The Severe Adverse Reaction to Vitamin K₁ Injection Is Anaphylactoid Reaction but Not Anaphylaxis

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Abstract

The severe adverse reaction to vitamin K₁ injection is always remarkable and is thought to result from anaphylaxis. Paradoxically, however, some patients administered vitamin K₁ injection for the first time have adverse reactions. Using beagle dogs, the present study tested the hypothesis that the response to vitamin K₁ is an anaphylactoid reaction. The results showed that serious anaphylaxis-like symptoms appeared in beagle dogs after the administration of vitamin K₁ injection for the first time. The plasma histamine concentration increased, and blood pressure decreased sharply. After sensitization, dogs were challenged with vitamin K₁ injection and displayed the same degree of symptoms as prior to sensitization. However, when the vitamin K₁ injection-sensitized dogs were challenged with a vitamin K₁-fat emulsion without solubilizers such as Tween-80, the abnormal reactions did not occur. Furthermore, there was no significant change in the plasma immunoglobulin E concentration after vitamin K₁ challenge. Following treatment with vitamin K₁ injection, the release of histamine and β-hexosaminidase by rat basophilic leukemia-2H3 cells as well as the rate of apoptosis increased. The Tween-80 group displayed results similar to those observed following vitamin K₁ injection in vivo. However, the dogs in the vitamin K₁-fat emulsion group did not display any abnormal behavior or significant change in plasma histamine. Additionally, degranulation and apoptosis did not occur in rat basophilic leukemia-2H3 cells. Our results indicate that the adverse reaction induced by vitamin K₁ injection is an anaphylactoid reaction, not anaphylaxis. Vitamin K₁ injection induces the release of inflammatory factors via a non-IgE-mediated immune pathway, for which the trigger may be the solubilizer.

Introduction

Vitamin K (VK) is an essential factor that is required for the post-translational modification of coagulation factors II, VII, IX and X, protein C and protein S (the natural inhibitors of coagulation). VK occurs naturally in two forms, VK₁ and VK₂. Since the synthesis of the first synthetically prepared fat-soluble VK₁ in 1953 [1], VK₁ injection has been the most commonly employed preparation of a coumarin antagonist and widely used in the treatment of hemorrhagic disease caused by VK₁ deficiency. Soon after its introduction, reports of severe adverse reactions began to appear, and the number of adverse reactions to VK₁ injection is always remarkable. The consistent symptoms include facial flushing, vague uneasy feelings of weakness, abdominal and low back pain, nausea, vomiting, dyspnea, and chest pain. In severe reactions, these symptoms are followed within minutes by cyanosis, loss of consciousness, and hypotension, with the potential for cardio-pulmonary arrest and death [2,3].

The adverse reaction to VK₁ injection is believed by many to be classified as anaphylaxis. In December 2011, the State Food and Drug Administration, China, and the National Center for Adverse Drug Reaction Monitoring, China, issued a notice raising concern regarding serious anaphylaxis resulting from VK₁ injection [4]. The database in the State Food and Drug Administration contained a total of 8146 cases of adverse reactions between January 2004 and May 2011. The adverse reactions were primarily associated with the intravenous administration of VK₁ (95.3%). The United States Pharmacopeia and the Mariandale Extra Pharmacopeia have reported that VK₁ can cause hypersensitization. The medical use instructions for VK₁ products from Merck, US, report that VK₁ can induce anaphylaxis. After reviewing the reaction description (CONSTART terms, “Coding symbols for a thesaurus of standard adverse reaction terms”) in the Food and Drug Administration SRSAR file, Louis found that the patients who experienced such a reaction were never identified as “anaphylactoid” [2].

