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Original Article

Baicalein improves the symptoms of polycystic ovary syndrome by mitigating oxidative stress and ferroptosis in the ovary and gravid placenta

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ABSTRACT

Background: Polycystic ovary syndrome is a metabolic and hormonal disorder that is closely linked to oxidative stress. Within individuals diagnosed with PCOS, changes occur in the ovaries, resulting in an excessive buildup of iron and peroxidation of lipids, both of which may be associated with the occurrence of ferroptosis. Baicalein, a flavonoid found in the roots of Scutellaria baicalensis and widely known as Chinese skullcap, is known for its anti-inflammatory and anti-ferroptotic properties, which protect against various diseases. Nevertheless, there has been no investigation into the impact of baicalein on polycystic ovary syndrome.

Purpose: This study aimed to correlate ferroptosis with polycystic ovary syndrome and to assess the effects of baicalein on ovarian dysfunction and placental development in pregnant patients.

Study design and methods: Polycystic ovary syndrome was induced in a rat model through the administration of dehydroepiandrosterone, and these rats were treated with baicalein. Oxidative stress and inflammation levels were assessed in serum and ovaries, and tissue samples were collected for histological and protein analyses. Furthermore, different groups of female rats were mated with male rats to observe pregnancy outcomes and tissue samples were obtained for histological, protein, and RNA sequencing. Then, RNA sequencing of the placenta was performed to determine the key genes involved in ferroptosis negative regulation (FNR) signatures.

Results: Baicalein was shown to reduce ovarian oxidative stress and pathology. Baicalein also ameliorated polycystic ovary syndrome by decreasing lipid peroxidation and chronic inflammation and modulating mitochondrial functions and ferroptosis in the ovaries. Specifically, glutathione peroxidase and ferritin heavy chain 1 were considerably downregulated in polycystic ovary syndrome gravid rats compared to their expression in the control group, and most of these differences were reversed after baicalein intervention.

Conclusions: Our findings, initially, indicated that baicalein could potentially enhance the prognosis of individuals suffering from polycystic ovary syndrome by reducing oxidative stress and ferroptosis, thus potentially influencing the formulation of a therapeutic approach to address this condition.

Introduction

Polycystic ovary syndrome (PCOS), a prevalent endocrine disorder that impacts the reproductive capabilities of women, has been extensively studied (Norman et al., 2007; Joham et al., 2022). PCOS is characterized by a multitude of symptoms encompassing irregular menstrual cycles, elevated androgen production, and the presence of ovarian cysts (Azziz et al., 2016; Dapas et al., 2022). Oxidative stress is a prerequisite marked by an imbalance between the body's antioxidant defenses and pro-oxidant molecules. This imbalance is pivotal in the development of subfertility in men and women (Hayashi et al., 2020; Evans et al., 2021). Typically, oxidative stress levels are higher in individuals with PCOS than in individuals without PCOS, probably due to the high oxidative stress damage in ovarian tissues caused by a reduc-

Abbreviations: ACSL4, long-chain fatty acid-CoA ligase 4; COX2, cyclooxygenase-2; DHEA, dehydroepiandrosterone; FTH1, ferritin heavy chain 1; GPX4, glutathione peroxidase; GSH, glutathione; MDA, malondialdehyde; MMP, mitochondrial membrane potential; PCOS, polycystic ovary syndrome; ROS, relative oxygen species

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tion in estrogen and progesterone levels. Additionally, insulin resistance in PCOS patients may lead to metabolic disorders and high oxidative stress (Murri et al., 2013; Liu et al., 2019). These findings point to a role for oxidative stress in PCOS pathogenesis.

The lipid peroxidation process central to ferroptosis can be initiated and exacerbated by oxidative stress. Excessive oxidative stress has the potential to cause elevated levels of lipid peroxides, thus facilitating ferroptotic demise (Park et al., 2021). The occurrence of ferroptosis is triggered by the buildup of lipid peroxides in an iron-reliant fashion, rendering it distinct from alternative types of controlled cellular demise(Dixon et al., 2014). Glutathione peroxidase (GPX4) play pivotal roles in regulating ferroptotic cell death (Dixon et al., 2012; Liu et al., 2023). Primarily, the overexpression of cyclooxygenase-2 (COX2), increase in malondialdehyde (MDA) levels, and reduction in glutathione function as pivotal markers signifying ferroptosis. These markers are critical indicators of the advancement and progression of ferroptotic cell death (Gao et al., 2015). Lipids, specifically polyunsaturated fatty acids, are susceptible to oxidation and play a vital role in ferroptosis (Xu et al., 2022). Ferroptosis is associated with various physiological and pathological mechanisms, including damage caused by ischemia–reperfusion, neurodegenerative ailments, and cancer (Stockwell et al., 2017). Numerous investigations have demonstrated that the regulation of ferroptosis, either through its stimulation or inhibition, can assist in the treatment of numerous disorders (Lei et al., 2021; Zeng et al., 2023). The primary pharmaceutical treatments for PCOS involve insulin-sensitizing agents, such as metformin, and anti-androgen medications, such as spironolactone. However, no medication is known to treat PCOS by inhibiting ferroptosis.

The placenta develops in the uterus during pregnancy and plays a vital role in supporting the growing fetus. The uterus is attached to the uterine wall and connected to the fetus by the umbilical cord. The placenta supplies oxygen and nutrients to the developing fetus and removes waste products and carbon dioxide. Hormones also regulate fetal development and prepare the mother's body for childbirth (Redline et al., 2023). The placenta is capable of producing significant amounts of free radicals and oxidative stress-related substances. It can also counteract oxidative stress by secreting antioxidant enzymes and other molecules to maintain its stability. High oxidative stress may damage placental cells, possibly affecting their function. Therefore, maintaining an optimal level of oxidative stress is essential for maintaining placental health and promoting normal placental development (Grzeszczak et al., 2023). A number of research investigations have documented that increased ROS levels combined with a lack of a corresponding increase in autophagy can lead to preterm labor and delivery (Schoots et al., 2018).

Baicalein, an extract found in the roots of Scutellaria baicalensis Georgi, a plant belonging to the Lamiaceae family, is a flavonoid compound. *Scutellaria baicalensis roots* are the primary source of baicalein for various scientific and medicinal purposes. *Scutellaria baicalensis* is recognized for its traditional use in herbal medicine, particularly in East Asian countries (Li-Weber, 2009; Chmiel and Stompor-Gorący, 2023). The reported bioactivities of baicalein encompass a diverse array of pharmacological effects. It is renowned for its anti-inflammatory properties, attributed to its ability to modulate signaling pathways involved in the inflammatory response. Additionally, baicalein has demonstrated antioxidant activity, contributing to its potential in combating oxidative stress. Studies have suggested its efficacy in inhibiting certain enzymes involved in cancer cell proliferation, highlighting its potential anti-cancer properties effects (Oliveira et al., 2015; Chmiel and Stompor-Gorący, 2023). Baicalein protects against ferroptosis by regulating multiple signaling pathways. For example, baicalein can prevent the accumulation of cellular iron ions, reduce peroxisome production, and promote glutathione production in cells; these changes can reduce lipid peroxidation reactions. Baicalein has the ability to hinder the action of crucial enzymes implicated in metabolic pathways of lipids, like NADPH oxidase. This compound leads to a reduction in the levels of unsaturated fatty acids found in cellular membranes. Additionally, it helps decrease the generation of free radicals derived from lipids and the occurrence of reactions related to lipid peroxidation (Yang et al., 2023; Dinda et al., 2017; Kushwah and Hu, 2010). The diverse bioactivities of baicalein have led to continued research and exploration of its therapeutic potential across various health domains.

The abovementioned studies indicated that the pathogenesis of PCOS might be influenced by lipid peroxidation and ferroptosis. The complete understanding of the main regulatory factors and signaling pathways implicated in PCOS remains unknown. To fill this knowledge gap, we investigated the relationships between ferroptosis and ovarian dysfunction and placental development in pregnant rats with PCOS and between ferroptosis and the effect of administering baicalein.

Experimental section

Chemicals and reagents

Baicalein (molecular formula: C15H10O5, relative molecular mass: 270.24, purity: >98 %) was purchased from Abmole (M6098, Shanghai, China). Ferrostatin-1 was purchased from MedChemExpress (HY100579, Shanghai, China). Dehydroepiandrosterone (DHEA) was purchased from Solarbio (D8950, Beijing, China). Sodium carboxymethyl cellulose (0.5 % —CMC-Na) was purchased from MedChemExpress (HY-Y0703, Shanghai, China). The primary antibodies used in the experiments were against GPX4, FTH1, ACSL4 and COX2 (ab125066, ab75972, ab205197, ab179800, Cambridge, U.K.). GAPDH was purchased from Proteintech (60004–1-Ig, Wuhan, China). The secondary antibodies were obtained from ZSGB-BIO (ZB2305, ZB2306, Beijing, China). A ferric test kit was obtained from Selleck (MAK025, Houston, USA). Interleukin-18 (IL-18), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) ELISA kits were purchased from Biolegend (430507, 741127, 430204, California, USA). GSH, MDA, SOD, ROS, estradiol (eE2), and follicle stimulating hormone (FSH) assay kits were purchased from Sino-UK Bio (HY-60006, HY-M0003, HY-M0001, HY-M0087, HY-M1899, and HY-10024T; Beijing, China). A cell counting kit-8 (CCK-8) and a bicinchoninic acid assay (BCA) were purchased from Beyotime (C0037, P0010, Beijing, China). RIPA lysis buffer was purchased from Solarbio (R0010, Beijing, China).

