Induction of immune responses and allergic reactions in piglets by injecting glycinin

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ABSTRACT

Glycinin, an antigenic glycoprotein found in soybeans, is the major cause of allergic reactions in young animals. The objective of this study was to evaluate the effects of a prior immunisation of sucking piglets with glycinin on their post-weaning growth performance, serum immunoglobulin contents, small intestinal histamine release and mucosal histology. Forty piglets (7 d of age) were randomly divided into four groups of 10 piglets each. Piglets of Group C (Control) received a physiological saline solution, Groups Im (Immunised) and Im+S (Sensitised) were immunised twice with 500 l g/kg of glycinin at 7 and 21-d-old. All piglets were weaned at 23 d; Groups Im+S and S were sensitised with 2500 l g/kg of glycinin at weaning. Compared with Group C, in Group S the average daily gain (ADG), average daily feed intake and gain:feed ratio were decreased, and serum levels of IgG and IgE were increased. Furthermore, in this group, the histamine levels in the duodenum and ileum were significantly decreased, and the structure of duodenal and ileal mucosa was severely damaged. On the contrary, in Groups Im and Im+S the ADG was increased, serum IgE levels were decreased, intestinal histamine levels were increased and the intestinal mucosa was not damaged. These findings suggest that prior immunisation with glycinin can protect the structural integrity of the intestinal mucosal epithelia and alleviate allergic reactions in piglets.

Introduction

Soybean is a high-quality protein due to its excellent nutritional, processing and functional properties and can be found in a wide range of food products (Golubovic et al. 2005; Sun et al. 2013; Taliercio et al. 2014). However, soybean is also a dietary allergenic source for humans (Foucard & Malmheden 1999), which threatens individuals with soybean-sensitive food allergies.

Previous studies showed that the presence of antigenic proteins mainly led to the allergenic properties of soybean. Antigenic proteins that were found in soybean include glycinin, α-conglycinin, β-conglycinin and γ-conglycinin, among which glycinin was the main antigenic component. Glycinin is made up of five subunits, and each subunit contains an acidic and a basic polypeptide linked by a disulphide bond (Hou & Chang 2004). Glycinin may cause allergic reactions in young children (Niggemann et al. 1999), piglets (Li et al. 1990, 1991a; Sun et al. 2008a), calves (Lallès et al. 1996) and lambs (Ouedraogo et al. 1998).

It has been demonstrated that glycinin causes changes in intestinal morphology and disorders of immune function, resulting in growth depression and diarrhoea in young animals (Dréau et al. 1994; Qiao et al. 2003; Sun et al. 2008b). The antigen causes the immune system to generate secretory IgA (sIgA) and serum IgA, IgG, IgM and IgE (Dréau et al. 1995). Studies showed that glycinin could induce Wuzhishan pigs to generate IgE-mediated type I allergic reactions through activation of intestinal mast cell degranulation and to increase histamine release (Sun et al. 2008b; Huang et al. 2010). In animals suffering from soybean allergy, mucosal lesions of the gastrointestinal tract were observed typically. Symptoms were related to abnormal bowel patterns, including accelerating crypt cell mitosis, plasma protein leakage into the intestine, goblet cell mucus exudation, intestinal villous atrophy, cell fusion and apoptosis due to allergy in piglets induced by glycinin (Li et al. 1991b; Guo et al. 2007; Chen et al. 2011).

In order to eliminate the allergic potential of soybean products, various methods were used to process
soybeans, and efforts were even made to modify the gene structure of soybean (Aoyama et al. 2001; Golubovic et al. 2005; Liu et al. 2010). To this day, there are limited reports about allergic reactions in piglets which are alleviated by immunisation with the antigen prior to feeding the soybean to the animal. By immunisation, we attempted to evaluate the effects of glycinin injection on potential immune protection, exploring a new way of preventing the allergic reaction in weaned piglets.

Materials and methods

The experimental use of animals and procedures followed were approved by the Anhui Agricultural University Animal Care Committee (Hefei, China).

Characterisation of glycinin

Purified glycinin powder was kindly donated by Professor Shuntang Guo from the Food Institute of China Agricultural University (patent number, 200,410,029,589.4, China). After lyophilisation, the protein content was measured and glycinin purity was analysed according to a previous report (Hao et al. 2009). The purity of glycinin was 90.6%.

