



Viaminate ameliorates *Propionibacterium acnes*-induced acne via inhibition of the TLR2/NF- κ B and MAPK pathways in rats

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Abstract

Viaminate, a retinoic acid derivative developed in China, has been clinically used for acne treatment to regulate and control keratinocyte cell differentiation and proliferation, inhibit keratinization, reduce sebum secretion, and regulate immune and anti-inflammatory functions; however, its potential molecular mechanism has not yet been elucidated. Therefore, we induced ear acne in rats using *Propionibacterium acnes* and sebum application. Symptoms of ear redness, epidermal thickening, inflammatory reaction, keratin overproduction, subcutaneous oil, and triglyceride (TG) accumulation improved significantly in acne model rats treated with viaminate for 30 days. Transcriptome analysis of rat skin tissues suggested that viaminate had significant regulatory effects on fatty acid metabolism and cellular keratinization pathways. Molecular target prediction suggested that toll-like receptor 2 (TLR2) may be a key target of viaminate's therapeutic mechanism. Western blotting results confirmed that viaminate inhibited the TLR2 and its downstream pathways, nuclear factor-kappa B (NF- κ B) [NF- κ B inhibitor alpha (I κ B α)/NF- κ B-p65] and mitogen-activated protein kinases (MAPKs) [MAPK p38/c-Jun N-terminal kinase (JNK)/extracellular regulated kinase 1/2 (ERK1/2)] in acne vulgaris rats. In vitro studies revealed that viaminate treatment attenuated *P. acnes* proliferation and *P. acnes*-induced inflammatory response in human keratinocytes and has an inhibitory effect on the activation of NF- κ B and MAPKs, while overexpression of TLR2 attenuated these effects. In conclusion, viaminate ameliorates *P. acnes*-induced acne by inhibiting the proliferation and inflammatory response of keratinocytes, ascribed to the deactivation of the TLR2-mediated NF- κ B and MAPK pathways.

Keywords Viaminate · Acne · *Propionibacterium acnes* · Keratinization · Proliferation

Introduction

Acne is a chronic skin condition, which usually occurs frequently in adolescence can also be observed in adults. The pathophysiology of acne involves the following three factors: excessive secretion of sebaceous glands, abnormal keratinization of hair follicles, and abnormal proliferation of *Propionibacterium acnes*. Current treatments for acne vulgaris include pharmacotherapy, optical therapy, and physical therapy. Drugs are the preferred mode of treatment for acne, and different drugs are prescribed according to the symptoms and severity. However, long-term application of

drugs is limited due to their toxicity and side effects (Leung et al. 2021). Therefore, further clarification of the targets and mechanism of these drugs in acne treatment may facilitate further drug discovery.

Retinoids (such as tretinoin, tazarotene, adapalene, and trifarotene) are a class of vitamin A derivatives, which are preferred for the treatment and maintenance of mild or moderate acne vulgaris (Leung et al. 2021). These drugs inhibit the proliferation of keratin-forming cells, thereby reducing follicle obstruction and preventing microcomedones formation. Viaminate, structurally classified as a retinoid, was developed in China in the 1980s, and it is synthesized from all-trans retinoic acid (ATRA) and ethyl para-aminobenzoate (Cao et al. 2008). Viaminate inhibits the activity of ornithine decarboxylase, diminishing its potential to induce tumorigenesis in the skin. Additionally, viaminate enhances the self-repair ability of keratin-forming cells, preferentially allowing the differentiation of normal keratin-forming cells

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while inhibiting the proliferation and differentiation of abnormal keratin-forming cells, restoring keratinization abnormalities, and exfoliating keratin (Cao et al. 2008; Yin et al. 2007). However, its specific mechanism of function in the healing of acne has not yet been clarified.

Toll-like receptors (TLRs) are a group of recognition receptors that identify pathogen-associated molecular patterns (PAMPs), shared by a large number of microorganisms, as well as endogenous alarm signals, such as apoptosis-associated membrane fragments and inflammatory ligands. Upon detecting these potentially harmful signals, TLRs initiate the body's immune response by triggering the production of inflammatory cytokines (El-Zayat et al. 2019). Ten TLRs have been identified in humans, among which keratin-forming cells can express TLRs 1–6. Studies have shown that acne is primarily caused by *P. acnes* (recently renamed *Cutibacterium acnes*), which triggers inflammatory skin response and can cause the deterioration of inflammatory acne lesions by activating TLRs to release chemokines (Hwang et al. 2020). TLR activation leads to the increase of proinflammatory cytokines, such as tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-1 β , which exacerbate acne development (Kim et al. 2002). Fragments of enzymes, such as lipase, protease, and hyaluronidase, secreted by *P. acnes* can also be recognized by TLR2 in keratin-forming cells near sebaceous glands. The recognition signal is then transmitted from TLR2 and TLR1/6 to the downstream bridging protein, MyD88, to further stimulate the activation of TRAF6, which in turn promotes the phosphorylation of IKK and the increase of nuclear factor-kappa B (NF- κ B) inhibitor alpha (I κ B α). NF- κ B is separated from I κ B and transferred to the nucleus and transcribes cytokines. Inflammatory acne was induced by cytokines in sebum-clogged pores, and persistently high levels of cytokines may cause the acute disease to evolve into chronic disease. In addition, *P. acnes* can also upregulate the expression of TLRs, including TLR2 and TLR4, and play a considerable role in acne-associated inflammation (Jugeau et al. 2005). Notably, ATRA inhibits acne progression by downregulating TLR2 and its coreceptor, CD14 (P. T. Liu et al. 2005). Therefore, the TLR system is a significant target in the treatment of acne, and viamine, the structural analog of ATRA, plays a role in acne treatment via the TLR.

At present, the underlying mechanism of viamine in acne treatment is not clear, and previous studies are based on the analysis of clinical samples, without further animal or cell experiments. This research investigated the therapeutic effects of viamine on acne treatment by establishing a rat acne model. The potential mechanism of action of viamine was analyzed by transcriptome analysis and target prediction and verified and confirmed by an in vitro keratinocyte model, which will lay the foundation to find anti-acne drugs.

Materials and methods

Bacterial culture

Propionibacterium acnes cultures (ATCC11827; Guangzhou Microbial Strain Conservation Center, China) were grown in herpes flesh medium (Basebio, China) at 37 °C under anaerobic conditions. Log phase bacterial cultures (OD 600 = 1.0) were obtained and centrifuged at 5000 g for 15 min at 4 °C. The culture was resuspended in phosphate-buffered saline (PBS) and the concentration was 8.0×10^7 CFU/20 μ L.

Viaminate preparation

Viaminate (>98% purity) was purchased from Psaitong Reagent Co., Ltd. Viaminate dosage was based on the tissue metabolism level of rats (Cao et al. 2008), and 1/3 \times and 3 \times of this standard dose were used in the low and high dose groups, respectively. Viaminate was dissolved in PBS containing 5% carboxypopyl cyclodextrin to obtain 1.7 (low), 5 (standard), and 15 (high) mg/mL solutions at 4 °C in the dark.

Animal models and administration

The rat acne model was constructed according to Stacey et al. (Kolar et al. 2019). Fifty male SD rats (250 \pm 20 g) were divided into 5 groups on average. Group 1 (normal control; NC) was injected with 20 μ L PBS and groups 2–5 were intradermally injected in the left auricle with 20 μ L *P. acnes* (dissolved in PBS, 8.0×10^7 CFU per mouse) for 4 consecutive days to induce the acne model. After the induction of the acne model (i.e., day 5), rats in groups 3, 4, and 5 were given a daily gavage of viamine at low (1.7 mg/kg), medium (5 mg/kg), and high (15 mg/kg) dose, respectively, for 30 days. Additionally, to accelerate the induction of acne formation, the left ears of the rats in groups 2–5 were smeared daily with artificial mixed sebum [17% oleic acid, 45% triglyceride (TG), 25% jojoba oil, and 13% squalene; 100 μ L/ rat; Shanghai Maclin Reagent Company]. The rats were euthanized using CO₂ asphyxiation method after 30 days of treatment.

