

Effect of roasted peanut allergen Ara h 3 protein on the sensitization of Caco-2 cells

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Abstract

Background: Roasted peanut is widely loved as a kind of food with rich taste. However, peanut allergy is one of the major threats to human health, which affects about 5% of children and 1.4-2% of adults in the world.

Results: To evaluate the sensitization mechanism of peanut allergen Ara h 3, Caco-2 cells as the model, which has the similar structure and function to differentiated small intestinal epithelial cells. Compared with Ara h 3-raw (purified from raw peanut) group, more significant results such as the inhibited Caco-2 cell viability and proliferation, the increased secretion of ROS and the decreased transepithelial electrical resistance were obtained in Ara h 3-roasted (purified from roasted peanut) group. Accordingly, oxidative stress and NF- κ B signaling pathway were more imbalanced, which lead to the increased of TSLP, IL-6, IL-8 and MCP-1. Then the gene expression of tight junction proteins ZO-1, occludin and JAM-1 were down more, which proved the integrity of Caco-2 monolayer barrier is severely damaged.

Conclusion: These finding identify the mechanisms of the allergenicity of roasted peanut allergy proteins are probably associated with intestinal uptake and cytokine-dependent allergies. The aggravated allergic reaction might be caused by the increment of TSLP, IL-6, IL-8 and MCP-1 due to the activated NF- κ B signaling pathway, and the enhanced transport of Ara h 3-roasted protein by Caco-2 monolayer.

Keywords: roasted peanut; Caco-2 cells; Inflammatory factor; Oxidative stress; NF- κ B signaling pathway

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the [Version of Record](#). Please cite this article as doi: [10.1002/jsfa.11180](https://doi.org/10.1002/jsfa.11180)

1. Introduction

Although the rich taste of roasted peanuts is widely loved, peanut allergy is one of the major threats to human health, accounting for 90% of all food allergy cases¹. As an immune response mediated by immunoglobulin E (IgE)², peanut allergy has attracted extensive attention because of its universality, high incidence, long-term effects on health, and low sensitization dose. Since the first case of peanut allergy was found in 1925, 17 kinds of peanut sensitizing proteins (Ara h 1–17) have been generally accepted and officially named. Main peanut allergens are defined as those which are found in high concentrations, and which demonstrate specific binding to IgE in the serum of more than 50% of allergic patients. Main allergens include Ara h 1, Ara h 2, Ara h 3, and Ara h 6³. In its natural state, Ara h 3 is a hexamer protein with multiple subunits⁴ and a molecular weight of more than 400 kDa. Four main bands of Ara h 3 can be observed using SDS-PAGE electrophoresis to denature and separate the protein subunits. These bands include three acidic subunits with molecular masses of 42–45 kDa, and a basic subunit with a molecular mass of 25 kDa⁵. Rao et al.⁶ found that after peanuts were roasted, the hydrophobic interaction between peanut proteins changed, and protein solubility decreased with time. These effects were observed strongly in Ara h 1 and Ara h 3. When the roasting temperature was over 140°C or the roasting time was more than 20 min, the hydrophobic index of the protein surface was the highest. Under these conditions, the allergen proteins showed a decrease in α -helices, an increase in β -folds and random coils, and a loose secondary structure. These conformational changes allow for exposure of the binding epitopes of allergens with IgE, which results in significantly enhanced binding ability to IgE. Consequently, the changes to peanut allergen proteins conferred by roasting lead to a significant increase in peanut allergenicity.

Caco-2 cells, which have been cloned from a line of human colorectal adenocarcinoma cells, have a similar structure and function to small intestinal epithelial cells. They have been widely used to predict the permeability of intestinal mucosa to allergen proteins in the past 20 years⁷. Studies by Roth-Walter et al. showed that soluble milk β -lactoglobulin allergens could induce Caco-2 cells to produce specific IgE antibodies, Th2 cytokines IL-6, IL-8, IL-15, and thymic stromal lymphopoietin (TSLP)⁸. In addition, studies show that Caco-2 cells can be cultured in a transwell system to form a monolayer^{9, 10, 34}. The integrity of the monolayer can be determined using transepithelial electrical resistance (TEER) and by looking at expression levels of tight junction protein (TJ), occludin, ZO-1, and JAM-1, which are closely

related to oxidative stress and protein transport¹¹. Caco-2 cells have been shown to be the best model to study intestinal allergen absorption and intestinal toxicity¹⁹. Hurley et al.²⁰ found that the intestinal permeability and allergen-passing capacity of sensitized individuals were enhanced compared with normal individuals. Therefore, evaluation of allergen transport through the intestinal barrier and its effect on epithelial cell permeability may be an important parameter for allergen risk assessment^{16, 21}.

In terms of oxidative stress, the catalytic subunit gp91phox (Nox2) of triphosphopyridine nucleotide (NADPH) oxidase and its homologs Nox1, Nox3, Nox4, Nox5, DUOX1, and DUOX2 are collectively referred to as the Nox family¹². These core subunits are key for the function of NADPH oxidase¹³. As a signal molecule, reactive oxygen species (ROS) produced by NADPH oxidase in non-phagocytic cells participate in the signal transduction pathway and regulate cell differentiation, proliferation, aging, and apoptosis¹⁴. With abnormal expression of Nox family proteins, ROS levels increase sharply, which can induce a number of changes, including activation of the NF- κ B signal pathway. This pathway is important for inflammatory diseases and immune response¹⁵⁻¹⁷. It is involved in several immune response processes, including malignant transformation, the development and survival of lymphocytes and lymphoid organs, and overall control of the immune response¹⁸. A series of immunoregulation processes of antigen entering Caco-2 cells, such as activation of NF signaling pathway and release of inflammatory factors such, are shown in Fig 1.

With an increasing number of roasted peanuts appearing in people's diets, the allergenicity of roasted peanuts has been widely studied. However, most of the studies are based on a single *in vitro* experiment, and no prior study has focused on the effect of roasted peanut allergens on intestinal epithelial cells of the gastrointestinal immune system. In view of this, Caco-2 cells were incubated with purified raw Ara h 3 protein (Ara h 3-raw) and purified roasted Ara h 3 protein (Ara h 3-roasted). The effects of Ara h 3 on the sensitization of Caco-2 cells were studied by measuring viability and proliferation of Caco-2 cells, expression of Nox family proteins, ROS content, activation of the NF- κ B signaling pathway, TSLP levels, presence of proinflammatory factors, and integrity of the cell monolayer barrier. Our research aims to clarify the mechanism of peanut allergy and provide a theoretical basis for the development of new desensitization methods.

2. Materials and methods

2.1 Peanut protein extraction

After removing the red coat surrounding the peanuts, peanut seeds (NuoQian, China) were weighed

and separated into five-gram groups, roasted at 150°C for 20 min, and then crushed with an HCP-200 high-speed grinder (Qijian, China). Each 5-gram group of peanut powder was added to 20 mL Tris-HCl buffer solution, stirred at 4°C for 30 min, and then centrifuged at 4000 x g for 30 min. The supernatant was collected and filtered through a 0.45 µm filtration membrane. The purified Ara h 3-raw and Ara h 3-roasted were obtained using an AKTA pure protein purifier (General Electric, USA) and a HiTrap Q HP precolumn (General Electric, USA). Peanut proteins were separated by anion exchange chromatography. The protein was filtered through a 0.22 µm filter (PerkinElmer, USA) and then loaded into a 5 ml sample loop (General Electric, USA), which was connected to the purifier. HiTrap Q HP precolumn (General Electric, USA) was then equilibrated with buffer A (20 mM Tris-HCl, pH 8.8). During the equilibrium process, the protein in the sample loop was loaded into the precolumn. Next, buffer A and buffer B (20 mM Tris HCl, 1 M NaCl, pH 8.8) were mixed to form a 0-1 M NaCl gradient to elute the protein in the precolumn. The whole purification process was carried out in 4°C chromatographic cabinet (General Electric, USA). The eluent with protein peak was collected to obtain purified Ara h 3-raw and Ara h 3-roasted protein.

