Effect of white mange mixture in a murine model of psoriasis

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Abstract. Psoriasis is an autoimmune disease with periods of remission or aggravation. Until now, no effective treatment has been developed. The aim of this study was to assess the effect of the traditional Chinese medicine white mange mixture in a murine model of vaginal psoriasis. Female mice (n=70) were randomly divided into seven groups as follows: negative control group, positive control group, acitretin group, Xiaoying granule group, high-dose white mange mixture group, medium-dose white mange mixture group, and low-dose white mange mixture group. After vaginal psoriasis mouse model design, the inhibition of keratinocyte (KC) cell proliferating cell nuclear antigen (PCNA) was achieved by SP immunohistochemical method, spleen T lymphocyte apoptosis detection was assessed by using electron microscopy and granulocyte colony stimulating factor (GM-CSF) levels were detected by ELISA method. According to our results, T lymphocyte nucleus appearance in the negative control group was normal whereas in all the doses of white mange mixture the nucleus significantly showed apoptotic trend. Compared with the negative control group, the amount of GM-CSF in the serum of the model was significantly increased (P<0.01) while administration of white mange mixture in different doses decreased the GM-CSF content significantly (P<0.01). White mange mixture can significantly inhibit vaginal psoriasis in a mouse model by decreasing the amount of epithelium KC cell PCNA and production of the inflammatory cytokines GM-CSF in serum.

Introduction

Psoriasis is a complex erythema scaly skin disease (1), and basically cellular immune disorders, genetic, environmental and infectious factors determine its pathogenesis (2,3). Psoriasis is an autoimmune disease, triggered with periods of remission or aggravation by certain infectious factors such as streptococcal infections (4,5). Although there are many studies on psoriasis, an effective treatment without many side effects has not been discovered yet.

Western medicine mainly focusses on symptomatic treatment and physical therapy (6,7), including inhibition of cell proliferation, immunosuppressive and anti-inflammatory effects (8). Recently, many biological agents have been designed with limited effects, but their usage is not efficient because of their unknown long-term effects (9-11). Currently, alternative medicine gives hope to patients with psoriasis disease by using various traditional herbal medicines.

Traditional chinese medicine (TCM) is a part of alternative medicine and extensively used in China. Recent studies proved that the application of TCM in psoriasis treatment shows acceptable results with less side effects than modern western treatment (12-14). Acitretin is a common drug used in the treatment of psoriasis (15). It can also regulate the epidermal keratinocytes (KCs) of the body by inhibiting the excessive proliferation of epithelial cells. In addition, the drug has significant immunomodulatory effects on abnormal epidermal cells, which can effectively lead to the reduction of inflammatory response in the skin tissue of the patients (16). Xiaoyin granule is a pure Chinese medicine agent (17). The main components of Xiaoyin granule are: Angelica, raw land, Radix Sophora flavescens, honeysuckle, Sophora flavescens and peony skin. TCM claims that psoriasis is mainly caused by blood stasis, blood heat and blood deficiency. Xiaoyin granule has remarkable effects on cooling and nourishing blood, dispelling cold, moistening dryness and alleviating itching. Each part of Xiaoyin granule has unique effects. The Radix Sophora flavescens activate blood circulation and they have also nourishing and cooling effects on blood (18). Radix Sophorae, honeysuckle, white fresh skin and windproof have detoxifying, antipruritic effects and also reducing effect on blood heat stress (19). Red flower and Angelica can promote blood circulation and dissipate blood stasis (20).

The excessive proliferation of KCs is an important feature of psoriasis, and proliferating cell nuclear antigen (PCNA) is the most reliable index to evaluate cell proliferation (21). PCNA is a nucleoprotein involved in DNA synthesis, which is may be related to the pathogenesis of psoriasis (22). Granulocyte colony stimulating factor (GM-CSF) is now considered to be an important natural immune activator, which activates mature white blood and immune cells and is involved in the chronic stage of inflammatory and autoimmune diseases. The expression of GM-CSF is abnormal in psoriasis and correlated with the severity of psoriasis (23,24).

