < .05$, ** $p < .01$ versus saline.

were terminated after 30 min by placing the plates on ice for 10 min, and the supernatant was collected and centrifugation at $1000 \times g$ for 10 min at 4°C . The remaining cells were lysed by assay buffer containing 1% (v/v) Triton X-100 prior to incubation with the substrate. Hexosaminidase activity was determined by incubating the supernatant and cell lysate with 1 mM β -hexosamine for 90 min at 37°C . The reaction was terminated by adding $0.1 \mu\text{M}$ $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ (9:1), and then, absorbance was measured at 405 nm using a microplate reader (Bio-Rad). The percent β -hexosaminidase released was calculated as follows: % β -hexosaminidase release = fluorescence of the drug supernatant/(fluorescence of the blank extracellular supernatant + fluorescence of the blank intracellular supernatant) $\times 100$.

Histamine release assay

This assay was carried out in a 96-well plate. RBL-2H3 cells at a density of 2×10^4 cells/well were incubated overnight at 37°C with 5% CO_2 . Cells were counted to normalize each sample. Histamine (HA) was purchased from Sigma, histamine-2HCl (A, A, B, B-D4, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (Cambridge, MA), and HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA). Mass spectrometry-grade formic acid was from Sigma. For LC-ESI-MS/MS method, an LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used. Histamine was evaluated on the system employing a HILIC column (Venusil HILIC, 2.1×150 mm, $3 \mu\text{m}$, Agela Technologies, Tianjin, China), and isocratic elution was conducted with

acetonitrile–water containing 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 ml/min.

Degranulation portion assay in RBL-2H3 cells

RBL-2H3 cells were seeded in 96-well microplates (5×10^4 cells/per well), incubated for 24 h at 37°C , and treated with various concentrations of HAR (50, 100 and $200 \mu\text{M}$) or C48/80 ($30 \mu\text{g/mL}$) for 30 min. The cell supernatant was collected to detect IL-6 and IP3 levels after HAR or C48/80 administration using ELISA kits according to the manufacturer instructions.

Statistical analyses

The data are presented as the mean $\pm$ standard error of mean and analyzed for statistical significance using analysis of variance. Two-tailed tests were used for 2-group comparisons. Differences were considered statistically significant at $p < .05$.

Results

Effect of HAR on mast cell degranulation

In this study, the MTT assay results showed that below a concentration $400 \mu\text{M}$, HAR was not cytotoxic (Figure 1(A)). We found that HAR increased intracellular calcium ion (Ca^{2+}) concentrations in RBL-2H3 cells (Figure 1(B)). To assess the effect of HAR on degranulation, we evaluated the release of two granule markers, β -hexosaminidase and histamine, from RBL-2H3 cells. RBL-2H3 cells were exposed to various concentrations of HAR. Treatment with $100 \mu\text{M}$ HAR increased