Although most of these episodes have been described as anaphylaxis in studies of clinical cases [2,5–8], the number of these reactions that were IgE-mediated is unclear. Furthermore, Yang [9] collected 46 cases of allergic shock for vitamin K₁ injection, and 19 patients (42%) were administered vitamin K₁ injection for the first time. These results are contradictory to the mechanism of anaphylaxis. Some reports have classified these adverse reactions as anaphylactoid reactions [10]. Rieger-Johnson has reported one case of an adverse reaction to VK₁ and concluded that this patient most likely experienced an anaphylactoid (non-IgE-mediated) hypersensitivity reaction [11]. Fiore has defined “anaphylactoid” as an adverse drug event in which at least one of the reported adverse drug reactions includes any of the following CONSTART entries: anaphylaxis, allergic reaction,
apnea, death, cardiac arrest, hypotension, shock or vasodilation [2]. The attempt to identify the nature of VK1 injection-induced adverse reactions as anaphylaxis or anaphylactoid reactions has clearly been relatively unsuccessful. Paradoxically, VK1 is required in the body to participate in coagulation but can also cause anaphylaxis. On the other hand, Tween-80, a non-ionic surfactant, is the most extensively used solubilizer in lipid-soluble drugs containing VK1. Growing evidence suggests that Tween-80 used as a solubilizer can induce anaphylactoid reactions [12–14]. Therefore, we considered whether VK1 injection-induced adverse reactions are anaphylactoid reactions triggered by the solubilizer. The objectives of the present study were to ascertain the nature of the adverse reactions to VK1 injection. Clearly distinguishing the types of adverse reactions and determining the trigger will be significant in finding solutions to prevent and reduce the adverse reactions.

Materials and Methods

Reagents

The following reagents were used in this study: VK1 injection (Cisen Pharmaceutical Co., Shandong, China), VK1-fat emulsion (VK1-FE) (Anjian Pharmaceutical Co., Xi’an, China), Tween-80 (Sigma Co., UK), adrenalin hydrochloride injection (Shanghai Harvest Pharmaceutical Co., Ltd, Shanghai, China), diphenhydramine hydrochloride injection (Tianjin Jinyao Amino Acid Co., Ltd, Tianjin, China), p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Co., UK), IgE ELISA kits (KeyGENBioTECH, Nanjing, China), histamine ELISA kits (KeyGENBioTECH, Nanjing, China), and Annexin V-FITC apoptosis detection kits (KeyGENBioTECH, Nanjing, China).

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocols for using beagle dogs were approved by the Animal Ethics Committee at Xi’an Jiaotong University, Xi’an, China (Permit Number: XJTU 2011-0045). All surgeries were performed under sodium pentobarbital anesthesia. Adrenaline hydrochloride injection and diphenhydramine hydrochloride injection were available and ready for use to ensure dogs safety.

Animals and cells

Beagle dogs were purchased from Xi’an Dilepu Biology Resources Development Co., Ltd. (Xi’an, China). Forty-two healthy beagle dogs (twenty females and twenty-two males), aged 2 to 3 years and with a body weight ranging from 8 to 10 kg, were included in the study. The dogs were housed in individual cages in a large colony room, with free access to water, and were fed a standard dry food twice a day. Breeding environment: The room temperature was 20–25 °C, with a relative humidity of 40%~70% and a day-night cycle of 12/12 h. The dogs were randomly divided into 7 groups as following (6 dogs in each group): control, ovalbumin, VK1 injection (0.25 and 0.085 mg/kg), VK1-FE (0.25 and 1 mg/kg), and Tween-80 (1 mg/kg). The dogs were anesthetized by the intravenous injection of pentobarbital sodium (30 mg/kg) and immobilized on the operating table while the blood pressure was measured via a BL-420 biological function system. The femoral artery was intubated, and the blood pressure was recorded for 30 min. In the anaphylaxis experiments, the dogs were sensitized with intravenous drugs every other day for a total of three administrations. On the 10th day after the last sensitization, the dogs were challenged intravenously, and their behaviors were observed. The challenge doses were twice the doses used for sensitization. A cross-challenge experiment was implemented, in which the dogs sensitized with VK1 injection were challenged with VK1-FE or the dogs sensitized with VK1-FE were challenged with VK1 injection. An assessment standard for anaphylaxis and anaphylactoid reactions (Table 1) was established with some modifications [14–18]. The sums of the scores were determined for each symptom after drug administration.

Blood pressure

The dogs were anesthetized by the intravenous injection of pentobarbital sodium (30 mg/kg) and immobilized on the operating table. The femoral artery was intubated, and the blood pressure was recorded in real time via a BL-420 biological function system.

ELISA

Before and 10 min after drug administration, blood samples were harvested in a tube containing heparin and centrifuged for 15 min at 2–8 °C. The plasma was collected and stored at −20 °C. Plasma histamine and IgE concentrations were quantified according to the manufacturers’ instructions (Thermo Electron Corporation, USA).

