

Note

Retinoic Acid Ameliorates the Severity of Food Allergy under Allergen Exposure in a Mouse Model with Food Allergy

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Summary Effectiveness of retinoic acid (RA) in treating food allergy is not yet clear. Using an allergic mouse model, we examined the amelioration of the severity of food allergy by daily RA intake with allergen or without. Female Balb/c mice were systemically sensitized to egg white (EW) and alum by intraperitoneal injection. Sensitized mice were provided diets supplemented with 0% (non-treated group), 0.1% EW (allergen group), 0.0017% RA (RA group), or 0.1% EW plus 0.0017% RA (RA+allergen group) with 20% casein for 4 wk. Oral food challenge (OFC) and allergic biomarkers were quantified. The decrease in rectal temperature post-OFC was significantly suppressed in the RA and RA+allergen groups compared to those in the non-treated and allergen groups, respectively. The plasma levels of ovalbumin-specific IgE, IgA and IgG1 at the study endpoint were higher in the allergen and RA+allergen groups than those in the non-treated and RA+allergen groups, respectively. Plasma ovalbumin-specific IgG2a levels at the study endpoint were significantly higher in the RA+allergen group than those in the RA groups. The supernatant concentrations of interleukin-10 and interferon- γ in the cultured spleen lymphocytes were highest in the RA+allergen group compared to those in the other groups. Thus, continuous intake of RA under allergen exposure ameliorated the severity of food allergy in a mouse model with food allergy.

Key Words diet, egg white, lymphocytes, interleukin-10, interferon- γ

In the Japanese guidelines for food allergy 2017 (1), food allergy is defined as “a phenomenon in which adverse reactions are caused through antigen-specific immunological mechanisms after exposure to a given food.” Principle of dietary and nutritional instruction is minimum avoidance of causative foods (1). This treatment is considered as “Although eliminating causative foods is the principal treatment for food allergies, caution should be exercised in not eliminating these foods excessively. Even for foods that demonstrate confirmed positive results in an oral food challenge (OFC), the patient is instructed to take in lower amounts or hypoallergenic forms (heated or cooked)” (1). The intake of oligosaccharides or herbal medicines under allergen exposure can ameliorate the severity of food allergy (2, 3). Petrarca et al. reported that the abundance of eosinophils in bronchoalveolar lavage fluid post-allergen challenge was decreased in the allergen+vitamin D3 treatment group compared to that in the allergen treatment and vitamin D3 treatment groups (4). However, few studies have examined the effects of nutrients under allergen exposure.

Retinoic acid (RA), a metabolite of vitamin A, plays important roles in immunity. Nagata et al. suggested that an endogenous increase in the RA concentration in

the lamina propria of the colon was related to improvements in food allergy (3). Dawicki et al. showed that intraperitoneal administration of allergen-specific mature RA-skewed dendritic cells suppressed anaphylactic responses to OFC in mice with food allergy (5). However, whether food allergy can be ameliorated by daily RA intake remains unclear.

We thus examined here, the amelioration of the severity of food allergy by daily RA intake and investigated the influence of allergen exposure in an allergic mouse model.

Materials and Methods

Animals, diets, and experimental protocol. The care and treatment of the experimental animals were performed according to the guidelines of Mukogawa Women's University (Hyogo, Japan) for the ethical treatment of laboratory animals (No. FSN-01-2017-01-A). Female Balb/c mice ($n=32$; 6-wk old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). All diets and freeze-dried egg white (EW) powder for the study were prepared in-house, and the supplement amounts of EW and all-*trans* RA (FUJIFILM Wako Pure Chemical Corporation, Ltd., Osaka, Japan) were 1.00 and 0.017 g/kg diet, respectively (Table 1).

