Flavonoid combination therapy regulating immune system's homeostasis via activation the FOXO3 signaling pathway for the cure of Propionibacterium acnes infection

Authors: Juan Wu[a]; Biao Le[a] Xiaocong Fu[a]; Li Hu[b]; Jinlu Zuo[c]; Jin Huang[d]*

Addresses：
[a]Sexually Transmitted Disease Institute, Shanghai Skin Disease Hospital, School of Medicine，Tongji University , Shanghai, 200443, China.
[b]Anesthesiology Department, Shanghai East hospital，Shanghai, 200120, China.
[c]Guangzhou Vocational School of Finance and Business, Datansha Campus, Guangzhou, 510410, China.
[d]Department of Pharmacy, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, China.

Juan Wu
Sexually Transmitted Disease Institute, Shanghai Skin Disease Hospital, School of Medicine，Tongji University , Shanghai, 200443, China.
Tel:0086-13661419368.
Email: 13661419368@163.com.

Juan Wu 13661419368@163.com. https://orcid.org/0000-0003-4213-7542
Biao Le 1755913503@qq.com
Xiaocong FU fuxiaocong2022@126.com
Li Hu huli20207@163.com
Jinlu Zuo 675979624@qq.com
Jin Huang john70550@163.com

Short Title: Flavonoid regulating immune system's homeostasis

*Corresponding Author:
Jin Huang
Department of Pharmacy, Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai, China.
Tel: 18917099081
Email: john70550@163.com

Abstract

In the present study, the in vitro and in vivo anti-acne activation by flavonoid combination therapy were investigated, and the role and relationship between flavonoid and FOXO3 signaling pathway were discussed. Three natural flavonoid such as quercetin, kaempferol, luteolin had certain anti-inflammation activity. Meanwhile, the combination therapy significantly outperforming using these flavonoids alone, which might be attributed to the synergistic bactericidal effect on the cells. Besides, it was also found that the FOXO3 pathway play an important role in P acnes induced skin inflammation. It's promoted the decreasing of FOXO3 signaling pathway activation and induced an increasing in sebum secretion. During the acne inflammatory response, the expression of pro-inflammatory cytokine was increased significantly. After flavonoid combination therapy, the pro-inflammatory cytokines expression levels such as TNF-α (19.36 ± 2.44 pg/ml), IL-8 (1392.52 ± 131.75 pg/ml), IL-1β (13.42 ± 4.54 pg/ml) and IL-6 (109.64±13.34 pg/ml) were significantly reduced. Flavonoid combination therapy effectively decreased the expression of pro-inflammatory cytokines and increased the secretion of the anti-inflammatory cytokines. In addition, the activation of the FOXO3 pathway was found to be inversely proportional to the expression of pro-inflammatory cytokines. In this study, the flavonoid combination therapy demonstrated a "synergistic effect" inhibiting the inflammatory process via the activation of the FOXO3 pathway. The enhanced synergy observed surpassed their individual effects when used in combination. Consequently, the "synergistic effect" of three flavonoid ingredients suggested a new approach for the development of anti-acne reagents as FOXO3 enhancers.

KEY WORDS: P.Acnes (Propionibacterium acnes), Flavonoid combination therapy (quercetin combination with kaempferol and luteolin), Forkhead boxes, class O (FOXO)，anti-inflammatory，pro- inflammatory , cytokines

Introduction

Acne is a chronic inflammatory skin disease affecting hair follicles sebaceous glands, including abnormal keratinization of hair follicle sebaceous gland vessels, excessive sebum secretion, proliferation of P acnes, inflammation and excessive immune response [1]. Pro-inflammatory cytokines such as TNF-a, IL-6, GM-CSF and IL-8 can produce keratinocytes by P acnes stimulation and induce neutrophil or lymphocyte aggregation to the pilosebaceous epithelium. Although the exact pathogenesis of acne is still unclear, the excessive growth of P acnes is considered a key factor in the formation of acne vulgaris [2,3]. Additionally, P acnes secretes various proteases and lipases, contributing to the degradation of the integrity of the hair follicle wall. Microbiota in hair follicles and/or abnormal lipids activate Toll-like receptors (TLRs), resulting in the production of interleukin-1α (IL-1α) and other inflammatory factors [4]. During the inflammatory reaction, P acnes triggers the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-8 in monocytes and keratinocytes [5]. In this case, it is considered one of the main strategies for treating acne induced inflammation to regulate the homeostasis and inflammatory response of the immune system.

