Anzi Heji Downregulates DNMT1 to Improve Anticardiolipin Antibody (ACA)-Positive Abortion by Regulating JAK/STAT Pathway

Li Miao¹ and Qibin Lu²

Abstract
Anzi Heji (AZHJ) is a traditional Chinese medicine compound prepared for long-term treatment of Anticardiolipin Antibody (ACA)-positive abortion, with small side effects and definite curative effect. Abortion was reported to be related to DNMT1, a methylation transferase regulated by JAK2 pathway, so this study aimed to explore whether AZHJ treated ACA-positive abortion by regulating the DNMT1. Cell proliferation estimation employed Cell counting kit-8 (CCK-8) and flow cytometry. Human β2-glycoprotein I (GPI) was used as an inducer to establish ACA-positive mice model. Western blot was applied to examine the expressions of DNMT1, FOXP3, IL-6, and JAK/STAT3 pathway-related proteins. ACA titers and IL-6 levels in peripheral blood were tested by enzyme-linked immunosorbent assay (ELISA). Placental tissue damage was assessed by hematoxylin and eosin (H&E) staining. Based on the findings from experiments, AZHJ could significantly inhibit apoptosis and regulate the proliferation activity of HTR-8/SVneo cells. AZHJ treatment reduced the expression levels of DNMT1, FOXP3, IL-6, and JAK/STAT3 signaling pathways-related proteins in HTR-8/SVneo cells and maternal–fetal interface (uterine decidua and placenta), and the titer of serum ACA was also significantly decreased. In addition, AZHJ effectively alleviated placental tissue damage caused by ACA-positive abortion compared with model group. To sum up, AZHJ may play a therapeutic role by inhibiting DNMT1 activation through Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, and then promoting FOXP3 expression in maternal–fetal interface of pregnant mice, thereby improving immune tolerance at the maternal–fetal interface, preventing and treating ACA-positive abortion.

Keywords
Anzi Heji, ACA-positive abortion, DNMT1, FOXP3, JAK/STAT3 pathway

Introduction
A previous study showed that 26.4% of adverse pregnancies are related to anticardiolipin antibody (ACA) positivity,¹ which mainly damages the placental villous vascular epithelial cells, causes clotting disorders, placental vascular embolism, and tissue infarction, and then trigger fetal abortion.²,³ At present, the main clinical treatment methods for ACA-positive abortion are immunotherapy and anticoagulant therapy. However, immunotherapy-related drugs such as glucocorticoid, immunoglobulin, aspirin, and low-molecular-weight heparin may cause fetal malformation, liver and kidney function damage, and other toxic and side effects.⁴,⁵ Similarly, anticoagulant therapy may also cause widespread bleeding tendency and gastrointestinal irritation, and other adverse reactions.⁶ Therefore, ACA-positive abortion is urgently needed to find new alternative drugs.

Traditional Chinese medicine has a variety of immunomodulatory powers and has been used to prevent miscarriage with few side effects.⁷,⁸ The aqueous extract from the Chinese medicine prescription Anzi Heji (AZHJ) has been approved as a prescription drug by the Jiangsu Province Hospital of Traditional Chinese Medicine, China (approval No. 051226). In recent years, it has been applied for the prevention of spontaneous abortion (particularly in ACA-positive cases) with proven efficacy, with an overall response rate of over 85%, and the rate of ACA-positive to ACA-negative was 90%.⁹ Our previous study showed that AZHJ could significantly reduce the serum ACA content in model mice and improve the proliferation activity of

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trophoblast cells, markedly ameliorate the clinical symptoms of ACA-positive pregnant women at risk of spontaneous abortion and improve pregnancy hormones levels. AZHJ could also regulate peripheral blood and maternal–fetal interface Treg cells, and effectively increase the proportion of peripheral blood Treg cells in patients with ACA-positive abortion. However, the therapeutic mechanism of AZHJ is not fully understood.

