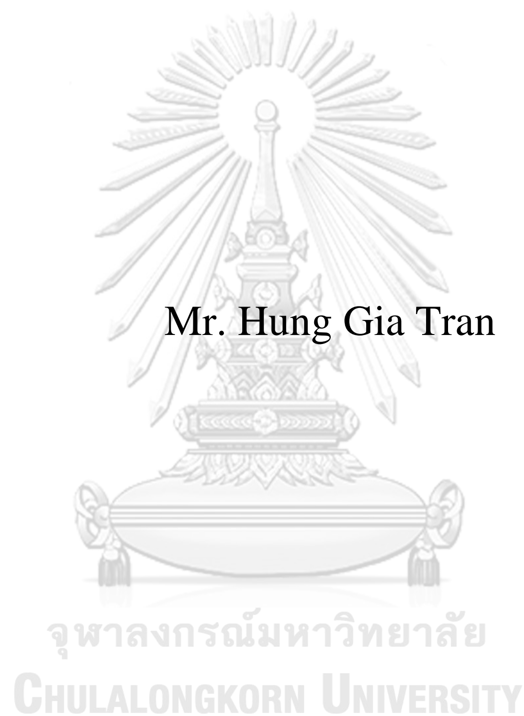
Effect of Oxyresveratrol on Inflammatory Skin Diseases



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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ผลของอ๊อกซีเรสเวอราทรอลกับโรคผิวหนังอักเสบ

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ฮุง เกีย ทราน : ผลของอ๊อกซีเรสเวอราทรอลกับโรคผิวหนังอักเสบ . (Effect of Oxyresveratrol on Inflammatory Skin Diseases) อ.ที่ปรึกษาหลัก : รศ. ดร.พญ.จงกลนี วงศ์ปิยะบวร, อ.ที่ปรึกษาร่วม : ดร.ปฏิภาค เขื่อนจินดา

ที่มา: สารออกซีเรสเวอราทรอล (oxyresveratrol(ORV)) เป็นหนึ่งในสารต้านอนุมูลอิสระ (antioxidant)ตัวใหม่ที่มีการศึกษาอย่างมากในช่วงไม่กี่ปีมานี้ หนึ่งในแหล่งที่มาสำคัญของสารออกซีเรสเวอราทรอล คือ มะหาด (Artocarpus lacucha) ซึ่งใช้ในการรักษาแพทย์แผนไทยมานานหลายสิบปี อย่างไรก็ดียังไม่มีรายงานถึงบทบาทของสารออกซีเรสเวอราทรอลกับการอักเสบที่ผิวหนัง ดังนั้นคณะผู้วิจัยจึงศึกษาผลการลดการอักเสบของสารออกซีเรสเวอราทรอล ในแบบจำลองโรคผิวหนังอักเสบ (dermatitis)

วัตถุประสงค์: เพื่อประเมินผลและกลไกของสารออกซีเรสเวอราทรอลต่อการลดการแบ่งตัว(anti-proliferation) ลดการอักเสบ (anti-inflammation) และการต้านเชื้อสแตปไฟโรคอคคัส ออเรส (*Staphyloccocus aureus*) ของเซลล์ผิวหนัง พร้อมทั้งแสดงประสิทธิภาพในการรักษาการอักเสบของผิวหนังในหนูทดลอง

วัสดุและวิธีการ: ทำการศึกษาผลของสารออกซีเรสเวอราทรอลในเซลล์ผิวหนังกำพร้ามนุษย์ชนิดเซลล์ไลน์ (immortalized keratinocyte cell line (HaCaT) และเซลล์ปฐมภูมิ (primary human keratinocytes (HEKa)) ภายหลังการกระตุ้นด้วยส่วนประกอบของเชื้อแบคทีเรีย ได้แก่ สารเปปติโดไกลแคน(peptidoglycan (PGN)) และสารไลโปโปลีแซคคาไลน์ (lipopolysaccharide (LPS)) โดยสารเปปติโดไกลแคน และสารไลโปโปลีแซคคาไลน์ถูกนำมากระตุ้นให้เกิดการอักเสบของ เซลล์ผิวหนังกำพร้ามนุษย์ชนิดเซลล์ไลน์ และเซลล์ปฐมภูมิ และทำการประเมินผลโดยการทดสอบ เอมทีที(MTT assay) เอนเนคซินห้า (Annexin V) การทดสอบพีไอ(PI assay) การวิเคราะห์วัฏจักรของเซลล์ (cell cycle analysis) เรียวทามพีซีอาร์ (RT-PCR) อีไลซา (ELISA) และ เวสเทอร์นบลอต (Western blot) ในห้องปฏิบัติการ และใช้สารสอง-สี่ ไดไนโตรคลอโลเบนซีน(2,4-Dinitrochlorobenzene (DNCB)) เพื่อกระตุ้นให้เกิดผื่นอักเสบที่ผิวหนัง (Dermatitis)ในหนูทดลองและทำการตัดชิ้นเนื้อย้อมสีเอชและอี (H&E) ย้อมอิมมูโนฮีสโตเคมีสทรี (immunohistochemistry) ต่อซีดีสาม(CD3) ซีดีสี่ (CD4)และซีดีแปด(CD8) เพื่อประเมินผลของสารออกซีเรสเวอราทรอลต่อการอักเสบที่ผิวหนังหนูเบลาซี(BALB/c mice) และวิธีดีสดีฟิสชั่น(Dsc diffusion) เพื่อประเมินการยับยั้งการโตของเชื้อ *Staphyloccocus aureus*

ผลการทดลอง: สารออกซีเรสเวอราทรอลสามารถลดการแบ่งตัวเซลล์ HaCaT โดยก่อให้เกิดการตายแบบอะพอโตสิส (apoptosis) ผ่านทางการกระตุ้นคาสเพสสาม(caspase-3) การให้ สารออกซีเรสเวอราทรอลก่อนกระตุ้นการอักเสบ ของเซลล์ HaCaT และเซลล์ HEKa สามารถยับยั้งการหลั่งสร้างและหลั่งสารไซโตไคน์ที่ก่อให้เกิดการอักเสบ (pro-inflammatory cytokines) ผ่านทางเส้นทางเอ็นเอฟเคปาบี (NF-κB pathway) ในหนูทดลองที่ถูกกระตุ้นให้เกิดผื่นอักเสบด้วย DNCB พบว่า สารออกซีเรสเวอราทรอลสามารถลดความรุนแรงของผื่น ลดความหนาของผิวหนัง และลดเซลล์ชนิด CD3, CD4 และ CD8 ที่ผิวหนังหนูทดลอง สารออกซีเรสเวอราทรอลร่วมกับยาปฏิชีวนะยังมีคุณสมบัติเสริมฤทธิ์กันในการการยับยั้งการเติบโตของเชื้อ

บทสรุป: ผลของความสามารถของสารออกซีเรสเวอราทรอลในลดการอักเสบทั้งในการทดลองในห้องปฏิบัติการและในสัตว์ทดลอง ช่วยบ่งชี้ถึงความเป็นไปได้ในการนำสารออกซีเรสเวอราทรอลมาใช้ในการรักษาโรคผิวหนัง โดยเฉพาะอย่างยิ่งโรคผิวหนังอักเสบ และสารออกซีเรสเวอราทรอลยังช่วยเพิ่มความสามารถในการต่อต้านเชื้อ *Staphyloccocus aureus* ของยาปฏิชีวนะอีกด้วย

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สาขาวิชา	เวชศาสตร์คลินิก	ลายมือชื่อนิสิต ....
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Hung Gia Tran : Effect of Oxyresveratrol on Inflammatory Skin Diseases. Advisor: Assoc. Prof. JONGKONNEE WONGPIYABOVORN, M.D., Ph.D. Co-advisor: Dr. Patipark Kueanjinda

Background: Oxyresveratrol (ORV) is one of the novel antioxidants having been extensively studied in recent years. One of the main sources of ORV is *Artocarpus lakoocha*, which has been used in traditional medicine in Thailand for decades. However, the role of ORV in skin inflammation has not been clearly demonstrated. Therefore, we investigated the anti-inflammatory effects of ORV on dermatitis model.

Objectives: To determine the effect and mechanism of ORV on anti-proliferation, anti-inflammation and anti-*Staphyloccocus aureus* with keratinocytes and to clarify the treating efficacy of ORV on skin inflammatory mouse model.

Materials and methods: The effect of ORV was examined on human immortalized and primary skin cells exposed to bacterial components including peptidoglycan (PGN) and lipopolysaccharide (LPS). PGN and LPS were used to induce inflammation on immortalized keratinocytes (HaCaT) and human epidermal keratinocytes (HEKa). We then performed MTT assay, Annexin V and PI assay, cell cycle analysis, real-time PCR, ELISA and Western blot in these in vitro models. On mouse model, 2,4-Dinitrochlorobenzene (DNCB) was used to induce dermatitis. H&E staining, immunohistochemistry (IHC) staining with CD3, CD4 and CD8 markers were used to evaluate the effects of ORV in in vivo model of skin inflammation using BALB/c mice. Disc diffusion method on *Staphyloccocus aureus* was used to test the anti-bacterial effect of ORV.

Results: Treatment with ORV on HaCaT cells showed the anti-proliferation effect through inducing apoptosis by activating caspase-3. Pretreatment of HaCaT and HEKa cells with ORV inhibited pro-inflammatory cytokine production through inhibition of NF-κB pathway. In DNCB-induced dermatitis mouse model, ORV treatment reduced severity of skin lesion, and skin thickness and numbers of CD3, CD4 and CD8 T cells in the sensitized skin of mice. The combination of ORV and several antibiotics at the dosage using on keratinocytes showed the synergestic effect on anti-*Staphyloccocus aureus* through the increase of inhibiting zone.

Conclusion: ORV treatment can ameliorate inflammation in the in vitro models of skin inflammation and in vivo models of dermatitis, suggesting a therapeutic potential of ORV for treatment of skin diseases particularly eczema. ORV can facilitate the anti-bacterial property of antibiotics on *Staphylococcus aureus*.

Field of Study:	Clinical Sciences	Student's Signature ....
Academic Year:	2022	Advisor's Signature ....
		Co-advisor's Signature ....

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CHAPTER 1: INTRODUCTION

Eczema is the most popular disease in dermatology, including Atopic dermatitis (AD), and Contact dermatitis (CD). Clinical manifestation consists of different stages of eczema. Acute eczematous lesions represent by erythematous papules and vesicles, with pinpoint crusting or weeping. While, subacute to chronic eczematous lesions typically reveal scale, excoriation, and lichenification (1, 2).

Atopic dermatitis is one of the most common eczematous skin diseases with complex mechanism and diverse progress. Clinical manifestation of AD may vary from acute stage with erythematous papulovesicles to chronic stage with lichenification. In addition, the symptoms of AD including itching, even burning in severe condition, and insomnia may cause significant impact on the quality of life of the patients (1). The pathogenesis of AD is the combination of several mechanisms inside such as gene mutation, immune dysregulation, barrier dysfunction, and microbiome dysregulation (3). Role of immune system in AD is categorized into two stages. The initial stage is induced by Th2 cells with IL-4 and IL-13 playing the main function; Th1 and Th17 cells will take part in the later stage with the release of IL-17 and IL-22 (4).

Allergic contact dermatitis (ACD) is a cell-mediated (type IV), delayed type hypersensitivity to the contact allergens. Naïve T cells, which are primed in lymphoid organ during the sensitization phase and grow to be memory CD4+ and CD8+T cell and infiltrate to the skin upon re-exposure to the haptens, play an important role in skin inflammation and cytotoxicity of keratinocytes. (5). Irritant contact dermatitis (ICD) may be the primary diagnosis, but it can also play a crucial role in predisposing to AD and ACD. In fact, ICD is the result of innate immune activation from various external stimuli and the interaction between barrier disruption, cellular changes and proinflammatory mediators (6).

Oxyresveratrol (ORV) is the extract of the heartwood of *Artocarpus lacucha* Buch-Ham. It has been immensely studied about the great antioxidant effect on skin. Therefore, it may possess a wide range of potential biological activities (7). Besides, several research on various kinds of cell also revealed the anti-inflammatory, anti-proliferative and apoptosis effects (8-10). Thus, ORV can be conceptualized to alleviate

inflammatory skin condition, especially eczema. Our aim is to demonstrate the evidences that ORV can be considered as the alternative therapy for eczema.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Eczema

Skin is the biggest barrier organ that separates the body from the outside environment. Besides, this barrier is not always integrating because of the invasion of many physical, chemical, and microbial agents. Eczema (dermatitis) are the group of common inflammatory skin disorders. Patients may present with acute, subacute or chronic eczematous skin lesions. Acute eczematous lesions represent by erythematous papules and vesicles, with pinpoint crusting or weeping. While, subacute to chronic eczematous lesions typically reveal scale, excoriation, and lichenification.

2.1.1. Prevalence of eczema

The proportion of cutaneous disorders may vary from countries, ethnic and ages. To assess the influence of skin diseases on life, a systematic study using the disability adjusted life year (DALY) metric showed that the burden of inflammatory skin condition had the first position among the other types of skin conditions. Particularly, inflammatory skin condition accounted for about 0.38 year of life lost (11). Ranking among the health problems worldwide with all ages, eczema was considered as the prominence, with 11% for white race, 10% for African America and black, 13% Asian and Pacific Islander, and 13% for Native American (12). For more detail, a 2014 global analysis performed a prevailing eczema rate in most countries (13). Notably, among various types of eczema and atopic dermatitis (atopic eczema) seemed to have reach the plateau in the countries with the highest prevalence such as the UK and New Zealand, AD continues to increase in prevalence, specifically in young children (age 6-7 as compared to age 13-14 years) and in low-income countries, such as Latin America or South East Asia (14). The worldwide prevalence of AD is about 2% but varies according to region. Asian and African have a lower proportion than the others (15). It was recorded in Taiwan with 30.3 per 100 000 people each year, and about 0.14% in east Asia in general (16), whereas up to 11% in Caucasian and Scandinavian populations (15). Among various kinds of eczema, atopic dermatitis and contact dermatitis are the most prevalent.

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2.1.2. Pathogenesis

Even though, the pathogenesis several common inflammatory skin diseases are not fully understood. Several skin diseases are attributed to multifactor disorders from the acquired immunity, innate immunity, and the skin barrier (17).

Atopic dermatitis

AD is one of the most common chronic inflammatory skin diseases. Genetic predisposition, epidermal barrier disruption, and dysregulation of the immune system are critical components of AD pathogenesis. An impaired skin barrier may be the initial stage in the development of AD, leading to skin inflammation, allergic sensitization, and even colonization of microorganisms. Type 2 cytokines, interleukin-17 (IL-17) and interleukin-22 (IL-22) have been demonstrated to be contributing factors in skin barrier dysfunction and AD development. New insights into the pathophysiology of AD have focused on epidermal lipid profiles, neuroimmune interactions, and microbial dysbiosis, leading to more precise treatment and AD prevention.