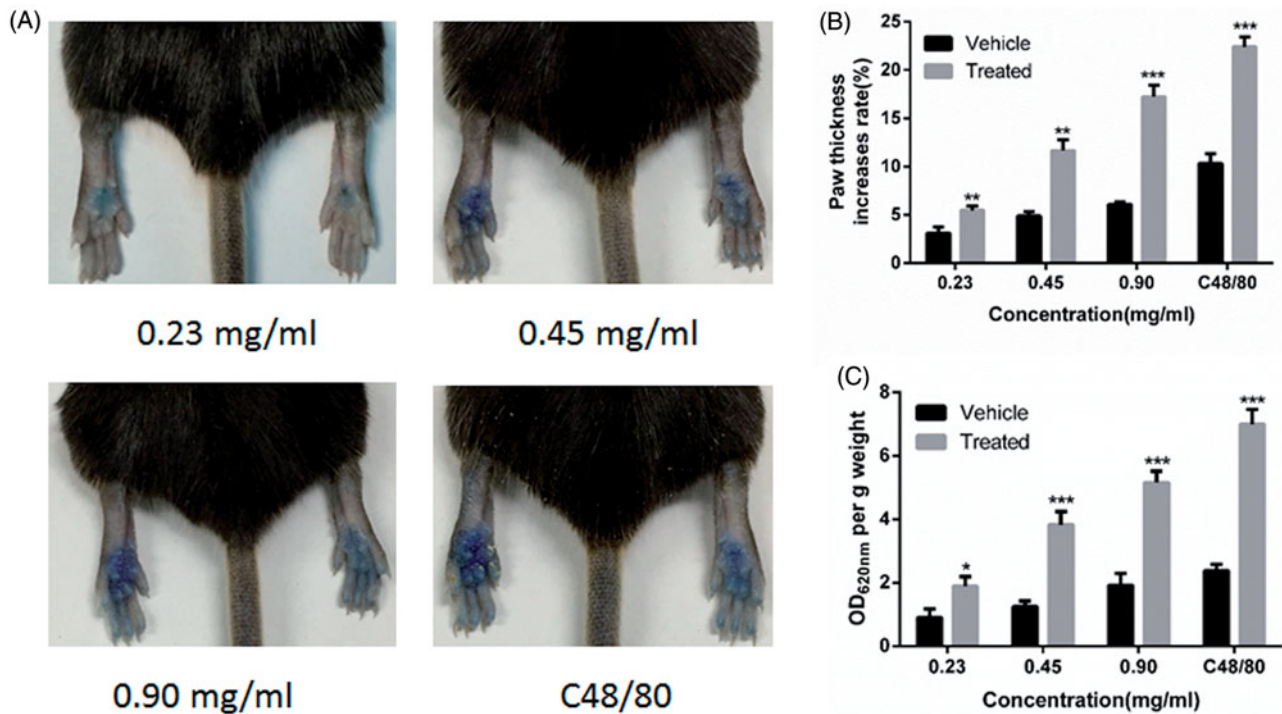


Figure 2. Representative images of Evans Blue-stained extravasation 15 min after intraplantar injection of 5 μ L of HAR (left paw), C48/80 (left paw), or saline (right paw). A: 0.23, 0.45, and 0.90 mg/mL of HAR and 30 μ g/mL of C48/80 caused local anaphylaxis in mice. B: Quantification of paw thickness increase in each group, with all animals compared to the saline paw thickness, respectively. C: Quantification of Evans Blue leakage into the paw after 15 min ($n=6$ in each group). * $p < .05$, ** $p < .01$ vs. saline.

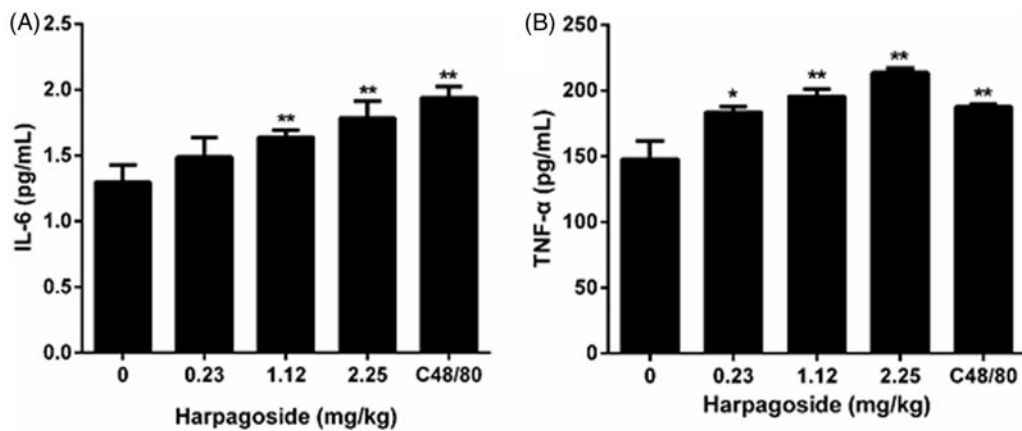


Figure 3. IL-6 and TNF- α levels in mouse serum after intravenous injection of HAR, C48/80, or saline. A: IL-6 level after treating mice with different doses of HAR ($n=6$). B: TNF- α level after treating mice with different doses of HAR ($n=6$). * $p < .05$, ** $p < .01$ vs. saline (0 in the figures).

release rate of β -hexosaminidase by 53.20% compared to in the control group (Figure 1(C)). Moreover, the increase in the release of histamine by HAR was greater than in the control group. This indicated that HAR caused RBL-2H3 cell degranulation *in vitro*, leading to dose-dependent release of β -hexosaminidase and histamine. Additionally, IP3 and IL-6 release was significantly increased by treatment with HAR (Figure 1(E, F)).

HAR-induced local anaphylaxis

To investigate the allergenic effects of HAR *in vivo*, HAR or C48/80 was subcutaneously injected to the right paw of the mice. Evans Blue was exuded from the paw vessel after treatment with HAR, and the paw showed different levels of

swelling and Evans Blue exudation. Treatment with HAR increased dye diffusion by 50%, and the thickness of the paw was increased by up to 20% when the dose of HAR was 0.90 mg/mL (Figure 2).

