

Effect of Dietary Low-molecular-weight Hyaluronic Acid on Ear Swelling in 2,4-Dinitro-1-fluorobenzene-treated BALB/c Mice

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Summary

Hyaluronic acid (HA) is a straight chain, glycosaminoglycan polymer composed of repeating units of the disaccharide [$\beta(1,4)$ -D-glucuronic acid- $\beta(1,3)$ -N-acetyl-D-glucosamine]_n that is found in the extracellular matrix. HA has been reported to have anti-inflammatory and immunosuppressive properties. However, little information is available on the anti-inflammatory effects of dietary HA. In this study, we evaluated the effect of dietary low-molecular-weight HA (LMW-HA) on 2,4-dinitro-1-fluorobenzene (DNFB)-induced contact hypersensitivity in BALB/c mice because the absorption of LMW-HA in the small intestine seems to be better than that of high-molecular-weight HA (HMW-HA). Dietary LMW-HA significantly inhibited ear swelling in mice. Analysis of cytokine mRNA expression in the spleen indicated that supplementation with dietary LMW-HA may improve the Th1/Th2 imbalance in DNFB-treated mice. Our results suggest that dietary LMW-HA exerts anti-inflammatory effects via improvement of Th1/Th2 balance.

Keywords: low-molecular-weight hyaluronic acid, dietary supplements, anti-inflammatory, ear swelling, Th1/Th2 balance, cytokines

Abbreviations: DNFB, 2,4-dinitro-1-fluorobenzene; HA, hyaluronic acid; LMW-HA, low-molecular-weight hyaluronic acid; HMW-HA, high-molecular-weight hyaluronic acid; IL-10, interleukin-10; IL-18, interleukin-18; IFN- γ : interferon-gamma; TNF- α , tumor necrosis factor-alpha; Th1, T-helper 1; Th2, T-helper 2

Introduction

Hyaluronic acid (HA) is a high-molecular-weight glycosaminoglycan found in the extracellular matrix, especially of soft connective tissues, that consists of repeating disaccharide units of *N*-acetyl glucosamine (GluNAc) and glucuronic acid¹⁾. The interest in HA has intensified in cell biology, pathology, and immunology, although its most important biological function has been regarded as the polymer for a space filler. The efficacy of HA in suppressing inflammation and immune responses has been reported^{2, 3)}. It was reported that HA suppresses β -hexosaminidase secretion from antigen-stimulated rat basophilic leukemia (RBL2H3) cells⁴⁾. HA was also shown to inhibit macrophage proliferation and cytokine release, thereby decreasing the inflammatory response in the early wound of a preclinical post-laminectomy rat model⁵⁾. Moreover, HA enhances proteoglycan synthesis, reduces the produc-

tion and activity of proinflammatory mediators and matrix metalloproteinase, and alters the behavior of immune cells⁶⁾.

High-molecular-weight HA (HMW-HA) is not absorbed into the body in its intact polymer form after oral ingestion. Moderate penetration of a differentiated Caco-2 cell monolayer was observed using low-molecular-weight HA (LMW-HA) (< 5 kDa), whereas HMW-HA (> 100 kDa) barely penetrated the cell monolayer⁷⁾. This suggests that dietary LMW-HA can be absorbed through the epithelial cells in the small intestine of humans. We previously reported that GluNAc, which along with glucuronic acid, constitutes HA, has *in vivo* anti-inflammatory effects, and suppresses the activation of mast cells⁸⁾. Hence, we speculated that dietary LMW-HA has anti-inflammatory effects, although many of the physiological effects of exogenous HA may be due to its molecular weight.

The objective of this study was to evaluate the effects of

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dietary LMW-HA on 2,4-dinitro-1-fluorobenzene (DNFB)-induced ear swelling in BALB/c mice and to provide a better understanding of how dietary LMW-HA acts on the balance between the proinflammatory and anti-inflammatory responses.

