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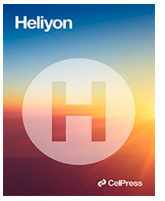

Research article

Effect of Hita Tenryo Water™, a natural mineral water, on allergic symptoms induced by cedar in mice

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ABSTRACT

The number of patients with allergies to pollen and food is increasing worldwide. In Japan, the prevalence of cedar pollinosis, a type I allergy, is nearly 30% and accounts of hay fever are rising. A potential natural remedy for these allergic diseases may be Hita Tenryo Water™ (referred to simply as Hita Tenryo water), water that is pumped from deep underground in the Hita region of Oita, Japan, which has been the subject of various research reports. Here, we investigated the potential of using Hita Tenryo water to suppress the onset of cedar pollinosis in a mouse model and explored the immunological mechanism of the suppression. Test model mice were given Hita Tenryo water ad libitum to drink and received intraperitoneal administration of (i) tap water (Hw1), (ii) 25% Hita Tenryo water (Hw2) or (iii) 100% Hita Tenryo (Hw3). There were no significant differences in body weight change, feed intake, or water intake among the groups during the experimental period. We examined nose rubbing and sneezing as allergic symptoms. The frequency of rubbing and sneezing tended to decrease in the Hw1 and Hw2 group, and significantly decreased in the Hw3 group compared to control. Total IgE levels in serum were also significantly reduced in Hita Tenryo water intraperitoneal administration groups. In vitro examination of the rate of release of β-hexosaminidase from BL-2H3 cells showed that there were no significantly differences between Hita Tenryo water-treated and control cells. In addition, measurement of Th2-related cytokine levels in concanavalin A-stimulated peripheral blood mononuclear cells revealed a significant decrease in IL-4, IL-6, and IL-10 levels in medium ($p < 0.01$). In contrast, production of IFN-γ significantly increased ($p < 0.01$). These results indicate that Hita Tenryo water may alleviate and/or suppress allergic symptoms.

1. Introduction

An increasing number of people around the world are developing allergic diseases such as allergic rhinitis and conjunctivitis, as well as allergies to pollen and food. Japanese cedar pollinosis is a common allergic disease in Japan caused by inhalation of cedar (*Cryptomeria japonica*) pollen. The prevalence of cedar pollinosis in Japan is nearly 30%, and the prevalence of hay fever is on the rise [1,2].

Cedar pollinosis is a typical type 1 allergy characterized by sneezing, watery nasal discharge, and conjunctivitis. Cry j 1 and Cry j 2 proteins have been identified as the major allergens associated with cedar pollinosis [2,3]. According to some reports, about 90% of people with Japanese cedar pollinosis are positive for Cry j 1-specific immunoglobulin E (IgE) [4,5]. Allergic reactions are classified

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into types I-IV. Type I allergic reactions are triggered by type 2 helper T (Th2) cells and/or group 2 innate lymphocytes (ILC2), which produce type 2 cytokines such as interleukin (IL)-4, IL-5, IL-13 and other inflammatory mediators [6]. Th2 cells activate B cells, which in turn produce IgE. The IgE comes into contact with allergens and activates FcεRI receptors, which are high-affinity IgE receptors on the plasma membrane of mast cells and basophils. Cross-linked IgE bound to the receptor then induces mast cells to release chemotransmitters, including β-hexosaminidase granules, a common marker of degranulation, leading to inflammation [6,7].

Many people suffer from long-term cedar pollinosis. Although new treatments such as targeted therapy exist, antihistamines and steroids remain the main drugs used. There is currently no treatment that effectively alleviates or cures the disease without side effects [2].

Japan is a country rich in water resources and one of few in the world where tap water tastes good and safe to drink. As an example of the safety of tap water, the results of measurements made by the tap water administration of Fukuoka City, Japan, are shown (sTable S1). This table shows the results of periodic water quality inspections at the water purification plants that supply our laboratory's tap water (which was used for this study). In Japan, water quality testing is carried out regularly 4 or 12 times a year at all of the more than 6000 water treatment plants in the country. Japan's stringent water quality standards for tap water offer a glimpse into the country's high level of water awareness [8]. Perhaps for this reason, research in Japan has examined the potential benefits of water with additive functions—so-called functional water—for human health [9]. A prominent example of functional water is reduced water, including electrochemically reduced water and natural reduced water (NRW), which has been shown increase the ability of cultured cells to scavenge reactive oxygen species (ROS) [10–12]. Reduced water has also been suggested to prevent or improve oxidative stress-related diseases such as diabetes, cancer, arteriosclerosis, neurodegenerative diseases, and the side effects of hemodialysis [9]. In particular, Hita Tenryo water™ (referred to simply as Hita Tenryo water), produced in the Hita region of Oita in Japan, despite being a purely natural water, has been the focus of several reports. We previously reported that Hita Tenryo water was a NRW [11,13,14]. Hita Tenryo water has been reported to have various physiologically effects, such as increasing aquaporin protein expression [15], activating natural killer cells [16], suppressing type 1 diabetes [11,14], suppressing mental anxiety [17] and anti-obesity effects [18]. Moreover, recent studies have reported that continuous consumption of Hita Tenryo water for 6 months shifts the intestinal microflora in a positive direction for health [19], and that continuous consumption of Hita Tenryo water has verified effects on cognitive function, body composition, and psychological function [20].