HAR-induced preinflammation factor release

To determine whether the allergic effect of HAR is related to inflammation, IL-6 and TNF- α protein levels in mouse serum were detected by ELISA 30 min after intravenous injection of HAR. As shown in Figure 3, IL-6 was less than 1.5 pg/mL when saline was administrated, while the TNF- α was 150 pg/mL; the levels of these factors were sustained at high level following HAR treatment in each group (Figure 3). Thus, HAR

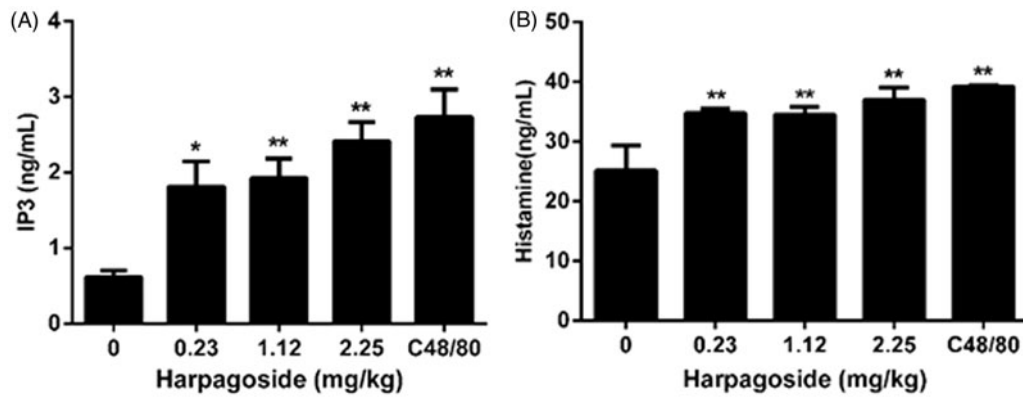


Figure 4. IP3 and histamine level in mouse serum. A C57 BL/6 mice ($n = 6$) were intravenously injected into the tail with different doses of HAR, saline, or C48/80. After 30 min, IP3 level was detected. B: Changes in histamine levels in mouse blood serum ($n = 6$), * $p < .05$, ** $p < .01$ vs. saline (0 in the figures).

