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Immunoregulatory influence of *Sargassum carpophyllum* polyphenol on the allergic reactions induced by shrimp tropomyosin

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ABSTRACT

Polyphenol is a kind of natural active substance with immunoregulatory activity. In this study, the immunoregulatory activities of *Sargassum carpophyllum* polyphenol (SCP) on the allergic reactions induced by shrimp tropomyosin were investigated. As the results, the SCP performed high hyaluronidase inhibition ability, which even greater than sodium chromoglycate (an anti-allergic medicine). SCP was able to affect the allergic reactions of RAW 264.7 macrophage cells via regulating the nuclear factor kappa-B (NF-κB), c-Jun N-terminal kinase 1/2 (JNK1/2), extracellular regulated protein kinase 1/2 (Erk1/2) and p38 mitogen-activated protein kinase (MAPK) cellular signaling pathways, weakened the degranulation degree and secretion of interleukin-4 (IL-4), tumor necrosis factor-α (TNF-α) and histamine, and promoted the secretion of interleukin-10 (IL-10) from RBL-2H3 mast cells. In BALB/c mouse model assay, after gavage of SCP, the mouse allergic symptoms got significantly alleviated, the cytokine (IL-4, IL-10 and interferon-γ (IFN-γ)) secretion of mouse spleen lymphocytes were significantly declined and the allergic lung damage was relieved, which indicated SCP performed strong both *in vitro* and *in vivo* anti-allergic functions. Therefore, SCP could serve as candidate anti-allergic bioactive substances for shrimp induced allergy via immunoregulation.

1. Introduction

Food allergy refers to the adverse immune reactions to food[1]. When food allergy occurs, the allergic symptoms appear in the skin, gastrointestinal tract, respiratory tract and even throughout the whole body[2-3]. The World Health Organization (WHO) has listed food allergy as one of the top three diseases to be prevented and treated in the 21st century, which has become a major health issue in the world[4]. However, up to now, there is no complete medical cure for food allergy, the general cure approach is still avoiding food allergen. Therefore, investigating the approaches to reduce and control food allergen has become an urgent issue. Currently, there are two major approaches to weaken food allergy. One is to reduce the allergenicity of food allergens through food processing techniques, and the other is to use natural anti-allergic active substances to control food allergic reactions through immune regulation[1]. However, considering the current food processing technology could not completely eliminate the allergenicity of food allergens[5], inhibiting food allergy reactions through natural anti-allergic active ingredients (e.g. polyphenol) has become a promising approach to prevent and control food allergy[6].

Polyphenols are active substances that have been proved with anti-allergic functions[6-7], which mainly performed anti-allergic activity through the following mechanisms: 1) Polyphenols could interact with allergic effector cells (e.g. mast cell and basophil) and inhibit the release of allergic mediators (e.g. histamine and cytokines)[8];

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2) Polyphenols as antioxidants, which could suppress the cell and organ damage induced by free radicals (e.g. reactive oxygen species) during allergic damage[9]; 3) Polyphenols could affect the generation of allergen-immunoglobulin E (IgE) complex[6,10]; 4) Polyphenols could influence the allergen-IgE complex bind to the receptor (Fc epsilon RI (FcεRI)) on the surface of mast cells and basophils[6]. Currently, there have been several studies proved algae polyphenol extracts have the effects of anti-food allergy[6,11-12]. Therefore, polyphenols could alleviate food allergic reactions.

Sargassum carpophyllum (SC) belongs to brown algae, which widely distributed in the coastal regions of China. As a kind of edible algae, SC has the characteristics of high safety, rich source and high polyphenol content[13]. However, currently, the main dietary utilization of SC is direct consumption, the development of bioactive polyphenol in SC is still lacking, which lead to the low value utilization rate of SC resource and economic loss. Therefore, the exploitation and utilization of SC polyphenol (SCP) could promote the high value utilization of SC resources. This work is purpose to investigate the anti-allergic functions and elucidate the anti-allergic mechanisms of SCP via immunoregulation, which could provide a novel insight into the prevention and treatment of shrimp induced food allergy via algae polyphenol.

2. Material and methods

2.1 Materials

SC was collected from Rongcheng, Shandong Province, China; Ethanol (CAS: 64-17-5) and trifluoroacetic acid were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); Shrimp tropomyosin was extracted from *Litopenaeus vannamei* according to our previous work[12]; AB-8 macroporous resin, phosphate buffered saline (PBS), citric acid buffer, Tyrode's solution and Triton X-100 were obtained from Solarbio Technology Co., Ltd. (Beijing, China); Anti-DNP-IgE was purchased from Sigma-Aldrich (St. Louis, MO, USA); DNP-bovine serum albumin (BSA) was obtained from Alpha Diagnostic International (San Antonio, TX, USA); MEM was purchased from Shanghai Yuanpei Biotechnology Co., Ltd. (Shanghai, China); Fetal bovine serum (FBS) was obtained from Moregate Biotech (Bulimba, Australia); Ethylenediaminetetraacetic acid (EDTA)-pancreatic enzyme was obtained from Biological Industries (Beit-Haemek, Israel); Pyrrolidinedithiocarbamate (PDTC), SP600125, U0126-EtOH, SB203580 and cell counting kit-8 (CCK-8) were purchased from MedChemExpress (Princeton, NJ, USA); Interleukin-4 (IL-4), interleukin-10 (IL-10), histamine, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) ELISA kits were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). All chemical reagents are analytical grade or better.

