

# Therapeutic potential of $\alpha$ -lipoic acid derivative, sodium zinc histidine dithiooctanamide, in a mouse model of allergic rhinitis

Toshiaki Nakano, PhD<sup>1,2</sup>, Li-Wen Hsu, PhD<sup>1</sup>, Chia-Yun Lai, MVS<sup>1,3</sup>, Yuki Takaoka, PhD<sup>1</sup>, Masafumi Inomata, MD, PhD<sup>4</sup>, Seigo Kitano, MD, PhD<sup>5</sup>, Chao-Long Chen, MD, PhD(Hon)<sup>1</sup> and Shigeru Goto, MD, PhD<sup>1,6,7</sup>

**Background:** Oxidative stress is involved in various diseases, including allergies. Several studies have pointed to the preventive and therapeutic potential of antioxidants in allergic disorders. However, little is known about the immunomodulatory effects of antioxidants in type I hypersensitivity. In this study we aimed to explore the impact of a water-soluble antioxidant and  $\alpha$ -lipoic acid derivative, sodium zinc histidine dithiooctanamide (DHL-HisZn), on mast-cell- and T-cell-mediated allergic and immune responses both in vitro and in vivo.

**Methods:** The therapeutic impact of DHL-HisZn on mast-cell-mediated type I hypersensitivity was evaluated by a mast-cell degranulation assay using bone marrow-derived mast cells and by a mouse model of ovalbumin (OVA)-induced allergic rhinitis. The effect of DHL-HisZn on the proportion of regulatory T cells (Tregs) was evaluated using flow cytometry.

**Results:** During the course of OVA-induced allergic rhinitis in mice, serum nitrate was elevated, suggesting the involvement of oxidative stress in allergic responses. DHL-HisZn not only suppressed mast-cell degranulation but also ameliorated OVA-induced nasal hypersensitivity,

with significant suppression of serum nitrate. DHL-HisZn treatment significantly suppressed OVA-specific immunoglobulin E (IgE) but enhanced OVA-specific IgG2a in OVA-sensitized and nasal-challenged mice. Furthermore, DHL-HisZn treatment suppressed interleukin-17 production in OVA-stimulated splenocytes. Finally, we demonstrated the induction of Tregs by DHL-HisZn in concanavalin A blasts.

**Conclusions:** These findings suggest that DHL-HisZn may regulate mast-cell-, T-helper 2 (Th2)-, and Th17-mediated allergic and immune responses by induction of Tregs. © 2017 ARS-AAOA, LLC.

**Key Words:** allergic rhinitis; hypersensitivity; type I;  $\alpha$ -lipoic acid; therapeutics

## How to Cite this Article:

Nakano T, Hsu L-W, Lai C-Y, et al. Therapeutic potential of  $\alpha$ -lipoic acid derivative, sodium zinc histidine dithiooctanamide, in a mouse model of allergic rhinitis. *Int Forum Allergy Rhinol.* 2017;XX:XXX-XXX.

<sup>1</sup>Graduate Institute of Clinical Medical Sciences, Chang Gung University College of Medicine, Kaohsiung, Taiwan; <sup>2</sup>Liver Transplantation Center and Department of Surgery, Division of Transplant Immunology, Institute for Translational Research in Biomedicine, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan; <sup>3</sup>Department of Veterinary Medicine, College of Veterinary Medicine, National Pingtung University of Science and Technology, Neipu, Pingtung, Taiwan; <sup>4</sup>Department of Gastroenterological and Pediatric Surgery, Faculty of Medicine, Oita University, Yufu, Oita, Japan; <sup>5</sup>Oita University, Oita, Japan; <sup>6</sup>Nobeoka Medical Check Center, Fukuoka Institution of Occupational Health, Nobeoka, Miyazaki, Japan; <sup>7</sup>Basic Medical Science of Nursing, Faculty of Nursing, Department of Nursing, Josai International University, Togane, Chiba, Japan

Correspondence to: Toshiaki Nakano, Ph.D. and Shigeru Goto, M.D., Ph.D. Graduate Institute of Clinical Medical Sciences, Chang Gung University College of Medicine, Liver Transplantation Center, Kaohsiung Chang Gung

Oxidative stress is involved in several diseases, including neurologic disorders,<sup>1</sup> autoimmune diseases,<sup>2</sup> and cancer,<sup>3</sup> as well as aging.<sup>4</sup> Involvement of oxidative stress in allergic disorders, such as asthma, rhinitis, and atopic dermatitis, has also been reported.<sup>5–7</sup> Oxidative stress is defined as an imbalance of reactive oxygen species (ROS) generation and natural antioxidant defense.<sup>8</sup> Excessive

Memorial Hospital, 123 Ta-Pei Road, Niao-Sung, Kaohsiung 833, Taiwan; e-mail: nakano33@mail.cgu.edu.tw; pochigoto0224@gmail.com

Funding sources for the study: Ministry of Science and Technology, MOST104-2320-B-182-030 and MOST105-2320-B-182-037 to T.N.; Chang Gung Medical Foundation, CMRPD8D1381 and CMRPD8F0911 to T.N. Potential conflict of interest: None disclosed. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Received: 29 March 2017; Revised: 26 June 2017; Accepted: 10 August 2017  
DOI: 10.1002/alr.22008  
View this article online at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).

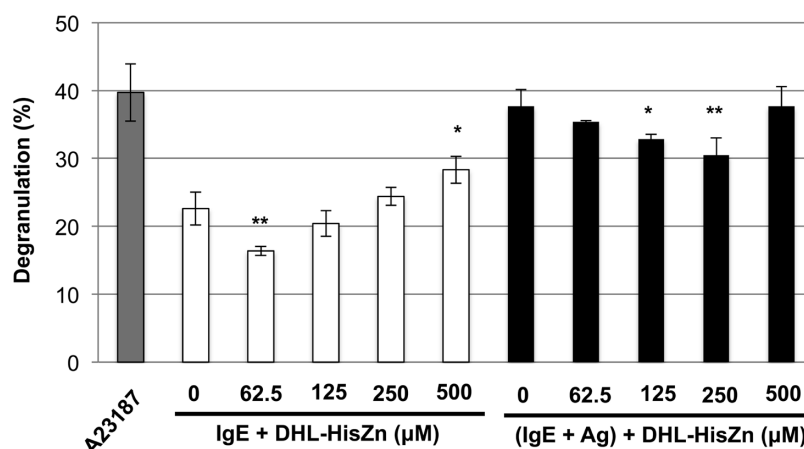


**FIGURE 1.** Impact of DHL-HisZn on mast-cell degranulation. The degranulation was evaluated by  $\beta$ -hexosaminidase release rate. Values are presented as the mean  $\pm$  standard deviation from 3 independent experiments. A23187 (10  $\mu$ mol/L) was used as a positive control for degranulation. White bar: anti-DNP IgE treatment only; black bar: anti-DNP IgE + antigen (Ag: DNP-BSA) treatment. \* $p < 0.05$  and \*\* $p < 0.01$  versus (IgE + Ag: 0  $\mu$ g/ml of DHL-HisZn) or (IgE + Ag: 0  $\mu$ g/ml of DHL-HisZn) (Student t-test). BSA = bovine serum albumin; DHL-HisZn = sodium zinc histidine dithiooctanamide; DNP = dinitrophenol; IgE = immunoglobulin E.