Moreover, acne patients exhibit nuclear defects of FoxO1 and increased cytoplasmic expression in the sebaceous glands, FoxO1 assumes a critical role in two pivotal processes of wound healing, i.e., attenuating the inflammatory response and enhancing keratinocyte migration protect cells from oxidative damage [6]. The functions of FOXO3 were to maintain blood vessel homeostasis, regulating the activity of mammalian target of rapamycin complex 1 (mTORC1), and related to human lifespan [7]. FOXO3, phosphorylated by the PI3K/Akt cascade, induces mTORC1 activation by downregulating adenosine monophosphate activated protein kinase (AMPK) [8]. Activated mTORC1 leads to the formation of acne and acne scars. These suggest that activating FOXO3 in the sebaceous glands may improve the inflammatory response caused by P acne [9].

The treatment of acne includes local treatment (erythromycin, clindamycin and retinoids) and systemic treatment (oral antibiotics and retinoids). However, the main disadvantage of these therapies currently is the significant increase in antibiotic resistance caused by antibiotic abuse [10]. Flavonoid combination therapy may provide new insights to address this issue. Quercetin is an antioxidant for treatment various inflammatory diseases [11], kaempferol with potent antibacterial and immunomodulatory effects [12], and pro-inflammatory mediators such as TNF-α, IL-6 and IL-1β can be inhibited by luteolin [13]. The strategy by using flavonoid for treatment of acne focuses on regulating the homeostasis of the immune system, but the mechanism is still unknow.

FOXO3 signaling pathway matters considerably in the cell proliferation, oxidative stress, inflammatory response and apoptosis. However, the “synergistic effect” of quercetin/kaempferol/luteolin combination therapy in acne inflammation induced by P acne has never been reported. P acnes induced skin inflammation model is one of the main models for studying the pathogenesis of acne. Therefore, a lentivirus-based FOXO signaling pathway activation fluorescence reporter system and P acnes induced skin

inflammation model was used to evaluate the “synergistic effect” of quercetin/kaempferol/luteolin on the antibacterial activity.

2. Materials and Methods

2.1. Flavonoid and reagents

Reagents used in the flow experiments included quercetin (Q111273-20mg), kaempferol (K107144-20mg) and luteolin (L107329-250mg) purchased from Shanghai Aladdin Biochemical Technology; Lentiviral vector pLV-5×FOXO-EGFP-Puro, packaging genome plasmid pMD2.G and envelope expression plasmid psPAX2 from Wuhan Miaoling Biology Co., Ltd.; human epidermal keratinocytes CP-H113 cell lines and 293T cell line from the Wuhan Procell Co., LTD.

Reagents used in the IHC experiments included Xylene (SCR,10023418), Absolution (GENERALREAGENT,G73537G), Hydrogen Peroxide Solution, 30%(SCR, 10011208), and Goat Serum, New Zealand Origin(Thermo Fisher, 16210072).

2.2. Determination the antibacterial effect of flavonoid on P acnes

Flavonoid ingredients such as kaempferol, quercetin and luteolin were dissolved at a concentration of 3mg/ml in 0.01% dimethyl sulfoxide (DMSO) of PBS, clindamycin as a positive control [14]. Flavonoid ingredients were infused with a diameter of 8mm filter paper and placed on the GAM agar plate containing of (10^7CFU/ml) P acnes, which were further incubated in an anaerobic gas generating pouch at 37°C for 48 h under the condition contained 5% CO_2. Then the diameter of inhibition zone was measured by vernier caliper.

2.3 Design of reporter constructs and transfection to 293T cell lines

To study the effect of flavonoids on the FOXO3 signaling pathway in vitro, a lentivirus-based FOXO signaling pathway activation fluorescence reporter system was designed. The self-inactivating lentiviral vector pLV-5×FOXO-EGFP-Puro, along with the packaging genome plasmid pMD2.G and envelope expression plasmid psPAX2, was co-transfected into 293T cells following the user manual guidelines, with EGFP serving as the reporter gene. After incubation, cell supernatants were collected and store at −80˚C until use.

2.4 Stimulation experiments and reporter assays

Human epidermal keratinocyte line CP-H113 was cultured in RPMI-1640 complete medium, and supplemented with 293T cells supernatant. The stable transgenic CP-H113 cell line was obtained via puromycin selection. CP-H113 cell line, equipped with an activation fluorescent reporter system for the FOXO-3 signaling pathway, demonstrated EGFP gene expression upon activation of the FOXO-3 signaling pathway induced by 10μm triciribine. The transfection effects were confirmed by inverted fluorescence microscope. The completion of the transfection process was positively correlated with the green fluorescence intensity per cell.