2.2 Purification of SCP

The purification of SCP was according to our previous method with few modifications[12]. Briefly, the fresh SC was air-dried at 60 ℃ for 24 h in baking oven, then placed in an ultra-micro crusher, the crushed powder was sieved through a 60-mesh sieve. Afterwards, the extraction of SCP were set as extraction time of 10 h, extraction temperature of 40 ℃ and ratio of SC:75% ethanol = 1:16 (*m/V*), then the extracts were centrifuged (4 ℃, 9 000 r/min, 15 min) and the supernatant was retained. The precipitate was re-dissolved in 75% ethanol (*V/V*) for the second extraction, the extracts were centrifuged (4 ℃, 9 000 r/min, 15 min) and the supernatant was kept, with two supernatants were mixed well and passed through the double-layered filter papers to obtain the SCP extract. The polyphenol content in SCP extract was determined. Afterwards, the SCP extract was purified using AB-8 macroporous adsorbent resin, then further purified using preparative high performance liquid chromatography (HPLC) (1260 Inffnity II Prepared Liquid Chromatography System, Agilent, Santa Clara, CA, USA) with the eluates harvested[12].

2.3 Hyaluronidase inhibition assay of SCP

The hyaluronidase inhibition rate of SCP was determined by measuring the amount of *N*-acetylglucosamine formed from sodium hyaluronic acid, according to the method described by Morgan-Elson method[14], to reflect the *in vitro* anti-allergic activity. The specific methods were as following: 4 different 5 mL tubes were taken and the parallel experiment was repeated 3 times in each group. 100 μL sodium acetate buffer (0.2 mol/L NaOAc, 0.15 mol/L NaCl, pH 4.6) and 50 μL hyaluronidase (3 mg/mL) were added to tube A, tube B was added to 150 μL sodium acetate buffer, tube C was added to 100 μL SCP sample and 50 μL hyaluronidase and tube D was added to 100 μL SCP sample and 50 μL sodium acetate buffer. After incubation at 37 ℃ for 20 min, adding 20 μL calcium chloride solution (2.5 mol/L $CaCl_2$) and incubate at 37 ℃ for 20 min. Then 50 μL sodium hyaluronate solution (3 mg/mL), 100 μL sodium acetate buffer (0.2 mol/L NaOAc, 0.1 mol/L NaCl, pH 4.6) and 250 μL ultrapure water were added, incubated at 37 ℃ for 40 min, and left at room temperature for 10 min. Add 100 μL alkaline borate solution (2.8 mol/L H_3BO_3, 1.4 mol/L KOH, 0.08 g/mL K_2CO_3), boiling water bath for 10 min, then ice water bath for 20 min, adding 1.5 mL *p*-dimethylaminobenzoic acid solution (0.268 mol/L *p*-dimethylaminobenzoic acid, 0.6 mol/L HCl, 16.625 mol/L HAc), incubate at 37 ℃ for 20 min, immediately read the absorption value at 585 nm ($OD_{585\,nm}$). The inhibition rate of hyaluronidase is calculated by the following formula:

$$\text{Hyaluronidase inhibition rate}\ (\%) = \frac{(A-B)-(C-D)}{A-B} \times 100$$

Where *A* was $OD_{585\,nm}$ of control solution (buffer solution was used instead of sample); *B* was $OD_{585\,nm}$ of blank solution (with buffer solution instead of sample and hyaluronic acid); *C* was $OD_{585\,nm}$ of the SCP sample; *D* was $OD_{585\,nm}$ of the blank sample (with buffer solution replaced enzyme solution and hyaluronic acid).

2.4 Determination of anti-allergic activity of SCP on RAW 264.7 cells

2.4.1 Effects of SCP on the TNF-α and NO secretion from RAW 264.7 cells

The RAW 264.7 cells were cultured in DMEM (Basal Media, Shanghai, China) containing FBS. CCK-8 was used to evaluate the cytotoxicity of SCP on RAW 264.7 cells. Then, the RAW 264.7 cells were pipetted (100 μL/well, 1×10^5 cells/well) in 96-well plates and incubated in a CO_2 cell incubator (Heal Force 90, Heal Force Bio-meditech Holdings Ltd., Shanghai, China) for 24 h. The plates were cultured with lipopolysaccharide (LPS) for 24 h, after washed with Tyrode's solution, the plates were incubated with 100 μL different concentrations of SCP (1, 5, 10, 25, 50 μg/mL) for 2 h. The secretion

of NO from RAW 264.7 cells were detected using the Griess method to detect the NO secretion[15], and the secretion of TNF-α from RAW 264.7 cells were detected using TNF-α ELISA kits (Boster Biological Technology Co., Ltd., Wuhan, China).

2.4.2 Effects of SCP on the cellular pathways of RAW 264.7 cells

The RAW 264.7 cells were plated in 96-well plates and incubated in a CO_2 cell incubator for 24 h. Then, the plates were washed with Tyrode's solution, pipetting 100 μL the following cellular pathway inhibitors to each well: PDTC (nuclear factor kappa-B (NF-κB) activation inhibitor, 100 mmol/L), SP600125 (c-Jun N-terminal kinase 1/2 (JNK1/2) inhibitor, 100 mmol/L), U0126 (extracellular regulated protein kinase 1/2 (Erk1/2) inhibitor, 10 mmol/L) and SB203580 (p38 mitogen-activated protein kinase (MAPK) inhibitor, 10 mmol/L), respectively, then incubated in a CO_2 cell incubator at 37 ℃ for 12 h. Afterwards, pipetting 100 μL SCP (10 μg/mL) to each well and incubate at 37 ℃ for 2 h. Then, taking the supernatant, using the Griess method to detect the NO secretion[15], and the secretion of TNF-α from RAW 264.7 cells were detected using TNF-α ELISA kits (Boster Biological Technology Co. Ltd., Wuhan, China). The secretion changes of NO and TNF-α were used to reflect the effects of SCP on the cellular pathways (NF-κB, JNK1/2 MAPK, Erk1/2 MAPK and p38 MAPK) of RAW 264.7 cells.

