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# Changes in allergenicity of ovalbumin *in vitro* and *vivo* on conjugation with quercetin

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## ABSTRACT

Previous study demonstrated decreased allergenicity *in vitro* of some food allergens after conjugation with polyphenols. However, little is known about how polyphenol conjugation with food allergens affects *in vivo* allergenicity. We conjugated a well-known food allergen, ovalbumin (OVA), with quercetin (QUE) to assess the potential allergenicity of OVA *in vitro* and *in vivo* in a BALB/c mouse model. QUE could covalently conjugate with OVA and changed the protein structure, which might destroy and/or mask OVA epitopes. Conjugation with QUE decreased IgE-binding properties and the release capacity of the conjugated OVA. *In vivo,* as compared with native protein, conjugation with QUE decreased the levels of IgE, IgG1, IgG, plasma histamine and mast cell protease-1 (mMCP-1) on the surface of sensitized mast cells, along with decreased $Fc\varepsilon RI^+$ and c-$kit^+$ expression. The levels of Th2-related cytokines (IL-4, IL-5, IL-13) decreased and that of a Th1-related cytokine (IFN-γ) increased slightly, which suggests that conjugation with QUE modulated the imbalance of the Th1/Th2 immune response. Conjugation of OVA with QUE could reduce OVA allergenicity *in vitro* and *in vivo*, which could provide information for reducing food allergenicity by conjugation with polyphenols.


KEYWORDS: OVA, Quercetin, Conjugation, Allergenicity, Mouse model

# INTRODUCTION

Food allergy is an important public health issue and affects about 8% of children and 5% of adults, with increasing incidence in the past few years [1]. Egg allergy is one of the most common food allergies, particularly in children under the age of 3 [2].

Ovalbumin (OVA, 45 kDa) is one of the major allergens in egg causing IgE-mediated allergic reactions, the most frequent. Processing techniques for destroying the allergenicity of OVA in egg white have wide interest [3]. Among the potential alteration methods, the interaction between polyphenols and allergens can reduce allergenicity by changing the protein structure or rendering the allergen less bioavailable [4].

Polyphenols can bind protein by non-covalent and covalent interaction [5, 6]. A number of studies have investigated the interaction between polyphenols and food allergens. Polyphenol interactions can reduce the immunogenicity and allergenicity of wheat gliadins [7]. Caffeic, chlorogenic acids and ferulic can bind to Ara h1 and Ara h2, which reduces the IgE binding of the protein [8]. The IgE-binding capacity of the major cherry allergen Pru av 1 was reduced after interaction with gallic acid, quercetin and epicatechin [9]. Previous studies have found reduced IgE-binding and degranulation capacities of peanut flour after interaction with polyphenolic extracts rich in pro-anthocyanidins and anthocyanins [10-12]. Furthermore, an in vivo study demonstrated that complexation of polyphenols to peanut flour inhibited specific IgE antibody production in peanut-sensitized mice [13]. Our previous report of the reduced IgE- and IgG-binding activities of β-lactoglobulin after catechin binding supports the

above findings [14]. Considering these findings, interaction with polyphenols might be a potential novel strategy for producing hypoallergenic food.

Non-covalent binding of epigallo-catechin 3-gallate (EGCG) to OVA resulted in structural changes in protein. However, EGCG did not prevent the IgE–OVA interaction [15]. As compared with covalent interactions between polyphenols and proteins, non-covalent interactions are reversible, with lower affinity [16], which suggests that reducing the allergenicity of food allergen by non-covalent interactions is limited in food processing. We have investigated the effect of covalent interactions with polyphenols on the allergenic capacity of OVA. After conjugation with polyphenols, the IgE-binding of OVA decreased, with increased emulsifying and foaming properties, antioxidant activity and digestibility [17-18]. However, whether covalent conjugation with polyphenols can reduce the allergenic properties of OVA needs to be confirmed by *in vivo* studies.

Quercetin (QUE) is a common dietary polyphenol in many plant foods. In this study, we prepared OVA–polyphone conjugates by covalent interaction with QUE and investigated the covalent interaction and structural changes. The changes in allergenicity of OVA *in vitro* were evaluated by IgE-binding capacity and human basophil leukemia (KU812) cell degranulation assay. Furthermore, we used a mouse model of orally induced OVA allergy to evaluate the allergenicity of OVA after conjugation with QUE *in vivo*, which could provide pre-clinical data for the development of innovative hypoallergenic food products.

# MATERIALS AND METHODS

## Materials

OVA (protein content >98 %), QUE (purity≥95%), 1,10-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteau phenol reagent, 3,3'-5,5'tetramethylbenzidine (TMB) and 2,2-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS) were from Sigma-Aldrich (St. Louis, MO, USA). Mouse IgG-antihuman IgE antibody and NeutrAvidin-HRP-conjugated antibody were from SouthernBiotech (Birmingham, AL, USA). Horseradish peroxidase (HRP)-conjugated goat anti-human IgE was from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA).

Serum samples from 12 children who were allergic to OVA were generously provided by Shenzhen Children's Hospital (Guangdong, China), and the availability was approved by the human ethics committee of Health Science Center at Shenzhen University (Permit No. 201606016). Each individual signed an informed consent. All anti-OVA–specific IgE levels in serum samples were > 10 kU/L. Pooled normal serum samples from individuals (n=5) without allergy to chicken egg were negative controls.