Given the accumulating evidence that consumption of Hita Tenryo water confers benefits to human health, we examined its potential benefit for allergy symptoms. As we have obtained statements from many consumers of Hita Tenryo water regarding an improvement in their allergy symptoms, we investigated Hita Tenryo water's ability to suppress the onset of hay fever in a mouse model of cedar pollinosis and the underlying immunological mechanism.

2. Materials and methods

2.1. Water samples

Hita Tenryo water (Hita Tenryo-Sui Co., Ltd., Oita, Japan) and commercial mineral water (Mw) were sterilized by filter sterilization, and Fukuoka City tap water was sterilized by autoclaving. In this animal study, Fukuoka city tap water was used as the control water, as it was considered unlikely to contain allergenic substances from the data provided (sTable 1). In addition, commercial mineral water, which is widely distributed, was randomly selected as another control water (water expected to be inactive against allergy). All solutions were prepared using ultrapure water produced by a Milli-Q System (Merck KGaA, Darmstadt, Germany). All chemicals were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan) unless otherwise indicated.

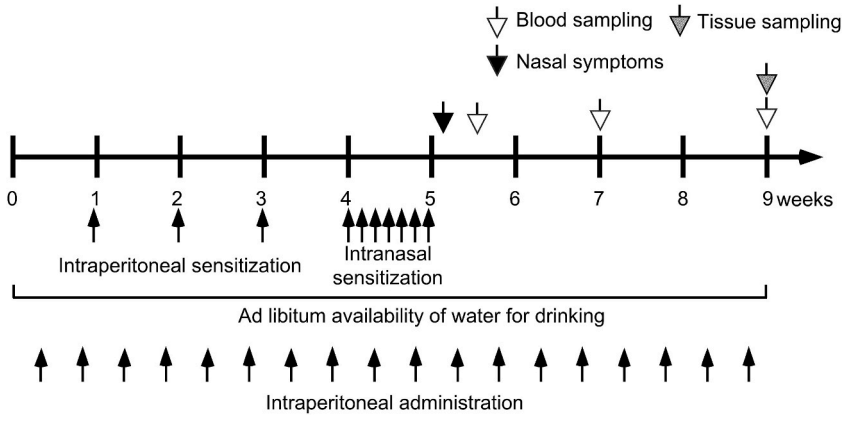

Fig. 1. Outline of the schedule used for mouse sensitization and water administration.

2.2. Experimental animal studies and creation of a cedar pollen allergy mouse model

All animal experimental procedures were reviewed by the Animal Welfare Committee of Kyushu University (approval numbers: A19-238-0 and A21-267-0) and were performed in accordance with relevant guidelines and regulations. We also followed the Reporting of in vivo Experiments (ARRIVE) guidelines (https://www.nc3rs.org.uk/arrive-guidelines) and the National Research Council's Guide for the Care and Use of Laboratory Animals.

Four-week-old female Balb/c SPF mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used in this study. The mice were kept under the following conditions throughout the study: temperature and humidity: 20–26 °C, 40–70%; ventilation: 10–15 times/hour; lighting: 12-h light/dark cycle with lights on from 8:00–20:00, 150–300 lux; and diet: CRF-1LID10 solid feed (Oriental Yeast Co., Ltd., Tokyo, Japan), ad libitum.