Enzyme-linked immunosorbent assay (ELISA)

Blood was drawn from the main abdominal vein of the rats and centrifuged to separate the serum. Thereafter, the serum levels of IL-6, TNF- α , and IL-1 β (RK00020, RK00029, and RK00009, respectively; Abclonal, China) were measured by an enzyme marker (CMax Plus, Molecular Devices, USA) following the manufacturer's instructions.

Histopathological analysis

The left ear tissues of the rats were paraffin-embedded, sectioned, dewaxed, and stained with hematoxylin and eosin (HE) to detect histopathological conditions. Additionally, the slides were stained with Masson staining using the modified Masson trichrome staining kit (G1346, Solarbio, China) following the manufacturer's instructions. A light microscope was used to observe and photograph.

Tissue immunofluorescence

Dewaxed rat ear tissue sections were placed in 0.01 M citrate buffer (121 °C, 20 min) then were put in 1% bovine serum albumin (BSA) for 20 min. Thereafter, the sections were incubated with rabbit anti-rat Ki67 (ab16667, Abcam) antibodies for 16 h at 4 °C and Alexa Fluor 488-coupled secondary antibodies (ab150077, Abcam) for 1 h at room temperature. Slides were then restained with 1 µg/mL Hoechst 33,342 dye and observed and photographed with the fluorescence microscope (DM500, Leica). The fluorescence intensity was quantified by Image-Pro Plus 6.0 (IPP 6.0) software, and the Ki67 positivity rate was counted in three different fields of view.

Immunohistochemistry (IHC)

Dewaxed rat ear tissue sections were placed in 0.01 M citrate buffer at 121 °C for 20 min, and 1% BSA for 20 min. Thereafter, the sections were combined with rabbit anti-rat keratin 7 (KRT7; ab181598, Abcam) and vimentin (ab92547, Abcam) antibodies separately for 16 h at 4 °C. After that, the sections were combined with goat anti-rabbit IgG-HRP (ab6721, Abcam) secondary antibody for 1 h. DAB and hematoxylin was used to stain the cells. The sections were photographed by optical microscope, and the positive chromogenic optical density values were quantified by IPP 6.0 software in three different fields of view.

Western blot

Rat ear tissue was homogenized by incubating with RIPA buffer for 5–10 min on ice. The protein lysates were boiled and denatured, separated by SDS-PAGE, and transferred to PVDF membranes, then incubated with 5% nonfat dry milk. Thereafter, the membranes were combined with primary antibodies (1:1000) at room temperature. Different rabbit anti-rat primary antibodies purchased from Abcam were used for western blotting: extracellular regulated protein kinases (ERK, ab184699); phosphorylated (*p*)-ERK (ab76299); c-Jun N-terminal kinase (JNK, ab179461); *p*-JNK (ab76572); p38 alpha/MAPK14 (p38, ab170099); *p*-p38 (ab4822); vimentin (ab92547); KRT7

(ab181598); patatin-like phospholipase domain containing 2 (PNPLA2, ab109251); lipoprotein lipase (LPL, ab91606); TLR2 (ab209217); nuclear factor kappa-B p65 subunit (NF-κB-p65, ab16502); *p*-NF-κB-p65 (ab76302); NF-kappa-B inhibitor alpha (IκBα, ab32518); and *p*-IκBα (ab92700). Thereafter, the membranes were combined with goat anti-rabbit IgG-HRP antibody (1:2000) for 2 h. ECL chemiluminescence substrate was added to emit light, and imaging was performed using the ChemiDoc-It Imaging System. The β-actin was used as the internal reference protein. The optical density values of protein bands were quantified and statistically analyzed using Image J software.

Transcriptome analysis

The left ear tissues of control, model, and Via^{Hi} rats were subjected to transcriptome sequencing analysis. GO, KEGG, and Reactome enrichment analyses were performed for differentially expressed genes (DEGs) associated with TLR gene expression.

Molecular simulation docking

Viaminate structure was from Pubchem (<https://pubchem.ncbi.nlm.nih.gov/>), and the 3D structural model of TLR2 was from PDB (<https://www.rcsb.org/>). AutoDockTools-1.5.6 software was used to calculate the potential molecular force of viaminate and TLR2 and determine the binding site, which was visualized using PyMOL V.2.3 software.

qPCR detection

Total RNA was extracted from 50 mg rat left ear tissue by the TRIzol, and the mRNA was reverse transcribed into cDNA with cDNA Synthesis Kit (Novoprotein, China). And the qPCR methods were according to SYBR qPCR SuperMix Plus kit (Novoprotein) and 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Inc.): 95 °C, 2 min, 95 °C for 10 s, annealing at 60 °C for 10 s; and extension at 72 °C for 30 s (40 cycles). The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method by using β-actin as an internal reference (Rao et al. 2013). The sequences of the primers are as follows: Human-*TLR2* Forward: 5'-CTT CAC TCA GGA GCA GCA AGC A-3', Human-*TLR2* Reverse: 5'-GCT TGG TGG TTT GCT ACG AC-3'; Human-*β-actin* Forward: 5'-GAG GGC AAG ATG GTG TCG GAT A-3', Human-*β-actin* Reverse: 5'-CTT CTC AGC CAG GTG TTC CAA G-3'; Rat-*TLR2* Forward: 5'-TGG AGG TCT CCA GGT CAA ATC-3', Rat-*TLR2* Reverse: 5'-ACC AGC AGC ATC ACA TGA CA-3'; and Rat-*β-actin* Forward: 5'-CAC CCG CGA GTA CAA CCT T-3'; Rat-*β-actin* Reverse: 5'-ATA CCC ACC ATC ACA CCC TGG-3'.

TG analysis

Approximately 0.1 g of left ear tissue was mixed with normal saline (1:9, m/v), homogenized in a tissue grinder, and centrifugation at 3000 g for 10 min in a refrigerated centrifuge. Subsequently, tissue TG levels were detected according to the kit's instructions (BC0625, Solarbio, China).

Detection of cell proliferation activity

Propionibacterium acnes (8.0×10^7 CFU/20 μ L) was heated at 80 °C for 30 min to get heat-killed *P. acnes* (HKP), which was then freeze-dried and stored at 4 °C. The human keratinocyte cell line, HaCaT (Zhejiang Ruyao Biotechnology Co., Ltd.), was grown in DMEM containing 10% FBS, 1% penicillin–streptomycin, and L-glutamine respectively cultured in a constant temperature incubator (37 °C, 5% CO₂). HaCaT cells were transferred to the 96-well plates (8000 cells/well) and treated with HKP (0, 50, 100, 200, 400, and 800 μ g/mL) or viamine (0, 62.5, 125, 250, 500, 1000, 2000, and 4000 nmol/L) for 48 h. The proliferation activity of HaCaT cells was detected by MTT assay (Wang et al. 2011). Additionally, 200 μ g/mL HKP was combined with 0.1, 0.5, and 1 μ mol/L viamine, and the influence of viamine on the HKP-induced proliferation of HaCaT cells was performed to observe with MTT assay.

In addition, the protein of Ki67 was detected by western blot after incubation of 200 μ g/mL HKP with viamine (0.1, 0.5, and 1 μ mol/L) for 48 h. The optical density values of the protein bands were quantified with Image J software.

Cell immunofluorescence

HaCaT cells were added into 24-well plates. After treatment, the coverslips were obtained and the cells were treated with 4% paraformaldehyde for about 15 min. After that, HaCaT cells were treated with 0.5% Triton X-100 for 10 min and blocked with 1% BSA for 30 min. Thereafter, the HaCaT cells were incubated overnight with the primary antibodies (vimentin and KRT7) at 4 °C then incubated with FITC-conjugated goat anti-rabbit secondary antibody for 1 h in dark condition. HaCaT cells were combined with DAPI for 10 min. A fluorescence microscope (Leica, Germany) was used to get images, and fluorescence staining intensity was quantified by IPP 6.0.