JAK/STAT is an essential pathway for signal transduction of many cytokines and extensively engaged in cell proliferation, differentiation, apoptosis, and immune regulation. JAK/STAT and its downstream signals are closely related to immune infertility, and make a difference in the proliferation and differentiation of trophoblasts. Hou et al noted that p-STAT3 could combine with FOXP3 promoter to reduce its activity in patients with autoimmune diseases. FOXP3 belongs to the transcription factor forkhead/wing-helix family and mainly regulates the function of transcription factors. Recently, it has been found that the methylation transferase DNMT1 may be regulated by STAT3 pathway, and veri

**Results**

**AZHJ Promotes Trophoblast Cell Proliferation**

AZHJ is composed of 12 kinds of Chinese medicinal materials such as mulberry, parasitic dodder, ramic, root daneshen, scutellaria, atrac-tylodes, radix pseudostellariae, and licorice. Early investigations have uncovered that baicalin, Salvianolic acid B, and glycyrrhizic acid could be used as effective active substances in the treatment of abortion. Therefore, high-performance liquid chromatography (HPLC) analysis of the components of AZHJ compound found that it also contained these 3 active components (Figure 1A). Subsequently, the impact of AZHJ on the viability of trophoblast cells was estimated. Cell counting kit-8 (CCK-8) showed that both low and high doses of AZHJ significantly promoted cell proliferation compared with untreated control group (Figure 1B). Further, flow cytometry indicated that both low and high-dose AZHJ resulted in a reduced apoptosis rate relative to the control group (Figure 1C and D), indicating that AZHJ has the effect of promoting trophoblast cell growth and the effect is similar to that of aspirin, which is also used to treat ACA-positive abortion. In addition, we also detected the level of IL-6, an inflammatory factor in the supernatant of cells, after the intervention of different groups. The results in Figure 1E demonstrated that no significant difference was observed in IL-6 level in each group, indicating that Chinese and Western drugs did not inhibit or activate cell inflammation under normal conditions.

**AZHJ Inhibits the Activation of DNMT1 in Vitro via JAK/STAT Pathway**

To further verify the regulation of AZHJ on JAK/STAT pathway and its influence on DNMT1, RT-PCR was performed to detect the gene expressions of JAK, STAT3, IL-6, and DNMT1 in HTR-8/SVneo cells treated with different drugs (Figure 2A). In contrast to the control group, AZHJ significantly inhibited the gene expressions of JAK, STAT3, IL-6, and DNMT1, and the inhibitory effect of high concentration AZHJ was better than low concentration AZHJ, similar to the positive drug aspirin. In addition, we further examined p-STAT3 and DNMT1 protein expression levels by Western blot (Figure 2B) and found that AZHJ inhibited p-STAT3 and DNMT1 protein expressions concentration-dependently.

**AZHJ Promotes the Expression of FOXP3 in Vivo by Preventing JAK/STAT Pathway to Activate DNMT1**

In the above cell experiments, we have demonstrated that AZHJ inhibits intracellular DNMT1 activity by inhibiting the JAK/STAT pathway. To further confirm this conclusion, we established ACA-positive mouse model with human β2-glycoprotein I (GPI) as an inducer. After treatment with different groups, the levels of STAT3, IL-6, DNMT1, and FOXP3 in mice uterine decidua were assayed employing reverse transcription-quantitative PCR (RT-qPCR) (Figure 3A). The findings illustrated that AZHJ conspicuously inhibited the expressions of STAT3, IL-6 and DNMT1 concentration-dependently relative to the model group, and the inhibitory effect was similar to aspirin. It is noteworthy that AZHJ effectively promoted FOXP3 mRNA expression level, which may benefit from AZHJ’s inhibitory effect on methyltransferase DNMT1. It can be seen in detail that AZHJ inhibited the protein level of DNMT1 and promoted the stable expression of FOXP3 (Figure 3B), thus facilitating the differentiation and proliferation.
of Treg cells and enhancing body’s immune tolerance. In addition, the placentas of mice were tested for all of these indicators and showed the same results (Figure 4A and B).

