

Effect of new zaogong erteng decoction on embryo implantation in mice following treatment of endometritis

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Abstract: Background: New zaogong erteng decoction (NZGETD) is a compound Chinese medicine modified from the Zaogong Erteng Decoction. Previous clinical observations suggested its potential efficacy against endometritis, an inflammatory condition that adversely impacts endometrial receptivity and embryo implantation. **Objectives:** In this study, the role and mechanism of NZGETD in improving embryo implantation were investigated in a mouse model of lipopolysaccharide-induced endometritis. **Methods:** Female mice were treated with 2.5 mg/mL lipopolysaccharide in the endometrium for 24 h to induce endometritis and were subsequently gavaged with NZGETD for 8 days. Pathological changes, inflammatory cytokine levels, uterine index, endometrial decidualization, microangiogenesis and hormone levels were assessed. Network pharmacology analysis was employed to identify potential active components and targets. The expression of key proteins related to endometrial receptivity and implantation was confirmed by western blotting. **Results:** NZGETD attenuated lipopolysaccharide-induced pathological changes and downregulated uterine levels of IL-1 β , IL-6 and TNF- α , indicating its anti-inflammatory effect in this endometritis model. NZGETD significantly increased the uterine index, promoted endometrial decidualization and microangiogenesis (upregulated CD31 on gestational day 4.5). Additionally, NZGETD significantly elevated uterine levels of estrogen and progesterone and increased the number of implantation sites on gestational day 8.5. Network pharmacology analysis screened beta-sitosterol, quercetin, kaempferol and stigmasterol as the common active components of NZGETD. Western blotting confirmed that NZGETD up-regulated ESR, PGR, Hoxa10, integrin β 3, LIF, HIF1 α and VEGF during embryo implantation. **Conclusion:** In a mouse model of lipopolysaccharide-induced endometritis, NZGETD promoted decidualization and microangiogenesis of the endometrium, enhanced endometrial receptivity and facilitated embryo implantation.

Keywords: Endometritis; Endometrial receptivity; Embryo implantation; New zaogong erteng decoction

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INTRODUCTION

Endometritis is a prevalent gynecological disorder characterized by inflammation of the endometrium, which adversely impacts female reproductive health (McQueen *et al.*, 2015). It is primarily caused by bacterial or viral infections. Mild endometritis may manifest with symptoms such as lower back pain, abdominal discomfort, hemorrhage, irregular menstrual cycles and abnormal vaginal discharge. In severe cases, endometritis can decrease endometrial receptivity and affect embryo implantation and reproduction, leading to miscarriage and even infertility (Hu *et al.*, 2024; Park *et al.*, 2016). Antibiotics, such as ceftriaxone, doxycycline and metronidazole, are commonly prescribed treatments for endometritis (Verdon, 2019). Although antibiotics are effective for managing inflammation, they are unable to repair endometrial damage, which may consequently affect conception. Furthermore, long-term administration of high doses of antibiotics may lead to antibiotic resistance and ultimately recurrent endometrial infections (Salmanov *et al.*, 2020).

Chinese herbal medicines are under active clinical research due to their good safety profile, low toxicity and minimal residue (Huang *et al.*, 2025). New zaogong erteng decoction (NZGETD) is a modified formulation

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based on the previously reported zaogong erteng decoction (Li *et al.*, 2025a). It is a compound Chinese medicine comprised of 15 Chinese herbal medicines, such as the spine of *Gleditsia sinensis* Lam. (*G. sinensis* Lam.), *Taraxacum officinale* (*T. officinale*), *Lonicerae Japonicae Caulis* (*L. Japonicae Caulis*), *Sargentodoxa cuneata* (*S. cuneata*), *Angelica sinensis* (*A. sinensis*) and the newly added *Citri Reticulatae Pericarpium* and *Citri Reticulatae Pericarpium Viride*. This prescription has shown promising clinical efficacy with incomparable advantages over other medications in the treatment of endometritis (Li *et al.*, 2025b). Preliminary observations suggest it may not only promote endometrial repair but also improve reproductive outcomes. In particular, the spine of *G. sinensis* Lam. is a detoxifying anti-inflammatory component that boosts uterine circulation and repairs endometrial injury (Li *et al.*, 2016). *T. officinale* possesses antibacterial, anti-inflammatory and hemostatic functions, effectively improving symptoms and preventing endometritis recurrence (Jeon *et al.*, 2008). *S. cuneata* has detoxifying and anti-inflammatory effects, along with the ability to enhance blood circulation, relieve pain and facilitate endometrial regeneration and improvement of menstrual irregularities (Hu *et al.*, 2020). *L. Japonicae Caulis* is abundant in anti-inflammatory and antioxidative flavonoids that can attenuate endometritis symptoms and effectively restore endocrine imbalance (Su *et al.*, 2021).

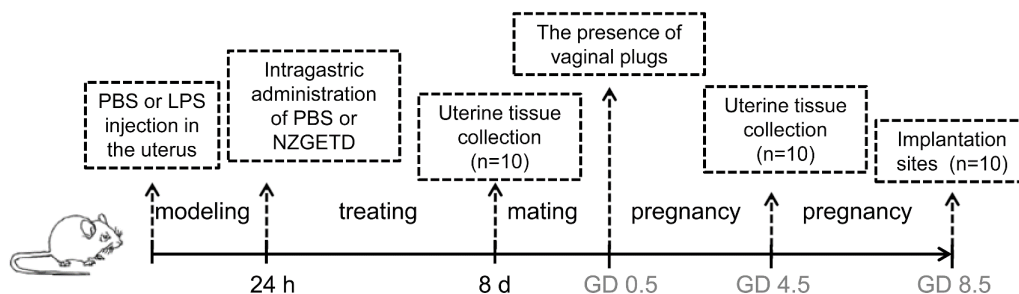


Fig. 1: Flow chart of the experimental procedure for animal treatment and grouping. PBS (phosphate-buffered saline); LPS (lipopolysaccharide); NZGETD (new zaogong erteng decoction).

Furthermore, *A. sinensis* can enhance blood circulation, nourish blood and prevent miscarriage (Nai *et al.*, 2021). The added *Citri Reticulatae Pericarpium* is known to warm the uterus and tonify Qi and blood, while *Citri Reticulatae Pericarpium Viride* promotes Qi circulation and resolves blood stasis, both of which are pertinent to the traditional Chinese medicine (TCM) pathogenesis of endometritis characterized by Qi stagnation and blood stasis (Liu *et al.*, 2025; Zhang *et al.*, 2025; Huang *et al.*, 2024). Considering the pharmacological actions of the above-mentioned herbs, it is speculated that after treating endometritis, the herbal components of NZGETD may synergistically promote uterine endometrial blood circulation, repair damaged endometrium and enhance embryo implantation.

This research examined how NZGETD influences endometrial growth and receptivity following endometritis treatment. The potential active components and targets of NZGETD that might aid in embryo implantation were also explored to better understand its mechanism of action. The results suggest that NZGETD could be a new therapeutic option for enhancing embryo implantation after endometritis.

MATERIALS AND METHODS

Study animals

Sexually mature, 8-week-old Kunming female mice (n=120; weight 30g-35g) were provided by Yisi Experimental Animal Technology Co., Ltd. (Changchun, China). Mice were housed under a 12-h light/dark cycle at a temperature of 22±2°C. The animal experimentation procedures received approval from the Ethics Committee of Jilin Medical College (approval number: 2023-LW001) and all experiments were conducted following applicable guidelines and regulations, adhering to the ARRIVE guidelines.

Preparation of NZGETD

The NZGETD was decocted from 12 g of *G. sinensis* Lam. spine, 30 g of *T. officinale*, 15 g of *L. Japonicae* Caulis, 15 g of *S. cuneata*, 12 g of *A. sinensis*, 9 g of *Cyperus rotundus* L. (*C. rotundus* L.), 15 g of *Poria cocos*, 15 g of *Fructus liquidambaris*, 12 g of *Radix*

Paeoniae Alba, 9 g of *Radix Bupleuri*, 9 g of *Carthamus tinctorius* L. (*C. tinctorius* L.), 9 g of *Semen Persicae*, 9 g of *Radix Linderae*, along with the newly added 12 g of *Citri Reticulatae Pericarpium* and 9 g of *Citri Reticulatae Pericarpium Viride*. Each Chinese medicinal herb was weighed, placed in a medicine casserole pot and boiled according to the TCM decoction methods (Fan, 1997). A high-dose NZGETD (2.24 g medicinal products in 1 mL solution) and a low-dose NZGETD (1.12 g medicinal products in 1 mL solution) were prepared. These NZGETDs were stored at 4°C for subsequent use.

