

Different thermal processing effects on peanut allergenicity

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Abstract

BACKGROUND: Peanut allergy is one of the most common food allergies worldwide. Studies have shown that the incidence of peanut allergies in Western-born Asians is higher than that in Asia-born Asians. Notably, Europeans and Americans mostly eat roasted peanuts, whereas Asians mostly eat boiled or fried peanuts.

RESULTS: BALB/c mice were sensitized using purified protein from raw, roasted or boiled peanuts, then fed the same by oral gavage. The relevant allergic reactions were studied using BALB/c mice model, including a rat basophilic leukemia (RBL) cell model, simulated gastric fluid experiments, and ultraviolet (UV) and circular dichroism (CD) spectral analysis. Serological studies showed increased levels of immunoglobulin E, interleukin-4 and interleukin-5, and pathological studies showed mast cell degranulation and inflammatory changes in jejunal tissues, with an increase in thymic stromal lymphopoietin (TSLP) gene expression in all treatment groups compared with the control group (phosphate-buffered saline). Compared with the raw peanut group, sera from the roasted peanut group produced a significant increase in RBL β -hexosaminidase A release *in vitro*, and roasted peanuts showed increased resistance to digestion in simulated gastric fluid experiments. Ultraviolet and CD spectral analyses showed that the roasting and boiling processes altered the structure of the major peanut allergens, which may have contributed to the differences observed in peanut allergenicity.

CONCLUSION: Our findings indicate that peanut allergies are related to peanut thermal processing methods. In our mouse model, the raw, roasted and boiled peanuts elicited different degrees of allergic response. Compared with raw peanut, roasted peanuts show a higher allergenicity, whereas the boiled peanuts show a lower allergenicity.

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Keywords: BALB/c mice; RBL-2H3 cells; Simulated gastric fluid; TSLP gene; CD spectrum; Peanut allergenicity

INTRODUCTION

Allergy is a common medical problem worldwide and peanuts are one of the most important food allergens.¹ It is estimated that about 2% of the population in western countries are allergic to peanuts and this number is increasing.² It was reported that about 0.8% of children and 0.6% of adults were allergic to peanuts in the USA in 2002, and that this rate in children had increased to 1.4% by 2008.³ In Canada, the percentage of children who are allergic to peanuts increased from 1.3% to 1.6% during 2007.⁴ The reasons for this increase are unclear, highlighting the need to develop new ways to prevent the activation of allergens that cause anaphylactic reaction, especially because up to 75% of people with known peanut allergies experience reactions as a result of accidental exposure (accounting for about 59% of allergy-related deaths reported).⁵ Furthermore, studies have shown that the incidence of peanut allergies in Western-born Asians is higher than that in Asians.⁶ Western populations are also more allergic to peanuts than Asian populations and are more likely to eat roasted peanuts, whereas Asians mostly eat boiled or fried peanuts.^{7,8} Therefore, different processing methods might affect the sensitization potential of peanut products. To confirm this hypothesis and determine the specific mechanism underlying this, further research is required.⁸ To date, 13 peanut allergen proteins have been recognized by the Allergen Nomenclature Sub-Committee of the International

Union of Immunological Societies.³ The number of these proteins or protein subunits was identified containing with more than half of plasma samples of specific peanut allergy immunoglobulin E (IgE) as the major allergen.⁹ The major allergens in peanuts are suggested to be Ara h 1, 2 and 3, as defined by frequent and prominent IgE binding from patient sera.¹⁰ The conformation of peanut proteins involves a primary structure (sequence of amino acids), secondary structure (formation of α -helices and β -sheets) and tertiary structure. The formation of α -helices and β -sheets is promoted by interactions between polypeptide chains linked together by hydrophobic and hydrophilic interactions, electrostatic interactions and disulfide bonds.¹¹ During thermal processing, these chemical interactions can change the conformation and structure of peanut proteins, including the allergens, which may protect consumers from threatening allergic reactions related to accidental peanut exposure.

