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Supplementation of Red Cabbage (*Brassica oleracea* L. var.) Juice Increases Serum Total Antibody Levels in Mice

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ABSTRACT

This study investigates the effects of supplementing unimmunized (non-specific) and ovalbumin (OVA)/complete Freund's adjuvant (CFA)-immunized mice with red cabbage (RC, *Brassica oleracea* L. var.) juice for 8 and 6 weeks, respectively. Changes in serum total immunoglobulin (Ig) A, IgE, IgG and IgM levels, as well as OVA-specific IgE, IgG1 and IgG2a titers were determined. The results showed that RC juice supplementation did not significantly affect the body weight, feed intake and feed efficiency in either unimmunized or OVA/CFA-immunized mouse models. Serum OVA-specific antibody titers in OVA/CFA-immunized mice were also not significantly affected. However, RC juice supplementation increased serum total antibody levels, especially IgM and IgE, in the sera of unimmunized mice. The present study suggests that RC juice has immunomodulatory effects *in vivo* via enhancing humoral immunity under unimmunized status.

Key words: *Brassica oleracea* L., ovalbumin (OVA)/complete Freund's adjuvant (CFA)-immunization, red cabbage juice, serum antibody

INTRODUCTION

Humoral immunity, especially the antibody secretion in the body, plays an important part in the defense against infectious diseases and maintains a memory of the same infectious agents *in vivo*[1]. Therefore, regulating humoral immune responses by foods, nutraceuticals or medicines is an interesting and important issue. Among foods, we found that fruits and vegetables seem to have potential in modulating immunity[2,3]. Recently, red cabbage (RC) has attracted our attention due to its potent anti-inflammatory effects *in vitro*[4].

Cabbage, which belongs to the Cruciferae family (including cultivated cabbage categorized into white cabbage, RC and savoy cabbage), is one of the most important vegetables grown worldwide[5]. It is found that acylated forms of the anthocyanins in RC are notably more stable than non-acylated forms[6]. The RC's dye has been used as a pH indicator in pharmaceutical formulations[7] and as a colorant in food systems[8]. The anthocyanins in RC inhibit amyloid β protein-induced neurotoxicity in neuron-like PC12 cells[9]. RC juice via gavage to ICR female mice displayed a protective effect on oxidative stress in the brains of mice administered (i.p.) with N-methyl-D-aspartate (NMDA)[10]. Our previous studies demonstrated that RC (*Brassica oleracea* L. var.) juice shows anti-inflammatory effects on LPS-stimulated murine splenocytes[4]. RC juice may serve as green medicine, and RC leaf extract for various medicinal purpose has been recognized as safe[11]. However, it is currently unclear whether RC juice affects humoral immunity *in vivo*. This study attempted to investigate the effects of RC juice supplementation on serum antibody levels in mice.

We hypothesized that RC juice has the ability to regulate humoral immunity *in vivo*. However, the humoral immunity in non-specific (unimmunized) and antigen-specific status is quite different *in vivo*. Hence, this study investigates the effects of RC (*Brassica oleracea* L. var.) juice supplementation on unimmunized and ovalbumin (OVA)/complete Freund's adjuvant (CFA)-immunized mice for 8 and 6 weeks, respectively. Changes in total serum immunoglobulin (Ig) A, IgE, IgG and IgM levels, as well as OVA-specific IgE, IgG1 and IgG2a titers were determined.

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MATERIALS AND METHODS

I. *Preparation of Red Cabbage (RC) Juice*

Red cabbage (*B. oleracea* L. var.) juice was prepared as described in our previous studies[4,12]. Briefly, RC was purchased from a local supermarket in Taichung, Taiwan. The fresh sample was immediately (without storage) squeezed to juice by a manual stainless screw squeezer (Vegetable & Fruit Grinder, manual type, Mei-Er-Then Co., Ltd., Taipei, Taiwan). The juice was centrifuged at 9,000 ×g (4°C) for 30 min, and the supernatant was collected using suction filtration through filter papers (Toyo No. 5B, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The extraction efficiency of RC juice was 69.1%. The filtrate was weighed, lyophilized and stored at -30°C for future use. The yield of lyophilized powder from the RC juice was 5.3%. The lyophilized powder of RC juice was weighed and freshly dissolved in deionized water at appropriate concentrations for tube feeding.