2.5 RBL-2H3 mast cell assay

2.5.1 Effects of SCP on cell viability of RBL-2H3 mast cells

RBL-2H3 mast cells were cultured in MEM (Basal Media, Shanghai, China), then the mast cells were pipetted into 96-well plates (100 μL/well, 1×10^6 cells/well), incubated at 37 ℃ for 24 h. Afterwards, the supernatants were removed, each well was washed with Tyrode's solution for twice and pipetted 100 μL/well of MEM containing different concentrations of SCP (1, 5, 10, 50, 100, 200, 400 and 800 μg/mL, respectively). After that, CCK-8 (10 μL/well) were pipetted and incubated for 1 h, the absorbance at 450 nm ($OD_{450\,nm}$) was determined using a 96-well plate reader (Powerwave XS, BioTek, Winoosk, VT, USA).

2.5.2 Effects of SCP on the degranulation of RBL-2H3 mast cells

RBL-2H3 mast cells were cultured in MEM in 96-well plates (1×10^6 cells/well), incubated at 37 ℃ for 24 h and then sensitized for 12 h with 100 μL of anti-DNP-IgE (10 μg/mL). After washing with Tyrode's solution, the RBL-2H3 mast cells were pipetted 100 μL/well of MEM containing different concentrations of SCP (1, 5, 10, 50 and 100 μg/mL, respectively), incubated at 37 ℃ for 2 h. After incubating with 100 μL/well of DNP-BSA (with final concentration as 0.25 μg/mL) as antigen for 1 h, the supernatant was collected. The *β*-hexosaminidase release rate was determined, to reflect the degranulation degree of RBL-2H3 mast cells[16].

2.5.3 Effects of SCP on the cytokine secretion of RBL-2H3 mast cells

As to cytokine (IL-4, IL-10 and TNF-α) determination, the mast cells were sensitized using anti-DNP-IgE (100 μL/well) for 12 h. Afterwards, each well was washed with Tyrode's solution for twice and pipetted 100 μL/well of MEM containing different concentrations of SCP (1, 5, 10, 50 and 100 μg/mL, respectively), incubated at 37 ℃ for 2 h. After incubating with 100 μL/well of DNP-BSA (with final concentration as 0.25 μg/mL) as antigen for 1 h, the supernatant was collected. The secretion of IL-4, IL-10 and TNF-α levels of RBL-2H3 mast cells were determined using rat IL-4, IL-10 and TNF-α ELISA kits (Boster Biological Technology Co., Ltd., Wuhan, China), respectively.

2.6 BALB/c mouse model assay

The BALB/c mice (female, 6–8 weeks) were purchased from Shandong Qinda Biological Co., Ltd. (Qingdao, China) and housed at the Animal Culture Center of Ocean University of China (Qingdao, China). The animal experimental design was approved by the Ethics Committee of Ocean University of China (Permit No: SPXY2023042010). The mice were randomly divided into 5 groups with 8 mice in each group (negative control group (PBS group), positive control group (tromoyosin (TM) group), high SCP dosage group: 20 mg/kg, medium SCP dosage group: 10 mg/kg and low SCP dosage group: 5 mg/kg). After 1 week of acclimatization, on days 0, 7, 14 and 21, the mice in the PBS group were intraperitoneally (i.p.) injected with 200 μL PBS, the mice in other groups were i.p. injected with 200 μL PBS containing 150 μg TM and 100 μL alumni adjuvant (Pierce Chemical, Rockford, IL, USA).

On day 28, each mouse in the PBS group were intragastric (i.g.) gavaged with 200 μL PBS, each mouse in other groups were i.g. gavaged with 200 μL PBS containing 2 mg TM. On days 29–35, each mouse in different SCP treatment groups were i.g. gavaged with different dosage of SCP. On day 36, each mouse in the PBS group were i.g. gavaged with 200 μL PBS and each mouse in other groups were i.g. gavaged with 200 μL PBS containing 2 mg TM. After 1 h of excitation, the anaphylactic scores of allergic symptoms and body temperature were tested. Then, the mice were anesthetized and killed by cervical dislocation, with the spleen and lung tissues were harvested under sterile conditions. The levels of IFN-γ, IL-4 and IL-10 from spleen lymphocyte were tested using the mouse IFN-γ, IL-4 and IL-10 ELISA kits (Boster Biological Technology Co., Ltd., Wuhan, China).

2.7 Statistical analysis

All data were presented as means ± standard deviation (SD). Data were analyzed by general linear model and One-way ANOVA, differences measured by Dunnett's multiple comparisons test between groups using GraphPad Prism software (GraphPad Software Inc., Version 10, San Diego, CA, USA). Each experiment was repeated at least three times.

3. Results

3.1 Hyaluronidase inhibition rate of SCP

The hyaluronidase inhibition assay is an effective method for rapid screening of anti-allergic substances *in vitro*[14], therefore it can be used to evaluate the *in vitro* anti-allergic ability of SCP. In this work, the it can be used to evaluate hyaluronidase inhibition rates of SCP at different concentrations (0.01, 0.05, 0.1, 0.2, 0.4 and 0.5 mg/mL,

respectively) were determined, and a curve was fitted to calculate the half maximal inhibitory concentration (IC_{50}) of SCP. The hyaluronidase inhibition rates of tea polyphenol (serve as positive control) and sodium cromoglycate (a typical anti-allergic medicine, serve as medicine control) at different concentrations (0.05, 0.1, 0.25, 0.5 and 1 mg/mL) were determined, and the curves were fitted to calculate the IC_{50} of tea polyphenols and sodium cromoglycate, respectively. As reported, the lower IC_{50} value indicated the better the hyaluronidase inhibitory activity, which suggested the better anti-allergic ability[17]. As shown in Fig. 1A, the IC_{50} of SCP to hyaluronidase was 0.031 mg/mL, the IC_{50} of tea polyphenol was 0.250 mg/mL, and the IC_{50} of sodium cromoglycate was 0.280 mg/mL, this indicated the IC_{50} of SCP was significantly lower than that of tea polyphenol and sodium cromoglycate, which suggested the strong *in vitro* anti-allergic ability of SCP.