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Based on history and unique green plant cover of China, TCM has great potential as treatment in chronic disease. The present study was designed to evaluate the protective effect of white mange mixture and Xiaoyin granules in comparison with regular treatment (acitretin) on the mouse vaginal psoriasis model. Epithelial KC cell PCNA, T lymphocyte apoptosis and GM-CSF were evaluated for the first time.

Materials and methods

Main reagents. Phosphate-buffered saline (PBS) (Changde Beekman Biotechnology Co., Ltd.); RPMI-1640 (Xibao Biotech Co., Ltd.); hematoxylin and eosin (H&E) stain solution (Shanghai Xinfan Biotechnology Co., Ltd.); mercuric oxide, aluminium potassium sulfate, 5% glacial acetic acid and 1% Hydrochloric acid solution (Hubei Xinkang Pharmaceutical Chemical Co., Ltd.); PCNA (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.); chromogenic agent (DAB) (code WK294; Beijing Baiaolaibo Technology Co., Ltd.); SP Immunohistochemical commercial assay kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.); and GM-CSF enzyme-linked immunosorbent assay (ELISA) kit (Shenzhen Dako Biotechnology Co., Ltd.).

Main experimental equipment. Micropipettes, electronic balances and surgical instruments (Tianjin Celiss Automation Technology Co., Ltd.); JEM-100CX II transmission electron microscope (JEOL Ltd.); microplate reader (Shanghai Flash Spectrum Biotechnology Co., Ltd.); BY-160C type medical centrifuge (Beijing Baiyang Medical Instruments Co., Ltd.).

Experimental animals. Seventy female BALB/c mice were purchased from Beijing Longmidas Science and Technology Development Co., Ltd. The animals were 6 to 8 weeks old, weighing 18 and 22 g at the beginning of the study. The animals were acclimatized for 2 weeks at standard conditions of $24\pm2^{\circ}$ C temperature, humidity between 40 and 60% and 12 h light/dark cycle. The animals had free access to tap water and food. The animal experiment has been approved by the Ethics Committee of the Guiyang University of Chinese Medicine (Guiyang, China).

Experimental groups and treatment of mice. Seventy mice were randomly divided into 7 groups (n=10) as follows: negative control group, positive control group, acitretin group, Xiaoying granule groups, high-dose white mange mixture group, medium-dose white mange mixture group and low-dose white mange mixture group. The doses of white mange mixture, Xiaoying granules and acitretin were settled based on the equivalent received by humans (25). For example, a person with the weight of 70 kg receives a dose of 1.610 mg white mange mixture per day. According to the proportion of body surface area of the human and the mice we conclude that mice of 10 g should receive a dose of 0.23 mg white mange mixture per day that is equivalent to 23 mg/kg/day. The drugs were dissolved in drinking water. The stock solution (10 ml) (acitretin group: 11.5 mg/ml; Xiaoying granule group: 2.438 mg/ml; high-, medium-, and low-dose of white mange mixture groups: 46, 23 and 11.5 mg/ml, respectively, was prepared. A mouse weighing 20 g received a dose of 0.2 ml from stock solution. Negative control group received every day the same amount of drinking water by gavage. Acitretin group received a dose of 5.75 mg/kg/day of and Xiaoying granule group 1.219 mg/kg/day, respectively. High-, medium- and low-dose of white mange mixture groups received 23, 11.5 and 5.75 mg/kg/day, respectively. The treatment was for 28 consecutive days. Experimental drugs used in the protocol are presented in Table I.

White mange mixture is a Chinese medicine which is effective in the treatment of psoriasis. It contains: 10 g of *Fructus amomi*, 20 g Figwort, 20 g Chinese Angelica, 20 g *Scutellaria baicalensis*, 20 g Madder, 15 g Radix Arnebiae, 25 g *Rhizoma imperatae*, 15 g Honeysuckle, 20 g Cortex Moutan, and 15 g Licorice (these drugs are made into powder and dissolved in water when they are used).

