



# Indirubin, a purple 3,2- bisindole, inhibited allergic contact dermatitis via regulating T helper (Th)-mediated immune system in DNCB-induced model

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## ABSTRACT

**Ethnopharmacological relevance:** Indirubin, isolated from *Indigo naturalis* (Apiaceae) is a purple 3, 2- bisindole and a stable isomer of indigo. Although it is known to have anti-inflammatory activities, its mechanism of action has not been elucidated.

**Materials and methods:** Seven-week-old female BALB/c mice were sensitized with 1-chloro-2, 4-dinitrobenzene (DNCB) to induce skin inflammation. Hematoxylin and eosin staining was performed to assess epidermal and dermal hyperplasia, which were determined by measuring the thicknesses of the epidermis and dermis, respectively. We also evaluated serum immunoglobulin E (IgE) levels and cytokines production, such as tumor necrosis factor (TNF)-α, interleukin (IL)-4, 6 and Interferon (IFN)-γ. In addition, we investigated nuclear factor (NF)-κB, IκB-α and mitogen-activated protein (MAP) kinase activities for verifying the molecular mechanism of inflammation.

**Results:** Indirubin treatment suppressed skin inflammation in DNCB-exposed mice. The skin lesions were significantly thinner in the Indirubin-treated group than in untreated controls, and the hyperkeratosis disappeared. Indirubin reduced the total serum IgE level and cytokines production. In addition, it normalized NF-κB, IκB-α and MAP kinase expression.

**Conclusions:** Indirubin might be a useful treatment for allergic contact dermatitis via regulating the co-expression of T helper (Th) 1 and 2 cell-mediated immune responses.

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## 1. Introduction

Severe skin inflammatory diseases, including contact dermatitis, are caused by inappropriate or unbalanced immune responses, also referred to as a T helper (Th) cell-mediated skin inflammatory reaction (Tomimori et al., 2005). Allergic contact dermatitis (ACD), which may be caused by contact hypersensitivity, results in skin inflammation due to exposure to contact allergens (Vocanson et al., 2006). To investigate the mechanism of ACD, mice with 1-chloro-2,4-dinitrobenzene (DNCB)-induced contact dermatitis are commonly used as an animal model (Zhang et al., 2009). The sensitization phase, induced by exposure of the skin to DNCB, is initiated by the activation of skin innate immunity.

A wide variety of plant-derived products and compounds show anti-inflammatory effects, but the molecular mechanisms of inhibitory action have only been assessed in a few of them

(Miyauchi-Hashimoto et al., 2005). In addition, many agents used to treat inflammatory diseases are associated with severe side effects. Thus, the search for less toxic efficacious compounds remains a worldwide challenge (McKellar et al., 2007). *Indigo naturalis* (Apiaceae), an herbal medicine, has been used to treat skin disorders including eczema, aphtha, furuncles, and psoriasis (Lin et al., 2009). Indirubin, which is isolated from *Indigo naturalis*, is a purple 3,2- bisindole and a stable isomer of indigo. Although this compound exhibits activity against various types of cancer and anti-inflammatory activities, its mechanism of action is poorly understood (Nam et al., 2005; Kim et al., 2007).

In the present study, we investigated the effects of Indirubin on DNCB-induced ACD in mice and its mechanism of action. To examine the protective effects of Indirubin on skin inflammation, we performed histopathological examination of dermatitis-like skin lesions in mice treated topically with DNCB. The total serum immunoglobulin E (IgE) and skin tissue cytokines production levels were measured. In addition, we analyzed nuclear factor (NF)-κB, IκB-α and mitogen activated protein (MAP) kinase expression in DNCB-treated mice by Western blotting.

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## 2. Materials and methods

### 2.1. Animal treatment

Seven-week-old female BALB/c mice (18–20 g) were purchased from SLC, Inc. (Hamamatsu, Japan). Animals were given an unlimited amount of water and feed throughout the duration of the experiment. They were kept at a constant temperature (23 °C) and humidity (55%) with a 12-h light/dark cycle. Animal care and experimental procedures were performed in accordance with the “Guide for the Care and Use of Laboratory Animals” (Department of Health, Education and Welfare, NIH publication #78-23, 1996).

