

Melatonin inhibits the development of 2,4-dinitrofluorobenzene-induced atopic dermatitis-like skin lesions in NC/Nga mice

Abstract: Atopic dermatitis (AD) is a common disease in children, and epicutaneous treatment with a chemical hapten such as 2,4-dinitrofluorobenzene (DNFB) evokes an AD-like reaction in NC/Nga mice under specific pathogen-free conditions. Melatonin (*N*-acetyl-5-methoxytryptamine) is synthesized by the pineal gland, has several different physiologic functions, which include seasonal reproduction control, immune system modulation, free radical scavenging, and inflammatory suppression. In the present study, we investigated whether melatonin suppresses DNFB-induced AD-like skin lesions in NC/Nga mice. The topical administration of melatonin to DNFB-treated NC/Nga mice was found to inhibit ear thickness increases and the skin lesions induced by DNFB. Furthermore, interleukin (IL)-4 and interferon (IFN)- γ secretion by activated CD4⁺ T cells from the draining lymph nodes of DNFB-treated NC/Nga mice were significantly inhibited by melatonin, and total IgE levels in serum were reduced. Our findings suggest that melatonin suppresses the development of AD-like dermatitis in DNFB-treated NC/Nga mice by reducing total IgE in serum, and IL-4 and IFN- γ production by activated CD4⁺ T cells.

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Introduction

Atopic dermatitis (AD), characterized by chronic and relapsing inflammatory dermatitis with immunologic disturbances, and pruritic and eczematous skin lesions, is one of the most common skin diseases. Furthermore, its incidence is increasing in industrialized countries and 10–20% of children worldwide are affected by AD [1]. The clinical symptoms of AD are varied and may change with disease progression [2]. In addition, it has been reported that sleep disturbances and AD might be associated [3]. AD is a multi-factorial disease with genetic, immunologic, and environmental components, and its pathogenic mechanisms have not been clearly defined [4]. It is a biphasic inflammatory skin disease, provoked by Th1/Th2 immune response [5]. Acute AD skin lesions exhibit Th2 type inflammation because of the infiltration of CD4⁺ T cells, which secrete interleukin (IL)-4, IL-5, and IL-13 [5], whereas, in contrast, the chronic phase is characterized by Th1 type immune responses and delayed-type hypersensitivity (DTH) reactions [5, 6]. DTH reactions are cell-mediated immune responses and usually evoked by chemical allergens [6]. Furthermore, it is known that 2,4-dinitrofluorobenzene (DNFB)-induced contact hypersensitivity is related to the activation of Th1 cells [7].

The NC/Nga mouse model is the most studied animal model of AD. This model is considered to be important for understanding and developing treatments for AD, as the symptoms displayed are similar to those observed in man [8]. Although NC/Nga mice spontaneously develop AD-like skin lesions under conventional conditions, the low

incidences of AD-like skin lesions are problematic [8]. In contrast, the administration of a chemical hapten, for example, DNFB, to these mice produces reproducible AD-like lesions [8]. In addition, repeated treatment with DNFB evokes AD-like skin lesions in NC/Nga mice under specific pathogen free (SPF) conditions [9, 10].

Melatonin (*N*-acetyl-5-methoxytryptamine) plays an important role in the regulation of seasonal reproduction and circadian rhythms [11, 12]. Melatonin is also synthesized in the gastrointestinal tract, and skin as well as in many other organs [13, 14]. Melatonin may also play a role in cutaneous biology as a melatonergic system has been shown to be fully expressed in rodent and human skin [15–20]. Furthermore, melatonin as well as several of its metabolites has antioxidant, anti-inflammatory, and anti-aging effects [21–29]. Recently, it was reported that melatonin and its precursor L-tryptophan inhibit the inflammatory response associated with contact hypersensitivity [30]. However, it has not been determined whether melatonin suppresses the development of AD. In the present study, we investigated whether melatonin suppresses DNFB-induced AD-like skin lesions in NC/Nga mice.

Materials and methods

Animals

Male NC/Nga mice, 8 wks of age, were obtained from Japan SLC (Shizuoka, Japan) and maintained under SPF conditions. Animals were housed in an air-conditioned animal room (25 ± 1°C; relative humidity 40 ± 5%) and

fed a laboratory diet and water. The experiments were complied with the guidelines issued by the ethical committee for animal welfare at Kyung Hee University and all procedures were conducted in accordance with the U.S. National Institute of Health guidelines.