Animal treatment

The female mice were randomized into the control, lipopolysaccharide (LPS), LPS+low NZGETD and LPS+high NZGETD groups (n=30 per group). The flowchart of treatment is presented in Fig. 1. The LPS, LPS+low NZGETD and LPS+high NZGETD groups received a unilateral injection of 20 µL of 2.5 mg/mL LPS (L2755, Sigma, St. Louis, MO, USA) in the uterine horn to induce endometritis, as described in previous studies (Jiang *et al.*, 2021; Zhang *et al.*, 2019), while those in the control group were unilaterally administered phosphate-buffered saline. At 24 h following LPS injection, animals were orally gavaged with 0.067 mL of high-dose NZGETD (equivalent to 7.5 g raw herbs/kg body weight) or 0.067 mL of low-dose NZGETD (equivalent to 3.7 g raw herbs/kg body weight) and phosphate-buffered saline in the control group. The doses were selected based on prior safety data for the related zaogong erteng decoction (Li *et al.*, 2025a) and were well tolerated in the present study. After 8 days, 10 mice from each group were euthanized by cervical dislocation and the uterus was collected. Five uterine tissue samples were fixed and the other five were stored at -80°C. The remaining 20 female mice in each group were paired at a 1:1 ratio with mature male mice and those with a vaginal plug on the following day were considered to be on gestational day 0.5 (GD0.5). On GD4.5, 10 random pregnant mice were chosen from each group, weighed and euthanized. Their uteri were weighed to determine the uterus index, using the formula uterus index (%) = (uterus weight/body weight) × 100%. Again, five uterus samples were subjected to fixation and the other five were stored at -80°C. On D8.5, the remaining 10 pregnant mice in each group were

euthanized and uterine tissues were collected for embryo implantation assessment.

HE staining

Uterine tissues were fixed, dehydrated, embedded in paraffin and sectioned. The sections were then stained with hematoxylin and eosin (HE) following standard protocols and observed under an Olympus BX53 microscope (Tokyo, Japan).

ELISA

Uterine tissue samples were homogenized in ice-cold normal saline at a ratio of 1:9 (weight: volume) using a tissue homogenizer. The homogenates were then centrifuged at 3000 r/min for 15 min at 4°C to collect the supernatant. This standardized homogenization protocol ensured a consistent matrix for the comparison of analyte concentrations across experimental groups. The levels of interleukin-1 β (IL-1 β) (YJ301814), estrogen (YJ063198), interleukin-6 (IL-6) (YJ063159), progesterone (YJ058395) and tumor necrosis factor- α (TNF- α) (YJ002095) were measured using the corresponding ELISA kits (Shanghai Yuanju Biotechnology Co., China). OD450 was determined using the CMax Plus (Molecular Devices, USA).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded uterine sections. After antigen retrieval and blocking, sections were incubated overnight at 4°C with a rabbit anti-cluster of differentiation 31 (CD31) polyclonal antibody (1:200 dilution; Cat# A19014, ABclonal, USA). Subsequently, sections were incubated with a biotinylated goat anti-rabbit IgG secondary antibody (BA1006, Wuhan Boster Biological Technology, China), followed by streptavidin-horseradish peroxidase complex and development with diaminobenzidine. Counterstaining was performed with hematoxylin. The mean optical density of CD31 expression was analyzed by ImageJ.

Analysis of active components and targets of NZGETD

Main chemical component screening and target prediction
The active components and potential targets of each Chinese medicinal component of NZGETD were screened using the TCMSP (<http://tcmsp.w.com/tcmsp.php>), HERB (<http://herb.ac.cn/>) and Swiss TargetPrediction (<http://www.swisstargetprediction.ch/>) with $\geq 30\%$ oral bioavailability (OB) and ≥ 0.18 drug-likeness (DL) as filter conditions. All target information related to TCM active components was standardized to gene names utilizing UniProt (<https://www.uniprot.org/>).

Prediction of NZGETD targets related to embryo implantation

Targets associated with embryo implantation were searched using the keyword "embryo implantation" in PharmGKB, OMIM and GeneCards. Venn analysis of targets of NZGETD active components and embryo implantation was performed to obtain common targets.

Network construction and KEGG analysis

Protein-protein interaction network analysis was conducted using the String 11.5 database (<https://cn.string-db.org/>), with "Homo sapiens" specified as the biological type and a minimum interaction threshold of "highest confidence (>0.95). Subsequently, Cytoscape 3.8.0 (<https://cytoscape.org/>) was used to screen the imported targets. The discrete nodes were excluded from further analysis. After two screenings, the common targets were selected based on betweenness centrality, local average connectivity, degree centrality, eigenvector centrality, network centrality and closeness centrality, all of which exceeded the median. Common targets between NZGETD active components and embryo implantation-related targets were imported into DAVID6.8 for KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis.

Western blot

Proteins were extracted from mouse uterine tissue and quantified. Following protein transfer, the membrane was probed with rabbit anti-estrogen receptor 1 (ESR1) (A16668, ABclonal), anti-estrogen receptor 2 (ESR2) (A2546, ABclonal), anti-progesterone receptor (PGR) (A22061, ABclonal), anti-homeobox A10 (Hoxa10, PB0215, Boster), anti-integrin $\beta 3$ (A19073, ABclonal), anti-leukemia inhibitory factor (LIF, BA1239-2, Boster), anti-hypoxia-inducible factor 1 α (HIF1 α) (PB9253, Boster) and anti-vascular endothelial growth factor (VEGF, A21647, ABclonal). Then, the membrane underwent incubation with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (31210, Thermo Scientific Pierce), enhanced chemiluminescence and visualization with the ChemiDOC XRS+ system (BioRad Laboratories, USA). The relative expression levels of ESR1, ESR2, PGR, Hoxa10, integrin $\beta 3$, LIF, HIF1 α and EGF were measured using Image J.

Statistical analysis

Data, described by mean $\pm$ standard deviation, were analyzed using SPSS 26.0. To compare differences, one-way ANOVA and LSD post hoc tests were employed. A $P < 0.05$ was considered statistically significant.

RESULTS

NZGETD attenuates pathological changes in endometritis

HE staining of uterine tissues showed that the LPS group had severe endometrial damage characterized by partial sloughing of the endometrial epithelium, multiple regions of hemorrhage and structural disruption of uterine glands (Fig. 2). In contrast, the control, LPS+low NZGETD and LPS+high NZGETD groups showed smooth and intact uterine epithelial and normal uterine gland structures without areas of hemorrhage and inflammatory cell infiltration. These data demonstrated that NZGETD significantly attenuated the pathological changes in LPS-induced endometritis.