ROS production leads to exhaustion of the antioxidant system, resulting in the elevation of nitric oxide (NO), nitrite/nitrate, and malondialdehyde, which are known as oxidative stress markers.<sup>9–11</sup>

To scavenge ROS and adjust the balance of ROS and natural antioxidant defense, various antioxidants, such as ascorbic acid (vitamin C),  $\alpha$ -tocopherol (vitamin E), and ubiquinol (coenzyme Q), are commercially available. The preventive and therapeutic effects of these antioxidants in allergic disorders have been reported,<sup>12–16</sup> but little is known about the immunomodulatory effect of antioxidants in type I hypersensitivity. One antioxidant,  $\alpha$ -lipoic acid, is a mitochondrial fatty acid involved in energy metabolism, and many studies have demonstrated the therapeutic potential of  $\alpha$ -lipoic acid in various diseases, including diabetes,<sup>17</sup> ischemia/reperfusion injury,<sup>18</sup> fibrosis,<sup>19</sup> and cancer.<sup>20</sup> Some reports also demonstrated the therapeutic impact of  $\alpha$ -lipoic acid on compound 48/80-induced anaphylaxis-like reaction<sup>21</sup> and 2,4-dinitrofluorobenzene (DNFB)-induced atopic dermatitis.<sup>22</sup> However, the effects of  $\alpha$ -lipoic acid on mast-cell- and T-cell-mediated allergic and immune responses, both in vitro and in vivo, are uncertain.

$\alpha$ -Lipoic acid possesses low water solubility, which may affect its utility in cell culture systems. Dihydrolipoyl histidinate zinc complex (DHL-HisZn) is a novel  $\alpha$ -lipoic acid derivative composed of  $\alpha$ -lipoic acid, histidine, and zinc.<sup>23</sup> DHL-HisZn is water-soluble and stable in aqueous solution. Moreover, histidine has been shown to scavenge both singlet oxygen ( $^1\text{O}_2$ ) and hydroxyl radicals.<sup>24,25</sup> In previous studies, we demonstrated the therapeutic impact of DHL-HisZn on cardiac dysfunction,<sup>26</sup> renal ischemia/reperfusion injury,<sup>23</sup> chemotherapy-induced alopecia,<sup>27</sup> and colon cancer growth.<sup>28</sup> In addition, a recent study demonstrated that DHL-HisZn may be useful in the prevention of insulin resistance and diabetes related to

metabolic disorders.<sup>29</sup> In this study, we explored the therapeutic potential of DHL-HisZn and its mechanism in a mouse model of ovalbumin (OVA)-induced allergic rhinitis.

## Materials and methods

### Ethics statement

Our experimental design was reviewed and approved by the institutional animal care and use committee of Kaohsiung Chang Gung Memorial Hospital (Approval No. 2014101601). The committee recognized that the proposed animal experiments were in accordance with the Animal Protection Law of the Council of Agriculture, Executive Yuan, R.O.C., and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, USA).

### Animals

Male Lewis rats and female BALB/c mice were obtained at 5 weeks of age from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). All animals were maintained under specific pathogen-free animal conditions with water and a commercial diet ad libitum. All serum samples were stored at  $-80^\circ\text{C}$  until analysis.

### Mast-cell degranulation

Lewis bone marrow-derived mast cells (BMMCs,  $1 \times 10^6$  cells/mL) were prepared and the effect of DHL-HisZn on mast-cell degranulation was evaluated by previously described protocols.<sup>30,31</sup> Briefly, BMMCs were sensitized with anti-dinitrophenol (anti-DNP) immunoglobulin E (0.5  $\mu$ g/mL; Sigma Co., St. Louis, MO, USA) for 18 hours at  $37^\circ\text{C}$ . They were then washed with Tyrode's buffer (10 mmol/L HEPES, 130 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L  $\text{CaCl}_2$ , 1 mmol/L  $\text{MgCl}_2$ , 5.6 mmol/L glucose,