Drugs

Mice were randomly assigned to one of four groups: the control, DNFB, DNFB + melatonin (10 mg/kg), and DNFB + melatonin (20 mg/kg). Each group consisted of five mice. The melatonin-treated mice received the indole (Sigma, St. Louis, MS, USA) at 10 or 20 mg/kg i.p. daily in the morning at 10:00 hours. Melatonin was dissolved in several drops of dimethyl sulfoxide and diluted with phosphate-buffered solution (PBS).

DNFB-induced dermatitis

Dermatitis was induced by the repeated application of 25 μ L of 0.15% DNFB in acetone/olive oil (3:1) to the outer and inner surfaces of mouse ears and 100 μ L of the same solution was applied to shaved back skin once on days 1 and 5. On days 9 and 13, sensitized mice were challenged by applying 0.2% DNFB to the skin surfaces. Controls were treated with the identical volumes of vehicle. Ear thicknesses were measured with a thickness gauge (Digimatic Indicator, Mitsutoyo, Tokyo) on days 1 (prior to treatment application), 2, 4, 5, 6, 8, 9, 10, 12, and 13. Macroscopic assessment of the severity of dermatitis was determined by a scoring method as described earlier [31–34]. The degree of each sign were scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). This scoring was based on the severity of erythema (hemorrhage), edema, excoriation (erosion), and dryness (scaling), as described previously. The total score (minimum 0 and maximum 12) from the five symptoms of each mouse was taken as the score for that mouse. Assessment was performed by an investigator who was blind about the grouping of the animals.

Histologic analysis

Ear samples were taken 24 hr after final DNFB painting and fixed in 10% buffered formalin, embedded in paraffin, sectioned at 3 μ m, and then stained with hematoxylin and eosin.

Serum IgE measurements

Total serum samples were prepared from blood collected 24 hr after DNFB application on day 13, and IgE concentrations in serum were quantified using BD OptEIA™ Set Mouse IgE ELISA kits (BD Pharmingen, San Diego, CA, USA). In brief, a 1:250 dilution of anti-mouse IgE monoclonal antibody in PBS was placed in each well of an immunoplate (Corning 3590 96-well EIA/RIA plate, Corning, NY, USA) and maintained overnight at 4°C. After washing wells with PBS containing 0.05% Tween 20 (washing buffer) three times, 250 μ L of PBS containing 1% bovine serum albumin (BSA in PBS) was placed in each well. After 1 hr at room temperature, wells were washed

three times with washing buffer and 200 μ L aliquots of serum samples, diluted 100–200-folds with BSA in PBS, were added to the wells. After further incubation for 2 hr at room temperature, wells were again washed five times with washing buffer, 100 μ L of streptavidin-horseradish peroxidase-conjugated detection antibody (SAv-HRP) diluted 250-fold with washing buffer was then added, and the plate was maintained 1 hr at room temperature. After washing seven times with washing buffer, the enzyme reaction was initiated by adding 100 μ L of substrate solution (0.1 M citric acid, 0.2 M Na₂HPO₄, o-phenylene diamine and H₂O₂) for 50 min at room temperature. The reaction was terminated by adding 50 μ L of 1 M H₂SO₄ to each well, and absorbance at 450 nm was measured immediately using an enzyme-linked immunosorbent assay (ELISA) reader (EL800) (Bio-Tek, Winooski, VT, USA). A standard curve was prepared using recombinant mouse IgE diluted with BSA in PBS.

Measurement of cytokine production in vitro

Lymph nodes were excised from mice and CD4⁺ T cells were purified using a MACS separation column (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. The purified CD4⁺ T cells (1×10^6 cells/mL) were then stimulated with plate bound anti-CD3 antibody (2C11; 5 μ g/mL) plus a soluble form of purified anti-mouse CD28 antibody (BD Pharmingen) (2 μ g/mL; 48 hr) in RPMI-1640 supplemented with 10% fetal bovine serum, 50 μ M of β -mercaptoethanol, and 2 mM of glutamine. The productions of IL-4 and IFN- γ after T-cell activation were quantified by ELISA (Biolegend, San Diego, CA, USA).

Statistical analysis

Results are expressed as mean $\pm$ S.E.M. The significant changes were evaluated using the Student's *t*-test. Differences between experimental groups were evaluated using analysis of variance. *P* values <0.05 were considered significant.