Cell transfection

The pCMV6-TLR2 (NM_003264; RC207597) and pCMV6-Entry control (PS100001) plasmids were from Origene. The plasmids were transiently transfected into HaCaT cells

(60–80% cell confluency before transfection) with the cationic lipid (X-TremeGene HP DNA transfection reagent, Roche). About 48 h of incubation, some cells were collected for qPCR detection of transfection efficiency.

Responsive experiment

The cells were divided into 4 groups: group 1 (control group); group 2 (model group): HaCaT cells were incubated with 200 μ g/mL HKP for 48 h; group 3 (Via group): HaCaT cells were incubated with 200 μ g/mL HKP and 1 μ M viamine for 48 h; and group 4 (TLR2 group): HaCaT cells (transfected with pCMV6-TLR2 plasmid) were incubated with 200 μ g/mL HKP and 1 μ M viamine for 48 h. After 48 h of incubation, cell proliferation and keratinization levels were tested by MTT, immunofluorescence, and western blotting.

Statistical analysis

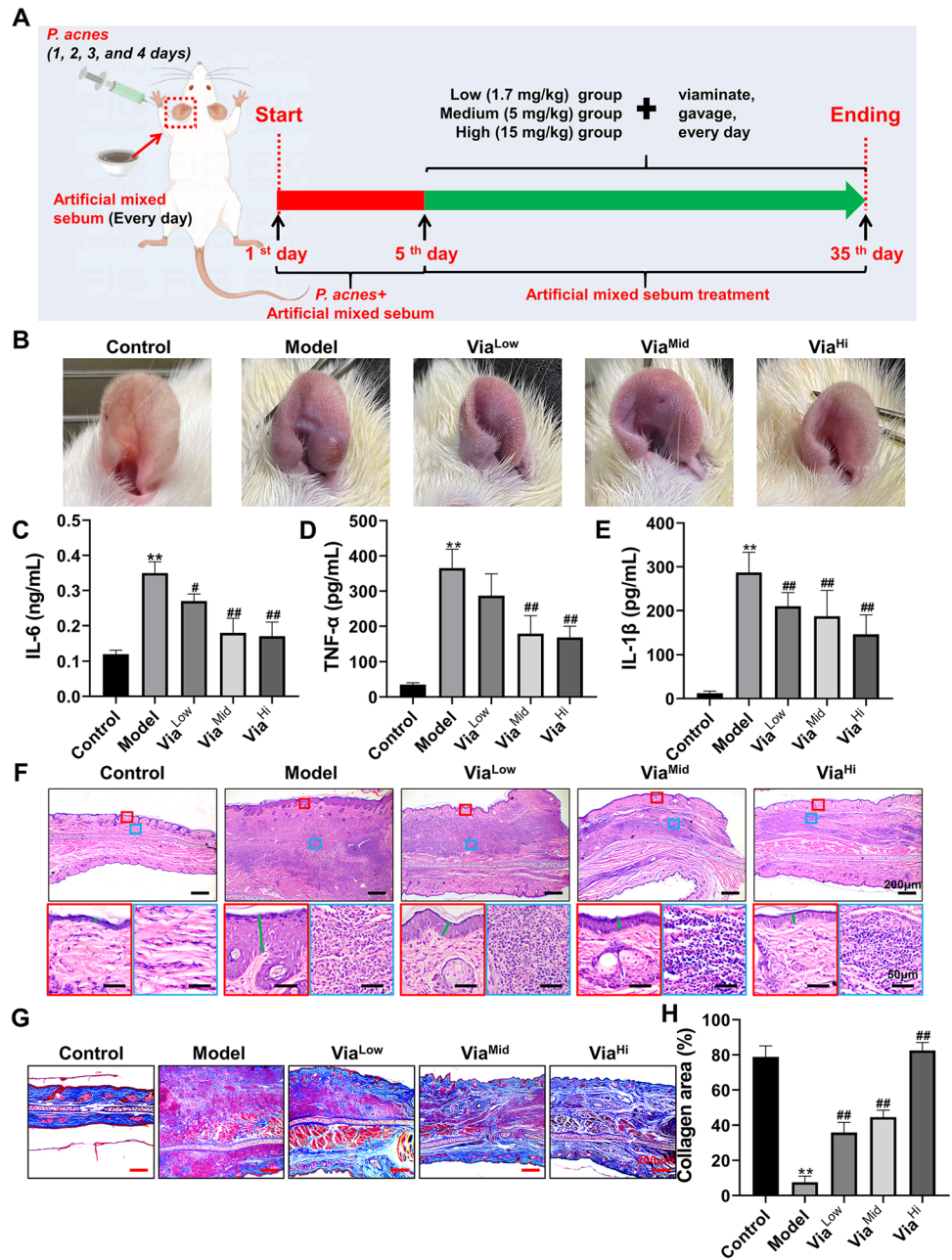
All data are presented as means \pm standard deviation based on three replicates. The results were calculated using GraphPad Prism 8 (GraphPad Software, Inc.). T-tests were used for comparisons between two groups, and $P < 0.05$ was considered statistically significant.

Results

Viamine can protect against *P. acnes*-induced inflammatory damage of ear skin in rat acne model

The skin surface of the rats was observed to examine the therapeutic effect of viamine on *P. acnes*-induced acne in rats. Figure 1A shows the flow chart of animal experimental modeling and treatment. The ear edema was prominent and the skin surface was rough in the rats of the acne model group; however, the ear edema improved post-treatment, with the Via^{Hi} group showing the best results (Fig. 1B). Subsequently, the inflammatory factors in the tissues showed that IL-1 β , IL-6, and TNF- α in the Via^{Med} and Via^{Hi} groups were evidently lower than the model group ($P < 0.01$; Fig. 1C–E). Pathological HE staining (Fig. 1F) revealed that in the model group, the structure of the skin epidermis (shown in the red box) and the thickness of the entire ear were increased, accompanied by much inflammatory cell infiltration (shown in the blue box). In contrast, the structure of the skin epidermis and the thickness of the entire ear were reduced and the structure of the sebaceous glands was normal in the viamine-treated group. Additionally, Masson staining results (Fig. 1G, H) showed the area of positive collagen staining in the ear post-viamine treatment was evidently higher than that in the model group, and a large area of collagen was lost in the model group.

Fig. 1 Viamineate reduces *Propionibacterium acnes*-induced inflammatory damage of ear skin in rat acne model. **A** Flow chart of rat acne model treatment experiments; **B** changes in the skin surface of the rat ear; **C–E** enzyme-linked immunosorbent assay to detect the secretion levels of IL-6, TNF- α , and IL-1 β in rat ear skin tissue; **F** HE staining to detect the pathological changes in rat ear skin tissue (red and blue boxes represent the enlarged epidermis and enlarged inflammatory cells, respectively); **G** Masson staining to detect changes in the tissue collagen levels; and **H** the proportion of Masson-stained collagen area (represented by blue). ** $P < 0.01$, compared with the control group and ### $P < 0.01$ and # $P < 0.05$, compared with the model group. Scale bar is 50 or 200 μm



Viamineate can inhibit the abnormal proliferation of epidermal tissue in rat acne model