Materials and Methods

Animals and diets

LMW-HA sodium salt (average molecular weight ~3 kDa) was provided by Nippon Shinyaku Co., Ltd. Female BALB/c mice (6-week-old, 15–20 g body weight) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Animals were cared for according to the Guide for the Care and Use of Laboratory Animals of Kyoto University, and the experimental protocol was approved by the Animal Care Committee of Kyoto University. Animals were housed at 25°C with a 12 h light/dark cycle in individual cages. After acclimation to a standard diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) for a week, the mice were divided into two groups ($n = 6$ per group) (day 0). The control group was fed AIN-93G with 10% cellulose, and the experimental group (LMW-HA group) was fed AIN-93G with 1% HA sodium salt and 9% cellulose (Table 1). All mice were allowed free access to food and water for 13 d. The body weight of each mouse was recorded every 3 d and food intake was monitored every 1 or 2 d.

The *in vivo* anti-allergic activity of LMW-HA was tested using the DNFB-induced contact hypersensitivity reaction^{8,9}. After feeding animals with the experimental or control diet for 7 d, the dorsal skin of each mouse was shaved, and 100 μ L of 0.5% DNFB in acetone-soybean oil (4:1) was applied to the area to sensitize the mice (day 7). After 6 d, both the right and left ears were challenged with 20 μ L of 0.5% DNFB in acetone-soybean oil (4:1) (day 13). The thickness of the right ear was measured with a Dial Thickness Gauge (Mitutoyo Co., Kanagawa, Japan) 0 h and 24 h after DNFB challenge. Ear swelling was reported as a percentage of the ear thickness measured before DNFB challenge. Twenty-four hours after DNFB treatment, blood was collected, and the mice were euthanized by isoflurane anesthesia. The right ear was fixed in formalin solution, and each paraffin-embedded section was stained by hematoxylin and eosin (HE) to observe the morphological changes using a microscope (Keyence Co., Osaka, Japan). The spleen of each mouse was immediately excised. Sections of the spleen were stored in RNAlater solution (Life technologies Japan, Tokyo, Japan) for mRNA quantification, and the remaining parts were frozen in liquid nitrogen and then stored at -80°C until use.

Table 1 Composition of experimental diets

Ingredient	Control group	LMW-HA group
	%	
Cornstarch	34.75	34.75
Casein	20.0	20.0
Dextrinized cornstarch	13.2	13.2
Sucrose	10.0	10.0
Soybean oil	7.0	7.0
Cellulose powder	10.0	9.0
LMW-HA	–	1.0
AIN-93G mineral mix	3.5	3.5
AIN-93 vitamin mix	1.0	1.0
L-Cystine	0.3	0.3
Choline bitartrate	0.25	0.25
Butyl hydroxyl toluene	0.0014	0.0014

Measurement of cytokines in the ear by ELISA

Amounts of interferon- γ (IFN- γ) and interleukin-10 (IL-10) in the homogenates of ears were quantified using Murine IFN- γ and Murine IL-10 ELISA kits (Diaclone Research, Besancon, France), respectively, according to the manufacturer's instructions. Levels of these cytokines in each supernatant were normalized to total protein content, which was determined using a DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Quantification of cytokine mRNA expression by real-time RT-PCR

Total RNA was extracted from the spleen using Sepasol reagent (Nacalai Tesque, Inc., Kyoto, Japan) according to the manufacturer's instructions. RNA was treated with RNase-free DNase (Invitrogen, Carlsbad, CA, USA) to remove genomic DNA. After inactivating DNase by heating at 65°C for 10 min, the RNA was reverse-transcribed to cDNA using SuperScript RNase II reverse transcriptase (Invitrogen) with random hexamers at 25°C for 10 min, and then at 42°C for 50 min. The reactions were stopped by incubation at 70°C for 15 min. Aliquots (6 μ L) of each cDNA, 10 μ L of iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 2 μ L of each cytokine primer (forward and reverse) were mixed to obtain a final reaction volume of 20 μ L. The primers used to quantify each gene are listed in Table 2. Primer pairs were selected to yield single gene-specific amplicons based on melting curve analyses. Real-time PCR was performed using the DNA Engine Option system (Bio-Rad Laboratories). The thermal cycling parameters were as follows: 3 min at 95°C, followed by 40 cycles of melting at 95°C for 15 s, and annealing and extension at 60°C for 30 s.