AZHJ Effectively Reduces ACA and IL-6 Expressions and Alleviates Tissue Damage Induced by β2-GPI

Subsequently, compared with the control group, the levels of ACA and IL-6 in model group were also elevated, indicating that the model was successfully established. However, AZHJ effectively reduced abnormally elevated ACA and IL-6 levels in mice treated with β2-GPI (Figure 5A and B). These findings explained that AZHJ can reduce the β2-GPI-triggered adverse response in pregnant mice with positive ACA. For the determination of the recovery influence of AZHJ on the tissue damage induced by the model, mice’s placenta was stained with hematoxylin and eosin (H&E). As shown in Figure 5C, β2-GPI-induced placenta tissues showed obvious shallow

Figure 1. Anzi Heji (AZHJ) promotes trophoblast cell proliferation. (A) High-performance liquid chromatography (HPLC) was used to detect the active components of AZHJ. (B) The cell viability after 24 h treatment with aspirin, low-dose or high-dose AZHJ was detected by cell counting kit-8 (CCK-8). (C-D) Cell apoptosis was detected by flow cytometry. (E) The expression level of inflammatory cytokine IL-6 in supernatant was detected by enzyme-linked immunosorbent assay (ELISA).

*P < .05, **P < .01, ***P < .001 versus Control.
Figure 2. Anzi Heji (AZHJ) inhibits the activation of DNMT1 in vitro via JAK/STAT pathway. (A) JAK, STAT3, IL-6, and DNMT1 mRNA expression in HTR-8/SVneo cells in the 4 groups were analyzed by utilizing reverse transcription-quantitative PCR (RT-qPCR) analysis. (B) The protein levels of DNMT1, STAT3, and P-STAT3 in HTR-8/SVneo cells were examined by adopting Western blot.

*P < .05, **P < .01 versus Control.

Figure 3. Anzi Heji (AZHJ) promotes the expression of FOXP3 in mice uterine decidua by inhibiting the activation of DNMT1 by the JAK/STAT pathway. (A) The mRNA expressions of JAK, STAT3, IL-6, and DNMT1 in uterine decidua of pregnant mice were analyzed by applying reverse transcription-quantitative PCR (RT-qPCR). (B) The protein levels of DNMT1 and FOXP3 in decidua of pregnant mice were detected by Western blot.

*P < .05, **P < .01 versus Control; #P < .05 and ##P < .01 versus Model.
staining, immune/inflammatory cell infiltrates and cell disruption compared with that in Control group. And, low and high-dose AZHJ effectively enhanced the staining of the tissues and the No. of cells was increased.

Discussion

AZHJ has achieved remarkable results in treating ACA-positive abortion by regulating the immune response of the body, but the molecular mechanism of its effect remains unclear. This study aimed to explore whether AZHJ treated ACA-positive abortion by regulating the DNMT1 through JAK/STAT3 signaling pathway. The results demonstrated that AZHJ could promote trophoblast cell proliferation and inhibit the activation of DNMT1 in vitro via JAK/STAT pathway, and AZHJ also could promote the expression of FOXP3 in vivo by preventing JAK/STAT pathway to activate DNMT1, thereby alleviating β2-GPI-induced tissue damage.

The JAK/STAT3 signaling is known to function in modulating the immune tolerance of maternal and fetal. In a recent study, hyperphosphorylation of STAT3 downregulated STAT5 and FOXP3 expression and impaired Treg cell function in unexplained recurrent abortion. Another study also showed an increased p-STAT3 level in unexplained spontaneous abortion, which was accompanied by increased levels of IL-23 and IL-6. On the basis of the findings from the current study, the expression of JAK/STAT3 pathway-related proteins was upregulated under the action of ACA, which indicated that one of the pathological mechanisms of pregnancy loss caused by ACA was the activation of JAK/STAT3 signaling pathway, which resulted in abnormal expression of related proteins and decreased proliferation activity of trophoblasts, thus causing damage to maternal–fetal interface tissue and resulting in pregnancy loss. This was in accordance with the outcomes of Liu et al.

Furthermore, studies have shown that infertility is related to the methylation level of decidual and trophoblast cells in early pregnancy. DNA methyltransferase (DNMT) is an enzyme promoting the methylation process in CNS2 region, which can inhibit the epigenetic effect of Treg cell activation. In patients with autoimmune diseases, p-STAT3 binds closely with DNMT1, thereby inducing an increase in upstream FOXP3 methylation and a decrease in FOXP3 promoter activity. It should be noted that the stabilization of FOXP3 motifs is the key to Treg cell production. Previously, we used CD4+ CD25+ FOXP3 as the marker to explore the role of Treg cells in maintaining pregnancy, which indicated that low FOXP3 expression might be related to repeated abortion. Therefore, the mechanism of AZHJ for ACA-positive abortion may be to reduce DNMT1 expression to promote the stable expression of FOXP3, and to increase Treg cells to enhance immune tolerance. This study also proved that AZHJ could inhibit the activation of DNMT1 by JAK/STAT pathway, and effectively improve the expression of FOXP3 in mice uterine decidua and placenta.