Xiaoyin Granules is a Chinese medicine for treating psoriasis, macular and itchy skin diseases. It has obvious anti-histamine, anti-inflammation, anti-bacterial and antivirus effects, and improves microcirculation. It contains: 10 g Chinese Angelica, 10 g *Rhizoma chuanxiong*, 10 g Mirabilite, 10 g *Polygonum multiflorum*, 15 g Dried Rehmannia Root, 15 g Radix Paeoniae Rubra, 15 g Madder, 15 g Cortex Dictam, 15 g *Tribulus terrestris*, 15 g *Flos sophorae*, 9 g *Flos carthami*, 6 g Licorice and 30 g *Lignum millettiae* (these drugs are made into powder and dissolved in water when they are used).

Murine model of vaginal psoriasis. The mice from experimental groups received estradiol (Guangzhou Baiyun Mountain Mingxing Pharmaceutical Co., Ltd.), intraperitoneally in doses of 5 mg/kg/day, for 3 consecutive days, while the mice from negative control group received saline solution. On the fourth day, each animal was weighed and treated for 28 consecutive days as mentioned above.

Blood sample collection. At the end of the treatment the blood samples were collected directly from heart of each mouse. The samples were centrifuged (BY-160C type medical centrifuge; Beijing Baiyang Medical instruments Co., Ltd.) at 671 x g for 10 min and the sera were stored at -80°C.

Histopathological lesions. The vaginal epithelial tissue was removed immediately, fixed in 10% neutral formalin (Zhongshan Kang Naixin Biomedical Science and Technology Co., Ltd.) for 24 to 48 h, and then processed to obtain paraffin blocks. Paraffin-embedded blocks were routinely processed; $5 \mu m$ thick sections were prepared (26).

PCNA evaluation. PCNA level was determined by using SP Immunohistochemical commercial assay kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.). The staining procedure was performed according to the protocol of the manufacturer. Briefly, after deparaffinization, the slides were immersed in antigen retrieval solution (pH 6.0) and heated in microwave for 15 min so as to unmask antigens. The sections were then incubated in 3% H₂O₂ for 10 min to block the activity of endogenous peroxidase. Each reagent (A, mouse PCNA antibody, B and C) was added consecutively and each step contained incubation of the samples at room temperature

Table I.	Experim	ental drug	zs.

Drugs ^a	Components	
White mange mixture	10 g <i>Fructus amomi</i> , 20 g Figwort, 20 g Chinese Angelica, 20 g <i>Scutellaria baicalensis</i> , 20 g Madder, 15 g Radix Arnebiae, 25 g Rhizoma Imperatae, 15 g Honeysuckle, 20 g Cortex Moutan, 15 g Licorice	
Acitretin	Acitretin	
Xiaoyin granules	10 g Chinese Angelica, 10 g Rhizoma Chuanxiong, 10 g Mirabilite, 10 g <i>Polygonum multiflorum</i> , 15 g Dried Rehmannia Root, 15 g Radix Paeoniae Rubra, 15 g Madder, 15 g Cortex Dictam, 15 g <i>Tribulus terrestris</i> , 15 g <i>Flos sophorae</i> , 9 g <i>Flos carthami</i> , 6 g Licorice, 30 g <i>Lignum millettiae</i>	

^aAll drugs were from Shaanxi Kanghui Pharmaceutical Co., Ltd. (Xianyang, China).

for 15 min and then washed by PBS (each step was repeated 3 times).

Finally, DAB (code WK294; Beijing Baiaolaibo Technology Co., Ltd.) was exposed to samples and then gray value tones were measured by Leica Q550CW image acquisition and analysis system (both from Leica Microsystem Trading Co., Ltd.). The gray mean value evaluation was measured by positive cell count/unit area. The system set the white gray value to 255 and black gray value to 0. The higher the gray value, the weaker the expression.

T lymphocyte isolation and evaluation. Under deep anesthesia (0.6% sodium pentobarbital; Shanghai Xinya Pharmaceutical Co., Ltd.) the spleen tissue was extracted at aseptic conditions and placed in a Petri dish. The tissue was rinsed off using PBS (0.1 mol/l, pH 7.4) (Changde Beekman Biotechnology Co., Ltd.) and then was centrifuged twice at 377 x g for 5 min. The red blood cells were suspended in RPMI-1640, 10% FBS and 1% antibiotics (Xibao Biotech Co., Ltd.) cultivating at 37°C in 5% CO₂ incubator for 24 h. Then, the non-adherent cell suspension (spleen lymphocytes) was collected (27).

