Original Article

The Pathogenesis of Rheumatoid Arthritis is Associated with Milk or Egg Allergy

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Abstract

Background: Rheumatoid arthritis (RA) is a very complicated autoimmune disease with apparent synovial hyperplasia and cartilage and bone destruction. **Aims:** In the present study, we aimed to determine whether the pathogenesis of RA correlates with food allergy and which allergen(s) are relevant. **Materials and Methods:** We used type-II collagen (CII) to induce arthritis (collagen-induced arthritis, CIA) model in Wistar rats, and the development of arthritis was evaluated accordingly by scoring system. Proinflammatory cytokine levels in plasma were measured by enzyme-linked immunosorbent assay (ELISA), and concentrations of circulating immune complexes (CICs) were analyzed by C1q solid phase method. Furthermore, food-specific immunoglobulin G (IgG) and immunoglobulin E (IgE) levels were determined in the CIA model. **Results:** In the CIA model, we found that levels of tumor necrosis factor-alpha (TNF-a), interleukin (IL)-1, IL-6, and IL-17, as well as CICs, were elevated significantly. Moreover, concentrations of milk- or egg-specific IgG and IgE were enhanced strikingly in CIA rats. **Conclusion:** The results suggest that pathogenesis of RA correlates closely to increased egg- or milk-specific antibodies.

Keywords: Egg allergen, food allergy, milk allergen, proinflammatory cytokines, rheumatoid arthritis (RA)

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease of multifactorial etiology characterized by synovial hyperplasia; autoantibody production; cartilage and bone destruction and joint malformation; and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders.[1] The prevalence of RA is between 0.3% and 1% in the general population.[2] Both clinical and experimental studies have identified the osteoclast as the predominant cell type mediating bone loss in RA. Histologically, RA synovium is characterized by hyperplasia of the synovial lining cells and infiltration of the synovium deep to the lining layer by lymphocytes, activated macrophages, plasma cells, and other cell types, and is accompanied by intense neovascularization. Inflammatory mediators released by cells in the synovium and invasive pannus contribute to the destruction of both the cartilage and bone tissues.[3]

The pathogenesis of RA is complicated and elusive. It is considered to involve genetic and external factors,[4]

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lifestyle factors, infectious agents, occupational exposures, and long-term living with pets.[5] Variable clinical responses to current therapies, such as tumor necrosis factor (TNF) blockers, T-cell costimulation inhibitors, and B-cell depletors, demonstrate that the disease is heterogeneous and probably lacks a single mechanism that is adaptive to all patients. Studies have indicated that T-cell lineage, B-cell lineage, autoantibodies such as anti-cyclic citrulline antibody, and the cytokine network all contribute to the occurrence of RA. Enhanced levels of proinflammatory cytokines such as TNF-α, interleukin (IL)-6, and IL-33, are key features in patients with RA.[6] Growing evidences support that TNF-α is primarily responsible for driving inflammation and IL-1 for the destruction of cartilage and bone.[7]

The correlation between RA and food allergy has been suspected since 1953,[8] and then reported by several groups.[9] Moreover, the effect of food restrictions in treating RA patients was proposed in many studies. For decades, dietary manipulation, including vegetarian or vegan diets, vegetarian and Mediterranean diets (typically plant foods), elemental diet plans (hypoallergenic, protein-free artificial diet), and elimination diets (nonallergenic), has been used for RA patients to possibly prevent the autoimmune response.[10] A possible explanation for improvement in RA may involve reduced food intolerance, decreased gastrointestinal permeability, and benefit from both weight loss and altered intake of substrates for prostaglandin production. Until 2006, Hvatum *et al.* were the only ones to intensively investigate intestinal immunity in RA patients, and they found that there is a general positive correlation between RA and food antibodies, especially in terms of immunoglobulin M (IgM) class.[11]

Using CII to induce arthritis (collagen-induced arthritis, CIA) in rats, this study was conducted with the aim to investigate whether allergy to "big eight" foods is correlated with the pathogenesis of RA.

