



PRELIMINARY REPORT

Inhibition of atopic dermatitis by topical application of silymarin in NC/Nga mice

Jong Soon Kang^a, Won Kee Yoon^a, Mi Hwa Han^a, Hyunju Lee^a,
Chang Woo Lee^a, Ki Hoon Lee^a, Sang-Bae Han^b, Kiho Lee^a,
Kyu-Hwan Yang^c, Song-Kyu Park^{a,*}, Hwan Mook Kim^{a,*}

^a Bioevaluation Center, KRIBB, Cheongwon, Chungbuk, Republic of Korea

^b College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea

^c Gachon BioNano Research Institute, Kyungwon University, Kyunggi, Republic of Korea

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Abstract

Silymarin has been known to inhibit chemical-induced irritant contact dermatitis. In the present study, we report that topical application of silymarin suppresses dust mite extract (DPE)-induced atopic dermatitis (AD) in NC/Nga mice. Repeated topical application of ears with DPE caused AD-like skin lesions in NC/Nga mice. However, silymarin reduced AD-like skin lesions in these mice, resulting in decreased ear swelling and leukocyte infiltration into the ear. Moreover, our results showed that mast cell infiltration into the ear was suppressed by silymarin treatment in DPE-treated NC/Nga mice. Silymarin also reduced plasma level of IL-4 and IgE in these mice. Further study demonstrated that the mRNA expression of IL-4 was increased and that of IFN- γ was decreased by DPE treatment in the ears of NC/Nga mice. However, DPE-induced changes in IL-4 and IFN- γ mRNA expression were reversed by silymarin. DPE-induced increase in TNF- α mRNA expression was also suppressed by silymarin treatment. The results presented in this report suggest that silymarin might be beneficial for the treatment of AD.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease which occurs mainly in childhood and a pervasive health

problem in developed countries. Both genetic and environmental factors are known to be involved in the pathogenesis of AD [1]. It has been reported that the incidence of AD in children is closely linked to the prevalence of AD in their parents [2]. However, a variety of environmental factors, such as environmental pollutants and microbial infection, are also implicated in the increased occurrence of AD [3,4]. House dust mites, such as *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, are the most common environmental allergens associated with human AD [5]. In

* Corresponding authors. Park is to be contacted at Tel.: +82 43 240 6521; fax: +82 43 240 6529. Kim, Tel.: +82 43 240 6520; fax: +82 43 240 6529.

E-mail addresses: spark123@kribb.re.kr (S.-K. Park),
hwanmook@kribb.re.kr (H.M. Kim).

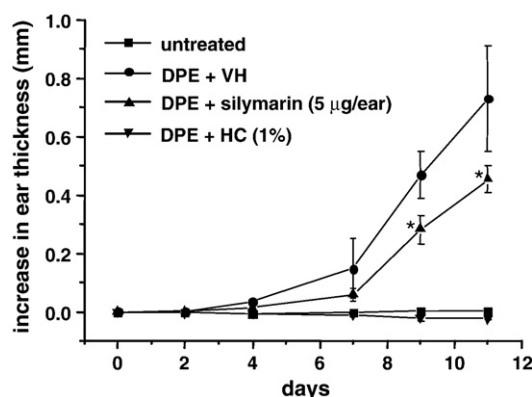


Figure 1 Effect of silymarin on DPE-induced increase in ear thickness in NC/Nga mice. Vehicle (AOO), silymarin (5 µg/ear) or HC (1%) was pretreated for 1 h every time before DPE treatment (10 mg/ml, 20 µl/ear). DPE was treated three times a week for 11 days. Ear thickness was measured every time before silymarin treatment and at the end of experiment. Data are presented as mean ± SD of six determinations. Significance was determined using Dunnett's *t*-test versus vehicle-treated group (**p* < 0.05). HC: hydrocortisone.

allergic diseases, it has been suggested that Th2 responses predominate over Th1 responses, showing increased expression of Th2 cytokines, including interleukin-4 (IL-4), IL-5, and IL-13 [6]. AD patients commonly exhibit hyperproduction of IgE and it has been reported that Th2 cytokines, such as IL-4, mediate IgE isotype switch in B lymphocyte [1]. Mast cells are known as key effector cells in IgE-mediated allergic disorders and activated by cross-linking of a high affinity IgE receptor, FcεRI. Upon activation, mast cells undergo degranulation and release a variety of biologically active substances, which play an important role in host defenses and allergic reactions [7]. TNF-α is also implicated in the pathogenesis of AD and plays an important role in the early stage of AD [6].