Spectrophotometry and fluorospectrophotometry

The release of β-hexosaminidase was measured by a fluorospectrophotometric assay. RBL-2H3 mast cells were treated with different concentrations of drugs for 30 min. Aliquots (50 μl) of the supernatants were collected and incubated with 30 μl of 1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) at 37 °C for 2 h. At the end of incubation, 250 μl of carbonate buffer containing 0.1 M Na2CO3 and 0.1 M NaHCO3 (pH 10) was added, and the absorbance resulting from the formation of p-nitrophenol was measured at 405 nm [19,20]. PBS was used as a blank, and the supernatant from cells stimulated with Triton was considered as the total β-hexosaminidase release. The β-hexosaminidase release rates were calculated as percentages relative to the β-hexosaminidase release from the control.

$$\beta - \text{hexosaminidase release rate} = \frac{\text{OD of tested sample}}{\text{OD of Triton}} \times 100\%$$
Table 1. An assessment norm for the anaphylactoid reactions and anaphylaxis in dogs.

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

Each symptom in the dogs received a score corresponding to that symptom. doi:10.1371/journal.pone.0090199.t001

Histamine release was measured by a fluorospectrophotometry assay [19], with some modifications. The supernatant (100 µl) from RBL-2H3 mast cells was added to 40 µl of 0.5 M NaOH and 20 µl of 2.5 mg/ml o-phthalaldehyde and incubated for 30 min. The reaction was terminated by the addition of 10 µl of 3 M HCl. The fluorescence intensity (FI) was measured at an excitation wavelength of 365 nm and an emission wavelength of 465 nm. PBS was used as a blank, and the supernatant from cells stimulated with Triton was considered to represent total histamine release. The histamine release rates were calculated as percentages relative to the histamine release from the control:

Histamine release rate = 
\[
\text{FI of tested sample/FI of Triton \times 100%}
\]

Fluorescence microscopy observations

According to the instructions provided with the Annexin V-FITC apoptosis detection kit, RBL-2H3 cells were treated with different concentrations of drugs for 30 min, washed twice with cold PBS, and resuspended in binding buffer containing Annexin V-FITC (5 µl) and propidium iodide (5 µl). After 10 min in the dark, the cells were examined and photographed at 400× magnification on an Olympus fluorescent microscope (Eastman Kodak, Rochester, NY, USA).

Flow cytometry

RBL-2H3 cells were harvested and treated as described above. After 10 min in the dark, the cells were analyzed with a flow cytometer (Guava easyCyte HT, Millipore Co., Hayward, USA).

Statistical analysis

All statistical analyses were performed using SPSS (Version 18.0). All data were represented as the mean ± SE, and p value < 0.05 was considered statistically significant. Data normality was assessed using the Shapiro-Wilk test. Then ANOVA with least significant difference (LSD) were used to analyze quantitative data, including the differences of the levels of histamine, IgE, β-hexosaminidase, the blood pressure, and the percentage of apoptotic cells among the groups. The Kruskal-Wallis test was used to analyze ordinal data, including the change of dog behaviors.

Results

Anaphylactoid reactions

Behavioristics. The behaviors of the dogs were observed and recorded within 30 min after the first intravenous administration of drugs. Table 2 shows that the dogs in the control and VK1-fat emulsion (VK1-FE) groups did not display any abnormal behavior. In the Tween-80 group, one dog displayed anaphylactoid grade III symptoms, including scratching his nose or head, ear swinging, skin rash, and gait disturbance; the rest of the dogs displayed grade IV symptoms of pawing at the ground, tumbling, sluggishness, somnolence, or hypodynamia. The average grade and anaphylactoid score were 3.8 ± 0.2 and 13.5 ± 1.1, respectively (P < 0.01 vs. control). In the 0.25 mg/kg VK1 injection group, four dogs displayed anaphylactoid grade IV symptoms, while the other two dogs displayed the grade V symptoms of gatism, hematemesis, hemafrica, breathlessness. Adrenaline hydrochloride injection 0.25 mg and diphenhydramine hydrochloride injection 10 mg were performed intramuscularly to treat these signs. The average grade and anaphylactoid score were 4.3 ± 0.2 and 23.2 ± 4.9, respectively (P < 0.01 vs. control). In the 0.085 mg/kg VK1 injection group, all of the dogs displayed anaphylactoid grade IV symptoms, and the anaphylactoid score was 17.0 ± 1.0 (P < 0.01 vs. control).

