Honokiol Improves Acne-like Lesions in a Rabbit Ear Model by Alleviating Hyperkeratosis and Sebum Secretion

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Abstract
The prevalence of acne vulgaris is high, but the topical retinoids used as the foundation of treatment have teratogenic and photosensitivity properties. Previous studies have suggested that honokiol, a small-molecule compound extracted from Magnolia officinalis, could effectively inhibit Cutibacterium acnes (C. acnes) and inflammation in vitro. However, the effect in vivo is unclear. The rabbit ear acne model that we created showed obvious comedones and hyperkeratosis. These lesions were repeatedly measured and recorded by dermatoscopy (ultraviolet light). Compared with the control group, topical 2.5% honokiol cream obviously improved the comedones and hyperkeratosis and effectively reduced sebum secretion, as shown by Oil Red O staining. The effects were equivalent to those of adapalene gel without local side effects. We added honokiol’s other functions to acne treatment in addition to antiinflammation, but further studies are needed.

Keywords
acne, honokiol, comedone, sebum, phenylpropanoid

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Introduction
Acne vulgaris (AV) is a common chronic pilosebaceous inflammatory disease, and the 4 major pathogenic factors contributing to the development of AV are hyperseborrhea, follicular hyperkeratinization, inflammation, and Cutibacterium acnes.\(^1\) The incidence rate of acne is high, and cases occur worldwide.\(^2,3\) AV affects 95% of teenagers aged 15-17 years old,\(^4\) and 20% of these cases are moderate to severe.\(^5\) AV can continue and recur until adulthood, and approximately 50% of adult patients still suffer from this disease.\(^1\) It was reported that AV was the eighth most prevalent disease globally by a systematic analysis study in 2010.\(^6\) AV lesions are mainly distributed in areas with abundant sebaceous units, such as the face, upper chest, and back. The primary lesions include comedones (open or closed) and inflammatory lesions, including papules, pustules, and nodules/cysts.\(^5\) AV can have secondary effects of scar formation and postinflammatory hyperpigmentation. AV affects the patient’s appearance, mental health, and quality of life.\(^7\) However, the treatment is full of challenges because it is difficult to find an efficacious, well-tolerated, long-term, safe acne therapy. Topical retinoids (tretinoin, adapalene, and tazarotene) have been used as the foundation for most AV treatment regimens.\(^7\) The main effect of retinoids is reducing follicular hyperkeratinization in keratinocytes, and inhibiting the genesis of comedones.\(^5\) In addition, retinoids can inhibit C. acnes and have antiinflammatory effects.\(^3\) However, all retinoids should be used with contraception when used by women of childbearing age because of their teratogenicity. Moreover, they are mildly photosensitizing and can induce different degrees of local reactions, including erythema, dryness, and peeling.\(^8\) These side effects often cause the treatment to be suspended, resulting in poor patient compliance.\(^8\) Therefore, a safer and more effective external substitute is needed.

Complementary and alternative medicine therapies for acne have increased in popularity in recent years. It has been reported that some natural products such as tea tree oil, green tea, resveratrol, and curcumin are beneficial for the treatment of acne.\(^9\) Honokiol (3′,5-di-(2-propenyl)-1,1′-biphenyl-2,4′-dio) is a small-molecule lipophilic compound extracted from Magnolia officinalis.\(^10\) It is a phenylpropanoid in the neolignan class, which have a para-allyl-phenol and an ortho-allyl-phenol joined together with ortho- and para-C-C-couplings (Figure 1). Honokiol has been proven to have diverse biological and pharmacological activities, including antiinflammatory, antioxidant, antitumor, antilipid peroxidation, antiangiogenic, and neuroprotective effects.\(^11,12\) Because of