Silymarin is a flavonoid isolated from the milk thistle, *Silybum marianum*. Silymarin has been used clinically for a long time to treat liver diseases in Europe [8,9]. Silymarin also has a variety of biological activity, including anti-cancer

and anti-inflammatory activities [10,11]. In addition, skin cancer chemopreventive effect of silymarin has been studied extensively [12,13]. Recently, we demonstrated that topical application of silymarin reduces chemical-induced irritant contact dermatitis in mice [14]. In the present study, we investigated the effect of topically applied silymarin on AD in mice. We examined the effect of silymarin on AD using dust mite extract (DPE)-induced AD model in NC/Nga mice, which is known to be genetically predisposed to develop AD-like skin lesions. In addition, the effect of silymarin on plasma level of IL-4 and IgE and mast cell infiltration was evaluated in DPE-treated NC/Nga mice. The effect of silymarin on DPE-induced mRNA expression of IL-4, IFN-γ and TNF-α was also determined in the ears of NC/Nga mice.

2. Materials and methods

2.1. Chemicals and animals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Five-week-old female BALB/c mice and NC/Nga mice were purchased from Koatech (Pyungtaek, Gyeonggi, Korea) and Charles River Japan (Kanagawa, Japan), respectively, and cared for as described previously [15]. All animal experiments were conducted using protocols approved by Institutional Animal Care and Use Committee at Korea Research Institute of Bioscience and Biotechnology. Silymarin (Sigma-Aldrich) was dissolved in AOO (acetone:olive oil = 4:1) for topical application. Crude extracts of *Dermatophagoides pteronyssinus* (DPE) was purchased from Woongbee Meditech, Inc. (Seoul, Korea) and dissolved in phosphate buffered saline (PBS) containing 0.5% Tween 20 as described previously [16].

2.2. Induction of AD-like skin lesions in NC/Nga mice

Induction of AD-like skin lesions using mite antigen was performed as described previously [16] with slight modifications. 20 µl of DPE (10 mg/ml) was painted onto the dorsal surface of ears three times a week for 11 days. Silymarin (5 µg/ear) was topically applied 30 min before DPE painting.

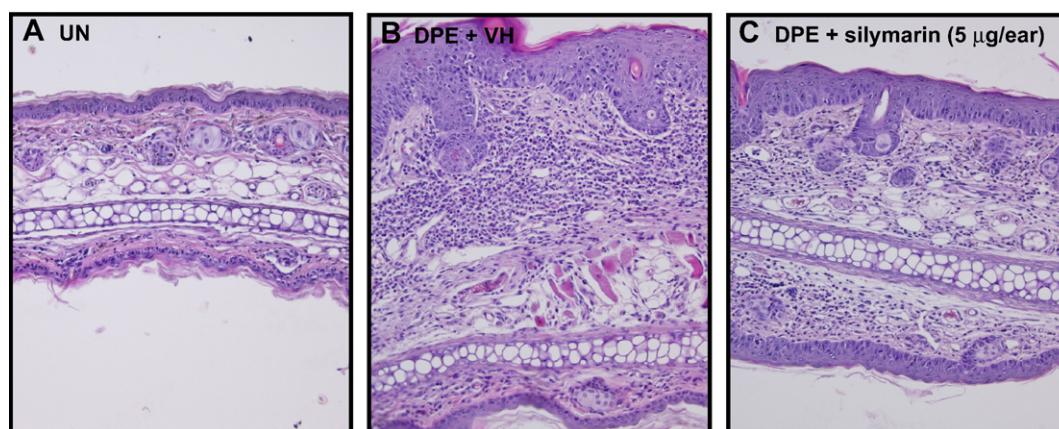


Figure 2 Histopathological analysis. The ears of untreated (A), DPE plus vehicle (AOO)-treated (B) and DPE plus silymarin (5 µg/ml)-treated (C) NC/Nga mice were excised, fixed with 10% formaldehyde, embedded in paraffin and thin sections were made. The skin sections were stained with hematoxylin and eosin.

Ear thickness was measured using dial thickness gauge immediately before silymarin treatment. At day 11, final ear thickness was measured and blood samples were collected by orbital puncture. Plasmas were prepared from blood samples and stored at -70°C for further analysis. After blood collection, ears were removed and used for histopathological analysis.

