Role and mechanism of matrine alone and combined with acitretin for HaCaT cells and psoriasis-like murine models

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Abstract

Background: Acitretin and matrine have been used in the treatment of psoriasis in China. This study was designed to investigate the role and related mechanisms of matrine alone and in combination with acitretin in the treatment of psoriasis *in vitro* and *in vivo*.

Methods: HaCaT cells were treated with matrine at different concentrations of 0 (blank control), 0.2, 0.4, 0.8, and 1.6 mg/mL for 24, 48, 72 h, respectively. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium cell viability assay was used to assess the growth and proliferation of HaCaT cells. Cell cycle and apoptosis were detected by flow cytometry. Expression of protein was detected by Western blotting. Autophagy was observed by transmission electron microscopy. Then HaCaT cells were assigned to normal saline (NS) control group, matrine (0.4 mg/mL) group, acitretin (10 μmol/L) group, and matrine plus acitretin group, and the above methods were repeated. In animal experiments, the cumulative score (erythema, scaling, thickening) as a measure of the severity of inflammation was used to measure the skin performance of mice after treated with matrine 50 mg/kg, acitretin 4.5 mg/kg or combination of the two drugs on the psoriasis-like mouse models, respectively. Pathological findings of the lesions were observed, and the protein expressions in the lesions were detected by immunohistochemistry.

Results: Cell proliferation inhibition was seen in HaCaT cells with treatment of matrine in a dose- and time-dependent manner ($P < 0.01$, respectively). Cell cycle G0/G1 phase arrest was observed in a dose-dependent way ($P < 0.01$). The expression of p21 ($P < 0.05$), LC3II/I ($P < 0.01$), and Beclin 1 ($P < 0.01$) increased and the expression of cyclin D1 ($P < 0.05$) decreased with increasing doses of matrine. Compared with the blank control, more autophagosomes were seen in HaCaT cells treated with matrine at 0.4 mg/mL by transmission electron microscopy (2.667 ± 1.202 *vs*. 21.33 ± 1.453, $t = 9.899$, $P < 0.01$). Cell proliferation inhibition and degree of the G0/G1 phase arrest was significantly higher in matrine plus acitretin group than those in matrine, acitretin, or the NS control group ($P < 0.01$, respectively). Compared with matrine or acitretin group, the expression of p21 ($P < 0.05$, $P < 0.05$) and LC3II/I ($P < 0.01$, $P < 0.05$) in matrine plus acitretin group increased significantly and the expression of cyclin D1 ($P < 0.01$, $P < 0.05$) and p62 ($P < 0.05$, $P < 0.05$) was reduced significantly. Compared with matrine or acitretin, matrine plus acitretin significantly down-regulated the phosphorylation of phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway ($P < 0.05$) and its downstream p-p70S6K ($P < 0.05$). In addition, the cumulative score of mice in the matrine plus acitretin group was significantly better than that in the matrine or acitretin group (1.480 ± 0.230 *vs*. 2.370 ± 0.241, $P < 0.01$; 1.480 ± 0.230 *vs*. 2.888 ± 0.341, $P < 0.01$). The expression of LC3 protein in the matrine plus acitretin group was also higher than that in the matrine, acitretin, or the NS control group ($P < 0.05$, respectively).

Conclusions: Matrine has therapeutic potentials for psoriasis. Matrine and acitretin show synergistic effect *via* cell cycle arrest and autophagy induction by PI3K/Akt/mTOR pathway.

Keywords: Psoriasis; Autophagy; Phosphoinositide 3-kinase; Protein kinase B; Mammalian target of rapamycin

Introduction

Psoriasis is a common, chronic inflammatory skin disease most often appearing in the form of well-demarcated, scaly plaques. These lesions highlight the fundamental processes underlying its pathogenesis, namely, inflammation and epidermal hyper-proliferation. The incidence is high, and the disease is prone to relapse. Its pathogenesis is complex and uncertain, which is thought to be associated with many factors, such as heredity, abnormal proliferation, and differentiation of keratinocytes and immune regulation disorders. The efficacy of traditional psoriasis treatment is not satisfactory. It mainly includes topical medicine, phototherapy, and systematic treatment, such as methotrexate and acitretin. Although the development and application of biological agents based on their cellular immunity mechanism have made rapid progress recently, there is still a lack of more long-term safety data. Acitretin is a mature system drug for the treatment of moderate and severe psoriasis. The treatment mechanism mainly involves the immune response, angiogenesis, inflammatory process,

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and cell hyperplasia of psoriasis, and it is not toxic and immunosuppressive. However, the efficacy of acitretin as a single drug in the treatment of chronic moderate to severe plaque psoriasis is not always good. In the range of therapeutic doses, the larger the dose, the better the efficacy is and the higher the risk of adverse reactions is. It is often used in combination with other methods to achieve better results. When acitretin is combined with other therapies, the dosage can be reduced as appropriate. It can be combined with topical preparations and phototherapy or other immunosuppressive agents. Therefore, it is necessary to investigate better combination treatments.[1,2]

Sophora is a traditional Chinese medicine, the root of the sophora plant, which is used to treat chronic hepatitis B, tumors, inflammation, abscess, diarrhea, and eczema and other itching skin diseases. Matrine and oxymatrine are the main active ingredients. They showed a great deal of pharmacological activities, including anti-inflammatory, anti-allergic, anti-virus, anti-fibrosis, and cardiovascular protective effects.[3,4] Matrine has extensive anti-cancer activities by blocking the cell cycle, accelerating apoptosis, inhibiting angiogenesis to suppress cancer cell proliferation, differentiation, metastasis and invasion, and reverse resistance. Meanwhile, it could prevent or reduce the toxicity of the other types of chemotherapy and radiotherapy when they are combined with chemotherapy drugs.[5] Matrine has been extensively studied for the treatment of viral hepatitis, cancer, cardiac diseases, and some kinds of skin diseases (such as atopic dermatitis and eczema). An increasing number of experiments have proven the importance of matrine in chronic hepatitis,[6] cancers,[7-9] and infectious diseases.[10]

Some studies have shown that it can inhibit cell proliferation and invasion and promote the secretion of anti-inflammatory factors in melanoma and other chronic inflammatory skin diseases in cell lines and animal models.[11-13] At the same time, research of gastric cancer, bladder cancer, and chronic hepatitis showed that combined use of matrine and other drugs have synergy to enhance curative effect, increase drug sensitivity, and reduce side effects.[14-16] It has been suggested that the combination of matrine and acitretin was superior to single-drug therapy in the treatment of psoriasis.[17] This is consistent with our clinical experience. However, little is known about its mechanism of action. Therefore, it is necessary to explore the concrete mechanism of matrine and feasible combination therapies of matrine in psoriasis.