The EW allergic mouse model was prepared as previously described (6). The experiment outline is illustrated

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Table 1. Composition of experimental diets.¹

Component (g/kg diet)	Non-treated (20% casein diet)	0.1% EW	0.0017% RA	0.1% EW plus 0.0017% RA
Vitamin free milk casein	200	199	200	199
Freeze-dried EW powder ²	0	1	0	1
DL-Methionine	2	2	2	2
Gelatinized cornstarch	453	453	452.983	452.983
Sucrose	200	200	200	200
Corn oil	50	50	50	50
Cellulose	50	50	50	50
Mineral mixture (AIN-93G)	35	35	35	35
Vitamin mixture (AIN-93)	10	10	10	10
All-trans RA	0	0	0.017	0.017

¹ Vitamin-free milk casein, gelatinized cornstarch, cellulose powder, AIN-93G mineral mixture, and AIN-93 vitamin mixture were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). DL-Methionine, corn oil, and all-trans RA were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Sucrose was purchased from Mitsui Sugar Co., Ltd. (Tokyo, Japan). All diets were prepared in-house.

² EW was diluted by 3-fold in water, mixed for 1 h, filtered through gauze, and freeze-dried.

in Fig. S1 (Supplemental Online Material). The mice were maintained under specific-pathogen-free conditions and individually housed at 22°C with 60% humidity and under a 12-h light (08:00–20:00)/dark (20:00–08:00) cycle. All mice were fed a 20% casein diet during preparation of the EW allergic mouse model. All mice were systemically sensitized by two intraperitoneal administrations of 100 µg EW in 100 µL of saline plus 100 µL of aluminum hydroxide gel (Imject™ Alum Adjuvant; Thermo Fisher Scientific, Waltham, MA, USA) at weeks –4 and –2. At week –1, the plasma was collected by submandibular bleeding and assayed for ovalbumin (OVA)-specific IgE to verify the sensitization to EW. Subsequently, all mice were administered 20 mg EW in 200 µL saline orally three times for 1 wk to induce allergic symptoms. A day before the treatment, all mice were fasted from the previous night, and were carried out OFC in the morning.

EW allergic mice ($n=32$) were divided into four groups and provided diets supplemented with 0% (non-treated group), 0.1% EW (allergen group), 0.0017% RA (RA group), or 0.1% EW plus 0.0017% RA (RA + allergen group) in their 20% casein diet for 4 wk. Submandibular blood was collected at the mid-point (2 wk). One mouse each from the non-treated and RA groups and two mice from the allergen group died before the study was complete. OFC was performed at 4 wk. At the study endpoint, the plasma, small intestine and spleen were harvested. Plasma samples were stored in the dark at –40°C.

OFC. OFC was performed as described previously (6). Briefly, after overnight fasting, the mice were orally challenged in the morning with 40 mg EW in 200 µL of saline. Immediately before and 15 min after OFC, the rectal temperature was measured using a thermometer (KN-91-AD-1687-M; Natsume Seisakusyo Co., Ltd., Tokyo, Japan).

Determination of the levels of OVA-specific antibodies

(IgE, IgA, IgG1, and IgG2a) in plasma, feces and small intestine. The plasma concentrations of OVA-specific IgE and IgA were determined by capture enzyme-linked immunosorbent assay (ELISA) (6). A mouse anti-OVA IgE monoclonal antibody E-G5 and mouse anti-OVA IgA monoclonal antibody 2G12E12 (Chondrex, Inc., Redmond, WA, USA) were used as standards to determine the concentration of OVA-specific IgE and IgA. Additionally, the levels of OVA-specific IgG1 and IgG2a were determined by indirect ELISA (6). The dilution ratios of OVA-specific IgG1 and IgG2 were 1 : 10,000 and 1 : 1,000, respectively. Horseradish peroxidase substrates of OVA-specific IgG1 and IgG2 were used 2,2'-azino-bis and *o*-phenylenediamine, respectively and the reaction time of OVA-specific IgG1 and IgG2 were 5 min and 10 min, respectively.

The fecal and small intestine concentrations of OVA-specific IgA and fecal levels of OVA-specific IgA immunocomplex (IgA IC) were determined by indirect and sandwich ELISA, respectively (7). The dilution ratio of OVA-specific IgA IC were 1 : 10. Horseradish peroxidase substrate of OVA-specific IgA IC was used *o*-phenylenediamine and the reaction time was 5 min.