2.2 Culture of Caco-2 cells

The human colorectal cancer cell line Caco-2 (ZhongQiao, China) was continuously passaged in a 25 cm² T25 plastic culture bottle (generations 20–40). The cell culture medium was composed of RPMI cell culture medium (Gibco, USA), 10% fetal bovine serum (FBS) and 1% double antibody (Aibo, China). The cells were cultured at 37°C and 5% CO₂. The cells were cultured for 2–3 days to achieve fusion, and to assist in the process of cell growth and differentiation, fresh culture medium was added daily. The growing cells were digested with trypsin and passaged continuously at a ratio of 1:2. All experiments were performed in serum-free and phenol red-free RPMI.

2.3 Detection of cell viability and proliferation of Caco-2 cells

2.3.1 Detection of Caco-2 cell viability

Caco-2 cells were seeded in a 96-well culture plate at a density of 1×10⁴ cells/well and grown in 100 µL of complete medium for 7 days. The medium was changed every 2 days and Caco-2 cells were starved for 2 h before the experiment. Cells were treated with different concentrations of Ara h 3-raw and Ara h 3-roasted (10, 20, 40, 80, 100 µg/mL) serum-free medium or separate medium (control). Cell viability was detected by Cell Counting Kit-8 (Sangon biotech, China). After 24 h of incubation, 10 µL of

Cell Counting Kit-8(CCK-8) solution was added to each well, and incubated for 2 h in the incubator. The absorbance (OD) at 450 nm was determined using a multi-functional optical absorption enzyme-labeled instrument (Molecular Devices, USA). Cell viability was expressed as a percentage using the following expression:

$$\text{Cell viability(\%)} = \frac{\text{ODt} - \text{ODb}}{(\text{ODc} - \text{ODb})} \times 100$$

Here, ODt and ODc are the optical densities (absorbances) of the treated and untreated cell dispersions, respectively, and ODb is the optical density of the cell-free solution.

2.3.2 Detection of Caco-2 cell proliferation

1) Detection of cell proliferation via fluorescence microscopy

The proliferation of Caco-2 cells was evaluated using an 5-Ethynyl-2'-deoxyuridine (EdU) cell proliferation assay kit (AmyJet, China). Caco-2 cells were seeded in 24-well plates at a density of 2.5×10^4 cells per well. They were grown in complete medium for 7 days and starved for 2 h. After removing the culture medium, five different concentrations of Ara h 3-raw and Ara h 3-roasted (10, 20, 40, 80, 100 $\mu\text{g/mL}$) were incubated for 24 h in serum-free medium. Additionally, a control group consisting of a single culture medium without any allergens was incubated for 24 h. After 24 h, the culture medium was removed, and 100 μL of medium containing 50 μL EdU was added into each well and incubated for 2 h. The medium was discarded, and the cells were washed with PBS. The cells were fixed with 4% formaldehyde and 100 μL $1 \times$ Apollo[®], and then stained with Hoechst 33258 after incubation for 30 min. Fluorescence images of EdU and Hoechst were observed by fluorescence microscopy (Olympus, Japan). The excitation wavelength was 350 nm and the emission wavelength was 461 nm. Using Caco-2 cells treated with a single medium as a blank control, the ratio of EdU-labeled Caco-2 cells in any unit (i.e., cell proliferation rate) was calculated and set to 100%. The proliferation rate of Caco-2 cells in the different treatment groups was compared, and the experimental data were analyzed using ImageJ software.

2) Detection of cell proliferation via flow cytometry

The treatment of Ara h 3-raw and Ara h 3-roasted was the same as above. After treatment, the cells were placed in EdU medium and incubated for 2 h. The cells were then digested and transferred into a 1.5 mL flow tube and fixed with formaldehyde and 100 μL $1 \times$ Apollo[®]. After 30 min of incubation, the cells were stained with Hoechst 33258, and then immediately observed using flow cytometry (Beckman Coulter MoFlo XDP, USA). The excitation wavelength was 350 nm and the emission wavelength was

461 nm. Caco-2 cells without EdU formed the negative control group (blank), while Caco-2 cells with EdU formed the positive control group (control). The experimental data were analyzed using FlowJo software.

2.4 Uptake of Ara h 3 by Caco-2 cells

1) FITC-labeled protein

According to the manufacturer's instructions (Sigma-Aldrich, USA), fluorescein isothiocyanate (FITC) was dissolved in NaCO₃-NaHCO₃ buffer solution at the concentration of 5:1, 10:1, and 20:1, and incubated with 5 mg/mL Ara h 3-raw and Ara h 3-roasted for 2 h. A Sephadex G-25M pre-affinity column (Sigma-Aldrich, USA) was used to separate FITC-labeled conjugate from unreacted FITC, and detected the absorbance at 280 nm and 495 nm. The proteins with A₂₈₀ between 0.2 and 1.0 were then collected. The absorption coefficients of 1.0 mg/mL protein solutions without FITC labeling were determined at 280 nm and were named E₂₈₀^{0.1%}. The concentration of the sample and the binding ratio of FITC to protein (molar F/P) were calculated by the formula below, and the sample was kept away from light at -20 °C until use.

$$C = \frac{MW_{Ara\ h\ 3} \times E_{280}^{0.1\%}}{389 \times 195}$$

$$\text{Molar F/P} = \frac{A_{495} \times C}{A_{280} - (0.35 \times A_{495})}$$

MW_{Ara h 3}: Molecular weight of Ara h 3 protein ; E₂₈₀^{0.1%}: Absorbance of protein (1.0 mg/ml) at 280 nm; C:Protein constant, it can be obtained by formula calculation; A₄₉₅: The absorption values of the conjugates at 495 nm; A₂₈₀: The absorption values of the conjugates at 280 nm ; Molar F/P: The molar ratio of FITC to protein in fluorescein protein conjugate, conjugates with Molar F/P values between 0.3 and 1.0 can be used in subsequent experiments.

2) Detection of Ara h 3 uptake via fluorescence microscopy

Caco-2 cells were seeded in 24-well plates at a density of 2.5×10⁴ cells per well for 7 days. Ara h 3-raw and Ara h 3-roasted proteins were labeled with FITC. After starvation for 2 h, samples of Ara h 3-raw and Ara h 3-roasted proteins were placed in serum-free medium at a concentration of 40 µg/mL and incubated for 12, 24, 36, 48 h. After removing the culture medium, the cells were washed three times with PBS buffer at 37°C. DAPI was used for nuclear staining, and stained samples were observed using fluorescence microscopy. When observed with a fluorescence microscope (Olympus, Japan), the excitation wavelength was 490 nm and the emission wavelength was 525 nm. The experimental data

were analyzed using ImageJ software.

3) Detection of Ara h 3 uptake via flow cytometry

Caco-2 cells were seeded in 24-well plates at a density of 2.5×10^4 cells per well for 7 days. Ara h 3-raw and Ara h 3-roasted proteins were labeled with FITC. After starvation for 2 h, samples of Ara h 3-raw and Ara h 3-roasted protein were placed in serum-free medium at a concentration of 40 $\mu\text{g/mL}$ and incubated for 12, 24, 36, 48 h. After removing the culture medium, the cells were washed three times with PBS buffer at 37 °C. DAPI was used for nuclear staining, and stained samples were observed using fluorescence microscopy. Flow cytometry (Beckman Coulter MoFlo XDP, USA) was used to detect the experimental results. Caco-2 cells treated with culture medium alone were set as the blank group, and the right area P2 of the negative peak was drawn. In the same region, the P2 value of the experimental group was the ratio of Caco-2 cells that absorbed Ara h 3 to all cells, that is, the rate of Ara h 3-positive cells.