The stable transgenic CP-H113 cells cultured in Corning®Costar®6 6well culture plate (1.0×10^5 cells/well) were supplemented with quercetin, kaempferol and luteolin alone or flavonoid combination therapy and clindamycin as a positive control at a

concentration of 3mg/ml for 2 h, which were then stimulated with 1.0×10^7 CFU/well of P acnes for 18 h. The expression of GFP intensity was analyzed by flow cytometry, and the degree from each cells expressed GFP green fluorescence, indicating the effects of the flavonoid combination therapy.

2.5 Enzyme-linked immunosorbent assay (ELISA)

CP-H113 (1.0×10^6 cells/well) cells were pre-treated with flavonoid alone and flavonoid combinations therapy, at a concentration of 3mg/ml for 2 h, respectively. Then collected the supernatant which was stimulated by P acnes (1.0×10^7 CFU/well) for 18h, and quantification of IL-6, IL-8, IL-1 β, TNF- α, IL-10 and TGF- β horizontal by ELISAs. In in vivo experiments, peripheral blood was collected from the left ventricular before sacrifice, then the expression level of inflammatory cytokines was detected by ELISA kits.

2.6 Experimental of animal model

All animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals and the Ethics Committee of Novopathway (ethical approval number 20230201001). Male ICR mice aged 6-8 weeks were housed 4-5 per cage (385x194x172 mm; Guangzhou Cleanroom Company)) in rooms at a temperature of 22-24˚C with a 12-h light/dark cycle. All animals had free access to food (Radiation sterilization of experimental mouse food; Diet, XieTong Co. Ltd) and water and were acclimated to these conditions for at least 7 days before experiments were initiated. During the animal experiment, the health status of the animals was monitored daily and no adverse events were observed. Researcher had not any exclusion decisions, until the mouse's weight over 20% of the average body weight.

On D0, P acnes (1.0×10^7 CFU in 10 μl PBS) was intradermally injected to mice, either in the left ears or dorsal skin. After the injection, randomly divide the mice into 10 groups with 6 mice in each, and 20 μL of 3mg flavonoids were applied to the surface of the injection area as follows: control: no treatment; model: treated with normal saline; luteolin, kaempferol and quercetin alone, QK: quercetin combination with kaempferol; QL: quercetin combination with luteolin; KL: kaempferol combination with luteolin; QKL: Quercetin combination with kaempferol and luteolin of 3mg/ml in 0.01% DMSO per 20 μl of PBS; clindamycin as a positive control.

Animals were sacrificed on D7, left ears or dorsal skin was weighed and the skin thickness was measured. Peripheral blood was collected, and the differences in cytokine levels between each group were analyzed using ELISA.

2.7 Histological analysis

Samples from left ears and dorsal skin sections were fixed in 10% formaldehyde, paraffin embedded and cut into 4-μm thickness slices. Subsequently, these sections were stained with hematoxylin and eosin (H&E) and examined using a light microscope to assess the presence of edema and inflammatory cell accumulation. Tree visual fields were

randomly selected for the evaluation of the severity of acne using Global acne grading score (GAGS) criteria.

2.8 Statistics

All experiments were performed independently in triplicate. Values are expressed as means with ± standard deviations (SD). The statistical significance of the result was analyzed by one-way ANOVA test using a GraphPad package (prism v8.0; GraphPad Inc., USA). P value ≤ 0.05 was considered significant.

Data were analyzed by one-way ANOVA test (* $p < 0.05$ versus control and ** $p < 0.05$ versus P acnes alone, n=5 each group). Abbreviation: ns, no statistically significant.

3. Result

3.1 Antimicrobial activity of flavonoid against P acnes

To investigate the bacteriostatic activity of flavonoids such as kaempferol, luteolin and quercetin, the antibacterial effect was evaluated by Kirby-Bauer test (Fig.1), and chemical constitution of flavonoid was shown in Figure. 1A. The mechanisms by flavonoid carry out main anti-inflammatory activities are shown in Figure.1C. Flavonoid was involved in the regulation and expression of various transcription factors like FOXO3, NF-κB, PPARγ, ERK/IκK, Akt/PI3k and activator protein-1 (AP-1) [15]. Herein, the inhibitory effect of flavonoids at concentrations of 3mg/ml against P acnes was determined using Kirby-Bauer test, as shown in Figure. 1B. Compared with other flavonoids, the flavonoid combination therapy group presented the best against P acne efficiency (30.6±2.1mm VS 29.8 ±2.1mm).