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At present, many methods are used to study food allergy. Verma *et al.* studied sensitization to Indian black bean protein by analyzing several serological indicators *in vivo*, including the concentration of specific antibodies (IgE and IgG) as well as interleukin (IL)-4.¹² Plundrich *et al.* explored the effects of protein combined with fruit tea polyphenols on antibodies and peanut allergens by analyzing β -hexosaminidase A (β -HXA) release in rat basophilic leukemia (RBL)-2H3 cells.¹³ Zhou *et al.* studied sensitivity to milk proteins by observing the pathological changes in spleen and jejunum of mice.¹⁴ Li *et al.* detected increased expression of the TSLP gene in mouse enterocytes, demonstrating that medium-chain triglycerides have a facilitative effect on oral sensitization.^{15,16} Yang *et al.* investigated the stability of soybean protein after the Maillard reaction using simulated gastrointestinal digestion.¹⁷ Cai *et al.* showed that heat treatment affects the structure of Ara h 2.02 using circular dichroism (CD) spectroscopy, demonstrating an influence of thermal processing on the allergenicity of raw peanuts.¹⁸ Beyer *et al.* showed that differences in methods of peanut preparation had an important effect on peanut allergenicity. In Beyer's study, immunoblots were probed with sera from patients with peanut allergy. In addition, we determined the stability of peanut proteins in thermal processing by simulated gastrointestinal digestion experiments. We also detected structural changes in the major allergens of raw, roasted and boiled peanuts by ultraviolet and CD spectra. Our study used a variety of models to explore the allergenicity, stability and structural changes of peanut allergen proteins *in vivo* or *in vitro* respectively, so that our research is more in-depth, innovative and comprehensive. In China, because of relevant laws and policies, human serum is very difficult to obtain. BALB/c mice are commonly used as allergic animal models, which are inbred high-IgE-response strains. Dearman's study shows that BALB/c mice can respond to specific IgE and IgG by intraperitoneal injection of allergen including peanut agglutinin and ovalbumin (OVA). However, BALB/c mice cannot produce specific IgE after injection of potato acid phosphatase (PAP), which has no immunogenicity.^{19–21} His study shows that BALB/c mice may be an effective food allergy animal model. We therefore chose the BALB/c mouse model in our study. The sensitization method has great influence on the experimental results. Moghaddam's study involved many kinds of sensitization methods, including intragastric gavage sensitization, subcutaneous combined with intragastric gavage sensitization and epicutaneous combined with intragastric gavage sensitization.²² After comparing the sensitizations mentioned above, we choose subcutaneous combined with intragastric gavage as the sensitization method for our experiment, which is easily controlled and could produce a high immune response. To determine the effect that roasting and boiling treatments have on peanut sensitization, we sensitized BALB/c mice to raw, roasted or boiled peanut extracts, then fed the corresponding peanut homogenate by oral gavage. Serological and pathological changes as well as changes in TSLP gene expression were studied. Allergic reactions of RBL-2H3 cells *in vitro* were analyzed. Simulated gastrointestinal digestion experiments were conducted to determine the stability of peanut proteins following thermal processing. Ultraviolet and CD spectra were used to detect structural changes in the major allergens of raw, roasted and boiled peanuts. From a biochemical perspective, thermal processing promotes conformational changes in food proteins, which may affect the digestion and absorption of proteins or peptides by the intestinal epithelium, as well as their recognition by RBL immune cells. The results of this multilevel study suggest that roasting can increase peanut allergenicity, and boiling can

decrease peanut allergenicity, by changing the structure of several major peanut allergens. It is also necessary to determine the effect of different thermal processing on peanut allergenicity.

MATERIALS AND METHODS

Peanut allergen preparations

1. Raw peanuts (Nuo Qianjia brand, Shanghai Wal-Mart Supermarket), after removal of the red membrane, were roasted at 170 °C for 15 min in an oven (roasted peanuts) or boiled at 100 °C for 20 min (boiled peanuts).
2. To obtain raw peanut powder, raw peanuts were crushed with an HCP-200-type high-speed multi-function crusher (Flag Arrow Shredders, Shanghai, China) and then ground to a powder using a mortar and pestle. This method was also implemented to obtain roasted or boiled peanut powder.
3. Respectively 5 g raw, roasted or boiled peanut powder was placed in 100 mL of 20 mmol L⁻¹ Tris-HCl (pH 8.2) with stirring for 4 h. The homogenate was collected in a high-speed refrigerated centrifuge (Thermo Heraeus, USA) at 5000 × g for 30 min. The supernatant was then collected for injection into BALB/c mice for sensitization in the immune process.
4. In addition, 0.2 g raw, roasted or boiled peanut powder was added to phosphate-buffered saline (PBS) buffer (20 mL) in a centrifuge tube and placed in an ultrasonic grinding mill (Misonix Ltd, USA) to blend the emulsion for feeding mice in the immune process.

BALB/c mice sensitization protocols

Thirty-five 4-week-old specific-pathogen-free (SPF) female BALB/c mice were sourced from the Animal Experiment Center at Fudan University and randomly divided into four groups according to body weight as follows: control group (PBS; $n = 5$; the preliminary experimental result showed that mice in the control group were treated only with PBS, equivalent to normal growth; the various related data change and differences were not very obvious, so only five mice were in the control group); raw peanut group ($n = 10$), roasted peanut group ($n = 10$) and boiled peanut group ($n = 10$). All mice were kept in a specific-pathogen-free animal room for 10 days, with free access to food and drinking water.

Mice from all four groups underwent an immune sensitization process (Fig. 1A). On day 1, the mice were injected with 100 μ L peanut purified protein (about 100 μ g peanut powder). Mice in the four groups were perfused with 0.5 mL (about 5 mg peanut powder) on days 2, 3, 7, 14, 21 and 28. Gavage of 25 mg peanut was performed on day 35.

Determination of specific antibodies

On day 35, blood was collected from each group of mice, stored in a tube containing anticoagulant (ethylenediaminetetraacetic acid), and left at 4 °C overnight. The serum was then centrifuged at 3000 × g for 20 min and stored at -20 °C.

Peanut-specific total IgG, IgG1 and IgG2a (Thermo Fisher Scientific, USA) were determined using ELISA²³ and a serum titration series. Briefly, 96-well ELISA plates were coated with 1 μ g protein, left at 4 °C overnight and washed three times with PBS-Tween 20 (PBST) buffer. Skim milk powder (300 μ L per well) was added for blocking, and the plates were incubated at 37 °C for 1 h. Then, sera (200 μ L per well, 1:1000 dilution) was added, and the plates were incubated at 37 °C for 1 h. HRP-goat anti-mouse IgE (200 μ L per well, 1:5000 dilution) was then added, and the plates were

incubated at 37 °C for 1 h and washed three times. Then, tetramethylbenzidine solution was added in darkness and the plates were incubated for 15 min before 2 mol L⁻¹ H₂SO₄ (50 µL) was added as a stop solution, and the absorbance determined at 450 nm using an EMax Plus single-function optical absorption microplate reader (Molecular Devices Corporation, USA).