Results

Repeated topical application of DNFB induces AD-like skin lesions in NC/Nga mice [9, 10], and in the present study, we investigated whether melatonin suppresses these lesions. DNFB-treated mice group exhibited AD-like skin lesions, which manifested as scaling, dried skin patches (Fig. 1). Melatonin (10 mg or 20 mg/kg i.p.) treated mice shows mildly reduced lesion severities as compared with the DNFB group (Fig. 1). Ear thicknesses were increased gradually with number of DNFB challenges (Fig. 2). Melatonin at 10 mg or 20 mg/kg significantly reduced DNFB-induced ear thickness increases (Fig. 2). Furthermore, DNFB-treated mice showed hypertrophy, hyperkeratosis, and dense inflammatory cell infiltration, and melatonin significantly inhibited these symptoms (Table 1 and Fig. 3), but at these levels no dose-dependent effect was observed (Figs 2 and 3).

An elevated serum IgE level is a hallmark of AD. After DNFB treatment on day 13, serum samples were prepared

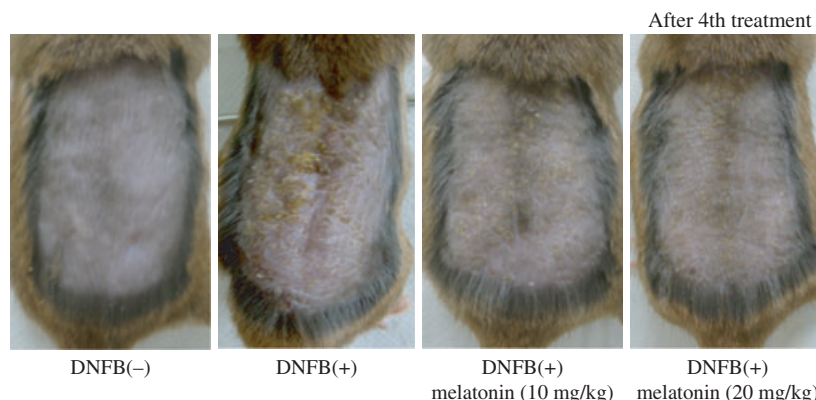


Fig. 1. Comparisons of 2,4-dinitrofluorobenzene (DNFB)-induced atopic dermatitis-like skin lesions on NC/Nga mice after melatonin administration. Dermatitis was induced by repeatedly painting 0.15% DNFB (acetone 3:olive oil 1) on shaved back skin once on days 1 and 5. On days 9 and 13 sensitized mice were challenged with 0.2% DNFB on same areas. Identical volumes of acetone/olive oil were painted on control mice. Melatonin was injected i.p. daily at 10:00 hours from the day of first DNFB application. The photographs above were taken after treatment completion. DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB + phosphate-buffered solution and dimethyl sulfoxide 1% treated, DNFB + melatonin (10 mg/kg) and DNFB + melatonin (20 mg/kg) treated. Five animals were allocated to each group.

and total IgE levels were determined by ELISA. Repetitive DNFB application was found to increase serum IgE levels compared with controls (Fig. 4), and serum IgE level in the melatonin treated groups was significantly lower than in the DNFB-treated group at this time (Fig. 4). AD skin lesions exhibit Th2 or Th1 dominant inflammation dependent on stage [5]. These inflammation types are dependent on CD4⁺ T cells in draining lymph nodes and skin lesions [35]. Therefore, we investigated whether melatonin regulated the Th1 and Th2 reactions of CD4⁺ T cells in draining lymph nodes. CD4⁺ T cell from draining lymph nodes in the four study groups were stimulated with anti-CD3 antibody plus anti-CD28 antibody for 48 hrs. Supernatants were then collected and IL-4 and IFN- γ levels were measured by ELISA. The production of IL-4 and IFN- γ was found to be

Table 1. Macroscopic assessment of severity of dermatitis

Treatment	DNFB(-) (n = 5)	DNFB(+) (n = 5)	DNFB(+)	
			Melatonin (10 mg/kg) (n = 5)	Melatonin (20 mg/kg) (n = 5)
1st treated	0	0	0	0
2nd treated	0	2.75 $\pm$ 1.25	1.5 $\pm$ 1.29	0.5 $\pm$ 0.57*
3rd treated	0	4.5 $\pm$ 2.08	2.25 $\pm$ 1.25	2 $\pm$ 0*
4th treated	0	8.25 $\pm$ 1.7	4 $\pm$ 0.81*	3.75 $\pm$ 0.95*

Each value was obtained from five mice per group. DNFB(-): treated with acetone : olive oil (3:1), DNFB(+): treated with DNFB + PBS and 1% DMSO, DNFB + melatonin (10 mg/kg) and DNFB + melatonin (20 mg/kg) treated. Data are mean $\pm$ S.E.M. (n = 5). *P < 0.05 versus DNFB(+) mice.