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its multifunctional activities, honokiol has been found to play an essential role in treating skin diseases.\textsuperscript{13} In particular, it has powerful antioxidant and antitumor effects. In research related to AV, Park et al. first proposed that honokiol could effectively inhibit \textit{C. acnes} and \textit{Propionibacterium granulosum}, with minimum inhibitory concentrations (MICs) of 4 and 3 \textmu g/mL, respectively. The authors found that honokiol reduced the interleukin-8 (IL-8) and tumor necrosis factor-\alpha (TNF-\alpha) levels induced by \textit{C. acnes} in Tohoku Hospital Pediatrics-1 (THP-1) cells.\textsuperscript{14} Furthermore, honokiol has no toxic effect on cells and caused no irritation in human external use experiments. Further research has indicated that honokiol can reduce oxygen radicals, such as 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and superoxide dismutase (SOD). Honokiol reduced the cyclooxygenase-2 induced by \textit{C. acnes} in THP-1 cells, with an effective inhibition concentration of 10-15 \textmu M. This anti-inflammatory effect of honokiol on AV could occur through the nuclear factor kappa B (NF-\kappa B) pathway.\textsuperscript{15} Fu et al. compared several natural products and indicated that honokiol results in greater inhibition of IL-8 and TNF-\alpha in HaCaT cells, with low cytotoxicity.\textsuperscript{16} However, the above studies were limited to cells in vitro. Moreover, the effects of honokiol on acne have only been reported in terms of bacteriostasis and antiinflammation. It is unclear whether honokiol impacts other pathogenic causes of acne.

We created a rabbit ear acne model through the external use of coal tar, which formed many acne-like rashes. Then, we found that 2.5\% honokiol cream could effectively eliminate these comedone-like rashes, and its effect was equivalent to that of adapalene gel. These changes were also observed in pathology. In addition, honokiol could reduce hair follicle hyperkeratosis and effectively alleviate sebum secretion. In addition to antiinflammation, we added honokiol’s other functions in acne treatment, providing a further basis for its application.

\section*{Results}

\textbf{The Rabbit Ear Acne Model Could Simulate Comedone and Hyperkeratosis of Hair Follicles Both Macroscopically and Microscopically}

After 14 days of modeling with coal tar, there were apparent patulous follicles, comedones, and skin thickening with greasy scales on the right ears of 6 rabbits in the model group, but these lesions were not found on the left ear (Figure 2a and d). The ultraviolet mode of the dermoscope was more conducive to counting the comedones (Figure 2c). Hematoxylin and eosin (H&E) staining showed local epidermal thickening, hyperkeratosis, and hypertrophy of the spinous cell layer. The sebaceous glands of the hair follicles were enlarged, and the infundibulum of hair follicles was filled with keratinocytes and expanded into a pot shape (Figure 2f). Moreover, the above changes were maintained for 28 days after stopping the external use of coal tar. These results showed that the rabbit ear acne model was successful.

\textbf{The 2.5\% Honokiol Cream was as Effective as Adapalene in Treating Acne-Like Lesions in the Animal Model}

There were 3 rabbits, 6 ears, and 18 dermoscopic fields in each group. Repeated measurement of analysis of variance showed that there were differences in the decrease in the comedone number ($F = 0.893, P < .01$) and reduction in keratotic area ($F = 0.454, P < .001$) among the 3 groups. There was no statistically significant difference in the number of comedones and the percentage of keratosis area (Area\%) among the 3 groups on Day 1 (comedones $P = .42$, Area \% $P = .528$) and Day 8 (comedones $P = .133$, Area \% $P = .80$). However, after 2 weeks of treatment, the numbers of comedones ($t = 6.483, P < .01$) and Area\% ($t = 3.799, P < .01$) among the 3 groups were significantly different (Figure 3). After we performed the $t$-test between any 2 groups, it was found that there was only a significant difference in the reduction of comedones between the honokiol (HNK) group and the negative control (NC) group ($P < .01$). However, there was no significant difference in the reduction in comedones between the ADA group and the control group ($P = .51$). Honokiol and adapalene significantly reduced the Area\% compared with the control group ($P < .01$ and $P < .01$, respectively). However, there was no significant difference between them ($P = .774$).

\textbf{Honokiol Effectively Treated Acne-like Lesions by Alleviating Hyperkeratosis and Sebum Secretion}

After treatment with honokiol for 2 weeks, the epidermal layer became prominently thinner than that in the control group ($P < .05$). However, there was no significant difference between the adapalene (ADA) group and the control group. Both honokiol and adapalene significantly reduced the number of inflammatory cells in the dermis ($P < .001$ and $P < .05$, respectively) (Figure 4). Oil Red O staining showed that, after 14 days of treatment, honokiol and adapalene significantly reduced sebum secretion ($P < .001$, and $P < .001$, respectively).