Piglets and diets

Forty crossbred piglets [(Duroc × Landrace) × Yorkshire] with an initial body weight (BW) of 2.98 (±0.07) kg were randomly selected from four litters in the Antai Agricultural Group in Anhui province (China). In the sucking period, 10 piglets were kept commingled in each pen, and the feedstuff was added from 7-d-old. After weaning, the piglets were housed in a mechanically ventilated nursery room with a lighting cycle of 12 h light and 12 h darkness, the temperature was maintained at 26–28 °C and the relative humidity was set at 50–60%. The room was equipped with a feeder and a waterer to allow ad libitum access to water and feed.

During the experiment, the diets were provided in two phases, from day 7 to 22 and from day 23 to 35. The diets for the pre-weaning and post-weaning piglets (Table 1) were formulated to meet or exceed nutrient requirements suggested by the National Research Council (NRC 1998).

Feed consumption and BW of individual pigs were determined at weaning (23 d of age) and at the end of the trial (35 d of age) in order to calculate average daily gain (ADG), average daily feed intake (ADFI) and the gain:feed ratio (G/F).

### Experimental diets

| Ingredients, % | Pre-weaning (Day 7–22) | Post-weaning (Day 23–35) |
|---------------|------------------------|--------------------------|
| Corn          | –                      | 60.85                    |
| Dried whole milk | 46.00                  | –                        |
| Skimmed milk powder | 42.00                  | –                        |
| Soybean meal (expanded) | –                      | 25.00                    |
| Whey powder   | 10.50                  | 5.00                     |
| Fish meal     | 5.00                   | 5.00                     |
| Calcium hydrogen phosphate | –      | 2.20                     |
| Limestone     | –                      | 0.69                     |
| Bran          | –                      | 0.37                     |
| Salt          | 0.30                   | 0.25                     |
| Premixb       | 1.00                   | 0.49                     |
| Choline chloride | –                      | 0.15                     |
| Total         | 100.00                 | 100.00                   |

**Nutrient levels**

| CP, %        | 27.90 | 20.80 |
| Ca, %        | 1.04  | 0.64  |
| P, %         | 0.89  | 0.51  |
| Ly, ‰        | 1.61  | 1.06  |
| DE, MJ/kg    | 14.50 | 13.50 |

aExpanded for eliminating the antigen proteins.
bPremix supplies per kg (pre-weaning/post-weaning): vitamin A, 7000/5250 IU; vitamin D3, 2000/1050 IU; vitamin E, 10/4.5 IU; vitamin K2, 2.2/1.2 mg; vitamin B1, 2.375/0.375 mg; vitamin B2, 4.8/1.8 mg; vitamin B6, 0.15/0.15 mg; vitamin B12, 17.5/7.5 μg; niacin, 16/6 mg; Ca-pantothenate, 5.75/3.75 mg; folic acid, 0.85/0.15 mg; biotin, 17.5/7.5 μg; lysine, 0.95/0.75 mg; antioxidant, 45/45 μg; enzyme preparation (mixture of amylase, protease, lipase and phytase), 1100/1000 mg; flavour agents, 45/40 mg; sweet agents, 45/40 mg; neomycin, 0/20 mg; Cu (as copper sulphate), 15/15 mg; Fe (as ferrous sulphate), 144/144 mg; Zn (as zinc sulphate), 110/110 mg; Mn (as manganese sulphate), 20.18/10.18 mg; I (as calcium iodide), 0.4/0.4 mg; Se (as sodium selenite), 0.35/0.3 mg; CP, crude protein; DE, digestible energy.

cThe digestible energy is calculated while the other nutrients are analysed.

**Experimental design and sample collection**

Forty piglets (7 d of age) were randomly divided into four groups with 10 piglets per group, i.e. Group C (control group), Group Im (immunised group), Group Im + S (immunised + sensitised group) and Group S (sensitised group). The piglets of Group C were injected with a physiological saline solution by hypodermic injection at 7 and 21 d. Meanwhile, two further groups of piglets (Group Im and Im + S) were immunised twice by hypodermic injection with 500 μg glycinin per kg BW at 7 and 21 d according to our previous report (Xu et al. 2010). Furthermore, the piglets of Group Im + S received an intramuscular injection of glycinin at 2500 μg/kg BW for sensitisation when they were 23 d old. The piglets of Group S were not immunised in advance but injected with a physiological saline solution as the Group C, and then were directly sensitised with 2500 μg glycinin per kg BW by intramuscular injection on the same day. All piglets were weaned at 23 d.

