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Anti-dermatitis effects of oak wood vinegar on the DNCB-induced contact hypersensitivity via STAT3 suppression

Chang Seok Lee^a, Eun Hee Yi^a, Hang-Rae Kim^b, Sang-Rok Huh^c, Sang-Hyun Sung^d, Myung-Hee Chung^a, Sang-Kyu Ye^{a,*}

^a Department of Pharmacology and Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, 103 Daehangno, Jongno-gu, Seoul 110-799, Republic of Korea

^b Department of Anatomy, Seoul National University College of Medicine, 103 Daehangno, Jongno-gu, Seoul 110-799, Republic of Korea

^c SR Tech corporation, 10B-21L, Banwol Industrial Complex, Wonsi-Dong, Danwon-Gu, Ansan-si, Gyeonggi-Do 425-090, Republic of Korea

^d College of Pharmacy, Seoul National University, San 56-1, Sillim-Dong, Gwanak-Gu, Seoul 151-742, Republic of Korea

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ABSTRACT

Aims of the study: The aim of this study was to evaluate the anti-dermatitis effects of oak wood vinegar (OWV) in a 2,4-dinitrochlorobenzene (DNCB)-induced contact dermatitis mice model.

Materials and methods: Immunoglobulin E (IgE) production, infiltration of immune cells (neutrophils, CD3+ cells), inducible nitric oxide synthase (iNOS) expression, skin thickness, and expression of phospho-rylated STAT3 (signal transducers and activators of transcription 3) protein were tested in a DNCB-induced contact dermatitis model. *In vitro* wound healing and proliferative assays were also performed.

Results: OWV showed anti-inflammatory effects on DNCB-induced dermatitis in mice, leading to inhibition of IgE production, immune cell infiltration, and iNOS expression. Skin thickness and the level of phospho-STAT3 were dramatically reduced by OWV. Using the HaCaT human keratinocyte cell line, we confirmed that constitutive STAT3 activation induced faster proliferation of epithelial cells. In addition, OWV suppressed HaCaT proliferative ability and phospho-STAT3 levels.

Conclusions: The study revealed that OWV has anti-inflammatory and anti-proliferative effects in a DNCB-induced contact dermatitis mice model. Furthermore, we showed that the mechanism by which OWV most likely inhibits epithelial proliferation is through STAT3 inactivation.

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1. Introduction

Oak tree (*Quercus acutissima*) is a temperate climate tree species found in Japan and the all region of South Korea. Oak wood vinegar (OWV), a byproduct from oak tree charcoal production, is a liquid generated from the gas and combustion of fresh wood burning in airless condition. Raw OWV is acid having more than 50% of organic acids and contains several types of organic compounds (>200), including acetic acid, and various kinds of phenols, carboxyl and alcohol compounds (Yatagai and Urinin, 1989; Kim et al., 2001). OWV has been evaluated for its various biological activities including as an insect repellent (Mitsuyoshi et al., 2001), a food preservative, an anti-bacterial and anti-fungal agent (Velmurugan et al., 2009). OWV is also used in traditional Asian medicine for its many health benefits to humans, such as blocking diarrhea, relieving pain and lowering cholesterol. In addition, the application of OWV to insect bites can help draw insect toxins out of the body. Notably, OWV has long been used to relieve symp-

toms of skin inflammatory diseases, such as cellulitis, athlete's foot and atopic dermatitis. In particular, OWV was found to suppress the expression of inducible nitric oxide synthase (iNOS) induced by lipopolysaccharide (LPS) in macrophages (Jung et al., 2007). Although these uses of OWV are based on traditional knowledge and use over the years, there are few reports on scientific research findings.

The skin has a central role in host defense because it is the body's largest and most exposed interface with the environment. The regulation of skin defense mechanisms is also crucial, as inappropriate or unbalanced immune responses cause a variety of severe skin inflammatory disorders, including psoriasis, and atopic and contact dermatitis (Kadunce and Krueger, 1996; Galli et al., 2003; Thomas et al., 2004). The prevalence of atopic dermatitis and allergic or irritant contact dermatitis has been increasing significantly in the general population (Pónyai et al., 2007). Contact dermatitis, also referred to as contact hypersensitivity, is a reaction to specific environmental antigens. Most contact allergens are themselves irritants, and they therefore provide both antigen and danger signals when they contact skin. Penetration of irritants or allergens into the living epidermal layers and keratinocyte membrane induces increased proliferation, which serves to remove the invad-

* Corresponding author. Tel.: +82 2 740 8281; fax: +82 2 745 7996.