Genetics

The *filaggrin (FLG)* gene is located on chromosome 1q2 and encodes FLG (filaggrin protein), which is a major structural protein in the stratum corneum (SC). The generation of FLG product, urocanic acid, and pyrrolidine carboxylic acid plays a crucial role in maintaining the cutaneous hydration and acidic pH (18). Loss-of-function mutation in the *FLG* gene causes ichthyosis vulgaris, the most common inherited disorder of keratinization. The odds ratio regarding loss-of-function mutations in FLG and AD has been estimated by meta-analysis to range from 3.12 to 4.78 (19). It associates with a more severe phenotype of X-linked recessive ichthyosis and pachyonychia congenital. The same *FLG* mutations are major risk factors for atopic dermatitis and other skin and allergic diseases, including irritant contact dermatitis, asthma, and food allergy (20). Subsequent genome-wide studies (GWAS) and meta-analyses performed in European and Asian populations have confirmed this association and identified additional susceptibility loci for AD (21-23).

The *Serine Peptidase Inhibitor Kazal type 5 (SPINK5)* gene encodes lympho-epithelial kazal-type related inhibitor (LEKTI), which regulates proteolysis in terminal keratinocyte differentiation. LEKTI inhibits a well-characterized protease stratum corneum chymotryptic enzyme (SCCE) involved in cleaving the intercellular

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attachments between corneocytes in the normal desquamation process. The consequences of LEKTI deficiency are upregulating SCCE function, which leads to the increasing cleavage of intercellular branches, reducing corneocyte cohesion, and compromising barrier function (24). A meta-analysis on SPINK5 Asn368Ser polymorphism also revealed that this gene may be a risk of AD with odd ratios varied from 1.1 to 1.7 among the allele model (25).

Polymorphisms of various immune pathway genes are associated with an increased risk of AD through alternations in the T-helper (Th) type 2 signaling pathway. The upregulation of interleukin (IL) 4 and IL-13 lowers FLG expression and follows skin barrier defects (26). Other immune-related genes contributing to AD development include *IL-31, IL-33, signal transducer and activator of transcription (STAT) 6, thymic stromal lymphopoietin (TSLP)* and its receptors, *interferon regulatory factor 2, Toll-like receptor 2, and high-affinity IgE receptor (FcεRI) α gene* in a specific population (27-29). Moreover, recent studies found that *vitamin D receptor* mutations and *cytochrome P450 family 27 subfamily A member 1 (CYP27A1)* changing also correlate with AD. CYP27A1 is a factor in the metabolism process of vitamin D3, so it is essential in modulating the immune system (30).

Immune dysregulation

Several studies showed that immune cytokines from Th2, especially IL-4 and IL-13, play a significant role in chemokine production, barrier dysfunction, suppression of antimicrobial peptides (AMPs), and allergic inflammation (31). Interestingly, IL-31 was reported to enhance brain-derived natriuretic peptides' release and production and coordinate cytokine and chemokine release from cutaneous cells, thus inducing itch in AD patients (32). There is a hypersensitive condition to environmental factors such as allergens, microorganisms, diesel exhaust, cigarette smoke, and chemical irritants. The higher expression of TSLP in the epidermis was demonstrated as an essential contributor.

Not as common as the type 2 pathway, IL-17 has been reported to reduce the expression of FLG and involucrin. More prominent Th17 activation was observed in blood and acute AD skin lesions in Asian patients than in European and American patients (33). IL-22 is also upregulated in the skin of AD patients and relates with skin barrier dysfunction and abnormal epidermal markers, such as keratin 6 and keratin 16

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(34). When it turns into the chronic phase, Th1-cell activation manifests along with sustained activation of Th2 and Th22 cells. In addition, the combination of TNF-α and Th2 cytokines reduce the level of long-chain free fatty acids (FFA) and ester-linked ω-hydroxy (EO) ceramides (35).

Recent studies revealed that skin-resident group 2 innate lymphoid cells (ILC2) could help the production of IL-5, IL-13 and positively enrich in the lesions of patients with AD. This leads to the release of type 2 cytokines to induce skin allergic inflammation. In contrast, epidermal ILC2s are inhibited by E-cadherin (36).

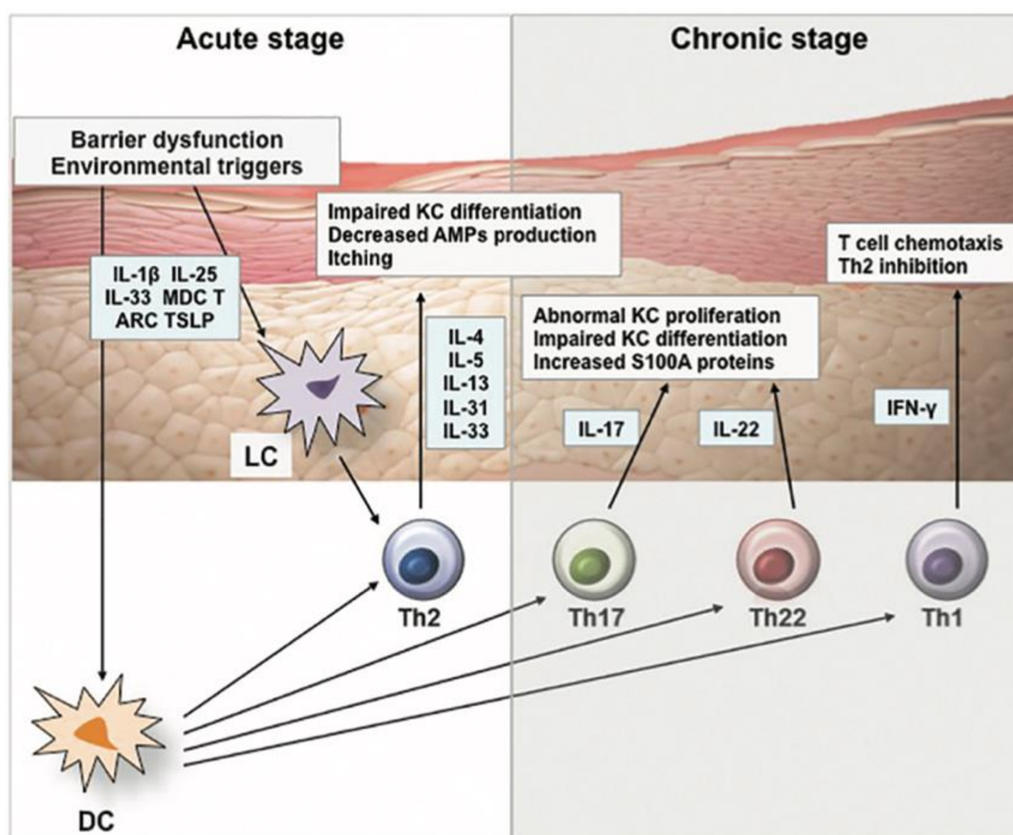

Figure 1. Effect of cytokines on the epidermis in AD (37)

Neuroimmune mechanisms

Histamine is a well-known factor, which can cause itching as well as inflammation. Thus, H1 antihistamines have been widely used for the treatment of itch because of urticaria. Also, clinicians choose those for control itch in AD, although the effects seem to be limited. Recently, more researches have been interested in the role of histamine-independent itch signaling pathways in which TSLP and type 2 cytokines,

such as IL-4, IL-13, and IL-31 (4). In addition, the activation of STAT3 in the astrocytes of the spinal dorsal horn has been reported to be involved in chronic pruritus via the generation of lipocalin-2 (38).

Epidermal dysfunction

IL-4, IL-13, IL-31, IL-33, and high-mobility group box 1 (HMGB1) downregulate epidermal barrier protein production, including FLG, keratins, loricrin, involucrin, and cell adhesion molecules. A damaged epidermal barrier leads to the development of AD and raises the risk of food allergy and airway hyperactivity (18, 39).

AMPs, including cathelicidin (LL37) and human β-defensins, are produced by keratinocytes and play a vital role for host defense as well as control physiologic functions, such as inflammation and wound healing. AMPs expression can be decreased by Th2 cytokines which induces a higher predisposition to S. aureus colonization and aggravates AD (40).

Lipids

A wide range of lipids, consisting of ceramides, long-chain free fatty acids (FFAs), and cholesterol, construct the lipid matrix organized in lamellar bodies and located between corneocytes. Subsequent enzymic processing produces the major lipid classes which are necessary to maintain the integrity of the epidermal barrier. In AD patients, the alteration in lipid composition is witnessed. Th2 cytokines suppress levels of long-chain FFAs and ester-linked omega-hydroxy acyl (EO) ceramides in a STAT6-dependent manner. Moreover, transepidermal water loss (TEWL) also is reverse-correlation with the levels of these ceramides (41).

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Microbiome

During the AD flares, bacterial diversity on skin has been decreased with reduction of commensal bacteria such as *Streptococcus, Propionibacterium, Acinetobacter, Corynebacterium*, whereas *Staphylococcus* highly increase (42). In contrast, a greater change in bacterial community has been observed after AD treatment with a rising abundance of *Staphylococcus epidermidis*, *Streptococcus*, *Corynebacterium*, and *Propionibacterium* (43). *S. aureus* colonization has pivotal roles in the development and exacerbation of AD. It can induce the expansion of B cells independent with T cells; upregulate proinflammatory cytokines such as TSLP, IL-4,

IL-12, and IL-22; and stimulate mast cell degranulation, which results in Th2 skewing and skin inflammation (44). Interestingly, MRSA colonization on AD skin can worsen the reduction of commensal bacteria. On an AD mice model, a recent study demonstrated that epidermal thickening and expansion of cutaneous Th2 and Th17 cells correlated with exposure to *S. aureus* isolated from AD patients.

Besides, the microbiome imbalance is not only limit to the skin but also in the intestinal tract. The previous study has revealed a lower number of *Bifidobacterium* and a higher number of *Staphylococcus* than that of healthy control subjects.

Table 1. Epithelial skin dysfunction in Atopic Dermatitis (37)

Epithelial Dysfunction	Abnormalities	Effects
Cornified envelope proteins	Decreased expression of filaggrin, transglutaminases, keratins, loricrin, involucrin, and intercellular proteins	↓ Skin hydration ↑ Skin pH ↑ Penetration of allergens and microbes ↑ Proinflammatory cytokines ↓ Inflammatory threshold levels
Tight junctions	Reduced claudins	↓ Skin hydration ↑ TEWL ↑ Penetration of allergens and microbes
Antimicrobial peptides	Decreased cathelicidin (LL-37) and human β-defensins	↑ Skin infections ↑ Cytokine production
Microbiome	S. aureus colonization and decreased bacterial diversity	↓ Expression of filaggrin, loricrin, desmocollin1, and keratins ↑ Proinflammatory cytokines ↑ Skin infections

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Epidermal lipids	Decreased long-chain FFAs and ceramides	↑ TEWL ↑ S. aureus infections

Contact dermatitis

Due to the urbanization, contact dermatitis becomes popular. As the outermost layer of the body, the skin has to incessantly face with physical or chemical factors from the environment. According to the pathophysiological mechanisms, contact dermatitis is classified into two main types: Allergic contact dermatitis (ACD) and Irritant contact dermatitis (ICD).

Allergic contact dermatitis

ACD is the type 4 hypersensitivity reaction to the contact allergens. Initially, naïve T cells, which are primed by antigen presenting cell in lymphoid organ during the sensitization phase and grow to be memory CD4+ and CD8+T cell. They infiltrate to the skin upon re-exposure to the contact allergens and play an important role in skin inflammation and cytotoxicity of keratinocytes. (5). To develop ACD, skin must have initial contact with the sensitizing agents to develop immunologic memory, then having subsequently contact to that substance which will elicit the immune response. Therefore, it would be categorized into three phases, including sensitization phase, elicitation phase and the regulation or resolution phase to withdraw the inflammation (5).

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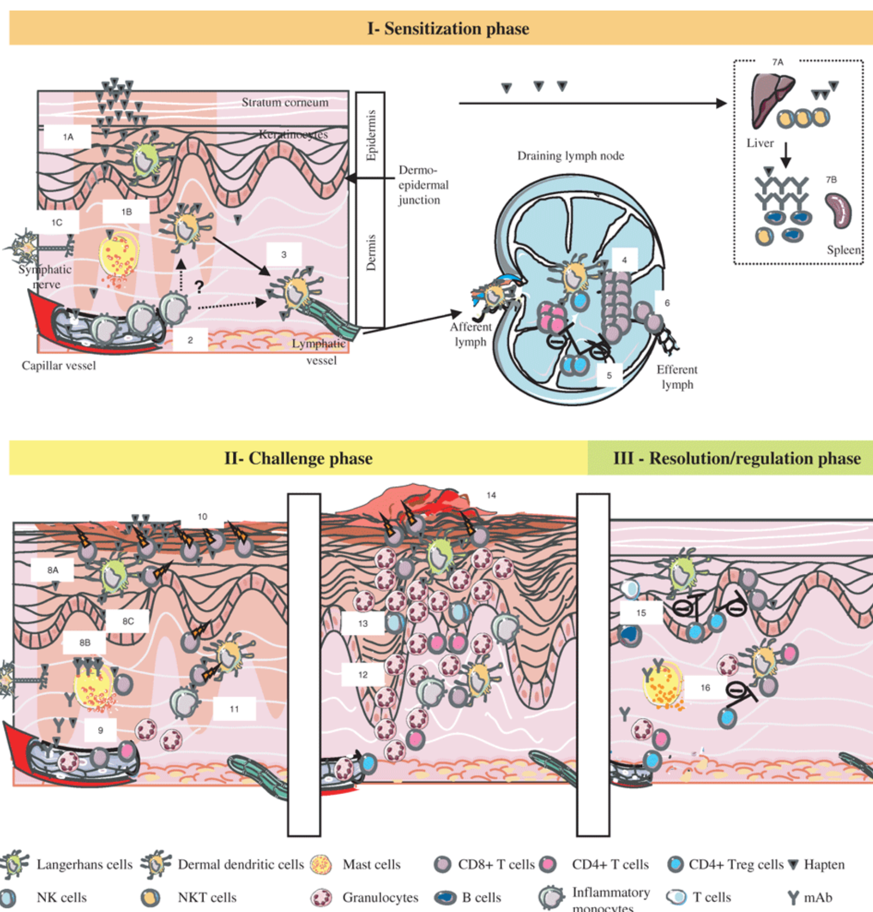

Figure 2. The scheme of pathophysiology of allergic contact dermatitis (5)

Sensitization phase

When having the first contact to the skin, the hapten will cause the generation of hapten-specific T cells in the lymph nodes (LNs). This special T cells will migrate to the skin to wait for the next exposure. There are two properties that can affect the potential causing sensitization of a hapten. Firstly, the proinflammatory properties, haptens will activate the innate immunity and induce the signal productions from IL-8, IL-18, TNF-α and granulocyte-macrophage colony-stimulating factor to recruit and promote the maturation of DCs (45). Secondly, through the binding with amino acid residues which modify as self-proteins, haptens can affect how the skin adapt to new antigenic (46).

The DCs will engulf the haptens and express the haptenated peptides via MHC class I and II on the cell surface. These DCs will migrate from the skin to regional LNs to interact with CD8+ and CD4+ T lymphocytes primed in the para-cortical area. After

differentiated process, the T cells will move out to the area between lymphoid organs and the skin. The most important population of cells in allergic sensitization is the Th1/Tc1 subset (IFN-γ producing) and Th17/Tc17 subsets has been described and may play a role in the sensitization phase of ACD. The afferent phase may last for 10-15 days in human with light manifestation or even no expression. After that, a primary skin inflammation will occur (figure 2) (47).