Blood samples were collected via the anterior vena cava at 7, 21 and 35 d in all of the piglets. The serum samples for analysis of immune function were
obtained by centrifugation at 2000 g for 15 min at 4°C and analysed immediately. At the end of the trial, five piglets were selected randomly from each group and euthanised by an intracardiac injection of sodium pentobarbital (50 mg/kg BW) followed by jugular exsanguination. Two portions of proximal duodenum, middle jejunum and proximal ileum were collected and flushed using physiological saline solution to remove the gut contents. One portion of each of them was transferred to −70°C freezers for preservation and detection of histamine levels, and other intestinal tissues were fixed in 3–5 volumes of 4% phosphate-buffered formaldehyde (pH 7.2) processed and embedded in paraffin for histological examination.

**Measurement of serum IgA, IgM, IgG antibody levels**

The serum IgA, IgM, IgG antibody levels were measured by using the swine immunoglobulin A, M, G kit (Shanghai Fosun Long March Medical Science Co., Ltd. Shanghai, China) and measured by a semi-automatic biochemistry analyzer (GF-D200, Shandong, China), according to the manufacturer’s instructions.

**Measurement of total IgE antibody levels**

The serum IgE antibody levels were determined by using the swine Enzyme-Linked Immunosorbent Assay Kit (ELISA kit; Rapid Bio Company, CA) and a microplate reader (Bio-Rad 680, Hercules, CA).

**Measurement of histamine contents**

To measure histamine concentrations in the small intestine, 4 cm sections of the duodenum, jejunum and ileum were processed according to the method of van Halteren et al. (1997). Protein lysates of the intestine were prepared using a cocktail of proteinase inhibitors and centrifuged at 10,000 g at 48°C for 10 min. Supernatants were collected for determination of histamine levels by an enzyme immunoassay kit (USCN Life Science, Double Lake, Missouri City, TX) as described by the manufacturer. Histamine contents were standardised to the weight of intestine tissue in each sample (Nakajima-Adachi et al. 2006).

**Investigation of the intestinal mucosal histology**

Different tissues prior to fixation were examined by histological paraffin sections. Six cross sections were obtained from each formalin-fixed segment and processed for histological examination and stained using the standard haematoxylin and eosin method. Villus height and crypt depth were evaluated according to Wu et al. (1996), under a light microscope (Olympus CK 40, Olympus Co., Ltd. Tokyo, Japan). Ten measurements of villus height and crypt depth were taken from each section on randomly selected microscopic fields. The histological analysis was performed by an investigator who was unaware of the anatomical origin of tissue sections.

**Chemical analysis of nitrogen, calcium and total phosphorus contents**

Samples of all feeds were analysed for their nitrogen, calcium and total phosphorus contents using the methods of the Association of Official Analytical Chemists (AOAC 1990). Nitrogen was analysed using the Kjeldahl method (AOAC official method 988.05), calcium by titration with 0.1 N KMnO₄ (AOAC official method 927.02), and the total phosphorus was determined colorimetrically using a molybdenum reagent (AOAC official method 965.17). Lysine contents in feed were determined using high-performance liquid chromatography (Shimadzu LC 10 Liquid Chromatograph, Kyoto, Japan).

**Statistical analyses**

All data were analysed using the general linear model procedures of SAS system (SAS 2002; version 9.1.3, SAS Institute, Inc., Cary, NC). A litter of piglets was selected as one group for convenient the immunisation of glycinin and measuring the initial BW. Data were presented as the mean ± SEM. The differences between the groups were examined using analysis of variance. Differences between means were considered statistically significant for values of p < 0.05. The statistical software GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA) was used in all histograms.

**Results**

**Effect of glycinin on growth performance**

The effects of soya bean glycinin on ADG, ADFI and G/F are shown in Table 2. Compared with Group C, the ADG in Group Im and Im+S was increased (p < 0.05), but ADG in Group S was significantly decreased (p < 0.01). In Group S, the ADFI and G/F were significantly reduced compared with Group C (p < 0.01) while
no differences were observed among Groups C, Im and Im + S.

**Effect of glycinin on serum IgG, IgA and IgM levels**

The immunoglobulin data are presented in Table 3. Compared with Group C, the serum IgG levels of piglets were significantly increased \((p<0.05)\) in Group Im and Im + S at day 21 and 35. On day 35, in Group C the serum IgG levels were lower than those in all other groups \((p<0.05)\). There were no significant differences in the IgA and IgM antibody levels among the groups throughout the experiment.

