

ORIGINAL ARTICLE

Other

Non-IgE-mediated hypersensitivity induced by multivitamins containing Tween-80

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Abstract

Multivitamins have been widely used for years. Adverse reactions, especially hypersensitivity, to multivitamins are becoming noteworthy. However, the classification of hypersensitivity is confusing, and the trigger is unknown. Multivitamins consist of two vials labelled vial 1 containing Tween-80 and vial 2. Multivitamins without Tween-80 were used as a contrast. Behaviouristics, histamine, IgE, and blood pressure of beagle dogs and guinea-pigs were investigated by observation, ELISA and sphygmomanometer, and degranulation and apoptotic of RBL-2H3 cells were assayed by spectrophotometry and flow cytometry. The results showed that dogs suffered from multiorgan anaphylactoid symptoms, and dramatically decreased blood pressure, and high plasma concentrations of histamine after the first administration of multivitamins and multivitamins vial 1, which contains Tween-80, compared to the control, multivitamins vial 2 or multivitamins without Tween-80. In anaphylaxis assay, guinea-pigs did not display any anaphylaxis symptoms and there were no changes in plasma histamine and IgE concentrations in the multivitamins and multivitamins vial 1 groups or in the multivitamins vial 2 and multivitamins without Tween-80 groups except ovalbumin. Compared to the control, the release of β -hexosaminidase and histamine, and the apoptosis of non-antigen-sensitized RBL-2H3 cells significantly increased in the Tween-80 and multivitamins and multivitamins vial 1 groups in a concentration-dependent manner. However, there was no alteration in multivitamins vial 2 and multivitamins without Tween-80 groups. The results indicate that the hypersensitivity induced by multivitamins may be anaphylactoid reaction, but not anaphylaxis. Multivitamin-induced release of inflammatory factors is triggered by Tween-80 through a non-IgE-mediated pathway.

KEYWORDS

anaphylactoid reaction, anaphylaxis, hypersensitivity reaction, multivitamin, Tween-80

1 | INTRODUCTION

Multivitamins contribute to the intake of essential vitamins to maintain the body's resistance and repair functions. Multivitamins are the most commonly used supplement and the use of multivitamins

is increasing.¹ Meanwhile, adverse drug reaction cases from multivitamins, especially severe and fatal hypersensitivity, are increasing and becoming noteworthy. Hypersensitivity induced by multivitamins is mainly involved in the intravenous administration route.²⁻⁵ Multivitamins are indicated as a daily oral vitamin maintenance supplement and as the intravenous administration for situations, such as extensive burns, surgery, severe infection and so on, with substantial

Mi and Yan contributed equally to this work.

changes in the body's metabolic demands and consequent nutrient consumption.²⁻⁵ Hypersensitivity induced by multivitamins is severe and fatal, and require our serious attention.

Hypersensitivity comprises up to approximately a third of adverse drug reactions.⁶ Depending on the activation mechanism of the immune system, drug hypersensitivity is classified as anaphylactoid reactions (formerly called "pseudoallergic" or "non-allergic hypersensitivity," which have a direct effect on the activation of mast cells), type I (anaphylaxis, which is an IgE-mediated activation of mast cells), type II, type III, and type IV reactions.^{7,8} Seventy-five percent of drug hypersensitivity are anaphylactoid reactions and anaphylaxis.⁷ In both IgE-mediated anaphylaxis and non-IgE-mediated anaphylactoid reactions, basophils and mast cells instantly release anaphylactic mediators such as histamine through different targets.⁹⁻¹¹ Hence, the symptoms of anaphylactoid reactions are similar to anaphylaxis, which leads to confusion in clinical reports. The adverse drug reactions induced by multivitamins are one example of this confusion in clinical reports. Although most of these reports have been described as anaphylaxis in clinical settings, the number of these adverse drug reactions involving IgE is unclear.^{2-4,12} Distinguishing the type of multivitamin-induced hypersensitivity has been unsuccessful.

Paradoxically, vitamins are required in the body to maintain the body's normal resistance, but they can cause anaphylaxis or anaphylactoid reactions. The products involved in multivitamins-induced hypersensitivity contain Tween-80, which is commonly used as a solubilizer in liposoluble drugs. In a sterile preparation of multivitamins, two single-dose vials were labelled vial 1 and vial 2. Vial 1 contained vitamin A, C, D3, B1, B2, B6, E, K₁, in which Tween-80 was used to dissolve fat-soluble vitamins A, D, E and K₁. Vial 2 contained folic acid, biotin, vitamin B12 and propylene glycol. Growing evidence suggests that Tween-80 can induce anaphylactoid reactions.¹³⁻¹⁵ Hence, we speculated that multivitamin-induced adverse drug reactions are triggered by Tween-80, and these are anaphylactoid

reactions, not anaphylaxis. Avoiding adverse drug reactions is thus mandatory from both an economic and an ethical viewpoint. The objective of the present study was to clearly distinguish the types of hypersensitivity induced by multivitamins and to determine the trigger, which will be of great significance in preventing and reducing adverse drug reactions from multivitamins.

2 | RESULTS

2.1 | Behaviouristics in the anaphylactoid reaction test

The behaviours of the beagle dogs were observed in the anaphylactoid reaction test. In the low dose of multivitamins group, two dogs suffered grade II symptoms, including scratching nose, head and ear and several skin rubeosis; one dog suffered grade III symptoms, including ear scratching and mania swinging, while the others had no abnormal behaviours in the anaphylactoid reaction test. The average grades and scores of the anaphylactoid reactions were higher than those in the control group ($P < 0.05$ vs control; Table 1). In the middle dose of the multivitamins group, one dog suffered from grade I symptoms; one dog suffered from grade III symptoms, and four dogs suffered from grade IV symptoms. The average grades and scores of anaphylactoid reactions were significantly higher than those in the control group ($P < 0.01$ vs control) (Table 1). Both in the high-dose multivitamins and vial 1 groups, all dogs suffered grade IV symptoms of tumbling, wheezing, hypodynamia, somnolence or mind sluggishness, and pawing the ground, and the average scores of the anaphylactoid reactions increased compared to the control group ($P < 0.01$ vs control). With the treatment of Tween-80, dogs displayed the same grade IV symptoms as in multivitamins group, including nose, head and ear scratching, several skin rubeosis, vomiting, skin rash, pawing the ground, tumbling, gait disturbance, hypodynamia, wheezing, and somnolence or mind sluggishness, and

TABLE 1 Grades and scores of behaviour assessment with the administration of multivitamins in beagle dogs