After a week of habituation, the mice were administered saline solution containing 1 μg Cry j 1 cedar pollen antigen (Cry j 1, Cedar Pollen Allergen, Purified, BioDynamics Laboratory Inc., Tokyo, Japan) and adjuvant (0.1 ml 2% aluminum hydroxide) for primary sensitization once a week for a total of 3 times (Fig. 1) by intraperitoneal administration. For secondary sensitization, 10 μl of PBS containing 0.5 μg of Cry j 1 was administered intranasally for 7 days. As shown in Table 1, the mice were allocated to five groups (N = 9 each) in which they received Hita Tenryo water (Hw), tap water (Tw), or commercial mineral water (Mw) throughout the study period of 9 weeks. In addition to a group that received Hita Tenryo water ad libitum to drink (Hw1), we also included two groups that received intraperitoneal administration of Hita Tenryo water (Hw2 and Hw3) to enhance the affect in mice. The Hw2 group was intraperitoneally administered 10 ml/kg saline in Hita Tenryo water and 30 mg/kg saline in tap water, and the Hw3 group was intraperitoneally administered 40 ml/kg saline in Hita Tenryo water. Both the Hw2 and Hw3 group received Hita Tenryo water ad libitum to drink. The unsensitized test group (Nc) did not receive sensitization and formed the negative control group, while the Tw group received sensitization and formed the positive control group. The Nc group administered intraperitoneal saline solution without Cry j 1 and adjuvant for primary sensitization and intranasal PBS for secondary sensitization. The Tw group was intraperitoneally administered a saline solution prepared with tap water and received tap water ad libitum to drink. The Mw group, another control group, was intraperitoneally administered Mw and received Mw ad libitum to drink. All intraperitoneal administrations were given at 40 mg/kg twice a week throughout the study period starting one week prior to sensitization. Body weight, food intake, and water consumption were measured once a week. At the end of the study, 9 weeks after the start of administration, blood samples were collected and erythrocyte and leukocyte counts were measured using a K-4500 multiparameter automated hematology analyzer (Symex Corp., Hyogo, Japan), and total IgE levels were measured from the obtained serum. In addition, blood samples were taken from the lateral tail vein twice after secondary sensitization for measurement of serum IgE levels. Liver and spleen were also collected and weighed.

2.3. Evaluation of allergic symptoms

Allergic symptoms were measured one day after secondary sensitization. Fifteen minutes after intranasal administration of 10 μl of PBS containing 0.5 μg of Cry j 1(BioDynamics Laboratory Inc.), the number of nose rubs and sneezes over a 15-min period were counted.

2.4. Total immunoglobulin E (IgE) levels in serum

Total IgE levels in serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Thermo Fisher Scientific K.K., Tokyo, Japan). Measurements were evaluated according to the manufacturer's protocol.

2.5. β-Hexosaminidase release assay

We examined the effect of each water type on β-hexosaminidase release from the rat basophilic leukemia cell line RBL-2H3 according to a previous report with some modifications [21]. In brief, RBL-2H3 cells were seeded in Eagle's Minimum Essential Medium (MEM; Toyobo Co., Ltd., Osaka, Japan) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific K.K.) at a concentration of 1×10^5 cells/well in a 24-well plate at 37 °C, 5% CO_2 and 95% humidity. After 1 day, the medium was replaced with 10% FBS MEM prepared with each water type. After another day, cells were sensitized by incubating with anti-DNP IgE (50 ng/ml) in MEM medium containing each water type for 120 min. Modified Tyrode (MT) buffer (20 mM HEPES, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$)

Table 1
Sensitization and water administration conditions for each group in the mouse study.

Group	Sensitization	Water type	
		Intraperitoneal	Drinking
Nc	−	–	Tap water
Tw	+	Tap water	Tap water
Mw	+	Mineral water	Mineral water
Hw1	+	Tap water	Hita Tenryo water
Hw2	+	75% Tap water, 25% Hita Tenryo water	Hita Tenryo water
Hw3	+	Hita Tenryo water	Hita Tenryo water

prepared with each water type was then added and the cells were incubated for 10 min. The tests were performed using MEM medium prepared with each water type (MQ: Milli-Q water, Mw: commercial mineral water, HW2: 25% Hita Tenryo water/75% Milli-Q water, HW3: 100% Hita Tenryo water). MT buffer containing 1 μM wortmannin (LC Laboratories, MA, USA) was used as a control to suppress degranulation release. Both the medium and cell lysate were treated with 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and the resulting colored solution was measured at 405 nm using a microplate reader (iMark, Bio-Rad Laboratories, Inc., CA, USA) to evaluate β-hexosaminidase activity.

2.6. Isolation of human lymphocytes

Peripheral blood mononuclear cells (PBMCs), consisting primarily of lymphocytes, obtained from heparinized venous blood collected from healthy male volunteers were purchased from KAC Co., Ltd. (Kyoto, Japan). The PBMCs were then washed twice with RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and used for subsequent experiments.

2.7. Evaluation of cytokine induction in PBMCs

PBMCs were seeded at 5×10^5 cells/ml in RPMI 1640 medium. After incubating at 37 °C, 5% CO_2 and 95% humidity for 24 h, the medium was replaced with RPMI 1640 prepared with each water type, and 5 μg/ml concanavalin A (ConA) was added. After incubating the cells for 72 h in a CO_2 incubator, the supernatant was collected and stored at −86 °C until analysis by ELISA. Concentrations of the cytokines IL-4, IL-6, IL-10, IL-13 and IFN-γ produced in the culture supernatant were determined using a commercially available ELISA kit (Thermo Fisher Scientific K.K.). Measurements were performed according to the manufacturer's protocol.