Elicitation phase

Re-exposure to the same hapten can evoke the inflammation within 24-72 hours. This phase is also known as efferent or challenge phase of ACD and have two waves. At first, haptens diffuse into the skin and induce low-grade non-specific inflammation through cellular stress. Also, toll-like receptors and nucleotide-binding oligomerization domain-like receptors are activated leading to the recruitment of leucocytes and effector T cells (47).

The second wave happens when the antigen-specific effector T cells immigrate to skin and interact with their target antigen indirectly through the antigen-presenting cells (LCs and DCs). In response the antigen-specific T cells will amplify specific immune response, leading to the release of cytokines (IFN-γ and TNF-α). Thus, it will attract more inflammatory cells while stimulating the macrophages and keratinocytes to secrete more cytokines (48).

Regulation phase

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The regulation phase or resolution phase of ACD consist of several mechanisms with the clearance of allergic agents from the skin and the activations of CD4+ regulatory T cells (Tregs). Interestingly, CD4+ Treg cells take part in both the priming/expansion of specific CD8+ T cells in lymphoid organs and the activation of CD8+ T cells in the skin (49). However, the exact mechanism and phenotype of Treg cells involving in the resolution phase of ACD still remain unclear (48, 50).

Irritant contact dermatitis

Irritant contact dermatitis (ICD) may be the primary diagnosis, but it can also play a crucial role in predisposing to AD and ACD. In fact, ICD is the result of innate immune activation from various external stimuli and the interaction between barrier disruption, cellular changes and proinflammatory mediators (6). Following the irritant insult, there is disruption of the epidermal barrier with the release of initial

proinflammatory mediators, which are IL-1 and TNF-α. This results in the production of further cytokines and chemokines from keratinocytes, fibroblasts, and endothelial cells. Also, there are the upregulation of adhesion molecules on endothelial cells, and the subsequent recruitment of leucocytes, thereby prolonging the inflammation (6).

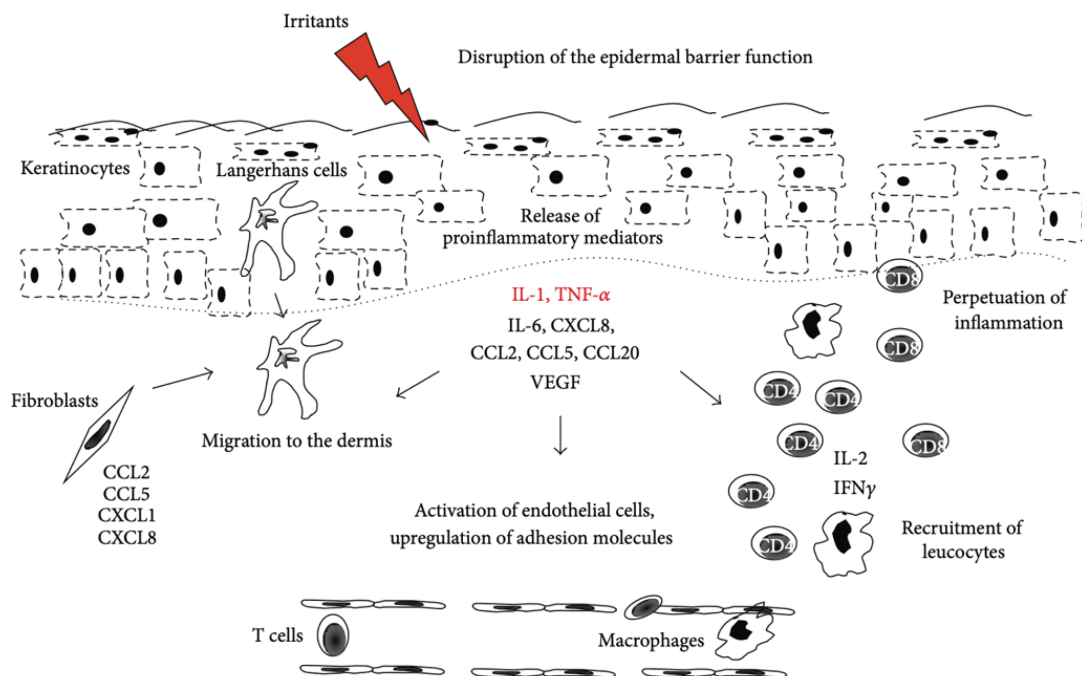

Figure 3. Mechanisms in Irritant contact dermatitis (6)

Comparison between ACD and ICD

Although having unique pathological characteristics, ACD and ICD also have some common features which manifest the outbreak of inflammation on the skin such as certain histopathology (cellular infiltration, vasodilation) and molecules (cytokines, chemokines, endothelial adhesion molecules). The initial phase of both types witnesses an expression of IL-1 and TNF-α from innate immune responses. In the subsequent phase of ICD, innate immune still own the main role for the inflammation. In contrast, adaptive immune responses involving antigen-specific T cells take responsibility to amplify the inflammation (51). In particular, K. Ouwehand et al. determined that CXCR4 and CCR7 would increase by allergen but not irritant exposure. Those chemokines can help to facilitate allergen-induced LC migration toward lymph vessels (52). In addition, CXCL9, CXCL10 and CXCL11 also join in the upregulation in ACD (51). It has been demonstrated that the production of IL-1β and CXCL8 in an ACD

model was higher than that compared to ICD. Also, considering to the gene expression, allergens but not irritants may lead to the activation of certain genes such as CCL23, CCL4, CYP27A1, NOTCH3, S100A4, and SLAM in DCs (6).

2.1.3. Role of some essential inflammatory pathways

NF-κB

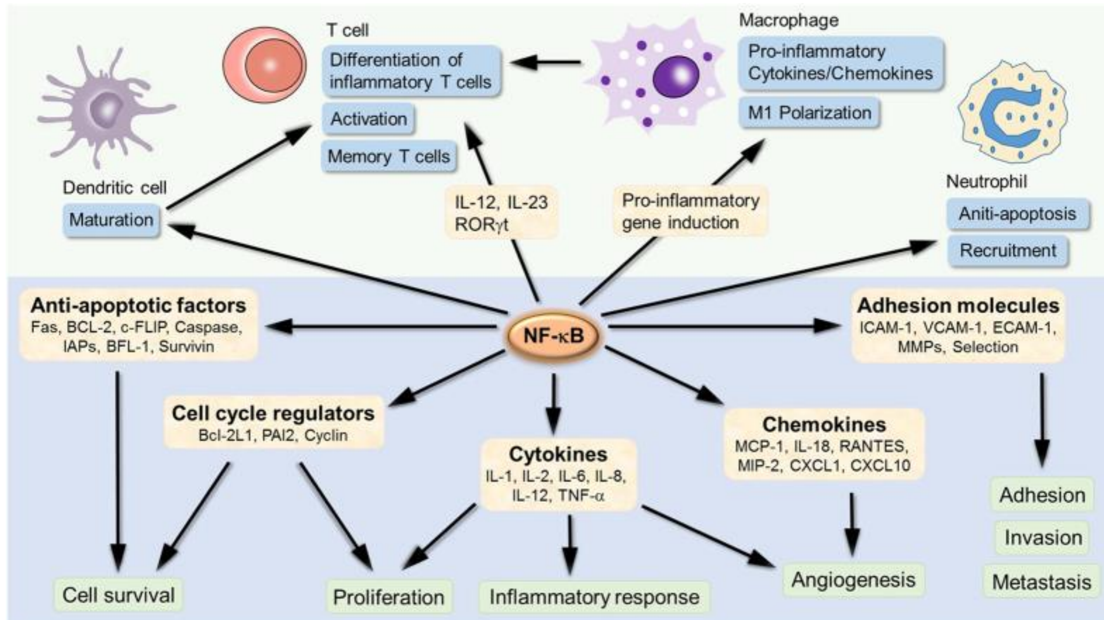

Figure 4. Role of NF-κB in inflammation development and progression (53)

Nuclear factor-κB (NF-κB) represents a family of inducible transcription factors, leading to a large number of genes taking part in the process of immune and inflammatory responses. This family is composed of five subsets, which are NF-κB1 (also named p50), NF-κB2 (also called p52), RelA (also named p65), RelB, and c-Rel. In resting condition, NFκB present in the cytoplasm with the inhibition of IκB. After stimulated by agents, the phosphorylation will happen and following with IκB degradation. There are two canonical and noncanonical pathways, which came from diverse stimuli. NFκB involves most of the immune response and inflammatory response (54). NFκB play a central role in both innate and adaptive immune system by inducing gen transcriptions to produce cytokines, chemokines, and adhesion molecules. Thus, it regulates cell proliferation, apoptosis, morphogenesis, and differentiation. A recent study showed that NFκB plays a pivotal role in forming the NLRP3 inflammasome, leading to the activation of IL-1β, a potent pro-inflammatory cytokine.

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This explains the importance of NFκB in for the host-response and resistance to pathogens, damaging effect during chronic disease and acute injury (55). For those reasons, NFκB still always an essential hallmark in immunology studies to develop novel therapeutic strategies.

In atopic dermatitis, NFκB is the center of interest. The acute inflammatory phase elevates the transcription of Th2 cytokines, such as IL-6, and adhesion molecules ICAM-1. In addition, the chronic phase mainly depends on the Th1 pathway, and NFκB cause the secretion of cytokines such as IFN-γ. The studies using an ointment containing oligodeoxynucleotides, and NFκB inhibitor showed promising results in treatment of mild AD in NC/Nga mice models (56).

JAK-STAT

JAK-STAT pathways play an essential role in the adaption to cytokines stimulation. They are also crucial in normal or abnormal conditions such as immune-mediated inflammatory diseases, including atopic dermatitis and psoriasis. There are four different types of JAK proteins (JAK1, JAK2, JAK3, and TYK2) and seven STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6).

STAT6

Signal transducer and activator of transcription (STAT) proteins are critical mediators of cytokine signaling. There are seven STAT proteins, and STAT6 is activated by IL-4 and IL-13, significantly correlated with AD. Also, STAT6 keep a predominant role in the immune system compared with the others. Once IL-4 and IL-13 bind to their receptors, the associated Janus Kinases (Jak) are activated. Then, the tyrosine residues on the receptor will be phosphorylated, follow by the combination of STAT6 to phosphorylate the conserved tyrosine-641. After that, STAT6 will transform homodimers and translocate to the nucleus, binding directly to DNA to regulate transcription (57).

STAT6 is critical for several responses in T-cells, including the development of Th2 and IL-4 proliferative responses. Through regulating Gata3, the master regulator for Th2 differentiation, STAT6 is required for Th2 functions (58). Besides, mice models that diminished STAT6 also see the loss of expression of Th2 cytokine including IL-4, IL-13, and IL-5 (59).

In B cells, STAT6 causes the switching of immunoglobulin to IgE and IgG1 and the cell surface molecules' expression for antigen presentation of B cells. The level of IgE and IgG1 were dramatically reduced in the models of STAT6 deficiency. IL-4 is also proved to have the mediating property of B cells' surface molecules, consisting of MHC II, CD80, and CD86. Moreover, IL-4 prevents apoptosis in B cells in a STAT6-dependent manner (59).

2.1.4. Treatment

Atopic dermatitis

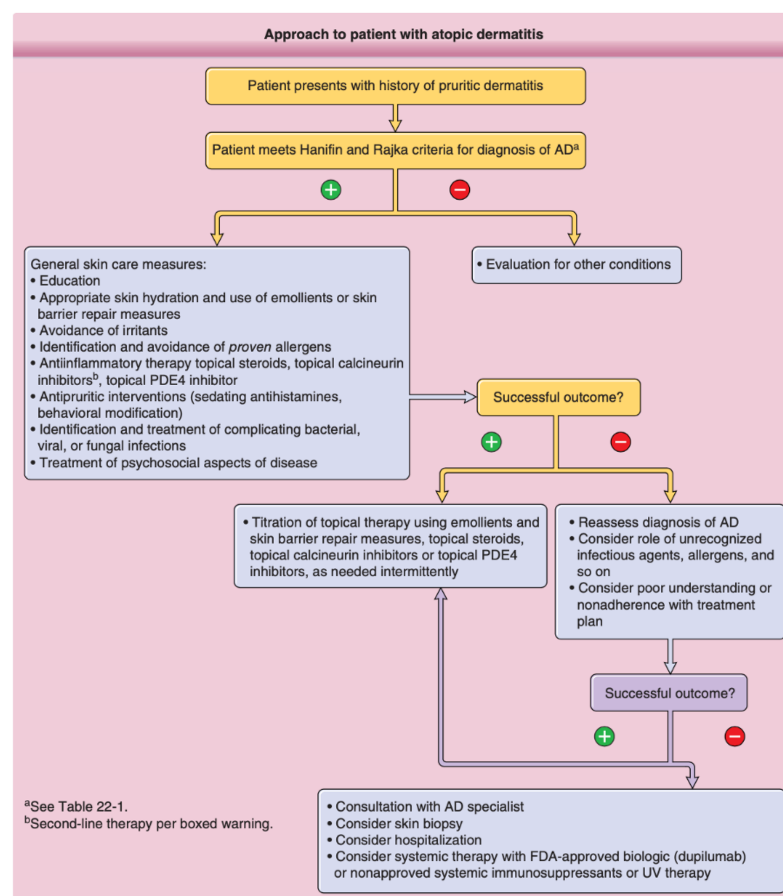

Figure 5. Treatment approach for atopic dermatitis (3)

Moisturizer

Moisturizers, which may contain physiologic mixtures, ceramides-dominant lipid, or petrolatum, have always been considered the first choice in AD treatment and post-treatment to reduce the recurrence of the disease. Many studies have proved the positive effects in lowering TEWL, increasing skin hydration, balancing the cutaneous microbiome, and upregulating AMPs (60, 61). Thus, the need for topical corticosteroids

also reduces. In addition, a recent study demonstrated that topical application of liver X receptor agonist (VTP-38543) improved epidermal differentiation and lipids in patients with mild to moderate AD (62).

Corticosteroids

Corticosteroids play as the cornerstone in the treatment of atopic dermatitis due to the potent anti-inflammatory, antiproliferative, and immunosuppressive properties. They are utilized in both topical and systemic therapy. The application must base on the site of lesions, ages, and even the severity of lesions. The early use corticosteroids can help normalize filaggrin, loricrin, decrease TEWL, increase epidermal hydration. In addition, using intermintently can also help to prevent the recurrence. However, long-term use of TCs brings numerous adverse effects, thereby most clinicians only use it to control the acute phase of AD (3).

Antibiotics

Both topical and oral antibiotics have been used to diminish colonization and infection in AD. However, long-term use may cause drug resistance and a negative impact on commensal microorganisms.

Topical Calcineurin inhibitors

Calcineurin inhibitors, such as tacrolimus and pimecrolimus, inhibit calcineurin-dependent T-cell activation, which leads to downregulation of proinflammatory cytokines. Additionally, tacrolimus has been reported to alleviate pruritis by suppressing sensory nerve activation. Previous study demonstrated that tacrolimus monotherapy could reverse dysbiosis in AD. In particular, it can help to increase the prevalence of some commensal genera such as *Dermacoccus, Pseudomonas, Corynebacterium, Proteus, Micrococcus luteus and Lactococcus* (63).