## Preparation of samples

The OVA–QUE conjugates were prepared by the alkaline method or free radical method. To prepare conjugates by the alkaline method as described previously [13], 0.25 g OVA was dissolved in 50 mL distilled $H_2O$ and the pH was adjusted to 9.0, then

samples were maintained at 25℃ for 2 h under atmospheric air. A 0.5-mmol amount of QUE was added to the mixture with continuous stirring for 24 h. The unreacted QUE was removed by dialysis at 4 °C for 48 h with Milli-Q water. The free radical method was performed as described [19] with modification. After dissolving 0.5 g OVA in 50 mL distilled $H_2O$, 1.0 mL 5.0 M $H_2O_2$ and 0.25 g ascorbic acid were added to the solution, then samples were maintained at 25°C. After 2 h, 0.35 mmol QUE was added to the solution and maintained at 25°C for 24 h. Unreacted QUE was removed as described above. Finally, the conjugates were obtained by freezing and drying samples.

The contents of conjugates were detected by the Foline-Ciocalteu method as we previously described [20], with QUE as a standard. Determining free amino groups in samples involved use of trinitro-benzene-sulfonic acid as previously described [21]. The contents of sulfhydryl thiol groups and tyrosine in samples were measured as we previously described [19].

**SDS-PAGE**

SDS-PAGE was performed on a 5% stacking gel and 12% separating gel. A 10-μl loading buffer (Solarbio Life Science, Beijing) was mixed with 40 μl samples (1 mg/mL) and heated at 95 ℃ for 5 min. Then aliquots were loaded on the gels. After staining with Coomassie Brilliant Blue R-250 for 0.5 h, gels were destained with 7.5% acetic acid and 5% methanol for 10 h and scanned.

**Structural analysis of conjugates**

*Fourier transform infrared (FTIR) spectroscopy*

The FTIR spectra were detected as previously described [22] by using a FTIR-8300PCS spectrometer (SHIMADZU Co., Kyoto, Japan) with KBr pellets. The samples were measured at 25 ℃ in the range of 400 to 4000 cm-1 with 32 scans and 4 cm-1 resolution.

*Circular dichroism (CD)*

CD was performed as previously described [23]. The ellipticity of samples (0.015 mg/mL) was recorded on a Jasco-810 spectrophotometer (Jasco Co., Japan) with resolution 0.2 nm and 100 nm/min speed step in the range of 190-250 nm at 25 ℃.

*Differential scanning calorimetry (DSC)*

DSC was used to examine the thermal stability of samples by using a DSC-60 calorimeter (Shimadzu, Tokyo). A 5-mg sample was placed in an aluminum pan that was sealed tightly. Using nitrogen as the transfer gas at a rate of 30 mL/min, the thermal analyses were performed from 30 to 180 ℃ with a constant rate of 5 ℃/min.

**In vitro allergenicity assessment**

Competitive inhibition of ELISA was used to measure the IgE-binding ability of samples.A-100 μL sample solution (2 mg/ml) was added to a 96-well microplate and incubated overnight at 4 ℃. After a wash with phosphate buffered saline (PBS)

containing 0.05% Tween-20 (PBST), plates were blocked with 2% fish gelatin in PBST for 1 h at 37°C, then washed with PBST. A 50-μL amount of serum pool from egg-allergic patients (diluted 1:200) was mixed with 50 μL of sample solution and added to each well. After incubation for 1 h, plates were washed. A 100-μL amount of HRP-conjugated goat anti-human IgE antibody (1:10,000) was added to wells and incubated for 40 min at 37 ℃. After a wash, 50 μL substrate was added. The reaction was stopped by adding 50 μL $H_2SO_4$ (2 M), and absorbance at 450 nm was measured.

**Antioxidant activity analysis of OVA–QUE conjugate**

*DPPH scavenging activity*

DPPH scavenging activity of OVA and OVA–QUE conjugates was measured as previously described [20]. In brief, 2 mL DPPH was mixed with 2 mL sample (0.5 mg/mL). The mixture was stored in the dark for 1 h. Finally, the absorbance at 517 nm was measured.

*ABTS+ scavenging activity*

ABTS+ scavenging activity of OVA and OVA–CHA conjugates was performed as previously described [20]. In brief, 3 mL ABTS reagent was mixed with 1 ml sample (0.5 mg/mL). The mixture was incubated for 1 h at 25 °C. Finally, the absorbance at 734 nm was measured.

*Cell model analysis*

The immature prebasophilic cell line (KU812) was purchased from the Chinese Academy of Sciences (Shanghai). Cells were cultured and the contents of histamine and IL-6 in cells were measured as described [24] with little modification. The cells were maintained in IMDM (Gibco, Shanghai) supplemented with 1% penicillinstreptomycin (100x stock; GibcoBRL, Grand Island, NY) and 5% fetal bovine serum (FBS, Gibco, Australia) and cultured in a humidified atmosphere with 5% $CO_2$ at 37 ℃. KU812 cells ($1\times10^7$ cells/mL) were pre-incubated with 100 μL serum from egg-allergic patients (v/v=5:1). Normal serum was a negative control. After incubation for 24 h, 10 μL protein sample (5 mg/ml) was added and incubated for 4 h. After centrifugation, supernatant was collected to detect the contents of histamine and IL-6 by using human histamine and human IL-6 ELISA kits (Elabscience Biotech, Wuhan, China).

**Animal model analysis**

*Mice*

All female BALB/c mice (5-6 weeks) were approved by SPF (Beijing) Biotechnology. Animal experiments were carried out according to protocols approved by the Animal Care and Use Committee of Health Science Center of Shenzhen University (Permit No. 201711003). We used 40 female BALB/c mice in this study. Before experiments, mice were housed and fed an OVA-free dietary for least 1 week. All mice were divided into four groups: control (healthy mice without stimulation),

OVA, and radical and alkaline OVA–QUE conjugate groups.