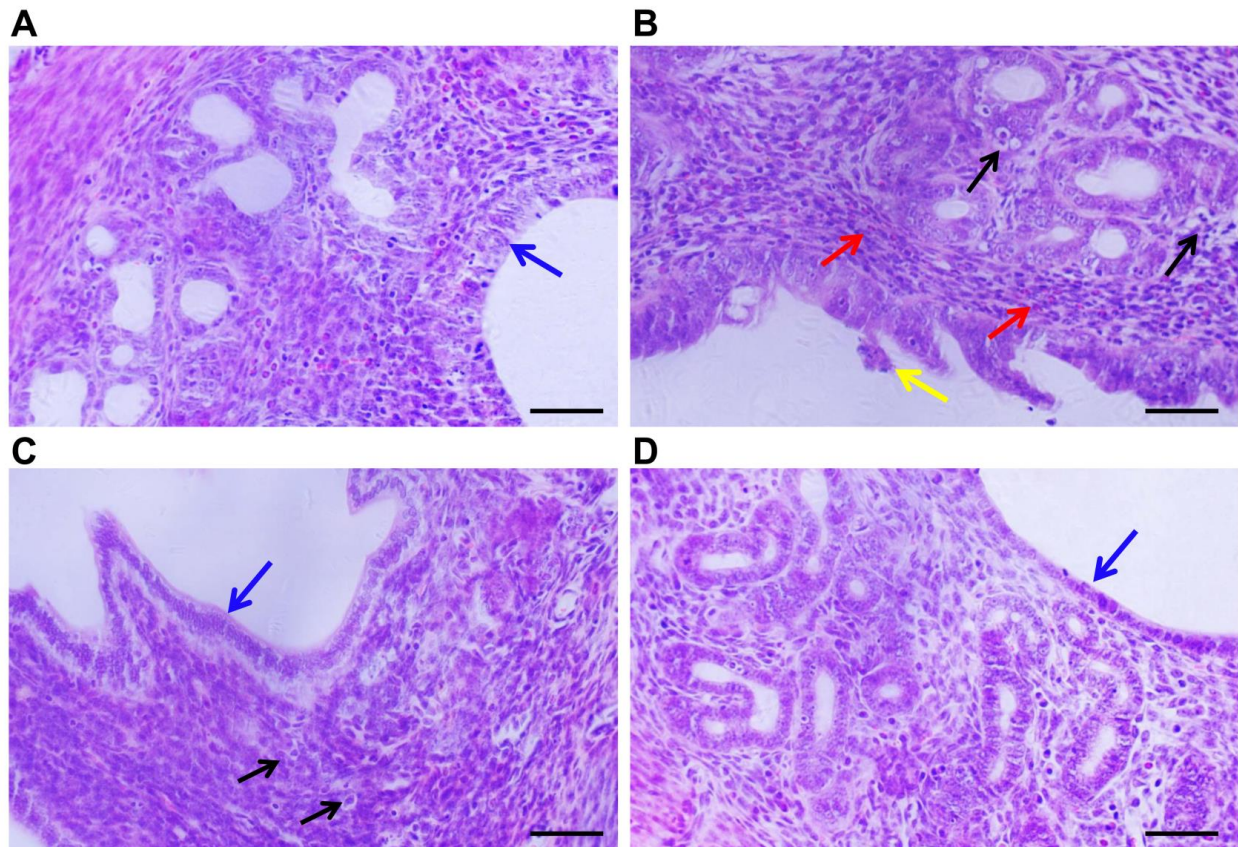


Fig. 2: HE staining of uterine tissues of NZGETD-treated mice (scale bar =100 μ m). (A) Control group; (B) LPS group; (C) LPS+low NZGETD group; (D) LPS+high NZGETD group. Blue arrows: Intact endometrial epithelium; Yellow arrows: Epithelial cell sloughing; Red arrows: Areas of hemorrhage; Black arrows: Infiltration of inflammatory cells.

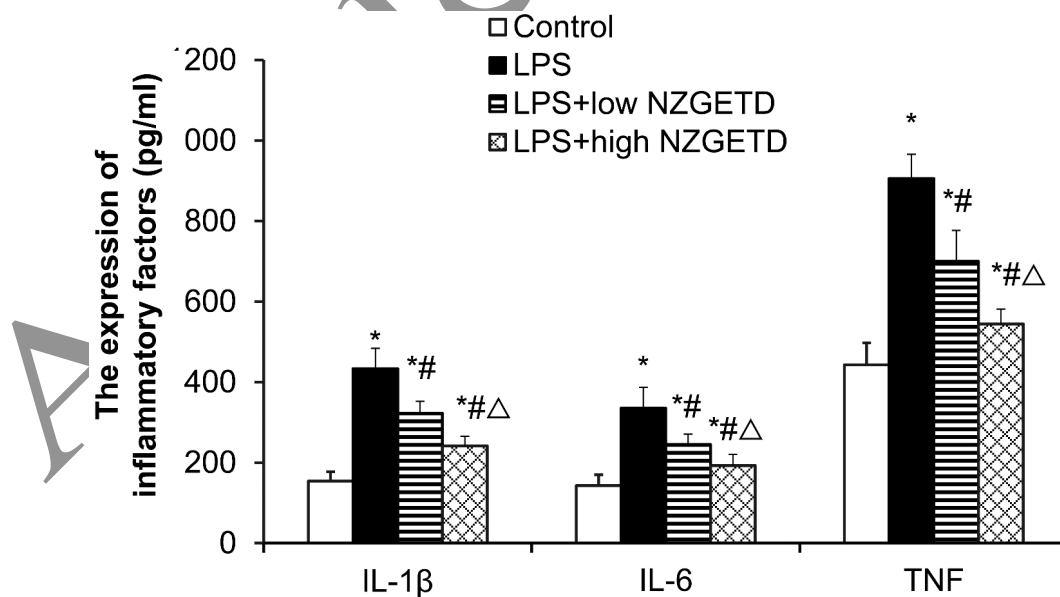


Fig. 3: IL-1 β , IL-6 and TNF- α expression in the uterus (N=5). IL-1 β , IL-6 and TNF- α expression were detected with ELISA. *P<0.05, compared with the control group; #P<0.05, compared with the LPS group; ΔP<0.05, compared with the LPS+low NZGETD group.

Table 1: Mouse uterine weight, body weight, and uterine index (mean $\pm$ standard deviation).

Group	Mean uterine weight (g)	Mean body weight (g)	Uterine index (%)
Control (n=5)	0.24 $\pm$ 0.02	35.20 $\pm$ 3.63	6.98 $\pm$ 0.59
LPS (n=5)	0.19 $\pm$ 0.02*	36.64 $\pm$ 2.90	5.21 $\pm$ 0.84*
LPS+low NZGETD (n=5)	0.21 $\pm$ 0.01*	33.90 $\pm$ 2.22	6.17 $\pm$ 0.61#
LPS+high NZGETD (n=5)	0.21 $\pm$ 0.01*	33.60 $\pm$ 3.29	6.32 $\pm$ 0.56#

Note: * P <0.05, compared to the control group; # P <0.05, compared to the LPS group.

NZGETD downregulates proinflammatory cytokine expression in the uterus

ELISA revealed that IL-1 β , IL-6 and TNF- α expressions were significantly upregulated in the uterine tissues of the LPS group (P <0.05) but significantly downregulated in those of the NZGETD groups (P <0.05) (Fig. 3). The cytokine levels in the LPS+high NZGETD group were significantly lower compared with the LPS+low NZGETD group (P <0.05). Thus, high-dose NZGETD is more potent in inhibiting proinflammatory cytokine production in endometritis.

NZGETD promotes endometrial development and decidualization

To assess the impact of NZGETD on endometrial development, the uterus of mice was weighed and the uterus index was calculated (Table 1). LPS treatment significantly reduced the uterus index, while this effect was attenuated by NZGETD treatment (P <0.05). No significant alterations were found between the NZGETD and control groups (P >0.05). Therefore, NZGETD may promote the development of the endometrium.

Next, the decidualization of the endometrium during the early stage of embryo implantation was examined to further ascertain the effect of NZGETD on endometrial receptivity and embryo implantation. The control group exhibited a loosely structured endometrium with abundant uterine glands and a wide gland lumen (Fig. 4A). The endometrial epithelium was arranged in an orderly manner and the epithelium was smooth, intact and thick with normal decidualization. In contrast, LPS treatment significantly reduced endometrial thickness (P <0.05) (Fig. 4B), along with a lower number of uterine glands with a narrower lumen and markedly inadequate decidualization (Fig. 4A). Statistically, both low and high doses of NZGETD reversed the effect of LPS on endometrial thickness, with the high-dose NZGETD having a more pronounced effect (P <0.05) (Fig. 4B). Furthermore, NZGETD-treated mice had increased uterine glands, wider gland lumen, relatively loose endometrial tissue and improved endometrial decidualization (Fig. 4A). These results suggest that NZGETD could promote the development and decidualization of the endometrium.

NZGETD enhances endometrial microangiogenesis

To examine endometrial microangiogenesis, CD31 protein expression in the endometrium was measured by immunohistochemistry. LPS treatment caused a decreased distribution of CD31-positive microvessels (Fig. 5A) and

significantly downregulated CD31 expression (P <0.05) (Fig. 5B). In contrast, both low-dose and high-dose NZGETD alleviated the effects of LPS treatment, as evidenced by significantly increased distribution of CD31-positive microvessels (Fig. 5A) and upregulated CD31 protein expression (Fig. 5B) (P <0.05). Notably, the effect of high-dose NZGETD on CD31 expression was more prominent than that of low-dose NZGETD (P <0.05) (Fig. 5B). This implies that NZGETD may enhance microangiogenesis in the endometrium.

NZGETD boosts estrogen and progesterone levels in the uterus

ELISA found that LPS and both doses of NZGETD significantly reduced estrogen and progesterone levels in the uterine tissues (P <0.05) (Fig. 6). Compared with LPS, high-dose NZGETD significantly upregulated estrogen and progesterone levels (P <0.05). Notably, high-dose NZGETD had a more pronounced effect than low-dose NZGETD. These results indicate that NZGETD boosts local uterine levels of estrogen and progesterone in mice.

NZGETD facilitates embryo implantation

Uterine tissues were collected from mice on GD8.5 to determine the number of embryo implantations. The number of embryo implantations was significantly different on the treated side, but not on the untreated side, of the uterus among the groups (Fig. 7A). The number of embryo implantations was significantly decreased by LPS treatment (P <0.05) (Fig. 7B). However, this effect was abolished by both doses of NZGETD, with high doses displaying a more pronounced effect (P <0.05) (Fig. 7B). Thus, NZGETD markedly promotes embryo implantation in mice.

