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OVULATION-INDUCING EFFECT OF ANGELICA SINENSIS EXTRACT ON AGED INFERTILE MICE AND ITS IMPACT ON FERTILITY CAPABILITY

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ABSTRACT

Although *Angelica sinensis* extract has been widely utilized for female reproductive health issues, its therapeutic effects on infertility are not established. This study aimed to assess the ovulation-inducing effects of *Angelica sinensis* extract in aged infertile mice and investigate its impact on fertility. An aged infertile mouse model was initially established and validated by monitoring estrous cycles. Subsequently, mice were randomly rolled into the control group (Group C) – receiving no intervention and being maintained under normal conditions; experimental group A (Group A) – establishing an infertility mouse model without any intervention; experimental group B (Group B) – constructing an infertility mouse model and administering *Angelica sinensis* extract as an intervention. Monitoring of hormonal levels, including estradiol (E2), anti-Mullerian hormone (AMH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), progesterone (P), and testosterone (T), follicle counts, expression of proliferating cell nuclear antigen (PCNA), and primordial follicle activation-related proteins (YAP, p-YAP, mTOR, p-mTOR, rpS6, p-rpS6, PTEN, p-PTEN) were carried out to evaluate the impact of *Angelica sinensis* extract on mouse ovarian function. Simultaneously, fertility experiments were conducted to record pregnancy rates, litter sizes, and litter numbers for both groups of mice to investigate the impact of *Angelica sinensis* extract on fertility. Group A exhibited greatly lower E2, AMH, P, follicle count, PCNA protein expression, p-YAP/YAP ratio, p-mTOR/mTOR ratio, p-rpS6/rpS6 ratio, and higher levels of FSH, LH, T, p-PTEN/PTEN ratio, and the number of atretic follicles versus Group A ($P \leq 0.05$). Group B showed higher levels of E2, AMH, P, PCNA protein expression, p-YAP/YAP ratio, p-mTOR/mTOR ratio, p-rpS6/rpS6 ratio, primordial follicles, primary follicles, and antral follicles, as well as lower levels of FSH, LH, T, p-PTEN/PTEN ratio, and the number of atretic follicles versus Group A ($P \leq 0.05$). Group A exhibited a significantly decreased pregnancy rate, litter size, and litter number compared to Group C, while Group B showed a higher litter size and number than Group A ($P \leq 0.05$).*Angelica sinensis* extract has a pronounced ovulation-inducing effect on aged infertile mice and can enhance their fertility. These findings suggest that *Angelica sinensis* extract might be a natural remedy for treating declining ovarian function.

Keywords: *Angelica sinensis* extract; aged; infertility; ovulation-inducing effect; ovaries; fertility capability.

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INTRODUCTION

Research has indicated that women's fertility capacity gradually declines with advancing age due to reductions in both egg quantity and quality (Chronopoulou *et al*., 2021). Infertility refers to the inability of couples to achieve pregnancy within a defined period (typically one year) under normal sexual conditions (Mir and Pal, 2023). Advanced maternal age infertility specifically pertains to women aged over 35 who experience infertility (Ubaldi *et al*., 2019). This condition may stem from various factors, including ovulatory issues, endometriosis, uterine fibroids, tubal abnormalities, and more (Bonavina *et al*., 2022; Freytag *et al*., 2021; Carson and Kallen, 2021).

Traditional Chinese medicine (TCM) has been widely employed in treating advanced maternal age infertility and has shown certain therapeutic efficacy (Zheng *et al*., 2020; Tong *et al*., 2017). Among them, *Angelica sinensis* can nourish blood, promote blood circulation, regulate menstruation, relieve pain, and nourish yin (Wei *et al*., 2016; Nai *et al*., 2021). Within gynecology, *Angelica sinensis* is commonly utilized to address issues such as menstrual irregularities, dysmenorrhea, postpartum weakness, and menopausal syndrome (Xia *et al*., 2021). It is believed to regulate women's physiological cycles, enhance blood circulation,

increase endometrial thickness, and improve female fertility (Yuan *et al*., 2019). Additionally, *Angelica sinensis* is employed to address pallor, palpitations, and insomnia symptoms due to its blood-nourishing properties (Gong *et al*., 2023). *Angelica sinensis* can be consumed as a standalone decoction or is often used with other herbal ingredients to enhance its therapeutic effects. *Angelica sinensis* extract is a naturally derived plant extract from *Angelica sinensis*, rich in various bioactive components, with the most crucial being pharmacologically active compounds (Batiha *et al*., 2022). The most common constituents of Angelica sinensis extract include sodium ferulate, isoligustrazine, puerarin, and anthocyanins (Hoshino *et al*., 2022; Kaur *et al*., 2020). These compounds confer unique pharmacological activities to Angelica sinensis extract, such as promoting blood circulation, nourishing yin and blood, and regulating menstruation. Additionally, *Angelica sinensis* extract is believed to have a role in balancing female endocrine functions (Zierau *et al*., 2014; Zeng *et al*., 2022). Nevertheless, despite the widespread use of *Angelica sinensis* in TCM for female reproductive health issues, its exact therapeutic effects on infertility have not been fully confirmed. Some studies suggest that *Angelica sinensis* may positively influence the regulation of the female reproductive system and hormonal balance, potentially aiding infertility patients to a certain extent (Ma *et al*., 2022; Gong *et al*., 2015). Nevertheless, current research is insufficient to draw definitive conclusions, and in clinical practice, *Angelica sinensis* extract is often utilized as an adjunctive therapy, combined with other traditional Chinese herbs or modern medical treatment approaches.

Hence, in this study, a model of aged infertile mice was initially established, which was treated with *Angelica sinensis* extract to demonstrate the influences of *Angelica sinensis* extract on ovulation and fertility capability in aged infertile mice. The objective was to provide insights for the clinical treatment of infertility in elderly female patients, offering potential therapeutic avenues for those facing infertility issues.