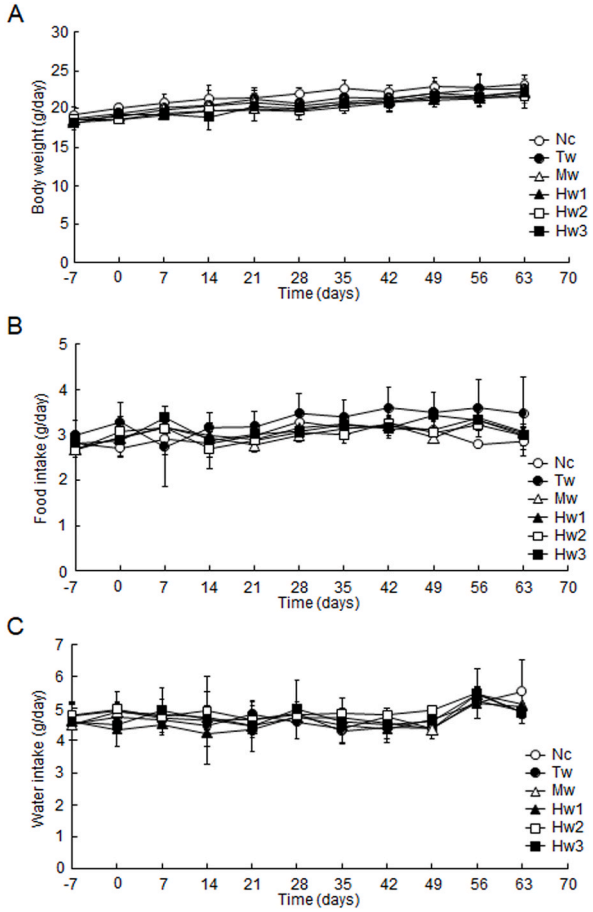

Fig. 2. Mean body weight, food consumption and water consumption in each test group. A. Body weight. B. Food consumption. C. Water consumption. Nc: unsensitized control, Tw: tap water, Mw: commercial mineral water, Hw1: Hita Tenryo water ad libitum, Hw2: 25% Hita Tenryo water administered intraperitoneally, Hw3: 100% Hita Tenryo water administered intraperitoneally.

2.8. Measurement of cell viability

Cell counts of the cultured cells were measured using the WST-8 assay kit (Dojindo Co., Tokyo, Japan), a cell counter (Countess3, Thermo Fisher Scientific K.K.), and hemacytometer, as needed. The WST-8 assay was performed by closely following the manufacturer's protocol. Briefly, 1 μl of WST-8 dye was added to each well of cultured cells and the cells were incubated for an additional 4 h. Viable cells were measured using a microtiter plate reader (iMark) at 450 nm.

2.9. Measurement of intracellular ROS

A detailed report of the method used to measure intracellular ROS is published elsewhere [12]. In brief, the human fibrosarcoma cell line HT1080 (CCL-121; American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM medium (Nissui) prepared with each water type supplemented with 10% FBS (Lot No. 12D168, Sigma-Aldrich Japan Co. LLC, Tokyo, Japan). HT1080 cells were seeded at a density of 0.5×10^5 cells/ml into a 96-well plate and incubated in a CO_2 gas incubator at 37 °C, 5% CO_2 and 95% humidity for 24 h. The cells were then treated with 30 or 60 μM hydrogen peroxide (H_2O_2) for 30 min, and then incubated for 120 min in DMEM medium prepared with each water type. The cells were then incubated with 2 μM 3′-*O*-acetyl-6′-*O*-pentafluorobenzenesulfonyl-2′, 7′-difluorofluorescein (BES–H_2O_2–AC) and 1 μg/ml Hoechst 33,342 for 15 min. The intracellular fluorescence intensity induced by each water type was probed with BES–H_2O_2–AC and detected using a BZ-9000 microscope (Keyence Corp, Osaka, Japan) with an excitation filter at 480 nm and an emission filter at 535 nm.

2.10. Statistical analysis

Microsoft Excel 2016 was used to calculate the mean, standard deviation (SD) or coefficient of determination in triplicate experiments. One-way or two-way ANOVA (STATMATE III for Windows) was used to determine the presence of statistically significant differences. Tukey test was performed as a post hoc test after ANOVA. Error bars indicate SD unless otherwise noted.

3. Results and discussion

3.1. Effects of water intake on standard health parameters

To examine the effect of Hita Tenryo water on allergic reactions, we established a mouse model of Cry j 1-induced pollinosis using Cry j 1, a major allergen of cedar pollen, as an antigen. The dose of Cry j 1 and the administration schedule were based on a previous report [21]. Fig. 1 and Table 1 show an overview of the experiment and conditions.

Fig. 2 shows the average body weight, food intake, and water consumption of individual mice in each group, measured at weekly intervals. There were no significant differences in body weight change, feed intake, or water intake between groups during the experimental period.