Methods

Cell culture

HaCaT cells, spontaneously immortalized, non-tumorigenic human skin keratinocyte cell lines purchased by Central Laboratory of Peking University Third Hospital, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Eggenstein, Germany) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), 1% penicillin and streptomycin at 37°C in a 5% CO_2 humidified atmosphere.[14]

Reagents

Matrine was purchased from the Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China), and its purity was >99% as assessed by high-performance liquid chromatography. The matrine stock solution was prepared in ddH_2O at 50 mg/mL. Log-phase growing cells were exposed to matrine at concentrations of 0 (negative control), 0.2, 0.4, 0.8, and 1.6 mg/mL.[18] Acitretin (JD20443; Shanghai Jingdu Biotechnology Co., Ltd., Shanghai, China) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, USA) and stored at –20°C until use. It was then diluted to a working concentration of 10 μmol/L. Subsequent dilutions were made in normal culture medium with a final DMSO concentration of 0.05%. The control was supplied with DMSO in a final concentration of 0.05%.[19]

Cell proliferation assays

Cell proliferation was detected using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. Briefly, HaCaT cells were seeded into 96-well microtiter plates (Costar 3596, Corning Inc., Corning, NY, USA) at a density of 2×10^3/well and incubated in complete medium. After 24 h, the HaCaT cells were treated with various concentrations of matrine, acitretin, or matrine plus acitretin, with DMEM as the negative control. When incubated for the indicated times, cells were treated with 20 μL of MTS for an additional 4 h. The absorbance value was measured at 490 nm *via* a 96-well plate spectrometer (Thermo Fisher Scientific, New York, USA). The cell viability = $(A_{\text{experimental group}} - A_{\text{blank group}})/(A_{\text{control group}} - A_{\text{blank group}}) \times 100\%$. Each experiment was executed in triplicate wells, and three biologic replicates were performed.[20]

Detection of cell cycle and apoptosis by flow cytometry

Cell cycle analysis was performed with 7-aminoactinomycin D (7-AAD) by flow cytometry. In short, 2×10^6 transduced and control cells were collected, rinsed, and fixed with cold 70% ethanol. The cells were re-suspended in 300 mL of phosphate-buffered saline (PBS) and stained with 5 mL 7-AAD overnight in –20°C. Cell cycle phases were detected by flow cytometry (BD, Franklin Lakes, NJ, USA). The fluorescence intensity of 7-AAD directly reflected the content of DNA in cells to divide each phase of the cell cycle into G0/G1 phase, S phase, and G2/M phase. Cell apoptosis was assessed by annexin-V-fluorescein isothiocyanate (FITC)/7-AAD staining (BD), according to the manufacturer's instructions. The data were obtained by ModFit and FlowJo software (Verity Software House, ME, USA).[21]

Transmission electron microscopy

To morphologically demonstrate the induction of autophagy in HaCaT cells treated with matrine, acitretin, matrine plus acitretin for 24 h, we performed an ultra-structural analysis. We made a cell suspension and abandoned supernatant by centrifuge after confirming abundant cells.

Then, cells were centrifuged into clumps after cleaning twice by PBS. Groups of cells were added to glutaraldehyde overnight at 4°C. Autophagic vacuoles at various time were detected by transmission electron microscopy (TEM, JEM-1230, JEOL, Japan).

Western blotting analysis

After treatment with matrine, acitretin, or matrine plus acitretin for different durations, the HaCaT cells were harvested and washed with PBS. The total proteins were extracted using radioimmunoprecipitation assay lysis buffer (including a protease inhibitor cocktail; Applygen Technologies, Beijing, China). They were subjected to Western blotting after the concentration of proteins was measured using a bicinchoninic acid assay kit (Thermo Scientific). Equal amounts of proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Applugen Technologies, Beijing, China). The membrane was blocked with 5% non-fat milk for 1 h at room temperature, and then incubated with primary antibodies at 4°C overnight. The membrane was incubated with IRDye 800CW-conjugated antibodies for 1 h and target protein was scanned on a LI-COR Odyssey® Imaging System (Lincoln, NE, USA). The density of the products was quantified using ImageJ software (NIH, Bethesda, MD, USA) by two examiners who were blind to the identity of the samples being studied. The primary antibodies used in this study included 1:1000 monoclonal rabbit anti-LC3B (3868S, CST, Beverly, MA, USA), 1:1000 monoclonal rabbit anti-Beclin (3495S, CST), 1:1000 monoclonal mouse anti-p62 (ab56416, Abcam, Cambridge, MA, USA), 1:1,000 monoclonal rabbit anti-p21 (ab109199, Abcam), 1:1000 monoclonal rabbit anti-cyclin D1 (2922S, CST), 1:1000 monoclonal rabbit anti-phosphoinositide 3-kinase (PI3K)-α (A5047, Selleck, Shanghai, China), 1:1000 monoclonal rabbit anti-PI3K (A5048, Selleck), 1:1000 monoclonal rabbit anti-protein kinase B (Akt, 4685S, CST), 1:1000 monoclonal rabbit anti-p-Akt (4060S, CST), 1:1000 monoclonal rabbit anti-mammalian target of rapamycin (mTOR, 2983S, CST), 1:1000 monoclonal rabbit anti-p-mTOR (5536S, CST), 1:1000 polyclonal rabbit anti-p70S6K (9202S, CST), 1:1000 monoclonal rabbit anti-p-p70S6K (A5033, Selleck), and 1:5000 monoclonal mouse anti-glyceraldehyde 3-phosphate dehydrogenase as an internal control (PPLYGEN, Beijing, China).

Mouse model and administration

The protocol and procedures employed were ethically reviewed and approved by the Peking University Biomedical Ethics Committee-Experimental Animal Ethics Branch. Female mice (BALB/c) at 8 to 11 weeks of age were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). All the mice were shaved on the back with a shaving machine. The skin area of the back was approximately 2 cm by 3 cm. Experimental animals received a daily topical dose of 62.5 mg of commercially available imiquimod cream (5%) (Mingxinlidi, Sichuan, China) on the shaved back for 7 consecutive days. Control mice were treated similarly with same amount of vaseline cream. Then, experimental mice were treated with a different daily dose of normal saline, matrine, acitretin, or a combination of matrine and acitretin. Matrine was intraperitoneally injected with 50 mg/kg, and acitretin was intra-gastrically administered at 4.5 mg/kg once a day for 7 consecutive days. The tissue of the skin lesions was collected for subsequent experiments. To score the severity of inflammation of the back skin, an objective scoring system was developed based on the clinical Psoriasis Area and Severity Index, except that for the mouse model the affected skin area is not taken into account in the overall score. Erythema, scaling, and thickening were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked. The cumulative score (erythema plus scaling plus thickening) served as a measure of the severity of inflammation (scale 0–12). In addition, hematoxylin-eosin (HE) staining was performed to observe the changes of skin histology in the imiquimod-induced psoriasis-like mice. It includes epidermal thickening, epidermal protrusion extension, stratum corneum differentiation, and inflammatory cell infiltration.[16] Scoring was done by other researchers who did not know the group of animals.