Apoptosis of T lymphocytes was determined by JEM-100CX II transmission electron microscope (JEOL Ltd.) (28). For this, $100 \,\mu$ l of cell suspension was added to each well of the culture plates (n=5). Then, 20 μ l freshly prepared 5% 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich; Merck KGaA) solution was added into each well followed by incubation for more than 4 h. Glutaraldehyde 2.5% was added to collect T lymphocytes for 2 h. After rinsing off the cells using PBS, they were fixed for 1 h using 1% osmium acid. The samples underwent dehydration of gradient ethanol and acetone (Changsha Zhenxiang Biotechnology Co., Ltd.) and then were soaked overnight in epoxy resin EMbed 812 (Electron Microscopy Sciences). Polymerization was performed at 40 and 60°C for 24 h. Finally, LKBV ultra-thin slicing machine (Guangzhou Jiuying Machinery Equipment Co., Ltd.), was used to prepare the samples at 500-600A thickness. After uranium acetate (Suzhou Weiboyi Chemical Co., Ltd.) and lead citrate double staining, the samples were observed using transmission electron microscope. The number of apoptotic cells was counted. There were 5 fields in each pore under high-power lens, and the average value was calculated and analyzed.

Granulocyte macrophage-colony stimulating factor evaluation. The level of GM-CSF was determined by commercially available ELISA kit (GM-CSF ELISA kit; Shenzhen Dako Biotechnology Co., Ltd.). The procedure was performed according to manufacturer protocol. Briefly, the absorbance (OD) value of samples was determined at 450 nm wavelength (n=3). The regression equation resulted from drawing standard curve using standard absorbance (OD) and its corresponding concentration. The absorbance value of the sample and the content of GM-CSF that was calculated according to the regression equation, were used for the statistical analysis.

Statistical methods. Statistical analysis was carried out by using SPSS statistical software (version 20.0; IBM SPSS, Armonk, NY, USA). Data are presented in mean \pm standard error (SE). Differences in measured parameters among the seven groups were analyzed with one way ANOVA test followed by Least Significant Difference post hoc. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of white mange mixture on PCNA. The PCNA protein expression is shown in Table II and Fig. 1. PCNA in negative control group showed the highest grey value with regard to the experimental groups. The mice in the positive control group (vaginal psoriasis model groups) showed lowest color level around 98.17. It was also observed that the grey value of the white mange mixture group was significantly increased by increasing the dose to 23 mg. In addition, the grey values of high- and medium-dose groups were very close. Also, average grey value in low white mange mixture, acitretin and Xiaoying granule groups were similar. However, the inhibitory effect of TCM in high- and-medium dose groups was more obvious than acitretin group and Xiaoying granules group.

The results suggested that white mange mixture is effective on vaginal psoriasis compared with acitretin and Xiaoying granules. Low-dose of white mange mixture had similar effect to acitretin and Xiaoying granules on inhibiting the cell proliferation of mouse vaginal epithelium KC PCNA.

T lymphocyte cell apoptosis. The T lymphocyte cell apoptosis is shown in Fig. 2. T lymphocytes in the negative control group

Table II. The expression of PCNA in the vaginal tissue of mice.

Groups	Dose (mg/kg/day)	Average grey value
Negative control group	-	180.03±11.24 ^{b,c,e}
Positive control group	-	98.17±5.91 ^{a,c,e}
Acitretin group	5.75	119.31±5.23 ^{a,b}
Xiaoying granules group	1.219	116.33±7.75 ^{a,b}
High-dose white mange mixture group	23	140.83±4.13 ^{a-c,e}
Medium-dose white mange mixture group	11.5	137.33±3.46 ^{a-d}
Low-dose white mange mixture group	5.75	$115.28 \pm 2.97^{a,b}$

^aP<0.01, compared to negative control group; ^bP<0.01, compared to positive control group; ^cP<0.01, compared to Acitretin group; ^dP<0.05 and ^cP<0.01, compared to Xiaoying granules group. PCNA, proliferating cell nuclear antigen.