Plasma histamine concentrations. The standard curve and regression equation for the plasma histamine concentrations were established:

\[ y = -0.0032x + 0.1775 \times 0.0571, R = 0.9996. \]

Table 3 shows that the plasma histamine concentration in the 0.25 mg/kg VK1 injection group increased from 6.41 ± 0.24 before administration to 7.51 ± 0.27 after administration (P < 0.05). However, compared to the levels before administration, the histamine concentrations in the other groups did not significantly change after administration.

Changes in blood pressure. Figure 1 shows both the systolic (Figure 1A) and diastolic (Figure 1B) blood pressure changes, which are presented as decreases in blood pressure from before to after drug administration. VK1 injection clearly affected the blood pressure. The systolic and diastolic blood pressures of dogs administered VK1 decreased to 62 ± 4.2% (P < 0.01) and 64 ± 6.8% (P < 0.01) at 5 min, respectively. Then, the blood pressure continued to decrease further. At 30 min, the systolic and diastolic blood pressures decreased to 17 ± 2.9% (P < 0.01) and 26 ± 4.3% (P < 0.01), respectively. In the control, VK1-FE (0.25 and 1.0 mg/kg) and Tween-80 groups, the blood pressure did not obviously change after drug administration when compared with before administration.
Anaphylaxis and cross-challenge

**Behavioristics.** The dogs were challenged with drugs on the 10th day after the last sensitization. The dogs in the control and VK1-FE (0.25 and 1.0 mg/kg) groups did not display any abnormal behavior (Table 4). As predicted, the dogs challenged with ovalbumin displayed obvious abnormalities in the digestive and nervous system behaviors, such as diarrhea, vomiting, tumbling, sluggishness or somnolence, and skin rash. The average grade and anaphylaxis score were 4.0±0.0 (P<0.01) and 10.0±1.7 (P<0.01), respectively (Table 4). The dogs that were both sensitized and challenged with VK1 injection displayed the same symptoms as those in the anaphylactoid experiments. The scores after sensitization three times with 0.25 mg/kg VK1 injection were 23.2±4.9, 16.8±1.5, and 15.4±2.1 (Table 5). The ordered decrease in the scores clearly implied that the dogs developed a tolerance to VK1 injection. Unfortunately, one dog was very sensitive to VK1 injection and presented with skin lesion and gastrointestinal signs such as skin rash, nodule, tumor, vomiting, diarrhea, gait disturbance and unsteadiness of gait. Adrenaline hydrochloride 0.25 mg and diphenhydramine hydrochloride 10 mg were intramuscularly injected to treat these signs. Although some signs faded, the dog remained ill and exhibited frailty and

### Table 2. Grades and scores for anaphylactoid reactions in dogs after the first intravenous administration.

| Groups               | Doses (mg/kg) | The number of dogs in different grades | Grades | Scores   |
|----------------------|---------------|----------------------------------------|--------|----------|
|                      |               | I           | II          | III         | IV          | V          | VI         | 0            | 1        | 2        | 3        | 4        | 5        | 6        |
| Control              | -             | 6           | 0           | 0           | 0           | 0           | 0          | 0±0.0       | 0±0.0    | 0±0.0    |
| Ovalbumin            | 2.5           | 6           | 0           | 0           | 1           | 5           | 0          | 3.8±0.2**   | 13.5±1.1**|
| Tween-80             | 1.0           | 6           | 0           | 0           | 0           | 0           | 0          | 0.0±0.0     | 0.0±0.0   | 0.0±0.0  |
| VK1-FE               | 1.0           | 6           | 0           | 0           | 0           | 0           | 0          | 0.0±0.0     | 0.0±0.0   | 0.0±0.0  |
| VK1-FE               | 0.25          | 6           | 0           | 0           | 0           | 0           | 0          | 0.0±0.0     | 0.0±0.0   | 0.0±0.0  |
| VK1 injection        | 0.25          | 0           | 0           | 0           | 0           | 4           | 2          | 4.3±0.2**   | 23.2±4.9**|
| VK1 injection        | 0.085         | 0           | 0           | 0           | 0           | 6           | 0          | 4.0±0.0**   | 17.0±1.0**|

Drugs were intravenously administered to the dogs via a micro-injection pump at 0.4 ml/min. Symptoms were observed and recorded for 30 min. The sums of the scores were determined for each symptom. The values are shown as the mean ± SE, n=6.

