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Research article

Leaf extracts of *Nandina domestica* Thunb ameliorate atopic dermatitis in NC/Nga mice treated with 1-chloro-2,4-dinitrobenzene (DNCB)

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Abstract: Atopic dermatitis (AD) is a skin disease. In this study, we observed the effects of *Nandina domestica* (ND) on AD-induced NC/Nga mice with 1-chloro-2,4-dinitrobenzene (DNCB)-treatment. ND significantly ameliorated the AD-skin signs with reducing of skin thickness and mast cell infiltration in the injuried skin of DNCB-induced AD model. Furthermore, ND reduced serum immunoglobulin E (IgE) levels and restored into normal condition the total cell number (TCN) in dorsal skin tissue, the axillary lymph node (ALN), and spleen after DNCB exposure. ND also decreased the number of CD23+/B220+ cells in the ALN and CD3+ cells in dorsal skin. Moreover, it decreased the interleukin (IL)-4, (IL)-5 and IL-13 levels but increased the interferon-γ levels in splenocytes. Immunohistofluorescence staining showed that ND significantly increased claudin1 and Sirt1 protein expressions in the AD-induced mice. Our results suggested that ND significantly ameliorates DCNB-induced AD.

Keywords: Atopic dermatitis; Nandina domestica; potential remedy; immunoglobulin E; dorsal skin tissue

1. Introduction

Atopic dermatitis (AD) is a atopic eczema showing inflammatory and intensely pruritic skin disease [1]. AD is raised by various interactions among environmental factors and disintergration of the immunity. AD showed various symptoms containing a fluctuating course, hyperactive immune response to environmental factors, skin barrier disintergration, drying, and itching [2].

AD exhibited skin thickening with characterstic infiltration of activated T cells and monocytes/macrophages within the derma [3]. Most of patients possessing AD show increased serum total IgE against various allergens [4]. Furethermore, the expression of T helper (Th) 2 cytokines (IL-4, IL-5, and IL-13) is increased in the AD-induced injured skin of AD [5], proposing that Th2 cells play important roles in disease progressing by AD.

In several reports, the pathogenesis and development of AD have been mostly attributed to the Th1/Th2 immune dysregulation. Accordingly, most therapies of AD have been conducted to reduce Th2-mediated immune responses [6]. However, raising many evidences have focused on a important role of structural abnormalities in the stratum corneum (SC) of AD pathophysiology [7]. Although any connection against the correlation of skin barrier disintergration and abnormal immune esponse still needs to be investigated, recent studies have reported the high synergism in dysregulation of immune response [8]. The reports show that both breaking skin barrier and abnormal immune responses are important factors in the progressing of AD and therefore, the multi-pronged treatment such as recovery of skin barriers and suppression of skin inflammation is essential for the effective method decreasing development of AD [9].

AD care is devoted on alleviating itching, repairing disintergration of skin barrier and attenuating the skin lesions. Most patients suffering from AD used emollients to improve health of skin and decrease itching of skin as well as anti-inflammatory agents such as glucocorticoids, cyclosporine A, azathioprine, and mycophenolate mofetil [10]. However, many patients limited current therapies becuase of their side effects [11]. Conventional products such as creams and ointments also were limited topical use on their skin. Therefore, passionate needs for novel topical agents generated to provide more effective treatment of AD skin lessions [12].

Nandina domestica (family of Berberidaceae) have its origin from China and Japan [13]. *N. domestica* has been used as a effective treatment to reduce dermatophytic infections [14]. Our previous study reported that *N. domestica* extracts possessing anti-oxidant property show anti-inflammatory effect by regulating mitogen activated protein kinases (MAPKs) signaling in lipopolysaccharide (LPS)-induced RAW264.7 cells [15]. In addition, *N. domestica* leaf extracts are known to possess anti-bacterial effect [16]. However, AD efficacy of the leaf extracts of *N. domestica* has not been adequately reported. Therefore, we explored the anti-atopic efficacy of leaf extracts of *N. domestica* and the related mechanisms.