MATERIALS AND METHODS

Modeling of anovulatory infertility in mice

Experimental animals: Mice and grouping were as follows. 30 female mice aged 47 weeks and weighing 20-30g (Beijing Vitalstar Biotechnology Co., Ltd. China) were purchased and acclimatized to appropriate environmental conditions of temperature, humidity, and lighting, with access to free water and diet. After one week of acclimatization, modeling commenced when the mice reached 48 weeks of age. Humane care according to the criteria expressed in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health, were followed throughout the experiment period. The ethics regulations followed were in accordance with national and institutional guidelines for the protection of animal welfare during the experiments. This study had been approved by ethics committee of Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University, Changsha, China) (No. 2023/06-18).

Mice were randomly rolled into the control group (Group C, n=10), which received no intervention and was maintained under normal conditions; experimental group A (Group A, n=10), which established an infertility mouse model without any intervention; and experimental group B (Group B, n=10), which constructed an infertility mouse model and administered *Angelica sinensis* extract as intervention.

Furthermore, 15 male mice (BioMice, China) with normal sexual behavior and weighing 30~40 g were also purchased for the follow-up study of pregnancy and farrowing of female mice. To avoid interference with fertility experiments due to the potential decline in reproductive performance of aged male mice, male mice utilized in fertility experiments were not older than six months.

Establishment of Model: For the mice in Group A and Group B, a single subcutaneous injection of 1.25 mg of testosterone propionate (Henan Kelun Pharmaceutical Co., Ltd., China) was administered to each mouse at the cervical region. The mice were maintained by experiment personnel under natural lighting conditions at approximately 25°C and a humidity level of around 50%. No vitamin supplements were applied to their diet. As for Group C, mice received a single subcutaneous injection of an equivalent amount of neutral tea tree oil (Shaanxi Zhengyi Pharmaceutical Excipients Co., Ltd., China) in the nape, while all other conditions remained the same as those for mice of group A and group B.

Estrous cycle monitoring: The estrous cycle was monitored starting from the first week of feeding and continued for two weeks. Each morning, between 9 AM and 11 AM, a suitable volume of physiological saline solution (10-20 μL) (Lefeke, Germany) was gently introduced into the vaginal cavity of the mice. The solution was repeatedly flushed back and forth until the saline appeared cloudy. The collected vaginal fluid was smeared onto glass slides, allowed to air-dry, and subjected to HE staining (Sigma-Aldrich, USA). The cell morphology and quantity of the vaginal smears were examined under a microscope (KEYENCE, Japan) to determine the stage of the estrous cycle in mice. Successful modeling was indicated by disrupted estrous cycles in all mice. Estrous cycle methodology (Yu *et al*., 2024) was determinated.

Intervention measures: No interventions were performed for Group C, and mice were maintained under normal conditions for four weeks.

For Group A, mice were raised for an additional 4 weeks following successful modeling.

For Group B, after successful modeling, mice were orally administered a suspension of 50 mg/kg^{-1} *Angelica sinensis* extract (Shaanxi Ruikang Biotechnology Co., Ltd., China) (prepared in physiological saline) via gavage. The administration was conducted in two equal doses daily for four consecutive weeks before evaluating the designated indicators. The estrous cycle was monitored following the intervention, and the average duration of the estrous cycle (in days) was recorded, along with the proportions of each phase, including proestrus, estrus, metestrus, and diestrus. The effect of *Angelica sinensis* extract on the estrous cycle was assessed.

Five randomly selected mice were further maintained from each group for subsequent fertility assessment experiments. The remaining mice (n=5/group) were euthanized by eyeball puncture under 1% pentobarbital anesthesia (Shanghai Xinya Pharmaceutical Co., Ltd., China). The ovaries were dissected under a stereomicroscope (KEYENCE, Japan), separating them from surrounding adipose tissue. Ovaries were extracted and photographed using a digital microscope camera (Beijing Surui Technology Co., Ltd., China). After 2 hours of ambient temperature incubation, the blood from the eyeballs was centrifuged (4°C, 3,000 rpm, 15 min). The resulting supernatant was collected and aliquoted into 1.5 mL Eppendorf (EP) tubes (Greiner Bio-One International GmbH, Austria) and stored at -80°C for further use. Half of the ovaries were frozen at -80°C, and the other half were fixed in 4% paraformaldehyde (Santa Cruz Biotechnology, USA), followed by subsequent embedding and sectioning.

Ovarian function assessment

Sex hormone level detection: Mouse serum levels of neutral hormones, including estradiol (E2), anti-Mullerian hormone (AMH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), progesterone (P), and testosterone (T), were quantified utilizing ELISA (Thermo Fisher Scientific, USA). The procedure followed the instructions provided for the assay kits. The control solution (100 µL) was applied to designated wells, while the test sample ((100 µL)) was applied to other wells. The plate was then covered and incubated for 90 minutes. After liquid removal and plate air-drying, biotinylated Ab working solution (100 µL) (Thermo Fisher Scientific, USA) was applied, followed by a 1-hour undercover incubation period. Subsequently, 350 µL of wash solution (R&D Systems, USA) was applied, and the plate was washed thrice. Next, the enzyme conjugate working solution (100 µL) (Thermo Fisher Scientific, USA) was applied, and the plate was covered and incubated at a suitable temperature for 30 minutes. Following five washes, substrate solution (90 µL) was applied, and the plate was covered and incubated in the dark for 15 minutes, which was stopped by adding stop solution (50 µL). Each well's absorbance value (A) was measured at a wavelength of 450 nm utilizing an ELISA reader (Sigma-Aldrich, USA). A standard curve was established to calculate the sample's mass concentration.

Ovulation function test: Ovarian tissues were immersed in 4% paraformaldehyde for at least 24 hours, followed by subsequent paraffin embedding and sectioning. Each ovary was serially sectioned at a thickness of 5 µm and sequentially placed on glass slides, with four ovarian sections per slide. After HE is staining, follicle counting was conducted. Every eighth ovarian section (equivalent to 40 µm) was examined, and one section was counted. Only follicles containing oocyte nuclei were considered, and follicle counting was performed for each developmental stage.