H4R antagonist, JNJ77777120 (JNJ) decreased the inflammatory cytokines and chemokines, such as IL-1β, TNF-α, MIP-2, and MCP-1 in the prophylactic and the

Figure 4. Anzi Heji (AZHJ) promotes the expression of FOXP3 in placentas of mice by inhibiting the activation of DNMT1 by the JAK/STAT pathway. (A) The mRNA expressions of JAK, STAT3, IL-6, and DNMT1 in placentas of mice were analyzed by utilizing reverse transcription-quantitative PCR (RT-qPCR). (B) The protein levels of DNMT1 and FOXP3 in placentas of mice were tested with the use of Western blot.

*P < .05, **P < .01, ***P < .001 versus Control; #P < .05 and ##P < .01 versus Model.
therapeutic treatments of rheumatoid arthritis. A2AR agonist improved neuroimmune dysfunction through the regulation of CD14+TLR2+ cells, CD14+TLR3+ cells, CD14+TLR4+ cells, and CD14+IL-27+ cells, and decreased the TLR2, TLR3, TLR4, and NF-κB p65 in brain tissue of BTBR T+Itpr3tf/J (BTBR) mouse. JNJ reduced the proportion of immune cell percentages (CD3(+), CD8(+), CD28(+), and CD4(+)CD28(+)) and the production of IL-2(+), IL-6(+), IL-9(+), IL-21(+), and IL-27(+) cytokines in stress-induced immune responses. The above studies indicated that the immune diseases could be improved by reducing immune cells and inhibiting inflammation. Here, AZHJ treatment effectively reduces immune cells and IL-6 level to alleviate the inflammation of placental tissue to varying degrees.

Although our study could provide valuable guidance for understanding the therapeutic mechanism of AZHJ, the actual findings revealed that AZHJ could remarkably repress DNMT1 and restore FOXP3 expression relative to the model group, but this effect was not obvious in terms of protein expression. In addition to the higher sensitivity and specificity of RT-qPCR compared with Western blot, the possible reason is that the process of translating mRNA into protein involves complex regulatory mechanisms, which requires further research. In addition, Chinese herbal compound preparations are often regulated by multiple targets in the treatment of diseases. This means that the other mechanisms of AZHJ in the treatment of ACA-positive abortion still need further research. Moreover, the underlying mechanisms need to be further investigated in transcription signaling. And, the present study was conducted at the cellular and animal levels, and the community should be focused on people in future study.

Figure 5. Anzi Heji (AZHJ) effectively reduces Anticardiolipin Antibody (ACA) and IL-6 levels in vivo and alleviates tissue damage induced by β2-glycoprotein 1 (GPI). (A and B) Effects of AZHJ on the levels of ACA and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA) in mice serum. (C) Hematoxylin and eosin (H&E) staining was adopted for estimating the effect of AZHJ on placental tissue injury induced by β2-GPI.

***P < .001 versus Control; #P < .05, ##P < .01, and ###P < .001 versus Model.
In conclusion, the present study demonstrated that AZHJ promoted trophoblast cell proliferation and inhibited the activation of DNMT1 via JAK/STAT pathway in vitro, and AZHJ also could promote the expression of FOXP3 in vitro by preventing JAK/STAT pathway to activate DNMT1, thereby alleviating β2-GPI-induced tissue damage. Our findings imply that DNMT1 and FOXP3 may be potential targets for clinical prevention and treatment of ACA-positive spontaneous abortion.