Specific IgG1 serum was diluted 200 000-fold and the secondary antibody was diluted at 1:80 000. Specific IgG2a serum was diluted 4 × 10⁴-fold and the secondary antibody was diluted at 1:5000.

Determination of cytokines, histamine and mouse mast cell protease-1 (mMCP-1)

IL-4, IL-5, interferon-γ (IFN-γ), histamine and mMCP-1 ELISA kits were purchased from Baoman Co. (Shanghai, China). Cryopreserved serum was retrieved and the concentrations of IL-4, IL-5, IFN-γ, histamine and mMCP-1 were measured by ELISA following the manufacturer's instructions.

Histopathological observation

Mice were sacrificed and blood was drawn after a large number of stimulations at 35 days. Hanks liquid (3 mL) was administered to the mice intraperitoneally, and the abdomen was gently rubbed for 1 min before the removal of Hank's liquid, which was used to observe degranulation in the intestinal fluid. Mice were exsanguinated and about 1 cm of jejunum was removed and placed in 4% paraformaldehyde. Hematoxylin and eosin (H&E) staining was performed to observe the infiltration of inflammation in the jejunum. Mast cells and basophils were stained with toluidine blue to observe changes in mast cell aggregation and degranulation in various tissues.²⁴

Determination of the relative expression of the TSLP gene in the small intestine of mice

Mice were dissected and total RNA was extracted from the intestine after thoroughly grinding the intestine with liquid nitrogen.²⁵ This total RNA was reverse transcribed to obtain cDNA, which was used as a template to measure the relative expression of the TSLP gene by real-time polymerase chain reaction.¹⁵

Determination of hexosaminidase release rate in RBL-2H3 cells

When the RBL-2H3 cells were in the logarithmic growth phase (5 × 10⁵ cells mL⁻¹), they were inoculated into 24-well plates (500 µL per well) and cultured for 24 h. After the cells were washed with PBS three times, serum samples (500 µL per well) were added and the cells cultured for 24 h. Cells were then washed three times with Tyrode's buffer and separated with Tyrode's buffer peanut protein solution (200 µL per well) for 60 min before placing in an icebox to terminate the reaction. Then, 50 µL of the supernatant was transferred to another 96-well plate, 5 mmol L⁻¹ pNAG (hexose) citric acid solution was added and the cells were incubated at 37 °C for 1 h. Sodium bicarbonate buffer (200 µL per well, pH 10.7) was then added to each well and the absorbance at 405 nm was measured with a microplate reader. A total release group was obtained by adding 1% Triton X-100 solution instead of peanut protein, and a blank control group was obtained by adding Tyrode's buffer instead of peanut protein-treated cells. The hexosaminidase release rate was calculated as follows: (test group absorbance – blank control group absorbance) / (released group absorbance – blank control group absorbance) × 100%.

Simulated gastric digestion of crude protein with different processed peanuts

For the first experiment, 190 µL of a 1 mol L⁻¹ pepsin solution (190 µL, pH 1.2) was added to a 1.5 mL centrifuge tube and kept at 37 °C in a water bath for 5 min; 10 µL of 5 g L⁻¹ sample protein and 70 µL of 0.2 mol L⁻¹ sodium bicarbonate solution were then added and the tube was vortexed and placed in an ice bath. Then, 135 µL loading buffer was added and the tube was incubated in a boiling water bath for 5 min before cooling at room temperature. For the second experiment, 1.9 mL of a 1 mol L⁻¹ pepsin solution (pH 1.2) was added to a 1.5 mL centrifuge tube and kept at 37 °C in a water bath for 5 min; 100 µL of 5 g L⁻¹ sample protein was then added, and the tube vortexed and placed in a 37 °C water bath for 60 min. At 15 s, 2 min, 30 min and 60 min, 200 µL of the reaction solution was transferred to a 1.5 mL centrifuge tube containing 70 µL of a 0.2 mol L⁻¹ sodium bicarbonate solution, and the tube was placed in an ice bath. Then, 135 µL of loading buffer was added and the tubes were boiled in a water bath for 5 min before being cooling at room temperature. SDS-PAGE samples were prepared for each time point.²⁰

Ultraviolet spectrum analysis

Ara h 1, Ara h 2 and Ara h 3 proteins were purified from raw, roasted and boiled peanuts. This involved anion exchange using a DEAE–Sephacrose Fast Flow column (16 × 250 nm), followed by 0.3–0.4 mol L⁻¹ NaCl linear gradient elution, three rounds of dialysis, and concentration using a 30KD Millipore membrane before storing at –20 °C. The proteins were analyzed using a UV–visible spectrophotometer (UV759CRT) with a scanning wavelength of 250–420 nm and a scanning rate of 100 nm min⁻¹.

CD spectroscopy

A Jasco J-815 spectropolarimeter (Jasco, MA, Japan) was used for CD measurements at room temperature. The concentration of Ara h 1, Ara h 2 and Ara h 3 was 10 µmol L⁻¹ for each protein in the far-UV region ranging from 190–250 nm. The ellipticity was recorded at a scanning speed of 500 nm min⁻¹, with three accumulations and a bandwidth of 0.1 nm. The experimental data were analyzed using CDpro software for protein secondary structure.