3.2 Anti-allergic activity of SCP on RAW 264.7 macrophage cells

3.2.1 The toxicity of SCP on RAW 264.7 cells

The toxicity of SCP on RAW 264.7 macrophage cells was determined using the CCK-8 toxic assay. As the results (Fig. 1B), 1–50 μg/mL of SCP had no toxic effects on the cell viability of RAW 264.7 cells, while higher contents of SCP (50–300 μg/mL) could reduce the viability of RAW 264.7 cells. Therefore, 10–50 μg/mL of SCP were safe for RAW 264.7 cells and could be selected for the following cellular experiments.

RAW 264.7 macrophage cell is a common cell line for analyzing allergic reactions[18]. In this work, the secretion of TNF-α and NO from LPS-stimulated RAW 264.7 cells was investigated to evaluate the anti-allergic effects of SCP. As shown in Fig. 1C, compared with the TNF-α content in RAW 264.7 cells without SCP treatment (positive control), the TNF-α concentrations in RAW 264.7 macrophage cells treated by different concentrations of SCP (1, 5, 10, 25, 50 μg/mL) decreased to 154.00, 197.70, 248.07, 415.48 and 565.85 pg/mL, respectively, which indicated SCP could significantly reduce the secretion of TNF-α. As to NO secretion, compared with the NO secretion from RAW 264.7 cells without SCP treatment (positive control), the NO concentrations (Fig. 1D) in RAW 264.7 cells treated by different concentrations of SCP (1, 5, 10, 25, 50 μg/mL) decreased to 11.75, 14.33, 20.00, 27.67 and 34.08 μmol/mL, respectively, this suggested SCP decreased the secretion of NO. After incubation with SCP, the secretions of TNF-α and NO were significantly lower than that of RAW 264.7 cells without SCP treatment, moreover, with the increase of SCP contents, the secretion of TNF-α and NO in RAW 264.7 cells performed an increased tendency. The similar results were also found by Gu et al.[19]. This might be due to that, once macrophages are activated, the secretion of NO and some cytokines (e.g. TNF-α) are increased. RAW 264.7 cells can spontaneously secrete cytokines in the resting state, and such secretion in RAW 264.7 cells was significantly promoted by LPS stimulation. The stimulation of LPS simultaneously promoted the secretion of NO and TNF-α of RAW 264.7 cells. The similar results were also found in the polysaccharide from *Sinonovacula constricta* on RAW 264.7 cells[20].

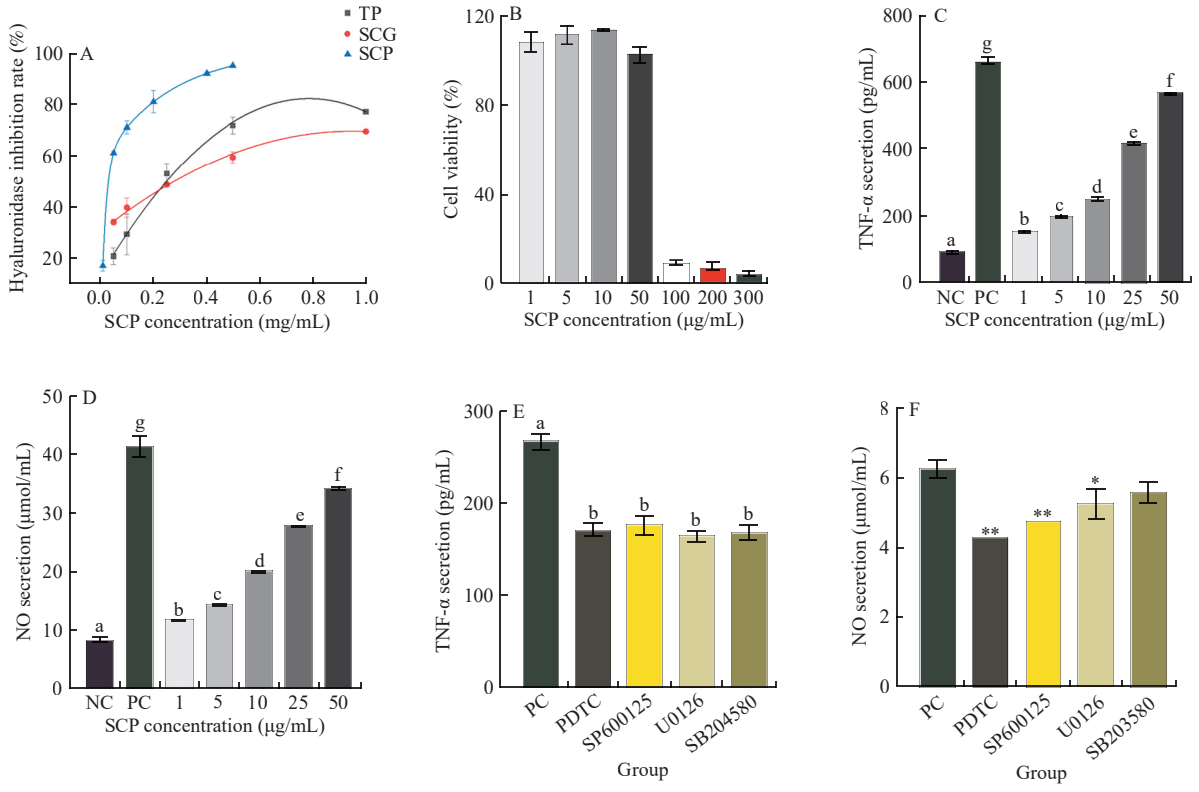

Fig. 1 Hyaluronidase inhibition rate of SCP (A); Effects of different SCP contents on the cell viability (B), TNF-α secretion (C) and NO secretion (D); Effects of different signaling pathway inhibitors on TNF-α secretion (E) and NO secretion (F) of RAW 264.7 cell. TP: tea polyphenol; SCG: sodium chromoglycate; NC: negative control (treated with Tyrode's solution); PC: positive control (treated with LPS).
Different superscript letters mean significant differences ($P < 0.05$). * means significant differences as compared with PC group, *$P < 0.05$, **$P < 0.01$.