Table 2 Real-time RT-PCR primers for quantification of mouse mRNA

Gene name		Sequence	Accession number
IL-10	Forward	GGTTGCCAAGCCTTATCGGA	NM_010548.2
	Reverse	ACCTGCTCCACTGCCTTGCT	
IL-18	Forward	CAGGCCTGACATCTTCTGCAA	NM_008360.1
	Reverse	TCTGACATGGCCCATTTGT	
IFN- γ	Forward	TCAAGTGGCATAGATGTGGAAGAA	NM_008337.3
	Reverse	TGGCTCTGCAGGATTTTCATG	
TNF- α	Forward	CATCTTCTCAAAATTCGAGTGACAA	NM_013693.3
	Reverse	TGGGAGTAGACAAGGTACAACCC	
GAPDH	Forward	AGGTCGGTGTGAACGGATTTG	NM_008084.3
	Reverse	TGTAGACCATGTAGTTGAGGTCA	

The values were normalized to GAPDH, which was used as an internal standard.

Statistical Analysis

Data are presented as mean \pm SEM. Differences between groups were evaluated by using Student's *t*-test.

Results

There was no significant difference in the food intake and body weight gain between the control and LMW-HA groups during the experimental period (Fig. 1). Challenging the mice with DNFB led to the induction of typical allergic contact dermatitis in their ears, with an increase in the thickness of the ears and visible congestion of the blood vessels. Dietary LMW-HA suppressed DNFB-induced ear swelling. As shown in Fig. 2, the ear swelling at 24 h was significantly lower in the LMW-HA group than in the control group ($p < 0.05$). Histochemical analysis with HE staining showed that expansion of dermal tissue in the ear due to lymphocyte infiltration was mitigated in the LMW-HA group (Fig. 3).

To evaluate the effect of dietary LMW-HA on DNFB-induced inflammation, especially on Th1/Th2 balance, levels of IFN- γ and IL-10 in the ear were measured as indicators of Th1 and Th2 cells, respectively, by ELISA. However, the IFN- γ and IL-10 levels of the LMW-HA group were not significantly altered (Fig. 4). We measured mRNA levels in the spleen by a real-time RT-PCR and found that the expression of IFN- γ was significantly increased whereas the expression of IL-10 was significantly suppressed in the LMW-HA group (Fig. 5). In addition, the expression of tumor necrosis factor-alpha (TNF- α) was significantly suppressed and IL-18 mRNA was significantly increased in the LMW-HA group.

Discussion

To evaluate the anti-inflammatory effect of dietary LMW-HA, we compared DNFB-induced ear swelling in BALB/c mice belonging to two experimental groups. Mice from the control group were fed ordinary AIN-93G and those from the experimental group were fed AIN-93 containing 1% LMW-HA for 13 d. In this study, we showed that dietary LMW-HA suppresses DNFB-induced ear swelling.