Statistical analysis

Data presented in the graphs are means ± standard error of the mean. Data analyses were performed using Graph Pad Prism software (La Jolla, CA, USA). Before statistical analysis, all data from *in vivo* studies were transformed logarithmically and checked for normality. Data were tested by one-way ANOVA with Bonferroni as a post hoc test, if not stated otherwise. Differences were considered significant when *P*-values were <0.05.

RESULTS

Effect of different processed peanuts on levels of specific antibodies and cytokines in mice

After mice were sensitized (Fig. 1A), levels of specific IgE and Th2-type cytokines, including IL-4 and IL-5, were upregulated in all peanut-fed mice, whereas IgG2a and Th1-type cytokines, such as IFN-γ, were downregulated compared with control mice (treated with PBS). There was a dominant IgG1/IgG2a bias (Fig. 1B–F). Compared with raw peanuts, roasted peanuts showed higher levels, and boiled peanuts showed lower levels, of specific IgE and Th2-type cytokines. This indicates that roasted peanuts

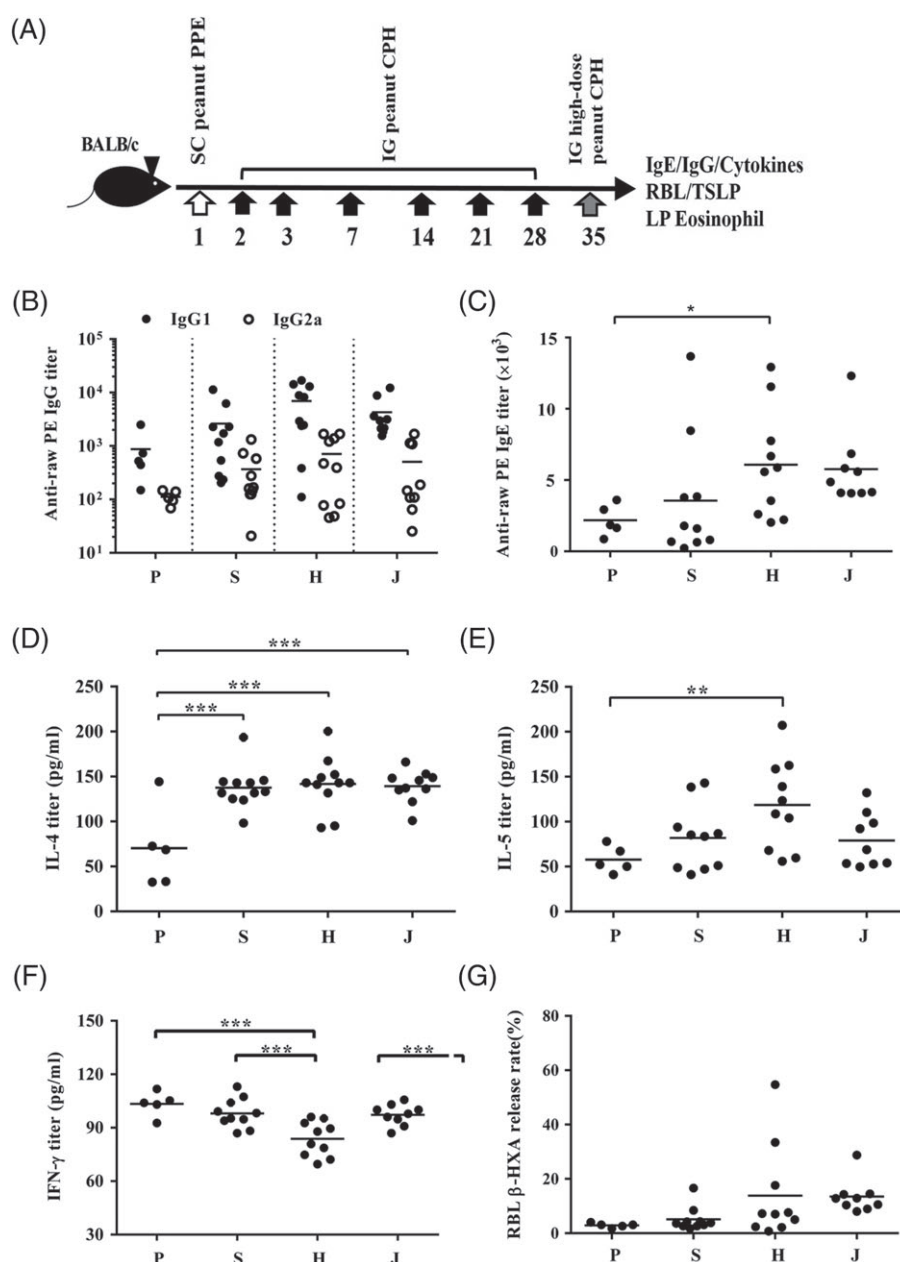


Figure 1. Effects of different processed peanuts on specific antibodies and cytokines in mice: (A) immunization procedure; levels of IgG1 and IgG2a; (B) IgE; (C) IL-4; (D) IL-5; (E) and IFN- γ ; (F) in the serum of BALB/c mice; (G) β -HXA release rate (%) in RBL cells. P, PBS; C, raw peanut; R, roasted peanut; B, boiled peanut (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.005$).

induced higher allergenicity and boiled peanuts induced lower allergenicity.