Detection of ovarian cell proliferation ability: Immunohistochemistry (IHC) was employed to evaluate the expressions of PCNA in mouse ovarian tissue. Fixed tissues were dehydrated, embedded, sectioned, and placed on glass slides. Subsequently, antigen retrieval was performed by adding an appropriate antigen retrieval solution (Sigma-Aldrich, USA) and subjecting the slides to microwave (Glen Dimplex, China) heating (5 min at high power followed by 15 min at low power). After antigen retrieval, tissue sections on slides were incubated with primary antibody (rabbit monoclonal PCNA antibody (Kinsray, USA)) and secondary antibody (horseradish peroxidase-conjugated antibody (Macklin, China)) to form complexes. A suitable amount of freshly prepared AEC (AEC Chromogen) substrate (Beijing Biolab Biotechnology Co., Ltd., China) was added for color development, maintaining consistent incubation times for antibody staining. The color development was halted using tap water. The slides were briefly placed in a hematoxylin staining solution for 30 seconds, followed by hydrochloric acid-alcohol differentiation and counterstaining with an alkaline lithium carbonate solution (Macklin, China). Following the completing of nuclear staining, the slides were left to air dry naturally and sealed with an aqueous mounting medium. Tissue sections were visualized under a microscope, captured images, and documented staining patterns. The protein average integrated optical density (IOD) was analyzed utilizing *Image-Pro Plus* (IPP) (Media Cybernetics, Inc., USA).

Detection of protein expressions related to primordial follicle activation: Expressions of primordial follicle activation-related proteins, including YAP, p-YAP, mTOR, p-mTOR, rpS6, p-rpS6, PTEN, and p-PTEN, in

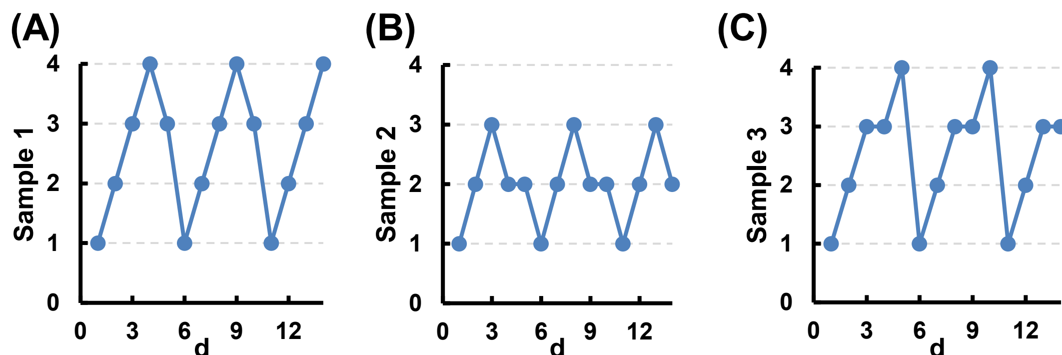
mouse ovarian tissue were examined using Western Blot analysis. Frozen ovarian tissue was placed in cell lysis buffer (Weiao Biotechnology Co., Ltd., China), applied with protease and phosphatase inhibitors (Sigma-Aldrich, USA), and homogenized to disrupt cells and release proteins. The resultant lysates were then centrifugated (4°C, 12,000 rpm, 20 min), and supernatant was collected into new 1.5 mL enzyme-free centrifuge tubes (Thermo Fisher Scientific, USA). A protein aliquot of 2 μL from each tube was utilized for protein quantification. Subsequently, protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Macklin, China) for separation based on protein molecular weight. Following electrophoresis, proteins were transferred from the gel to a polyvinylidene fluoride membrane (Arkema, France) utilizing an electrophoretic transfer apparatus. The membrane was then blocked with bovine serum albumin (BSA) (Pronease Life Technologies Co., Ltd., China). Tissue sections on slides were incubated with primary antibodies: rabbit monoclonal yap polyclonal (YAP) antibody, rabbit monoclonal p-YAP antibody (Cambridge Bioscience Ltd., China), rabbit monoclonal mammalian target of rapamycin (mTOR) antibody, rabbit monoclonal p-mTOR antibody (Sigma-Aldrich, USA), rabbit monoclonal rpS6 (ribosomal protein S6) antibody, rabbit monoclonal p-rpS6 antibody (Sigma-Aldrich, USA), rabbit monoclonal phosphatase and tensin homolog (PTEN) antibody, and rabbit monoclonal p-PTEN antibody (Shanghai Yanjin Bio-Tech Co., Ltd., China). After incubation with primary and secondary antibodies, unbound antibodies were washed away. Target bands were incubated in an electrochemiluminescence (ECL) detection solution (Nanjing Jiangyuan Biotechnology Co., Ltd., China) for 2-3 minutes and visualized using a Bio-Rad imaging system. The band intensities were quantified using *Image J*, and protein relative levels were calculated using GAPDH as an internal reference.

Fertility assessment: Female mice (n=5/group) were co-housed with male mice at a female-to-male ratio 3:2. Female mice exhibiting evident abdominal distension were individually isolated upon detection, and pregnant mice were housed separately. Parameters were meticulously recorded for each maternal generation, including maternal pregnancy rate, litter size per dam, and number of litters. Separation of the A1 generation pups occurred at postnatal day 21. Subsequently, female mice were re-cohabitated, and seven successive mating experiments (H1, H2, H3, H4, H5, H6, H7) were conducted. The experimental protocol culminated in euthanasia after the completion of all trials.

Statistical analysis: Data analysis was performed using *SPSS 23.0*. The data were presented as mean ± standard deviation (mean ± SD). For comparisons between two independent groups, independent sample t-tests (Student's t-test) were used; for comparisons among multiple groups, one-way analysis of variance (ANOVA) was employed. To ensure the accuracy and reliability of the statistical results, post hoc multiple comparison corrections were performed following one-way ANOVA. Specifically, the Bonferroni or Tukey's HSD method was applied to control for the risk of false positives (Type I error) due to multiple comparisons. Additionally, power analysis was conducted prior to data collection, and the appropriate sample size was determined based on effect size and significance level ($\alpha = 0.05$) to ensure a statistical power of 80% or higher. Furthermore, normality tests (e.g., Shapiro-Wilk test) and homogeneity of variance tests (*e.g.,* Levene's test) were performed prior to conducting t-tests and ANOVA to verify the suitability of the data. A *P*-value of less than 0.05 was considered statistically significant for all tests.