Immunohistochemistry

Paraffin-embedded sections (5-μm) were obtained. The tissues were incubated in a 1:200 dilution of rabbit monoclonal antibody against LC3B (ab48394; Abcam) overnight at 4°C. Following the primary stain, the tissues were stained with goat anti-rabbit (PV-6001; Zhongshan Golden Bridge, Beijing, China) detection kits. To evaluate the immunoreactive score, micrographs were captured (Nikon E600; Nikon, Tokyo, Japan). We multiplied the staining intensity by the percentage of positive cells to produce the immunoreactive score. Scores were obtained by three blinded dermatologists.[22]

Statistical analysis

Statistical analyses were conducted using the SPSS20.0 software (SPSS Inc., IL, USA). Student's t test was used to analyze the data that conforms to the normal distribution in the statistical analysis of pairwise comparison (such as the difference of autophagosome number between the control group and matrine treated cells by TEM, and the comparison of cumulative scores between the control group and the mice treated with imiquimod). Repeated measurements of cell proliferation were done at different time points using repeated analysis of variance (ANOVA). One-way ANOVA was used for the analysis and comparison of multiple observation points within the group. Least significant difference test was used for pairwise comparison between the groups. Data were expressed as mean ± standard deviation. A P value less than 0.05 indicated statistically significant difference.

Results

Effects of matrine on cell proliferation in HaCaT cells in vitro

HaCaT cells were seeded on a 96-well plate with a density of about 2×10^3 cells/well and cultured in a complete medium for 24 h before adherent to the wall. Cells were

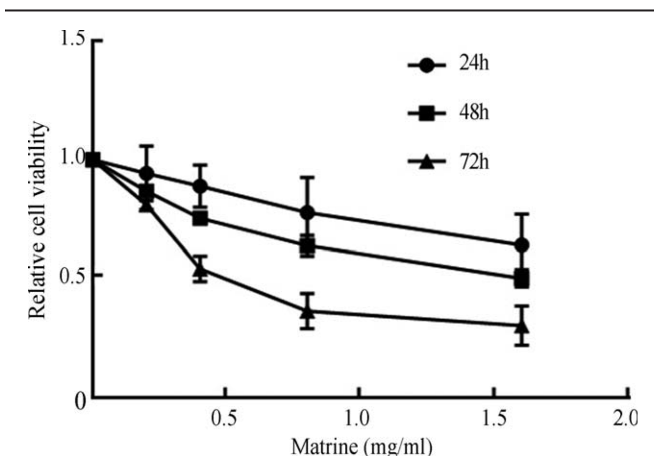

Figure 1: Matrine inhibited the viability of HaCaT cells in a dose- and time-dependent manner after the cells were treated with matrine at 0.2 to 1.6 mg/mL for 24, 48, and 72 h.

incubated with various concentrations of matrine for 24, 48, and 72 h. DMEM was used as the negative control. Then, cell viability was determined by MTS assays. The results showed that matrine inhibited the viability of HaCaT cells in a dose- and time-dependent manner after the cells were treated with matrine at 0.2 to 1.6 mg/mL for 24, 48, and 72 h ($F_{dose} = 15.392, P < 0.01; F_{time} = 95.656, P < 0.01$) [Figure 1]. After the treatment of matrine on HaCaT cells for 24 h, the proliferation percentages of the 0.2, 0.4, 0.8, 1.6 mg/mL matrine groups were 94.36% ± 11.60%, 89.00% ± 8.98%, 77.80% ± 14.72%, and 64.42% ± 13.19%, respectively. After 48 h, the proliferation percentages of the four concentration groups were reduced to 86.78% ± 7.60%, 75.40% ± 1.56%, 63.94% ± 4.43%, and 50.21% ± 3.61%, respectively. They were reduced to 81.05% ± 2.12%, 54.15% ± 5.47%, 36.45% ± 7.38%, 30.36% ± 8.36% after 72 h.

Effects of matrine on cell cycle, apoptosis, and protein expression in HaCaT cells in vitro

Matrine inhibited the G0/G1 stage of HaCaT cells in a dose-dependent manner after the cells were treated with matrine at 0 to 1.6 mg/mL for 24 h ($F = 42.514, P < 0.01$) [Figure 2A]. At 24 h, the inhibition rate of 0, 0.2, 0.4, 0.8, 1.6 mg/mL matrine in HaCaT cells during G0/G1 phase were 38.80% ± 4.00%, 47.40% ± 6.91%, 60.08% ± 1.08%, 79.21% ± 4.90%, and 82.31% ± 6.33%, respectively.

Matrine did not promote apoptosis in the range of 0 to 0.8 mg/mL after 24, 48, or 72 h. However, it did promote apoptosis in 1.6 mg/mL. So the matrine in the range of 0 to 0.8 mg/mL could be able to inhibit cell proliferation instead of cytotoxicity ($P > 0.05$) [Figure 2B].

In addition, the expression of LC3II/I, Beclin1 and p21 was increased and the expression of cyclin D1 was decreased in a dose-dependent way (LC3II/I, $F = 17.087, P < 0.01$; Beclin1, $F = 8.684, P < 0.01$; p21, $F = 5.817, P < 0.05$; cyclin D1, $F = 3.546, P < 0.05$) [Figure 2C and 2D]. Furthermore, compared with blank control, more autophagosomes could be seen in HaCaT cells treated with matrine at 0.4 mg/mL by TEM (2.667 ± 1.202 *vs.* 21.33 ± 1.453, $t = 9.899, P < 0.01$) [Figure 2E]. 3-Methyladenine (3-MA) is an effective autophagy and PI3K inhibitor, which can block autophagy by inhibiting the activity of the PI3K pathway. When HaCaT cells were pre-treated with 3-MA, autophagy was induced by starvation and matrine was attenuated by 3-MA, as reflected by Western blotting ($P < 0.01$) [Figure 2F and 2G]. According to the above results, 0.4 mg/mL of matrine was used as the working concentration in subsequent experiments.

Effects of matrine, acitretin, matrine plus acitretin on HaCaT cells in vitro

At 24 h, the percentages of proliferation of HaCaT cells in matrine and acitretin group were 86.89% ± 3.96% and 82.83% ± 2.61%, respectively, and the percentage of proliferation in the matrine plus acitretin group was 72.19% ± 5.11%. At 48 h, the percentage of cell proliferation in the matrine plus acitretin group was 58.67% ± 2.16%, which was significantly better than that in the acitretin group (79.95% ± 1.77%) or matrine group (74.40% ± 2.37%). Pairwise comparison of the data of each group showed that the cell proliferation inhibition was statistically significant with extending the action time of the drug ($F = 18.424, P < 0.01$) [Figure 3A]. The combined treatment group had a more significant effect on the inhibition of cell proliferation than the single treatment group ($F = 90.969, P < 0.01$) [Figure 3A]. Similarly, the percentage of G0/G1 stage arrest in matrine plus acitretin group (74.96% ± 4.20%) was larger than that in matrine (64.77% ± 5.48%) or acitretin (43.66% ± 3.84%) group ($P < 0.01$) [Figure 3B].

In addition, compared with matrine or acitretin group, the expression of p21 ($P < 0.05, P < 0.05$) and LC3II/I ($P < 0.01, P < 0.05$) in matrine plus acitretin group increased significantly and the expression of cyclin D1 ($P < 0.01, P < 0.05$) and p62 ($P < 0.05, P < 0.05$) was reduced significantly [Figure 3C and 3D]. Moreover, compared with matrine or acitretin group, more autophagosomes were observed in combined treatment group (21.33 ± 1.856 *vs.* 40.33 ± 1.453, $P < 0.01$; 18.00 ± 1.155 *vs.* 40.33 ± 1.453, $P < 0.01$) [Figure 3E and 3F].