Spleen lymphocyte culture and assessment of cytokine secretion. Spleen lymphocytes were isolated and cultured as previously reported (6). They were isolated from spleen homogenates using Lympholyte-M [Lympholyte(R)-M Cell Separation Media, Cedarlane Laboratories, ON, Canada]. Cells from each mouse were plated at a density of 1.5×10^7 /well in Roswell Park Memorial Institute-1640 complete medium in 24-well plates. Spleen lymphocytes were stimulated with 2 mg EW per well and incubated at 37°C with 5% CO₂. The culture supernatants were collected after 48 h and then stored at –40°C until analysis. The concentrations of interleukin-4 (IL-4), IL-10, and interferon-γ (IFN-γ) were determined according to the manufacturer's instructions (BioLegend, San Diego, CA, USA).

Table 2. Nutritional effect by diets supplemented with EW and/or RA.¹

	Non-treated	Allergen	RA	RA+allergen	p value		
					Allergen	RA	Interaction
Body weight at day 1 (g)	22.8±1.6	23.3±1.0	24.1±1.9	23.6±1.1	N.S.	N.S.	N.S.
Body weight at day 28 (g)	23.1±2.4	23.8±1.3	22.1±1.9	23.4±1.8	N.S.	N.S.	N.S.
Body weight change during 4 wk (g)	0.3±1.4	0.5±1.8	-2.0±1.9*	-0.2±1.8	N.S.	p<0.05	N.S.
Daily food intake (g)	2.8±0.2	2.8±0.2	2.7±0.4	2.9±0.2	N.S.	N.S.	N.S.

¹ Values are presented as the mean±standard error (n=6–8). Significant differences were assessed using a two-way ANOVA followed by Bonferroni's multiple comparison test. When two-way ANOVA indicated the presence of an allergen-RA interaction, one-way ANOVA was conducted followed by Tukey's multiple-comparison test among all columns and superscript letters without a common letter indicate significant differences (p<0.05). When the interaction was not significant, different superscript asterisks (* (non-treated vs. RA or allergen vs. RA+allergen) in the RA or RA+allergen groups indicate significant differences (p<0.05). N.S.: not significant.

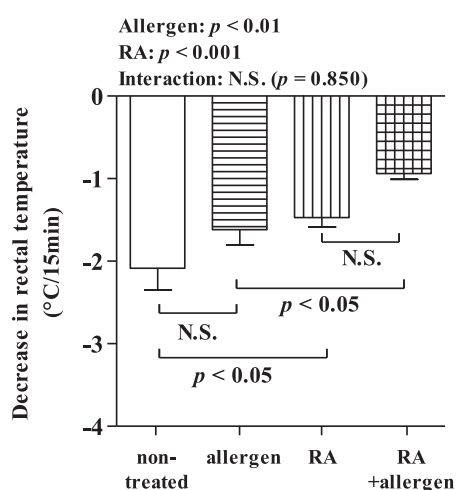


Fig. 1. Decrease in rectal temperature post-OFC after 4-wk the treatment period. Value are shown as the mean±standard error (n=6–8). Significant differences were assessed using a two-way ANOVA followed by Bonferroni's multiple comparison test. p values less than 0.05 were considered as significant. N.S.: not significant.

Statistical analysis. Significant differences were assessed using a two-way ANOVA followed by Bonferroni's multiple comparison test. When two-way ANOVA indicated the presence of an allergen-RA interaction, one-way ANOVA was conducted followed by Tukey's multiple-comparison test among all columns. p values less than 0.05 were considered as significant. All analyses were conducted using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

Results

Nutritional effect of diets supplemented with EW and/or RA

Body weights at days 1 and 28 were comparable in the all groups (Table 2). Body weight change during the treatment term significantly decreased in the RA group than in the non-treated group (Table 2). Daily food

intakes were comparable in all the groups (Table 2).

Improvement of food allergy by RA intake with or without allergen exposure

In a two-way ANOVA analysis of OFC at 4 wk, the each factors of allergen and RA significantly differed although the interaction was not indicated (Fig. 1). The decrease in rectal temperature post-OFC was significantly suppressed in RA and RA+allergen groups than the non-treated and allergen groups, respectively (Fig. 1). Moreover, the decreased temperatures in rectum were lower in the RA+allergen group than those in the RA group although not significant (Fig. 1, 95% confidence interval of difference: -0.02 to 1.09).

Determination of OVA-specific antibodies in plasma, feces and small intestine

At 2 wk, the plasma levels of OVA-specific IgE, IgG1 and IgG2a were comparable among all groups (Table 3). The plasma OVA-specific IgA levels were significantly higher in the allergen group than that in the non-treated group (Table 3).