Materials and Methods

Animals

Female Wistar rats (body weight 80-120 g) were purchased from Vital River Laboratories (Beijing, China) and housed in groups of 4 per cage in a controlled environment with a photoperiod of 12 h light/12 h dark and a temperature of 20 ± 2°C. Sanitary controls were performed for all major rodent pathogens, and the results of these tests were uniformly negative. All the animal experimental procedures were approved by the Animal Care and Use Committee of Shenzhen University and carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH; publication no. 85-23, revised 1996).

Preparation of bovine type-II collagen

Bovine CII was isolated from articular cartilage after removing proteoglycans by guanidine hydrochloride, digestion by pepsin, salt precipitation by sodium chloride, and purification with cellulose column chromatography as previously described, with slight modification.[12]

Collagen-induced arthritic model

The CIA model was induced in the present study, as previously reported.[13] The rats were randomly assigned to one of three groups: Control group, incomplete Freund's adjuvant (IFA) group, and CIA group. Purified bovine CII was dissolved in 0.01 mol/L acetic acid and emulsified with an equal volume of IFA (Sigma, MO, USA). For the CIA group, 1 mL of emulsion (4 mg/mL CII) was intradermally injected into several spots on the backs of the rats as the primary immunization. After 7 days, the rats were given a booster injection at the same sites with 0.5 mL emulsion of CII and IFA. The control group received the injection of an equal volume of 0.01 mol/L acetic acid at the same location, while the IFA group was injected with the same volume of acetic acid:IFA (1:1).

Evaluation of the development of arthritis

All animals were tested daily. The arthritis index (AI) was recorded before first immunization and every 2 or 3 days beginning on the day when arthritic signs were first visible. The rats were inspected daily for the onset of arthritis, characterized by edema and/or erythema in the paws. The incidence and the severity of arthritis were evaluated using a system of arthritic scoring, measuring bi-hind ankle diameter and paw volumes. Scores indicate the range and degree of joint swelling and deformation, and were recorded according to the following criteria: Score 0, there was no swelling; score 1, single swelling site was affected in paw or in paw pad; score 2, two or more sites were affected in paw, paw pad, or ankle joint; score 3, the whole paw was affected; score 4, there was severe swelling causing ankylosis, deformity, and functional disturbance. The maximum score of a single paw was 4 and of a single rat was 16.[14]

Based on previous experience, arthritis was observed approximately on Day 12 and by Day 28 there was significant joint pathology. Thus, the animals were sacrificed 28 days after the first immunization and plasma was collected for the determination of inflammatory cytokines. The right hindpaw was obtained for histological observation. The results of ankle

diameter, and paw volumes were presented on day 0, day 14, and day 28.

C1q solid phase immunoassay

The circulating immune complexes (CIC) in plasma were detected by C1q solid phase assay in duplicate by a modification of the method described by Hardin *et al*.[15] In brief, each well of the enzyme-linked immunosorbent assay (ELISA) plate was coated with 200 uL C1q solution 10 mg/L in phosphate-buffered saline (PBS) overnight at 4°C. After three washes with PBS-Tween, all the wells were incubated with 0.1% bovine serum albumin (BSA) in PBS for 2 h. Then the plates were washed with PBS-Tween for three times and 200 uL of samples serum containing CIC were added into the well. The plates were incubated for 1 h at 37°C and for 20 h at 4°C, and unbound proteins were then removed by washing three times with cold PBS-Tween. CICs bound to the C1q-coated wells were detected by incubating the tubes with 1 mcg of purified horseradish peroxidase (HRP)-labeled IgG.

Food allergens detection by homemade ELISA or western blot

Relative levels of IgG and IgM antibody activities against "big eight" food allergens in the serum of rats were detected by ELISA. Briefly, total proteins from fish, shrimp, egg, wheat, peanut, bean, milk, and hazelnut were extracted by removing lipid with acetone, as previously described. ELISA plates were coated with the above eight kinds of total proteins (1 ug/mL) respectively. Serum from CIA rats was used as the first antibody and HRP-labeled IgG was the second antibody.

IgE measurement by radioallergosorbent test (RAST)

Food-specific IgE in plasma was determined by RAST as previously described.[16]

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). When two comparisons were obtained, Student's unpaired two tailed *t*-test was used. When multiple comparisons were obtained, the analyses consisted of one-way analysis of variance (ANOVA) for repeated measures and the Student-Newman-Keuls multiple comparison test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Type-II collagen extraction

After isolating from bovine articular cartilage, CII was dissolved with 0.5M acetic acid (AcOH) by vortexing overnight at a concentration of 10 mg/mL or 5 mg/mL. The purity of the extracted collagen was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining [Figure 1].