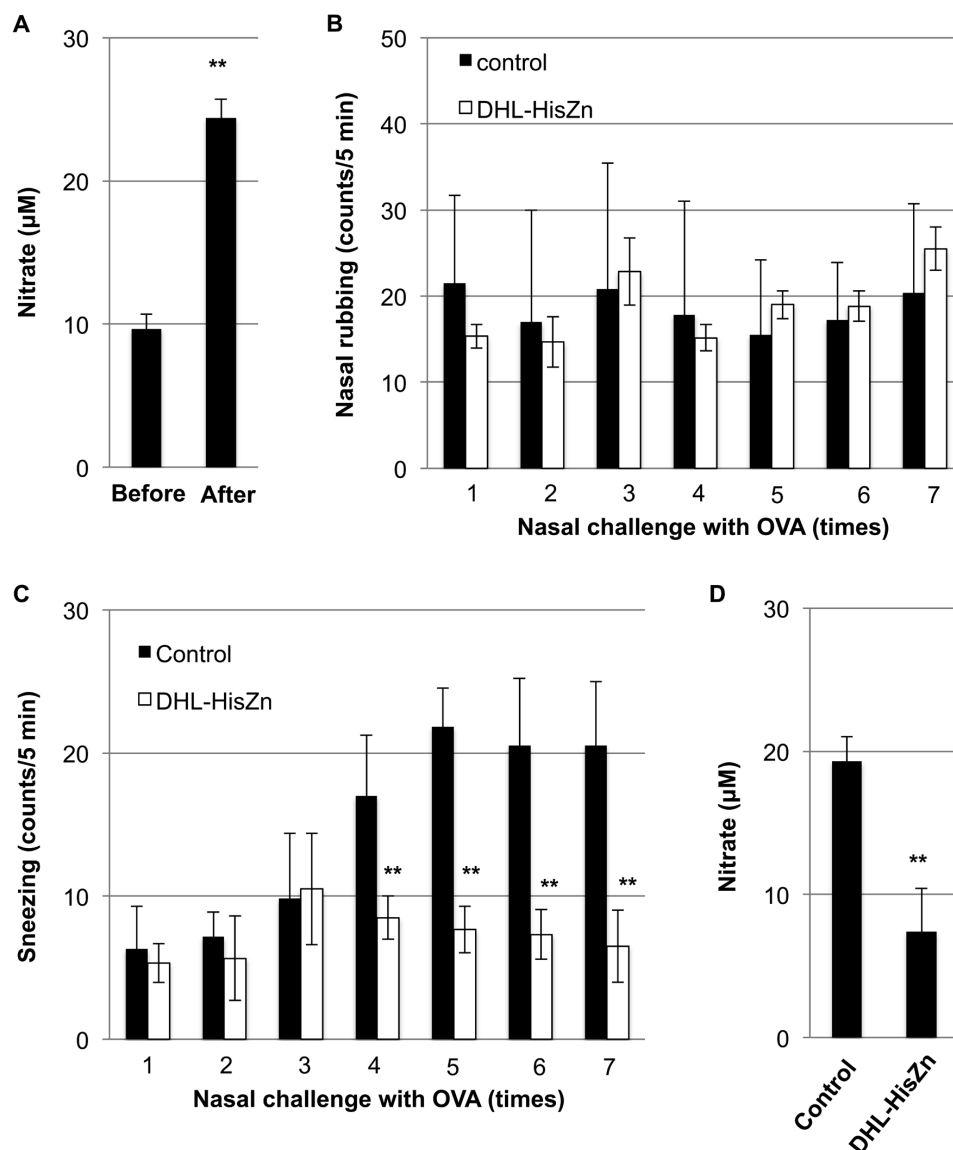


**FIGURE 2.** Impact of oxidative stress on type I hypersensitivity and its amelioration by DHL-HisZn. (A) Serum nitrate levels before and after OVA/alum sensitization. Values are presented as mean  $\pm$  standard deviation from 6 individuals. \*\* $p < 0.01$  (Student t-test). (B, C) Prevention of allergic rhinitis-like symptoms by DHL-HisZn. Mice were immunized with OVA/alum ( $n = 12$ ) and divided into 2 groups for intraperitoneal administration of control treatment (normal saline,  $n = 6$ ) or DHL-HisZn (10 mg/kg,  $n = 6$ ) before daily OVA nasal challenge. To evaluate allergic rhinitis-like symptoms, the instances of nasal rubbing (B) and sneezing (C) were counted for 5 minutes immediately after OVA nasal challenge. \*\* $p < 0.01$  (Student t-test). (D) Serum nitrate levels in control and DHL-HisZn treatment groups. Values are presented as mean  $\pm$  standard deviation from 6 individuals. DHL-HisZn = sodium zinc histidine dithiooctanamide; OVA = ovalbumin.

0.1% bovine serum albumin [BSA], at pH 7.4), and  $2.5 \times 10^6$  cells/mL of BMDCs were incubated in the presence of DHL-HisZn (62.5, 125, 250, and 500  $\mu\text{mol/L}$ ) or control vehicle (normal saline) for 1 hour at  $37^\circ\text{C}$ . To induce degranulation, 0.5  $\mu\text{g/mL}$  of DNP-BSA (Invitrogen, Carlsbad, CA, USA) was added. The supernatants were collected, and 0.5% Triton X-100 solution in Tyrode's buffer was added to quantify the enzymatic activity remaining in the cells. To quantify  $\beta$ -hexosaminidase activity, 20  $\mu\text{L}$  of substrate solution (1.3 mg/mL *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosaminide in 0.04 mol/L sodium citrate at pH 4.5) was added to each sample (30  $\mu\text{L}$ ) and incubated for 1 hour at  $37^\circ\text{C}$  under gentle rotation. To stop the

enzymatic reaction, 150  $\mu\text{L}$  of stop solution (0.2 mol/L glycine at pH 10.0) was added, and absorbance at 405 nm was determined using a plate reader (Victor X4 Multilabel Plate Reader, Perkin Elmer, Waltham, MA, USA). The percentage of degranulation was calculated using the following formula: Degranulation rate (%) =  $100 \times \text{absorbance of supernatant} / (\text{absorbance of supernatant} + \text{absorbance of cell lysate})$ .

#### Ovalbumin-induced allergic rhinitis model

Immunization and nasal challenge with ovalbumin (OVA; Sigma) were performed by previously described protocols,

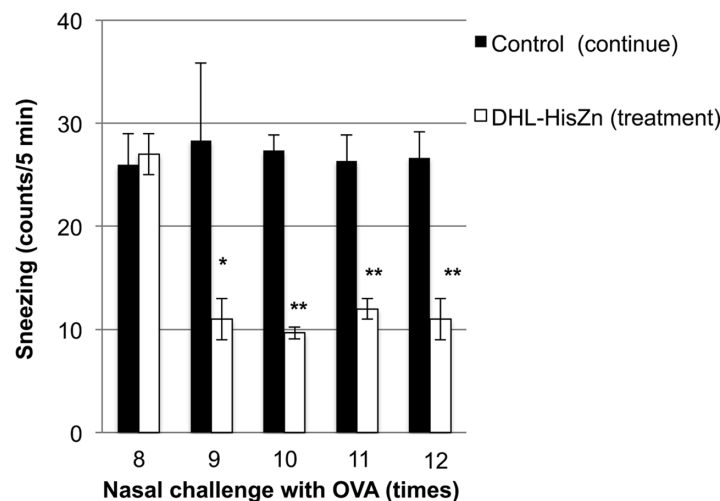


**FIGURE 3.** Therapeutic potential of DHL-HisZn after establishment of OVA-induced allergic rhinitis. The mice with allergic rhinitis after 7 injections of OVA ( $n = 6$ ) were divided into 2 groups: a control group (continue,  $n = 3$ ) and a DHL-HisZn treatment group (control shift to DHL-HisZn, 10 mg/kg,  $n = 3$ ). \* $p < 0.05$  and \*\* $p < 0.01$ , respectively (Student t-test). DHL-HisZn = sodium zinc histidine dithiooctanamide; OVA = ovalbumin.

with some modification. Briefly, BALB/c mice ( $n = 18$ ) were immunized twice intraperitoneally with a precipitate (100  $\mu$ L/mouse) of alum (2 mg; Thermo Fisher Scientific, Rockford, IL, USA) and OVA (20  $\mu$ g), with a 2-week interval. To evaluate the preventive potential of DHL-HisZn, 10 mg/kg of DHL-HisZn ( $n = 6$ ) or normal saline ( $n = 6$ ) was intraperitoneally administered before the daily nasal challenge with OVA (50 mg/mL, 10  $\mu$ L/mouse). To evaluate the therapeutic potential of DHL-HisZn, we prepared allergic rhinitis mice by seventh nasal challenge with OVA, and 10 mg/kg of DHL-HisZn ( $n = 3$ ) or normal saline ( $n = 3$ ) was intraperitoneally administered before the continual daily nasal challenge with OVA (50 mg/mL, 10  $\mu$ L/mouse). To evaluate the allergic rhinitis-like symptoms, sneezing and nasal rubbing were counted for 5 minutes after OVA nasal challenge.