After 10 days of acclimatization, fur on the upper back was shaved with a shaver. Mice were assigned to one of three groups (five mice each): normal (vehicle-treated group); DNCB (negative control group), mice sensitized with DNCB; and Indirubin, mice sensitized with DNCB and treated with Indirubin at doses of 1 and 100 µg/mL. Indirubin was obtained from Santa Cruz Biotechnology (cat. no. sc-201531). The molecular weight of indirubin is 262.3. The treated concentrations of indirubin in this experiment are 381 nM and 38.1 µM, respectively.

Barrier disruption was achieved by applying 100 µL 4% sodium dodecyl sulfate (SDS) to the shaved dorsal skin 3 h before the application of DNCB. Induction of ACD was achieved by topical application of 100 µL 1% DNCB in 4:1 (v/v) acetone/olive oil once a day to the shaved dorsal skin. These procedures were repeated for 3 days (days 0–2), after which mice did not receive any treatment for 5 days (days 3–7). Then the treatment was repeated with 0.5% DNCB once a day (days 8–14). After SDS and DNCB treatment, mice in the Indirubin group were additionally treated with 100 µL Indirubin in 4:1 (v/v) acetone/olive oil once a day (1 and 100 µg/mL, separately). Normal and DNCB groups received acetone/olive oil (4:1, v/v). Following challenge for 10 days, the mice were sacrificed (day 17).

### 2.2. Histology

Skin sections were stained with hematoxylin and eosin (H&E) to assess epidermal and dermal hyperplasia, which was measured by the thickness of the epidermis and dermis, respectively, in normal, DNCB-treated, and Indirubin 1 and 100 µg/mL-treated mouse skins. In brief, pieces of skin (1 × 0.5 cm) were fixed through incubation in 10% neutralized formalin for 24 h, embedded in paraffin, and sectioned to a thickness of 4 µm. Digital images were obtained and thicknesses were measured using Leica Application Suite (LAS) Microscope Software (Leica Microsystems Inc., IL, USA). The magnification was × 200.

### 2.3. Enzyme-linked immunosorbent assay (ELISA)

ELISA analysis was used for measurement of total IgE production in blood samples and cytokines production in dorsal skin tissue protein. Mouse total serum samples were prepared from blood collected by cardiac puncture into heparinized tubes. The blood was centrifuged at 14,000 rpm for 30 min, after which the supernatant and serum were collected. Serum IgE concentrations were quantified using a Mouse IgE ELISA kit (Shibayagi, Shibukawa, Japan) according to the manufacturer's protocol. A 100 mg dorsal skin samples ( $n=5$  per group) were weighted and homogenized in 1 mL of T-PER tissue protein extraction reagent (Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN). After incubating 15 min on ice, protein was extracted by centrifugation at 12,000 ×  $g$  for 15 min at 4 °C. The supernatant was obtained. Tissue cytokine concentrations were quantified using a Mouse TNF- $\alpha$ , IL-4, 6 and IFN- $\gamma$  ELISA kit (BD Bioscience, San Jose, CA, USA) according to the manufacturer's instructions. Cytokine levels were normalized with a protein

assay kit and calculated as pg per mg total protein. Color development at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Downingtown, PA).