Collagen-induced arthritic model

In the present study, the CIA rats showed apparent clinical signs of arthritis and significant higher arthritis score on day 28 [Figures 2 and 3i]. The results also showed that ankle diameters [Figure 3ii] and paw volumes [Figure 3iii] were significantly increased in CIA rats. Antigen-specific IgGs are important mediators in the pathogenesis of RA. Thus, we wonder if CII-specific (anti-CII) IgG was elevated in the CIA group. The results indicated that an enhanced level of anti-CII IgG was detected in the serum of CIA rats [Figure 3iv].

Levels of proinflammatory and Th2-type cytokines were increased in CIA model

It has been reported that TNF-α and other proinflammatory cytokines lead to synovial fibroblast hyperplasia, destruction of the extracellular matrix, and eventual damage to the affected joints.[17,18] In this study, the concentrations of proinflammatory cytokines, including TNF-α, IL-1, IL-6, and IL-17, in the blood serum were determined on day 28 by ELISA. It was found that all these cytokines levels were twofold higher in the CIA group, when compared to vehicle-treated control animals [Figure 4i]. In parallel, Th2-type cytokines were also dramatically elevated in CIA rats [Figure 4ii].

Circulating immune complexes (CIC) analysis

CICs are produced during the immune response in RA, which can activate the complement system by both the classical and the alternative pathways. In this investigation, we used C1q solid phase method to determine the concentration of CIC in plasma. The

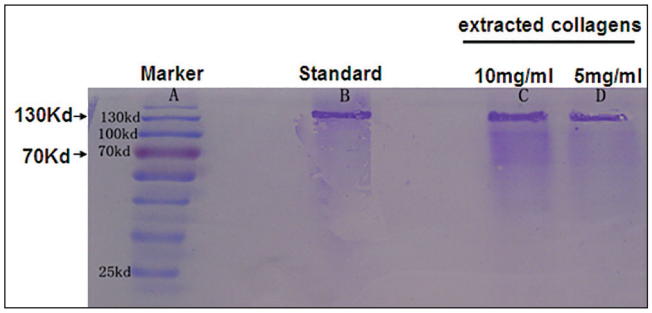

Figure 1: Coomassie Blue staining of extracted collagen. Purity of the isolated CII was confirmed by SDS-PAGE using Coomassie Blue staining. Commercial purified collagen was used as standard

results showed that the CIC level was twofold higher than that of the control group (210 ± 25% vs 100 ± 13% of control) [Figure 5].

The correlation between food allergy and RA

Recent studies have recognized that intestinal immune reactions might be associated with the articular inflammation. Taking into consideration the fact that IgG is the most important antibody playing a role in the pathogenesis of RA, in the present study we measured IgG antibody activities against the "big eight" food antigens using ELISA. As shown in Figure 6i, the results indicated that occurrence of RA is more related to egg- or milk-specific IgG. Furthermore, egg- or milk-specific IgE was determined by RAST and significant elevated concentrations of specific IgE (sIgE) were observed in CIA rats [Figure 6ii].

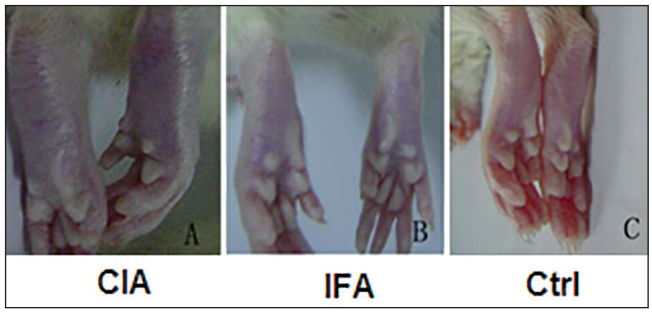

Figure 2: Clinical manifestation of arthritic rats, CIA = Collagen-induced arthritis rats, IFA = Incomplete Freund's adjuvant-treated rats, Ctrl = Vehicle-treated rats

Discussion

In the present study, we used CII, which is a major and specific protein isolated from articular cartilage, to induce arthritis model in rats. The severity of arthritis was evaluated by scoring paw edema and erydema, ankle diameter and volume, as well as proinflammatory cytokines production. By detecting food-specific antibodies in the serum from CIA rats, the crossreactivity of food allergy and rheumatoid arthritis was observed.