\section*{Adverse Events}

There were no obvious side effects in either group. We only observed local erythema on several rabbit ears, but all of them were transient, and they were normal by the next day.
During the use of honokiol, no local skin ulcerations or erythema were found, and no discomfort, such as irritability, ear shaking, or scratching was found in the experimental rabbits.

Discussion

Honokiol is a natural phenylpropanoid. It has been studied extensively in recent years, especially in tumors, and liver and nervous system diseases. In the study of antiacne effects, only inhibition of acne pathogenic bacteria and inhibition of the inflammatory response induced by $C.\text{acnes}$ have been reported. However, these effects have not been confirmed by in vivo experiments. Our study found that topical honokiol could effectively decrease the number of inflammatory cells in the dermis of the rabbit ear acne model. The effect was not significantly different from that of adapalene gel. Thus, the previously reported antiinflammatory effects were verified in an animal experiment.

Our study also observed that topical honokiol and adapalene had the same effect on relieving hyperkeratosis in animal acne models, but the antim comedone effect of honokiol was better. Comedones are enlarged hair follicles filled with sebum, keratinous material, and bacteria. Colonization by $C.\text{acnes}$ is not only related to an inflammatory reaction, but also to keratinization and comedone formation. Therefore, the antibacterial effect of honokiol might be involved in the treatment of comedones. Then, we observed that honokiol could effectively alleviate hyperkeratosis of rabbit ears and reduce sebum secretion. Summarizing the above research, we found that honokiol could act on all causes of comedone formation. In addition, the microcomedone, a small comedone that is invisible to the naked eye, is considered to be the cornerstone of the pathogenesis and treatment of acne. Therefore, if microcomedones are effectively treated, acne can be controlled. Given the great antimcomedone effects of honokiol in animal models, we speculate that it could be used as a basic drug for the topical treatment of acne. However, this research focused on animal experiments and lacked progressive mechanistic research and cell experiments.

Recently, it has been reported that honokiol can downregulate the NF-$\kappa B$ pathway to reduce the inflammatory reaction and lipid metabolism disorders of the liver. Honokiol can improve hepatic steatosis by acting on sterol regulatory element-binding protein (SREBP) targets. Moreover, this inhibition of the SREBP-1 effect might be mediated by the liver kinase B1 (LKB1)-AMPK signaling pathway and reduce the expression of acetyl-CoA carboxylase (ACC). SREBP-1 and ACC also play important roles in sebum metabolism. Inhibiting the maturation and expression of these molecules can effectively inhibit sebum secretion. In addition, hormones, especially androgens, are also important factors affecting sebum secretion. Antiandrogen therapy is effective for male and female acne patients. Bernard et al. indicated that honokiol could effectively inhibit 5-alpha-reductase type 1. After 56 days of continuous
topical application to the faces of men, wrinkles were reduced because honokiol affected hormone levels. Therefore, honokiol could significantly reduce sebum secretion in animal models, which might be due to the above reasons. For some objective reasons, we have not been able to conduct more in-depth research. We look forward to further research on the effects of honokiol on the sebaceous glands.

Methods and Materials
Reagents and Preparation
Honokiol (≥98% pure) was purchased from Aladdin Biochemical Technology Co., Ltd. The cream is the standard cosmetic matrix. Honokiol was dissolved in the cream matrix by stirring it in a 50 °C water bath to form a 2.5% honokiol cream. The adapalene gel was purchased from Sichuan Med Shine Co., Ltd.

Animal Models and Treatments
Male New Zealand white rabbits (2-2.5 kg, 16 weeks old) purchased from Sichuan Experimental Animal Special Committee were used in the animal experiments. Each rabbit was kept in a separate cage and fed standard food and water ad libitum. Six of 15 rabbits were assigned to the model group, and the remaining 9 were assigned to the treatment groups. Each side ear of the model group rabbit and bilateral ear of the treatment group rabbit were coated with 0.5 mL of crude coal tar every day for 2 weeks. The application position was near the ear tube with an opening range of 2 cm × 2 cm.

