

Assessing the effect of 5-hydroxymethylfurfural on selected components of immune responses in mice immunized with ovalbumin

Effect of 5-HMF on immune response

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Abstract

BACKGROUND: 5-hydroxymethylfurfural (5-HMF) is one of the most important products of the maillard reaction. In recent years, many profitable biological effects of this compound have been demonstrated. This study sought to elucidate the anti-allergic effect of 5-HMF by investigating some selected

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components of the immune response in BALB/c mice immunized with ovalbumin (OVA).

RESULTS: Immunized animals had an increased level of serum total and OVA-specific antibodies when compared to the control ($P<0.01$). We found that the OVA-induced increase in serum IgE and OVA-specific IgE were significantly suppressed in the groups treated with 5-HMF ($P<0.05$). Moreover, IL-4 and IFN- γ were significantly reduced in a dose-independent manner when compared to the sensitized group ($P<0.05$).

CONCLUSION: 5-HMF inhibited the up-regulation of total and OVA-specific IgE through the suppression of the Th2-type immune response in immunized BALB/c mice. 5-HMF could therefore be a novel therapeutic approach for the prevention of IgE-mediated allergic diseases.

Key words: 5-HMF; antibodies; cytokines; ovalbumin; mice

INTRODUCTION

The worldwide prevalence of allergic diseases has increased during the last three decades.¹ Immunoglobulin E (IgE) is a central mediator in pathogenesis of many allergic diseases such as allergic asthma,² allergic rhinitis,³ allergies to certain foods⁴ and atopic dermatitis⁵. Various therapeutic interventions have been

investigated to modulate the immune system and inhibit IgE synthesis⁶, with the immune-modulating and anti-allergic effects of food receiving more attention over the past years^{7,8}.

5-hydroxymethylfurfural (5-HMF) is one of the most important products of the non-enzymatic maillard reaction, where the presence of acidic condition with high temperatures induces a reaction between a reducing sugar and an amino acid.^{9,10} A variety of foods and beverages provide 5-HMF; including cereal products,¹¹ instant coffee,¹² dried fruit,¹³ bread,¹⁴ pasta,¹⁵ milk¹⁶ and honey¹⁷. However, food processing conditions such as temperature, duration and water activity affects the amount of 5-HMF in these foods.¹⁴ Estimated daily food intake of 5-HMF indicated that it was commonly up to 150 mg/day¹⁸, and there are wide ranges in many studies.^{19,20}

In the past, foods with high 5-HMF were known as a risk factor for human health and many studies indicated that this compound may elicit genotoxic, mutagenic and carcinogenic activities.²⁰⁻²² In spite of this, a long term animal study demonstrated that different doses of 5-HMF had no neoplastic effect.²³ The carcinogenic activity of 5-HMF were rejected by direct or indirect methodologies²⁴. In recent years, experiments have revealed further health promoting effects of 5-HMF. Some studies indicated that 5-HMF acts as a novel natural antioxidant with potential applications in cancer,²⁵ high glucose-induced oxidative stress reduction²⁶ and many other beneficial physiological effects such as neuroprotective effects²⁷ and anti-hypoxia²⁸. An *in vitro* study indicated that 5-HMF as an anti-allergic compound, acting by suppressing IgE-mediated chemical mediator release.²⁹

As mentioned above, recent experiments illustrated that enhancement of the immune response must be a profitable effect of 5-HMF. Therefore, the aim of this study was to examine the anti-allergic effects of 5-HMF on BALB/c mice sensitized with ovalbumin (OVA). In this study, we have evaluated the effects of 5-HMF, in two different doses on total and OVA-specific IgE response in the serum and two selected Th-1 and Th-2 derived cytokines in the culture supernatants of spleen cells from after stimulation with OVA.

MATERIALS AND METHODS

Animals and diet

The study protocol was conducted with approval from the research ethics committee of Tabriz University of Medical Sciences, Tabriz, Iran (reference number: TBZMED.REC.1394.8). Eight-week-old female BALB/c mice (30-35 g) were maintained under standard conditions (temperature, 22-23 °C; humidity, 50-60 %; 12-h light/dark cycle) and specific pathogen-free circumstances. After acclimation for one week, the animals were allocated to four weight-matched groups (8 animals per cage). Mice in the control and sensitized groups were fed with normal chow-diet (Table 1) throughout the study, and the other two groups were fed diets containing low and high doses of 5-HMF (Matrix Scientific, Pontiac Business Center Drive, Elgin, USA) five days per week. The doses of 5-HMF were selected to supply a daily intake of 188 (LHMF) and 750 (HHMF) mgkg⁻¹ of body weight during the experiments. The animals were fed pellet diets and water *ad libitum*.