2. Materials and methods

2.1. Animal studies

Seven-week-old ALC NC/Nga Tnd mice were purchased from Orient (Sungnam, South Korea) and the mice were kept in specific pathogen free conditions. To start the animal studies, the mice were randomly divided into 4 groups including 5 mice per each group [no treatment (NC/Nga_Nr), dinitrochlorobenzene (DNCB) treatment (DNCB-CTL), dexamethasone plus DNCB (DNCB_Dexa), *Nandina domestica* Thunb extracts plus DNCB (DNCB_ND)]. All mice keeped in a cage without

pathogen and provided ad libitum access to foods and waters. Our animal studies were conducted in accordance with relevant guidelines, and regulations and procedures related with mice were approved by the Institutional Animal Care and Use Committee (IACUC) of Daejeon University, republic of Korea (DJUARB2018-041).

2.2. Sensitization and treatment

To progress AD-skin lesions, the back skin of mice (week-8) were shaved. The mice were sensitized twice per week by applying 200 μL of 1% DNCB in acetone (3) : olive oil (1). Seven days later, the second challenge was conducted with 150 μL of 0.4% DNCB three times per week for 5 weeks in order for the mice to retain the disease. The sensitized mice were classified into four groups: NC/Nga_Nr is normal control. DNCB_CTL is DNCB-treated control. DNCB_Dexa is 3 mg/kg dexamethasone. DNCB_ND is 300 mg/kg ND. Control mice were colored with acetone : olive oil (3:1) ND suspended in 0.5% carboxymethyl cellulose (CMC) was treated by oral administration daily for 5 weeks (300 mg/kg). Dexamethasone was used as a positive control.

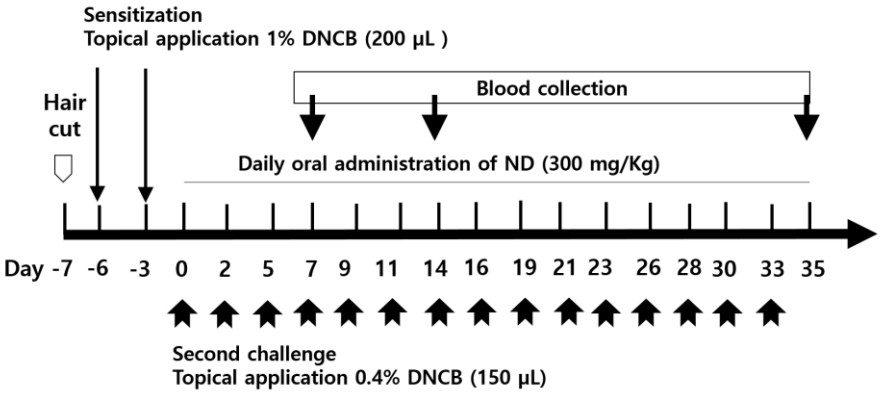

Figure 1. Experimental scheme of AD model. After remove of back hair, the 1% DNCB (200 μL) was applied to the dormal back (day -6 and day -3). The animals were applied with 0.4% DNCB thrice a week for five weeks (days 0 to 35). ND was orally administrated one a day for five weeks (days 0 to 35).

2.3. Clinical skin score and histopathological analyses of skin

Clinical observation to evaluate changes in the skin of ALC NC/Nga Tnd mice was performed at 1, 2 and 5-week for 5 weeks. The harshness of AD-skin lesions was examined by a previsously reported method based on skin symptoms such as scarring/dryness and excoriation/erosion symptoms [17]. Briefly, the skin clinical scoring combined an assessment of skin disease extrent with three clinical characters: erythema/hemorrhage, scarring/dryness and excoriation/erosion. The features are assessed on a representative area for a given intensity item, also on a scale of 0–3 (0 is none, 1 is mild, 2 is moderate and 3 is severe) .The back skin of the mice were biopsied. After embedding by paraffin wax, the back skin cut in 4 μm thickness. The analysis of the AD-skin was conducted by hematoxylin and eosin (H&E) and toluidine blue (TB) staining. All samples were investigated under an inverted microscope (Nikon Eclipse Ti, Nikon, Tokyo, Japan).