Figure 3. The 2.5% honokiol cream was effective as an adhesive gel in treating acne-like lesions in an animal model. (a) Typical photographs of a naked eye view and a dermoscopic ultraviolet view of the control, HNK, and ADA groups on the 1st, 8th, and 15th days of treatment. Honokiol had significant efficacy in relieving comedones and hyperkeratosis. After 2 weeks of treatment, the number of comedones (b) and Area% (c) in the HNK group were significantly lower than those in the NC group. Adapalene gel reduced the number of comedones, but there was no significant difference compared with the control group.

Abbreviations: ADA, adapalene; H&E, hematoxylin and eosin; HNK, honokiol; NC, negative control.
Nine of the treatment group rabbits were randomly assigned to 3 groups: the NC group (cream without a drug), the HNK group (2.5% honokiol cream), and the ADA group. The 3 groups were topically treated with 0.25 g of the corresponding products once per day for 14 days. All of the animal experiments were approved by Sichuan University in Chengdu, China (Protocol 2020309A).

**Evaluation**

The numbers of comedone-like rashes and the percentages of hyperkeratosis area were recorded at baseline and after 7 and 14 days of treatment. Each rabbit ear was counted in 3 dermoscopic fields with the naked eye and by dermoscopic ultraviolet examination. Each field was approximately 1 cm² in size. The fields were 1.5 cm (point A) and 2.5 cm (point B) outward from the junction of the central dorsal ear artery and the protruding cartilage of the external auditory canal, while point C was on the inside of points A and B. The connection of points A, B, and C formed an equilateral triangle (Figure 5). A Skiary-K3 hand-held dermatoscope was purchased from Beijing Xiangzhe Technology Co., Ltd and used to count the number of comedones in every dermoscopic field. The percentages of the keratinized area were analyzed by ImageJ analysis software (NIH).

**Histology and Oil Red O Staining**

Animals were sacrificed after the last observation, and the skin lesions were completely removed, including points A, B, and C, and cut into 2 pieces. One part was collected and fixed in 4% paraformaldehyde, and another part was immediately frozen and stored at −80 °C. The tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Then, tissues were sectioned at 3 μm thickness and stained with H&E. Frozen tissues were sectioned at 8 μm thickness and rinsed briefly in distilled water. Then, the tissues were rinsed in 60% isopropanol for 20-30 s. The Oil Red O working solution was prepared with a BASO Oil Red O Stain Kit (Zhuhai Baso Biotechnology Co., Ltd), and the samples were stained for 5-10 min. The slide was rinsed in 60% isopropanol for several seconds to remove excess stain, followed by washing it in distilled water. Then, the slides were stained with hematoxylin solution and differentiated in 1% hydrochloric acid for a few seconds. After washing the slide with water 3 times, it was dried and sealed. Images were captured using an NIS-Elements AR (Nikon).
and analyzed using ImageJ analysis software. The region of interest for Oil Red O staining was the epidermis and dermis.

**Statistical Analysis**

The statistical analysis was performed using SPSS software, version 23.0 for Windows, and GraphPad Prism software, version 7.04 (GraphPad Software Inc.). Repeated measurement of analysis of variance was utilized. The \( t \)-test was used for continuous variables, and \( P < .05 \) was considered statistically significant.

**Conclusions**

Honokiol could reduce the number of comedones in a rabbit ear model of acne, with major implications for topical acne treatment. Its therapeutic effect is achieved by reducing local hyperkeratosis and sebum secretion. These effects are comparable to those of adapalene gel and are less irritating and safer. We hope that honokiol provides a new choice for acne treatment.

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**Authors’ Contributions**

YZ contributed toward conceptualization, methodology, and writing—original draft. LZ contributed to visualization and writing—review & editing. XZ contributed to conceptualization, funding acquisition, supervision, and writing—review & editing.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethical Approval**

This study was approved by the Administration Committee of Experimental Animals, Sichuan University, Chengdu, China (Protocol 2020309A).

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**Statement of Human and Animal Rights**

All of the experimental procedures involving animals were conducted in accordance with the Institutional Animal Care guidelines of Sichuan University, China, and approved by the Administration Committee of Experimental Animals, Sichuan University, Chengdu, China.

**Statement of Informed Consent**

There are no human subjects in the article and informed consent is not applicable.

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