Phosphodiesterase-4 inhibitor

Crisaborole, which is a steroid-free substance and approved by FDA in 2016 for patients aged from 3 months in the US and from 2 years in Canada, Israel, and Australia (64). It is a phosphodiesterase-4 inhibitor that works primarily on phosphodiesterase 4B (PDE4B). Crisaborole is a phenoxybenzoxaborole chemically. PDE4B inhibition appears to reduce the production of cytokines such as tumor necrosis factor alpha (TNF), interleukin-12 (IL-12), IL-23, and other proteins involved in the immune response and inflammation. Crisaborole 2% ointment is indicated for mild to

moderate AD. Thus, it can help to suppress the secretion of proinflammatory cytokines. Therefore, crisaborole represents a safe and efficacious novel nonsteroidal option to treat mild to moderate AD (65).

Immunosuppressive agents

Cyclosporine was first approved to prevent body rejection to organ transplant, and the FDA approved this medication for treatment in dermatology in 1992. The major actions of cyclosporine are on T-cells; it also affects the other cell types. By binding to cyclophilin, this complex inhibits calcineurin, then prevents the cytokine gene transcription (66). Cyclosporine can effectively treat AD patients with a severe level and resist conventional treatment, and it can also improve the quality of life. However, it must be used in the short-term of 12 to 16 weeks to reduce the risks of serious side effects (3).

Antimetabolite drugs include methotrexate (MTX), mycophenolate mofetil, and azathioprine. MTX and azathioprine can help reduce about 42% of disease severity scores in adult patients (67). However, due to hepatoxicity and hematologic abnormalities, MTX and mycophenolate mofetil should not be used for long-term treatment. Also, azathioprine is noted for the potential in myelosuppression (3).

Phototherapy

In AD treatment, various UV light methods have been approached, including broadband UVB, broadband UVA, narrowband UVB (311 nm), UVA-1 (340 to 400 nm), and combined UVAB. UV light has been demonstrated its photo-immunologic mechanisms, which can be useful in treating AD. With or without psoralene, epidermal LCs and eosinophils are the targets of UVA. On the other hand, UVB can reveal immunosuppressive effects via blocking the function of antigen-presenting of LCs and regulating the production of keratinocyte cytokines. These methods are used for patients with severe and widespread AD (3).

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Biologic treatment

Various targeted biologics therapy which blocks the immune pathways have been incessantly developed for patients with moderate to severe AD. Dupilumab, a humanized monoclonal antibody (mAb) to block IL-4 and IL-13, has been approved by the FDA (68). Because of the identification of Th17 and Th22 upregulation on AD patients, secukinumab and a human monoclonal antibody against IL-17 also in the

progress of investigation (37). Besides, topical tofacitinib (JAK1/JAK3 inhibitor) and oral baricitinib (JAK1/JAK2 inhibitor) were reported to effectively reduce skin inflammation and pruritus in AD patients (69, 70), and omalizumab targeting IgE limits mast cell degranulation and inhibits the release of inflammatory mediators.

Other therapies

The other therapies for treating AD may vary and remain controversial, such as interferon-γ for downregulating Th2 proliferation and function, allergen immunotherapy, probiotics, herbal medication, or oral Vitamin D (3).

Recent studies in microbiome transplantation using *S. hominis* and *S. epidermidi*s or *Roseomonas mucosa*, can help decrease *S. aureus* colonization and AD severity. These can be promising therapies that need more profound studies for wider application (71, 72).

Contact dermatitis

To manage patients with contact dermatitis, the priority is to identify and avoid the causative substances. Especially, with allergic dermatitis, optimal management of ACD requires knowledge of specific allergens, their sources, and the mechanisms by which individuals are exposed to the allergens (48). A commodity management should follow step by step.

The primary prevention includes educating the patients to be aware and identify which exposure to allergens or irritants is likely. Thus, they will have good practice in protecting themselves from contacting to those agents such as wearing protective clothing, using barrier creams, choosing appropriate products for personal hygiene, and even inducing the motivation for patients to keep practicing the protective methods (73).

The secondary prevention needs more approach from the clinicians thorough medical history and careful clinical examination of the patients to figure out the possible exposures from work, home and hobbies. Then, the patch test including suspicious environment allergens can help to identify allergic components of contact dermatitis (73).

The final step or the treatment aims at controlling the inflammation, adjusting the impairment of the skin. Localized acute CD lesions are successfully treated with mid or high potency topical steroid according to the sites. If the condition getting worse

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with the generalization of lesions, systemic steroid therapy is required and offers relief within 12-24 hours. Prednisolone with the initial dosage at 0.5 to 1.0mg/kg is recommended. The clinicians can consider reducing the dosage base on the reduction of severity. Oral antihistamines can be prescribed to control the pruritus associated with CD. Finally, emollients, moisturizers, or barrier creams may be applied as secondary prevention strategies to help avoid continued exposure (74).

In the project of determining the effect of Oxyresveratrol (ORV) on skin diseases, we would like to approach its impacts on dermatitis, which is the most common skin problem.

2.2. Characteristics and bioactivity of oxyresveratrol

Oxyresveratrol (2,4,3',5'-tetrahydroxystilbene) is a phytoalexin present in large amounts in the heartwood of *Artocarpus lakoocha*, also known as moket fruit or monkey jack. It is a tropical evergreen tree species of the family Moraceae. In Thailand, partials of Artocarpus lakoocha have been used as traditional treatment for various kinds of disease for a long time.

Figure 6. Atocarpus lakoocha (Wikipedia)

Like the previous well-known resveratrol, Oxyresveratrol (ORV) is sensitivity and undergo oxidative degradation in aqueous solution. The aqueous solubility of ORV is about 0.6mg/ml. Although having higher water solubility than resveratrol (0.04mg/ml), ORV performed low bioactivity in the aqueous suspension. Therefore, ORV is recommended to suspend in the organic solvents to ensure the highest bioactivity performance (75).

Over the years, ORV and its derivates have attracted lots of research attention, including antioxidant, antibacterial, antiviral, and anticancer activities (7). Also, ORV does not affect cell viability in a higher concentration than resveratrol. The previous studies on macrophages and glial cells demonstrated that ORV is almost safe with the dose ≤80μM/L. While resveratrol, a previously well-known antioxidant, may cause cytotoxicity at 50μM/L after 24h incubation (76, 77).

Although the benefits have been shown on cells, applying this substance as an oral therapy for antioxidants, antivirus, and antiinflammation needs more innovative study. The compound was reported to undergo extensive hepatic metabolism and rapid urinary elimination, resulting in a short-half lifetime, hence restricting its clinical use (78)

2.2.1. Antioxidant activities

It has been proven that oxygen-derived radicals play a role in acute inflammation. To compare ORV ability in antioxidative activities, examine the inhibitory effect against $FeSO_4/H_2O_2$-induced lipid peroxidation in rat liver microsomes and the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging influence had been executed. Subsequently, ORV exhibited more potent antioxidative activity than resveratrol, mulberroside A, also known as antioxidative substances (76). Besides, ORV showed better DNA protection than Trolox, an antioxidant like Vitamin E (7).

2.2.2. Anti-inflammatory activities

Inflammation is an essential response which the body attempts to protect itself from the invasion of foreign agents. However, when this condition is disproportionate, it may lead to many autoimmune diseases. Macrophages, the crucial immune cells of innate immunity, can kill pathogens directly and indirectly via phagocytosis and the secretion of various proinflammatory mediators, including prostanoids, reactive oxygen and nitrogen species, metalloproteinase, cyclooxygenase 2 (COX2), and inflammatory cytokines (8). In a typical model to investigate the potential of ORV on the inflammatory process, the cell line RAW264.7 (monocyte/macrophage-like cells, originating from Abelson leukemia virus transformed cell line derived from BALB/c mice) was chosen, and lipopolysaccharide (LPS) was considered as the main stimulator. Exposure of LPS-stimulated cells to ORV inhibited nitrite accumulation in the culture medium. ORV also inhibited the increase of inducible nitric oxide synthase (iNOS)

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expression in a concentration manner. Also, it significantly suppressed LPS-evoked nuclear translocation of NF-κB and COX2 activity in macrophages (8, 76). Furthermore, IL-6 was also impeded about 70%, with the concentration at 40μM/L (8).

Neuroinflammation can lead to several different neurodegenerative conditions, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis (79). It has been demonstrated that ORV strongly decreased the release of IL-6 and MCP-1 from Human Microglial Cells (HMC3) stimulated with IL-1β (80). Along with protecting microglial cells, ORV could help reserve the spinal cord's integration in a model of spinal cord injury murine (77).

2.2.3. Antibacterial activities

Staphylococcus aureus infected condition is one of the most prevalent infections worldwide. Through many assays, ORV revealed a promised ability as an antibiotic. In the previous study, the minimum inhibitory concentration (MIC) of ORV against all *S. aureus* was 125μg/ml (81). At this value, ORV may cause toxicity on the macrophages. However, in a combination of ORV + antibiotics, ORV performed an adequate response at only 62.5μg/ml of MIC, less than twice when using it alone and safer to the cell viability. Also, the combination models could successfully prevent the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) species. It also demonstrated that ORV sufficiently enhanced membrane permeability and ATPase inhibition. Besides, cell morphology of ORV-treated cells was observed using transmission electron microscopy, which confirmed the weakening of the cell wall and lytic effects of ORV on the *S. aureus* (81, 82).

2.2.4. Antivirus activities

ORV and its derivates were first tested against Herpes simplex virus type 1. Then, the cumulative studies exhibited that ORV inhibited the early and late phases of viral replication. In a pretreatment model, ORV affected both HSV-1 and HSV-2. Nearly 100% inhibition of virus replication was achieved for both types after 24h with the concentration of 50μg/ml, which there was no cytotoxic happen (83). In the microemulsion, 20-30% ORV cream had as effective as 5% acyclovir cream when applying five times a day on cutaneous HSV-1 mouse infection (84). Due to antivirus potential, topical ORV products can be considered a promising therapy for treating

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HSV-1 disease in humans. Also, with the ability to inhibit tyrosinase, a cream containing ORV can prevent post-inflammatory hyperpigmentation.

2.2.5. Antiproliferation

In the immune-mediated chronic inflammatory disease, the proliferative property also plays a crucial role. Notably, in atopic dermatitis, an excess proliferation may cause desquamation or even lichenification if the disease prolongs. ORV also has the antiproliferative characteristic like the other anti-inflammatory substances. Through some previous studies, people have proved the antiproliferative effect through cancer cell models. However, its antiproliferative effect varies base on the cell types.

In research using HepG2 (hepatic cancer cell line) and MCF-7 (human breast cancer cell line), the half maximal inhibitory concentration (IC50) of ORV were $36.37 \pm 1.50 \mu M$ and $60.86 \pm 1.56 \mu M$, respectively. It was just slightly differ to resveratrol (RSV) with $39.14 \pm 1.55 \mu M$ and $56.07 \pm 1.51 \mu M$ (85). A study on HT-19 (colon cancer cell line) revealed a more substantial antiproliferative effect of ORV than RSV with the IC50 $74.4 \pm 10.1 \mu M$ and $152.1 \pm 17.6 \mu M$, respectively (9).

Another study with squamous cell carcinoma cell lines (HSC-3, HN-8, and HN-30) demonstrated that resveratrol and ORV could effectively inhibit cell survival through a dose-dependent manner. With ELISA and Real-time PCR methods, ORV could also inhibit vascular endothelial growth factor (VEGF) at both mRNA and protein levels. Although it was found that ORV possesses the anticancer potential, its efficacy is lower compared with that of resveratrol. The IC50 values of ORV were slightly higher than RSV with HN-8 and HN30, while it was higher than that of RSV for HSC-3 (86).

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2.2.6. Apoptosis

Apoptosis or programmed cell death plays a crucial role in regulating inflammation and may be the result of inflammation. To maintain homeostasis, it is necessary to recognize and remove invading microbial organisms and the dying cells. In a study with human neuroblastoma cells (SH-SY5Y), ORV treatment at 120μM promoted the phosphorylation of p38 MAPK and induced autophagy and apoptosis. ORV can cause the decrease of PI3K/AKT/mTOR signaling in the targeted cells, thereby increasing the autophagy level. Interestingly, the process of apoptosis in this research was not the direct result of ROS. It was because of the upregulating caspase-3

and caspase-9, which are the hallmark of the apoptosis pathway in neuroblastoma cells (10). Another study also revealed the enhanced ability of ORV on caspase-3 and caspase-9 through and reduced mitochondrial membrane potential on osteosarcoma cells (Saos-2). Besides, the phosphorylation of STAT3 was attenuated in Saos-2 cells after treatment with ORV, inducing the inhibition of cell viability and apoptosis (87). In contrast to the above studies, a research on breast cancer cell line (MDA-MB-23) showed that ORV could generate a remarkable level of ROS at 80μM (88).

2.2.7. Toxicity of Oxyresveratrol

To determine the safety profile of ORV, a study on rat has been generated recently. ORV at greater than 2000 mg/kg through oral gavage did not cause any mortality to the rats. Also, there was no change in behavior or locomotion during the study period (89). In addition, a sub-chronic toxicity test was performed in nine weeks with ORV at 50, 100 and 150 mg/kg by daily oral administration. In general, there was no significant changes in body weight, food consumption and water intake among the groups. However, when compared to the control group, the 150 mg/kg oxyresveratrol group showed changes in several biochemical and hematological parameters, as well as changes in the pathophysiology of cardiac, hepatic, and renal tissues. In conclusion, no observed adverse or harmful effect of ORV at 100 mg/kg in rats (89). On human, there are no toxicity studies reported on ORV long term use until now.

In conclusion, ORV has essential characteristics that need in antiinflammation. However, the effect relies on the cell type, a dose-dependent manner and time-dependent manner. Also, the previous studies on cutaneous cells mainly focused on cosmetic aspect, which is antioxidant effect. Therefore, we need more profound research on skin disease models to approach more safe and novel therapies.

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CHAPTER 3: OBJECTIVES

Eczema is the most prevalent condition in dermatology. To treat this kind of condition, beside the conventional treatment by corticosteroid in the exacerbating phase, the alternative and adjunctive treatments have been always considered in the maintenance phase to prevent the relapse of the diseases.

Oxyresveratrol is the extract from Thai herb, isolated from the heartwood of *Artocarpus lakoocha* Buch-Ham. The previous studies on cutaneous cells have mainly focused on the antioxidant effect and revealed the significant benefits. However, there were some single studies on several kind of cells showed that ORV also possessed anti-pathogenic characteristics. Also, the performance also varied among the cell types. Therefore, our study will focus to clarify the beneficial effects and mechanisms of ORV in controlling the inflammatory skin diseases.

Objective

1. To determine the effect and mechanism of ORV on anti-proliferation, anti-inflammtion with keratinocytes.
2. To determine the treating efficacy of ORV on skin inflammatory mouse model.
3. To determine the antibacterial effect of ORV on *Staphylococcus aureus*.

Hypothesis

Oxyresveratrol (ORV) has beneficial effects on eczematous skin diseases by its antiproliferative, anti-inflammatory, and antibacterial properties on keratinocytes.

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CHAPTER 4: SIGNIFICANCE OF THE RESEARCH

Although the studies about the mechanisms and treatments in inflammatory skin diseases have always been conducting, sufficient understanding in order to apply the best treating and managing methods have not been optimal. Nowadays, the products from herbal extracts are becoming more common. In addition, utilizing these therapeutics for mild to moderate conditions may offer positive subsequent as well as the conventional methods in the official treating guideline.