### 2.4. Protein extraction

To analyze the NF- $\kappa$ B and I $\kappa$ B- $\alpha$  protein level, a 100 mg sample ( $n=5$  per group) of the frozen tissue was homogenized with the cytoplasmic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.15% Nonidet P-40, 50 mM  $\beta$ -glycerophosphate, 10 mM NaF and 5 mM Na3VO4) containing protease inhibitors cocktail and incubated on ice for 15 min. The resulting homogenate was centrifuged at 12,000 ×  $g$  for 30 min at 4 °C and the supernatant was carefully collected without disturbing the fluffy nuclear pellet and labeled cytoplasmic protein fraction. The pellet was added to the nuclear buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.50% Nonidet P-40, 50 mM  $\beta$ -glycerophosphate, 10 mM NaF and 5 mM Na3VO4) containing protease inhibitors cocktail and then incubated 15 min on ice. Nuclear protein was extracted by centrifugation at 12,000 ×  $g$  for 15 min at 4 °C. The supernatant was collected. On the other hand, total skin lesion was extracted with total protein buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) containing protease inhibitors cocktail to evaluate the levels of extracellular signal-regulated kinase (ERK), Jun NH2-terminal kinase (JNK), and p38 MAP kinase. A 100 mg tissue ( $n=5$  per group) was homogenized. The resulting homogenate was incubated on ice for 15 min, and after immediate vortexing for 15 s. The process was repeated four times. Protein fractions were obtained as supernatants after centrifugation at 12,000 ×  $g$  for 30 min at 4 °C. The cytoplasmic, nuclear and total protein fractions were stored at –80 °C until use.

### 2.5. Western blotting analysis

Activation of NF- $\kappa$ B, I $\kappa$ B- $\alpha$ , ERK, JNK and p38 MAPK was analyzed by Western blotting. The protein concentration was measured using the Bradford method and 50 µg protein was denatured with SDS buffer. The denatured protein was separated on a 15% polyacrylamide gel and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Non-specific labeling was blocked through incubation with TBS-T buffer containing 5% skim milk for 2 h. The blots were incubated overnight with primary antibody (anti-NF- $\kappa$ B, I $\kappa$ B- $\alpha$ , –ERK, –JNK, or –p38 MAPK; dilution 1:800 in TBS-T; cell signaling, USA). Anti-rabbit alkaline phosphatase-conjugated secondary antibody (dilution 1:2000 in TBS-T; Santa Cruz, CA, USA) was used to detect the target proteins. After incubation for 2 h at room temperature, bound antibody was visualized using an enhanced chemiluminescence (ECL) detection reagent (Amersham Pharmacia, Piscataway, NJ, USA).  $\beta$ -actin was used as an internal loading control. The relative band density was determined using a computerized densitometry system and normalized to the  $\beta$ -actin signal from a blot developed under similar conditions.

### 2.6. Statistical analysis

Significance was determined by one-way analysis of variance (ANOVA) and nonparametric tests. In all analyses,  $P < 0.05$  was taken to indicate statistical significance.

### 3. Results

#### 3.1. Histopathological effects of Indirubin

To investigate the effects on skin thickness, histological analysis was performed. None of the animals died during the study and no noticeable adverse effects were observed in any animal. Representative photographs of specimens that had been stained with H&E are shown in Fig. 1. The skin was significantly thicker in DNCB-treated mice than in the normal group. Compared to the negative control group, Indirubin-treated mice showed less thickening and hyperplasia. Notably, treatment with 100  $\mu\text{g/mL}$  Indirubin significantly reduced the thicknesses of both the epidermis and dermis.

#### 3.2. Effects of Indirubin on serum IgE level

ACD is characterized by elevated total serum IgE levels. We evaluated the total serum IgE level in DNCB-induced mice.

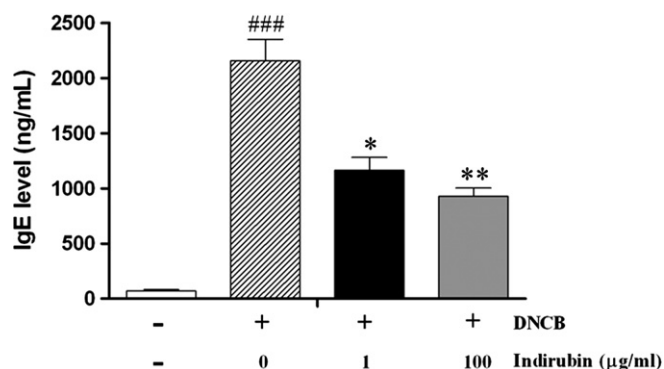
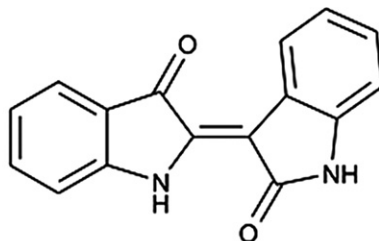