Groups	Doses (mL/kg)	No. of dogs					Scores	Grades
		0	I	II	III	IV		
Control	-	6	0	0	0	0	0.0 ± 0.0	0.0 ± 0.0
Multivit	0.01	3	0	2	1	0	2.5 ± 1.3 ^a	1.2 ± 0.5 ^a
Multivit	0.04	0	1	0	1	4	12.2 ± 3.4 ^b	3.3 ± 0.5 ^b
Multivit	0.16	0	0	0	0	6	24.8 ± 2.5 ^b	4.0 ± 0.0 ^b
Multivit vial 1	0.16	0	0	0	0	6	19.0 ± 2.3 ^b	4.0 ± 0.0 ^b
Multivit vial 2	0.16	6	0	0	0	0	0.0 ± 0.0	0.0 ± 0.0
Tween-80	0.16	0	0	0	0	6	24.3 ± 3.1 ^b	4.0 ± 0.0 ^b
Multivit w/o 80	0.16	6	0	0	0	0	0.0 ± 0.0	0.0 ± 0.0

Beagle dogs were intravenously administered to drugs in the anaphylactoid reaction tests.

Behaviours were observed for 30 min.

The values are expressed as the mean ± SEM, n = 6.

Multivit: multivitamins; Multivit w/o 80: multivitamins without Tween-80.

^a $P < 0.05$.

^b $P < 0.01$ vs control.

the anaphylactoid score was significantly increased compared to the control group ($P < 0.01$ vs control) (Table 1). However, the dogs in the control group, the multivitamins vial 2 group, and the multivitamins without Tween-80 group did not display any abnormal symptoms (Table 1 and Figure 1).

2.2 | Changes in blood pressure in anaphylactoid reactions

Multivitamins, multivitamins vial 1 and Tween-80 notably lowered the blood pressure in beagle dogs (Figure 2). With the administration of multivitamins, the systolic blood pressure of dogs declined sharply in 30 min ($P < 0.01$ vs before administration, Figure 2A), and the diastolic blood pressure also decreased ($P < 0.01$, Figure 2B). Multivitamins vial 1 and vial 2 were, respectively, administered to dogs. The blood pressure of dogs treated with multivitamins vial 1 declined significantly ($P < 0.01$ vs before administration), while the blood pressure of dogs treated with multivitamins vial 2 did not show a notable change. The blood pressure of dogs administered with Tween-80 had the same decreasing tendency of blood pressure as the dogs in the multivitamins vial 1 group ($P < 0.01$ vs before administration). However, there were no significant changes in blood pressure after the administration of the multivitamins without Tween-80 compared to before administration (Figure 2).

2.3 | Plasma histamine concentrations in anaphylactoid reactions

The concentrations of plasma histamine-fluorescence intensity curve and regression equation were established as follows: $y = 386.9x + 76.405$, $R^2 = 0.9967$. Table 2 shows that the concentrations of plasma histamine in the high-dose multivitamins group significantly increased after administration when compared to before administration ($P < 0.05$). Similarly, the plasma histamine concentrations significantly increased after the administration in the multivitamins vial 1 group and the Tween-80 group ($P < 0.05$ vs before

administration). However, the concentrations of plasma histamine did not significantly change both in the multivitamins vial 2 group and the multivitamins without Tween-80 group, when compared to before administration.

2.4 | Plasma IgE concentrations

The standard curve and regression equation for the plasma IgE concentration were established as follows: $y = 0.0084x - 0.0366$, $R^2 = 0.9983$. The plasma IgE concentrations showed no significant changes in dogs among the control, multivitamins, multivitamins vial 1, multivitamins vial 2, Tween-80 and multivitamins without Tween-80 groups (Table 3).

2.5 | Behavioristics in anaphylaxis

Guinea-pigs were sensitized and then challenged with drugs to assess anaphylaxis. In the ovalbumin group, the guinea-pigs displayed grade III strongly positive anaphylaxis symptoms, including alysmus, piloerection, trembling, scratching nose, sneezing, sough, ecphy-sis, defecation, dyspnea, wheezing rale, instability of gait, pant, convulsion, or Cheyne-Stokes sign breathing ($P < 0.01$ vs control; Table 4). The guinea-pigs occasionally displayed alysmus, trembling or scratching nose among the control, multivitamins, multivitamins vial 1, multivitamins vial 2, Tween-80 and multivitamins without Tween-80 groups. These behaviours were believed to be ways of reacting to emotional tension. Additionally, there were no differences in grades and scores for anaphylaxis among the control, multivitamins, multivitamins vial 1, multivitamins vial 2, Tween-80 and multivitamins without Tween-80 groups (Table 4).

2.6 | Plasma IgE concentrations in anaphylaxis

A standard curve of the concentration of plasma IgE vs the absorbance was established as follows: $y = 0.0912x - 0.0078$, $R^2 = 0.996$. Compared to the control group, the concentration of plasma IgE in



FIGURE 1 The symptoms of the beagle dogs after the first administration of multivitamins. The photographs were taken within 30 min after the administration, and they present a representative from six dogs. Multivit: multivitamins; Multivit w/o 80: multivitamins without Tween-80

the guinea-pigs significantly increased after challenge with ovalbumin ($P < 0.05$, Figure 3A). However, there were no significant differences in the concentrations of plasma IgE among the control,

multivitamins, multivitamins vial 1, multivitamins vial 2 and multivitamins without Tween-80 groups.

2.7 | Plasma histamine concentrations in anaphylaxis

A standard curve of the concentration of plasma histamine was established as follows: $y = 0.0008x + 0.0597$, $R^2 = 0.9995$. In the ovalbumin group, the concentration of plasma histamine was higher than that in the control group in guinea-pigs ($P < 0.05$, Figure 3B). There were no significant changes in the concentrations of plasma histamine among the control, multivitamins, multivitamins vial 1, multivitamins vial 2 and multivitamins without Tween-80 groups.

2.8 | Degranulation of RBL-2H3 cells

Figure 4 shows that the degranulation of RBL-2H3 cells activated by multivitamins. 1.4% Tween-80 induced a clear increase in the releases of β -hexosaminidase and histamine when compared to the control ($P < 0.05$ or $P < 0.01$), and its effect was concentration-dependent (Figure 4A,B,G). Multivitamins and multivitamins vial 1 containing Tween-80 also induced the release of β -hexosaminidase of RBL-2H3 cells and had a same increase tendency as the β -hexosaminidase release induced by Tween-80 (Figure 4C,D). The degranulation of RBL-2H3 cells could be completed in 15 minutes. In contrast, there was no significant difference in the release of β -hexosaminidase among in the multivitamins vial 2 and the multivitamins without Tween-80 and the control groups (Figure 4E,F).