2.5 Detection of ROS content and NADPH oxidase gene expression

2.5.1 Detection of ROS content

Lipopolysaccharide (LPS) was used as a positive control to induce the immune response of Caco-2 cells. Caco-2 cells were treated with 40 $\mu\text{g/mL}$ Ara h 3-raw or Ara h 3-roasted, or 1 $\mu\text{g/mL}$ LPS for 30, 60, 90, 120, 150, 180 min. Next, 10 $\mu\text{mol/L}$ 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA, ROS-sensitive probe) was added to Caco-2 cells and the resulting solution was incubated for 30 min. The cells were washed with PBS buffer three times, digested with trypsin, and resuspended in PBS. The fluorescence intensity was detected by flow cytometry. The excitation wavelength was 488 nm, and the emission wavelength was 525 nm. The experimental data were analyzed using Flow J software.

2.5.2 Detection of the expression of the NADPH oxidase gene

Caco-2 cells were treated with 40 $\mu\text{g/mL}$ Ara h 3-raw or Ara h 3-roasted, or 1 $\mu\text{g/mL}$ LPS for 24 h. Total RNA was extracted using a Trizol kit (CwBiotech, China), and reverse transcriptase was added to reverse the transcription of extracted RNA into cDNA. The primer sequences are listed in **Table 1**. An RT-PCR kit (Takara, China) was used to detect the relative expression levels of Nox1, Noxa1, Nox2, p67phox, and p47phox genes in the NADPH oxidase protein family.

Table 1 Primer sequences used in RT-PCR experiments

Gene	Sense	Antisense
Nox1	TAACAGCACGCTGATCCTG	CTGGAGAGAATGGAGGCAAG
Noxa1	TTCCAGGAGGCTCTGTCTGACTTC	AGAGAGGAGCCTGTTTGCCAACTT
Noxo1	CCCGATACCCAGTTTCAGTG	CGTGTCTCTGGGTACAGAAGG
Nox2	TCACTCCCCACCA AAACC	CACCTTCTGTTGAGATCGCC
p67phox	AATGAAATGGCACGTC	GGTGAAGGTCGGAGTCAACG
p47phox	CAAGAGTACCGCGACAGACA	TACATGGACGGGAAGTAGCC

2.6 Detection of NF- κ B pathway-related proteins

Caco-2 cells were treated with LPS (1 μ g/mL), Ara h 3-raw, or Ara h 3-roasted (40 μ g/mL) for 24 h. The cytoplasmic and nuclear proteins of Caco-2 cells were extracted using a nuclear and cytoplasmic extraction kit (Aibo, China). The protein was separated using a 10% SDS electrophoresis kit (Yamei, China). We used a gel with a thickness of 1.0 mm for electrophoresis. An upper gel voltage of 80 V was applied for a duration of 20 min, and a lower gel voltage of 120 V was applied for a duration of 60 min. The gel was retained and soaked in transfer buffer (25 mM Tris-HCl, 125 mM glycine, 20% v/v methanol) for 10 min. The protein on the gel was transferred to a nitrocellulose membrane (PVDF) using a Mini-PROTEAN 3 system set at 300 Ma for 250 min. Then, the proteins were blocked with 5% skimmed milk for 2 h, and incubated with human anti-IKK α , p-IKK α , IkB α , p-IkB α , p65, and p-p65 polyclonal antibodies (diluted to 1:2000) at 4°C. The membrane was washed twice with Tween20/Tris buffer solution, and then incubated with rabbit anti-human horseradish peroxidase (HRP)-bound anti-IgG antibody (diluted to 1:10000) for 2 h at room temperature. Then, the membrane was washed with Tween20/Tris buffer 3 times, each time for 10 min. The color reaction was performed with β -actin antibody as a control. The immunoreactive bands were detected by enhanced chemiluminescence (Tanon, USA) and quantified by ImageJ densitometry analysis.

2.7 Detection of Caco-2 cytokines

Caco-2 cells were seeded in a 6-well plate at a density of 2.5×10^5 cells/mL. After 7 days of culture, Caco-2 cells were incubated with 40 μ g/mL of Ara h 3-raw or Ara h 3-roasted or 1 μ g/mL LPS for 24 h. According to the manufacturer's instructions, total RNA was extracted and transcribed into cDNA by reverse transcription kit (Lianke, China). Real-time quantitative RT-PCR was used to detect the target

gene TSLP. The primer sequences used in this experiment are shown in **Table 2**.

Caco-2 cells were seeded in a 96-well plate at a density of 2.5×10^5 cells/mL. Caco-2 cells were incubated with Ara h 3-raw and Ara h 3-roasted samples at concentrations of 10, 20, 40, 80, 100 $\mu\text{g/mL}$, or with 1 $\mu\text{g/mL}$ LPS for 24 h. The concentrations of IL-6, IL-8, and MCP-1 were determined using an ELISA kit (Lianke, China). The OD values at 425 nm and 630 nm were measured using a microplate reader (Molecular Devices, USA). The OD value after calibration was 450 nm measured value minus 630 nm measured value, and the result was calculated using a standard curve.

Table 2 Primer sequences used in RT-PCR experiments

Gene	Sense	Antisense
GAPDH	CCACCCATGGCAAATTCC	TGGGATTTCATTGATGACCAG
TSLP	CCAGAGCCTAACCTTCAATCC	GTTGTGACTTTCCTTTTCTCCTC

2.8 Preparation and viability of Caco-2 monolayer

Caco-2 cells were seeded at a density of 2×10^4 cells/mL in the Transwell chamber side (AP) of a 6-well plate, and 2.6 mL of cell-free medium was added to the basal side (BL) and cultured at 37°C with 5% CO_2 for 21 days. Cell resistance was measured every 3 days using a cell resistance meter. Before being measured, the medium was replaced with preheated HBSS, and the cells were incubated at 37°C for 15 min. The cell transmembrane (TEER) was measured using a cell resistance meter (JinGong, China), and was calculated using the following formula:

$$\text{TEER}(\Omega \cdot \text{cm}^2) = (\text{Rm} - \text{Ri}) \times A$$

Here, Rm represents transmembrane resistance, Ri represents intrinsic resistance of a cell-free media, and A represents the surface area of the membrane in cm^2 . When Caco-2 cells formed a complete monolayer, TEER values were in the range of $500 \Omega \cdot \text{cm}^2$ to $1000 \Omega \cdot \text{cm}^2$. Therefore, in this study, cell monolayers with TEER values greater than $600 \Omega \cdot \text{cm}^2$ were selected and any cells lower than this value were discarded.

2.9 Detection of Caco-2 cell monolayer

2.9.1 Determination of barrier integrity

On the AP side of the transwell chamber, 1.5 mL Ara h 3-raw or Ara h 3-roasted (40 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$), or 1 $\mu\text{g/mL}$ LPS medium were added and treated for 3 h. With the same volume of fresh medium as the blank, TEER was recorded before and after treatment. After the medium was changed, the culture

was continued for 24 h, and then the TEER was recorded using a cell resistance meter (JinGong, China).

2.9.2 Detection of tight junction protein genes

Caco-2 cells were treated with Ara h 3-raw or Ara h 3-roasted (40 µg/mL or 100 µg/mL), or 1 µg/mL LPS for 24 h. Total RNA was extracted, and cDNA was synthesized. An RT-PCR kit (Takara, China) was used to detect the relative expression of tight junction proteins ZO-1, occludin, and JAM-1. The primer sequences used in this experiment are shown in **Table 3**.