Measurements of body weight, food intake and immunization

The body weight of the mice was measured in all groups on first day of the study and at one week intervals. Food consumption was determined every other day throughout the experiments. Sensitization of the animals to OVA (grade V; Sigma–Aldrich, St. Louis, MO, USA) was performed according to methods described in a previous study.³⁰ Briefly, the mice were immunized intraperitoneally with 100 µg of alum-precipitated OVA on day seven. The OVA inoculum was prepared with an equal volume of alum suspension, and the final concentration of OVA in the 100 µg alum-precipitated OVA was 100 µg ml⁻¹ with the final concentration of alum being 1 mg ml⁻¹. The mice were boosted with the same doses of the antigen on day 28, three weeks after the first immunization. 100 µg of PBS was used in the control group.

Preparation of blood serum and spleen cell suspensions

One week after the last immunization, the mice were anesthetized using pentobarbital (50 mg kg⁻¹) and blood was collected via the inferior vena cava and centrifuged at 4000 rpm for 30 minutes at 4°C. An incision was made into the abdominal wall to remove the spleen. The spleens were then immediately removed for weight and spleen index calculations, and immersed in prepared RPMI-1640 media (Sigma) containing an antibiotic-antimycotic combination (Gibco-BRL Life Technologies, MD, USA). Spleen cell suspensions were prepared following the procedure previously described.³¹ Briefly, splenocytes were washed in the media by centrifugation, followed by treatment with 0.83% ammonium chloride in Trishydrochloride (Sigma–Aldrich, St. Louis, MO, USA) (pH 7.4) at 37°C for 5 min to remove the red blood cells. The splenocytes were then washed three subsequent times with the culture media.

Ovalbumin specific cytokine production

A suspension of spleen cells (5×10^6 cells/well) was prepared in RPMI-1640 medium containing an antibiotic-antimycotic combination (10,000 units mL^{-1} of penicillin, 10,000 $\mu\text{g mL}^{-1}$ of streptomycin, and 25 $\mu\text{g mL}^{-1}$ of Gibco Amphotericin B), 25 mM Hepes buffer, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal bovine serum (Sigma–Aldrich, St. Louis, MO, USA); and cultured in triplicate wells with 100 $\mu\text{g mL}^{-1}$ ovalbumin (OVA) under air containing 5% carbon dioxide. After 72 h, the supernatants of the cultures from each mouse were collected and pooled for enzyme-linked immunosorbent assay (ELISA) analysis of interferon gamma (IFN- γ) and interleukin (IL)-4.

Total and OVA-specific IgE

IgE levels in the serum were measured using a double-antibody sandwich ELISA kit (Hangzhou Eastbiopharm Co. Ltd.; Hangzhou, China) according to the manufacturers' instructions. Briefly, wells pre-coated with mouse IgE monoclonal antibody were incubated with serially diluted purified mouse IgE (standards) or sera and IgE antibodies labeled with biotin; and then combined with streptavidin-HRP to form immune complexes. Finally, chromogen solutions were added to the plates followed by stop solution after 15 min incubation in the dark. Absorbance was measured at 450 nm in a microplate reader (Model 680; Bio-Rad Laboratories, Inc., USA). IgE antibody levels against OVA were determined by an indirect ELISA method. Briefly, OVA was dissolved at a concentration of 100 $\mu\text{g mL}^{-1}$ in a 50 mM carbonate buffer (pH 9.6), and 200 μL of this was applied to each well of the plates (Greiner