As to potential signaling pathways, matrine plus acitretin significantly down-regulated the expression of PI3K-α ($P < 0.01, P < 0.05$), p-Akt ($P < 0.05, P < 0.05$), p-mTOR ($P < 0.05, P < 0.05$), and downstream substance p-p70S6K ($P < 0.05, P < 0.05$) than matrine or acitretin [Figure 3G and 3H].

Effect of matrine, acitretin, matrine plus acitretin on imiquimod-induced psoriasis-like mice

Compared with the control mice, the model group mice treated with imiquimod on day 7 showed typical psoriasis-like changes in both manifestations and HE staining (cumulative score: 0.200 ± 0.058 *vs.* 7.667 ± 0.333, $t = 22.07, P < 0.01$) [Figure 4A and 4B]. The skin lesions and HE staining of the four different treatment groups are shown in Figure 4C. The score of the combined treatment

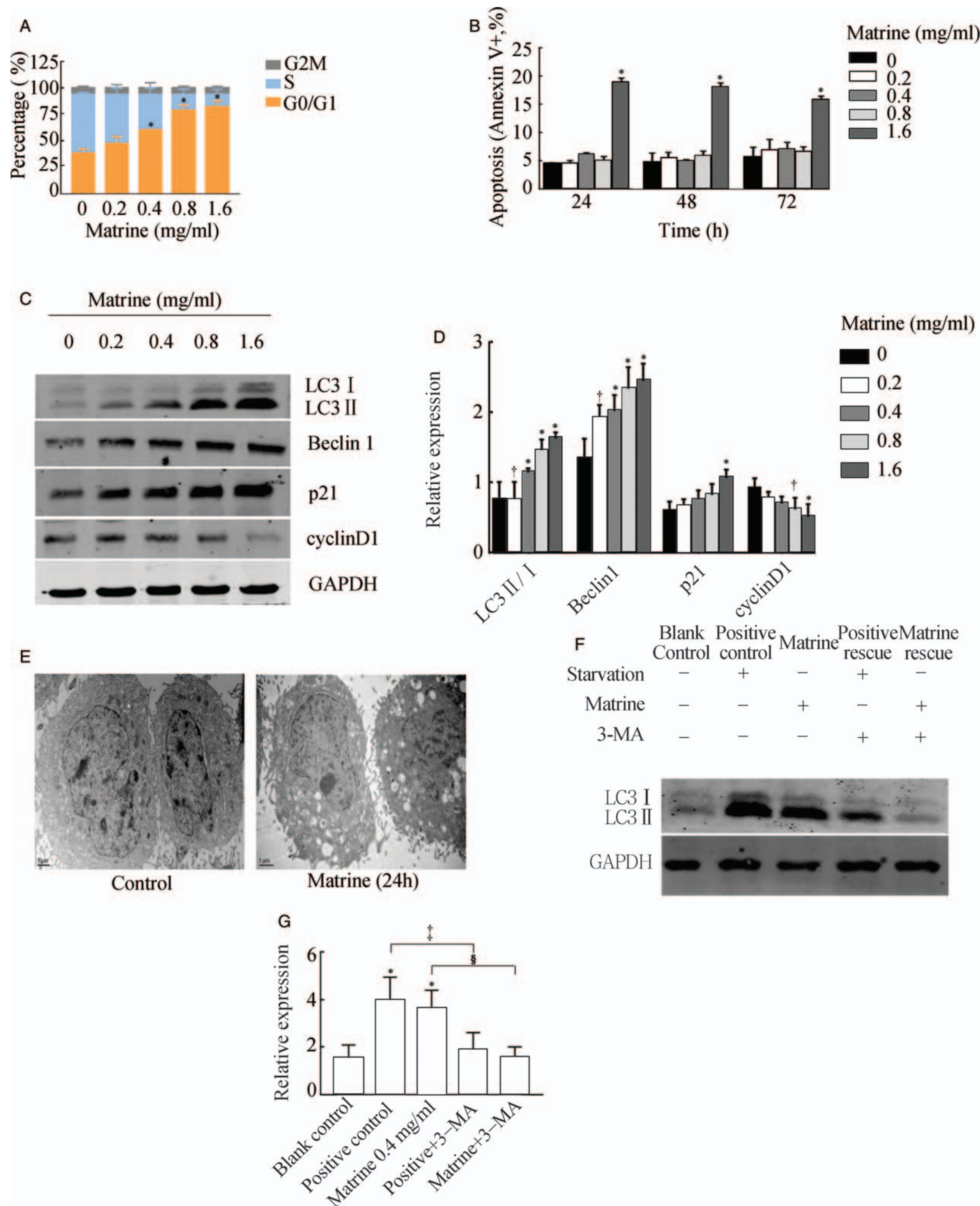

Figure 2: Effects of matrine alone in HaCaT cell lines *in vitro*. (A) An increase in the G0/G1 stage and a decrease in the S or G2/M stage was seen in HaCaT cells that were incubated with various concentrations of matrine for 24 h by 7-AAD analysis. (B) Matrine did not promote apoptosis in the range of 0 to 0.8 mg/mL after 24, 48, or 72 h. However, it did promote apoptosis in 1.6 mg/mL. (C, D) Expression of LC3, Beclin1 and p21 was increased and the cyclin D1 was decreased in a dose-dependent way. Cells were pre-incubated at different concentrations for 48 h before detection. (E) More autophagosomes could be seen in HaCaT cells treated with matrine at 0.4 mg/mL by TEM (bar = 1 μm). (F, G) Cells were starved for 12 h and treated as the autophagy positive control. Autophagy induced by starvation and matrine were attenuated by 3-MA reflected by Western blotting when pretreated with 3-MA in HaCaT cells. Each bar represents the mean $\pm$ standard deviation from three independent experiments. $^{*}P < 0.01$, $^{\dagger}P < 0.05$, *vs.* blank control. $^{\ddagger}P < 0.01$ *vs.* positive control. $^{\S}P < 0.01$ *vs.* matrine 0.4 mg/mL group. 3-MA: 3-Methyladenine; 7-AAD: 7-Aminoactinomycin D; TEM: Transmission electron microscope.