The CIA model is a well-established RA experimental model in susceptible strains of rodents by immunization with CII. Compared to other experimental arthritic models, CIA is demonstrated to more closely resemble human RA in terms of clinical, histological, and immunological features, as well as by genetic linkage.[14]

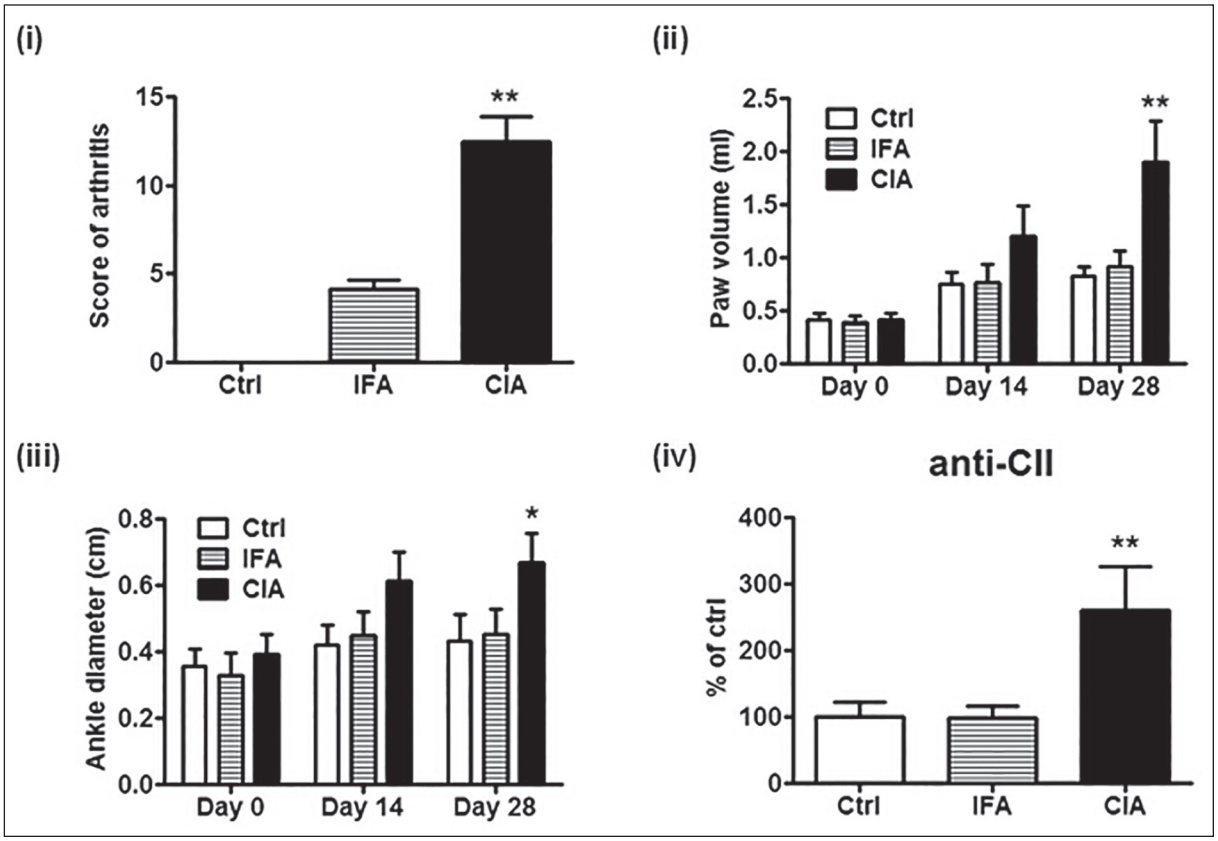

Figure 3: Arthritic changes in CIA rats: (i) Arthritis score changes in rats (ii) Ankle diameter changes in rats (iii) Paw volume changes in rats (iv) Serum level of CII-specific (anti-CII) IgG. The rats were killed at 28 days following the primary immunization Data were expressed as mean ± SEM, n = 6-9. *P < 0.05, **P < 0.01 vs ctrl