#### Oxidative stress markers

Serum nitrate levels were measured using an oxide analyzer (Sievers Nitric Oxide Analyzer NOA 280i, GE Analytical Instruments, Boulder, CO, USA) according to the manufacturer's instructions.

#### OVA-specific immunoglobulins

The serum levels of OVA-specific IgG1, IgG2a, and IgE were determined by enzyme-linked immunoassay (ELISA), as described previously.<sup>31</sup> Briefly, OVA solution (50 mg/mL, in phosphate-buffered saline [PBS]) was used to coat 96-well microplates (Nalgene Nunc International, Roskilde, Denmark) and incubated overnight at 4°C. The plate was then blocked with 1% (w/v) BSA/PBS for 2 hours at 37°C, and serum samples (IgG1: 10,000 $\times$  dilution; IgG2a: 1000 $\times$  dilution; IgE: 10 $\times$  dilution with blocking buffer) were added to each well and incubated for 2 hours at 37°C. Next, biotin-conjugated antimouse IgG1, IgG2a, or IgE (250 $\times$  dilution; BD Biosciences, San Jose,

CA, USA) was added and incubated at 37°C for 1.5 hours. Streptavidin-horseradish peroxidase (200 $\times$  dilution; R&D Systems, Minneapolis, MN, USA) was then added and incubated at 37°C for 1 hour, followed by the addition of substrate solution (1-Step Ultra TMB, Thermo Fisher, Waltham, MA, USA). The absorbance at 450 nm was then measured using a plate reader (Victor X4 Multilabel Plate Reader, PerkinElmer).

#### Cytokine production in splenocytes

Splenocytes ( $1 \times 10^5$  cells) from mice after final OVA nasal challenge were cultured for 72 hours at 37°C in the presence of OVA (200  $\mu$ g/mL) in RPMI-1640 medium (Invitrogen) supplemented with penicillin-streptomycin (Invitrogen) and 10% FBS (Invitrogen). The supernatants were then collected, and interferon (IFN)- $\alpha$ , interleukin (IL)-4, IL-5, IL-10, IL-13, and IL-17 levels were measured using ELISA development kits (DuoSet, R&D Systems) according to the manufacturer's protocols.

#### Characterization of regulatory T cells

Splenocytes ( $1 \times 10^6$  cells) from naive animals were cultured for 72 hours at 37°C in the presence of DHL-HisZn (50, 100, 200 and 250  $\mu$ mol/L) and 2.5  $\mu$ g/mL concanavalin A (Con A). Fc receptors were blocked with unconjugated anti-CD32 (Fc $\gamma$  II receptor; BD Pharmingen, San Diego, CA, USA) for 30 minutes. Cells were incubated with antigen-presenting-cell (APC)-conjugated anti-CD4 antibody (eBioscience, Thermo Fisher, San Diego, CA, USA) and phycoerythrin (PE)-conjugated anti-CD25 antibody (eBioscience) for 30 minutes at 4°C. Intracellular staining for Foxp3 was performed using a BD Cytofix/Cytoperm fixation/permeabilization solution kit with BD GolgiPlug (BD Bioscience) and Alexa Fluor 488-conjugated anti-Foxp3 antibody (eBioscience, Thermo Fisher, San Diego, CA, USA). The proportions

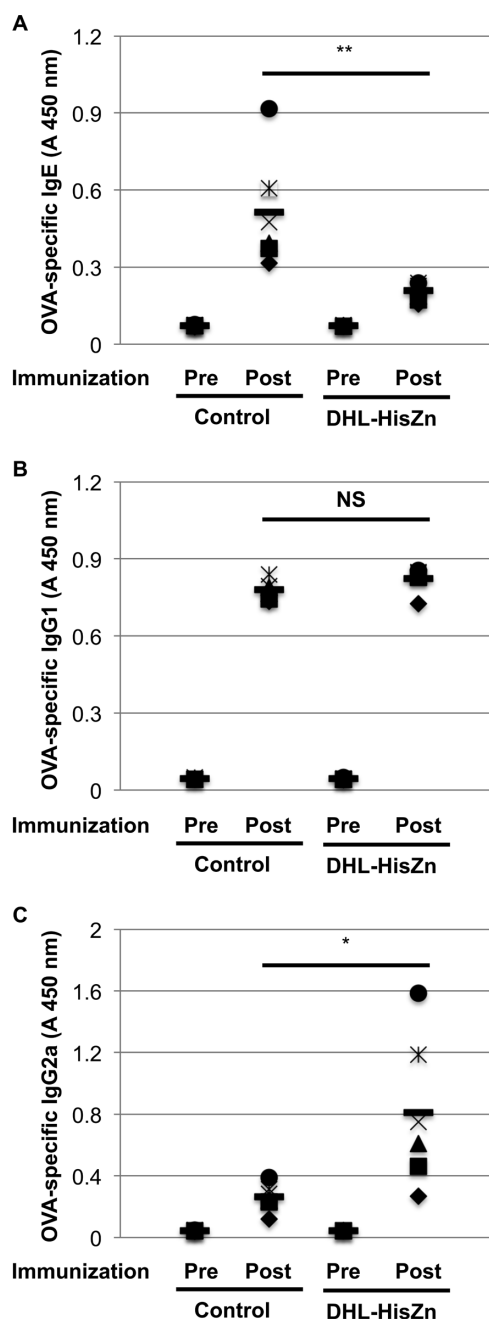


**FIGURE 4.** Impact of DHL-HisZn on OVA-specific antibodies. Peripheral blood was obtained from control ( $n = 6$ ) and DHL-HisZn-treated groups ( $n = 6$ ) before OVA immunization and after final OVA nasal challenge. Serum OVA-specific IgE (A), IgG1 (B), and IgG2a (C) titers were determined by ELISA. Each symbol indicates an individual mouse, and bars show the mean values. \*\* $p < 0.01$  (Student  $t$ -test). DHL-HisZn = sodium zinc histidine dithiooctanamide; ELISA = enzyme linked immunoassay; Ig = immunoglobulin; NS = not significant; OVA = ovalbumin.

of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs) were determined by using a flow cytometer (BD LSRII, BD Biosciences). The production of transforming growth factor-beta (TGF- $\beta$ ) was quantified using a TGF- $\beta$ 1 rat ELISA kit (eBioscience, Thermo Fisher, San Diego, CA, USA) according to the manufacturer's instruction.