RESULTS

Modeling results: Upon microscopic observation of smears, in this study, the estrous cycles of Group C mice exhibited regular patterns and were preserved for reference. In both Group A and Group B, irregular changes in estrous cycles occurred, indicating the successful establishment of the model. These observations were also preserved for future reference (Fig.1A-I).



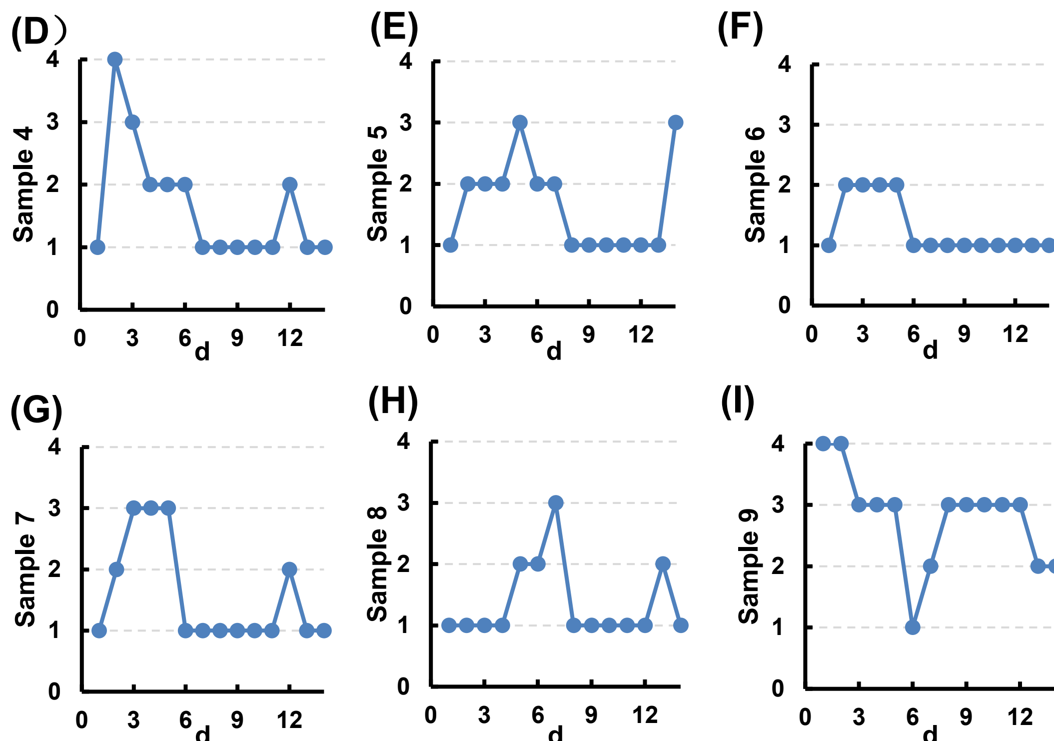


Fig.1 Example of typical estrous cycle evaluation results. Ordinates 1-4 represent pre-estrous period, post estrous period, and interval in sequence; A-C represents normal samples in Group C; D-F represents samples in Group A; G-I represents samples in Group B.

Changes in the estrous cycle after intervention: Table 1 shows the changes in the estrous cycle of mice in each group over the 4-week intervention period. Group C maintained a normal estrous cycle, with a balanced distribution across all phases and a cycle length of 4-5 days. In contrast, the average duration of the estrous cycle in Group A was significantly prolonged, with a marked increase in the proportion of the proestrus phase, while the proportions of estrus and metestrus were significantly reduced. After treatment with *Angelica sinensis* extract, Group B showed a trend toward recovery. The proportion of proestrus decreased, and the proportions of estrus and metestrus gradually returned to within the normal range.

Table 1. Changes in the duration and proportions of different estrous cycle phases in mice of different groups.

Group	Average estrous cycle duration (days)	Proestrus (%)	Estrus (%)	Metestrus (%)	Diestrus (%)
Group A	7.5 ± 1.2	40.0 ± 5.1	30.0 ± 4.2	20.0 ± 3.3	10.0 ± 2.1
Group B	5.2 ± 0.8	27.5 ± 4.2	34.5 ± 4.0	26.0 ± 3.5	12.0 ± 2.3
Group C	4.8 ± 0.5	30.0 ± 3.5	35.0 ± 3.5	25.0 ± 4.0	10.0 ± 2.0

Evaluation results of mouse ovarian function

Sex hormone level test results: The test results revealed that in comparison to Group C, E2, AMH, and P, drastically declined in ovarian tissue in mice of Group A and Group B, whereas those of FSH, LH, and T were notably elevated ($P \leq 0.05$). Additionally, the E2, AMH, and P levels in mice in Group B were dramatically superior to those in Group A, while those of FSH, LH, and T were markedly inferior to Group A ($P \leq 0.05$) (Fig.2A-F).

Ovulation function evaluation results: The follicle statistics revealed that the number of primordial follicles, preantral follicles, and antral follicles were all notably reduced in mice of Group A and B versus Group C, while the number of atretic follicles was considerably superior in Group A and B ($P \leq 0.05$). In Group A, the count of primordial follicles (43.76 ± 3.19), preantral follicles (13.62 ± 1.14), and antral follicles (13.41 ± 1.62) of mice were markedly inferior to Group B, and the count of atretic follicles (1.75 ± 0.13) of mice was dramatically superior to Group B ($P \leq 0.05$) (Fig. 3).