*Oral sensitization and challenge of mice*

Mice were sensitized orally with a lavage needle on days 0, 7, 14,21 and 28 with 5 mg native OVA dissolved in PBS, which was mixed with 15 μg cholera toxin (Sigma–Aldrich, St. Louis, MO, USA) as an oral sensitization (Figure 4A). The mice of the control group were sensitized with 15 μg cholera toxin dissolved in PBS, then challenged with PBS (500 μl) alone on day 35. The other three mouse groups — OVA and radical and alkaline OVA–QUE — were orally challenged on day 35 with 20 mg OVA or radical or alkaline OVA–QUE conjugate in 500 μl PBS, respectively. After 1 h, a validated anaphylactic scoring (Table 1) was used to determine the allergic or anaphylactic symptoms of mice. Then mice were euthanized and blood, peritoneal fluid and spleens were collected for measuring the following biomarkers.

*Determination of allergen-specific immunoglobulins (Ig)*

IgE, IgG and $IgG_1$ were determined by ELISA. In brief, 100 μl of 6 mg/mL ELISA coating buffer (pH 9.6) was added to 96-well plates and incubated overnight. Serum from each mouse group (n =5/group) was pooled in equal volumes (100 μl) before analyses. Each diluted serum sample (1:20 for specific IgE, 1:8000 for specific IgG and IgG1) was added to plates. HRP-labeled goat anti-mouse IgE, IgG and $IgG_1$ (1:2000) antibodies (BD Pharmingen, Mississauga, ON, Canada) were added to determine the binding of IgE, IgG and IgG1. Finally, o-phenylenediamine substrate

(Sigma–Aldrich, St. Louis, MO, USA) was added for color development.

*Detection of mouse mast cell protease-1 (mMCP-1) in serum*

Serum was collected from mice (n=5/group) after 1-h challenge. The content of mMCP-1 in serum was analyzed by using an mMCP-1 ELISA kit (Elabscience Biotech, Wuhan, China) in accordance with the manufacturer's instructions.

*Morphological structure of duodenum*

After 1-h challenge with OVA or radical and or OVA–QUE, 4 mice in each group were euthanized. A 4-cm segment of the duodenum was removed from the mice immediately, then fixed with 10% formalin, and processed by standard histological techniques. Samples sectioned at 5-μm thickness were stained with eosin and hematoxylin. Finally, a light microscope (Nikon Ti Microscope, Japan) was used to observe the morphological structure of the small intestine.

*Cytokine analysis*

Individual spleens were aseptically removed from mice (n=6/group). After gently grinding spleens by using a syringe plunger, the contents were passed through a 100-μm nylon membrane cell strainer. After erythrocyte lysis, spleen cells were cultivated in complete medium (RPMI-1640 containing 2 mM/L-glutamine, 10% fetal bovine serum, 25 mM HEPES buffer, 100 mg/mL streptomycin and 100 IU/mL penicillin). Cells ($2\times10^6$/well) were cultured in 48-well plates for 72 h in the presence

of OVA or radical or alkaline OVE-QUE conjugate (50 μg/ml). The supernatant was collected, then IL-4, IL-5, IL-13 and IFN-γ contents were measured by using ELISA kits (Elabscience Biotech, Wuhan, China).

*Histamine levels in mouse plasma*

Plasma was collected from mouse blood (n=5/group) and centrifuged for 10 min at 4000 g. Plasma histamine was measured by using a histamine ELISA kit (Elabscience Biotech, Wuhan, China).

*Identification of peritoneal mast cells in mice*

After challenge, peritoneal mast cells were collected from mice (n=6/group). In the initial step, 5 ml RPMI-1640 containing 10% fetal bovine serum was injected into the mouse's peritoneal cavity. After the mouse's abdomen was massaged softly for 1 min, peritoneal fluid containing mast cells was collected. After centrifugation at 150 g for 10 min, cells were suspended in flow cytometry staining buffer.

The expression of the IgE receptor (FcεRI) and c-kit was detected in mast cells. After staining with APC-conjugated anti-mouse-FcεRI antibody (0.25 μg/test, clone MAR-1, Biolegend, San Diego, CA) and PE-conjugated anti-c-kit antibody (0.06 μg/test, clone 2B8, Biolegend, San Diego, CA) for 30 min in the dark at 4℃, cells were washed twice with flow cytometry staining buffer before flow cytometry.

### Statistical analysis

All sample determinations were carried out in triplicate. All data are expressed as mean ± SD. Differences were determined by using ANOVA and the Duncan least significant difference test. $P < 0.05$ was considered statistically significant.

## RESULTS AND DISCUSSION

### Profile of the OVA–QUE conjugates

The band of OVA was about 45 kD after conjugation with QUE (Figure 1A). As compared with native OVA, the bands of radical and alkaline OVA–QUE conjugates migrated up, which suggested that conjugation with QUE increased the molecular weight of OVA. The polyphenol content of radical and alkaline OVA–QUE was increased (Table 2), which suggests the conjugation of OVA with QUE.

The content of thiol groups was decreased in OVA with QUE conjugation (Table 2), which suggested that QUE covalently conjugated with some free amino groups in OVA [20]. The DPPH and ABTS+ values for OVA conjugates were 2 to 3 times higher than that for unmodified protein, because of more hydroxyl groups in QUE introduced on the protein.

The above results indicate the covalent interaction between QUE and OVA.