3.2. Allergic symptoms

Following nasal administration of Cry j 1 on day 28, allergic symptoms were analyzed by measuring the frequency of nose rubbing movements and sneezing over a 15-min period in sensitized and unsensitized mice on day 36. The nose rubbing frequency was 22.4 ± 4.7 in the Nc group, 81.2 ± 12.1 in the Tw group, 83.3 ± 11.3 in the Mw group, 73 ± 16.5 in the Hw1 group, 73.4 ± 12.3 in the Hw2 group and 50.1 ± 8.7 in the Hw3 group. The number of sneezing events was 1.5 ± 0.5 in the Nc group, 8.6 ± 1.5 in the Tw group, 8.9 ± 1.3 in the Mw group, 7.3 ± 1.3 in the Hw1 group, 7.3 ± 1.3 in the Hw2 group, and 4.6 ± 1.3 in the Hw3 group. The number of nose rubbing events was significantly higher in the Tw and Mw groups than the Nc group ($p < 0.01$; Fig. 3A). Likewise, the number of

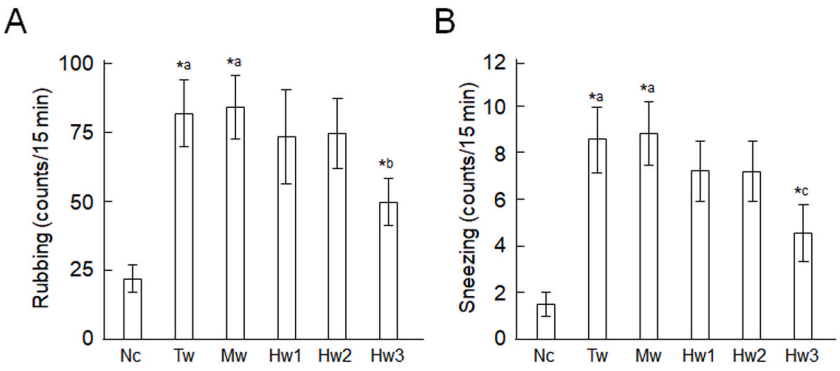

Fig. 3. Nasal symptom scores. A. Rubbing. B. Sneezing. *a: $p < 0.01$ vs. Nc,*b: $p < 0.01$ vs. Mw and Tw,*c: $p < 0.05$ vs. Mw and Tw. Error bars indicate standard error (SE). Other notations are as described for Fig. 2.

sneezing events was also significantly higher in the Tw and Mw groups compared to the Nc group ($p < 0.01$; Fig. 3B). Both nose rubbing and sneezing frequency tended to decrease in the Hw-treated group, and were significantly lower in the Hw3 group compared to the control group ($p < 0.05$).

3.3. Serum total IgE

Levels of total IgE in serum, which is expected to be increased by the development of cedar pollen allergy, were measured by ELISA from serum collected at 3 days, 2 weeks and 4 weeks (end of the animal study) after secondary sensitization, respectively. In the case of blood samples taken 3 days after secondary sensitization, IgE was significantly higher in the Tw (3.80 ± 1.33 μg/ml) and Mw (3.86 ± 0.34 μg/ml) groups than the Nc group (0.86 ± 0.22 μg/ml, $p < 0.01$; Fig. 4). While IgE levels showed a trend towards being lower in the Hw1 group (3.52 ± 1.02 μg/ml) than in the Tw and Mw control groups, they were significantly reduced the Hw2 group (2.90 ± 0.91 μg/ml, $p < 0.01$) and the Hw3 group (1.92 ± 1.48 μg/ml, $p < 0.01$). Thus, injection of Hita Tenryo water appears to have a dose-dependent effect on IgE. Significant differences were confirmed only for Hw3 ($p < 0.05$ vs Tw, sFig. S1), but the same trend was observed in the measurements of the samples after 2- and 4-weeks days after secondary sensitization.

Tap water and commercial mineral water were used as control groups in the present animal study. There were several candidates for the control water, but these were chosen with an important focus on comparing them with the water we normally drink. Another reason for selecting them was that they are soft water, as well as Hita Tenryo water. As expected, tap water and randomly selected commercial mineral water showed similar results. In addition, since the behavior of serum IgE with these control water was similar to previous studies using mouse models prepared under similar conditions, we conclude that our results indicate that Hita Tenryo water suppressed the amount of IgE triggered by sensitization [22]. It should also be mentioned that preliminary tests did not identify any significant changes in allergic symptoms between Fukuoka City tap water and ultrapure water (Data not shown).

3.4. Degranulation of RBL-2H3 cells

Given that our experiments in the pollinosis model mice suggested that intake of Hita Tenryo water suppresses pollinosis symptoms, we next investigated the immunological mechanism of pollinosis suppression by Hita Tenryo water. A major factor in the occurrence of allergy is the production of various lipid mediators and/or cytokines, including the degranulation response, which arises following activation of FcεRI aggregation on the surface of mast cells [23]. We thus speculated that exposure to Hita Tenryo water could affect the degranulation response. As such, we examined the effect of Hita Tenryo water on mast cell degranulation using RBL-2H3 cells.