Table 3 Primer sequences used in RT-PCR experiments

Gene	Sense	Antisense
ZO-1	TAAGCCTCCTGCACACATTG	GGATGGGTTCATAGCGTTTC
Occludin	CCACCTATCACTTCAGATCAAC	TCAGCAGCAGCCATGTACTC
JAM-1	CAGAGGTGATTCATGGCTCTGTG	TTCCAGGCTGGCAATAACTGAC

2.9.3 Immunofluorescence observation of ZO-1 protein

Caco-2 cells were incubated with fresh medium, Ara h 3-raw (40 µg/mL), Ara h 3-roasted (40 µg/mL), or LPS (1 µg/mL) for 24 h. The cells were washed with HBSS buffer solution three times and fixed with 4% paraformaldehyde for 30 min. The cells were then permeabilized with 0.25% Triton X-100 for 3 min, and sealed with 5% bovine serum at 37°C for 30 min. Antibody ZO-1 (diluted at 1:3000) was incubated overnight at 4°C. The cells were then incubated in the dark with goat anti-rabbit IgG (diluted at 1:1000) at 37°C for 2 h. The cells were washed with HBSS buffer three times and stained with DAPI. After washing with HBSS three times, the cell images were obtained using a fluorescence microscope (Olympus, Japan). The excitation wavelength was 488 nm and the emission wavelength was 525 nm.

2.10 Transport of Ara h 3 by Caco-2 monolayer

Caco-2 cells were cultured continuously for 21 d to form a monolayer. After 21 d, the medium was removed, the HBSS buffer was rinsed, and 1.5 mL of either HBSS (control), Ara h 3-raw HBSS solution (40 µg/mL), or Ara h 3-roasted HBSS solution (40 µg/mL) was added to the AP side of the transwell chamber. The BL group received 2.6 mL of HBSS solution. The cells were cultured in a 37°C incubator, and the plate was gently shaken at 50 r/min. During incubation, 0.5 mL of buffer solution on the BL side of each treatment group was collected at intervals of 1 h, 2 h, 3 h, 12 h, 24 h, and 48 h. Immediately afterwards, the same amount of fresh HBSS solution was added back to the BL side. The intensity of the fluorescence of the collected solution was recorded using a multi-functional fluorescent enzyme analyzer

(Molecular Devices, USA). The excitation wavelength was 488 nm and the emission wavelength was 525 nm.

2.11 Statistical analysis

The data are expressed as the mean $\pm$ SE, and all assays from every independent treatment were carried out in triplicate. Appropriate data were analyzed to determine statistical significance using ANOVA (SPSS version 13.0, USA). The t test and Fisher's least significant difference (LSD) test were used to examine differences between group means. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3. Results

3.1 Effects of Ara h 3 on the viability and proliferation of Caco-2 cells

After treatment with different concentrations of Ara h 3-roasted, viability and proliferation of Caco-2 cells was documented. From **Table 4**, it can be seen that the viability of Caco-2 cells decreased with increasing Ara h 3 concentrations. When the concentrations of Ara h 3-raw and Ara h 3-roasted were lower than 40 $\mu\text{g/mL}$, the viability of Caco-2 cells was more than 95%. This indicates that a low concentration of Ara h 3 has no obvious cytotoxicity. However, when the concentration was higher than 40 $\mu\text{g/mL}$, the inhibition of Ara h 3-roasted on Caco-2 cells was significantly higher than that of Ara h 3-raw.

The proliferation of Caco-2 cells was detected using fluorescence microscopy. The results show that cell proliferation was inhibited when allergen protein concentration increased. Compared with the control group, 40 $\mu\text{g/mL}$ Ara h 3-roasted inhibited the proliferation of Caco-2 cells. A similar trend was observed using flow cytometry, and the inhibition of cell proliferation detected by flow cytometry was observed to be greater under the same concentration of Ara h 3-roasted. At a concentration of 100 $\mu\text{g/mL}$, the proliferation ability of the Ara h 3-roasted group was only 58.3% of Ara h 3-raw.

Table 4 Effects of Ara h 3 on the viability and proliferation of Caco-2 cells

Protein concentration (µg/mL)		10	20	40	80	100	
Cells viability (%)	Raw	99.6±0.4	98.8±0.5	97.2±0.2	90.1±0.3	86.7±0.2	
	Roast	98.9±0.2 ^{ns}	97.7±0.2*	95.6±0.3*	87.2±0.4**	82.8±0.2**	
Cells proliferation (%)	Fluorescence microscope	Raw	95.0±0.5	93.5±0.2	91.2±0.3	88.1±0.4	82.6±0.2
		Roast	94.6±0.4 ^{ns}	90.5±0.3 ^{ns}	85.6±0.2*	75.2±0.1*	68.3±0.3*
	Flow cytometry	Raw	77.8±0.2	70.0±0.2	63.5±0.5	55.0±0.3	46.8±0.2
		Roast	74.3±0.3 ^{ns}	64.3±0.2*	57.4±0.2*	42.0±0.1**	27.3±0.2***

Note: Cell viability and cell proliferation were observed after cells were incubated with different concentrations of protein for 24 hours. Each sample was an independent experiment in triplicate, and data are expressed as the mean $\pm$ SE(n=3). Under the same treatment concentration, the roasted peanut treatment group was significantly compared with the raw peanut treatment group. Appropriate data were analyzed to determine statistical significance using ANOVA (SPSS version 13.0, USA). T-tests and Fisher's least significant difference (LSD) were used to examine differences between group means. ns: no significant difference, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.2 Uptake of Ara h 3 by Caco-2 cells

The uptake of Caco-2 cells was observed by fluorescence microscopy. The cells were incubated with Ara h 3-raw or Ara h 3-roasted for 12, 24, 36, 48 h,. Nuclei were stained with DAPI. As shown in **Fig. 2**, when the incubation time was 12 h, there was only a small amount of Ara h 3 in Caco-2 cells. With the prolongation of incubation time, the uptake of Ara h 3 by Caco-2 cells increased gradually. Compared with Ara h 3-raw, more Ara h 3-roasted cells absorbed Caco-2 cells at the same incubation time, which suggested that roasting can promote the uptake of Ara h 3 protein by Caco-2 cells.

Flow cytometry was used to verify the above results (see the supplementary document **Fig. S1**). Similar to the results of fluorescence microscopy, the rate of Ara h 3-positive cells increased when the incubation time reached 24 h. At the same incubation time, there were more Ara h 3-roasted positive Caco-2 cells than Ara h 3-raw positive cells. These results indicate that Ara h 3-roasted is more easily absorbed by Caco-2 cells than is Ara h 3-raw.

3.3 Effect of Ara h 3 on oxidative stress response of Caco-2 cells

After incubation with Ara h 3-roasted for 30, 60, 90, 120, 150, 180 min, the ROS content in Caco-2 cells was detected by flow cytometry. The results are shown in **Fig. 3a**. With the prolongation of incubation time, the content of ROS in the cells treated only with culture medium showed no significant change. When cells were incubated with Ara h 3-roasted, the ROS content increased from 33.87% to 58.08%, which was higher than that in the control group and the Ara h 3-raw group. This suggests that Ara h 3-roasted can induce high levels of ROS production in Caco-2 cells. Ara h 3-roasted is more likely to induce an imbalance in the redox state of Caco-2 cells and promote the oxidative stress reaction of Caco-2 cells.