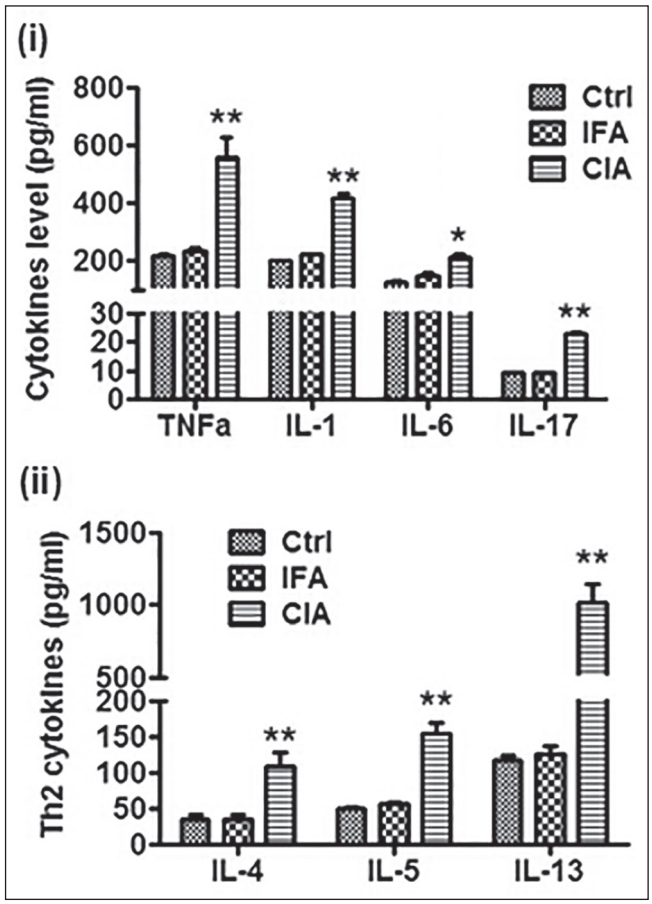

Figure 4: Proinflammatory cytokines levels in CIA rats. The serum from all the animals was collected and the levels of cytokines were measured by ELISA, Data were expressed as mean ± SEM, n = 6-9. *P < 0.05, **P < 0.01 vs ctrl

Therefore, we established the CIA model in Wistar rats accordingly and RA was induced in 100% of the animals, showing apparent paw edema and/or erydema, and increased ankle diameters and paw volumes.

The amounts of TNF-α, IL-1β, and IL-6 produced by monocytes, macrophages, and synovial fibroblasts increased significantly in the synovial fluid and joint tissues of RA patients.[19] These proinflammatory cytokines are found to play a vital role in the pathogenesis and disease progression of RA. Recent research has uncovered that proinflammatory cytokines such as TNF-α and IL-1β play key roles in the development of RA, while the mechanisms regarding them are not clear.[20,21] In the present study, we found that proinflammatory factors including TNF-α, IL-1, IL-6, and IL-17 were significantly increased in CIA rats, which is consistent with previous research.

The formation of immune complexes and the activation of the complement system are known features of RA. The identity of antigens incorporated into immune complexes provides the information that in the future may facilitate

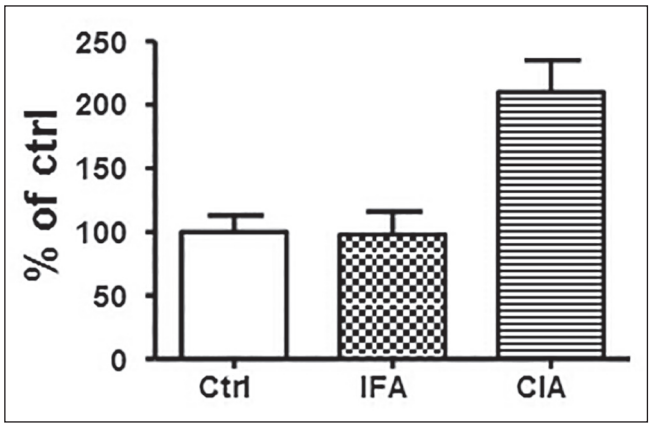

Figure 5: The concentration of CIC in plasma of CIA rats. The serum from all the animals was collected and the levels of CIC were measured by C1q solid phase immunoassay, Data were expressed as mean ± SEM, n = 6-9. *P < 0.05, **P < 0.01 vs ctrl

the development of diagnosis and treatment strategies for autoimmune diseases, and this information might be more relevant than the information on free antigens. In this study, we observed that CICs, as measured by the determination of C1q binding activity, were significantly increased in arthritic rats.