E-mail addresses: sangkylu@snu.ac.kr, cslee2010@naver.com (S.-K. Ye).

ing pathogens (Proksch et al., 2006). 2,4-Dinitrochlorobenzene (DNCB)-induced contact dermatitis in mice is a commonly used animal model for studying the pathogenesis of contact dermatitis (Zhang et al., 2009).

STAT3 (signal transducers and activators of transcription 3) is a latent cytoplasmic protein involved in transmitting extracellular signals to the nucleus upon stimulation with interleukin-6 (IL-6), epidermal growth factor (EGF) and many other growth factors (Levy and Darnell, 2002). STAT3 undergoes activation through phosphorylation of Tyr705 and in turn activates downstream genes involved in cell proliferation, survival and cell migration (Hirano et al., 2000). In particular, STAT3 activation in keratinocytes is essential for skin wound healing (Sano et al., 1999), and activated STAT3 is found in the epidermis at the wound edge in humans and mice. There is also a report that STAT3 is constitutively activated in epidermal keratinocytes in human psoriatic lesions (Sano et al., 2008). Furthermore, another report showed that STAT3 plays a role in epidermal cell proliferation and survival following exposure to ultraviolet B (UVB) irradiation and is constitutively active in UVB-induced skin tumors from both mice and humans (Kim et al., 2007). Thus, inhibition of STAT3 activation ameliorated both pathological conditions (i.e., skin carcinogenesis and dermatitis).

In the present study, we show that OWV has anti-inflammatory and anti-epidermal proliferative activities against DNCB-induced contact dermatitis and provide evidence to suggest that epidermal thickening and proliferation of keratinocytes are suppressed via STAT3 inactivation.

2. Materials and methods

2.1. Materials and cells

OWV (derived from *Quercus acutissima*), a gift from Dematopy Biotech (Gyeonggi-do Province, South Korea), was verified to have no heavy metals (e.g., Hg, Pb, As and Cd) through heavy metal analysis. Briefly, OWV liquid is made from the cooling gas which is generated by burning oak wood. Then, OWV is refined and filtered more than 10 times, according to the manufacturing methods of Dematopy Biotech. Any solvent is not added to OWV in the process of production. IgE production was tested with an enzyme-linked immunosorbent assay (ELISA) Kit (R&D Systems, MN, USA). The antibody against phospho-STAT3 for immunohistochemistry was purchased from Cell Signaling Technology (MA, USA), and antibodies against phospho-STAT3, α -tubulin and glyceraldehyde phosphate dehydrogenase (GAPDH) for Western blotting were purchased from Santa Cruz Biotechnology (CA, USA). HaCaT cells (a human keratinocyte cell line) were cultured at 37 °C in a humidified 5% CO₂ environment in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% fetal bovine serum (FBS).

2.2. DNCB-induced contact dermatitis mice model

Six-week-old female BALB/c mice were obtained from Samtako (Osan, Korea) and kept under pathogen-free conditions. All animal procedures were performed in accordance with procedures documented in the Seoul National University Laboratory Animal Maintenance Manual. The DNCB-induced contact dermatitis mice model was developed by first shaving the backs of mice with an electronic razor. Two groups, DNCB ($n=6$) and OWV ($n=6$) groups, were epicutaneously treated with 1% DNCB on days 0 and 3, and then with 0.5% DNCB on days 7, 10, 21 and 24. The OWV group received a topical application of 100 μ l oak wood vinegar liquid on the back twice daily from day 7 to day 24. Mice were sacrificed on day 25.

2.3. IgE ELISA assay

Concentrations of IgE in serum isolated from sacrificed mice ($n=6$ per a group) were measured with a specific ELISA kit according to the manufacturer's instructions (R&D Systems).

2.4. Histological analysis

Skin specimens were obtained on day 25 and fixed in 4% formaldehyde. Tissues were cut mid-sagittally, embedded in paraffin 24 h after fixation and serially sectioned for histological analysis. Tissue sections were stained with hematoxylin and eosin (H&E) for analysis of general morphology. For immunohistochemistry, sections were deparaffinized with xylene and washed in ethanol. For antigen unmasking, slides were incubated in 10 mM sodium citrate buffer (pH 6.0) and maintained at a sub-boiling temperature for 10 min. Endogenous peroxidase activity was inhibited by incubating with a 3% hydrogen peroxide solution for 10 min. Slides were then incubated with blocking solution for 1 h at room temperature. After removing blocking solution, primary antibody, diluted in antibody diluent (Invitrogen, USA), was applied to slides and slides were incubated at 4 °C for 24 h. After washing three times with phosphate-buffered saline containing Tween-20 (PBST), slides were incubated with a biotinylated secondary antibody, diluted in antibody diluent, for 30 min. Slides were then incubated with ABC reagent (Vector Labs, CA, USA) for 30 min at room temperature and treated with diaminobenzidine (DAB) as the chromogen (DakoCytomation, CA, USA) for 1–3 min. As soon as the sections developed, slides were immersed in distilled water and counterstained with hematoxylin. Slides were then mounted using Canada balsam reagent.