Cell culture and viability assay

Human ovarian granulosa cells (CTCC-003–0105) were purchased from Meisen, China, and grown in Dulbecco's modified Eagle's medium (11995; Solarbio, Beijing, China) supplemented with 10 % fetal bovine serum, 1 % penicillin and streptomycin in a humidified atmosphere containing 5 % CO_2 at 37 °C. KGN cells were cultured, Fer-1 (10 μmol/l) and baicalein (20 μmol/l) were added for 5 h, and DHEA (20 μmol/l) was used to stimulate KGN cells for 24 h, 48 h, or 72 h. Cell viability was assessed using a CCK-8 assay.

Experimental animals

All the experiments followed the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Vital River supplied three-week-old female Sprague Dawley rats (Beijing, China). Standard food and water were given to the rats kept under SPF conditions (24 ± 1 °C, 40–80 % relative humidity) at Beijing Obstetrics and Gynecology Hospital (Ethics Code. BOGH21–2305–5, Apr. 26, 2023.). The blank control group included seven rats. These rats received a daily injection of 0.2 ml of sesame oil. To induce PCOS, the rats were subcutaneously injected daily with 60 mg/kg DHEA (dissolved in 0.2 ml of sesame oil). After the PCOS rat model was estab-

lished, seven rats were randomly assigned to either the PCOS group or the baicalein group. After treatment for 21 days, the rats in the PCOS group were treated with 0.2 ml of 0.5 % —CMC-Na, whereas those in the baicalein group were administered 25 mg/kg, 50 mg/kg or 100 mg/kg baicalein (baicalein was dissolved in 0.2 ml of 0.5 % —CMC-Na, the concentration of which was 1 μg/μl) via intraperitoneal injection for 28 days. Pregnancy was established by pairing proestrus female rats with fertile male rats of the same strain at a 2:1 ratio. Successful mating was confirmed by the presence of a vaginal plug the following morning, which was considered gestational day 0.5. On gestational day 15.5, all rats were killed, and serum samples and the ovaries, uterus, and placenta were collected. These specimens were subsequently either fixed for morphological and immunohistochemical analyses or promptly frozen in liquid nitrogen and stored at –80 ˚C for Western blot analysis and RNA sequencing.

Hematoxylin and eosin staining

The ovarian and placental tissues of the rats were immediately fixed in 4 % paraformaldehyde (BL539A, Biosharp, Beijing, China). After paraffin embedding, the tissues were cut into 4 μm slices. Next, the tissue sections were immersed in a hematoxylin solution, which selectively stains the nuclei a blue–purple color. After staining, the tissue was differentiated in an acid alcohol solution to remove excess stain and achieve the desired contrast between the nuclei and other tissue components. To enhance the color and contrast of the hematoxylin-stained nuclei, the tissue sections were briefly exposed to a weak alkaline solution, such as running tap water, in a process called “bluing”. Following bluing, the tissue underwent eosin staining, which imparted a pink color to the cytoplasm and other extracellular components. This step is relatively short compared to hematoxylin staining. After staining, the tissue sections were dehydrated using a series of alcohol baths of increasing concentration to remove water. Then, the slides were cleared in xylene to prepare for mounting. Finally, the tissue sections were mounted on a slide with a coverslip using a mounting medium. Finally, the ovarian and placental tissues were prepared for microscopic examination (EVOS M7000, Thermo Fisher Scientific, Waltham, USA). This procedure allowed the visualization of tissue structures; the cell nuclei appeared blue, which facilitated the histological analysis of the ovarian and placental tissues.

Perls’ histochemical reaction

Iron deposition in the placenta was evaluated by diaminobenzidine (DAB)-enhanced Perls’ staining. Following deparaffinization and rehydration, the tissue sections were submerged in a mixture of equal volumes of potassium ferrocyanide solution and hydrochloric acid solution for 1 h at room temperature. The sections were rinsed with PBS five times for 5 min each, exposed to DAB for 10 min , and finally treated with pararosaniline solution for 2 min . Images of the distribution of iron were taken using a microscope (EVOS M7000, Thermo Fisher Scientific, Waltham, USA).

Immunohistochemistry of placental tissue

The tissues were fixed for 24 h in 4 % paraformaldehyde at 4 ˚C, embedded in paraffin, and subsequently sectioned into slices with a thickness of 4 μm. Next, the tissues were blocked in a 0.5 % bovine serum albumin solution and incubated with primary antibodies against FTH1 (1:200) and COX2 (1:200) overnight. Next, the sections were incubated with biotin-conjugated secondary antibodies (1:300) for 1 h at 25 ˚C for visualization. The coverslips were counterstained using hematoxylin and eosin for 5 min at 25 ˚C. Finally, the samples were examined under a fluorescence microscope (EVOS M7000, Thermo Fisher Scientific, Waltham, USA).

Transmission electron microscopy

The KGN cell pellets and ovarian tissue were fixed with 2.5 % glutaraldehyde at room temperature. The plates were incubated first for 2 h and then for 24 h (overnight) at 4 ˚C. The following day, the KGN cells and ovarian tissue were dehydrated with ethanol and cut into 100 nm sections. Next, the sections were treated with uranyl acetate and lead citrate. Finally, the sections were analyzed via transmission electron microscopy (Leica, Weztlar, Germany).

JC-1 staining

JC-1 is an effective fluorescent probe that is extensively utilized for detecting the mitochondrial membrane potential (MMP). The cells treated with the drug were stained with JC-1 working solution for 20 min , followed by two washes with the prepared JC-1 staining buffer. Subsequently, 2 ml of cell culture medium was added, and the cells were observed under a laser confocal microscope (Olympus, Japan). The fluorescence intensity ratio of green to red was used to indicate the MMP.

ROS staining

The cell fluorescence probe 2′,7′-dichlorodihydrofluorescein diacetate was used to determine oxidative stress. We used this method to assess the intracellular ROS levels in KGN cells. Dichlorodihydrofluorescein diacetate working solution was added to the DHEA-treated cells, which were subsequently incubated at 37 ˚C for 30 min. The cells were subsequently washed with a serum-free culture medium. Subsequently, the fluorescence signal of dichlorofluorescein was measured using a fluorescence microscope, which reflects the intracellular ROS levels.

Dihydroethidium is a probe that can enter cells freely and be oxidized by ROS to form oxyethidium. This product binds to DNA and generates a red fluorescent signal. The intensity of the red fluorescence detected in living cells was used to determine the level of ROS and changes in the cells. Frozen tissue sections of the rat placenta were placed in DHE working solution. The sections were fully immersed in a staining solution to allow DHE to penetrate the tissue. These sections were incubated at 37 ˚C for 30 min and then washed with PBS to remove any unabsorbed DHE and dimethyl sulfoxide (DMSO). Finally, the sections were observed, and images were collected under a Nikon fluorescence microscope.

Iron assay

Levels of ferrous iron were quantified by means of an iron assay kit. First, a 10 μl aliquot from the 100 mM standard solution was diluted, and a 1 mM standard solution (0, 2, 4, 6, 8, and 10 μl) was introduced into a 96-well plate to create standards with concentrations of 0, 2, 4, 6, 8, and 10 nmol/well per well. Iron assay buffer was added to each well, and the volume was 100 μl. Next, 5 μl of iron reducer was added to each standard well. The tissues and cells were swiftly homogenized in 4–10 vol of iron assay buffer. Subsequently, the samples were centrifuged at 16, 000 × *g* for 10 min at 4 ˚C to eliminate insoluble material. Subsequently, the samples were dispensed into 96-well plates. After incubating at 37 ˚C for 1 hour, the absorbance was measured at 593 nm using an automatic microplate reader (Promega, Madison, USA).

Detection of ROS, SOD, MDA, and GSH

Blood samples from the rats were subjected to centrifugation at 3000 rpm for 15 min for biochemical analysis. The levels of ROS, SOD, MDA, and GSH were subsequently determined using the corresponding assay kits following the manufacturer's instructions. The tissues or cells were homogenized with PBS. Following homogenization or lysis, the

samples were centrifuged at 10, 000 × *g* - 12, 000 × *g* for 10 min , after which the resulting supernatant was collected for subsequent analysis. We determined the protein concentration of tissue or cell samples after preparation with a BCA protein concentration determination kit. Using this method, we calculated the protein content in tissue or cells per unit protein weight. The optical density was quantified at 450 nm using an automatic microplate reader (Promega, Madison, USA).

ELISA

We performed IL-6, IL-18, and TNF-α assays using suitable kits. A blank control hole and standard holes were set; then, 50 μl of serum was directly added to test each test well. Next, 50 μl of enzyme-labeled antigens and antibodies were added to all the wells, the contents were thoroughly mixed, the sealing plate mold was added, and the mixture was incubated at 37 ˚C for 1 hour. Then, the plate was washed manually, developer A and developer B were added to each well, and the treated samples were incubated at 37 ˚C for 15 min . Finally, a termination solution was added, and the optical density was quantified at 450 nm using an automatic microplate reader (Promega, Madison, USA).

Western blot

Protein extraction was carried out by lysing tissues or cells in RIPA buffer which was supplemented with protease and phosphatase inhibitors. To determine protein concentrations, BCA assays were conducted. Equal amounts of proteins were then loaded onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) for separation. Subsequently, the separated proteins were transferred onto polyvinylidene difluoride membranes. Following the addition of 5 % skim milk (Solarbio, Beijing, China), the membranes were incubated overnight at 4 ˚C with primary antibodies. Next, the sections underwent incubation with secondary antibodies for a duration of one hour. Finally, the utilization of a ChemiDoc MP Imaging System from Bio-Rad (California, USA) allowed for visualization of the signal.