An RBL cell degranulation assay confirmed these results. Compared with the raw peanut group, the roasted peanut group had a significantly greater titer of IgE, which led to RBL degranulation and increased β -HXA release, whereas the boiled peanut group had a lower titer of IgE and decreased β -HXA release. This demonstrates that roasted peanuts significantly increased allergenicity, and boiled peanuts reduced allergenicity *in vitro* (Fig. 1G).

Effect of different processed peanuts on mast cells from the intestine of mice

Cells from the solution of intestinal tissue were examined using toluidine blue staining. As shown in Fig. 2(A), basophils and mast

cells showed significant degranulation in the roasted and boiled peanut groups. In addition, mucosal mast cell degranulation was assessed by measuring serum mMCP-1.²⁶ Degranulation of basophils and mast cells causes the release of histamines, and in the sera we found that titers of mMCP-1 and histamine in the roasted peanut group were the highest, and those of the boiled peanut group were the lowest, of all groups (Fig. 2B,C). This further indicates that roasting enhances peanut allergenicity and boiling reduces peanut allergenicity.

Effect of different processed peanuts on TSLP gene expression in mice

Mouse jejunal tissues were sectioned and stained with H&E for examination under a microscope. Greater numbers of intestinal

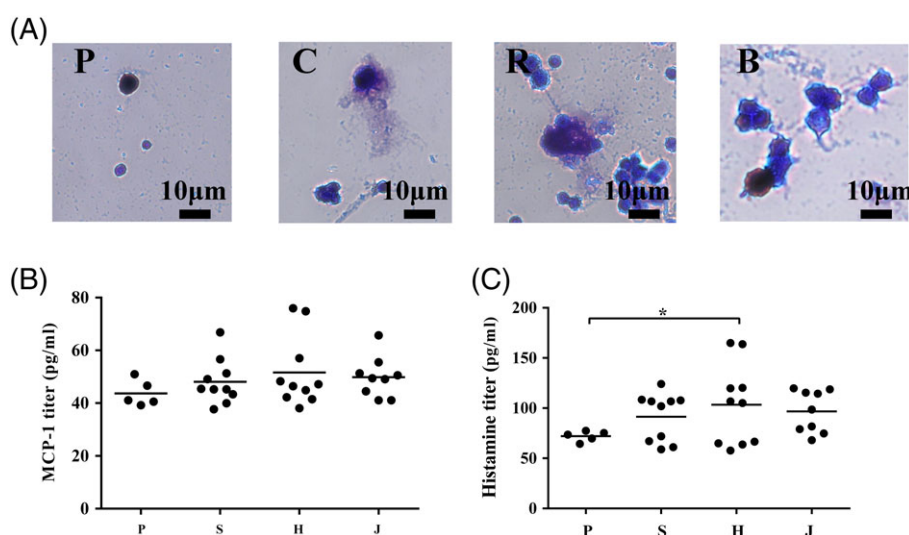


Figure 2. Effects of different processed peanuts on the degranulation of mast cells in mice. (A) Degranulation of mast cells under a microscope. Blue cells are mast cells and purple cells are basophils ($\times 200$). (B) Levels of mMCP-1 in the serum of BALB/c mice. (C) Levels of histamine in the serum of BALB/c mice (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.005$). P, PBS; C, raw peanut; R, roasted peanut; B, boiled peanut.

villus tips were ulcerated in the roasted peanut group compared with the other groups (Fig. 3A). Recent reports indicate that TSLP released by intestinal epithelial cells contributes to allergic sensitization by releasing Th2 type cytokines.²⁷ To determine whether the effect of roasted peanuts was associated with increased TSLP expression, we measured intestinal epithelial *Tslp* mRNA in jejunal epithelia. As shown in Fig. 3(B), in contrast to mice administered boiled peanuts, mice administered roasted peanuts had significantly higher *Tslp* mRNA values in jejunal epithelial cells than mice administered raw peanuts, indicating that roasting contributes to allergic sensitization.

Differences in the stability of different crude peanut proteins by simulated gastrointestinal digestion

The crude protein of raw, roasted and boiled peanuts was digested in simulated gastric fluid to observe the effect on peanut sensitizing proteins (Fig. 4). These crude proteins underwent varying degrees of degradation by simulated gastrointestinal digestion. Ara h 3 (14–45 kDa) in raw peanut protein was significantly degraded by simulated gastric fluid by 15 s, and Ara h 2 (17–20 kDa) was most degraded after digestion for 60 min (Fig. 4A). Ara h 1 (65 kDa) and Ara h 3 in roasted peanuts were significantly more resistant to digestion compared with those in raw peanuts, and Ara h 3 was not digested after 2 min of digestion.¹⁰ Ara h 2 in roasted peanuts was observed after digestion for 60 min (Fig. 4B) and a new enzyme-resistant band was present at 25 kDa after 30 min of digestion. Ara h 1 and Ara h 3 in boiled peanuts were significantly degraded by simulated gastric fluid for 15 s, and Ara h 2 (17–20 kDa) was most degraded after digestion for 60 min (Fig. 4C). This indicates that the sensitizing proteins in roasted peanuts were more resistant to pepsin digestion than those of raw and boiled peanuts, indicating that roasted peanuts have a greater sensitization potential.