The previous evidence has implicated that ORV has anti-inflammatory and anti-proliferative potentials in several cell types. However, these characteristics have not been adequately clarified in keratinocytes associated with inflammatory skin models yet. Our study will contribute the remaining effects of ORV beside the antioxidant effect. Therefore, ORV can be considered as a promising therapeutic for controlling and managing inflammatory skin diseases.

Furthermore, antibiotic resistance is a worldwide issue. Investigations into novel antibiotics, bactericidal agents, or compounds capable of increasing the antibacterial activity of existing antibiotics have always remained interesting and developed. If ORV has an antimicrobial impact, this discovery could help with dermatitis therapy.

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CHAPTER 5: RESEARCH METHODOLOGY

5.1. Experiment method

5.1.1. Culture of HaCaT cells and Primary Keratinocyte HEKa cells

HaCaT was maintained in Dulbecco's Modified Eagle Medium (DMEM, high glu-cose, pyruvate; #11995), supplemented with 10% fetal bovine serum (#10270), 0.01 M HEPES (#15630) and 100 U/mL penicillin-streptomycin (#15140) and was incubated at 37 °C in humidified incubator containing 5% CO2. All the media and reagents for HaCaT cell culture were purchased from Gibco (Grand Island, NY, USA). A night before treatment process, the cells were maintained in serum-free and antibiotic-free media. Cells were then pre-treated with ORV for 24 h before exposed to PGN (Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

Human Epidermal Keratinocytes (HEKa) derived from normal human adult foreskin were purchased from ATCC (PCS-200-011). HEKa cells were cultivated in dermal cell basal medium containing supplements from a keratinocyte growth kit (ATCC, PCS-200-040) and incubated at 37 °C in a humidified incubator containing 5% CO2. Cells were then treated as described and collected for further experiments.

5.1.2. Oxyresveratrol

ORV (2,4,3′,5′-tetrahydroxystilbene) extracted from *Artocapus lakoocha*, was kindly given by the Thailand's Ministry of Public Health. The purity of ORV was analyzed by using ultra-performance liquid chromatography and had a purity greater than 95%. Commercial ORV purchased from Sigma-Aldrich (St. Louis, MO, USA) was used for comparison. For in vitro cell culture experiment, the compound was dissolved in DMSO and diluted with PBS to achieve the desired working concentration, whereas the final concentration of DMSO in the solution was 1%. For in vivo experiment in dermatitis mouse model, a cream containing ORV, which was also developed by the Ministry of Public Health and Chulalongkorn University's Faculty of Medicine, were determined for an optimal formulation using DNCB-induced dermatitis mouse model.

5.1.3. Peptidoglycan (PGN) and lipopolysaccharide (LPS)

PGN of Staphylococcus aureus (#77140) and LPS of Escherichia coli O111:B4 (#L4391) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Each compound

was dissolved in sterile distilled water and further diluted with PBS for in vitro cell culture experiments.

5.1.4. Cell viability assay

To detect the cytotoxicity effect of ORV on HaCaT and HEKa cell viability, we seeded 0.5×10^4 cells/well in a flat bottom 96-well plate (SPL Life Sciences, Gyeonggi-do, Korea) with DMEM containing 10% FBS, 1% penicillin and streptomycin at 37% in humid 5% CO2 atmosphere for 24 h. Then, cells were treated with ORV at several concentration for 24 h. 50 μL MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (ab211091, Abcam Plc., Aibo Trading Co., Ltd., Shanghai, China) was added to each well and incubate in 5% CO2 incubator at 37 °C for 3 h. Next, optical densities (OD) were measured at a wavelength of 490 nm using Varioskan microplate reader (Thermofisher Scientific, Grand Island, NY, USA).

5.1.5. Apoptosis assay

The apoptosis assay was performed on HaCaT cells using APC Annexin V Detection kit with PI (#640932, BioLegend, San Diego, CA, USA) according to the manufacturer's instructions. Before analysis, HaCaT cells were seeded at 0.5×10^6 cells/well in a 6-well plate (SPL Life Sciences, Gyeonggi-do, Korea) and incubated overnight. On the next day, the cells were treated with ORV for 24 h. The cells were then trypsinized and washed twice with Cell Staining buffer (#420201, Biolegend, San Diego, CA, USA). Annexin V binding buffer at a concentration of 1×10^6 cells/mL was added to the cells that were later stained with APC annexin V and propidium iodine (PI) for 15 min at room temperature. Cells were measured for annexin V and PI intensity suing BD® LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the data were analyzed using FlowJo 10 cytometry analysis software (FlowJo, Ashland, OR, USA).

5.1.6. Cell cycle analysis

For cell cycle analysis, HaCaT cells were trypsinized after treatment with ORV for 24 h. The cells were then washed with PBS buffer and fixed using 70% ethanol at −20 °C. After cell fixation, the cells were washed twice with cold PBS buffer and treated with 50 μL of 100 μg/mL RNAse to remove RNA. Finally, the cells were stained with 200 μL of Propidium Iodine (PI) and collected data of PI-stained cells using BD

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LSR II flow cytometer (BD Biosciences). Data were analyzed by FlowJo 10 cytometry analysis software (FlowJo, Ashland, OR, USA).

5.1.7. RNA isolation, complementary DNA synthesis, and Quantitative Real-time PCR analysis

Total RNA from cells and mice skin specimens was isolated using TRIzol reagent (Gibco, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, the first strand of cDNA was synthesized from a starting concentration of total RNA at 500 ng using iScript™ cDNA Synthesis kit (#1708891, Biorad, Hercules, CA, USA). PCR was performed using EmeraldAmp® GT PCR Mastermix (RR310A, Takarabio, Kusatsu, Shiga, Japan). The optimal PCR condition was as follows: 98 °C for 10 s, 60 °C for 10 s and 72 °C for 60 s for 30 cycles. For Real time-PCR, the amplification steps were performed using PowerUp™ SYBR™ Master Mix (A25742, Applied Biosystem, Vilnius, Lithuania) as follows: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min.

Table 2. Primers in the study

Gene	Sequence	T_m (°C)	Length (base pairs)
GAPDH	Fw: 5'-ACC CAC TCC TCC ACC TTT-3' Rv: 5'-CAC CAC CCT GTT GCT GTA G-3'	60	108
IL-1β	Fw: 5'-ACA GAT GAA GTG CTC CTT CCA-3' Rv: 5'-GTC GGA GAT TCG TAG CTG GAT-3'	60	73
IL-6	Fw: 5'-GGC ACT GGC AGA AAA CAA CC-3' Rv: 5'-GCA AGT CTC CTC ATT GAA TCC-3'	60	85
IL-8	Fw: 5'-GAG AGT GAT TGA GAG TGG ACC AC-3' Rv: 5'-CAC AAC CCT CTG CAC CCA GTT T-3'	60	112
hBD3	Fw: 5'-AGC CTA GCA GCT ATG AGG ATC-3' Rv: 5'-CTT CGG CAG CAT TTT GCG CCA-3'	60	206
LL37	Fw: 5'-GCC CAG GTC CTC AGC TAC AAG G-3' Rv: 5'-CTA GGA CTC TGT CCT GGG TAC AAG-3'	60	426

5.1.8. IL-6 and IL-8 Cytokine Measurement

The levels of supernatant cytokines were measured using human IL-6 and IL-8 ELISA kits (#88-7066 and #8086-22, respectively; Thermofisher Scientific, Carlsbad, CA, USA). Cell cultures were performed in triplicates under each treatment condition and their supernatants were collected and processed for cytokine detection according to the manufacturer's instructions. The optical densities were measured at 450 nm and 570 nm with Varioskan Microplate reader (Thermofisher Scientific, Grand Island, NY, USA). The concentrations were calculated from the standard curve generated by a curve-fitting program.

5.1.9. Western blot analysis

Western blot analysis was utilized to detect intracellular proteins, NF-κB, phosphorylated-NF-κB, and cleaved caspase-3. Cells were lysed for protein lysate using RIPA buffer (#9806, Cell Signaling Technology, Danvers, MA, USA) containing protease/phosphatase inhibitor cocktail (#5872, Cell Signaling Technology, Danvers, MA, USA). For detection of GAPDH, NF-κB, and phosphorylated-NF-κB, 20 μg of protein lysate was used. For detection of cleaved caspase-3, 50 μg of the protein lysate was used. The protein lysates were loaded into 12% SDS-PAGE and run at 100 volts for 90 min to separate the proteins. After that, the proteins were transferred to nitrocellulose mem branes (Amersham, Arlington Heights, IL, USA) at 70 volts for 75 min. The membranes were incubated in blocking solution for 60 min and then in blocking solution containing the following antibodies: NF-κB p65 (D14E12) XP rabbit mAb (#8242), Phospho-NF-κB p65 (Ser536) (93H1) rabbit mAb (#3033), Cleaved caspase-3 (Asp175) (5A1E) rabbit mAb (#9664), and GAPDH (D16H11) XP rabbit mAb (#5174). Then, the membranes were washed twice in PBS buffer to remove excessive antibodies and incubated in blocking buffer containing HRP-conjugate anti-rabbit secondary antibody (#7074) followed by washing steps. All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). The protein bands were detected using UltraScience Pico Plus Western Sub-strate (Bio-Helix, New Taipei City, Taiwan). GAPDH expression levels were used as in-ternal control. The band intensity of proteins of interest was quantified from triplicate Western blot images using Image Lab software version 6.1 (Bio-Rad Laboratories, Her-cules, CA, USA).

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5.1.10. Dermatitis Animal Model

Seven-to-nine-week-old BALB/c mice were purchased from Nomura Siam (Bangkok, Thailand). The animals were maintained according to the standard animal care protocol approved by the Animal Care and Use Committee of the Faculty of Medicine, Chulalongkorn University (No. 032/2565; approved on December 2022). Mice were housed in a husbandry unit with 12 h light/dark cycle under Thermos-regulated (22 ± 2 °C) and humidity-controlled (50 ± 10%) condition and provided with standard diet and water ad libitum. All mice were randomly assigned into four groups: no treatment, DNCB, DNCB + cream base, DNCB + ORV, consisting of 3 mice in each group.

Dermatitis was induced in mice by application of 2,4-Dinitrochlorobenzene (DNCB) on their skin as described previously with some modifications (90, 91). Briefly, the dorsal hairs of mice were completely removed. And on the next day, 100 µL of 1% DNCB in acetone/1 cm2 were applied twice a week at the bare skin to sensitize the mouse skin. Then, 100 µL of 0.5% DNCB was applied to the sensitized skin areas twice a week to induce dermatitis. In ORV treatment group, ORV was applied regularly on the skin. On day 23, mice were sacrifice and skin specimens of the mice were collected for pathological and immunohistochemistry assessments.

5.1.11. Evaluation of Severity Using Clinical Skin Score

Manifestations of dermatitis were collected twice per week for four weeks. Four symptoms, including erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion, were graded on a scale from 0 to 3 (none, 0; mild, 1; moderate, 2; severe, 3) to determine the severity of dermatitis (90). The clinical skin score, ranging from 0 to 12, was defined as the sum of the scores from each symptom.

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5.1.12. Pathology Observation and Immunohistochemistry

The dorsal skins were fixed in 4% paraformaldehyde for 24 h before paraffinization. The paraffin-embedded skin sections were then embedded on glass slides, and they were stained with hematoxylin and eosin (H&E) to examine skin histology and skin thickness. Immunohistochemistry staining was performed with CD3, CD4, and CD8 markers to identify the recruitment of lymphocytes into the skins.

5.1.13. Antimicrobial susceptibility testing

To check the susceptibility of bacteria to ORV, the Kirby–Bauer disc diffusion method was used according to the Clinical and Laboratory Standard Institute (CLSI) guidelines of 2011. *Staphylococcus aureus* (ATCC-12600) was used. Each bacterial suspension was adjusted based on the McFarland standard value of 0.5, swabbed onto Luria–Bertani (LB) broth, and incubated in the presence of antibiotic discs at 37 ^{o}C for 18 h. There were three groups of discs: antibiotic-only, ORV-only, and antibiotic-ORV. Diameters of the inhibition zones were measured to determine the resistance or susceptibility of each isolate to ORV. The antibiotic discs used in this study included erythromycin (SD013-5CT), dicloxacillin (SD052-5CT), cloxacillin (SD008-5CT), and fusidic acid (SD171-5CT), purchased from HiMedia Laboratories (Thane, MS, India).

5.2. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.0. For multiple comparison test, one-way ANOVA was utilized. A pairwise comparison was also used to test the statistical significance of different ORV concentration groups of samples. Unless otherwise stated, the data included measurements collected from triplicate samples, and their numerical values were presented in graphical formats as mean ± standard deviation (SD). The difference is considered statistically significant when the *p* value is less than 0.05.

CHAPTER 6: RESULTS AND DISCUSSION

6.1. Results

6.1.1. Effects of Oxyresveratrol on Human Immortalized (HaCaT) and Primary (HEKa) Keratinocytes

To investigate the effect of ORV on skin, in vitro experiments on immortalized and primary keratinocytes (HaCaT and HEKa, respectively) were conducted. After treating the cells with various concentrations of ORV, cell viability was determined. There was no significant difference in the cell viability of HaCaT between our ORV extract and the commercial ORV. The IC50 values of ORV for the two cell types HaCaT and HEKa were 81.68 µg/mL and 266.8 µg/mL, respectively (Figure 6). Until the ORV concentration reached 10 µg/mL, neither type of keratinocyte died. Therefore, all of the following experiments were conducted with ORV concentrations less than 10 µg/mL unless stated otherwise.

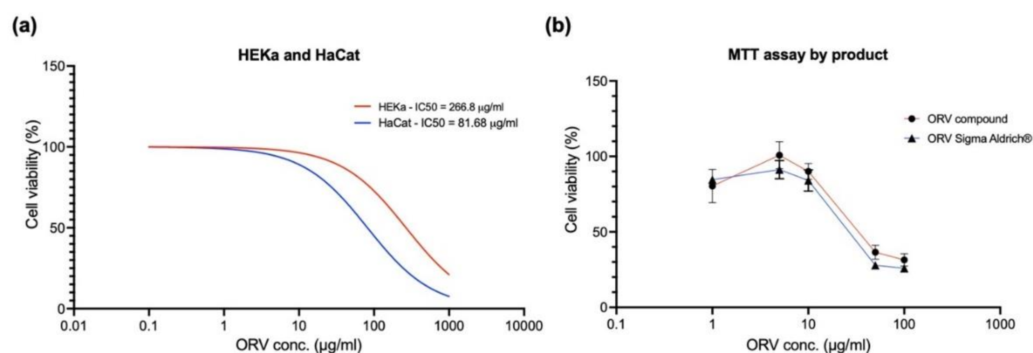

Figure 7. Impact of ORV on the viability of cells.
*HaCat and HEKa cells were exposed to the different concentrations of ORV for 24 h. MTT assay was used to determine cell viability. GraphPad Prism was utilized to calculate the IC_{50} of ORV on each cell type **(a)**. HaCat cell viability was determined using MTT assay after exposed to different concentrations of commercial ORV compare with ORV extraction for 24 h. **(b)** Commercial ORV purchased from Sigma-Aldrich (St. Louis, MO, USA) was used for comparison on HaCaT.*

After determining the optimal concentration for in vitro experiments, we further investigated the proliferation and apoptosis effects of ORV on HaCaT. HaCaT cells were treated with ORV at concentrations of 1, 5, and 10 µg/mL for 24 h before being

stained for apoptosis markers with annexin V and propidium iodine (PI). Apoptotic cells in both early and late apoptosis increased approximately two-fold in all ORV-treated groups compared to the control group, according to flow cytometer analysis of annexin V and PI stained cells (Figure 7a; quadrant (Q) 2 indicates late apoptosis and Q3 indicates early apoptosis). However, there was no statistically significant difference in the number of apoptotic cells between cell groups treated with different ORV concentrations.