2.9 | Apoptosis of RBL-2H3 cells

Cells appeared green because of the binding of Annexin V-FITC during the early period of apoptosis. Cells appeared red, which was attributed to propidium iodide passing through the cytomembrane and embedding in the DNA during the middle and late periods of apoptosis. While normal cells were colorless. As Figure 5 shows, cells were yellow when stimulated by multivitamins and vial 1 and Tween-80

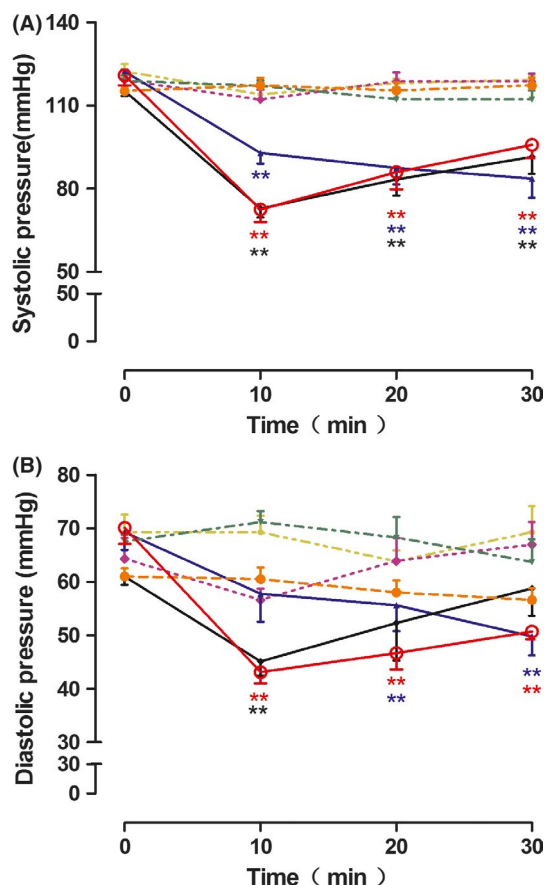


FIGURE 2 Effect of multivitamins on blood pressure in beagle dogs. The values of blood pressure are shown as the mean $\pm$ SEM, $n = 6$. $**P < 0.01$ vs before administration. Multivit: multivitamins; Multivit w/o 80: multivitamins without Tween-80. (A: systolic pressure; B: diastolic pressure) (Yellow dotted line), 0.01 mL/kg Multivit; (Purple dotted line), 0.04 mL/kg Multivit; (Blue solid line), 0.16 mL/kg Multivit; (Green dotted line), Multivit w/o 80; (Black solid line), Multivit vial 1; (Orange dotted line) Multivit vial 2; (Red solid line), Tween-80

TABLE 2 The changes in the concentrations of plasma histamine with the treatment of multivitamins in beagle dogs

Groups	Doses (mL/kg)	Concentrations of histamine ($\mu\text{g/L}$)	
		Before administration	After administration
Control	-	3.59 ± 0.50	4.11 ± 0.70
Multivit	0.16	3.60 ± 0.50	5.77 ± 0.86^a
Multivit vial 1	0.16	3.17 ± 0.47	6.26 ± 1.03^a
Multivit vial 2	0.16	5.70 ± 0.93	5.76 ± 1.01
Tween-80	0.16	4.19 ± 0.75	7.52 ± 0.73^a
Multivit w/o 80	0.16	4.45 ± 0.86	5.63 ± 0.79

The results are shown as mean $\pm$ SEM, $n = 6$.

Multivit: multivitamins; Multivit w/o 80: multivitamins without Tween-80.

^a $P < 0.05$ vs before administration.

when compared to the control. The results indicated that both multivitamins and multivitamins vial 1 which contains Tween-80 obviously induce the apoptosis of RBL-2H3 cells. Conversely, RBL-2H3 cells displayed only a spot of yellow with the treatment of multivitamins vial 2 and multivitamins without Tween-80. There was no significant increase in the apoptosis of RBL-2H3 cells when compared to the control.

The concentration-response relationships between the treatment of multivitamins and apoptosis were assayed by flow cytometry. Figure 6 showed that the rates of apoptotic cells treated with 1.4% Tween-80 and multivitamins vial 1 containing 1.4% Tween-80 clearly increased ($P < 0.05$ or $P < 0.01$) compared to that in the control group. Nevertheless, there were no significant differences in the rates of apoptotic cells both in the multivitamins vial 2 and multivitamins without Tween-80 groups compared to the control group (Figure 6). The results indicated that Tween-80 and multivitamins vial 1, which contains Tween-80, could induce apoptosis on RBL-2H3 cells in a concentration-dependent manner.

3 | DISCUSSION

Adverse drug reactions are a crucial cause of the increase in morbidity and mortality. Adverse drug reactions occur in approximately 17% of patients.¹⁶ Avoiding adverse reactions is thus mandatory from both economic and ethical points of view.⁷ Hence, it is very important to clearly distinguish the types of adverse reactions of multivitamins and to ascertain the trigger to determine solutions to prevent and reduce adverse drug reactions. In this study, we discriminated a non-IgE-mediated anaphylactoid reactions induced by multivitamins, and the trigger was its solubilizer, Tween-80.

Drug-induced anaphylactoid reactions and anaphylaxis are related to drug delivery route, drug identity, metabolism, hereditary capacity, immune status, and other factors. It is difficult to completely simulate these responses in vitro. Hence, selecting a suitable animal model is important. Dogs are more similar to humans in the

TABLE 3 The change in plasma IgE concentrations with the treatment of multivitamins in beagle dogs

Groups	Doses (mL/kg)	Concentrations of IgE ($\mu\text{g/L}$)
Control	-	63.3 ± 2.9
Multivit	0.16	70.9 ± 5.3
Multivit vial 1	0.16	68.9 ± 5.0
Multivit vial 2	0.16	72.1 ± 3.4
Tween-80	0.16	71.1 ± 4.4
Multivit w/o 80	0.16	63.4 ± 4.2

Plasma samples were harvested in a tube containing heparin at 10 min after drug administration.