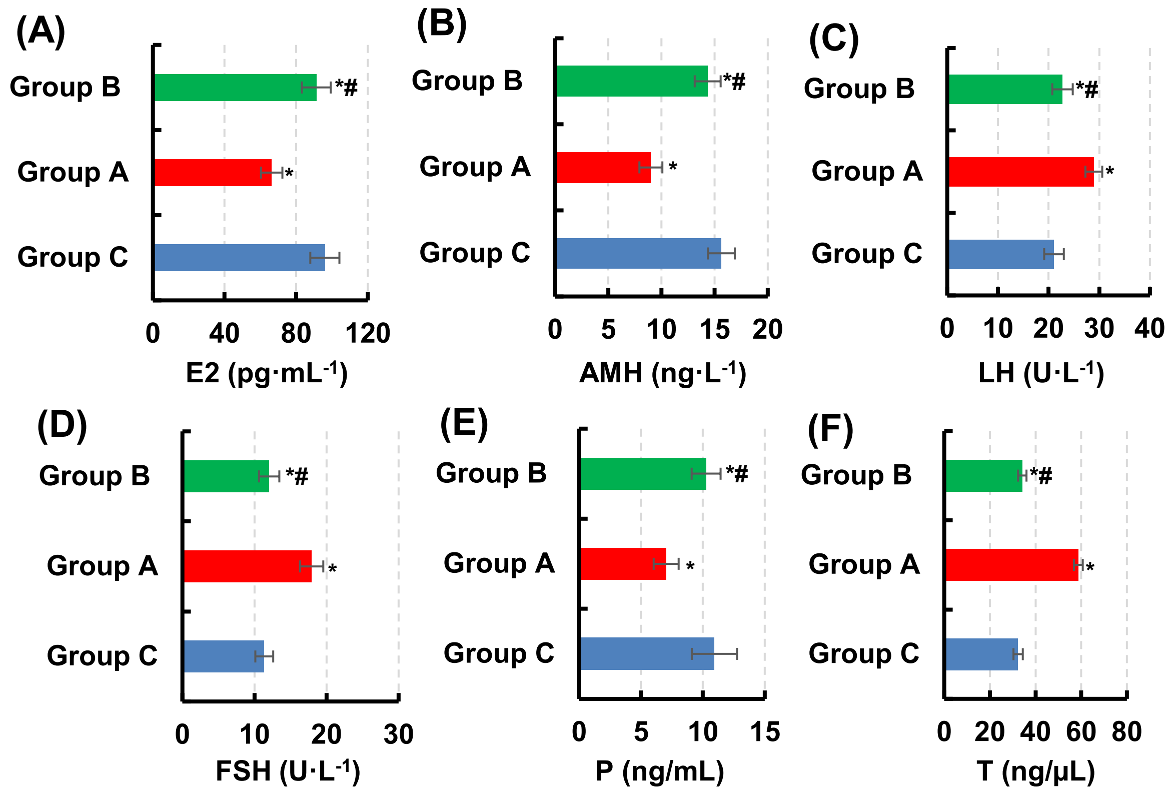


Fig. 2 Comparison of sex hormone levels in each group. A-F represents E2, AMH, LH, FSH, P, and T levels in sequence. *P≤0.05 vs. Group C; #P≤0.05 vs. Group A.

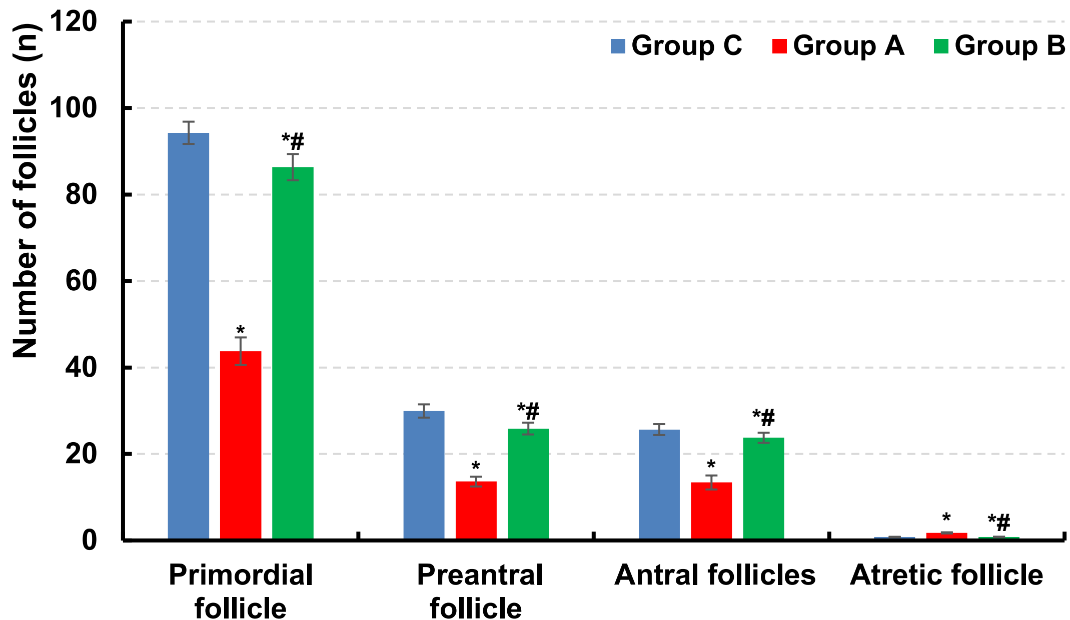


Fig.3 Comparison of the number of follicles. *P≤0.05 vs. Group C; #P≤0.05 vs. Group A.

Results of PCNA detection: The assessment of PCNA protein levels showed that, compared to Group C, the levels of PCNA in the ovarian tissue of mice in the Group A and Group B were significantly reduced (P≤0.05). This result was visually confirmed by IHC. Figure 4A presents the PCNA staining images of ovarian tissues from mice in different groups. In Group C, PCNA staining exhibited a strong red reaction, indicating a high number of PCNA-

positive cells and reflecting elevated cell proliferation activity. In contrast, the intensity of PCNA staining was markedly reduced in the ovarian tissue of Group A and Group B mice, with a significant decrease in the red-stained areas, indicating a lower level of cell proliferation. Further quantitative analysis revealed that the PCNA protein IOD value in Group A (11.07 ± 5.14) was significantly lower than that in Group B (21.37 ± 5.29) ($P \leq 0.05$) (Fig. 4B).