2.5. Transfection and establishment of stable cell lines

HaCaT cells were seeded at 1×10^6 cells in 60-mm dishes and cultured for 1 day. Cells were then transfected with vector only (CMV-HaCaT) or a constitutively activated STAT3 construct (ST3-HaCaT) using Effectene transfection reagent (Qiagen) following the manufacturer's instructions. After selection with 800 μ g/ml G418 for 4 weeks, resistant clones were isolated and cultured. The final clones stably expressed activated STAT3.

2.6. Wound-healing assay

For scratch wound-healing assays, HaCaT cells were seeded into 6-well plates (5×10^5 cells per well) in DMEM containing 5% FBS and grown to confluence (2 days). A wound was made by scratching the monolayer with a 200- μ l pipette tip, after which cells were incubated with the indicated concentration of OWV or AG490. After 24 h, wounded areas of monolayers were photographed using an Olympus microscope.

2.7. Cell proliferation assay

Cell proliferation assays were performed using a CCK-8 (Cell Counting Kit-8), as described by the manufacturer (DOJINDO, Tokyo). Briefly, HaCaT cells (0.5×10^4 cells) were seeded in 96-well culture plates and incubated for 24 h at 37 °C and 5% CO₂, after which cells were incubated with the indicated percentage of OWV. Finally, CCK-8 solution was added to each well and incubated for 4 h, and then absorbance at 450 nm was measured using a microplate reader. Data are representative of three independent experiments.

2.8. Western blot analysis

Cells were washed twice with cold PBS, and then lysed in ice-cold modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl) containing protease inhibitors (2 mM phenylmethylsulfonylfluoride, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 0.5 mM Na_3VO_4 , 2 mM EDTA and 1 mM NaF). Lysates were microcentrifuged at 13,000 rpm for 20 min at 4 °C, and supernatants were collected. Proteins in lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies, washed, treated with peroxidase-conjugated secondary antibodies, rewashed, and then visualized using an enhanced chemiluminescence system (Amersham, NJ, USA).

2.9. Statistical analysis

Data were analyzed using Microsoft Excel 2007 software, and are expressed as mean $\pm$ SEM. Statistical comparisons were made using Student's *t*-test.

3. Results

3.1. Anti-inflammatory effect of OWV on the DNCB-induced contact dermatitis in mice

To define the effect of OWV on skin inflammation, we developed a DNCB-induced contact dermatitis mice model, as described in Section 2. Mice were challenged by multiple applications of the indicated doses of DNCB to the back skin at appropriate time intervals (Fig. 1A). When mice were sacrificed on day 25, we found that DNCB-stimulated mice developed strong skin inflammation as evidenced by scarring and edema; notably, the scarring and edema of DNCB-stimulated mice were more severe than those of OWV-treated mice. Excoriation and formation of crusty scales in the epidermis of DNCB-stimulated mice were slightly alleviated by OWV (Fig. 1B).

Prior to histopathological analyses, we measured production of IgE, a potential marker of atopic dermatitis, in serum. As shown in Fig. 1C, DNCB increased IgE levels nearly 14-fold in our mice model; this dramatic increase was attenuated in the OWV group, which exhibited approximately a 38% decrease in IgE production compared to the DNCB group. Next, we investigated tissue sections for the presence of neutrophils and T cells as an indicator of immune cell infiltration by immunohistochemistry using antibodies against a neutrophil marker protein and CD3 (Fig. 1D). The data showed that many neutrophils and CD3T cells infiltrated into the dermis and hypodermis in DNCB-stimulated skin tissues; however, OWV inhibited the infiltration of immune cells into inflamed skin tissue (Fig. 1D). Moreover, immunohistochemical analyses revealed that expression of iNOS, a key enzyme involved in inflammation, was induced in inflamed skin cells by DNCB, and this effect was powerfully suppressed by OWV (Fig. 1E).