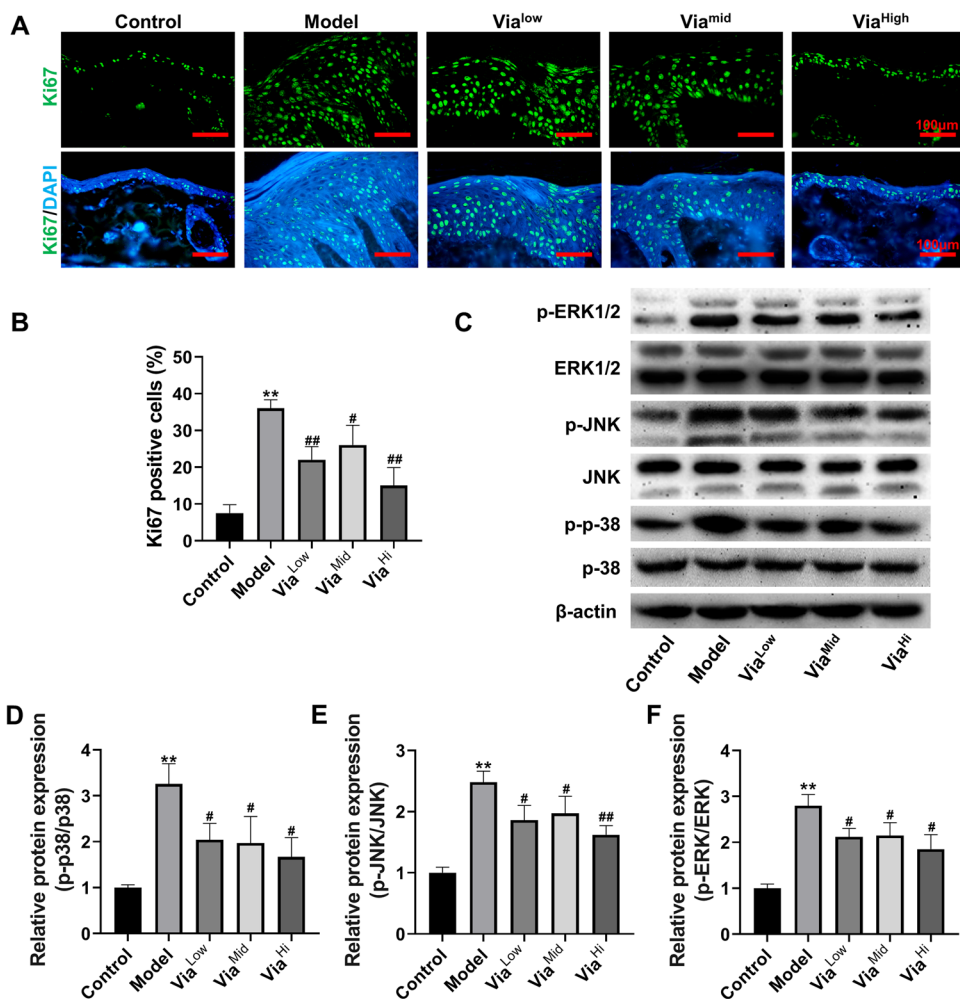
Acne is usually accompanied by abnormal proliferation of the epidermal cells. To determine the influence of viamineate on epidermal cell proliferation, Ki67, a cell proliferation-associated protein was detected by tissue immunofluorescence (Fig. 2A). It showed that the Ki67-positive rate was $36 \pm 2.3\%$ in the model group, while that in the Via groups were significantly lower ($22 \pm 3.6\%$, $26 \pm 5.4\%$, and $15 \pm 4.9\%$ in the Via^{Low}, Via^{Med}, and Via^{Hi} groups, respectively; $P < 0.05$; Fig. 2B). Additionally, western

blotting (Fig. 2C–F) results indicate that compared with the model group, gradually doses of viamineate markedly inhibited the phosphorylation level of p38/JNK/ERK proteins ($P < 0.05$).

Viamineate inhibits the epidermal keratinization in rat acne model

Excessive keratinization of the skin epidermis is also an important factor in acne progression; therefore, analysis of skin epidermis keratinization will facilitate in evaluating acne progression. Immunohistochemistry (Fig. 3A–D)

Fig. 2 Viaminate can inhibit abnormal proliferation of epidermal tissue in rat acne model. **A, B** Tissue immunofluorescence detection of Ki67 expression and statistical quantification of Ki67-positive rate; **C** western blot detection of the expression levels of p38/extracellular regulated kinase (ERK)/c-Jun N-terminal kinase (JNK) and its phosphorylated proteins; and **D–F** statistical quantification of western blot band optical density values. $**P < 0.01$, compared with the control group, and $###P < 0.01$ and $\#P < 0.05$, compared with the model group. Scale bar is 100 μm



and western blotting results (Fig. 3E–G) showed that the cell keratin-associated proteins, vimentin and KRT7, were substantially upregulated in the model group ($P < 0.05$) compared with the control group. However, vimentin and KRT7 levels were evidently lower in the epidermal tissues of the acne-prone rats in the Via^{Med} and Via^{Hi} groups ($P < 0.05$) compared with the model group.

Transcriptome analysis of the viaminate-treated rat acne model

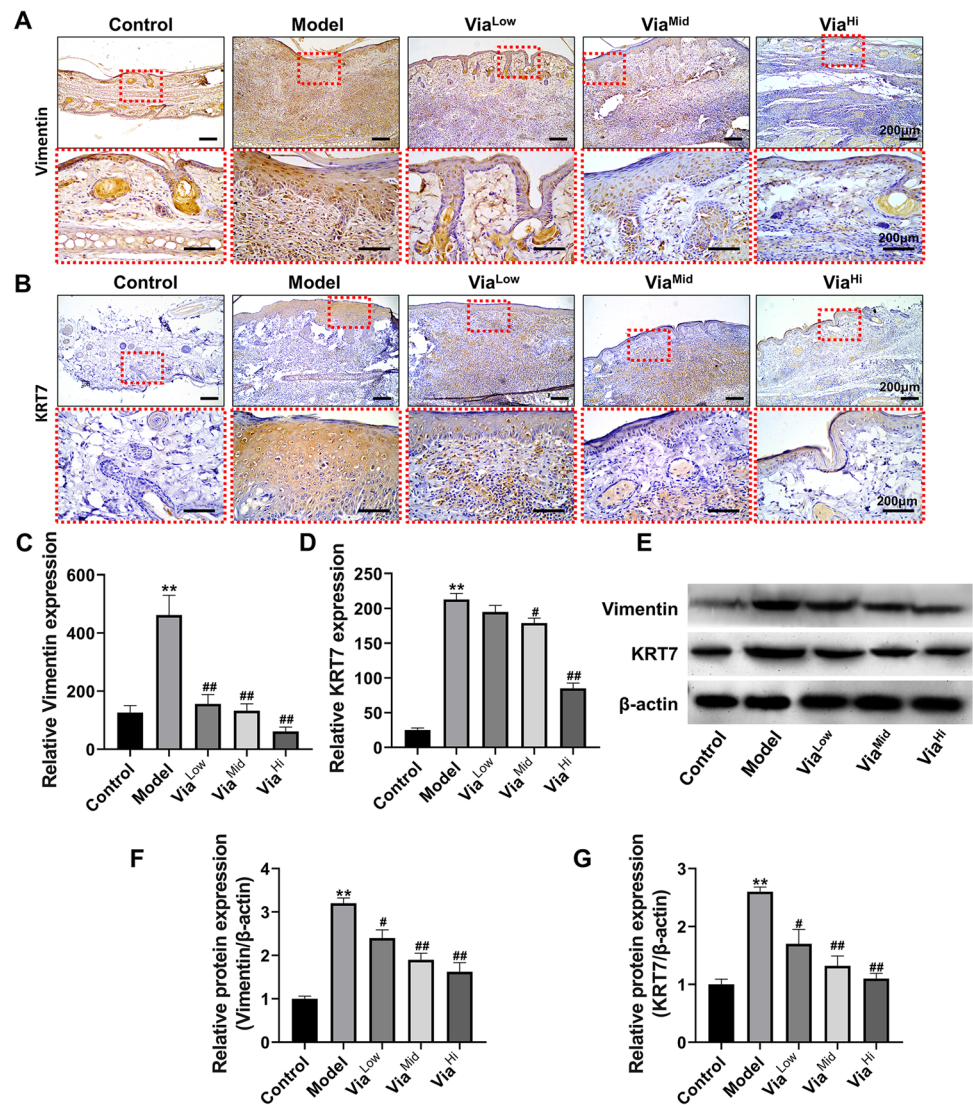
Although viaminate is effective in the treatment of acne, its specific therapeutic mechanism remains to be elucidated. Therefore, we analyzed the ear tissues of the normal, model, and viaminate-treated rats to explain the therapeutic mechanism of viaminate with transcriptome analysis. As seen in Fig. 4A, B, compared with the control group, 3581 and 1813 genes were evidently downregulated in the skin tissue of the model group. Additionally, compared with the model group, Via^{Hi} upregulated 2401 genes and downregulated 862 genes. Figure 4C is a heatmap of gene expression. KEGG

(Fig. 4D) and Reactome (Fig. 4E) enrichment analyses were performed on the genes that were evidently inhibited in the viaminate group and significantly upregulated in the model group. The results suggested that post-viaminate treatment, the genes were significantly enriched during the regulation of B lymphocyte activation, fatty acid metabolism, and cell keratin pathways. Transcriptome analysis revealed that the TLR2 gene was most significantly upregulated in the model group (Fig. 4F); however, TLR2 expression decreased after viaminate treatment. Furthermore, molecular docking simulations suggest a possible binding between viaminate and TLR2, with the binding energy of -9.6 kcal/mol (Fig. 4G).