2.3. Histopathology

The ears were fixed with 10% formaldehyde, embedded in paraffin and thin sections were made. After the skin sections were stained with hematoxylin and eosin, the microscopic examination was carried out and photographed. For measurement of mast cell infiltration, skin sections were stained with toluidine blue and the number of mast cells in five sites chosen at random was counted.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The level of IL-4 and IgE in blood was measured by ELISA. The amount of IL-4 in plasma was measured by sandwich immunoassays using a protocol supplied by R&D Systems (Minneapolis, MN, USA). IgE levels were measured using IgE

ELISA kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to manufacturer's instruction.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

The expression of mRNA transcripts of IL-4 (forward: 5'-gaatgtaccaggagccatc-3', reverse: 5'-ctcagtactacgagtaatcca-3'), IFN- γ (forward: 5'-agcggctgactgaactcagattgtag-3', reverse: 5'-gtcacagtttctcagctgtatagg-3'), TNF- α (forward: 5'-cctgtagcc-cagctcgtagc-3', reverse: 5'-ttgacctcagcgtgagttg-3') and β -actin (forward: 5'-tggaatcctgtggcatccatgaaac-3', reverse: 5'-taaaacgcagctcagtaacagtcgc-3') was determined by real-time RT-PCR. Total RNA was isolated using TRIzol[®] Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) as described previously [17]. Equal amounts of RNA were reverse transcribed into cDNA using oligo(dT)₁₅ primers. iQ[™] SYBR[®] Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and iCycler iQ[™] Real-time PCR Detection System (Bio-Rad Laboratories, Inc.) were used for real-time PCR analysis. For amplification, samples were heated to 94°C for 8 min and cycled 45 times at, 94°C for 30 s, and 56°C for 30 s, and 72°C for 45 s. Using standards, the amount of IL-4, IFN- γ and TNF- α cDNA was determined and normalized by the amount of β -actin cDNA.