Collectively, these results suggest that OWV suppresses inflammatory responses, including IgE production, immune cell infiltration and iNOS expression in DNCB-induced contact dermatitis mice.

3.2. Anti-proliferative effect of OWV in DNCB-induced contact dermatitis mice

Severely inflamed skin tissues cause changes in epidermal histology, changes that include increased epidermal proliferation caused by disturbed differentiation. To address this, we measured the effect of OWV on DNCB-induced skin thickness (Fig. 2A). As expected, DNCB increased skin thickness, notably generating a new

necrotic epidermal layer. However, in OWV-treated mice, the thickness of all layers, including the necrotic epidermis (a), epidermis (b), dermis (c) and hypodermis (d), was substantially reduced. These results indicate that OWV exerts a potent anti-proliferative activity against keratinocyte hyperplasia and/or fibrosis.

3.3. Inhibitory effect of OWV on keratinocyte proliferation through inhibition of STAT3 activation

Next, to elucidate the mechanism underlying the inhibitory effect of OWV on epidermal proliferation, we focused on activated STAT3 expression, because STAT3 activation is considered to be crucial in epithelial cell proliferation and differentiation (Sano et al., 2008). Immunohistochemistry using an antibody against phospho-STAT3, the active form of STAT3, revealed that STAT3 was dramatically activated in DNCB-stimulated epidermis (Fig. 2B). However, phospho-STAT3 levels were significantly reduced in OWV-treated mice group, suggesting that OWV suppresses epidermal hyperplasia through its inhibitory effects on STAT3 activation.

To confirm that OWV blunts epithelial proliferation by inhibiting active STAT3, we firstly confirmed that active STAT3 causes keratinocyte proliferation *in vitro* using HaCaT cells, a human keratinocyte cell line. For these experiments, we developed a stable cell line expressing constitutively activated-STAT3 (Fig. 3A), as described in Section 2. As expected, ST3-HaCaT cells grew faster than CMV-HaCaT cells, indicating that STAT3 activation promotes epithelial cell proliferation. Next, we determined the effect of OWV on HaCaT cells proliferation using wound-healing (Fig. 3B) and proliferation assays (Fig. 3C). The results demonstrated that OWV decreased the proliferation rate in a dose-dependent manner compared to control cells. AG490, a JAK/STAT inhibitor, also inhibited HaCaT cell proliferation. Finally, we tested the inhibitory effect of OWV on STAT3 activation in HaCaT cells (Fig. 3D). To induce STAT3 activation *in vitro*, we incubated cells with IL-6, a representative STAT3 activator, for 30 min prior to OWV treatment. Using Western blot, we confirmed that OWV inhibited phospho-STAT3 expression in a dose-dependent manner, as was observed in *in vivo* experiments. Taken together, these data suggest that OWV suppresses keratinocyte proliferation via STAT3 inactivation.

4. Discussion

OWV is activated charcoal made from the bark of an oak and contains wood vinegar liquid including organic acids such as formic acid, acetic acid and propionic acid. OWV has been used to control microbes in plants and pests in agriculture (Radhakrishnan et al., 2002; Yatagai et al., 2002), and is also used as a folk medicine for skin disease (Kimura et al., 2002). In this latter context, the anti-dermatophyte activity of phenolic compounds in OWV was previously reported by Ikerami et al. (1992). Some phenolic acids have their anti-oxidant capacity, ability to scavenge peroxy radicals. These have been described as chain-breaking anti-oxidants, which are related to their hydrogen or electron donating capacity, and their ability to delocalize/stabilize the resulting phenoxyl radical within the structure. It has been reported that the number of phenolic groups in phenolic acids influences anti-oxidant activity (Silva et al., 2001), and has been shown to possess anti-inflammatory properties (Kono et al., 1997). OWV also contains phenolic acid, and has similar properties above described.

Some companies in Korea, Japan and the USA make the wood vinegar into a powder or herb-mixed liquid and use it to alleviate skin inflammation, such as that associated with insect-bites, infected wounds and cellulitis. Although many benefits of organic wood vinegar have been described, little is known about the mechanism(s) underlying its helpful effects on human disorders, including