Inhibition activity of flavonoids against P acne was analyzed, as shown in Table.1. The clear zones of inhibition observed for quercetin, kaempferol, luteolin, and clindamycin individually against P acnes were 24.9±1.7 mm, 22.9±1.5 mm, 21.3±1.3 mm, and 29.8±2.3 mm, respectively. However, flavonoid combination therapy was significantly better compared to quercetin, kaempferol, luteolin and clindamycin alone, which might benefit from the synergistic bacteriostatic effect of flavonoid.

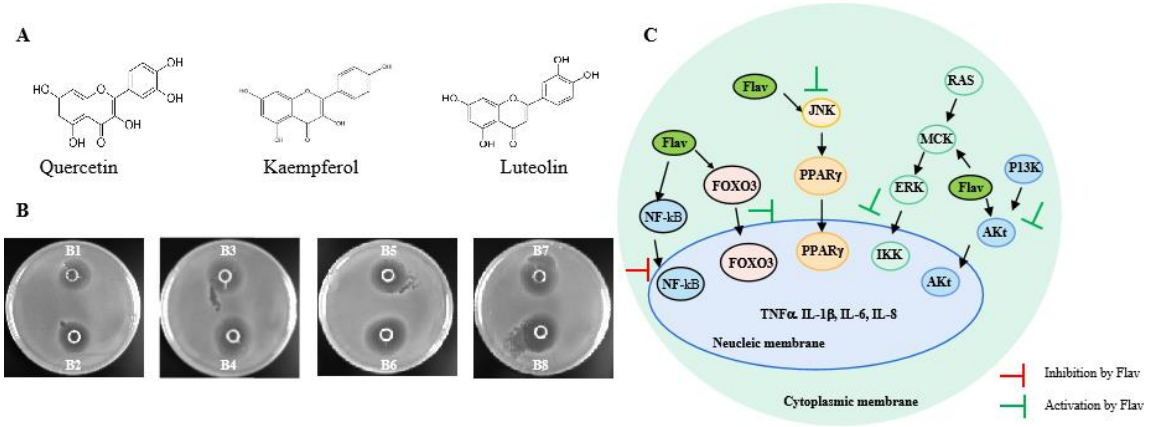

Fig. 1 Antibacterial activity of quercetin, kaempferol and luteolin against P acnes. (A) Structure of quercetin, kaempferol and luteolin; (B) The inhibitory effect of flavonoid against P acnes. (B1) kaempferol, (B2) luteolin, (B3) quercetin, (B4) kaempferol combination with luteolin, (B5) kaempferol combination with quercetin, (B6) luteolin combination with quercetin, (B8) quercetin combination with kaempferol and luteolin.（B7) Clindamycin as a positive control, respectively.（C） Major inflammatory pathways targeted by flavonoid.

Table1

Drugs	Symbol	Zone of inhibition (mm) mean ± SD
		Propionibectarium acnes
		1mm
Kaempferol alone	B1	22.9±1.5
Luteolin alone	B2	21.3±1.3
Quercetin alone	B3	24.9±1.7
Kaempferol + Luteolin	B4	25.9±1.7
Kaempferol + Quercetin	B5	26.8±1.8
Quercetin + Luteolin	B6	28.4±2.0
Clindamycin alone	B7	29.8±2.1
Quercetin + Kaempferol + Luteolin	B8	30.6±2.1

Table.1 Diameters of zones of bacterial inhibition by using Quercetin, Kaempferol, Luteolin and flavonoid combination against P acnes. Values represent mean ± SD (n >3)

3.2 Effect of flavonoids by activation of FOXO3 signaling pathway in invitro study

Herein, a lentivirus-based FOXO-3 signaling pathway activation fluorescence reporter system was designed, as shown in Fig.2A, and was co-transfected into 293T cells according to the user manual. 293T Cell supernatant was added to human epidermal keratinocytes CP-H113 cell lines, and the stable transgenic CP-H113 cell line was then obtained by puromycin selection. CP-H113 cells were equipped with the EGFP reporter system of FOXO-3 signaling pathway. After activating the FOXO-3 signaling pathway by 10 μm triciribine, which enhanced the transcriptional activity of FOXO3 by inhibiting the phosphorylation of PKB/Akt signaling channels in CP-H113 cells [13]. The expression of the EGFP signaling were activated, and the transfection results were confirmed by using inverted fluorescence microscope (Fig. 2B). The completion of the transfection process was positively correlated with the green fluorescence intensity per cell.