Because ORV inhibited cell growth, we wanted to know which cell cycle phase it inhibited. To answer this question, we used PI staining of HaCaT cells after treatment with various concentrations of ORV and performed cell cycle analysis. After treatment with 1 μg/mL, 5 μg/mL, and 10 μg/mL of ORV, cells were arrested at G0/G1 (76.83 ± 0.83%), G2/M (14.20 ± 0.36%), and S phase (11.33 ± 0.25%), respectively, indicating that there was a shift in cell cycle arrest in HaCaT cells as the concentration of ORV increased (Figure 7b). To determine how ORV affected cell death, we looked at the cleaved form of caspase-3, a key initiator of apoptosis signal, in HaCaT cells after 24 h of treatment with 5 μg/mL of ORV. The level of cleaved caspase-3 was nearly threefold higher (3.29 ± 0.18, p value < 0.05) in the ORV treatment group than in the untreated group (Figure 7c). According to our findings, ORV may activate caspase-3 and induce apoptosis, as evidenced by cell cycle arrest in human keratinocytes, which can shift depending on ORV concentration.

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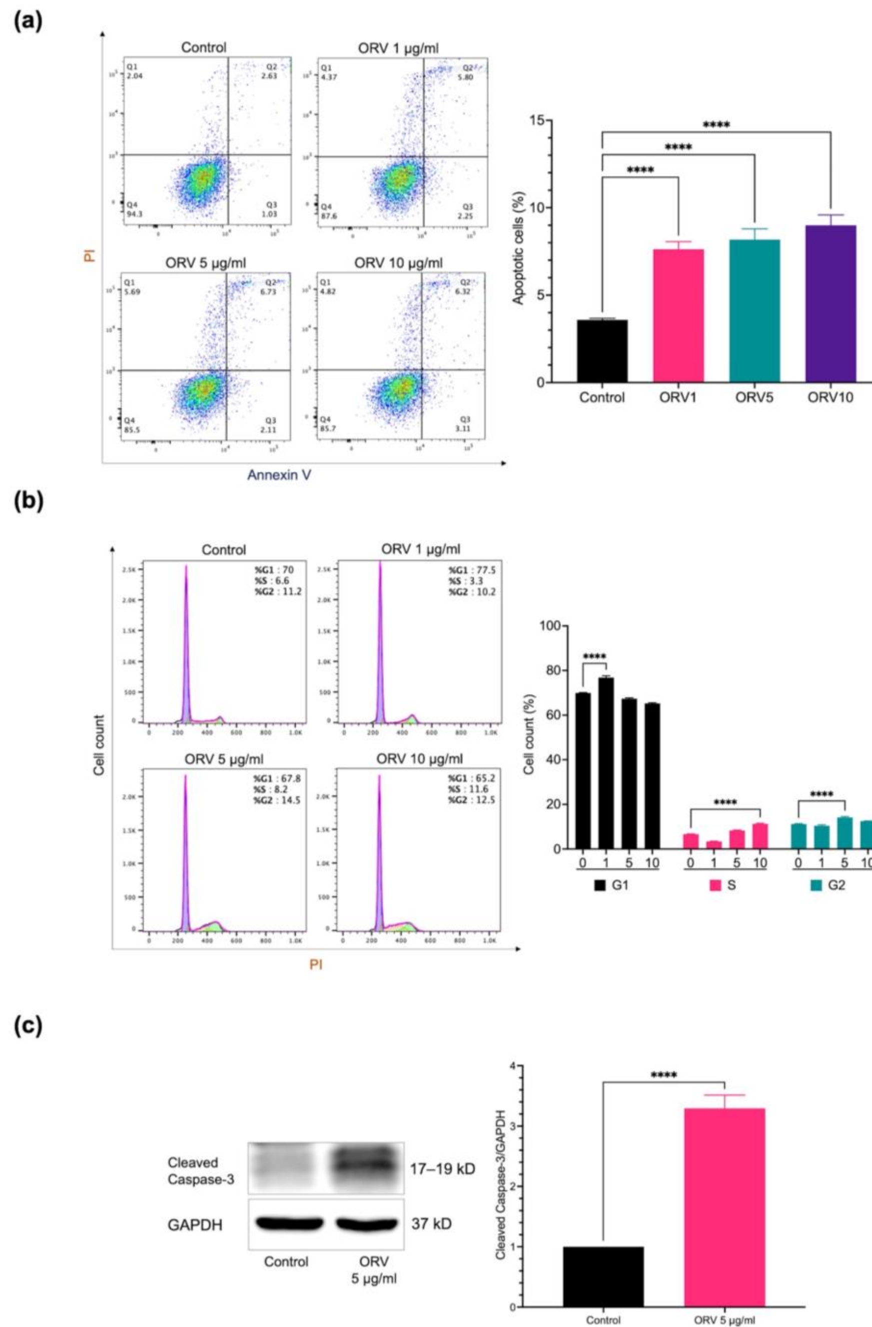

Figure 8. The apoptosis effect of ORV in HaCaT cells.

*(**a**) The prevalence of apoptotic keratinocytes (Q2 and Q3) after treatment with ORV for 24 h. (**b**) The analysis of cell cycle on HaCaT after treatment with ORV. (**c**) Cleaved caspase-3 proteins (left) and their quantified levels (right). HaCaT cells were treated with ORV 5 μg/mL for 24 h. GAPDH was used as an internal control. Data were present as the mean ± SD. Asterisks denote significant differences compared to untreated cells. **** $p < 0.0001$.*

6.1.2. Anti-Inflammatory Effect of Oxyresveratrol on HaCaT Cells

Microbial colonization on the skin is one of the causes of skin inflammation. Once the normal flora population is disrupted, the inflammatory microbial population can over-whelm. Bacterial cell wall components such as PGN and LPS can bind to their cognate receptors on the cells composed of the skin, thereby stimulating keratinocyte inflam-mation. After assessing effect of ORV on cell proliferation, we next investigated its effect on inflammatory regulation in both keratinocyte cell line (HaCaT) and primary keratinocyte (HEKa). HaCaT cells were stimulated with varying concentrations of PGN and monitored for the levels of pro-inflammatory cytokines. We found that 5 µg/mL of PGN was sufficient to induce expression of inflammatory cytokines in HaCaT cells without affecting the cell viability (Figure 8).

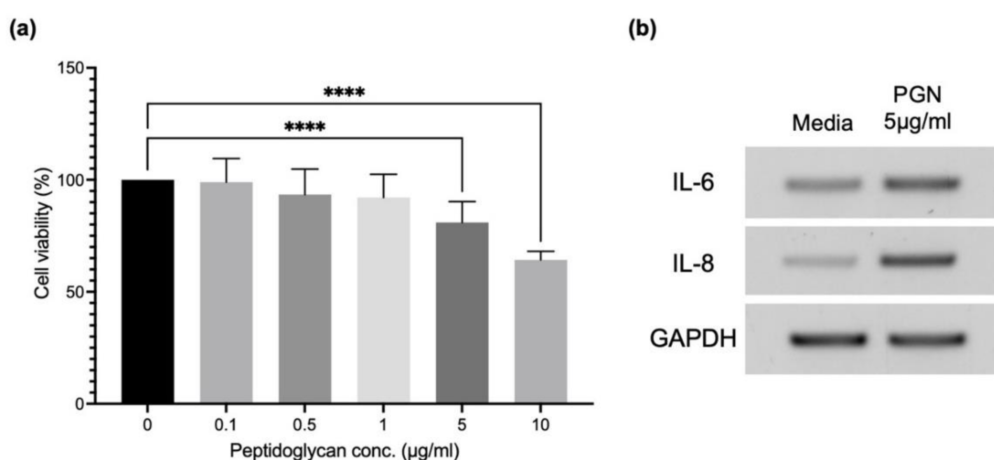

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Figure 9. Cell viability and production of inflammatory cytokines (IL-6 and IL-8) by HaCat cells after treatment with PGN.

*PGN concentrations were varied in order to find an optimal concentration that was greater than IC50 and still produced a substantial amount of IL-6 and IL-8 cytokines. PGN at 5 µg/ml exerted a modest effect on HaCat viability as measured by MTT assay (**a**). Also, PGN at 5 µg/ml can stimulate substantial amount of IL-6 and IL-8 expression as measured by PCR (**b**).*

To examine the effect of ORV on prevention of inflammation, HaCaT cells were pretreated with various concentrations of ORV before being stimulated with PGN. The results showed that pretreatment at 1, 5 and 10 µg/mL of ORV significantly inhibited IL-6 and IL-8 cytokine production at mRNA and protein levels (Figure 9a–d; $p < 0.0001$); however, there was no significant difference on the cytokine expression levels

between the ORV-pretreated groups. Because NF-κB is one of the central regulators of inflammatory signaling cascades, we therefore sought to verify that ORV treatment inhibited NF-κB transcription factor acti-vation in keratinocytes. Adding PGN to HaCaT cells significantly increased NF-κB transcription factor activation, as indicated by an increase in phosphorylated NF-κB p65 subunit (1.53 ± 0.10; $p < 0.05$) (Figure 9e). However, an ORV treatment prior to PGN stimulation significantly reduced the level of phosphorylated NF-κB in HaCaT cells (0.86 ± 0.31; $p < 0.05$) (Figure 9e).

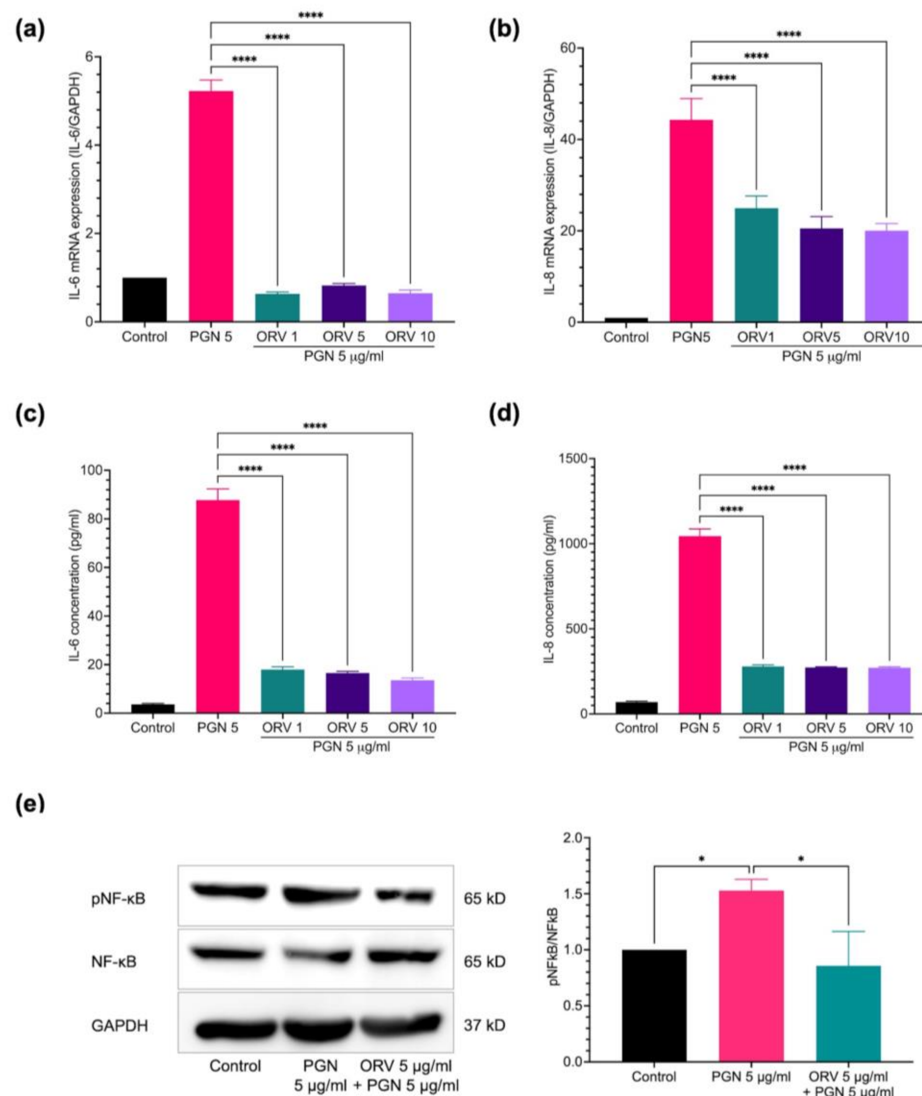

Figure 10. ORV inhibited PGN-induced inflammation in immortalized keratinocytes.

*HaCaT cells were treated with ORV for 24 h prior to stimulation of 5 μg/mL PGN for another 24 h. Culture supernatants were collected for ELISA analysis and cells were collected for RT-PCR analysis. Expression levels of IL-6 **(a)** and IL-8 **(b)** mRNA and*

*cytokine levels of IL-6 (**c**) and IL-8 (**d**) in the culture supernatants. (**e**) Phosphorylation of NF-κB, total NF-κB, and GAPDH proteins. GAPDH gene and protein are used as internal controls for mRNA and protein expression experiments. Quantitative analysis of protein expression levels is presented as the mean ± SD. .* $p < 0.05$; **** $p < 0.0001$.*

In addition, there was also no significant difference in the suppressive effect of IL-6 and IL-8 mRNA expression between commercial ORV and our ORV extract (Figure 10).

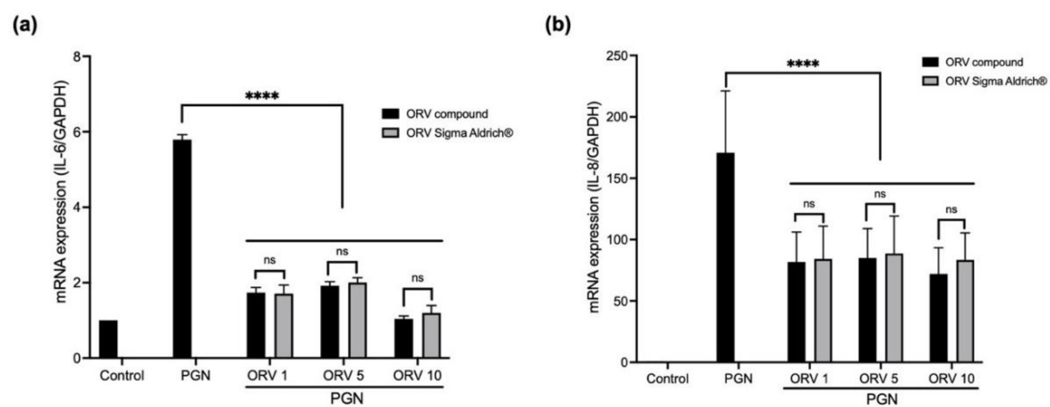

Figure 11. Comparison of anti-inflammatory effect between the ORV compound and commercial ORV from Sigma-Aldrich.