**Fig. 2.** Effects of Indirubin on serum IgE level. Results are presented as mean  $\pm$  S.D. # and ### indicate that the mean differs significantly between normal group and DNCB group ( $p < 0.05$  and  $p < 0.001$ , respectively). \* and \*\* indicate that the mean differs significantly between Indirubin 1  $\mu\text{g/mL}$ , Indirubin 100  $\mu\text{g/mL}$ -treated group and DNCB group ( $p < 0.05$  and  $0.01$ , respectively).



Indirubin

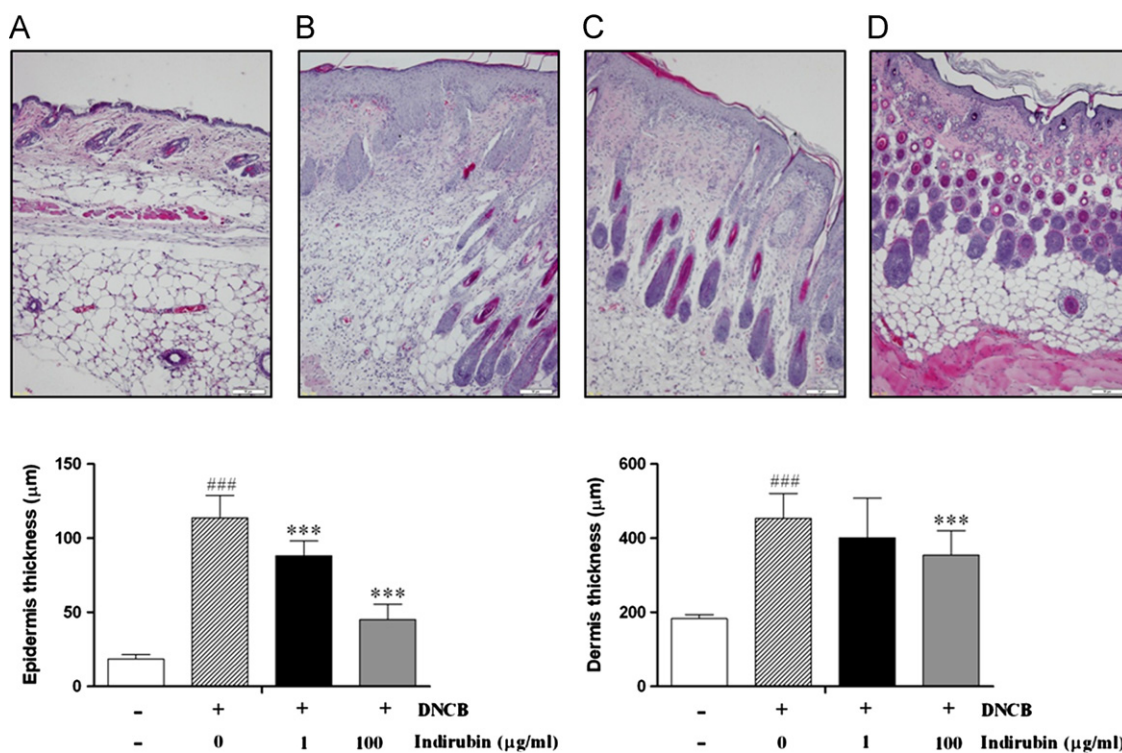


**Fig. 1.** Histopathological findings by hematoxylin/eosin staining of skin sections ( $n=5$ , magnification:  $\times 200$ ). No contact dermatitis induction as normal mice (A), contact dermatitis induction as control mice (B), Indirubin 1  $\mu\text{g/mL}$  topical application following contact dermatitis induction (C), Indirubin 100  $\mu\text{g/mL}$  topical application following contact dermatitis induction (D). Results are presented as mean  $\pm$  S.D. ### indicates that the mean differs significantly between normal group and DNCB group ( $p < 0.001$ ). \*\*\* indicates that the mean differs significantly between Indirubin 1  $\mu\text{g/mL}$ , Indirubin 100  $\mu\text{g/mL}$ -treated group and DNCB group ( $p < 0.001$ ).