### Structural analysis of the OVA–QUE conjugates

Information on changes in the structure of OVA before and after QUE conjugation was obtained by using FTIR and CD spectroscopy and DSC. FTIR

spectra can help provide information about the changes in the protein secondary structure. The FTIR spectra of samples are in Figure 2A. Protein amide I (1600–1700 $cm^{-1}$) and amide II (≈ 1530 $cm^{-1}$) have a relationship with the secondary structure of protein. As compared with native OVA, the peak positions of the amide I band for radical and alkaline OVA–QUE conjugates moved from 1653.08 to 1657.05 and 1651.90, respectively, and peak positions of the amide II band moved from 1537.85 to 1534.83 and 1538.07, respectively. The results suggested that the secondary structure of the OVA was changed after conjugation with QUE.

CD spectra were used to further analyze the changes in OVA secondary structure after conjugation with QUE. Figure 2B shows that all spectra exhibited a negative band (222 nm), which is characteristic of α-helical structures in protein. As compared with native OVA, the absolute θ values of this negative band for the conjugates decreased, which indicates loss of some α-helical structures in the conjugates [19]. A similar result was obtained previously [25], with decreased α-helical structures of OVA after conjugation with catechin.

The protein structure is related to the thermal stability of the protein. We used DSC to study the thermal stability of the protein. Figure 2C shows that the thermal denaturation temperatures of OVA and radical and alkaline OVA–QUE conjugates were 98.4, 94.9 and 66.6 ℃ respectively, so the tertiary conformation stability of OVA was decreased after covalent conjugation with QUE.

The structural analysis suggested that covalent conjugation with QUE changed the protein secondary structure and caused the unfolded protein structure of OVA. In

a previous study, we also found that covalent conjugation with EGCG and chlorogenic acid caused an unfolded OVA structure [17-18] .

**In vitro assessment of allergenicity**

The *in vitro* allergenic capacity of the native OVA and conjugates was analyzed by ELISA to assess the IgE binding capacity. Figure 3A shows a lower IgE binding capacity for OVA after conjugation with QUE. ELISA cannot predict the ability of allergens to trigger degranulation of effector cells [24]. The release of histamine, IL-6 and other cytokines is a characteristic and critical function of basophils [26]. Therefore, we used human basophilic KU812 cells to test the ability of samples to elicit cell degranulation. As compared with native protein, the conjugates showed lower levels of histamine (Figure 3B) and IL-6 (Figure 3C) with conjugation, which suggests that QUE conjugation reduced the ability of OVA to trigger cell degranulation. These results agree with IgE binding capacity testing by ELISA.

**In vivo assessment of allergenicity**

A mouse model of orally induced OVA allergy was used to assess the *in vivo* allergenicity of OVA after conjugation with QUE.

*Anaphylactic shock score*

Anaphylactic shock symptoms were observed in BALB/c mice after challenge with native OVA (Figure 4B). However, as compared with native OVA treatment, the

anaphylactic shock symptoms with both radical and alkaline OVA–QUE conjugate were less severe (Figure 4C), with no significant difference between the radical and alkaline OVA–QUE groups.

*Assessment of IgE, IgG, IgG1, histamine and MCP-1 in mouse serum.*

We detected serum-specific IgE, IgG, IgG1, histamine and mMCP-1 in mouse serum to evaluate the allergenic potential of the OVA after conjugation with QUE. As compared with OVA alone, radical and alkaline OVA-QUE conjugate groups showed reduced serum levels of IgE, IgG, IgG1, histamine and mMCP-1 (Figure 4C, 4D, 4E, 4F and 4G).

*Assessment of cytokines in splenic cells*

Levels of Th2-related cytokines (IL-13, IL-4 and IL-5) and a Th1-related cytokine (IFN-γ) were detected in mouse spleen to further determine whether OVA conjugation with QUE could affect cytokine production in the immune response. As compared with OVA alone, radical and alkaline OVA–QUE conjugate groups showed a marked reduction in IL-13, IL-4 and IL-5 cytokine production in splenic cells, with a slight increase in IFN-γ level (Figure 4H, 4I, 4J and 4K).

*Histopathological assessment of duodenum*

Histopathological assessment of duodenum is shown in Figure 5. As compared with the control group, in mice sensitized with OVA and OVA–QUE conjugates, the

intestines were damaged. However, unmodified OVA-treated mice showed more severe allergic symptoms in duodenum than both radical and alkaline OVA–QUE conjugate groups.

*FcεRI$^+$ and c-kit$^+$ expression on peritoneal mast cells*

We collected peritoneal mast cells to test c-kit$^+$ and FcεRI$^+$ cells by flow cytometry to assess the attenuation of mast cell activation after challenge with OVA and its conjugates. The proportion of c-kit$^+$ and FcεRI$^+$ cells was greater with OVA challenge than control treatment and radical and alkaline OVA–QUE treatment (Figure 6).

Changes in the allergenicity of food allergen by conjugation with dietary polyphenols have been reported; however, research on the effect in *vivo* remains unclear. In this study, we evaluated the effect of QUE conjugation with OVA on sensitization and the allergic response in a BALB/c mouse model. On *in vivo* assessment of allergenicity in BALB/c mice, different levels of allergic symptoms were triggered by OVA and its conjugates, which suggests lower allergenicity *in vivo* with OVA–QUE conjugates than the native protein.

After conjugation with QUE, OVA relieved anaphylactic symptoms in mice. As compared with native OVA, histamine secretion with OVA–QUE treatment was reduced, which was consistent with the *in vitro* study. The IgE binding capacity of an allergen is directly related to its IgE binding epitopes. Conjugation with QUE alters OVA structures, then affects the conformational epitopes of the protein. Furthermore,

348 in our previous studies, we found that polyphenols conjugated on the surface of the
349 protein, which might affect linear IgE epitopes [17-18]. Therefore, after conjugation with
350 QUE, conformational and linear IgE epitopes in OVA may have been affected,
351 thereby reducing the IgE binding capacity, then led to decreased release of histamine.
352 Histamine will be released from basophil degranulation and mast cells and then
353 induce an allergic reaction [27].