RNA sequencing analysis

RNA extraction and quantification were performed using the HiPure Total RNA Mini Kit (R4111–02; Magen, Guangzhou, China). All samples with an OD 260/280 ratio above 1.8 were subsequently sent to Novogene (Tianjin, China) for sequencing. After rRNA was removed, the library was constructed. The constructed library was checked using an Agilent 2200 and a Qubit 3.0 fluorometer and then sequenced using the Illumina HiSeq X Ten platform after passing the test. First, low-quality and adapter-contaminated reads were removed, and the retained data were considered to be clean reads. The clean reads were aligned to the reference genome, after which new transcript prediction, short sequence variation detection, fusion gene detection, and difference DNA testing were performed. We started by incorporating new transcript sequences with protein-coding potential into the existing reference transcript sequences to create a comprehensive reference sequence. Next, we quantified the gene expression levels. When evaluating multiple samples, we initially identified differential expression genes (DEGs) between these samples and subsequently conducted cluster analysis and functional enrichment analysis on the identified DEGs.

Statistical analysis

The values are expressed as the mean ± SEM. One-way analysis of variance (ANOVA) followed by multiple comparisons was used for statistical analysis (GraphPad Prism 9.0; San Diego, CA, USA). All comparisons were considered to be statistically significant at $p < 0.05$.

Results

Baicalein restores DHEA-induced lipid peroxidation in KGN cells

The KGN cell line, derived from human ovarian granulosa cells, provides valuable insights into the biological characteristics and functions of these cells (Gao et al., 2021). In our in vitro study, we first stimulated KGN cells with DHEA and subsequently treated them with Fer-1 or baicalein. Fer-1, a potent inhibitor of ferroptosis, was utilized to assess the effects of ferroptosis (Dixon et al., 2012). Cell viability was determined by the CCK-8 assay. In the DHEA group, the viability of KGN cells was reduced compared to that in the control group. In contrast, treatment with Fer-1 and baicalein increased cell viability (Fig. 1B). Moreover, we examined the effect of baicalein on oxidative stress in KGN cells. We evaluated the contents of MDA and GSH (Fig. 1C, Fig. 1I). Intracellular ROS generation was determined by —DCFH-DA staining. Treatment with DHEA caused excessive ROS production, while Fer-1 and baicalein treatment reduced ROS levels (Fig. 1D-E). We determined the effect of baicalein on the MMP in KGN cells by JC-1 staining, which serves as a metric of mitochondrial function and reflects the extent of cell death. We found that the MMP of cells stimulated by DHEA decreased considerably, whereas the MMP of KGN cells increased substantially after treatment with Fer-1 and baicalein (Fig. 1F-G). We also measured the content of intracellular iron ions and found that intracellular Fe^{2+} accumulated after DHEA stimulation, whereas baicalein intervention decreased intracellular Fe^{2+} levels (Fig. 1H). Jiang et al. reported that ROS and lipid peroxidation increased in KGN cells after DHEA stimulation, which is consistent with our findings. Moreover, we found that baicalein mitigated lipid peroxidation in KGN cells.

Baicalein reduces DHEA-induced KGN cell ferroptosis

Concerning ferroptosis, Dixon et al. reported that ferroptosis is linked to significant morphological alterations in mitochondria characterized by mitochondrial fragmentation and enlargement of cristae structures, including reduced size, increased membrane density, reduced or disappearing cristae, disruption of the outer membrane, and less pronounced alterations in nuclear morphology (Dixon et al., 2012). Thus, we examined the morphological changes in KGN cells using a transmission electron microscope. We cultured cells in groups as described above and found distinct differences in cell morphology between the cells in different groups. Most cells in the control group had regular morphological characteristics, with slight irregularities in the nuclear structure. The cytoplasm contained uniform particles, and the mitochondria appeared as short rod-shaped structures with a clear inner ridge and an intact structure. The rough endoplasmic reticulum had a well-defined structure. After the DHEA intervention, the cells were slightly irregular, but their nuclei were regular. Homogeneous particles were visible in the cytoplasm. The mitochondria in the cytoplasm appeared oval, with swelling, partial internal ridge rupture, and vacuolation. The rough endoplasmic reticulum was short and clear in appearance. After baicalein and Fer-1 were administered, the morphological characteristics of the cells and mitochondria were restored to a normal state compared to the morphological characteristics recorded in the DHEA group (Fig. 2A).

We also performed Western blotting assays to investigate the key regulatory molecules implicated in the induction of ferroptosis in KGN cells to elucidate the underlying mechanism of ferroptosis induced by DHEA. GPX4, a crucial enzyme, is instrumental in preventing ferroptosis by diminishing lipid peroxides. ACSL4 can regulate the levels of PUFA-containing phospholipids in cell membranes; these phospholipids are highly susceptible to oxidation and can generate lipid peroxides that trigger ferroptosis (Doll et al., 2017). To further investigate the associated changes, we conducted experiments on dif-

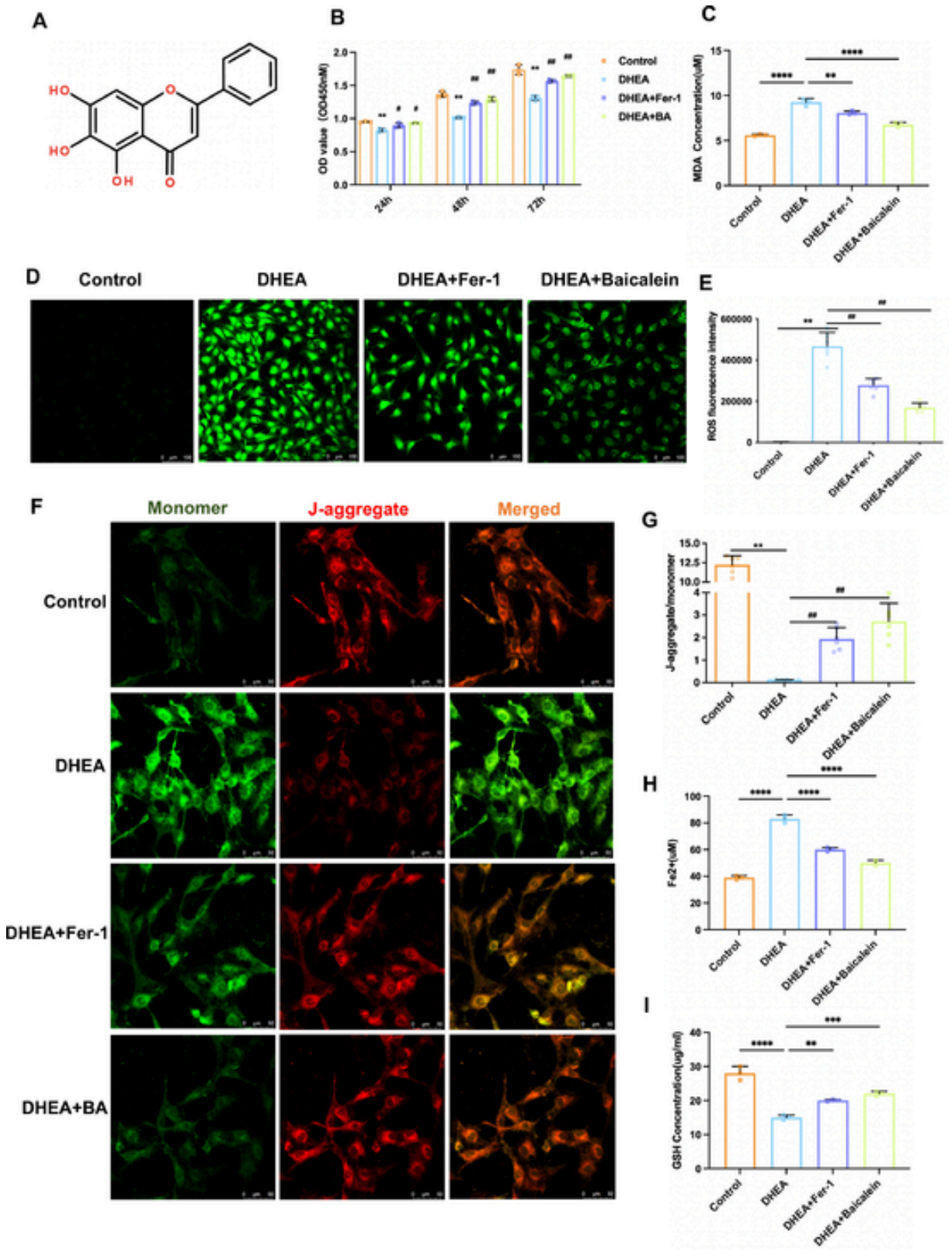

Fig. 1. Baicalein restores DHEA-induced lipid peroxidation in KGN cells

KGN cells were treated with Fer-1 (10 μmol/l) or baicalein (20 μmol/l) for 5 h, and DHEA (20 μmol/l) was used to stimulate KGN cells for 24 h, 48 h, or 72 h. In all plots, the values are expressed as the means ± SEMs. The p values for selected comparisons are indicated as * $p < 0.05$, # $p < 0.05$, * *$p < 0.01$, # #$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. A. Chemical structure of baicalein. B. Cell viability was measured at an OD of 450 nm after culture for different durations. C. The level of MDA activity in KGN cells was determined. D-E. ROS distribution and fluorescence intensity were determined via laser confocal

Fig. 1.—*continued*
imaging. F-G. Changes in the MMP of KGN cells were detected via laser confocal microscopy. H. The level of Fe^{2+} was assessed using an iron reagent test kit. I. The level of GSH activity in KGN cells was determined.