**P<0.01 vs. control.

VK1: vitamin K1; VK1-FE: vitamin K1-fat emulsion.

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### Table 3. The change in plasma histamine concentrations after the first intravenous administration.

| Groups               | Doses (mg/kg) | Histamine concentration (μg/L) |
|----------------------|---------------|---------------------------------|
|                      |               | Before administration | After administration |
| Control              | -             | 5.48±0.16 | 5.90±0.41 |
| Ovalbumin            | 2.5           | 6.39±0.55 | 6.56±0.18 |
| Tween-80             | 1.0           | 4.77±0.28 | 4.89±0.55 |
| VK1-FE               | 1.0           | 6.94±0.26 | 7.07±0.22 |
| VK1-FE               | 0.25          | 4.74±0.15 | 4.82±0.46 |
| VK1 injection        | 0.25          | 6.41±0.24 | 7.51±0.27* |
| VK1 injection        | 0.085         | 6.85±0.39 | 6.86±0.16 |

Blood samples were taken before and 10 min after drug administration. The plasma histamine concentrations were quantified by ELISA. The values are shown as the mean ± SE, n=6.

**P<0.05 vs. control.

VK1: vitamin K1; VK1-FE: vitamin K1-fat emulsion.

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### Figure 1. Effect of vitamin K1 (VK1) injection on systolic blood pressure (A) and diastolic blood pressure (B) in Beagle dogs.

The change in blood pressure is presented as the decrease in blood pressure from before to after drug administration. The values are shown as the mean ± SE, n=6. *P<0.05, **P<0.01 vs. control. VK1-FE: vitamin K1-fat emulsion.

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weight loss over the following days. Unfortunately, treatment proved ineffective, and the dog died during the night of the fifth day after the challenge. Although death did not occur within 30 minutes of administration, we believe that it should be included in the list of adverse reactions. The rest of the dogs displayed grade IV symptoms after VK1-FE challenge. The score after challenge with 0.25 mg/kg VK1 injection was 14.0±1.6. To distinguish whether the reaction was the result of anaphylaxis or an anaphylactoid reaction, a cross-challenge was performed. When the dogs sensitized with VK1 injection were challenged with VK1-FE, no abnormal behavior was observed. However, when dogs sensitized with VK1-FE were administered VK1 injection, severe grade IV symptoms were observed, with a score of 14.2±2.4 (P<0.01) (Table 6).

**Plasma IgE concentrations.** A standard curve and regression equation for the plasma IgE concentration were established: y = 3.4×10^{-5}x^2+0.0182x−0.0332, R = 0.9998. The plasma IgE concentration in the dogs increased from 48.6±3.0 to 62.7±4.1 (P<0.05) after challenge with ovalbumin (Table 7). However, no significant change in the plasma IgE concentrations was observed in the VK1 injection and VK1-FE groups.

**Effect on degranulation in RBL-2H3 cells**

**β-hexosaminidase release.** Figure 2 shows that VK1 injection treatment directly stimulated RBL-2H3 cells to release β-hexosaminidase. Treatment with 2, 20, or 200 μg/ml VK1 injection induced a significant increase in β-hexosaminidase release to 2.9±0.3%, 3.2±0.4%, and 4.8±0.4%, respectively (P<0.05 or P<0.01), when compared with the release from the control (1.5±0.3%). Tween-80 (10–1000 μg/ml) also induced β-hexosaminidase release from 3.4±0.4% to 18.8±0.4%. In contrast, no significant difference in the β-hexosaminidase release rate was observed between the VK1-FE group and the control group.

**Histamine release.** The effect of VK1 on histamine release from non-antigen-stimulated RBL-2H3 cells was evaluated. Figure 3 shows that the histamine release in the treatment groups treated with 2, 20, or 200 μg/ml VK1 injection was 14.8±1.6%, 26.0±8.3%, and 45.7±2.4%, respectively, indicating that VK1 injection increased histamine release in a concentration-dependent manner. Similarly, Tween-80 (1–1000 μg/ml) increased the histamine release from 12.7±2.2% to 46.6±2.0% when compared with the release by control cells (4.9±8.3%) [P<0.05 or P<0.01]. As expected, and in agreement with the previous results, VK1-FE did not induce histamine release.