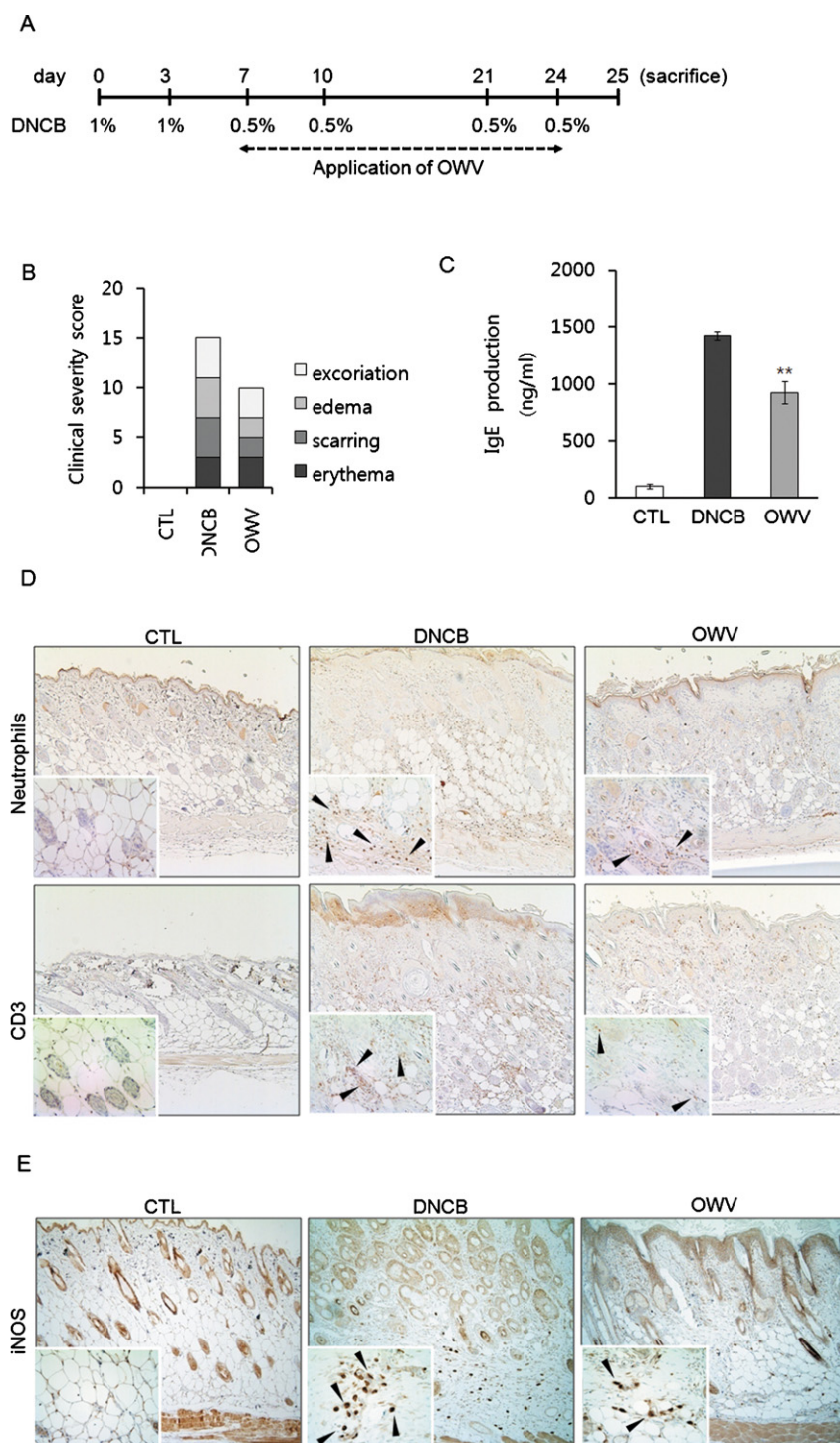


Fig. 1. Morphological and biochemical alteration in OWV-treated mice. (A) The DNCB and OWV treatment schedule used in this study. Mice backs were painted with DNCB, as described in Section 2. (B) Clinical severity scores were graded from scales 0 to 4 (0, not present; 1, minimal; 2, slight; 3, moderate; 4, marked) on day 25. (C) IgE production in each group. Data are presented as mean $\pm$ SEM (** $p < 0.01$ compared to mice treated with DNCB alone; $n = 6$). (D) Neutrophils and CD3+ T cells and (E) iNOS proteins were detected by immunohistochemical staining of back skins of mice treated with or without OWV. The black arrows indicate stained cells. Six mice per group were used, and a representative slide for each group is shown.

skin diseases. Given this absence of information, we sought to study the mechanism by which wood vinegar exerts its biological effects, focusing initially on the anti-inflammatory and anti-proliferative effect of wood vinegar on skin inflammation.