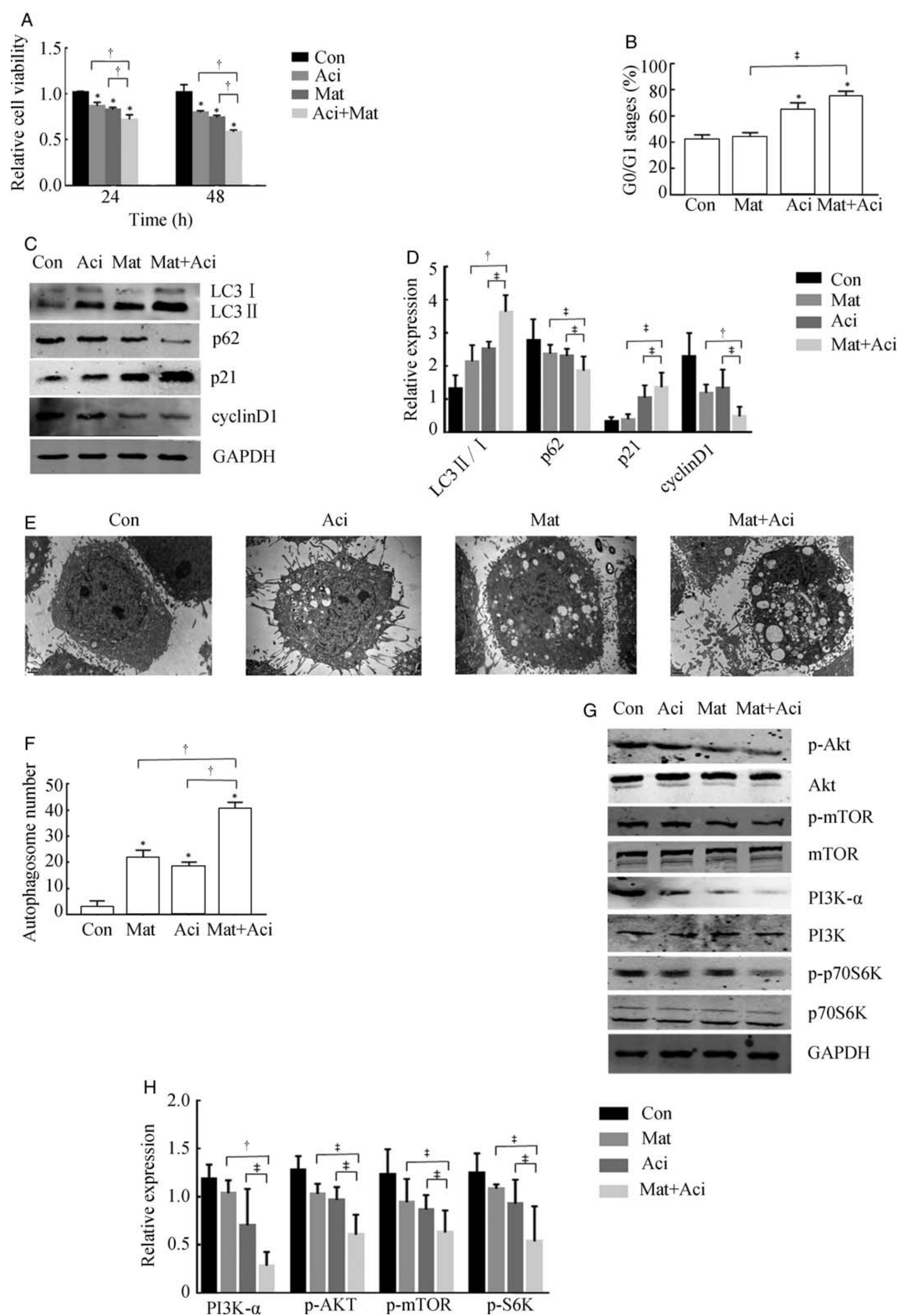

Figure 3: Effects of matrine, acitretin, matrine plus acitretin on HaCaT cell lines *in vitro*. (A) The effect of matrine plus acitretin was better than matrine or acitretin alone in the viability inhibition in HaCaT cells. (B) The percentage of G0/G1 stage arrest in HaCaT cells treated with matrine plus acitretin was larger than that in HaCaT cells treated with matrine or acitretin alone. (C, D) The effect of matrine plus acitretin was better than matrine or acitretin alone in the expression of LC3, p62, p21, and cyclin D1 by Western blotting. (E, F) Autophagosomes were induced more significantly in matrine plus acitretin group than other groups. (G, H) The effect of matrine plus acitretin was better than matrine or acitretin alone in the downregulation of PI3K-α, p-Akt, p-mTOR, and downstream substance p-p70S6K. Each bar represents the mean ± standard deviation from three independent experiments. $^{*}P < 0.01$ *vs*. control group. $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.05$, *vs*. matrine 0.4 mg/mL group or acitretin 10 μmol/L group. Aci: Acitretin; Con: Control; Mat: Matrine; Mat + Aci: Matrine plus acitretin.