2.4. Isolation of axillary lymph nodes, the spleen, and dorsal skin cells

Axillary lymph nodes (ALNs) and spleens were isolated from mice. After crushing, they were filtered by a cell strainer (70 μm). The filtered cells were conducted centrifugation at 3000× g for 5 min, primary spleen cells were put at ACK lysis buffer (Red Blood Cell Lysis, Thermo Scientific, Waltham, MA, USA) to elinimate red blood cells (RBC) for 5 min. They were washed twice with RPMI-1640 medium containing 10% FBS. After isolation of primary ALNs and spleen cells, they were resuspended in RPMI-1640 medium containing 10% FBS. Briefly, the dorsal skin was eliminated from the mice. After mincing with scalpels, it was incubated in PBS including 1 mg/ml collagenase IV (Sigma-C5138, Sigma, St. Louis, MO, USA) at 37 ℃ for 40 mins. The each samples were actively down to further separate the remaining tissue clumps by pipetting. The cell suspensions were filtered with a pore nylon cell strainer (BD Falcon, Bedford, MA, USA) of 70 mm and centrifuged for 20 min (450× g). The cell pellets were gathered. After washing twice, total cell number was collected with a hemocytometer chamber (Thermo Fisher Scientific, Grand Island, NY, USA). The flow cytometry analysis was conducted with suitable antibodies after staining of the cells.

2.5. Splenocyte isolation and culture

Spleens from mice were excluded. To gain single-cell suspensions, the RBC were excluded using RBC lysis buffer. The isolated splenocytes were incubated with or without anti-CD3 antibody (0.5 μg/mL) (eBioscience, San Diego, CA, USA). After incubation for 24 h, the supernatant was gathered to decide the IL-4, IL-5, IL-13, and IFN-γ levels.

2.6. Fluorescence-activated sell sorting

The minced dorsal skins of mice were incubated in PBS containing collagenase IV (1 mg/mL) and dispase (2 mg/mL) for 40 min at 37 ℃. After staining the cells with nine antibodies (anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD23, anti-CD69, anti-B220, anti-CCR3 and anti-CD11b; BD Biosciences Pharmingen, San Diego, CA, USA) in staining buffer (PBS containing 1% v/v fetal bovine serum and 0.01% w/v sodium azide) for 30 min below ice, the cells were analyzed by a fluorescence-activated cell sorting analyzer with Cell-Quest software (BD Biosciences).

2.7. Enzyme-linked immunosorbent assay

The IgE levels were assessed by ELISA kits in plasma, and IL-4, IL-5, IL-13 and IFN-γ levels (R&D Systems, St. Louis, MO, USA) were assessed by ELISA kits in the supernatant of cultured splenocytes. The ELISA kits were used with the manufacturer's protocols.

2.8. Statistical analysis

Data are dispalyed as the means ± standard errors (SEMs). One-way analysis of variance (ANOVA) and Duncan's test were analyzed with Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Effects of ND on AD-skin lesion in a ALC NC/Nga Tnd mice and mast cell infiltration

To assess the potential of the ND on AD-like skin lesion, skin clinical scores were obtained by the AD-skin features. As shown in Figure 2a, the skin clinical scores of DNCB-control(CTL) were significantly high after the treatment of DNCB and DNCB-Dexa mice showed significantly reduced. Interestingly, after week-5, skin clinical scores of DNCB-ND mice went into significantly reverse compared to the NDCB-CTL mice.