3.2.2 Effects of SCP on RAW 264.7 cellular pathways

MAPK cellular pathway play key roles during allergic reactions[21]. In this work, four different cellular pathway inhibitors (PDTC served as NF-κB signaling pathway inhibitor, SP600125 served as JNK1/2 MAPK signaling pathway inhibitor, U0126-EtOH served as Erk1/2 MAPK signaling pathway inhibitor, SB203580 served as p38 MAPK signaling pathway inhibitor) were used to investigate the effects of SCP on the cellular pathways to affect the secretion of TNF-α and NO from RAW 264.7 cells.

As the results (Figs. 1E-F), SCP could affect the secretion of TNF-α and NO by 4 different cellular pathway inhibitors (PDTC, NF-κB signaling pathway inhibitor; SP600125, JNK1/2 MAPK signaling pathway inhibitor; U0126-EtOH, Erk1/2 MAPK signaling pathway inhibitor; and SB203580, p38 MAPK signaling pathway inhibitor) to investigate the effects of SCP on cellular pathways of RAW 264.7 cells. The results indicated that the secretion of TNF-α (Fig. 1E) was regulated by NF-κB, JNK1/2 MAPK, Erk1/2 MAPK and p38 MAPK signaling pathways, and the secretion of NO (Fig. 1F) was regulated by NF-κB, JNK1/2 MAPK and Erk1/2 MAPK signaling pathways, while could not be significantly affected by p38 MAPK pathway. These studies showed that the SCP could play an immunomodulatory role by regulating cellular signaling pathways during allergic reactions.

3.3 Anti-allergic activity of SCP on RBL-2H3 mast cells

3.3.1 The toxicity of SCP on RBL-2H3 mast cells

The toxicity of SCP on RBL-2H3 mast cells was determined using CCK-8 toxic assay. As the results (Fig. 2A), 1–100 μg/mL of SCP had no toxic effects on the cell viability of RBL-2H3 mast cells, while higher concentrations of SCP (100 − 800 μg/mL) could reduce the cell viability of RBL-2H3 mast cells. Therefore, 1–100 μg/mL of SCP were safe for RBL-2H3 mast cells and could be selected for the following cellular experiments.

3.3.2 Effects of SCP on degranulation of RBL-2H3 mast cells

The degranulation of mast cell is an important factor for inducing allergic reactions, the allergic biomarker (e.g. histamine and *β*-hexosaminidase) got released during degranulation, which induced allergic reactions. As shown in Fig. 2B, compared with the histamine content in positive control (127.42 pg/mL), after treated by different concentrations of SCP (1, 5, 10, 50, 100 μg/mL), the histamine concentrations from RBL-2H3 mast cells got reduced to 62.79, 48.50, 31.72, 19.71 and 14.04 pg/mL, respectively. The degranulation of RBL-2H3 mast cells was measured by determining the enzymatic activity of *β*-hexosaminidase in the cell supernatant. As shown in Fig. 2C, compared with the control (43.26%), the *β*-hexosaminidase activity of RBL-2H3 mast cells got decreased with SCP treatment, and higher contents of SCP (1, 5, 10, 50, 100 μg/mL) induced weaker enzymatic activity of *β*-hexosaminidase, which indicated the *β*-hexosaminidase activity was negatively related to the contents of SCP, SCP weakened the degranulation of RBL-2H3 mast cells.

3.3.3 Effects of SCP on cytokine secretion of RBL-2H3 mast cells

During degranulation, the mast cells released various cytokines, which included the IL-4, IL-10 and TNF-α. As shown in Fig. 2D, compared with the IL-4 concentration in positive control (42.43 pg/mL), after treated by different concentrations of SCP (1, 5, 10, 50, 100 μg/mL),