Figure 1. PCNA protein expression in the vaginal tissue in mice (DAB chromogenic). (A) Negative control group, (B) positive control group, (C) acitretin group, (D) Xiaoying granules group, (E) high-dose white mange mixture group, (F) medium dose white mange mixture group, and (G) low-dose white mange mixture group. Magnification, x200. PCNA, proliferating cell nuclear antigen.



Figure 2. The effect of white mange mixture on T lymphocyte apoptosis in mice (uranium acetate, citrate double staining). (A) Negative control group, (B) positive control, (C) acitretin group, (D) Xiaoying granules group, (E) high-dose white mange mixture group, (F) medium-dose white mange mixture group, and (G) low-dose white mange mixture group. Magnification, x400.

had normal appearance; the nucleus did not show any segmentation. T lymphocyte cells in positive control group showed normal but large nucleus compared to negative control group. In all the white mange mixture groups cell apoptosis was significantly observed but apoptosis ratio in high-dose group $(4.20\pm3.0 \text{ cells})$ and medium-dose group $(4.00\pm2.0 \text{ cells})$

Table III. GM-CSF levels in serum of each group of mice.

Groups	GM-CSF (pg/ml)
Negative control group	10.41±0.36 ^{b,d,f}
Positive control group	$21.83 \pm 0.47^{a,d,f}$
Acitretin group	12.02±0.41 ^{a,b}
Xiaoying granules group	14.29±0.36 ^{a,b}
High-dose white mange mixture group	$14.41 \pm 0.81^{a,b}$
Medium-dose white mange mixture dose	14.92±0.42 ^{a-c}
Low-dose white mange mixture group	$16.87 \pm 0.36^{a,b,d,e}$

^aP<0.01, compared to negative control group; ^bP<0.01, compared to positive control; ^cP<0.05 and ^dP<0.01, compared to Acitretin group; ^cP<0.05 and ^fP<0.01, compared to Xiaoying granule group. GM-CSF, granulocyte colony stimulating factor.

(nucleus was big and circular and heterochromatin was distributed in the periphery) were increased compared to low-dose group (4.0 ± 2.0 cells). Apoptosis was also observed in Xiaoying granules (2.00 ± 1.0 cells) and acitretin groups (2.20 ± 1.0 cells) but apoptotic trend was decreased compared with white mange mixture groups (Fig. 2).

Serum GM-CSF levels. Serum GM-CSF levels are shown in Table III. Serum GM-CSF levels in negative control and positive groups were 10.41 and 21.83 pg/ml, respectively. Compared with the negative control group, the level of GM-CSF in the serum of the psoriasis model increased significantly (P<0.01). In our results, acitretin group showed decrease in serum GM-CSF levels to minimum compared to all treatment groups. GM-CSF serum levels were decreased in Xiaoyin granules and white mange mixture group, at high- and medium-dose, but there was no significant difference between these groups (P>0.05). The results indicated that the effect of white mange mixture, acitretin and Xiaoyin granules was similar on inhibiting the inflammatory cytokine GM-CSF serum levels.

Discussion

Basic clinical signs of psoriasis are erythema, papule, differentiation and proliferation of KCs (29-31). The vaginal epithelial hyperplasia can be used as a murine model of vaginal psoriasis (32). In this model, estrogen (which can simulate the rapid growth of epidermal hyperplasia) ejection significantly increases mitosis and cell proliferation (33,34).

SP immunohistochemistry results showed that white mange mixture in different doses had inhibitory effect on mouse vaginal epithelium PCNA protein expression and the effect was better than that of Xiaoying granules and acitretin. This finding leads to the conclusion that white mange mixture had inhibitory effect on mouse vaginal epithelium PCNA expression. Zhang *et al* (35) detected the effect of white mange mixture on mitosis and PCNA expression in vaginal epithelium of mice with psoriasis in estrogen stage by using immunohistochemistry methods. They confirmed that white mange mixture can promote the formation of granular layer in the tail scale model of mice, and reduce the expression of PCNA in the vaginal epithelium of the female mice. In our study white mange mixture reduced also the expression level of PCNA in the vaginal epithelium.

Liu *et al* (36) studied the effect of Xiaoyin granules on histopathology of skin lesions and PCNA content in guinea pigs with psoriasis. The results of studies showed that Xiaoyin granules can play an important role in the treatment of psoriasis by improving the histopathological score and inhibiting the expression of PCNA. Results of Liu *et al* were similar to our Xiaoyin granule results on serum PCNA expression.