**Effect of glycinin on total serum IgE levels**

At day 7 and 21, the IgE levels of experimental groups were not different (Figure 1). But at day 35, compared with Group C the serum IgE levels in Group S were significantly increased \((p<0.05)\) and in Groups Im and Im + S they were significantly decreased \((p<0.05)\). However, the histamine levels among groups showed no significant differences in the jejunum.

**Effect of glycinin on small intestinal mucosa epithelium histology**

As shown in Figures 3–5, the structure of intestinal mucosa was damaged at varying degrees, intestinal villus was shortened obviously, crypt was descended and

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**Table 2. Effects of glycinin on growth performance in the experimental groups.**

| Item     | Experimental groups* | SEM | \(p\) Value |
|----------|-----------------------|-----|-------------|
| ADG, g/d | C 320.14^A           |      |             |
|          | Im 358.27^B           |      |             |
|          | Im + S 353.31^B       |      |             |
|          | S 215.02^B            |      |             |
| ADFI, g/d| C 461.25^A            |      |             |
|          | Im 486.06^A           |      |             |
|          | Im + S 490.40^A       |      |             |
|          | S 386.37^B            |      |             |
| G/F      | C 0.69^A              |      |             |
|          | Im 0.74^a             |      |             |
|          | Im + S 0.72^a         |      |             |
|          | S 0.56^a              |      |             |

* C, control group; Im, immunised group; Im + S, immunised and sensitised group; S, sensitised group; SEM, standard error of the mean \((n=10, \text{number of replicates}); ADG, average daily gain; ADFI, average daily feed intake; G/F, ratio of gain to feed.

Different letters in the same row denote significant differences \((p<0.05)\).

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**Table 3. Effects of glycinin on serum immunoglobulin G, A, M levels in the experimental groups (g/L).**

| Item | Age (day) | Experimental groups* | SEM | \(p\) Value |
|------|-----------|----------------------|-----|-------------|
| IgG  | 7 2.10     | C 2.21               |      |             |
|      |           | Im 1.99              |      |             |
|      |           | Im + S 2.18          |      |             |
|      | 21 2.18    | S 2.32               |      |             |
|      | 21 2.11^a  | Im 2.11^a            |      |             |
|      | 21 2.11^b  | Im + S 2.11^b        |      |             |
| IgA  | 7 0.31     | C 0.30               |      |             |
|      |           | Im 0.29              |      |             |
|      |           | Im + S 0.31          |      |             |
|      | 21 0.31    | S 0.34               |      |             |
|      | 21 0.34^a  | Im 0.34^a            |      |             |
|      | 21 0.34^b  | Im + S 0.34^b        |      |             |
| IgM  | 7 0.86     | C 0.84               |      |             |
|      |           | Im 0.80              |      |             |
|      |           | Im + S 0.85          |      |             |
|      | 21 0.48    | S 0.50               |      |             |
|      | 21 0.50^a  | Im 0.50^a            |      |             |
|      | 21 0.50^b  | Im + S 0.50^b        |      |             |
|      | 35 0.17    | C 0.18               |      |             |
|      |           | Im 0.17              |      |             |
|      |           | Im + S 0.16          |      |             |

* C, control group; Im, immunised group; Im + S, immunised and sensitised group; S, sensitised group; SEM, standard error of the mean \((n=10, \text{number of replicates); IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.}

Different letters in the same row denote significant differences \((p<0.05)\).
lots of eosinophils infiltrated duodenal lamina propria, and the ileal aggregate lymphatic nodule was damaged in piglets from Group S. Intestinal villi adhered to one another, mucosal epithelium was atrophied and a few eosinophils were detected in duodenal lamina propria, and the ileal aggregate lymphatic nodule was loose in Group C. Intestinal mucosal epithelium was clear and lined up in order, the structure of epithelium was intact and the nucleus was clear, and the ileal aggregate lymphatic nodule was compact in Group Im + S.

**Effect of glycinin on small intestinal villus height and crypt depth**

To investigate the effects of glycinin on intestinal mucosal reactions in piglets, the villous height and crypt depth were determined (Figures 6 and 7). Compared with Group C, in Groups Im and Im + S the villous height in duodenum and ileum was increased ($p < 0.05$), and the crypt depth was reduced ($p < 0.05$). At the same time, in Group S the villous height in duodenum and ileum was reduced ($p < 0.05$), and the crypt depth was greater ($p < 0.05$). In the jejunum of piglets, no significant differences concerning villous height and crypt depth were observed.