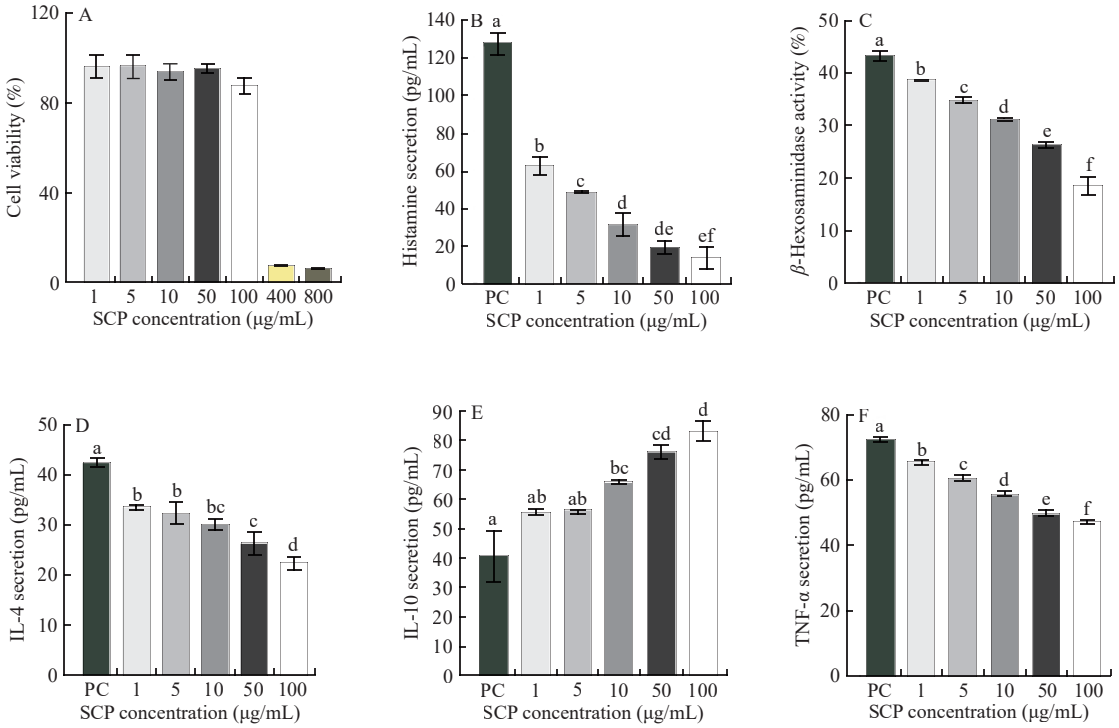

Fig. 2 Effects of different contents of SCP on the cell viability (A), histamine secretion (B), *β*-hexosaminidase activity (C), IL-4 (D), IL-10 (E) and TNF-α (F) of RBL-2H3 mast cell. PC: positive control (treated with anti-DNP-IgE and DNP-BSA). Different superscript letters mean significant differences ($P < 0.05$).

the IL-4 concentrations from RBL-2H3 mast cells got decreased to 33.54, 32.36, 30.14, 26.35 and 22.30 pg/mL, respectively. As for the secretion of IL-10 (Fig. 2E), compared with the IL-10 concentration in positive control (40.85 pg/mL), after treated by different concentrations for SCP (1, 5, 10, 50, 100 μg/mL), the IL-10 concentrations from RBL-2H3 mast cells got increased to 55.46, 56.23, 65.85, 76.23 and 83.15 pg/mL, respectively. As for the secretion of TNF-α (Fig. 2F), compared with the TNF-α concentration in positive control (72.60 pg/mL), after treated by different concentrations of SCP (1, 5, 10, 50, 100 μg/mL), the TNF-α concentrations from RBL-2H3 mast cells got reduced to 65.72, 60.84, 55.79, 49.77 and 47.18 pg/mL, respectively.

3.4 *In vivo anti-allergic ability of SCP*

The *in vivo* anti-allergic activity of SCP was analyzed using the BALB/c mouse model according to Fig. 3A. As showed in Fig. 3B, compared with the negative control (PBS group), the mouse anaphylactic scores showed a significant increase in the positive control group (treated without SCP), after i.g. gavaged with SCP, the mouse anaphylactic scores in SCP treated group performed significant reduction, and the reduction degree of anaphylactic scores was positively related to the SCP dosage, which indicated the decrease of anaphylactic scores was SCP dosage-dependent.

As to the body temperature (Fig. 3C), compared to the blank group (PBS group), the mouse body temperature in the positive control group (treated with TM) had a significant increase; compared with the mouse body temperature in the positive control group (treated with TM), the mouse body temperature in the SCP treated groups performed reduction.

As an important immune organ, the spleen played a key role during allergic reactions, and the cytokine secretion from spleen lymphocytes (SLP) were used to characterize the allergic reactions. As shown in Fig. 4A, compared with the IFN-γ secretion (363.67 pg/mL) from SLP in mice of positive control (tropomyosin group), the IFN-γ secretion from SLP in mice gavaged by high dosage SCP, medium dosage SCP and low dosage SCP reduced to 52.00, 138.67 and 309.50 pg/mL, respectively. As shown in Fig. 4B, compared with the IL-4 secretion (63.21 pg/mL) from SLP in mice of positive control (tropomyosin group), the IL-4 secretion from SLP in mice gavaged by high dosage SCP, medium dosage SCP and low dosage SCP reduced to 6.65, 29.88 and 38.31 pg/mL, respectively. As shown in Fig. 4C, compared with the IL-10 secretion (141.63 pg/mL) from SLP in mice of positive control (tropomyosin group without SCP treatment), the IL-10 secretion from SLP in mice i.g. gavaged by high dosage SCP, medium dosage SCP and low dosage SCP reduced to 20.65, 57.90 and 80.25 pg/mL, respectively. As showed in Figs. 4A-C, the secretion of IFN-γ, IL-4 and IL-10 in SLP showed the same tendency after treated by SCP. The cytokine (IFN-γ, IL-4 and IL-10) secretion in the SLP from positive control group (without SCP) was significantly higher than that in the negative group (PBS group), and the cytokine secretion of SCP treated group was significantly lower than that in the positive control group (without SCP) in a dosage-dependent manner.