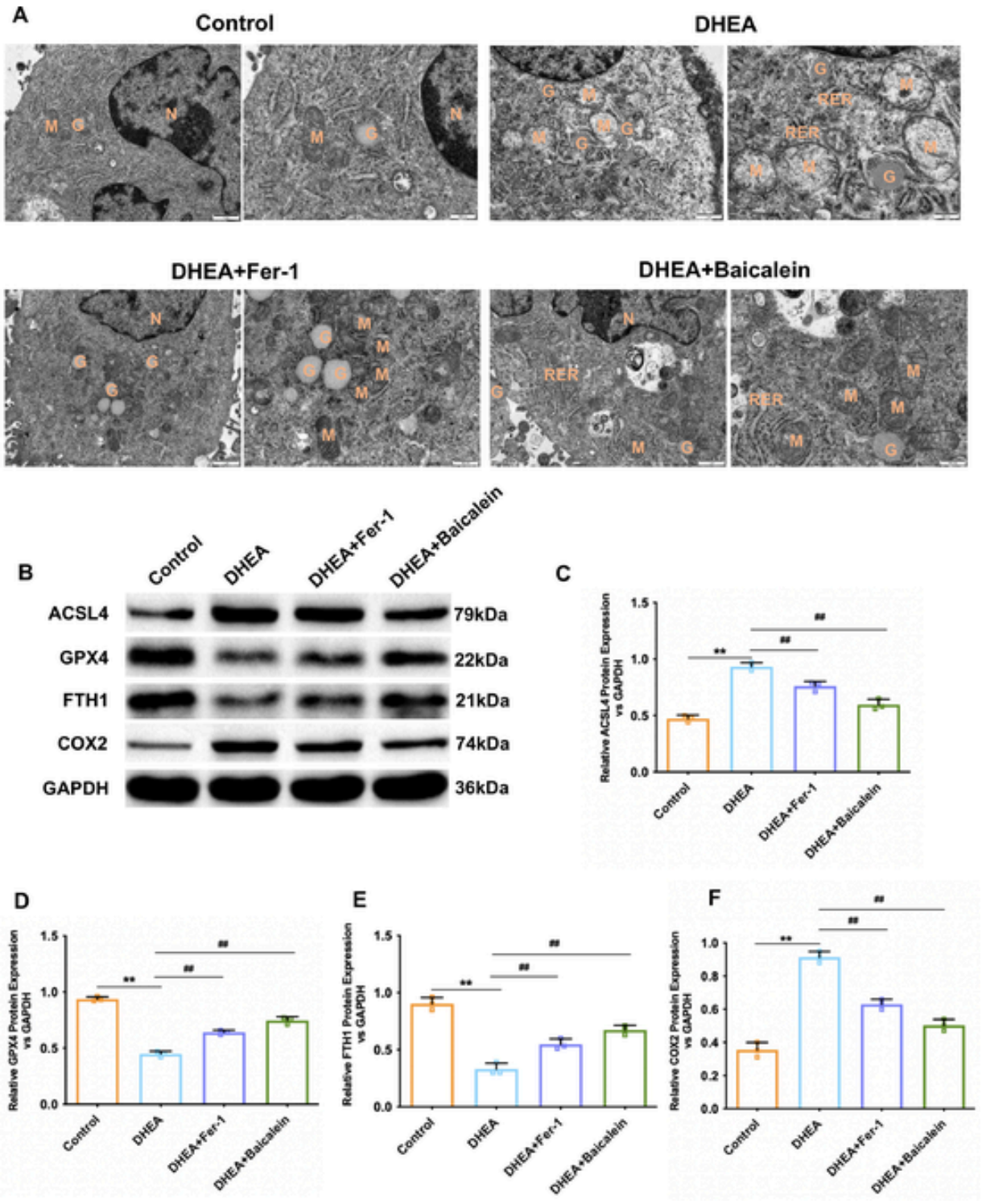

Fig. 2. Baicalein reduces DHEA-induced KGN cell ferroptosis
The KGN cells were cultivated and grouped into control, DHEA, DHEA + Fer-1, and DHEA + baicalein groups. In all plots, the values are expressed as the mean ±SEM. The p values for selected comparisons are indicated as * $^{*}p < 0.01$, and # $^{\#}p < 0.01$. A. Images of KGN cell morphology and mitochondrial morphology were observed under an electron microscope. B-F. The protein levels of ACSL4, GPX4, FTH1, and COX2 in KGN cells.

ferent groups of KGN cells. These findings showed a marked decrease in expressing GPX4 and FTH1 upon stimulation with DHEA. Concurrently, there was an upregulation of ACSL4 and COX2 expression. However, following treatment with Fer-1 or baicalein, GPX4 and FTH1 protein significantly increased, ACSL4 and COX2 expression decreased (Fig. 2B-F).

Based on the above findings, we inferred that when KGN cells were exposed to DHEA, ferroptosis increased significantly. However, treatment with Fer-1 or baicalein effectively decreased ferroptosis in KGN cells.

Baicalein alleviated ovarian dysfunction in DHEA-induced PCOS rats

Fig. 3A outlines the experimental procedures. The rats were injected with sesame oil as a sham control. Rats were randomly assigned to six groups: the oil + 0.5 % —CMC-Na group, DHEA + 0.5 % —CMC-Na group, DHEA + Fer-1 group, DHEA + baicalein (25 mg/kg) group, DHEA + baicalein (50 mg/kg) group, and DHEA + baicalein (100 mg/kg) group. At the conclusion of the present study, the PCOS group demonstrated a significantly greater rat weight than the control group. Fer-1 or baicalein at 100 mg/kg reduced the weight of the PCOS rats(Fig.3C). Subsequently, alterations in ovarian pathology were assessed among the different groups(Fig.3B). We also measured luteinizing hormone (LH), E2, and testosterone (T) levels. Some research have showed that baicalein can inhibit ovarian cancer, while no studies explored the effects of baicalein on PCOS (Chuang et al., 2023). Our data showed that serum sex hormone levels were significantly higher in PCOS rats after treatment with baicalein (Fig. 3D-G). Overall, Fer-1 and 100 mg/kg baicalein had promising effects on mitigating histological changes and improving ovarian function.

Baicalein attenuated lipid peroxidation and chronic inflammation in DHEA-induced PCOS rats

Baicalein has known anti-inflammatory and anti-oxidative stress properties. It is reported that can be used to treat osteoarthritis(Wan et al., 2023). For evaluation of the impact of baicalein on oxidative stress, we examined the serum and ovarian tissue levels of oxidative stress markers and antioxidant substances. Compared with control rats, PCOS rats exhibited significantly greater serum ROS and MDA levels but lower serum SOD and GSH levels. After Fer-1 intervention, the levels of ROS and MDA decreased significantly, while SOD and GSH levels did not change. Administering baicalein at 100 mg/kg significantly decreased the serum ROS and MDA levels and increased the serum SOD and GSH levels (Fig. 4A–I). Next, we measured the inflammatory factor levels in the serum and found that 100 mg/kg baicalein significantly decreased the IL-18, IL-6, and TNF-α levels (Fig. 4J–L). These findings suggested that in PCOS rats, lipid peroxidation and inflammation increase. However, these effects diminished following baicalein treatment.

Baicalein mitigated ovarian ferroptosis in DHEA-induced PCOS rats

Increased iron accumulation and lipid peroxidation have been shown in PCOS rats (Macut et al., 2013). We investigated the key regulatory molecules of ferroptosis in the ovary via Western blotting analysis and assessed morphological changes in ovarian granulosa cells through transmission electron microscopy. The results indicated that rats with PCOS exhibited reduced expression of GPX4 and FTH1, elevated expression of ACSL4 and COX2, fewer mitochondrial cristae, and greater membrane density; these changes indicated ferroptosis and mitochondrial dysfunction. However, treatment with Fer-1 and baicalin at 100 mg/kg partially inhibited ferroptosis (Fig. 5A, B). These findings suggest that appropriate doses of baicalein may have beneficial effects on ferroptosis and mitochondrial dysfunction in PCOS rats.

Genes associated with ferroptosis are altered in the placenta

To assess whether baicalein impacts pregnancy outcomes in patients with PCOS, we established a pregnant rat model of PCOS. This choice was made because PCOS can substantially affect the reproductive capabilities of women of childbearing age. The experimental processes are illustrated in Fig. 6A. RNA sequencing of placental tissue from the three groups of rats revealed that the gene expression differed significantly among the placental tissues (Fig. 6B, C). The results of the gene enrichment analysis were visualized using GO and KEGG pathway diagrams. The results indicated that the signaling pathways were associated with the cell cycle, iron ion transport, reactive oxygen species metabolism, and the insulin response (Fig. 6D, E). According to the FerrDb database, ferroptosis-related genes were classified into two categories: ferroptosis-positive regulatory signatures, which promote ferroptosis, and ferroptosis-negative regulatory signatures, which suppress ferroptosis. Furthermore, we compared the transcriptome sequences of rat placental tissue and the genes listed in the FerrDb database to identify the common genes found in both datasets. The occurrence of ferroptosis was more intense in the placental tissues of PCOS rats, and this change was subsequently alleviated by administering baicalein.

