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Allergic Identification for Ginkgo Kernel Protein in Guinea Pigs

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Abstract *Ginkgo biloba* L. can cause allergic reactions when consumed. In this paper, an allergy test to guinea pig was investigated. Guinea pigs were sensitized with 50 mg/mL of ginkgo kernel protein orally on days 1, 3, and 5, and intraperitoneally challenged with 100 mg/mL of the protein on day 7 after the last sensitization. The volume of sensitization and challenge was 0.20 mL/100 g weight. The results showed the average allergy grade for guinea pigs reached four and the allergy rate was 100%. The immunoglobulin G and E levels in sera were significantly higher than those in the controls. Footpads swelled distinctly, and the passive cutaneous allergy test manifested a positive response. There were inflammatory changes in the lungs and intestines. In conclusion, the present results may indicate that gingko kernel protein has an allergenic capacity.

Keywords: ginkgo kernel, protein, allergy, guinea pig

Introduction

Ginkgo biloba L. is the sole survivor of the Ginkgocae family of gymnosperms. It is considered to be a living fossil native to China. Its edible kernel is called Baiguo in China. Baiguo is rich in protein, amino acids, fats, sugars, vitamin C, and riboflavin as well as other functional components such as flavone and lactone. The ginkgo kernel has exhibited special medical functions such as antihypoxia, antifatigue, and antiaging effects (1,2). However, raw, or even cooked, consumption of the ginkgo kernel has been shown to cause allergic reactions and even death. It has been indicated that children are more sensitive to ginkgo kernel. The allergic latent period is 1-12 h. The clinical manifestations are generalized as nausea, vomiting, bellyache, diarrhea, narcolepsy, dysphoria, coma, and then decompensation, pupil dilation with slow reaction to light, and death (1,3).

To date, 4-O-methylpyridoxine (MPN) has been considered to be the main component responsible for allergic reactions. Kobayashi *et al*. (4) and Yasushi *et al*. (5) reported that MPN was the main poisonous substance in ginkgo seeds. Many other researchers have suggested that ginkgolic acids (GAs) in the ginkgo seeds are the allergic factors. Ahlemeyer *et al*. (6), Helke *et al*. (7), and Koch *et al*. (8) pointed out that GAs were potentially hazardous constituents in *Ginkgo biloba* extract. However, Yang *et al*. (9) found other allergen or allergic mechanisms. It is common that proteins can provoke allergic reactions. Volcheck (10) reported that protein was the main allergen in tree nuts, soybeans, peanuts, etc. Some seeds such as tree nuts, soybeans, and peanuts are typical allergens (11,12). There is rich protein content in gingko kernel; therefore, it is necessary to clarify the allergenicity of gingko kernel protein. Presently, there are no reports on the allergenic capacity of the ginkgo kernel protein. With the development of food processing technologies, food composition has become more complex, and some adverse responses to food result in many health problems. Therefore, the identification of food allergies has been one of the hottest research topics in food safety fields. Presently, food allergy assay technologies include a serum immunoglobulin (Ig) E level test; skin prick tests; double-blind, placebo-controlled (DBPC) studies; and *in vivo* animal tests (13). Canines, swine, guinea pigs, mice, and rats have been used as food allergy animal models (14-17). Guinea pigs are very sensitive to allergens and can undergo a rapid allergic reaction with manifest symptoms. Therefore, they are always used as test animals in allergy research. In this paper, to assess ginkgo protein allergenicity, guinea pigs were exposed to the ginkgo protein. The obtained results are not only useful to identify new ginkgo allergens but also are beneficial for the development of the ginkgo-based food industry.

Materials and Methods

Preparation of ginkgo kernel protein Ginkgo kernel protein was extracted from ginkgo kernels (cultivar Dafozhi, Taixing, China). After being shelled, the kernels were freeze-dried and pulverized into flour in an ice bath. The flour was defatted with petroleum ether, which was discarded after the flour was fully defatted. The defatted kernel

flour was suspended in 0.20 mol/L Tris-HCl buffer adjusted to pH 7.4 (TBS), with a flour to buffer ratio of 1:10 (m/v) at 4°C for 24 h. The slurry was centrifuged at 4,024x*g*, 4°C for 30 min. The supernatant was precipitated with ammonium sulfate (saturation degree 40-80%). The precipitation was dialyzed with a dialyzing bag (Huamei Bioengineering Co. Ltd., Shanghai, China, molecular cut 3.5 kDa) in pH 7.4 TBS at 4°C until ammonium sulfate was not detected with $BaCl_2$. The inner substance in the dialyzing bags was lyophilized and stored at 20°C for animal tests.