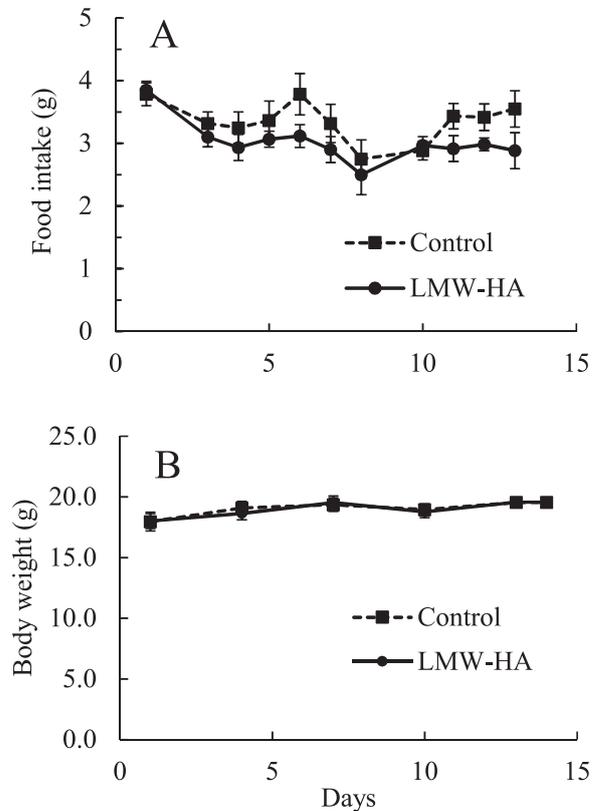


Fig. 1 Changes of food intake (A) and body weight (B) during the experimental period. Values are reported as mean \pm SEM, $n = 6$.

The results demonstrated that the cytokine levels in the ear of LMW-HA group were not significantly different from that of the control group. However, the cytokine mRNA levels in the spleen indicated that dietary LMW-HA shifted the Th1/Th2 balance in the immune response toward Th1. In addition, the expression of the pro-inflammatory cytokine TNF- α was also downregulated in the spleen. It appears that dietary LMW-HA may exert an anti-inflammatory effect by promoting the expansion of Th1 cells, which can induce the production of inflammatory cytokines and antibodies.

Naïve helper T cells mature in the thymus and differentiate mainly into two types of cells: Th1 cells, which drive cellular immunity, and Th2 cells, which steer humoral immunity. These two subsets show distinct cytokine repertoires. Th1 cells secrete an array of cytokines including IFN- γ and IL-2, whereas Th2 cells secrete IL-4, IL-5, and IL-10⁽⁰⁾. It is well known that IFN- γ is the principal mediator of Th1 responses, whereas IL-4 and IL-10 are predominant during Th2 responses. IFN- γ not only induces Th1 differentiation but also suppresses Th2 differentiation, whereas IL-10 suppresses Th1 differentiation. It has been reported that IL-18 induces IFN- γ mRNA expression in Th1 cells⁽¹⁾. In the present study, we found that expression of IL-10 was suppressed and that of IFN- γ and IL-18 was induced in the splenocytes. Based on these results, dietary LMW-HA may promote the development of Th1 cells. Th2 cells predominantly secrete IL-4 and probably

other cytokines, all of which help recruit B cells, mast cells, and eosinophils. Antigen-stimulated B cells synthesize IgE antibodies against ubiquitous antigens. Secreted IgE antibodies attach to basophils and mast cells through their surface IgE receptors. Activation of IgE-sensitized cells releases various mediators such as histamine, leukotrienes, and prostaglandins, all of which play a central role in the induction of allergic symptoms⁽²⁾. We assume that the dominant Th1 cells suppressed the inflammatory response during the humoral immune response in BALB/c mice treated with DNFB.

The spleen is the most important organ for antibacterial and antifungal immune reactivity because it controls both the innate and adaptive immune systems and has highly organized lymphoid compartments. One of the most important roles of the spleen in immunity is the maturation of T, B, and plasma cells⁽³⁾. In a previous study using rabbits, intravenously administered HA was taken up and mainly degraded in the liver and was concentrated in immune tissues, such as the bone marrow, lymph nodes, and spleen⁽⁴⁾. Moreover, Zhou *et al.* found the receptor for endocytosis of HA and suggested that HA could be absorbed by the spleen as well as the liver and kidneys⁽⁵⁾.