Zhou and Yu (37) observed the clinical effect of Xiaoyin capsule on psoriasis vulgaris blood heat syndrome and explored its effect on Ki-67 and PCNA expression in skin lesions, showing that the expression of PCNA in the skin lesions of the patients before treatment was strongly positive, and the expression intensity was significantly higher than in normal skin (P<0.05). After treatment, the expression intensity was significantly decreased, showing a weak positive expression or near normal skin expression. Therefore, Xiaoyin capsule can achieve therapeutic effect by inhibiting the proliferation of epidermal cells.

T lymphocytes have a key role in the pathogenesis of psoriasis (38). Many studies have shown that dermal infiltration of T lymphocytes is an important pathological feature of psoriasis (39,40). The relation between cytokines and psoriatic lesions is unclear and requires further research (41,42). According to our electron microscope findings, apoptotic appearance was observed in the high and middle white mange mixture groups. The cause of psoriasis lies mainly in the abnormality of T lymphocyte activation. Yuan and Li (43) studied the activation and regulation of T cells in the model of guinea pig with psoriasis and proved that the white mange mixture can improve the excessive proliferation of skin in guinea pigs with psoriasis. Their data show correlation with our findings. According to our results, white mange mixture induced apoptosis of T cells, as pictured by the atomic force microscope. Chen et al (44) investigated the effects of white mange mixture on the expression level of IL-6 and CXCR2 protein ratio in HaCa T cells. Their results showed that white mange mixture inhibits the secretion of IL-6 and reduce the expression of IL-6 mRNA and CXCR2 protein during psoriasis treatment. Xu et al (45) detected T lymphocyte subsets in peripheral blood before and after treatment with white mange mixture and the results showed that white mange mixture has few side effects and low relapse rate in treatment of psoriasis.

GM-CSF is now considered to be involved in the chronic phase of inflammatory and autoimmune diseases (46,47). GM-CSF has a role in the regulation of neutrophils (48,49). In addition, GM-CSF promotes the secretion of IL-1 cytokines and is involved in the pathogenesis process of psoriasis (50). This research adopted the ELISA method to detect mouse serum GM-CSF concentrations. The results showed that GM-CSF concentration in mouse serum of the psoriasis model controls were higher than the negative control group showing that GM-CSF have a key role in the pathogenesis of psoriasis. GM-CSF levels of Chinese medicine group with different doses, were significantly lower than the positive control group. The data showed that white mange mixture inhibited the expression of GM-CSF serum levels mainly in high- and medium-doses. Yang *et al* (51) detected the level of cytokine IL-2, -6 and -8 in the skin lesion of the guinea pig psoriasis model by radioimmunoassay and the effect of white mange mixture on the cytokine in the skin lesion was observed. The results showed that white mange mixture may inhibit the proliferation of KCs and regulate cellular inflammatory factors. Studies showed that the levels of GM-CSF in psoriatic lesions and serum are increased (52,53). Cai *et al* (54) studied the effect of Xiaoyin granules on the expression of monocyte chemoattractant protein-1 (MCP-1), macrophage colonystimulating factor (M-CSF) and macrophage inflammatory protein-1 α (MIP-1 α) in patients with psoriasis and its results showed that Xiaoyin granules can improve the expression of MCP-1, M-CSF and MIP-1 α in patients with psoriasis, by improving the state of oxidative stress and inhibiting the local inflammatory responses.

In conclusion, our study showed that the white mange mixture has some unique effects: i) inhibition of proliferation of mouse vaginal epithelium; ii) KC cell PCNA protein expression; iii) regulation and induction of apoptosis to T lymphocytes; and iv) decrease in GM-CSF serum levels. However, the mechanism of action is not yet completely revealed, and further research is needed to unmask intracellular pathways.

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Availability of data and materials

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

Authors' contributions

JG contributed to the conception and design of the study. JG and JL were responsible for the collection and assembly of the data, and wrote the manuscript. JL was involved in the data analysis and interpretation. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Guiyang University of Chinese Medicine (Guiyang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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