Effects of thermal processing on the structure of major peanut allergens

Ultraviolet spectra were used to analyze the structures of Ara h 1, Ara h 2 and Ara h 3 in raw, roasted and boiled peanuts (Fig. 5A–C). Absorbance values of Ara h 1, Ara h 2 and Ara h 3 at 280 nm

were increased after roasting and reduced after boiling, indicating that roasting causes extensive denaturation and destruction of the structure, and might expose amino acid residues embedded in these proteins. Far-UV CD spectra were used to analyze the secondary structures of Ara h 1, Ara h 2 and Ara h 3 (Fig. 5D–F). The percentage of conformational units was calculated using CDPro software (Fig. 5G–I). Compared with raw peanuts, the content of different conformational units changed after roasting. In roasted peanut Ara h 1, a significant shift in was observed with a decrease in the content of α -helices, β -sheets, turns and random coils from 5.23% to 4.32%, 43.2% to 37.1%, 22.4% to 22.1% and 27.3% to 25.3%, respectively. Roasted peanut Ara h 2 showed a decrease in the content of α -helices and random coils from 6.61% to 5.04% and 30.7% to 20.6%, respectively, and an increase in the content of β -sheets and turns from 43.3% to 55.6% and 19.3% to 25.1%, respectively. Roasted peanut Ara h 3 showed a decrease in the content of turns and random coils from 25.3% to 24.1% and 35.2% to 23.9%, respectively, and an increase in the content of α -helices and β -sheets from 4.49% to 17.4% and 30.1% to 38.1%, respectively. Compared with raw peanuts, the content of different conformational units also changed after boiling. Boiled peanut Ara h 1 showed an increase in the content of α -helices, β -sheets, turns and random coils from 5.23% to 7.03%, 43.2% to 50.5%, 22.4% to 24.1% and 27.3% to 27.6%, respectively. Boiled peanut Ara h 2 showed a decrease in the content of β -sheets from 43.3% to 19.8% and an increase in the content of α -helices, turns and random coils from 6.6% to 13.9%, 19.3% to 30.1% and 30.7% to 37.6%, respectively. Boiled peanut Ara h 3 showed a decrease in the content of β -sheets, turns and random coils from 30.1% to 29.6%, 25.3% to 22.9% and 35.2% to 29.1%, respectively, and an increase in the content of α -helices from 4.49% to 18.7%. These results show that the secondary structures of these proteins were affected after roasting and boiling.

DISCUSSION

In this study, mice sensitized to raw, roasted or boiled peanuts and then administered the same by oral gavage showed a dominant IgG1/IgG2a bias and higher IgE titers compared with the

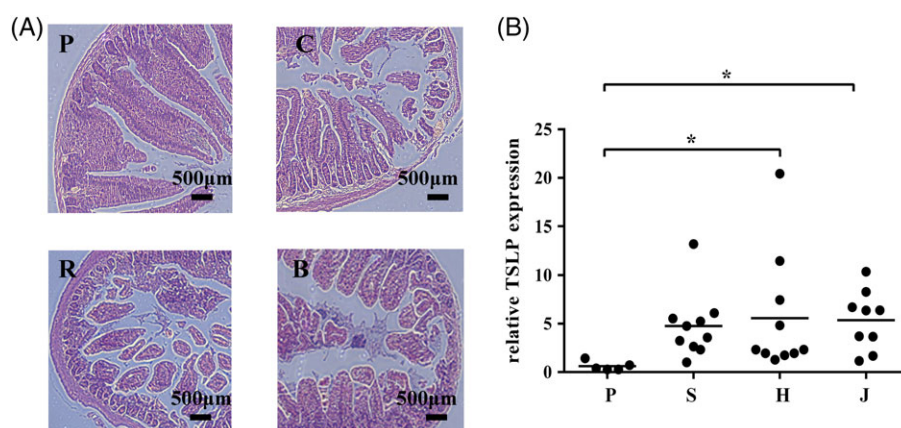


Figure 3. Effects of different processed peanuts on jejunum structure and relative expression of the TSLP gene. (A) Pathological sections of jejunum (H&E staining, $\times 200$). (B) Relative expression of TSLP (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.005$). P, PBS group; C, raw peanut group; R, roasted peanut group; B, boiled peanut.

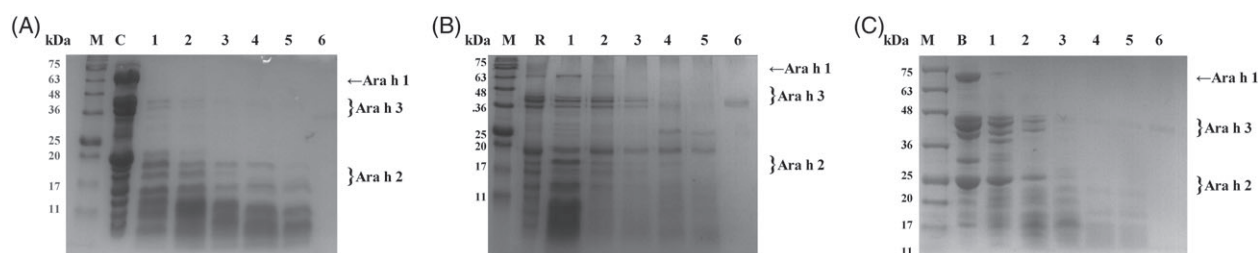


Figure 4. Differences in the simulated gastric fluid digestion of peanut products. SDS-PAGE analysis of (A) raw peanuts, (B) roasted peanuts, (C) boiled peanuts. M, molecular weight markers. Numbers indicate the profile at specific time points during digestion. C, raw peanut group; R, roasted peanut group; B, boiled peanut.