II. *Experimental Procedure*

Female BALB/c ByJNarl mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, Taiwan and maintained in the Department of Food Science and Biotechnology at the College of Agriculture and Natural Resources of National Chung Hsing University in Taichung, Taiwan. The animal room was kept in a 12-h light and 12-h dark cycle. Constant temperature (25 ± 2°C) and humidity were maintained. Every 3 mice were housed in a stainless steel cage and kept on a chow diet (laboratory standard diet) to acclimatize for 2 weeks before being fed with the AIN-76 experimental diet. The experimental diet was prepared according to the recommendations from the American Institute of Nutrition AIN-76[13]. After this equilibration period, the mice were randomly divided into groups for experiments. The animal use protocol listed in this study was reviewed and approved by the Institutional Animal care and Use Committee (IACUC) of National Chung Hsing University, Taiwan.

III. *Grouping and Supplementation in the Unimmunized Mouse Model*

The mice (8 weeks old) were grouped into control (0 mg RC juice/0.5 mL deionized water/mouse/day, 0 mg/kg bw), low dose (6 mg/mouse/day, 300 mg/kg bw) of RC juice (coded as RL), and high dose (240 mg/mouse/day, 12 g/kg bw) of RC juice (coded as RH) groups in the unimmunized mouse model. There were 12 mice per group. Four sets of three mice in each group were respectively earmarked and housed in separate stainless steel cages. The supplemented low dose RC juice lyophilized powder (6 mg/mouse/day, 300 mg/kg bw) to mice was equal to 60 g fresh RC/day in humans according to an appropriate conversion ratio at 1 : 387.9 for mice (20 g) to human (70 kg)[14]. Each group was fed with AIN-76 feed and supplemented with RC juice once per day with intragastric gavage using a stainless steel feeding syringe for 8 consecutive weeks. Mean feed intake and body weight change in mice were measured twice a week during the study period. At the time of completion of the study, the mice (16 weeks old) were weighed, anaesthetized with isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane, Sigma, MO, USA) and immediately bled using retro-orbital venous plexus puncture to collect the blood for later determination of non-specific antibody titers, including IgA, IgG, IgM and IgE. Immediately after blood collection, the animals were sacrificed using CO_2 inhalation. The serum was collected and assayed.

IV. *Grouping and Supplementation in the OVA-Immunized Mouse Model*

The mice (8 weeks old) were grouped into non-immunized control [treated with phosphate buffered saline (PBS) and complete Freund's adjuvant (CFA, Sigma A-5881, MO, USA), coded as PBS/CFA], dietary control (treated with OVA and CFA, coded as OVA/CFA), OVA/CFA-RL [treated with OVA/CFA and supplemented with low dose (6 mg/mouse/day, 300 mg/kg bw) RC juice] and OVA/CFA-RH [treated with OVA/CFA and supplemented with high dose (240 mg/mouse/day, 12 g/kg bw) RC juice] groups. There were 12 mice per group. Four sets of three mice in each group were respectively earmarked and housed in separate stainless steel cages. The control groups received 0 mg RC juice/0.5 mL deionized water/mouse/day. Each group was fed with AIN-76 feed and supplemented with RC juice once per day with intragastric gavage using a stainless steel feeding syringe for 6 consecutive weeks. There were 4 mice in the PBS/CFA group. There were 12 mice in each OVA/CFA-immunized group. Mean feed intake and body weight change in mice were measured twice a week during the study period.

During the study period, the experimental mice were immunized with OVA in the OVA-immunized mouse model. They were immunized by an intraperitonal injection (i. p.) of 100-μL aliquots of CFA-precipitated antigen containing 2 μg of OVA (albumin chicken egg grade III; Sigma, MO, USA) to induce primary immunity after supplementation with RC juice for one week. Two booster injections of this CFA-OVA mixture containing 6 μg of OVA were given after 14 and 28 days, respectively. Non-immunized control mice received CFA-phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 0.2 μm filtered) only. The mice (14 weeks old) were weighed one week later, anaesthetized with isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane; Sigma, MO, USA) and bled immediately using retro-orbital venous plexus puncture to collect the blood. Immediately after blood collection, the animals were sacrificed using CO_2 inhalation. The serum was collected and assayed. The organs were collected and weighted. To determine the changes of serum antibody titers during the experimental period, the animals were also anaesthetized with isoflurane, on days 0, 14 and

28, to collect a small amount of whole blood (about 200 μL) for later determination of OVA-specific IgG1, IgG2a and IgE antibody titers[14].