*The anti-inflammatory effect of ORV on HaCaT was compared with that of a commercial compound from Sigma-Aldrich on IL-6 (**a**) and IL-8 (**b**). GAPDH was used as the internal control. Asterisks indicate statistical significance compared to PGN-stimulated cells. ****, $p < 0.0001$.*

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6.1.3. Anti-Inflammatory Effect of Oxyresveratrol on Primary Epidermal Keratinocytes

Furthermore, the anti-inflammatory properties of ORV in primary human epidermal keratinocytes (HEKa) were examined. We found that PGN and LPS stimulated different profiles of pro-inflammatory cytokines in HEKa cells (Figure 11)

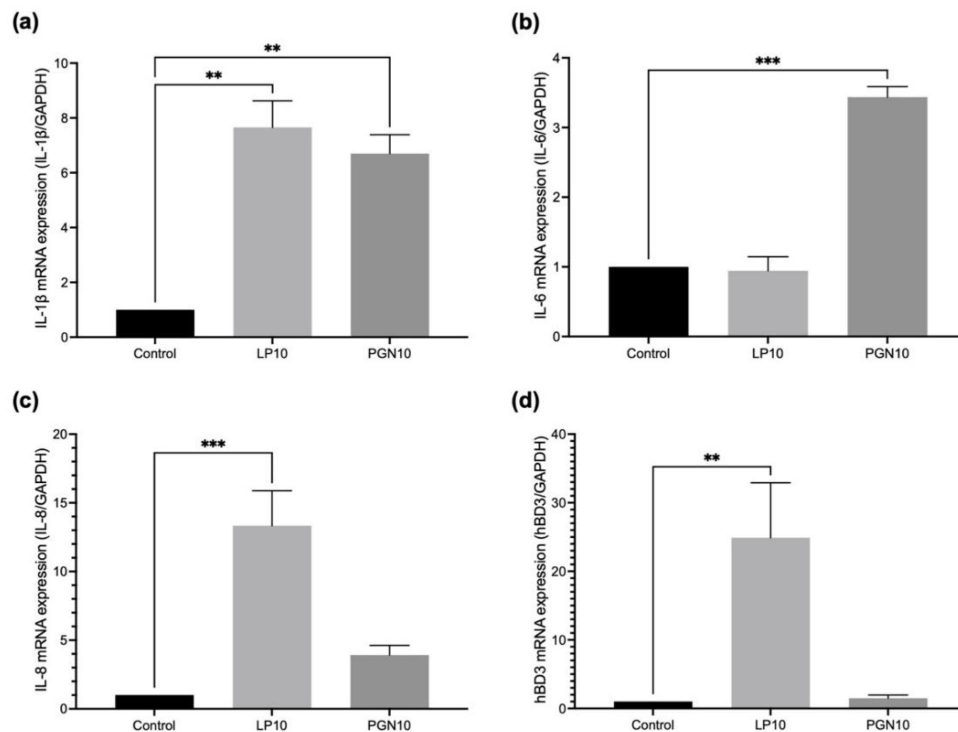

Figure 12. Expression levels of genes in HEKa cells after LPS and PGN treatment. *PGN and LPS at 10 μg/ml were used to treat HEKa cells and mRNA expression levels of IL-1β (**a**), IL-6 (**b**), IL-8 (**c**), and hBD3 (**d**) were measured. GAPDH was used as the internal control. Asterisks indicate statistical significance compared to medium-treated control. **, $p < 0.01$; ***, $p < 0.001$.*

LPS stimulation, for instance, increased the expression of *IL-1β*, *IL-8*, *hBD3*, and *LL37* genes and pretreatment with ORV significantly reduced their expression (Figure 12). Similar results were observed when the cells were pretreated with ORV before PGN stimulation (Figure 13). However, *IL-8* expression level was not suppressed by ORV pretreatment when the cells were stimulated with a high dose of PGN (10 μg/mL) (Figure 13b). In summary, our results showed that pretreatment with low doses of ORV prior to stimulation can significantly reduce the expression of pro-inflammatory cytokine genes.

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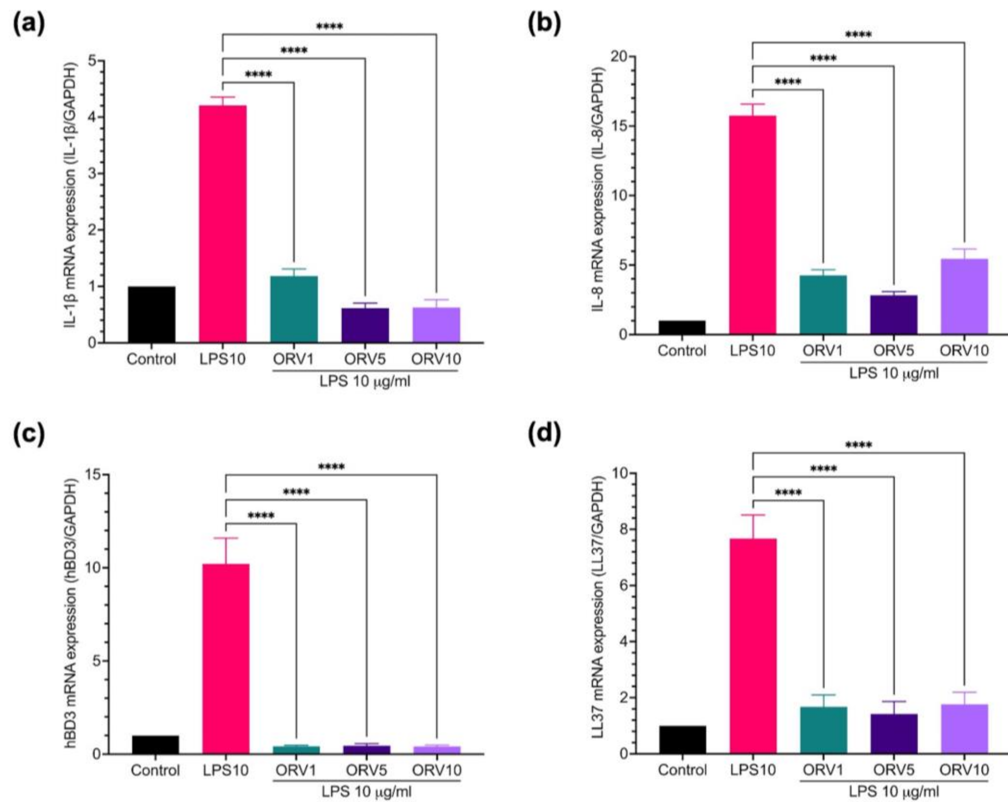

Figure 13. ORV inhibited LPS-induced inflammation in primary human keratinocytes.

*HEKa cells were treated with ORV for 24 h prior to stimulation of 5 μg/mL LPS for another 24 h. Cells were col-lected for RT-PCR analysis. Expression levels of IL-1β (**a**), IL-8 (**b**), hBD3 (**c**), LL37 (**d**) mRNA are shown. GAPDH was used as a house keeping gene. Asterisks denote significant differences com-pared to LPS-stimulated cells. **** $p < 0.0001$.*

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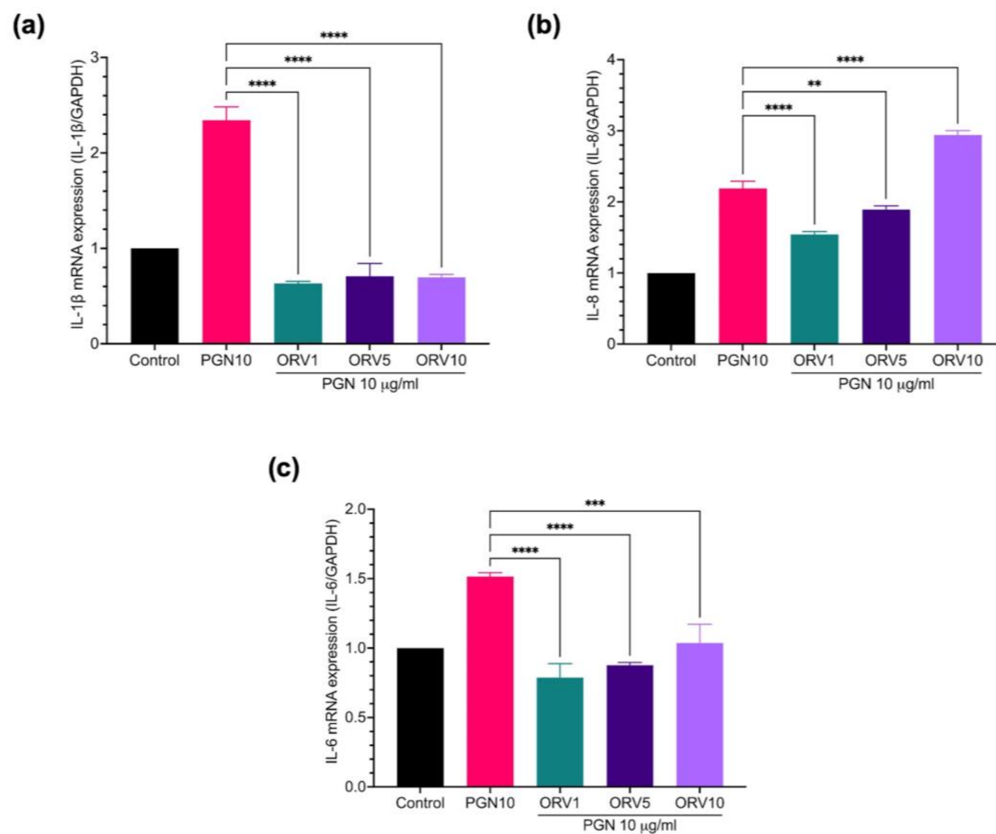

Figure 14. ORV inhibited PGN-induce inflammation in primary human keratinocytes.

*HEKa cells were treat-ed with ORV for 24 h prior to stimulation of 10 μg/mL PGN for another 24 h. Cells were collected for RT-PCR analysis. Expression levels of IL-1β (**a**), IL-8 (**b**), IL-6 (**c**) mRNA are shown. GAPDH was used as a house keeping gene. Asterisks denote significant differences compared to PGN-stimulated cells. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.*

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6.1.4. Oxyresveratrol Treatment Alleviates Skin Inflammatory Manifestation on Mice

We previously showed that ORV could not only induce apoptosis but also inhibit inflammatory cytokine production in immortalized and primary human keratinocytes in vitro. Because of its clearly strong anti-inflammatory effect, we wanted to use ORV as a topical treatment for dermatitis. We used DNCB to induce skin inflammation in BALB/c mice, followed by regular applications of ORV-based emollient cream to the mice's inflamed skin (Figure 14a, top panel). Clinical manifestations were documented and scored (see Materials and Methods). On day 8 to 11, we observed clinical manifestations of DNCB-induced dermatitis on the mouse skin, including erythema,

scarring/dryness, edema, and excoriation/erosion. For the next 10 days, emollient cream with or without ORV was applied on the mouse skin once every 2 days. Finally, mice were sacrificed on day 23 and their skins were collected for pathological and immunohistochemical analysis. As expected, clinical scores were significantly lowered as demonstrated by alleviated clinical manifestations in mice regularly treated with ORV for 10 days (0.5 ± 0.7) compared to the group receiving base emollient cream without ORV (3.5 ± 0.7; $p < 0.05$) and to the non-treatment group (5.5 ± 0.7; $p < 0.01$) (Figure 14a, bottom panel).

In addition to severe clinical manifestations on the skin, epidermal thickness also increases as the skin becomes chronically inflamed. Pathological examination of the skin tissues from the DNCB-treated mice revealed significantly increased epidermal thickness when compared to that of the control group (Figure 14b). Topical treatment with base emollient cream alone, on the other hand, significantly reduced epidermal thickness of the skin. However, the epidermal thickness of mice topically treated with ORV-containing emollient cream was reduced to levels comparable to the control group.

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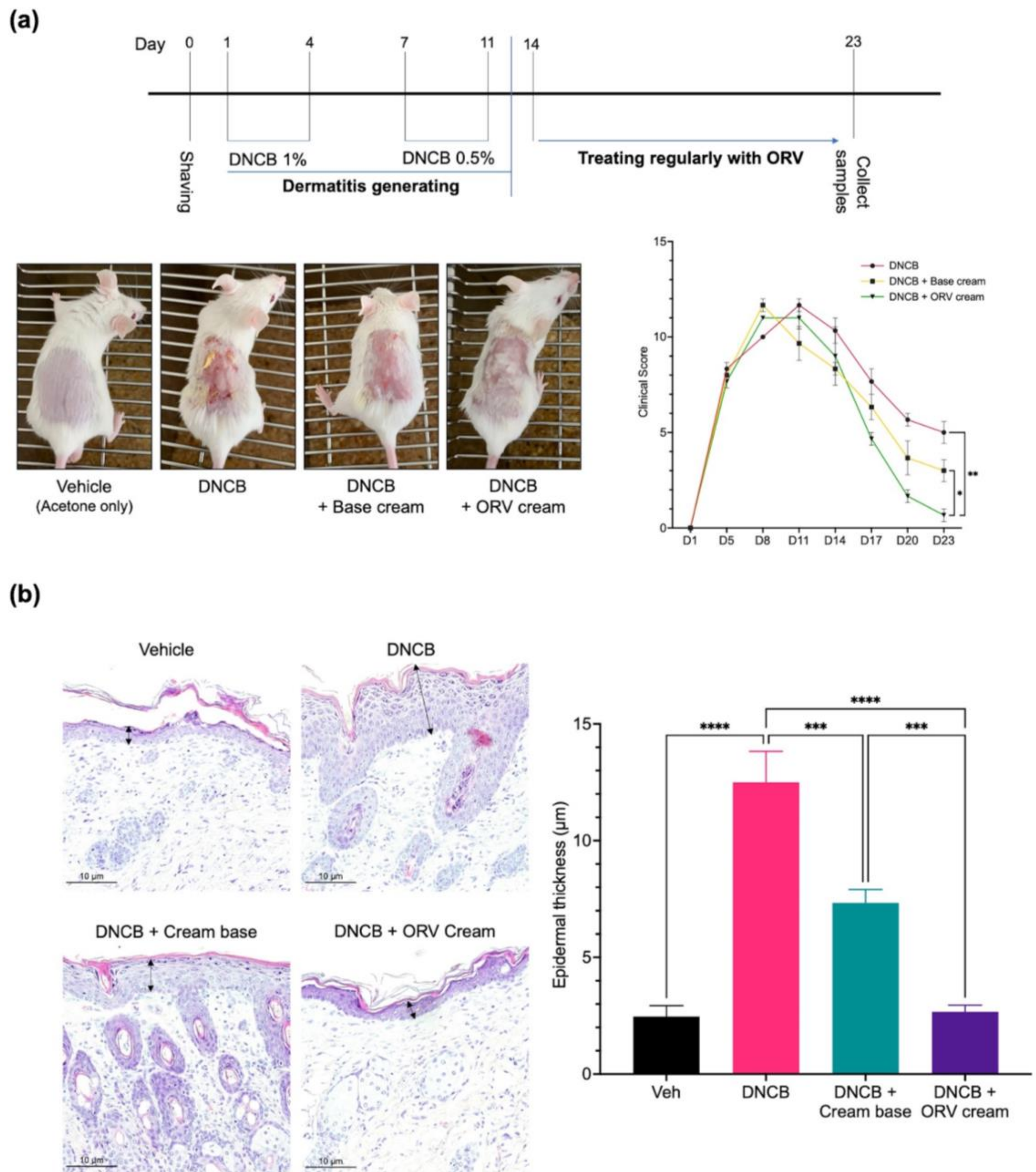

Figure 15. ORV alleviated inflammatory manifestations on mice skin.