control group (Fig. 1A, B). In addition, Th2 cells in BALB/c mice were overactivated after the gavage, and showed IgG1-mediated allergy and hypersensitivity with mast cell degranulation as well as increased IgE titers.²⁸ Notably, mice fed with roasted peanuts had higher IgE and IgG titers, and those fed with boiled peanuts had lower IgE and IgG titers, than those fed with raw peanuts (Fig. 1A, B). This indicates that roasted peanuts have a stronger immunogenic potential, and boiled peanuts have a weaker immunogenic potential, than raw peanuts. Compared with the raw peanut group, the roasted peanut group also had an increased titer of Th2 cytokines (IL-4 and IL-5) and a decreased titer of a Th1 cytokine (IFN- γ) (Fig. 1D–F). Generally, during anaphylactic reactions, the release of Th2 cytokines such as IL-4 and IL-5 are increased and Th1 cytokines such as IFN- γ are decreased.²² Therefore, these results indicate that roasted peanuts are stronger sensitizers of peanut allergy than boiled peanuts.

Antibody and cytokine titers in sera are indicators of anaphylactic reactions. For example, IgE has a critical role in immune pathways in combination with mast cells and basophilic granulocytes.²⁹ Here, mast cell and basophil degranulation were clearly observed. Moreover, the concentration of mMCP-1 and histamine was highest in the roasted peanut group and lowest in the boiled peanut group (Fig. 2). Allergic reactions usually depend on the activation of the peanut-specific IgE receptor (Fc ϵ RI). When peanut allergens enter the body, they crosslink Fc ϵ RI on mast cells and initiate a signaling cascade that eventually results in degranulation, cytokine and chemokine production (mMCP-1), and histamine release. Mucosal mast cell degranulation was assessed by measuring the concentration of mMCP-1 in serum.²⁶ Histamine is present in skin cells, the bronchial mucosa, intestinal

mucosa and nervous system. Mediators (such as histamine and leukotriene) released by mast cells and basophilic granulocytes cause intestinal villus fracture, jejunal damage and intestinal villus disappearance.³⁰ Our pathological studies and TSLP gene expression analyses showed inflammation in jejunal tissues and upregulated TSLP gene expression in mice receiving raw, roasted or boiled peanuts, but especially in those receiving roasted peanuts (Fig. 3A), indicating a higher sensitization potential for roasted peanuts. To determine the mechanisms involved in the anaphylactic reactions of mice, gene-level analysis was performed. TSLP, a gene expressed by intestinal epithelial cells, contributes to Th2-type immunoreactions.³¹ We found that TSLP gene expression was increased in peanut-treated groups compared with the control group, and was greater in the roasted peanut group than the other groups (Fig. 3B), indicating that larger amounts of cytokines might be produced.

To further investigate the allergenicity of roasted and boiled peanuts, an *in vitro* experiment was performed. The RBL-2H3 cell line, which is similar to mast cells and basophilic granulocytes *in vivo*, is widely used to evaluate the immunogenicity of allergens because of its ability to combine with IgE and to release mediators (histamine and β -HXA).²² In this experiment, serum samples from mice from the roasted peanut group induced significantly increased β -HXA production compared with those from the raw and boiled peanut groups (Fig. 1G). β -HXA release is usually accompanied by histamine release, and therefore used to determine mast cell and basophil degranulation. This result also supports the above conclusions.

Our results show that roasting increases peanut allergenicity and boiling decreases peanut allergenicity. Protein digestion is