Flavonoids at a concentration of 3mg/ml was supplemented to CP-H113 cells, and clindamycin as a positive control, respectively, which were then stimulated with 1.0×10^7 CFU/well of P acnes for 18 h. The induction of EGFP expression levels in CP-H113 cells treatment by each flavonoid was assessed using flow cytometry (Figure. 2C). After treatment with quercetin, kaempferol, luteolin alone, the expression level of EGFP increased to about 50% (46.3%, 58.8%, and 54.1%, respectively). Compared with positive control clindamycin, the flavonoid combination treatment group had the higher expression of EGFP (85.5% vs. 63.3%). These results indicated that the flavonoids effectively inhibited P acne infection by activating the FOXO3 signaling pathway in in vitro study (Fig. 2C).

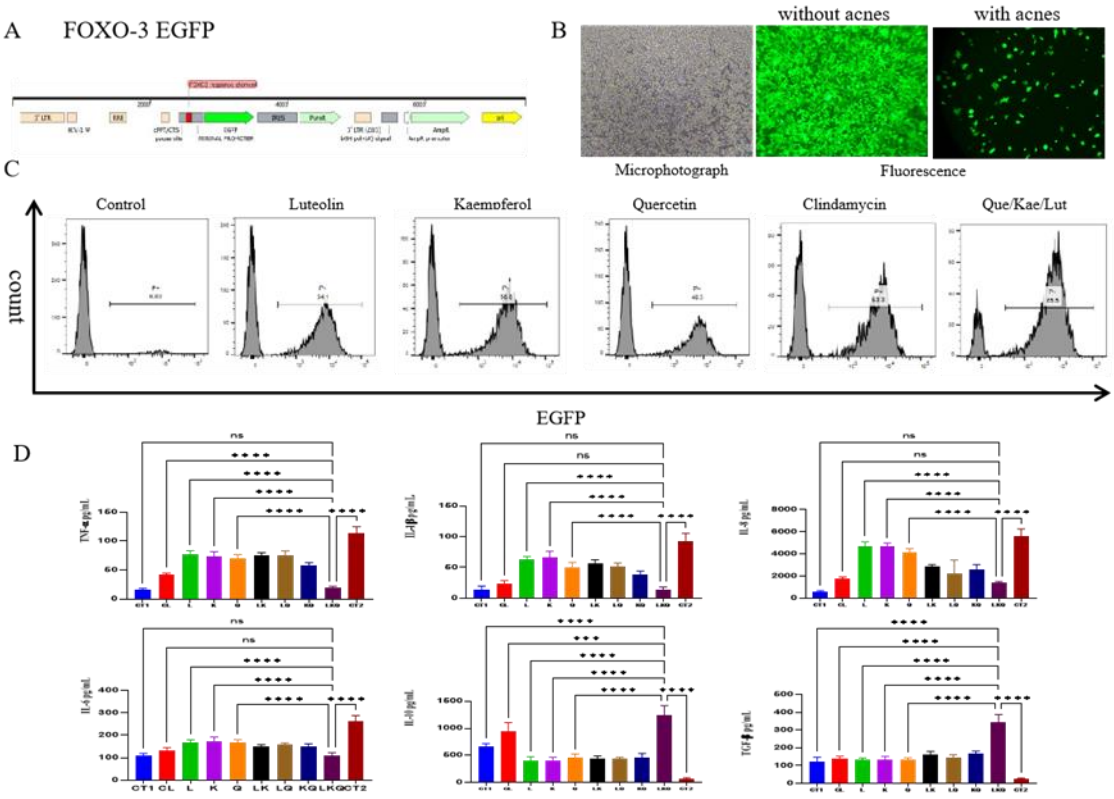

Fig.2 Generation and characterization of the lentivirus-based FOXO3 signaling pathway activation fluorescence reporter system. A) Schematic illustration of reporter constructs encoding FOXO-3 EGFP with restriction enzyme recognition sites. B) Fluorescent inverted microscope results of CP-H113 cells, FOXO-3 signaling pathway was activated by 10μm triciribine for 1hr. After incubation, stimulated with or without 1.0 × 10^7 CFU/well of P acnes for 18 h. C) Histogram representation to measure the effect on the transcription factor activation of FOXO3 signaling pathway. Numbers show geometric mean of fluorescence intensity (EGFP). D) Effect of flavonoid against P acnes-induced cytokines in CP-H113 cells. (CT1) control, (L) luteolin, (K) kaempferol, (Q)quercetin, (LK) luteolin combination with kaempferol, (LQ) luteolin combination with quercetin, (KQ)kaempferol combination with quercetin, (LKQ) quercetin combination with kaempferol and luteolin. A control experiment without P acnes-stimulation was conducted in CT2, and CL(Clindamycin) as a positive control, respectively. Data were analyzed by one-way ANOVA test (* $p < 0.05$ versus control and ** $p < 0.05$ versus P acnes alone, n=5 each group). Abbreviation: ns, no statistically significant.