NZGETD active components

The common active components of each medicinal component of NZGETD were subsequently analyzed through the TCMSP platform, with $\geq 30\%$ OB and ≥ 0.18 DL as filter conditions. Through this analysis, 11 active components were identified in *G. sinensis* Lam. spine, 5 in *S. cuneata*, 23 in semen persicae, 22 in *C. tinctorius* L., 18 in *C. rotundus* L., 2 in *A. sinensis*, 9 in radix linderiae, 5 in citri reticulatae viride, 5 in citri reticulatae pericarpium, 4 in *Fructus liquidambaris*, 17 in radix bupleuri, 17 in radix paeoniae alba and 15 in *Poria cocos*. Using the HERB database with molecular weight ≤ 500 , cLogP ≤ 5 , Hbond donor ≤ 5 and HBondacc ≤ 10 , 8 active components were identified in *T. officinale* and 5 active components in *L. Japonicae* Caulis.

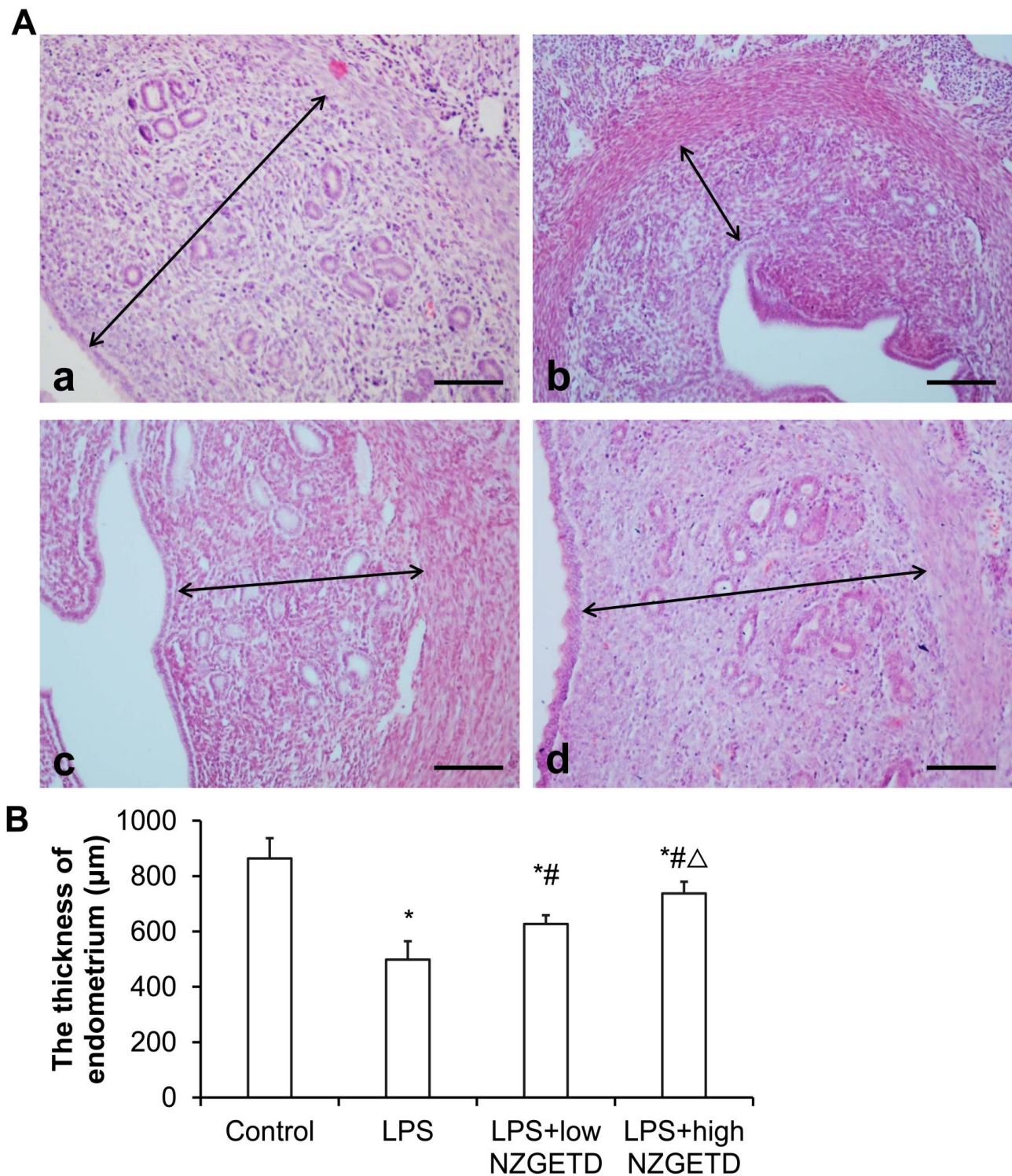


Fig. 4: HE staining of the uterine tissues of pregnant mice on GD4.5. (A) Representative HE staining images (scale bar = 200 μm). (Aa) Control group, (Ab) LPS group, (Ac) LPS+low NZGETD group and (Ad) LPS+high NZGETD group. The straight line with double arrows indicates endometrial thickness; (B) Statistical analysis of endometrial thickness (N=5). * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with the LPS group; Δ $P < 0.05$, compared with the LPS+low NZGETD group.

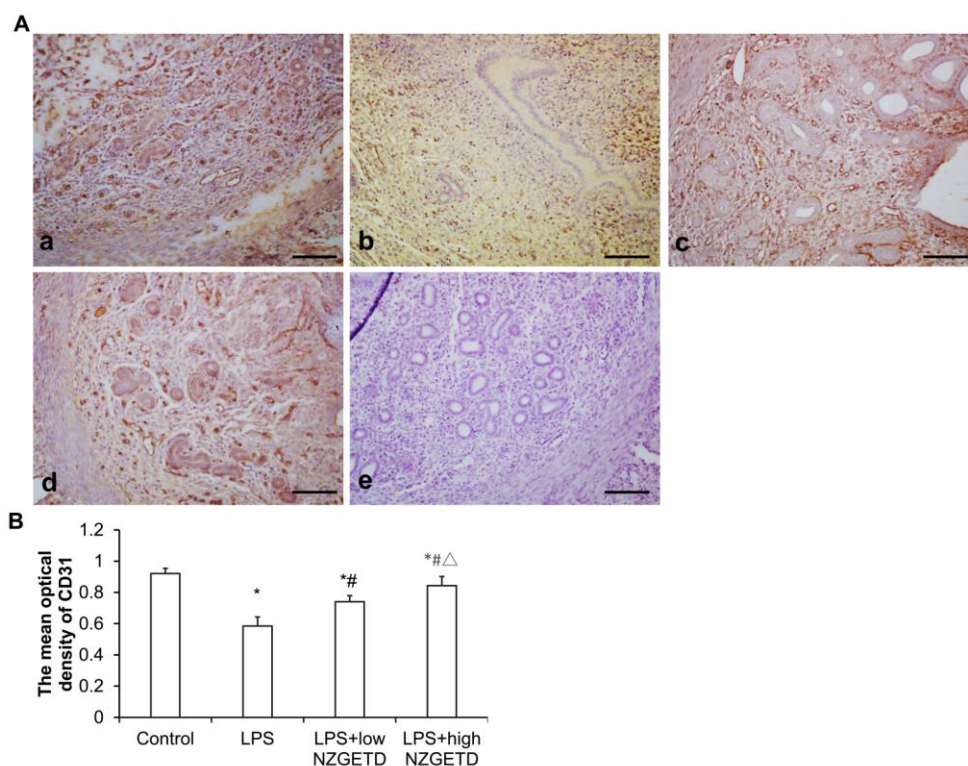


Fig. 5: Immunohistochemical staining of CD31 in the uterine tissues of pregnant mice on GD4.5. (A) Representative immunohistochemical staining images (scale = 200 μ m). (Aa) Control group, (Ab) LPS group, (Ac) LPS+low NZGETD group, (Ad) LPS+high NZGETD group and (Ae) Negative control group. (B) Mean optical density of CD31 expression (N=5). *P<0.05, compared with the control group; #P<0.05, compared with the LPS group; ΔP<0.05, compared with the LPS+low NZGETD group.

Table 2: The common active components of the medicinal herbs in the NZGETD.