Viaminate reduces oil accumulation in the ear skin tissue of rat acne model

Transcriptome data showed that viaminate affected sebum synthesis in the ears of acne model rats. Transcriptome results indicated (Fig. 5A) that, in comparison with the control group, the TG levels degradation-associated genes, PNPLA2 and LPL, were decreased in the model

Fig. 3 Viaminate inhibits the level of epidermal keratinization in rat acne model. **A, B** Immunohistochemical (IHC) detection of vimentin and keratin 7 (KRT7) protein expression levels; **C, D** statistical quantification of IHC results; **E** western blot detection of vimentin and keratin 7 (KRT7) protein expression levels; and **F, G** statistical quantification of western blot band optical density values. $**P < 0.01$, compared with the control group and $##P < 0.01$ and $\#P < 0.05$, compared with the model group. Scale bar is 200 μm



group but increased in the Via^{Hi} group. These results were also confirmed by western blotting assay (Fig. 5B–D). Analysis of TG content in rat ear tissues (Fig. 5E) showed that the TG content increased significantly in the model group, while it was in a dose-dependent decline in the viaminate-treated group ($P < 0.05$).

Viaminate inhibits *P. acnes*-induced abnormal proliferation and keratinization of HaCaT cells

We conducted MTT assays to consider the influence of viaminate on the proliferation and keratinization of human immortalized keratinocytes. MTT assays (Fig. 6A–C) revealed that 200 $\mu\text{g}/\text{mL}$ HKP had a significant promoting effect on HaCat cell viability ($P < 0.05$), compared with the 0 $\mu\text{g}/\text{mL}$ group, while viaminate from 0 to 1000 nM did not have any significant cytotoxic effect on HaCat cells

($P > 0.05$). However, treatment with 0.5 and 1 μM viaminate significantly inhibited the promoting effect of HKP on HaCaT cells ($P < 0.05$), in comparison with the model group. The western blotting results of Ki67 showed that (Fig. 6D) different doses of viaminate prominently inhibited the HKP-induced proliferation of HaCaT cells ($P < 0.05$), compared with the model group. Additionally, immunofluorescence assay of vimentin and KRT7 indicated (Fig. 6E, F) that viaminate inhibited the HKP-induced expression of keratinization-associated proteins of HaCaT cells ($P < 0.05$).

TLR2 overexpression attenuates the inhibitory effect of viaminate on HaCaT cell proliferation and keratinization

qPCR (Fig. 7A) confirmed that 0.1, 0.5, and 1 μM viaminate had a prominent inhibitory influence on TLR2 gene

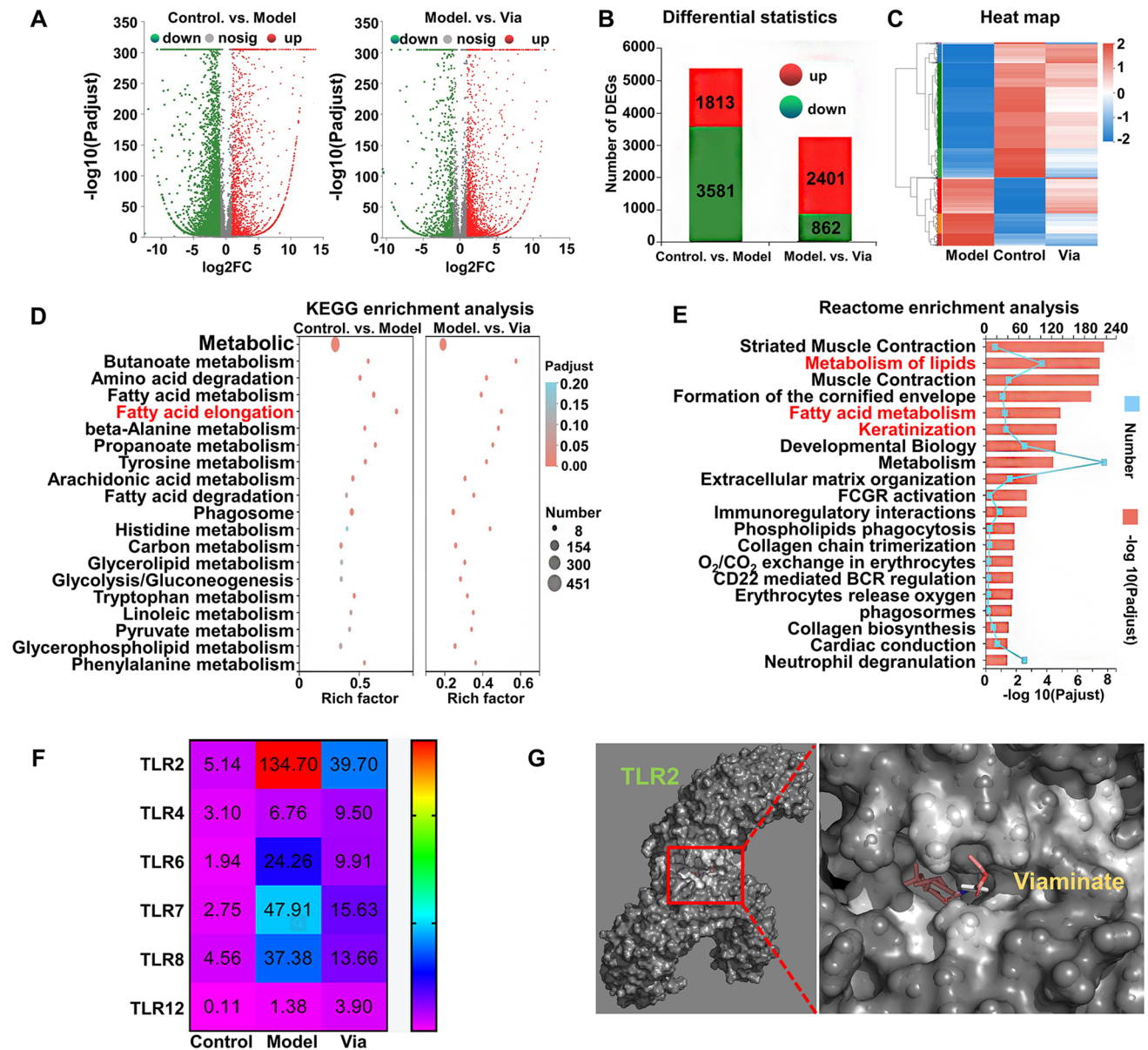
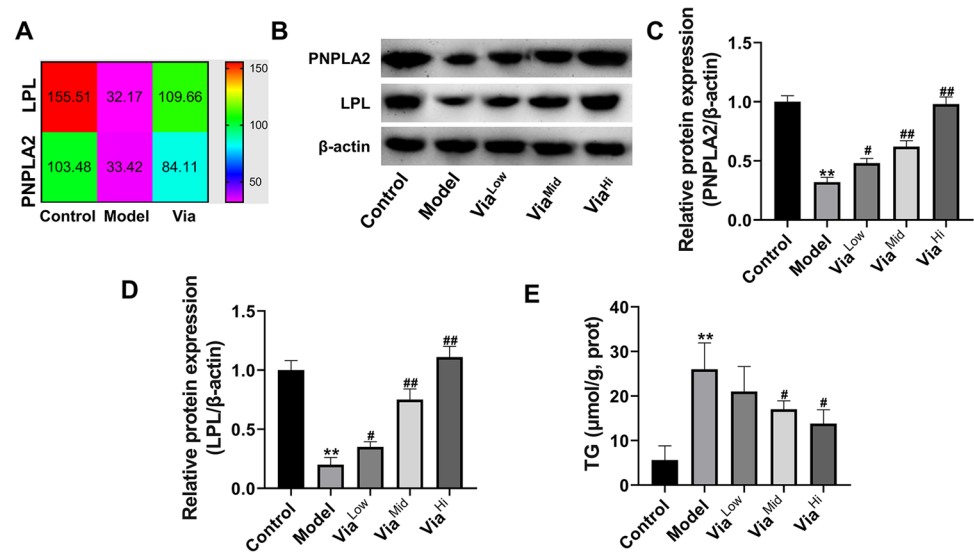