In a two-way ANOVA analysis of OVA-specific IgE concentrations at the study end point, the significant interaction between allergen and RA was indicated (Table 3). The plasma levels of OVA-specific IgE, IgA and IgG1 were significantly higher in the allergen and RA+allergen groups than those in the non-treated and RA groups, respectively (Table 3). The plasma levels of OVA-specific IgG2a were not significantly difference among the non-treated and allergen groups (Table 3). However, the plasma levels of OVA-specific IgG2a in the RA+allergen group were significantly higher than those in the RA group (Table 3).

In a two-way ANOVA analysis of OVA-specific IgA and IgA IC in feces, the significant interactions between allergen and RA were indicated (Table 3). The fecal concentrations of OVA-specific IgA were higher in the RA group than those in the other groups (Table 3). The fecal levels of OVA-specific IgA IC were higher in the RA+allergen group than those in the other groups (Table 3). The OVA-specific IgA concentrations in the small intestine were significantly higher in the allergen and RA+

Table 3. Levels of OVA-specific antibodies in plasma, feces and small intestine.¹

	Non-treated	Allergen	RA	RA + allergen	p value		
					Allergen	RA	Interaction
Plasma at 2 wk							
OVA-specific IgE (μg/mL)	15.0±1.8	22.3±4.5	14.7±1.9	18.6±1.6	p<0.05	N.S.	N.S.
OVA-specific IgA (μg/mL)	15.3±2.7 ^b	43.7±12.0 ^a	16.6±2.8	33.3±4.0	p<0.01	N.S.	N.S.
OVA-specific IgG1 (Abs 405 nm)	0.375±0.017	0.355±0.027	0.376±0.011	0.381±0.019	N.S.	N.S.	N.S.
OVA-specific IgG2a (Abs 492 nm)	0.302±0.047	0.191±0.023	0.414±0.089	0.625±0.197	N.S.	p<0.05	N.S.
Plasma at endpoint							
OVA-specific IgE (μg/mL)	25.6±2.8 ^b	45.8±3.3 ^a	29.2±2.2 ^b	39.1±1.4 ^a	p<0.001	N.S.	p<0.05
OVA-specific IgA (μg/mL)	17.7±2.7 ^b	73.5±19.8 ^a	21.0±1.3 ^B	86.4±13.7 ^A	p<0.001	N.S.	N.S.
OVA-specific IgG1 (Abs 405 nm)	0.244±0.015 ^b	0.357±0.027 ^a	0.281±0.011 ^B	0.390±0.011 ^A	p<0.001	p<0.05	N.S.
OVA-specific IgG2a (Abs 492 nm)	0.333±0.043	0.585±0.110	0.380±0.068 ^B	0.772±0.116 ^A	N.S.	p<0.01	N.S.
Freeze-dried feces at 4 wk							
OVA-specific IgA (μg/g of feces)	5.61±1.30 ^b	3.39±0.94 ^b	14.8±2.0 ^a	4.34±0.90 ^b	p<0.001	p<0.001	p<0.01
OVA-specific IgA IC (Abs 492 nm)	0.020±0.005 ^c	1.72±0.20 ^b	0.007±0.005 ^c	2.11±0.06 ^a	p<0.001	N.S.	p<0.05
Small intestine at endpoint							
OVA-specific IgA (μg/g of tissue)	24.1±2.1 ^b	110±21 ^a	43.4±7.2 ^B	120±6 ^A	p<0.001	N.S.	N.S.

¹ Values are presented as the mean $\pm$ standard error ($n=6-8$). Significant differences were assessed using a two-way ANOVA followed by Bonferroni's multiple comparison test. When two-way ANOVA indicated the presence of an allergen-RA interaction, one-way ANOVA was conducted followed by Tukey's multiple-comparison test among all columns and superscript letters (a, b, c) without a common letter indicate significant differences ($p<0.05$). When the interaction was not significant, different superscript letters (a, b (non-treated vs. allergen) and A, B (RA vs. RA + allergen)) indicate significant differences ($p<0.05$). N.S.: not significant.