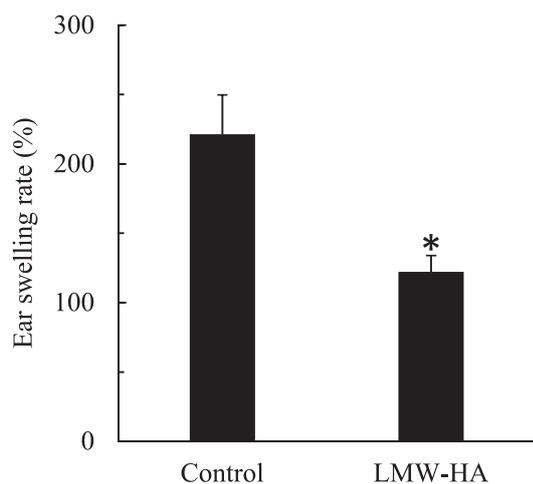


Fig. 2 Effect of dietary LMW-HA on DNFB-induced ear swelling in BALB/c mice. The thickness of the ears of BALB/c mice sensitized with dietary LMW-HA was measured at 24 h after challenge with DNFB, and the values are presented as a percentage of the thickness of the ear measured before DNFB challenge. Values are reported as mean \pm SEM, n = 6. Asterisks indicate values that are significantly different from the control, p < 0.05.

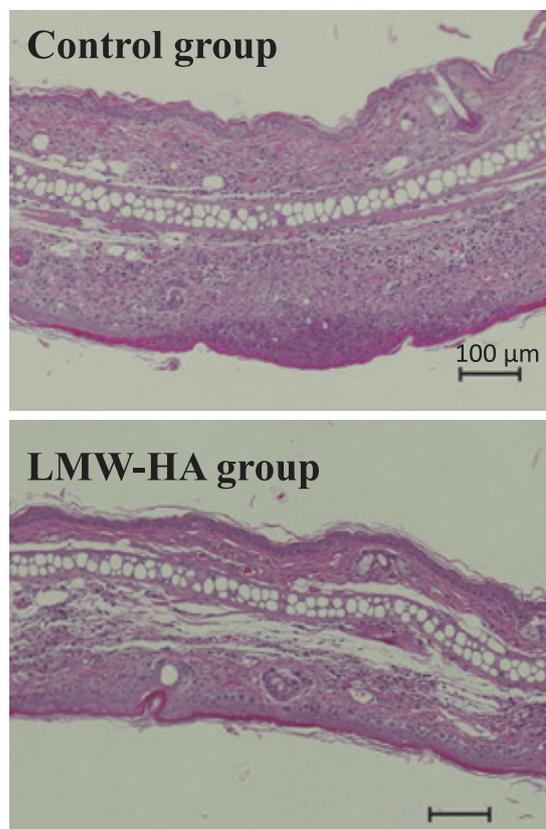


Fig. 3 Photographs of ear section 24 h after DNFB challenge. The ear was fixed in a formalin solution, and paraffin sections were stained with hematoxylin and eosin.

Lymphatic vessel endothelial HA receptor-1 (LYVE-1) was identified as a lymph-specific HA receptor¹⁶). Therefore, in our study, the dietary LMW-HA may have been taken up by the spleen and other lymphatic tissues.

There are several studies on the intestinal absorption of HA. Balough *et al.* reported that 86.7–95.6% of the ingested ^{99m}technetium-labeled HMW-HA was recovered from the urine and feces of rats¹⁷). It was also shown that serum HA levels were elevated after its oral administration¹⁸). In addition, LMW-HA (< 5 kDa) can penetrate a Caco-2 cell monolayer, a model of the intestinal epithelium, via the paracellular pathway more easily than HMW-HA (> 100 kDa)⁷). Thus, it can be assumed that the LMW-HA used in the present study, with an average molecular mass of ~3 kDa, was absorbed through the intestine.

The biological functions of HA are dependent on its molecular weight. Anti-inflammatory and immunosuppressive effects have been reported for HMW-HA. HMW-HA can coat cell surfaces, which prevents ligand access to surface receptors and inhibits phagocytosis by monocytes, macrophages, and polymorphonuclear leukocytes¹⁹). In contrast, small polymer fragments of HA are thought to be involved in the body's alarm system. Horton *et al.* reported that HA that is < 10 kDa can induce inflammatory responses²⁰). In this study, the increase in Th1 cells in mice following the administration of dietary LMW-HA might be an immune response to bacterial or viral signals.