Number	ID	Component	OB (%)	DL	Medicinal herb
1	MOL000358	Beta-sitosterol	36.91	0.75	Fructus liquidambaris, Cynanchum otophyllum schneid., Gleditsia sinensis Lam. spine, semen persicae, Cyperus rotundus L., Sargentodoxa cuneata, Angelica Sinensis, Carthamus tinctorius L., Radix linderae
2	MOL000098	Quercetin	46.43	0.28	Gleditsia sinensis Lam. spine, Cyperus rotundus, Bupleurum chinense, Carthamus tinctorius L., Taraxacum officinale, Radix linderae
3	MOL000422	Kaempferol	41.88	0.24	Cynanchum otophyllum Schneid., Gleditsia sinensis Lam. spine, Cyperus rotundus, Bupleurum chinense, Carthamus tinctorius L.
4	MOL000449	Stigmasterol	43.83	0.76	Gleditsia sinensis Lam. spine, Cyperus rotundus, Angelica sinensis, Bupleurum chinense, Carthamus tinctorius L.
5	MOL000006	Luteolin	36.16	0.25	Caulis Lonicerae japonicae, Cyperus rotundus, Carthamus tinctorius L., Taraxacum officinale
6	MOL004328	Naringenin	59.29	0.21	Citri reticulatae viride pericarpium, Pericarpium citri reticulatae
7	MOL005828	Nobiletin	61.67	0.52	Citri reticulatae viride pericarpium, Pericarpium citri reticulatae
8	MOL000354	Isorhamnetin	49.60	0.31	Cyperus rotundus, Bupleurum Chinense
9	MOL000296	Hederagenin	36.91	0.75	Semen persicae, Poria cocos

Note: OB: oral bioavailability; DL: drug-likeness.

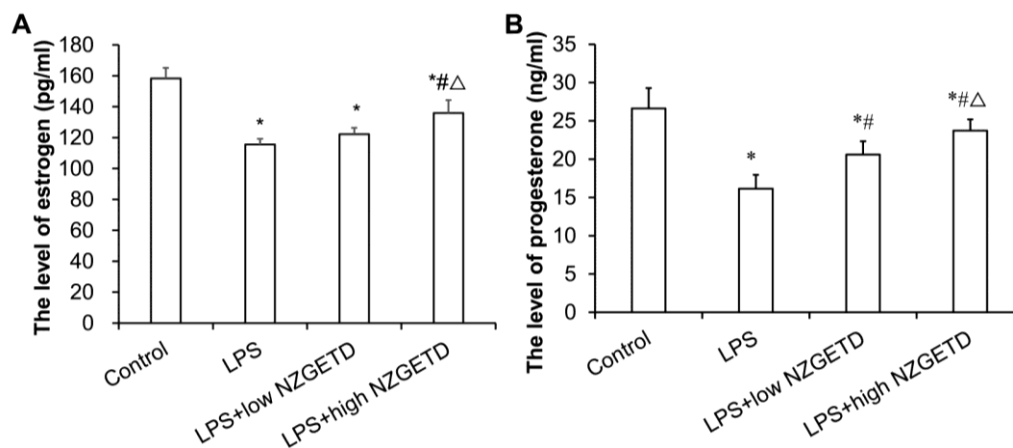


Fig. 6: Estrogen and progesterone levels in the uterine tissues of pregnant mice on GD4.5. (A) Representative bar graph showing concentrations of estrogen across experimental groups; (B) Representative bar graph showing concentrations of progesterone across experimental groups. * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with the LPS group; $\Delta P < 0.05$, compared with the LPS+low NZGETD group.

After compiling and removing duplicate active components, 166 active components in NZGETD were obtained, 9 of which were common among the different medicinal components, including beta-sitosterol, quercetin, kaempferol, stigmasterol, luteolin, naringenin, nobiletin, isorhamnetin and hederagenin. (Table 2).

NZGETD targets, embryo implantation-related targets and signaling pathway analysis

A total of 3712 target genes for “embryo implantation” were identified in GeneCards, OMIM and PharmGkb. Subsequently, a Venn Diagram analysis was performed between the 328 targets of NZGETD active components (obtained from TCMSP and SwissTargetPrediction) and the 3712 target genes related to “embryo implantation”. This analysis identified 209 common targets, including ESR1, HIF1A, VEGFA, ESR2, PGR, etc (Fig. 8A and Table 3). Protein-protein interaction network analysis revealed that of the 172 nodes identified, 19 key nodes were obtained after two screenings (Fig. 8B) and the top 10 key nodes included ESR1, myelocytomatosis viral oncogene homolog (MYC), AKT serine/threonine kinase 1 (AKT1), heat shock protein 90 alpha family class A member 1 (HSP90AA1), HIF1A, epidermal growth factor receptor (EGFR), Fos proto-oncogene, AP-1 transcription factor subunit (FOS), mitogen-activated protein kinase 1 (MAPK1), tumor protein 53 (TP53) and MAPK3 (Fig. 8C). In particular, ESR1 was the most predominant target protein. Furthermore, KEGG analysis of the 209 common targets identified 181 signaling pathways that were significantly associated with the targets ($P < 0.05$) (Fig. 8D). The top 20 signaling pathways associated with embryo implantation were listed in Fig. 8D, which included the HIF1, VEGF and estrogen signaling pathways, etc.

NZGETD upregulates ESR and PGR expression in the endometrium

To further validate the NZGETD targets, ESR and PGR

expression in the endometrium was measured by Western blotting. The results revealed that ESR1, ESR2 and PGR expression were significantly reduced by LPS treatment ($P < 0.05$) (Fig. 9). This reduction was reversed by NZGETD treatment, with the high-dose NZGETD demonstrating a more significant effect compared to the low dose ($P < 0.05$) (Fig. 9). This suggests that NZGETD may promote the upregulation of ESR and PGR expression.

NZGETD upregulates the expression of endometrial receptivity-related proteins

To investigate the regulation of embryo implantation pathways by NZGETD targets, the expression of embryo implantation-related proteins was detected by Western blotting. The findings indicated that LPS treatment led to significant decreases in the protein expressions of Hoxa10, integrin $\beta 3$, LIF, HIF1 α and VEGF ($P < 0.05$) (Fig. 10). However, this reduction was reversed with NZGETD treatment and the high-dose NZGETD had a more marked effect than the low dose ($P < 0.05$) (Fig. 10). These findings demonstrate that NZGETD may markedly upregulate the expression of endometrial receptivity-related proteins.

Analysis of NZGETD active components that are associated with confirmed targets involved in embryo implantation

The active ingredients of NZGETD and the validated embryo implantation-related targets (ESR, PGR, HOXA10, integrin $\beta 3$, LIF, HIF1 α and VEGF) were imported into Cytoscape for network analysis. As shown in table 4, there were 15 corresponding Chinese medicinal components and 39 active components in NZGETD. Notably, beta-sitosterol, quercetin, kaempferol, stigmasterol, luteolin, naringenin, nobiletin, isorhamnetin and hederagenin were important common active components that were involved in the regulation of embryo implantation-related protein expression.