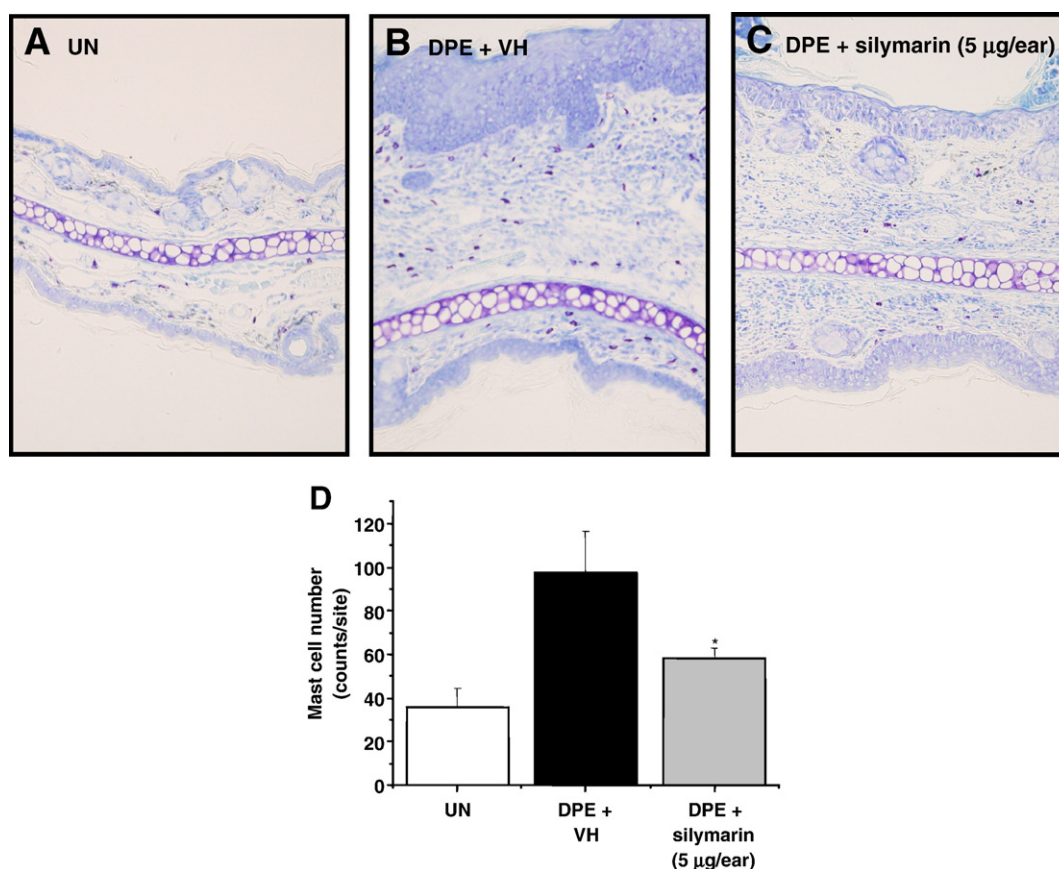


Figure 3 Effect of silymarin on DPE-induced infiltration of mast cells into the ear of NC/Nga mice. The ears of untreated, DPE plus vehicle (A00)-treated and DPE plus silymarin (5 µg/ml)-treated NC/Nga mice were excised, fixed with 10% formaldehyde, embedded in paraffin and thin sections were made. (A) The skin sections were stained with toluidine blue. (B) The number of mast cells in five sites chosen at random was counted. Data are presented as mean $\pm$ SD of triplicate determinations. Significance was determined using Dunnett's *t*-test versus vehicle-treated group (* $p < 0.05$).

2.6. Statistical analysis

The results are expressed as mean $\pm$ S.D. One-way ANOVA and Dunnett's *t*-test was used for multiple comparisons using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). The criterion for statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of silymarin on DPE-induced AD-like skin lesions in NC/Nga mice

To examine the effect of silymarin on AD in mice, we used dust mite extract-induced AD model in NC/Nga mice. As shown in Fig. 1, repeated topical application of DPE significantly increased ear thickness in NC/Nga mice. However, treatment with 5 μ g/ear of silymarin significantly suppressed DPE-induced increase in ear thickness (Fig. 1). Silymarin (5 μ g/ear) caused 40% and 38% inhibition of DPE-induced ear swelling at day 9 and day 11, respectively (Fig. 1). Hydrocortisone (HC) was used as positive control and Fig. 1 shows that HC completely blocked DPE-induced ear swelling in NC/Nga mice until day 11. Histopathological analysis revealed that DPE increased the thickness of

both epidermis, the outermost layer of the skin, and dermis, a layer of skin beneath the epidermis (Fig. 2). Fig. 2 also shows that excessive leukocyte infiltration into the dermis was observed in DPE-treated ear. In consistent with the result of Fig. 1, histopathological data also demonstrated that silymarin reduced DPE-induced ear swelling (Fig. 2). Moreover, Fig. 2 shows that DPE-induced leukocyte infiltration was also blocked by silymarin treatment.

3.2. Effect of silymarin on DPE-induced mast cell infiltration in NC/Nga mice

To further investigate, we assessed the effect of silymarin on the infiltration of mast cells, an important effector cell involved in AD, into the ears. As shown in Fig. 3, treatment with DPE increased the infiltration of mast cells. However, this was significantly suppressed by silymarin treatment (Fig. 3).

3.3. Effect of silymarin on plasma level of IL-4 and IgE and mRNA expression of IL-4, IFN- γ and TNF- α in the ear

We also examined the effect of silymarin on plasma level of IL-4 and IgE. DPE treatment significantly increased plasma level of IL-4 and IgE. As shown in Fig. 4, plasma level of IL-4 was increased 2.2-fold and that of IgE was increased 6.9-fold by DPE treatment versus baseline level. Silymarin treatment caused 85% and 72% inhibition of plasma level of IL-4 and IgE, respectively (Fig. 4). Moreover, the mRNA expression of IL-4 was increased by DPE treatment and silymarin significantly suppressed DPE-induced mRNA expression of IL-4 (Fig. 5A). In contrast, Fig. 5B shows that IFN- γ mRNA level was decreased by DPE treatment and this was reversed by silymarin treatment. In addition, the mRNA expression of a proinflammatory cytokine, TNF- α , was markedly increased and silymarin significantly inhibited TNF- α expression in DPE-treated NC/Nga mice (Fig. 5C).