V. *Serum Preparation*

The whole blood was collected into a 1.5-mL vial and allowed to stand for 2 h at room temperature, then centrifuged at 12,000 ×g for 15 min at 4°C to separate the serum. The sera were collected and stored at -30°C for analysis.

VI. *Non-Specific Antibody Quantification by an ELISA*

Serum antibody levels of IgA, IgE, IgG and IgM were analyzed using the mouse IgA, IgE, IgG and IgM ELISA quantization kit (catalog number: E90-103, E90-115, E90-131 and E90-101; Bethyl Laboratories, Inc., TX, USA). The serum samples were appropriately diluted. The protocol has been described in the previous study[1].

VII. *OVA-Specific IgE, IgG1 and IgG2a Assays*

An ELISA protocol was used to determine the OVA-specific IgE, IgG1 and IgG2a antibody levels in the serum. Aliquots of 200 μL/well of OVA (10 μg/mL dissolved in 0.1 M of $NaHCO_3$ (*Wako* Pure *Chemical* Industries, Ltd., Osaka, Japan), pH 8.2) were pipetted into the 96-well EIA/RIA microplate (Nunc). The plates were incubated overnight at 4°C. They were then carefully washed thrice with PBS. Aliquots of 200 μL/well of blocking solution [1% bovine serum albumin (BSA; Sigma, MO, USA) in PBS buffer] were pipetted into the 96 microplate wells to block the free sites on the wells. After incubation for 2 h at room temperature, the plates were carefully washed thrice with PBST buffer [0.05% Tween 20 (polyoxyethylene (20) sorbitan monolaurate; Wako, Osaka, Japan) in PBS]. The serum samples were appropriately diluted in PBST buffer before they were added to the plate wells. Pooled sera from non-immunized and OVA-immunized mice were also included in each assay and served as the negative (blank) and positive controls, respectively. Aliquots of 100 μL/well of diluted serum samples were pipetted into the 96 microplate wells and incubated overnight at 4°C (IgE detection plates) or incubated for 2 h at room temperature (IgG1 and IgG2a detection plates). After incubation, the plates were carefully washed with PBST buffer five times. The biotin-conjugated rat anti-mouse IgE monoclonal antibody (BD PharMingen, CA, USA), biotin-conjugated rat anti-mouse IgG1 monoclonal antibody (BD PharMingen, CA, USA) and biotin-conjugated rat anti-mouse IgG2a monoclonal antibody (BD PharMingen, CA, USA) were diluted 1 : 2000 in blocking solution. Aliquots of 100 μL/well of diluted biotin-conjugated rat anti-mouse IgE, IgG1 and IgG2a monoclonal antibody were respectively pipetted into the 96 microplate wells and incubated for 1 h at room temperature. After incubation, the plates were carefully washed with PBST buffer six times. The streptavidin-conjugated horseradish peroxidase (R&D Systems, Minneapolis, MN, USA) was diluted 1 : 250 in blocking solution. Aliquots of 100 μL/well of diluted streptavidin-conjugated horseradish peroxidase were pipeted into the 96 microplate wells and incubated for 20 min at room temperature. After incubation, the plates were carefully washed with PBST buffer six times. Then, aliquots of 100 μL/well of tetramethylbenzidine (TMB; R&D Systems, Minneapolis, MN, USA), the substrate of horseradish peroxidase, were pipetted into the 96 microplate wells. After moderate incubation to develop the color, aliquots of 50 μL/well of stop solution (2 N H_2SO_4) were pipetted into the 96 microplate wells to stop the reaction. The plates were finally read on a plate reader (ASYS Hitech GmbH, Austria) at 450 nm. Absorbency (A) was measured at 450 nm with the ELISA reader and optical densities were converted into arbitrary ELISA units[14,15]. The ELISA unit = $(A_{sample} - A_{blank})/(A_{positive} - A_{blank})$.

VIII. *Statistical Analysis*

Values are expressed as mean ± SD. Data were analyzed statistically using one-way ANOVA, and if justified by the statistical probability ($p < 0.05$), followed by Dunnett's test in the same experiment. Differences between dietary control and experimental groups in the same experiment were considered statistically significant if $p < 0.05$. Statistical tests were performed using SPSS version 12.0.