Baicalein ameliorated placental development in DHEA-induced PCOS gravid rats

FTH1 can protect cells against ferroptosis by sequestering excess intracellular iron, thus preventing its involvement in iron-dependent lipid peroxidation reactions (Mi et al., 2023; Zhang et al., 2021). Several studies have reported that COX2 might play a protective role in ferroptosis by promoting anti-inflammatory and cytoprotective effects, while others have suggested that COX2 can contribute to ferroptosis by facilitating lipid peroxidation and oxidative stress (Kohandel et al., 2021; Wang et al., 2022). Based on the results of the sequencing analysis, we performed immunohistochemistry (IHC) on rat placental tissues to assess the expression of ferroptosis regulatory molecules, specifically FTH1 and COX2. The results of the analysis confirmed the patterns observed in the sequencing data (as shown in Fig. 7A, B). We also investigated the changes in ferroptosis-related molecules in the ovaries, placenta, and uterus in each experimental group. The results indicated a significant decrease in GPX4 expression in the PCOS group, whereas the expression of ACSL4 substantially increased. After treatment with baicalein, the expression of the GPX4 protein increased significantly, but the expression of ACSL4 decreased significantly (Fig. 7C–E). As shown in Fig. 7F, placental ROS levels in the PCOS group were considerably greater than those in the control group. However, after administering baicalein, ROS production decreased significantly in placental tissue. Next, we conducted prussian blue staining to visualize iron deposits in ovarian and placental tissues and investigate the occurrence of ferroptosis. Our results indicated that iron deposition in the PCOS group was greater than that in the control group, and baicalein reversed these changes. Iron deposition may contribute to the initiation and progression of ferroptosis in placental tissues, and baicalein may have therapeutic effects by reducing iron deposition and inhibiting ferroptosis (Fig. 7G, H). These findings indicated that baicalein might ameliorate gravid placental development by mitigating ferroptosis.

Discussion

In this study, we presented two new findings. First, PCOS is correlated with oxidative stress, ferroptosis, and chronic inflammation.

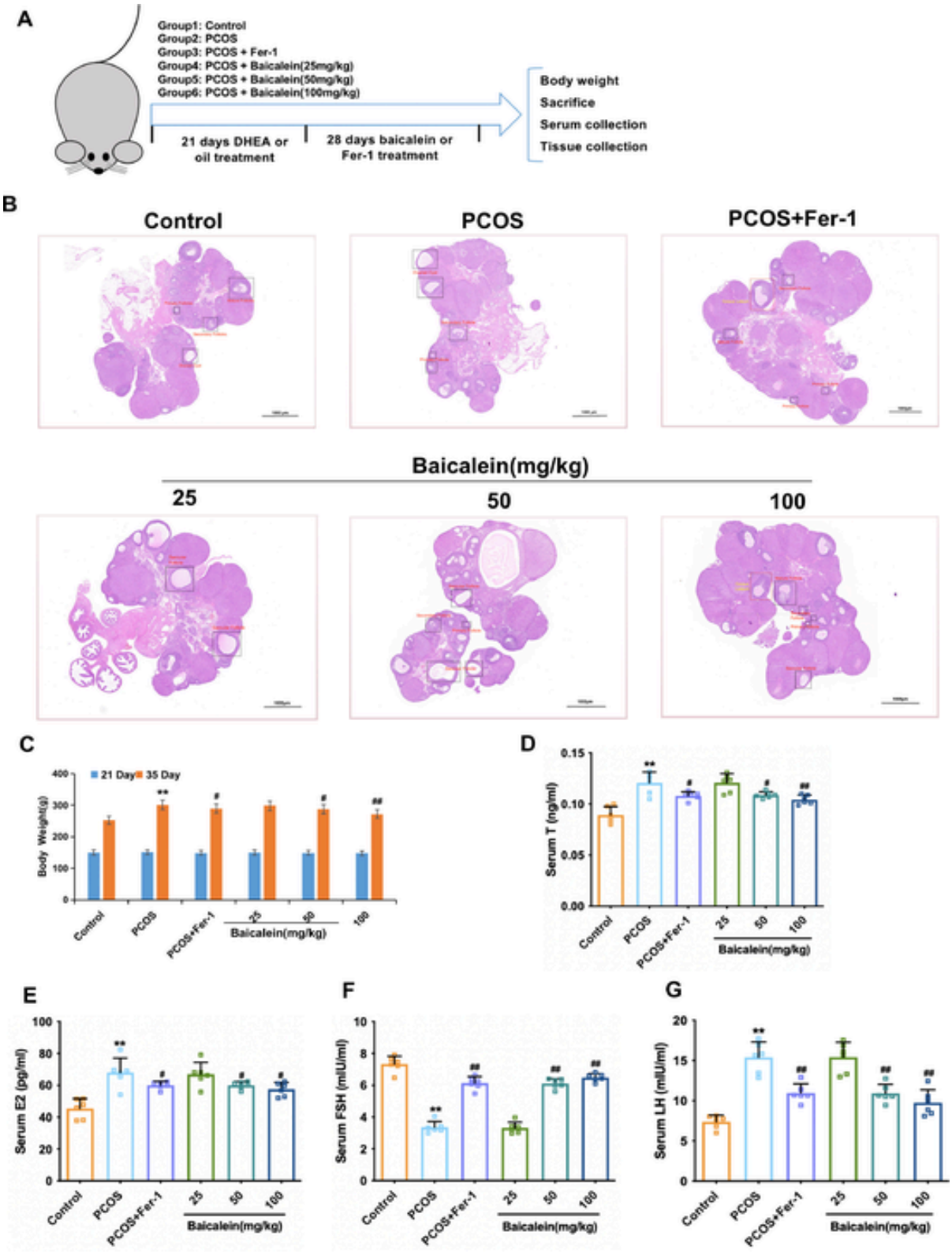

Fig. 3. Baicalein alleviated ovarian dysfunction in DHEA-induced PCOS rats

In all plots, the values are expressed as the means ±SEMs. The p values for selected comparisons are indicated as * $p < 0.05$, # $p < 0.05$, * *$p < 0.01$, and # #$p < 0.01$.A. The experimental processes are illustrated. Since DHEA was dissolved in sesame oil during the experiment, the sesame oil-treated group was used as a sham control. The rats were randomly divided into six groups: the oil + 0.5 % —CMC-Na, DHEA + 0.5 % —CMC-Na, DHEA + Fer-1, DHEA + baicalein (25 mg/kg), DHEA + baicalein (50 mg/kg) and DHEA + baicalein (100 mg/kg) groups. The rats were weighed on days 21 and 35. Each

Fig. 3.—*continued*
group included seven rats ($n = 7$). B. The ovaries were stained with H&E. C. The rats were weighed on days 21 and 35. D. Serum T levels in each group. E. Serum E2 levels in each group. F. Serum FSH levels in each group. G. Serum LH levels in each group.

Second, baicalein effectively attenuated DHEA-induced ovarian dysfunction and gravid placental development in rats with PCOS (Fig. 8).

PCOS, a prevalent metabolic disorder marked by metabolic issues, such as hyperandrogenaemia, chronic inflammation, and oxidative stress. These factors can also disrupt ovulation and impair the functions of ovaries (Escobar-Morreale, 2018). Clinically, as the etiology and pathogenesis of PCOS remain unknown, the main treatment administered is symptomatic relief (Kauffman et al., 2015). Baicalein has antibacterial, diuretic, anti-inflammatory, antiallergic, and spasmolytic effects (Liao et al., 2021; Palierse et al., 2021; Yarla et al., 2016). Baicalein is an effective component in the body and can quickly be converted into baicalin and other metabolites in the blood (Pan et al., 2021; Banik et al., 2022; Chuang et al., 2023). The role of baicalein in ferroptosis related to tumors, sepsis, and hypoxic kidney injury was investigated, and baicalein was found to be a promising therapeutic agent for reducing ferroptosis-related tissue damage (Kong et al., 2021; Wan et al., 2023). However, research examining the direct impact of baicalein on PCOS is scarce. This objective of this study were investigating the influence of baicalein on ovarian function. Our study delineates the novel therapeutic potential of baicalein in addressing both metabolic disorders and inflammatory responses, establishing a significant breakthrough in the field. Our results revealed a compelling correlation between baicalein administration and the amelioration of metabolic irregularities, as evidenced by the substantial improvement in body weight dynamics and the notable reduction in inflammatory cytokine levels in rats with PCOS. This dual-action effect of baicalein presents a pioneering avenue for addressing multifaceted aspects of PCOS-related pathophysiology. Moreover, our investigation reveals the unprecedented efficacy of baicalein in mitigating ovarian dysfunction and effectively countering the pathological damage observed in the ovarian tissues of PCOS-afflicted rats. This nuanced insight underscores the innovative potential of baicalein as a therapeutic agent with a comprehensive impact on both systemic metabolic health and specific ovarian functionality in the context of PCOS. However, additional research is needed into the mechanism of action of this drug, which is the subject of this report.

Approximately half of the patients with PCOS are obese, and they have high total free fatty acids, including arachidonic acid, which can alter the cellular mitochondrial distribution and increase ROS production (Ma et al., 2022; Lin et al., 2020). A notable increase in markers of the oxidative cycle is regarded as a potential initiator of the pathogenesis of PCOS (Mohammadi, 2019). In the metabolic process, oxygen-free radical reactions and lipid peroxidation reactions play crucial roles. Under normal circumstances, they exist in a well-coordinated and dynamic equilibrium, contributing to several physiological, biochemical, and immune responses (Zhang et al., 2021). During lipid peroxidation, ROS oxidize the components of biomembranes, including the side chains of nucleic acids, polyunsaturated fatty acids and other macromolecules associated with ROS and phospholipids. Membrane receptors and enzymes in the biomembrane are affected by lipid peroxidation, which leads to the formation of lipid peroxidation products such as MDA and 4-hydroxynonenoic acid. These changes, in turn, alter the permeability and fluidity of the cell membrane, ultimately resulting in changes in the structure and function of cells (Jaganjac et al., 2022; Li et al., 2022; Mahoney-Sanchez et al., 2021). A dynamic balance between oxidation and antioxidation occurs within the follicular microenvironment in the ovary, and this balance is closely associated with follicle maturation. In PCOS, ovarian oxidative stress can result in the loss of gonadotropin receptors, an increase in reactive nitrogen species, and the accumulation of ROS. These events are associated with lipid peroxidation of the follicle cell membrane and contribute to ovarian dysfunction (Jiang et al., 2021a; 2021b). The degree of lipid peroxidation in cells is determined by the location and number of polyunsaturated fatty acids, which in part control ferroptosis. Li et al. suggested that tempol improves the PCOS phenotype by reducing intestinal oxidative stress(Li et al., 2021). Additionally, other studies show Chinese herbal medicine improves oxidative stress to relieve PCOS (Zhang et al., 2023). In this investigation, we meticulously documented the dysregulation of mitochondrial dynamics, elevated oxidative stress levels, and manifestations of inflammatory responses within the context of PCOS. These pronounced alterations observed in our PCOS model were notably reversed upon treatment with a ferroptosis inhibitor. Intriguingly, the administration of baicalein had analogous beneficial effects, demonstrating its potential for inhibiting critical pathophysiological mechanisms, specifically lipid peroxidation and ferroptosis. Our identification of baicalein's capacity to mitigate these detrimental processes presents a groundbreaking perspective in PCOS management.