3.3 "Synergistic effect" of flavonoids on the production of inflammatory cytokines

Flavonoids at a concentration of 3mg/ml was supplemented to CP-H113 cells due to investigate the "synergistic effect", and clindamycin as a positive control, respectively, then stimulated with 1.0 × 10^7 CFU/well of P acnes for 18 h. The culture supernatant was collected, and expression level of anti-inflammatory and Pro-inflammatory cytokines was detected by ELISA kits (Fig. 2D). In this study, flavonoid combination therapy shown the expression level of IL-8 (1392.52 ± 131.75 pg/mL), IL-1β (13.42 ± 4.54 pg/mL). TNF-α

(19.36 ± 2.44 pg/mL) and IL-6 (109.64 ±13.34pg / mL), respectively. On the other hand, flavonoid combination therapy significantly increased the secretion of the anti-inflammatory cytokine IL-10 (1240.54 ± 181.58 pg/mL) and TGF-β (342.34 ± 37.05 pg/mL). These results suggested that compared to the use of drugs alone, flavonoid combination therapy effectively decreased the expression of pro-inflammatory cytokines and increased the secretion of anti-inflammatory cytokine in CP-H113 cell lines stimulated with P acnes in in vitro.

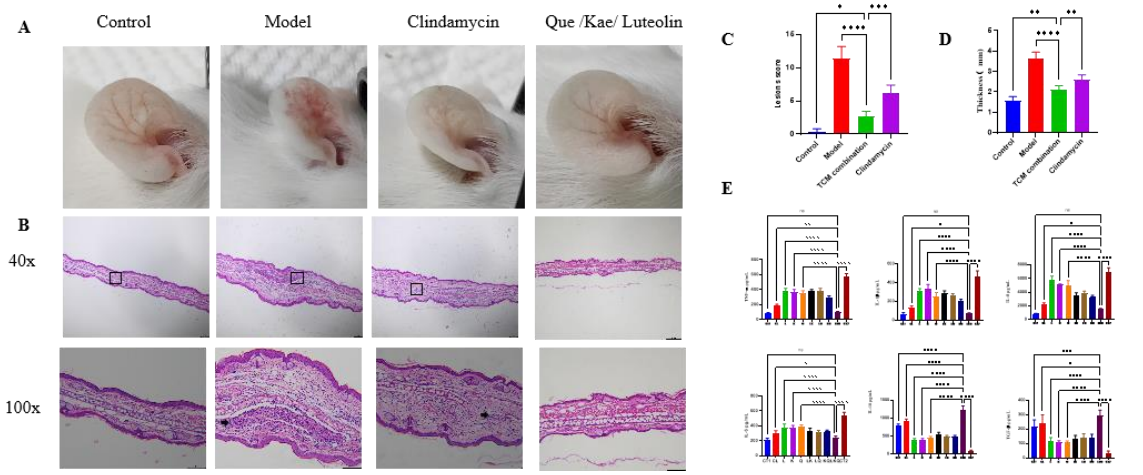

Figure 3. Effect of flavonoid combination therapy on P acnes-induced skin inflammation in vivo. P acnes were intradermally injected into the ears for 24 h. Control (non-treatment) mice were similarly treated with PBS and without P acnes inflammation, while the model group with P acnes. Clindamycin as a positive control, respectively. (A) Effect of flavonoid combination therapy on P acnes-induced skin inflammation. Ear thickness, swelling, erythema, and inflammatory response after treatment; (B) Paraffin sections of left ear were stained with hematoxylin and eosin observed by microscope (40× and 100× magnified); (C) Lesions score, and (D) Auricle thickness. (E) Effect of flavonoid compounds against P acnes-induced cytokines in ears models. Data were analyzed by one-way ANOVA test (* $p < 0.05$ versus control and ** $p < 0.05$ versus P. acnes alone, n=5 each group). Abbreviation: ns, no statistically significant.)