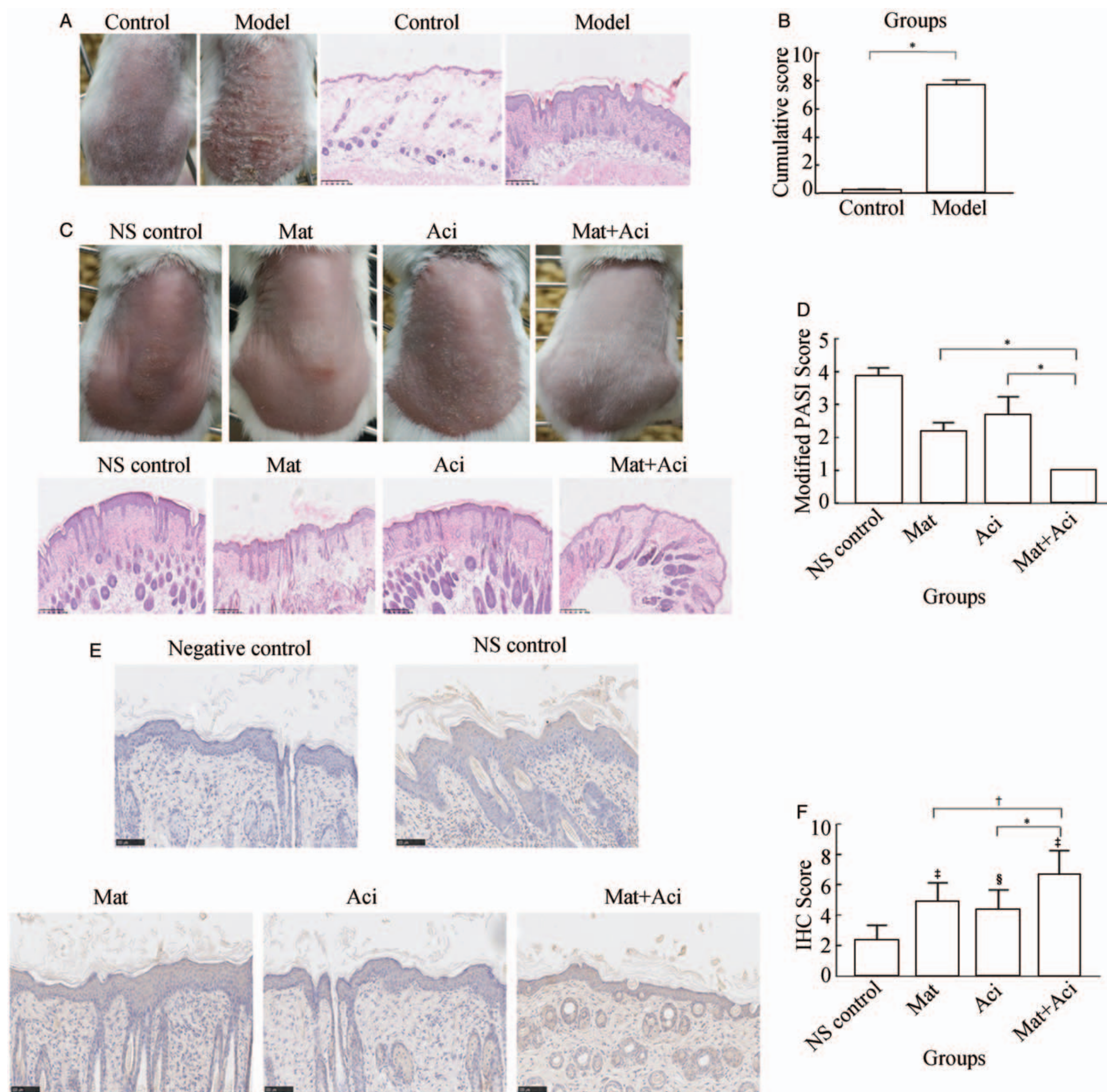

Figure 4: Effect of matrine, acitretin, matrine plus acitretin on imiquimod-induced psoriasis-like mice. (A) The manifestations and pathological findings of skin lesions in imiquimod-induced psoriasis-like mice (hematoxylin-eosin staining, original magnification ×100). (B) The cumulative score (erythema plus scaling plus thickening) on the 7th day indicated the severity of inflammation. (C) The manifestations and pathological findings of skin lesions in the mice after treatment with NS, matrine, acitretin, matrine plus acitretin (hematoxylin-eosin staining, original magnification ×100). (D) The cumulative scores of the above four groups. (E, F) The expression of LC3 in the combined drug group was significantly higher than that of the single-drug group (immunohistochemical staining, original magnification ×200). Each bar represents the mean ± standard deviation from three independent experiments. $^*P < 0.01$, $^{\dagger}P < 0.05$ *vs.* Control, matrine 0.4 mg/mL group or acitretin 10 μmol/L group. $^{\ddagger}P < 0.01$, $^{\S}P < 0.05$ *vs.* NS control group. Aci: Acitretin; Con: Control; Mat: Matrine; Mat + Aci: Matrine plus acitretin; NS: Normal saline.

group was significantly lower than that of the matrine or acitretin group (1.480 ± 0.230 *vs.* 2.370 ± 0.241, $P < 0.01$; 1.480 ± 0.230 *vs.* 2.888 ± 0.341, $P < 0.01$) [Figure 4D]. The immunohistochemical results were scored by three dermatologists, according to the method in Supplementary Table 1, http://links.lww.com/CM9/A84. The expression of LC3 in the combined treatment group was significantly higher than that of the matrine or acitretin group (4.333 ± 1.366 *vs.* 6.833 ± 1.602, $P < 0.05$; 4.833 ± 1.329 *vs.* 6.833 ± 1.602, $P < 0.01$) [Figure 4E and 4F].

Discussion

Matrine is often used in the treatment of psoriasis in China. Yang *et al*[22] achieved satisfactory results using hyperbaric oxygen combined with compound matrine to treat 11 cases of psoriasis vulgaris. Compound matrine injection is effective and safe in the treatment of erythrodermic psoriasis.[23]

To explore the effect and related mechanisms of matrine, we first studied the effect of matrine on cell proliferation and the cell cycle on HaCaT cells. Our findings suggest that

matrine could suppress the cell proliferation in a time- and dose-dependent manner. Moreover, proportions of G0/G1 phase HaCaT cells were significantly increased after treatment with matrine in a time- and dose-dependent manner. In addition, more autophagosomes could be seen in matrine-treated HaCaT cells by TEM. Our data demonstrated that autophagy induction and cell cycle G0/G1 arrest may be related to the effect of matrine on psoriasis. This theory is validated by the dose-dependent expression of autophagy-related protein LC3II/I and cell cycle arrest-related protein p21 and cyclin D1 after treatment with matrine. Previous reliable evidence has been provided that the pathogenesis of psoriasis is related to autophagy injury.[22-29] Autophagy is a phenomenon involving intracellular lysosomal phagocytosis, degradation of damaged or superfluous organelles and mutant proteins. The main role of autophagy is to realize the self-protection of cells under emergency conditions, such as starvation, so that cells can continue to survive. Abnormal levels of autophagy of psoriasis keratinocytes may be involved in the psoriasis pathological mechanism.[27] Cell cycle arrest also plays an important role in pathogenesis of psoriasis.[30] Meanwhile, other studies have also confirmed that matrine can induce autophagy of liver cancer,[31-33] breast cancer,[34] glioma,[35] and leukemia.[36] These conclusions are supportive of the establishment of our theory.

Interestingly, Jing *et al*[17] observed the clinical effect of matrine glucose injection combined with acitretin capsule in the treatment of psoriasis vulgaris. They found that treatment of matrine glucose injection combined with acitretin capsule has a definite curative effect on psoriasis, which can significantly improve the curative effect and reduce the incidence or the degree of adverse reactions compared with acitretin capsule alone. This is consistent with our clinical experience. However, the mechanisms involved have not been studied yet.

Acitretin, an active metabolite of etretinate, is the most widely used systemic retinoid in the treatment of psoriasis. The specific mechanism of action of acitretin also remains unknown. Acitretin has multiple effects on epidermal cell growth and differentiation possibly responsible for its therapeutic action in psoriasis. The overall effect of acitretin on psoriatic epidermis results in reduction of the proliferation rate in the acanthotic epidermis by down-regulating the number of cycling cells, promoting terminal differentiation of keratinocytes, regulating desquamation of corneocytes, and decreasing the thickness of stratum corneum and inflammation in epidermis and dermis. In addition, the immunomodulatory and antiangiogenic action of retinoids may contribute to their efficacy.[37] Acitretin has the best curative effect on pustular psoriasis and the second is erythrodermic psoriasis. However, the treatment of psoriasis vulgaris with acitretin is not always good. Furthermore, mass use for a long period of time increases the risk of adverse effects. It has been reported that acitretin modulates HaCaT cell proliferation through signal transducer and activator of transcription 1- and signal transducer and activator of transcription 3-dependent signaling,[38] but there is no relevant literature exploring whether acitretin plays a role in autophagy and cell cycle arrest in HaCaT cells. To explore whether matrine and acitretin have synergistic effects in the treatment of psoriasis and related mechanisms, we studied the effect of matrine plus acitretin on HaCaT cell lines and imiquimod-induced psoriasis-like models for further study.