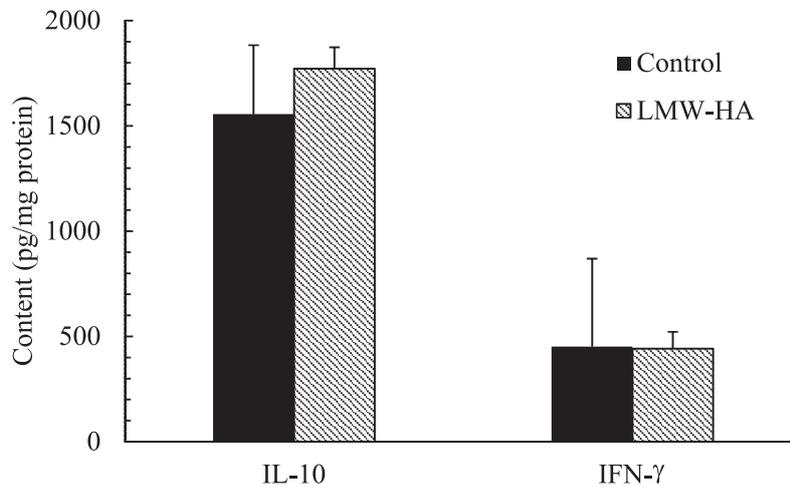


Fig. 4 Effect of dietary LMW-HA on the cytokines in the ear of DNFB-challenged mice. IL-10 and IFN- γ were measured in the ear using ELISA. Values are presented as mean \pm SEM, n = 6.

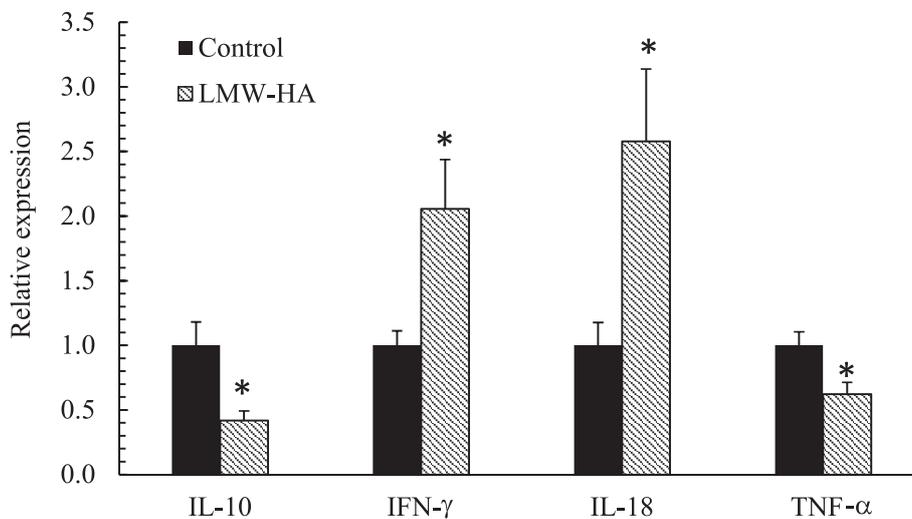


Fig. 5 Effect of dietary LMW-HA on the mRNA expression of cytokines in the spleen of DNFB-challenged mice. The mRNA expression of various cytokines was measured in the spleen using quantitative real-time PCR. Values are presented as mean \pm SEM, n = 6. Asterisks indicate values that are significantly different from the control, p < 0.05.