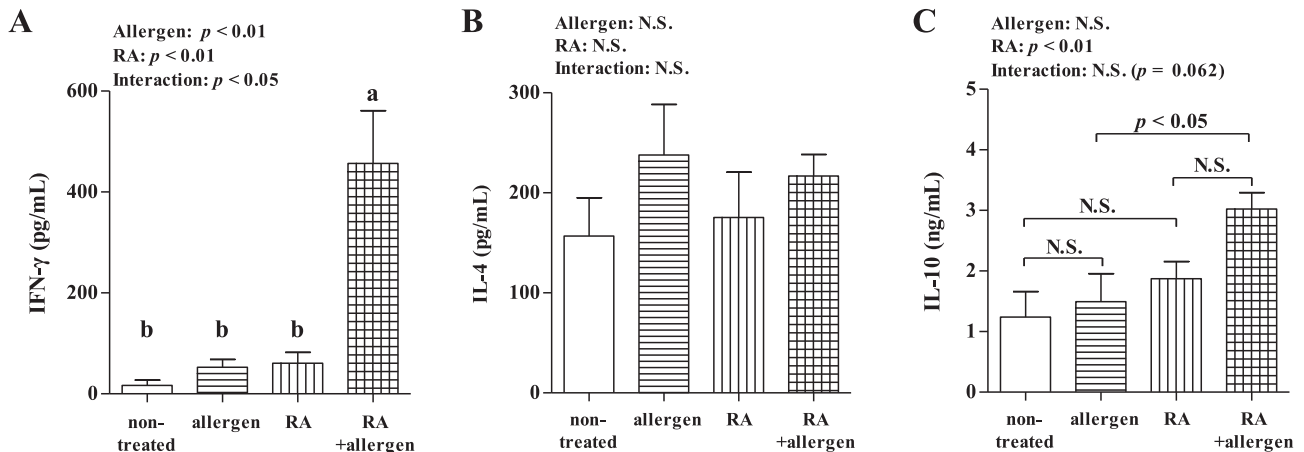


Fig. 2. Concentrations of IFN- γ (A), IL-4 (B), and IL-10 (C) following in vitro stimulation of spleen lymphocytes with EW. Values are shown as the mean $\pm$ standard error ($n=6-8$). Significant differences were assessed using a two-way ANOVA followed by Bonferroni's multiple comparison test. When two-way ANOVA indicated the presence of an allergen-RA interaction, one-way ANOVA was conducted followed by Tukey's multiple-comparison test among all columns. p values less than 0.05 were considered as significant. N.S.: not significant.

allergen groups than those in the non-treated and RA groups, respectively (Table 3).

Assessment of cytokine secretions with in-vitro EW-stimulation of spleen lymphocytes

The concentrations of IFN- γ showed the significant interaction between allergen and RA, and were significantly higher in the RA+allergen group than in the other groups (Fig. 2A). IL-4 concentrations were comparable among all groups (Fig. 2B). In a two-way ANOVA analysis of IL-10, the RA factors significantly differed although the interaction was not indicated (Fig. 2C). IL-10 concentrations in the RA+allergen group were significantly higher than those in the allergen group (Fig. 2C). Moreover, the IL-10 concentrations in the RA+allergen group were higher than those in the RA group although not significant (Fig. 2C, 95% confidence interval of difference: -0.02 to 2.32). The relationship between the decrease in rectal temperature post-OFC and IFN- γ and IL-10 concentrations were significantly correlations (Spearman $r=0.643$ [$p<0.001$] and 0.499 [$p=0.007$], respectively) (Fig. S2A and S2B, Supplemental Online Material).

Discussion

RA effects a wide range of physiological processes. However, it is believed that the most of adverse symptoms by excessive intake of vitamin A is caused to the increase of RA. The AIN-76 and -93G diet was formulated to contain 4 IU retinol activity equivalents (RAE)/g of meal and the commercial diet (Mouse diet (Cat 5015); PMI Nutrition) contain 18 IU RAE/g of meal. Goverse et al. reported that C57BL/6 mice orally administrated RA (100 $\mu\text{g/g}$ of dry food) for a week exhibited an increased percentage of CD4 $^{+}$ Foxp3 $^{+}$ cells within the mesenteric lymph node (8). Therefore, we determined that the supplemented amounts of RA were 17 $\mu\text{g/g}$ of diet (16 IU RAE/g of diet), which the total dietary amount of vitamin A and RA in diets contained

approximately 20 IU.