RT-PCR was used to detect the relative expression of Nox family protein genes of the NADPH oxidase system in Caco-2 cells. The results are shown in **Fig. 3B**. Compared with the control group, the relative expression levels of Nox1, Noxa1, and Noxo1 in Caco-2 cells increased after treatment for 5 min or more. When the treatment time was prolonged to 10 min, the relative expression levels of Nox1, Noxa1, Noxo1, NOX2, p67phox, and p47phox increased to 3.19, 5.4, 3.86, 1.64, 1.98, and 1.74 respectively, which were higher than those of the Ara h 3-raw treatment group. These results suggest that compared with Ara h 3-raw, Ara h 3-roasted may promote ROS production by upregulating NADPH oxidase content in Caco-2 cells.

3.4 Effect of Ara h 3 on NF- κ B pathway in Caco-2 cells

Western blotting was used to detect the relative expression of IKK α in the NF- κ B pathway, and the results are shown in **Fig. 4**. Compared with the control group, the relative expression of IKK α protein in Ara h 3-raw and Ara h 3-roasted groups decreased from 95% to 56% and 29%, respectively, and the relative expression of p-IKK α increased from 4% to 34% and 48%, respectively. These results indicate that the IKK α protein was activated by Ara h 3. In addition, the relative expression of p-IKK α in the cells treated with Ara h 3- roasted was higher than that of Ara h3-raw cells, indicating that Ara h 3-roasted promoted the phosphorylation of IKK α in Caco-2 cells, thus enhancing the effect of Ara h 3 on the NF- κ B pathway. Similarly, compared with the blank control group, a decrease in I κ B α and an increase in p-I κ B α were observed in the cells incubated with Ara h 3, particularly when incubated with Ara h3-roasted. This suggests that Ara h 3-roasted promotes the phosphorylation of I κ B α protein. Compared with Ara h 3-raw, the expression of p65 protein in the cytoplasm of Caco-2 cells was downregulated and

the expression of p-p65 in the nucleus was upregulated after Ara h 3-roasted treatment, indicating that p65 protein entered the nucleus after phosphorylation. These results indicate that Ara h 3-roasted increases activation of the NF- κ B pathway in Caco-2 cells.

3.5 Effect of Ara h 3 on the secretion of proinflammatory factors in Caco-2 cells

The expression of the TSLP gene in Caco-2 cells was detected by RT-PCR, and the results are shown in **Fig. 5A**. Compared with Ara h 3-raw, the expression of the TSLP gene, the promoter of Th2-type allergic reactions, was upregulated after Ara h 3-roasted treatment.

The secretion of Th2 cytokines, IL-6, IL-8, and MCP-1 was detected by ELISA. As shown in **Fig. 5B-D**, the secretion levels of IL-6, IL-8, and MCP-1 in Caco-2 cells after treatment with Ara h3-roasted were higher than in the Ara h3-raw treatment group, especially when protein concentration was greater than or equal to 40 μ g/mL.

3.6 Effect of Ara h 3 on monolayer barrier integrity of Caco-2 cells

The integrity of the Caco-2 monolayer barrier can be evaluated by the TEER value of the cell monolayer (**Fig. 6a**). After treatment with Ara h 3-roasted for 3 h, the TEER value of Caco-2 cells decreased significantly. When the Ara h 3 protein was replaced with fresh medium, the TEER value increased after 24 h of incubation, but was still lower than that of the control group. This suggests that Ara h 3-roasted can damage Caco-2 cell monolayers. The fluorescence intensity of the BL side medium showing Caco-2 cell monolayer transport of Ara h 3 was observed across several time intervals using a fluorescent enzyme analyzer, and the results are shown in **Fig. 6B**. The transport of Ara h 3-raw and Ara h 3-roasted in Caco-2 cell monolayers was time-dependent, and reached a stable state after 24 h incubation. Compared with the Ara h 3-raw group, high levels of fluorescent proteins were detected on the BL side of the Caco-2 cells treated for 12 h with Ara h 3-roasted. This indicates that Caco-2 cell monolayers transported more Ara h 3-roasted than Ara h 3-raw. After treatment with Ara h 3-roasted, the relative expression levels of tight junction proteins ZO-1, occludin, and JAM-1 in Caco-2 cell monolayers were detected by RT-PCR. The results are shown in **Fig. 6C**. Compared with the control group, Ara h 3-roasted treatment decreased the gene expression level of tight junction proteins in Caco-2 cells, especially ZO-1 protein. The effects of Ara h 3-raw and Ara h 3-roasted on tight junction protein ZO-1 in Caco-2 cells were observed by immunofluorescence microscopy. As shown in **Fig. 6D**, ZO-1 in

the control group was evenly distributed on the membrane of Caco-2 cells and had an obvious continuous ring structure. After 24 h of treatment with Ara h 3-roasted, the expression of ZO-1 on the membrane decreased, took on a zigzag shape, and featured interruptions in continuity, all of which indicate that the polygonal morphology of the cells was destroyed.

3. Discussion

Because of its high rate of incidence combined with the lack of a cure, we wanted to explore the mechanism of peanut allergy to provide a theoretical basis for the development of hypoallergenic products. The response of intestinal epithelial cells to allergens can not only regulate the type of immune response, but also regulate the severity of the immune response, which is closely related to the clinical phenotype of allergen sensitization²². Caco-2 cells can be used to evaluate the biological activity of food allergens on human intestinal epithelial cells, which is reflected in cell proliferation, cytokine release, oxidative stress capacity, and intestinal barrier integrity²³. Our results showed that roasting treatment led to an increase in the uptake of Ara h 3 in Caco-2 cells and an inhibition of cell viability and proliferation. This may be due to the fact that Ara h 3 is easily aggregated into complex polymer during roasting, which is easier to be absorbed by cells, more allergens are absorbed in cells, then cell viability and proliferation were inhibited. Moreover, Ara h 3 epitopes were exposed to the protein surface after roasting, so that more epitopes could be recognized by Caco-2 cells, which promoted uptake of Ara h 3. Aggregation after processing seems to be a common feature of food allergens²⁴. For example, the aggregation of milk allergens after pasteurization helps to increase the uptake of allergens by intestinal cells and enhances their potential sensitization⁸.

NADPH oxidase appears to be a major contributor to superoxide production and subsequent downstream ROS production under various disease conditions^{25, 26}. From **Fig. 3-4**, it can be confirmed that Ara h 3-roasted significantly increased the expression of Nox family proteins and the amount of ROS in Caco-2 cells, compared with Ara h 3-raw. As an indispensable second messenger in the NF- κ B signaling pathway, the presence of ROS activates NF- κ B signaling, which results in the complete degradation of I κ B α and translocation of p65 protein to the nucleus. In turn, this regulates the expression of growth-, immune-, and inflammatory-related genes²⁷. The results show that when Ara h 3-roasted was ingested by Caco-2 cells, it led to an increase in ROS levels. Meanwhile, Ara h 3-roasted had a stronger stimulative effect on Caco-2 cells than Ara h 3-raw, and more proinflammatory factors such as IL-6, IL-8,

and MCP-1 were secreted (**Fig. 5**). This may be because Caco-2 cells absorb more Ara h 3-roasted, thus producing more ROS, which promotes greater activation of the NF- κ B signal pathway, which leads to an increased release of proinflammatory factors. The increase in proinflammatory factors may be a kind of cellular reaction that compensates for the anti-proliferation effect of the protein. Teodorowicz et al.²⁸ found that after heat treatment of peanut 7S globulin (Ara h 1), the release of IL-8 in Caco-2 cells was significantly increased, and the release was temperature-dependent. This is consistent with the effect of Ara h 3-roasted. The results confirm that Ara h 3-roasted has a stronger ability to induce local inflammatory reactions in the intestinal mucosa, indicating that roasting treatment improves the potential sensitization of Ara h 3.