## Statistical analysis

Unpaired 2-tailed Student  $t$ -tests in Excel (Microsoft Corp., Redmond, WA, USA) were used to determine the significance of the difference between the normally distributed means of value in the 2 groups. Each sample was tested in triplicate, and the results are presented as mean  $\pm$  standard deviation.  $p < 0.05$  was considered statistically significant.

## Results

### U-shaped dose response of DHL-HisZn on mast-cell degranulation

To demonstrate the effect of antioxidant DHL-HisZn on mast-cell-mediated allergic response in vitro, BMMCs were sensitized with anti-DNP IgE and then treated with DHL-HisZn (62.5, 125, 250, and 500  $\mu$ mol/L) in the absence or presence of DNP-BSA. As shown in Figure 1, DHL-HisZn treatment resulted in a U-shaped dose response on mast-cell degranulation, suggesting potential adverse effects of higher doses of DHL-HisZn ( $> 500$   $\mu$ mol/L) on mast-cell degranulation. Therefore, relatively lower doses of DHL-HisZn (10 mg/kg in vivo and up to 250  $\mu$ mol/L in vitro) were applied in the following studies.

### Preventive effect of DHL-HisZn on allergic rhinitis

To explore the induction of oxidative stress over the course of allergen sensitization in vivo, serum nitrate levels before and after OVA/alum sensitization were evaluated. As shown in Figure 2A, OVA/alum-sensitized mice ( $n = 9$ ) expressed significantly higher levels of serum nitrate, suggesting involvement of oxidative stress in type I hypersensitivity.

To evaluate the preventive effect of antioxidant DHL-HisZn on mast-cell-mediated allergic responses in vivo, OVA/alum-sensitized mice were divided into 2 groups ( $n = 6$  per group), and DHL-HisZn (10 mg/kg) or control vehicle (normal saline) was intraperitoneally administered before the daily nasal challenge with OVA. Although no obvious effect of DHL-HisZn was observed on nasal rubbing (Fig. 2B), DHL-HisZn significantly ameliorated the symptom of OVA-induced sneezing compared with controls (Fig. 2C). The elevation of serum nitrate in OVA/alum-sensitized mice was significantly suppressed by DHL-HisZn (Fig. 2D).

### Therapeutic effect of DHL-HisZn on allergic rhinitis

We next explored the therapeutic potential of DHL-HisZn on mice with allergic rhinitis. We prepared allergic rhinitis mice by seventh nasal challenge with OVA (Fig. 2, control). We then divided these control mice ( $n = 6$ ) into 2 groups: a control group ( $n = 3$ ) and a DHL-HisZn treatment group ( $n = 3$ ). As shown in Figure 3, OVA-induced sneezing was maintained at a higher level by continual nasal challenge with OVA in the control group ( $n = 3$ ), whereas it was dramatically

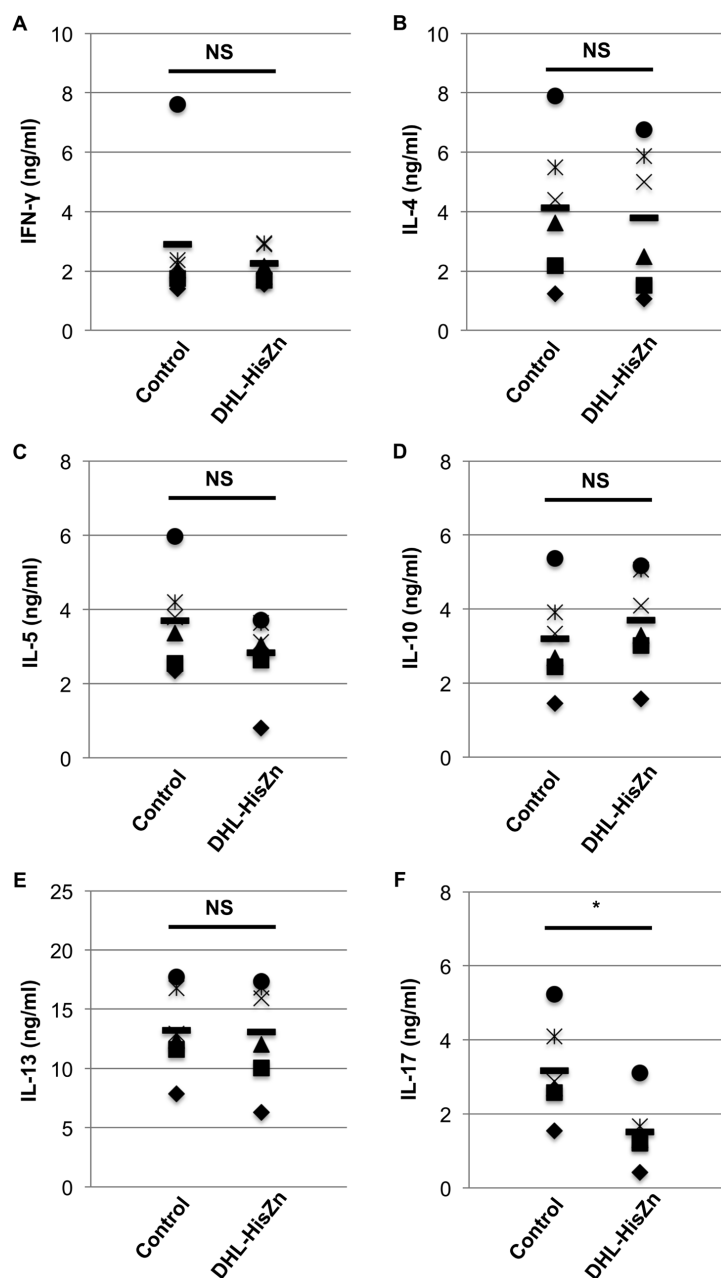


**FIGURE 5.** Impact of DHL-HisZn on cytokine release from total splenocytes stimulated with OVA. After final OVA nasal challenge, the mice were euthanized, and splenocytes were obtained from control ( $n = 6$ ) and DHL-HisZn-treated groups ( $n = 6$ ). Splenocytes were stimulated with OVA for 72 hours, and cytokine levels in culture supernatants were determined by using specific ELISA kits: (A) IFN- $\gamma$ ; (B) IL-4; (C) IL-5; (D) IL-10; (E) IL-13; and (F) IL-17. Each symbol indicates an individual mouse, and bars indicate mean values. \* $p < 0.05$  (Student  $t$ -test). DHL-HisZn = sodium zinc histidine dithiooctanamide; ELISA = enzyme linked immunoassay; NS = not significant; OVA = ovalbumin.

suppressed after a second injection of DHL-HisZn in the treatment group ( $n = 3$ ).