RESULTS AND DISCUSSION

I. *Changes in Feed Intakes, Feed Efficiencies and Body Weights of Mice Supplemented with RC Juice in Unimmunized and OVA-Immunized Models*

Mice were supplemented with RC juice through 8 and 6 weeks in two experiments. Feed intakes and feed efficiencies of unimmunized and OVA-immunized female BALB/c mice were measured and the results are shown in Table 1. The mean feed intake in different groups supplemented with RC juice through the experimental period ranged from 3.47 ± 0.52 to 4.68 ± 1.42 g/day/mouse. The OVA-immunized treatment slightly decreased feed intake (Table 1). However there was no significant difference ($p > 0.05$) among groups within the same experiment. The mean feed efficiency in different groups supplemented with RC juice ranged from 1.21 ± 1.62 to 2.80 ± 1.76% (Table 1). However, there was no significant difference among groups within the same experiment. The body weight and experimental procedure during the experimental periods are shown in Figure 1. The mean body weight of all mice increased slightly when the experimental period was extended. However, there were no significant differences among groups within the same experiment. The results suggest that supplementation with RC juice, even with the highest dosage (12 g/kg bw/day in mice, 2400 g fresh RC/day in humans), is safe and has no toxic side effects on feed intake, feed efficiency and body

Table 1. Effects of RC juice supplementation on feed intake and feed efficiency of unimmunized and OVA/CFA-immunized BALB/c mice through 8 and 6 weeks, respectively

Treatment	Groups	Feed intake (g/day)	Feed efficiency (%)
Unimmunized	Control	3.84 ± 1.66	1.78 ± 1.04
	RL	4.68 ± 1.42	1.73 ± 1.31
	RH	4.68 ± 1.42	1.89 ± 1.07
OVA/CFA -Immunized	PBS/CFA	4.14 ± 0.54	1.21 ± 1.62
	OVA/CFA	3.47 ± 0.52	2.80 ± 1.76
	OVA/CFA-RL	3.86 ± 0.82	1.33 ± 0.49
	OVA/CFA-RH	3.54 ± 0.28	2.46 ± 0.25

Values are presented as mean ± SD. Data within the same column in the same experiment are analyzed using one-way ANOVA, followed by Dunnett's test. There are no significant differences among groups within the same column in the same experiment. RL, low dose (300 mg/kg bw) of RC juice; RH, high dose (12 g/kg bw) of RC juice. Feed efficiency (%) = [body weight gain (g)/food intake (g)] × 100.

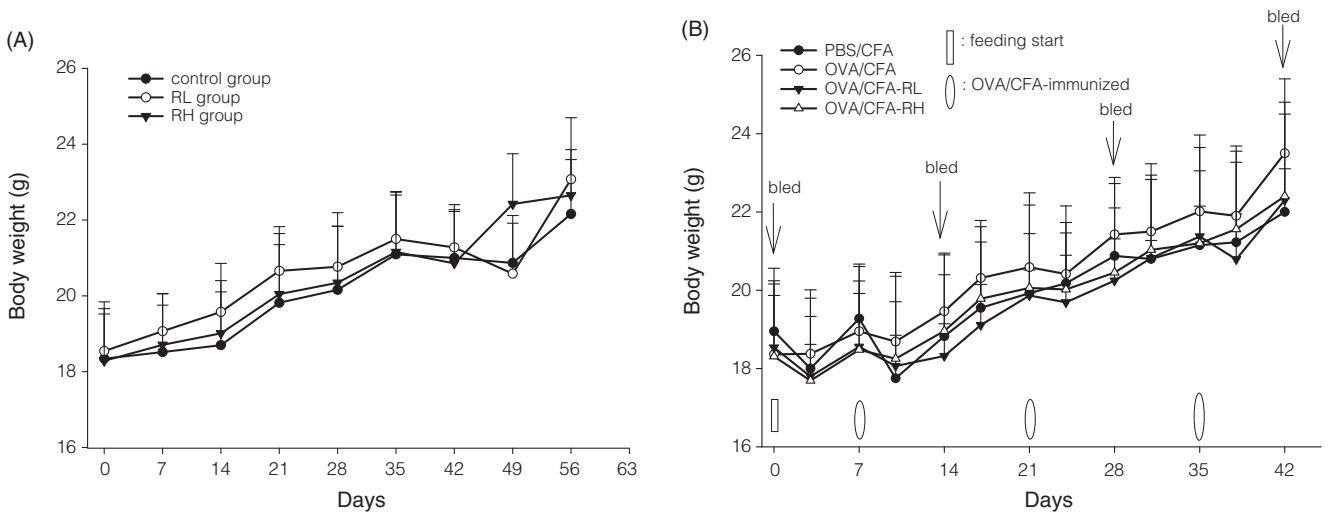

Figure 1. Effects of RC juice supplementation on body weights of unimmunized mice for (A) 8 weeks and (B) OVA/CFA-immunized female BALB/c mice for 6 weeks. Values are presented as mean ± SD. Data at same experimental points are analyzed using one-way ANOVA, followed by Dunnett's test. There are no significant differences among groups at the same experimental points in the same experiment. In the OVA-immunized mouse model, the experimental mice were immunized with OVA at 7, 21 and 35 days, and bled at 0, 14, 28 and 42 days, respectively.