The cell monolayer is closely related to oxidative stress and protein transport²⁹. The transepithelial electrical resistance value and tight junction protein expression levels can be used to detect its integrity³⁰. **Fig. 6** shows that the TEER value of the Caco-2 cell monolayer decreased after exposure to Ara h 3-roasted. The relative expression of ZO-1, occludin, and JAM-1 genes changed after incubation with Ara h 3-roasted³¹. These results suggest that the decrease in TEER value is not caused by cell death, but by the change in tight junction protein expression. ZO-1 protein is an important junction between occludin and the cytoskeleton³², and its destruction by Ara h 3-roasted can promote morphological changes in Caco-2 cells. There are complex interactions between tight junction proteins and membrane-related structures³³. The loss of ZO-1 led to an increase in permeability of Caco-2 cells (**Fig. 6D**), and increased transport of Ara h 3-roasted (**Fig. 6B**) by Caco-2 can lead to the occurrence of an inflammatory reaction. In the above results, the significant difference between the roasted peanut group and the raw peanut group was lower than that between the two groups and the control group, indicating that the most effective treatment for peanut allergy patients is to avoid taking peanut allergens as far as possible. Understanding the mechanism of peanut allergy is of great significance for the prevention and treatment of peanut allergy. In future experiments, other processing methods, such as enzyme treatment, should be studied to determine potential efficacy in reducing the allergenicity of peanut products.

4. Conclusion

We used Caco-2 cell model to evaluate the change of peanut allergenicity after roasting and its mechanism. The results showed that the allergenicity of Ara h 3 protein was increased after roasted. Compared with the Ara h 3-raw group, the Caco-2 cells viability and proliferation in Ara h 3-roasted group were more inhibited, and the secretion of ROS was more increased, which led to the imbalance of

oxidative stress and activation of NF- κ B signaling pathway. At the same time, the content of TSLP, IL-6, IL-8 and MCP-1 were more increased, and the transepithelial electrical resistance of Caco-2 cells were seriously decreased, while the gene expression of tight junction proteins ZO-1, occludin and JAM-1 were down more, which higher destroyed the distribution of ZO-1 on Caco-2 cells and the integrity of Caco-2 monolayer barrier. As consequence, the transport of Ara h 3-roasted protein by Caco-2 monolayer was more enhanced, and the allergic reaction was aggravated. In the future, animal models can be used to further study the mechanism of the allergenicity of roasted peanut, or other processing methods (such as adding edible enzymes to treat protein) can reduce the allergenicity of roasted peanut, so as to provide theoretical basis for the production of low allergenic peanut food.

Acknowledgement

This study is supported by the Project of Central Guidance for Local Science and Technology Development (19395810100), Science and Technology Joint Project of the Yangtze River Delta (19395810100). We would like to thank Editage (www.editage.cn) for English language editing.

Conflict of interest

This article is an original work, and no portion of the study has been published or is under consideration for publication elsewhere. None of the authors has any potential conflicts of interest related to this manuscript. All authors have contributed to the work and have agreed to submit the manuscript.

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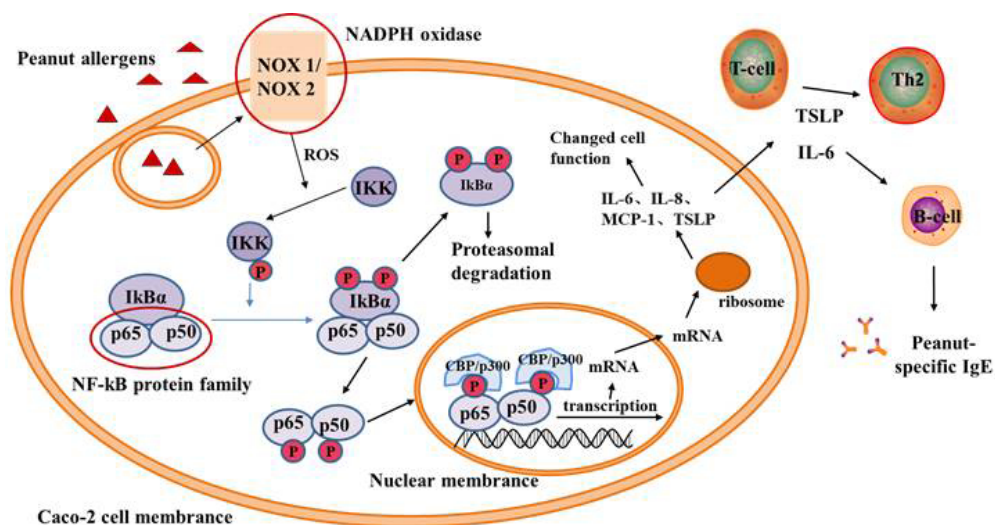


Fig.1 NF-κB pathway in Caco-2 cells

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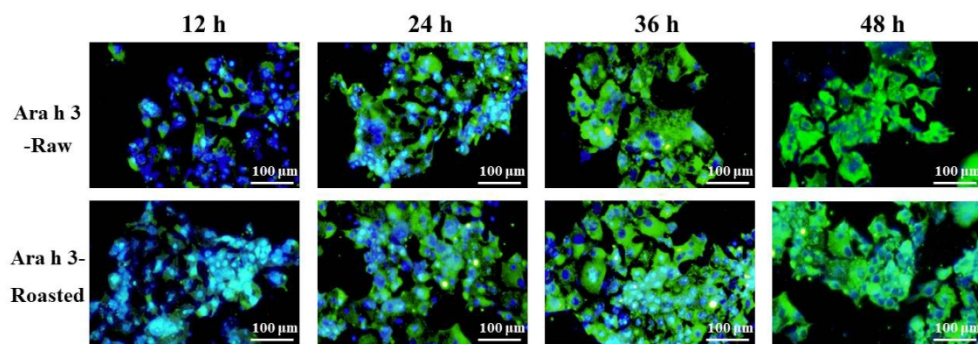


Fig. 2 Fluorescence microscopic observation of the uptake for Ara h 3 by Caco-2 cells. The fluorescence microscope showed that the cells were blue and the protein intake was green.