References

- 1) Laurent TC, Fraser RE (1992) Hyaluronan. *FASEB J* 6: 2397-2402.
- 2) Delmage JM, Powars DR, Jaynes PK, Allerton SE (1986) The selective suppression of immunogenicity by hyaluronic acid. *Ann Clin Lab Sci* 16: 303-310.
- 3) McBride WH, Bard JB (1979) Hyaluronidase-sensitive halos around adherent cells. Their role in blocking lymphocyte-mediated cytotoxicity. *J Exp Med* 149: 507-515.
- 4) Kim Y, Lee YS, Hahn JH, Choe J, Kwon H, Ro JY, Jeoung D (2008) Hyaluronic acid targets CD44 and inhibits FcεRI signaling involving PKCδ, Rac1, ROS, and MAPK to exert anti-allergic effect. *Mol Immunol* 45: 2537-2547.
- 5) Schimizzi AL, Massie JB, Murphy M, Perry A, Kim CW, Garfin SR, Akeson WH (2006) High-molecular-weight hyaluronan inhibits macrophage proliferation and cytokine release in the early wound of a pre-clinical postlaminectomy rat model. *Spine J* 6: 550-556.
- 6) Moreland LW (2003) Intra-articular hyaluronan (hyaluronic acid) and hylans for the treatment of osteoarthritis: mechanisms of action. *Arthritis Res Ther* 5: 54-67.
- 7) Hisada N, Satsu H, Mori A, Totsuka M, Kamei J, Nozawa T, Shimizu M (2008) Low-molecular-weight hyaluronan permeates through human intestinal Caco-2 cell monolayers via the paracellular pathway. *Biosci Biotechnol Biochem* 72: 1111-1114.
- 8) Sakai S, Sugawara T, Kishi T, Yanagimoto T, Hirata T (2010) Effect of glucosamine and related compounds on the degranulation of mast cells and ear swelling induced by dinitrofluorobenzene in mice. *Life Sci* 86: 337-343.
- 9) Maeda A, Beissert S, Schwarz T, Schwarz A (2008) Phenotypic and functional characterization of ultraviolet radiation-induced regulatory T cells. *J Immunol* 180: 3065-3071.
- 10) Kidd P (2003) Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern Med Rev* 8: 233-246.
- 11) Yang J, Murphy T, Ouyang W, Murphy K (1999) Induction of interferon-gamma production in Th1 CD4+ T cells: evidence for two distinct pathways for promoter activation. *Eur J Immunol* 29: 548-555.
- 12) Singh VK, Mehrotra S, Agarwal SS (1999) The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. *Immunol Res* 20: 147-161.
- 13) Mebius RE, Kraal G (2005) Structure and function of the spleen. *Nat Rev Immunol* 5: 606-616.
- 14) Fraser JR, Appelgren LE, Laurent TC (1983) Tissue uptake of circulating hyaluronic acid. A whole body autoradiographic study. *Cell Tissue Res* 233: 285-293.
- 15) Zhou B, Weigel JA, Fauss L, Weigel PH (2000) Identification of the hyaluronan receptor for endocytosis (HARE). *J Biol Chem* 275: 37733-37741.
- 16) Banerji S, Ni J, Wang S X, Clasper S, Su J, Tammi R, Jones M, Jackson DG (1999) LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 144: 789-801.
- 17) Balough L, Polyak A, Mathe D, Kiral R, Thuroczy J, Terez M, Janoki G, Ting Y, Bucci LR, Schauss AG (2008) Absorption, uptake and tissue affinity of high-molecular-weight hyaluronan after oral administration in rats and dogs. *J Agric Food Chem* 56: 10582-10593.
- 18) Huang S, Ling P, Zhang T (2007) Oral absorption of hyaluronic acid and phospholipids complexes in rats. *World J Gastroenterol* 13: 945-949.
- 19) Forrester JV, Balazs EA (1980) Inhibition of phagocytosis by high molecular weight hyaluronate. *Immunology* 40: 435-446.
- 20) Powell JD, Horton MR (2005) Threat matrix: low-molecular-weight hyaluronan (HA) as a danger signal. *Immunol Res* 31: 207-218.