There was an increase in the IgE level in the DNCB group. Indirubin reduced the serum IgE level compared to the DNCB group in a dose-dependent manner (Fig. 2).

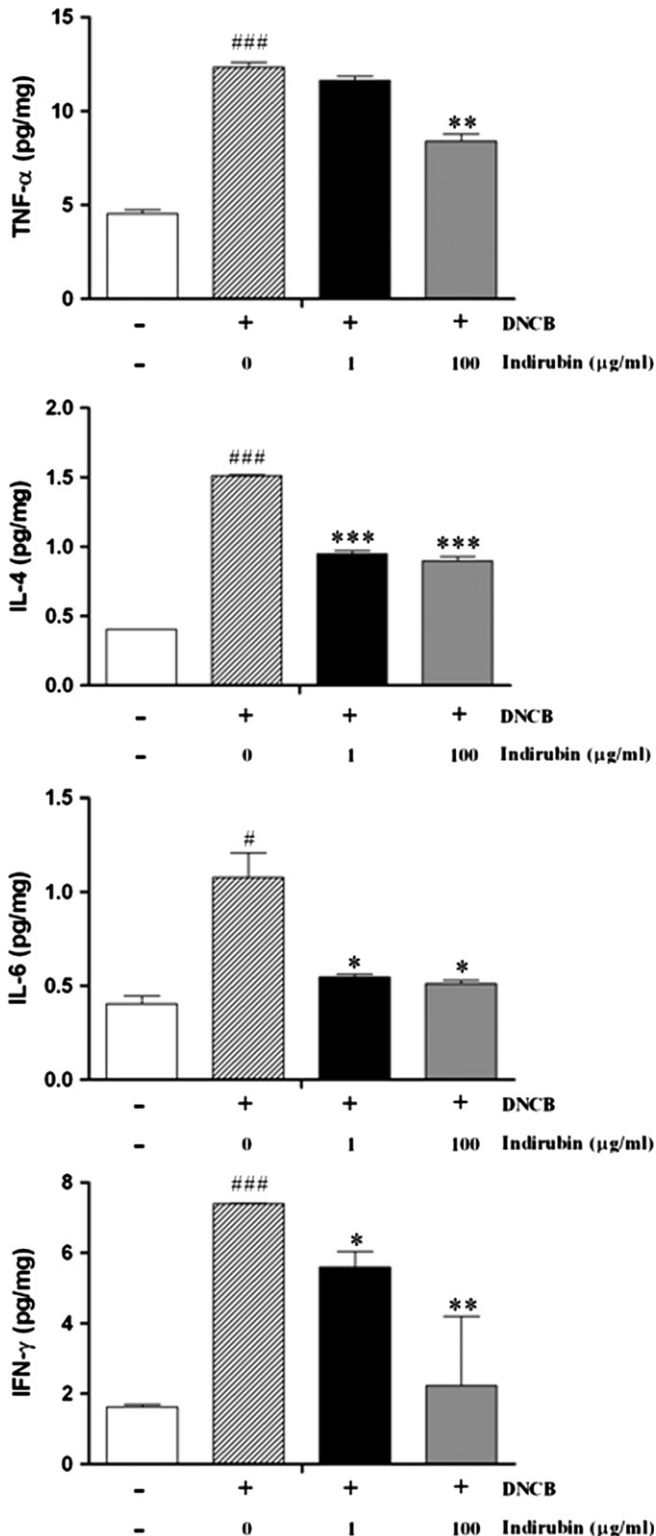


**Fig. 3.** Effects of Indirubin on skin tissue cytokines production. Results are presented as mean  $\pm$  S.D. # and ## indicate that the mean differs significantly between normal group and DNCB group ( $p < 0.05$  and  $p < 0.001$ , respectively). \*, \*\* and \*\*\* indicate that the mean differs significantly between Indirubin 1  $\mu$ g/mL, Indirubin 100  $\mu$ g/mL-treated group and DNCB group ( $p < 0.05$ , 0.01 and 0.001, respectively).