Labrotechnik, GmbH). The microplate wells were incubated first for 30 min at room temperature with shaking and subsequently overnight at 4°C. To block the remaining binding sites on the well surface, the wells were treated at room temperature for 2 h with 300 µl of 3% (w/v) BSA dissolved in Tris-HCl buffer (25 mM, pH 7.4) containing 0.14 M NaCl and 5 mM KCl. They were washed four times with PBS-T, with 100 µl of test sera diluted 10, 50 and 100 fold in buffer containing 20 mM Tris-HCl, pH 7.2, PMSF and 10⁻⁵ M leupeptin added and incubated for 2 h. They were washed again in a similar way before the addition of 100 µl of biotinylated mouse anti-mouse IgE monoclonal antibodies (Yamasa Corporation) diluted 1:1000. After incubation for 90 min at room temperature, the wells were washed and treated with 100 µl of avidin-HRP for 1 h. The wells were finally washed seven times and supplemented with 100 µl of a solution of TMB as the substrate of HRP, and the color was allowed to develop for 30 min at room temperature. The reaction was stopped by adding 50 µl of 1.0 M H₂SO₄, and the absorbance (OD) was read at 450 nm using a microplate reader.

Assessment of cytokines in the culture supernatants

Levels of IFN-γ and IL-4 in the splenocyte culture supernatants were measured using a commercially available kit (Hangzhou Eastbiopharm Co. Ltd.; Hangzhou, China). The microtitre plates were measured for absorbance at 450 nm in an ELISA reader. The limits of detection were 2.53 ng/ml for IL-4 and 2.43 ng/ml for IFN-γ.

Statistical analysis

All data were tested for normal distribution and equal variance by the Kolmogorov-Smirnov test. Comparison among groups was performed using one-way ANOVA with Tukey's correction for multiple comparisons or the Kruskal-Wallis test for nonparametric values. SPSS 13.5 software was used for statistical analysis with $P < 0.05$ indicating a statistically significant difference.

RESULTS

Food intake, body weight and spleen index

Mice from different groups did not differ in body weight at the beginning of the study. There was a significant trend of increased food intake (Table 2) during the experiments among the animals treated with 5-HMF compared with the sensitized and control groups ($P < 0.05$), so that final body weight of mice in the low and high 5-HMF groups were significantly ($P = 0.03$, $P = 0.04$, respectively) greater than the sensitized group. (Table 2)

The spleen index of mice (mg g^{-1} body weight) in the sensitized group was markedly higher than in the control ($P < 0.001$). The spleen index in the LHMF and HHMF groups were also significantly lower than in the sensitized group ($P < 0.005$) (Table 2).

The effect of 5-HMF on total and OVA-specific IgE levels in the serum

To investigate the effect of 5-HMF administration to OVA-sensitized mice on the induction of humoral immune responses, mice sera were collected one week after the second immunization and analyzed for total and OVA-specific IgE using ELISA, with the results shown in Fig. 1. Data indicated a marked increase in the serum level of total and OVA-specific IgE in the sensitized group, as

compared to the control groups ($P < 0.01$). As illustrated, the treatment of mice with low and high doses of 5-HMF effectively prevented an increase in serum total IgE ($P = 0.03$, $P = 0.01$, respectively) and OVA-specific IgE ($P = 0.04$, $P = 0.01$, respectively) levels compared to the sensitized group. Levels of these immunoglobulins in the 5-HMF treated groups were not significantly different from the control group ($P > 0.05$).

The effect of 5-HMF on cytokine production from splenocytes

To determine whether 5-HMF influenced cytokine production, we investigated the effects of 5-HMF dietary intake on the secretion of two cytokines in the culture supernatant of isolated splenocytes after stimulation with OVA. As shown in Fig. 2, there were significant differences in splenocyte IL-4 production between the control and sensitized groups ($P = 0.02$). In LHMF and HHMF groups, the production of IL-4 were significantly dose-independently inhibited when compared to the sensitized group ($P = 0.00$, $P = 0.04$, respectively). On the other hand, as shown in Fig. 2, production of IFN- γ was not significantly different between the control and sensitized groups ($P > 0.05$). However, treatment with low and high 5-HMF significantly inhibited IFN- γ secretion by splenocytes when compared to the sensitized group ($P = 0.01$, $P = 0.04$, respectively). In addition, IFN- γ levels were lower in the LHMF group compared to the control ($P = 0.03$).

DISCUSSION

This study revealed that 5-HMF reduced the total and OVA-specific IgE levels in the sera of OVA-sensitized mice, followed by a remarkable suppression of IL-

4, a key cytokine which drives Th-2 immune responses. Inhibition of IgE function, by either neutralization or inhibition of production has been considered as an approach for the treatment of IgE-mediated allergic diseases.³²⁻

³⁴ There is evidence that the level of total serum IgE has a relative positive correlation with the severity of allergic diseases.^{35,36} However, the application of anti-IgE drugs in patients is limited, since large doses of usually expensive drugs are needed to down-regulate high serum IgE levels.^{37,38} Thus, despite some concerns about the safety of foods containing 5-HMF, it seems that 5-HMF may be valuable in the alleviation of IgE-mediated food allergy and in the better management of IgE mediated allergies.