Materials and Methods

AZHJ Preparation and HPLC Analysis

The extraction and quality control of AZHJ were carried out based on our previous research.10 HPLC analysis was performed on a Thermo Accure AQ (50 mm × 2.1 mm, maintained at 35°C. The injection volume was 5 µL. The mobile phase (1.0 mL/min) consisted of 60% acetonitrile (A) and 0.1% formic acid aqueous solution (B). The following gradient program was employed: 10% B (0-5 min), 70% B (5-15 min), 100% B (15-60 min), and the UV detection wavelengths were set at 254 nm.

Cell Culture

HTR-8/SVneo cells were provided by Wuhan Punosai Life Technology Co., LTD. Cells were kept in culture in Roswell Park Memorial Institute (RPMI)-1640 (Gibco; Thermo Fisher Scientific, Inc.) to which 5% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Beyotime Biological Technology Co., LTD) were added and then placed in a humidified incubator at 37°C with 5% CO2. The medium was changed every 2 days and the cells were passaged at a ratio of 1:3. Cells in the logarithmic growth phase were harvested for follow-up experiments.

CCK-8 Assay

96-well plates were adopted to load the HTR-8/SVneo cells (1 × 10^5 cells/well) and incubated together at 37°C and 5% CO2. When cell growth fusion reached approximately 80%, cells were incubated with low-dose AZHJ (3.77 g/mL), high-dose AZHJ (7.54 g/mL), and positive control aspirin (0.975 mg/mL) for 24 h. Then, 10 µL CCK-8 solution (Beyotime Biological Technology Co., LTD) was employed to culture the cells for 4 h. Optical density measurement was carried out at 450 nm with the application of a microplate reader (Multiskan FC, Thermo Fisher Scientific, Inc.).

Flow Cytometry Analysis of Cell Apoptosis

HTR-8/SVneo cells were inoculated in 6-well culture plates (1 × 10^5 cells/well). After adherence, these cells were incubated with low-dose AZHJ (3.77 g/mL), high-dose AZHJ (7.54 g/mL), and positive control aspirin (0.975 mg/mL) for 24 h, respectively. The medium was discarded and washed utilizing PBS. Each group of cells was subjected to 5 min of centrifugation at 1000 g. Annexin V-FITC Apoptosis Detection Kit (Beyotime Biotechnology Co., cat. No. C1062S) was employed to estimate cell apoptosis. 200 µL Annexin V-FITC and 5 µL PI were added into the HTR-8/SVneo cells at 20-25°C for 20 min. The samples were then analyzed by flow cytometry (BD, Franklin Lakes, NJ) on ice.

Western Blotting Analysis

Total protein was obtained by lysis of HTR-8/SVneo cells, placental tissues, or uterine membrane making use of ice-cold RIPA lysis buffer (Beyotime Biotechnology Co., cat. No. P0013C), followed by an identification of protein concentration with BCA protein assay kit (Bio-Rad Laboratories, Inc.). After that, protein samples (20 µg/lane) were subjected to isolation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and moved on the polyvinylidene difluoride (PVDF) membranes, which were then blocked utilizing 5% nonfat milk for 1 h at room temperature. Subsequently, the membranes were co-incubated overnight at 4°C with primary antibodies (all provided by Abcam) against DNMT1 (dilution, 1:1000; ab188453), STAT3 (dilution, 1:1000; ab68153), p-STAT3 (dilution, 1:1000; ab267373), FOXP3 (dilution, 1:1000; ab20034), and GAPDH (dilution, 1:1000; ab181602), followed by adding goat anti-rabbit IgG secondary antibodies (dilution, 1:2000; ab6721) that horseradish peroxidase conjugated for 1 h at room temperature. Following 3 washes in Tris-buffered saline with Tween 20, the membranes were mixed with an enhanced chemiluminescence reagent (GE Healthcare) to make visible the protein blots, which were semi-quantified with the help of ImageJ v1.48 (National Institutes of Health), taking GAPDH as a loading control.