**Table 4. The grades and scores of symptoms in dogs after the challenge with vitamin K1 (VK1) injection and vitamin K1-fat emulsion (VK1-FE).**

| Groups         | Doses (mg/kg) | The number of dogs in different grade |
|----------------|---------------|--------------------------------------|
|                |               | 0 | I–III | IV | V | VI |
| Control        | -             | 0 | 0     | 0  | 0 | 0  |
| Ovalbumin      | 2.0           | 0 | 0     | 0  | 0 | 0  |
| VK1 injection  | 0.085         | 0 | 0     | 0  | 0 | 0  |
| VK1 injection  | 0.25          | 0 | 0     | 5  | 0 | 1  |
| VK1 injection  | 0.25          | 0 | 0     | 6  | 0 | 0  |

The dogs were intravenously stimulated with double doses of drug via a micro-injection pump at 0.4 ml/min, and symptoms were then observed and recorded for 30 min. The sum of the scores was determined for each symptom. The values are shown as the mean ± SE, n=6.

**Table 5. The scores of the symptoms shown by the dogs after three sensitizations with vitamin K1 (VK1) injection and vitamin K1-fat emulsion (VK1-FE).**

| Groups         | Doses (mg/kg) | Sensitization |
|----------------|---------------|---------------|
| VK1 injection  | 0.25          | VK1-FE        |
| VK1 injection  | 0.085         | VK1-FE        |
| VK1 injection  | 0.25          | VK1-FE        |

The dogs were sensitized by intravenous drugs every other day for a total of three administrations via a micro-injection pump at 0.4 ml/min; symptoms were then observed and recorded for 30 min. The sum of the scores was determined for each symptom. The values are shown as the mean ± SE, n=6.

**Table 6. Grades and scores for the symptoms presented by the dogs in a cross-challenge experiment.**

| Sensitization | Stimulation | Grades | Scores |
|---------------|-------------|--------|--------|
| Drugs         | Doses (mg/kg) | Drugs | Doses (mg/kg) | |
| VK1 injection | 0.25         | VK1-FE | 0.5     | 0.0±0.0 | 0.0±0.0 |
| VK1-FE        | 0.25         | VK1   | 0.25    | 4.0±0.0**| 14.2±2.4**|

Dogs sensitized with 0.25 mg/kg vitamin K1 (VK1) injection were challenged with 0.25 mg/kg VK1 injection. Symptoms were observed and recorded for 30 min. The sum of the scores was determined for each symptom. The values are shown as the mean ± SE, n=6.

**Apoptosis of RBL-2H3 cells.** The morphology of apoptotic cells was assessed by fluorescence microscopy using the Annexin V-FITC and propidium iodide staining method. During the early
stages of apoptosis, the membrane phospholipid phosphatidylserine translocates from the inner to outer leaflet of the cytomembrane. Annexin V-FITC binds to cells with exposed phosphatidylserine, causing the cells to appear green. During the middle-late stages of apoptosis, the integrity of the cytomembrane is disrupted, and propidium iodide (a reactive dye) can pass through the cytomembrane and embed in the DNA, causing the cells to appear red. Thus, normal cells are colorless, early apoptotic cells are bright green, and middle to late apoptotic cells are yellow (representing equal amounts of red and green). The results indicated that both VK1 injection and Tween-80 significantly induced the apoptosis of RBL-2H3 cells compared with the control (Figure 4). In contrast, VK1-FE treatment caused only a spot of yellow, and no significant apoptosis was detected compared with the control (Figure 4).

Flowcytometry was used to explore the concentration-response relationship between VK1 injection treatment and apoptosis.

Table 7. The change in plasma IgE concentrations after the challenge with vitamin K1 (VK1) injection and vitamin K1-fat emulsion (VK1-FE).

| Groups        | Doses (mg/kg) | IgE concentration (µg/ml) |
|--------------|--------------|---------------------------|
|              | Before sensitivity | After stimulation |
| Control      | -            | 46.9±0.6                  |
| Ovalbumin    | 2.5          | 48.6±3.0                  |
| VK1-FE       | 0.5          | 67.4±4.5                  |
| VK1-FE       | 2.0          | 51.1±1.0                  |
| VK1 injection| 0.25         | 56.1±3.0                  |
| VK1 injection| 0.085        | 45.5±1.6                  |

Blood samples were collected into a tube containing heparin at 10 min after drug administration. The plasma IgE concentrations were determined by ELISA. The values are shown as the mean ± SE, n = 6.