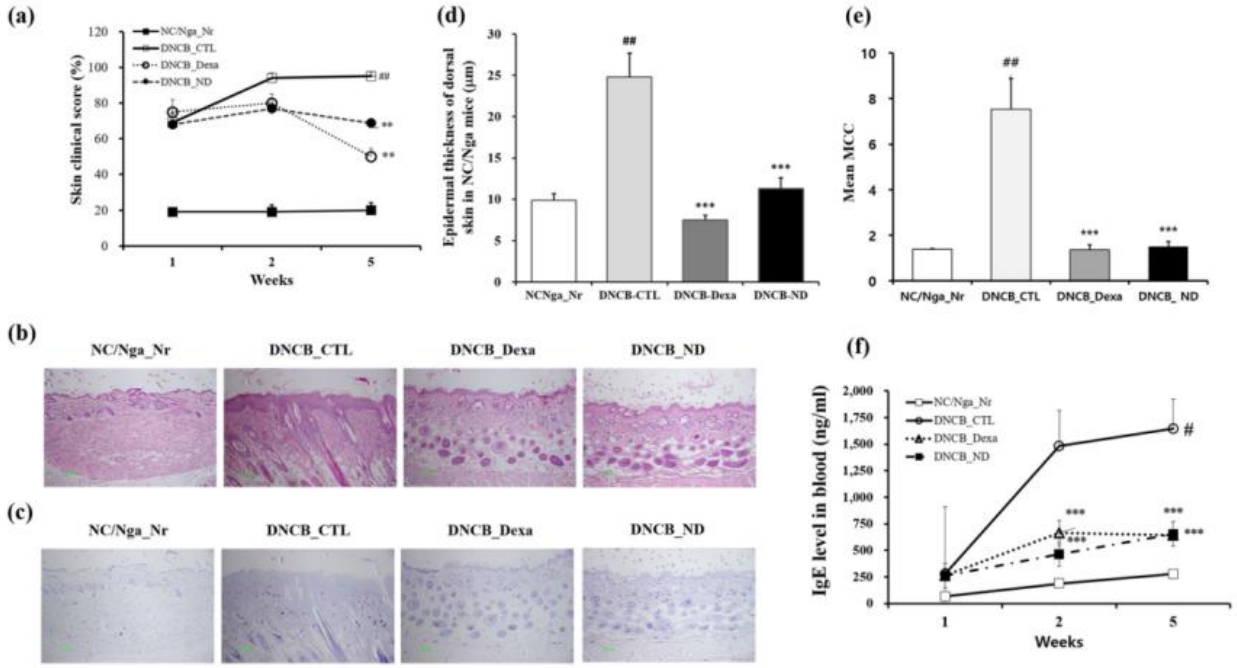

Figure 2. (a) Induction and treatment of AD in the dorsal skin of NC/Nga mice and skin clinical score; (b) Microphotographs stained with Hematoxylin and eosin; (c) Toluidine blue staining; (d) Epidermal thickness; (e) Mast cell count (MCC). The effects of *Nandina domestica* Thunb extracts (ND) on the plasma levels of immunoglobulin E (IgE) in NC/Nga mice with atopic dermatitis; (f) Total IgE levels in plasma were evaluated by ELISA. The bloods gethered from each mice at 1, 2, and 5 weeks after administration of ND. NC/Nga_Nr is normal control. DNCB_CTL is DNCB-treated control. DNCB_Dexa is 3 mg/kg dexamethasone. DNCB_ND is 300 mg/kg ND. Values are present as the means ± SEMs (n = 5). # $p < 0.05$ and ## $p < 0.01$ compared with NC/Nga_Nr; ** $p < 0.01$ and *** $p < 0.001$ compared with DNCB_CTL.

To evaluate the efficacy of ND on hypertrophy and granulocyte infiltration of AD skin, the images were obtained by microscopy after applying with ND (Figure 2b,d). The repeated treatment with DNCB was increased skin thickening of AD-mice. However, ND was able to retrieval dermal thickness to almost normal mice compared to that in the NDCB-CTL group, showing that it decreases the hyperkeratinization of the skin in AD-mice and improves the histological expression of skin lesions of AD-mice. As shown in Figure 2c,e, the dorsal skin stained with TB exhibited a

decrease in the infiltration of mast cells in the ND-treated group. The mast cell count (MCC) in ND-treated group was significantly smaller than DNCB_CTL group. These findings indicate that ND has anti-allergic activity.

3.2. Treatment of ND decreases serum IgE level in AD mice

To evaluate whether ND exert their efficacy through a IgE, serum levels of total IgE were gathered. Treatment repeated with DNCB caused a remarkable increment of total IgE (Figure 2f). However, serum levels of total IgE in ND treated mice reduced significantly more than that of the DNCB_CTL. In the total IgE, ND showed almost equal potency as compared to that of DNCB-Dexa. These results indicate that ND may play a important role in suppression of IgE production during the progress of AD.