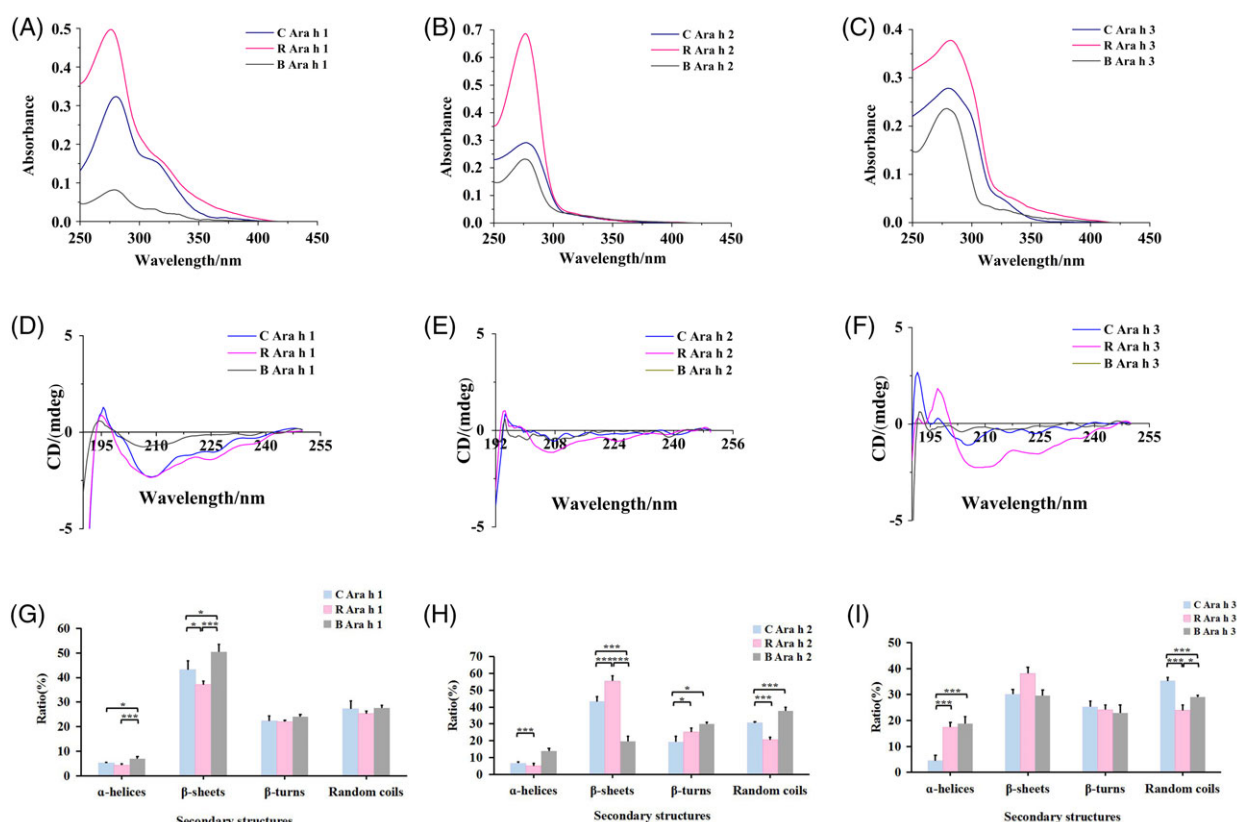


Figure 5. Analysis of heating treatment on the major allergen structures of peanuts. (A–C) Absorbance values at 280 nm of Ara h 1, Ara h 2 and Ara h 3. (D–F) CD spectra of Ara h 1, Ara h 2 and Ara h 3. (G–I) The altered conformational units of Ara h 1, Ara h 2 and Ara h 3. C, raw peanut group; R, roasted peanut group; B, boiled peanut.

commonly evaluated to assess allergenicity.³² Roasted peanut proteins showed greater stability than raw or boiled peanut proteins, and boiled peanut proteins showed a lower stability in our simulated gastric fluid (SGF) experiment (Fig. 4). This high stability of roasted peanut proteins might explain its higher allergenicity, and likewise the low stability of boiled peanut proteins might explain its weaker allergenicity. Furthermore, as protein structure is thought to be the basis of protein function, changes in allergen conformation might alter allergenicity.³³ UV spectra were used to analyze the structure of major peanut allergens (Ara h 1, Ara h 2 and Ara h 3) from raw, roasted and boiled peanuts. The absorbance values of these allergens at 280 nm were increased after roasting and decreased after boiling compared with the raw state (Figs. 5A–C), indicating that roasting caused extensive denaturation and destruction of the structure, and may have exposed parts of the amino acid residues. CD spectra used to analyze the secondary structure of raw, roasted and boiled peanut allergens indicated that the secondary structure of raw peanuts was affected by roasting or boiling (Figs. 5G–I). This may also explain the difference in allergenicity between roasted and boiled peanuts. In conclusion, our results indicate that roasting and boiling cause a marked change in the structure of the major peanut allergens (Ara h 1, Ara h 2 and Ara h 3), which increased and decreased allergenicity, respectively. We performed a series of experiments involving BALB/c mice sensitized to raw, roasted or boiled peanuts and then fed the same by oral gavage, and recognition of their sera by RBL immune cells. Serological and pathological changes as well as changes in TSLP gene expression were studied, and allergic reactions of RBL-2H3 cells *in vitro* were

analyzed. Simulated gastrointestinal digestion experiments were conducted to determine the stability of peanut proteins after thermal processing. UV and CD spectra were used to detect structural changes in the major allergens of raw, roasted and boiled peanuts. Therefore, we performed a variety of experiments to discuss the effect of different thermal processing on peanut allergenicity. In the BALB/c mouse model, serological and pathological changes were studied. Allergic reactions of RBL-2H3 cells *in vitro* were analyzed. Finally, our study shows that thermal treatment can affect the allergenicity of peanut through changing the structure of major peanut allergen. In conclusion, compared with raw peanut, roasted peanuts show a higher allergenicity, whereas boiled peanuts show a lower allergenicity. This may be one of the reason why Westerners, who tend to eat roasted peanuts and peanut butter made from roasted peanuts, were more likely to have allergies. Furthermore, those people who are sensitive to peanuts should instead try eating boiled peanuts to decrease their allergy risk.

CONCLUSIONS

This study shows that raw, roasted and boiled peanuts have sensitization potential in BALB/c mice compared with raw peanuts, roasted peanuts having higher, and boiled peanuts lower, sensitization potential as shown using animal models, cell models and TSLP gene expression levels. On the basis of our simulated gastric fluid experiments and UV and CD spectra, we speculate that these differences in allergenicity result from changes in peanut allergen structure caused by the roasting and boiling processes. The results of simulated gastric fluid experiments

show that roasted peanuts may contain some digestion-resistant allergenicity protein, which is probably the reason for roasted peanuts having stronger sensitization. Figure 4 also shows that a new molecular fragment appears, so in the future the allergenic study of roasted peanuts could revolve around the roasted peanut protein structure.

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