Animals Healthy adult Hartly guinea pigs were purchased from Qinglongshan Animal Breeding Corporation (Nanjing, China). All animals were male with a weight of 200-250 g. They were fed with a standard animal feed that was ginkgo-free, and they were housed in an animal room maintained at 20-25°C, with a relative humidity of approximately 70%, and a light/dark cycle of 12 h/12 h.

Guinea pigs were grouped into five groups (*n*=5). The sensitization and challenge concentration of protein was as follows: Group 1 was given 10 mg/mL protein orally to sensitize and 50 mg/mL protein orally to challenge; Group 2 was given 10 mg/mL protein orally to sensitize and 50 mg/mL protein intraperitoneally to challenge; Group 3 was given 50 mg/mL protein orally to sensitize and 100 mg/mL protein orally to challenge; Group 4 was given 50 mg/mL protein orally to sensitize and 100 mg/mL protein intraperitoneally to challenge; and Group 5 (CK) was given TBS orally to sensitize and TBS intraperitoneally to challenge. Each group was sensitized on days 1, 3, and 5, and challenged seven days after the last sensitization. The sensitization and challenge volume was 0.20 mL/100 g weight. Allergy reactions were observed after the challenges.

Evaluation of guinea pig allergies The allergic reaction grade of guinea pigs challenged with ginkgo kernel protein was evaluated according to the symptoms listed in Table 1.

It was considered that there was an allergic reaction when the average allergic reaction grade was up to 2. The average allergic reaction grade and allergy rate were calculated according to the formulas (1) and (2).

$$\overline{A}_d = \frac{\Sigma(A_d \times n)}{5} \quad (1)$$

$$RA = \frac{n'}{5} \times 100 \quad (2)$$

In (1) and (2), $\overline{A}_d$ was the average allergic reaction grade, A_d was the allergy grade, *n* was the number of guinea pigs with the allergic reaction grade, RA was the allergy rate, and n was the number of all allergic guinea pigs.

Guinea pig footpad challenge test According to the results of the allergic reaction, the protein concentration was given orally to guinea pigs three times on days 1, 3, and 5. Therefore, the protein resulted to the highest average allergic reaction grade. The challenge was administered at the same concentration seven days later from the last gavage by subcutaneous injection of the two hind footpads. The challenge dose was 0.10 mL/100 g weight for each footpad. TBS was used with the same procedure as for CK. Three guinea pigs were treated for protein and CK. The thickness of the footpad was measured before the challenge and 1 h afterward. Measurement of the thickest part in the limb was performed by one experimenter with a micrometer. A two-fold increase in thickness was considered to be an indicator of allergic reaction.

Table 1. Allergy reaction grade

Symptoms	Classification	Grade
No any abnormity	-	0
Pilo-erection, pruriginous, inquietude	±	1
Except for the above, trembling, sneering, polypnea, etc.	+	2
Except for the above two, gatism, dyspnea, aimless circumduction and dysphoria	++	3
Except for the above three, muscle convulsion, lie on side but without death	+++	4
Except for the above all, death	++++	5

Separation of sera Forty minutes after the challenge, blood samples were taken from the retro-orbital plexus. After coagulation for 3 h at room temperature, blood samples were centrifuged at 1,776x*g*, 4°C for 10 min to obtain sera. The sera were stored at 20°C for analysis of IgG and IgE.

Detection of IgG and IgE in sera Specific IgG and IgE levels in serum were measured using enzyme linked immunosorbent assay (ELISA). Microtiter plates were coated with 0.5 μg/well (5.0 μg/mL) of dilution of ginkgo kernel protein in carbonate/bicarbonate buffer (pH 9.6) and incubated at 37°C for 90 min. Serum samples were diluted in phosphate-buffered saline (PBS; 0.010 mol/L, pH 7.4) with 0.50% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) and 0.05% (v/v) Tween 20. Plates were washed three times with 0.05% PBS-Tween 20. Non-specific binding sites were blocked with 200 μL/well of PBS and 1.0% (w/v) BSA at room temperature for 90 min. Afterwards, serum samples were added to the wells (diluted 1:100 for IgG and 1:20 for IgE) and incubated overnight at 4°C. The sera from guinea pigs treated with TBS were used as native controls. After washing, bound antibodies were detected by adding a biotin conjugated rat monoclonal anti-guinea pig IgG and IgE (Serotec, Raleigh, NC, USA) at room temperature for 5 h followed by 2.5 mg/mL avidin-peroxidase (Serotec) for 30 min. The secondary antibodies were used at 1:500 dilutions. The peroxidase substrate (3,3,5,5-tetramethylbenzidine, TMB; Sigma-Aldrich) was added, and the optical density was determined by an ELISA reader (Thermo Fisher Scientific, Waltham, MA, USA) at 492 nm.