ACSL4 and GPX4 serve as promoters and inhibitors of ferroptosis, respectively (Mohammadi, 2019). GPX4 takes central stage in preventing lipid peroxidation. This role involves the conversion of harmful lipid hydroperoxides into nontoxic lipid alcohols, thereby reducing ROS levels and preventing the initiation of ferroptosis (Yant et al., 2003; Yang et al., 2014; Seibt et al., 2019). The availability of cellular GSH determines the proper function of GPX4. GPX4 is inactivated after GSH depletion, which can be triggered by erastin (Jiang et al., 2021). In contrast, ACSL4 catalyzes the acetylation of long-chain polyunsaturated fatty acids, producing lipid peroxides that become esterified by interacting with membrane phospholipids, ultimately resulting in ferroptosis (Yang et al., 2016; Kagan et al., 2017). Studies have found that PCOS drugs work by inhibiting ferroptosis, such as metformin (Peng et al., 2023). Our findings revealed a distinctive pattern of ovarian GPX4 and ACSL4 expression in rats with PCOS, marking a pivotal shift in our understanding of ferroptosis modulation in this context. Remarkably, baicalein administration resulted in increased levels of GPX4 and FTH1 while concomitantly reducing ACSL4 expression. These compelling outcomes not only highlight baicalein's role in orchestrating a transformative response but also help elucidate the complex mechanisms involved in mediating ferroptosis in PCOS patients. This nuanced exploration illustrates the pivotal involvement of ACSL4 in mediating ferroptosis within the landscape of PCOS, particularly through its active participation in lipid peroxidation reactions. The ability of baicalein to modulate intracellular lipid peroxidation while mitigating iron accumulation has emerged as a groundbreaking discovery.

Studies on the placental aspects of pregnancies in PCOS women are limited. PCOS is characterized by high levels of pregnancy hormones and insulin resistance. These factors are significant in preventing placental dysfunction, thus contributing to an increase in the rate of maternal and fetal complications. More research is necessary for developing effective strategies to prevent adverse maternal and protecting their offspring (Bahri Khomami et al., 2019; Hoch et al., 2019). Zhang et al. (2020) discovered that insulin resistance and hyperandrogenism influence ferroptosis in the uterine and placental tissues of PCOS rats during pregnancy. This aligns with our research findings. Beharier et al. (2021) showed that relatively high levels of 5-lipoxygenase and 15-lipoxygenase in the placenta may promote hydroxyl peroxidation of phosphatidylethanolamine-containing polyunsaturated fatty acid chains, thereby elevating the risk of ferroptosis. Excessive iron accumulation can inhibit the production or activity of maternal hepcidin, resulting in the continuous absorption of dietary iron. This phenomenon may increase blood viscosity, obstruct blood vessels, and decrease placental perfusion, thus affecting pregnancy outcomes. Several studies have highlighted the key role of ferroptosis in placental development

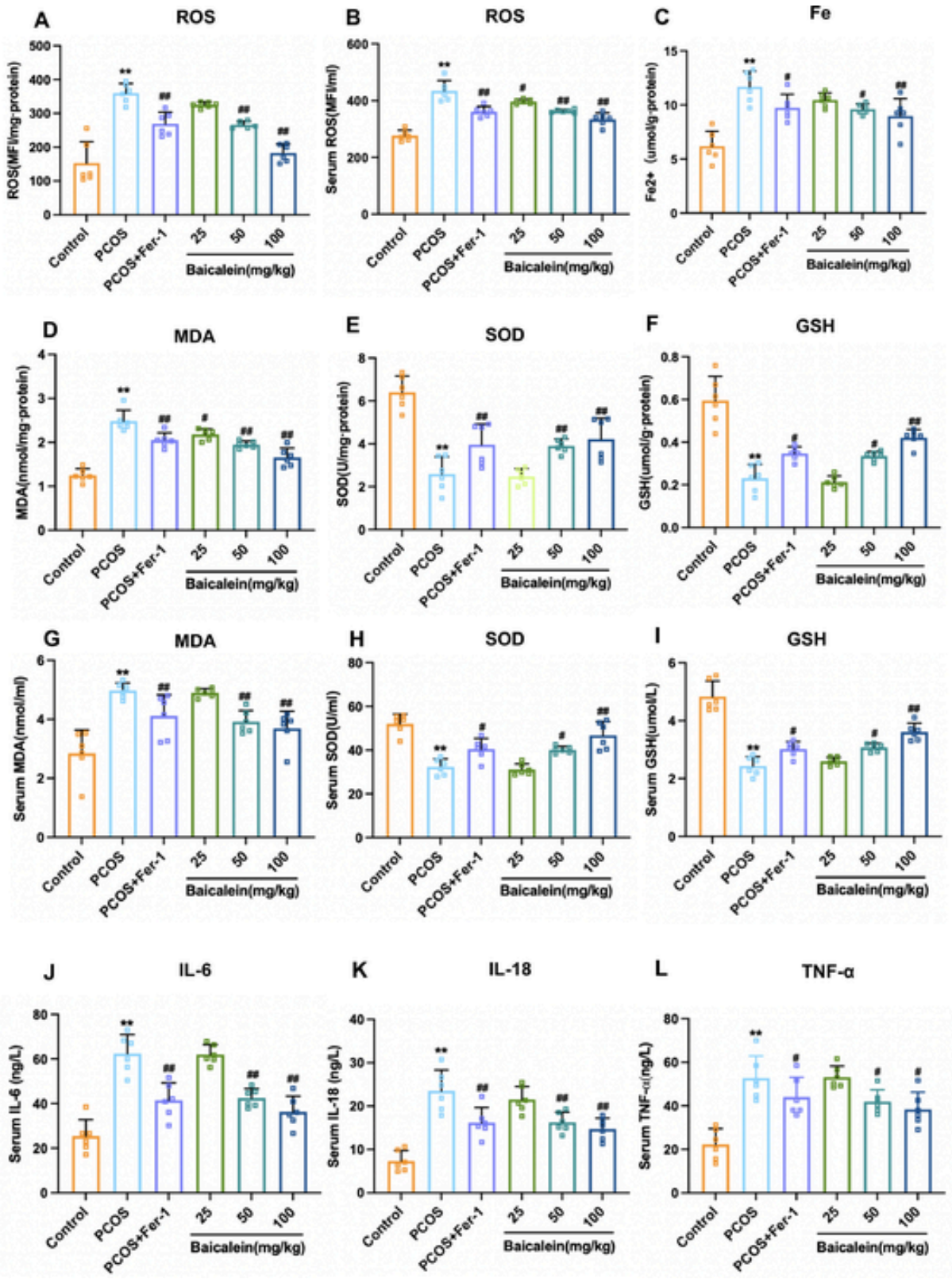

Fig. 4. Baicalein attenuated lipid peroxidation and chronic inflammation in DHEA-induced PCOS rats

In all plots, the values are expressed as the mean ±SEM. The p values for selected comparisons are indicated as * $p < 0.05$, # $p < 0.05$, * *$p < 0.01$, and # #$p < 0.01$. * represents comparison with the control group, # represents comparison with the PCOS group. A. The ROS content in the ovaries of the different groups was determined via ELISA. B. The serum ROS concentration in the different groups was determined via ELISA. C. The Fe^{2+} concentration in the ovaries of different groups was determined. D. The MDA content in the ovaries of the different groups was determined via ELISA. E. The SOD content in the ovaries of the different groups was determined via ELISA. F. The GSH content in the ovaries of the different groups was determined via ELISA. G. The serum MDA concen-

Fig. 4.—*continued*
tration in the different groups was determined via ELISA. H. The serum SOD concentration in the different groups was determined via ELISA. I. The serum GSH concentration in the different groups was determined via ELISA. J. The serum IL-6 concentration in the different groups was determined via ELISA. K. The serum IL-18 concentration in the different groups was determined via ELISA. L. The TNF-α concentration in the serum of the different groups was determined via ELISA.