The plasma IgE concentrations were quantified by ELISA. The values are shown as the mean $\pm$ SE, $n = 6$.

experience of symptoms,¹⁷ and the beagle dog is more sensitive to anaphylactoid reactions than others, such as guinea-pig and mouse.¹⁸

The present study showed that beagle dogs suffered multiorgan anaphylactoid symptoms, the concentrations of plasma histamine were elevated, and the blood pressure greatly declined with the first administration of multivitamins and multivitamins vial 1 containing Tween-80. However, the above levels were not significantly changed in beagle dogs treated with multivitamins vial 2 and multivitamins without Tween-80. Undoubtedly, the results demonstrated that multivitamins induce an anaphylactoid reaction and that the trigger is Tween-80.

On the other hand, guinea-pig is a classical anaphylaxis animal model that is used for the safety assessment of drugs.¹⁹ In the active systemic anaphylaxis assay, guinea-pigs did not suffer anaphylaxis symptoms, and there were no increases in plasma histamine and IgE concentrations in the multivitamins and vial 1 containing Tween-80 groups or in the multivitamins vial 2 and multivitamins without Tween-80 groups. The results indicated that the hypersensitivity induced by multivitamins may not be anaphylaxis.

We further studied the underlying mechanisms by which multivitamins induced anaphylactoid reactions. RBL-2H3 cells have

Groups	No. of guinea-pigs in different grades					Scores	Grades
	0	I	II	III	IV		
Control	1	5	0	0	0	2.2 ± 0.7	0.8 ± 0.2
Ovalbumin	0	0	0	6	0	62.0 ± 8.0^a	3.0 ± 0.0^a
Multivit	2	4	0	0	0	3.0 ± 0.8	0.7 ± 0.2
Multivit vial 1	1	5	0	0	0	3.2 ± 1.0	0.8 ± 0.2
Multivit vial 2	1	5	0	0	0	2.2 ± 0.9	0.8 ± 0.2
Multivit w/o 80	1	5	0	0	0	3.5 ± 0.8	0.8 ± 0.2

TABLE 4 Grades and scores for anaphylaxis with the challenge of multivitamins in guinea-pigs

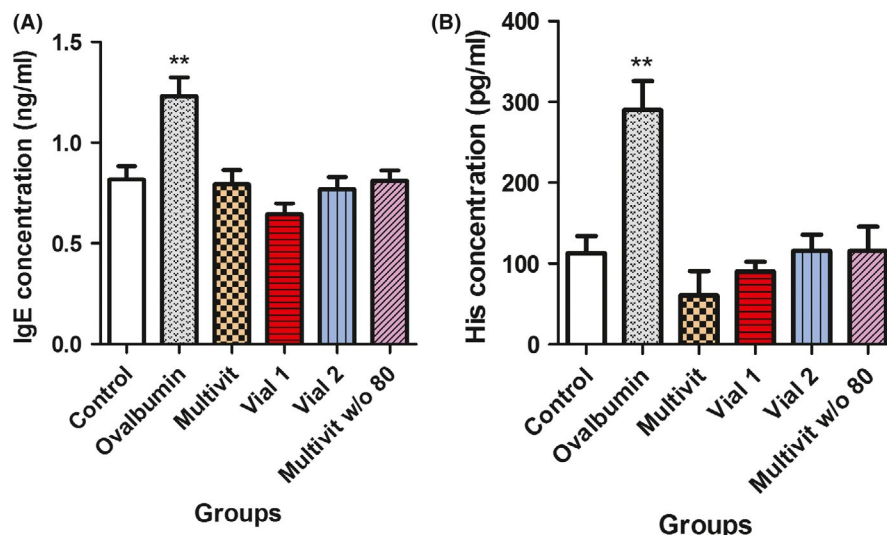
In the active systemic anaphylaxis assay, behaviours of the guinea-pigs were observed for 30 min after the challenge.

The values are expressed as the mean $\pm$ SEM, $n = 6$.

Multivit: multivitamins; Multivit w/o 80: multivitamins without Tween-80.

^a $P < 0.01$ vs control.

FIGURE 3 Changes in the concentrations of plasma IgE (A) and histamine (B) in guinea-pigs challenged with multivitamins. The concentrations are expressed as the mean $\pm$ SEM, $n = 6$. ** $P < 0.01$ vs control. Multivit: multivitamins; Multivit w/o 80: multivitamins without Tween-80



been widely used to study signalling pathways of the activation of mast cells which are involved in non-IgE- or IgE-induced degranulation.⁹⁻¹¹ In the present study, the treatment of multivitamins and multivitamins vial 1 containing Tween-80 directly increased the degranulation of the non-antigen-sensitized RBL-2H3 cells. The results indicated that multivitamins-induced anaphylactoid reactions were caused by the release of inflammatory factors, which cause pruritus, acute inflammation, vasodilatation and hypotension through a non-IgE-mediated pathway. Simultaneously, the increase in apoptosis demonstrated that the release of inflammatory factors in RBL-2H3 cells induced by multivitamins and multivitamins vial 1 containing Tween-80 resulted from an increase in cell membrane permeability. Multivitamins vial 2 and multivitamins without Tween-80 groups did not induce the degranulation and apoptosis of RBL-2H3 cells, indicating that the degranulation was not triggered by the vitamins. Nevertheless, Tween-80 caused serious degranulation and apoptosis of RBL-2H3 cells depending on the concentration, suggesting that the degranulation of RBL-2H3 cells induced by multivitamins may be due to Tween-80. It has been reported that Tween-80 can activate the complement system through classical and alternative pathways both in vivo and in vitro.^{15,20} However, the degranulation of RBL-2H3 cells was directly activated by Tween-80, which implies that another pathway exists for the activation of mast cells. Recent studies have shown that the MAS-related G-protein-coupled receptor-X2 is a newly found target of anaphylactoid reactions that can activate mast cells triggered by many small molecule drugs.^{11,21} Whether MAS-related G-protein-coupled receptor-X2 is the targets of Tween-80 that activates mast cells is still unknown and will be a starting point for further studies.

In conclusion, the adverse drug reactions induced by multivitamins are anaphylactoid reactions which are due to the degranulation of mast cells or basophilic granulocytes by the release of inflammatory factors triggered by Tween-80 through a non-IgE-mediated pathway.