Homogeneous passive cutaneous anaphylaxis Six adult guinea pigs were grouped into three groups (two animals per group): the controlled serum group, 1:1 dilution serum group, and 1:2 dilution serum group. Guinea pigs were denuded with a shaver. The hair on the back was cut with scissors at first, soaked with warm soap water, and shaved. Four points were chosen as injection locations. Two points, 1.5 cm apart, were distributed on one side of the backbone, and the other two points were located on the other side of the backbone.

Sera with the highest IgE levels were diluted at the ratio of 1:1 with normal saline and then 0.20 mL of sera sample per point was injected intradermally. The sera from the TBS treated animal was used as controlled sera. After 48 h, 1.0 mL ginkgo kernel protein (100 mg/mL, containing 0.5% Evans Blue, with a 1:1 ratio) was injected with a mainline in the hind legs. After 30 min, the guinea pigs were sacrificed by vertebral dislocation, and the skin of the back was peeled off to measure the blue spot diameter.

Preparation of histopathological slices of organs One hour after the challenge, guinea pigs from Group 5 and the group with the highest average allergic reaction grade were sacrificed by vertebral dislocation. Livers, lungs, kidneys, and intestines were harvested and fixed in neutral formalin solution (100 mL/L). After dehydration treatment, the above organs were embedded in paraffin and sliced continuously in sections that were 3 mm thick. All the materials were stained with hematoxylin and eosin (H&E), and micrographs were observed.

Statistical analysis Data was corresponded to the average±SD of three repeats. The Student's *t*-test was used to analyze the data. *p* value of <0.05 was considered to be statistically significant, and *p* value of <0.01 was extremely significant. Lowercase letters in Duncan's multiple range test denote a significant difference ($p<0.05$), and capital letters denote an extremely significant difference ($p<0.01$).

Results and Discussion

Reaction grade and allergy rate in guinea pigs Different sensitization and challenge concentrations of the protein were given to guinea pigs orally or intraperitoneally. The average allergic reaction grade and allergy rates were obtained (Table 2). Fifteen minutes after challenge, the guinea pigs began to show different allergic symptoms such as pilo-erection, pruriginous, polypnea, and twitching. Two guinea pigs died in Group 4 after 2 h, whereas there were no typical allergy manifestations in Group 5 (CK). The allergy rate was directly proportional to the protein concentration for sensitization and challenge. After being sensitized with 50 mg/mL and challenged with 100 mg/mL, the allergy rate was 100%. When guinea pigs were sensitized and challenged with lower concentrations of the protein, the average allergic reaction grade was lower. As to the method of sensitization and challenge, at any protein concentration, the average allergic reaction grade that was challenged intraperitoneally was higher than that when challenged orally. Because of the immunity barrier via the digestive system when orally ingested, oral effect was not as fast as intraperitoneal challenges. In oral ingestion, some allergenic protein may be digested in the gastrointestinal gut, which can lead to lower effective concentrations (17). Therefore, the guinea pigs that were challenged orally were not allergic. When guinea pigs were challenged intraperitoneally, the protein effectively reached to the target sites. de Jonge *et al*. (18) reported the types of IgG and specific IgE varied between oral and intraperitoneal administration, and this may be related to different allergy symptoms. In the present study, ginkgo kernel protein induced guinea pigs to manifest allergy symptoms. There was a great difference in the allergy grade of guinea pigs with variable sensitization and challenge parameters.