Skin inflammatory diseases, such as contact dermatitis, atopic dermatitis and psoriasis, have features in common, including the recruitment of immune cells and epidermal hyperplasia. DNCB is a representative irritant that induces a contact dermatitis that

depends on type-1 immune responses. However, several reports have recently revealed that Th1 and Th2 immune responses are co-expressed in chemical-induced contact dermatitis (Ulrich et al., 2001; Vanoirbeek et al., 2006). Other reports have shown that DNCB induces symptoms of atopic dermatitis, such as production of IgE and Th2 cytokines, depending on treatment times and doses (Kitagaki et al., 1997; Friedmann, 2006). Based on these previous reports, we investigated IgE production in plasma, infiltration of

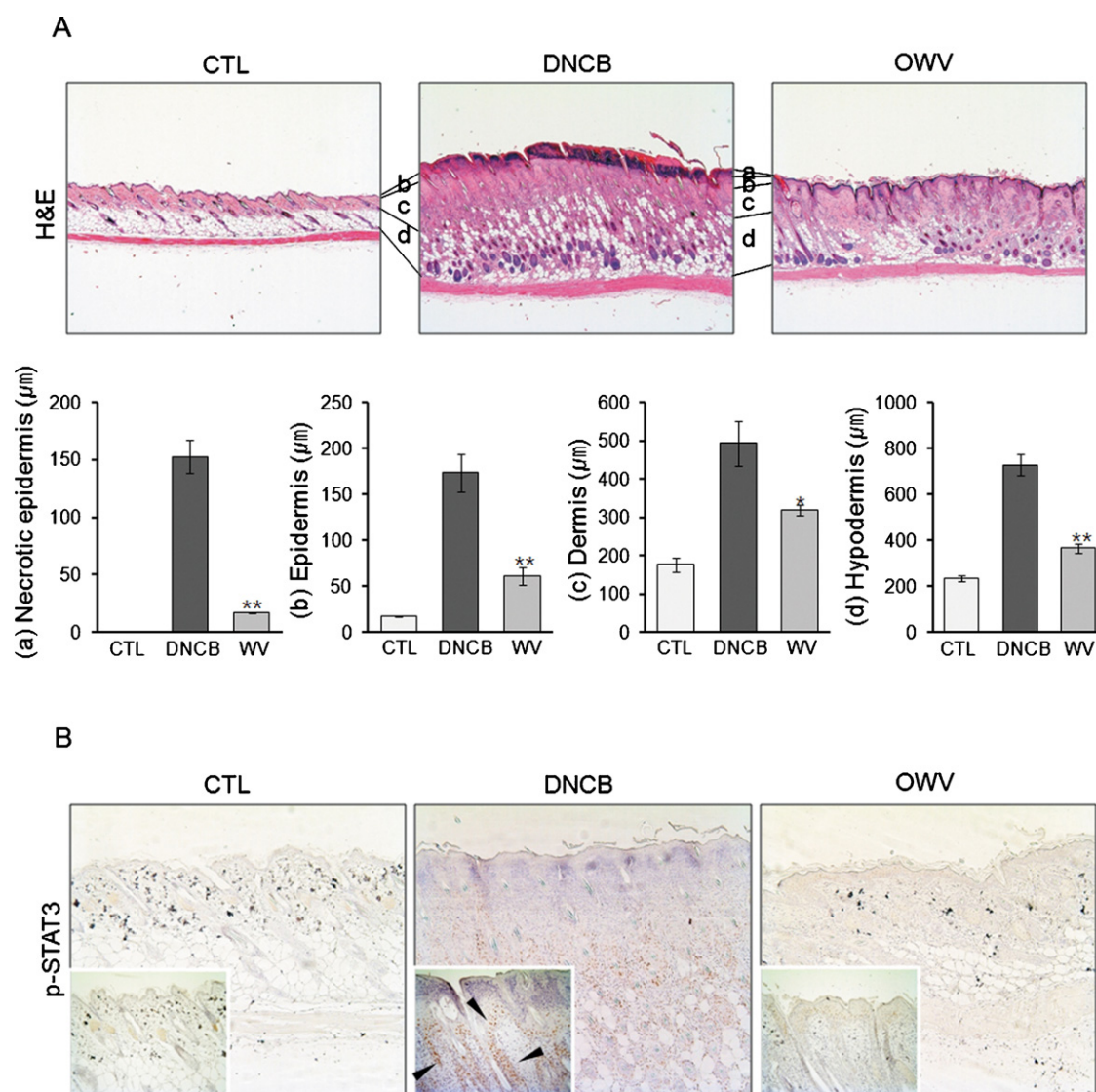


Fig. 2. Comparison of back skin thickness and phospho-STAT3 levels in DNCB-stimulated mice treated with or without OWV. (A) Back skin thickness was determined from representative H&E-stained sections. Skin thickness was calculated according to the skin layers: a, necrotic epidermis; b, epidermis; c, dermis; and d, hypodermis. Each value shown is the mean $\pm$ S.D. (* $p < 0.05$ and ** $p < 0.01$ compared to mice treated with DNCB alone; $n = 6$). (B) Detection of phospho-STAT3 by diaminobenzidine (DAB) staining of the back skins of mice treated with or without OWV. The black arrows indicate stained cells. Six mice per group were used, and a representative slide for each group is shown.

immune cells into inflamed back skin and iNOS expression levels for evidence of the anti-inflammatory effects of wood vinegar on contact dermatitis. In our model, we found that DNCB dramatically induced IgE production and that OWV inhibited DNCB-induced production of IgE. For the detection of immune cell infiltration, we immunostained tissues of back skin with antibodies against neutrophil and CD3T cells marker antibodies. As shown in Fig. 1C, infiltration