Since the inflammatory cell infiltration and goblet cell hyperplasia are the main trigger of allergic reactions, the lung tissue

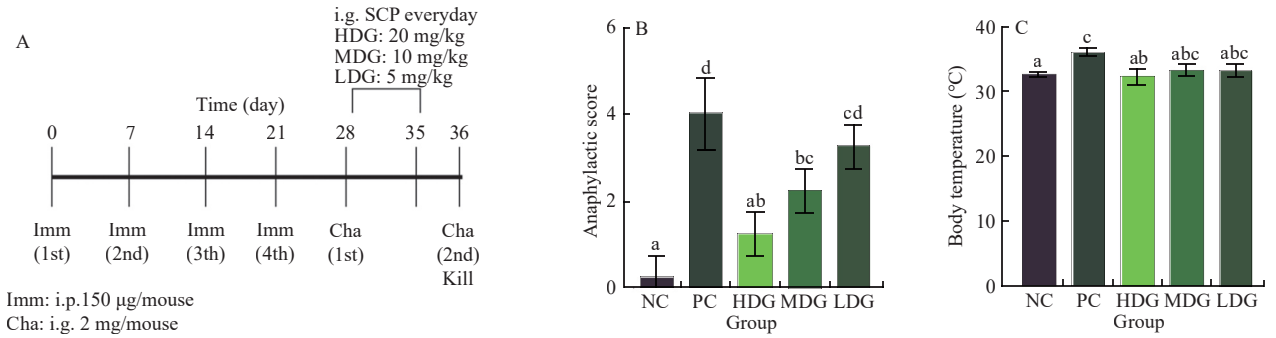

Fig. 3 Allergy sensitization and challenge protocol of BALB/c mouse model (A), anaphylactic score (B) and body temperature (C) treated by SCP. NC: negative control (treated with PBS); PC: positive control (treated with tropomyosin); HDG: high dosage group of SCP; MDG: medium dosage group of SCP; LDG: low dosage group of SCP. Different superscript letters mean significant differences ($P < 0.05$).

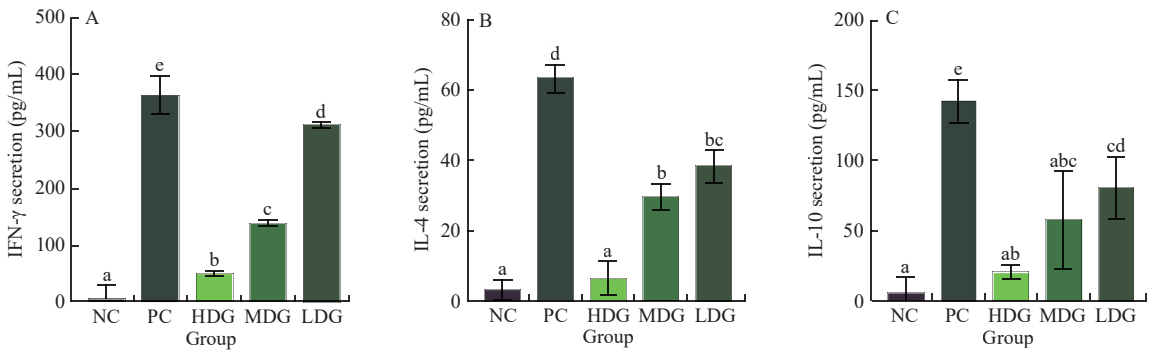

Fig. 4 Effects of different dosages of SCP on the IFN-γ (A), IL-4 (B) and IL-10 (C) in the spleen lymphocytes of BALB/c mice, hematoxylin-eosin (HE) staining of lungtissue (10×) frommouse treated by NC (D), PC (E), HDG (F), MDG (G) and LDG (H). NC: negative control (treated with PBS); PCG: positive control (treated with tropomyosin); HDG: high dosage group of SCP; MDG: medium dosage group of SCP; LDG: low dosage group of SCP. Different superscript letters mean significant differences ($P < 0.05$).

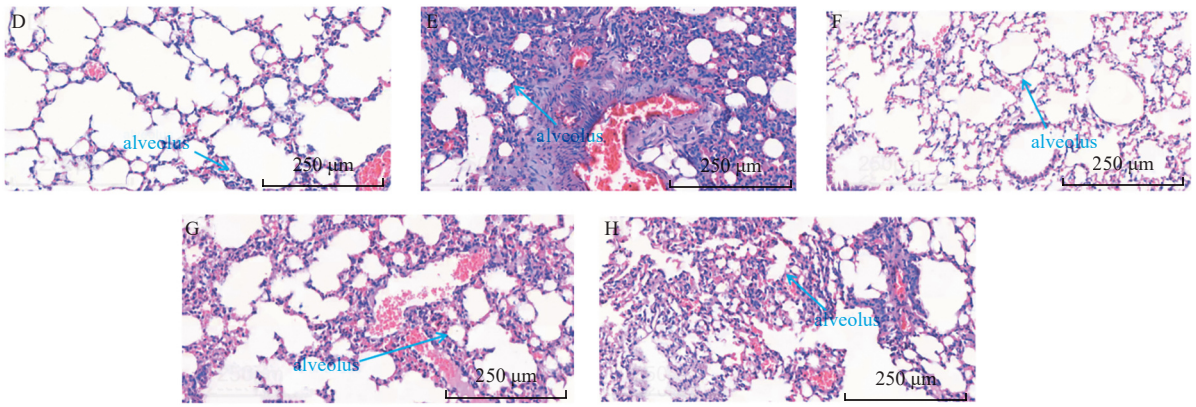

Fig. 4 (*Continued*)

slices were used to characterize the degree of allergic reactions. As showed in Figs. 4D-H, a large number of inflammatory cell infiltration and goblet cell hyperplasia appeared in the lung tissue of mice in the allergic mice, the mice in the high dosage group, medium dosage group and low dosage group showed a dose-dependent reduction of inflammation. The results of inflammatory cell infiltration in the high dosage group were almost similar to that in the negative control group, which proved that SCP had excellent anti-allergic effects on allergic reactions in lung. These results showed that SCP performed excellent *in vivo* anti-allergic activity.

4. Discussions

SC is an edible brown algae, which is widely distributed in the coastal regions of China with high production[22]. In the past decades, the health benefits of polyphenols (e.g. immunoregulatory, anti-inflammatory and anti-oxidant functions) have been intensively investigated[23-25]. SC is rich in various nutrition (e.g. polyphenol), while the bioavailability and utilization of SC resource is low, and up to now, there are very few investigations and reports on the anti-allergic ability of SCP.