In contrast to matrine or acitretin alone, the growth inhibition by matrine plus acitretin was more significant. Our findings suggest that the combination of matrine and acitretin was more effective than matrine or acitretin in autophagy induction and cell cycle G0/G1 arrest in psoriasis-like cell line HaCaT cells. Moreover, the expression of LC3II/I was reversed when treated with the autophagy inhibitor 3-MA. These data indicated that matrine and acitretin have synergistic effects by inducing autophagy and inhibiting the G0/G1 stage of the cell cycle in the treatment of psoriasis. These results suggested that the combined effect may be the mechanism by which matrine can improve the efficacy and reduce its side effects of acitretin. Autophagy, as a novel and promising drug target involved in a wide range of human diseases, can be modulated by many traditional Chinese medicine-derived agents, indicating that autophagy modulation may be an important mechanism underlying the therapeutic effect of traditional Chinese medicine in treating diseases.[39]

The process of autophagy and cell cycle arrest is well-regulated, and the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathway plays a key role in this process.[40-42] To further dissect the underlying molecular mechanism of matrine or acitretin induced autophagy and cell cycle arrest, we detected the activity of the phosphorylated PI3K/Akt/mTOR pathway in HaCaT cells upon matrine, acitretin, or matrine plus acitretin treatment. The results showed that with treatment of matrine, acitretin, or matrine plus acitretin, the PI3K/Akt/mTOR pathway was down-regulated in HaCaT cells. Furthermore, the effect of matrine plus acitretin was more significant than matrine or acitretin alone. These results suggested that matrine plus acitretin exhibited synergetic effects of autophagy induction and cell cycle G0/G1 stage arrest by negatively regulating the phosphorylation of PI3K/Akt/mTOR signaling pathway activities.

Blockage of the PI3K/Akt/mTOR signaling pathway plays a critical role in regulating autophagy. The inactivation of the phosphorylation of Akt, mTOR, and their downstream substance, p70S6K, were found in matrine-treated HaCaT cells. Thus, the autophagic promotion and cell cycle arrest induction of matrine might be a promising therapeutic strategy for psoriasis. Matrine-related autophagy and cell cycle arrest induction may reduce the drug resistance of acitretin in psoriasis. Traditional Chinese medicine has not been widely used in the world due to its main characteristics of empirical treatment and lack of evidence. Our study provides evidence for better and more scientific application of matrine in the treatment of psoriasis. The combination of matrine with acitretin should double the efficacy and halves the side effects to patients in clinical practice. We explored the combined application and related possible mechanism of matrine and acitretin in the treatment of psoriasis. We will continue to conduct further research to make matrine and its combined therapy with

acitretin in the treatment of psoriasis more evidence-based and reliable.

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Conflicts of interest

None.

References

1. Guenther LC, Kunynetz R, Lynde CW, Sibbald RG, Toole J, Vender R, *et al*. Acitretin use in dermatology. J Cutan Med Surg 2017;21:2S–12S. doi: 10.1177/1203475417733414.
2. Kolios AG, Yawalkar N, Anliker M, Boehncke WH, Borradori L, Conrad C, *et al*. Swiss S1 guidelines on the systemic treatment of Psoriasis Vulgaris. Dermatology 2016;232:385–406. doi: 10.1159/000445681.
3. Hwang GB, Lee JE, Nho CW, Lee BU, Lee SJ, Jung JH, *et al*. Short-term effect of humid airflow on antimicrobial air filters using Sophora flavescens nanoparticles. Sci Total Environ 2012;421-422:273–279. doi: 10.1016/j.scitotenv.2012.01.060.
4. Jin JH, Kim JS, Kang SS, Son KH, Chang HW, Kim HP. Anti-inflammatory and anti-arthritic activity of total flavonoids of the roots of Sophora flavescens. J Ethnopharmacol 2010;127:589–595. doi: 10.1016/j.jep.2009.12.020.
5. Wang W, You RL, Qin WJ, Hai LN, Fang MJ, Huang GH, *et al*. Anti-tumor activities of active ingredients in compound Kushen injection. Acta Pharmacol Sin 2015;36:676–679. doi: 10.1038/aps.2015.24.
6. Long Y, Lin XT, Zeng KL, Zhang L. Efficacy of intramuscular matrine in the treatment of chronic hepatitis B. Hepatobiliary Pancreat Dis Int 2004;3:69–72.
7. Fang R, Wu R, Zuo Q, Yin R, Zhang C, Wang C, *et al*. Sophora flavescens containing-QYJD formula activates Nrf2 anti-oxidant response, blocks cellular transformation and protects against DSS-induced colitis in mouse model. Am J Chin Med 2018;4:1–15. doi: 10.1142/S0192415X18500829.
8. Guo S, Chen Y, Pang C, Wang X, Shi S, Zhang H, *et al*. Matrine is a novel inhibitor of the TMEM16A chloride channel with antilung adenocarcinoma effects. J Cell Physiol 2019;234:8698–8708. doi: 10.1002/jcp.27529.
9. Jiang JH, Pi J, Jin H, Yang F, Cai JY. Chinese herb medicine matrine induce apoptosis in human esophageal squamous cancer KYSE-150 cells through increasing reactive oxygen species and inhibiting mitochondrial function. Pathol Res Pract 2018;214:691–699. doi: 10.1016/j.prp.2018.03.015.
10. Ji R, Cui W, Liang RW, Guan ZY, Li RF. Effect of radix Sophorae flavescentis (RSF) mixture on mast cells in jejunal mucosa of mice infected by Cryptosporidium (in Chinese). Chin J Schistosomiasis Control 2013;25:280–283. doi: 10.3969/j.issn.1005-6661.2013.03.016.
11. Wei YP, Wang XH, Liu G, Zhang JF, Yang YX, Zhang J, *et al*. Matrine exerts inhibitory effects in melanoma through the regulation of miR-19b-3p/PTEN. Int J Oncol 2018;53:791–800. doi: 10.3892/ijo.2018.4414.
12. Zhaowu Z, Xiaoli W, Yangde Z, Nianfeng L. Preparation of matrine ethosome, its percutaneous permeation in vitro and anti-inflammatory activity in vivo in rats. J Liposome Res 2009;19:155–162. doi: 10.1080/08982100902722381.
13. Liu JY, Hu JH, Zhu QG, Li FQ, Wang J, Sun HJ. Effect of matrine on the expression of substance P receptor and inflammatory cytokines production in human skin keratinocytes and fibroblasts. Int Immunopharmacol 2007;7:816–823. doi: 10.1016/j.intimp.2007.02.003.
14. Wang X, Lin H, Zhang R. The clinical efficacy and adverse effects of interferon combined with matrine in chronic hepatitis B: a systematic review and meta-analysis. Phytother Res 2017;31:849–857. doi: 10.1002/ptr.5808.
15. Liao XZ, Tao LT, Liu JH, Gu YY, Xie J, Chen Y, *et al*. Matrine combined with cisplatin synergistically inhibited urothelial bladder cancer cells via down-regulating VEGF/PI3K/Akt signaling pathway. Cancer Cell Int 2017;28:124. doi: 10.1186/s12935-017-0495-6.
16. Hu MJ, Zeng H, Wu YL, Zhang YP, Zhang S, Qiao MM, *et al*. Synergistic effects of matrine and 5-fluorouracil on tumor growth of the implanted gastric cancer in nude mice. Chin J Dig Dis 2005;6:68–71. doi: 10.1111/j.1443-9573.2005.00201.x.
17. Jing WX, Zhang QQ, Tian Y. Curative effect of matrine glucose injection combined with avia capsule in the treatment of psoriasis vulgaris (in Chinese). Med Innovation China 2011;36:41–43. doi: 10.3969/j.issn.1674-4985.2011.36.022.
18. An Q, Han C, Zhou Y, Li F, Li D, Zhang X, *et al*. Matrine induces cell cycle arrest and apoptosis with recovery of the expression of miR-126 in the A549 non-small cell lung cancer cell line. Mol Med Rep 2016;14:4042–4048. doi: 10.3892/mmr.2016.5753.
19. Lin XY, He CD, Xiao T, Jin X, Chen J, Wang YK, *et al*. Acitretin induces apoptosis through CD95 signalling pathway in human cutaneous squamous cell carcinoma cell line SCL-1. J Cell Mol Med 2009;13:2888–2898. doi: 10.1111/j.1582-4934.2008.00397.x.
20. Wang Y, Gu X, Li W, Zhang Q, Zhang C. PAK1 overexpression promotes cell proliferation in cutaneous T cell lymphoma via suppression of PUMA and p21. J Dermatol Sci 2018;90:60–67. doi: 10.1016/j.jdermsci.2017.11.019.
21. Chen Y, Yan H, Song Z, Chen F, Wang H, Niu J, *et al*. Downregulation of TNIP 1 expression leads to increased proliferation of human keratinocytes and severer psoriasis-like conditions in an imiquimod-induced mouse model of dermatitis. PLoS One 2015;10:e0127957. doi: 10.1371/journal.pone.0127957.
22. Yang Y, Yang Y. Hyperbaric oxygen combined with compound matrine in the treatment of 11 cases of submarine crew with psoriasis (in Chinese). J Navy Med 2015;2:156–157. doi: 10.3969/j.issn.1009-0754.2015.02.022.
23. Wang J, Feng YY, Liu MZ. Assessment of efficacy of compound matrine injection in the treatment of erythrodermic psoriasis (in Chinese). Chin J Leprosy Skin Dis 2014;5:292–294.
24. Akinduro O, Sully K, Patel A, Robinson DJ, Chikh A, McPhail G, *et al*. Constitutive autophagy and nucleophagy during epidermal differentiation. J Invest Dermatol 2016;136:1460–1470. doi: 10.1016/j.jid.2016.03.016.
25. Yin H, Wu H, Chen Y, Zhang J, Zheng M, Chen G, *et al*. The therapeutic and pathogenic role of autophagy in autoimmune diseases. Front Immunol 2018;9:1512. doi: 10.3389/fimmu.2018.01512.
26. Feng L, Song P, Xu F, Xu L, Shao F, Guo M, *et al*. cis-Khellactone inhibited the proinflammatory macrophages via promoting autophagy to ameliorate imiquimod-induced psoriasis. J Invest Dermatol 2019;13:31316–31318. doi: 10.1016/j.jid.2019.02.021.
27. Varshney P, Saini N. PI3K/AKT/mTOR activation and autophagy inhibition plays a key role in increased cholesterol during IL-17A mediated inflammatory response in psoriasis. Biochim Biophys Acta Mol Basis Dis 2018;1864:1795–1803. doi: 10.1016/j.bbadis.2018.02.003.
28. Mahil SK, Twelves S, Farkas K, Setta-Kaffetzi N, Burden AD, Gach JE, *et al*. AP1S3 mutations cause skin autoinflammation by disrupting keratinocyte autophagy and up-regulating IL-36 production. J Invest Dermatol 2016;136:2251–2259. doi: 10.1016/j.jid.2016.06.618.
29. Tian R, Li Y, Yao X. PGRN suppresses inflammation and promotes autophagy in keratinocytes through the Wnt/ β-catenin signaling pathway. Inflammation 2016;39:1387–1394. doi: 10.1007/s10753-016-0370-y.
30. Kim SA, Ryu YW, Kwon JI, Choe MS, Jung JW, Cho JW. Differential expression of cyclin D1, Ki-67, pRb, and p53 in psoriatic skin lesions and normal skin. Mol Med Rep 2018;17:735–742. doi: 10.3892/mmr.2017.8015.
31. Xie SB, He XX, Yao SK. Matrine-induced autophagy regulated by p53 through AMP-activated protein kinase in human hepatoma cells. Int J Oncol 2015;47:517–526. doi: 10.3892/ijo.2015.3023.
32. Yang J, Yao S. JNK-Bcl-2/Bcl-xL-Bax/Bak pathway mediates the crosstalk between matrine-induced autophagy and apoptosis via interplay with beclin 1. Int J Mol Sci 2015;16:25744–25758. doi: 10.3390/ijms161025744.
33. Xie BS, He XX, Ai ZL, Yao SK. Involvement of β-catenin in matrine-induced autophagy and apoptosis in WB-F344 cells. Mol Med Rep 2014;9:2547–2553. doi: 10.3892/mmr.2014.2107.
34. Ren LL, Wang LL, Wang XJ. Relationship between matrine induced autophagy and mTOR in human breast cancer Bcap-37 cells (in Chinese). Zhejiang J Integrated Traditional Chin Western Med 2016;9:783–786. doi: 10.3969/j.issn.1005-4561.2016.09.002.
35. Chi G, Xu D, Zhang B, Yang F. Matrine induces apoptosis and autophagy of glioma cell line U251 by regulation of circRNA-