*A schematic diagram of BALB/c mouse skin sensitization using 1% and 0.5% DNCB to induce skin inflammation followed by treatment with ORV cream or vehicle cream **(a, top)**. Skin images were taken at the end of the experiment **(a, bottom left)** and evaluated for clinical skin severity score of dermatitis **(a, bottom right)**. Histological images of mouse skin epidermis from all treatment groups are shown (200× magnification) **(b, left)**. Arrows indicated the epidermal thickness. The thickness data from each mouse group in **(b, left)** are presented quantitatively as mean ± SD **(b, right)**. Asterisks denote*

*significant differences compared to DNCB-stimulated group. ***, $p < 0.001$; ****, $p < 0.0001$.*

The skin of dermatitis patients has been studied and reportedly found to be colonized with skin-infiltrating lymphocytes. T cell populations such as CD3+, CD4+, and CD8+ T cells can secrete cytokines that promote the inflammatory response and contribute to the chronic state of the disease. We performed immunohistochemical staining and identified CD3, CD4 and CD8 T cells in dissected mouse skin tissues. Mice treated with DNCB alone had a higher number of T cells in their skin compared to the control group (Figure 15). The number of T cells in the skin of mice treated with base emollient cream alone decreased significantly when compared to the DNCB-treated group. The number of T cells was even lower in the skin from mice treated with ORV-containing emollients, but not as low as the number of T cells found in the control group. These findings suggest that ORV treatment can help alleviate dermatitis symptoms by reducing clinical manifestations, skin thick-ness, and the number of skin-infiltrating lymphocytes.

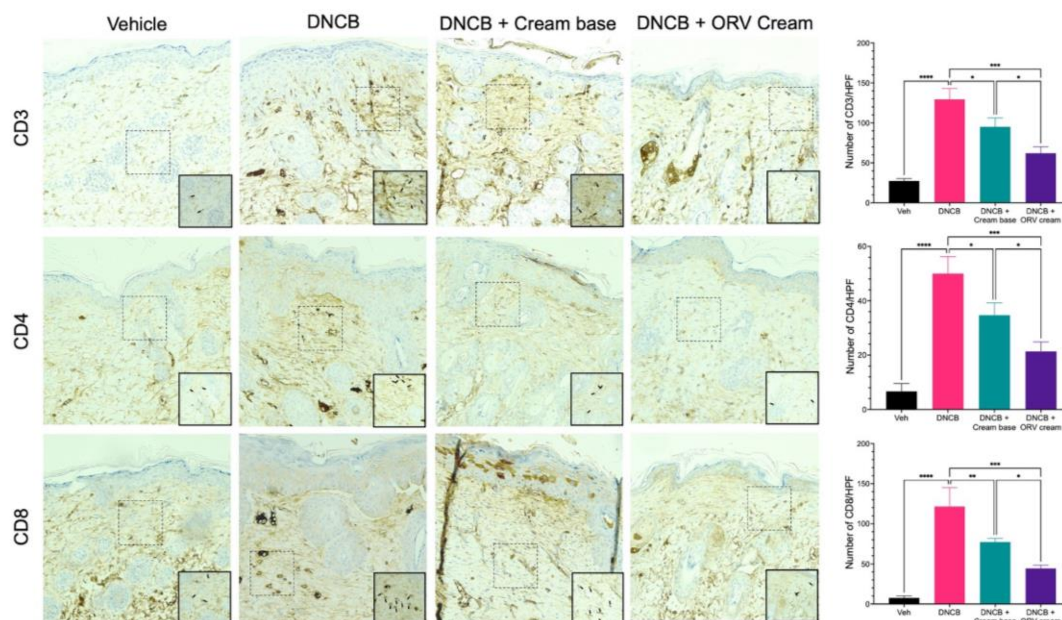

Figure 16. ORV reduced dermal infiltration of inflammatory cells

Cross-sectional images of mouse skins stained with antibodies specific to CD3 (upper panel), CD4 (middle panel) and CD8 (lower panel) to identify CD3+, CD4+, and CD8+ T cells, respectively. The tissue sections were examined under a microscope at a magnification of 400×. The number of cells stained with respective anti-body were

*spotted, counted, and displayed in bar graphs on the right. Quantitative data of T cells are displayed as mean ± SD. Asterisks denote significant differences compared to DNCB-stimulated group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.*

6.1.5. The Anti-bacterial Effect of Oxyresveratrol on Staphylococcus aureus

We next continued with the antibacterial effect of Oxyresveratrol on *S. aureus* with ORV at 10 µg per disc. Treatment with ORV alone did not have an antibacterial effect on *S. aureus*. However, the combination of ORV with antibiotic discs, including erythromycin, fusidic acid, dicloxacillin, and cloxacillin, significantly increased the inhibition zone of these antibiotics (Figure 16).

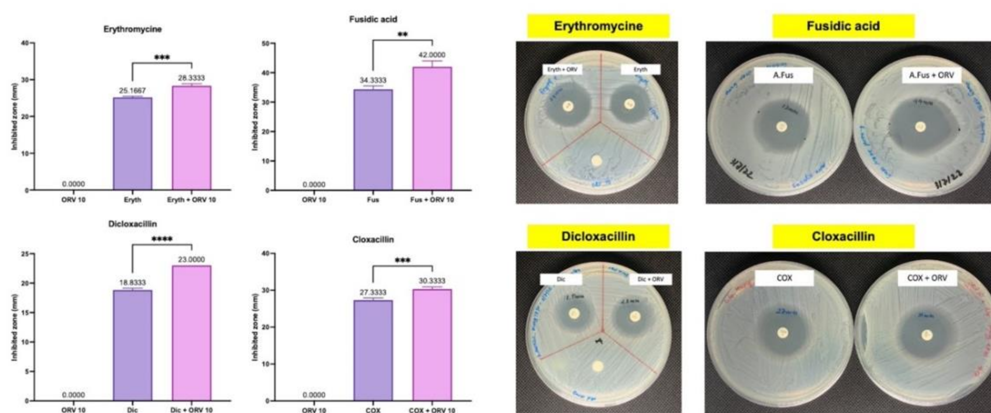

Figure 17. ORV increases the antibacterial potential of antibiotics.

*ORV 10 µg alone did not reveal an antibacterial effect. The combination of antibiotics and ORV increased the zone of inhibition on Staphylococcus aureus. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.*

6.2. Discussion

Eczema is a type of skin inflammation in which skin permeability and barrier functions are compromised. Inflammation is accompanied by a concurrent increase in epidermal proliferation and disruption of epidermal differentiation, resulting in clinical manifestations such as erythema, scales and lichenification (92). In addition, eczema is one of the most common dermatological disorders that requires both immediate treatment and a long-term management strategy. ORV has long been reported as an antioxidant reagent with potential dermatological applications (93, 94). In addition, it has anti-proliferative effects on various cancer cell lines (HepG2, MCF-7, HT-19, HSC-3, HN-8 and HN-30) as well as anti-inflammatory effects on macrophages (RAW264.7)

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and human microglial cells (HMC3) (8, 9, 76, 85, 86). In this study, the anti-inflammatory efficacy of ORV on dermatitis models in vitro and in vivo was investigated using human keratinocyte cells and BALB/c mice, respectively.

Our study found that ORV reduced cell viability proportionally to its concentration in both HaCaT and HEKa cells. It had no effect on the viability of either cell type at concentrations of 10 μg/mL and lower, which is consistent with previous studies in macrophage cell lines and HaCaT cells (40 μM = 9.76 μg/mL) (8, 95). Despite having low cytotoxicity, our ORV still induced cleaved caspase-3 in HaCaT cells. The consequences of activating caspase-3 are the degradation of multiple cellular proteins, morphological changes and DNA fragmentation (96). Interestingly, we observed a shift in cell cycle arrest in HaCaT cells after treatment with increasing doses of ORV. The cell cycle shift could be caused by ORV interference in cell proliferation at different levels, from DNA replication to cell division, which suggest that ORV is a cell cycle non-specific anti-proliferation compound (97). Because our ORV showed apoptosis-induced anti-proliferation property, this property is in line with that of glucocorticoid, one of the most commonly used therapeutic reagents for dermatitis, suggesting the potential use of ORV to reduce cellular proliferation in dermatitis (98).

In addition to anti-proliferative properties, we also investigated anti-inflammatory properties of ORV because skin inflammation is commonly observed in dermatitis. Initially, the anti-inflammatory effect of ORV was investigated using an immortalized keratinocyte cell line (HaCaT). The immortalized cell line, resulting from in vitro modification, may have some different properties from primary keratinocyte cell (99, 100). Besides, it has been accepted that HEKa is a better model for studying human keratinocyte because it is the primary cell. Thus, the anti-inflammatory effect of ORV was further extensively studies using HEKa. Our results revealed that ORV inhibited a variety of pro-inflammatory cytokines in keratinocyte in vitro model. In particular, IL-6 and IL-8 cytokines, which are reportedly produced by keratinocytes during the acute phase of skin inflammation in dermatitis and other skin diseases, were significantly reduced (101). The master regulator of inflammatory signaling, NF-κB, was also disrupted, as evidenced by a decrease in phosphorylation of NF-κB p65 subunit, which is belonged to the canonical pathway, in ORV-treated keratinocytes. The canonical NF-κB is activated by various stimuli, transducing a rapid but transient

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transcriptional activity, to regulate the expression of numerous proinflammatory genes and function as the essential mediator of the inflammatory response (102). Although our results suggest that ORV may inhibit pro-inflammatory cytokines via canonical NF-κB p65 signaling pathway, the non-canonical arm of NF-κB and other downstream genes causing other morphological changes such as cell proliferation, apoptosis, morphogenesis, and differentiation should be further investigated to elucidate the effect of ORV treatment (53).

Furthermore, ORV also effectively inhibited the production of hBD3 and LL37, antimicrobial peptides secreted by keratinocytes and other innate immune cells when stimulated with bacterial antigens such as LPS or PGN. These antimicrobial peptides have been reported to protect skin from infection by gram-positive and gram-negative bacteria, fungi, and viruses (103, 104). On the other hand, the overexpression of hBD3 and LL37, in turn, stimulates keratinocytes to produce pro-inflammatory cytokines (IL-6, interferon gamma-induced protein (IP)-10) and chemokines (MCP-1, MIP3-α, and RANTES) that play an important role in recruiting activated T cells, macrophages, and other immune cells from the peripheral blood into sites of tissue inflammation, which consequently promote proliferation and inflammation in eczema (104-106). The LL37 and hBD3 production, as well as pro-inflammatory cytokines, may be regulated by the canonical NF-κB p65 signaling pathway. Therefore, ORV treatment can suppress both mechanisms in keratinocytes, according to our findings.

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Current treatment approach for eczema includes using of oral or topic agents containing corticosteroids, calcineurin inhibitors, antihistamines, immune suppressants, or biologics and moisturizer (107). We developed anti-inflammatory cream containing ORV for topical use and determined the therapeutic effect on dermatitis mouse model. Recently, the induction of dermatitis in mice with DNCB has mainly focused on the atopic dermatitis model (90, 91, 108). However, this model also represents other types of dermatitis such as contact dermatitis (109). Thus, DNCB-treated animal model suggests a potential anti-inflammatory effect in general. When applied regularly for 10 days, ORV cream could effectively reduce eczema as evidenced by lower clinical manifestation in DNCB-induced dermatitis mouse model. In addition, skin tissues examination also revealed that ORV-containing cream reduced epidermal thickness as well as the number of skin-infiltrating lymphocytes, particularly

CD3, CD4, and CD8 T cells. While the CD3 receptor is generally associated with antigen recognition, signal transduction, and activation of immunocompetent T lymphocytes (110), $CD4^+$ Th_2 cells are associated with atopic dermatitis initiation and $CD8^+$ T cells play a pivotal role in both contact dermatitis and the chronic phase of atopic dermatitis (111, 112). Our findings that ORV treatment reduced epidermal thickness and skin-infiltrating lymphocytes in vivo clinically prove the anti-proliferation and anti-inflammation properties previously reported. Future studies in humans should be conducted to support the safety and efficacy of ORV therapy on inflammatory skin diseases especially eczematous skin diseases. Finally, we believe we are the first to demonstrate that ORV has an anti-inflammatory effect of ORV that can improve dermatitis and ORV could be utilized as a topical agent for treatment of dermatitis and other inflammatory skin diseases.

Staphylococcus aureus, a gram-positive bacterium, is one of the most common pathogen in skin infections such as abscesses, cellulitis, and furuncle (113). In eczema, colonization of *S. aureus* together with decrease of the diversity of the skin microbiome flora result in reduction of filaggrin, loricrin, desmocollin, and keratins expression (37). In addition, superantigens (sAgs) and toxins produced by *S. aureus* are significant factors that trigger eczema severity (114). Thus, the combination of antibiotics, such as fusidic acid, with hydrocortisone or betamethasone, plays an important role in eczema treatment. We further investigated the effect of ORV on *S. aureus* at a dose applicable to human cells (10 μg/ml). Our results showed that 10 μg does not have an effect on *S. aureus*. This result is consistent with a previous study by Joung et al. (2016), which determined the MIC of ORV against *S. aureus* to be 125 μg/ml (82). However, they also revealed that ORV at a lower dose 62.5 μg/ml can increase the permeability of S. aureus membranes, thus increasing the bactericidal ability of detergents such as Triton X-100 (TX), sodium azide (NaN3), and N,N'-dicyclohexylcarbodiimide (DCCD) (82). We combined ORV with antibiotics, including fusidic acid, erythromycin, cloxacillin, and dicloxacillin, which are common in skin infection treatments. Thus, the expansion of the inhibition zone compared to the antibiotic alone may indicate that ORV can enhance the permeability of *S. aureus* cells even at very low doses.

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CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

Our study revealed that oxyresveratrol possesses antiproliferative and anti-inflammatory properties. In a keratinocyte inflammation model with PGN and LPS stimulation, ORV effectively reduced the production of inflammatory cytokines at both the mRNA and protein levels. The mechanism of anti-inflammatory action involves regulation of the canonical NF-κB p65 signaling pathway.

In the mouse eczema model, ORV cream significantly attenuated eczematous lesions and recovered mouse dermatitis lesion after 1-week treatment compared to the control group. The beneficial effect was also determined through skin thickness and infiltration of lympho T CD3, CD4, and CD8 cells in the skin.

In the future, the beneficial effects of ORV on eczema should be further studied using other inflammatory signaling pathways, such as JAK/STAT. Moreover, future clinical trial in human is necessary to conduct to validate the therapeutic efficacy and safety ORV cream in eczematous treatment.

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