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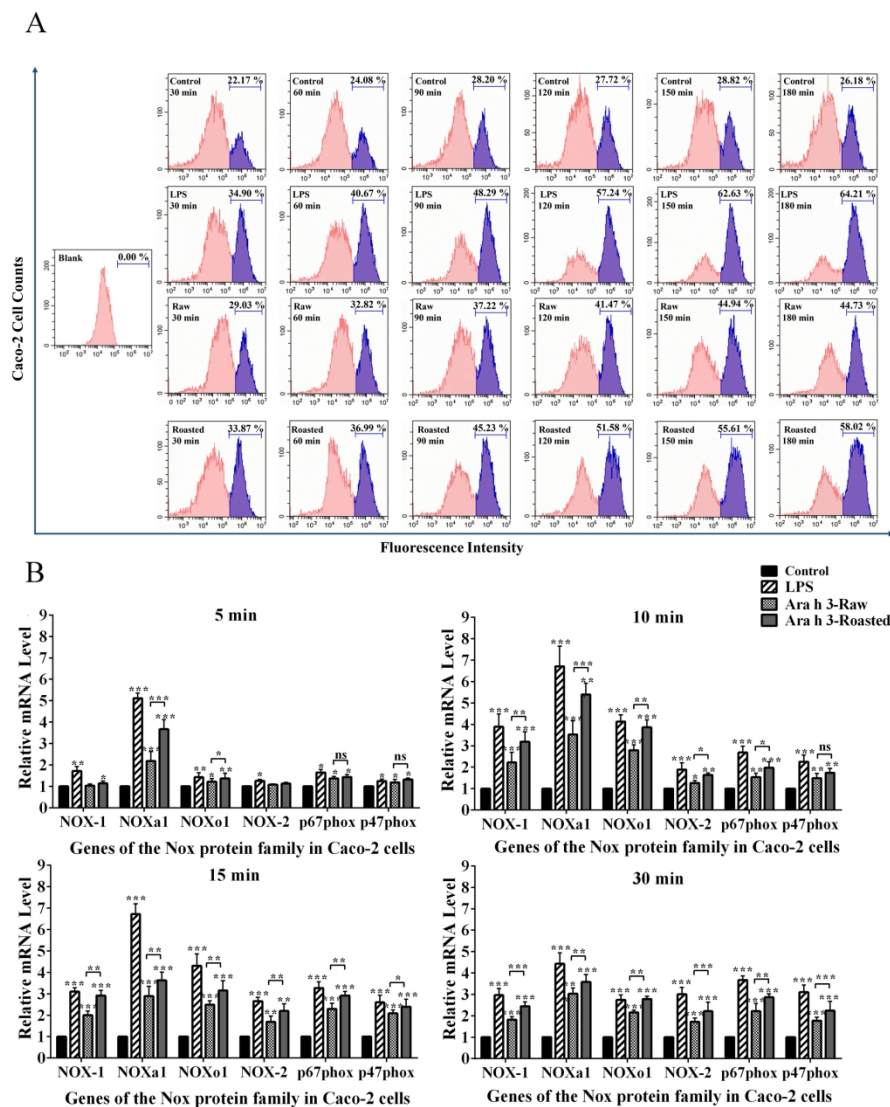


Fig. 3 Detection of ROS and Nox protein family in Caco-2 cells. (A) The content of ROS; (B) The expression of Nox family genes in the cells. Data were expressed as means $\pm$ SD from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

95x127mm (600 x 600 DPI)

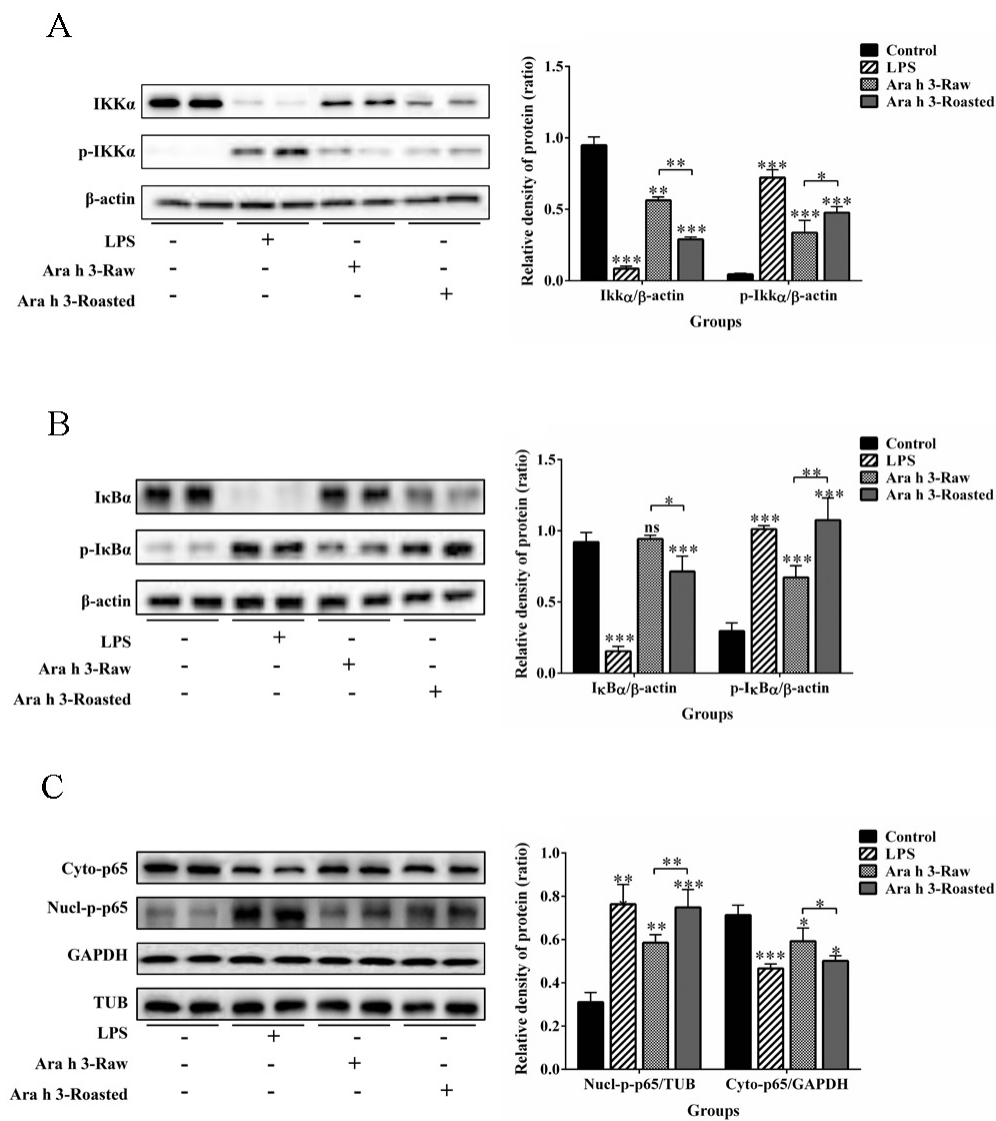


Fig.4 Analysis of IKK α , I κ B α and p65 protein expression in Caco-2 cells. (A) The expression level of IKK α protein; (B) The expression level of I κ B α protein; (C) The expression level of p65 protein in nucleus and cytoplasm. Data were expressed as means $\pm$ SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

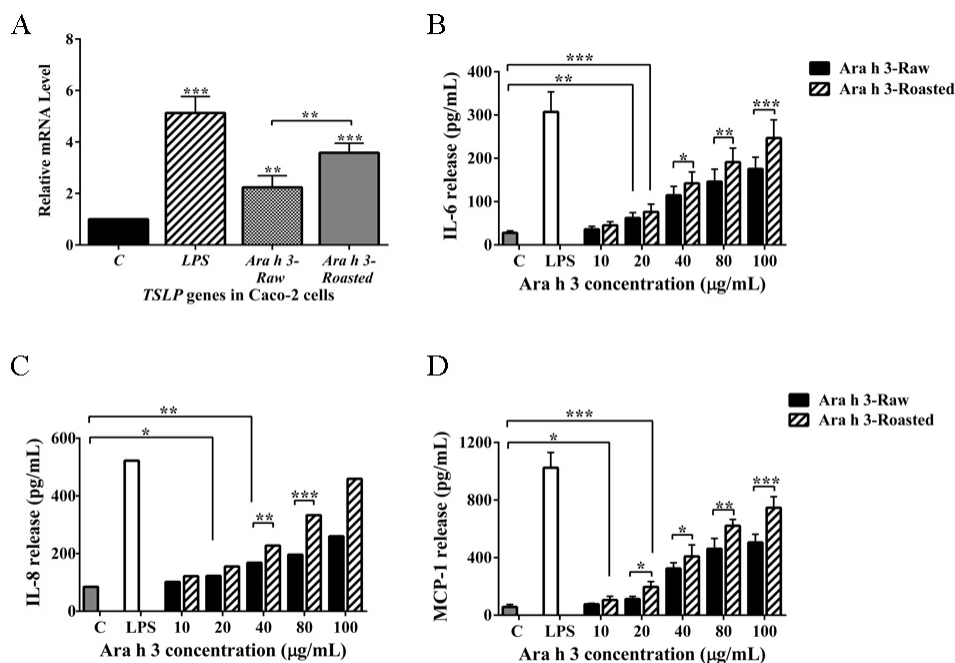


Fig.5 Contents of TSLP, IL-6, IL-8 and MCP-1 in Caco-2 cells before and after treatment. (A) The expression of TSLP gene in Caco-2 cells; (B) The secretion level of Th2 cytokine IL-6; (C) The secretion level of Th2 cytokine IL-8; (D) The secretion of MCP-1. Data were expressed as means $\pm$ SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