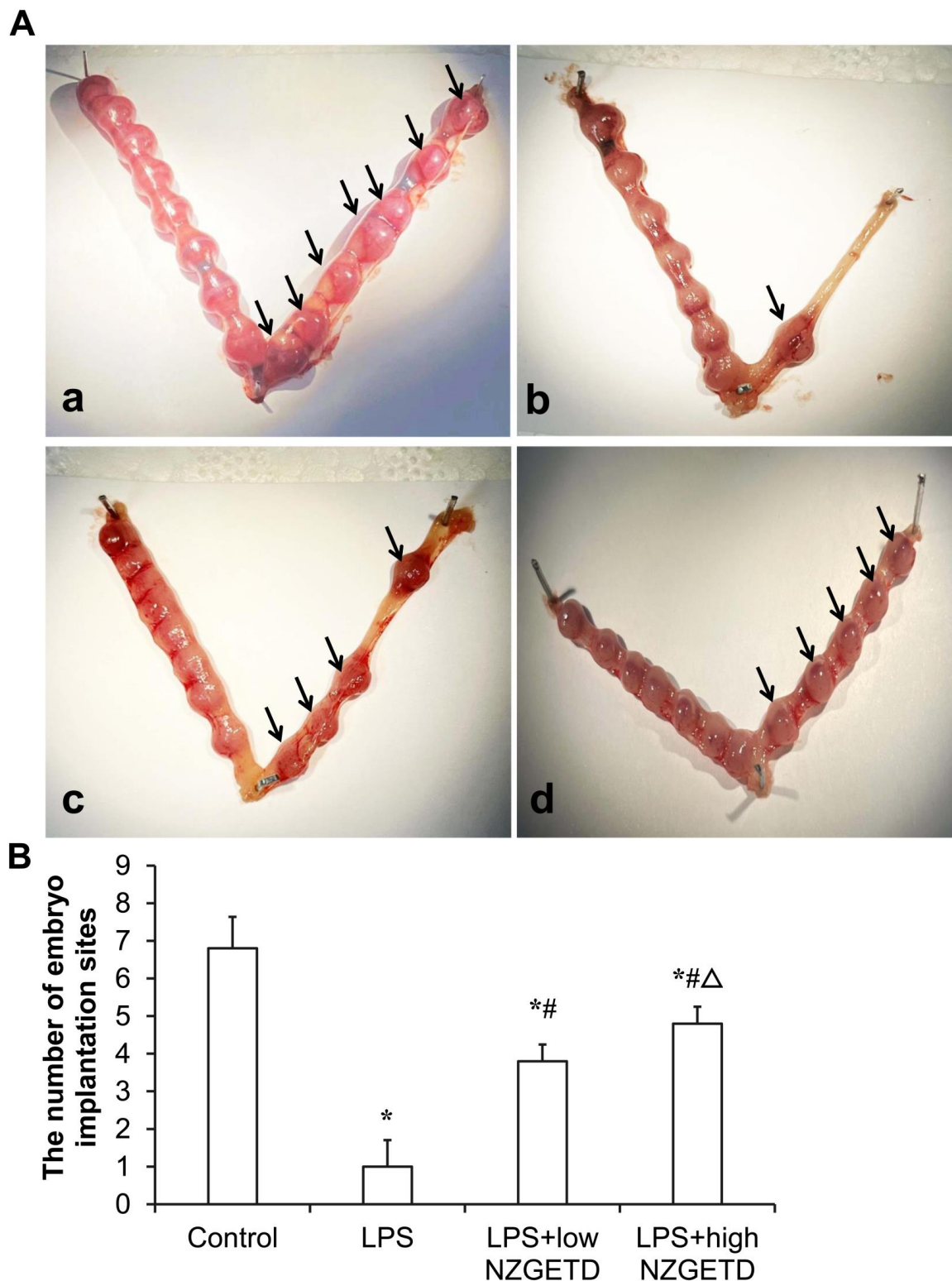


Fig. 7: Number of embryo implantations in the uterine tissues of pregnant mice on GD8.5. (A) Gross morphology of the uterus. (Aa) Control group, (Ab) LPS group, (Ac) LPS+low NZGETD group and (Ad) LPS+high NZGETD group. Arrows indicate the site of embryo implantation. (B) Number of embryo implantations in the uterine horn of the LPS-treated side (N=5). * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with the LPS group; $\Delta P < 0.05$, compared with the LPS+low NZGETD group.

Table 3: The common targets of NZGETD and embryo implantation.

PTGS2	DPP4	F2	MDM2	BAD	SPP1	IL2RA	CASP8	KCNH2	STAT1	APEX1
NCOA2	MAPK14	AURKB	APP	APOB	RUNX2	CCNB1	TOP1	MMP3	CDK1	CDK5R1
RXRA	GSK3B	ADORA1	PCNA	HMGCR	E2F1	PLAT	RAF1	EGFR	HMOX1	ESRRA
PGR	HSP90AA1	GLO1	MCL1	CYP19A1	CTSD	THBD	SOD1	VEGFA	CYP3A4	TTR
NR3C2	CDK2	PIK3R1	IL4	GSR	IGFBP3	SERPINE1	PRKCA	CCND1	CYP1A2	MMP12
ADH1C	CCNA2	ADORA2A	XIAP	ABCC1	IGF2	COL1A1	HIF1A	BCL2L1	CYP1A1	CFTR
NCOA1	PPARD	SRC	PTGES	ADIPOQ	CD40LG	IFNG	HSPA5	FOS	ICAM1	PFKFB3
SLC6A2	CHEK1	PTK2	MET	CES1	IRF1	IL1A	ERBB2	CDKN1A	SELE	TERT
SLC6A3	ACHE	HSD17B2	RASA1	SOAT1	PON1	MPO	ACACA	CASP9	VCAM1	PLG
ADRB2	GRIA2	MMP13	KDR	TIMP1	HK2	TOP2A	CAV1	MMP2	NRII2	
PLAU	RELA	PLK1	HTR3A	CREB1	NCOA3	NFE2L2	MYC	MMP9	CYP1B1	
MAOB	OLR1	CSNK2A1	DRD2	PLA2G4A	CD14	NQO1	F3	MAPK1	HAS2	
MAOA	IKBKB	NEK2	MMP7	EPHB2	PTGS1	PARP1	GJA1	IL10	GSTP1	
ADRB1	AKT1	CXCR1	CCND2	FOSL2	DRD1	COL3A1	IL1B	EGF	AHR	
SCN5A	BCL2	ALK	CDC25C	CYCS	SLC6A4	CHEK2	CCL2	RB1	SLC2A4	
NOS2	BAX	ABCB1	CD36	NFATC1	OPRM1	CLDN4	PTGER3	IL6	NRII3	
ESR1	TNF	BACE1	PTPN1	EGLN1	MAP2	PPARA	CXCL8	TP53	INSR	
AR	CASP3	AKR1A1	XDH	APOD	CAT	HSF1	BIRC5	NFKBIA	PPP3CA	
PPARG	MAPK8	MAPT	IGFIR	CTNNB1	MAPK3	CXCL10	NOS3	POR	GSTM1	
ESR2	MMP1	PIK3CG	FLT3	CDK4	FASN	CHUK	HSPB1	ODC1	SLPI	

Note: The highlighted targets in the table are targets with a Network pharmacology degree ≥ 10 in the drug-active component-target network.

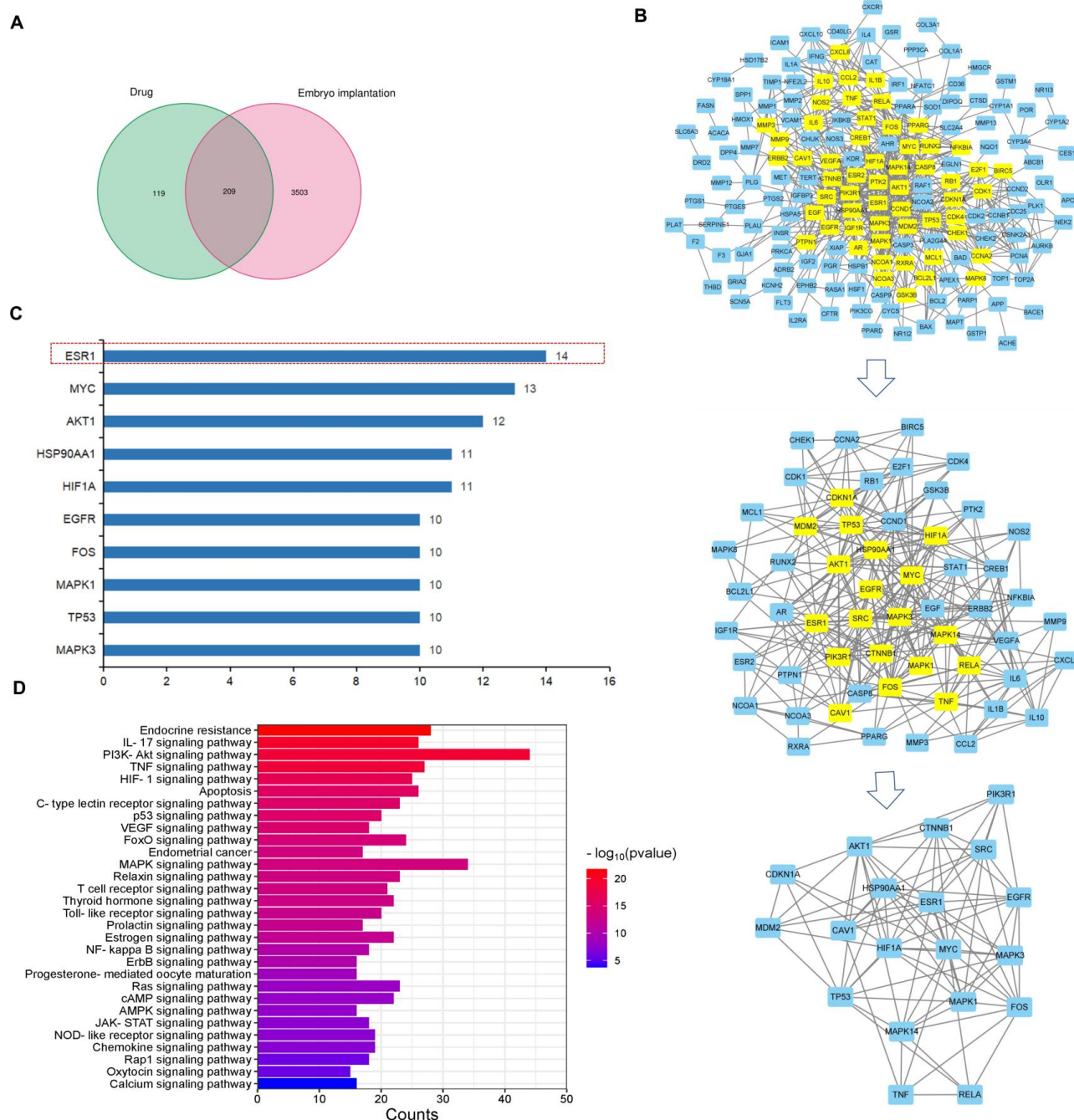


Fig. 8: Target analysis of NZGETD active components involved in embryo implantation. (A) Venn diagram of targets of NZGETD active components and embryo implantation; (B) Protein-protein interactions among common targets and screening of core proteins. The highlighted proteins in yellow are the selected proteins; (C) The top 10 common targets; (D) KEGG analysis of common targets.