Table 2. Average allergy reaction grade and allergy rate of guinea pigs

Group	Allergy grade						Average allergy grade $\overline{A}_d$	Rate of allergy, RA (%)
	0	1	2	3	4	5		
Group 1	3	1	1	0	0	0	0.60	40
Group 2	3	1	0	1	0	0	0.80	40
Group 3	0	1	2	1	1	0	1.80	100
Group 4	0	0	0	2	1	2	4.00	100
Group 5	5	0	0	0	0	0	0	0

IgG and IgE in guinea pig sera The immune response is evoked and special antibodies (IgG and IgE) are produced when guinea pigs are exposed to allergenic protein. As shown in Fig. 1, after being sensitized orally by ginkgo kernel protein, guinea pigs that were challenged intraperitoneally had higher IgG and IgE levels in sera. When establishing the food allergy animal model, animals were given adjuvant therapy together with protein to enhance the allergic reaction and levels of IgG and IgE. However, in some reports on BALB/c mice and brown Norway rat sensitization oral models, IgE and IgG levels were insignificant when adjuvant therapy was given (18,19). In this test, adjuvant therapy was not used in order to simulate food ingested in humans. Although the levels of IgG and IgE were low, it was statistically significant ($p<0.01$). If an IgG response was induced, the protein was immunogenic. On this basis, when an IgE antibody was produced, the protein showed immunoreactive responses (20). In our present study, IgE levels from four groups treated with protein were significantly different from Group 5 ($p<0.05$), and there was an extremely significant difference between Group 4 and Group 5 ($p<0.01$). Therefore, ginkgo kernel protein was shown to be not only immunogenic but also immunoreactive. When exposed to the human body, IgE-mediated allergic reactions would be induced.

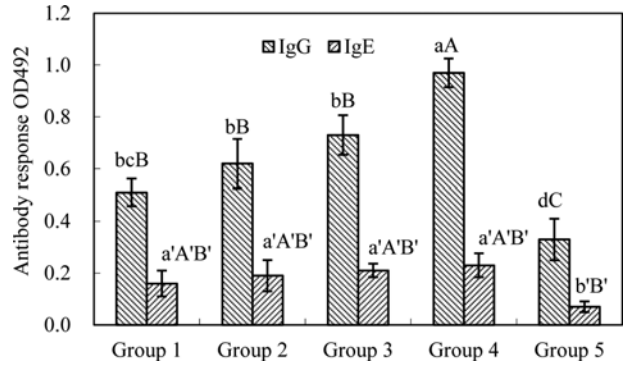

Fig. 1. IgG and IgE of guinea pigs after different treatment.

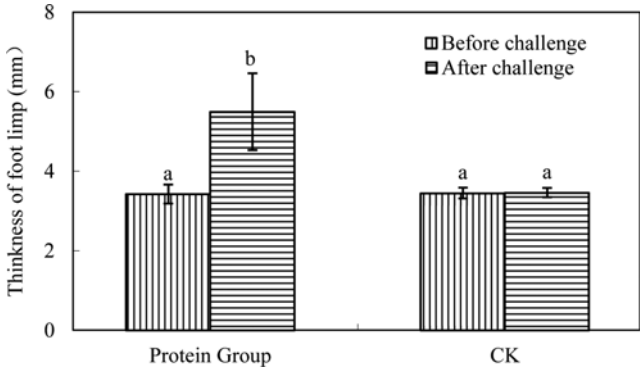

Fig. 2. Footpad thickness of guinea pigs before and after challenge.

Guinea pig footpad tests Many organs are involved in food allergies, including derma, stomach and intestines, respiration tract, and capillary vessel system (21). Helm and Burks (11) studied the derma of pigs and found that red and swollen spots appeared after the guinea pigs were exposed to ovomucoid orally. Chanettee *et al*. (14) also showed red and swollen spots in the derma of dogs when air dust and insects were injected subcutaneously. After guinea pigs were injected with ginkgo kernel protein subcutaneously, the footpads swelled significantly ($p<0.05$) while there was no swelling in the footpads of the controls (Fig. 2). Therefore, the injected ginkgo kernel protein may combine with already produced protein-specific IgE, causing the release of an active mediator to elicit cutaneous swelling. This correlated with the results on footpad swelling of BALB/c mice sensitized by fish parasite protein (22).

Homogeneous passive cutaneous anaphylaxis In the test, blue spots were observed on the recipient skin after ginkgo kernel protein and Evans Blue were injected. The spot areas from the sera with a 1:1 dilution and 1:2 dilution were 5.5±0.8 and 2.9±0.4 mm, respectively (Fig. 2). The degree of allergic reaction was proportional to the area of blue skin. However, there were no blue spots in CK guinea pigs. Rebecca and Ian (20) measured the titer of IgE antibodies in sensitized mice sera by homogeneous passive cutaneous allergy (PCA) test and found that the blue spot area and numbers of mice with positive responses were smaller with a thinner dilution of sera.