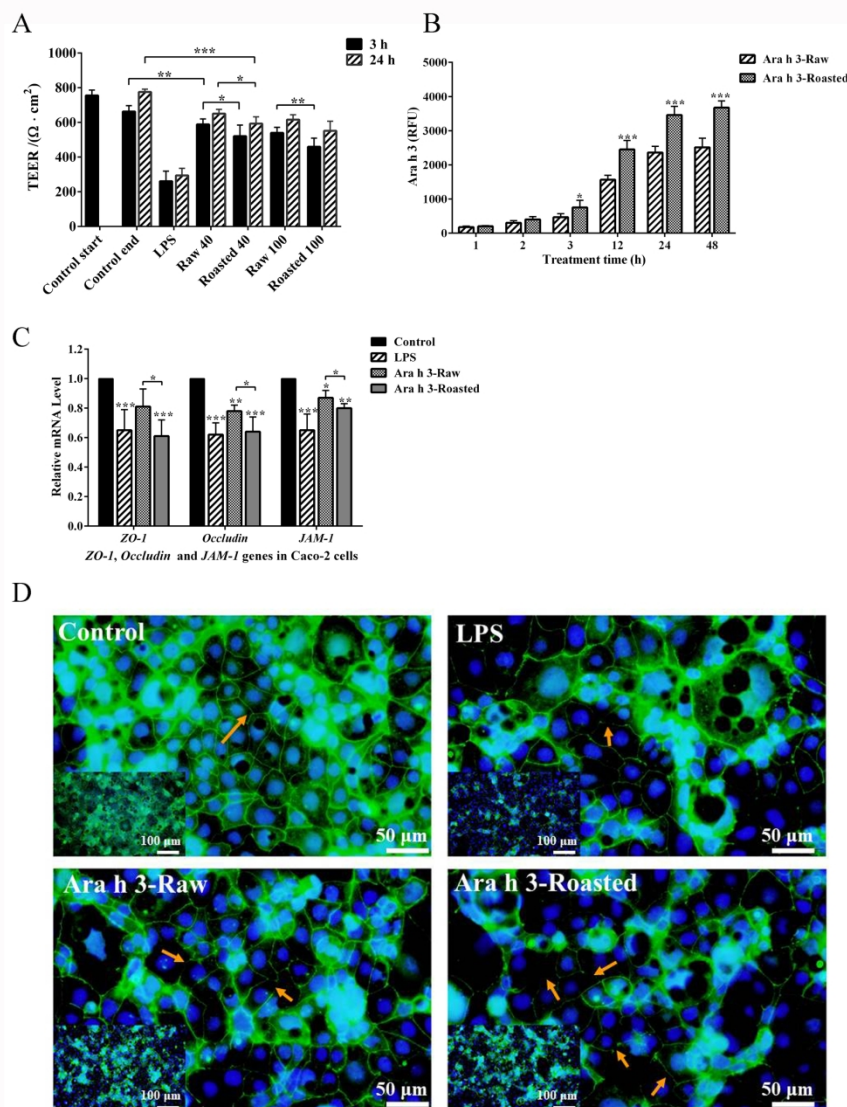


Fig. 6 Protein transport and integrity of Caco-2 cell monolayer barrier. (A) TEER value of cell monolayer; (B) The amount of cell transport protein after the protein is incubated with cells for different times; (C) The relative expression of tight junction protein ZO-1, occludin and JAM-1 in Caco-2 cells; (D) The effect of Ara h 3-row and Ara h 3-roasted on ZO-1 in Caco-2 cells. Data were expressed as means $\pm$ SD from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

99x134mm (600 x 600 DPI)

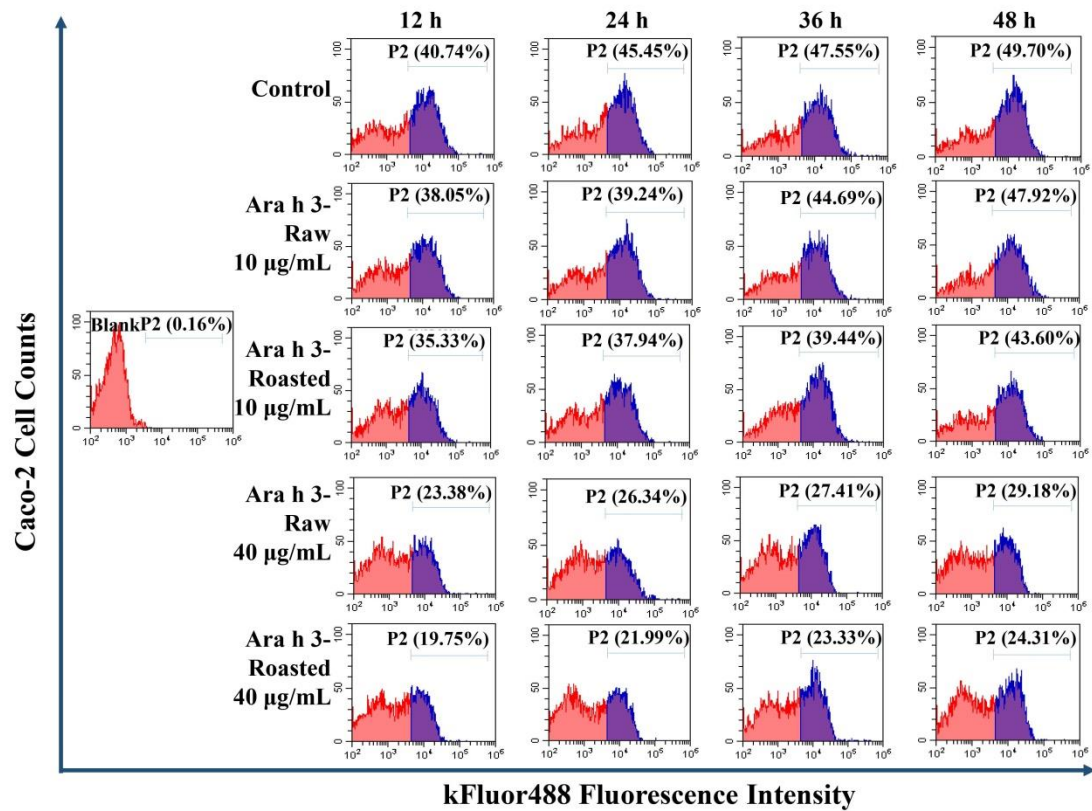


Fig.s1 Effects of Ara h 3-roasted incubation for different time on the proliferation of Caco-2 cells

Flow cytometry was used to detect the effect of Ara h 3 protein incubation time on the proliferation of Caco-2 cells. The results were shown in Fig. S1. Under different incubation time, the proliferation rate of Caco-2 cells treated with 40 µg/mL Ara h 3 protein was significantly lower than that of 10 µg/mL Ara h 3 protein treatment. With the prolongation of Ara h 3 protein incubation time, the proliferation of Caco-2 cells was not significantly inhibited. The results showed that Ara h 3-roasted significantly inhibited the proliferation of Caco-2 cells in a concentration dependent manner, but not in a time-dependent manner.