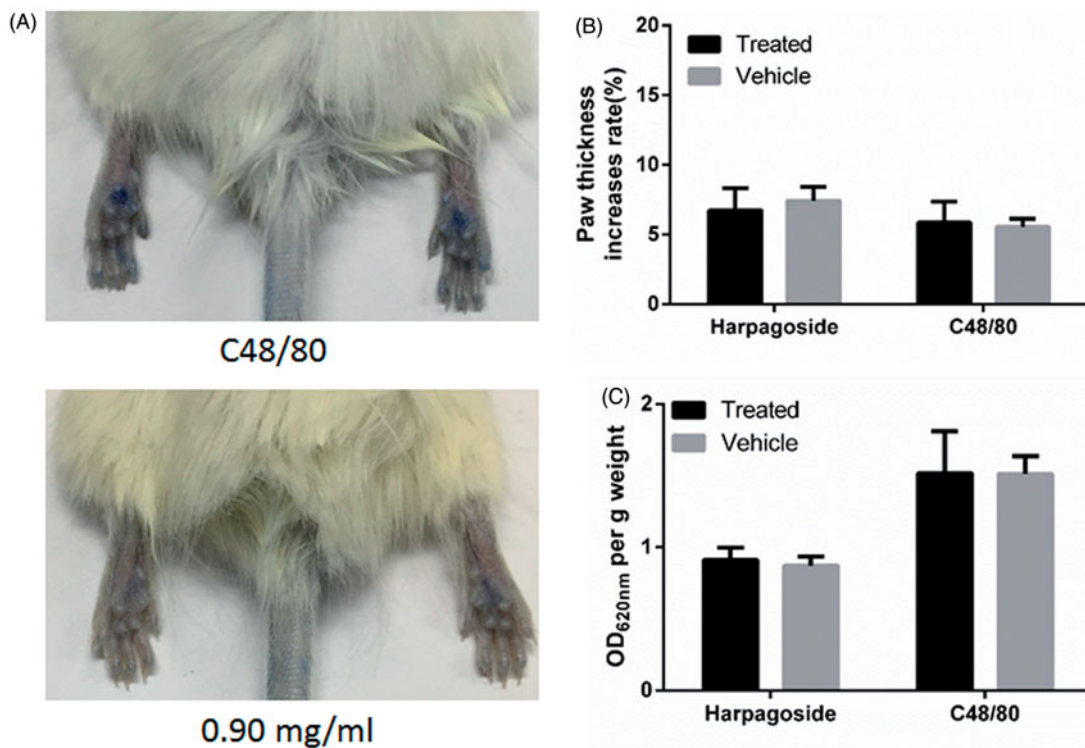


Figure 5. Representative images of Evans Blue stained extravasation 15 min after intraplantar injection of 5 μ L of HAR (left paw), C48/80 (left paw), or saline (right paw). A: 0.90 mg/mL of HAR and 30 μ g/mL of C48/80 caused local anaphylaxis in mice. B: Quantification of paw thickness increase in each group, with all animals compared to the saline paw thickness, respectively. C: Quantification of Evans Blue leakage into the paw after 15 min ($n = 6$ in each group). * $P < .05$, ** $P < .01$ vs. saline.

induced an allergy reaction accompanied by pre-inflammation factor release in mice.

HAR increased allergic factor release in mouse blood serum

To determine whether HAR induced allergic factor release in mice, changes in the levels of IP3 and histamine in the serum were detected after HAR administration. Figure 4 shows that HAR clearly induced both IP3 and histamine release after HAR administration in a dose-dependent manner.

Effect of HAR on STOCK Kit^{W-sh}/HNihrJaeBsmJNju mice

STOCK Kit^{W-sh}/HNihrJaeBsmJNju mice are gene knockout mice lacking mast cell function. These mice were treated in the same manner as wild-type C57 BL/6 mice. The results showed that neither C48/80 nor HAR induced local anaphylaxis (Figure 5). Both the degree of swelling (Figure 5(B)) and Evans Blue dye (Figure 5(C)) exudation were similar as in the negative control. Because the functions of mast cells in these mice were deficient, HAR leads to a pseudo-allergic reaction, possibly via mast cells.

Discussion

The goal of this study was to test whether HAR induces anaphylaxis in mice. Our data showed that HAR directly caused mice local anaphylaxis. HAR induced paw swelling and vascular permeability, resulting in exudation of histamine, IL-6 and other molecules and induced an allergic and inflammation reaction. *In vitro* assays indicated that HAR induced RBL-2H3 cell degranulation. Thus, HAR induced anaphylaxis and RBL-2H3 cell activation and is a potential allergenic compound in mice.

Anaphylaxis is a serious allergic reaction that can have a rapid onset and occasionally be fatal. The prevalence of allergic disease is increasing, as is the frequency of allergic reactions, ranging from mild to fatal anaphylaxis. Anaphylaxis is an evolving area of research that has shown great advances in recent years¹¹. Central to anaphylaxis is the production of IgE and its interaction with receptors (mainly FcεRI), predominantly on mast cells and to a lesser extent on basophils. Subsequent exposure to allergen allows IgE to cross-linking which activates FcεRI on the surface membranes of mast cells and basophils, resulting in cell activation and ultimately mediator release¹². This is known as the 'classical pathway' of anaphylaxis¹³. Another recently reported pathway involves mast cell activation of antibody-independent reactions by a range of cationic substances, collectively known as basic secretagogues, including inflammatory peptides and drugs associated with allergic-type reactions^{12,14,15}.

Our data showed that HAR induced RBL-2H3 cell degranulation and anaphylaxis in mice; in both *in vitro* and *in vivo* assays, allergic symptoms appeared immediately after HAR administration, indicating that the allergic reaction was directly activated in an antibody-independent manner. The pathway of this activation requires further analysis.

HAR induced local anaphylaxis in wild-type C57 BL/6 mice, but not in STOCK Kit^{W-sh}/HNIhrJaeBsmJNju mice. Thus, HAR caused anaphylactoid reactions via mast cells.

Interestingly, after intraplantar injection of both C48/80 and HAR into the left paw of C57 BL/6 mice, serious local anaphylactoid reactions were observed; with increasing drugs concentrations, the right paws also showed a certain degree of swelling and Evans Blue dye exudation. This was also reported in previous studies examining antimicrobial-induced drug anaphylactoid reactions¹⁶. However, the causes of these reactions are unclear and require further analysis.

In summary, our results demonstrate that HAR induced allergy both *in vitro* and *in vivo*, providing a reference for the clinical safety of radix scrophulariae injection and its main constituent HAR.

Disclosure statement

The authors have no conflict of interest to declare.

Funding

This work was supported by the National Natural Science Foundation of China [Grant Number: 81503321], China Postdoctoral Science Foundation [Grant Number: 2016M592808] and Natural Science Foundation of Shaanxi Province [Grant Number: 2016JQ8025].

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