We induced degranulation in anti-DNP IgE-sensitized RBL-2H3 cells by treating them with DNP-HSA and subsequently exposed some of these cells to 25% or 100% Hita Tenryo water, or Mw or MQ (controls). We then measured the rate of release of β-hexosaminidase, a marker of mast cell degranulation. Untreated cells showed significantly lower degranulation levels (40%) than anti-DNP-treated cells (100%) (Fig. 5A). Wortmannin-treated cells also showed lower degranulation levels (37%, $p < 0.01$ vs MQ). However, degranulation in stimulated RBL-2H3 cells treated with Mw, 25% Hita Tenryo water, and 100% Hita Tenryo water was not significantly different to those treated with MQ (Fig. 5A). The viability of cells treated with Hita Tenryo water, as measured using a WST-8 assay, was likewise not significantly different to those treated with MQ. Similarly, no significant differences were observed in intracellular β-hexosaminidase production between cells treated with Hita Tenryo water and the other water types (data not shown). These results suggest that Hita Tenryo water does not suppress the degranulation process in mast cells that could explain the reduction in allergic symptoms observed in the mouse model of pollinosis. Although not verified in this study, there may have been changes in IL production by the stimulated mast cells. Further studies are needed to explore this theory. Based on the current findings, Hita Tenryo water does not seem to impair the degranulation process required for defense against infection.

3.5. Cytokine release by PBMCs

As the results of the degranulation activity assay using RBL-2H3 cells suggested that treatment with Hita Tenryo water had little

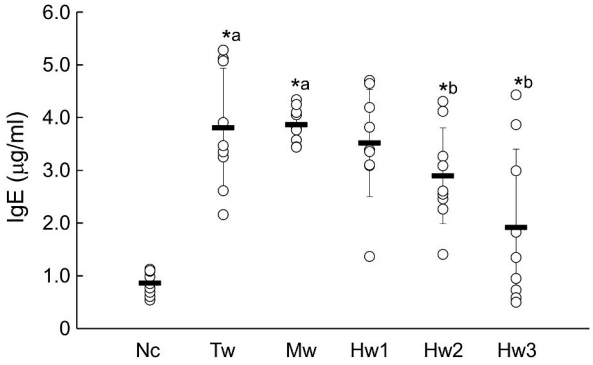

Fig. 4. IgE in serum. *a: $p < 0.01$ vs. Nc,*b: $p < 0.01$ vs. Tw and Mw. Other notations are as described for Fig. 2.

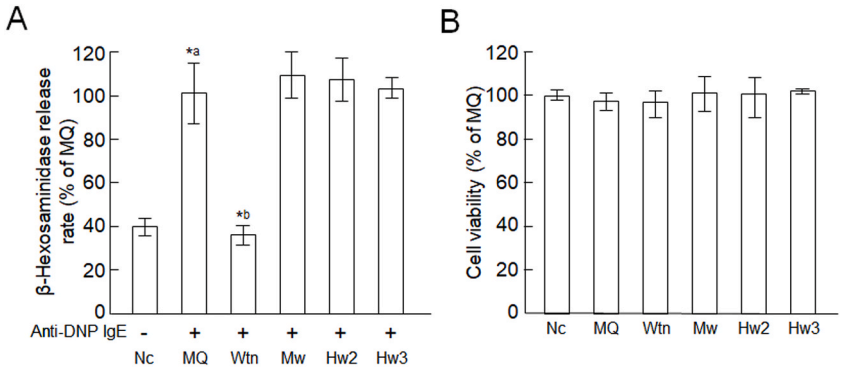

Fig. 5. Degranulation in BL-2H3 cells. A. β-hexosaminidase release rate. B. Viability of cells tested in A. *a: $p < 0.01$ vs. Nc,*b: $p < 0.01$ vs. MQ. MQ: Milli-Q-filtered water, Wtn: 1 μM wortmannin in Milli-Q-filtered water, Hw2: 25% Hita Tenryo water in 75% MQ water, Hw3: 100% Hita Tenryo water. Other notations are as described for Fig. 2.

influence on mast cell degranulation, we speculated that the water may instead affect Th2 cells, which play a major role in antibody production. Hence, we tested the effect of Hita Tenryo water on the immune response using PBMCs by measuring the amount of cytokine production. PBMCs collected from healthy subjects were treated with MQ water, Mw or Hw for 3 days with and without ConA stimulation, and the production of cytokines involved in Th2 function, namely IL-4, IL-6, IL-10, IL-13, and IFN-γ, were measured by ELISA.