Anaphylaxis is most often triggered by repeated exposure to allergens such as drugs, which can stimulate the body to produce antibodies through an IgE-mediated immune response. Meanwhile, drugs can cause anaphylactoid reaction which displays the same clinical manifestations as anaphylaxis. However, a different mechanism exists. Anaphylactoid reactions are non-IgE-mediated and do not require a history of exposure. In both IgE- and non-IgE-mediated reactions, mast cells and basophils rapidly release histamine, β-hexosaminidase and tryptase via different trigger mechanisms [19,21]. Because anaphylaxis and anaphylactoid reactions are clinically indistinguishable, reports of anaphylactoid reactions and anaphylaxis are confused. The nature of the adverse reaction to VK1 injection is one example of this uncertainty.

The anaphylaxis and anaphylactoid reactions induced by drugs are related to hereditary capacity, immune status, the drug delivery route, the identity of the drug, and metabolism, among other factors. Completely simulating these responses in vitro is difficult. Therefore, selecting and establishing the appropriate animal model is very important. Compared with other experimental animals, dogs and humans are more likely to experience similar symptoms [22,23], and dogs are more sensitive to anaphylactoid reactions than any other animals. The degranulation of basophils or mast cells is an important element in the study

Annexin V-FITC/PI staining indicated that both VK1 injection and Tween-80 induced apoptosis in RBL-2H3 cells in a concentration-dependent manner. Figure 5 showed that the percentage of apoptotic cells in the control was 5.3±0.6%. The percentage of apoptotic cells in the Tween-80 (1–1000 µg/ml) group clearly increased from 8.0±1.8% to 15.1±2.0% (P<0.01). The percentage of apoptotic cells in the groups treated with 2, 20, and 200 µg/ml VK1 injection increased significantly to 8.2±1.9%, 9.1±1.4%, and 14.7±3.4%, respectively (P<0.05 or P<0.01), when compared with the control. However, VK1-FE did not induce apoptosis at concentrations of 0.2–20 µg/ml, although apoptosis was observed at 200 µg/ml.

Discussion

Anaphylaxis is most often triggered by repeated exposure to allergens such as drugs, which can stimulate the body to produce antibodies through an IgE-mediated immune response.
of anaphylaxis and anaphylactoid reactions. RBL-2H3, a continuous rat cell line that is useful for in vitro studies [24], has been used extensively to study signaling pathways involved in degranulation [25] and IgE-FcεRI interactions.

Behavioristics is considered to be a vital, simple and intuitive method to determine the type of adverse reaction. The present study showed that dogs experienced serious multiorgan symptoms, increases in plasma histamine concentrations, and sharp decreases

**Figure 3. Effect of vitamin K₁ (VK₁) injection on histamine release in RBL-2H3 cells.** RBL-2H3 cells were treated with the different drugs for 30 min. The supernatants were collected and measured by spectrofluorometry. PBS was used as a blank, and the supernatant from cells stimulated with Triton was considered to represent the total histamine release. The histamine release rates are shown as the mean ± SE, n = 6. *P<0.05, **P<0.01 vs. control (conl). VK₁-FE: vitamin K₁-fat emulsion.

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**Figure 4. Effect of vitamin K₁ (VK₁) on apoptosis of RBL-2H3 cells by fluorescence staining.** AnnexinV-FITC-stained cells appear green at the early stage of apoptosis. PI caused the cells to appear red during the middle to late stages of apoptosis. The magnification is 400×. The merged green and red images represent total apoptosis. VK₁-FE: vitamin K₁-fat emulsion.