Fig. 4 Transcriptome analysis of the viaminate-treated rat acne model. **A** The left image shows the volcano plot of differentially expressed genes (DEGs) compared with the model group. The right image shows the volcano plot of DEGs compared with the viaminate-treated (via) group; **B** histograms of the DEGs in the volcano plot; **C** heatmap comparing the DEGs of the three groups; **D** KEGG enrichment analysis results; **E** Reactome enrichment analysis results; **F** differential expression levels of genes associated with the toll-like receptor (TLR) family in the transcriptome data; the results are presented as heatmap; and **G** docking of viaminate and TLR2 protein structures visualized by PyMOL software

expression ($P < 0.01$), compared with the model group. To prove that viaminate exerts inhibitory effects on HaCaT cell hyperproliferation and keratinization via TLR2, the TLR2 expression plasmid was transfected into the HaCaT cells. qPCR results indicated that the TLR2 gene level was prominently increased in the pCMV6-TLR2-transfected HaCaT cells ($P < 0.01$; Fig. 7B), and TLR2 transfection reversed the inhibitory effect of viaminate on the TLR2 gene significantly

($P < 0.01$; Fig. 7C, compared with the Via group). The cell growth curve was tested by MTT assay, and the results suggested that viaminate could attenuate HKP-induced HaCaT proliferation, while overexpression of TLR2 could effectively reverse the effect of viaminate (Fig. 7D). Western blotting method was used to verify the $\text{I}\kappa\text{B}/\text{NF-}\kappa\text{B}$ p65 and MAPK p38/JNK/ERK pathways downstream of TLR2, and the results showed that overexpression of TLR2 could

Fig. 5 Viaminate reduces oil accumulation in the ear skin tissue of rat acne model. **A** relative expression levels of the TG degradation-related genes, patatin-like phospholipase domain containing 2 (PNPLA2) and lipoprotein lipase (LPL), in the transcriptome data; the results are presented as heat-map; **B** western blot detection of PNPLA2 and COX-2 protein expression levels; **C–D** statistical quantification of western blot band optical density values; **E** Triglyceride (TG) content in the rat ear skin tissue; $**P < 0.01$, compared with the control group, and $\#P < 0.05$, compared with the model group



significantly reverse the inhibitory effect of viaminate on $\text{I}\kappa\text{B}/\text{NF-}\kappa\text{B}$ p65 and MAPK p38/JNK/ERK pathway protein phosphorylation ($P < 0.05$; Fig. 7E, F), compared with the Via group. Immunofluorescence results indicated (Fig. 7G, H) that with the inhibition of the MAPK p38/JNK/ERK pathway, the secretion levels of vimentin and KRT7 proteins in HaCaT cells were inhibited post-viaminate treatment; however, vimentin and KRT7 were expressed after TLR2 overexpression. Figure 8 shows the therapeutic mechanism of visticamic acid on a rat model of acne.

Discussion

To study the specific treatment mechanism of viaminate in vivo, we established a rat model of acne, similar to human acne lesions, using *P. acnes*. Within 3 days of *P. acnes* inoculation, the ears of the acne model rats were swollen and red (Kolar et al. 2019). However, viaminate intervention treatment for 30 days significantly relieved the inflammatory symptoms in the ears of the acne model rats, reduced the proliferation of keratinocytes, the symptoms of hyperkeratosis, and the number of infiltrating inflammatory cells in skin lesions. Although research on the applications of viaminate in animal acne models is lacking, the anti-hyperkeratotic and anti-inflammatory functions of viaminate have been confirmed in clinical acne treatments (Ma et al. 2021; Nabatanzi et al. 2020); however, its mechanism of action remains unclear. Therefore, we determined a specific regulatory mechanism of viaminate using the transcriptome data of acne model rats. Considering the importance of the TLR receptor family in acne disease progression, we focused on the regulation of TLR members in transcriptome data. Although many TLRs are significantly regulated

by viaminate, the TLR2 gene had the highest background expression level in the model group. Based on this, it was speculated that TLR2 may be associated with acne progression and thus, targeted by viaminate. In addition, viaminate targets were also predicted by PyMOL molecular simulation docking software, and the results suggested a potential binding between TLR2 and viaminate.

During acne progression, activation of TLR2 in skin cells can trigger innate immunity that promotes inflammatory lesions of acne (Köllisch et al. 2005). In vitro, *P. acnes* stimulates keratinocytes and monocytes to activate TLR2, resulting in improving the secretion of proinflammatory cytokines (IL-1 β , IL-6, TNF- α , etc.) (Graham et al. 2004; Grange et al. 2009). In animal experiments, TLR2 gene was upregulated together with NF- κ B in acne lesions, suggesting that *P. acnes* activates TLR2 (Hwang et al. 2020). Coralie et al. revealed that TLR-2 could recognize *P. acnes* CAMP factor 1 (Lheure et al. 2016), and in this study, TLR2/NF- κ B pathway proteins were also observed to be activated in the ear skin tissue of acne model rats. The TLR2/NF- κ B pathway is primarily responsible for the regulation of inflammatory factors, such as IL-6, TNF- α , and IL-1 β (Balkrishna et al. 2020; Franz et al. 2022). ELISA and western blotting results indicated that viaminate prominently inhibited the phosphorylation levels of the inflammatory factors, $\text{I}\kappa\text{B}\alpha$ and NF- κ B p65. However, TLR2 overexpression could restore the effect of viaminate, indicating that viaminate could exert an anti-inflammatory effect through the TLR2/NF- κ B pathway.

Notably, the results of KEGG and Reactome enrichment analyses of DEGs indicate that viaminate may act a major role to regulate abnormal cellular keratinization and fatty acid metabolism in acne treatment. In keratinocytes, bacterial lipopeptide-mediated activation of TLR2 can trigger the