Several studies have demonstrated the potential of antioxidants for the prevention of various allergic diseases.^{8, 39, 40} Recently, many studies confirmed the antioxidant activity of 5-HMF. A cell line *in vitro* study by Zhao *et al.* showed that 5-HMF exhibited novel antioxidant activity by scavenging ABTS and DPPH free radicals. The assay was used in the antioxidant activity assay of water-soluble substances. On the other hand, antioxidant enzyme activity such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) were increased in erythrocytes pretreated with 5-HMF.²⁵ Lipid peroxidation and the evaluation of antioxidant defenses in mouse brain tissue showed that pretreatment with 5-HMF reduced malondialdehyde (MDA) content and augmented the activity of SOD⁴¹; protecting against the oxidative damage induced by cerebral ischemia in rats.⁴² Yong-Xin *et al.* suggested that the functional groups of the 5-HMF structure including double bonds, an aldehyde oxygen atom and an oxygen existing in the furan ring may exhibit antioxidant

effect by attracting electrons.⁴³ Therefore, the antioxidant activity of 5-HMF may have important role in the suppression of IgE-mediated allergy.

IgE-mediated reactions produce various inflammatory mediators such as tumor necrosis factor (TNF)- α and many other cytokines.⁴⁴ Thus, we examined cytokine production in the culture supernatant of isolated cells from the spleen after stimulation with OVA. Two different subtypes of T-helper cells including Th1 and Th2 secrete a variety of cytokines. Among the Th2 cytokines, our main attention focused on interleukin IL-4; since one of its main functions is the production of IgE. On the other hand, interferon- γ as a Th1 cytokine which promotes cell-mediated immune responses.⁴⁵ These cytokines, especially interleukin-4 (IL-4), play a central role in initiating IgE-mediated type 1 hypersensitivity reactions.⁴⁶ Our experiments indicated that 5-HMF treatment dose-independently inhibited IL-4 and IFN- γ production. Suppression of IL-4 by antioxidants diminished IgE levels in allergic mice, as our study indicated.⁴⁷

This study revealed that 5-HMF possesses anti-inflammatory effects via the down-regulation of pro-inflammatory cytokines. In line with this observation, an *in vitro* study by Kim *et al.* demonstrated the anti-inflammatory properties of 5-HMF through the inhibition of NF- κ B transcription factor activation and vascular cell adhesion molecule-1(VCAM-1) expression in human umbilical vein endothelial cells (HUVECs) stimulated by tumor necrosis factor- α (TNF- α).⁴⁸ 5-HMF and its derivative also impeded TNF- α and IL-1 β production.⁴⁹ Thus, it is speculated that this suppression takes place through the inhibition of ROS production. It seems that the mechanism of action by which 5-HMF inhibits cytokine production, especially IFN- γ , is influenced by preventing NF- κ B production. Activation of this signal could induce the expression of many

cytokines, chemokines and adhesion molecules that mediate the activation of immune cells.⁵⁰ NF- κ B play a key role in the production of IL-12, which is induces Th1 cells to produce IFN- γ .^{51, 52} Therefore, the mechanisms mentioned above could somewhat justify the reduction of IFN- γ in 5-HMF treated mice in a mechanism separate to the reduction in ROS. In addition, William *et al.* demonstrated that selective inhibitors of NF- κ B can reduce allergic airway inflammation⁵³ in a manner that is in accordance with the above mentioned anti allergic effects of 5-HMF. It is concluded that inhibition of IFN- γ by 5-HMF in this study could possibly be the result of NF- κ B transcription factor suppression; since many studies have indicated that NF- κ B inhibitors could suppress production of cytokines such as IFN- γ and IL-4.^{51, 52} However, future research is needed to reinforce this conclusion.