4. Discussion

In this study, we investigated the effect of silymarin on AD-like skin lesions using DPE-induced AD model in NC/Nga mouse. NC/Nga mouse model has been considered as one of the most valuable mouse model representing human AD [5,18,19]. This model has been used for the evaluation of drug candidates developed for the treatment of AD [20,21]. Previously, we established a modified NC/Nga mouse model and suggested that this model might be a useful tool to evaluate drug candidates of AD [16]. Here, we clearly demonstrated that typical manifestations of AD can be observed in NC/Nga mice by repeated topical application of DPE. Ear swelling and eczematous skin lesion were observed and plasma level of IL-4 and IgE was significantly increased. Histopathological analysis also showed that infiltration of leukocytes, especially mast cells, into the ear was evident. However, treatment with silymarin significantly inhibited ear swelling induced by DPE treatment in NC/Nga mice, suggesting that silymarin exerts a beneficial effect on DPE-induced AD in NC/Nga mice. Considering the potential adverse effects of long-term treatment of steroids, such as hydrocortisone, it is assumed that treatment of atopic patients with silymarin alone or in combination with other agents might be helpful although the effect of silymarin is

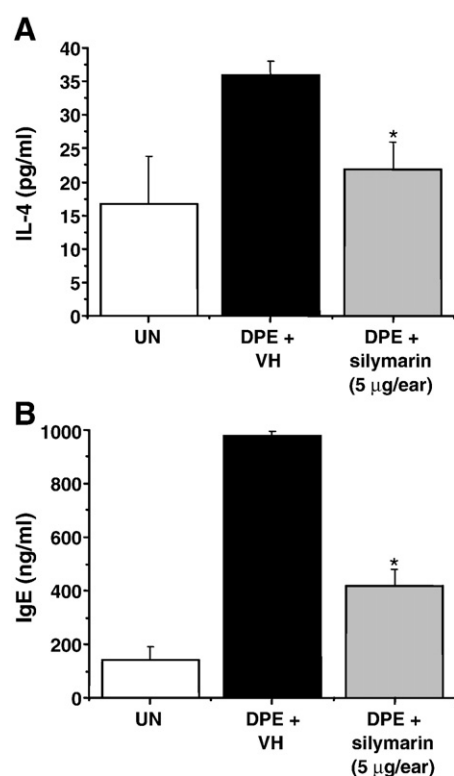


Figure 4 Effect of silymarin on DPE-induced increase in plasma level of IL-4 and IgE in NC/Nga mice. The blood samples of untreated, DPE plus vehicle (A00)-treated and DPE plus silymarin (5 mg/ml)-treated NC/Nga mice were collected by orbital puncture and plasma levels of IL-4 (A) and IgE (B) was quantified by ELISA. Data are presented as mean $\pm$ SD of triplicate determinations. Significance was determined using Dunnett's *t*-test versus vehicle-treated group (* $p < 0.05$).