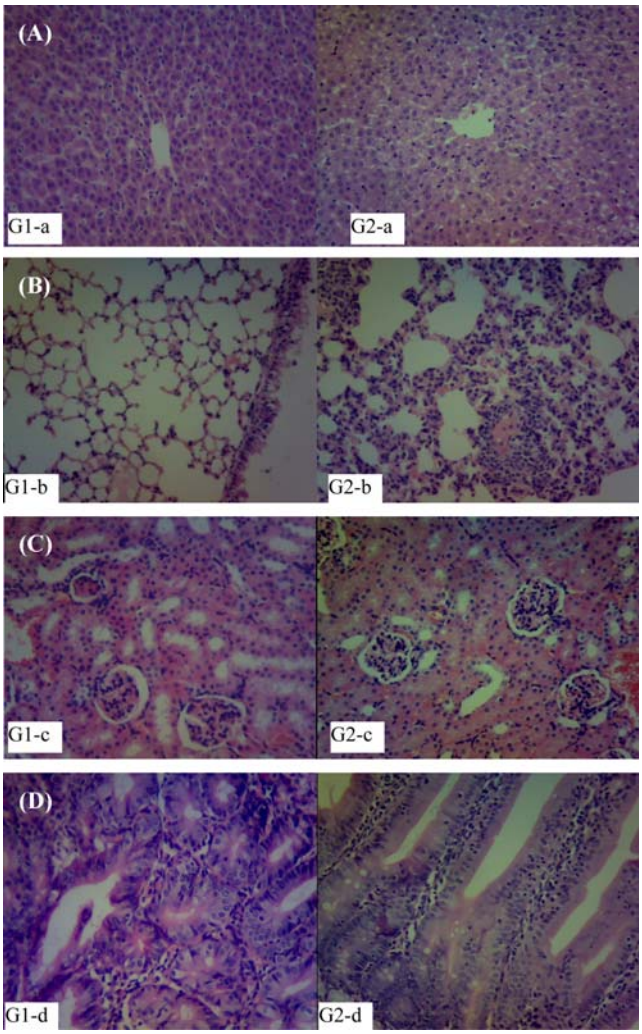

Fig. 3. Representative sections of livers, lungs, kidneys, and intestines taken from CK (G1) and sensitized guinea pigs by ginkgo kernel protein (G2), embedded with paraffin and stained with H&E. (A) livers, (B) lungs, (C) kidneys, (D) intestines (magnification, ×100).

Therefore, in our present studies, specific IgE production in guinea pigs sensitized with ginkgo kernel protein was induced, and this protein may be allergenic.

Analysis of organs histopathology Various inflammatory pathologic changes appeared in the livers, lungs, kidneys, and intestines of guinea pigs in this test (Fig. 3). There was no abnormal manifestation in the organs of the control guinea pigs. In organs treated with the protein, there were pulmonary interstitial hyperplasia and inflammatory cell soakage reactions. Epithelium desquamation from the trachea, as well as mucus secretion and inflammatory cell soakage, were found. There was also more inflammatory cell soakage in the intestine mucous membranes. No pathological changes in the morphology of the livers and kidneys of guinea pigs were obtained. Because of its resistance to digestion, protein cannot be degraded and absorbed well. The large proteins may traverse the alimentary

tract and are transported to other parts, such as the skin and other organs, where inflammation occurs (23). Inflammatory cell soakage was observed in the lungs of guinea pigs after the antigens were injected intraperitoneally (24). When mice were sensitized with OVA intraperitoneally and challenged inhalationally, there was an increase in monocytes and leukocytosis peripheral lung blood vessels (25). It could be concluded that the lung is another vulnerable organ. By analyzing the patient's lung histopathology, we can know whether foods are allergenic. It was interesting that no pathological changes in livers and kidneys were observed in our study, although they are detoxification organs in the body. There is little relevant information on the two viscera. They may not be vulnerable in food allergies, but they should be studied further.

In conclusion, **s**ymptoms of allergic reactions happened to the guinea pigs exposed to ginkgo kernel protein. The main manifestations were piloerection, polypnea, pruriginous, twitching, and death. The average allergy grade was four, and the allergy rate was 100%. IgG and IgE levels in sera derived from guinea pigs administered with ginkgo kernel protein were significantly higher than the controls, and footpads swelled significantly compared to the controls. PCA results showed a positive response on the skin of the guinea pigs treated with ginkgo kernel protein. There were inflammatory changes in the guinea pigs' lungs and intestines after being challenged with the protein. The present results showed the allergenic capacity of gingko kernel protein.

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Disclosure The authors declare no conflict of interest.

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