In this study, body weight or body weight gain was higher in 5-HMF treated mice than the sensitized group in a dose-independent manner. Similar to these results, Matsushita *et al.* reported that the final body weight of mice in a group treated with 5-HMF (188 mg kg⁻¹bw) was significantly increased.⁵⁴ In opposition to this, a National Toxicology Program study indicated that the body weight gain of male mice (1,500 mg kg⁻¹bw) were significantly lower than in the sensitized group.²³ Dissimilar to the most of previous studies, we administered 5-HMF through pellet diets, instead of gavage, which could affect the results of food intake and therefore body weight. As our results showed, mice in 5-HMF treated groups had a notably higher intake of food. It appears that the odor and flavor of pellets containing 5-HMF might stimulate the appetite of mice. Furan compounds are important aroma chemicals with attractive flavor properties.⁵⁵ These compounds are major contributors to the flavor of coffee, wine and a

variety of fruits^{56, 57} and are widely used for their pleasant sensory properties in the flavor industry.⁵⁸ In spite of this, more investigations are needed to detect the physiological effect of 5-HMF on appetite.

According to many reports, immunization with OVA is associated with a remarkable increase in the spleen size of the animals.^{59,60} Interestingly, our experiments indicated that 5-HMF prevented this increment of spleen weight due to immunization with ovalbumin, which was in accordance with a study by Li *et al.* that 5-HMF treatment decreased the spleen index in mice compared to a group supplied with alcohol.⁶¹ In addition, a lower spleen index together with a reduction of IgE and cytokine levels in 5-HMF treated groups could be result of a the antioxidant and anti-inflammatory properties of this compound.

The effect of 5-HMF on spleen cellularity is currently unknown. To investigate this, the spleen index was calculated to help understand the effect of 5-HMF on spleen mass which in part reflects the white blood cell count of the spleens. In addition, to address the confounding effect of spleen cellularity, the number of cells were carefully adjusted within the treatment groups. However, the inclusion of additional groups to determine the distinct effect of 5-HMF on spleen cellularity may further strengthen the conclusions of the current study and warrants further study.

In conclusion, the results of this study suggest for the first time that 5-HMF inhibited the up-regulation of serum total and OVA-specific IgE; possibly through the suppression of the Th2-type immune response in immunized BALB/c mice. Further studies may allow us to use 5-HMF as a novel supplement or medicine for the prevention of IgE-mediated allergies. Further

investigations are needed to explore the precise mechanisms involved in the suppression of the IgE mediated immune response.

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Table 1. Composition of experimental diet

Constituents	Amount (g kg ⁻¹ of diet)
Casein	200
Carbohydrate ^a	670
Cellulose	20
Mineral mixture ^b	50
Vitamin mixture ^c	10
Corn oil	50

^aStarch: sucrose, 2:1 ratio.

^bMixture (mg kg⁻¹diet): Calcium hydrogen orthophosphate dihydrate, 7280; potassium dihydrogen orthophosphate, 12,860; sodium dihydrogen orthophosphate, 4680; sodium chloride, 2330; calcium lactate, 17,550; ferrous citrate, 1590; magnesium sulfate, 3590; zinc carbonate, 55; MnSO₄·4-6H₂O, 60; copper sulfate pentahydrate, 15; potassium iodide, 5.

^cComposition (vitamin units or mg kg⁻¹diet): thiamine-hydrochloride, 12; riboflavin, 40; pyridoxine-hydrochloride, 8; vitamin B12, 0.005; ascorbic acid, 300; D-biotin, 0.2; folic acid, 2; calcium pantothenate, 50; *p*-aminobenzoic acid, 50; niacin, 60; inositol, 60; choline chloride, 2000; retinol acetate, 5000; ergocalciferol, 1000 IU; tocopherol acetate, 50; menadione, 52.