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Figure 5. Effect of vitamin K₁ (VK₁) injection on the apoptosis of RBL-2H3 cells by flow cytometry. Apoptotic cells were identified by Annexin V-FITC and PI staining. The concentrations of Tween-80 are 1000, 100, and 10 μg/mL, from top to bottom. The values are shown as the mean ± SE, n = 6. *P<0.05, **P<0.01 vs. control (conl). VK₁-FE: vitamin K₁-fat emulsion.
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in blood pressure after the first intravenous VK₁ injection. Undoubtedly, the results demonstrate that VK₁ injection induces an anaphylactoid reaction. VK₁ is a lipid-soluble substance. For preparation as an injection, the use of a solubilizer such as Tween-80 is required. When 1 mg/kg Tween-80 was administered to the dogs, anaphylactoid reactions appeared; these results are in agreement with previous reports [12,14,26]. Other drugs that require solubilizers containing Tween-80, such as qingkailing injection, shengmai injection, the anti-neoplastic agent paclitaxel and the immunosuppressant cyclosporine, also cause obvious anaphylactoid reactions [18,27,28]. However, it is the low dose of Tween-80 (1 mg/kg) that did not lead to the significant changes in the plasma histamine concentration and blood pressure. The plasma histamine levels sharply increased in the dogs upon the administration of 25 mg/kg Tween-80 [13].

VK₁-FE is a preparation in which VK₁ is dissolved in lecithin and is a steady O/W emulsion without any solubilizer. This preparation can be used to help explain the effect of the solubilizer on adverse reactions. Abnormal behaviors were not observed in dogs administered VK₁-FE without Tween-80. Furthermore, no significant change in plasma histamine was observed in these dogs. The results revealed that VK₁-FE does not induce an anaphylactoid reaction. Therefore, VK₁ is not the trigger that initiates the anaphylactoid reaction in response to VK₁ injection.

Upon challenge with VK₁, the dogs showed the same abnormal multisystem symptoms as dogs that had been sensitized with VK₁ injection. Determining whether the multisystem symptoms were indicative of anaphylaxis was difficult. Therefore, we devised a cross-challenge experiment. The results showed that abnormal behaviors were not observed in dogs sensitized with VK₁ injection that were later challenged with VK₁-FE. In dogs sensitized with VK₁-FE that were challenged with VK₁ injection, severe abnormal behaviors were observed. Furthermore, no significant change in plasma IgE concentrations was observed in the VK₁ injection and VK₁-FE groups. The results indicated that the abnormal behaviors of the dogs administered VK₁ injection in the anaphylaxis experiment (during both sensitization and challenge) were due to anaphylactoid reactions, not anaphylaxis.

We further studied the mechanisms underlying the anaphylactoid reactions. It is well-known that measurements of histamine and β-hexosaminidase release are common methods for detecting the activation of RBL-2H3 cells stimulated in vitro. In the present study, VK₁ injection treatment directly resulted in the degranulation of the non-antigen sensitized RBL-2H3 cells in a concentration-dependent manner. These results demonstrated that the anaphylactoid reaction resulting from VK₁ injection treatment was due to the release of histamine, β-hexosaminidase, and other inflammatory factors through a non-IgE-mediated pathway, and these factors are known to cause pruritus, acute inflammation, and vasodilatation. Simultaneously, the percentages of both early and late-stage apoptotic cells markedly increased in the VK₁ injection treatment group. The results demonstrate that the release of histamine and other inflammatory factors induced by VK₁ injection is the result of phosphatidylycerine exposure and an increase in cell membrane permeability in RBL-2H3 cells. VK₁-FE did not induce apoptosis in RBL-2H3 cells, suggesting that apoptosis is not induced by VK₁. However, RBL-2H3 cells treated with Tween-80 displayed severe apoptosis in a concentration-dependent manner, suggesting that the apoptosis induced by VK₁ injection may be due to the presence of Tween-80 in the formulation.

In conclusion, VK₁ injection induces anaphylactoid reactions, not anaphylaxis. However, VK₁ itself is not involved in the anaphylactoid reaction. The trigger may be the solubilizer. This conclusion provides a basis for generating VK₁ preparations without severe adverse reactions. Altering the VK₁ preparation, decreasing the solubilizer dosage, or using a highly safe solubilizer may be good strategies to reduce or eradicate the anaphylactoid reactions caused by treatment with VK₁ injection. Properly discerning the category and pathogenesis of adverse drug reactions will aid our ability to prevent and reduce adverse reactions to VK₁ preparations.

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Author Contributions
Conceived and designed the experiments: YXC. Performed the experiments: YNM NNP XX YBZ JL. Analyzed the data: YNM. Contributed reagents/materials/analysis tools: YXC. Wrote the paper: YNM YXC.

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