### 3.3. Effects of Indirubin on cytokine production

In allergic contact dermatitis, immunologic and inflammatory processes are regulated by inflammatory mediators and cytokines. We examined the effect of Indirubin on cytokine production in dorsal skin tissues. The levels of TNF- $\alpha$ , IL-4, 6 and IFN- $\gamma$  in DNCB-treated group were significantly increased than those in normal group. By treating Indirubin, the cytokine levels of TNF- $\alpha$ , IL-4, 6 and IFN- $\gamma$  in dorsal skin were reduced in a dose-dependent manner (Fig. 3).

### 3.4. Effects of Indirubin on NF- $\kappa$ B and I $\kappa$ B- $\alpha$ activation

To investigate the anti-inflammatory mechanism of Indirubin, its effects on activation of NF- $\kappa$ B and I $\kappa$ B- $\alpha$ , which are important transcription factors that mediate the transcription of many

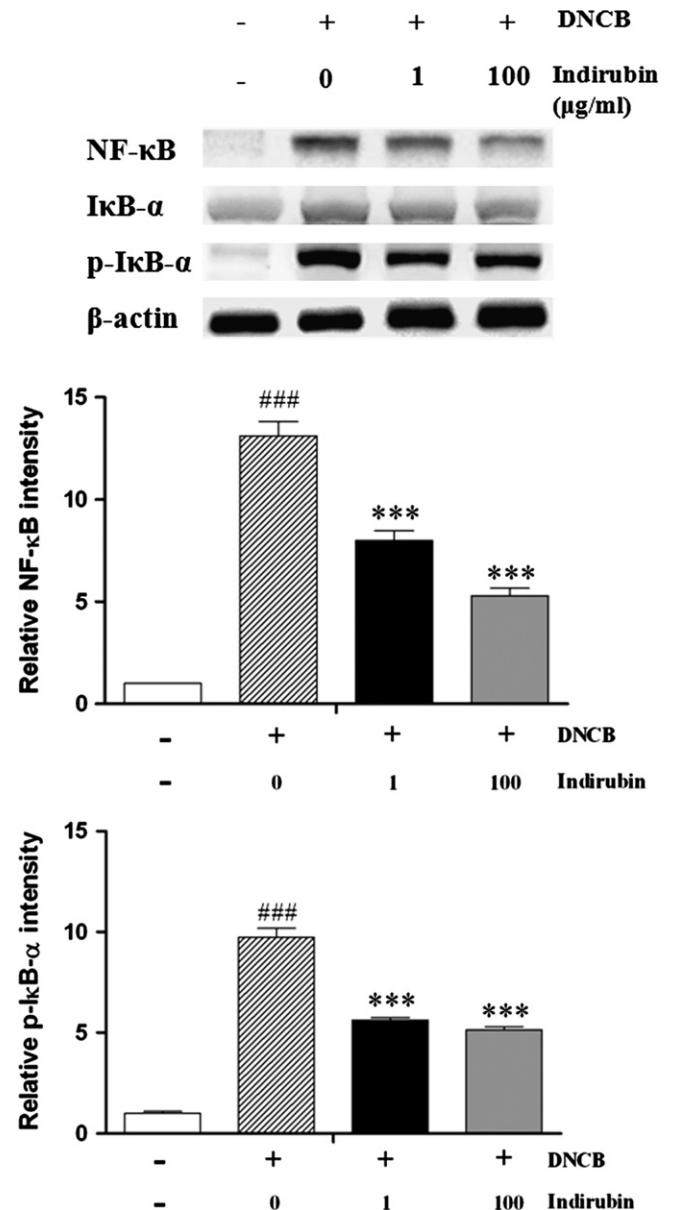


**Fig. 4.** Effects of Indirubin on NF- $\kappa$ B and I $\kappa$ B- $\alpha$  activation. Results are presented as mean  $\pm$  S.D. ### indicates that the mean differs significantly between normal group and DNCB group ( $p < 0.001$ ). \*\*\* indicates that the mean differs significantly between Indirubin 1  $\mu$ g/mL, Indirubin 100  $\mu$ g/mL-treated group and DNCB group ( $p < 0.001$ ).

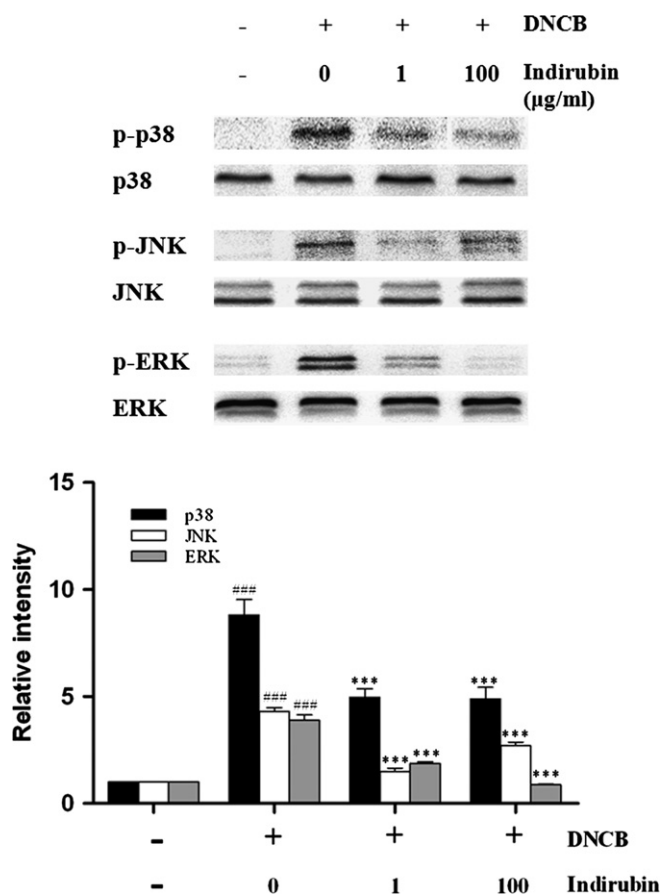


**Fig. 5.** Effects of Indirubin on MAP kinases activation. Results are presented as mean  $\pm$  S.D. ### indicates the mean differs significantly between normal group and DNCB group ( $p < 0.001$ ). \*\*\* indicates that the mean differs significantly between Indirubin 1  $\mu$ g/mL, Indirubin 100  $\mu$ g/mL-treated group and DNCB group ( $p < 0.001$ ).

pro-inflammatory cytokine genes, were examined by Western Blotting. Indirubin treatment significantly reduced the expression of NF- $\kappa$ B in the nucleus as well as inhibited I $\kappa$ B- $\alpha$  degradation in the cytoplasm compared to the DNCB group in a dose-dependent manner. In addition, Indirubin significantly inhibited the phosphorylation of I $\kappa$ B- $\alpha$  (Fig. 4).

### 3.5. Effect of Indirubin on MAP kinase activity

MAP kinase pathways have been reported to play major roles in biological responses, including cytokine expression. Therefore, we examined whether Indirubin reduced the phosphorylation of three MAP kinases in skin tissues from DNCB-treated mice. Indirubin blocked the increases in phosphorylated MAP kinase levels (Fig. 5). Notably, treatment with 100  $\mu$ g/mL Indirubin significantly reduced the expression of ERK.