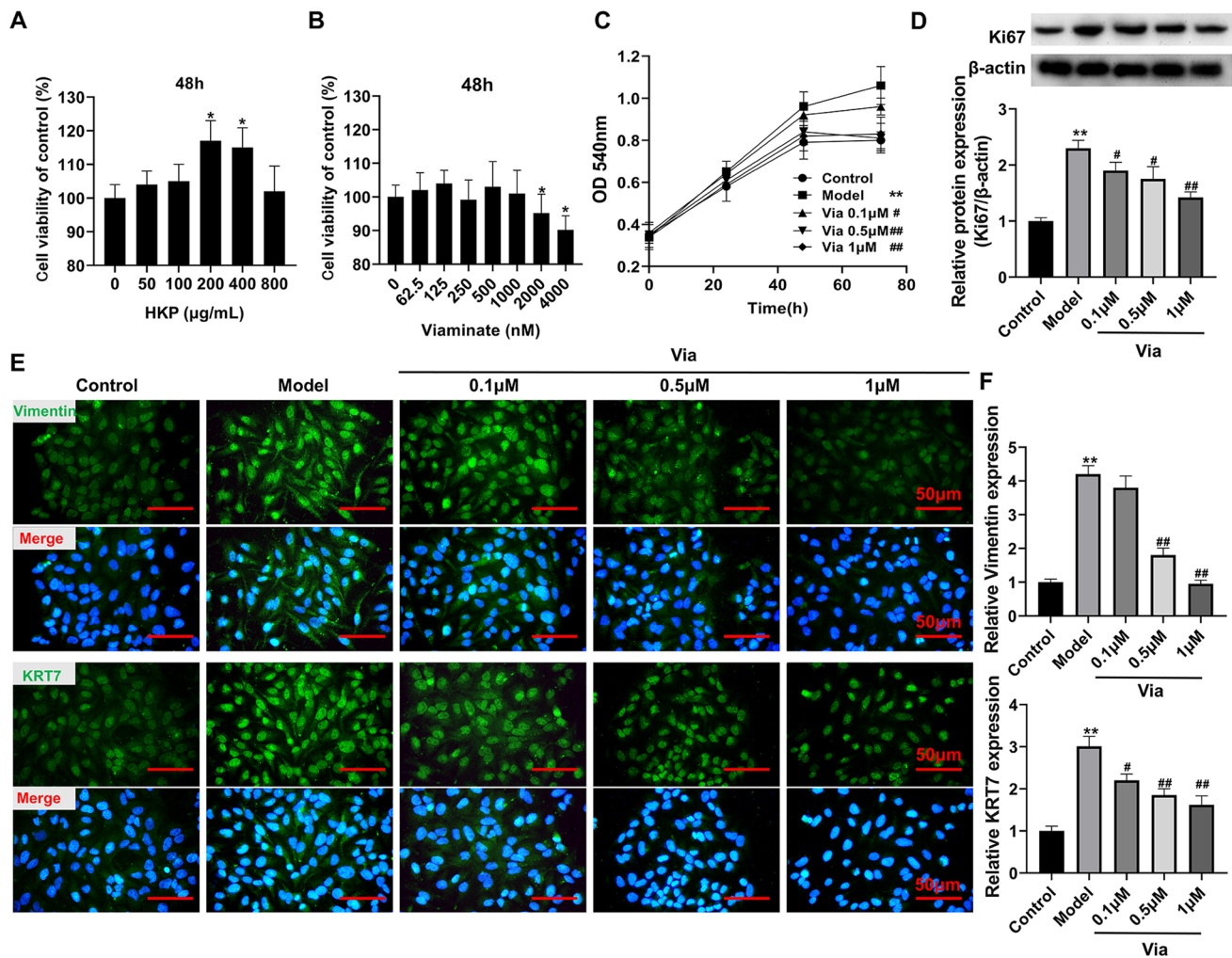


Fig. 6 Viamine inhibits *Propionibacterium acnes* (*P. acnes*)-induced abnormal proliferation and keratinization of HaCaT cells. **A–C** MTT assay was used to screen the concentrations of heat-killed *P. acnes* (HKP) and viamine and to determine the regulatory effect of viamine on the HKP-stimulated activity of HaCaT cells; **D** western blotting to detect Ki67 protein expression and quantification of the

optical density values; and **E, F** immunofluorescence assay to detect the expression and localization of vimentin and keratin 7 (KRT7) proteins in the cells. ** $P < 0.01$, compared with the control group, and ### $P < 0.01$ and # $P < 0.05$, compared with the model group. Scale bar is 50 µm

production of proinflammatory cytokines and enhance the tight junction barrier function of the epidermis upon pathogen invasion simultaneously, even enhances the level of cytokeratinization (Meisgen et al. 2014; Yuki et al. 2011). Moreover, a positive correlation between keratinocyte keratinization levels and TLR2 is often observed in acne disorders (Zhu et al. 2019).

Propionibacterium acnes-activated TLR2 further activates the MAPK p38 pathway in addition to affecting the NF-κB pathway (Huang et al. 2015). MAPK family members, such as JNK and MAPK p38, activate the MAPK p38/JNK/ERK pathway to promote keratinocyte proliferation (Kim et al. 2018). Simultaneously, the above pathway can also promote the formation of cytoke- ratin and reduce the decomposition of the keratin filament network

(Wöll et al. 2007). Inhibition of MAPK p38 reduces the secretion of keratinization-related proteins from the epidermal keratinocytes (Cursons et al. 2015; Jonak et al. 2011; Meng et al. 2018; Usuki et al. 2022). Therefore, it is speculated that the inhibition of epidermal hyperkeratosis by viamine via TLR2 under acne conditions is caused by MAPK p38. The in vitro and in vivo data of our study indicated that *P. acnes* activated the MAPK p38/JNK/ERK pathway, while overexpression of TLR2 prominently reversed the active repressions of viamine on the MAPK p38/JNK/ERK pathway and promoted HaCaT cell proliferation. Secreted keratinization-related proteins, vimentin and KRT7. Vimentin stabilizes the keratin filaments and prevents keratin degradation (Keeling and Gavara 2020). Additionally, western blotting and

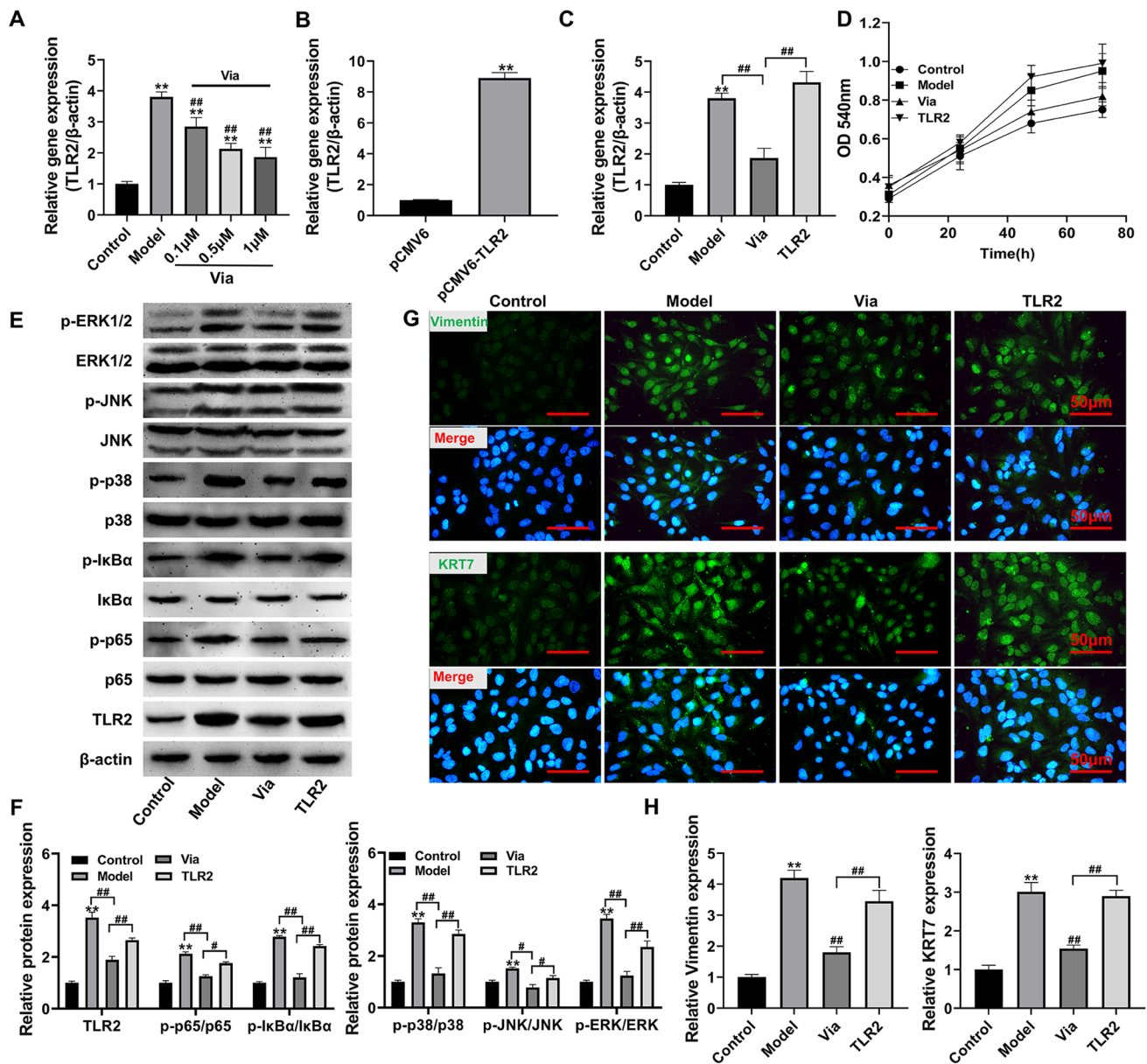


Fig. 7 Toll-like receptor 2 (TLR2) overexpression attenuates the inhibitory effect of viamine on HaCaT cell proliferation and keratinization. **A–C** qPCR detection of TLR2 gene expression level; **D** MTT detection of cell viability; **E** western blot detection of nuclear factor-kappa B (NF-κB) inhibitor alpha IκBα/NF-κB p65 and mitogen-activated protein kinases (MAPKs) p38/JNK/ERK pathway