Table 2. Effects of RC juice supplementation on total serum antibody levels of unimmunized BALB/c mice through 8 weeks

Groups	IgG (μg/mL)	IgA (μg/mL)	IgM (μg/mL)	IgE (μg/mL)
Control	1653 ± 338	734 ± 323	466 ± 299	1.90 ± 0.67
RL	1987 ± 1041	773 ± 291	782 ± 343*	2.52 ± 0.82*
RH	2069 ± 893	748 ± 244	588 ± 130	4.34 ± 1.51*

Values are presented as mean ± SD. Data within the same column are analyzed using one-way ANOVA, followed by Dunnett's test. Asterisk (*) means significantly different ($p < 0.05$) from the control group within the same column. Sera were respectively diluted to 1/100 for IgE, 1/5,000 for IgA & IgM, 1/10,000 for IgG determinations.

weight in unimmunized or OVA/CFA-immunized mice. The present study also suggests that the lyophilized powder of RC juice may be an appropriate exploitation mode in the future.

II. *Changes in Total Serum Antibody Levels in Unimmunized BALB/c Mice or OVA-Specific Antibody Titers in OVA-Immunized BALB/c Mice Supplemented with RC Juice*

The changes in serum total IgG, IgA, IgM and IgE levels of unimmunized mice supplemented with RC juice are given in Table 2. Serum IgG, IgA, IgM and IgE concentrations increased in the RC juice-supplemented mice at all experimental doses used. Serum IgM level significantly

($p < 0.05$) increased at low dose supplementation (300 mg RC juice/kg bw). Serum IgE levels significantly ($p < 0.05$) increased at both low (300 mg/kg bw) and high doses (12 g/kg bw) supplementation. Serum IgE levels increased in a dose-response manner as RC juice supplementation. The results indicated that RC juice supplementation increased serum total antibody levels of IgM and IgE. B lymphocytes proceed with isotype switching *in vivo* and switch the immunoglobulin (Ig) class from IgM to IgG, IgE, or IgA isotype during an immune response[16]. Different antibody isotypes participate in differential humoral immune responses in body. The present study indicated that RC juice supplementation indeed increased serum total antibody levels, including IgG, IgA, IgM and IgE, especially IgM and IgE (Table 2). IgM antibodies can form a pentamer and frequently recognize repetitive epitopes such as those on bacterial cell-wall polysaccharides. The IgE antibody *in vivo* plays an important role in resistance to parasite infection[17]. Lee *et al.* reported that all purple and red vegetables and fruit juices exert anti-bacterial activities in dilutions ranging from 1 : 2 to 1 : 16 *in vitro*[18]. This study further suggests that RC juice supplementation may enhance humoral immunity under unimmunized status. In general, the IgE level is low in sera compared to the IgG level. The higher serum IgE level may be an indicator of allergy. However, we hypothesized that the IgE level (0.11% - 0.21% of IgG) of experimental mice in this study was still within a normal range (Table 2). It is rarely reported that supplementation with raw vegetable juices cause adverse effects such as diarrhea or allergy.