104075/BCL-9. Chem Biol Interact 2019;308:198–205. doi: 10.1016/j.cbi.2019.05.030.
36. Wu J, Hu G, Dong Y, Ma R, Yu Z, Jiang S, *et al*. Matrine induces Akt/mTOR signalling inhibition-mediated autophagy and apoptosis in acute myeloid leukaemia cells. J Cell Mol Med 2017;21:1171–1181. doi: 10.1111/jcmm.13049.
37. Dogra S, Yadav S. Acitretin in psoriasis: an evolving scenario. Int J Dermatol 2014;53:525–538. doi: 10.1111/ijd.12365.
38. Qin X, Chen C, Zhang Y, Zhang L, Mei Y, Long X, *et al*. Acitretin modulates HaCaT cells proliferation through STAT1- and STAT3-dependent signaling. Saudi Pharm J 2017;25:620–624. doi: 10.1016/j.jsps.2017.04.034.
39. Wang SF, Wu MY, Cai CZ, Li M, Lu JH. Autophagy modulators from traditional Chinese medicine: mechanisms and therapeutic potentials for cancer and neurodegenerative diseases. J Ethnopharmacol 2016;194:861–876. doi: 10.1016/j.jep.2016.10.069.
40. Lee HJ, Venkatarame Gowda Saralamma V, Kim SM, Ha SE, Raha S, Lee WS, *et al*. Pectolinarigenin induced cell cycle arrest, autophagy, and apoptosis in gastric cancer cell via PI3K/AKT/mTOR signaling pathway. Nutrients 2018;10:E1043. doi: 10.3390/nu10081043.
41. Nys K, Agostinis P, Vermeire S. Autophagy: a new target or an old strategy for the treatment of Crohn's disease? Nat Rev Gastroenterol Hepatol 2013;10:395–401. doi: 10.1038/nrgastro.2013.66.
42. Coutte L, Dreyer C, Sablin MP, Faivre S, Raymond E. PI3K-AKT-mTOR pathway and cancer. Bull Cancer 2012;99:173–180. doi: 10.1684/bdc.2011.1384.

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