3.3. The effects of ND on the absolute numbers of immune cell subtypes in ALNs, the spleen, and dorsal skin

The total cell numbers in the ALNs, spleen, and dorsal skin tissue statistically increased by DNCB and decreased by ND (Table 1). ND also decreased the absolute numbers of CD23+/B220+ double-positive cells. The absolute numbers of CD3+/CD19+, CD4+/CD8+, and CD4+/CD69+ cells didn't change in ALNs and the spleen. Infiltration of inflammatory cells - was lower in the dorsal skin in the DNCB_ND group, although CCR3+/CD11b+ cell infiltration didn't suggest significant change. These results show that ND reduces allergic cell numbers

Table 1. Immune cell subtypes by fluorescence-activated cell sorting analysis (FACS) in the axillary lymph nodes (ALNs), spleens, and dorsal skin tissues of DNCB-atopy dermatitis mice.

Cell Phenotypes		NC/Nga-Nr	DNCB_CTL	DNCB_Dexa	DNCB_ND
ALN	Total ALN cells ($\times10^4$/mL)	5.3 ± 1.6	184.0 ± 22.5###	38.3 ± 12.2***	94.0 ± 9.0**
	CD3+/CD19+ ($\times10^5$ Cells)	0.69 ± 0.31	28.3 ± 5.15###	5.21 ± 1.17***	27.46 ± 4.76
	CD4+/CD8+ ($\times10^5$ Cells)	0.69 ± 0.13	23.84 ± 0.27###	6.27 ± 0.73***	21.60 ± 0.50
	CD4+/CD69+ ($\times10^5$ Cells)	0.59 ± 0.15	4.10 ± 1.72###	2.70 ± 0.66**	3.70 ± 1.11
	CD23+/B220+ ($\times10^5$ Cells)	0.97 ± 5.50	140.0 ± 12.72###	27.4 ± 5.12***	50.0 ± 4.15**
Spleen	Total spleen cells ($\times10^4$/mL)	665.0 ± 99.1	1585.0 ± 231.3##	810.0 ± 143.9**	850.0 ± 83.5
	CD3+/CD19+ ($\times10^5$ Cells)	269 ± 0.31	563 ± 115.2#	321.0 ± 72.4	467.0 ± 32.4
	CD4+/CD8+ ($\times10^5$ Cells)	118 ± 0.13	284 ± 32.7#	227 ± 73.0	160.0 ± 0.50
	CD4+/CD69+ ($\times10^5$ Cells)	7.72 ± 37.79	18.50 ± 7.49	12.57 ± 4.07	12.5 ± 3.67
	CD23+/B220+ ($\times10^5$ Cells)	373.25 ± 86.49	616.21 ± 331.61#	448.81 ± 65.24	575 ± 57.35
Dorsal skin	Total dorsal skin cells ($\times10^4$/mL)	3.50 ± 1.10	27.5 ± 2.5###	9.0 ± 1.0***	9.5 ± 1.5**
	CD3+($\times10^5$ Cells)	0.30 ± 4.46	3.20 ± 0.31###	1.80 ± 0.46**	2.2 ± 0.16*
	CCR3+/CD11b+ ($\times10^5$ Cells)	0.89 ± 0.06	2.86 ± 0.59##	2.07 ± 0.03	1.26 ± 0.10

*Note: Each value represents mean ± SEM values for five mice. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ compared with normal group (NC/Nga_Nr); * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with only DNCB group (DNCB-CTL).