3.4 "Synergistic effect" of flavonoids in in vivo study

To evaluate the anti-inflammatory effect of flavonoid combination therapy, mouse left ears or dorsal skin were intradermally injected with P acnes (1.0×10^7CFU in 10µl in PBS) for 24 h. Left ear and dorsal skin thickness was used to indirectly assess the inflammation degree. By contrast, the combination treatment with flavonoids resulted in noticeably reduced erythema, skin thickness, swelling, and inflammation in both the ears and dorsal skin model (Fig. 3A and Fig. 4A). Histological observation was shown in Fig. 3B and Fig. 4B. The lesions score was analyzed following GAGS standards. The average score of left ears model was 12.3 ± 3.1, clindamycin (6.2 ± 0.8) and flavonoid combination therapy (5.3 ± 0.7), respectively (Fig. 3C). The average thickness of left ears model was 4.3 ± 0.8 mm;

control (2.3 ± 0.3 mm); clindamycin (3.2 ± 0.6 mm) and flavonoid combination therapy (2.8 ± 0.5 mm), respectively (Fig. 3D).

For the easy to observe the initial process of inflammation caused by acne, P acnes infection model was established in dorsal skin of mice. The average lesion score of the dorsal skin model was 15.3 ± 2.5, while it was (8.1 ± 1.3) for clindamycin and (4.5 ± 0.6) for the flavonoid combination, respectively (Fig. 4C). The average thickness of dorsal skin model was 5.6 ± 0.8 mm, control (2.3 ± 0.4 mm), clindamycin (3.9 ± 0.7 mm) and flavonoid combination therapy (2.6 ± 0.5 mm) (Fig. 4D). These results suggested that dorsal skin model had an advantage in indirectly evaluation the therapeutic effects of flavonoids.

To evaluate the "synergistic effect" of flavonoid combination therapy, pro-inflammatory cytokines IL-6, IL-8, IL-1β, TNF-α, and anti-inflammatory cytokine IL-10 and TGF-β was measured. (Fig. 3E and Fig.4E). The "synergistic effect" shown by compared with the control group, flavonoids not only decreased pro-inflammatory markers but also increased the expression of anti-inflammatory factors. In this study, flavonoid combination therapy significantly decreased the expression level of IL-8 (1478.5 ± 139.6 vs 1270 ± 261.8 pg/mL), IL-1β (67.9 ± 20.3 vs 95.7±15.9 pg/mL), TNF-α (98.1±13.6 vs 106.2 ± 12.1 pg/mL) and IL-6 (235.6 ±28.7 vs 222.8±18.6 pg/mL), and increased the expression level of TGF-β (294.7± 30.4 vs 322.7±49.6 pg/ml) and IL-10 (98.1±13.6 vs 89.6 ± 10.8 pg/mL), in the left ears or dorsal skin models. Therefore, the "synergistic effect" of flavonoids regulation P acnes-induced inflammation by decreasing pro-inflammatory cytokines and increasing the secretion of anti-inflammatory cytokine, both left ear and dorsal skin mice model.