4 | METHODS

4.1 | Materials

Reagents were obtained as follows: multivitamins (multivit) for infusion (Sandoz Canada Inc., Boucherville, Canada) consists of two single-dose vials labelled vial 1 and vial 2. Multivitamins vial 1 contains 3300 IU vitamin A, 200 mg vitamin C, 200 IU vitamin D3, 6 mg vitamin B1, 3.6 mg vitamin B2, 6 mg vitamin B6, 10 IU vitamin E, and 150 μ g vitamin K₁, in which 1.4% Tween-80 was used to dissolve fat-soluble vitamins A, D, E and K₁. Multivitamins vial 2 contains 600 μ g folic acid, 60 μ g biotin, 5 μ g vitamin B12 and 30% propylene glycol. Multivitamins without Tween-80 (multivit w/o 80, Libang Zhaoxin Biotechnology Co., Ltd, Xi'an, China) contain the same amount of vitamin A, C, D3, B1, B2, B6, E, K, B12, folic acid, biotin as multivitamins but without Tween-80 and propylene glycol. Firstly, multivitamins without Tween-80 was dissolved in 5 mL physiological saline and formed microemulsion. This microemulsion was diluted with physiological saline to different concentrations according to the requirements. Tween-80 (Sigma-Aldrich, Dorset, UK), p-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma-Aldrich), o-phthalaldehyde (Sigma-Aldrich), guinea-pig IgE ELISA kits (Xitang BioTECH, Shanghai, China), guinea-pig histamine ELISA kits (Xitang BioTECH), and FITC-Annexin V apoptosis detection kits (7seaBioTECH, Shanghai, China).

4.2 | Animals and treatments

Animals were handled in accordance with the criteria outlined in the Guide for Care and Use of Laboratory Animals. All experimental protocols were approved by the Animal Ethics Committee at Xi'an Jiaotong University according to the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Forty-eight healthy beagle dogs, half females and half males, weighing 9–13 kg, were utilized in this study and were purchased from Xi'an Dilepu Biology Resources (Shaanxi, China). Every beagle dog was

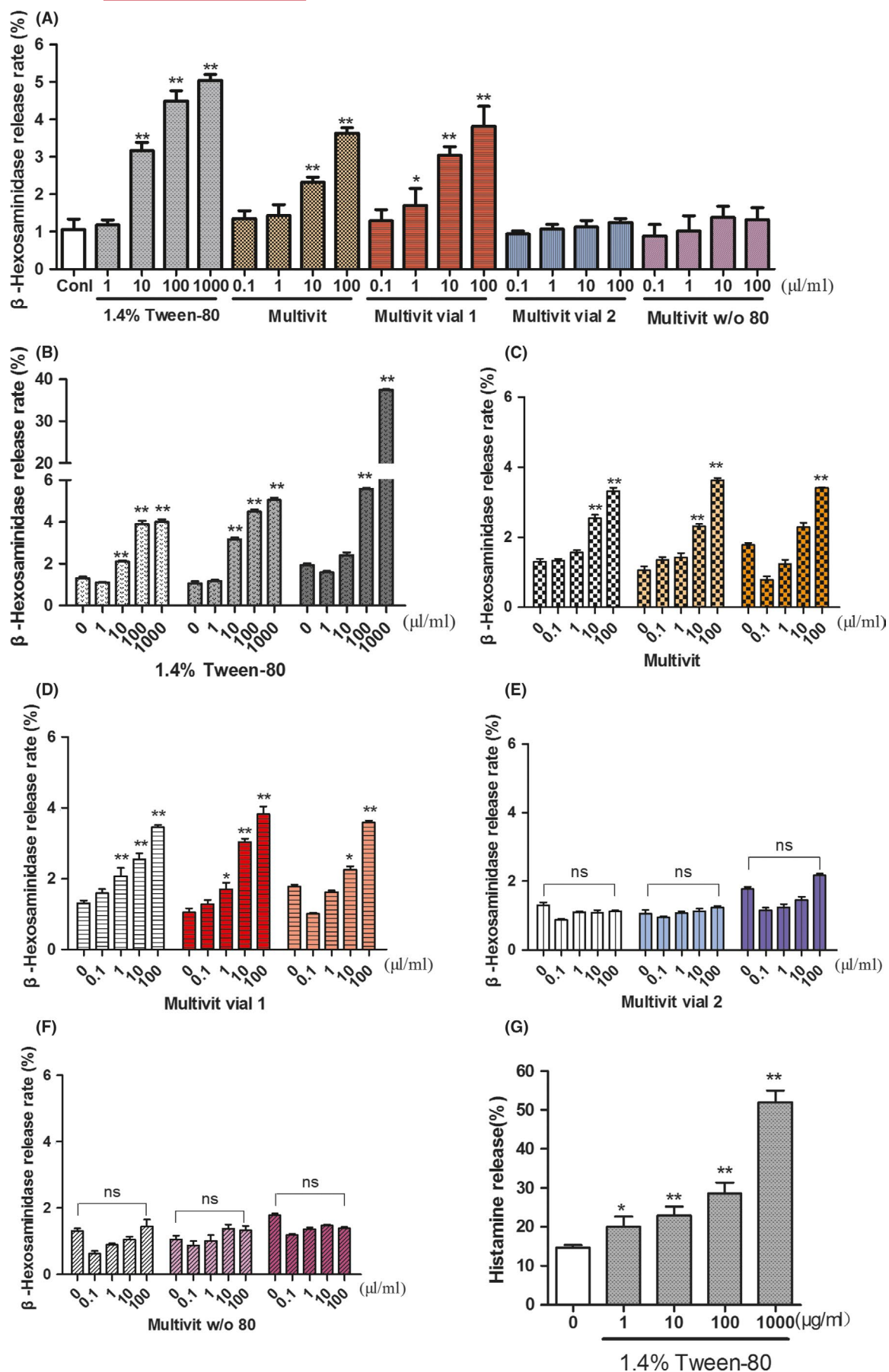
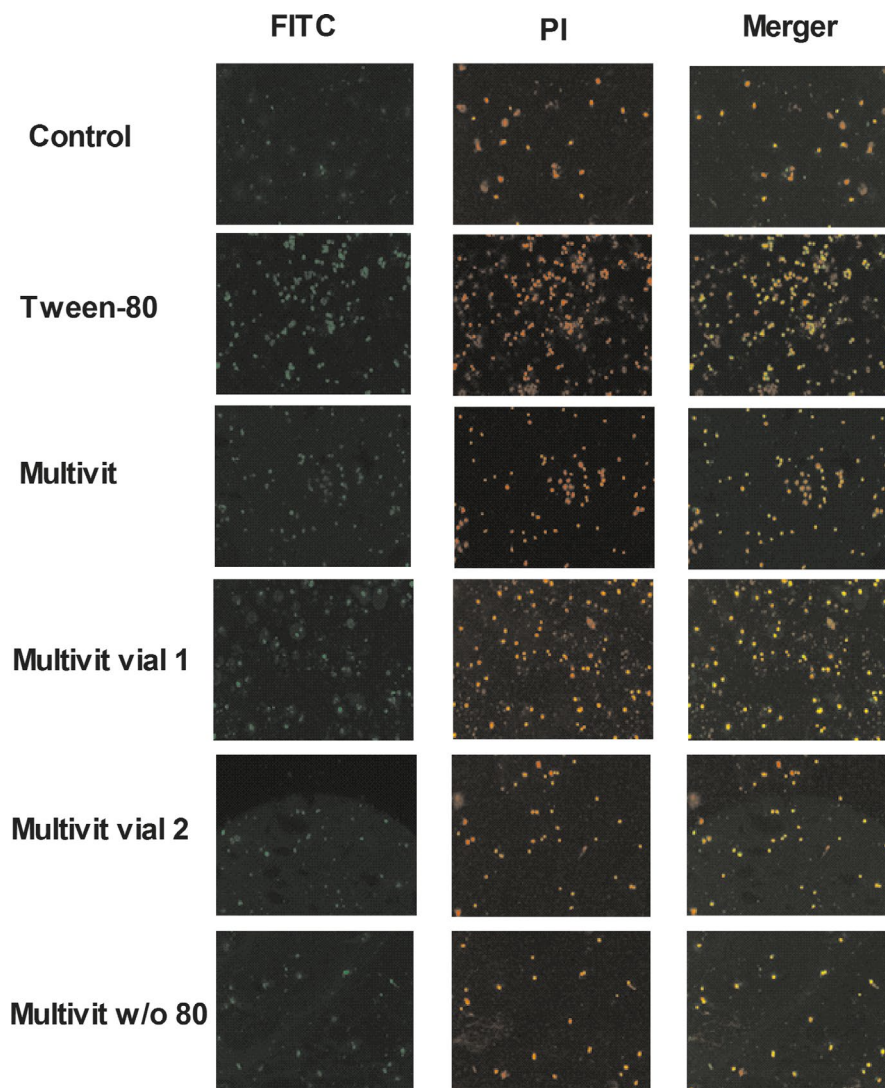