of neutrophils and CD3T cells was suppressed by OWV. In addition, iNOS, a crucial inflammation molecule, was apparently expressed by several cell types in inflamed skin tissue; however, OWV decreased the number of cells expressing iNOS proteins. These data indicate that OWV can alleviate symptoms of skin diseases, such as atopic or contact dermatitis, providing evidence of its anti-inflammatory functions.

Furthermore, we demonstrated that the anti-proliferative action of OWV is likely mediated by STAT3 inactivation. STAT3, a major transcription factor for genes involved in cell survival, is well known to participate in cell proliferation, differentiation and migration (Leonard and O'Shea, 1998). STAT3 is activated in

the regenerating epidermis (Sano et al., 2005) and is required for UVB-induced proliferation of follicular keratinocytes and epidermal thickening (Kim et al., 2007). Thus, we focused on STAT3 activation in this study because we supposed that active STAT3 is involved in epidermal hyperplasia in DNCB-induced contact dermatitis. Our data showed clearly that STAT3 was hyper-activated in DNCB-induced epidermal thickness and OWV potentially suppressed STAT3 activation and hyperplasia (Fig. 2B). Using *in vitro* studies, we further showed that STAT3 mediates keratinocyte proliferation and demonstrated that OWV suppresses keratinocyte proliferation and STAT3 activation in a dose-dependent manner. These results suggest the possibility that STAT3 mediates DNCB-induced epidermal hyperplasia and OWV suppresses epidermal hyperplasia via STAT3 inactivation *in vivo*. However, further investigation will be required to fully elucidate the relationship between STAT3 activation and epidermal hyperplasia in contact dermatitis. Additionally, it is well known that STAT3 induces the expression of inflammatory molecules, such as iNOS and COX-2, on inflammatory responses (Kim et al., 2008; Lai et al., 2009). These reports give us the pos-