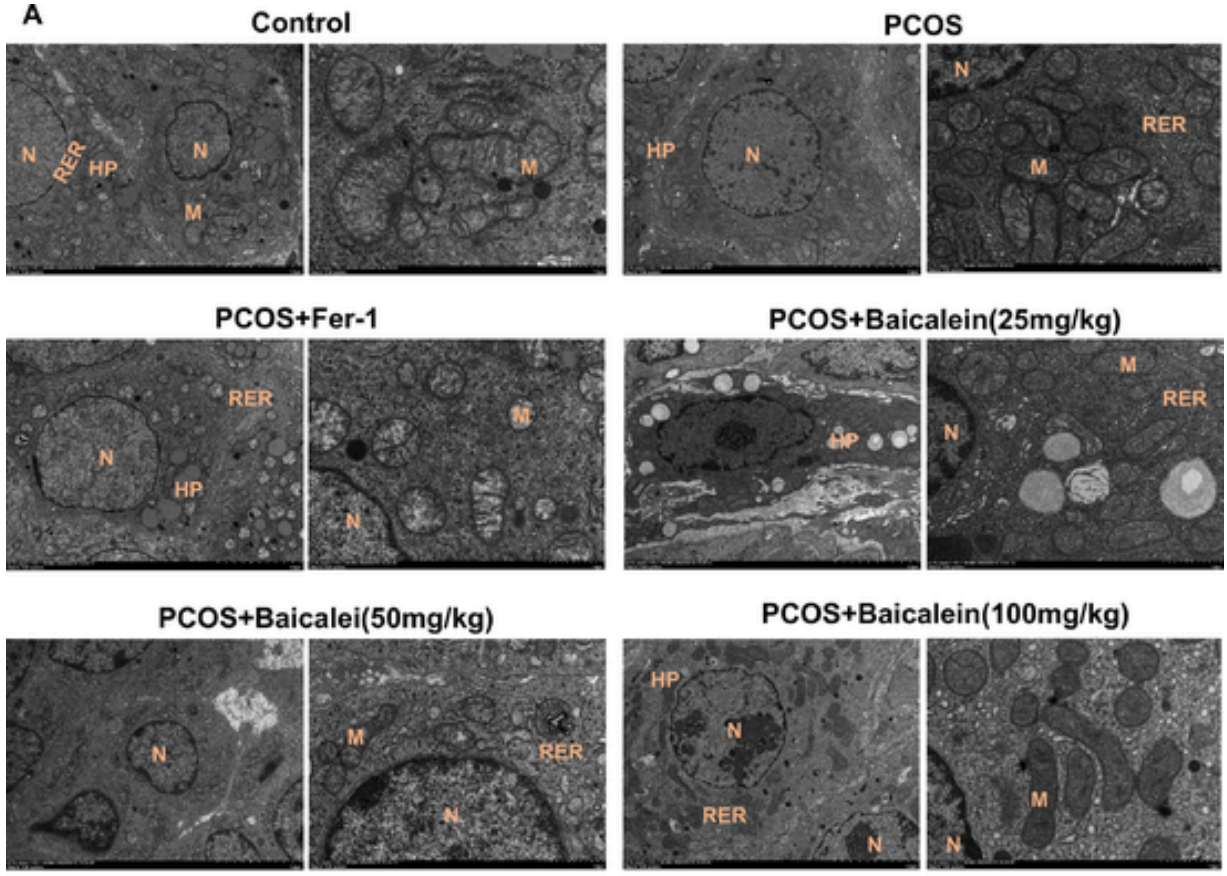

Fig. 5. Baicalein mitigated ovarian ferroptosis in DHEA-induced PCOS rats
A. Images of changes in the cellular and mitochondrial morphology of rat ovary cells were captured under an electron microscope. B. The protein levels of ACSL4, GPX4, FTH1, and COX2 in the ovaries were determined via Western blotting assays.

(Yang et al., 2022; Hu et al., 2021). We also investigated the efficacy and underlying mechanism by which the antioxidant baicalein can reverse ferroptosis of the placenta in pregnant rats exposed to DHEA. We established a PCOS rat pregnancy model, and on the 15.5th day of pregnancy, the placenta was analyzed through RNA sequencing to detect DEGs. Then, we conducted a comparative analysis between the transcriptome sequencing data of the placental tissue of rats and the gene list in the FerrDb database. Through this analysis, we identified the genes that were shared between the datasets.

Fer-1 can cause liver toxicity in some animal models, and it may cause unintended consequences by exerting off-target effects on other cellular processes. Dong et al. (2022). Baicalein is a widely available natural flavonoid compound. It is considered safe and associated with a low risk of side effects. The gestational placenta is composed of various

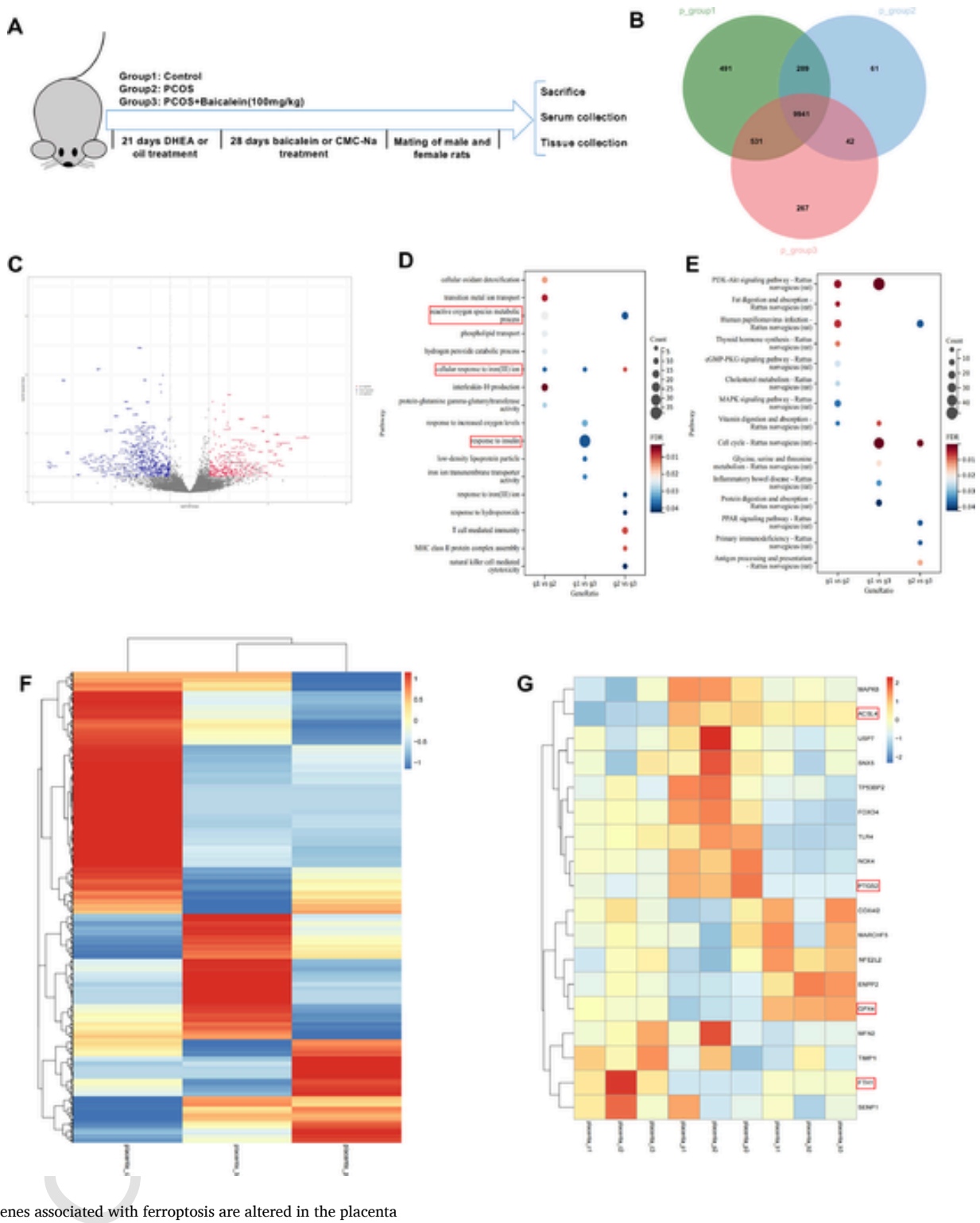

Fig. 6. Genes associated with ferroptosis are altered in the placenta

A. The experimental processes are illustrated. Since DHEA was dissolved in sesame oil during the experiment, the sesame oil-treated group was used as a sham control. The rats were randomly divided into three groups: the oil + 0.5 % —CMC-Na, DHEA + 0.5 % —CMC-Na, and DHEA + baicalein (100 mg/kg) groups. Each group included seven rats ($n = 7$). B. Venn diagram showing the number of common genes and the number of DEGs among the three groups of placental tissues. P-group 1: the control group; P-group 2: the DHEA group; P-group 3: the DHEA + baicalein group. C. A volcano plot of differentially expressed genes in the control and PCOS groups. D. GO pathway enrichment analysis. E. KEGG pathway enrichment analysis. F. A heatmap of the DEGs in the placentas of the three different groups of rats. G. A heatmap of the differential expression of common genes according to the transcriptome sequencing of rat placental tissue and the FerrDb database.

types of cells, each of which has a different gene/protein expression pattern that may be sensitive to baicalein treatment. Ferroptosis pathways in isolated placental regions or isolated cell types in baicalein-treated rat placentas should also be analyzed in future studies.