FIGURE 4 The degranulation of RBL-2H3 cells activated by multivitamins. A, The β -hexosaminidase release rate for 30 min; B, The β -hexosaminidase release rate of Tween-80 at different times. (White bar chart), 15 min; (Light gray bar chart), 30 min; (Dark gray bar chart), 60 min; C, The β -hexosaminidase release rate of multivitamins at different times. (White bar chart), 15 min; (Light yellow bar chart), 30 min; (Dark yellow bar chart), 60 min. D, The β -hexosaminidase release rate of multivitamins vial 1 at different times. (White bar chart), 15 min; (Red bar chart), 30 min; (Orange bar chart), 60 min. E, The β -hexosaminidase release rate of multivitamins vial 2 at different times. (White bar chart), 15 min; (Blue bar chart), 30 min; (Purple bar chart), 60 min. F, The β -hexosaminidase release rate of multivitamins without Tween-80 at different times. (White bar chart), 15 min; (Light red bar chart), 30 min; (Dark red bar chart), 60 min. G, The release of histamine in RBL-2H3 cells triggered by Tween-80 at different times. The rates of β -hexosaminidase or histamine release are expressed as the mean $\pm$ SEM, $n = 6$. * $P < 0.05$, ** $P < 0.01$ vs control (conl or 0). Multivit: multivitamins; Multivit w/o 80: multivitamins without Tween-80

FIGURE 5 Effect of multivitamins on the apoptosis of RBL-2H3 cells by fluorescence staining. RBL-2H3 cells were treated with 100 μ l/ml 1.4% Tween-80, multivitamins, multivitamins vial 1, multivitamins vial 2, and multivitamins without Tween-80 for 30 min, respectively. Green represents cells during the early period of apoptosis. Red represents cells during the middle to late period of apoptosis. The magnification is 400 $\times$. Multivit: multivitamins; Multivit w/o 80: multivitamins without Tween-80



kept in a separate cage. Thirty-six guinea-pigs weighing 260–340 g were purchased from the Animal Center of Xi'an Jiaotong University (Shaanxi, China). Three guinea-pigs were housed in one cage. Animals were allowed access to food and water ad libitum unless otherwise stated. The animals were raised in an environment with 20–25°C room temperature, 40%–70% humidity and a 12/12 h day-night cycle.

In order to calculate conveniently the dose of multivitamins expressed in a volume, a mixture of multivitamins was used. The dose of 0.16 mL/kg in beagle dogs is equivalent to the clinical dose for humans. Beagle dogs were randomized into eight groups,

including control group (normal saline), multivitamins 0.01 mL/kg group (0.01 mL/kg multivitamins vial 1 + 0.01 mL/kg multivitamins vial 2), multivitamins 0.04 mL/kg group (0.04 mL/kg multivitamins vial 1 + 0.04 mL/kg multivitamins vial 2), multivitamins 0.16 mL/kg group (0.16 mL/kg multivitamins vial 1 + 0.16 mL/kg multivitamins vial 2), multivitamins vial 1 group (0.16 mL/kg), multivitamins vial 2 group (0.16 mL/kg), 1.4% Tween-80 group (0.16 mL/kg), and multivitamins without Tween-80 group (0.16 mL/kg). Six animals were in each group. Drugs were diluted with physiological saline and then slowly intravenously injected to beagle dogs according to the dose