## 4. Discussion

The present study examined the effects of Indirubin on ACD. DNCB-induced skin inflammation is accompanied by changes in IgE and cutaneous cytokine production (Leung and Bieber, 2003). In this study, topical Indirubin treatment in BALB/c mice markedly improved inflammatory skin lesions, which were significantly thinner than those in untreated skin, and the hyperkeratosis disappeared. Serum IgE levels in mice increased with repeated topical applications of DNCB, while Indirubin reduced the total serum IgE level in a dose-dependent manner.

ACD is attributed to co-expression of Th 1 and 2 cell-mediated immune responses (Watanabe et al., 2002). Previous reports have suggested that the predominant expression of Th2-type cytokines, which promotes B cell proliferation, systemic IgE switching, and then augments IgE secretion are accompanied by both acute and chronic ACD while the chronicity of ACD lesions is characterized by the Th1-type cytokine, such as IFN- $\gamma$  (Yamamoto et al., 2007; Choi et al., 2010). Furthermore, the cytokine TNF- $\alpha$  is known to regulate dermal–epidermal interactions and to stimulate IL-6 with the enhancement of IL-4-induced IgE production (Sumimoto et al., 1992; Segawa et al., 2008). IL-4, which is hallmark of Th 2 cell-mediated response, mediates an IgE isotype switch in B cells (Jung et al., 2010). Here, we confirmed the anti-inflammatory effects of Indirubin through interaction with cytokines and IgE production. Indirubin-treated skin lesions affected by DNCB significantly inhibited the production of TNF- $\alpha$  which induced decreased IL-6 to a reduction of IL-4. And then, reduced IL-4 induced decreased serum IgE level. In addition, IFN- $\gamma$  production was significantly inhibited by Indirubin. Indirubin suppressed immune response by inhibiting both of Th1 and Th2-type cytokines production.

NF- $\kappa$ B is a key transcription factor that regulates the expression of genes involved in immune and inflammatory responses (Hammer et al., 2005). Transcription factors of the NF- $\kappa$ B family have also been implicated in the arrest of proliferation and initiation of differentiation in epidermal keratinocytes. A thin and hypoplastic epidermis results in forced activation of NF- $\kappa$ B in the basal cell layers (Seitz et al., 1995). We demonstrated that activation of NF- $\kappa$ B in mice with DNCB-induced dermatitis results in skin thickening, and that Indirubin reduced hyperkeratosis in a mouse model by suppressing activation of NF- $\kappa$ B by DNCB. In addition, we have observed that the translocation of NF- $\kappa$ B to nucleus by Indirubin was inhibited, as shown by a marked decrease NF- $\kappa$ B in the nucleus and a decrease phosphorylated I $\kappa$ B- $\alpha$  in the cytoplasm.

MAP kinase signaling is active in inflammatory skin diseases and inhibitors of this pathway reduce skin inflammation in various rodent models of human skin diseases (Ipaktchi et al., 2006). Activation of p38 MAPK is associated with cell growth and differentiation, cell death, and inflammation (Kyriakis and Avruch, 2001; Pearson et al., 2001; Kaminska, 2005). JNKs are involved in the regulation of cell proliferation and survival and activation of the ERK pathway controls several cellular functions, including cell cycle progression (Wagner and Nebreda, 2009). Previous studies have suggested that MAP kinases regulate the expression of inflammatory cytokines (Venuprasad et al., 2006). In the present study, the increased activities of MAP kinases following DNCB challenge are consistent with the results of previous studies. Indirubin inhibited the ERK, JNK, and p38 signaling pathways. These data suggest that inhibition of MAP kinases by Indirubin may contribute to its anti-allergic and anti-inflammatory activities.

In conclusion, Indirubin reduced skin inflammation and induced tissue remodeling through regulation of serum IgE levels, skin tissue cytokines production, NF- $\kappa$ B, I $\kappa$ B- $\alpha$  activation, and MAP kinase signaling. Our results suggest that Indirubin might be an alternative or complementary therapeutic option for ACD. More in-depth studies would be necessary to clarify its molecular mechanisms of action.

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