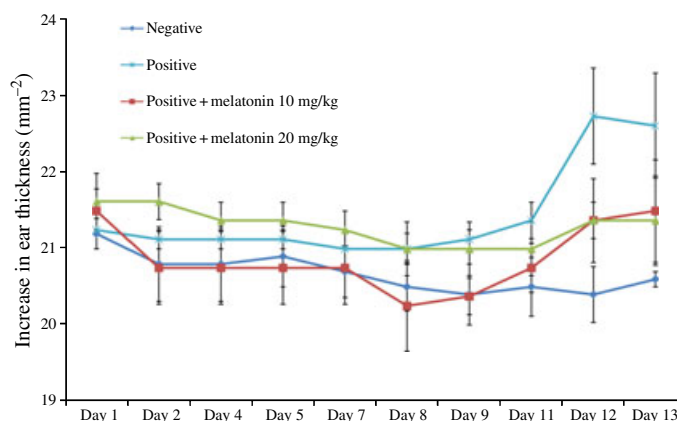


Fig. 2. Effect of melatonin on ear skin swelling induced by repeated 2,4-dinitrofluorobenzene (DNFB) treatment in the NC/Nga mouse atopic dermatitis model. Dermatitis was induced by repeatedly painting 0.15% DNFB (acetone 3:olive oil 1) on outer and inner mouse ears once on days 1 and 5. On days 9 and 13 sensitized mice were challenged with 0.2% DNFB on both sides of each ear. An identical volume of acetone/olive oil was painted onto control mice. Melatonin was injected i.p. daily at 10:00 hours from the day of first DNFB application. Skin thicknesses were determined as described in Materials and methods. DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB + phosphate-buffered solution and dimethyl sulfoxide 1% treated, DNFB + melatonin (10 mg/kg) and DNFB + melatonin (20 mg/kg) treated. Ear thicknesses were measured after each DNFB application. Values are mean $\pm$ S.E.M. of five animals per group.