Although the association between RA and food allergy has been suspected for a long time, there are only a few studies regarding this topic, and the detailed link and mechanism is far from known. Among them, most of the studies focused on the effect of diet restriction on RA therapy. According to a questionnaire survey, 33.33% of the patients with RA reported aggravation of diseases symptoms after intake of certain foods.[22] A case study in 1 RA patient displayed that the exclusion of milk and cheese from the diet resulted in a pronounced improvement in synovitis and reduction in immune complexes, IgE antibodies, and heat-damaged red cell clearance rates.[23] In 2006, the first extensive investigation of intestinal food antibodies in RA patients was conducted; it investigated five types of foods, i.e., milk, cereals, egg, fish, and meat,[11] while our study has expanded the number of foods to eight types.

Using a well-known experimental RA model induced by CII, we investigated the "big eight" food-specific antibodies in the serum of CIA rats. A close association between pathogenesis of RA and egg- or milk-specific antibodies was observed for the first time by our group.

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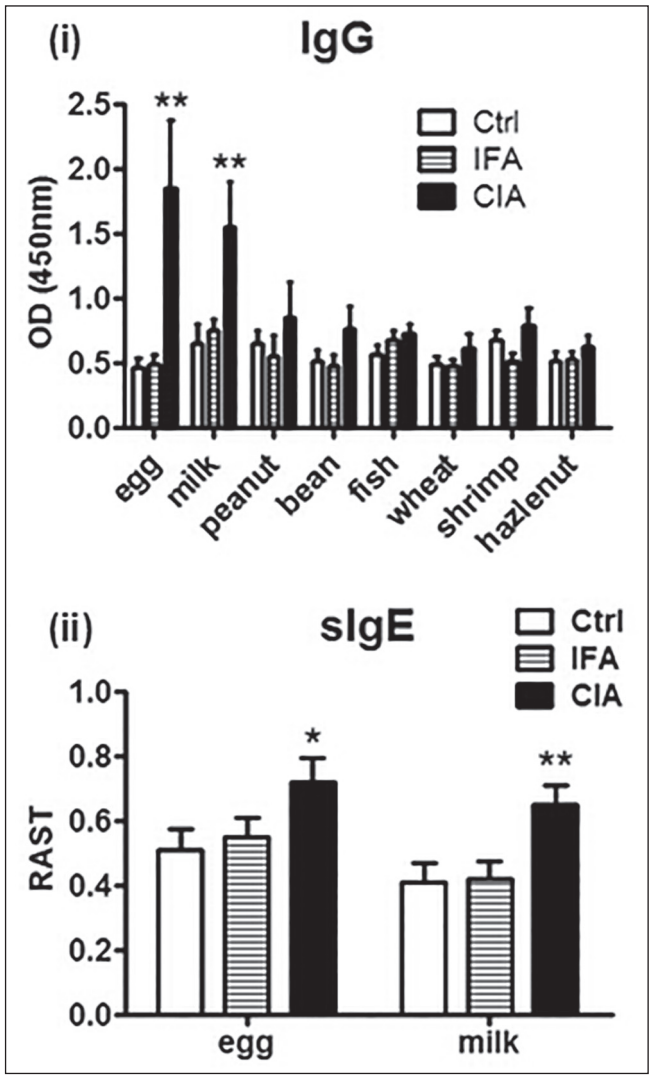

Figure 6: The concentration of food-specific IgG and IgE were checked in the plasma of CIA rats. The serum from all the animals was collected and the levels of food-specific IgG (i) and IgE (ii) were measured by ELISA and RAST, respectively, Data were expressed as mean ± SEM, n = 6-9. *P < 0.05, **P < 0.01 vs ctrl

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Nil.

Conflicts of interest

There are no conflicts of interest.

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