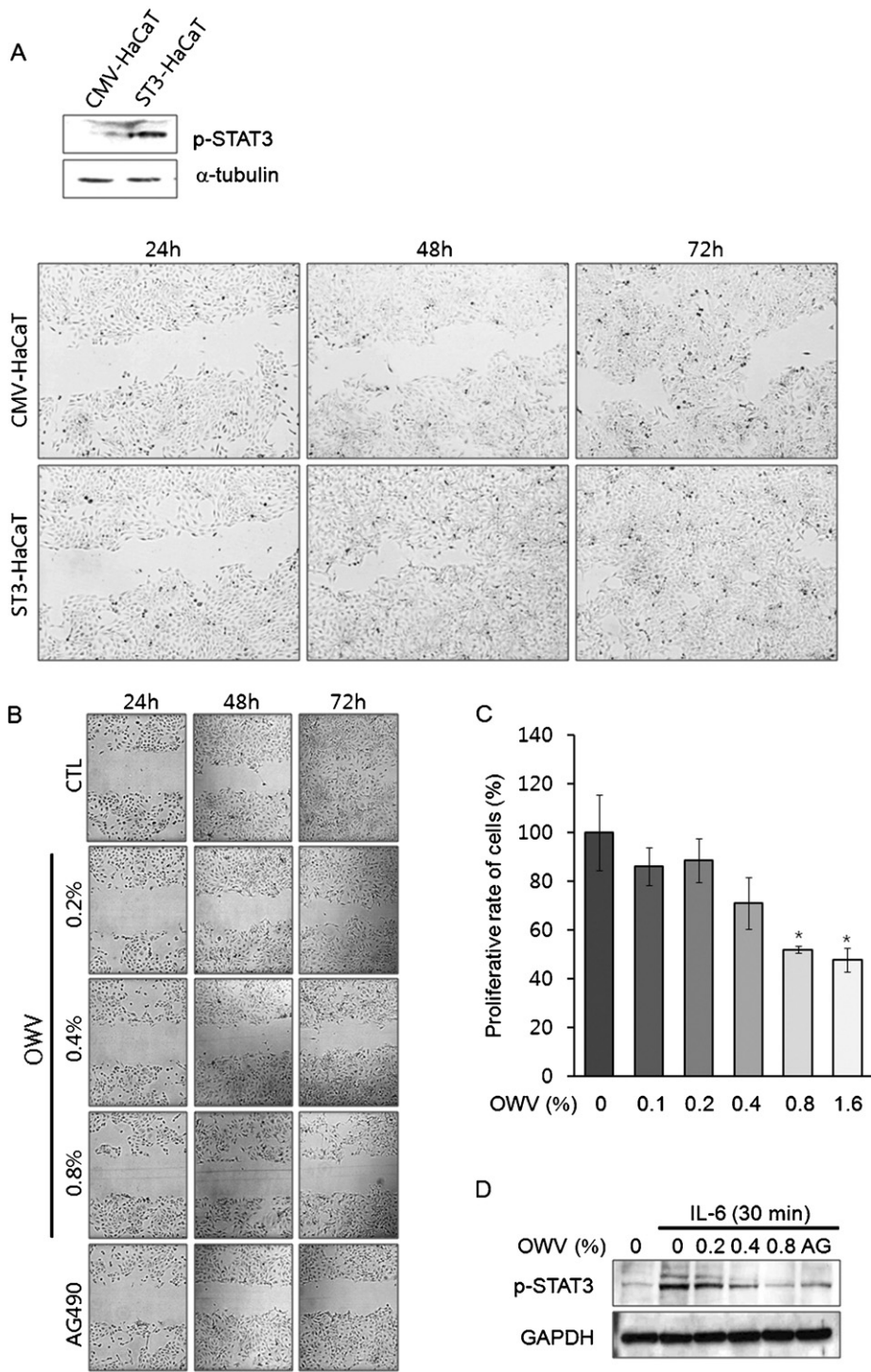


Fig. 3. Effects of OWV on proliferation and phospho-STAT3 levels in HaCaT cells. (A) An HaCaT cell line stably expressing constitutively activated STAT3 (ST3-HaCaT) was established as described in Section 2. The scratched fields were photographed immediately after wounding and 24, 48 and 72 h later. (B) HaCaT cells were incubated with the indicated concentrations of OWV or 25 μ M AG490. The scratched fields were photographed immediately after wounding and 24, 48 and 72 h later. (C) Proliferation assays were performed using a CCK-8 kit, as described by the manufacturer. Each value shown is the mean $\pm$ S.D. (* p < 0.05 compared to control samples; n = 3). (D) Determination of the effect of OWV on phospho-STAT3 status in IL-6-treated HaCaT cells by Western blotting. After HaCaT cells were incubated with the indicated percentages of OWV or 25 μ M AG490, cells were treated with 20 μ g/ml IL-6 for 30 min.

sibility that inhibition of iNOS expression by OWV is mediated by STAT3 inactivation (Fig. 1E). Consistently with these results, the modulation of the STAT3 dependent pathway of DNCB-induced dermatitis mice provides new insight to understand the mode of action of OWV for its anti-inflammatory and anti-proliferative activities.

Taken together, our results show that OWV has anti-inflammatory and anti-proliferative effects on contact dermatitis that are likely mediated through STAT3 inactivation. This finding proposes the possibility that OWV could be clinically used for the treatment of inflammatory diseases by understanding its underlying mechanism. However, further analysis is necessary to

clarify what compositions of OWV have an influence on the anti-dermatitis effects and STAT3 signaling pathway.

5. Conclusion

In the present study, we investigated the anti-inflammatory and anti-proliferative effects of OWV on the skin tissues against DNCB-induced contact dermatitis as well as the underlying STAT3 signaling. We found that OWV exerts anti-dermatitis against DNCB-induced contact dermatitis and the suppression of STAT3 plays an important role in mediating the anti-inflammatory and anti-proliferative effects of OWV.

Q3 Uncited reference

Kupper and Fuhlbrigge (2004).

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