### Effects of DHL-HisZn on OVA-specific immune responses

To determine the impact of DHL-HisZn on OVA-specific immune responses, OVA-specific immunoglobulin levels were evaluated before immunization and after the final nasal challenge. As shown in Figure 4, DHL-HisZn

significantly suppressed OVA-specific IgE responses, yet it enhanced OVA-specific IgG2a response.

To identify the effect of DHL-HisZn on cytokine profiles, we next evaluated cytokine secretion after the OVA stimulation of splenocytes from OVA-sensitized and OVA-challenged mice. As shown in Figure 5, there was no obvious difference between control-treated and DHL-HisZn-treated mice on IFN- $\gamma$ , IL-4, IL-5, IL-10, and IL-13 levels. On the other hand, IL-17 levels were significantly suppressed by DHL-HisZn treatment.

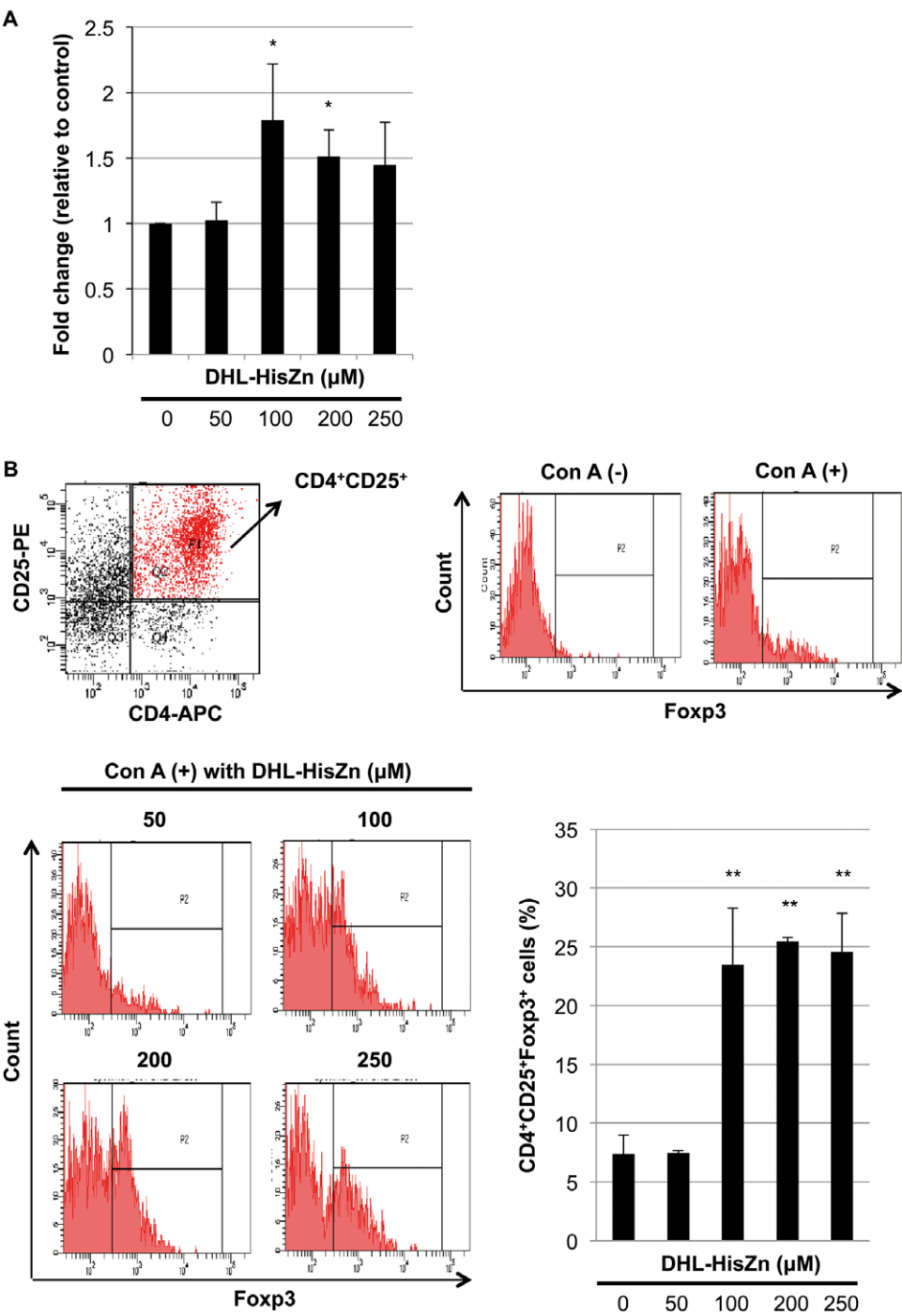


**FIGURE 6.** Impact of DHL-HisZn on the proportion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Tregs). Splenocytes were stimulated with Con A in the presence of DHL-HisZn (50, 100, 200, and 250 μmol/L) for 72 hours, and the secretion of TGF-β (A) and Treg phenotype (B) were evaluated. (A) TGF-β levels were quantified by ELISA and the values are presented as the mean ± SD of three independent experiments. (B) A dot plot represents splenic levels of CD4<sup>+</sup> and CD25<sup>+</sup> cells in Con A blasts with DHL-HisZn (250 μmol/L). The histograms of Foxp3<sup>+</sup> cells within CD4<sup>+</sup>CD25<sup>+</sup> cells are representative of 3 independent experiments. The proportions of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs are shown in the right panel. \**P* < 0.05; \*\**P* < 0.01, respectively (Student *t*-test). Con A = concanavalin A; DHL-HisZn = sodium zinc histidine dithiooctanamide; ELISA = enzyme linked immunoassay; TGF-β = transforming growth factor-beta; Tregs = regulatory T cells.

Taken together, these data suggest that DHL-HisZn may affect not only mast-cell-mediated type I hypersensitivity but also T- and B-cell-mediated immune responses in the course of the allergic response.

**Induction of Treg phenotype by DHL-HisZn**  
To determine the impact of DHL-HisZn on the generation of Treg phenotypes in vitro, splenocytes were stimulated with Con A in the presence of DHL-HisZn (50, 100, 200

and 250  $\mu\text{mol/L}$ ). Although the induction of TGF- $\beta$  production was limited (Fig. 6A), the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg population was significantly increased by DHL-HisZn in a dose-dependent manner (Fig. 6B).