Analysis of NZGETD active components that are associated with confirmed targets involved in embryo implantation

The active ingredients of NZGETD and the validated embryo implantation-related targets (ESR, PGR, HOXA10, integrin $\beta 3$, LIF, HIF1 α , and VEGF) were imported into Cytoscape for network analysis. As shown

in Table 4, there were 15 corresponding Chinese medicinal components and 39 active components in NZGETD. Notably, beta-sitosterol, quercetin, kaempferol, stigmasterol, luteolin, naringenin, nobiletin, isorhamnetin and hederagenin were important common active components that were involved in the regulation of embryo implantation-related protein expression.

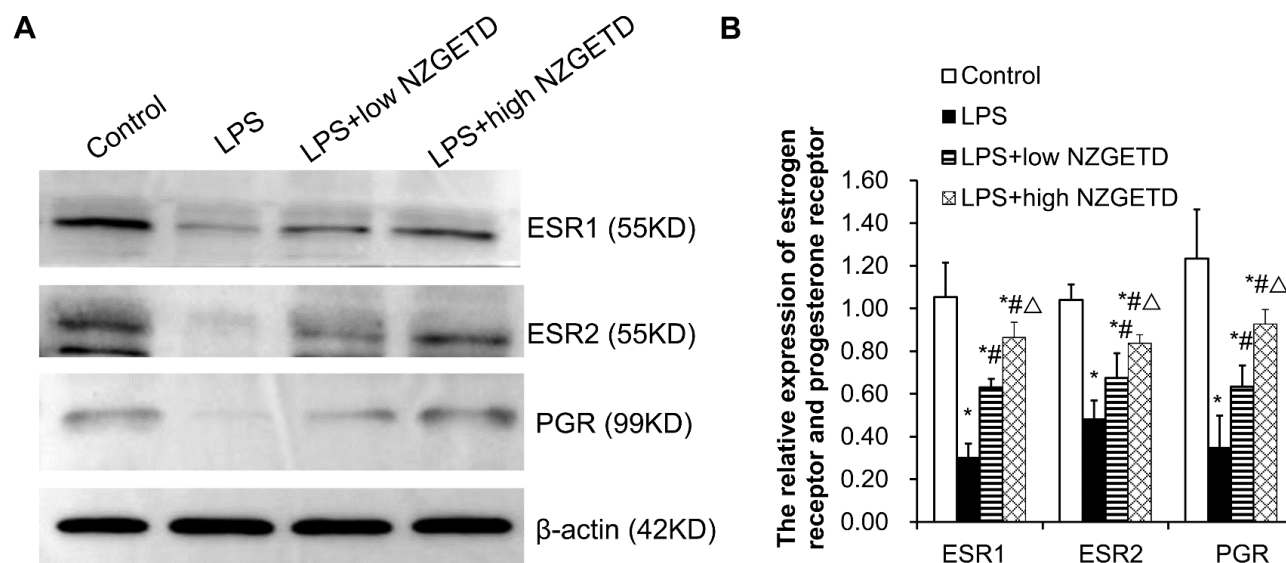


Fig. 9: ESR1, ESR2, and PGR expression in uterine tissues. (A) Endometrial ESR1, ESR2, and PGR expression was detected by western blotting; (B) Relative endometrial expression of ESR1, ESR2 and PGR. * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with the LPS group; $\Delta P < 0.05$, compared with the LPS+low NZGETD group.

Table 4: Traditional Chinese herbs and active components in NZGETD corresponding to embryo implantation target genes.

Gene	Active components	Medicinal herb
ESR1	(+)-Catechin, isorhamnetin, 3,5,6,7-tetramethoxy-2-(3,4,5-trimethoxyphenyl)chromone, naringenin, nobiletin, 2-(4-hydroxyphenyl)ethyl (E)-3-(4-hydroxyphenyl)prop-2-enoate, (-)-catechin, lignan, isostyracin epoxide, coniferin, 6,7-dimethoxy-2-(2-phenylethyl)chromone, DMPEC, chryseriol, 8-isopentenyl-kaempferol, ent-epicatechin, luteolin, apigenin, coumestrol	Paeoniae radix alba, Radix bupleuri, Citrus reticulata, Sargentodoxae Caulis, Carthami flos, Fructuslipuidambaris, Citri reticulatae Pericarpium Viride, Linderac Radix, Cyperi rhizoma, Gleditsiae Spina, Lonicera japonica, Taraxacum mongolicum
ESR2	Isorhamnetin, 3,5,6,7-tetramethoxy-2-(3,4,5-trimethoxyphenyl)chromone, areapillin, petunidin, nobiletin, Sinensetin, 6,7-dimethoxy-2-(2-phenylethyl)chromone, luteolin, quercetin, apigenin, coumestrol	Radix Bupleuri, Citrus Reticulata, Citri Reticulatae Pericarpium Viride, Linderac Radix, Cyperi Rhizoma, Lonicera japonica, Taraxacum mongolicum
PGR	(3S,5R,8R,9R,10S,14S)-3,17-Dihydroxy-4,4,8,10,14-pentamethyl-2,3,5,6,7,9-hexahydro-1H-cyclopenta[a]phenanthrene-15,16-dione, mairin, beta-sitosterol, sitosterol, kaempferol, stigmasterol, α -spinasterol, ergosta-7,22E-dien-3beta-ol, ergosterol peroxide, hederagenin, poriferast-5-en-3beta-ol, CLR, sitosterol alpha1, GA121-isolactone, GA122-isolactone, 4a-formyl-7alpha-hydroxy-1-methyl-8-methylidene-4aalpha,4bbeta-gibbane-1alpha,10beta-dicarboxylic acid, campesterol, stigmasterol glucoside_qt	Paeoniae Radix Alba, Radix Bupleuri, Citrus Reticulata, Angelicae Sinensis Radix, Sargentodoxae Caulis, Poria Cocos(Schw.) Wolf., Carthami Flos, Fructuslipuidambaris, Persicae Semen, Linderac Radix, Cyperi Rhizoma, Gleditsiae Spina
VEGFA	Quercetin, baicalein, beta-carotene, luteolin	Radix Bupleuri, Carthami Flos, Linderac Radix, Cyperi Rhizoma, Gleditsiae Spina
HIF1A	Quercetin, baicalein	Radix Bupleuri, Carthami Flos, Linderac Radix, Cyperi Rhizoma, Gleditsiae Spina

DISCUSSION

Compound Chinese medicines have shown notable advantages over antibiotics in treating endometritis, including symptom relief, endometrial repair and

improved conception rates (Peng *et al.*, 2023; Ji, 2022). NZGETD, used in this study, comprises 15 medicinal herbs and was found to promote endometrial angiogenesis, receptivity and embryo implantation in a mouse model of LPS-induced endometritis.