3.4. The Effects of ND on the production of Th2 cytokines (IL-4, IL-5, and IL-13) and Th1 cytokine (IFN-γ) by cultured splenocytes

We investigated whether ND may regulate the cytokines secreted by of Th1/Th2 cells in the AD mice. As shown in Figure 3, ND reduced the IL-4, IL-5 and IL-13 levels in cultured splenocytes. The level of IFN-γ increased compared to those in the NC/Nga_Nr. These results indicate that ND can improve AD-producted cytokines by regulating Th1 and Th2 cytokines

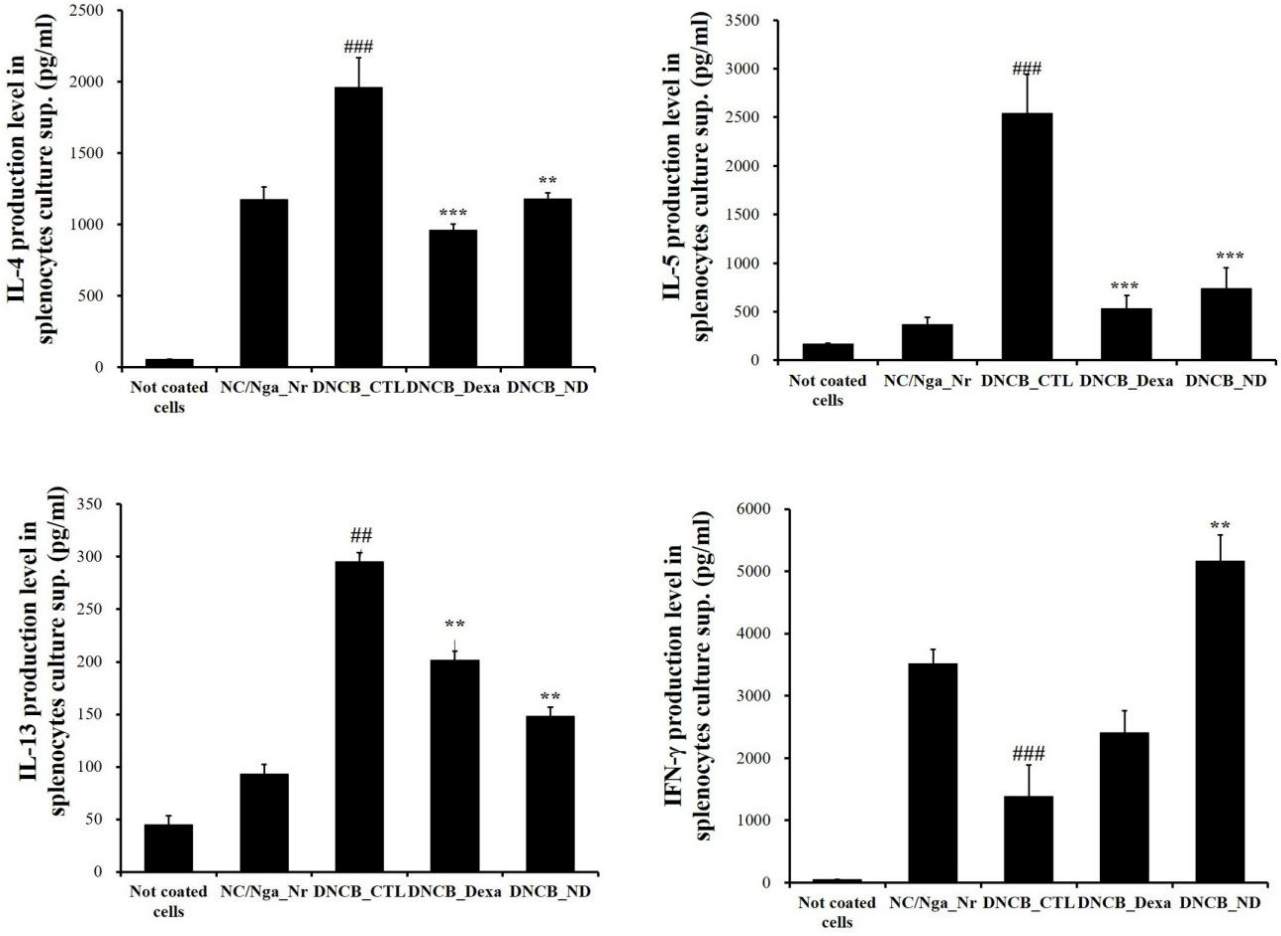

Figure 3. The effects of *Nandina domestica* Thunb extracts (ND) on the production of Th2 and Th1 cytokines (IFN-γ) by cultured splenocytes in NC/Nga mice. The IL-4, IFN-γ, IL-5, and IL-13 levels in the culture supernatant of cell were evaluated by ELISA. After atopic dermatitis (AD) induction by DNCB and subsequent treatment with ND or dexamethasone, splenocytes were cultured with anti-CD3 antibody–coated 96-well plates at 15 weeks of age for 48 h at 1×10^5 cells/well. The non-coated cells (no exposure to the anti-CD3 antibody) displayed as the negative control. NC/Nga_Nr is normal control. DNCB_CTL is (DNCB-treated control. DNCB_Dexa is 3 mg/kg dexamethasone. DNCB_ND is 300 mg/kg ND. Values are expressed as the means ± SEMs (n = 5). ## $p < 0.01$ and ### $p < 0.001$ compared with NC/Nga_Nr; ** $p < 0.01$ and *** $p < 0.001$ compared with DNCB_CTL.