With or without ConA stimulation, the concentration of IL-4 in PBMC cultures was statistically significantly lower ($p < 0.01$) on day 3 after treatment with Hw compared to MQ and Mw (Fig. 6A). Moreover, release of IL-6 (Fig. 6B) and IL-10 (Fig. 6C) was also statistically significantly lower ($p < 0.01$) in the Hw-treated group that received ConA stimulation compared to MQ and Mw controls. In contrast, no differences were observed in IL-13 concentrations (Fig. 6D) with or without ConA stimulation between the Hw group and MQ and Mw controls. Meanwhile, IFN-γ concentrations in the culture medium were statistically significantly higher ($p < 0.01$) in ConA-treated cultures in the Hw group.

Thus, overall, treatment with Hita Tenryo water suppressed the production of cytokines involved in B cell proliferation and activation. Specifically, levels of IL-4, which plays a central role in inducing antibody isotype switching and stimulates IgE production, IL-6, which is involved in lymphocyte and monocyte differentiation and plays an essential role in the eventual differentiation of B cells into Ig-secreting cells, and IL-10, which enhances B cell survival, proliferation and antibody production (Fig. 6A–C), decreased. However, Hita Tenryo water treatment caused no significant changes in IL-13 compared to control. While it is unclear exactly how

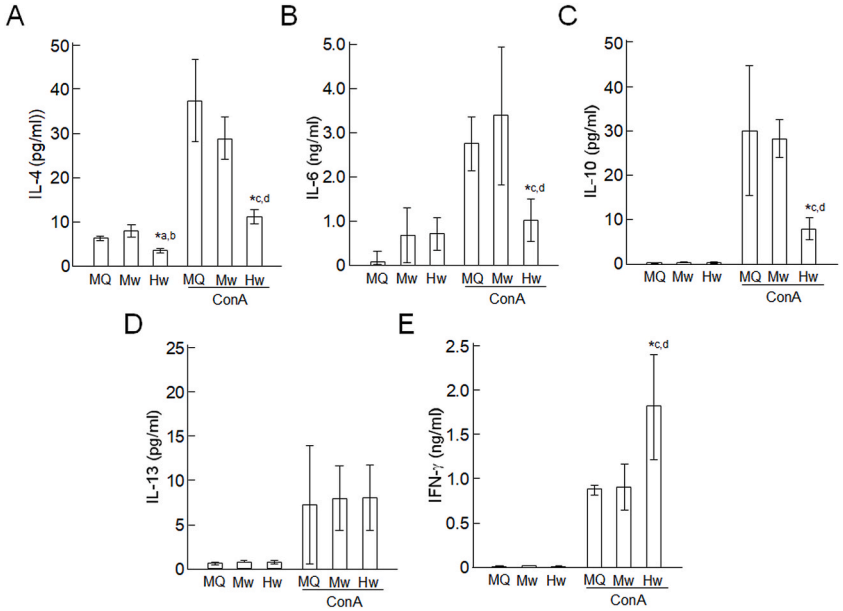

Fig. 6. Cytokine release from cultured PBMCs with and without stimulation with ConA. A. IL-4 production. B. IL-6 production. C. IL-10 production. D. IL-13 production. E. IFN-γ production. Hw: Hita Tenryo water, ConA: concanavalin A. Other notations are as described for Fig. 5. *a and *b indicate $p < 0.01$ vs. non-stimulated MQ and Mw, respectively. *c and *d indicate $p < 0.01$ vs. ConA-stimulated MQ and Mw, respectively.

these effects arise, they may be related to the mechanism by which Hita Tenryo water activates Th2. Specifically, we observed a significant increase in IFN-γ secretion (Fig. 6E), which suppresses CD40 ligand expression in Th2 cells and IgE antibody production, and a decrease in IL-10, which downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules in macrophages. As both IFN-γ and IL-10 suppress IgE production, their reduction may be due to a shift to Th1 dominance, which, according to the Th1/Th2 theory, may tip the balance toward suppression of allergy development. However, given that we observed IL release by whole PBMCs, exactly which cell types become activated and release greater amounts of IL remain to be determined. These results may be explained by the anti-inflammatory effects of Hita Tenryo water, since Hita Tenryo water have intracellular ROS scavenging activity [11,13,14]. Note that the PBMCs used in this study were from healthy subjects, and since we chose ConA stimulation (not Cry j1 stimulation) in this cellular study, it is not known whether the results reflect those of pollinosis patients. Thus, further validation is needed in pollinosis patients. Furthermore, cytokine experiments using primary cultures of mouse spleen cells are needed to determine the relevance of this experimental result in PBMCs to the allergic symptom-relieving phenomenon obtained in animal studies.