Reverse Transcription-Quantitative PCR

Following the extraction of total RNA from HTR-8/SVneo cells, placental tissues, or uterine membrane employing TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in keeping with the standard procedures of the supplier, RNA concentration was assessed with the aid of a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was generated by reverse transcription of RNA (2.5 µg) with an EasyScript® First-Strand cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd). Subsequently, qPCR was run on a Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific, Inc.) under the conditions: 95°C for 10 min; 40 cycles of 95°C for 10 s and 60°C for 60 s. The primers (GenScript) were presented below: STAT3 forward, 5′-GAGGAGTTGCGACAAAAAG-3′; and reverse, 5′-TGTGTTTGTGAGCCAAATGT-3′; IL-6 forward, 5′-AAC CTGAACCTTCAAAGTG-3′; and reverse, 5′-TCCGAACTTTGAAGGAGC-3′; IL-8 forward, 5′-CTGAGTTGCGACAAAAAG-3′; and reverse, 5′-GGAGTTGCGACAAAAAG-3′; and DNMT1 forward, 5′-AAC CTGAACCTTCAAAGTG-3′; and reverse, 5′-CTGAGTTGCGACAAAAAG-3′; and FOXP3 forward, 5′-CAAGTTCC
AACAATGGACGAC-3' and reverse, 5'-ATTGAGTGTCCGCTGCTTCT-3'; and GAPDH forward, 5'-AGCCACATCGCTGTCAGACAC-3' and reverse, 5'-GCCCAATACGACCAATCC-3'. The quantification of mRNA expressions was undertaken in accordance with the $2^{-\Delta\Delta C_{T}}$ method and GAPDH was deemed to be a standard internal control.

**Enzyme-Linked Immunosorbent Assay**

IL-6 level in serum samples and the levels of ACA and IL-6 in placental tissues were estimated with the utilization of ELISA kits (Shanghai Joyee Biotechnics, Co.) strictly comply with the description of the producer.

**Animal Experiments**

Eight-week-old BALB/c female (25 ± 2 g) and male (30 ± 2 g) mice were supplied by the Comparative Medical Center of Yangzhou University (Yangzhou, China). These mice were kept in specific pathogen-free cages at 20 to 25°C and provided with 50% to 70% humidity and a 12-hour light/dark cycle. During this period, the mice were supplied with standard chow and water.

After the mice adapt to the laboratory environment, the ACA-positive abortion mouse model was established. A total of 20 randomly selected BALB/c female mice were administered with an injection of 400 g/mL β2-GPI for 18 days to establish ACA-positive pregnancy at risk of spontaneous abortion. Another 5 female BALB/c mice were given normal saline for 18 days, which served as normal controls. The female and male mice were randomly chosen to be kept together in cages in a 1:1 ratio, and the onset of pregnancy was determined when vaginal plug appeared and was recorded as day 0. The above BALB/c mice were classified into 5 groups (n = 5): Normal control group (normal saline 100 ml/kg d), model group (normal saline 100 mL/kg d), low-dose AZHJ group (37.7 g/kg d), high-dose AZHJ group (75.4 g/kg d), and aspirin group (100 mL/kg d) were intragastrically administrated for 15 consecutive days. Mice were killed after on day 15, and the expressions of ACA and IL-6 from mice’s peripheral blood were tested by making use of ELISA. After that, placenta tissues were subjected to fixation employing 4% paraformaldehyde and the H&E staining, and fresh placenta and uterus tissues were subjected to Western blot and RT-qPCR detection. All animal-related experiments in this study were carried out in accordance with the approval of the Ethics Committee of the Second Affiliated Hospital of Nanjing University of Traditional Chinese Medicine.

**Statistical Analysis**

Statistical data were generated with the use of SPSS version 19.0 (IBM Corp.). One-way ANOVA with Tukey’s post-hoc test was used for multiple group comparisons of differences, and Student’s t-test was taken to assess differences between the 2 groups. All outcomes were expressed in the form of mean ± standard deviation. P-value below .05 was considered to indicate a statistically significant difference.

**Author’s Contribution**

QB conceptualized and designed critically the current study. LM performed the analysis and interpretation of the experiment data. LM and QB drafted the manuscript and revised it critically for important intellectual content. All authors agreed to be held accountable for the current study in ensuring questions related to the integrity of any part of the work are appropriately investigated and resolved. All authors read approved the final manuscript.

**Availability of Data and Materials**

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

**Declaration of Conflicting Interests**

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

**Ethical Approval**

All animal experiments in this study got approval from the Ethics Committee of the Second Affiliated Hospital of Nanjing University of Traditional Chinese Medicine.

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