3.5. Effects of ND on claudin 1 and sirt 1 expression

Topical application with DNCB on the skin of mice significantly reduced expression of claudin 1 and sirt 1 proteins due to damage of the skin barrier. DNCB-ND significantly increased expression of claudin 1 and sirt 1 proteins. A weaker increment of two proteins was appeard in the DNCB-Dexa compared to the DNCB-ND (Figure 4). These results indicate that ND alleviates disruption of claudin 1 and sirt 1 proteins related with skin barrier.

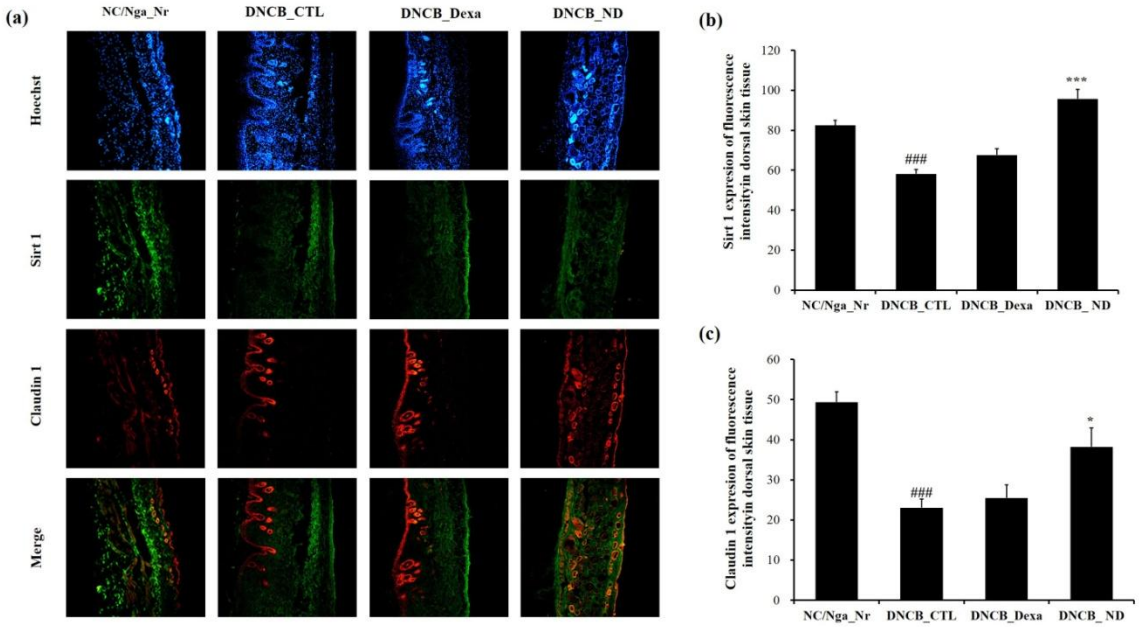

Figure 4. Anaysis of immunohistofluorescence staining for claudin 1 and Sirt1 protein expression in NC/Nga mice dorsal skin tissue. (a) Hoechst (blue), Sirt1 (green), Claudin1 (red) and Merge of claudin 1 and Sirt1 (mixture of green and red). NC/Nga_Nr is normal control. DNCB_CTL is DNCB-treated control. DNCB_Dexa is 3 mg/kg dexamethasone. DNCB_ND is 300 mg/kg ND. Densitometric quantification of Sirt1 (b), and claudin1 (c) in dorsal skin tissue of mice. After fluorescence staining, the values quantified by image J program are expressed as bars. Values are expressed as means ± SEMs (n = 5). ## $p < 0.01$ and ### $p < 0.001$ compared with NC/Nga_Nr; ** $p < 0.01$ and *** $p < 0.005$ compared with DNCB_CTL.