3.6. Cytotoxicity

Based on the results of this study and previous reports [13–20], Hita Tenryo water appears to induce beneficial physiological effects on cells. However, whether it also has adverse effects is unclear. Although we did not specifically evaluate toxicity, treatment with Hita Tenryo water did not lead to any significant differences in liver and spleen weight, or red and white blood cell number compared to the other groups after 9 weeks of continuous administration (sFigs. S2 and 3). In addition, we did not observe any effects on the proliferation of PBMCs, RBL-2H3, or TH1080 cells (sFig. S4 and Fig. 5B). Furthermore, Hita Tenryo water has been sold commercially in Japan for about a quarter of a century and has been tested in accordance with the Japanese Waterworks Law [8], and there are no known reports of toxicity to date. Together, this evidence suggests that Hita Tenryo water is unlikely to have any toxic effects.

3.7. Active substance(s) in Hita Tenryo water

Many studies have reported the uniqueness of Hita Tenryo natural water [13–20]. One characteristic of note is its intracellular ROS scavenging activity, which makes it a NRW. We previously showed that Hita Tenryo water suppressed the ROS damage induced by alloxan treatment in HIT-T15 cells and reduced intracellular ROS levels [14]. We further confirmed the intracellular ROS scavenging activity of Hita Tenryo water in HT1080 cells using a previously reported method [12], and found a significant decrease in intracellular ROS following treatment with Hita Tenryo water in a reproducible manner (sFig. S5). Although there are no reports of ROS stimulation altering IL production in Th2 cells, ROS production has been shown to promote IL-6 production in macrophages [24] and IL-4 production in mast cells [25]. In addition, treatment with the antioxidant N-acetylcysteine has been shown to reduce Th2-derived IL-4 production, suggesting that depletion of reduced glutathione promotes Th2-related responses [26,27]. Despite the differences in the cell types tested, these prior findings suggest that the intracellular ROS-scavenging activity of Hita Tenryo water may cause a change in the intracellular glutathione balance, resulting in reduced Th2 cellular response and activation.

The active substances involved in the series of biological effects induced by Hita Tenryo water have yet to be identified. Interestingly, the mineral content of Hita Tenryo water, which is pumped from deep underground in the Hita region of Oita, Japan, is not as noteworthy as that of other Mw [20]. However, natural waters, including groundwater, surface water, tap water, and various brands of Mw, are known to contain a variety of nanoparticles [28,29]. Natural water contains amorphous silicate minerals in the form of nanoparticles which may exhibit antioxidant properties due to the presence of adsorbed hydrogen anions [30,31]. When Hita Tenryo water is thawed and allowed to stand while its minerals re-dissolve after being frozen, a colloidal substance is observed (sFig. S6). This phenomenon is not observed in commercial mineral water, but rather is a characteristic of Hita Tenryo water. Kitagawa et al. reported increased water permeability of aquaporins in Hita Tenryo water, which are deactivated by freeze-drying and lost by boiling [16]. The same processes lead to nanoparticle deactivation, where nanoparticles without protective materials lose their activity due to aggregation [32]. Thus, we speculate that NRW such as Hita Tenryo water contains mineral nanoparticles and mineral hydrides that confer antioxidant activity, which may contribute to the immunological effects of Hita Tenryo water [9]. The identification of the active substance was not verified in this study, but we are currently trying to identify the active substance in a study using dialysis and fractionation focusing on nanoparticles.

4. Conclusions

This is the first study to show that administration of Hita Tenryo water reduces pollinosis allergy symptoms. Continuous administration of Hita Tenryo water significantly reduced cedar pollinosis allergy symptoms such as nose rubbing and sneezing frequency without affecting body weight, food intake, or water intake in mice. It also significantly reduced serum total IgE levels. Degranulation assays showed that treatment with Hita Tenryo water had no effect on degranulation in stimulated RBL-2H3 cells. In PBMC experiments, Hita Tenryo water significantly reduced Th2-mediated cytokine release from stimulated PBMCs with or without ConA stimulation and concentrations of IL-4 in culture medium. Hita Tenryo water also significantly reduced IL-6 and IL-10 concentrations in ConA-treated PBMC cultures and increased IFN-y concentrations in ConA-treated cultures, but had no effect on IL-13. These results suggest that consumption of Hita Tenryo water could potentially suppress the onset of allergies and alleviate and/or suppress allergic symptoms in humans. However, it is necessary to note the limitation that the PBMC experiments were carried out on samples from healthy subjects. Therefore, further trials are needed to determine the effect of Hita Tenryo water consumption on allergic symptoms in

humans.

Ethics declarations

This study was reviewed and approved by Animal Welfare Committee of Kyushu University, with the approval number: A19-238-0 and A21-267-0.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Takeki Hamasaki: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Kiichro Teruya:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Yoshinori Katakura:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Takeki Hamasaki reports financial support was provided by Tenryo-Sui Co., Ltd.

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Appendix A. Supplementary data

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