354 Our OVA–QUE conjugates induced histamine significantly less than did the
355 native protein, which may be related to the weak systemic allergic symptoms.

356 FcεRI$^+$ and c-kit$^+$ were expressed on the surface of sensitized mast cells. Flow
357 cytometry revealed significantly higher expression of c-kit$^+$ and FcεRI$^+$ in
358 OVA-challenged mice than in controls, with the expression significantly reduced in
359 the OVA–QUE conjugate groups.

360 The level of mMCP-1 is key indicator for detecting mastocyte degranulation.
361 The level of mMCP-1 was higher in OVA-challenged mice than controls. As
362 compared with OVA challenge, with OVA–QUE conjugate challenge, the level of
363 mMCP-1 was significantly reduced. The results of mMCP-1 measurement were
364 consistent in the results of FcεRI$^+$ and c-kit$^+$ detection, which illustrates that after
365 conjugation with QUE, mastocyte degranulation was alleviated, thereby reducing the
366 allergenicity of OVA.

367 IFN-γ secreted by Th1 cells can inhibit IgE production [28]. However, cytokines
368 including IL-13, IL-4, and IL-5 can provoke Th2 cells to produce IgE and induce an
369 IgG isotype switch to IgG1. IgG1 and IgE can induce systemic anaphylaxis [29] and

Th2-dependent antibodies [30]. In this study, as compared with serum from native OVA challenge, that from OVA–QUE challenge showed significantly reduced levels of IgE and IgG1.

Moreover, conjugation with QUE downregulated the Th2-related cytokines (IL-13, IL-4 and IL-5) in spleen cells but upregulated the Th1 cytokine (IFN-γ), which agreed with the detection of antibodies in mice. Thus, conjugation with QUE may have suppressed the OVA allergic reaction by modulating the Th1/Th2 imbalance: suppressing Th2 immune response and promoting the Th1 immune response.

We prepared OVA–QUE conjugates by radical and alkaline methods. The mechanisms of these two methods are different. In the free radical method, hydroxide radicals are generated by oxidation-deduction reactions between redox pair components, then the radicals attack the amino acid in protein to produce radical species, which react with polyphenols, inducing a covalent bond [31]. In the alkaline method, polyphenols are oxidized to form a semiquinone, which may react with some residues such as histidine, cysteine and lysine residues in the chains of the protein to form a covalent bond [31]. However, the allergenicity of the radical and alkaline OVA–QUE conjugates did not significantly differ. Thus, both radical and alkaline methods could be used to prepare OVA–QUE conjugates with weaker allergenicity than native OVA. The weaker allergenicity of OVA–QUE conjugates may be due to QUE destroying and/or masking OVA epitopes.

To our knowledge, the interaction of polyphenols with proteins can alter the immunological properties of protein by changing the protein structure and/or masking

392 epitopes and their direct effect on allergic effector cells [4]. In addition, their
393 endogenous antioxidant ability limits the extent of cellular injury from free radicals
394 during the allergic insult [4]. Some polyphenols show inhibitory properties against some
395 digestive enzymes, such as pepsin, which would affect the digestibility of food
396 allergens [32]. In most cases, in the preparation of non-covalent protein–polyphenol
397 complexes, the unreacted polyphenols are not removed, and the polyphenols binding
398 on the protein may be released under certain conditions. So, the free polyphenols in
399 the non-covalent protein–polyphenol complex may have an important anti-allergy
400 role.

401 QUE has been investigated for its anti-allergic effect in different disease models
402 [33-35]. In this study, unreacted QUE was removed by dialysis. Furthermore, the protein–
403 polyphenol covalent conjugate was more stable than the non-covalent protein–
404 polyphenol complex. The QUE binding on the covalent conjugate hardly moved away
405 from the conjugate. Therefore, there was no free QUE in the test samples, which may
406 not directly affect the immune cell and biological pathways in the allergic immune
407 response by the free QUE. Whether QUE covalent binding on the protein has the
408 same anti-allergic effect as free QUE deserves further investigation.

409 In conclusion, QUE could covalently conjugate with OVA and led to a changed
410 protein structure of OVA, which might destroy and/or mask OVA epitopes. The
411 conjugates showed lower allergenicity *in vitro* and *in vivo.* Conjugation with QUE
412 reduced the ability for IgE binding and triggering cell degranulation of OVA *in vitro,*
413 and the levels of specific IgE, IgG1, IgG, plasma histamine, and mMCP-1 as well as

414 FcεRI$^{+}$ and c-kit$^{+}$ expression on the surface of sensitized mast cells were suppressed.
415 Furthermore, conjugation with QUE modulated the imbalance of the Th1/Th2
416 immune response, which might suppress the OVA-induced allergic reaction.
417 Therefore, conjugation OVA with QUE could reduce OVA allergenicity, which may
418 help in producing hypoallergenic food.
419

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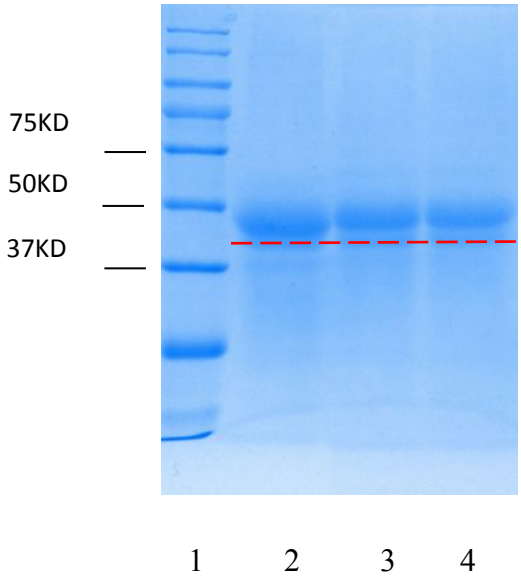
## Figure Legends