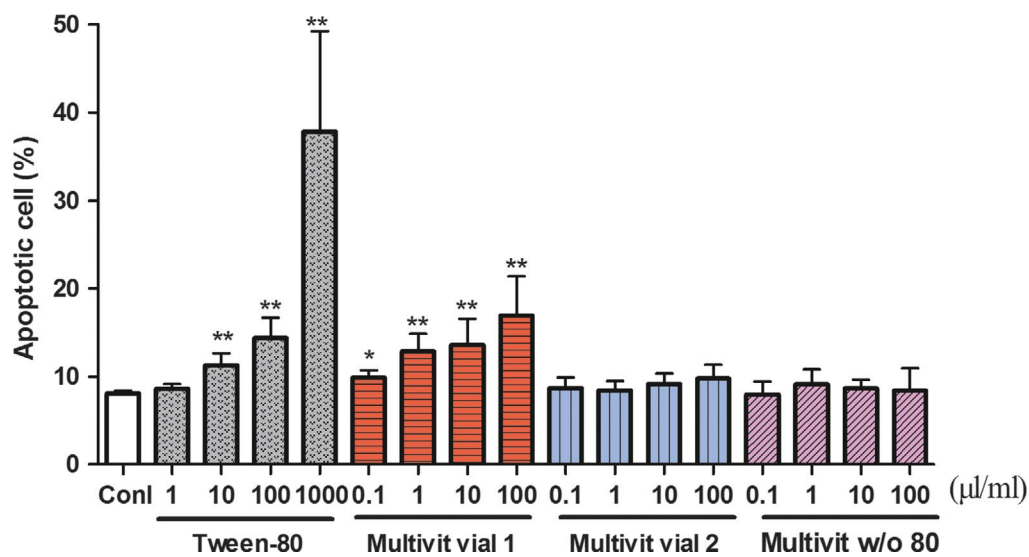
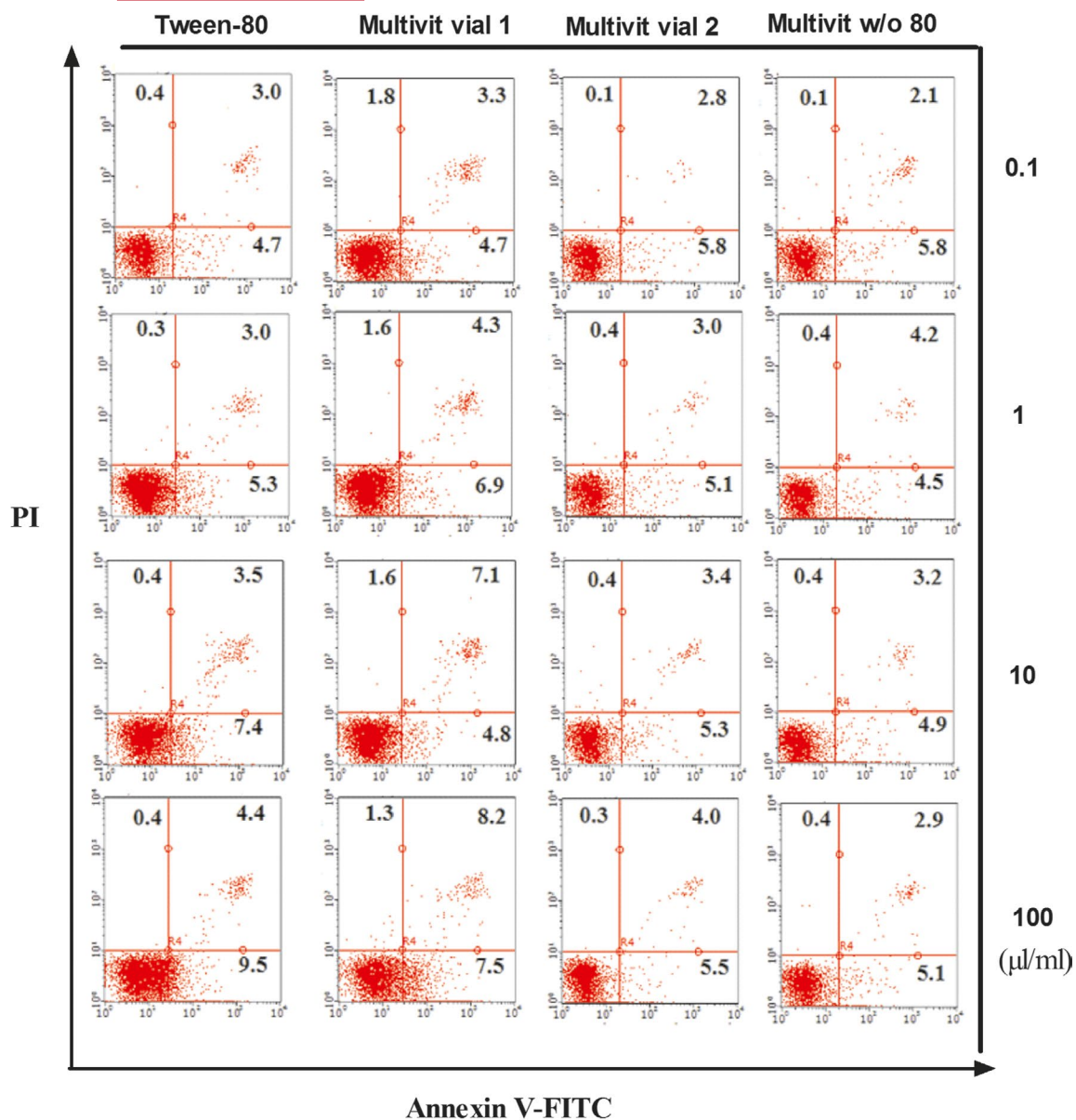


FIGURE 6 Effect of multivitamins on the apoptosis of RBL-2H3 cells by flow cytometry. The cells were treated with 1.4% Tween-80, multivitamins, multivitamins vial 1, multivitamins vial 2, and multivitamins without Tween-80 for 30 min, respectively. The apoptosis rates of RBL-2H3 cells are shown as the mean $\pm$ SEM, $n = 3-11$. * $P < 0.05$, ** $P < 0.01$ vs. control (conl). Multivit: multivitamins; Multivit w/o 80: multivitamins without Tween-80

volume of 1 mL/kg. Adrenaline injection and diphenhydramine injection were on standby to ensure the safety of the dogs when they suffered life-threatening reactions.

The guinea-pigs were randomized into six groups as follows: control group (normal saline), ovalbumin group (5 mg/kg), multivitamins group (0.5 mL/kg multivitamins vial 1 + 0.5 mL/kg multivitamins vial 2), multivitamins vial 1 group (0.5 mL/kg), multivitamins vial 2 group (0.5 mL/kg), and multivitamins without Tween-80 group (0.5 mL/kg); 6 guinea-pigs in each group. The dose of 0.5 mL/kg on guinea-pigs is equivalent to the clinical dose. Drugs were diluted with physiological saline and then intravenously injected to guinea-pigs according to the dose volume of 1 mL/kg. In the active systemic anaphylaxis assay, guinea-pigs received three times sensitization with the intravenous administrations of drugs every other day. Fourteen days after the sensitization, the guinea-pigs were intravenously challenged with a double dose of drugs. The behaviours of guinea-pigs were observed for 30 minutes.