## Discussion

The impact of oxidative stress on allergic responses is well documented,<sup>5–7</sup> yet little is known about the immunomodulatory effect of antioxidants in type I hypersensitivity. In this study, we have reported for the first time on the preventive and therapeutic potential of the  $\alpha$ -lipoic acid derivative, DHL-HisZn, in a mouse model of OVA-induced allergic rhinitis, and that this antioxidant may act through the promotion of Tregs to suppress mast-cell-, Th2-, and Th17-cell-mediated allergic responses.

Although some reports have demonstrated the therapeutic impact of  $\alpha$ -lipoic acid in allergic responses,<sup>21,22</sup> the guidelines for an  $\alpha$ -lipoic acid supplement noted several adverse effects, including allergic skin rashes and gastrointestinal distress.<sup>32</sup> Interestingly, our in-vitro data revealed a U-shaped dose response of DHL-HisZn on mast-cell degranulation (Fig. 1). The ideal dosage of an  $\alpha$ -lipoic acid supplement is not clearly described in the current guideline, but our findings suggest the necessity to test for the optimal effective dose of an  $\alpha$ -lipoic acid supplements in clinical settings.

In this study, relatively lower doses of DHL-HisZn were applied for in-vivo and in-vitro assays. Our in-vivo study clearly demonstrated the preventive and therapeutic potential of DHL-HisZn in a mouse model of OVA-induced allergic rhinitis (Figs. 2C and 3). In the course of OVA/alum sensitization, we have confirmed the elevation of serum nitrate (Fig. 2A). The elevation of NO and its metabolites (nitrite and nitrate) is a hallmark of allergy.<sup>33–36</sup> Therefore, a potential mechanism for amelioration of type I hypersensitivity by DHL-HisZn is the reduction of oxidative stress caused by OVA/alum sensitization (Fig. 2D). NO production caused by oxidative stress is known to lead to the relaxation of blood vessels in the nasal mucosa and deteriorates allergic rhinitis.<sup>37,38</sup> In addition, oxidative stress acts on the sympathetic nervous system (SNS) and hypofunctioning of the SNS is associated with allergic rhinitis.<sup>39,40</sup> Indeed, modulation of the SNS is one of the therapeutic

options currently in use in the clinic. One recent study demonstrated the impact of  $\alpha$ -lipoic acid on the modulation of sympathoexcitation in high-salt-induced hypertension.<sup>41</sup> This evidence strongly supports the therapeutic potential of antioxidant DHL-HisZn in allergic rhinitis, partly through the reduction of oxidative stress and modulation of the SNS.

In addition, our data suggest another potential mechanism of DHL-HisZn, through the modulation of T-cell-mediated allergic responses. Briefly, DHL-HisZn may modulate the Th1/Th2 balance through inhibition of Th2-mediated IgE production and induction of Th1-mediated IgG2a production (Fig. 4). In addition, DHL-HisZn treatment suppressed IL-17 production in OVA-stimulated splenocytes obtained from OVA/alum-sensitized and OVA-challenge mice (Fig. 5). Although splenic IL-10 levels in DHL-HisZn-treated mice were similar to those of the control group (Fig. 5), our in-vitro data clearly demonstrated the induction of Treg phenotype in a dose-dependent manner (Fig. 6). Similar to our study, Wang et al identified suppression of Th17 cells and the enhancement of splenic Tregs by  $\alpha$ -lipoic acid in a mouse model of experimental autoimmune encephalomyelitis.<sup>42</sup> Furthermore, recent studies demonstrated the induction of Tregs by zinc supplementation in vitro.<sup>43,44</sup> These results suggest that the  $\alpha$ -lipoic acid derivative DHL-HisZn may modulate the Th17/Treg balance, resulting in the amelioration of type I hypersensitivity.

## Conclusions

We have demonstrated the preventive and therapeutic potential of a novel  $\alpha$ -lipoic acid derivative, DHL-HisZn, on mast-cell-mediated type I hypersensitivity, partly through its antioxidant activity as well as its modulation of the Th1/Th2 and Th17/Treg balances.  $\alpha$ -Lipoic acid is widely recognized as a nutritional health supplement. Therefore, we expect broad application of this water-soluble  $\alpha$ -lipoic acid derivative for prevention and treatment of allergic disorders in the future. 

## Acknowledgments

The authors thank the staff at the Center for Laboratory Animals of Kaohsiung Chang Gung Memorial Hospital for providing excellent support.

## References

- Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr Neuropharmacol*. 2009;7:65–74.
- Perl A. Oxidative stress in the pathology and treatment of systemic lupus erythematosus. *Nat Rev Rheumatol*. 2013;9:674–686.
- Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med*. 2010;49:1603–1616.
- Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature*. 2000;408:239–247.
- Bowler RP, Crapo JD. Oxidative stress in allergic respiratory diseases. *J Allergy Clin Immunol*. 2002;110:349–356.
- Kelly FJ, Sandstrom T. Air pollution, oxidative stress, and allergic response. *Lancet*. 2004;363:95–96.
- Okayama Y. Oxidative stress in allergic and inflammatory skin diseases. *Curr Drug Targets Inflamm Allergy*. 2005;4:517–519.
- Betteridge DJ. What is oxidative stress? *Metabolism*. 2000;49(2, Suppl 1):3–8.
- Tsukahara H. Biomarkers for oxidative stress: clinical application in pediatric medicine. *Curr Med Chem*. 2007;14:339–351.
- Fatani SH. Biomarkers of oxidative stress in acute and chronic bronchial asthma. *J Asthma*. 2014;51:578–584.
- Aldakheel FM, Thomas PS, Bourke JE, et al. Relationships between adult asthma and oxidative stress markers and pH in exhaled breath condensate: a systematic review. *Allergy*. 2016;71:741–757.
- Abdala-Valencia H, Berdnikovs S, Soveg FW, Cook-Mills JM.  $\alpha$ -Tocopherol supplementation of allergic female mice inhibits development of CD11c<sup>+</sup>CD11b<sup>+</sup> dendritic cells in utero and allergic inflammation in neonates. *Am J Physiol Lung Cell Mol Physiol*. 2014;307:L482–496.
- Basketter DA, White IR, Kullavanijaya P, et al. Influence of vitamin C on the elicitation of allergic contact