**Figure 1**. SDS-PAGE analysis of OVA and OVA–QUE conjugates.




1: Marker, 2: Native OVA, 3: Radical OVA–QUE conjugate, 4: Alkaline OVA–QUE conjugate

**Figure 2**. Fluorescence (A), CD (B) and DSC (C) spectra for OVA and OVA–QUE conjugates.

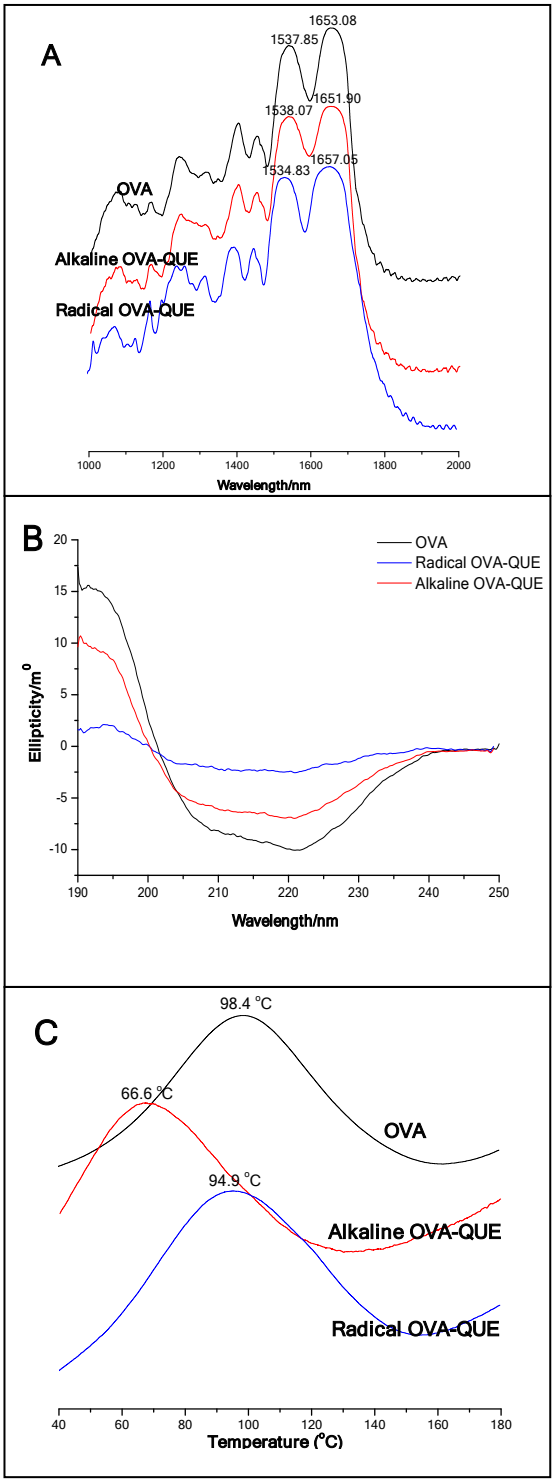
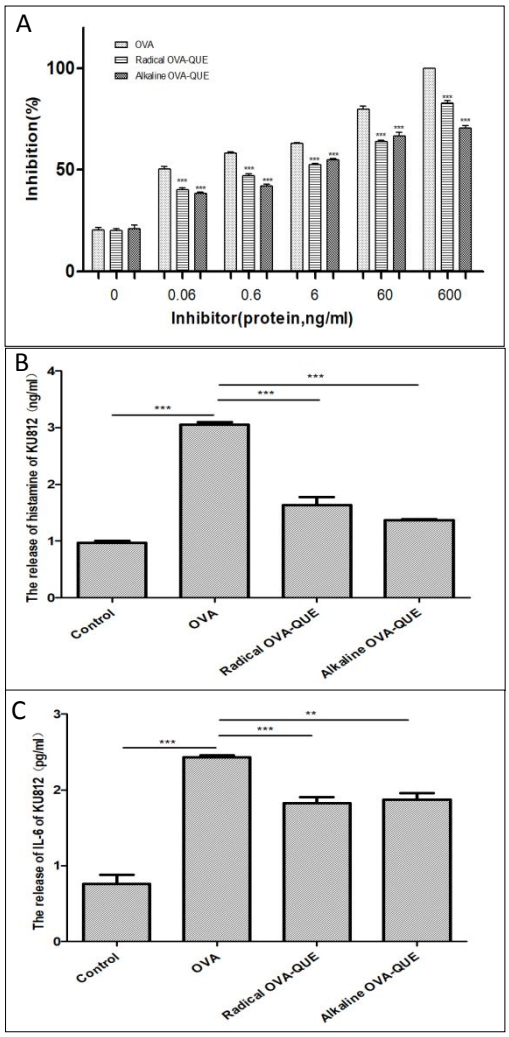


**Figure 3**. IgE binding ability detected by ELISA (A), basophil histamine release with KU812 cell degranulation (B) and IL-4 release with KU812 cell degranulation (C) with OVA or radical or alkaline OVA–QUE conjugates.