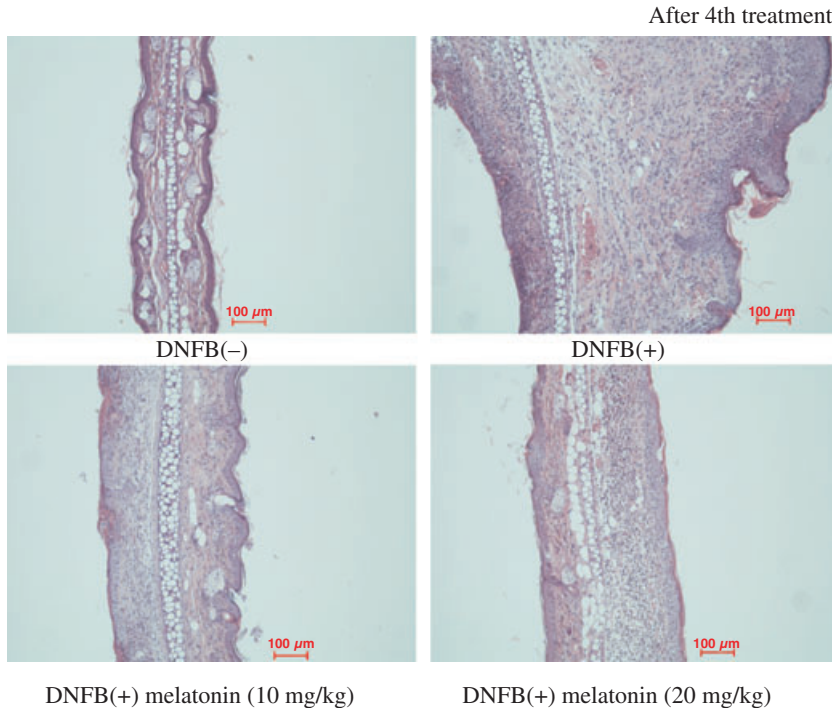


Fig. 3. Histologic analysis of ear skin lesions on 2,4-dinitrofluorobenzene (DNFB)-treated NC/Nga mice after melatonin administration. Dermatitis was induced by repeatedly painting of 0.15% DNFB (acetone 3:olive oil 1) on outer and inner ear surfaces once on days 1 and 5. On days 9 and 13 sensitized mice were challenged with 0.2% DNFB on surfaces. An identical volume of acetone/olive oil was painted on control mice. Melatonin was injected i.p. daily at 10:00 hours from the day of first DNFB application. The sections were prepared as described in Materials and methods. DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB + phosphate-buffered solution and DMSO 1% treated, DNFB + melatonin (10 mg/kg) and DNFB + melatonin (20 mg/kg) treated. Ear sections were stained with hematoxylin and eosin.

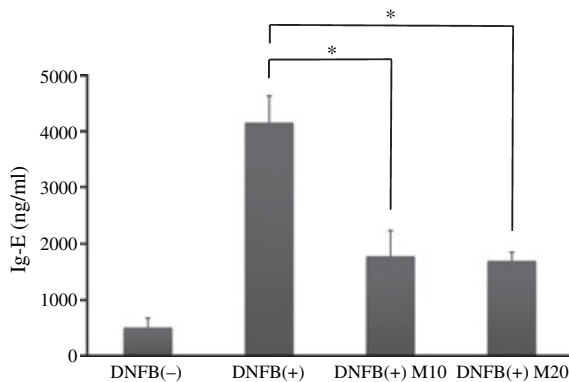


Fig. 4. Melatonin inhibited IgE level of 2,4-dinitrofluorobenzene (DNFB)-sensitized NC/Nga mice. The IgE levels were determined by enzyme-linked immunosorbent assay in serum obtained from DNFB-sensitized NC/Nga mice. Mice were painted on days 1 and 5 with 0.15% DNFB on both sides of ears and shaved back skin. On days 9 and 13 sensitized mice were challenged with 0.2% DNFB on same areas. Identical volumes of acetone/olive oil were painted on control mice. Melatonin was injected i.p. daily at 10:00 hours from the day of first DNFB application. Total IgE levels in the indicated groups were measured 24 hr after final administration. DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB + phosphate-buffered solution and dimethyl sulfoxide 1% treated, DNFB(+)M10; DNFB + melatonin (10 mg/kg) and DNFB(+)M20; DNFB + melatonin (20 mg/kg) treated. Data are mean $\pm$ S.E.M. (n = 5). * P < 0.05 versus DNFB(+) mice.

greater in the activated CD4⁺ T cells of the DNFB group than in those of the control group (Fig. 5). IL-4 and IFN- γ productions by activated CD4⁺ T cells were found to be significantly inhibited by melatonin treatment (Fig. 5).

Discussion

Atopic dermatitis is the most commonly encountered skin disease. Its clinical symptoms vary, but the condition is characterized by erythema, edema, excoriation, and scaling [1]. In addition, it has been reported that, depending on its severity, AD causes sleep disturbances [3]. On the other hand, melatonin is considered to regulate the sleep-waking cycle and other biologic rhythms as well as inflammatory reactions [12, 36]. Mammalian skin contains the entire molecular and biochemical machinery necessary to transform L-tryptophan to melatonin, through serotonin and *N*-acetylserotonin intermediates [17, 20]. Recently, it was reported that serum melatonin levels are reduced in AD patients, and that melatonin and its precursor L-tryptophan inhibit the inflammatory response associated with contact hypersensitivity [30] and melatonin prevents ultraviolet radiation-induced erythema and skin damage [17, 20, 37, 38]. Herein, we found that melatonin reduces the severities of AD-like skin lesions in DNFB-treated NC/Nga mice.

Increased total serum IgE levels are a hallmark of AD, and it is well known that IL-4 (a Th2-type cytokine) induces the class switching to IgM secreting B cells to IgE secreting B cells. Furthermore, elevated serum IgE levels have several effects on skin inflammation [39–41]. IgE expression is known to cause both acute and chronic phase skin symptoms, and it has been reported that repeated painting with DNFB increases serum IgE in NC/Nga mice [5, 7, 9, 10]. Consistent with these reports, we found that total serum IgE levels were significantly increased by repeated DNFB treatment in NC/Nga mice, as was IL-4 production by activated CD4⁺ T cells from these mice (Figs 4 and 5A). However, melatonin treatment significantly reduced levels of total serum IgE in DNFB-treated mice and IL-4 production by activated CD4⁺ T cells (Figs 4 and 5A).