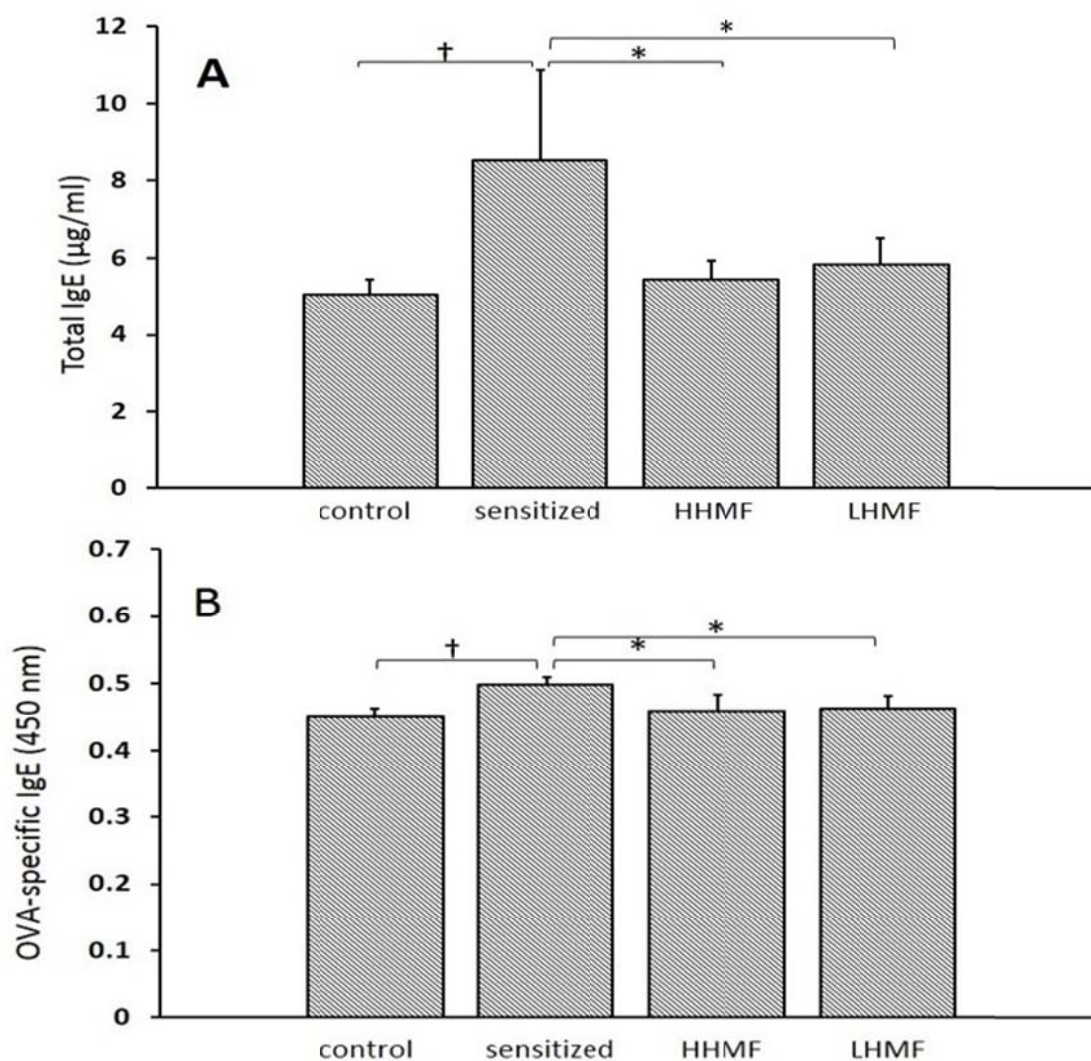
Table 2. Body weight, spleen index and food consumption.

Variable	Groups			
	Low HMF	High HMF	sensitized	Control
Initial body weight (g)	30.87±0.78	31.25±0.55	31.25±0.75	31.37±0.59
Final body weight (g)	34.62±1.03*	34.50±0.65*	31.50±0.59	33.75±0.61
Body weight gain (g)	3.75±0.97*	3.25±0.55*	0.25±0.75	2.37±0.59
Spleen index (mg/g bw)	5.76±0.41*	5.98±0.60*	7.11±0.58	5.54±0.58*
Food intake (g/day)	7.57±0.06*†	7.22±0.04*†	6.27±0.02	6.20±0.11

Values are presented as mean±SD. *P<0.05, compared with sensitized; †P<0.05, compared with control

Legends:

Legend for figure 1. The effect of low and high dose 5-HMF administration on total (A) and ovalbumin-specific IgE (B) in 10-fold diluted serum samples of ovalbumin-sensitized mice. Group data were expressed as means $\pm$ standard deviation (SD). Single asterisks and Single daggers represent p values less than 0.05.



Legend for figure 2. The effect of low and high dose 5-HMF administration on levels of IL-4 (A) and IFN- γ (B) in the culture supernatant of isolated cells from the spleen. Group data were expressed as means $\pm$ standard deviation (SD). Single and double asterisks represent p values less than 0.05 and 0.01, respectively and Single daggers indicate p values less than 0.05.