**Discussion**

Allergic reactivity of various soybean products represents a significant health problem, which occurs more frequently in the early life of humans and animals (Herian et al. 1993). Past studies typically observed immune function disorders and frequently associated diarrhoea (Li et al. 1990; Guo et al. 2007). In this study, a swine model was established for inducing an allergic
reaction to investigate the allergenic properties of soybean proteins. It was found that in Group S the performance of piglets was significantly reduced. This result referred to ADG, ADFI and G/F as well. On the contrary, in Groups Im and Im + S the ADFI and G/F were not decreased and ADG was significantly increased compared with the control group. The results showed that high levels of glycinin decreased the growth performance of piglets, yet the administration of glycinin in advance can alleviate allergic reactions effectively.

The blood of experimental piglets with diarrhoea contained high levels of anti-soybean-protein antibodies, mainly IgG (Dréau et al. 1995). In the present study, the serum IgG levels were significantly increased in 35-d-old piglets sensitised by glycinin. This indicated that glycinin peptides were absorbed into the blood and produced large quantities of IgG antibody in the serum, and they played an important role in the systemic immune response, which had been verified in previous studies (Helm et al. 2002; Gizzarelli et al. 2006).

Gastrointestinal food allergy is a food-stimulated IgE-mediated event. Soybean-allergic patients often have increased IgE antibodies in the sera (Shibasaki et al. 1980; Burks et al. 1988). IgE also plays a vital role in most food-induced allergies (Metcalfe et al. 1996). In the present experiment, in piglets of Group S which were directly injected with a high dose of glycinin, the serum total IgE levels were significantly increased. This indicated that a high dose of glycinin could enhance the secretion of serum IgE, which was consistent with results reported by Sun et al. (2008b). However, the serum IgE levels were significantly decreased in piglets immunised in advance by glycinin. This reaction was probably due to specific protective antibody IgG combined with the inhibitory receptor, which inhibited the secretion of IgE and eliminated the allergic reactions (Sun et al. 2008b).

Histamine release can be a criterion for the diagnosis of food allergy (Crockard & Ennis 2001) as patients manifest increased the release of histamine from the gastrointestinal mucosa after being sensitised by food allergen (He et al. 2004). Intracellular histamine levels can be used as an indicator of the sensitivity to histamine release, which means that lower intracellular histamine levels, more histamine is released by proliferative mast cells, and more histamine participates in the metabolism of the small intestine (Sun et al. 2008b).

In the present experiment, after glycinin sensitisation, the histamine levels of duodenum and ileum were significantly decreased in piglets, i.e. the histamine release was significantly increased, which was consistent with the previous results (Sun et al. 2008b; Huang et al. 2010). Excessive histamine release will promote the expansion and infiltration of local capillaries, which result in absorption of glycinin and cause damage to the digestive tract. However, the histamine release of duodenum and ileum was significantly reduced in piglets immunised in advance, which indicated that histamine release can be controlled by a prior immunisation with glycinin.

The damage to intestine integrity is a common phenomenon in animals consuming soybean proteins. Li et al. (1990, 1991a) reported that pigs had lower villus height but greater crypt depth after the ingestion of soybean protein. Dréau et al. (1994) observed epithelial cell hyperplasia in pigs fed soybean products.

In this study, glycinin caused various degrees of damage to the morphological structure of intestinal villi in weaned piglets. A similar observation was obtained by Chen et al. (2011), where glycinin could induce intestinal villous atrophy. These results indicate
that the occurrence of food allergy may correlate with the immune responses in the intestinal mucosa. However, after the piglets were immunised with glycinin prior to injecting soybean, the structural integrity of the intestinal mucosa was protected effectively. In addition, the damage phenomena of intestinal mucosa can be only observed in duodenum and ileum, which suggested the two parts of intestinal tracts were the main impairing position induced by glycinin.

Conclusions

This study showed that a direct sensitisation by glycinin caused a decrease of growth performance, an increase of serum IgG and IgE levels, and damage to intestinal mucosal integrity in piglets. However, after prior immunisation of piglets with glycinin the allergic reactions were reduced. This effect includes decreased serum IgE levels, improved ADG and a protection of the intestinal mucosa. The precise immune mechanism is not very clear and further studies are needed.

Disclosure statement

The authors report no conflicts of interest. The Authors alone are responsible for the content and writing of this article.

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