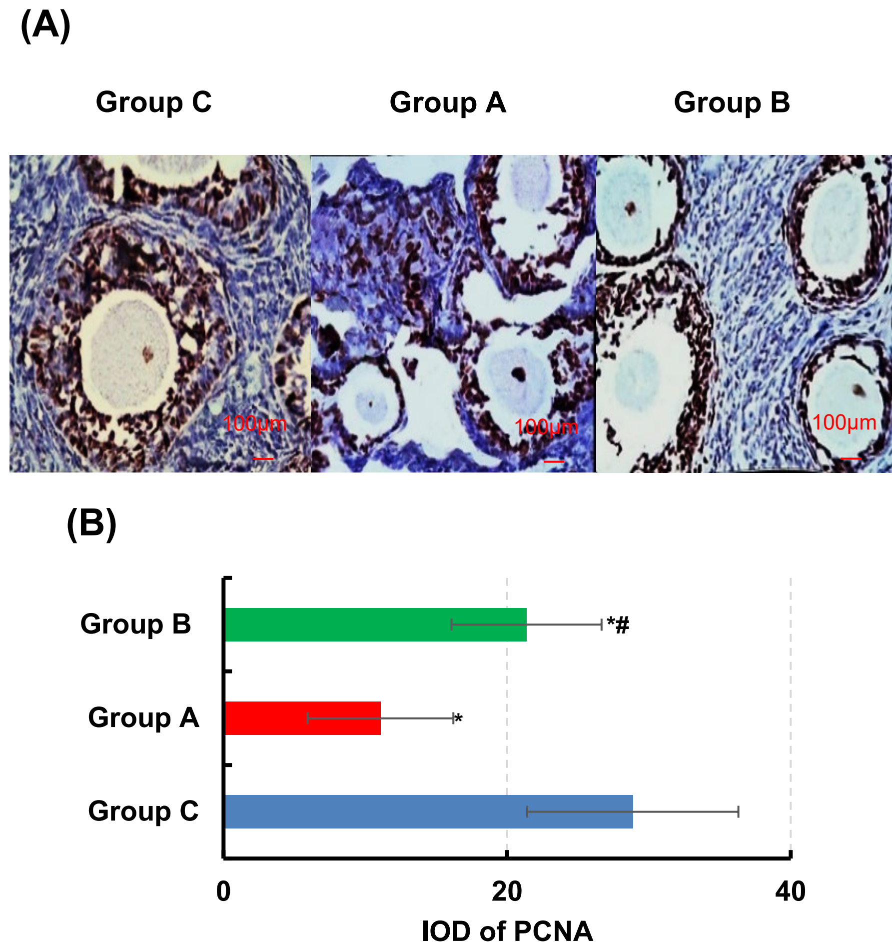


Fig.4 Comparison of PCNA protein levels in each group. A: IHC staining results (× 400); B: IOD value. *$P \leq 0.05$ vs. Group C; #$P \leq 0.05$ vs. Group A.

Detection results of protein expression levels related to primordial follicle activation: The results of protein immunoblotting experiments demonstrated that the levels of p-PTEN/PTEN were notably elevated in mice of Group A and Group B versus Group C, while those of p-YAP/YAP, p-mTOR/mTOR, and p-rpS6/rpS6 were notably reduced in mice of Group A and Group B ($P \leq 0.05$). Additionally, when contrasting Group B with Group A, a marked reduction in p-PTEN/PTEN levels and a prominent increase in p-YAP/YAP, p-mTOR/mTOR, and p-rpS6/rpS6 levels were observed in Group B ($P \leq 0.05$) (Fig.5A-B).

Fertility assessment results: The results of seven consecutive mating trials (H1, H2, H3, H4, H5, H6, H7) showed that, except for H3, Group C had a higher pregnancy rate in each round compared to Group A and Group B. When the pregnancy rates from H1 to H7 were averaged (M), the average pregnancy rate in Group C was significantly higher than that in Group A and Group B ($P \leqslant 0.05$), with Group B showing a significantly higher pregnancy rate than Group A ($P \leqslant 0.05$). In the evaluation of female mice reproductive capacity, compared to Group C, both Group A and Group B showed a significant reduction in the number of pups per litter and the number of litters ($P \leqslant 0.05$). Furthermore, Group B had significantly more pups per litter and more litters than Group A ($P \leqslant 0.05$) (Fig.6A-C).