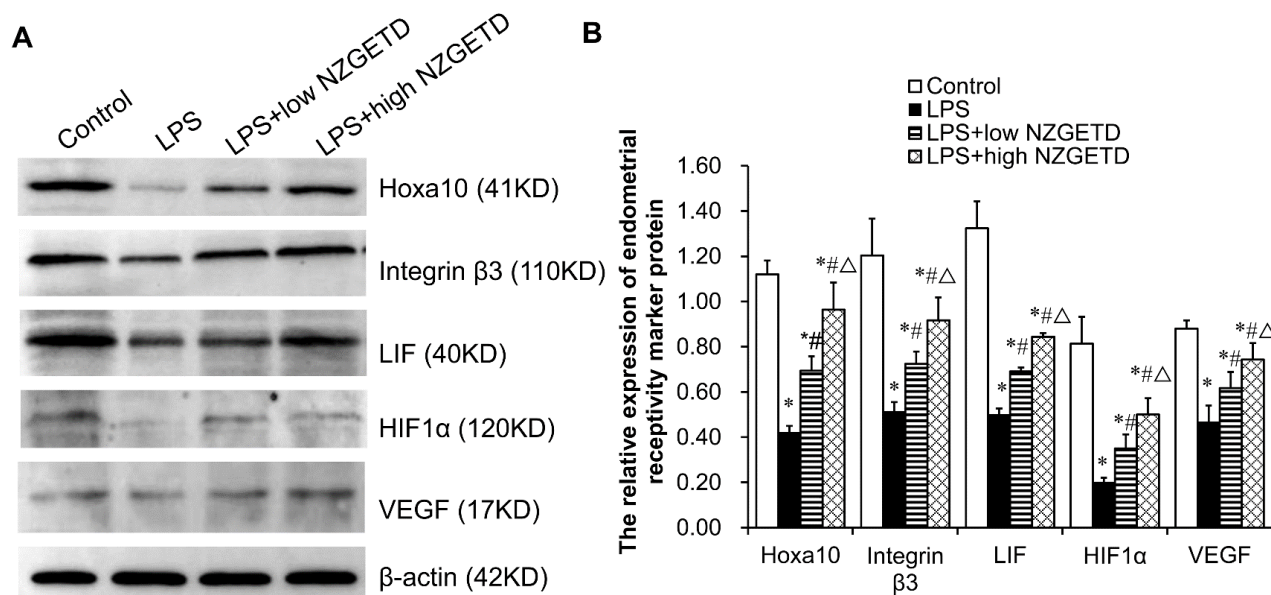


Fig. 10. Endometrial receptivity protein expression. (A) Hoxa10, integrin β 3, LIF, HIF1 α , and VEGF protein expression in the endometrium were measured by Western blot; (B) Relative endometrial expression of Hoxa10, integrin β 3, LIF, HIF1 α , and VEGF. * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with the LPS group; $\Delta P < 0.05$, compared with the LPS+low NZGETD group.

LPS, a key component of Gram-negative bacteria such as *Escherichia coli*, triggers endometrial inflammation by activating neutrophils and macrophages, leading to excessive production of TNF- α , IL-6 and IL-1 β (Li *et al.*, 2015b; Dong *et al.*, 2021; Oladejo *et al.*, 2022). One previous study showed that inhibition of TNF- α , IL-6 and IL-1 α significantly attenuated LPS-induced endometritis (Yin *et al.*, 2022). Consistently, the data in this study showed that oral gavage of NZGETD in LPS-treated mice significantly ameliorated endometritis, possibly by downregulating endometrial TNF- α , IL-6 and IL-1 α expression, decreasing inflammatory cell accumulation and hemorrhage in the endometrium and effectively inhibiting LPS-induced inflammation.

Inflammation can impair endometrial development and angiogenesis, affecting subsequent embryo implantation (Zhang *et al.*, 2018). Endometrial repair and decidualization are predominantly regulated by estrogen and progesterone (Gellersen and Brosens, 2014). Estrogen acts on ESRs to induce endometrial growth, increase endometrial thickness, improve blood supply and promote endometrial repair (Bai *et al.*, 2020). In addition, progesterone suppresses uterine smooth muscle contraction, promotes endometrial gland development and secretion and facilitates endometrial decidualization (Patel *et al.*, 2015). Data from the mouse model suggest that NZGETD synergistically elevated local uterine levels of both hormones, upregulated their receptors (ESR and PGR) and promoted endometrial decidualization and embryo implantation. This effect may be linked to several

active components within NZGETD. For example, *T. officinale* extracts promote granulosa cell hormone secretion (Wang *et al.*, 2018), sesquiterpenes from *C. rotundus* L. act as phytoestrogens (Park *et al.*, 2019) and beta-sitosterol can enhance progesterone activity and endometrial receptivity (Yu *et al.*, 2021). It should be noted that estrogen and progesterone levels in this study were measured in uterine tissue homogenates to assess the local hormonal environment. While this provides insight into tissue-specific bioavailability, future studies should include measurements of serum hormone concentrations to correlate systemic endocrine changes with the local effects observed here.

Angiogenesis is critical for embryo implantation (Fournier *et al.*, 2021). The results showed that NZGETD significantly upregulated CD31 expression and promoted microangiogenesis in the endometrium of pregnant mice. This pro-angiogenic effect may be attributed to the coordinated actions of several medicinal components within NZGETD. For instance, *C. tinctorius* L. is known to repair vascular endothelial injury and promote angiogenesis (Xu *et al.*, 2023). Similarly, *A. sinensis* extracts have been shown to promote VEGFR2 phosphorylation and upregulate HIF1 α and VEGF-A expression, thereby facilitating revascularization (Chen *et al.*, 2022). Additionally, *Radix Paeoniae Alba* extracts contribute to stabilizing microcirculation (Li *et al.*, 2017). The presence of these components in NZGETD likely underlies its observed ability to enhance endometrial vascularization, thereby improving blood supply and

creating a more supportive environment for embryo implantation.

Endometrial receptivity involves the precisely coordinated expression of specific molecular markers (Craciunas *et al.*, 2019). In this study, the focus was placed on Hoxa10, integrin $\beta 3$, LIF, HIF1 α and VEGF, all of which were significantly upregulated following NZGETD treatment. Network pharmacology analysis predicted that NZGETD would target key regulatory nodes within related pathways, such as ESR1, PGR, HIF1A and VEGFA. Experimental validation confirmed not only the upregulation of these predicted targets but also the increased expression of the associated receptivity marker proteins, linking predictive analysis with functional outcomes. The regulatory relationships among these markers help explain NZGETD's integrative effects. Hoxa10, a transcription factor whose expression is positively regulated by estrogen and progesterone, plays a critical role in endometrial stromal decidualization and directly modulates integrin $\alpha \beta 3$ expression, which is essential for embryo adhesion (Lim *et al.*, 1999; Li *et al.*, 2015a; Fraser *et al.*, 2021). VEGF, a central angiogenic factor, along with its upstream regulator HIF1 α , supports endometrial vascular remodeling, a process vital for implantation (Sivridis *et al.*, 2002; Hu *et al.*, 2021). Meanwhile, LIF, a cytokine released during the implantation window, promotes trophoblast invasion and helps establish local immune tolerance, both necessary for successful embryo embedding (Dimitriadis *et al.*, 2006; Kabir-Salmani *et al.*, 2005). The upregulation of this panel of proteins by NZGETD was thus closely associated with the observed improvements in endometrial decidualization, angiogenesis and embryo attachment.

Several common active components of NZGETD were identified as being associated with these target genes, including beta-sitosterol, quercetin, kaempferol, stigmasterol, luteolin, naringenin, nobiletin, isorhamnetin and hederagenin. These components have been reported to exhibit phytoestrogenic, anti-inflammatory, or pro-angiogenic activities in other systems, suggesting plausible biological routes through which they might contribute to the observed effects (Park *et al.*, 2019; Yu *et al.*, 2021). It is worth noting that while the network pharmacology analysis utilized human databases to predict potential targets of NZGETD, a common strategy for clinically oriented herbal formula research, the key pathways and targets identified (e.g., ESR1/PGR, HIF-1 α /VEGF) are functionally conserved in murine reproductive physiology. Subsequent experimental validation in a mouse model confirmed the regulatory effects of NZGETD on these conserved targets. Nevertheless, future studies incorporating murine-specific omics data could further refine these predictions and enhance translatability. Furthermore, the mechanisms by which the identified common active components regulate

target gene expression and promote embryo implantation warrant further investigation.

CONCLUSION

The findings demonstrate that in a mouse model of LPS-induced endometritis, NZGETD treatment was associated with a significant promotion of embryo implantation. The collective data suggest that the identified active components of NZGETD may contribute to this effect by promoting endometrial development, increasing microangiogenesis, elevating local estrogen and progesterone levels and upregulating the expression of hormone receptors (ESR, PGR) and key receptivity markers (Hoxa10, integrin $\beta 3$, LIF, HIF1 α , VEGF). These coordinated changes likely foster an endometrial environment conducive to decidualization and embryo attachment. The targets validated in this mouse model provide mechanistic insights that may inform future research on NZGETD, while acknowledging that findings from animal models may not fully recapitulate human pathophysiology. In future studies, the mechanisms of action of these active components will be further investigated to facilitate the simplification and standardization of such compound TCM formulations.

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None.

Authors' contributions

Shuang Li: Conceptualization, methodology, investigation and writing - original draft; Huiling Liu: Investigation, formal analysis and data curation; Nan Kang: Resources and validation; Xiaoyan Pan: Supervision, project administration, funding acquisition, writing - review and editing.

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Data availability statement

All data generated or analysed during this study are included in this published article.

Ethical approval

The animal experimentation procedures received approval from the Ethics Committee of Jilin Medical College (approval number: 2023-LW001) and all experiments were conducted following applicable guidelines and regulations, adhering to the ARRIVE guidelines. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interest

The authors declare no conflict of interest.

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