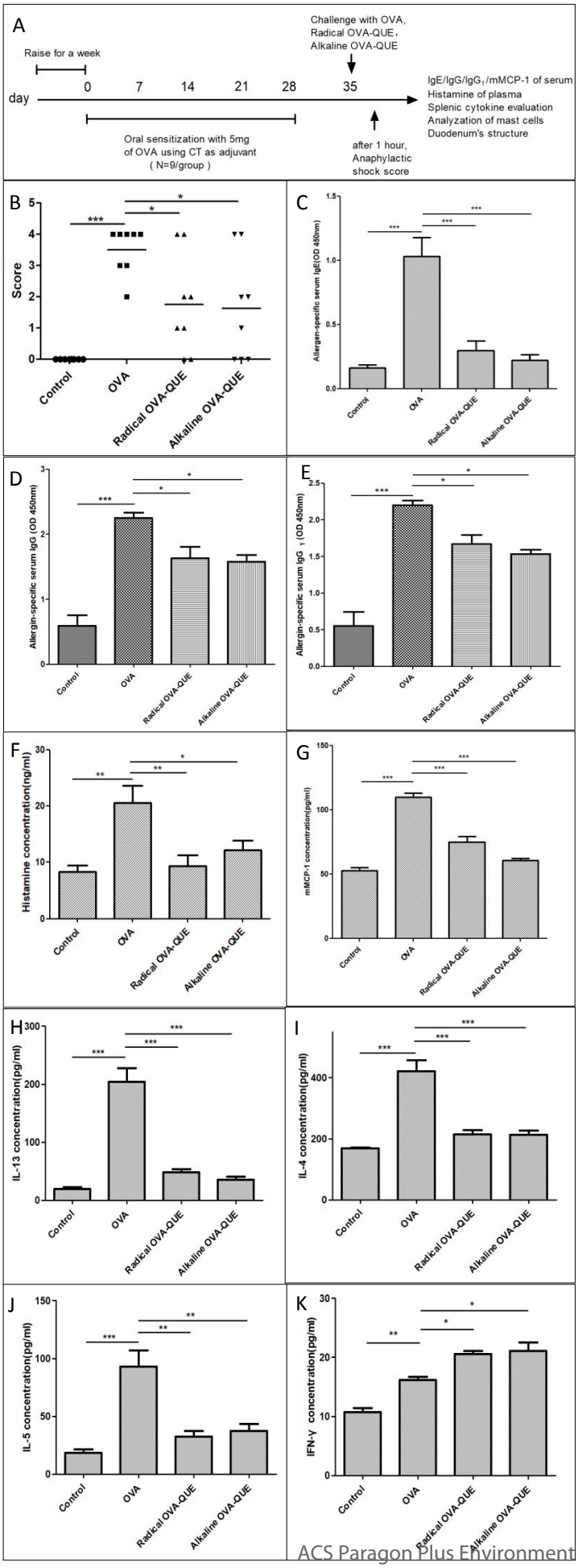



**Figure 4**. Oral immunization of BALB/c mice with OVA or radical or alkaline OVA–QUE conjugates (A). Hypersensitivity symptoms (B) scored on a scale from 0 (no symptoms) to 5 (death) as described in Table 1. Levels of IgG (C), IgG1 (D), IgE (E), plasma histamine (F) and mMCP-1 (G) in serum from mice. Levels of cytokines IL-13 (H), IL-4 (I), IL-5 (J) and γ-IFN (K) in mouse spleen. Results are expressed as mean±SD. ***p < 0.001, **P < 0.01, *P < 0.05 compared with OVA group.

**Figure 5.** Effects of control (A), OVA (B), radical OVA–QUE (B) and alkaline OVA–QUE (D) treatment on morphological structure of duodenum in mice.