4.3 | Cell culture

Rat basophilic leukemia-2H3 cells (RBL-2H3) cells (the Chinese Academy of Sciences Cell Bank, Shanghai, China) were cultured using Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum, 80 units/mL penicillin and 0.08 mg/mL streptomycin at 37°C in a 5% CO₂ atmosphere. RBL-2H3 cells (3.5×10^5 /mL, 0.5 mL/well) were seeded on 24-well flat-bottomed tissue culture plates and grown for 24 hours. Then, the cells were treated with a series of final concentrations of multivitamin (0.1, 1, 10, and 100 μ L/mL), multivitamins vial 1 (0.1, 1, 10, and 100 μ L/mL), multivitamins vial 2 (0.1, 1, 10, and 100 μ L/mL), 1.4% Tween-80 (1, 10, 100, and 1000 μ L/mL), multivitamins without Tween-80 (0.1, 1, 10, and 100 μ L/mL), 0.1% Triton, and phosphate buffer solution (PBS), and incubated for 15, 30, and 60 minutes, respectively.

4.4 | Behaviouristics

In the anaphylactoid reaction tests, the symptoms of beagle dogs were continuously observed for 30 minutes after the administrations. The levels of anaphylactoid reactions were assessed depending on an assessment standard with some modifications (Table S1).^{13,22} In the active systemic anaphylaxis assay, symptoms of the guinea-pigs were observed for 30 minutes after the challenge. The levels of anaphylaxis were assessed depending on an established assessment standard of anaphylaxis on guinea-pigs (Table S2).²³

4.5 | Blood pressure

Blood pressure was assayed by electronic blood pressure monitor (HEM-8611, OMRON (Dalian) Corporation, Dalian, China) at 0,

10, 20 and 30 minutes after the drug administration. Dogs without anaesthesia were fixed at the decubitus position. Dogs were required to wear the cuff of the electronic blood pressure monitor on the left arm. The assay was repeated three times at each time point.

4.6 | ELISA

Blood samples of guinea-pigs were harvested in tubes containing heparin before and 30 minutes after drug administration and then centrifuged for 10 minutes. The plasma samples were separated. Following the instructions of the ELISA kit, the concentrations of plasma IgE and histamine in guinea-pigs were assayed by a Multiskan Spectrum.

4.7 | Spectrophotometry

The release of β -hexosaminidase was assayed by a spectrophotometric method.¹¹ The supernatants (50 μ L) of RBL-2H3 cells stimulated with drugs were incubated with 0.1 mol/L sodium citrate containing 0.001 mol/L p-nitrophenyl-N-acetyl- β -D-glucosaminide (50 μ L, pH 4.5) at 37°C for 2 hours. Finally, the carbonate buffer (250 μ L, pH 10) consisted of 0.1 mol/L Na₂CO₃ and 0.1 mol/L NaHCO₃, which was used to terminate the reaction. The absorbance was measured at 405 nm. The absorbance of PBS was used as a blank, and the absorbance of Triton was taken as the total amount of β -hexosaminidase of RBL-2H3 cells. The β -hexosaminidase release rate of samples was expressed as a data that the absorbance of sample divide the absorbance of Triton.

4.8 | Fluorospectrophotometry

Histamine release of RBL-2H3 cells and the concentrations of plasma histamine in beagle dogs were measured by a fluorospectrophotometry assay. Plasma samples (300 μ L) were deproteinized with ethyl alcohol (700 μ L). After vortex mixing, the samples were centrifuged at 12 000 g. The samples or supernatant of RBL-2H3 cells (100 μ L) was added to 0.5 mol/L NaOH (40 μ L) and 2.5 g/L o-phthalaldehyde (20 μ L) and then incubated for 30 minutes. At the end of the incubation, 3 mol/L HCl (10 μ L) was added. The fluorescence intensity was assayed by fluorescent plate reader at 365 nm excitation wavelength and 465 nm emission wavelength. Standard curves were obtained by linear regression of the fluorescence intensity vs a series of concentrations of histamine standard substances to calculate the concentrations of plasma histamine in beagle dogs. The fluorescence intensity of PBS was used as a blank, and the fluorescence intensity of Triton represented total histamine release in RBL-2H3 cells. The histamine release rate of the sample was expressed as a data that the

fluorescence intensity of sample divide the fluorescence intensity of the Triton.

4.9 | Fluorescence microscopy observations

Rat basophilic leukemia-2H3 cells were incubated with drugs for 30 min and then washed three times with cold PBS. RBL-2H3 cells were resuspended in binding buffer containing Annexin V-FITC (5 μ L) and propidium iodide (5 μ L) and then incubated while being protected from light for 10 minutes. An inverted fluorescence microscope (Eastman Kodak, Rochester) was used to observe and photograph the apoptosis of RBL-2H3 cells at 400 $\times$ magnification.

4.10 | Flow cytometry

Rat basophilic leukemia-2H3 cells were treated with a series of drugs for 30 minutes and then washed three times with cold PBS. The cells were trypsinized and resuspended in binding buffer. Then Annexin V-FITC (5 μ L) and propidium iodide (5 μ L) were added, and cells were incubated away from light for 10 minutes. A flow cytometer (Millipore Co., Hayward) was used to analyze the apoptosis of RBL-2H3 cells.

4.11 | Statistical analysis

Data are expressed as the mean $\pm$ SEM. Data normality was analyzed by the Shapiro-Wilk test. Individual differences were determined by ANOVA with the least significant difference (LSD), and a P value <0.05 was considered statistically significant.

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DISCLOSURE

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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TABLE S1. An assessment norm for the anaphylactoid reactions in beagle dogs.

Grade	Reactions	Score
0	Normal	0
I	Nose, head, or ear scratching (within 3 min);sneezing; coughing; skin rubeosis	1
II	Nose, head, or ear scratching (greater than 3 min); several skin rubeosis; drooling	2
III	Skin rash; vomiting; diarrhea; mania; gait disturbance;	3
IV	Pawing the ground; tumbling; mind sluggishness; somnolence; hypodynamia; wheezing	4
V	Gatism; hematemesis; hemaecia; breathlessness	5
VI	Doom	6

Each reaction in the dogs received a score corresponding to that symptom.

TABLE S2. Criterion of active systemic anaphylaxis in guinea-pigs.

Score	Grade	Anaphylaxis intensity
0	0	Negative
1 - 4	I	Weakly positive
5 - 10	II	Positive
11 - 19	III	Strongly positive
20	V	Extremely positive

Anaphylactic score and symptoms as follows: 0 Normal, 1 Alysms, 2 Piloerection, 3 Trembling, 4 Scratching nose, 5 Sneezing, 6 Cough, 7 Ecphysexis, 8 Micturition, 9 Defecation, 10 Lacimation, 11 Dyspnea, 12 Wheezing rale, 13 Peliosis, 14 Instability of gait, 15 Leap, 16 Pant, 17 Convulsion, 18 Circumgyration, 19 Cheyne-Stokes sign, 20 Death.