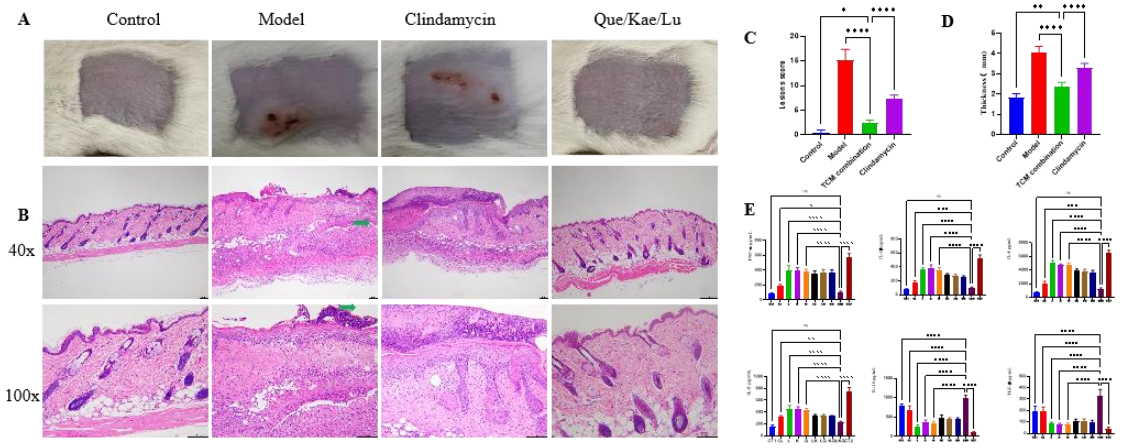

Figure 4. In vivo anti-inflammatory activity of flavonoid against P acnes. P acnes was intradermally injected into the dorsal skin. (A) Effect of flavonoid combination therapy on P acnes-induced skin inflammation. (B) Paraffin sections of the back skin were stained with hematoxylin and eosin, observed by microscope (40× and 100×magnified); (C) Lesions score and (D) Auricle thickness. (E) Effect of flavonoid against P acnes-induced cytokines in dorsal skin models. Data were analyzed by one-way ANOVA test (* $p < 0.05$ versus control and ** $p < 0.05$ versus P acnes alone, n=5 each group). Abbreviation: ns, no statistically significant

Discussion

Although natural product flavonoids shown effects in preventing and treating inflammatory diseases, but it still exhibits some limitations, including unclear of their mechanisms [15]. In this study, a lentivirus-based FOXO3 signaling pathway activation fluorescence reporter system was designed to detect the mechanism of flavonoid combination therapy. The activation of FOXO3 signaling pathway was decreased by P acne infection, and induced sebum production and keratinization. Under flavonoid combination therapy, the FOXO3 signaling pathway was activated, and the expression level of EGFP increased (Fig. 2B, 2C). Quercetin had significant antioxidant activation, while kaempferol exists anti-inflammatory properties, and luteolin not only inhibits pro-inflammatory mediators, but also regulates the IKK and JNK-PPARγ signaling pathway, and exerting a protective effect by reducing Th2 cytokines [16]. To determine the mechanism of flavonoid ingredients in P acne induced inflammatory response will contribute to the application of flavonoid combination therapy in the prevention and treatment of P acne.

The imbalance of pro-inflammatory and anti-inflammatory cytokine levels is the principal reason for P acne induced inflammatory pathogenesis and severity. Flavonoid combination therapy for acne focuses on regulating the immune system's homeostasis. Therefore, the pro-inflammatory and anti-inflammatory effects of flavonoid ingredients in inflammatory responses had been discussed in detail. Pro-inflammatory cytokines contributed to control and remove the occurrence and transmission of inflammation, while anti-inflammatory cytokines contributed to the resolution and recovery of inflammation or acute autoimmune diseases. In this study, flavonoid combination therapy significantly decreased the release of pro-inflammatory cytokines (TNF-α, IL-1β, IL-8 and IL-6) induced by P acnes in skin inflammation, and increased the secretion of anti-inflammatory cytokines (TGF-β and IL-10).

Pro-inflammatory cytokines TNF-α play a key role in mediating inflammatory responses and innate immunity [17], while IL-1β was an effective inducer for pro-inflammatory cytokines. During skin inflammation induced by P acne, the high expression of TNF-α promotes the production and secretion of cytokines such as IL-1, IL-6, IL-8, and IL-12 by activating T cells. Inhibiting of cytokine secretion may be an effective therapeutic strategy for skin inflammatory diseases [18].

Due to evaluate the anti-inflammatory effect of flavonoid combination therapy in vivo, left ears or dorsal skin mice model was established. The pathology score and skin thickness measurement results shown that the dorsal skin model had an advantage in indirectly evaluating the therapeutic effect of flavonoid ingredients. (Fig. 3A,3C and Fig. 4A,4C)

Final, the experimental results indicated that the flavonoid combination therapy could significantly decreased pro-inflammatory and increased anti-inflammatory cytokines to regulate immune system's homeostasis, and reduce sebum production and excessive keratinization by activation the FOXO3 signaling pathway. Therefore, flavonoid combined therapy could be recognized as a promising treatment strategy for autoimmune diseases related to inflammatory skin diseases.

Disclosure

The authors declare that is no conflicts of interest in this work.

Ethical Statement

Ethical approval was not sought for the present study because there are no human subjects in this article and informed consent is not applicable.

Animal Welfare

All animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals and the Ethics Committee of Novopathway (ethical approval number 20230201001).

Conflict of Interest Statement

The authors declared that they have no conflicts of interest to this work.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Author contributions

JW: Design and plan, responsible for the accuracy, completeness of the work and approving it.
BL: Wrote initial manuscript and make significant contributions to the research concept.
XF: Significant contributions to acquisition of research data, approving final version.
LH: Critical revisions for intellectual content.
JL: Substantial contribution to design of work and acquisition of data for work.
JH: Critical revisions for intellectual content as a senior author, approved final version, and agreed to be accountable for all aspects of work.

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