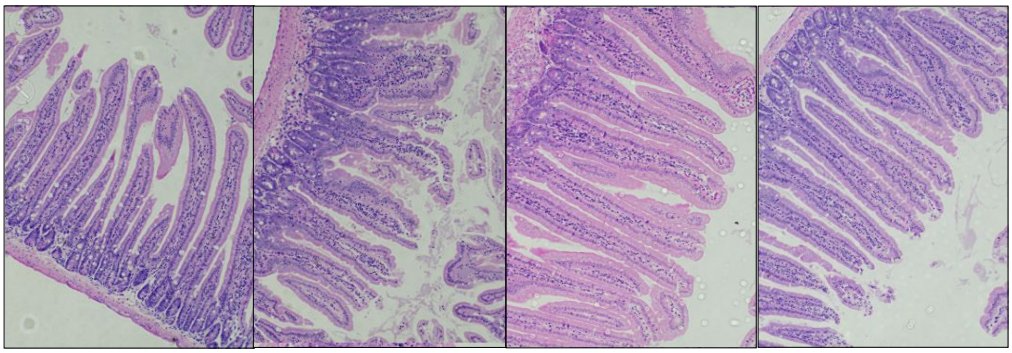
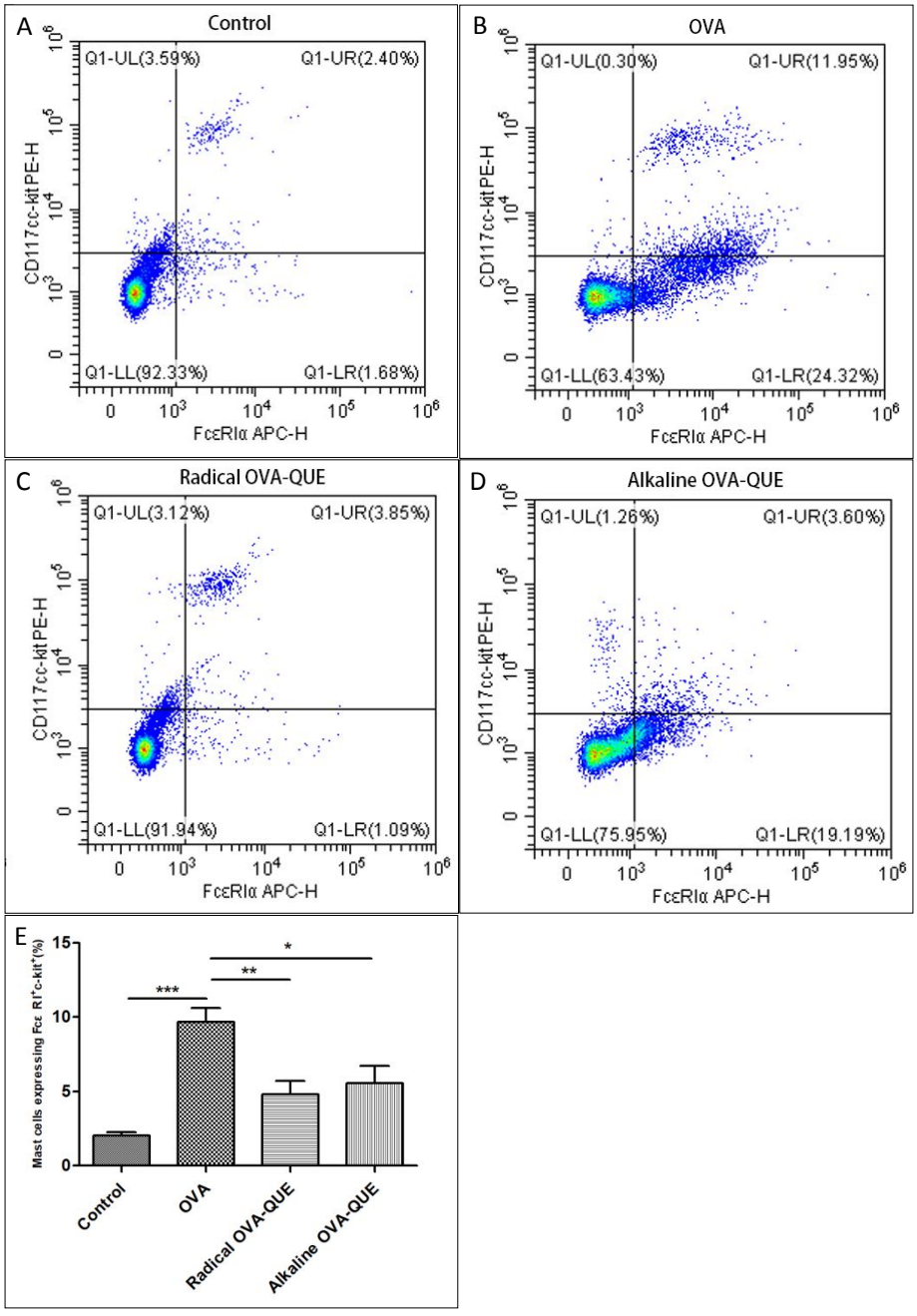


**Figure 6**. FcεRI and c-kit expression on peritoneal mast cells with control (A), OVA (B), radical OVA–QUE (B) and OVA–QUE (D) treatment. The proportion of FcεRI and c-kit cells was quantified (E). ***p < 0.001, **P < 0.01, *P < 0.05 significant difference as compared with the OVA group.



# Tables

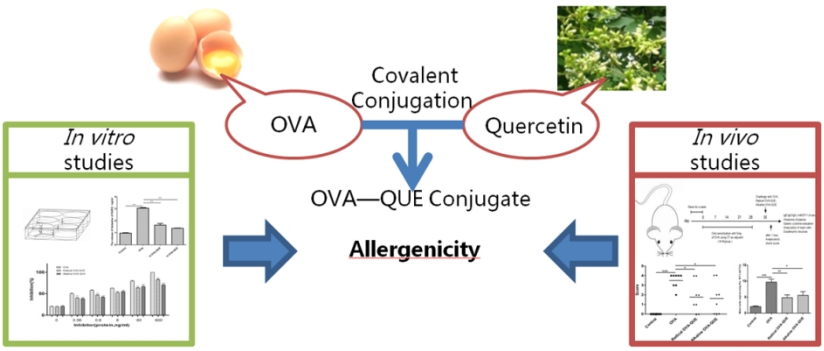
**Table 1**. Anaphylactic symptom scoring

| Score | Symptoms |
|---|---|
| 0 | No symptoms |
| 1 | Scratching nose and mouth |
| 2 | Swelling around the eyes and mouth; pillar erection; reduced activity; higher breathing rate |
| 3 | Shortness of breath; blue rash around the mouth and tail; higher breaching rate |
| 4 | No activity after stimulation, shivering and muscle contractions |
| 5 | Death by shock |

**Table 2**. Characterization of OVA and OVA-QUE conjugates.

| Protein/sample | Native OVA | Radical OVA-QUE | Alkaline OVA-QUE |
|---|---|---|---|
| Polyphones bound (mg/g) | — | 38.61±2.10*** | 21.55±1.91*** |
| Free amino group (nmol/mg) | 1.17±0.007 | 0.64±0.017*** | 0.92±0.003*** |
| Thiol group (nmol/mg) | 3.08±0.104 | 2.76±0.061* | 1.56±0.070** |
| DPPH scavenging activity (μmol Trolox/g sample) | 1.53±0.1996 | 5.27±0.085*** | 4.88±0.028*** |
| ABTS+ scavenging activity (μmol Trolox/g sample) | 3.24±0.146 | 6.44±0.600** | 6.84±0.214*** |

***p < 0.001, **P < 0.01, *P < 0.05 significant difference as compared with the native OVA.

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