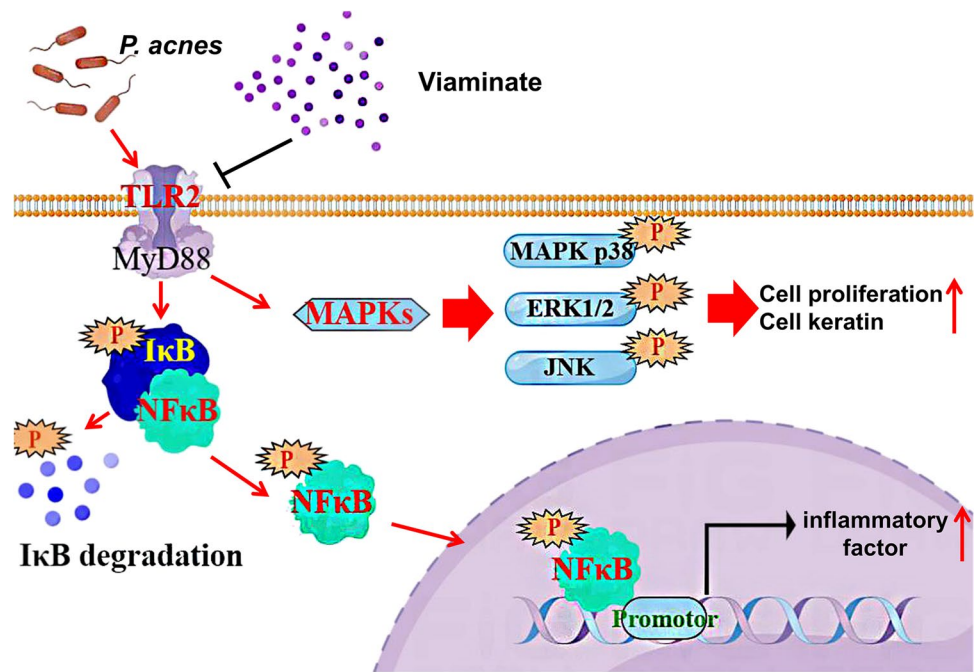
protein expression levels; **F** statistical quantification of western blot band optical density values; and **G, H** immunofluorescence detection of vimentin and KRT7 protein expression and subcellular localization. ** $P < 0.01$ and * $P < 0.05$, compared with the control group, and ### $P < 0.05$ and # $P < 0.05$, compared with the model group. Scale bar is 50 μm

Ki67 tissue immunofluorescence assays revealed that viamine inhibits MAPK p38/JNK/ERK1/2 activation and Ki67 expression. These results demonstrate that viamine can simultaneously inhibit the NF-κB and MAPK p38 pathways via TLR2, exert anti-inflammatory effects, and inhibit keratinocytes proliferation and keratinization.

In addition to the abnormal proliferation and hyperkeratinization of keratinocytes, acne formation is also associated with skin oil secretion disorders. *P. acnes* can activate the

TLR2 pathway in sebocytes, thereby mediating inflammation and simultaneously upregulating the level of adipokines (Kovács et al. 2020; Töröcsik et al. 2018). Combined with the transcriptome data in our research results, it was suggested that viamine treatment significantly inhibited the activity of the fatty acid metabolism pathway. The results also confirmed that viamine can reduce oil accumulation and TG synthesis and simultaneously upregulate the PNPLA2 and LPL levels, which are associated with TG

Fig. 8 Acne treatment mechanism of viaminate in rat acne model. In acne models, viaminate exerts an anti-inflammatory effect by inhibiting the toll-like receptor 2 (TLR2)/NF- κ B pathway. Additionally, viaminate inhibits keratinocyte proliferation and hyperkeratinization via the TLR2/mitogen-activated protein kinases (MAPKs) [MAPK p38/JNK/ERK 1/2] pathway



degradation (Jha et al. 2014; C. Liu et al. 2018). Therefore, we hypothesized that inhibition of TLR2 by viaminate can reduce the level of fatty acid metabolism. MAPK p38 is reported to be an adipogenesis signaling molecule, and specific p38 inhibitors block adipogenesis and the expression of fatty acid synthases (Ji et al. 2015). Blockade of MAPK p38 reduces the induction of lipid accumulation in human monocytes by TRL agonists (Lopez et al. 2013). Simultaneous activation of p38 MAPK/JNK signaling and NF- κ B signaling in human sebocytes can be induced by thermal radiation, thereby upregulating inflammation and adipogenesis (Kwon et al. 2019). Accordingly, it is believed that viaminate may inhibit excessive sebum secretion in acne by inhibiting the TLR2/MAPK p38 pathway. However, since there is no suitable source of sebocytes in China, verification studies have not been performed at the sebocyte level. Therefore, the regulatory influence of viaminate on the MAPK p38 pathway been confirmed only in animal experiments and HaCaT cells. There are limited experimental studies on fatty acid metabolism. In this study, the observed phenomenon is explained only by the characteristics of the TLR2/MAPK p38 pathway. Further research on fatty acid metabolism in sebaceous gland cells is required to deepen our cognition of the therapeutic effect of viaminate on acne treatment.

This study has the following limitations. Firstly, the data in this study are based on transcriptome analysis of rat ear tissues, and the regulatory mechanisms analyzed based on this may lack specificity. This is due to the presence of many complex cell populations in the ear tissue, such as epidermal keratinocytes, fibroblasts, sebaceous gland cells, and various lymphocytes. However, this study primarily focuses on the

mechanism of epidermal keratinocytes. Thus, the mechanistic studies of viaminate may have only uncovered the tip of the iceberg. In addition, transcriptome data revealed that viaminate has a regulatory effect on B lymphocyte recruitment, indicating that viaminate has a definite function on the chemotaxis of B lymphocytes. However, our lab is currently not equipped to carry out further research in this regard, and this will be pursued in the future research.

Summarily, this research verified the therapeutic ability of viaminate on *P. acnes*-induced epidermal inflammation, abnormal fatty acid metabolism, and abnormal proliferation and keratinization of epidermal cells in acne model rats. Additionally, transcriptome and experimental data proved that viaminate may play a role in regulating TLR2 and its downstream pathways, NF- κ B and MAPK p38. Furthermore, in vitro experiments using HaCaT cells proved that viaminate inhibits the excessive proliferation and keratinization of HaCaT cells via the TLR2/MAPK p38/JNK/ERK pathway. This study conducted a specific mechanism of viaminate in the treatment of acne and effectively filled the gap in the understanding its therapeutic mechanism in acne treatment.

Author contribution JC and SX contributed to the study conception and design. All authors collected the data and performed the data analysis. All authors contributed to the interpretation of the data and the completion of figures and tables. All authors contributed to the drafting of the article and final approval of the submitted version. The authors declare that all data were generated in-house and that no paper mill was used.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval Ethical approval was given by the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University (No.2022–1469).

Competing interests The authors declare no competing interests.

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