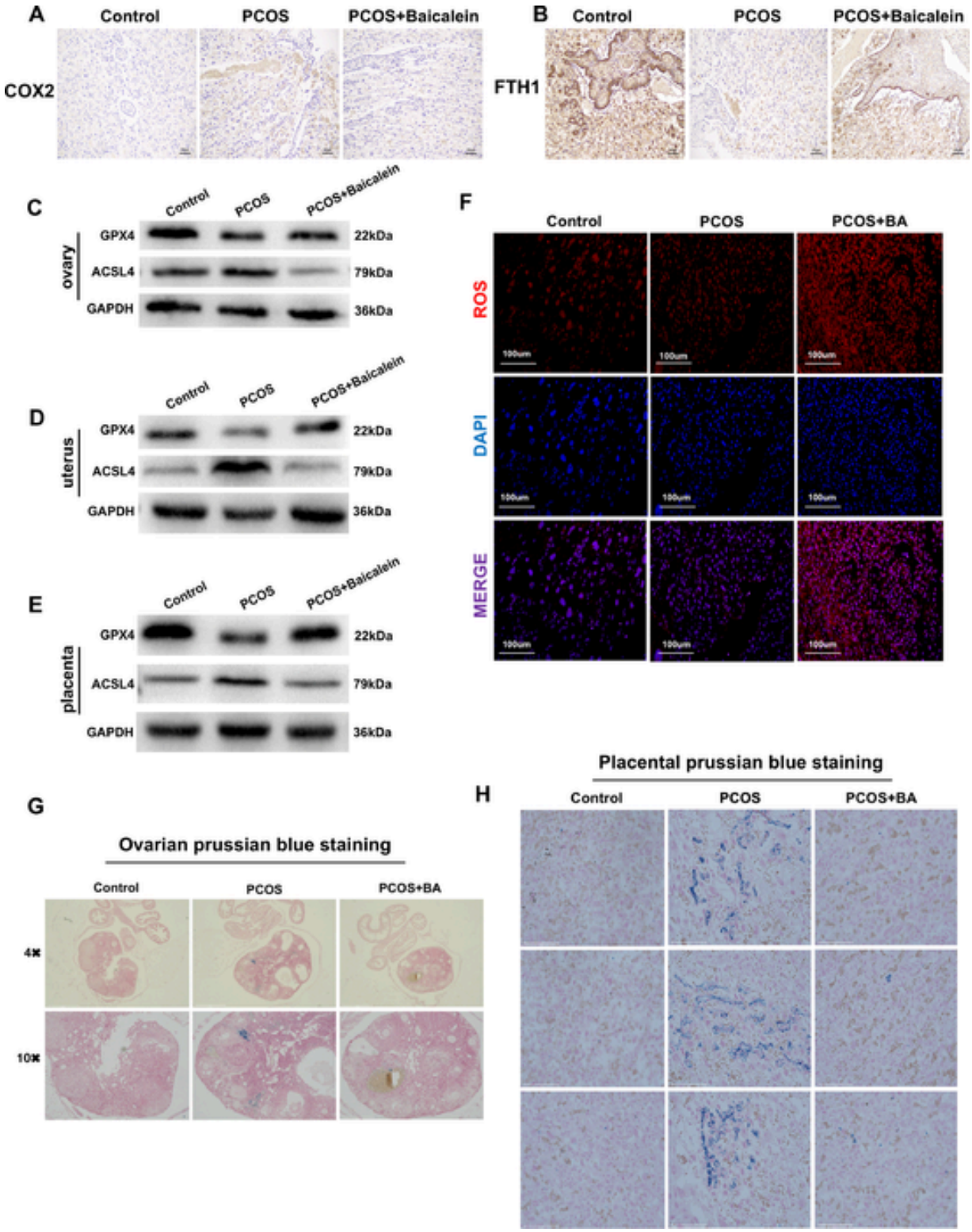

Fig. 7. Baicalein ameliorated placental development in DHEA-induced PCOS gravid rats
A. The expression of COX2 in the placental tissue of rats was evaluated by immunohistochemistry. B. The expression of FTH1 in the placental tissue of rats was determined by immunohistochemistry. C. GPX4 and ACSL4 protein levels in ovarian tissue were assessed via Western blotting. D. GPX4 and ACSL4 protein levels in the uterine tissue of rats were assessed via Western blotting. E. The expression of GPX4 and ACSL4 in the placental tissue of rats was evaluated by Western blotting. F. The level of ROS in the placenta of the rats in the three groups was determined using a fluorescence microscope. G. Iron deposition in the placenta was detected by DAB-enhanced Perls' staining. H. Iron deposition in the gravid placenta was detected by DAB-enhanced Perls' staining.

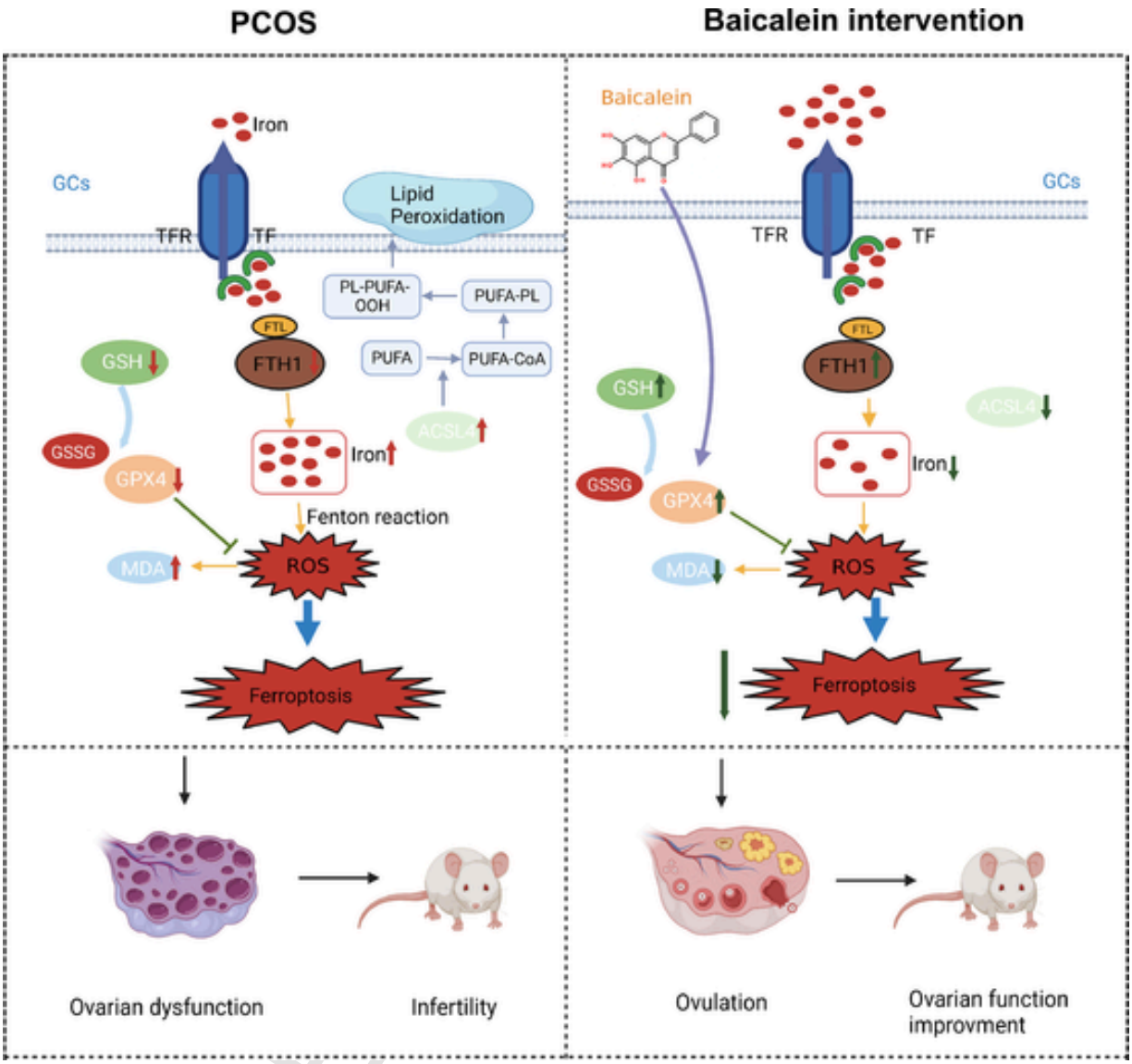

Fig. 8. A schematic depicting the mechanisms by which baicalein mediates antiferroptosis activity.
Abbreviations: GCs, Granulosa cells; TFR, Transferrin Receptor; TF, Transferrin; PUFA, Polyunsaturated fatty acid; PUFA-CoA, Polyunsaturated Fatty Acid-Coenzyme A; PUFA-PL, Polyunsaturated Fatty Acid-Phospholipid; PL-PUFA-OOH, Phospholipid Polyunsaturated Fatty Acid Hydroperoxide; GSSG, Glutathione disulfide.

Conclusions

In summary, our study investigated the mode of action of baicalein in PCOS, revealing novel insights that distinguish our research. The novelty of our study lies in uncovering the upregulation of ferroptosis in PCOS patients and suggesting that the therapeutic effect of baicalein may stem from its inhibition of this process. Ferroptosis, which acts as a regulator of oxidative stress pathways, adds a new dimension on PCOS pathogenesis and represents a potential treatment target. However, acknowledging the limitations of our study is crucial. We acknowledge that the animal model may not fully replicate the complexities of PCOS in humans and is influenced by various genetic and environmental factors. Additionally, while baicalein shows promise, more research needs to be done to find out how it works and establish its safety and efficacy in clinical settings. Moreover, we did not assess the pregnancy or live birth rates of the rats. To address these limitations, future research could incorporate larger sample sizes and diverse populations. During the course of our investigation, we encountered difficulties, mainly in the design and execution of animal experiments. To overcome these challenges, we carefully plan experimental designs and work with laboratory teams to demonstrate our commitment to methodological rigor and robustness in our study. Future investigations could focus on refining animal models to better mimic human PCOS and exploring additional interventions or combination therapies that may enhance treatment outcomes. Furthermore, clinical trials are necessary to determine baicalein's translational potential for PCOS in humans. These endeavors will help to better understand PCOS and develop effective treatments.

Access to Data

All of the data were in-house data only, and no use was made of paper mills. All authors agree that they are responsible for all aspects of the work and are guarantors of the integrity and accuracy of the work.

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CRediT authorship contribution statement

Ying-ying Li: Writing – original draft. **Yi-qiu Peng:** Methodology. **Yu-xi Yang:** Methodology. **Ting-juan Shi:** Investigation. **Rui-xia Liu:** Writing – review & editing. **Ying-yi Luan:** Writing – review & editing. **Cheng-hong Yin:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2024.155423.

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