- dermatitis to p-phenylenediamine. *Contact Dermatitis*. 2016;74:368–372.
14. Hayashi D, Sugaya H, Ohkoshi T, et al. Vitamin E improves biochemical indices associated with symptoms of atopic dermatitis-like inflammation in NC/Nga mice. *J Nutr Vitaminol (Tokyo)*. 2012;58:161–168.
  15. Allan KM, Prabhu N, Craig LC, et al. Maternal vitamin D and E intakes during pregnancy are associated with asthma in children. *Eur Respir J*. 2015;45:1027–1036.
  16. Gvozdzakova A, Kucharska J, Bartkovjakova M, et al. Coenzyme Q10 supplementation reduces corticosteroids dosage in patients with bronchial asthma. *Biofactors*. 2005;25:235–240.
  17. Golbidi S, Badran M, Laher I. Diabetes and alpha lipoic acid. *Front Pharmacol*. 2011;2:69.
  18. Dulundu E, Ozel Y, Topaloglu U, et al. Alpha-lipoic acid protects against hepatic ischemia-reperfusion injury in rats. *Pharmacology*. 2007;79:163–170.
  19. Min AK, Kim MK, Seo HY, et al. Alpha-lipoic acid inhibits hepatic PAI-1 expression and fibrosis by inhibiting the TGF-beta signaling pathway. *Biochem Biophys Res Commun*. 2010;393:536–541.
  20. Novotny L, Rauko P, Cojocel C. alpha-Lipoic acid: the potential for use in cancer therapy. *Neoplasma*. 2008;55:81–86.
  21. Choi YH, Chai OH, Han EH, et al. Lipoic acid suppresses compound 48/80-induced anaphylaxis-like reaction. *Anat Cell Biol*. 2010;43:317–324.
  22. Kim GD, Kim TH, Jang AH, et al. alpha-Lipoic acid suppresses the development of DNFB-induced atopic dermatitis-like symptoms in NC/Nga mice. *Exp Dermatol*. 2011;20:97–101.
  23. Koga H, Hagiwara S, Kusaka J, et al. New alpha-lipoic acid derivative, DHL-HisZn, ameliorates renal ischemia-reperfusion injury in rats. *J Surg Res*. 2012;174:352–358.
  24. Lee JW, Miyawaki H, Bobst EV, et al. Improved functional recovery of ischemic rat hearts due to singlet oxygen scavengers histidine and carnosine. *J Mol Cell Cardiol*. 1999;31:113–121.
  25. Obata T, Kubota S, Yamanaka Y. Protective effect of histidine on para-nonylphenol-enhanced hydroxyl free radical generation induced by 1-methyl-4-phenylpyridinium ion (MPP+) in rat striatum. *Biochim Biophys Acta*. 2001;1568:171–175.
  26. Hagiwara S, Teshima Y, Takahashi N, et al. New lipoic acid derivative drug sodium zinc dihydrolipoyl-histidinate prevents cardiac dysfunction in an isolated perfused rat heart model. *Biochim Biophys Acta*. 2001;39:506–511.
  27. Hagiwara S, Uchida T, Koga H, et al. The alpha-lipoic acid derivative sodium zinc dihydrolipoyl-histidinate reduces chemotherapy-induced alopecia in a rat model: a pilot study. *Surg Today*. 2011;41:693–697.
  28. Kono Y, Inomata M, Hagiwara S, et al. Antiproliferative effects of a new alpha-lipoic acid derivative, DHL-HisZnNa, in HT29 human colon cancer cells in vitro. *Expert Opin Ther Targets*. 2012;16(Suppl 1):S103–109.
  29. Chen CC, Hsu LW, Nakano T, et al. DHL-HisZn, a novel antioxidant, enhances adipogenic differentiation and antioxidative response in adipose-derived stem cells. *Biomed Pharmacother*. 2016;84:1601–1609.
  30. Nakano T, Lai CY, Goto S, et al. Immunological and regenerative aspects of hepatic mast cells in liver allograft rejection and tolerance. *PLoS One*. 2012;7:e37202.
  31. Nakano T, Kamei R, Fujimura T, et al. Impact of histone H1 on the progression of allergic rhinitis and its suppression by neutralizing antibody in mice. *PLoS One*. 2016;11:e0153630.
  32. Alpha-lipoic acid. Monograph. *Altern Med Rev*. 2006;11:232–237.
  33. Taniuchi S, Kojima T, Hara Mt K, et al. Increased serum nitrate levels in infants with atopic dermatitis. *Allergy*. 2001;56:693–695.
  34. Unal M, Eskandari HG, Ercetin N, et al. Serum nitrite/nitrate and arginase levels in patients with allergic rhinitis. *ORL J Otorhinolaryngol Relat Spec*. 2007;69:113–115.
  35. Syk J, Malinowski A, Borres MP, et al. Parallel reductions of IgE and exhaled nitric oxide after optimized anti-inflammatory asthma treatment. *Immun Inflamm Dis*. 2016;4:182–190.
  36. Castro-Rodriguez JA, Molina RO, Caceres M, Recabarren A. Correlation between nitrites in induced sputum and asthma symptoms in asthmatic schoolchildren. *Pediatr Pulmonol*. 2014;49:214–220.
  37. Sato M, Fukuyama N, Sakai M, Nakazawa H. Increased nitric oxide in nasal lavage fluid and nitrotyrosine formation in nasal mucosa—indices for severe perennial nasal allergy. *Clin Exp Allergy*. 1998;28:597–605.
  38. Mizutani N, Nabe T, Imai A, et al. Markedly increased nasal blockage by intranasal leukotriene D4 in an experimental allergic rhinitis model: contribution of dilated mucosal blood vessels. *Jpn J Pharmacol*. 2001;86:170–182.
  39. Ishman SL, Martin TJ, Hambrook DW, et al. Autonomic nervous system evaluation in allergic rhinitis. *Otolaryngol Head Neck Surg*. 2007;136:51–56.
  40. Emin O, Esra G, Ufuk E, et al. Autonomic dysfunction and clinical severity of disease in children with allergic rhinitis. *Int J Pediatr Otorhinolaryngol*. 2012;76:1196–1200.
  41. Su Q, Liu JJ, Cui W, et al. Alpha lipoic acid supplementation attenuates reactive oxygen species in hypothalamic paraventricular nucleus and sympathoexcitation in high salt-induced hypertension. *Toxicol Lett*. 2016;241:152–158.
  42. Wang KC, Tsai CP, Lee CL, et al. alpha-Lipoic acid enhances endogenous peroxisome-proliferator-activated receptor-gamma to ameliorate experimental autoimmune encephalomyelitis in mice. *Clin Sci (Lond)*. 2013;125:329–340.
  43. Rosenkranz E, Metz CH, Maywald M, et al. Zinc supplementation induces regulatory T cells by inhibition of Sirt-1 deacetylase in mixed lymphocyte cultures. *Mol Nutr Food Res*. 2016;60:661–671.
  44. Maywald M, Rink L. Zinc supplementation induces CD4+CD25+Foxp3+ antigen-specific regulatory T cells and suppresses IFN-gamma production by up-regulation of Foxp3 and KLF-10 and downregulation of IRF-1. *Eur J Nutr*. 2017;56:1859–1869.