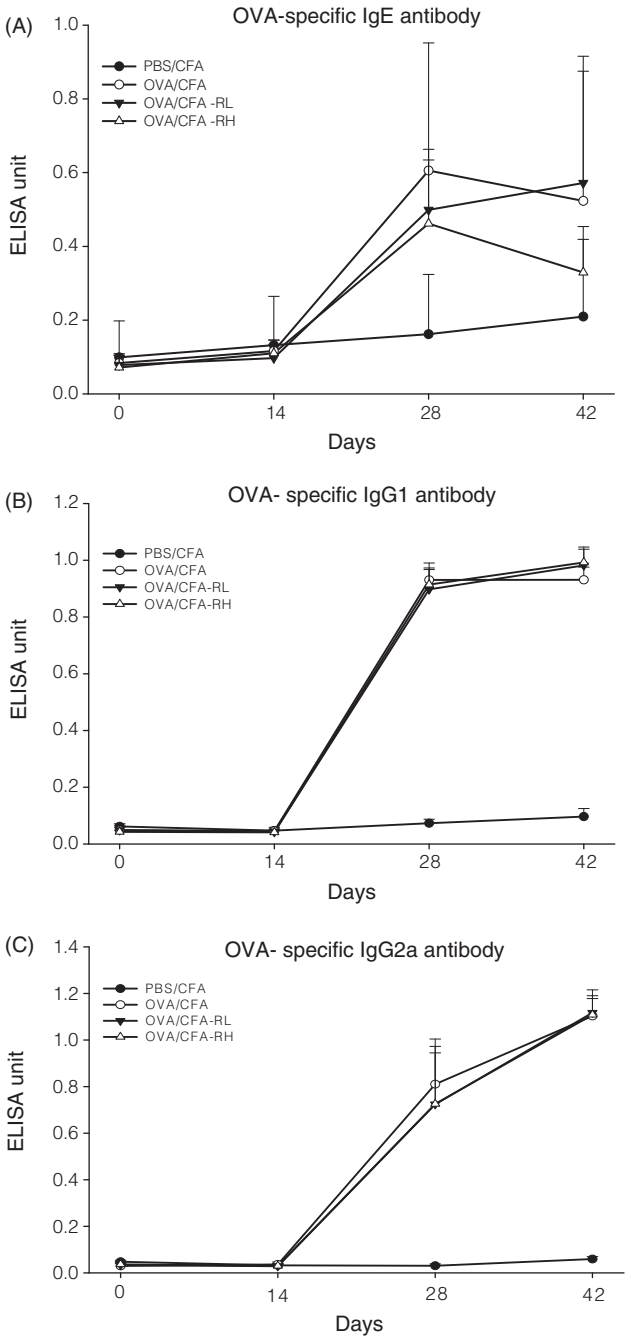

Figure 2. Effects of RC juice supplementation on serum antibody titers of (A) OVA-specific IgE, (B) OVA-specific IgG1 and (C) OVA-specific IgG2a from OVA/CFA-immunized female BALB/c mice for 6 weeks. Values are presented as mean ± SD. Data at same experimental points are analyzed using one-way ANOVA, followed by Dunnett's test. There are no significant differences among OVA/CFA-immunized groups at the same experimental points. Sera were respectively diluted to 1/50 for IgE, 1/1,000 for IgG2a and 1/5,000 for IgG1 determinations.

The changes in serum OVA-specific IgE, IgG1 and IgG2a titers in mice supplemented with RC juice through the 6-week experimental period are given in Figure 2. The results showed that OVA-immunized treatment markedly ($p < 0.05$) increased OVA-specific IgE, IgG1 and IgG2a (Figure 2). Although RC juice supplementation at the indicated high dose (12 g/kg bw) slightly decreased the OVA-specific IgE titer, there was no significant difference among OVA-immunized groups (Figure 2A). RC juice supplementation at both low and high doses did not significantly affect OVA-specific IgG1 and IgG2a titers in OVA-immunized groups (Figures 2B and 2C). In OVA-specific immune responses, the OVA-specific IgE titer is an important indicator of allergy. Unfortunately, our results suggest that RC juice supplementation could not significantly affect OVA-specific antibody levels, although it slightly decreased the IgE titer at high dose administration under OVA-immunized treatment (Figure 2). In the present study, RC juice supplementation exhibited a differential effect on the humoral immunity in non-specific (unimmunized status) and OVA-specific animal models. The total serum antibody levels in OVA-immunized mice were not determined in this study because we thought that antigen-specific antibodies were the better indicator than non-specific antibodies under an antigen-immunized experimental status. However, OVA-specific antibody titers might indeed reflect specific immune responses under the OVA-immunized status. It seems to be universal in different experimental models due to differential effects of immunomodulators in foods on immune cells under different status. However, the real bio-active immunomodulatory components in RC juice remain to be further studied.

CONCLUSIONS

Taken together, supplementation of raw RC juice increased serum antibody levels, especially IgM and IgE levels in unimmunized mice, but did not significantly affect OVA-specific antibody levels in OVA/CFA-immunized mice. This study suggests that RC juice has immunomodulatory effects *in vivo* via enhancing humoral immunity under unimmunized status.

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