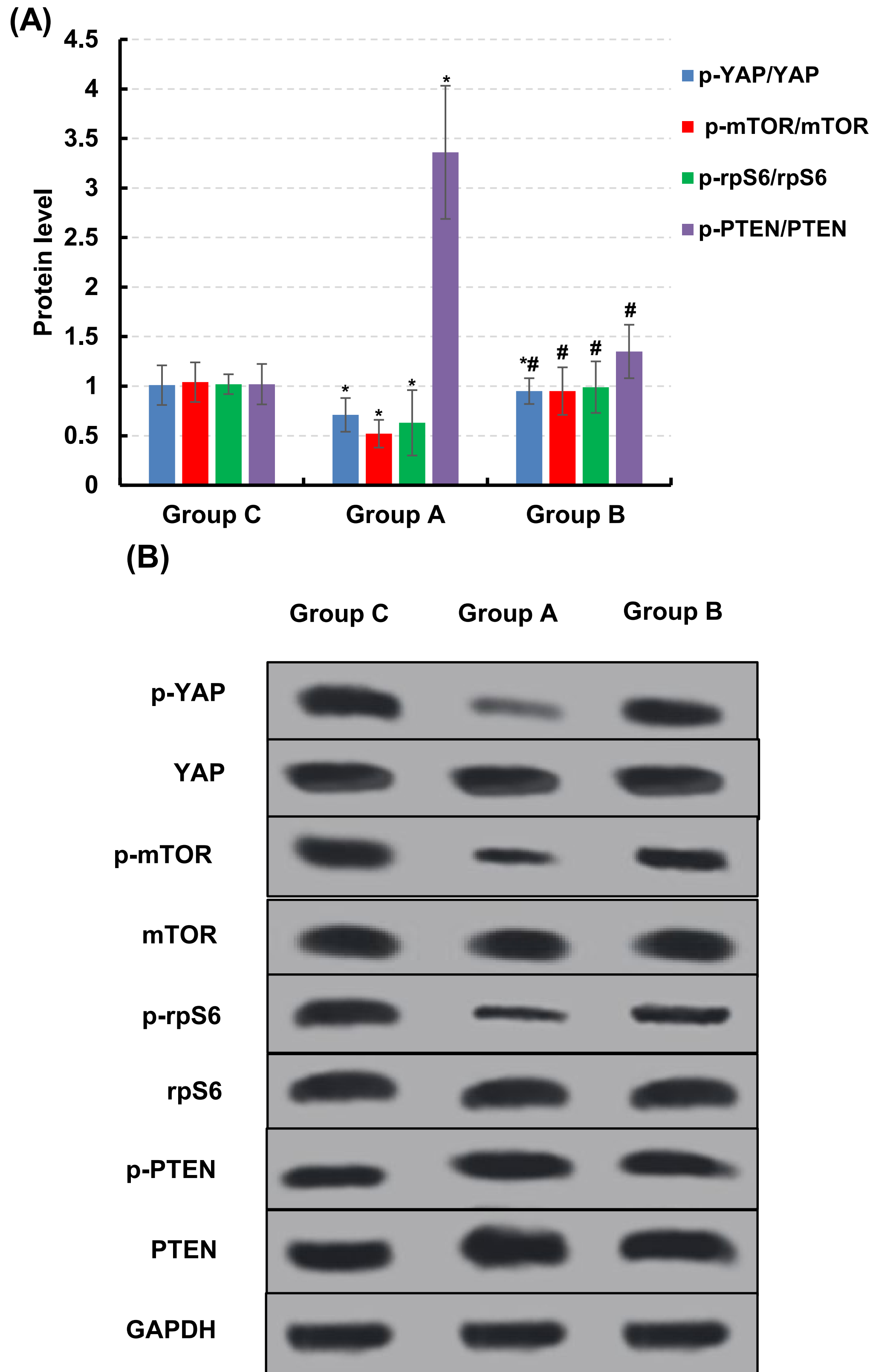


Fig. 5 Comparison of expression levels of primordial follicle activation-related proteins in each group. A: comparison of related protein expression levels; B: Western blot. *$P \leq 0.05$ vs. Group C; #$P \leq 0.05$ vs. Group A.

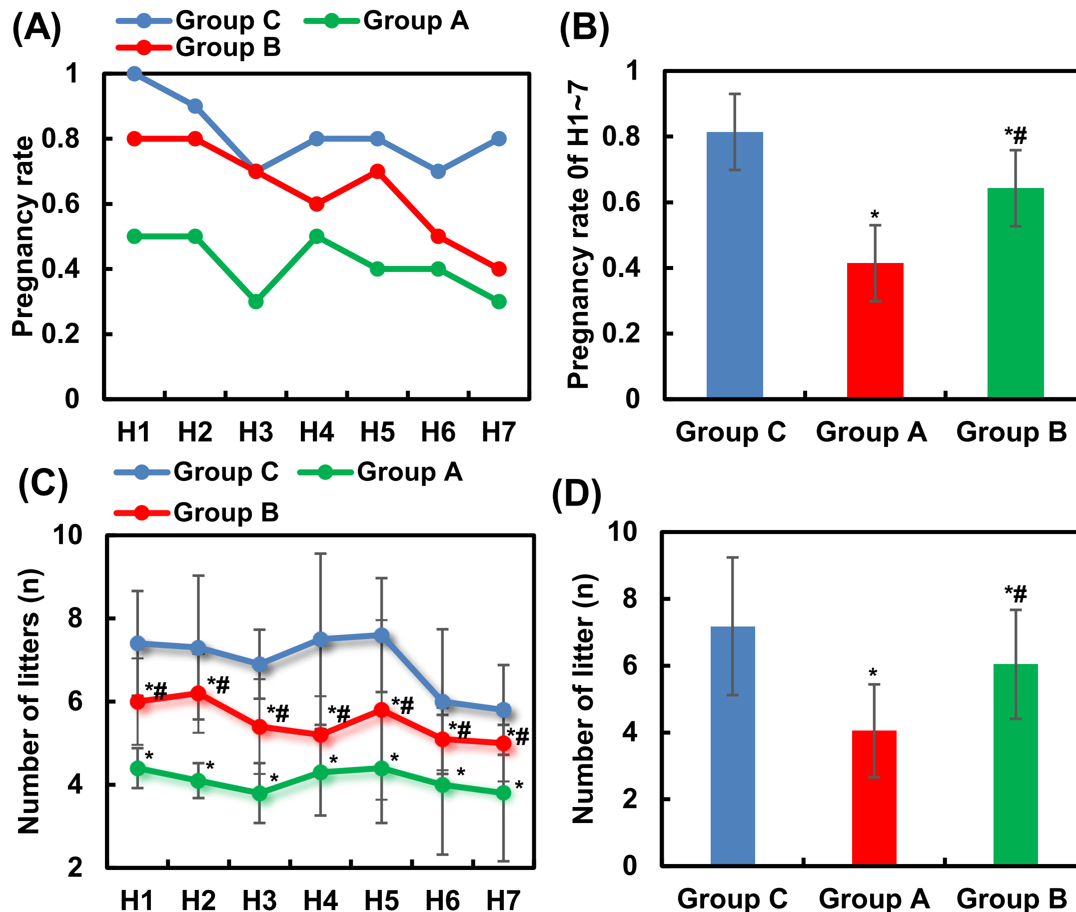


Fig.6 Evaluation results of mouse fertility. A: pregnancy rate; B: the average pregnancy rate from H1 to H7; C: litter size; D: litter size. *P≤0.05 vs. Group C; #P≤0.05 vs. Group A.