4. Discussion

AD is characterized by exacerbations and remissions of eczematous skin [18]. AD showed a chronic skin disorder with an interplay of migrating lymphocytes and epidermal keratinocytes (KC) [19]. AD-induced skin lesions are appeard dermal mononuclear infiltration and spongiosis in the epidermis of skin [20]. In this study, we examined whether ND could improve AD symptoms such as skin lesion and infiltration of mast cells. ND exhibited efficacy on improvement of skin clinical score and on obstruction of tissue mast cells infiltration with ameliorating the hyperkeratosis or thickening of the stratum corneum.

AD is generally showed increased allergen-specific IgE, mast cell activation and Th2 cytokine production in dermal lesions after continuous exposures to a special allergen [21]. Furthermore, IgE as a unique antibody possessed ability increasing the production of Th2-type cytokines as well as activation of mast cells, pointing its importance in makting a evil circle inducing to a chronic status in AD-patients [22]. In our study, ND showed inhibition of IgE and Th2 cytokines (IL-4, IL-5 and IL-13) levels but increment of Th1 cytokine (INF-γ). Furthermore, consistent with the reduced mast cell infiltration, ND potently ameliorated the levels of serum IgE and modulated Th2 cytokines and Th1 cytokine.

AD displayed accumulation of activated T cells in the skin and increased IgE production [23]. AD is generally characterized by increased numbers of infiltrating T cells and activated CD4+ or CD8+ T cells in the dermis. These lymphocytes are involved in the promotion of cell-mediated and humoral immunity through the secretion of various cytokines [24]. The problem, however, occurs when cell numbers are elevated because of the continuous presence of AD-inducing agents. At a previous study, double positive (DP) T cells expressing both CD4 and CD8 have been described in several pathological conditions as well as in normal individuals and a subset of DP T cells expressing high levels of both CD4 and CD8ab heterodimer (CD4hiCD8hi), has been identified in autoimmune and chronic inflammatory disorders [25]. The main finding of this study show that ND reduced the levels of CD3+ cells infiltration in the skin dorsal and lowered the numbers of CD4+/CD8+ cells in the ALNs and spleens. Simultaneously, ND restored the levels of IFN-γ secretion by splenocytes, as in the NC/Nga_Nr. However, ND lowered the levels of IL-4, IL-5 and IL-13 secreted by splenocytes.

Tight junctions (TJs) were involved in a cell-to-cell adhesion in the cells forming the epithelium and endothelium [26]. The TJ barrier is displayed below the stratum corneum barrier, and its constitutive proteins (occludin, claudin-1, claudin-4, and ZO-1) are displayed by colocalization in the granular layers [27].

Sirtuin 1 (Sirt1), which is an NAD-dependent protein deacetylase, relate with cell survival, metabolism, senescence, and stress response in several cells and it conduct a important role in tumorigenesis and homeostasis of skin [28,29]. The claudin1 and sirt1 related abnormalities of barrier in the development of AD. In this study, ND increased protein expression levels of claudin 1 and Sirt1 decreased by AD in dorsal skin tissue, indicating that ND may ameliorate skin barrier functioning in AD.

5. Conclusions

ND improved AD symptoms and it reduced the infiltration of mast cells, the levels of inflammatory cells infiltration in the skin dorsal, the numbers of CD4+/CD8+ cells in the ALNs and spleens, the levels of IgE and Th2 cytokines in AD-mice. It also improved skin barrier functioning with increasing of claudin 1 and Sirt1 protein expression levels. Therefore, this report suggest that ND significantly ameliorates DCNB-induced AD.

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Conflict of interest

The authors have declared no conflict of interest.

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