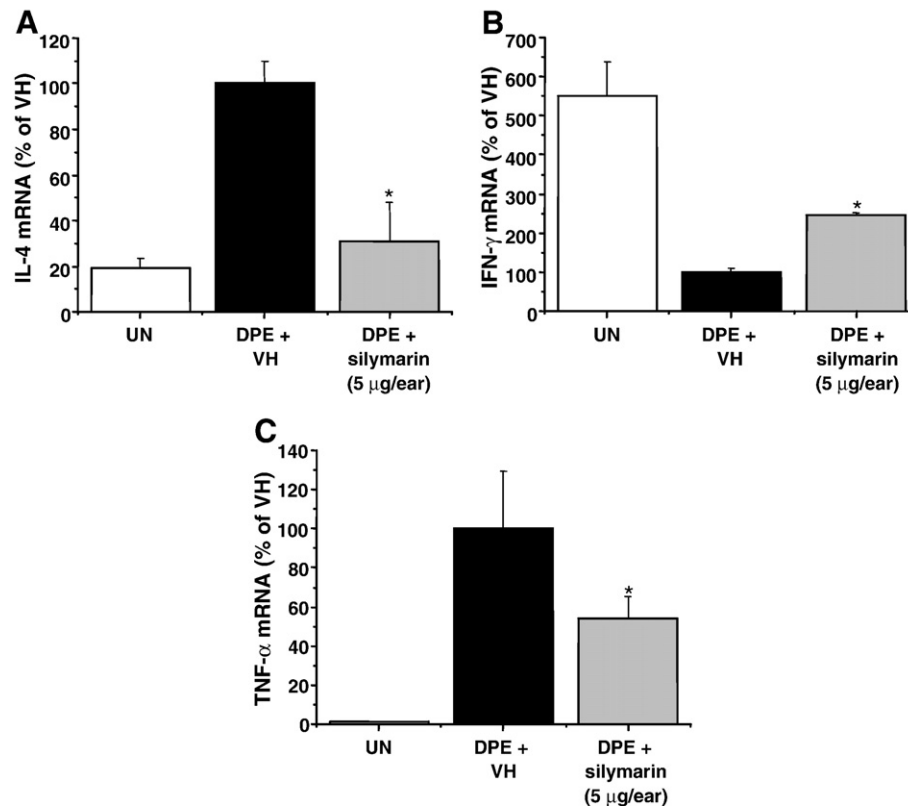


Figure 5 Effect of silymarin on the mRNA expression of IL-4, IFN- γ and TNF- α in DPE-treated NC/Nga mice. The ears of untreated, DPE plus vehicle (A00)-treated and DPE plus silymarin (5 mg/ml)-treated NC/Nga mice were excised and total RNA was isolated. Quantitative RT-PCR was performed as described in Materials and methods. Data are presented as mean $\pm$ SD of triplicate determinations. Significance was determined using Dunnett's *t*-test versus vehicle-treated group (* $p < 0.05$).

weaker than that of steroids. As described previously, mast cells are key effector cells involved in the development of various allergic diseases, including AD. Matsuoka and coworkers suggested that the infiltration of mast cells into the dermis is a necessary factor to define an appropriate animal model for AD [5]. In this report, we clearly demonstrated that repeated topical application of DPE resulted in mast cell infiltration into the dermis in the ears of NC/Nga mice, suggesting that the animal model of AD used in this study satisfy the conditions previously suggested. However, topical application of silymarin significantly inhibited the infiltration of mast cells induced by DPE treatment. These results demonstrate that treatment with silymarin suppresses DPE-induced symptoms of AD in NC/Nga mice, suggesting silymarin as a promising topical agent for AD patients.

For the past decades, the Th1/Th2 paradigm has been applied to explain the pathogenesis of immune diseases, including AD. It has been reported that the balance between Th1 and Th2 response is important for appropriate immune function and disruption of Th1/Th2 balance leads to various immune-related diseases, such as autoimmune diseases and allergic diseases [1]. In general, AD patients show Th2-dominant phenotype, showing increased expression of Th2 cytokines, such as IL-4, and increased serum level of IgE. Therefore, we examined the effect of silymarin on plasma level of IL-4 and IgE in DPE-treated NC/Nga mice. Our results demonstrated that silymarin significantly suppressed DPE-

induced increase in IL-4 and IgE level in plasma in NC/Nga mice. Further study demonstrated that the mRNA expression of IL-4 was increased and that of IFN- γ was decreased by DPE treatment in the ears of NC/Nga mice, showing that Th1/Th2 balance was shifted to Th2 response in DPE-treated NC/Nga mice. However, topical application of silymarin reversed DPE-induced Th2 shift in NC/Nga mice, suggesting that silymarin might inhibit DPE-induced AD-like skin lesions by restoring Th1/Th2 balance. Our unpublished data showed that silymarin has no direct effect on IL-4 signaling in splenic B cells. From these results, we can assume that the inhibitory effect of silymarin on DPE-induced AD in NC/Nga mice might be mediated, at least in part, by suppressing Th2 response and subsequent down-regulation of IgE and mast cell activation.

The production of proinflammatory cytokines, such as TNF- α , by epidermal cells has been known as one of the main events mediating the initiation of AD [6]. TNF- α and other proinflammatory cytokines produced at the initiation stage of AD induce the expression of a variety of chemokines and adhesion molecules which direct the recruitment, proliferation and survival of leukocytes within the skin [6]. Silymarin has been known to exert anti-inflammatory activity and inhibit TNF- α expression [14,22]. Zi and coworkers demonstrate that topical application of silymarin blocked phorbol ester- or okadaic acid-induced TNF- α expression in mouse epidermis [22]. We also previously demonstrated that 2,4-

dinitrochlorobenzene-induced TNF- α expression in mice skin and proinflammatory cytokine-induced TNF- α mRNA expression in human keratinocytes were suppressed by silymarin treatment [14]. Therefore, it is assumed that the inhibitory effect of silymarin on leukocyte infiltration in our models of AD might be mediated, at least in part, by blocking TNF- α expression and subsequent down-regulation of chemokines and adhesion molecules. However, further studies are required to elucidate the exact mechanism responsible for the inhibitory effect of silymarin on DPE-induced AD-like skin lesions.

In summary, the results presented in this report demonstrate that silymarin suppresses DPE-induced AD-like skin lesions in NC/Nga mice and this might be mediated, at least in part, by suppressing Th2 response and TNF- α expression. Our results suggest that silymarin might be a potential therapeutic agent for prevention and treatment of AD.

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