The *in vitro* inhibition of hyaluronase is one of the main testing methods for anti-allergic activity of SCP. When the allergen enters the human body for the second time, the IgE antibodies that already attached to the surface of mast cells will combine with it, and FcεRI cross-linking will occur, thus sending signals to activate various enzymes (e.g. hyaluronase) in cells and triggering allergic reactions. Hyaluronic acid is an important component in tissue matrix that restricts the diffusion of extracellular substances and water. After hyaluronic acid was hydrolyzed by hyaluronase, the intercellular stickiness decreases, resulting in mast cell degranulation and rapid allergic reactions. Therefore, inhibiting the activity of hyaluronic acid in mast cells could protect hyaluronic acid from decomposition and maintain the normal physiological functions of cells and the immune system[26]. Previous studies have confirmed that the enzyme activity of hyaluronase is positively correlated with the secretion of histamine from mast cells, and inhibiting the secretion of hyaluronase could also effectively reduce the histamine content in human body[27]. Moreover, previous investigations have shown that hyaluronidase does play a role in type I allergic reactions and immune inflammation[28]. In this study, it was found that the inhibition rate of hyaluronidase of the purified SCP was better than that of disodium cromoglycate and slightly higher than that of tea polyphenols (Fig. 1A), which proved that SCP performed excellent anti-allergic ability.

Polyphenols could serve as an anti-allergic active substance, regulating immune cells to play anti-allergic functions. Among these immune cells, macrophages are key players in the immune system and could release cytokines by connecting with other cells and working with T and B cells[29]. Macrophages secrete NO and TNF-α during allergic reactions, while polyphenols could dosage-dependently regulate the secretion of NO and TNF-α and play immunomodulatory functions[30]. Moreover, many immune response genes are transcribed by the transcription factor NF-κB, and when macrophages are stimulated, ubiquitinated degradation takes place and p65 in NF-κB get phosphorylated, which improves the transcription level of target genes[31]. Furthermore, three kinases, p38, JNK and ERK, make up the MAPK pathway and play crucial regulatory roles for activated macrophages[32]. As shown in Figs. 1E-F, when SCP were added, NF-κB, JNK1/2 MAPK and Erk1/2 MAPK signaling pathway inhibitors could prevent RAW 264.7 cells from secreting NO, p38 MAPK pathway inhibitor could not affect NO secretion. On the other hand, four pathway inhibitors (NF-κB, JNK1/2 MAPK, Erk1/2 MAPK and p38 MAPK) could prevent the secretion of TNF-α. It has been shown that SCP could modulate the immune system by regulating cellular signaling pathways.

In addition to RAW 264.7 cells, the anti-allergic effects of SCP were also proved by RBL-2H3 mast cells in this work. RBL-2H3 mast cell is a commonly used *in vitro* cell line of mast cells that could be used to characterize the allergic reactions[33]. As an inflammatory mediator in mast cell lysosomes, *β*-hexosaminidase is a specific enzyme that marks mast cell activation for degranulation[34]. Moreover, TNF-α is the primary cytokine released during mast cell degranulation, promotes eosinophil and neutrophil generation, and mediates eosinophil activity, which prolongs chronic inflammation[35]. Furthermore, IL-10 is a powerful immunosuppressive cytokine that blocks TNF-α action[36]. Histamine enhances the arterial wall's permeability during allergic reactions, enabling plasma to flow into the tissues and resulting in localized tissue edema[37]. In this work (Figs. 2A-F), SCP was able to suppress the allergic reactions of RBL-2H3 mast cells, weakened the degranulation degree, reduced the secretion of IL-4, TNF-α and histamine, and promoted the secretion of IL-10 from RBL-2H3 mast cells.

BALB/c mouse is a commonly used animal model to investigate allergic reactions. The mouse spleen is an immune organ, and its

lymphocyte cytokines can be used to evaluate the process of allergy[38]. IL-4, IL-10 and IFN-γ are all commonly used cytokines during allergic reactions. IFN-γ is a cytokine that could inhibit the transformation of Th2 cells. However, previous studies have found that the transformation of Th2 cells is reduced, which could lead to allergic reactions[39-40]. The lung is also known to be a complex immune organ that responds to allergens, so the study of lung tissue slices could also be used to characterize the degree of allergic reactions[41]. In this investigation, SCP were found could significantly reduce the secretion of allergic cytokines as well as lung inflammation in a dose-dependent manner, weakened the shrimp tropomyosin induced allergic reactions (Figs. 3-4). A similar previous investigation also indicated algae polyphenols could reduce allergic reactions in mice[42]. Considering the prevention effects of SCP on shrimp tropomyosin induced food allergy, the evaluation of SCP effects on food allergy before sensitization would be further investigated in the future.

In summary, SCP could reduce the allergenicity of tropomyosin, weaken the allergic reactions of RBL-2H3 mast cell, RAW 264.7 cell and mouse model, which indicated the anti-allergic ability of SCP. In conclusion, these results suggested that SCP could serve as potential anti-allergic substance for the prevention and treatment of shrimp tropomyosin induced food allergy.

5. Conclusions

In this work, SCP could affect the allergic reactions of RAW 264.7 cells through cellular signaling pathways, reduced the degranulation degree and the secretion of IL-4, TNF-α and histamine from RBL-2H3 mast cells, promoted the secretion of IL-10 from RBL-2H3 mast cells. As to BALB/c mouse, after i.g. gavage of SCP, the cytokine (IL-4, IL-10, IFN-γ) secretion of mouse SLP were declined significantly and the allergic lung damage was alleviated, which indicated SCP performed strong anti-allergic functions and could serve as candidate anti-allergic substances via immunoregulation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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