DISCUSSION

This study aimed to establish an aged infertile mouse model to investigate the ovulation-promoting impacts of *Angelica sinensis* extract and its impact on fertility. Experimental results demonstrated that *Angelica sinensis* extract can improve ovarian function and fertility in aged infertile mice, as evidenced by modulation of sex hormone levels, promotion of follicular development, and increased ovarian cell proliferation activity.

This study adopted the method proposed by Wang *et al.* (Wang *et al.*, 2019) to establish a model of age-related infertility in mice, which was induced by hormonal treatment. The results indicated irregular estrous cycles in both Group A and Group B mice. Clinical studies emphasized that regular estrous cycles are crucial indicators of reproductive system health, and disruption of estrogen/testosterone levels can lead to infertility (Hamilton *et al.*, 2017; Amir *et al.*, 2021). Therefore, the successful establishment of this model supports its utility for future investigations.

This study evaluated changes in ovarian function among different Group A and Group B of mice. It was found that compared to Group A, mice in Group B exhibited higher levels of E2, AMH, and P in ovarian tissue, while levels of FSH, LH, and T were lower. Li and Shi (2021) also reported in their study that ferulic acid (FA), an active component of *Angelica sinensis*, downregulates LH and E2 expression levels in serum. Additionally, mature follicles (primordial, primary, and antral follicles) markedly decreased in the Group A and Group B, whereas the number of atretic follicles significantly increased. A study by Zhang *et al.* (2021) demonstrated that high levels of exogenous hormones can lead to follicular development disorders. These studies suggest that hormonal regulation is a key mechanism through which *Angelica sinensis* extract promotes follicular development, potentially improving ovarian function by modulating the levels of specific hormones. Furthermore, compared to Group A, mice in Group B exhibited a drastic increase in primordial, preantral, and antral follicles, with a notable decrease in atretic follicles. Chen *et al.* (2022) proposed in their research that *Angelica sinensis* extract promotes vascularization of

chicken follicles, thereby increasing follicle numbers, which aligns with findings from our study. E2 promotes follicular development and endometrial proliferation (Chauvin *et al*., 2022), AMH reflects ovarian reserve and regulates follicle growth (Anderson *et al*., 2022), while LH and FSH modulate reproductive cycles and follicular development (Liu *et al*., 2019). Progesterone (P) maintains pregnancy and embryo development. Changes in these hormones are closely associated with infertility, underscoring their critical importance to female fertility and health (Kolatorova *et al*., 2022; Borrás *et al*., 2021; Qiu *et al*., 2022). These results suggest that *Angelica sinensis* intervention has a positive effect on hormone production and regulation, thereby maintaining normal follicular development.

Furthermore, the study found that compared to Group A, mice in Group B exhibited a significant increase in PCNA protein expression in ovarian tissue. Additionally, p-YAP/YAP, p-mTOR/mTOR, and p-rpS6/rpS6 levels were markedly elevated in Group B mice, while p-PTEN/PTEN levels were significantly reduced. PCNA serves as a crucial marker of cell proliferation, and its decreased expression in ovarian tissue of Group A and Group B indicates a significant decline in ovarian cell proliferation activity (Sirotkin *et al*., 2021), consistent with the previously mentioned reduction in follicle numbers. YAP, mTOR, and rpS6 are key proteins in cellular signaling pathways involved in regulating important physiological processes, including cell proliferation, metabolism, and survival (Wang *et al*., 2023; Cho *et al*., 2021). PTEN functions as a critical tumor suppressor gene, regulating cell proliferation, apoptosis, and metabolism in various physiological and pathological conditions (Shi *et al*., 2022). These signaling pathways are closely associated with cell proliferation, and *Angelica sinensis* extract may promote ovarian cell proliferation and improve ovarian function by regulating these pathways. Based on the results of this study, it is evident that *Angelica sinensis* extract protects ovarian tissue from damage by inhibiting the abnormal activation of these signaling pathways, thereby promoting normal follicular development and the maintenance of physiological function. To further validate this mechanism, future studies could utilize pathway inhibitors or gene knockout models to explore the action mechanism of *Angelica sinensis* extract in greater detail.

The proper functioning of the ovaries is crucial for female fertility, as normal follicular development and ovulation form the basis for successful conception. Observing fertility in female mice, it was found that various fertility indicators significantly improved in Group B aged infertile mice, indicating a positive effect of *Angelica sinensis* extract on ovarian function and fertility in aged infertile mice. This improvement is likely related to *Angelica sinensis* extract's ability to enhance follicular development and ovulation in aged infertile mice effectively. However, the specific therapeutic effects may also depend on dosage and intervention duration. Therefore, to further elucidate the specific mechanisms by which *Angelica sinensis* extract affects ovarian function, future research should employ more refined molecular biological techniques and explore the optimal treatment protocols and dosages.

Conclusion: This study aimed to assess the impact of *Angelica sinensis* extract on ovarian function, ovulatory function, and reproductive capacity in aged infertile mice through a comparative analysis of various interventions. The results of this investigation demonstrated remarkable enhancements in ovarian function, ovulatory capacity, and reproductive vigor following intervention with *Angelica sinensis* extract. This enhancement was evidenced by a notable increase in pregnancy rates, litter sizes, and number of offspring per litter.

Authors' Contributions: Wen Jiang and Bingru Luo designed experiments; Weihui Yang and Li Li analyzed data; Weihui Yang and Li Li collected samples; Weihui Yang and Li Li performed experiments; Wen Jiang and Bingru Luo wrote the manuscript. All authors agreed to publish this article.

Animal rights statement: All animal experiments were approved by the Animal Ethics Committee of Hunan Provincial People's Hospital (The First Affiliated Hospital of Hunan Normal University, Changsha, P.R. China), in compliance with Chinese national guidelines for the care and use of animals.

Conflict of interests: None

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