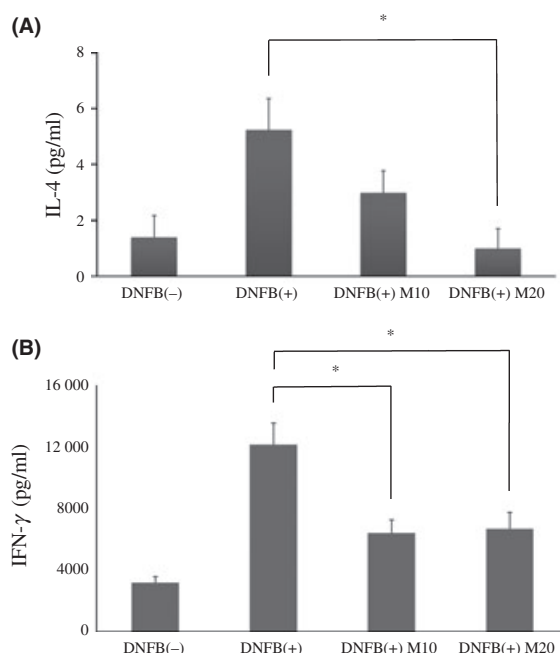


Fig. 5. Effect of melatonin on the productions of interleukin (IL)-4 (A) and interferon (IFN)- γ (B) by CD4⁺ T cells collected from the lymph nodes of DNFB-treated NC/Nga mice. Lymph nodes were excised 24 hr after final DNFB administrations and CD4⁺ T cells were purified. Cells (1×10^6 cells/mL) were then stimulated with plate bound anti-CD3 antibody (2C11; 5 μ g/mL) plus a soluble form of purified anti-mouse CD28 antibody (2 μ g/mL) for 48 hr. The productions of IL-4 and IFN- γ after T-cell activation were quantified by enzyme-linked immunosorbent assay. DNFB(-); acetone:olive oil (3:1) treated, DNFB(+); DNFB + phosphate-buffered solution and dimethyl sulfoxide 1% treated, DNFB(+) M10; DNFB + melatonin (10 mg/kg) and DNFB(+) M20; DNFB + melatonin (20 mg/kg) treated. Data are mean $\pm$ S.E.M. (n = 5). * P < 0.05 versus DNFB(+) mice.

The cytokines produced by activated CD4⁺ T cells determine their differentiations to the Th1 or Th2 cell lineage [42–46]. Furthermore, Th1 and Th2 cell ratio is known to be related to the overall pattern of cytokine production of IFN- γ or IL-4 and to clinical outcome in a variety of pathologic states [42–46]. In the present study, repeated DNFB treatment enhanced IFN- γ and IL-4 production from activated CD4⁺ T cells of draining lymph nodes in NC/Nga mice (Fig. 5B). It is known that IFN- γ (a polarized Th1-type cytokine) plays a role in determining the chronicity of AD lesions, and the major skin infiltrating T cells secrete IFN- γ in chronic AD patients, whereas IL-4 (a polarized Th2-type cytokine) and IgE are elevated in acute AD patients [28–30]. Furthermore, it has been reported that repeated exposure to DNFB induces IFN- γ (a polarized Th1-type cytokine) [7], and that melatonin promotes Th cell proliferation and improves their ability to secrete IL-4, but downregulates the levels of IL-2 and IFN- γ . These results suggest that melatonin acts by selectively activating Th2-like immune response [47].

In addition, we also found that IFN- γ production by activated lymph node CD4⁺ T cells is upregulated in DNFB-treated NC/Nga mice, and this too, was significantly inhibited by melatonin (Fig. 5B). However, in a

previous report, melatonin was found to activate human Th1 lymphocytes by increasing the productions of IL-2 and IFN- γ in vitro. On the other hand, Th2 cells appear to be unaffected by melatonin, as IL-4 levels were not modified by melatonin treatment. Several suggestions have been made regarding the mechanism of immune response regulation by melatonin [48], and further study is required to resolve this issue.

In conclusion, melatonin was found to reduce the development of AD-like skin lesions in DNFB-treated NC/Nga mice. The present study suggests that the mechanism responsible involves reduced IL-4 and IFN- γ production by activated CD4⁺ T cells.

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