In current experiment, the body weight change in the RA group significantly decreased more than that in the non-treated group. The intake amount of RA in mice fed the RA or RA+allergen diet was approximately 2 $\mu\text{g/g}$ of body weight in mouse (17 $\mu\text{g/g}$ of diet $\times$ 2.8 g diet/d/mouse $\times$ 23 g/mouse). Yamaguchi reported that oral administration of RA (5 $\mu\text{g/g}$ of body weight in mouse) suppressed body weight gain by high-fat diet in mice and increased energy expenditure and fat combustion (<https://kaken.nii.ac.jp/ja/file/KAKENHI-PROJECT-23700919/23700919seika.pdf>). Moreover, Hernandez et al. showed that RA stimulates uncoupling protein-1 mRNA in mouse adipocytes (9). We considered that the decrease in body weight in the RA group was related to an increase in energy expenditure.

Type I allergic responses are due to the cross-linked structure between IgE on Fc ϵ RI in mast and basophilic cells and the allergen. These cells then release various mediators, which induce decreases in blood pressure and body temperature due to vascular relaxation. Thus, in a mouse model with food allergy, a decreased rectal temperature post-OFC can quantitatively evaluate the severity of food allergy. We present three hypotheses for the suppression of decrease in rectal temperature post-OFC by RA and allergen intake. First, RA inhibits the type I allergic reaction. Second, RA reduces reactivity to the allergen. Third, RA induces anti-allergic mediators.

First, we evaluated whether RA inhibits the type I allergic reaction. In in vitro experiments using the rat basophilic leukemia (RBL-2H3) cells, RA did not inhibit degranulation by antigen stimulation (Supplemental Online Material, Fig. S3A). Therefore, long-term intake of RA and allergen was considered to decrease the severity of food allergy but not through inhibition of the type I allergic reaction via IgE-Fc ϵ RI interactions.

The production of allergen-specific antibodies from B-cell is promoted by allergen stimulation. We predicted

that reactivity to allergen could be evaluated by measuring the production of allergen-specific antibodies. The plasma levels of OVA-specific IgE, IgG1, and IgA were comparable among the allergen and RA+allergen groups. Moreover, OVA-specific IgA levels in the homogenate of the small intestine did not differ between the allergen and RA+allergen groups. However, the decrease in rectal temperature post-OFC was significantly suppressed in the RA+allergen group compared to that in the allergen group. These results suggested that the consecutive intake of RA and allergen does not reduce the reactivity to allergen.

IFN- γ and IL-10 are anti-inflammatory cytokines involved in asthma and allergy (10, 11). Moreover, IFN- γ induces class-switching to IgG2a production in B cell by allergen-stimulation (11). Petrarca et al. reported that allergen-specific IgG2a levels in the allergen+vitamin D3 treatment group were higher than those in the allergen treatment and vitamin D3 treatment groups (4). In this study, the plasma OVA-specific IgG2a levels and IFN- γ secretion in the RA+allergen group was higher than those in the other groups. IL-10 is involved in inducing specific anergy (10). Bakdash et al. reported that RA induces IL-10-producing regulatory T cells (12). In this experiment, IL-10 secretion in spleen lymphocytes was higher in the RA+allergen group than those in the other groups. Pierkes et al. reported that after wasp venom immunotherapy, IFN- γ and IL-10 partially suppressed the releases of histamine and sulfidoleukotriene through allergen-stimulation (13). Furthermore, the severity of food allergy and IFN- γ and IL-10 secretions showed significantly correlations. Therefore, the IFN- γ and IL-10 production by allergen-stimulation may have influenced the suppression of the decrease in rectal temperature post-OFC.

In conclusion, continuous intake of RA under allergen exposure ameliorated the severity of food allergy in a mouse model and may promote the induction of anti-inflammatory cytokines such as IFN- γ and IL-10.

Authorship

AM and KT designed the study. AM, RK, and MM analyzed the samples. AM and KT drafted the manuscript. All authors approved the final manuscript.

Disclosure of state of COI

The authors have no conflict of interest to declare.

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Supporting information

Supplemental online material is available on J-STAGE.

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